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The effect of hypothermia on outcome and neurologic injury after prolonged cardiac arrest treated by emergency preservation and delayed resuscitation

Vliv hypotermie na úspěch resuscitace a neurologické postižení po dlouhodobé srdeční zástavě léčené metodou Emergency Preservation and Resuscitation

Disertační práce

Školitel: Doc. MUDr. Robert Jech, PhD.

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TD

Summary:

Currently, the outcomes from traumatic exsanguination cardiac arrest (CA) show that over 50% of deaths due to trauma occur at the scene, where medical care is limited. Less than 10% of patients who become pulseless from trauma survive. However, in an appropriate setting, some of those traumatic injuries could be surgically repairable.

Emergency preservation and resuscitation (EPR) is a novel approach for resuscitation of exsanguination CA victims. EPR uses deep hypothermic preservation for prolonged CA to buy time for transport, damage control surgery, and delayed resuscitation with cardiopulmonary bypass (CPB). Initially, we used a dog model to maximize clinical relevance. We showed that the efficacy of EPR is related to the depth of hypothermia and duration of CA. Pharmacologic adjuncts tested to augment hypothermia generally failed. Extended hemorrhagic shock did not prevent the success of EPR vs. conventional resuscitation if extended post-resuscitative hypothermia was provided. Oxygenation of the flush allowed extending of survivable duration of deep hypothermic CA.

Because of the lack of molecular tools available for use in dogs, we developed a rat EPR model to study the cellular and molecular mechanisms underlying deep hypothermic neuroprotection to allow us to define specific targets for future interventions, assess markers of reversibility, and screen novel therapies. We showed that (1) rat EPR model with miniaturized CPB was feasible; (2) shorter durations of CA and deeper hypothermia yielded better outcome; (3) extended durations of normothermic CA prior to induction of hypothermia resulted in worse outcome, extensive neuronal death and neuroinflammation; (4) blood-brain barrier was not permeable even in insults with poor outcome; (5) three neuroprotective pharmacological strategies failed to confer additional benefits to hypothermia; (6) neuronal degeneration and neuroinflammation after EPR exhibited a characteristic temporo-regional pattern that may require selective therapeutical approaches.

Souhrn:

Úspěšnost resuscitace obětí srdeční zástavy je stále neuspokojivá. Srdeční zástava způsobená traumatem a následným vykrvácením má za použití konvenčních technik resuscitace velmi špatnou prognózu. Většina těchto pacientů umírá přímo na místě, přestože poranění by mohla být při patřičném vybavení chirurgicky ošetřitelná.

Emergency Preservation and Resuscitation (EPR) je nová resuscitační metoda, která využívá masivní infuze ledové tekutiny do tepenného řečiště k navození hypotermie jako hlavního ochranného mechanismu (Emergency Preservation). Navozená hypotermie pro období srdeční zástavy umožňuje získat čas pro převoz raněného do zdravotnického zařízení. Po chirurgické kontrole krvácení je odložená resuscitace následně zahájena s využitím mimotělního oběhu (Resuscitation), obdobně jako je tomu v srdeční chirugii.

Ve výzkumu jsme nejprve využívali model na psu, který nejlépe odpovídá klinické praxi. Zjistili jsme, že hloubka hypotermie koreluje s dobou zástavy, po níž je ještě možné docílit příznivého neurologického výsledku. Co nejčasnější navození hypotermie po nastalé zástavě je spojeno s příznivějšími výsledky. Úloha léků se jeví jako omezená. Prodloužená doba krvácení před zástavou není překážkou úspěchu EPR. Krevní deriváty a energetické substráty mohou mít kladný vliv. V nově vyvinutém modelu na kryse jsme prokázali, že kratší doba zástavy a hlubší hypotermie mají lepší výsledky, delší prodleva před navozením hypotermie vede k odúmrti neuronů a k zánětlivé reakci, přičemž mozkomíšní bariéra zůstává neporušena. Úloha farmak a role mikroglií na výsledném klinickém i histologickém nálezu je zřejmě omezená. Produkce cytokinů a odezva centrálního nervového systému na inzult je zřetelně odlišná v jednotlivých mozkových strukturách. Hypotermie příznivě ovlivňuje profil zánětu. Vývoj nových léřivých přípravků by měl být cílený pro jednotlivé oblasti mozku.

V současné době se metoda EPR dostává do stádia klinických zkoušek.

Abbreviations:

3-NT = 3-nitrotyrosine

BBB = blood-brain barrier

CA = cardiac arrest

CA1 = cornu ammonis region 1

CCI = controlled cortical impact

CNS = central nervous system

CPB = cardiopulmonary bypass

CPCR = cardiopulmonary-cerebral resuscitation

CPR = cardiopulmonary resuscitation

DAPI = 4’,6-diamidino-2-phenylindole

DHCA = deep hypothermic circulatory arrest

EPR = Emergency Preservation and Resuscitation

ExCA = exsanguination cardiac arrest

FJB = Fluoro-Jade B

H&E = hematoxylin/eosin

HDS = Histological Damage Score

HR = heart rate

HS = hemorrhagic shock

ICP = intracranial pressure

IL - interleukin

LEC = liposome-encapsulated clodronate

MAP = mean arterial pressure

MOF = multiple-organ failure

OPC = overall performance category

PARP = poly (ADP-ribose) polymerase

PBS = phosphate-buffered saline

PMN = polymorphonuclear neutrophils

ROSC = return of spontaneous circulation

RT = resuscitation time

TBI = traumatic brain injury

TNF-α = tumor necrosis factor alpha

Tpa = temperature at pulmonary artery

Tr = rectal temperature

Tty = tympanic temperature

VF = ventricular fibrillation

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1. Introduction

1.1. The history of resuscitation

Until early 1950's there did not exist any effective treatment for airway obstruction or cardiac arrest (CA) for laypersons. At the late 50's there were described isolated steps to establish a patent airway (A), provide mouth-to-mouth breathing (B) and restore circulation (C) with chest compressions. Tying those steps together into an A-B-C sequence became the basis of physiologically effective cardiopulmonary-cerebral resuscitation (CPCR), as the method was called originally.[1]

Although single steps proved to be effective, the outcomes of out-of-hospital CA treated by CPCR were not encouraging from the very start. The efforts provided by bystanders and medical personnel came often "too little too late".[2] The rapid developments in the field of cardiology in the following decades focused mostly on the circulatory part of resuscitation. Restoration of hemodynamically effective cardiac rhythm became the main criterion for "successful resuscitation", while brain as the key and target organ was somewhat left behind, at least in the efforts of the public health organizations responsible for promotion of "cardio-pulmonary resuscitation" (CPR), a newly coined term, now debrided of the cerebral component.

Survival and restoration of neurocognitive functions after normovolemic CA presumably of cardiac origin, are closely associated with early initiation of chest compressions assuring at least minimal blood flow until advanced medical treatment is available. The majority of CA is caused by ventricular fibrillation (VF). It is thus not surprising that the mainstay of further medical research focused mainly on the heart. Restoration of cardiac rhythm became an essential centerpiece of the resuscitation efforts. As early as in 1960's, EMS ambulances in Prague, Czechoslovakia and Moscow, Soviet Union

became equipped with the first defibrillators that occupied almost the entire trunk of the car. Technical developments in the following decades enabled to miniaturized the devices and their simplified versions known as automatic external defibrillators (AEDs) are now publicly available at the places of mass gatherings, i.e. airports or stadiums.[3]

It is clear that the significant improvement of the survival of CA victims were enabled by technological developments generally aimed to support the failing heart. The emergence of percutaneous coronary interventions and mechanical devices supporting the failing heart granted the extra time to recover cardiac function in patients who would not have the same chance several years ago.

The other line of research was focused on improving the circulation in victims in whom the perfusing rhythm cannot be restored. The complexity of the devices ranged from simple add-ons to standard devices, e.g. impedance threshold valve mountable on standard endotracheal tube,[4] to circulation-enhancing compression devices providing more effective cardiac massage.[5,6] The “ideal” resuscitation device based on miniaturized cardiopulmonary bypass (CPB) providing both circulation and oxygenation in the pre-hospital setting is still to be successfully introduced into clinical practice. Several reports documenting the feasibility of the method and effectiveness of prototypes have already been published from both experimental and clinical settings.[7-10]

Even if the most effective methods to preserve circulation are employed, not always there are enough reserves to combat evolving brain ischemia (becoming critical approximately after 5 min) and cardiac ischemia (critical after 20 min). While restoration of spontaneous circulation could be claimed in more than half of the CA victims, long-term survival has remained in single-digit numbers for decades. A major breakthrough in resuscitation science was achieved when two seminal papers published back-to-back in the

New England Journal of Medicine showed that prolonged mild hypothermia applied to comatose survivors of VF induced CA improved survival and neurologic outcome.[11,12]

These reports sparked a new enthusiasm in resuscitation research. However, the fact that hypothermia could be protective in multiple settings has been known for decades, if not centuries.

Many earlier examples of potential value of the use of hypothermia in acute life threatening insults exist. Mild or moderate hypothermia was recognized as being potentially beneficial in trauma victims even by Napoleon's respected surgeon, Baron Dominique-Jean Larrey, in 1814.[13] Similarly, Dr. Charles Phelps in 1897 (Phelps, 1897) recommended the use of the "ice cap" in his treatise, "Traumatic Injuries of the Brain and Its Membranes." T our knowledge, the initial series of therapeutic hypothermia applied to victims of CA of various origin (respiratory failure, trauma) was published in 1958.[14] Surprisingly, the target temperatures and duration of cooling (30 to 34 °C for 24 to 72 hours) closely resembled current recommendations (32 to 34 °C for 12-24 hours).[15] In 1959, Benson et al. reported the first case series of in-hospital CA patients. Their data revealed favorable neurologic recovery in 50% of hypothermic patients vs. 14% of normothermic patients.[16] Many others support the use of hypothermia in CNS insults in the 1950s and 1960s.[14,17]

The key role of therapeutic hypothermia in comatose patients after CA, for example is also readily seen in the historic first "ABCs" of resuscitation, published by Safar in 1964.[18] In that document, Safar presciently states that "hypothermia should be started within 30 minutes if there is no sign of CNS recovery." Indeed, Safar built upon a body of work already established by Rosomoff,[19] and the early work of Williams and Spencer on the use of therapeutic hypothermia in clinical CA, also in the 1950s.[14] In that era, Rosomoff and Safar routinely used therapeutic hypothermia as outlined in their incredible treatise from 1965 on

management of the comatose patient.[20] In that review, Rosomoff and Safar provided a roadmap for therapeutic hypothermia and temperature control in neurointensive care that in many ways holds up remarkably well by today's standards. On the topic of fever control after CNS insults, they indicated that "hyperpyrexia is disastrous if uncontrolled" and suggested that "we prevent temperature rises above 38 °C by the use of external cooling and/or vasodilators." For comatose patients, they suggested that "hypothermia seems indicated in any patient who has brain damage severe enough to produce unconsciousness -- usually the temperature is kept at 32 °C." On the topic of rewarming, they indicated that "re-warming should be accomplished by simply removing hypothermia blankets" and indicated that "patients may take 12–48 hours."

This is in a remarkable agreement with the current recommendations of the American Heart Association and European Resuscitation Council for post-resuscitative therapeutic hypothermia.

1.2. The Challenge of Traumatic CA in Resuscitation

While many efforts have been put to advancement of the techniques supporting the failing heart after CA, other forms of CA in which the heart was not the "weakest link in the chain" remained overlooked. The classic example is traumatic CA following either blunt or penetrating injury, often accompanied by hemorrhage. Both military and civilian trauma CA victims are most likely to represent a population characterized by "heart and brains too good to die".

The outcomes of resuscitation of exsanguination CA victims are especially dismal. The situation is complicated by the fact that unlike in normovolemic CA, the missing volume needs to be repleted to enable resuscitation efforts to be effective. Despite the fact that direct cardiac massage via thoracotomy is more effective than external chest compressions, those

patients rarely survive.[21,22] The acute mortality from trauma had remained unchanged for decades.[23]

In the civilian settings, 50% of deaths due to trauma occurred at the scene of the accident, with another 30% within a few hours of injury.[24] In the military settings, the majority of the US soldiers killed in action in Vietnam without brain trauma had penetrating truncal injuries and exsanguinated to CA within minutes (Figure 1).[23] Technically, the injuries were surgically repairable. Unfortunately, to such a large portion of acute mortality in both civilian and military settings, no effective methods have been established to reliably improve the survival. The technical obstacles seemed insurmountable. Shortening rescue and transport time can barely race against the irreversible deterioration of the brain that occurs 5 min after CA, let alone finding time for surgical repair; the conventional CPR is futile because of a volume-depleted and trauma-disrupted circulatory system. More aggressive treatments with thoracotomy and aortic cross clamping did not change the gloomy outcome either.[25]

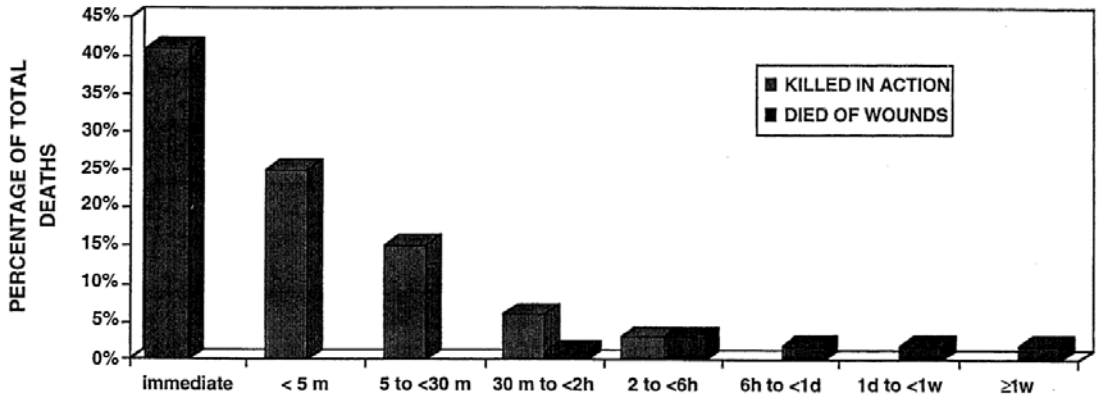


Figure 1. Time to clinical death in combat casualties. From the Wound Data and Munitions Effectiveness Team study in Vietnam. Two thirds of fatally shot soldiers died within 5 min.

The extremely grim prognosis has led the National Association of EMS physician Standard and Clinical Practice Committee and the American College of Surgeons Committee on Trauma to publish the guidelines for termination of resuscitation in pre-hospital traumatic cardiopulmonary arrest. If patients with either blunt or penetrating trauma are found apneic, pulseless, and absent of vital signs, such as pupilary reflexes, spontaneous movement, termination of resuscitation efforts is recommended.[26] If resuscitation of such patients was pursued, the observed resuscitation rate in a recently published series was one in 294 patients (0.3%).[27] In the military settings, resuscitation efforts will be stopped if patients fail to respond to 1 L of hetastarch.[28]

However, those victims are likely to represent a group of otherwise healthy personnel, "whose hearts and brains are too good to die". Most of the deaths associated with trauma occur at the site of the accident where medical care is limited.[23] This is supported by data from both recent military conflicts and civilian settings. Many of those injuries would be surgically repairable if the victim was treated in the medical care facility.[29]

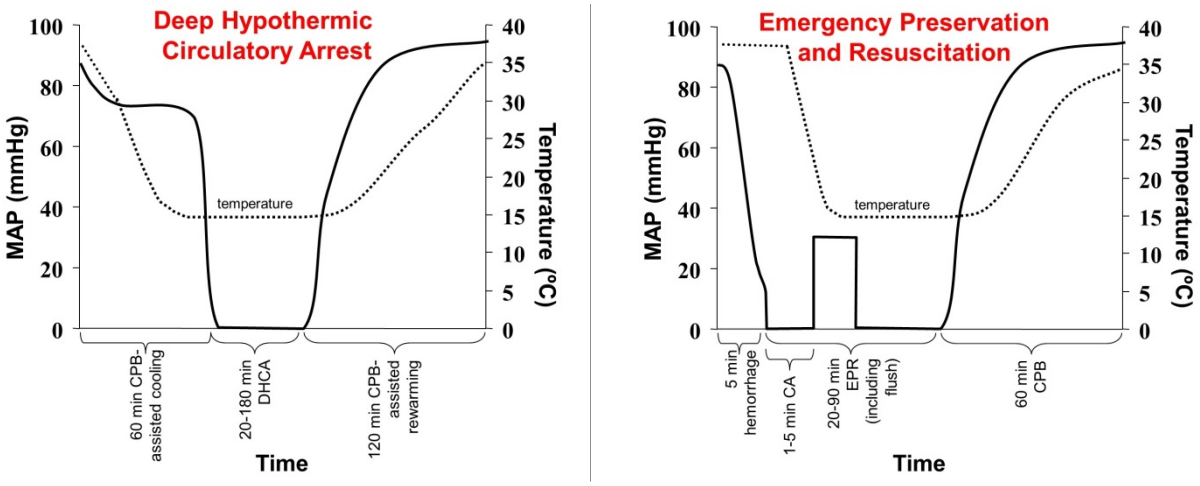
Few studies have tried to address this problem recently. For example, a group of emergency medicine researchers had proposed the use of intra-aortic balloon catheter to stop bleeding beyond the descending aorta and infuse blood or other solutions into the aorta above

the balloon to avoid ischemic injuries in the heart and the brain.[30] Its mechanism is similar to thoracotomy and aortic cross-clamping, but less traumatic. No outcome studies have been conducted to compare the effectiveness of this technique with thoracotomy.

1.3. The Concept of Emergency Preservation and Resuscitation

Facing the challenge of traumatic CA, late Dr. Peter Safar, the Father of Modern Resuscitation, and Col. Dr. Ronald Bellamy jointly created a new concept of care for such patients. While there was not much potential left in trying to restore the disrupted circulation, they rather focused on the long neglected part of equation – while supplies are limited and could not be increased, consumption must be decreased to maintain balance till the supply could be re-established. They envisioned to put the victim into a state of "suspended animation" that would buy time for transport, damage-control surgery and delayed resuscitation using CPB.[31] Instead of trying to restart circulation in vain, preservation was proposed as the initial intervention that would buy time for transport and damage control surgical repair of injuries. After an anatomical deficit in the circulation is repaired, delayed resuscitation could then be pursued using CPB.

The key component of the emergency preservation and resuscitation (EPR) method, as it was recently renamed, is imposing a state of deep hypothermia. Hypothermia is induced by a rapid retrograde infusion of a cold solution into the arterial circulation. This allows temporary slowing of metabolic processes. Deep hypothermia has been used in rather preventive than resuscitative fashion with great success in cardiac surgery to create bloodless field and allow to repair both congenital and acquired cardiac diseases (Figure 2).[32,33]



The EPR method builds upon the concept of deep hypothermic circulatory arrest (DHCA) used in cardiac surgery to repair congenital defects of the heart in infants, or acquired pathologies of the aorta in adults. Deep hypothermia, induced by CPB-assisted cooling, allows decreasing the metabolic demands of the organism to a level that enables to sustain a significant period of time without a circulation. Surgical procedure could then be accomplished with limited neurologic sequelae. In a traditional DHCA scenario, the protective cooling precedes the insult, i.e. a period of no-flow under hypothermia. In contrast, a clinically-relevant EPR model should have 1) preexistence of insults at normal or near normal body temperatures, including trauma, hemorrhage, and CA; 2) trauma-disrupted circulatory system that makes fluid replacement and chest compression futile. Any technology that relies on intact circulatory system, such as CPB, cannot be relied on for induction of hypothermia or administration of drugs/fluids in selected cases. Because of its complexities, a step-by-step approach had been taken in the establishment of EPR models.

1.4. Development of EPR Experimental Models

By the end of 20th century, a large series of experiments, mostly conducted at the Safar Center for Resuscitation Research, demonstrated that EPR is feasible in a clinical

setting, and with the advent of parallel efforts, EPR may eventually revolutionize the resuscitation of traumatic CA victims.[23]

1.4.1. 1980s: Feasibility of organism preservation after hemorrhagic shock

In the late 1980s, Tisherman *et al* conducted a ground-breaking series of EPR experiments. The preexisting insults were 30 or 60 min of hemorrhagic shock (HS) at a mean arterial pressure (MAP) 30 or 40 mmHg. Hypothermic preservation was induced by closed-chest CPB with hemodilution by crystalloids. At the end, delayed resuscitation was performed with CPB. Profound cerebral hypothermia induced at the beginning of exsanguination CA improved neurologic outcome after 60–120 min of CA, compared with that with deep hypothermia.[34-37] This series established the essential premise for the EPR concept by showing that the preexisting HS did not obviate the chance for hypothermic preservation.

1.4.2. 1999-2002 Clinically relevant EPR model: without trauma

The second series took one step closer towards the ideal EPR model in that 1) EPR was initiated after CA, assuming that most victims would have CA when approached by paramedics, and people would be more willing to initiate EPR at this moment; 2) 2-5 min of CA was allowed to elapse, assuming the time that was required for cannulation; and 3) one-way flush was used, assuming the existence of the disrupted circulatory system would not allow CPB to function properly. The solutions were perfused into the aorta, and drained out from the right atrium via an external jugular catheter.

Typically, an EPR model had 3 phases: 1) Hemorrhage and CA phase: 5 min rapid exsanguination followed by 2-5 min CA; 2) Preservation phase: up to 3 h of preservation; and 3) Delayed resuscitation phase: initiated with 2 h of CPB for rewarming and return of

spontaneous circulation, followed by up to 96 h of intensive care. The final outcome was assessed at 72 or 96 h using an Overall Performance Categories (OPC, 1-5) and Neurologic deficit score (NDS). Histological Damage Scores (HDS) was used for a semi quantification of tissue injuries in 19 brain regions.

1.4.3. 2002-2006 Clinically-relevant EPR model: with trauma

The success of EPR paradigm in non-trauma models prompted an exploration of applicability of EPR in trauma settings.

Nozari et al. added trauma in the forms of thoracotomy, laparotomy, and splenic transection into the above EPR model.[38] Splenectomy was performed during hypothermic arrest. As expected, the coagulopathy due to hemodilution, hypothermia, and ischemia were greatly worsened by trauma, even with use of fresh donor blood during resuscitation. Nevertheless, 60 min of CA plus severe trauma could be reversed to intact survival in about 50% of the animals. In the rest, multiple organ failure (MOF) occurred, prohibiting an evaluation of neurologic functions. Neuropathological assessment showed that there was almost no definitive histological damage in the brains of all animals, suggesting that should the extracerebral injuries be ameliorated, that animals could recover neurological function.

Plasma exchange was shown previously to decrease the microangiopathy seen in some patients with sepsis and MOF. In a following study by Nozari et al., plasma exchange not only decreased the MOF seen after trauma and EPR, but also had improved neurologic outcomes after 2 h of CA.[39]

2004 and beyond: Development of a rodent model of EPR

The dog model was used from the beginning of the EPR experiments to maximize clinical relevance. However, certain limitations are pertinent to that model. First, there are

few molecular tools available for dogs. That limits the evaluation of impact of neurologic injury on the cellular and subcellular basis. Understanding molecular mechanism beyond ischemia-reperfusion injury would allow us to assess markers of reversibility and define specific molecular targets for future interventions. Secondly, the cost and labor-intensiveness of the experiment pose a severe obstacle to rapid screening of the drugs that would seem promising to provide additional brain and extracerebral tissues preservation beyond the effects of hypothermia itself.

Regarding this, we have decided to design a rat model of EPR that would eliminate the abovementioned drawbacks. The cornerstone of the successful establishment of the model was development of a miniaturized CPB machine. The device should be capable to provide adequate circulatory support along with oxygenation and rewarming, with minimal priming volume that would limit the need for a donor blood.

In the past, there were multiple attempts to develop a heart-lung machine for small animals; the first one used in cats as early as in 1937. Over the past sixty years, tremendous improvements have been achieved. Both pulsatile and non-pulsatile models of CPB have been described and tested on various large animals – dogs, cows, sheep, pigs and rabbits. Development of a rat CPB has been driven by reduction of costs and equipment needs. Many models tested perfusion on isolated organs, while only a few actually implemented CPB with organs in situ. During the history of a rat CPB development, only several papers reported successful weaning and separation of the study animal from bypass. Frequently utilized open-chest cannulation prevented long-term survival.

Recently, several centers reported successful establishment of a rat CPB, both pulsatile[40] and non-pulsatile.[41] The reported flows ranged between 13 ml/min to 65 ml/min. Since cardiac output of a rat was found to be between 160 and 200 ml/kg/min, any model that

does not match this flow is unlikely to represent a full CPB. For rats weighing 300-400 g that are commonly used, full flow CPB would be in the range of 60-70 ml/min. Unfortunately, many of those reports exist only in the form of an abstract, and full reports from those centers were reported so far.

The absence of commercially available standard CPB machine for rodents remains another limiting factor. Static priming volume even of the smallest pediatric oxygenators ranges around 40 ml, approximately twice the total blood volume of a rat. Transfusion among rats is not a problem of compatibility, but the less overt transfusion-related injury may remain underappreciated. Donor blood for the circuit prime is still necessary, even for the custom-made devices now available, with priming volumes less than 10 ml.

In our center we have critically evaluated the existing literature on rodent CPB and identified several prototypes of rodent CPB that could be considered as a resuscitation tool for EPR. We were looking for a miniaturized device with a small priming volume that would provide oxygenation, full-flow circulation support and have capacities for rapid cooling and rewarming via an embedded heat exchanger. The volume of the cannulae and tubing should not significantly increase priming volume. Closed chest cannulation should be feasible to allow weaning from CPB and long-term survival to assess neurologic deficits in outcome models. After testing several devices, we chose a custom-designed CPB model used previously and tested for clinical efficacy under normothermic conditions.[41]

In our model, resuscitation from the DHCA and/or EPR was attempted using miniaturized CPB with small priming volume (~ 8-10 ml). The oxygenator is made from Plexiglas chamber that contains three-layer membrane made of microporous capillaries.[42] The circuit contains an open reservoir that includes ports for drug administration (Figure 2).

We have modified previously described cannulation sites and techniques that mirrored

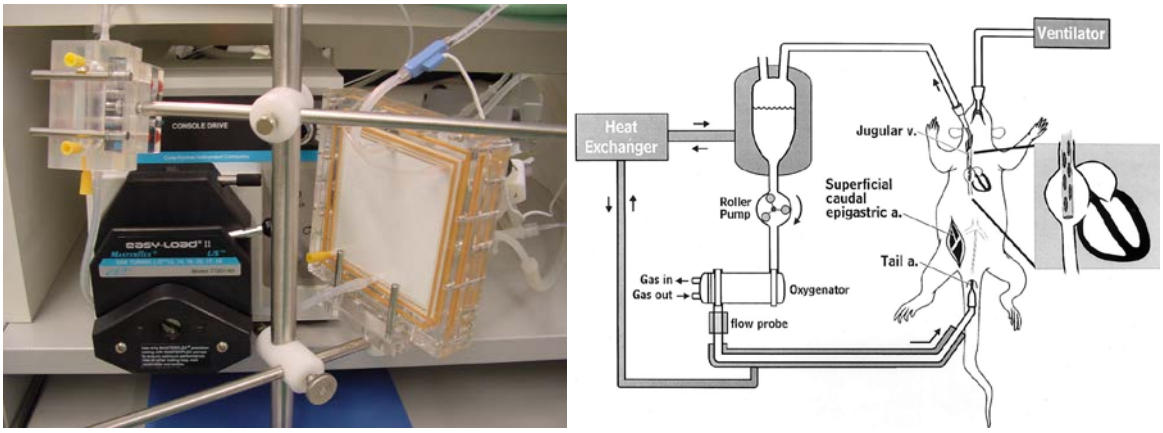


Figure 3. Custom-designed CPB setup. Left, reservoir; center, roller pump; right, oxygenator. Total priming volume is ~10 mL. The experimental setup in the right panel is modified from Grocott et al. (Perfusion 2001).

settings used in our dog EPR model and obviated the need for a vacuum-assisted drainage, a feature that would not be applicable in the EPR setting (Figure 4). Twenty gauge (G) cannula inserted in femoral artery serves as an arterial inlet, and modified multi-orifice 14G catheter advanced via jugular vein to the right atrium serves as venous drainage cannula, connected to the reservoir. Pilot experiments using this device with appropriate modifications showed that gas flow of 95%O_2/5%CO_2 mixture at 200 ml/min provided paO_2 > 300 mmHg after 60 min of full-flow CPB in non-ventilated normothermic rats.

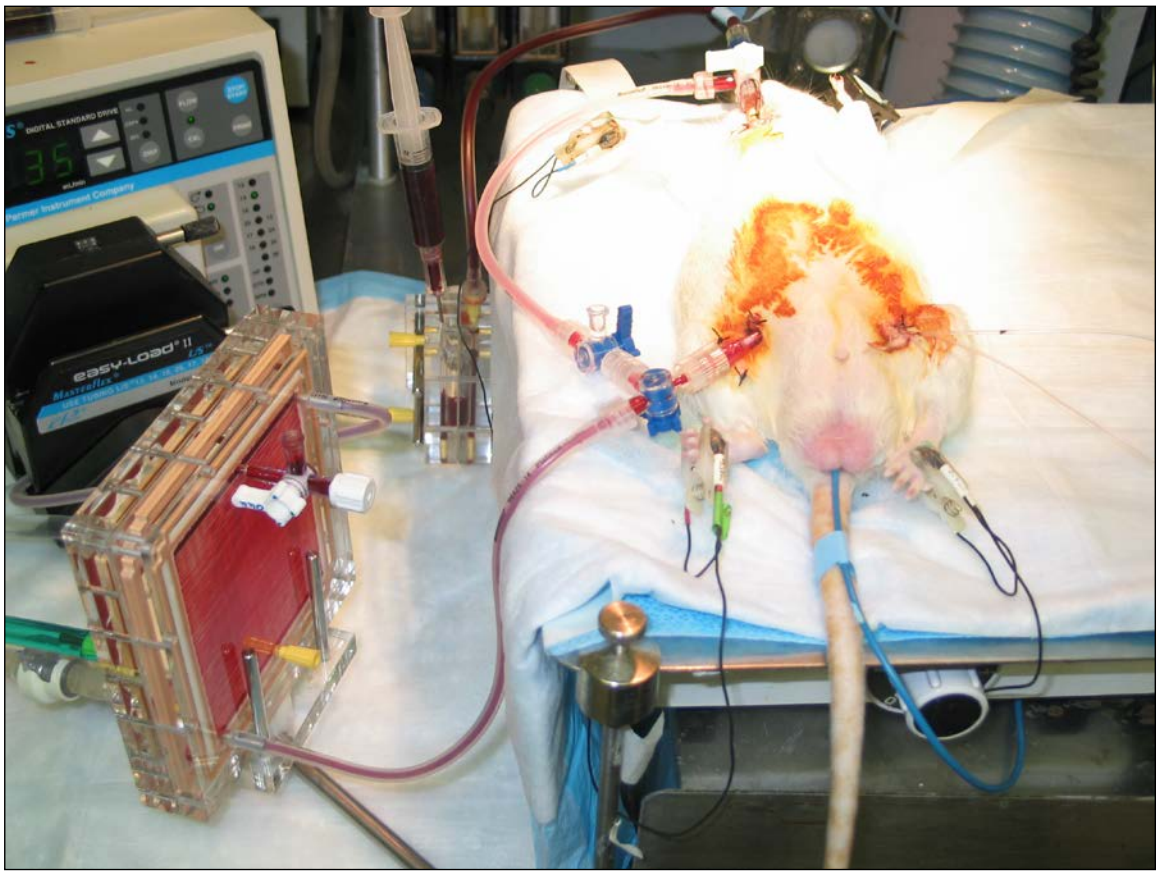


Figure 4. Experimental setup used at the experiments at the Safar Center for Resuscitation Research.

Previous studies using this device showed 75 min of normothermic CPB without any additional insult caused neurologic and neurocognitive impairment in rats.[41,43] However, these deficits were not associated with increased neuronal death in the hippocampus compared to controls.[43] CPB also triggers mesenteric endothelial dysfunction,[44] and acute lung injury[45] as part of systemic inflammatory response syndrome. Mechanical ventilation itself leads to endothelial damage, especially on top of ischemia-reperfusion injury. These observations are in accordance with the effects of CPB seen in humans. While the leading cause of the neurologic injury associated with clinically used CPB is microembolism, this may not be the case in rats. The cause of the abovementioned injuries remains to be elucidated. While our group was focused on using the CPB as a resuscitation tool rather than

study the effect of CPB itself, we acknowledged these limitations and hypothesized that the rather limited adverse effects of the use of the CPB would be overshadowed by the abnormalities associated with HS and prolonged CA.

In pilot studies we were able to demonstrate that the custom-designed CPB machine is capable of providing excellent oxygenation and heat exchange allowing the control of the body temperature during the experiment. Additional methods of cooling and rewarming (heating pad, forced-air) were also fine-tuned.

In the pilot studies designed to mirror dog studies, we hypothesized that 30 minutes EPR would be achievable in rats. We also tested the hypothesis that Plasma-Lyte A pH 7.4 (PL group) would be a more favorable flush solution than normal saline (NS group).

HS was induced with rapid exsanguination (12.5 ml) over 5 min, followed by KCl-induced CA. After 2 min of no-flow, cooling was initiated with ice-cold flush and surface cooling. Target temperature was set to 10 °C. After 30 min of EPR, reperfusion and re-warming were achieved via CPB over 60 min. Rats were extubated 2 hours later. The study showed that the different **pH of the flush solutions did not affect the pH of the animal** at 5 min resuscitation time (RT) (PL, 6.87±0.04 vs. NS 6.88±0.07, P=0.75), suggesting a dominating effect of the insult over the type of flush solution inducing hypothermia. Four out of seven rats survived to 24 h at both groups. Favorable outcome (OPC 1) with minimal neurologic impairment on clinical assessment (NDS) were achieved in both groups, with better results in the PL group (4/7 rats) vs. NS group (2/7 rats). Microscopic alterations within the brain sections were minimal to non-existent. Animals with normal saline flush showed more severe heart, lung and kidney injury.

To our knowledge, this was the first description of CPB in a rat that included a complex paradigm consisting of HS, CA and hypothermia, within the concept of EPR.[46]

1.5. Therapeutic Windows for initiation of EPR

It is well established that the durations of CA prior to initiation of resuscitation efforts and ultimately achieving ROSC is linked to survival and neurological deficits. A similar hypothesis was tested in the EPR model. Two studies had been conducted to explore the extent of preexisting injuries on efficacy of EPR.

CA durations

In a 30 min (total) CA model, initiation of 2 °C normal saline flush was delayed by 2, 5 or 8 min from the onset of CA. All animals received 100 ml/kg normal saline flushed into the thoracic aorta via a balloon catheter over 4 min. Delays in flushes did not change the efficacy of brain cooling. When cooling was delayed by 2 or 5 min, all 12 dogs regained consciousness after delayed resuscitation. In contrast, those with 8 min delay remained comatose and severely disabled.[47]

1.6. The Development of Hypothermic Approaches for EPR

Hypothermia so far is the most reliable and potent approach for preservation during CA. Behringer *et al* had conducted a series of experiments that systemically explored the potential efficacy of hypothermic approach.[47-50] These experiments documented that there is a clear linear relationship between brain temperatures and effective preservation durations (r=0.97). For 20 min preservation, cooling to brain temperature of 34 °C is needed; for 30 min, 28 °C should be reached; for 60 min, 15 °C appeared sufficient; and for 90 min, 10 °C probably should be targeted. The efficacy of ice-cold saline for cooling, however, decreased dramatically as the brain temperature decreased. For example, from 37 to 15 °C, 1 ml/kg ice-cold saline reduced brain temperature by 0.16 °C in average; from 15 to 10 °C, however, an identical volume of cold fluid showed only 10% efficiency (0.018 °C/ml/kg). When Tty was

cooled to 7 °C, the effective preservation time appeared to be 2-3 h, but the requirement of large amount of fluid limits its application on the field.

The method of cooling underwent considerable changes. The initial physiologic-based approach was based on the assumption that the “critical organs”, i.e. those suffering from the lack of oxygen most, needed to be cooled first. A special balloon catheter was developed to be advanced via the femoral artery to the descending aorta. When the position of the tip of the catheter was ascertained, a balloon occluding the distal flow was inflated, and the ice-cold flush through the internal lumen of the catheter was initiated, targeting brain and the heart. After the initial cooling, occluding balloon was deflated and the catheter was slightly withdrawn to allow cooling of the gut and lower extremities. While the catheter needed to be long and thin, it produced a significant resistance to the flow. In the later experiments, we realized that shorter and thicker catheter that would be positioned in the femoral artery would allow similar flow and cooling rates while providing simultaneous preservation for all organs at once.

Alam et al.[51] studied the impacts of cooling rates on outcome in a large blood vessel laceration model in pigs. Thirty min after vascular injuries, cooling with CPB was initiated to achieve a pharyngeal temperature of 10 °C at an average speed of 0.5, 0.9, and 1.35 °C/min. The CA lasted for 60 min, followed by rewarming and delayed resuscitation. Six weeks later, the survival rates were 37.5%, 62.5%, and 87.5% respectively, supporting the notion that the faster the cooling, the more effective it would be. However, it is doubtful that cooling at even faster rate (>2 °C/min) would further improve outcome. We found that one-way flush with ice-cold saline induced faster cooling than recirculation with CPB. At ~1.2 L/min with one-way flush, the cooling speed (to 10 °C) was about 2.5 °C/min.[50] Surprisingly, cooling with CPB (recirculation) appeared to have better neurological outcome.[52] Although there were

other factors that may be responsible for the tangible differences in outcomes, some transplantation researchers believed flush solution at very low temperatures can be detrimental for organ preservation.[53,54] It remains unclear what is the optimal cooling rate for induction of hypothermic EPR.

Rewarming from profound hypothermic EPR at graded speeds also significantly influenced the outcome. Using CPB, Alam et al. induced 10 °C hypothermic CA in their pig vascular injury model.[55] When pigs were rewarmed at a technically-feasible fastest rate (~ 0.52-0.8 °C/min depending on core temperatures), the 6 week survival rate was only 30%, in contrast to 90% survival with rewarming at 0.5 °C/min. Surprisingly, slow rewarming at 0.25 °C/min also had worse outcome (6 week survival rate 50%). In our laboratory, we did not set a target rewarming rate. Instead, the CPB water bath was set 5 °C higher than the core blood temperatures. Usually in 45-60 min the dogs would be rewarmed to 34 °C from deep hypothermic levels ~ 10-18 °C. This approach with an average rate of rewarming 0.4-0.53 °C/min may be more appropriate.

1.7. The Exploration of Pharmacological Adjuncts to Hypothermia

The use of pharmacologic adjuncts to augment the effects of hypothermic preservation is a highly appealing concept that could have remarkable implications. First, drugs can be easily delivered into the circulation; second, the small amounts that are usually clinically used could be made readily available for the use on the field in both military and civilian pre-hospital settings. The purported value of small-volume pharmacologic adjuncts becomes apparent when compared to exclusively hypothermic approach that relies on aortic cannulation, and requires a large quantity of cold saline.

Based on the evidence from a scientific literature and our consultants' experiments, we had tested the effects of **14 different drugs**. We used a model with rapid exsanguination CA followed by limited-volume flush (25 ml/kg) at ambient temperature, which achieved only mild cerebral hypothermia. The total duration of CA was 20 min. In controls, saline flush started at 2 min of CA achieved survival with brain damage. In groups of 3 to 6 experiments per drug, various doses were flushed into the aortic arch via an intra-aortic balloon catheter, and in some experiments, additional intravenous medication was given during reperfusion with CPB. The drugs tested could be divided into the following **six mechanistic strategies**:

1) delaying energy failure – adenosine, thiopental, thiopental plus phenytoine and fructose biphosphate;

2) protecting cell membrane integrity – MK-801 and nimodipine;

3) preventing structural degradation – cycloheximide;

4) regulating protein synthesis – Ca^{2+} calmodulin antagonist W-7;

5) preventing reoxygenation injuries – tempol; and

6) preserving mitochondria – cyclosporine A.

Unfortunately, none of the 14 tested therapeutical adjuncts granted a breakthrough effect, except the antioxidant tempol.[56] Tempol is available and inexpensive and penetrates the blood–brain barrier (BBB), but it is not approved for a clinical use by the U.S. Food and Drug administration. All eight dogs that received 150–300 mg/kg tempol via the aortic arch flush 2 min after CA achieved OPC 1 or 2 (normal or near normal), whereas none of the eight control animals achieved consciousness. Of concern, however, is that histological damage was not significantly mitigated by tempol.[56]

The goal of that series was to screen for a breakthrough drug. Unfortunately, a breakthrough effect was not apparent with any of the tested compounds. However, the study groups were limited in size and it cannot be ruled out that certain valuable properties of individual drugs for preservation could have been missed. Synergistic effects of various drugs were not explored either. In addition, it was not tested if any of the above drugs would provide significant effects at lower temperatures.

1.8. The Exploration of Novel Preservation Solutions

In the history of organ preservation for transplantation, preservation solutions used during harvesting of organs played a revolutionary role. However, in our pilot experiments, various solutions seemed to offer only marginal effects.

Tisherman et al explored the effects of University of Wisconsin (UW) solution on protection both in deep and profound hypothermia. Unfortunately, no benefit of using this solution instead of normal saline for flush on neurologic outcome was observed.

In a 30 min CA model, neither 5-25% albumin nor Unisol (Organ Recovery Systems Inc., Pittsburgh, PA) improved outcome. A combination of polynitroxylated albumin (Synzyme, Irving, CA) and tempol significantly reduced neurological and histological outcome compared to Unisol. In a 120 min CA model, hypothermia was induced with Normosol, followed by Unisol-I (Intracellular)+Tempol to fill the circulation during the preservation phase. Upon start of delayed reperfusion, Unisol-E (Extracellular) was used to replace Unisol-I. Using this strategy, five out of six dogs regained consciousness, compared to three out of six dogs in the group that used normal saline for preservation.[47]

In a closely related field of cardiac surgery, Aoki et al. found that intermittent flush of UW solution via the carotid artery over 2 h of a profound hypothermic (15 °C) CA in piglets

improved recovery of cerebral blood flow and ATP during early reperfusion, compare to the saline flush group.[57] In the following outcome study, however, the piglets that received 50 ml/kg UW solution flush via the carotid artery, was associated with similar OPC and NDS on day 5, but had worse histological deficit, compared to the control that did not receive any flush during CA.[58] Robbins et al. found that oxygenated "cerebroplegia" solution, which contained 2.5% glucose, 12.5% mannitol, 22mEq sodium bicarbonate, 25 mEq/L lidocaine, 0.5ug/L nitroglycerin, and 5 mg/L calcium chloride, flushed intermittently via the carotid artery during hypothermic CA substantially delayed brain energy depletion.[59] There was no outcome study published.

More recently, Taylor et al. developed an asanguineous solution for a whole-body perfusion during profound hypothermic CA. The choice of the solution components were carefully selected based on the then-current evidence, and satisfactory outcome after 3 h CA was achieved. However, it is not clear if the solution has any specific brain preservation properties, since the main problems observed in the controls but absent in the treatment group were cardiac or peripheral nervous injuries/dysfunctions.[60]

1.9. Mechanistic Studies

The most vulnerable organ during prolonged CA, with or without hypothermia, is the brain. When the whole body was cooled to ~10 °C for preservation of up to 3 h of CA, lethal extra-cerebral organ injuries were extremely rare using our delayed resuscitation protocol. The mechanisms of the brain injuries are multifactorial. Even when the brain temperature was at 8 °C, the oxygen consumption is still around 11% of the baseline,[61] and ATP and creatine phosphate in the brain were depleted in 60-90 min under hypothermia (12-15 °C).[59] Given that 2 min of normothermic CA precedes the induction of hypothermia in a large animal EPR model, energy depletion would occur sooner and may play a major role in the brain injuries.

Tseng et al. reported that release of excitatory amino acids and nitric oxide were also important factors responsible for neuronal injuries during profound hypothermic CA.[62]

Jenkins et al. in our center have focused on the degradation of proteins in the brain. The preliminary results showed that 30 min at 37 or 10 °C after decapitation caused only minimal protein degradations as assessed with 2D gel electrophoresis. Although the sensitivity of the technique could be in question, this finding is consistent with Fountoulakis's report in which moderate protein degradations were not detected until 24 h after death.[63] The ongoing studies of reperfusion phase are expected to reveal more profound changes in protein degradations. In 1960s, White et al. preserved the dog brains at 2 °C for hours to days. After recirculation and rewarming, EEG signals, pupil light reflex and rhythmic gasping were observed in the heads that were preserved for 4 h or shorter. However, these neurological functions eventually disappeared as reperfusion at 34 °C went beyond 6 h.[64] It was speculated that "reperfusion injuries" was the key that hindered successful reanimation of the preserved brain.

1.10. Other Possible Applications of EPR

In a broad sense, EPR can be viewed as a strategy that bridges the challenged vital organisms over a life-incompatible insult. Besides traumatic CA, other life-incompatible insults include extreme temperatures, chemical and biological toxicities, etc. When the milieu is improved by plasma exchange, administration of antidotes or the anatomic structure is corrected by surgery, delayed resuscitation can be started.

A large animal model of normovolemic VF CA has been established recently to simulate a scenario in which conventional CPR for prolonged CA appears to be futile.[65]A combination of EPR with limited flow produced by CPR seems to yield the best outcome.[66]

1.11. Alternative approaches to induce hypothermia

Future studies focused at better organ preservation are currently pursued in many centers. So far, diverse drugs such as PDE-4 inhibitor,[67] xenon,[68] cannabionoid-receptor agonists[69] or anesthetic agents[70] have shown promising results in terms of ameliorating CPB-induced injury or ischemic injury, respectively. Other promising drugs, as delta-opioid agonists[71] or hibernation-induction triggers,[72] emerge on the horizon. While several drugs were able to produce a variable degree of hypothermia, none has so far achieved to do with a rate comparable to the one-way flush or CPB assisted cooling.

Most recently, Blackstone, Roth et al. successfully induced a suspended animation state including hypothermia in mice inhaling hydrogen sulfide.[73] After discontinuing the hydrogen sulfide, mice spontaneously rewarmed. However, the same effect could not be replicated in higher species.[74,75] In contrast, a recent report indicated that hydrogen sulfide could be protective in HS in pigs, but this effect was not mediated by induction of hypothermia.[76]

1.12. The future of EPR – the launch of a clinical trial

While spontaneous hypothermia in trauma victims is associated with poor outcome, induction of therapeutical hypothermia could be beneficial. The potential of induced hypothermia and the purported mechanisms and caveats associated with this approach have been recently discussed at the workshop organized by the National Heart, Lung, and Blood Institute and the US Army Medical Research and Material Command as a forum for exchange of ideas among experts from diverse fields. The specific workshop goals were to (1) identify state-of-the-art and needs in knowledge of biology of hypothermia and hemostasis in the setting of significant traumatic injury; (2) provide an interdisciplinary forum to enhance knowledge regarding early detection of traumatic shock and monitoring of the level and

effect of controlled hypothermia in severe trauma settings; and (3) identify future research directions of the role of therapeutic-oriented hypothermia and hemostasis in trauma with severe blood loss. The report of the HYPOSTAT workshop has just been published.[77] It rightfully highlights the potential of the EPR method in the care ExCA victims and identifies future fields of experimental and clinical research.

Given this extensive pre-clinical background in clinically relevant large animal models in our laboratory and several others in the USA and Europe, the first multi-center clinical feasibility trial of EPR in humans was approved by the Food and Drug Administration, funded by the Department of Defense (NCT01042015; PI: Tisherman; Co-PI: Drabek; http://clinicaltrials.gov/ct2/show/NCT01042015?term=EPR&rank=2). The training sessions are currently underway and we expect to start enrolling patients in early 2013.

2. Aims and Hypotheses

We envisioned that a successful establishment of the technically demanding rodent EPR model should facilitate application of molecular tools to study effects of DHCA and/or EPR and reperfusion on neuronal death and organ injury, with relevance to cardiac surgery and organ preservation, within the frame of transplantation medicine. Rodent model of EPR should promote a rapid screening of pharmacological adjuncts to augment or replace hypothermic preservation. Pharmacologic strategies and hemodynamic management will need to be optimized to prevent further damage of the tissues during the reperfusion phase, to allow for a long-term favorable outcome.

In the following series of large and small animal studies we have sequentially pursued several hypotheses that fit within the overall goals of the aforementioned resuscitation research.

2.1. Study I – Prolonged hemorrhagic shock in dogs

EPR was shown to be effective after rapid exsanguination hemorrhage with limited duration of CA prior to the preservation phase. We hypothesized that EPR will be effective after prolonged hemorrhage resulting in CA, while conventional resuscitation will fail to achieve good outcome.

2.2. Study II – "Cold energy" in prolonged EPR in dogs

Drugs generally failed to improve outcome in an EPR model using rapid hemorrhage limited flush resulting in mild-moderate hypothermia, and relatively short preservation phase. We hypothesized that addition of energy substrates (oxygen and/or glucose) during an induction of preservation phase will be beneficial in a prolonged deep hypothermic CA.

2.3. Study III – Rat EPR feasibility study

In prior studies of EPR we used a dog model with prolonged intensive care to maximize clinical relevance. Because of the lack of molecular tools available for use in dogs, development of a rat EPR model would enable study of the molecular mechanisms of neuronal injury in ischemia-reperfusion injury from exsanguination CA. Understanding the impact of deep hypothermia and reperfusion on these cascades would allow us to define specific targets for future interventions and to assess markers of reversibility. The rodent model would also allow economical screening of pharmacological strategies for EPR. We hypothesized that survival from rapid exsanguination CA followed by 20 min of deep hypothermic EPR including CPB-assisted resuscitation is achievable and that survival with normothermic EPR and CPB-assisted resuscitation is unlikely.

2.4. Study IV – 60 vs. 75 min EPR in rats

The outcome of EPR strategy in dogs was associated with the depth of hypothermia and durations of no-flow. In rats, we hypothesized that extending durations of no-flow preservation state will result mortality and morbidity in a time-dependent manner.

2.5. Study V – Nitration and ribosylation in EPR in rats

Extended EPR in rats is associated with substantial mortality and impaired neurological outcome in survivors. We hypothesized that extended EPR would be associated with activation of two potential secondary injury cascades in brain as reflected by protein nitration and poly (ADP-ribose) polymerase (PARP) activation.

2.6. Study VI – DADLE in EPR in rats

Delta-opioid receptor agonists were shown to induce hibernation in naturally hibernating species, and were protective against ischemia-reperfusion injury in multiple

organs. Our model with extended duration of EPR showed MOF. Given certain similarities between hibernation and EPR, we hypothesized that addition of a delta-opioid agonist would confer additional protection in individual organs, and improve overall outcome.

2.7. Study VII – Deep vs. moderate hypothermia and minocycline in EPR in rats

The rapid exsanguination and a limited duration of normothermic CA prior to the initiation of induction of deep hypothermia yields favorable outcome. Extending the period of normothermic CA prior to preservation and different levels of intra-arrest hypothermia may result in neurologic deficits and neuroinflammation. Minocycline was shown to be beneficial in neuroinflammatory diseases as well as in ischemia-reperfusion syndromes. We hypothesized that deeper levels of hypothermia and minocycline will attenuate neuronal death and improve outcome after exsanguination CA with prolonged normothermic CA.

2.8. Study VIII – Blood-brain barrier integrity in rats

The effect of drugs in our EPR model has been limited. One of the explanations for the lack of effect could be a limited transport of the tested agents across the blood-brain barrier (BBB). The permeability of the BBB in our model is unknown. We hypothesized that BBB will not be disrupted even in models that are associated with poor outcome.

2.9. Study IX – Clodronate-induced depletion of microglia

Hippocampal neuronal damage was similar after EPR at moderate (28 °C) vs. deep (21 °C) intra-arrest hypothermia. However, neurologic outcome was improved with deep hypothermia. This was associated with attenuated microgliosis. Activated microglia could be an independent factor aggravating neurologic injury. Clodronate is a macrophage-

depleting drug. Given the links between macrophages and microglia, we hypothesized that intracerebrally injected clodronate could deplete microglia, and provide us with a model that would elucidate the role of microglia in post-CA neuroinflammation.

2.10. Study X – Cytokines in multiple brain regions after EPR in rats

We showed previously that prolonged cardiac arrest (CA) produces neuronal death with microglial proliferation. Microglial proliferation, but not neuronal death, was attenuated by deeper hypothermia. Microglia are a major source of cytokines. In this study, we tested the hypotheses that 1) CA will result in highly specific regional- and temporal-increases in brain tissue cytokine levels; and 2) the increases in cytokine levels will be attenuated by deeper hypothermia.

3. Research Part

This section contains the excerpts of the experiments that were outlined in the previous section Aims and Hypotheses, and were tested in individual studies. The sections that are most pertinent to neuroscience are highlighted here, along with additional information from pilot studies that may not be included in the published work. For full description of the experiments, please refer to the published papers.

3.1. Study I – Prolonged hemorrhagic shock in dogs

Wu X, **Drabek T**, Kochanek PM, Henchir J, Stezoski SW, Stezoski J, Garman RV, Tisherman SA
Induction of Profound Hypothermia for Emergency Preservation and Resuscitation Allows Intact Survival From Cardiac Arrest Resulting from Prolonged Lethal Hemorrhage and Trauma in Dogs
Circulation 2006 Apr 25;113(16):1974-82.

3.1.1. Summary

While all initial experiments used rapid HS (5 min), it was not clear if EPR would be effective if induced in the setting of a prolonged hemorrhage that would result in a delayed CA. We used extended volume-controlled HS with limited fluid resuscitation that resulted in CA after 1.5 to 2.5 h. After 2 min of CA, either conventional CPR or EPR was initiated. Conventional CPR included chest compressions, pressure-controlled ventilation, resuscitative medications and vigorous volume replacement for 60 min, followed by CPB-assisted resuscitation. In contrast, ice-cold flush in the EPR paradigm resulted in deep hypothermia (13 °C), maintained for 60 min, and followed by CPB-assisted resuscitation. While all dogs treated with CPR died before 16 h from MOF, all but one dog treated with EPR survived to >72 h. Surprisingly, to produce intact neurological outcome in this model, it was necessary to extend mild post-resuscitative hypothermia (34 °C) for 36 h -- the group that received post-resuscitation mild hypothermia for only 12 h later deteriorated. Thus, prolonged HS prior to

CA did not preclude the possibility of survival with intact neurological outcome with EPR treatment.

3.1.2. Background

Conventional resuscitation, including open cardiac massage, is often unsuccessful after exsanguination CA in trauma victims,[23,25,26] particularly when it results from prolonged HS. A novel approach is needed.

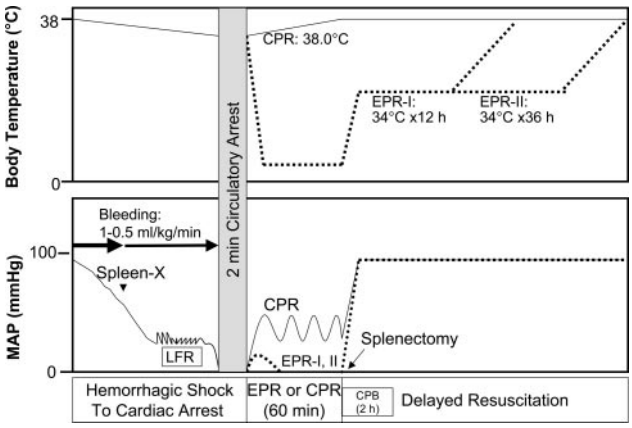
Previously, we reported the success of inducing EPR with profound hypothermia in animal models.[78] The goal of EPR is to "buy time" for transport and resuscitative surgery during pulselessness, followed by delayed resuscitation. EPR of up to 2 hours was induced with a rapid aortic flush with ice-cold (2°C) saline to induce profound hypothermia, followed by delayed resuscitation with CPB.[39,50] We used EPR to achieve intact survival of dogs after rapid hemorrhage (over 5 minutes) and CA.[39,48-50,56] We speculated that prolonged HS before CA may decrease the efficacy of EPR. Longer durations of HS may cause severe tissue acidosis and exhaust reserves. Although the CNS may be damaged only minimally during HS,[79] superimposing transient, normothermic CA and a period of EPR on prolonged HS may substantially complicate efforts to save trauma victims.

In this study, we designed a model relevant to military and civilian trauma, characterized by rate-controlled bleeding,[80,81] trauma (laparotomy and spleen transection), limited (hypotensive) fluid resuscitation during HS,[28] and CA. Allowing volume depletion and circulatory decompensation to cause CA was intended to create an insult nonsalvageable with conventional CPR. We hypothesized that EPR could allow survival with good neurological outcomes in this setting.

3.1.3. Materials and methods:

3.1.3.1. **Experimental Design**

The animal model (Figure 5) included 3 phases: (1) the HS and CA phases: bleeding was continued until CA; (2) the CPR/EPR phase: 2 minutes after CA, dogs were treated with conventional CPR or EPR; and (3) the delayed resuscitation phase including 2-hour CPB, and 72- to 96-hour intensive care. Trauma included spleen transection during HS. To simulate life-saving surgery, a splenectomy was performed during resuscitation.



Animals were assigned to 1 of 3 groups at the end of the HS and CA phase: (1) The CPR group was resuscitated with conventional CPR as per Advanced Cardiac Life Support guidelines plus aggressive fluid resuscitation; (2) the EPR-I group was resuscitated with an arterial flush with 20 L of ice-cold saline, followed by delayed resuscitation with CPB, 12 hours of post-EPR mild systemic hypothermia (34 °C), and euthanization 60 hours after initiation of rewarming (resuscitation time [RT], 72 hours); and (3) the EPR-II group was identical to the EPR-I group, except that the post-EPR mild hypothermia lasted for 36 hours and was reversed by slower rewarming, and euthanizing occurred 60 hours after rewarming (RT, 96 hours). The latter group was added to the study after 5 experiments each had been performed in the other groups. For the first time in our extensive experience with dog CA experiments, several animals in the EPR-I group had neurological deterioration with seizures. We hypothesized that this was due to delayed neuronal injury, which might be ameliorated by prolonging the post-resuscitation mild hypothermia.

3.1.3.2. **Anesthesia and Preparation**

Custom-bred, male hunting dogs (n=21; 21.1 to 26.6 kg) (Rotz Kennel, Shippensburg, Pa) were housed for at least 3 days before the experiment. Dogs were fasted with free access to water for 12 hours. Ketamine 10 mg/kg and atropine 0.4 mg were administered intramuscularly.

After anesthesia induction with 4% halothane by face mask, endotracheal intubation (internal diameter, 8 to 9 mm) was performed. Continuous anesthesia was provided with =1% halothane, titrated during preparation with O2:N2O, 50%:50%. Controlled ventilation (Piston Ventilator Model 613, Harvard Apparatus, South Natick, MA) was initiated with a tidal volume of 12 to 15 mL/kg, a positive end-expiratory pressure of 2 cm H2O, and a frequency of 20 to 25/min, titrated to maintain a PaCO2 of 35 to 45 torr. ECG lead II was continually monitored. A cannula (18 G) was inserted into a peripheral vein, and fluid infusion (D5W/0.45% NaCl at 4 mL/kg/h) was started. A Foley catheter was placed. Sterile cutdowns were performed in both groins and the right side of the neck. Temperature probes were inserted for measuring rectal, esophageal, and both tympanic membrane temperatures (Tty). A PE-90 catheter was inserted into the left femoral artery for blood pressure monitoring and blood sampling. A pulmonary artery catheter (7.5F) was inserted via the left femoral vein to monitor pressure, cardiac output, and core temperature (Tpa). A CPB arterial cannula (7 or 9 gauge) was inserted into the right femoral artery. A multiple-hole cannula (16F) was inserted into the inferior vena cava via the right femoral vein for blood withdrawal. Another multiple-hole cannula (19F) was inserted 10 cm into the right external jugular vein. This cannula was advanced into the right atrium when MAP was 30 mm Hg during HS. The cannulas were flushed intermittently with dilute, heparinized saline. The CPB system, including an oxygenator (Medtronic, Grand Rapids, MI) and centrifugal pump (Biomedicus, Eden Prairie, MN), was primed with shed blood (30 mL/kg) and Plasma-Lyte A (Baxter, Deerfield, Ill). In the CPR group, 500 U of heparin was added to the solution.

After baseline measurements were taken, a midline laparotomy (15 cm) was performed and the spleen mobilized medially. The abdominal wound was temporarily closed. Halothane was transiently decreased, and return of spontaneous respiration was achieved. FiO2 was set at 0.25 with O2:N2O at 25%:75%. Halothane was titrated to ensure anesthesia with spontaneous breathing.

3.1.3.3. **Hemorrhagic Shock and Cardiac Arrest Phase**

All heating sources were stopped. At HS time 0 minutes, continuous venous blood withdrawal via the right femoral vein catheter was set at 1 mL/kg/min over 40 minutes. Withdrawn blood was anticoagulated with 0.125 mL/kg/min citrate delivered through a PE-60 catheter inside the femoral vein catheter lumen. The tip of the citrate catheter was 3 cm from the tip of the femoral vein catheter. At HS time 40 minutes, the spleen was transected, and the blood withdrawal rate was decreased to 0.5 mL/kg/min. Halothane was decreased to 0.5% when MAP was <50 mm Hg. When MAP reached <30 mm Hg, limited fluid resuscitation (simulating field resuscitation) was started with bolus infusions of lactated Ringer's solution (100 mL over 2 minutes), with a maximum volume of 500 mL. CA was defined as either an MAP<10 mm Hg and severe bradycardia (<20 bpm) or asystole or VF.

3.1.3.4. **CPR/EPR Phase**

Two minutes after CA, dogs were randomized into the CPR or EPR groups. In the CPR group, conventional Advanced Cardiac Life Support protocols were initiated. In brief, chest compressions with a mechanical thumper (Michigan Instruments, Grand Rapids, MI) were started at 60/min; the compressing distance was adjusted to generate a systolic blood pressure of 100 mm Hg. Ventilation with 100% O2 was provided at 12 breaths/min, with the peak airway pressure set at 40 cm H2O. Epinephrine (0.01 mg/kg IV) was administered every 5 minutes as needed for a maximum of 5 doses. After epinephrine administration, defibrillation

with 150 J was attempted if VF was present and then increased in increments of 50 J after 2 unsuccessful shocks. Sodium bicarbonate and CaCl2 were administered for base deficit >6 mmol/L and ionized calcium <1 mmol/L, respectively. At CPR 0 minutes, lactated Ringer's solution (1 L) was infused over 10 minutes, followed by infusion of shed blood (30 mL/kg) over 5 minutes. Up to 3 additional boluses of lactated Ringer's solution (250 mL over 15 minutes) were administered per Advanced Trauma Life Support recommendations.

In the EPR groups, the lungs were inflated with air to maintain an airway pressure of =10 cm H2O during EPR. An aortic flush of 20 L of 2°C saline via the right femoral arterial cannula was initiated at 1.6 L/min with use of a roller pump (Ardiem, Indiana, Pa). The flush solution was drained through the external jugular catheter. The dog was then covered with ice.

3.1.3.5. **Delayed Resuscitation Phase (RT 0 to 2 Hours)**

Sixty minutes after the onset of aortic flush or CPR, CPB was started.5 Just before CPB, additional heparin (1500 U) and sodium bicarbonate (2 mEq/kg) were injected into the circuit. Dogs were paralyzed with pancuronium. CPB was started at 100 mL/kg/min. Reinfusion of shed blood in the EPR groups was titrated to achieve a central venous pressure of 10 to 15 mm Hg. Repetitive doses of epinephrine (0.01 mg/kg) were given when necessary to increase MAP to 100 mm Hg. O2 flow through the CPB oxygenator was adjusted to keep the PaCO2 at 30 to 35 mm Hg. Ventilation at a rate of 8 to 10/min was resumed to prevent atelectasis. IV fluids were restarted at 100 mL/h. A base deficit of >6.0 mEq/L was corrected with sodium bicarbonate. CPB flow was reduced to 75 mL/kg/min at 60 minutes and to 50 mL/kg/min at 90 minutes. During CPB, activated clotting times were maintained at >300 seconds with heparin.

At RT 0 minutes, a splenectomy was performed, and the abdomen was packed with gauze to simulate the clinical management of a trauma victim with a ruptured spleen. An abdominal drainage catheter was placed through the abdominal wall. The abdominal wound was closed. Tpa in the CPR group was maintained at 38.0±0.5°C. Dogs in the EPR groups were rewarmed to 34 °C over 1 hour. Defibrillation was again attempted, when necessary, when the splenectomy was completed in the CPR group or when the Tpa reached 32°C in the EPR groups. CPB was stopped at 2 hours.

3.1.3.6. **Intensive Care Management (RT 2 to 24 Hours in the CPR and EPR-I Group or 48 Hours in the EPR-II Group)**

Neuromuscular blockade was maintained with intermittent doses of pancuronium (0.1 mg/kg). Sedation and analgesia were provided with N2O/O2 (50%:50%) plus IV boluses of morphine (0.1 to 0.3 mg/kg) and diazepam (0.1 to 0.2 mg/kg) to prevent signs of wakefulness, e.g. mydriasis. Severe hypertension (MAP>150 mm Hg) despite adequate analgesia was controlled with IV boluses of labetalol (0.25 to 0.5 mg/kg) or hydralazine (0.1 to 0.2 mg/kg). Hypotension (MAP<70 mm Hg) was treated by normalization of filling pressures by administration of lactated Ringer's solution and titrated norepinephrine. The dogs received cefazolin (250 mg IV) every 8 hours for infection prophylaxis. Intensive care unit (ICU) care, including mechanical ventilation, was provided for at least 24 hours in the CPR and EPR-I groups and for 48 hours in the EPR-II group to ensure an equivalent period of post-rewarming intensive care. At 20 hours, the abdominal packing was removed and the abdominal wall closed. In the CPR group, body temperature was maintained at 37.5°C to 38.5 °C throughout the experiment. In the EPR-I group, body temperature was maintained at 34 °C until RT 12 hours, which was followed by self-rewarming and, when needed, external heating with blankets and a heater (target rewarming rate, 1 °C/h) to 37.5 °C. In the EPR-II group, rewarming was delayed to 36 hours and was deliberately slower (0.3 °C/h).

3.1.3.7. **Outcome Evaluation**

Functional outcomes[50,82] were evaluated after discontinuing sedation according to overall performance categories (OPC, 1=normal [able to eat and walk]; 2=moderate disability [able to eat and sit but not stand]; 3=severe disability [responds to pain but unaware of the environment]; 4=coma [minimal response to pain; positive pupillary light reflex; running movements and opisthotonus common]; and 5=death) and Neurological Deficit Scores (NDS). The NDS is based on assessment of 5 facets of neurological function (level of consciousness, breathing pattern, cranial nerve function, sensory and motor function, and behavior), each with a maximum value of 20% (neurologic deficit score 0% to 10%=normal; 100%=brain death). Evaluations were agreed on by at least 2 team members. Because of the number of team members needed to conduct these experiments and the differences in observation time between groups, there was no practical way for the evaluations to be blinded. In previous experiments, interobserver agreement has been excellent. Results at 60 hours after initiation of rewarming were taken as the final measurements in each group. Blood samples were obtained at baseline and every 24 hours for cardiac (troponin I, creatine phosphokinase MB fraction), and liver (transaminases and bilirubin) enzymes. At 72 hours (EPR-I) or 96 hours (EPR-II), animals were re-anesthetized with ketamine and halothane. A left thoracotomy was performed. Perfusion-fixation of the brain was accomplished with aortic infusion of 4% paraformaldehyde. A gross necropsy was performed. The brain was removed ~ 1 to 2 hours after perfusion-fixation and retained in 10% neutral buffered formalin until dissection.

3.1.3.8. **Neuropathology**

Whole perfusion-fixed brains were divided into multiple coronal slices. Six coronal brain slices plus 3 transverse sections of the medulla oblongata and upper cervical cord were

selected for microscopic evaluation. These slices were taken at the following levels: (1) optic chiasm; (2) anterior thalamus; (3) posterior thalamus; (4) midbrain; (5) posterior portions of the occipital lobes; (6) middle of the cerebellum and underlying brain stem; and (7) medulla oblongata and upper cervical cord. Brain slices were processed for paraffin embedding, resulting in 20 tissue blocks per brain. Blocks were sectioned at 5 µm, and the sections were stained with hematoxylin/eosin (H&E) and Fluoro-Jade B (FJB).16 The examining neuropathologist (R.G.) was blinded to treatment. A total of 25 neuroanatomic regions were examined. Each region with damage on microscopic examination received a pathological grade ranging from 1+ (minimal) to 5+ (severe). Each affected region on each side of the brain received separate scores in H&E-stained and FJB–stained sections. In each region, scores for edema were multiplied by 1, and scores for neuronal degeneration were multiplied by 2. Edema was not scored on the FJB sections. Thus, the total possible scores for each region were 5X1 plus 5X2 (total, 15) for the H&E stain and 5X2 (total, 10) for the FJB stain. Total histological damage scores were determined by totaling these individual scores (i.e., for each region with each stain). The maximum score was 1250 ([15+10 maximum per region]X25 regionsX2 sides of the brain).

3.1.3.9. **Statistical Analysis**

Data are presented as mean±SD unless otherwise stated. A repeated-measures ANOVA was performed, followed by Bonferroni post hoc tests to identify differences in hemodynamic parameters, temperature, and neurological deficit and histological damage scores (with ranked data). ANOVA was performed for other physiological variables. The Mann-Whitney *U* test was used for the final neurological deficit score and the total histological damage score. The Fisher exact test was used to assess differences in OPC proportions (i.e., normal outcome [OPC 1] versus abnormal outcome) among groups. A probability value <0.05 was considered significant.

3.1.4. Results

3.1.4.1. Baseline

Baseline hemodynamics, hematological and biochemical parameters, and acid-base status were similar among groups.

3.1.4.2. Hemorrhagic Shock and Cardiac Arrest

The hemorrhage time before CA was 124±16 minutes and did not differ among groups (Study I Table 1). Samples of arterial blood gases and chemistries taken 1 minute after CA were markedly abnormal but did not differ among groups (Study I Table 1).

3.1.4.3. Resuscitation of the CPR Group

During chest compressions, MAP was maintained at >50 to 60 mm Hg (Study I, Figure 2). However, return of spontaneous circulation (ROSC) was not achieved by CPR in any dog.

During CPB, ROSC was achieved with defibrillation (mean total defibrillation energy, 157±181 J) in all dogs 15±16 minutes after initiation of CPB. However, substantial fluid losses from the rectum, orogastric tube, and intraperitoneal drain occurred during the resuscitation phase (Table 2; all $P<0.01$ versus the EPR groups). Progressive hypotension developed despite massive fluid resuscitation and vasoactive support. Lactate levels decreased transiently but increased sharply again until death (Figure 3; $P<0.01$ versus the EPR groups). Tty decreased slightly during CPR to ~36°C and then increased to 38°C with CPB (Figure 4).

3.1.4.4. Resuscitation of EPR Groups

The perfusion pressure during aortic flush was ~ 20 mm Hg, with no difference between the EPR groups (Study I, Figure 3). At the end of the aortic flush, Tty had decreased to similar levels, with little change during circulatory arrest (Study I, Figure 4).

EPR dogs were rewarmed to 34 °C within 1 hour by CPB. When Tpa reached 32°C, defibrillation yielded ROSC in all dogs at 30±12 minutes in the EPR-I group and at 32±23 minutes in the EPR-II group (*P*=NS). The total defibrillation energy required was 229±225 J in the EPR-I group and 264±326 J in the EPR-II group (*P*=NS).

At RT 12 hours, hematocrit was lower in the CPR group (19±9%) compared with the EPR-I (33±7%, *P*=0.006) and EPR-II (28±9%, *P*=0.16) groups. Final hematocrit values were 29±3% at RT 24 hours in the EPR-I group and 30±6% at RT 48 hours in the EPR-II group (*P*=NS versus the EPR-I group).

3.1.4.5. **Final Outcome of All Groups**

All CPR dogs died, with a median survival time of 14.7 hours (range, 11.5 to 16.5 hours; *P*<0.01 versus the EPR groups). In contrast, 6 of 7 EPR-I dogs survived to 72 hours; 1 died at RT 29.5 hours due to circulatory collapse. Similarly, in the EPR-II group, 6 of 7 dogs survived to 96 hours; 1 dog unexpectedly died at RT 66 hours. This dog was extubated at 48 hours and was in OPC 1. At necropsy, gastric contents were found inside the trachea and

	CPR	EPR-I	EPR-II
5 Dead	●●●● *	●	●
4 Coma		●	●
3 Severe Disability		●●●	
2 Moderate Disability		●	
1 Normal		●	●●●●● **

Figure 6. Overall performance categories (OPC) after prolonged hemorrhagic shock leading to cardiac arrest with resuscitation by CPR or EPR. *P<0.01, survival in the CPR group vs the EPR-I and EPR-II groups; **P<0.06, normal (OPC 1) vs abnormal (OPC 2 to 5) in the EPR-II vs the EPR-I group.

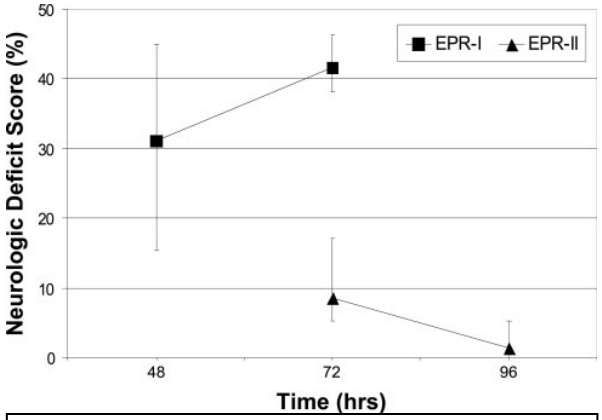


Figure 7. Neurological deficit scores after prolonged hemorrhagic shock leading to cardiac arrest with resuscitation by EPR, with or without prolonged resuscitative mild hypothermia. Median and interquartile ranges are shown. *P<0.09 for repeated-measures ANOVA; P<0.04 for final neurological deficit scores by the Mann-Whitney *U* test.

bronchus, suggesting aspiration.

Thirty-six hours after rewarming (RT 24 hours in the EPR-I group or RT 48 hours in the EPR-II group), 3 of 6 surviving dogs in the EPR-I group and 4 of 6 surviving dogs in the EPR-II group were in OPC 1 or 2. In the EPR-I group, however, 2 dogs developed recurrent generalized seizures 24 hours after extubation. Another had seizures shortly after extubation at RT 24 hours. Neurological function deteriorated in these animals. There was only 1 dog that recovered to OPC 1. In contrast, none of the EPR-II survivors exhibited seizures, and 5 of 6 survivors continued to improve and regained normal function (P=0.06 versus the EPR-I group; Figure 6). The NDS of the survivors in the EPR-II group was numerically better over time compared with the EPR-I group (P=0.09), although the final NDS were better (P=0.04; Figure 7).

3.1.4.6. Brain Histology

In the EPR-I group, the brains exhibited prominent, acute eosinophilic neuronal degeneration within the frontal, parietal, temporal, and occipital cortices (Figure 7). In the most severely affected regions, a laminar band of necrosis with associated neuropil spongiosis was evident. Marked to severe neuronal degeneration was present in the **caudate** region, with slightly less degeneration in the **putamen**. Within the caudate, degeneration was primarily present in the medium spiny neurons. The larger interneurons were relatively unscathed. In the hippocampus, moderate to marked neuronal degeneration was present primarily within the **CA1 region**, although degeneration often extended to the CA3 and CA4 regions. In the cerebellum of the EPR-I group, FJB staining revealed small numbers of degenerating **Purkinje neuron** cell bodies and greater numbers of dendrites. Degrees of neuronal degeneration were scored slightly higher in FJB–stained sections than in H&E-stained sections.

In contrast to the EPR-I group, the EPR-II group had only mild damage in the neocortex (Figure 8A; $P<0.05$ versus EPR-I in the frontal and temporal lobes). In the hippocampus of the EPR-II group, damage severity as assessed by FJB staining was also less (Figure 8B; $P=0.065$). Furthermore, FJB staining in the hippocampus of EPR-II group dogs was primarily restricted to neuronal processes within CA3 and CA4. Degenerative changes in the caudate and putamen of EPR-I and EPR-II groups were similar (Figure 8). Within the cerebellum of the EPR-II group, FJB staining revealed scattered Purkinje neurons with degenerative dendrites.

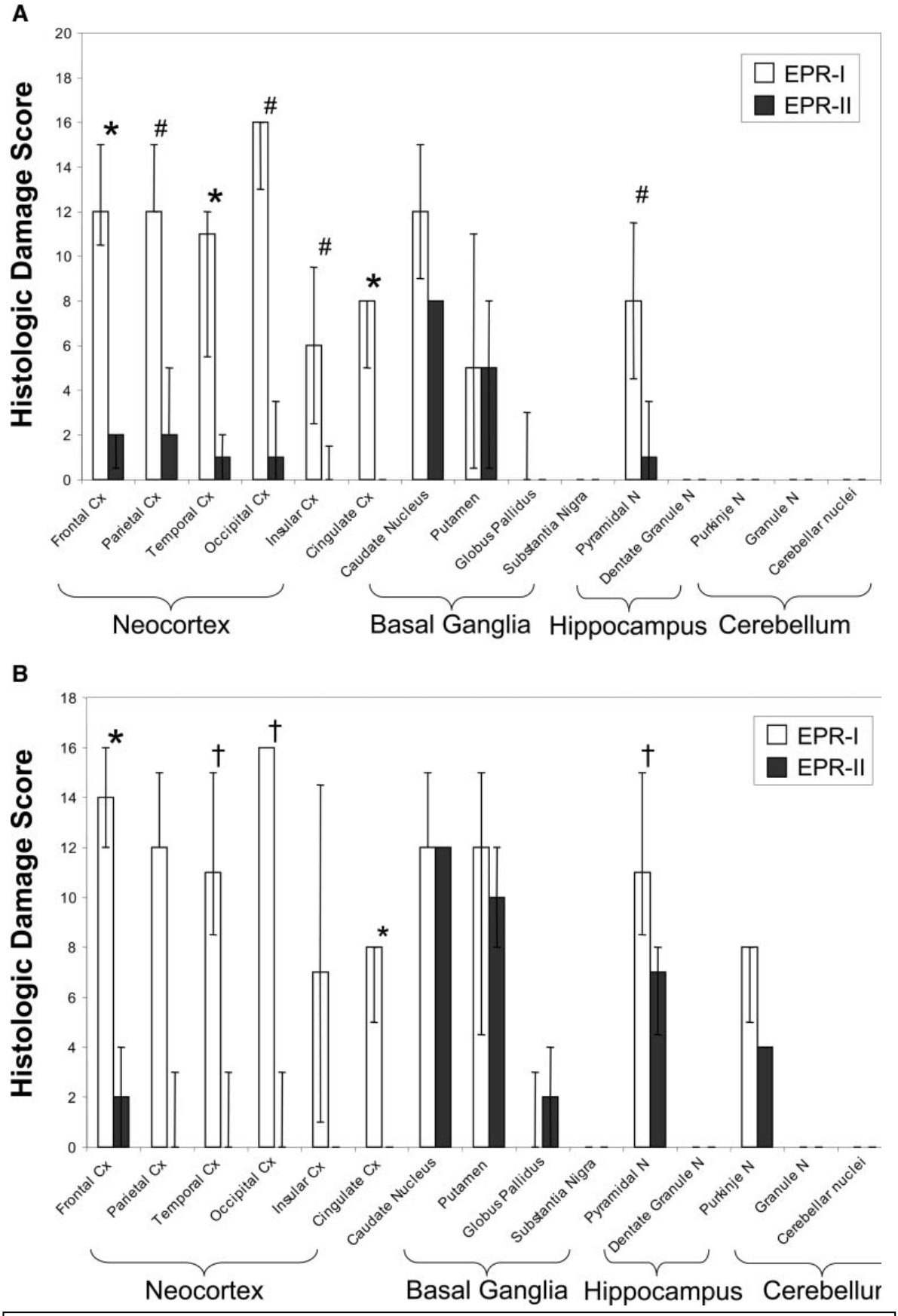


Figure 8. Brain histological damage scores (refer to text for details) after prolonged hemorrhagic shock leading to cardiac arrest with resuscitation by EPR, with or without prolonged postresuscitation mild hypothermia. Brain regions with no or minimal neuronal changes are not included. A, Hematoxylin/eosin staining; B, Fluoro-Jade B staining. Bars represent medians and interquartile ranges. Cx indicates cortex; N, neurons.
* $P<0.05$ for comparison between EPR-I and EPR-II; # $P=0.09$, † $P=0.07$.

Figure 8 includes only those brain regions that had greater than minimal degrees of degenerative changes. All other brain regions, i.e. the piriform cortex, entorhinal cortex, septal region, basal forebrain, anterior thalamus, posterior thalamus, amygdala, midbrain, pons, and medulla oblongata, were characterized by no or only minimal degrees of damage, with no differences between groups. By repeated-measures ANOVA for brain regions, the HDS were higher in the EPR-I group than in the EPR-II group (P=0.006). The total histological damage scores in the EPR-I group (226±111) were numerically higher than those in EPR-II (102±87; P=0.15).

3.1.4.7. **Extracerebral Organ Injury**

The heart and liver enzyme levels in the EPR groups were markedly increased after resuscitation. They decreased gradually after 24 hours but remained increased at 72 or 96 hours, with no difference between groups.

At necropsy, in the CPR group, all dogs had generalized edema, severe endocardial hemorrhage, lung edema, and bloody ascites. Although the serosal side of the intestine had mild hemorrhage, the intestinal mucosa was sloughed over the entire length of the intestine. In the EPR groups, the most consistent findings were mild to moderate endocardial hemorrhage and extensive hemorrhage in the gallbladder wall. Occasionally, hemorrhagic spots were found on the serosal surface of the intestine, but there was no necrosis. Lung edema was found only in 1 EPR-I dog.

3.1.5. **Discussion**

We established an exsanguination CA model that is unsalvageable by contemporary conventional resuscitation. At the time of CA ~ 60% to 90% of the estimated blood volume was removed. Arterial blood gases taken at the beginning of CA revealed severe acidemia

(pH<7.0), hyperkalemia, and hyperlactemia. As expected, none of the dogs could be resuscitated with CPR, despite vigorous blood and fluid replacement, standard drug therapy, and chest compressions with an MAP at 50 to 60 mm Hg. Such favorable hemodynamic responses would be difficult to achieve in traumatic, exsanguination CA victims. Although all of the dogs could be resuscitated with CPB, all subsequently died of severe MOF, including cardiovascular dysfunction, renal failure, and extensive gastrointestinal mucosal necrosis. This pattern is anticipated after prolonged HS and CA.[26] Such trauma victims similarly develop irreversible shock, including vasodilatation unresponsive to vasopressors and massive capillary leak, presumably secondary to the systemic inflammatory response. Thus, it was anticipated that animals in the conventional-resuscitation arm would exhibit considerable gastrointestinal fluid loss and ascites. We suspect that the large amounts of fluids administered in an attempt to sustain these animals led to hemodilution, as reflected in lower hematocrit values at 12 hours; there were no intergroup differences in blood loss. In contrast, EPR with delayed resuscitation was superior to conventional CPR for resuscitation of traumatic, exsanguination CA.[23,25,26] Twelve of 14 dogs survived without severe extracerebral organ damage.

Capone et al.[34] also compared EPR with conventional resuscitation. That investigation differed from the current study in that before induction of EPR, the period of severe hemorrhage was shorter (60 minutes), and there was no period of normothermic CA. After profound HS (MAP of 30 mm Hg for 60 minutes), survival was similar after conventional resuscitation or after addition of 1 hour of EPR at 10°C. The addition of more prolonged HS and brief CA before standard resuscitation in the current study is 100% lethal with standard care, although EPR can yield normal recovery.

Dogs in the EPR-I group had severe neurological deterioration after an initial recovery. Three dogs had generalized seizures, although 2 had regained consciousness

initially. This is a unique pattern that we had not previously encountered during years of experience with exsanguination and normovolemic CA models. Extensive neocortical laminar necrosis was found, along with cerebellar synaptic injury. This is in sharp contrast to the lack of histological brain damage after rapid exsanguination CA in our prior EPR report.[38] Given that the EPR and delayed resuscitation protocols were almost identical between these 2 studies, the preexisting prolonged HS probably set the stage for this delayed neurological deterioration.

We speculate that the mechanism of this delayed neurological deterioration may be cytotoxic brain edema that peaks at ~48 hours after reperfusion. Different from rapid exsanguination CA, blood glucose levels at the time of CA in this model were >500 mg/dL in most dogs, likely related to the stress of prolonged HS.[83] On the basis of a histological pattern similar to that found after hyperglycemia-associated brain injury in forebrain ischemia models[84] and previous reports that hyperglycemia exacerbates neurological dysfunction after CA,[85] we speculate that hyperglycemia may have contributed to the delayed neurological deterioration. Hypothermia appears to be very effective in protecting against ischemic brain injury during hyperglycemia.[86] However, rapid rewarming of the traumatically injured brain can markedly exacerbate injury.[87] This suggests the need to carefully optimize the use of mild hypothermia and rewarming.

The effects of prolonged post-EPR mild hypothermia were impressive. Similar to our finding, Gunn et al[83] documented that delayed brain edema that peaked 48 hours after 30 minutes of cerebral ischemia in fetal lambs was abolished by prolonged (72-hour) hypothermia; 48-hour cooling was associated with rebound seizures. Likewise, Colbourne and Corbett[88] found that to salvage CA1 neurons after 5 minutes of ischemia in gerbils, hypothermia (32 °C), induced 1 hour after ischemia, had to be maintained for 24 hours. In our study, with hypothermia for 36 hours and slow rewarming, 5 of 7 dogs regained

consciousness, and seizures were not observed. One dog died of probable aspiration. Although mild hypothermia is recommended for comatose survivors of CA[89] and laboratory studies suggest the benefit of mild hypothermia during resuscitation from HS,[90] more research on the optimal timing and rate of rewarming is needed.

This study has some limitations. First, it was not fully randomized. Seven EPR-II dogs were added after 5 experiments had been performed in each of the other groups because we had observed delayed neurological deterioration and we hypothesized that pro- longed mild hypothermia with slow rewarming would prevent this deterioration. However, these studies were carried out in sequence, contiguous with those in the previous dogs by the same experienced research team. In addition, outcomes in the CPR and EPR-I experiments performed after the addition of the EPR-II group mirrored the earlier experiments.

Second, final outcome was determined at different times in the EPR-I and EPR-II groups. On the basis of our previous experience, we thought it essential to compare functional outcomes after identical postrewarming and extubation periods in the EPR-I and EPR-II groups. This mandated that we compare outcomes at 72 and 96 hours in the EPR-I and EPR-II groups, respectively. We recognize that for histological evaluations, delayed neuronal death may occur 3 days to 1 week after global ischemia,[91] which may have biased the outcome toward worse damage at 96 hours than at 72 hours in the EPR-II group. Despite these differences, we still found significantly better brain histology results in the EPR-II group.

Third, external chest compressions are not standard care for trauma victims who experience CA. Emergency department thoracotomy with open chest cardiac massage is indicated because of the potential for treating a surgical cause of CA, e.g. pericardial tamponade.[25] External chest compressions generated excellent blood pressures with

aggressive fluid resuscitation. Despite this, there were no long-term survivors in the CPR group.

Fourth, systemic heparinization was used, because standard CPB equipment was utilized. This precluded studies of coagulation. In the massively traumatized patient, however, one could avoid systemic heparin by using a heparin-bonded CPB system.

Fifth, vessel cannulation for EPR within 2 minutes of CA may be difficult in the field, although it is reasonable for the scenario in the Emergency Department by a trauma surgeon. Also, large amounts of fluid are currently required to achieve the desired brain temperature. Despite these limitations, we believe that clinical application of EPR should be studied in trauma victims who have experienced exsanguination CA. Ideally, this would be applied in the field. Ambulances could carry large amounts of fluid for the aortic flush. Given the complexity of the procedure with currently available equipment, however, we believe that the first study should be conducted in Emergency Departments of major trauma centers. This could also facilitate rapid initiation of CPB for delayed resuscitation in this situation, although this is less of an issue, because preparations for CPB can proceed simultaneously with resuscitative surgery.

EPR is remarkably superior to conventional CPR in facilitating survival and neurological recovery in a model of otherwise unresuscitable prolonged hemorrhage with exsanguination CA. Extended application of mild hypothermia with slow rewarming during ICU care after EPR was critical in achieving intact neurological outcomes. Use of mild hypothermia and/or slow rewarming may also have implications for optimal neuroprotection in conventional DHCA.[92]

3.2. Study II – "Cold energy" in prolonged EPR in dogs

Wu X, **Drabek T**, Tisherman SA, Henchir J, Stezoski SW, Culver S, Stezoski J, Jackson E, Garman RH, Kochanek PM
Emergency Preservation and Resuscitation with Profound Hypothermia, Oxygen, and Glucose Allows Reliable Neurological Recovery after 3 h of Cardiac Arrest From Rapid Exsanguination in Dogs
J Cereb Blood Flow Metab 2008 Feb;28(2):302-11.

3.2.1. Summary:

In prior studies exploring the limits of our EPR paradigm for exsanguination CA victims we observed that 2 h but not 3 h of preservation could be achieved with favorable outcome using ice-cold normal saline flush to induce profound hypothermia. We tested the hypothesis that adding energy substrates, specifically **oxygen** and **glucose**, to saline during induction of EPR would allow intact recovery after 3 h CA. Dogs underwent rapid ExCA. Two minutes after CA, EPR was induced with arterial ice-cold flush delivered via CPB circuit. Four treatments (n = 6/group) were defined by a flush solution with or without 2.5% glucose (G+ or G-) and with either oxygen or nitrogen (O+ or O-) rapidly targeting tympanic temperature of 8 °C. At 3 h after CA onset, delayed resuscitation was initiated with CPB, followed by intensive care to 72 h. At 72 h, all dogs in the O+G+ group regained consciousness, and the group had better neurological deficit scores and overall performance categories than the O-groups (both $P < 0.05$). In the O+G- group, four of the six dogs regained consciousness. All but one dog in the O-groups remained comatose. Brain histopathology in the O-G+ was worse than the other three groups ($P < 0.05$). We conclude that EPR induced with a flush solution containing oxygen and glucose allowed satisfactory recovery of neurological function after a 3 h of CA, suggesting benefit from substrate delivery during induction or maintenance of a profound hypothermic CA.

3.2.2. Background

For induction of EPR in a series of prior reports, we have used ice-cold normal saline. Although pharmacological adjuncts added to the flush solution could have theoretical advantages in preserving vital tissues, long-term neurological outcome in our models was not improved versus normal saline with a number of mechanism-based pharmacological approaches[93,94] except for the antioxidant tempol, which had a modest effect.[56] Similarly, the use of conventional or novel alternative flush solutions such as albumin, Unisol,[95] or the UW solution[96] did not augment the protection afforded by profound hypothermia. Taking a different approach, Taylor et al.[97] demonstrated that continuous perfusion with an asanguineous preservation solution allowed satisfactory recovery over 3 h of ultraprofound hypothermia (< 5 °C). Alam et al.[98] have had success with a similar approach in pigs with traumatic hemorrhage. However, it is unlikely that in clinical trauma scenarios continuous perfusion will be an option.[23,96] Using an ice-cold saline flush for induction of EPR, preservation efficacy was improved with the use of either lower temperature or with faster cooling rates; however, efficacy reached its plateau at a core temperature between 7 and 10 °C [50,98] or when cooling rate was maximized.[51] However, at the maximal cooling rate that could be achieved, it still took between 12 and 15 mins to achieve a brain temperature of ~ 10 °C in dogs.[50] This 12–15-min period required to reach target temperatures might represent a key limiting factor in the ultimate success of EPR based on the report that the brain oxygen demand in pigs remains ~ 50% of baseline at 28 °C, ~ 19% at 18 °C, and ~10% at 8 °C, respectively.[61] Logically, we speculated that providing energy substrates during induction of EPR might either avoid further energy depletion, or even restore energy reserves that would probably be reduced during the 5 mins period of shock, 2 mins normothermic CA, and cooling duration—before achieving profound hypothermia. Perfusion of dissolved oxygen at deep hypothermia (without circulatory arrest) can deliver considerable substrate, particularly in a setting of markedly reduced metabolic demands.[99] Robbins et al.[59] reported that

intermittent flush of energy substrates into the brain during profound hypothermic CA delayed ATP and creatine phosphate depletion in brain. However, there is no solution that has been convincingly shown to improve neurological outcome in a prolonged CA model, without intermittent perfusion during the arrest. Using our modified EPR model,[52] the current study was designed to test if profound hypothermia induced by aortic flush with a solution that was enriched with energy substrates, that is oxygen and glucose, could successfully produce intact long-term neurological outcome despite a prolonged (3 h) CA.

3.2.3. Materials and methods

3.2.3.1. Experimental Design

The model included three phases: (1) exsanguination (5 min) and CA (2 min); (2) EPR (3 h); (3) delayed resuscitation, including CPB (2 h) and intensive care (72 h). At the end of the exsanguination and CA phase, dogs were randomized into four groups based on the specific additives in the ice-cold normal saline flush solution, namely (1) oxygen + glucose (O+G+), (2) oxygen without glucose (O+G-), (3) glucose alone (O-G+), and (4) neither oxygen nor glucose (O-G-).

3.2.3.2. Anesthesia and Preparation

The methods were identical to Study I. For a detailed description, please See Study II, Section Methods.

3.2.3.3. Exsanguination and Cardiac Arrest Phase

Rapid exsanguination was conducted stepwise to a mean arterial pressure of 20 mm Hg at 4 mins. At 5 mins, VF was induced to ensure no-flow.

3.2.3.4. EPR Phase

Two minutes after the onset of CA, flush solution (80 mL/kg) at 21 °C was infused into the aorta at a rate of 80 mL/kg per min using the CPB pump. Closed-chest CPB was then

initiated for induction of hypothermia until Tty reached 8 °C. Either 100% oxygen or nitrogen was supplied to the oxygenator throughout the flush interval and induction of hypothermia. The gas flow to the CPB oxygenator was adjusted to maintain PaCO2 35 to 45 mm Hg. Once Tty of 8 °C was reached, the CPB was stopped. The entire body was covered with ice from the onset of flush to the end of 3 h of CA.

3.2.3.5. Delayed Resuscitation Phase

CPB: After 3 h of CA, reperfusion and rewarming was started with CPB that was primed with shed blood. When Tpa reached 32 °C, defibrillation was attempted. MAP was maintained at 90-150 mm Hg. The CPB flow rate for assisted circulation was sequentially reduced and fully discontinued at 120 mins.

ICU: The details of life support, including mechanical ventilation, hemodynamic monitoring and support, and correction of acid–base or electrolyte abnormalities, were published previously.[50] Body temperature was kept at 34 °C until 36 h of resuscitation, followed by slow controlled rewarming (0.3 °C/h) to 36.5 °C, as per our previous Study I. Consequently, mechanical ventilation was continued to 48 h, when neuromuscular blockade was reversed, and sedation and analgesia were discontinued. Dogs were then weaned from mechanical ventilation. After extubation, they were transferred to the step down unit where continuous intravenous fluids and vital sign monitoring were provided until 72 h.

3.2.3.6. Outcome Evaluation

Functional outcomes were evaluated every 6 h using OPC and NDS similarly to Study I.

3.2.3.7. Neuropathology

Whole perfusion-fixed brains were divided into multiple coronal slices. Six coronal brain slices plus three transverse sections of the medulla oblongata and upper cervical cord were selected for microscopic evaluation. These represented entire brain slices taken at the following levels: (1) the optic chiasm; (2) the anterior thalamus; (3) the posterior thalamus;

(4) the midbrain; (5) posterior portions of the occipital lobes; (6) middle of the cerebellum and underlying brainstem; (7) medulla oblongata and upper cervical cord. These slices were processed and scored as in Study I. The HDS in each region were compared with other groups.

3.2.4. Results

3.2.4.1. Induction of EPR

The total EPR induction time to reach Tty 8 °C was similar between groups. During the induction of EPR, glucose levels in the G+ groups were approximately five times higher than in the G- groups ($P < 0.01$), and the PaO2 values in the O+ groups were ~ 20 times higher than in the O- groups ($P < 0.01$) (Study II Table 1). The brain temperatures did not differ among four groups over 3 h of CA (Study II, Figure 1).

3.2.4.2. Resuscitation

After 3 h of CA, rewarming and stable MAP (> 60 mm Hg without need for vasopressors) were achieved in all dogs with CPB. When Tpa reached 32 °C (40 to 50 mins after delayed resuscitation with CPB), ROSC was achieved in all dogs with 1 to 2 defibrillation attempts. CPB was then weaned off at 2 h in all dogs.

During the delayed resuscitation phase, the lactate levels were significantly higher in the two O- groups ($P < 0.05$) (Study II, Figure 2). All dogs were maintained with stable vital signs to 72 h.

3.2.4.3. Functional Outcome

At 72 h, all dogs in the O+G+ group regained consciousness with a significantly better OPC (Figure 9) and better NDS (Figure 10) (both $P < 0.05$), compared with the O- groups. In the O+G- group, four of the six dogs regained consciousness (NS versus other groups). In contrast, 11 of the 12 in the O- groups remained comatose.

	O+G+	O+G-	O-G+	O-G-
5 Dead		p<0.05		
4 Coma		*	***	**
3 Severe Disability		*	***	***
2 Moderate Disability	****	**		*
1 Normal	**	**		

Figure 9 Final overall performance category (OPC) at 72 h after 3 h of cardiac arrest. O+G+: 100% O2 with 2.5% glucose in normal saline; O+G-: 100% O2 with normal saline; O-G+: 100% N2 with 2.5% glucose in normal saline; O-G-: 100% N2 with normal saline.

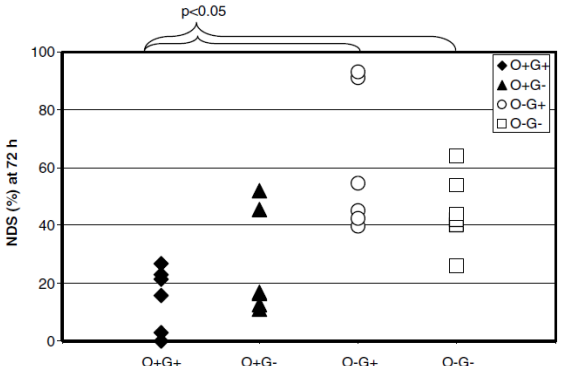


Figure 10 Final Neurologic Deficit Score (NDS) at 72 h after 3 h of cardiac arrest. O+G+: 100% O2 with 2.5% glucose in normal saline; O+G-: 100% O2 with normal saline; O-G+: 100% N2 with 2.5% glucose in normal saline; O-G-: 100% N2 with normal saline.

3.2.4.4. **Neuropathology**

Total HDS combining scores in all regions on both sides was significantly higher (worse) in the O-G+ group compared with the other groups ($P < 0.05$): **H&E staining** (median and range): O-G+: 145 (120;150), O+G+: 51 (10;76), O+G-: 57 (27;70), and O-G-: 48 (10; 112); **FJB staining:** O-G+ : 132 (106;174), O+G+ : 56 (38;74), O+G-: 36 (0;86), and O-G-: 57 (2;96). Of 25 brain regions, there were 11 regions that had substantial brain injury in at least one group. Histological damage scores of the O-G+ group was consistently worse than all other groups in all involved brain regions ($P < 0.05$) (Figures 11A and 11B). The results with H&E and FJB staining were significantly correlated in all 25 brain regions ($r = 0.52$ to 1.0, all $P < 0.01$).

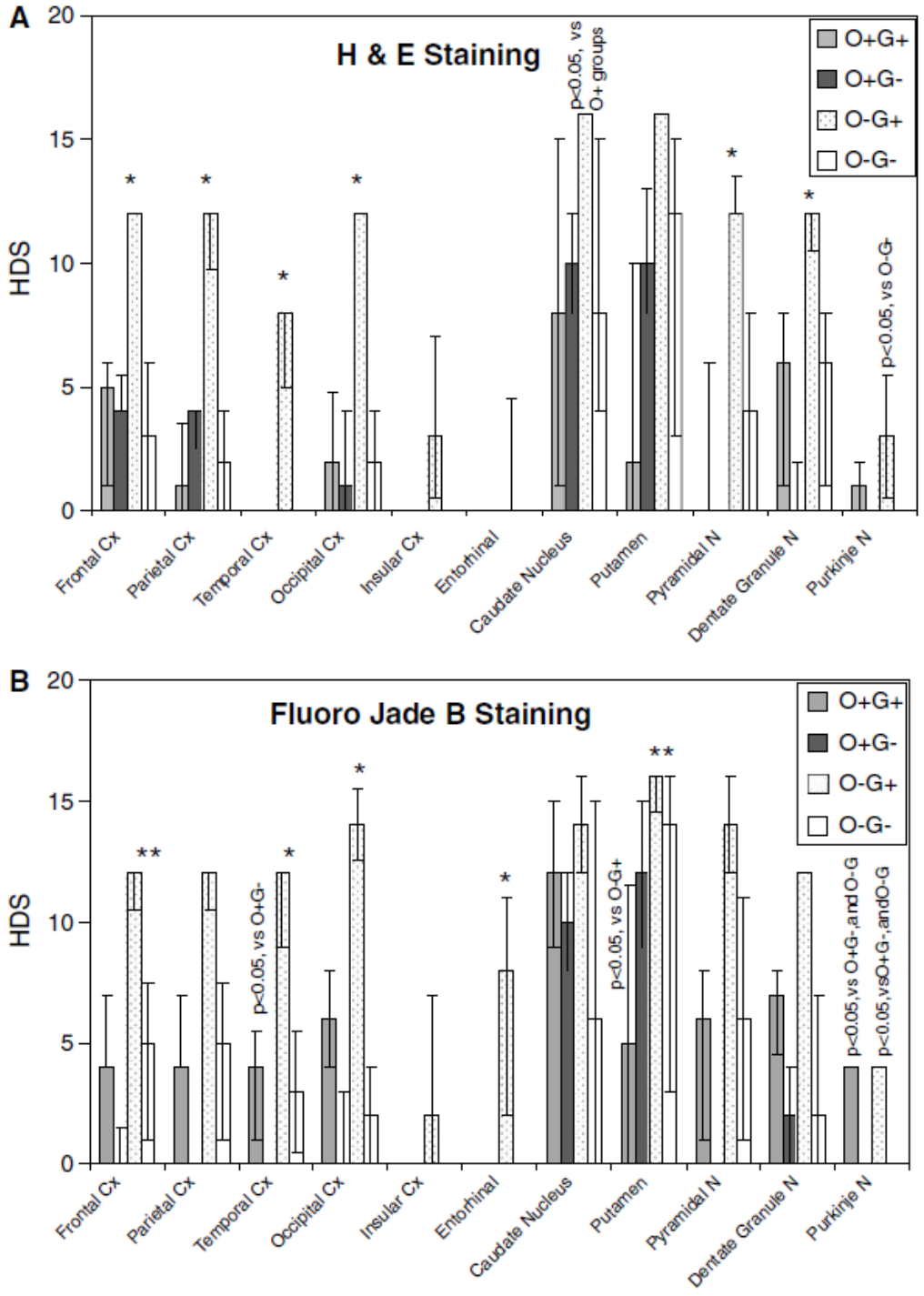


Figure 11 (A) Brain Histological Damage Score (HDS) at 72 h after 3 h of cardiac arrest (median, 25-75% IQR). **H&E staining**: O+G+: 100% O2 with 2.5% glucose in normal saline; O+G-: 100% O2 with normal saline; O-G+: 100% N2 with 2.5% glucose in normal saline; O-G-: 100% N2 with normal saline. N: neurons; Cx: cortex; *P < 0.05 compared with other three groups. **Figure (B)** Brain Histological Damage Score (HDS) at 72 h after 3 h of cardiac arrest (median, 25-75% IQR). **FJB staining:** O+G+: 100% O2 with 2.5% glucose in normal saline; O+G-: 100% O2 with normal saline; O-G+: 100% N2 with 2.5% glucose in normal saline; O-G-: 100% N2 with normal saline. N: neurons; Cx: cortex; *P < 0.05 compared with other three groups.

3.2.5. Discussion

With the induction of profound hypothermia for EPR with ice-cold saline following rapid exsanguination to CA, we have previously been able to achieve good outcomes in dogs after up to 2 h CA. In certain clinical situations of the exsanguinated trauma victim, more time may be needed for transport, resuscitative surgery, and initiation of delayed resuscitation. Thus, there is a clinical need to extend the safe duration of preservation. In the current study, an energy preservation strategy with oxygen and glucose allowed good recovery after 3 h of CA.

The significance of this finding could be demonstrated when it is placed along our many trials to improve EPR efficacy. In our pursuit of ***pharmacological preservation***,[93,94,96] only tempol improved outcome in a 20-min CA EPR model;[56] in the pursuit of ***hypothermic preservation,*** which was reliable, we came to realize that the maximal effect of profound hypothermia alone could allow consistent intact neurological outcome, but only to 2 h of CA.[39,47] Given this background, our current study, showing consistently good functional outcome after a 3-h ExCA (2.5 h of no flow), represents a significant step of advance in neuropreservation in EPR studies.

Although we have benefited from the earlier explorations in cryobiology and DHCA for neuropreservation, EPR studies have distinct challenges and goals. The normothermic hypotension and subsequent normothermic CA before induction of hypothermia, modeling ExCA, probably increased the difficulties in achieving successful preservation. In contrast, as early as 1986, successful recovery of neurological functions was achieved after 3 h profound hypothermic circulatory arrest in some healthy dogs.[100] For DHCA, 3 h circulatory arrest is rarely indicated clinically, and thus seldom addressed in labs. While EPR studies target survival with satisfactory neurological functions (OPC: 1 to 2) as an acceptable goal in trauma victims who would otherwise have near 100% mortality after ExCA,[25] DHCA studies,

however, target the reduction of neurological morbidity after ~ 60 mins of bloodless surgery.[101] In fact, the 'safe' duration of DHCA appears to be as short as 20 to 30 mins in patients who underwent thoracic cardiac surgery.[102] Neurological recovery after up to 3 to 3.5 h of profound hypothermic CA has been reported using continuous perfusion with an experimental tissue preservation solution.[97] However, using the same solution significantly worsened NDS and OPC were noticed when intermittent no flow (circulatory arrest) was allowed in a 100-min DHCA model.[103]

The decision to add oxygen and glucose to the perfusate was a logical step in our pursuit of more effective neuropreservation. First, we reported previously that despite using an arterial flush catheter of maximal diameter, maximal flush rate, and concurrent surface cooling, target temperature of 7 to 10 °C could not be achieved for 12 to 15 mins in dogs. Second, metabolic demands are much greater during cooling than at profound hypothermia.[61] Third, in our EPR model, previously screened 14 pharmacological adjuncts failed to augment the benefits of hypothermia, suggesting the need to consider alternative approaches. Similar disappointing results were observed by Aoki et al.[57] with addition of MK-801 to profound hypothermia in DHCA in piglets. Fourth, 2 h of DHCA (12 to 15 °C) in sheep could be achieved with preservation of high-energy phosphate levels in brain (to ~60% of baseline) via intermittent infusion of a crystalloid solution containing dissolved oxygen and 2.5% dextrose.[59] That solution was called 'cerebroplegia' and also contained lidocaine, sodium bicarbonate, nitroglycerine, and mannitol.

Fifth, as temperature decreases below 37 °C, the affinity of hemoglobin for oxygen is greatly enhanced, restricting delivery, and increasing the importance of the dissolved oxygen component.[99] Sixth, the solubility of oxygen in saline nearly doubles between 37 and 18 °C.[104] Grist et al. suggested that the use of hyperoxia before DHCA can take advantage of enhanced oxygen solubility and reduced metabolic demands of hypothermia to prevent tissue

injury. Hyperoxic perfusion during induction of hypothermia has been suggested to attenuate tissue acidosis in the clinical use of DHCA.[104] Finally, our flush rates of 20 L delivered over ~ 20 mins suggest that substrate delivery during the flush could be substantial, particularly in the setting of reduced metabolic demands. Thus, it was logical to propose that we could meet better metabolic demands with oxygen and glucose added to the flush solution during the induction of hypothermia. Different from DHCA, clinical application of EPR in management of ExCA would only be feasible after a normothermic ExCA has occurred. Brain energy reserve is depleted ~5 mins after normothermic CA.[105,106] Thus, to postpone energy failure, it may be important for preservation strategies to prevent energy depletion in brain and restore energy levels during induction of hypothermia. Based on our favorable outcomes in the dogs flushed with oxygen (with or without glucose) and on the aforementioned study by Robbins et al. in which the cerebral ATP depletion was attenuated with the addition of dissolved oxygen and glucose in the perfusate, flush with oxygen and glucose solution after ExCA may have prevented the development of critical energy depletion. Additional studies of ATP or energy charge would be needed to prove that hypothesis.

Recently, important work by Vereczki et al. demonstrated deleterious effects of hyperoxic reperfusion after a 10-minVF CA in dogs.[107] In contrast, the powerful favorable effect of oxygen in our study probably relates to the fact that it is used to mitigate energy failure during cooling—before it results in cellular disturbances that set the stage for oxidative reperfusion injury. This intriguing hypothesis also needs to be further evaluated.

The best functional outcome was achieved only with the combination of oxygen and glucose in our model. It is possible that added glucose is important in delaying energy depletion during the prolonged hypothermic CA. However, the effects of glucose in cerebral ischemia are complex. On one hand, high glucose may enhance energy production via glycolysis during anoxia/hypoxia,[108] and/or provide beneficial osmolar effects as did

mannitol in a cat middle cerebral artery occlusion model.[109] However, it is not clear how much glucose was transferred across the BBB during hypothermia induction in our model. The G+ groups did not have increased arterial lactate levels either at the end of flush or during early reperfusion. Instead, lactate levels were consistently higher only in both O- groups during early reperfusion, and there was no difference in lactate levels between two O- groups. Cerebral lactate production was not examined in this study. It is certainly possible that cerebral effects were masked by systemic effects. We recognize that it is possible that the choice of 2.5% dextrose does not represent the optimal concentration and could be excessive.

Alternatively, glucose could be detrimental during cerebral ischemia,[110] with higher tissue lactate levels, acidosis, oxidative stress, glutamate, DNA fragmentation, and other deleterious effects.[111] It is possible that a net benefit of glucose is seen if energy failure is prevented, while injury exacerbation dominates if frank ischemia occurs. Consistent with a potential dichotomous effect of glucose depending on whether energy failure is prevented, in our EPR model, the combination of oxygen and glucose produced the best functional outcomes, whereas the O-G+ group exhibited the worst histological injury. Additional studies of brain glucose utilization and energy charge would be helpful. Further studies would also be important to define the possible benefits of the osmotic effects of glucose in this model.[112] The findings of hyponatremia and decreased hemoglobin levels in the glucose groups suggest that the systemic osmotic effects were substantial. The effects on the brain, however, are unclear.

Given the dichotomous effects of additional glucose, the ability to successfully achieve 3 h of preservation might be attributed mostly to the addition of oxygen to profound hypothermia. Post-resuscitation lactate was lower in both groups with oxygen added to the flush versus those without oxygen, and there was a trend toward improved functional outcome in the O+G- group. Also, the O+G- and O+G+ groups had similar HDS. Based on

the observed OPC and HDS, our study was insufficiently powered to test for differences between groups with and without glucose in the presence of oxygen in the flush. The variable effects of glucose on histology in our model also suggest the possibility that alternative fuels such as β-hydroxybutyric acetate may be worthy of investigation as an adjunct to dissolved oxygen in EPR.[113]

Unlike our previous EPR studies where we used one-way flush for induction, we used CPB to induce profound hypothermia in the current study. The hematocrit during induction of EPR was around 5 to 10% in all groups. This deviation from one-way flush with saline could be important. On one hand, higher hematocrit (> 10%) during induction of hypothermia before CA was associated with better energy reserve and neurological outcome.[114] If so in the current study, it again suggests the importance of supporting oxygen delivery during induction of hypothermia.

However, after cooling to 10 to 12 °C, hemodilution to < 5% improved neurological function.[115] It is therefore difficult for us to predict the impact of the residual hemoglobin without further studies.

Among limitations, the lack of biochemical data (i.e., brain ATP, energy charge, glucose, and lactate levels) suggests the need for caution in making conclusions about a clear relationship between energy metabolism and improved outcome. In addition, it is important to recognize that it would be technically difficult to induce profound hypothermia in the field with CPB—as used in this proof of concept investigation. We also recognize that it is impossible to determine in this work whether beneficial effects of oxygen and glucose added to the flush are being produced during the induction of hypothermia, during the period of no flow, during reperfusion, and/or any combination of these time intervals. Biochemical studies of both ATP preservation and osmolar effects during induction and maintenance of hypothermia, and of secondary injury mechanisms during reperfusion are necessary to

understand further this intervention and optimize its application. Finally, we recognize that hypothermia can delay the appearance of damage after cerebral ischemia and that assessment of brain histopathology after longer outcome intervals would be valuable.

In summary, EPR using a combination of oxygen and 2.5% glucose plus profound hypothermia allowed satisfactory recovery of neurological function after 3 h of CA. Prevention or reversal of energy failure and other mechanisms may be responsible for the benefit. Adding oxygen and possibly glucose in the cooling solution might augment the efficacy of either resuscitative or elective deep hypothermia.

3.3. Study III – Rat EPR feasibility study

Drabek T, Stezoski J, Garman RH, Wu X, Tisherman SA, Stezoski SW, Fisk JA, Jenkins LW, Kochanek, PM
Emergency preservation and delayed resuscitation allows normal recovery after exsanguination cardiac arrest in rats: a feasibility trial
Crit Care Med 2007;35:532-7.

3.3.1. Summary

In prior studies of EPR we used a dog model with prolonged intensive care to maximize clinical relevance. Because of the lack of molecular tools available for use in dogs, development of a rat EPR model would enable study of the molecular mechanisms of neuronal injury in ischemia-reperfusion injury from exsanguination CA. Understanding the impact of deep hypothermia and reperfusion on these cascades would allow us to define specific targets for future interventions and to assess markers of reversibility. The rodent model would also allow economical screening of pharmacological strategies for EPR.

In this feasibility study in rats, we showed that survival from rapid exsanguination CA followed by 20 min of deep hypothermic EPR (15 °C) including CPB-assisted resuscitation is achievable with a favorable outcome, while an identical insult treated with normothermic

EPR and CPB-assisted resuscitation is lethal. This study documented feasibility of closed-chest CPB as a resuscitation tool usable in our EPR model.

3.3.2. Background

The dog model was used from the beginning of the EPR experiments to maximize clinical relevance. However, certain limitations are pertinent to that model. First, there are few molecular tools available for dogs. That limits the evaluation of impact of neurologic injury on the cellular and subcellular basis. Understanding molecular mechanism beyond ischemia-reperfusion injury would allow us to assess markers of reversibility and define specific molecular targets for future interventions. Secondly, the cost and labor-intensiveness of the experiment pose a severe obstacle to rapid screening of the drugs that would seem promising to provide additional brain and extracerebral tissues preservation beyond the effects of hypothermia itself.

Regarding this, we have decided to develop a rat model of EPR that would eliminate the abovementioned drawbacks.

In our pilot study, we determined that survival after rapid HS followed by 30 min CA with cooling to 10 °C is survivable, with favorable neurologic outcome in survivors.

In the follow-up study, we attempted to decrease the amount of flush in trade of higher body temperature during the preservation phase, improve survival rate and still be able to achieve favorable neurological outcome in survivors.

In our feasibility study, we hypothesized that survival from rapid exsanguination CA followed by 20 min of hypothermic EPR including CPB-assisted resuscitation is achievable and that survival with normothermic EPR and CPB-assisted resuscitation is unlikely. We assessed survival and neurologic outcome as primary outcome parameters and markers of organ injury as secondary outcome parameters. Based on the previous reports that CPB is associated with neurologic impairment, we also included CPB-only control group.

3.3.3. Materials and methods

Adult male Sprague-Dawley rats (350-400 g; Hilltop Lab Animals; Scottdale, PA) were housed in the facility for at least three days before the experiment with unrestricted access to food and water. On the day of the experiment, rats were anesthetized with 4% isoflurane in oxygen, intubated with a 14G intravenous cannula (Becton Dickinson; Sandy, UT) and mechanically ventilated (Harvard Ventilator Model 683, Harvard Rodent Apparatus; South Natick, MA). Anesthesia was maintained with 1-1.5% isoflurane in FiO2 0.5. Utilizing asepsis, the left femoral artery and vein were cannulated. EKG, respiratory rate, arterial and central venous pressure were continuously monitored and recorded (Polygraph; Grass Instruments, Quincy, MA). The right femoral artery was cannulated with a 20G Angiocath (Becton Dickinson; Sandy, UT), that served as an in-flow CPB cannula. The right jugular vein was cannulated with modified five-hole 14G cannula advanced to the right atrium. That cannula was used for venous drainage during the hemorrhage phase and later as a venous out-flow CPB cannula. Rectal and tympanic probes were used to monitor the temperature. After instrumentation, rats were allowed to breathe spontaneously 2% isoflurane in FiO2 0.25. Heparin was administered to achieve activated clotting time (ACT) >400 s (Haemochron Jr. Signature, ITC; Edison, NJ). After a 5 min equilibration period, a rapid exsanguination (12.5 ml of blood over 5 min) was performed via the internal jugular catheter. After the exsanguination phase, CA was ensured with administration of 1 ml of potassium chloride (2 mEq) intravenously. The no-flow duration was one minute.

According to the randomization, **three groups were studied**: (1) hypothermic EPR (H-EPR, 0°C flush with Plasma-Lyte A, n=6); (2) normothermic EPR (N-EPR, 37°C flush, n=6); (3) controls (anesthesia and surgical preparation identical to EPR groups followed by 60 min of CPB at 37°C, no CA, n=6). The flush solution was instilled via the right femoral artery catheter at 50 ml/min using a roller pump, and was drained from the jugular vein catheter. For the H-EPR group, a target tympanic temperature of 15°C was achieved with a combination of 270 ml of flush and surface cooling. The same flush volume was used for the N-EPR group. After 20 min of H-EPR or N-EPR, resuscitation was started with CPB. (Figure 12)

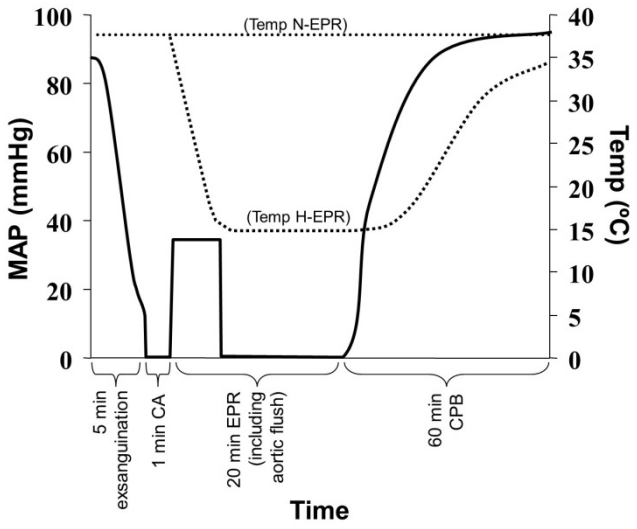


Figure 12. Experimental protocol including rapid hemorrhagic shock followed by 1 min of cardiac arrest (*CA*) and emergency preservation and resuscitation (*EPR*) using cardiopulmonary bypass (*CPB*). *Solid line*, mean arterial pressure (*MAP*) for hypothermic EPR (*H-EPR*) and normothermic EPR (*N-EPR*) groups; *dashed line*, temperature in H-EPR group; *dotted line*, temperature in N-EPR and control groups and MAP for a control group.

In brief, the CPB circuit consisted of a custom-designed oxygenator, an open reservoir (Ing. Martin Humbs, Ingenierburo fur Feinwerktechnik, Munich, Germany), tubing, and a roller pump (Masterflex, Barnant; Barrington, IL). The oxygenator contained a 3-layer capillary membrane sufficient to provide oxygen levels > 400 mmHg. Isoflurane was used for maintenance of anesthesia during the CPB phase. Heating and cooling were achieved with a circulating water bath and a forced-air blower (Life-Air 1000, Progressive Dynamics, Marshall, MI). The temperature gradient between the water bath and the body core was not allowed to exceed 10 °C.

Immediately before starting resuscitation, the CPB circuit was primed with collected shed blood, and mechanical ventilation with FiO2 0.3 was restarted to re-expand the lungs

and prevent atelectasis. For the H-EPR group, flow rate was gradually increased according to the core temperature,6 targeting attainment of full flow (160-180 ml/kg/min) at 25 °C (30 min CPB time). For the N-EPR and CPB group, full flow was maintained during the entire CPB course. Acid-base management followed alpha-stat principles. pH and electrolyte values outside of the normal range were corrected during CPB and ICU phases. Additional blood obtained from a donor rat was used, if needed, to maintain hematocrit level > 25%.

CPB support was discontinued after 60 min. Mechanical ventilation with a FiO2 of 1.0 was continued while maintaining normocapnia. Using a midline laparotomy incision, a Mini-mitter probe (Mini-Mitter Co; Sunriver, OR) was introduced into the peritoneal cavity to allow post-operative temperature control and continuous monitoring of vital functions. Surviving rats were extubated 2 h later after removal of catheters, and placed separately in a temperature-controlled cage (34.5 °C for 3 h) with supplemental oxygen for 18 h. Weight and neurologic status were assessed daily, using OPC (1=normal, 2=mild disability, 3=moderate disability, 4=severe disability, 5=death or brain death) and a modified NDS (0-50=normal, 500=maximum deficit). Blood samples were obtained from survivors on day 7, and rats were killed with an isoflurane overdose and perfused with normal saline followed by 10% formalin for histologic evaluation. The brains were divided into multiple coronal slices. Representative slices were also prepared from extracerebral organs. The tissue samples were processed and sectioned at 5 micrometers. All sections were stained with H&E. Additional (duplicate) sections of brain were stained with FJB. The tissue sections were examined by a neuropathologist (RHG). Each anatomic region with evidence of damage on microscopic examination received a subjective pathologic grade ranging from 1+ (minimal) to 5+ (severe). A total of thirty-five regions were evaluated in the brain. Each affected region on each side of the brain (right and left) received separate scores for the degrees of neuropathologic damage detected in H&E-stained and FJB-stained sections. HDS was

determined by adding up all of these individual scores (i.e., for each neuroanatomic region with each stain).

3.3.3.1. Statistical analysis

Data are presented as mean ± standard deviation unless otherwise stated. Repeated measures ANOVA with post-hoc Bonferroni correction were used to compare the biochemical data (pH, base excess, lactate). Fisher's exact test was used to assess differences in OPC proportions. To distinguish normal vs. other outcomes, OPC was dichotomized (OPC 1 vs. 2-5). NDS was analyzed using Mann-Whitney U test. Kruskal-Wallis test was used to compare baseline values for 3 groups. Mann-Whitney test was used for comparing two groups if Kruskal-Wallis test indicated differences between all three groups existed or if data were available only for two groups. The Wilcoxon signed rank test was used separately for each group to determine if values changed from baseline to final. Statistical software SPSS 13.0.1 for Windows was used. A p value <.05 was considered statistically significant.

3.3.4. Results

Baseline physiologic variables were similar between groups.

3.3.4.1. EPR and CPB Phases

MAP observed during the flush was 41±14 mmHg in the H-EPR group vs. 22±6 mmHg in the N-EPR group, respectively (p=.011). During reperfusion, the N-EPR group had significantly lower pH compared to the H-EPR group (Study III Fig. 2, top panel). The lactate level in the N-EPR group was significantly elevated compared to the H-EPR group (Study III Fig. 2, bottom panel).

3.3.4.2. Survival

All rats in H-EPR and control groups survived. None of the rats in N-EPR group had ROSC during 60 min of CPB, which prevented separation from CPB. After discontinuation of isoflurane and a 10 min wash-out period during continuing CPB support, no spontaneous

neurologic activity was observed and no reflexes could be elicited. EEG was also isoelectric in each rat. CPB was then stopped and rats were perfused with formalin.

3.3.4.3. Neurologic outcome:

All rats in the H-EPR and control groups achieved OPC 1 beginning on day 2. (Figure 13)

NDS was also normal or near normal in all rats and did not differ between H-EPR and controls (5 ± 8.4 vs. 7.5 ± 8.8, respectively; p=0.62). (Figure 14)

	H-EPR (0°C)	N-EPR (37°C)	control
OPC 5		●●●●●●	
OPC 4			
OPC 3			
OPC 2			
OPC 1	●●●●●●*		●●●●●●

Figure 13 Survival and Overall Performance Categories (OPC) at day 7 after hemorrhagic shock and emergency preservation and resuscitation (EPR) with hypothermic flush (H-EPR), normothermic flush (N-EPR) and in control groups. OPC 1 = normal, OPC 2 = mild disability, OPC 3 = moderate disability, OPC 4 = coma, OPC 5 = death or brain death. * p=.002, OPC in the H-EPR group vs. N-EPR group.

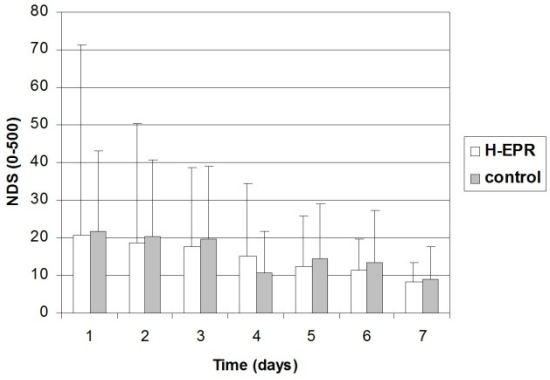


Figure 14. Neurologic deficit score (NDS) after hemorrhagic shock and emergency preservation and resuscitation (EPR) in hypothermic flush (H-EPR) and in control groups. No differences between groups.

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3.3.4.4. Histologic Evaluation

HDS did not differ between H-EPR and control groups in any region of the brain or extra-cerebral organs (p>.05). (Study III Table 1) HDS in brains of individual animals were 0; 0; 0; 0; 0; 0 (%) and 2.2; 0; 0; 0.2; 0.8; 0.4 (%) in the control and H-EPR groups, respectively.

Brain lesions:

All brains from the rats in the N-EPR group were characterized by multifocal edema, and some had evidence of peracute neuronal necrosis. Brains from the H-EPR group were normal in appearance, excepting for one rat. However, minimal degrees of FJB staining were

present in a minority of rats. No lesions were present in the brains of rats in the control group. Because of the early deaths of rats in the N-EPR group, brain histology in this group could not be compared to the other groups.

3.3.4.5. Biochemistry and Hematology

Enzymatic markers of organ injury (alanine transaminase - ALT, aspartate transaminase - AST, creatine phosphokinase - CPK, creatine phosphokinase myocardial band - CPK-MB, urea, creatinine) were normal in H-EPR and control rats on day 7. (Study III Table 2)

3.3.5. Discussion

To our knowledge, this is the first description of a successful resuscitation from exsanguination CA treated by an emergency preservation with hypothermic flush followed by a delayed resuscitation with CPB in rats. The long (20 min) duration of the CA prevented successful resuscitation of normothermic animals despite CPB-assisted circulatory support and re-establishing homeostasis with resuscitative drugs and multiple transfusions of the donor whole blood, a measure that is not readily available in large animal models.

Based on this feasibility study, it seems that EPR is achievable in rats and can produce intact neurologic outcome and normal brain histopathology. As expected, after exsanguination CA, the N-EPR protocol did not yield favorable outcome.

Importantly, there were no observed significant differences in any marker of outcome including OPC, NDS and HDS between survivors in H-EPR and controls. Minimal impairments in NDS were mostly motor deficits, presumably caused by peripheral (femoral) nerve injury during surgery; however, central origin or spinal cord injury could not be ruled out.

Histologically, the brains from the N-EPR group rats were characterized by extensive edema and peracute neuronal degeneration was suspected in many of the brains from this

group based on the contracted morphology of neurons in certain regions. However, it is well recognized that 70 min is an extremely early time after CA to appropriately evaluate neuronal death[116]. It is expected that the degree of neuronal degeneration/necrosis seen in the rats from the N-EPR group would have been greater if these rats had survived as long as the rats in the other treatment groups. Evidence of degeneration within the brains of H-EPR group rats was minimal. Although one rat in the H-EPR group had a significant degree of neuronal degeneration within multiple brain regions, these lesions were restricted to the right side of the brain and, therefore, probably resulted from vascular occlusion. No thrombi or emboli were found in the brain sections to account for these lesions. However, non-cellular intravascular material was found within selected non-nervous system tissues in other H-EPR group rats, and one rat had a small granuloma within its frontal cortex. These lesions are likely to be the result of the CPB. No brain lesions were present in control rats.

The higher perfusion pressures during flush in the H-EPR group could have contributed to the protective effect of hypothermia and better outcome. Recently it has been shown that a sufficient arterio-venous gradient of cold aortic flush allowing decrease of brain temperature can be achieved only when a vasopressor is added to the flush solution.[117] It is not clear, however, that increasing a perfusion pressure in the N-EPR group could favorably affect the outcome after this severe insult.

There are certain limitations to this study. Due to the complex nature of the model, the investigators could not be blinded. However, the investigators who assessed OPC and NDS were blinded to the protocol assignment of the tested animal. Histologic evaluation was not blinded in the initial steps of model development. In future studies of EPR of longer duration, where brain injury is anticipated and therapies are tested, it will be critical to mask the neuropathologist to treatment groups.

The use of CPB itself has been reported to produce neurologic deficits. In our study, we did not detect major deficits, and there were no differences between the H-EPR group and CPB controls. However, we did not have a sham-operated group in our study that would expose CPB-induced neurologic deficits. More sophisticated tests focused on spatial learning memory[118] might have revealed subtle differences between H-EPR and control groups. We also acknowledge that the reperfusion time for rats was different – 70 min for rats in N-EPR group vs. 7 d for survivors in H-EPR and controls. However, overwhelming injury in the N-EPR group prevented long-term survival, despite CPB-assisted resuscitation, due to cardiovascular collapse and brain death, as described in similar scenarios elsewhere.[119,120]

In conclusion, we have established an EPR model in rats that shows no neurologic injury despite exsanguination CA followed by emergency preservation with hypothermic flush, 20 min of asanguineous CA, and delayed resuscitation using a miniaturized CPB.

3.4. Study IV - 60 vs. 75 min EPR in rats

Drabek T, Stezoski J, Garman RH, Han F, Henchir J, Tisherman SA, Stezoski SW, Kochanek PM
Exsanguination cardiac arrest in rats treated by 60 min but not 75 min emergency preservation and delayed resuscitation is associated with excellent outcome
Resuscitation 2007;75:114-123.

3.4.1. Summary:

Previous studies in dogs demonstrated the clinical feasibility of the EPR method for resuscitation of exsanguination CA victims. We have then successfully established a rodent EPR model with a limited duration of the preservation phase under deep hypothermia. This model showed excellent survival and absent neurologic deficits, as well as absent brain histological damage. To be able to screen novel therapies, we needed to develop a model that would have neurologic deficits. We hypothesized that extending

the preservation phase we would reach a point when hypothermic preservation alone would not be adequate to protect from energy failure, and neuronal death. In a series of experiments based on our prior study, we serially extended the duration of hypothermic CA. The duration of 30-60 min produced intact survival and no deficits, while extended duration to 75 min resulted in significant mortality (only 2 out of 7 rats survived) and neurologic deficits in survivors. Surprisingly, extracerebral organ injury predominated in the group subjected to 75 min CA.

3.4.2. Background

In our previously published feasibility trial, we established a rat model of EPR utilizing rapid exsanguination CA followed by 20 min of preservation and subsequent delayed resuscitation using CPB with excellent survival and neurologic recovery[121].

We hypothesized that survival and neurological outcome is dependent on the duration of EPR. To test this hypothesis, we assessed survival and neurologic outcome after exsanguination CA followed by extended duration of deep hypothermic preservation phase. In pilot studies, we have demonstrated that durations of up to 60 min are associated with survival and favorable neurologic outcome. We have also optimized our method of induction of CA. We have noticed that excessive amount of KCl used to induce CA causes local tissue necrosis. Thus, we replaced the KCl-based cardioplegia with a combination of esmolol, a potent short-acting beta-blocker, with a limited dose of KCl. For this study, we assessed survival and neurological outcome after 60 or 75 min EPR as primary outcome parameters; histological damage score (HDS) served as a secondary outcome parameter.

3.4.3. Materials and methods

The methods were in general identical to Methods of Study III. We have replaced KCl-induced CA (1 mL = 2 mEq KCl) with a combination of esmolol (0.9 mL = 9 mg) and

KCl (0.1 mL = 0.2 mEq) to avoid KCl-induced tissue necrosis. For full description of Methods, please see Study IV / Methods.

3.4.4. Results

There were no differences in hemodynamic or biochemical measurements between the groups at the baseline, including MAP, heart rate (HR), temperature, Hct, arterial pH, pO2, pCO2, base excess, and serum Na, K, Cl, Ca, Mg, urea, or lactate levels.

3.4.4.1. EPR, CPB and ICU Phases

During the flush, temperature decreased in both groups similarly and was maintained at 15ºC for the required duration. During rewarming, the temperature increased to 34.5ºC similarly in both groups (Study IV Figure 2). During reperfusion, HR recovered similarly in both groups (Study IV Figure 3). Rats in the 75 min EPR group had higher MAP during the later phase of the CPB course (p=0.026) (Study IV Figure 4). The lactate levels were markedly increased vs. baseline in both groups; however, rats in the 75 min EPR group had significantly higher lactate levels compared to 60 min (p=0.012) (Figure 15). There were no changes in Hct between groups (p=0.837). To correct the metabolic acidosis during the CPB phase, more bicarbonate was administered to the 75 min EPR group vs. the 60 min EPR group (4.0±0.1 ml vs. 3.6±0.4 ml, p<0.05).

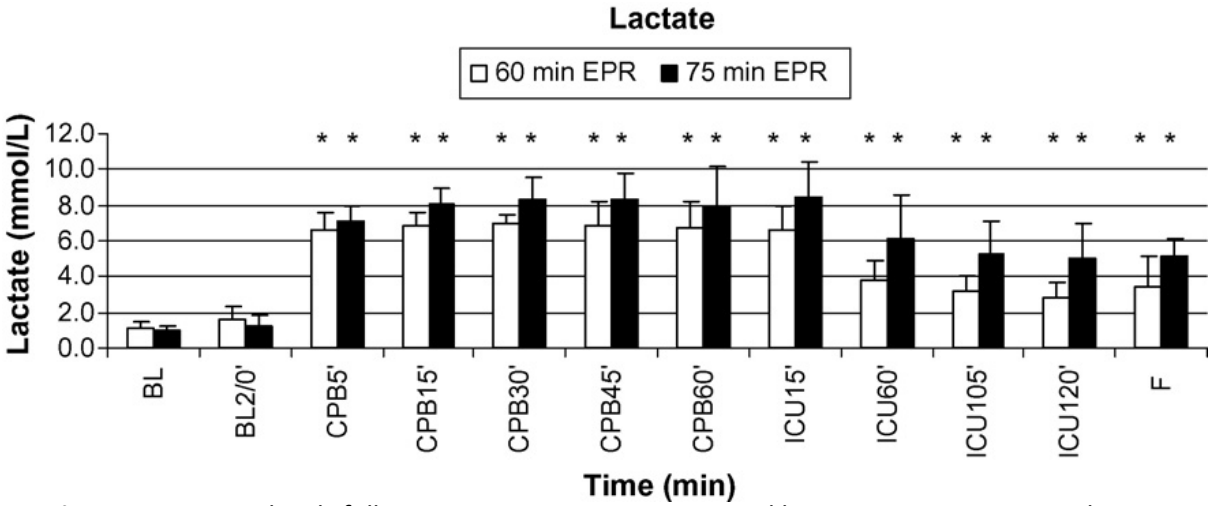

Figure 15. Lactate levels following exsanguination CA treated by 60 min or 75 min EPR during the CPB and ICU phases. BL = baseline, F = final (72 h). P = 0.012 overall between 60 min vs 75 min EPR. * P<0.05 vs respective baseline.

3.4.4.2. Survival

All rats in the 60 min EPR survived, while only 2 out of seven rats in the 75 min EPR group survived until day 3 (survival time was longer in the 60 min vs. 75 min EPR groups, *p*=0.007) (Figure 16).

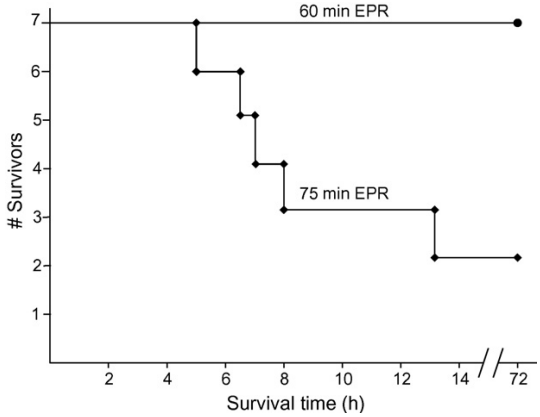

Figure 16. Survival following exsanguination CA treated by 60 min or 75 min EPR. p=0.007, 60 min EPR vs. 75 min EPR groups.

Neurologic outcome:

All rats in the 60 min EPR group achieved OPC 1 by day 3, while the two surviving rats in the 75 min group achieved only OPC 3 (*p*=0.01) (Figure 17).

NDS was normal in all rats in the 60 min group on day 3 (i.e. NDS < 10%), while both survivors in the 75 min EPR group were neurologically impaired (NDS 40% and 42%, respectively). NDS was significantly better in the 60 min EPR group vs. 75 min EPR group (2.4 ± 2.3 vs. 83.1 ± 28.8, *p*=0.001). (Figure 18)

	60 min EPR	75 min EPR
OPC 5 Death / brain death		●●●●●
OPC 4 Severe disability		
OPC 3 Moderate disability		●●
OPC 2 Mild disability		
OPC 1 Normal	●●●●●●●	

Figure 17. Overall performance categories (OPC 1-5) at 72 h after exsanguination CA treated by 60 min or 75 min of EPR. Each dot represents one rat. p=0.01 60 min EPR vs 75 min EPR groups.

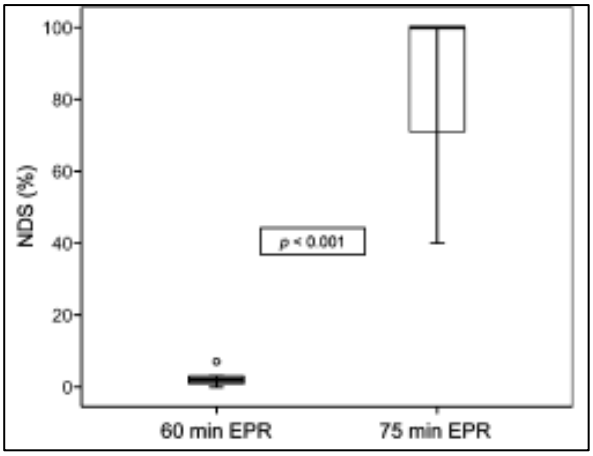

Figure 18. Neurologic deficit score (NDS, 0-100%) at 72 h after exsanguination CA treated by 60 min or 75 min of EPR. Boxes represent interquartile ranges. The line across each box indicates the median, and the whiskers are the highest and lowest values. The round marker represents an outlier.

3.4.4.3. **Histologic Evaluation**

HDS did not differ between 60 and 75 min groups in any **individual region of the brain** or in the **brain overall**. HDS between 60 and 75 min EPR groups was not different in the heart, lung, and liver. HDS was significantly worse in kidneys in the 75 min vs. 60 min EPR group ($p<.05$) (Study IV Table 1). There was a strong trend toward greater injury in all extracerebral organs – median (range) - total extracerebral organ injury score 6 (3,7) vs. 8 (4,13) for 60 min vs. 75 min EPR, respectively ($p<0.06$). The most frequently-present microscopic lesion was myocardial degeneration (or frank myocardial fiber necrosis) coupled with a pattern of subacute myocarditis. Some rats also had evidence of either peracute to acute tubular necrosis within the kidney or acute degeneration within the liver. The thymic tissues of some rats were mildly to moderately atrophic.

3.4.5. Discussion

Having achieved intact neurologic outcome with EPR durations up to 120 min in our well-established canine model[37,50,56,93,94,122], we aimed to assess the limits of EPR in rats.

Modifying our previously described paradigm of EPR in rats, we have extended the time allowing intact survival from 20 to 60 min. Surprisingly, further extension of EPR duration to 75 min was associated with renal injury, multi-organ failure, high mortality rate and unfavorable neurological outcome in survivors.

Rapid exsanguination resulted in MAP < 20 mmHg in all rats, suggesting minimal residual cardiac output. Previously, we used highly concentrated KCl to terminate cardiac activity. While a shorter duration of EPR (20 min) using this approach allowed survival with intact neurological recovery[121], pilot studies with longer durations of EPR revealed lung injury that prevented successful weaning from the ventilator (data not shown). One possible explanation for this finding comes from transplantation medicine literature on the optimal preservation of lung tissue. Perfusion with a solution enhanced with either high or low potassium concentrations (K+ 115 or 10 mmol/L) yielded worse results than perfusion with K+ 40 mmol/L.[123] This is in agreement with recent studies showing that use of highly concentrated potassium produced tissue damage and hindered post-ischemic recovery[124,125].

The short acting beta-blocker esmolol was recently tested as a CA-inducing agent in both clinical[126] and experimental settings[127-130]. Esmolol-treated hearts showed better recovery than those receiving potassium in terms of +/- dP/dt, left ventricular systolic pressure, and left ventricular developed pressure.[131] As an additive to cardioplegia in an isolated Langendorff model, esmolol only provided sustained protection when administered as a multi-dose or continuous infusion at constant pressure.[129] In light of these findings, in the current study we used esmolol plus a low dose of KCl to induce CA and minimize additional damage.

Previous parallel studies in a dog model with 60 min of EPR at 15°C or 90 min at 10°C showed excellent outcome at 72 h (OPC 1, NDS <10%).[50] With increased temperature or extended duration of EPR (up to 3 h), neurologic outcome worsened although survival was

still achieved. After transient multiple organ dysfunction syndrome, the survivors did not show any permanent extra-cerebral organ damage. In contrast, rats showed high early mortality with multiple organ failure dominated by renal failure (oliguria, anuria) subsequently confirmed by histological findings of acute renal tubular and liver necrosis. In addition, cardiac lesions were consistent with those seen in the brain-heart syndrome[132] (*i.e.* cardiac degeneration developing as a result of catecholamine release from damaged brain tissue) but could have other pathogenic mechanisms. A recent study in rats revealed variations in responses of individual organs to hemorrhagic shock and resuscitation and that the liver was the most vulnerable organ.[133] The rate of ATP loss in rat hearts was shown to be six-times higher compared to that in dogs[134], possibly limiting the resuscitability of the rats not due to neurologic injury after prolonged CA but due to depletion of energy sources, and ensuing cardiovascular failure.

In a pig model of EPR, profound hypothermia (10ºC) prevented neuronal and glial injury observed in normothermic controls[135] and allowed intact neurologic recovery after 60 min of low-flow state. Both the rates of cooling[51] and rewarming[55] were critical in order to achieve favorable outcome. However, EPR durations longer than 60 min were not evaluated in that model. Immediately-initiated EPR was superior to CPR followed by CPB even after prolonged HS[51,136]. EPR was also shown to be feasible after normovolemic CA[117].

Biochemical reactions that occur after an ischemic event are complex and are not yet completely understood. Energy depletion and cell membrane depolarization with ion fluxes occur within minutes after the onset of a global ischemic insult and are followed by formation of free oxygen radicals, altered gene expression[137], activation of caspases and other apoptotic pathways[138-140], inflammation[141], and release of excitatory amino-acids in brain[142]. Deep or profound hypothermia, the key component of EPR, exerts its beneficial effects mainly via reduction of metabolic rate and oxygen consumption[32], thus preserving the tissue energy state

and intracellular pH in both heart and brain[143]. The level of hypothermia that is used is critical to the spectrum of mechanisms of protection that is produced. The time to initiation of hypothermia[144], hypothermia level[98] and its duration[136] are all critical factors in limiting injury after CA.

The global effects of hypothermia on metabolism are defined by Q_{10}, which is for the whole human body about 2.0 (i.e. 50% reduction in metabolism per 10ºC decrease in temperature). The Q_{10} for the brain in adults is 2.3[145], but in neonates, infants and children it was determined to be 3.65[146]. Also, Q_{10} is not linear. In a canine model, brain Q_{10} was 2.23 between 37 and 27°C, was doubled to 4.53 between 27 and 14°C[147], and returned to 2.19 below 14°C[148]. In a rat model, overall brain Q_{10} for temperatures 38-28°C was 5.2, with a two-component response: Q_{10} was 12.1 in 38-30°C, and 2.1 in 30-28°C[149]. Those data suggest that Q_{10} is different between species, between age groups, and also between individual organs in one body. Hence, hypothermia may confer different level of protection for the same organ between species, resulting in different outcomes despite similar insults.

Current guidelines endorse extended hypothermia (32-34°C for 12-24 h) for victims resuscitated from CA[15]. In our model, we have used hypothermia for only 6 h. In pilot studies, we have found that longer durations of post-operative hypothermia are not feasible without sedation or neuromuscular blockade - the rats attempt to spontaneously rewarm by shivering, increasing the oxygen consumption. This could potentially aggravate the preceding insult. After the 6 h of controlled hypothermia, we opted to allow the temperature of the rat to drift spontaneously, avoiding active rewarming[150].

There are certain limitations to this study. We used esmolol to ensure that profound hemorrhage resulted in CA. Previous studies have showed that a single dose of esmolol followed by 40 min ischemia did not influence myocardial recovery after reperfusion, when compared to ischemia only.[129] In our protocol, cardioplegic solution was likely to be rapidly

eliminated from cardiac tissue after one min by the large volume of hypothermic flush. Thus, it is likely that our approach provided limited protection that would interfere with the evaluation of novel therapeutic adjuncts to EPR.

HDS was assessed in brain only in survivors excepting for one rat from the 75 min EPR group that died 5 h after resuscitation. Except for this rat, the brains from the rats that died prematurely could not be evaluated histologically due to autolysis. However, the histologic injury in brain does not appear to be appreciably aggravated when compared to survivors from the 75 min group (data not shown).

After the ICU period, we continued to monitor heart rate, but we did not follow other hemodynamic parameters. Thus, we cannot rule out the presence of a period of hypoperfusion in non-survivors that contributed to the aggravated extracerebral organ damage in the 75 min EPR group.

Unlike in our dog model, long-term intensive care was not provided to rats. We cannot rule out that extended intensive care would improve the outcome of the 75 min EPR group, thus allowing assessing the neurologic injury in current non-survivors at the scheduled time of sacrifice. Given this limitation, it would be important to strongly consider testing of promising agents in the definitive dog model.

We acknowledge the fact that starting flush after 5 min HS and one min of no-flow would be challenging in most out-of-hospital clinical scenarios. In hospital, arterial cannulation for insertion of a balloon catheter can be accomplished in less than 5 min[151]. To further investigate clinical applicability of EPR, studies focused on longer duration of CA before initiation of EPR are warranted. Also, targeting deep hypothermic levels requires large amount of fluid that could be a limiting factor for the clinical applicability of the method. Small volume additives that would enable flush volume to be reduced and/or provide further protection would be highly desirable. When testing potential promising therapeutical

adjuncts, we have to consider that pharmacokinetics of many drugs are altered under hypothermia.

In conclusion, rapid exsanguination CA followed by one min of no-flow can be successfully treated by EPR with hypothermic flush, 60 min of asanguineous CA, and delayed resuscitation using a miniaturized CPB allows normal survival. Surprisingly, further extension of asanguineous CA to 75 min is associated with MOF, high mortality and unfavorable neurologic outcome in survivors. This model can be used to study effects of hypothermic preservation and reperfusion on cell death cascades underlying multi-organ injury, as well as to test novel drugs that may enhance the outcome of EPR strategy.

3.5. Study V - Nitration and ribosylation in EPR in rats

Han F, **Drabek T**, Stezoski J, Janesko-Feldman K, Stezoski SW, Clark RSB, Bayir H, Tisherman SA, Kochanek PM
Protein nitration and poly-ADP-ribosylation in brain after rapid exsanguination cardiac arrest in a rat model of emergency preservation and resuscitation
Resuscitation 2008 Nov;79(2):301-310.

3.5.1. Summary

After successfully establishing the rat EPR model (Study III) and testing its limits or survivability (Study IV) we sought to explore what biochemical cascades may play role in the neurologic deficits. We used our previous experience showing that 60 min EPR in rats is achievable with favorable outcome, while 75 min is associated with substantial mortality and impaired neurological outcome in survivors. We hypothesized that 75 min but not 60 min of EPR would be associated with activation of two potential secondary injury cascades in brain as reflected by 1) protein nitration and 2) poly (ADP-ribose) polymerase (PARP) activation. Protein nitration and poly-ADP-ribosylation were assessed by Western blotting and immunohistochemistry for 3-nitrotyrosine and poly-ADP ribose polymers, respectively, in multiple brain regions. As expected, neurologic outcome was better in the 60 min vs. the 75

min EPR group. Nitration and PARP activation were significantly increased in hippocampus, cortex and striatum in the 75 min EPR group vs. other groups. However, there were no differences in cerebellum. Analysis of the full protein spectrum showed significantly increased PARP activation only in hippocampus in the 75 min EPR group vs. other groups. Extending the duration of EPR beyond the limit that can yield favorable recovery in rats was associated with increased nitration and ribosylation of selected proteins in selectively vulnerable brain regions. The impact of these mechanisms on the outcome remains to be determined.

3.5.2. Background

An extensive series of experiments in dog, swine and rat models have demonstrated EPR efficacy and clinical feasibility. The importance of optimizing EPR in experimental models has taken on greater significance. In rats, EPR of 60 min CA is survivable, while 75 min CA is associated with substantial mortality and impaired neurological outcome insurvivors.[152] In this report, we initiated studies to explore the possible molecular mechanisms at the threshold of failure of EPR. Two mechanisms of secondary injury that have been suggested to play important roles in cerebral ischemia are nitration and poly (ADP-ribose) polymerase (PARP) activation. Richards et al.[153] recently reported an important role for nitration of pyruvate dehydrogenase (PDH) and other targets in standard resuscitation after CA, while PARP activation has been shown to have a key role in limiting cerebral recovery across a variety of insults.[154,155] We, thus, hypothesized that nitration and PARP activation would be increased after 75 min CA treated by EPR compared to 60 min EPR or controls. We also explored whether CPB alone was associated with nitration or PARP activation.

3.5.3. Materials and methods

In general, the methods were identical as described in Study IV. For this study, we have also included a control group subjected to a normothermic CPB without HS or CA.

In brief, after rapid lethal HS and 1 min of normothermic no-flow, deep hypothermia (target Tr 15 °C) was induced by a one-way aortic flush. After 60 min (60 min EPR group) or 75 min (75 min EPR group) CA, resuscitation was started with CPB. CPB controls (without exsanguination, flush, or no-flow period) were also studied. Identical doses of KCl and esmolol were used to induce asystole, followed immediately by 60 min of CPB at 34 °C.

OPC and NDS were evaluated at 24. Rats were then euthanized with an isoflurane overdose and perfused with either normal saline (for Western blotting) or with normal saline followed by 10% formalin for immunohistochemistry.

3.5.3.1. Western blotting

Western blots were used to evaluate nitration and ribosylation of proteins. 3-Nitrotyrosine (3NT) is a marker of protein nitration while poly-ADP ribose polymers are a marker of PARP activation. Brain samples were homogenized in lysis buffer containing 0.1 M NaCl, 0.01 M Tris—HCl, 1 mM EDTA, 1 µg/ml aprotinin, 100 µg/ml phenylmethylsulfonylfluoride (PMSF), 1 mM dithiothreitol (DTT) and protease inhibitors, and centrifuged for 30 min at 14,000 g. Supernatants were collected and protein concentration was read using the BCA method (Pierce, IL). Sixty micrograms of protein were run on a 12% SDS—polyacrylamide gel and separated electrophoretically. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, CA) overnight. Membranes were blocked in 5% dry milk and then incubated overnight at 4 °C with the monoclonal primary antibody against 3NT (AB7048-50, AbCam, MA) or antibody against poly (ADP-ribose) (PADPR) polymers (SA-216, Biomol, PA) (1:1000). The activation of PARP was measured by poly-ADP-ribosylation. Membranes were washed for 1 h in 1X PBS containing 0.1% Tween-20 and

then incubated in the appropriate secondary antibody (1:3000) for 1 h at room temperature. Membranes were washed repeatedly for 40 min and then incubated with chemiluminescence reagent and exposed to an X-ray film. Relative optical density (ROD) was quantified using MCID Imaging System (Ontario, Canada).

3.5.3.2. **Immunohistochemistry**

Brain tissues were processed for paraffin embedding. Five-micrometer sections were deparaffinized in xylene, rehydrated through graded alcohols and rinsed in PBS. Endogenous peroxide was blocked for 45 min with 0.3% H2O2. Slides were rinsed and sections were blocked with 5% normal horse serum for 30 min. Sections were incubated with a 1:200 dilution of a mouse monoclonal antibody against either 3NT (AB7048-50, AbCam, MA) or PADPR polymers (SA-216, Biomol, PA) overnight at 4 °C. Sections were rinsed twice for 10 min each and then incubated in a 1:500 dilution of horse anti-mouse IgG antibody conjugated with fluorescein isothiocyanate for 1 h at room temperature. After three 5 min rinses, coverslips were placed on the slides for microscopic viewing.

3.5.3.3. **Statistical analysis**

Data are presented as mean ± standard deviation unless otherwise stated. One-way ANOVA was performed, followed by Student-Newman-Keuls post hoc tests to identify differences between groups in physiological parameters and to evaluate the Western blotting quantification analysis. The chi-square test was used to test the differences in proportions of OPC values between groups (favorable vs. unfavorable outcome, OPC 1-2 vs. OPC 3-5). Kruskal-Wallis test was used to compare NDS between groups. Mann-Whitney U test was used to compare groups if Kruskal-Wallis test indicated an overall effect. Statistical software SPSS for Windows was used. A P value <0.05 was considered statistically significant.

3.5.4. Results

Thirty-four of 41 rats remained in protocol for the duration of the study. One rat from the 60 min EPR group was excluded from the protocol due to failure of the membrane oxygenator. Six rats were excluded from the protocol in the 75 min EPR group due to technical difficulties with either (1) the flush delivery or (2) air embolism.

3.5.4.1. **Physiology**

Tty, MAP and blood gas results are shown in Study V, Figures 1 and 2 and in Table 1. Baseline hemodynamics, hematological and biochemical parameters, acid-base status and body temperature were all in the normal physiological range in all groups. After cooling, Tty decreased to 12.6 ± 1.24 °C in the 60 min EPR group and 12.6 ± 1.00 °C in the 75 min EPR group as expected during preservation. Lactate levels were significantly increased in both EPR groups after CA (controls, 2.4 ± 1.4; 60 min EPR, 7.9 ± 1.2; 75 min EPR, 9.8 ± 2.4 mmol/l). Cardiac activity was restored after 5-10 min of CPB in controls.

3.5.4.2. **Survival and Neurological Outcome**

One rat died at RT 20 h in the CPB control group. One rat died at RT 22 h in the 60 min EPR group with pulmonary hemorrhage at necropsy. Eight rats died in the 75 min EPR group at RT 4-7 h. Neurologic outcome was significantly better in the 60 min EPR vs. the 75 min EPR group (OPC, $P < 0.001$; NDS, $P = 0.001$) (Figures 19 and 20).

	Control	60minEPR †	75minEPR *
OPC 5 (Death)	•	•	••••••••
OPC 4 (Severe disability / Coma)			
OPC 3 (Moderate disability)			••••••
OPC 2 (Mild disability)		••••••	
OPC 1 (Normal)	••••••		

Figure 19 OPC at 24 h after resuscitation. *P < 0.001, 75 min EPR group vs. other groups; † P = NS, 60 min EPR group vs. control group (favorable vs. unfavorable outcome, OPC 1-2 vs. OPC 3-5). Each dot represents one rat.

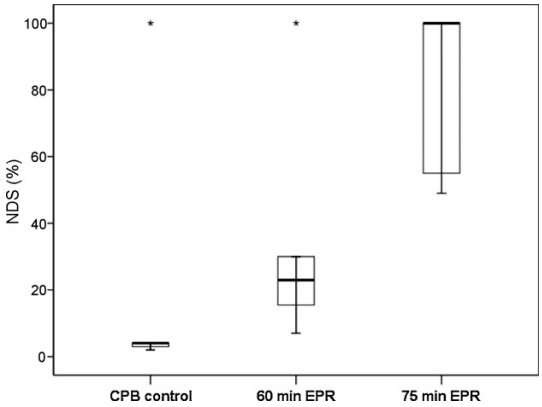

Figure 20 NDS at 72 h after ExCA treated by 60 min or 75 min of EPR. Boxes represent interquartile ranges. The line across each box indicates the median, and the whiskers are the highest and lowest values. The asterisks represent outliers. P < 0.01 75 min EPR groups vs. 60 min EPR group or control group.

3.5.4.3. **Nitration and PARP activation**

Semi-quantification of ROD for Western blotting for 3NT and PADPR is shown in Figure 21. Evaluating the major 64 kD band, nitration and ribosylation increased significantly in hippocampus and cortex in the 75 min EPR group vs. all other groups. In striatum, there was an increase in nitration of 64 kD band compared to CPB controls only. A more modest decrease in global protein nitration and ribosylation was also found in hippocampus in CPB controls, reaching statistical significance for PADPR in cortex. There were no differences in protein ribosylation or nitration in cerebellum both in total protein spectrum analysis and separate analysis of the 64 kD band.

In general, the pattern of changes observed in cerebellum and striatum were similar to those seen in cortex. **Hippocampus showed a unique pattern corresponding to the increasing severity of the insult.**

3NT and PADPR expression were also higher in 75 min EPR group than in other groups by immunohistochemistry which corroborated Western blotting results (Study IX Figures 9 and 10). **3NT and PADPR was predominantly detected in vascular endothelium** by immunohistochemical staining.

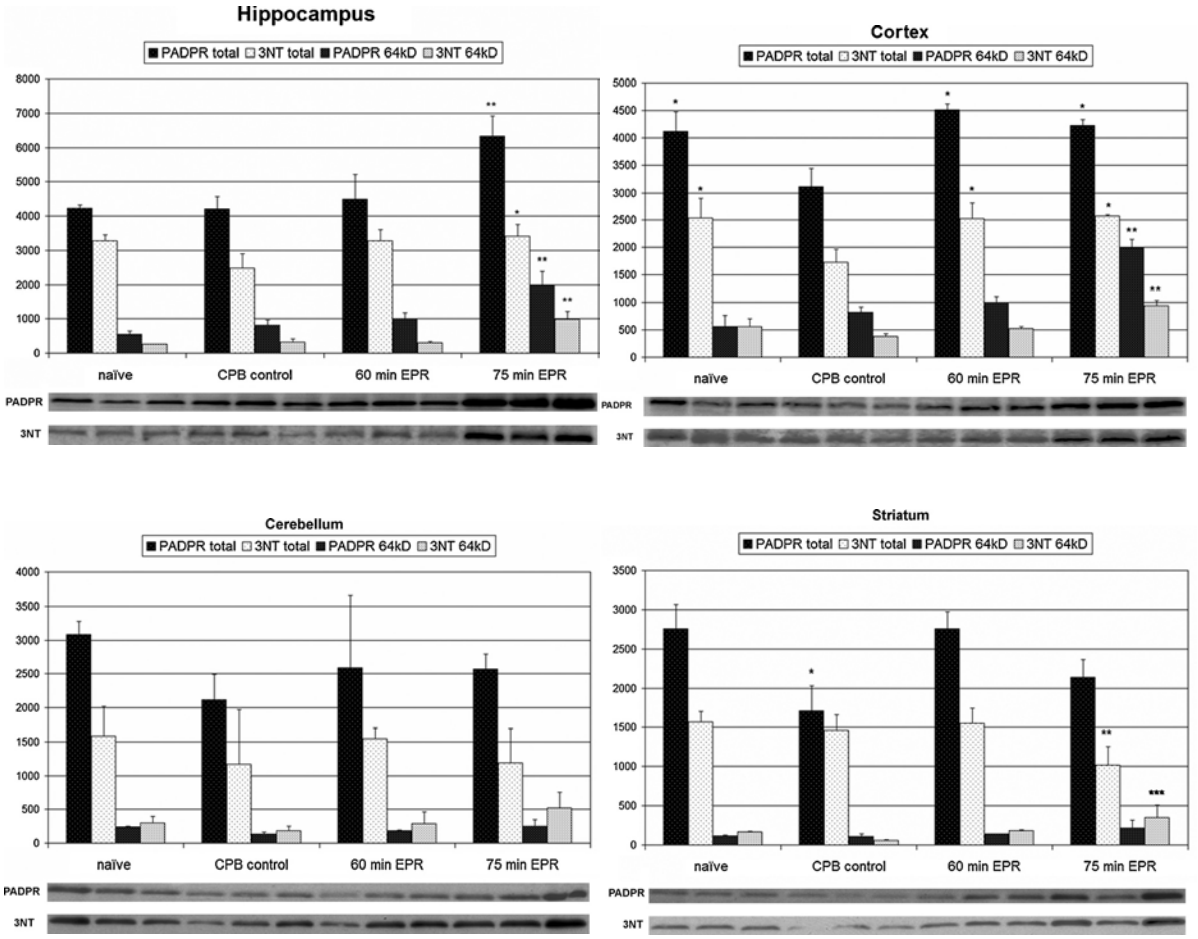

Figure 21 Relative optical densities of Western blotting analyses of nitrotyrosine (3NT) and poly (ADP-ribose) polymers (PADPR) in individual brain regions of each group. **P* < 0.05 vs. naïve and 60 min EPR group; ***P* < 0.05 vs. naïve; ****P* < 0.05 vs. CPB group. Bottom panel shows the Western blot of the 64 kD band. CPB, cardiopulmonary bypass; EPR, emergency preservation and resuscitation.

3.5.5. **Discussion**

In this study, we demonstrated that 75 min of EPR was associated with significant mortality and impaired neurological outcome in rats. Seventy-five minutes EPR was also associated with increases in nitration and ribosylation in brain——consistent with activation of these two secondary injury mechanisms in selected proteins assessed in selectively vulnerable brain regions, namely hippocampus. Our data suggest a possible role for these two important secondary injury mechanisms (nitration and ribosylation) in limiting the successful duration

of EPR that can be tolerated. However, the direct cause-effect of these mechanisms on the outcome remains to be determined in the future studies.

One possibility is that deep hypothermia protects against these secondary injury mechanisms during EPR, but this protective effect was lost when the ischemic time was extended to 75 min EPR in our rat model. Given that we did not see neuronal death in our model, we cannot rule out the possibility that nitration and/or ribosylation could be either protective or an epiphenomenon. Surprisingly, CPB alone was associated with a modest decrease in the degree of nitration and ribosylation compared with other groups. CPB alone has been associated with cognitive deficits and possibly neuronal death in rat models of CPB.[156] CPB alone in dogs was recently shown to activate PARP in gut, with impaired vascular reactivity.[157] Most rats in the 75 min EPR group in this study died between 4 and 7 h after resuscitation and necrotic regions in gut were seen on necropsy (data not shown). Complete histological analysis of gut or other extra-cerebral organs in our study was not carried out given our initial focus on brain injury. It seems that extra-cerebral organs are vulnerable to the effects of prolonged EPR in the rat. However, based on the results from our previously published study using the identical model, we can speculate that the cause of death was probably MOF, with cardiac failure as the terminal event.

Of interest, in an identical model, cardiac lesions were consistent with those seen in the brain—heart syndrome (i.e., cardiac degeneration developing as a result of catecholamine release from damaged brain tissue) but other pathogenic mechanisms may be operating. Immunohistochemistry showed increased 3-NT and PADPR staining mostly in the perivascular regions of the brain. This finding has some support in the literature—Szabo et al. reported reduction of endothelium-dependent vasodilation after 90 min of moderately hypothermic CPB, while PARP inhibitor INO-1001 prevented those effects. Endothelium-independent vasodilatation remained unaffected.[157]

Protective hypothermia, induced and reversed with CPB, is clinically used for some elective operations on heart or brain but has not been performed yet for emergency scenarios such as EPR. Elective therapeutic hypothermia has been shown to protect the brain and entire organism in animals or patients for up to 15 min of CA at brain temperature of about 35 °C (mild hypothermia),[158,159] for up to 20 min of CA at about 30 °C (moderate hypothermia),[160] for up to 30 min of CA at about 20 °C (deep hypothermia),[161] for up to 60—150 min of CA at 5—10 °C (profound hypothermia),[34,37,162-164] and perhaps even for longer CA with ultra-profound hypothermia.[100,165,166]

Treatment induced before arrest (protection) and maintained during arrest (preservation) is more likely to mitigate post-ischemic brain damage than when induced after arrest (resuscitation). Mild hypothermia has been recommended in post-resuscitation treatment of VF CA. The benefit derived from mild hypothermia after ROSC for cerebral recovery has been well documented.[11,125,167-169] Using a mouse model of CA, Abella et al.[125] reported improved outcome when cooling was induced during CA but not after ROSC. Similarly, in a clinically relevant large animal model of CA, survival with full neurological recovery after 40 min of VF was reported if mild or moderate hypothermia was initiated during ROSC attempts, but not after ROSC.[170]

Many potential mechanisms are believed to be responsible for neuronal damage after prolonged circulatory arrest. Nitration is believed to be an important secondary injury mechanism early after the insult. Nitration of protein tyrosine residues results in diverse pathologies. This reaction decreases the p*K*a of the tyrosine hydroxyl group, altering protein structure and function. Protein nitration also results in cytoskeletal damage and disturbances in energy production and signal transduction.[171] Nitration of protein tyrosine residues is a stable modification that can be detected immunochemically. 3NT immunoreactivity has been reported in acute and chronic CNS disease.[172] Several key nitration targets have been

suggested including PDH and manganese superoxid dismutase.[153,173] 3NT immunoreactivity is often localized to neurons, infiltrating neutrophils, and microvasculature after CNS injury.[155,171,172,174,175] Our findings are consistent with a possible role for nitration at the threshold between favorable and unfavorable neurological outcome. It is interesting that both protein nitration and poly-ADP-ribosylation were seen in brain in our model despite the paucity of neuronal death in surviving rats in the 75 min EPR group. It is possible that poor outcome reflects either neurological dysfunction or has an important extra- cerebral component, or is a response to prolonged EPR but has not functional consequence.

A second footprint of oxidative and nitrative injury is activation of the DNA repair enzyme PARP. This enzyme, when activated, adds multiple ribose moieties to proteins which can be detected with antibodies against PADPR. PARP activation may represent a deleterious effect by consuming NAD+ (the PARP Suicide Hypothesis) or via ribosylation of protein targets.[176] A key role for PARP-1 activation after acute brain injury was established using PARP-1 knockout mice, where deletion of PARP-1 was found to confer significant protective effects in histologic and behavioral outcome in several relevant models.[154,155,177]

Our study has some limitations. First, CPB flow rates were not identical between groups given the wide variance in other physiological conditions between CPB control and EPR insults. A possible influence of CPB flow rate on nitration or ribosylation cannot be excluded. Second, immunohistochemistry results showed increase of 3NT and PADPR especially in the 75 min EPR group in brain but not specifically in hippocampus as shown in Western blots.

In our study, we performed a separate analysis of total protein spectrum and separate analysis of the most prominent 64 kD band. A similarly prominent 64 kD band was detected by our group after traumatic brain injury. Using a 2D gel electrophoresis to identify the

protein, the authors hypothesized that it is a mitochondrial heat-shock protein HSP60.[178] It is evident from our study that hippocampus is selectively vulnerable to increased duration in ischemia as reflected by increased ribosylation and nitration in evaluation both total protein and the most prominent 64 kD band, while other regions show more variable injury pattern.

Finally, fluorescent labeling appeared to be predominantly perivascular in origin—— rather than in the vascular lumen. We cannot rule out the possibility of some contribution of autofluorescence by erythrocytes, however, the perfusion-fixation protocol was identical for all groups.

We conclude that protein nitration and PARP activation are seen in brain at the threshold of EPR durations associated with unfavorable neurological outcome. These two mechanisms may represent therapeutic targets to improve outcome in EPR and other CNS insults associated with DHCA.

3.6. Study VI - DADLE in EPR in rats

Drabek T, Han F, Garman RH, Stezoski J, Tisherman SA, Stezoski SW, Morhard RC, Kochanek PM.
Assessment of the delta opioid agonist DADLE in a rat model of lethal hemorrhage treated by emergency preservation and resuscitation.
Resuscitation 2008 May;77(2):220-8.

3.6.1. Summary

We reported previously that 20-60 min EPR in rats was associated with intact outcome, while 75 min EPR resulted in high mortality and neurological impairment in survivors. Given the fact that the cause of death in the 75 min was multifactorial with likely combination of MOF and incipient brain injury, we were looking for a drug that could ameliorate these complex injuries. The delta opioid agonist DADLE ([D-Ala(2),D-Leu(5)]-enkephalin) was shown

previously to be protective against ischemia-reperfusion injury in multiple organs, including brain. We hypothesized that DADLE could augment neurological outcome after EPR in rats. After rapid lethal hemorrhage, EPR was initiated by perfusion with ice-cold crystalloid to induce hypothermia (15°C). After 75 min EPR, resuscitation was attempted with CPB. Three groups were studied: DADLE 0 mg/kg (D0), 4 mg/kg (D4) or 10 mg/kg (D10) added to the flush and during reperfusion. Survival and neurologic outcome (OPC and NDS) and Histological Damage Score (HDS) were assessed in survivors on day 3. In D0 group, 2/10 rats survived, while in D4 and D10 groups, 4/10 and 5/10 rats survived, respectively (p=NS). Survival time (h) was 26.7±28.2 in D0, 36.3±31.9 in D4 and 47.1±30.3 in D10 groups, respectively (p=0.3). OPC, NDS and HDS were not significantly different between groups. In conclusion, DADLE failed to confer substantial benefit on functional or histological outcome in our model of prolonged rat EPR.

3.6.2. **Background**

Neurological outcome after EPR is dependent on the duration of CA and temperature in both large (dog, swine) and small animal models. Given the logistic challenges of inducing hypothermia and potential risks of hypothermia, testing of potential pharmacological adjuncts that would allow extension of the period of CA is warranted.

The delta receptor agonist (D-Ala2, D-Leu5 enkephalin – DADLE) has been recently evaluated as a possible link to hibernation.[179] DADLE possesses organ preservation properties evaluated on liver,[180] heart [181-185] and lungs,[186] or the organs harvested *en bloc*.[187] The vast majority of the experiments revealed positive results, extending ischemic time while preserving post-reperfusion organ function. Neuroprotective properties were also observed in multiple scenarios, including global brain ischemia[188] or pharmacologically-induced brain injury.[189,190] Moreover, DADLE effects were exerted even under hypothermia.[181,186]

In our previous studies, we established a rat model of EPR with excellent survival and neurological recovery after 20-60 min CA.[121] Further extension of the EPR duration to 75 min resulted in high mortality resulting from multi-organ failure, and neurological impairment in survivors.[191]

Using this model, we hypothesized that DADLE would augment survival and neurological outcome after exsanguination CA followed by 75 min EPR. To test this hypothesis, we evaluated two doses of DADLE added to the flush and administered during reperfusion. We assessed survival rate, survival time and neurological outcome as primary outcome measurements. Histological damage score (HDS) served as a secondary outcome measurement.

3.6.3. Materials and methods

We used our previously described model with extended EPR time to 75 min (Study IV).

In this study, rats were randomized into three groups (n=10 per group): DADLE 0 mg/kg (D0), 4 mg/kg (D4) or 10 mg/kg (D10) added to the flush and during reperfusion. Rats in the D0 group received the same volume of vehicle (Plasma-lyte A) as rats in the D4 and D10 groups. Surviving rats were sacrificed on day 3. We used OPC and NDS to evaluate the neurological outcome, and HDS in 35 brain regions to assess histological outcome. Given the high mortality observed across groups, a separate HDS subgroup analysis was performed for those rats that were sacrificed at 72 h and those that died before the planned sacrifice.

3.6.4. Results

There were no differences in hemodynamic or biochemical measurements between the groups at baseline, including MAP, HR, temperature, Hct, arterial pH, pO2, pCO2, base excess, and serum Na, K, Cl, Ca, Mg, urea, or lactate levels.

3.6.4.1. EPR, CPB and ICU Phases

During the flush, temperature decreased in all groups similarly and was controlled at 15 ºC for 75 min. During rewarming, the temperature was gradually increased to 34.5 ºC similarly in all groups. During the CPB phase, HR recovered similarly in all groups. There were no differences in MAP during CPB or ICU phases between groups. The lactate levels were increased markedly vs. baseline in all groups after the insult. Rats in the D0 group had slightly higher lactate levels (p=0.156) (Study 6 Figure 2). There were no significant differences in pH, Na, K, Ca, Mg, Hct, BUN or glucose levels between groups during resuscitation.

3.6.4.2. Survival

Two out of 10 rats survived in the D0 group, while 4 out of 10 and 5 out of 10 rats survived in the D4 and D10 groups, respectively (p=NS). Survival time was 26.7±28.2 h in D0, 36.3±31.9 h in D4 and 47.1±30.3 h in D10 groups, respectively (p=0.315) (Figure 22).

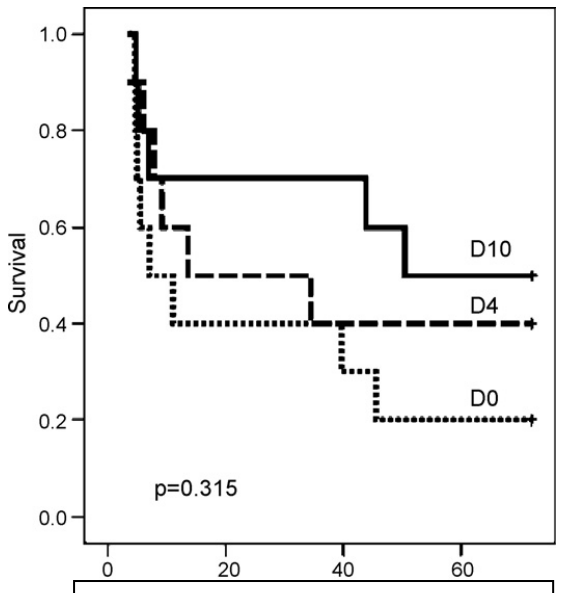

Figure 22 Survival following ExCA treated by EPR. No differences between groups.

3.6.4.3. Neurological Outcome

Few of surviving rats achieved OPC 1 in any group (Figure 23). Six out of eleven survivors remained neurologically impaired (OPC 2-3), and there was no significant

difference between groups for either OPC (p=0.496) or NDS (p=0.515) (Figure 23).

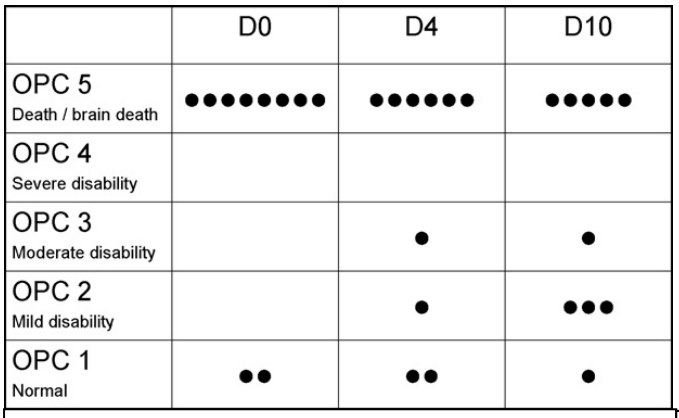

Figure 23 OPC at 72 h after ExCA treated by EPR. Each dot represents one rat. No differences between groups.

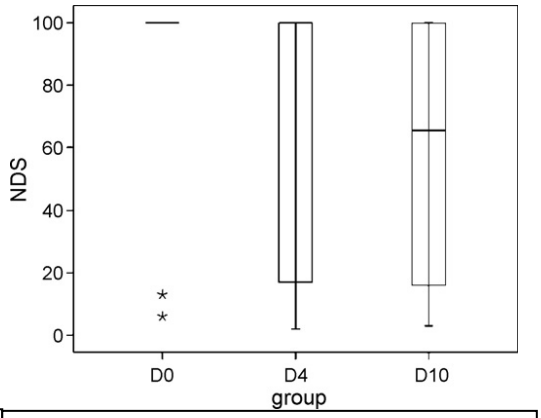

Figure 24 NDS at 72 h after ExCA treated by EPR. Boxes represent interquartile ranges. The line across each box indicates the median, and the whiskers are the highest and lowest values. The asterisk marker represents an outlier. No differences between groups.

3.6.4.4. Histological Evaluation

Inter-group differences in HDS were not found for the brain or for any of the visceral organs. (Table 1) Distribution of degenerative lesions in the brain also did not differ by treatment group.

Table 1 Histological damage score (HDS) after exsanguination cardiac arrest treated by emergency preservation and resuscitation (EPR)

	Brain	Heart	Lung	Liver	Kidney
D0	17 ± 15	2 ± 1	2 ± 1	1 ± 1	1 ± 1
D4	33 ± 39	1 ± 1	2 ± 2	0 ± 1	1 ± 1
D10	26 ± 16	1 ± 2	1 ± 1	1 ± 1	1 ± 2
p	0.38	0.61	0.5	0.26	0.28

Scale: brain, 0–720; extracerebral organs, 0 = no damage, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked and 5 = severe

The most frequent microscopic finding in the brain was peracute to acute neuron degeneration characterized in H&E-stained sections by neurons with bright eosinophilic cytoplasm and nuclei that were shrunken and darkly-stained (Figure 25A). In sections stained with FJB, these dying neurons were a bright yellowish-green color (Figure 25B). Neuron degeneration was most frequently found within various sectors of the hippocampus (especially the CA4 sector) and within the corpus striatum (caudate-putamen and globus pallidus) but, in some rats, also involved portions of the cerebral (especially frontal and piriform) and cerebellar cortices.

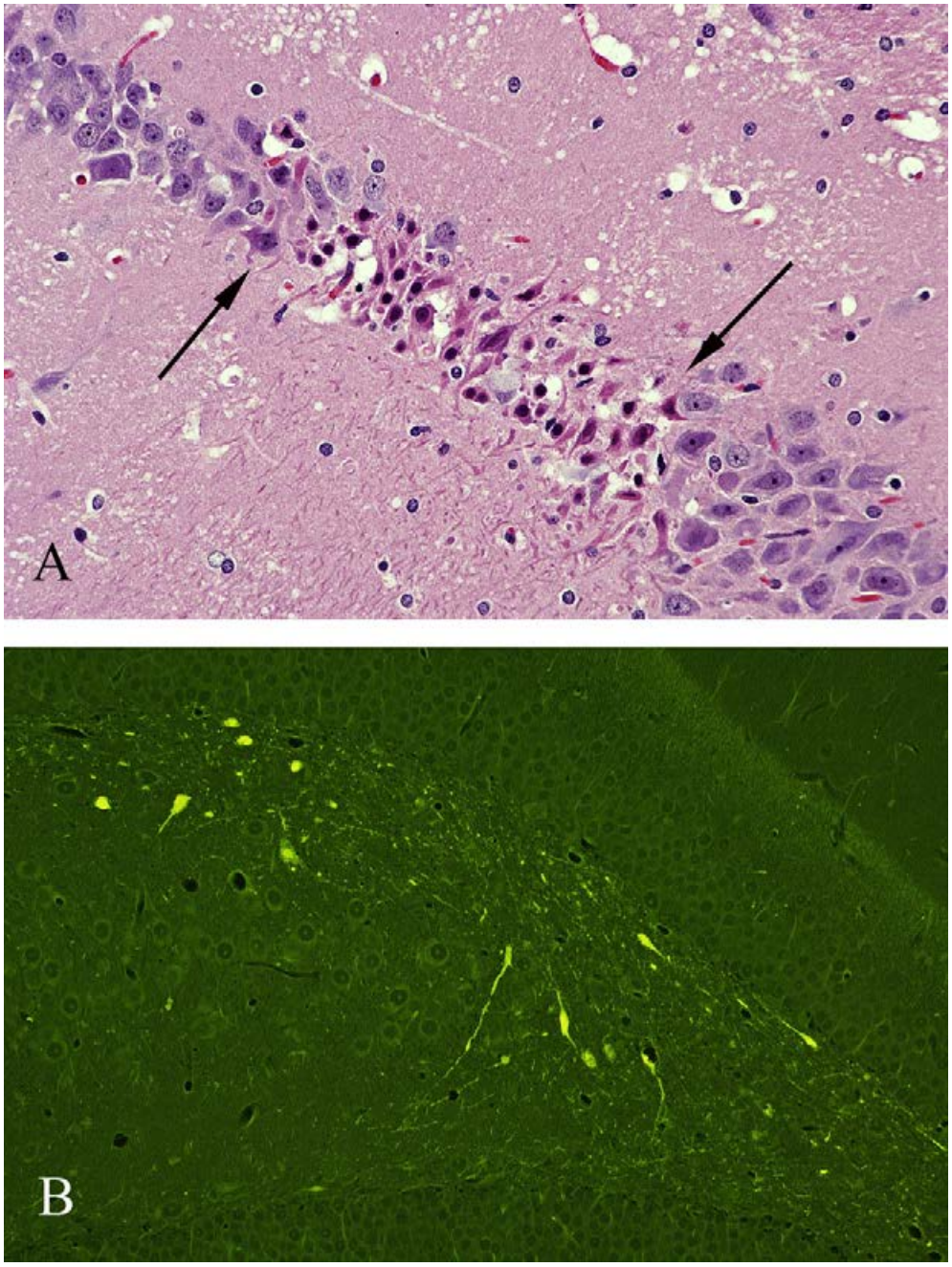

Figure 25 (A) H&E-stained section of the dorsal hippocampus from a rat in the D10 treatment group. (This rat died on study.) Within the CA2 sector of the pyramidal neuron layer (between the arrows), numerous degenerating neurons are present. These neurons have brightly stained eosinophilic cytoplasm and dark condensed nuclei. **(B)** FJB-stained section of the dorsal hippocampus from a rat in Group D4. Within the hilar or CA4 sector (i.e. between the blades of the dentate gyrus), numerous dying neurons are stained a bright yellow-green color. This was the most common pattern of hippocampal degeneration seen in this study.

The principal procedure-related microscopic finding for the heart was peracute to acute myocardial necrosis (Study VI Figure 7A). For the kidneys, the principle finding was

peracute proximal tubule degeneration/necrosis (Study VI Figure 7B) suggesting a pathogenesis of ischemia. In a separate analysis of animals that died before the planned sacrifice, cardiac lesions (i.e. acute cardiac degeneration or myocardial necrosis) were more severe in the D0 group vs. the other groups (D0, 1±1; D4, 0±0; D10, 0±0; p=0.035). However, in the same subgroup analysis, there was a trend for worse HDS in kidneys (tubular degeneration or necrosis) in both D4 and D10 groups (D0, 1±1; D4, 2±1; D10, 2±1; p=0.17).

3.6.5. Discussion

In our model of 75 min EPR associated with high mortality with brain injury and MOF, we considered DADLE as a promising candidate to be tested in our rat EPR model. Despite reports of protection in models of ischemic injury and organ damage, DADLE added during flush and reperfusion in this paradigm yielded no significant benefits to the protective effects of hypothermia in our EPR paradigm.

The delta opioid agonist DADLE, a 44-kDa protein, has been implicated as a novel hibernation-inducing trigger (HIT)[192] that also possesses organ-protective capabilities.[179] The underlying mechanisms of action of DADLE are probably complex. Primarily, DADLE stimulates both delta-1 and delta-2 receptors, and opens potassium-linked ATP (K_{ATP}) channels. Delta receptors activate multiple signaling pathways mediated by various protein kinases, most importantly protein kinase C,[193,194] extracellular signal-regulated kinase (ERK)[195,196] and p38 MAP kinase.[197] Those effects are similar to those elicited by ischemic or anesthetic preconditioning.[181,182,194,198] DADLE also has antioxidant properties.[190,199]

In an isolated rat heart model, DADLE conferred a similar level of protection as that of classic ischemic preconditioning.[181] However, differences between species may exist. In a swine model of cardiac ischemia, DADLE (1 mg/kg iv) did not confer cardioprotection and increased the incidence of arrhythmia during ischemia, while other delta-opioid agonists

showed protective effects.[200] The coadministration of a kappa-antagonist and DADLE was cardioprotective, and arrhythmogenic effects were completely abolished. Studies in an isolated working swine heart model also reported no protection from DADLE.[201]

DADLE added to Euro-Collins solution enhanced hypothermic lung preservation in rats in a living-rat lung transplant model.[186] Similarly, pre-treatment with DADLE protected against ischemia-reperfusion injury in hepatocytes, but not in the sinusoidal endothelial cells of the liver in rats. An anti-oxidative effect was suggested to mediate this effect.[180] In a multi-organ block preparation for long-term organ preservation (heart and lungs, liver, pancreas, kidneys, and a small portion of intestine en bloc),[202] DADLE (1 mg/kg q 2 h) extended survival time.[187]

Neuroprotective properties of DADLE have been tested in several models. Pretreatment with DADLE (2 and 4 mg/kg) dose-dependently blocked methamphetamine-induced long-term dopamine transporter loss.[190] In vitro, pretreatment with DADLE (0.0025, 0.005 and 0.01 g/ml) dose-dependently enhanced cell viability of cultured primary rat fetal mesencephalic cells. In addition, DADLE administration (4 mg/kg i.p. four doses 2 h apart) in adult rats prior to 6-hydroxydopamine lesions of the medial forebrain bundle significantly reduced the severity of neuronal loss.[203] In contrast, DADLE (0-16 mg/kg 30 min before the insult) in a rat model of bilateral carotid occlusion combined with hypotension showed no benefit of any dose on neurologic recovery or hippocampal neuron loss.[204] This could be partially explained by the reportedly poor BBB penetration of DADLE.[205]

Moreover, recent studies showed that another delta-opioid agonist pentazocine, acting via delta receptors and K_{ATP} channels, improved outcome and post-resuscitation myocardial performance in a rat CA model.[206,207]

We have observed no, or possibly only a very limited, effect of DADLE on the outcome variables, including post-resuscitation hemodynamic and biochemical data. Previously, DADLE (1 mg/kg) was reported to produce a 50% reduction in the arterial blood pressure in dogs, most likely due to vasodilatation. We did not observe any hemodynamic effects of DADLE in our study. Lactate levels were the lowest in the D10 group at all time points, while the highest levels were observed in the D0 group. However, statistically this was only a trend.

Histologically, the principle treatment-related changes in visceral organs were degeneration and/or necrosis of the cardiac muscle fibers and, similarly, degeneration/necrosis of tubular epithelial cells within the kidney. The renal lesions could represent the results of ischemia (*e.g.* secondary to the cardiac lesions) or could be related to CPB. However, for those rats in which degenerative lesions were present in both the heart and kidneys, the cardiac lesions were considered to be of slightly greater duration. While some rats with renal degeneration did not have evidence of a concurrent cardiac injury, the cardiac lesions were often small in size. Furthermore, since only two sections (one transverse and one longitudinal) were prepared from each heart, some degenerative foci could have been missed.

There are limitations to this study. We did not determine drugs levels in blood or target tissues. The BBB penetration of DADLE was shown to be limited.[205] While the integrity of BBB in our study was not assessed, BBB has been shown to be dysfunctional after normothermic CPB and/or DHCA in infant lambs,[208] suggesting that BBB might be permeable for DADLE in our paradigm. Nevertheless, injury was seen with and without treatment both in and outside of the CNS.

The dose regimen of DADLE used in our study was adopted from previous studies. We chose to use the intravenous route instead of more commonly used intraperitoneal route of administration, to obtain rapid onset of action. Also, we have repeated the same dose only in the immediate post-resuscitation period without extending the therapy further. Unfortunately, no detailed studies on the metabolism of DADLE exist. The only limited information is provided in a study of multi-organ block preservation, which showed rapid accumulation of DADLE in the liver and subsequently in the heart. Two hours later, DADLE was excreted in bile and in urine. No accumulation of DADLE was seen in lungs, where the protective effects were maximally pronounced.[187] Using those limited data, we assumed that DADLE is rapidly eliminated, at least under normothermic conditions.

Most of the studies focused on preconditioning and/or exploring different cell signaling pathways used pre-insult administration of DADLE over variable time periods, up to 48 h before the insult. Continuous infusion of DADLE was shown to induce hibernation in summer-active ground squirrels, in a similar time-dependent manner as a single injection of a known hibernation-induction trigger (HIT), plasma from hibernating woodchucks. Typically, it took 4-7 days for the first animals to start hibernating, with the maximal incidence of hibernation at 21 days, mirroring the effect of HIT[192].

Thus, the timing of DADLE administration in our study might not be optimal in terms of providing sufficient time for the drug to induce protection. On the other hand, pre-treatment in the CA models would lack clinical relevance. However, another study testing the delta-receptor agonist pentazocine showed benefit even after a single injection of the drug during resuscitation.[206]

A longer duration of CA before starting the flush (representing warm ischemia) might be needed to model the trauma scenario more realistically. It has been shown previously in a clinical setting that cannulation of a femoral artery can be accomplished in less than 5 min[151].

In our study, a number of rats died before their scheduled necropsy time points. The lack of prolonged intensive care could play a role here. The cause of death in many rats remained unclear. While some of the brains fixed post-mortem were relatively well preserved and showed little evidence of postmortem alteration, other brains were characterized by moderate degrees of postmortem autolysis. This made scoring these brains for neuronal degeneration difficult. Therefore, for some of these brains, the degrees of neuronal degeneration might be higher than actually indicated in the HDS. The model could be producing more extracerebral damage that could mask a neurological effect. Previous EPR studies in large animal models have shown that adding trauma resulted in more frequent and prolonged requirement of intensive care, and worse short term neurological outcome despite normal brain histopathology[38]. In a swine model of vascular, splenic and colon injuries, induction of profound hypothermia preserved the viability of key organs during repair of lethal injuries. After transient neurological decline, long-term neurological outcome was similar despite escalating complexity of injuries and without any considerable increase in postoperative complication rates[209]. The addition of extracerebral trauma to our model could aid in assessing the potential for clinical translation of new therapies.

We did not assess biochemical markers of organ injury that could serve as additional outcome variables to assess organ damage further in our model studying the effects of DADLE. This would have required maintaining intravascular access with tunneled catheters to allow serial sampling. In order not to influence our primary outcome parameter - i.e. neurological outcome - we chose to perform histological evaluation to assess organ damage. In a separate series of experiments using preventive administration of DADLE (0, 4 or 10

mg/kg i.v., n=6 per group) 30 min before deep hypothermic circulatory arrest (90 min at 15°C), ALT, AST, creatinine, CPK and troponin were increased 2 h after CPB in all groups vs. their respective baselines ($p<0.05$ for all). While no differences in ALT, AST and CPK between groups existed, creatinine and troponin were significantly more increased in the DADLE 10 mg/kg group vs. other groups ($p=0.011$ and 0.039, respectively). It is possible that the higher dose of DADLE exhibits some degree of toxicity.

Using deeper levels of hypothermia during preservation period[50] and/or addition of energy substrates[210] acting via other pathways than opioid receptors could further enhance the protection during CA, and improve outcome.

In conclusion, in our model of prolonged CA treated by EPR, DADLE failed to confer benefit on either functional or histological outcome. Future studies are needed to evaluate other potential adjuncts to hypothermia in the EPR paradigm.

3.7. Study VII – Deep vs. moderate hypothermia and minocycline in EPR in rats

Drabek T, Tisherman SA, Beuke L, Stezoski J, Janesko-Feldman K, Lahoud-Rahme M, Kochanek PM
Deep hypothermia attenuates microglial proliferation independent of neuronal death after prolonged cardiac arrest in rats
Anesth Analg 2009 Sep;109(3):914-23.

3.7.1. Summary

In previous studies we have systematically explored the effect of extending duration of EPR on the neurologic outcome, and explored some of the underpinning mechanisms. As expected, the extended durations were characterized with high mortality and neurologic impairment in survivors. We observed a relative lack of isolated neurologic injury even in

prolonged deep hypothermic preservation. We also considered the concerns that induction of EPR may not be realistically possible within one minute as used in our previous models, and that induction of deep hypothermia (15 °C) may not be always clinically feasible. Thus, we decided to modify our model in two ways to induce substantial neurologic injury without aggravating extracerebral organ damage: first, we have increased the duration of normothermic CA prior to the induction of hypothermic flush from; second, we have explored if higher intra-arrest temperatures would provide us with a model with substantial neurologic deficits. We have explored two different flush temperatures, e.g. ice-cold flush and room-temperature flush, to induce intra-arrest hypothermia, without surface cooling. We hypothesized that higher intra-arrest temperatures would be associated with worse neurologic and histologic damage. Minocycline, an antibiotic with anti-inflammatory properties, was then tested as an adjunct to the room-temperature flush. We hypothesized that minocycline would attenuate neuroinflammation that independently confer secondary neurologic damage, outside neuronal death. After 5 min HS and 5 min CA, room-temperature (RT) flush induced moderate hypothermia (28 °C), while ice-cold (IC) flush induced deep hypothermia (21 °C). Minocycline added to the RT flush (RT-M) did not alter the intra-arrest temperature.

Neurologic outcome was improved with deep vs. moderate hypothermia, with an extensive hippocampal neuronal death in corpus ammonis zone 1 (CA1) and dentate gyrus (DG), similar at both hypothermia levels. Surprisingly, microglia proliferation as a marker of neuroinflammation was improved with deep vs. moderate hypothermia. Minocycline did not confer additional benefits. We concluded that neuroinflammation may act as an independent mechanism of a secondary neuronal injury contributing to worse outcome early after the insult.

3.7.2. Background

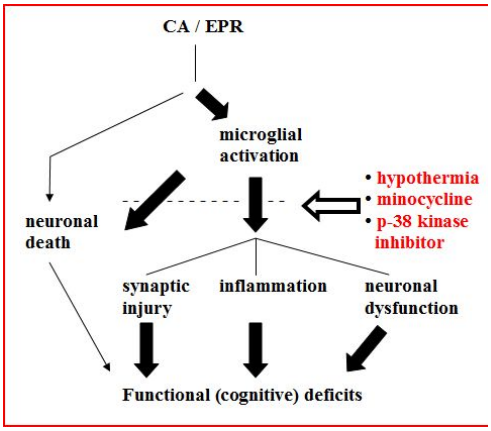

Figure 26. Inflammation-mediated synaptic injury and/or neuronal dysfunction could contribute to the cognitive deficits even in the absence of overt neuronal death. Pharmacological modulation of microglial intervention in the cascade of events that lead to neuronal necrosis may help to improve the structural and functional outcome following CA.

While the early brain injury in CA is initiated by energy failure and resultant neuronal death cascades, microglial activation has been suggested to be an additional mechanism of delayed neuronal death, most likely through releasing neurotoxic substances (Figure 26).[211] Pharmacological modulation of microglial proliferation may help to improve outcome following CA. Recently, studies in several CNS insults have shown benefit from treatment with minocycline, an agent that attenuated microglial activation and proliferation.[212]

We hypothesized that deeper levels of intra-arrest hypothermia would improve functional outcome, attenuate neuronal death and attenuate microglial proliferation compared to more moderate hypothermia. We also hypothesized that minocycline would further augment the hypothermic protection via attenuating microglial activation, neuronal death and improve outcome.

3.7.3. Materials and methods

We have used our previously described model of EPR (Study IV) with following modifications: (1) the duration of normothermic CA was extended from 1 min to 5 min before the initiation of the flush; (2) surface cooling was not utilized; (3) the total duration of CA was decreased to 20 min.

Three groups were studied: 1) Ice-cold (IC) flush group (n=7), 2) room-temperature (RT) flush group (n=9), and 3) room-temperature flush group followed by minocycline treatment 20 mg/kg 1 h after resuscitation and 90 mg/kg i.p. at 24 and 48 h (RT-M, n=11).

Rats in the RT and IC groups received the same volume of vehicle (phosphate buffered saline, PBS).

Final OPC and NDS were assessed as previously described at 72 h after resuscitation.

3.7.3.1. Histology

The tissue samples were processed for embedding in paraffin. The resulting paraffin blocks were sequentially sectioned at 5 micrometers. All sections were stained with FJC to detect neuronal degeneration.[213] For the Iba-1 staining detecting microglia, sections were washed in PBS, incubated in 0.3% H_2O_2 in methanol for 30 min to inhibit endogenous peroxidase activity, washed in PBS, and blocked in PBS containing 1.5% normal goat serum and 1% BSA for 2 h at RT. The sections were then incubated with a rabbit anti-Iba1 polyclonal antibody (1:500, Serotec) overnight at 4°C, washed in PBS, and incubated with a FITC-conjugated goat anti-rabbit IgG antibody (Invitrogen) for 2 h at room temperature. For control staining, normal rabbit IgG was used as the primary antibody. After the reaction, the sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI), dehydrated in ethanol steps, and mounted.

Adjacent sections obtained at approximately 4.3 mm from bregma were used for assessing neuronal death and microglial activation within the selective brain regions. A photograph of representative sections of dentate gyrus and CA1 region was taken under 10 x magnification. FJC positive neurons and Iba-1 positive activated microglia (characterized by ameboid cell body and retracted processes without thin ramifications)[214] were then counted using National Institutes of Health Image-J software by an observer masked to the treatment group.

3.7.3.2. Statistical analysis

Repeated measures ANOVA was performed, followed by Student-Newman-Keuls post hoc tests, to identify differences in hemodynamic and arterial blood gas parameters and temperature between groups. One-way ANOVA was used to compare histologic damage among groups. The chi-square test was used to test the differences in proportions of OPC among groups. Kruskal-Wallis H test was used to compare NDS among groups. Mann-Whitney U test was used to compare two groups if Kruskal-Wallis H test indicated differences between groups existed. Pearson and Spearman tests were used to test for correlations between variables as appropriate. A P value < 0.05 was considered statistically significant.

3.7.4. Results

After cooling, rats in the IC group had significantly lower temperature during CA vs. other groups (tympanic, 21 °C vs. 28 °C; rectal, 20-25 °C vs. 27-30 °C, P<0.001) (Fig. 27).

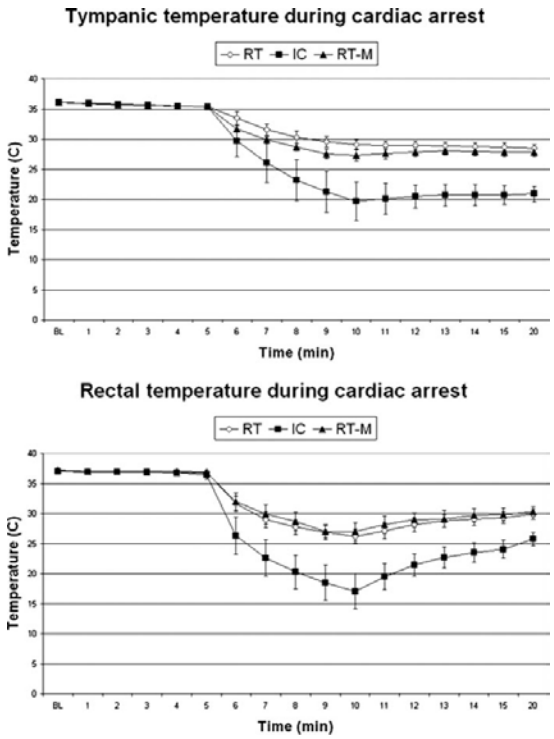

Figure 27. Tympanic and rectal temperatures during CA. P < 0.001 ice-cold (IC) flush group versus other groups.

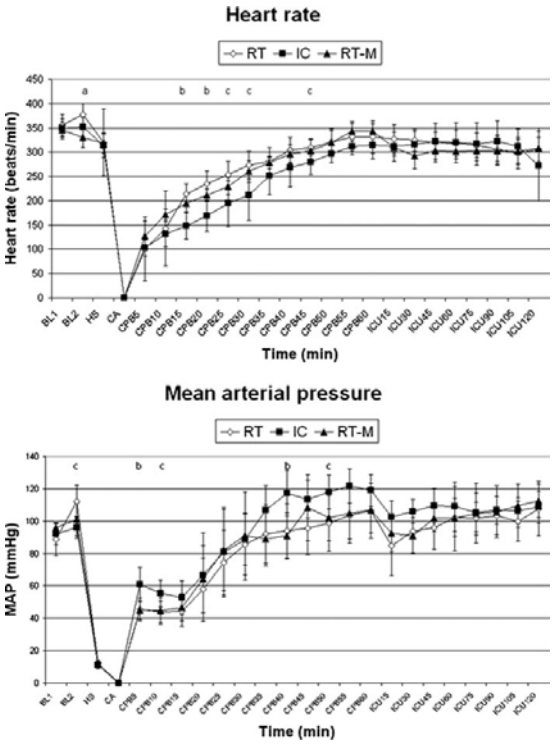

Figure 28. HR (top panel) and MAP (bottom panel). a=P<0.05 room-temperature (RT) flush vs. room-temperature flush followed by minocycline (RT-M) group; b=P<0.05 ice-cold (IC) vs. RT and RT-M groups; c =P < 0.05 RT vs. IC group.

Rats in the IC group had higher MAP during flush (35±5 vs. 27±6 mmHg, $P<0.05$). HR increased more slowly during resuscitation in the IC vs. RT group ($P<0.01$) (Fig. 28, top panel). MAP was higher in the IC group vs. RT group over time ($P<0.05$; Fig. 28, bottom panel). After discontinuation of temperature control, body core temperature increased more rapidly in the IC group. This steady increase reached statistical significance vs. RT group but not RT-M group ($P<0.05$ IC vs. RT group, Study VII Fig. 3). All groups achieved normothermia at 24 h after resuscitation. While pH and base excess were similar at 5 min after the start of resuscitation, lactate was lower in the IC group vs. other groups ($P<0.05$) (Study VII Table 1). Survival rate was not different between groups. Neurological outcome was significantly better in the IC vs. other groups ($P<0.05$) (Table 2, Fig. 29).

	RT	IC	RT-M
OPC 5 Death	●●●	●	●●●●●
OPC 4 Severe disability			
OPC 3 Moderate disability	●●●●		●●
OPC 2 Mild disability	●●	●●	●●●
OPC 1 Normal		●●●●	●

Table 2. OPC after 20-min CA treated with EPR. No differences among groups in survival rate ($P> 0.05$). Favorable neurological outcome (assessed by OPC) was significantly better in the IC group versus other groups ($P < 0.05$). IC = ice-cold flush group; RT = room-temperature flush group; RT-M = RT flush followed by minocycline treatment group. Each dot represents one rat.

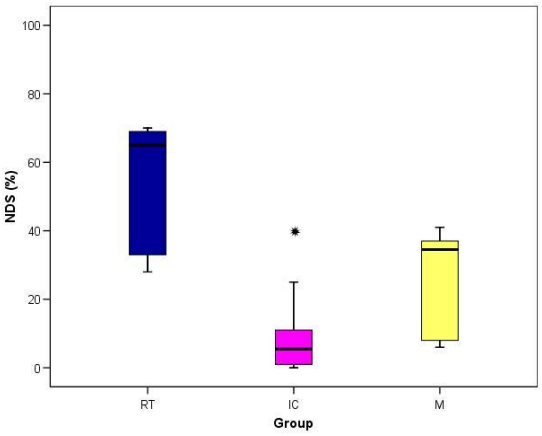

Figure 29. NDS after 20-min CA treated by EPR. Boxes represent interquartile ranges. The line across each box indicates the median, and the whiskers are the highest and lowest values. *$P <$ 0.05, room-temperature (RT) versus ice-cold (IC) group.

The markers of microglial activation but not neuronal death were attenuated in the IC vs. other groups (Fig. 30-32). Selective vulnerability of CA1 neurons and hilar neurons in the

dentate gyrus was observed (Fig. 30 and 31), with proliferated microglial cells with ameboid shaped cell bodies and shortened, retracted processes. Microglial activation was attenuated in the IC group (Fig 30, panel E, Fig 31, panel E). We found a positive correlation between OPC and neuronal death (r=0.566, *P*=0.018). Similarly, we found a correlation between FJC and Iba-1 (r=0.513, *P*=0.035). In contrast, there was only a trend for a correlation between Iba-1 and neurologic outcome (Iba-1 vs. OPC, r=0.345, *P*=0.176; Iba-1 vs. NDS, r=0.393, *P*=0.119). (Figure 32)

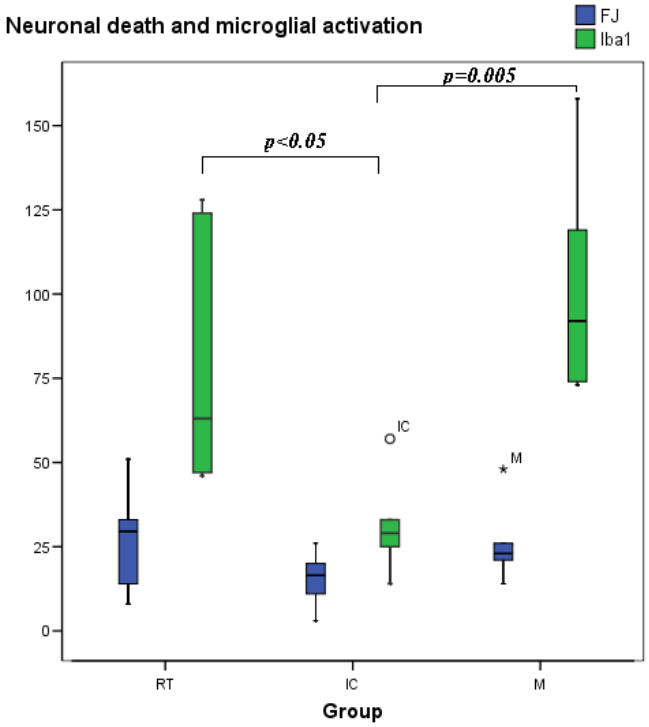

Figure 32. Neuronal death and microglial activation after 20-min CA treated with EPR with either room-temperature (RT) flush, ice-cold (IC) flush, or room-temperature flush followed by minocycline treatment (M) in the dentate gyrus region of hippocampus. The line across each box indicates the median, and the whiskers are the highest and lowest values. The round marker and the asterisk represent outliers of the respective groups.

We performed a formal necropsy on all rats that died before completion of the study. However, we were not able to determine the cause of death in all rats. The common findings were pulmonary edema and/or lung hemorrhage. The timepoints of death were between 15 and 45 h of resuscitation time in all groups (RT, 29 h 29 min ± 14 h 5 min; IC, 13 h 4 min, RT-M 23 h 56 min ± 7 h 28 min).

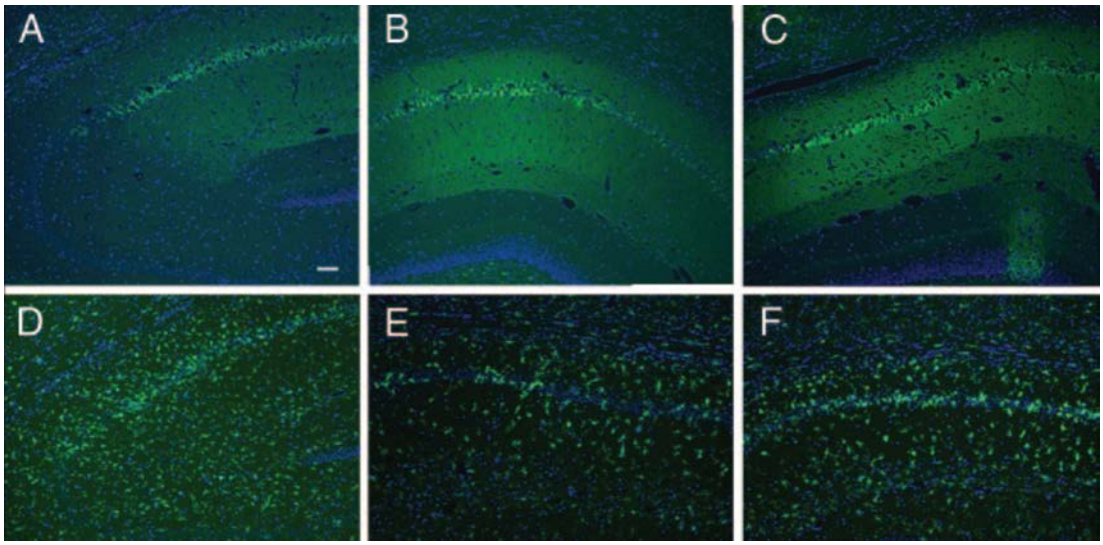

Figure 30. Neuronal death and microglial proliferation after ExCA and EPR with either room-temperature (RT) or ice-cold (IC) flush in the **CA1 region of hippocampus.** Blue staining is DAPI, identifying neurons, and green staining is FJC, Panels A–C, identifying dying neurons, or anti-Iba-1 staining visualizing microglia (Panels D–F). Microglial activation is attenuated in the IC group. Representative samples from each group are shown. A, 10X: Hippocampal neuronal loss in a rat from the RT group. Full CA1 loss. B, 10X: CA1 region in a rat from the IC group. Intensive neuropil staining between CA1 and dentate gyrus (DG). C, 10X: Hippocampal neuronal loss in a rat from RT-M group. D, 10X: Microglial activation in CA1-CA2 regions of hippocampus in a rat from the RT group. E, 10X: Microglial activation was attenuated in a rat from the IC group. F, 10X: Microglial activation was marked in a rat from RT-M group despite high dose minocycline treatment. Scale bar in Panel A = 80 μm.

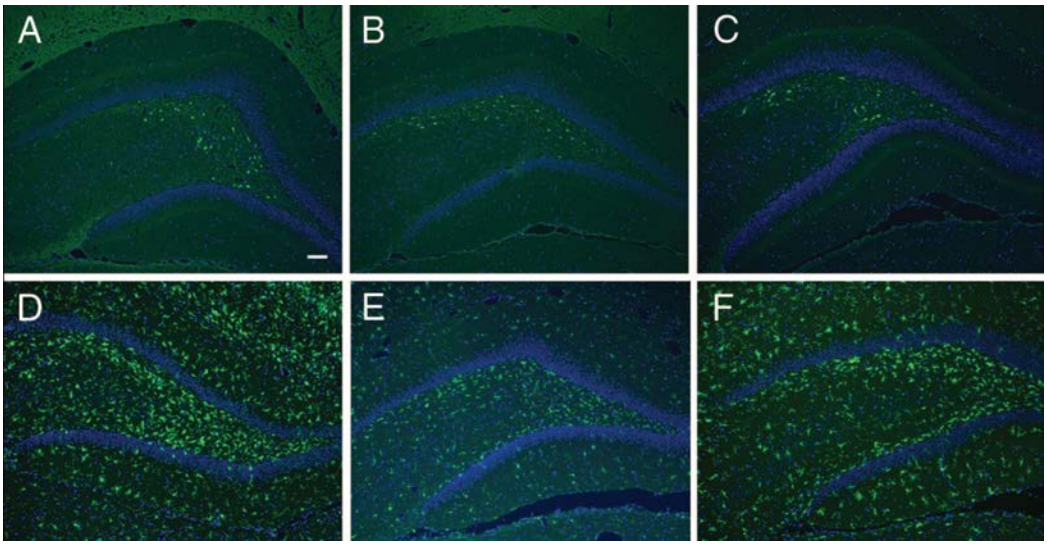

Figure 31 Neuronal death and microglial activation after ExCA and EPR with either room-temperature (RT) or ice-cold (IC) flush in the **dentate gyrus (DG) region of hippocampus**. Blue staining is DAPI, identifying neurons, and green staining is FJC, identifying dying neurons (Panels A–C), or anti-Iba-1 staining visualizing microglia (Panels D–F). Microglial activation (Panels D–F) is attenuated in the IC group (Panel E). Representative samples from each group are shown. Hippocampal neuronal loss in DG. Hilar neurons are selectively injured in all groups (A, 10X, RT group; B, 10X, IC group; C, 10X, RT-M group, respectively). D, 10X: Microglial activation in hippocampal DG in a rat from the RT group. E, 10X: Microglial activation was attenuated in a rat from the IC group. F, 10X: Microglial activation was marked in a rat from RT-M group despite high dose minocycline treatment. Scale bar in Panel A = 80 μm.

3.7.5. Discussion

Traditionally, DHCA has been used in cardiac surgery to provide a bloodless field and enable repair of the congenital cardiac malformations or acquired pathologies with considerable success. Hypothermia for DHCA is used in a protective rather than a therapeutic fashion; the use of hypothermia in EPR represents much more challenging situation. In resuscitation of ExCA, including EPR, the rapid onset of cooling can only be initiated after a period of normothermic CA. Adjuncts to hypothermia would thus be of great potential benefit. Recently, we modified the rat EPR model to produce a screening tool to study mechanisms of neuronal death and evaluate novel therapeutic adjuncts to hypothermia. Also it should be recognized that the use of a normothermic control group is not feasible since the rats would not survive the insult if maintained normothermic throughout the period of emergency preservation.

In our paradigm, we use 5 min of HS followed by ice-cold or room-temperature flush initiated 5 min after CA. This represents a clinically relevant delay that would allow cannulation of a large vessel.[151] Flushing with either IC or RT saline resulted in a brain temperature of 21 or 28 °C, respectively. Better protection achieved after IC flush was likely reflected by lower lactate levels at 5 min after reperfusion and better neurologic function in survivors at 72 h. The latter was not affected by minocycline treatment. Despite the functional benefit with IC flush, neuronal death seen in traditionally selectively vulnerable brain regions did not differ between groups. We also noted robust microglial activation surrounding the dying neurons. Surprisingly, deep hypothermia (21 °C) was able to attenuate microglial activation but not neuronal damage. It is possible that hypothermia-induced attenuation of microglial activation contributes to the improved neurologic outcome in the IC group.

After discontinuation of the post-operative hypothermia, rats in the IC group spontaneously rewarmed more quickly than rats in other groups. The attenuation of microglial activation in the IC group thus could not be explained by unintentional prolonged post-operative hypothermia.

While the early brain injury in CA is believed to result from release of excitatory mediators, energy failure, oxidative stress, damage to mitochondria and endoplasmic reticulum, and cell signaling pathway disturbances in neurons, secondary damage could also be triggered by microglia, that transform into phagocytes. Microglial activation starts immediately after ischemia and thus precedes the morphologically detectable neuronal damage.

Microglial activation has been suggested to contribute to delayed neuronal death, most likely through releasing neurotoxic substances, including reactive oxygen radicals, nitric oxide, and pro-inflammatory cytokines.[211] Microglial activation could contribute to neuronal death or microglial-mediated synaptic injury and/or neuronal dysfunction – which could mediate cognitive deficits even in the absence of overt neuronal death. Additional studies focused on these secondary injury mechanisms in our model are warranted. Microglia could also have a protective role,[215-219] possibly in delayed repair after injury via elaboration of growth factors. Thus, there may be a specific time window for benefit from inhibition of the early microglial contribution to damage. Recent studies also suggested that the severity of neuronal injury determines microglial release of "toxic" versus "protective" effectors.[216] To visualize microglia, we chose to use anti-Iba-1 staining. Iba-1 is a calcium-binding protein expressed specifically in activated microglia,[220] with its peak occurring at 4–7 days after injury.[221] While resident microglia exist in a ramified state, after brain injury they migrate toward the lesion, their cell body becomes ameboid-shaped, the processes shorten and become virtually undistinguishable from macrophages.

Minocycline is a widely used antibiotic with anti-inflammatory and anti-apoptotic properties which has been tested in several models of neurologic injury, including global[222-224] and focal brain ischemia,[225-228] traumatic brain injury,[229,230], spinal cord injury,[231,232] and intracerebral hemorrhage.[233] Most recently, minocycline showed favorable results in a clinical trial in acute stroke patients.[234] It penetrates the blood-brain barrier,[235] reduces tissue injury and improves functional recovery.[222,236,237] The primary effect of minocycline is probably inhibition of activation of microglia.[222,223,225,231,238] Surprisingly, minocycline was also reported to be more protective than brief hypothermia after focal cerebral ischemia.[227,228] Specifically, inhibition of p38 MAP kinase activation in microglia has been suggested as a key mechanism underlying minocycline anti-inflammatory effects, although other mechanisms may also be involved.

In preliminary studies, we did not observe a beneficial effect with a lower dose of minocycline (3 mg/kg IV followed by 45 mg/kg i.p.; data not shown). Thus, we chose to use the high-dose minocycline (20 mg/kg IV followed by 90 mg/kg i.p.), used previously by others in similar settings.

In our study, hypothermia attenuated microglial activation. Temperatures used in our study (21-28°C) were generally lower than used in other studies of mild-to-moderate hypothermia. Post-ischemic hypothermia (32°C for 24 h) suppressed microglial activation after hypoxic-ischemic injury in the developing brain.[239] Even a brief period of hypothermia (33°C for 2 h) attenuated neuroinflammation after experimental stroke and brain inflammation induced by intravenous injection of lipopolysaccharide (LPS).[240] A similar effect of hypothermia was observed in microglial cell cultures stimulated by LPS.[241]

While many studies utilized minocycline as an agent suppressing microglial activation, we did not see any effect of minocycline on microglia activation or neuronal

death. This striking lack of effect could be potentially explained by the fact that minocycline was added to augment the protective effects of pre-existing moderate hypothermia (28°C). It is possible that minocycline could not add further benefit to hypothermia. Moderate hypothermia in the 28°C group was limited to the intra-ischemic time, followed by mild hypothermia for 6 h. Previous studies suggested that the onset of microglial activation starts at 24 h and peaks at 4 to 7 days.[221] In our study, we administered minocycline up to 72 h. However, we cannot rule out that hypothermia delayed or modified the course of microglial activation, and therefore the dosing regimen or assessment time were not optimal.

The lack of effect of minocycline in our EPR paradigm is not entirely surprising. Previously, we tested 14 pharmacological adjuncts to hypothermia. Using our similar moderate-hypothermia canine model with 20 min CA, only the antioxidant tempol showed some benefit.[56]

Recently there has been growing line of evidence suggesting neuroprotective role of microglia in CNS pathologies. Selective ablation of microglial cells before cerebral ischemia *in vivo* revealed a marked neuroprotective potential of proliferating microglia, serving as an endogenous pool of neurotrophic molecules such as IGF-1.[217] Microglia cells were also shown to protect neurons by direct engulfment of invading neutrophil granulocytes, that infiltrate ischemic lesions, in an *in vitro* model.[219] Despite robust microglial proliferation, we have not however observed neutrophil accumulation in our model.

In our study, NDS assessments were not very tightly coupled to hippocampal cell loss. Rats in the IC group that achieved favorable OPC and NDS scores still had substantial neuronal injury. An advanced neurobehavioral testing will be needed in further experiments to define the association between hippocampal neuronal cell loss, microglial activation and neurocognitive outcome. Our exploratory study was focused on the histological markers of

injury. Previously we have shown that motor deficits observed in this complex model persist up to day 7.[242] This would require delaying the period of water maze tests till after day 7. The time of completion of water maze tasks would then fall out of the peak microglial activity.

We have observed a significant correlation between neuronal death and neurological outcome. Given the limited numbers of animals in our exploratory study, we could demonstrate only a trend for correlation between microglial activation and neurologic outcome. We cannot rule out that injuries in other brain regions or extracerebral injuries played a role and influenced the neurologic outcome.

In conclusion, deeper levels of hypothermia compared to moderate hypothermia (21 °C vs. 28 °C) induced by aortic flush resulted in better neurologic outcome in survivors. Surprisingly, hypothermia attenuated microglial activation but not hippocampal neuronal death. Minocycline did not improve either neurologic outcome or attenuate microglial activation in brain. Our preliminary findings suggest a potentially novel effect of hypothermia on microglial activation during deep hypothermia. Further studies with comprehensive neurobehavioral testing will be needed to further elucidate the role of microglia on functional outcome.

3.8. Study VIII – Blood-brain barrier integrity in rats

Lahoud-Rahme M, Stezoski J, Kochanek PM, Melick J, Tisherman SA, **Drabek T**
Blood-brain barrier integrity in a rat model of emergency preservation and resuscitation
Resuscitation 2009 Apr; 80(4):484-488.

3.8.1. Summary

The effect of drugs in our EPR model has been limited. One of the explanations for the lack of effect could be a limited transport of the tested agents across the blood-brain barrier (BBB). The permeability of the BBB in our model is unknown. In this study, we hypothesized that BBB will not be disrupted even in models that are associated with poor outcome. We chose to study the permeability to Evans Blue (EB) in EPR models that are associated with poor outcome, i.e. (1) 75 min CA with deep hypothermic preservation (please see Study IV), and (2) 20 min CA with moderate hypothermic preservation after extended period of normothermic no-flow (please see Study VII). We also included (3) a positive control group subjected to traumatic brain injury (TBI) and (4) a negative control. Rats in EPR groups were subjected to rapid hemorrhage, followed by CA, and delayed resuscitation via CPB within the EPR paradigm. Rats in the TBI group had a controlled cortical impact to the left hemisphere. Naïves were subjected to the same anesthesia and surgery. One hour after the insult, rats were injected with EB, a marker of BBB permeability for albumin. Rats were sacrificed after 5 h and EB absorbance was quantified in brain samples. TBI produced an approximately 10-fold increase in EB absorbance in the left (injured) hemisphere vs. left hemisphere for all other groups (p=0.001). In contrast, EB absorbance in either EPR group did not differ from sham. We conclude that BBB integrity to albumin is not disrupted early after resuscitation from prolonged CA treated with EPR. Neuroprotective adjuncts to hypothermia in this setting should focus on agents that penetrate the BBB. These findings also have implications for DHCA.

3.8.2. Background

The most vulnerable organ during CA is the brain. Although hypothermia is a potent neuroprotective intervention and the key platform for EPR, current research is focused on the development of adjuncts to hypothermia, toward additional neuroprotection. It is unclear,

however, whether BBB penetration is a requisite for therapies added during the induction of hypothermic preservation in EPR.

The BBB is a complex system with transport and metabolic functions. Barrier breakdown occurs with stroke, head trauma, sepsis, exposure to inflammatory mediators, among many other causes. Remarkably, the status of the BBB in both CA and deep hypothermia remains unclear.[208,243-246] In this study, we sought to investigate the BBB status in our EPR rat model, and we chose specifically two EPR paradigms that were associated with mortality and neurologic impairment in survivors.[152,247,248] One includes prolonged CA under deep hypothermia (15 $^{\circ}$C) and one includes brief CA under only moderate hypothermia (28 $^{\circ}$C). These paradigms thus represent scenarios where adjuncts to hypothermia are needed. In addition, we chose a traumatic brain injury (TBI) model with known disruption of the BBB as a control. We hypothesized that the BBB would be disrupted after TBI, but intact after prolonged hypothermic circulatory arrest.

3.8.3. Materials and methods

Four groups were studied:

1) EPR-RT (room temperature) flush (n=4), 5 mins of no flow, hypothermia (28 ºC) initiated using room temperature flush. The total duration of CA was 20 min.

2) EPR-IC (ice-cold) flush (n=3), 1 min of no flow, hypothermia initiated using ice-cold flush to target a temperature of 15 ºC. The total duration of CA was 75 min.

3) TBI (n=3), controlled cortical impact (CCI) at normothermia.

4) Sham (n=3), subjected to the same anesthesia and surgery, and maintained normothermic.

For EPR groups, the respective protocols have been described previously (please refer back to Study VII and Study IV, respectively).

3.8.3.1. TBI protocol

TBI (n=3): Isoflurane anesthetized rats were endotracheally intubated and mechanically ventilated similarly to the EPR groups, then positioned in a stereotaxic frame (David Kopf, Tujunga, CA), and a 6x6-mm left parietal craniotomy was made by using a high-speed dental drill. They were then subjected to CCI using a pneumatically driven 3-mm metal impactor tip at a velocity of 6 m/sec and a depth of penetration of 1.2 mm.[249] The rats were kept normothermic.

3.8.3.2. BBB Studies

To determine BBB integrity, after CPB or 1 h after TBI or 1 h after cannulation in shams, rats were injected intravenously with 2% Evans Blue (EB) at 4 ml/kg (Sigma).[250] EB was allowed to circulate for 5 h. The animals were then perfused with 200 ml of ice cold normal saline 0.9%, through the left cardiac ventricle at a pressure of 40 mmHg until colorless fluid was obtained from the right atrium. Afterwards, the brains were removed and dissected. Each hemisphere was weighed and the samples were placed in 1 ml of formamide and stored for 72 h in the dark. The supernatant was spun for 10 min at 7000 rpm. The absorbance of the supernatants for EB dye was measured at 620 nm with a spectrophotometer (DU 800 UV/Beckman Coulter). EB dye content is expressed as ng/g of brain tissue against a standard curve.

In addition, we performed separate experiments in sham, EPR-IC and TBI group (n=1 per group) to assess macroscopic penetration of EB into the brain tissue. After completion of the respective protocol, the rats were sacrificed and perfusion-fixed with 200 ml of 10%

formalin. After 72 h, the brains were removed from the skull and 1 mm thick coronal slices were prepared.

Comparison of groups was performed using one-way ANOVA with post hoc Tukey's test. Values of $p < 0.05$ were considered statistically significant.

3.8.4. Results

Results are summarized in Figure 33. EB absorbance in either EPR group did not differ from sham. In contrast, TBI resulted in obvious EB extravasation as visualized in a photograph of coronal brain sections taken through the dorsal hippocampus (Fig. 34). TBI produced an approximately 10-fold increase in EB absorbance in the left (injured) hemisphere at 5 h after injury vs. left hemispheres of all other groups (p=0.001), and a more modest albeit readily detectable increase in the right (uninjured) hemisphere vs. EPR-IC or EPR-RT groups (p<0.05).

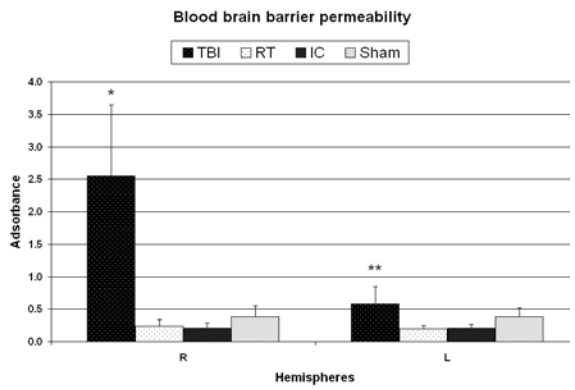

Figure 33. Absorbance, reflecting Evans Blue concentration in rat brain TBI, EPR–RT flush (RT), EPR-IC flush (IC) and shams. TBI produced a significant increase in EB absorbance in the left hemisphere 5 h after injury vs. left hemispheres of all other groups (*p = 0.001), and a more modest albeit readily detectable increase in the right (uninjured) hemisphere vs. EPR-IC or EPR-RT groups (**p < 0.05). Y bars represent 1 standard deviation of the measurements.

Figure 34. Photograph of coronal brain sections at the level of the dorsal hippocampus that reveal obvious Evans Blue extravasation after TBI (left panel), but not in EPR-RT flush (central panel) or sham groups (right panel).

3.8.5. Discussion

The blood vessels of the brain have a unique barrier function that ensures an optimally controlled homeostasis of the brain's internal environment. Disruption of the BBB is seen in

many conditions such as sepsis, TBI and stroke, among other conditions.[251] This allows passage of neurotransmitters,[252] hormones and large proteins that might be a contributing factor to the evolution of secondary injury process – such as inflammation and edema.[253] Our EPR model is complex, since it includes exsanguination, CA, DHCA and CPB. Review of the literature revealed conflicting reports of the status of the BBB in large animals and in rodents who underwent insults that represent one or more of the interventions that constitute our EPR model.[208,243,244,254-256]

Remarkably few studies have addressed the BBB interruption in HS, CA, DHCA, or even CPB – and much of that work is in large animal models. Initial studies in pigs showed no changes in BBB permeability after 2 h of normothermic CPB,[243] or 3 h of normothermic non-pulsatile CPB.[256] Using pulsatile CPB, minute foci of extravasated serum proteins appeared.[256] In a follow-up study comparing 2 h of hypothermic vs. normothermic CPB in a pig model, neither technique induced significant changes in BBB permeability.[244] The extent of brain damage is also CPB flow- and pressure-dependent – either modest decreases in CPB flow (from 70 to 50 ml/kg.min) or even high flow at lower MAP were associated with increased BBB permeability to serum proteins.[257] On the contrary, Cavaglia et al showed universal BBB dysfunction after 15-120 min normothermic CPB in infant lambs, further aggravated by superimposing 2 h of DHCA.[208] In humans, cerebral swelling was observed after both normothermic and hypothermic (28 °C) CPB using magnetic resonance imaging.[258,259] Thus, the anticipated findings with regard to BBB permeability from CPB alone are controversial.

The surrogate marker of BBB integrity, S-100β protein, an astroglial cell marker, has been recently monitored in CPB and DHCA. Increased serum concentrations of S-100β were found immediately after reperfusion and termination of CPB following 60 min DHCA in a

rabbit model, suggested a reperfusion injury to the astroglial cell complex that forms the BBB.[260] Serum S-100β also correlated well with the histopathologic outcome after prolonged DHCA in pigs.[261] This technique potentially allows one to advance the assessment of BBB function after CPB or DHCA to the bedside. How increases in S-100β compare to albumin/EB permeability, however, are not clear. It is certainly possible that release of S-100β could occur at a level of insult below the threshold for detection of albumin permeability.

However, it has been shown that other extracerebral organs release S-100β when subjected to ischemia. In a Langendorff isolated heart model, both S-100β and troponin increased after cardiac ischemia.[262] Previously we have documented in our hypothermic CA model a marked increase in cardiac troponin at 3 h after reperfusion (baseline, 0.45±0.71 ng/mL, post-reperfusion 11.43±5.10 ng/mL; $p<0.01$).[263] While S-100β could be an additional end-point in our study, it would be difficult to precisely interpret the data in our complex model.

In experimental CA, BBB permeability was not observed at 4 h after a 10 min normothermic CA in dogs.[264] Surprisingly, studies in a pig model showed preserved BBB integrity in the initial post-CA phase, but delayed BBB permeability.[265] In a mouse model, a brief CA did not result in BBB permeability to albumin.[245] In a study using rat global ischemia model, BBB permeability was disrupted early after reperfusion (up to 6 h) but restored at 24 h.[266] However, germane to our findings, the BBB disruption could only be detected with a small-molecule tracer (amino-isobutyric acid), but not with EB. Studies in a global cerebral ischemia model in a rat showed no difference of EB extravasation vs. sham at 40 min, but significant differences could be found at 24 and 48 h.[250] This was associated with

poly (ADP-ribose) polymerase activation, which was also shown to be increased in our 75 min EPR model.[267]

The few studies addressing the integrity of BBB during HS revealed that the BBB is disrupted during "decompensated" phase of HS, i.e., MAP 40 mmHg. A significant increase in BBB permeability was observed using sodium fluorescein, but not EB.[255]

In contrast, BBB disruption is well-recognized after TBI, and this was confirmed in our current work.[268,269]

In our rat EPR model, we have previously shown that a 60 min DHCA is well tolerated with CPB resuscitation.[152] However, intact neurological outcome is not achievable for insults of 75 min or beyond. Thus we chose to explore a 75 min insult in this initial work to examine the BBB given that it is beyond the threshold for good outcome. Our data suggest that BBB permeability does not contribute to the poor outcomes after 75 min of EPR.

We also did not assess BBB permeability at after longer follow-up periods (beyond 5 h). While some studies in DHCA show immediate disruption of BBB, we may speculate that BBB disruption could be delayed in the setting of prolonged post-resuscitation hypothermia. Increased BBB dysfunction could be detected in rats subjected to 10 min CA even after 2 months,[254,270] or 1 year.[271,272] Given the conflicting data in CA previously discussed, assessment at more delayed time points after DHCA is warranted. However, if resuscitative therapies are needed to be added to the flush and used for neuropreservation, agents that are permeable to an intact BBB are likely to be required. Using our canine model, we have previously tested fourteen of the most promising agents based on their potential role in preventing or ameliorating neuronal damage after ischemic injury. In accordance with our current results, only the BBB penetrating antioxidant tempol showed benefit.[31,56]

To study the status of BBB integrity, a variety of BBB markers have been described. In circulation, EB is tightly bound to albumin, thus serving as a readily identifiable tool that has been used in hundreds of studies of BBB.[244,250,256,269,271,273] Given the track record for use of EB as a BBB marker and the inflammation known to be associated with use of CPB,[244,256] we believe that albumin was the appropriate first approach to assess BBB in our model. However, other smaller molecules, such as amino isobutyric acid,[274] or gadolinium-based agents[274] could be more sensitive to subtle injury. In our study we found that BBB integrity to albumin is not disrupted early after resuscitation from prolonged CA treated with moderate or deep hypothermic EPR.

The positive control used in this study is represented by the TBI model, that shows extensive disruption of BBB, as shown in Figure 33, that quantifies the disruption, and in Figure 34, that allows visual comparison of the injured vs. non-injured hemisphere vs. representative samples of brains in the EPR models. Although the number of the animals per group was small, we were able to demonstrate dramatic differences between groups and germane to the question of our ability to detect subtle BBB injury with the sample size used, we detected a modest increase in BBB permeability in the hemisphere contralateral to the injury in the TBI group vs. EPR groups with an n=3-4 per group. Of note, in the hemisphere contralateral to impact in our TBI model there is no neuronal death and routine neuropathology reveals a normal appearance.[275] Thus, we were able to detect BBB permeability with statistical significance in our study even in brain regions with rather subtle damage. We believe that this strongly supports our findings even with a modest sample size.

We acknowledge the fact that this model may not represent the typical pre-hospital setting in which prolonged hemorrhage and/or no-flow time before intervention could be anticipated. However, data from military setting suggest that two-thirds of wounded soldiers

died within five minutes from the injury.[23] Previously, we have shown that prolonged HS (over 120 min) before CA does not prevent successful resuscitation using EPR.[136]

If needed, cannulation of a femoral artery could be established in less than 5 mins,[151] and cannulation of a saphenous vein in less than 1 min,[276] allowing rapid initiation of hypothermia. Alternative approach via thoracotomy could also be considered. Obviously, the overall success of resuscitation is related to the delay between CA and onset of hypothermia.[144,277] Other factors like depth of hypothermia, duration and reperfusion strategies may also play a role.

Our results could also be applied to the cardiac surgery setting, in which DHCA is used to facilitate complex repair of aortic pathologies without preceding ischemic insult.

In conclusion, resuscitation from ExCA with EPR using deep or moderate hypothermia does not increase BBB permeability to albumin. Our data suggest that BBB damage does not appear to contribute to the impaired outcome in these models. In addition, the development of neuroprotective adjuncts to hypothermia that are designed to enhance the outcome of EPR and/or DHCA should focus on agents that penetrate the BBB.

3.9. Study IX – Clodronate-induced depletion of microglia

Drabek T, Janata A, Jackson EK, End B, Stezoski J, Vagni VA, Janesko-Feldman K, Wilson CD, van Rooijen N, Tisherman SA, Kochanek PM
Microglial depletion using intrahippocampal injection of liposome-encapsulated clodronate in prolonged hypothermic cardiac arrest in rats
Resuscitation 2012 April; 83(4):517-26.

3.9.1. Summary

Hippocampal neuronal damage was similar after EPR at moderate (28 °C) vs. deep (21 °C) intra-arrest hypothermia. However, neurologic outcome was improved with deeper

level of hypothermia. This was associated with attenuated microgliosis. This led us to a speculation that activated microglia could be an independent factor aggravating neurologic injury. Pharmacological modulation of microglia may improve outcome following CA. Liposome-encapsulated clodronate (LEC) is a macrophage-depleting drug when administered systemically. Given the links between macrophages and microglia, we hypothesized that intracerebrally injected clodronate could deplete microglia, and provide us with a model that would elucidate the role of microglia in post-CA neuroinflammation. To test the hypothesis that intrahippocampal injection of LEC would attenuate local microglial proliferation after CA in rats, we administered LEC or PBS into the right or left hippocampus, respectively. After rapid exsanguination and 6 min no-flow, hypothermia was induced by ice-cold (IC) or room-temperature (RT) flush. Total duration of CA was 20 min. Pre-treatment (IC, RTpre) and post-treatment (RTpost) groups were studied, along with shams (cannulation only) and CPB controls. On day 7, shams and CPB groups showed neither neuronal death nor microglial activation. In contrast, the number of microglia in hippocampus in each individual group (IC, RTpre, RTpost) was decreased with LEC vs. PBS by ~34-46% ($p<0.05$). Microglial proliferation was attenuated in the IC vs. RT groups ($p<0.05$). Neuronal death did not differ between hemispheres or IC vs. RT groups. Thus, intrahippocampal injection of LEC attenuated microglial proliferation by ~ 40%, but did not alter neuronal death. This suggests that microglia may not play a pivotal role in mediating neuronal death in prolonged hypothermic CA. This novel strategy provides us with a tool to study the specific effects of microglia in hypothermic CA.

3.9.2. Background

Prolonged CA results in neuronal death and a reactive glial response. Specifically, microglial activation and proliferation has been linked to delayed neuronal death, presumably

via releasing neurotoxic substances, including reactive oxygen radicals, nitric oxide (NO), and pro-inflammatory cytokines.[211] Microglial activation could contribute to neuronal death or microglial-mediated synaptic injury and/or neuronal dysfunction – which could mediate cognitive deficits even in the absence of overt neuronal death.

Microglia could also have beneficial effects, contributing to delayed repair after injury via elaboration of growth factors,[217] or their presence could represent an epiphenomenon. The effect of microglia could also depend on the severity of the primary insult, resulting in neurotoxicity vs. neuroprotection. Thus, there may be a specific time window for benefit from inhibition of the microglial contribution to damage, as well as specific scenario in which inhibiting microglia could be helpful. Therapeutical modulation of the microglial response for insults even less than the threshold for neuronal death may help to improve outcome following global brain ischemia.[278]

Pharmacological modulation of microglial proliferation may help to improve outcome following CA. Recently, studies in several CNS insults have shown benefit from treatment with minocycline, an agent that attenuated microglial activation and proliferation.[212,279]

LEC is an agent that – when used systemically – depletes macrophages[280] and has been shown to deplete microglia *in vitro*, including brain slices.[281] In brain ischemia, however, the local inflammatory response is predominated by microglial rather than macrophage accumulation.[282] Since LEC does not cross the BBB, we hypothesized that intraparenchymal injection of LEC into the brain would selectively deplete microglia and attenuate hippocampal neuronal degeneration.

3.9.3. Materials and methods

We used the rat EPR model described in detail previously (Study IX Figure 1).[283]

Five groups were studied: 1) rats pre-treated 24 h prior to CA and subjected to deep hypothermia during CA using ice-cold (IC) flush (IC, n=6); 2) rats pre-treated 24 h prior to CA and subjected to moderate hypothermia during CA using room-temperature (RT) flush (RTpre, n=3); 3) rats injected 24 h after CA and subjected to moderate hypothermia during CA using RT flush (RTpost, n=3); 4) shams (n=4), subjected to the same cannulations and duration of anesthesia; 5) CPB controls (n=3), subjected to the same cannulations, anesthesia and 60 min of normothermic CPB.

3.9.3.1. Intrahippocampal Injections

Adult male Sprague-Dawley rats (350-375 g) were obtained from Hilltop Lab Animals (Scottdale, PA) and housed for at least three days before the experiment under 12-h light/dark cycle with unrestricted access to food and water. Rats were anesthetized with 4% isoflurane in a transparent acrylic jar. After tracheal intubation with a 14 G intravenous catheter (Becton Dickinson; Sandy, UT), rats were mechanically ventilated using a piston ventilator (Harvard Ventilator Model 683, Harvard Rodent Apparatus; South Natick, MA) with a tidal volume of 0.8 ml/100 g and a frequency 36-42/min to maintain normocapnia, and a positive end-expiratory pressure (PEEP) of 4 cmH_2O. Anesthesia was maintained with 1.5-2% isoflurane in FiO_2 0.5.

Using a stereotaxic frame, burr holes (diameter 0.45 mm) were created bilaterally (-4.3 mm dorsoventral, -2.0 mm lateral from bregma). A 27 G needle was then inserted 3.5 mm deep into the hippocampus. Each rat received simultaneous intrahippocampal injections of either 5 µL of liposome-encapsulated phosphate-buffered saline (PBS) (left hemisphere) or 5 µL of LEC (right hemisphere) over 10 min via a 27G needle connected by a polyethylene tubing to a 10 µL Hamilton syringe (Hamilton, 701 N) and an infusion pump (Harvard Apparatus; South Natick, MA). Clodronate was encapsulated in liposomes as described

previously.[284] Using a different treatment in each hemisphere, each rat served as its own control. After a 3 min additional period with the needle in place to allow distribution of the compound, the needle was withdrawn at the rate of 1 mm/min to prevent leakage through the burr hole.

In addition, we tested if intrahippocampal injections of 10 µl would cause an increase in intracranial pressure (ICP), and thus potentially alter our model. ICP was monitored in selected rats (n=4) via a 1 French intraparenchymal ICP probe (SPR-1000; Millar Instruments, Houston, TX) inserted from a separate burr hole in the frontal lobe. The ICP monitoring was discontinued and the probe was withdrawn after the completion of the injections.

After completion of the injections and ICP monitoring, the burr holes were sealed with a bone wax and skin was closed by layers using 2.0 silk. Anesthesia was discontinued; rats were extubated and allowed to recover in the cage.

Rats underwent the EPR protocol similar to Study VII, and sacrificed at 7 days after resuscitation.

3.9.3.2. Histology

The tissue samples were processed for embedding in paraffin. The resulting paraffin blocks were sequentially sectioned at 5 micrometers. All sections were stained with FJC to indicate neuronal degeneration[213] and with anti-Iba-1 staining visualizing microglia as described previously (see Study VII and Study IX section Methods).

In addition, colorimetric visualization of Iba-1 immunostaining using diaminobenzamide (DAB) (Vector, CA) was used as a secondary confirmatory method to visualize microglia. In short, sections were processed the same as for fluorescent labeling on

day 1, using a 1:250 dilution of anti-rabbit Iba-1 overnight at 4 °C. Sections were washed with TBST, incubated at RT for 1 h with a biotinylated anti-rabbit IgG, followed by 1 h of avidin-biotin complex binding using an ABC kit (Vector, CA). Sections were washed and incubated for 10 minutes with DAB followed by hematoxylin counterstaining. Tissue was dehydrated, cleared and coverslipped for microscopic analysis. For control staining, normal rabbit IgG was used as the primary antibody.

Adjacent sections obtained at approximately 4.3 mm from bregma were used to assess neuronal degeneration and microglial proliferation within the CA1 region of the hippocampus (Fig 35). A photograph of the representative section of the CA1 region was taken under 10 x magnification. FJC positive neurons and Iba-1 positive activated microglia (characterized by ameboid cell body and retracted processes without thin ramifications)[214] were then quantitated morphometrically by two independent researchers (KJ, CDW) in a CA1 region of the hippocampus marked in Fig. 34 using the National Institutes of Health Image-J software. No automated features of the software were used. Image-J was used solely to track the cell counts and provide a controlled feedback between the independent evaluators.

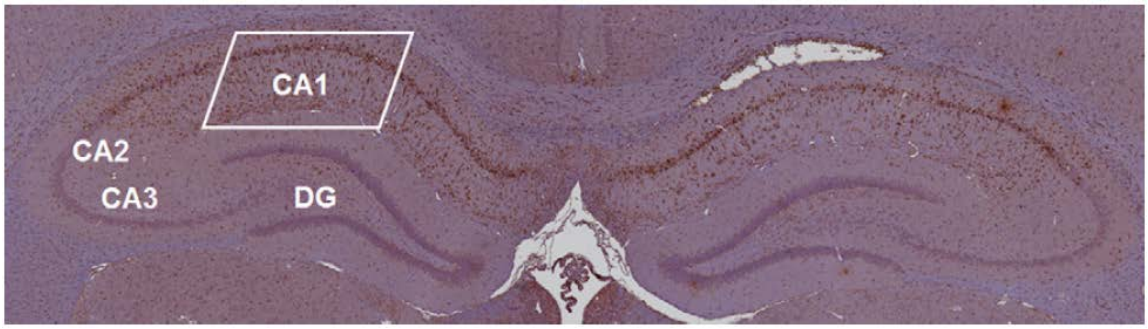

Figure 35. A microscopic pan-scan of both left and right hippocampi with identification of its main regions. A rectangle in the CA1 area shows the area of interest where cell counts were performed. The DAB staining used in this slide shows activated microglia. CA, cornu ammonis; DG, dentate gyrus.

3.9.3.3. Statistical analysis

Repeated measures ANOVA was performed, followed by Tukey post hoc tests, to identify differences in hemodynamic parameters and temperature between groups. For the aforementioned comparisons, data from RTpre and RTpost groups were pooled since they did not differ. One-way ANOVA was used to compare histologic damage, biochemical and hematologic data between groups. Kruskal-Wallis H test was used to compare NDS among groups. Mann-Whitney U test was used to compare two groups if Kruskal-Wallis H test indicated differences between groups existed. A P value < 0.05 was considered statistically significant.

3.9.4. Results:

There were no differences in baseline characteristics between individual study groups. Induction of hypothermia IC flush resulted in lower intra-arrest temperature compared to the RT groups (P < 0.05 IC vs. RT). Physiologic parameters (HR, MAP) and temperature profiles during resuscitation are shown in Study IX Figures 3-6. Prolonged CA in IC and RT flush groups resulted in marked physiologic derangements in the acid-base status with extremely low pH, BE up to -20, and increased lactate up to 6 mmol/L. These changes were gradually improved during resuscitation and were largely ameliorated by the end of the ICU phase. Minimal changes between groups were observed after 7 d (Study IX Table 1).

All rats improved in neurologic status over time but only rats from both RTpre and RTpost groups exhibited persistent neurologic deficits (P < 0.01 vs. IC, shams or CPB group, respectively; OPC, Table 2; NDS, Figure 36).

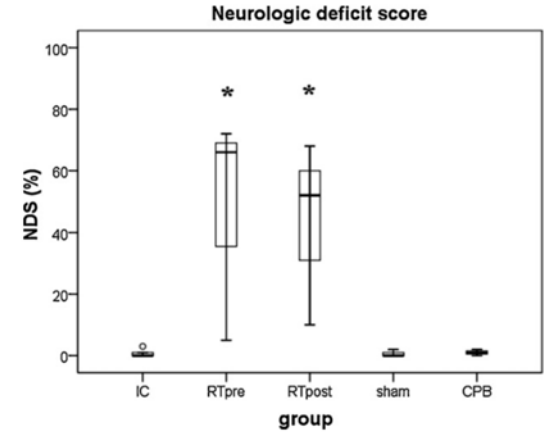

Figure 36. NDS between groups. Boxes represent interquartile ranges. The line across each box indicates the median, and the whiskers are the highest and lowest values. The round marker represents an outlier. * $P < 0.05$ vs. IC, sham or CPB group, respectively.

	IC	RTpre	RTpost	sham	CPB
OPC 5 Death / brain death					
OPC 4 Severe disability					
OPC 3 Moderate disability		●●	●●		
OPC 2 Mild disability			●		
OPC 1 Normal	●●●●●●	●		●●●●	●●●

Table 3. OPC at 7 d after prolonged hypothermic CA and in control groups. Each dot represents one rat. IC, ice-cold flush; RTpre, pre-treatment, room-temperature flush; RTpost, post-treatment, room-temperature flush; CPB, cardiopulmonary bypass control group.

At 7 d after the insult a robust microglial response was seen in the hippocampus in groups subjected to CA. Minimal microglial proliferation was observed in the CPB group. No activated microglia were visualized in shams**. The number of microglia in hippocampus was decreased in the hemisphere injected with LEC vs. PBS in all individual groups and when pooled together ($P < 0.05$).** The number of FJC-positive neurons, however, did not differ between hemispheres in individual groups ($P = 1.0$) (Figure 37 , Table 4). ICP did not

staining	**neuronal death** FJC			**microglial proliferation** FITC			DAB		
treatment/ratio	PBS	LEC	ratio	PBS	LEC	ratio	PBS	LEC	ratio
Treated groups:									
IC, n=6	892	921	1.03	1520	1011	0.67	1731	993	0.57
RTpre, n=3	453	405	0.89	677	406	0.6	1474	838	0.57
RTpost, n=3	312	327	1.04	346	187	0.54	560	362	0.65
Sum of all treated groups:	1657	1653	1	2543	1604	0.63*	3765	2193	0.58*
	0% difference in FJC			**37% attenuation per FITC**			**42% attenuation per DAB**		
Control groups:	-	-		-	-		-	-	
sham, n=4	0	0		22	26		43	35	
CPB, n=3	0	0		67	54		186	193	

Table 4. Cell counts showing neuronal degeneration and microglial proliferation after prolonged cardiac arrest and in control groups. Control groups did not receive intrahippocampal injection. The numbers represent the sum of all counted cells from all animals in individual groups. IC, ice-cold flush; RTpre, pre-treatment, room-temperature flush; RTpost, post-treatment, room-temperature flush; CPB; cardiopulmonary bypass control group. * $P < 0.05$ PBS vs. LEC.

significantly differ before (6±1 mmHg) and after (5±2 mmHg) LEC injection into hippocampus. Continuous ICP monitoring for 20 min during the LEC injection is shown (Study IX Figure 10).

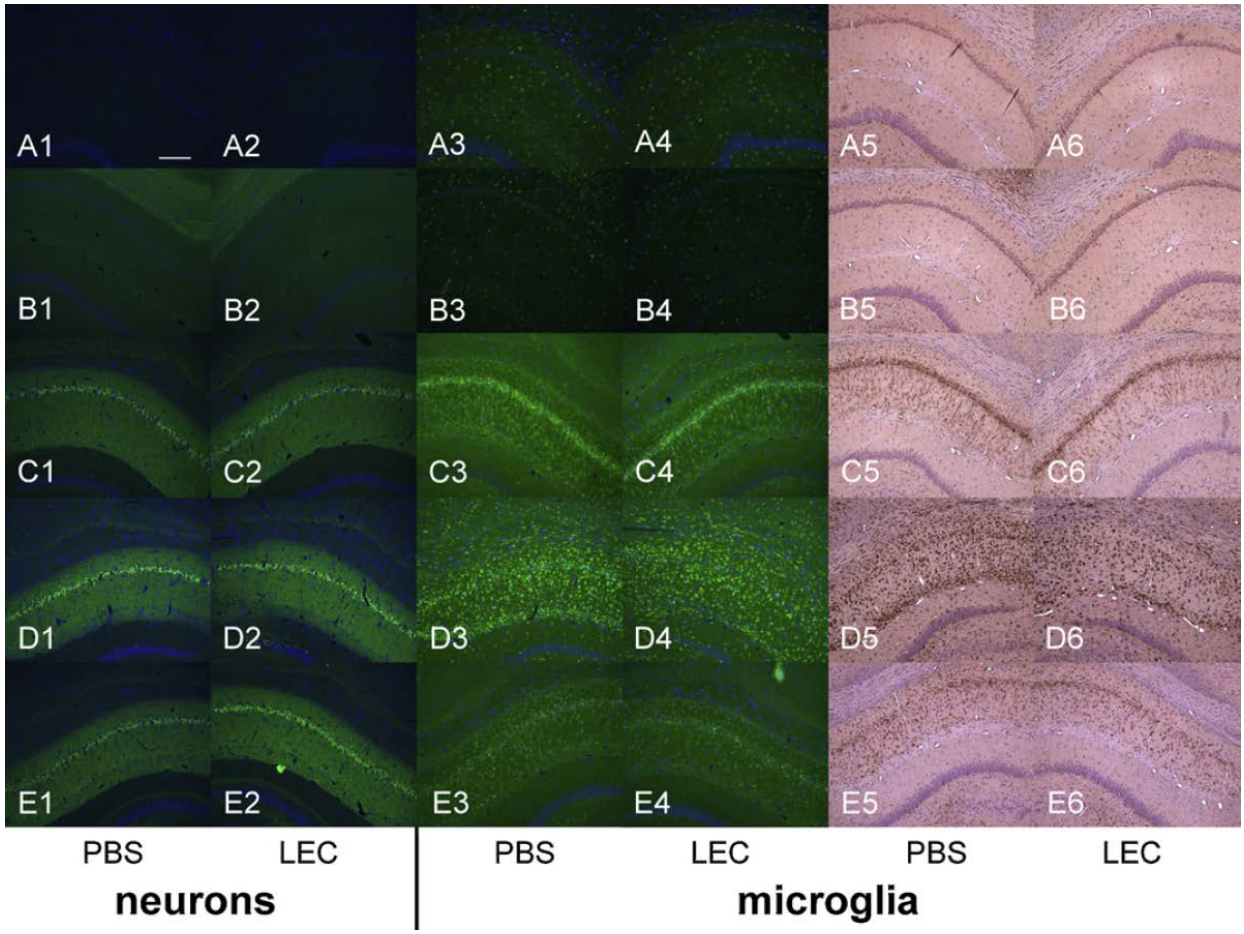

Figure 37. Histological outcome after prolonged hypothermic cardiac arrest. Representative samples of five animals are displayed, each row showing one animal from each individual group (A-E): A, shams; B, CPB controls; C, IC group; D, RTpre group; E, RTpost group. The two left columns (1-2) show degenerating neurons as visualized by the FJC staining, in the left and right CA1 regions of hippocampus, respectively. The four right columns show microglial proliferation and activation detected by anti-Iba-1 FITC staining and visualized by fluorescence (columns 3 and 4) or with DAB and hematoxylin counterstaining (columns 5 and 6). Both shams and CPB controls showed no neuronal death and minimal microglial staining. All groups subjected to cardiac arrest showed extensive neuronal degeneration with no differences between groups and between left and right hemispheres in the individual groups. The microglial proliferation was significantly attenuated in all groups subjected to cardiac arrest in the right hippocampus, the site of LEC injection, vs. the corresponding left hippocampus, where PBS was injected. Blue staining in columns 1-4 is DAPI, identifying nuclei of intact neurons. Scale bar in panel A1 = 200 µm.

3.9.5. Discussion

Using our established, clinically-relevant model of EPR to study prolonged hypothermic CA in trauma resuscitation we found that both pre- and post-treatment with direct injection of LEC into the brain attenuates local microglial proliferation in hippocampus. This effect was not associated with a decrease in neuronal loss or a change in ICP.

The role of microglia in neuroinflammation in prolonged CA remains poorly defined. There is a large body of evidence documenting that microglia are a source of multiple potentially cytotoxic substances including NO, free radicals and pro-inflammatory cytokines, especially tumor necrosis factor α and interleukin-1β.[285] Attenuation of microglial activation has shown benefit in multiple CNS injuries and neuroinflammatory diseases.[286] In contrast, ablation of microglia in transgenic mice in stroke models showed detrimental effects,[217] and administration of exogenous microglia had neuroprotective effects after ischemia,[287,288] possibly linked to the production of neurotrophic factors like insulin-growth factor-1 or brain-derived neurotrophic factor.[289] It has been postulated that the microglial reaction is dependent on the severity of the insult, and their role could be either "toxic" or "protective".[216,290] The tetracycline derivative minocycline has been used traditionally to attenuate microglial activity in multiple studies and showed potential as a neuroprotective agent.[212] However, minocycline is non-specific.[291] We specifically targeted microglia with local LEC administration.

Clodronate (dichloromethylene bisphosphonate – Cl_2MDP) has been developed to eliminate macrophages in order to permit "in vivo" studies of their function. Clinically, it has been used in the treatment of osteoporosis,[292] including prevention of skeletal events in patients with breast cancer.[293] Prolonged administration of clodronate (oral, intravenous or intramuscular) seems to be safe.[292]

The exact mechanism of effects of clodronate is not yet fully elucidated. Systemically injected liposomes including encapsulated clodronate are ingested by macrophages which are then destroyed following phospholipase-mediated disruption of the liposomal bilayers and release of clodronate via a so-called macrophage "suicide" technique (Figure 38).[294]

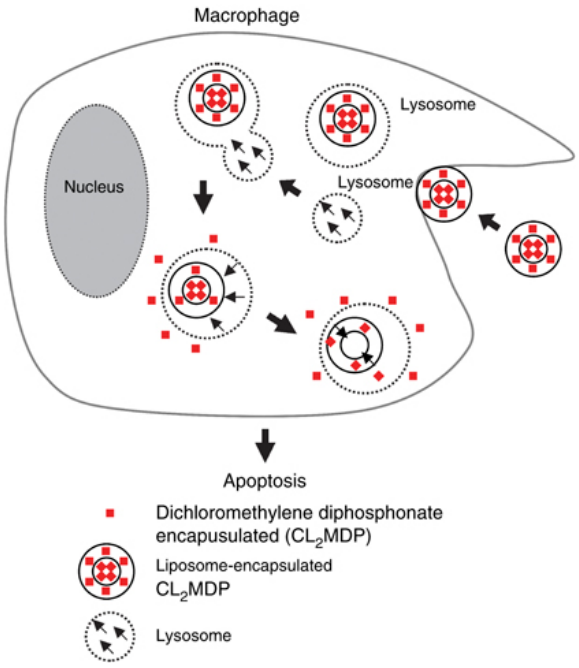

Figure 38. Macrophage 'suicide' technique. Liposomes, encapsulating the clodronate molecules, are ingested by macrophages via endocytosis. After fusion with lysosomes containing phospholipases, the latter disrupt the bilayers of the liposomes triggering apoptosis.

Clodronate released from the liposomes has a short half-life that allows prompt removal from the circulation.[295] Depletion of macrophages occurs rapidly (within 24 h after intravenous administration) and lymphocytes are not depleted. The effect persists for as long as one month after a single injection.[296] A second dose was used to deplete bone-marrow residing macrophages and optimize the depletion.[297] Despite profound effects, an increase in infectious complications has not been reported in long-term outcome models. Microglia remain unaffected because liposomes do not cross the BBB.[298] We previously showed that our model of EPR is not associated with BBB disruption.[299] Intraventricular injection of LEC resulted in a selective depletion only of perivascular and meningeal macrophages in the CNS. The macrophages started to repopulate in the given areas 14 d after the LEC depletion.[300] Thus, we chose to target microglia in hippocampus with a direct intrahippocampal injection of LEC.

On a subcellular level, after its phagocytosis by macrophages, clodronate causes collapse of the mitochondrial membrane potential via inhibition of the ADP/ATP translocase by its metabolite AppCCl2p, resulting in delayed apoptosis.[301]

The effects of direct injection of LEC into tissues have not yet been fully explored. An intraparenchymal injection of LEC was used to induce macrophage depletion in rat testes. After local injection, the number of residing macrophages in testes were reduced at least by 90-97% at 14 d after injection, with repopulation observed at 60 d.[302] These results suggest that the local spread of the liposomes and the depletion of tissue macrophages could be slower compared to rather rapid uptake of liposomes by circulating macrophages.

Stereotactic administration of LEC into hippocampus allowed us to selectively deplete resident microglia either before or after the insult. However, although successful, microglial depletion did not attenuate neuronal degeneration in CA1 region of hippocampus – a site of selective vulnerability that is accompanied by robust microglial activation and proliferation in our model.[283] While we achieved significant microglial depletion at the area of injection, the exact distribution of LEC-induced microglia depletion could not be determined in our model.

It is plausible that microglia could mediate bi-phasic effects – initially contributing to secondary injury, while later being neuroprotective. In an *in vitro* study, LEC depleted microglia and inhibited microglial secretion of pro-inflammatory cytokines and NO in excitotoxically injured organotypic hippocampal slice cultures.[303] Our newly developed method will enable us to study in the future the effects of microglia in temporal sequence.

Other methods of microglial depletion have been tested previously. Ganciclovir-treated transgenic mice that express a mutant form of herpes simplex virus type I thymidine kinase driven by a myeloid-specific CD11b promoter show 75% reduction in proliferating microglia after nerve injury.[304] Using this technique in a brain ischemia model, a ~40%

reduction of Iba-1 immunoreactivity was noted at 72 h, which is similar to the ~40% reduction observed with our technique. Mac-2, an alternative marker of activated/proliferating microglia, showed even higher reduction rate, up to 65%.[217] However, technical limitations of currently available experimental CPB techniques do not allow CPB-assisted resuscitation, an integral part of our paradigm, in a mouse model. Rats are currently the smallest animal that can be resuscitated with CPB. Thus, it was necessary for us to explore alternative methods of microglial depletion to study the effects of microglia after CA in our rat model.

Minocycline has been widely used to deplete microglia in models of neuroinflammatory diseases and brain ischemia in rats.[212] While the effects of minocycline in neuroinflammation have been generally positive, the outcomes of studies in brain ischemia models have been controversial. We and others showed minocycline-induced neuroprotection in both experimental[222,279,305] and clinical settings.[234] In contrast, others reported that selective ablation of proliferating microglial cells exacerbates ischemic brain injury.[217] Minocycline effects are also non-specific. In a spinal cord injury model, minocycline reduced delayed oligodendrocyte death and attenuated axonal dieback, thus improving functional outcome.[231] However, the effect of minocycline on oligodendrocytes could affect the neurological outcome by mechanisms other than microglial depletion.[236,306] We have previously reported that our model of hypothermic CA is associated with persistent neurological and motor deficits.[242,283] Minocycline was only marginally beneficial.[283] These controversies underscore the need for a treatment paradigm that would enable us to study selective microglial depletion in a rat model.

Brain ischemia can also result in an early post-insult of the ischemic lesion by polymorphonuclear neutrophils (PMN) that could aggravate the injury. Microglia were previously shown to be neuroprotective against invading PMNs via their engulfment in an *in*

vitro model.[219] It is not clear whether LEC had any effect on PMNs in our model, but it seems unlikely given the small dose of LEC used in our study. Studies aimed at systemic depletion of PMN used repeated doses of LEC to achieve complete depletion of PMN.

We explored both pre-treatment and post-treatment with LEC in a brain region that previously showed an extensive damage in our model, using a proof-of-concept approach in which each animal served as its own control (only one hemisphere was treated with LEC). Only the intra-arrest temperature, but not the timing of the microglial depletion, had an effect on outcome. There may exist a certain window of opportunity for post-insult treatment given the fact that even delayed treatment with minocycline improved outcome after focal brain ischemia.[226,229,236]

Consistent with the lack of a role for microglia in affecting neuronal death in hypothermic CA, we reported that attenuation of microglial proliferation with deep hypothermia during CA in our model vs. moderate hypothermia resulted in improved neurological outcome, despite not preventing neuronal loss.[283] We were not able to demonstrate this effect in the current study. It is possible that the intrahippocampal injections, with either clodronate or PBS containing liposomes, affected the microglial activation and proliferation in all groups, and the net effect of different levels of hypothermia were not as marked as they were in intact tissues. We did not use intrahippocampal injections in our control groups. We cannot exclude the possibility that intrahippocampal injections alone would produce a certain degree of gliosis and/or neuronal degeneration.

We did not explore the possibility of neurogenesis in our model. It has been postulated that delayed neuronal death is complete within 5-7 days. Several studies in both global and focal cerebral ischemia reported neurogenesis and/or synaptogenesis after an ischemic insult.[307,308] However, neurogenesis starts at the subgranular zone of the dentate

gyrus, and becomes maximal at two weeks after the insult.[309] Based on these studies, we chose a 7 day outcome as a timepoint when neuronal loss is complete but repopulation of CA1 is unlikely. However, the role of neurogenesis in our model and the impact of hypothermia and microglia on this process remain to be determined in future studies, using bilateral LEC-induced depletion and complex neurobehavioral assessment with Morris water maze tasks.

In this feasibility trial, we showed that either pre- or post-treatment with direct injection of LEC into the hippocampus 1) attenuated local microglial proliferation in hippocampus by ~ 40%, and 2) did not acutely increase ICP. However, depletion did not alter neuronal degeneration in the hippocampus in our model of hypothermic CA. This suggests that microglia do not play a pivotal role in mediating neuronal death. However, LEC at the dose and treatment time chosen did not cause total ablation of microglial activity. An optimized dose and a timing of pretreatment may be needed to achieve higher depletion rate. Also, our insult produces marked loss of neurons and thus we cannot rule out the possibility that depletion of microglia could further exacerbate neuronal loss. It is also possible that the insult was too severe for LEC to have a robust impact. A detailed topographical map of microglial depletion needs to be characterized in future experiments. This novel strategy provides us with a tool to study the effects of microglia comprehensively in hypothermic CA, and in other models of neuroinflammation.

3.10. Study X - Cytokines after hypothermic CA in rats

Drabek T, Wilson CD, Janata A, Stezoski J, Janesko-Feldman K, Tisherman SA, Kochanek PM
Unique brain region dependent cytokine signatures after prolonged hypothermic cardiac arrest in rats

3.10.1. Summary

We showed previously that prolonged cardiac arrest (CA) produces neuronal death with microglial proliferation. Microglial proliferation, but not neuronal death, was attenuated by deeper hypothermia. Microglia are a major source of cytokines. In this study, we tested the hypotheses that 1) CA will result in highly specific regional- and temporal-increases in brain tissue cytokine levels; and 2) the increases in cytokine levels will be attenuated by deeper hypothermia. After rapid exsanguination (5 min) and normothermic no-flow (6 min), different levels of hypothermia were induced by either ice-cold (IC) or room-temperature (RT) aortic flush. After 20 min CA, rats were resuscitated with CPB, and sacrificed at 6 or 24 h. Rats subjected to CPB only (no CA) and shams (no CPB or CA) served as controls. Cytokines were analyzed in cerebellum, cortex, hippocampus, and striatum. Intra-CA temperature was lower after IC vs. RT flush (21 ºC vs. 28 ºC, $p<0.05$). At 6 h, striatum showed a massive increase in interleukin (IL)-1α and tumor necrosis factor (TNF)-α (>100-fold higher than in hippocampus) which was attenuated in the IC vs. RT group. In contrast, IL-12 was 50-fold higher in hippocampus vs. striatum. Surprisingly, IL-10 was higher in IC vs. RT at 6 h. At 24 h, cytokines decreased. CPB controls showed a unique global increase of IFN-ɣ. Our results suggest that important temporo-spatial differences in the brain cytokine response to hypothermic CA exist, with a novel role of striatum. Hypothermia showed protective effects. New therapeutic strategies to reduce CNS damage may need to target early regional neuroinflammation.

3.10.2. Background

Ischemic insults including CA trigger a variety of biochemical cascades that may result in delayed neuronal degeneration. While early ischemic brain injury is the result of energy failure, it has been suggested that neuroinflammation could contribute, or even represent a major cause of delayed neuronal death and injury.

Microglia are the resident phagocytic cells of the CNS. It has been suggested that microglia are a major cause of delayed neuronal death and injury.[211] Microglial activation starts immediately after ischemia and precedes detectable neuronal damage. Activated microglia are a major source of both pro-inflammatory and anti-inflammatory cytokines, including IL-1α, IL-1β, IL-6, IL-10, TNF-α and others, shown to be up-regulated in cerebral ischemia.[310] It has been shown previously that TNF-α and IL-6 expression in the hippocampus are attenuated by mild hypothermia following hypoxic insult.[311] In addition, we previously reported a decrease in microglial activation in the hippocampus following prolonged CA when treated with deep hypothermia compared to moderate hypothermia.[283]

The underlying mechanisms of hypothermic preservation are not yet fully understood. Early studies exploring the protective effects of hypothermia focused mainly on hippocampus, a brain region with selective vulnerability to ischemia. However, it is well known that CA induces neuronal death patterns that are brain region dependent[312,313] and thus the neuroinflammatory response may not be uniform. CNS regional and temporal profiles of regional changes have not yet been fully explored. If important differences in neuroinflammatory reactions exist, different therapeutic strategies may be required. We used our established model of exsanguination CA that includes prolonged hypothermic CA and delayed resuscitation with CPB to explore regional and temporal patterns of brain tissue cytokines in a rat CA model. We hypothesized that 1) CA will result in highly specific regional- and temporal-increases in brain tissue levels of cytokines; and 2) the increases in cytokine levels will be attenuated by deep hypothermia. To test our hypotheses, we used multiplex cytokine array to evaluate region-specific levels of cytokines at 6 and 24 h after the insult in four selected brain regions.

Because both the surgical interventions and the use of CPB have been linked to neuroinflammation, we also included a control group subjected to CPB without CA, along

with sham-operated rats subjected to the identical surgical procedures and anesthesia, without CPB or CA.

3.10.3. Materials and Methods

We used our previously established model described in Study VII, using extended normothermic CA period prior to induction of either deep (21 °C) or moderate (28 °C) hypothermia. The animals were sacrificed at either 6 or 24 h after the insult. The rats were then decapitated, brains removed and dissected into four regions of interest: cortex (CTX), striatum (STRI), hippocampus (HIP) and cerebellum (CEREB). The samples were snap-frozen in liquid nitrogen and then stored at -70 °C freezer until further processing.

Since others indicated that exposure to artificial materials of an experimental CPB circuit used in our study induces systemic and cerebral inflammation,[314,315] we included a control group that was subjected to the same duration (60 min) of CPB but without preceding hemorrhage or CA. The temperature profile of this group was designed to mirror the RT group. We have also studied sham animals subjected to the same cannulation and anesthesia, without ischemic insult or CPB. This group was kept normothermic.

Six groups were studied, n=6 per group: 1) Ice-cold (IC) flush group, 6 h outcome (IC6); 2) room-temperature (RT) flush group, 6 h outcome (RT6); and 3) CPB controls, 6 h outcome (CPB) 4) sham-operated rats, 6 h outcome (sham), 5) IC flush group, 24 h outcome (IC24); and 6) RT flush group, 24 h outcome (RT24).

3.10.3.1. Assessment of cytokine levels

The samples were collected and processed for multiplex cytokine array. Cytokine levels were quantified using a Luminex kit (10-Plex, Luminex, Invitrogen, Austin, TX, USA), which included 10 cytokines for the simultaneous measurement of rat GM-CSF, IL-

1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12 (p40⁄p70), IFN-γ, and TNF-α. The tissue was homogenized in PBS by using Dounce homogenizer for 20 strokes. The homogenate was then sonicated for 10 seconds for three times with an interval of 20 seconds, followed by centrifugation at 16,000g for 30 minutes. The supernatant was used for cytokine analysis. Protein levels in the supernatants were measured using the BCA protein kit (Thermo Fisher Scientific Inc., Rockford, IL, USA).

3.10.3.2. Statistical analysis

Repeated measures ANOVA was performed, followed by Student-Newman-Keuls post hoc tests, to identify differences in temperature between groups. One-way ANOVA was used to compare cytokine levels among groups. Mortality was compared using Fisher's exact test. A p value < 0.05 was considered statistically significant.

3.10.4. Results

3.10.4.1. Temperature / survival

IC flush resulted in lower intra-CA temperature than RT flush (tympanic, 21 °C vs. 28 °C; rectal, 17-25 °C vs. 27-30 °C, $P<0.001$) (Fig. 1). The mortality rate was 2/14 in the IC group and 7/19 in RT group, respectively (P=n.s.). There were no deaths in either sham or CPB groups.

3.10.4.2. Regional differences

Striatum showed an early specific increase in pro-inflammatory cytokine levels. TNF-α levels after CA in the striatum were over 100-fold higher than in the hippocampus. IL-1α levels were 4-fold and 26-fold higher in striatum than in hippocampus, respectively, in the IC and RT groups (Fig. 39). In contrast, IL-12 was specifically increased only in the hippocampus, with levels 40- to 60-fold higher than in striatum (Fig. 38A).

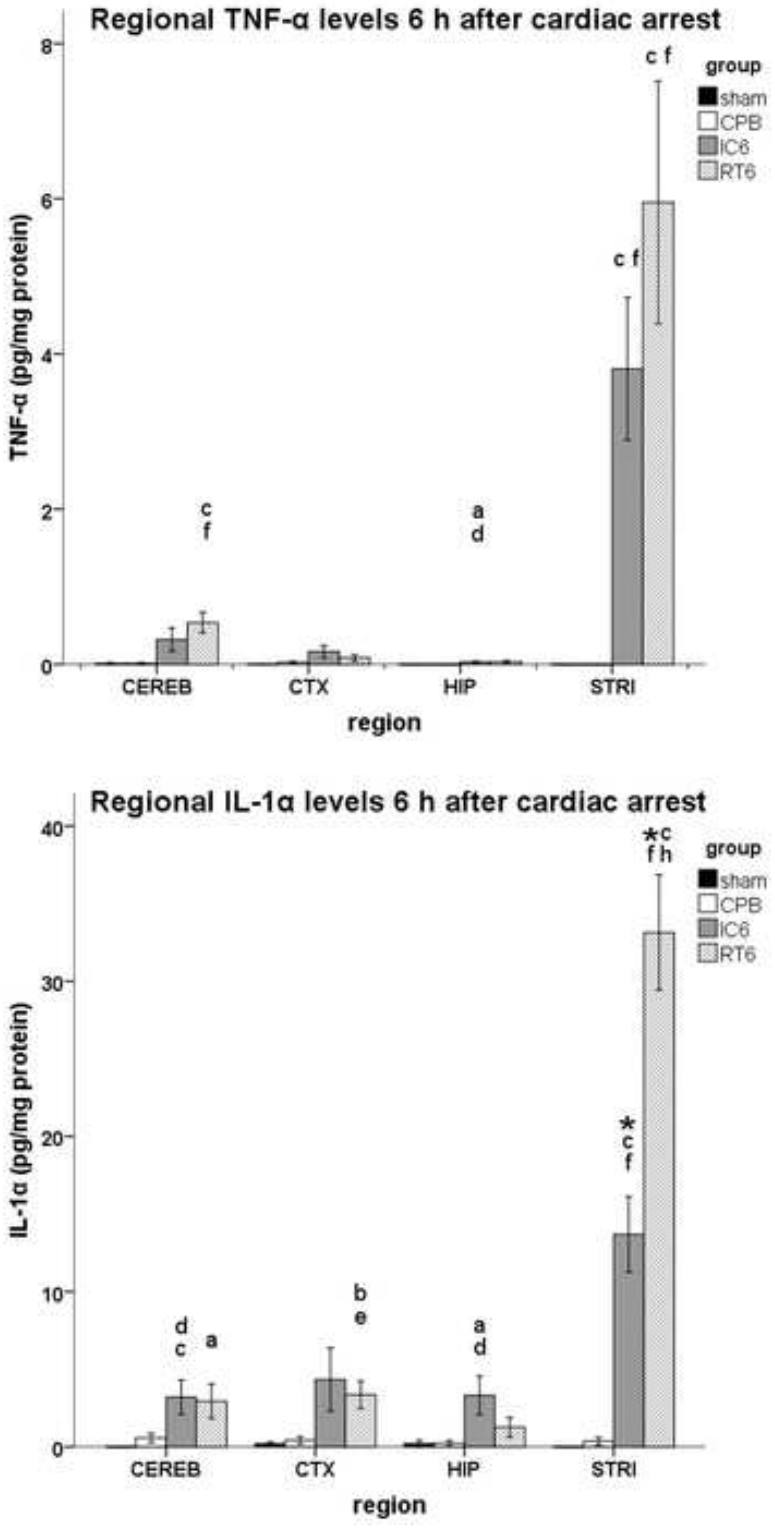

Figure 39. Regional TNF-α and IL-1α levels at 6 h after prolonged cardiac arrest. Marked isolated early increase of TNF-α was observed in striatum in both cardiac arrest groups. This was attenuated by deeper hypothermia. Mean±SEM. CEREB, cerebellum; CTX, cortex; HIP, hippocampus; STRI, striatum. a, p<0.05 vs. sham; b, p<0.01 vs. sham; c, p<0.001 vs. sham; d, p<0.05 vs. CPB; e, p<0.01 vs. CPB; f, p<0.001 vs. CPB, h, p<0.001 vs. IC; * p<0.05 IC6 vs. RT6.

3.10.4.3. Temporal expression of cytokines

Results at 6 h

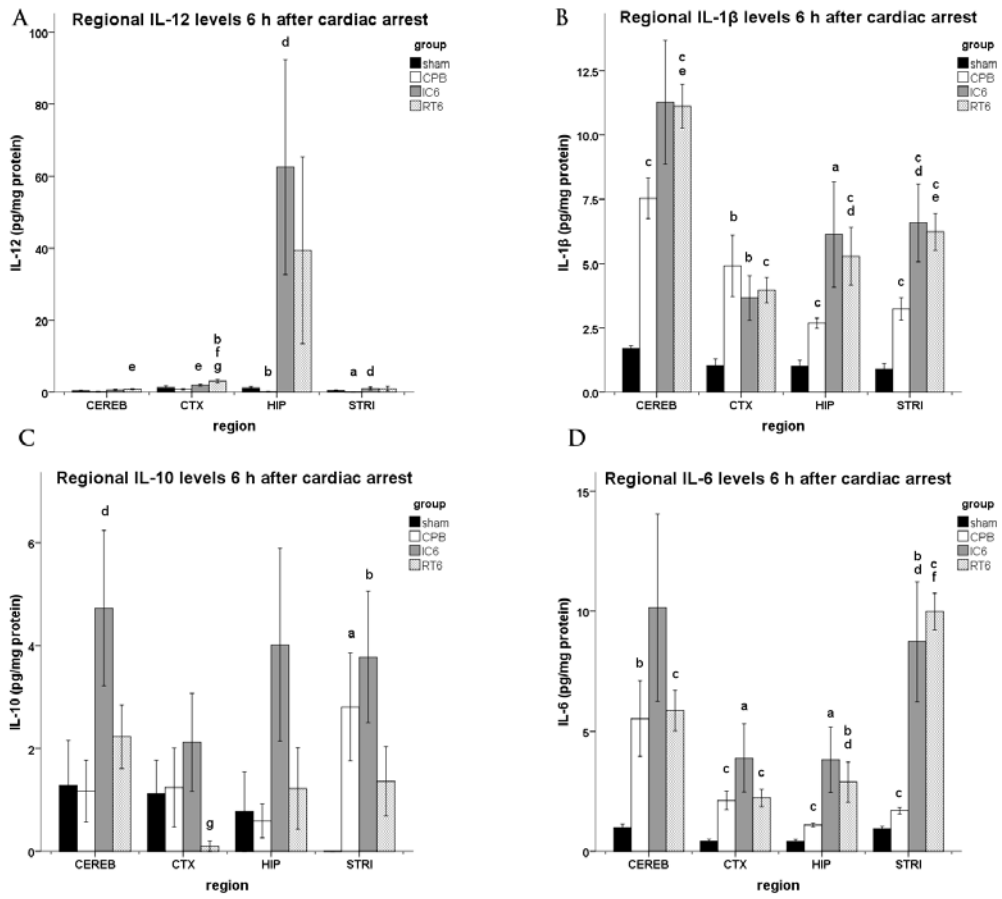

Figure 40. Regional IL-12, IL-1β, IL-10 and IL-6 levels at 6 h after prolonged cardiac arrest. (A) Marked isolated increase of IL-12 in hippocampus after CA was observed, with no added benefit of deeper hypothermia. (B) Both CPB and CA induced IL-1β increase in all regions with no effect of hypothermia. (C) Globally, taken all regions together, IL-10 levels were higher in the IC6 group vs. RT6 group, suggesting a selective upregulation of anti-inflammatory cytokine despite hypothermia-induced metabolism down-regulation. (D) IL-6 was increased by both CPB and CA, with highest levels seen in the striatum. Mean±SEM. CEREB, cerebellum; CTX, cortex; HIP, hippocampus; STRI, striatum. a, p<0.05 vs. sham; b, p<0.01 vs. sham; c, p<0.001 vs. sham; d, p<0.05 vs. CPB; e, p<0.01 vs. CPB; f, p<0.001 vs. CPB; g, p<0.05 vs. IC, h, p<0.001 vs. IC; * p<0.05 IC6 vs. RT6.

Groups subjected to CA

CA induced a global increase in cytokine levels compared to shams and CPB controls. Levels of TNF-α and IL-1α were markedly increased, especially in the striatum. TNF-α levels in the striatum were increased >100-fold over the levels in the hippocampus. This increase was attenuated by deeper levels of hypothermia (Fig. 39). IL-1β levels were increased after CPB

or CA in all regions vs. shams, but there were no differences between IC6 and RT6 groups (Fig. 40B). IL-10 levels were increased with deeper hypothermia ($p<0.002$ IC6 vs. RT6; Fig. 40C). In contrast, IL-12 levels were markedly increased in hippocampus (Fig. 40A).

There were no differences in IL-4 levels between groups and regions (data not shown). The levels of GCSMF were not detectable.

Control groups

In general, shams had extremely low levels of cytokines compared to rats subjected to CPB or CA at either temperature.

Contrasting CA, the increase of cytokines in CPB were in a global pattern across brain regions although to levels intermediate in magnitude vs. CA. CPB induced a global increase of IL-1β and IL-6 ($p<0.05$ vs. sham in all individual regions) (Fig. 40B,D). CPB failed to induce an increase of IL-1α, TNF-α or IL-12 in brain. IL-10 was two-fold higher in striatum than in other regions where levels were similar to shams (Fig. 40C). In shams, levels of IL-10 were undetectable. Surprisingly, rats subjected to CPB had 2-fold higher levels of IFN-γ in all brain regions compared to both shams and CA groups (Fig. 41).

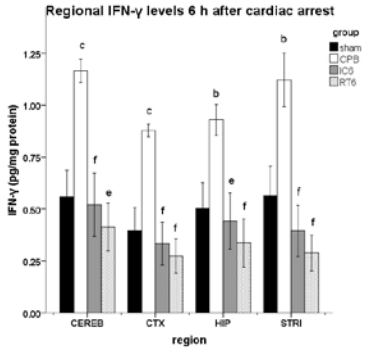

Figure 41. Regional IFN-γ levels after prolonged cardiac arrest. Selective two-fold increase was seen in the CPB group. Mean±SEM. CEREB, cerebellum; CTX, cortex; HIP, hippocampus; STRI, striatum. b, $p<0.01$ vs. sham; c, $p<0.001$ vs. sham; e, $p<0.01$ vs. CPB; f, $p<0.001$ vs. CPB.

Results at 24 h

In general, all cytokine levels at 24 h decreased vs. 6 h. TNFα and IL-1α levels were undetectable in most regions. IL-1β levels were lower in RT24 vs. IC24 in CTX but similar in other regions (Fig. 42). IL-6 levels decreased 10-fold between 6 h and 24 h with no differences between groups (data not shown). Levels of IL-10 remained similar

without differences between regions or levels of hypothermia. Levels of IL-12 decreased 4- to 6- fold over time, but the selective increase in HIP observed at 6 h was still present at 24 h (Fig. 42). No effect of a deeper level of hypothermia on IL-12 in HIP was observed. GMCSF again was not detected.

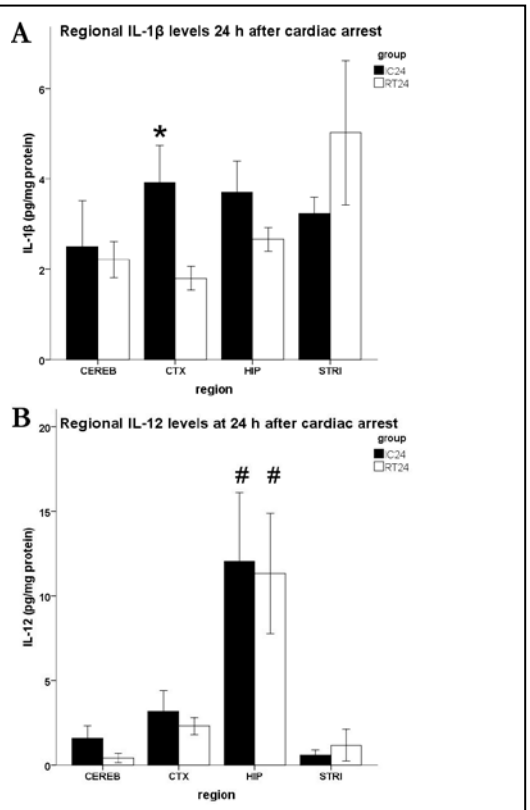

Figure 42. Regional IL-1β and IL-12 levels at 24 h after prolonged cardiac arrest. Mean±SEM. CEREB, cerebellum; CTX, cortex; HIP, hippocampus; STRI, striatum. *p<0.05 IC24 vs. RT24. #p<0.05 vs. other regions.

3.10.4.4. The effect of different levels of hypothermia

Hypothermia had marked and very specific effects on the individual cytokine levels. Deeper levels of hypothermia decreased the IL-1α spike in striatum, but had no effect in any other brain region studied (Fig. 39). A similar pattern was observed for TNF-α in striatum, but the attenuation did not reach statistical significance (Fig. 39). Deeper hypothermia had no effect on IL-1β, IL-6, IL-12 and IFN-γ. In contrast, deeper hypothermia surprisingly increased IL-10 levels across all regions ($p<0.002$ IC6 vs. RT6).

3.10.5. Discussion

The role of the cytokines in CNS insults is still poorly understood but their paramount role in neuroinflammation is supported by a large body of literature. Cytokines, produced by glial cells and neurons within the brain, or by peripheral immunocompetent cells, contribute

to the complex autonomic, neuroendocrine, metabolic, and behavioral responses to CNS injuries,[316] representing a valid therapeutic target that needs to be explored.

We previously reported that this model of prolonged CA results in extensive hippocampal neuronal death at both of the temperature levels used in this study (i.e., 21 ºC or 28 ºC). Deeper levels of hypothermia, however, attenuated the hippocampal microglial response but not neuronal death.[283] We now report (1) early increases in brain tissue levels of cytokines that showed pronounced regional differences, with markedly increased levels of cytokines generally considered to be pro-inflammatory (TNF-α, IL-1α) in striatum, and (IL-12) in hippocampus. Other than the striatum, TNF-α levels were low. Surprisingly, the highest TNF-α levels were seen in striatum, a >100-fold increase vs. hippocampus. (2) A powerful effect of hypothermia on brain tissue TNF-α and IL-1α levels in striatum was seen but surprisingly no effect of hypothermia on IL-12 was seen in hippocampus. (3) Levels of IL-10, a cytokine with anti-inflammatory properties, were increased, with deeper hypothermia, indicating that hypothermia is not only decreasing levels of pro-inflammatory cytokines in striatum, but also selectively increasing levels of an anti-inflammatory cytokine. (4) CPB uniquely increased IFN-γ levels across all regions. These results indicate a highly region-dependent cytokine response to global cerebral ischemia and hypothermia.

Although this unique cytokine response in brain after CA is novel, consistent with our overall hypothesis, region-dependent microglial proliferation (preferentially in striatum and cortex) has also been reported after global ischemia in gerbils and could be associated with selective regional cytokine production.[278] Similarly, region-dependent upregulation of several neurotrophic factors has been seen with hypothermia after CA.[317,318] Expressions of heat shock proteins and other cell death cascade messengers after CA showed also significant regional differences[319] that were further modulated by hypothermia.[320]

Few studies, however, have explored regional production of cytokines in cerebral ischemia models. In a gerbil model of bilateral carotid artery occlusion for 5 or 10 min, TNF-α levels were increased at 6 h after ischemia in hippocampus and striatum, but not in cortex, thalamus or cerebellum vs. shams. The magnitude of the increase was proportionate to the duration of ischemia. TNF-α levels were higher in hippocampus than in striatum. IL-6 and IL-1β showed similar pattern.[321] Finding much higher TNF-α levels in the striatum vs. other brain regions after deep hypothermic CA was surprising but does have some support in the literature. While most studies of neuroinflammation after CA focus on the selectively vulnerable hippocampus,[322-324] there are prior reports of increased TNF-α levels in the striatum.[325,326] In a transient focal cerebral ischemia model with core lesion in striatum and penumbra in cortex, TNF-α levels in striatum and cortex were both increased vs. shams. Surprisingly, hypothermia markedly increased TNF-α levels in striatum more than in cortex, contrasting our findings, but attenuated IL-1β levels in both regions.[325] In a hippocampal stab wound model, surprisingly, TNF-α was initially increased in the striatum at 24 h, followed by delayed increase in striatum and thalamus at day 6, and in the striatum, thalamus, cortex, and hippocampus on days 9-12. Lesions in other brain regions did not produce a similar response.[326] Thus, it is unclear whether the striatal increase in cytokines in our model reflects a response to remote selective damage in hippocampus or local damage in striatum.

Cerebral cytokine mRNA expression after a 7 min CA in pigs also increased IL-1β, IL-6, IL-10 and TNF-α in cerebral cortex at 24 h. This increase was attenuated by mild hypothermia (33 °C). Unfortunately, other brain regions or blood levels of cytokines were not assessed. In rats, global brain ischemia induced hippocampal neuronal degeneration accompanied by reactive microgliosis. A distinct temporal cytokine production was studied only in hippocampus, with increased levels observed after 7 d.[324] In focal cerebral ischemia model, striatal injection of IL-1α receptor antagonist mitigated both striatal and cortical

neuronal loss, suggesting that striatal circuits influence pathological processes in connected yet distant brain regions.[327] The critical role of IL-1 was shown also by attenuation of cytokine production (IL-1α, TNF-α) and improved neurologic outcome in mice lacking IL-1 receptors subjected to hypoxia/ischemia.[328]

The temporal pattern of individual cytokine mRNA expression and cytokine production is not necessarily coupled in brain after CA and can depend on the individual cytokine, among other factors. In a mouse model of cerebral ischemia, hippocampal TNF-α mRNA peaked early at 3 h, with a transient decline and a secondary peak at 24 h. IL-1β mRNA increased steadily, peaking at 12 h, while IL-6 mRNA peaked at 6 h and then again at 24 h. The temporal pattern of cytokine mRNA expression in individual cytokine gene knock-out mice was altered, suggesting that increases in cytokine levels are interdependent, interactive, and possibly modulate each other.[329]

Our paradigm includes the use of CPB as an essential tool used to resuscitate the animal from prolonged deep hypothermic CA. We thus included controls without CA to determine cytokines levels after an identical duration of exposure to CPB. TNF-α, IL-1α or IL-12 were not induced by CPB alone. IFN-γ levels were higher after CPB than in both CA groups. This is a surprising and novel finding that could be important since IFN-γ can trigger production of TNF-α and caspase-mediated apoptosis. IL-1β and IL-6 were increased vs. shams, although much more modestly than after CA.

The use of CPB has been linked to increased expression of cerebral TNF-α mRNA[315] and nuclear factor-kappa B (NF-κB) in hippocampal neurons.[314] This is important because NF-κB is a transcription factor that is activated after cerebral ischemia and is involved in multiple inflammatory responses that could potentiate ischemic injury by activating genes linked to production of pro-inflammatory cytokines. Some of the protective effects of

hypothermia may be conferred by attenuating NF-κB translocation from cytoplasm and binding activity.[330] Others suggested that inhibition of NF-kB after cerebral ischemia is protective independent of cytokine production.[331]

We did not identify the source of the cytokines in our study. Microglia are viewed as a major source of cytokine production triggered by ischemia, namely IL-1β and TNF-α.[289,332-334] Other glia[322,323,334,335] and neurons[336] can produce cytokines, depending on the type of insult.[337] There is some evidence that blood-borne cytokines might enter CNS by crossing the BBB. Systems with limited capacities to transport cytokines from blood to the CNS have been described for IL-1α, IL-1β, IL-6, and TNF-α. However, the amount of blood-borne cytokines entering the brain is modest and unlikely to explain the regional differences we observed, particularly the marked increase of TNF-α and IL-1α in striatum. We previously reported that our model is not associated with BBB disruption.[299] Similar to our results, the BBB was impermeable for TNF-α in a mouse CA model.[245] Thus, the cytokines detected in our study likely originate from cells within the CNS.

We showed previously that hippocampal TNF-α was significantly increased at 24 h in pediatric asphyxial CA model in rats. The increase of TNF-α and other cytokines using the same multiplex used in our study was not mitigated by minocycline, an agent that targets activated microglia, suggesting that there may be other sources of TNF-α than microglia (35).[338] Immunohistochemical studies revealed that focal cerebral ischemia in adult rats increases TNF-α mRNA and protein in ischemic neurons, rather than glia in the evolving infarct at 6 and 12 h.[336] TNF-α neutralization improved focal cerebral ischemia in a rat stroke model.[339] Focal ischemia is, of course, different from DHCA. Thus, it will be important to define the cell types involved in cytokine production in our model. Whether or not the specific cytokines contribute to neuronal death or recovery in striatum or hippocampus remains to be determined and is a subject of our current work.

Hypothermia induced specific region-dependent changes including decrease of pro-inflammatory cytokines (TNF-α, IL-1α) in striatum and a global increase of anti-inflammatory cytokine IL-10. Hypothermia mitigated neuroinflammation in several studies.[239,320,340] The increase in IL-10 seen in our study contrasts prior findings that hypothermia reduced IL-10 levels in LPS-treated microglia culture.[341] This may be explained by a different insult and/or a different source of cytokines in our model. Serum cytokines were not affected by hypothermia in asphyxial CA model in rats.[342]

Our study has limitations. We were unable to study a normothermic group because we showed previously that a similar insult is not survivable under normothermic conditions.[121] Thus, both insult groups were subjected to deep hypothermia, at different levels. Our model used CPB to resuscitate the rats. The use of CPB has been linked to inflammation in some prior studies. However, our control group subjected to CPB revealed a distinct and in general modest pattern of cytokine production that may or may not contribute to the ischemic insult. Based on prior studies, we have explored cytokine levels at two time points that seem to be representative of the neuroinflammatory course. However, we could not rule out that there may be increases beyond 24 h with distinct pattern,[322,324,334] possibly triggered by delayed neuronal death also characteristic for this model.

In conclusion, TNF-α and IL-1α are markedly increased early after CA with previously unreported unique finding of selective neuroinflammatory response in the striatum. This may represent an alternative, selectively vulnerable region in the prolonged hypothermic CA setting. In contrast, the IL-12 is increased in hippocampus. The anti-inflammatory cytokine IL-10 was increased with hypothermia. New therapeutic strategies to reduce the extent of tissue damage in CNS may need to target regional neuroinflammation early after the insult.

4. Conclusions

EPR is a novel method for resuscitation of exsanguination CA victims utilizing hypothermia to induce preservation during prolonged CA, and delayed resuscitation with CPB. Its clinical feasibility and superiority to conventional CPR have been demonstrated in a series of large animal studies, building upon previous experience from DHCA, commonly utilized in cardiac surgery.

In this series of experiments, we have sequentially explored several hypotheses:

Study I: Rapid HS (5 min) resulting in ExCA treated by EPR is associated with favorable outcome, while conventional CPR fails. We confirmed our hypothesis that an ***extended HS (~ 2 hours) treated by EPR could result in a favorable outcome*** if prolonged postoperative therapeutical hypothermia is provided. Conventional CPR followed by CPB-assisted resuscitation resulted in ROSC but 100% early mortality from MOF.

Study II: Durations of EPR up to 120 min were survivable with one-way aortic flush of ice-cold normal saline. Providing energy substrates, i.e. oxygen and/or glucose may be instrumental in successful extending the duration of EPR beyond 120 min. After rapid HS, ***addition of oxygen allowed favorable neurologic outcome after 180 min EPR*** (2 ½ hour of no-flow) at 8 °C. Addition of glucose did not confer additional benefit.

Study III: A rat model of EPR needed to be established in order to explore mechanisms of ischemia reperfusion injury, identify therapeutical targets and rapidly screen drugs. In this feasibility study, we established that rapid HS followed by deep hypothermic preservation were associated with intact neurologic outcome, confirming our hypothesis that ***EPR in rats is feasible.*** In contrast, identical insult treated with normothermic EPR or normothermic CPB-assisted resuscitation was lethal.

Study IV: Based on our experience from the large animal model, we hypothesized that extension of the hypothermic preservation phase would result in neurologic deficits. We confirmed our hypothesis and demonstrated that ***60 min but not 75 min of EPR is associated with favorable outcome***. However, the suboptimal outcome with extended duration of EPR was most likely due to MOF, since comprehensive neuropathology showed only limited CNS damage.

Study V: Extended EPR in rats results in substantial mortality and impaired neurological outcome in survivors. We hypothesized that extended EPR would be associated with activation of two potential secondary injury cascades in brain, namely ***protein nitration and PARP activation.*** This was confirmed ***in selected brain regions,*** i.e. hippocampus, cortex and striatum, but not in cerebellum.

Study VI: Delta-opioid receptor agonists were shown to induce hibernation in naturally hibernating species, and were protective against ischemia-reperfusion injury in multiple organs. Our model with extended duration of EPR (75 min) showed neurologic injury and MOF. We hypothesized that addition of a delta-opioid agonist would confer additional protection in individual organs, and improve overall outcome. ***DADLE failed to confer benefit*** on functional or histological outcome in our model of prolonged rat EPR, disproving our hypothesis.

Study VII: Rapid ExCA and a short duration of no-flow prior to the initiation of induction of deep hypothermic preservation yields favorable outcome. We hypothesized that extending the period of normothermic CA prior to flush, and increasing the intra-arrest temperature from deep to moderate hypothermia would result in neurologic deficits, neuronal degeneration and neuroinflammation. Minocycline was reported to be beneficial in neuroinflammatory diseases as well as in ischemia-reperfusion syndromes. We hypothesized that deeper levels of

hypothermia and minocycline will attenuate neuronal death and/or neuroinflammation, thus improving outcome after EPR. We demonstrated that ***deep hypothermia was associated with attenuated microglial activation independent of extensive hippocampal neuronal death*** that was similar across groups. ***Minocycline failed*** to confer additional benefits in our EPR model.

Study VIII: The effect of drugs in our EPR model has been limited. One of the explanations for the lack of effect could be a limited transport of the tested agents across the BBB. The permeability of the BBB in our model is unknown. We hypothesized that BBB will not be disrupted even in models that are associated with poor outcome. This was confirmed as ***BBB was not permeable*** to albumin early after the insult. Future studies should focus on therapeutical adjuncts that readily cross the BBB.

Study IX: Improved neurologic outcome was linked with deeper levels of hypothermia. This was associated with attenuated microgliosis but not neuronal death. Thus, we speculated that activated microglia could be an independent factor aggravating neurologic injury. Clodronate is a macrophage-depleting drug. We hypothesized that intracerebrally injected clodronate could deplete microglia, and provide us with a model that would elucidate the role of microglia in post-CA neuroinflammation. We achieved ~ ***40% microglia depletion by clodronate*** in the hippocampus. This regional, incomplete microglial ablation ***did not alter the neurologic outcome*** or ***hippocampal neuronal degeneration***, suggesting a limited role of microglia in this setting.

Study X: We showed previously that prolonged CA produces neuronal death with microglial proliferation. Microglial proliferation, but not neuronal death, was attenuated by deeper hypothermia. Microglia are reportedly a major source of cytokines. In this study, we tested the hypotheses that 1) CA will result in highly specific regional- and temporal-increases in

brain tissue cytokine levels; and 2) the increases in cytokine levels will be attenuated by deeper hypothermia. We documented ***significant temporo-spatial differences in cytokine signatures.*** Surprisingly, we also identified ***different cell types as sources of individual cytokines*** across regions. This suggests that the underpinning mechanisms of ischemia-reperfusion injury in EPR may require a targeted region-specific therapy.

In summary, induction of deep hypothermia early after normothermic ExCA seems superior to conventional CPR which is almost inevitably futile. The drugs pale to the powerful protective effects of hypothermia. This could be explained by the lack of BBB permeability. The neurologic outcome after EPR is determined by a complex interaction between extracerebral and CNS injuries that show a unique temporo-spatial pattern. A development of novel, target-specific BBB penetrating molecules to augment the effects of hypothermia may be warranted. This research also has direct links to cardiac surgery, brain ischemia, and organ preservation in transplant medicine.

Both small and large animal studies confirmed the clinical feasibility of the method even in complex models of multiple injuries. EPR method now proceeds to a multi-center clinical trial.

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Induction of Profound Hypothermia for Emergency Preservation and Resuscitation Allows Intact Survival After Cardiac Arrest Resulting From Prolonged Lethal Hemorrhage and Trauma in Dogs

Xianren Wu, Tomas Drabek, Patrick M. Kochanek, Jeremy Henchir, S. William Stezoski, Jason Stezoski, Kristin Cochran, Robert Garman and Samuel A. Tisherman

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Induction of Profound Hypothermia for Emergency Preservation and Resuscitation Allows Intact Survival After Cardiac Arrest Resulting From Prolonged Lethal Hemorrhage and Trauma in Dogs

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Background—Induction of profound hypothermia for emergency preservation and resuscitation (EPR) of trauma victims who experience exsanguination cardiac arrest may allow survival from otherwise-lethal injuries. Previously, we achieved intact survival of dogs from 2 hours of EPR after rapid hemorrhage. We tested the hypothesis that EPR would achieve good outcome if prolonged hemorrhage preceded cardiac arrest.

Methods and Results—Two minutes after cardiac arrest from prolonged hemorrhage and splenic transection, dogs were randomized into 3 groups (n=7 each): (1) the cardiopulmonary resuscitation (CPR) group, resuscitated with conventional CPR, and the (2) EPR-I and (3) EPR-II groups, both of which received 20 L of a 2°C saline aortic flush to achieve a brain temperature of 10°C to 15°C. CPR or EPR lasted 60 minutes and was followed in all groups by a 2-hour resuscitation by cardiopulmonary bypass. Splenectomy was then performed. The CPR dogs were maintained at 38.0°C. In the EPR groups, mild hypothermia (34°C) was maintained for either 12 (EPR-I) or 36 (EPR-II) hours. Function and brain histology were evaluated 60 hours after rewarming in all dogs. Cardiac arrest occurred after 124±16 minutes of hemorrhage. In the CPR group, spontaneous circulation could not be restored without cardiopulmonary bypass; none survived. Twelve of 14 EPR dogs survived. Compared with the EPR-I group, the EPR-II group had better overall performance, final neurological deficit scores, and histological damage scores.

Conclusions—EPR is superior to conventional CPR in facilitating normal recovery after cardiac arrest from trauma and prolonged hemorrhage. Prolonged mild hypothermia after EPR was critical for achieving intact neurological outcomes. **(*Circulation*. 2006;113:1974-1982.)**

Key Words: cardiopulmonary bypass ■ cardiopulmonary resuscitation ■ heart arrest ■ hemorrhage ■ hypothermia

Conventional resuscitation, including open cardiac massage, is often unsuccessful after exsanguination cardiac arrest in trauma victims,[1–3] particularly when it results from prolonged hemorrhagic shock. A novel approach is needed. Previously, we reported the success of inducing emergency preservation and resuscitation (EPR) with profound hypothermia in animal models.[4] The goal of EPR is to "buy time" for transport and resuscitative surgery during pulselessness, followed by delayed resuscitation. EPR of up to 2 hours was induced with a rapid aortic flush with ice-cold (2°C) saline to induce profound hypothermia, followed by delayed resuscitation with cardiopulmonary bypass (CPB).[5,6] We used EPR to achieve intact survival of dogs after rapid hemorrhage (over 5 minutes) and cardiac arrest.[5–9]

The success of EPR relies on the timely initiation of preservation during cardiac arrest. When 30-minute EPR was delayed for 2 or 5 minutes after cardiac arrest, all dogs survived with good neurological function. However, when EPR was delayed by 8 minutes, none had good outcomes.[10] We speculated that prolonged hemorrhagic shock before cardiac arrest may decrease the efficacy of EPR. Longer durations of hemorrhagic shock may cause severe tissue acidosis and exhaust reserves. Although the central nervous system may be damaged only minimally during hemorrhagic shock,[11] superimposing transient, normothermic cardiac arrest and a period of EPR on prolonged hemorrhagic shock may substantially complicate efforts to save trauma victims.

In this study, we designed a model relevant to military and civilian trauma, characterized by rate-controlled bleeding,[12,13] trauma (laparotomy and spleen transection), limited (hypoten-

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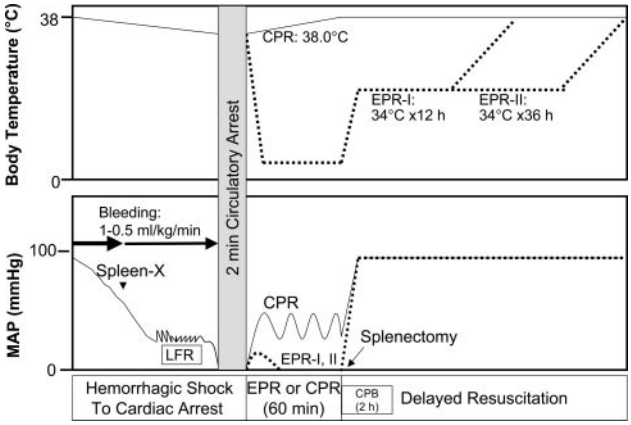

Figure 1. Experimental protocol including prolonged hemorrhagic shock with limited fluid resuscitation (LFR) to cardiac arrest with resuscitation by CPR or preservation by EPR followed by resuscitation with CPB. Splenic transection (spleen-X) was performed during hemorrhagic shock and splenectomy, during resuscitation. Temperature and MAP values are shown.

sive) fluid resuscitation during hemorrhagic shock,[14] and cardiac arrest. Allowing volume depletion and circulatory decompensation to cause cardiac arrest was intended to create an insult nonsalvageable with conventional cardiopulmonary resuscitation (CPR). We hypothesized that EPR could allow survival with good neurological outcomes in this setting.

Methods

The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Experimental Design

The animal model (Figure 1) included 3 phases: (1) the hemorrhagic shock and cardiac arrest phase: bleeding was continued until cardiac arrest; (2) the CPR/EPR phase: 2 minutes after cardiac arrest, dogs were treated with conventional CPR or EPR; and (3) the delayed resuscitation phase, including 2-hour CPB, and 72- to 96-hour intensive care. Trauma included spleen transection during hemorrhagic shock. To simulate life-saving surgery, a splenectomy was performed during resuscitation.

Animals were assigned to 1 of 3 groups at the end of the hemorrhagic shock and cardiac arrest phase: (1) The CPR group was resuscitated with conventional CPR as per Advanced Cardiac Life Support guidelines plus aggressive fluid resuscitation; (2) the EPR-I group was resuscitated with an arterial flush with 20 L of ice-cold saline, followed by delayed resuscitation with CPB, 12 hours of post-EPR mild systemic hypothermia (34°C), and euthanization 60 hours after initiation of rewarming (resuscitation time [RT], 72 hours); and (3) the EPR-II group was identical to the EPR-I group, except that the post-EPR mild hypothermia lasted for 36 hours and was reversed by slower rewarming, and euthanizing occurred 60 hours after rewarming (RT, 96 hours). The latter group was added to the study after 5 experiments each had been performed in the other groups. For the first time in our extensive experience with dog cardiac arrest experiments, several animals in the EPR-I group had neurological deterioration with seizures. We hypothesized that this was due to delayed neuronal injury, which might be ameliorated by prolonging the postresuscitation mild hypothermia.

Anesthesia and Preparation

Custom-bred, male hunting dogs (n=21; 21.1 to 26.6 kg) (Rotz Kennel, Shippensburg, Pa) were housed for at least 3 days before the experiment. Dogs were fasted with free access to water for 12 hours. Ketamine 10 mg/kg and atropine 0.4 mg were administered intramuscularly. After anesthesia induction with 4% halothane by face mask, endotracheal intubation (internal diameter, 8 to 9 mm) was performed. Continuous anesthesia was provided with ≈1% halothane, titrated during preparation with O_2:N_2O, 50%:50%. Controlled ventilation (Piston Ventilator Model 613, Harvard Apparatus, South Natick, Mass) was initiated with a tidal volume of 12 to 15 mL/kg, a positive end-expiratory pressure of 2 cm H_2O, and a frequency of 20 to 25/min, titrated to maintain a $PaCO_2$ of 35 to 45 torr. ECG lead II was continually monitored. A cannula (18 gauge) was inserted into a peripheral vein, and fluid infusion (D_5W/0.45% NaCl at 4 mL · kg^{-1} · h^{-1}) was started. A Foley catheter was placed. Sterile cutdowns were performed in both groins and the right side of the neck. Temperature probes were inserted for measuring rectal, esophageal, and both tympanic membrane temperatures (Tty). A PE-90 catheter was inserted into the left femoral artery for blood pressure monitoring and blood sampling. A pulmonary artery catheter (7.5F) was inserted via the left femoral vein to monitor pressure, cardiac output, and core temperature (Tpa). A CPB arterial cannula (7 or 9 gauge) was inserted into the right femoral artery. A multiple-hole cannula (16F) was inserted into the inferior vena cava via the right femoral vein for blood withdrawal. Another multiple-hole cannula (19F) was inserted 10 cm into the right external jugular vein. This cannula was advanced into the right atrium when mean arterial pressure (MAP) was 30 mm Hg during hemorrhagic shock. The cannulas were flushed intermittently with dilute, heparinized saline. The CPB system, including an oxygenator (Medtronic, Grand Rapids, Mich) and centrifugal pump (Biomedicus, Eden Prairie, Minn), was primed with shed blood (30 mL/kg) and Plasma-Lyte A (Baxter, Deerfield, Ill). In the CPR group, 500 U of heparin was added to the solution.

After baseline measurements were taken, a midline laparotomy (15 cm) was performed and the spleen mobilized medially. The abdominal wound was temporarily closed. Halothane was transiently decreased, and return of spontaneous respiration was achieved. FiO_2 was set at 0.25 with O_2:N_2O at 25%:75%. Halothane was titrated to ensure anesthesia with spontaneous breathing.

Hemorrhagic Shock and Cardiac Arrest Phase

All heating sources were stopped. At hemorrhagic shock time 0 minutes, continuous venous blood withdrawal via the right femoral vein catheter was set at 1 mL · kg^{-1} · min^{-1} over 40 minutes. Withdrawn blood was anticoagulated with 0.125 mL · kg^{-1} · min^{-1} citrate delivered through a PE-60 catheter inside the femoral vein catheter lumen. The tip of the citrate catheter was 3 cm from the tip of the femoral vein catheter. At hemorrhagic shock time 40 minutes, the spleen was transected, and the blood withdrawal rate was decreased to 0.5 mL · kg^{-1} · min^{-1}. Halothane was decreased to 0.5% when MAP was <50 mm Hg. When MAP reached <30 mm Hg, limited fluid resuscitation (simulating field resuscitation) was started with bolus infusions of lactated Ringer's solution (100 mL over 2 minutes), with a maximum volume of 500 mL. Cardiac arrest was defined as either an MAP<10 mm Hg and severe bradycardia (<20 bpm) or asystole or ventricular fibrillation.

CPR/EPR Phase

Two minutes after cardiac arrest, dogs were randomized into the CPR or EPR groups. In the CPR group, conventional Advanced Cardiac Life Support protocols were initiated. In brief, chest compressions with a mechanical thumper (Michigan Instruments, Grand Rapids, Mich) were started at 60/min; the compressing distance was adjusted to generate a systolic blood pressure of 100 mm Hg. Ventilation with 100% O_2 was provided at 12 breaths/min, with the peak airway pressure set at 40 cm H_2O. Epinephrine (0.01 mg/kg IV) was administered every 5 minutes as needed for a maximum of 5 doses. After epinephrine administration, defibrillation with 150 J was attempted if ventricular fibrillation was present and then increased in increments of 50 J after 2 unsuccessful shocks. Sodium bicarbonate and $CaCl_2$ were administered for base deficit >6 mmol/L and ionized calcium <1 mmol/L, respectively. At CPR 0 minutes, lactated Ringer's solution (1 L) was infused over 10 minutes, followed by infusion of shed blood (30 mL/kg) over 5 minutes. Up

to 3 additional boluses of lactated Ringer's solution (250 mL over 15 minutes) were administered per Advanced Trauma Life Support recommendations.

In the EPR groups, the lungs were inflated with air to maintain an airway pressure of ≈10 cm H_2O during EPR. An aortic flush of 20 L of 2°C saline via the right femoral arterial cannula was initiated at 1.6 L/min with use of a roller pump (Ardiem, Indiana, Pa). The flush solution was drained through the external jugular catheter. The dog was then covered with ice.

Delayed Resuscitation Phase (RT 0 to 2 Hours)

Sixty minutes after the onset of aortic flush or CPR, CPB was started.[5] Just before CPB, additional heparin (1500 U) and sodium bicarbonate (2 mEq/kg) were injected into the circuit. Dogs were paralyzed with pancuronium. CPB was started at 100 mL · kg^{-1} · min^{-1}. Reinfusion of shed blood in the EPR groups was titrated to achieve a central venous pressure of 10 to 15 mm Hg. Repetitive doses of epinephrine (0.01 mg/kg) were given when necessary to increase MAP to 100 mm Hg. O_2 flow through the CPB oxygenator was adjusted to keep the $Paco_2$ at 30 to 35 mm Hg. Ventilation at a rate of 8 to 10/min was resumed to prevent atelectasis. IV fluids were restarted at 100 mL/h. A base deficit of >6.0 mEq/L was corrected with sodium bicarbonate. CPB flow was reduced to 75 mL · kg^{-1} · min^{-1} at 60 minutes and to 50 mL · kg^{-1} · min^{-1} at 90 minutes. During CPB, activated clotting times were maintained at >300 seconds with heparin.

At RT 0 minutes, a splenectomy was performed, and the abdomen was packed with gauze to simulate the clinical management of a trauma victim with a ruptured spleen. An abdominal drainage catheter was placed through the abdominal wall. The abdominal wound was closed. Tpa in the CPR group was maintained at 38.0±0.5°C. Dogs in the EPR groups were rewarmed to 34°C over 1 hour. Defibrillation was again attempted, when necessary, when the splenectomy was completed in the CPR group or when the Tpa reached 32°C in the EPR groups. CPB was stopped at 2 hours.

Intensive Care Management (RT 2 to 24 Hours in the CPR and EPR-I Group or 48 Hours in the EPR-II Group)

Neuromuscular blockade was maintained with intermittent doses of pancuronium (0.1 mg/kg). Sedation and analgesia were provided with N_2O/O_2 (50%:50%) plus IV boluses of morphine (0.1 to 0.3 mg/kg) and diazepam (0.1 to 0.2 mg/kg) to prevent signs of wakefulness, eg, mydriasis. Severe hypertension (MAP>150 mm Hg) despite adequate analgesia was controlled with IV boluses of labetalol (0.25 to 0.5 mg/kg) or hydralazine (0.1 to 0.2 mg/kg). Hypotension (MAP<70 mm Hg) was treated by normalization of filling pressures by administration of lactated Ringer's solution and titrated norepinephrine. The dogs received cefazolin (250 mg IV) every 8 hours for infection prophylaxis.

Intensive care unit (ICU) care, including mechanical ventilation, was provided for at least 24 hours in the CPR and EPR-I groups and for 48 hours in the EPR-II group to ensure an equivalent period of postrewarming intensive care. At 20 hours, the abdominal packing was removed and the abdominal wall closed. In the CPR group, body temperature was maintained at 37.5°C to 38.5°C throughout the experiment. In the EPR-I group, body temperature was maintained at 34°C until RT 12 hours, which was followed by self-rewarming and, when needed, external heating with blankets and a heater (target rewarming rate, 1°C/h) to 37.5°C. In the EPR-II group, rewarming was delayed to 36 hours and was deliberately slower (0.3°C/h).

Outcome Evaluation

Functional outcomes[5,15] were evaluated after discontinuing sedation according to overall performance categories (1=normal [able to eat and walk]; 2=moderate disability [able to eat and sit but not stand]; 3=severe disability [responds to pain but unaware of the environment]; 4=coma [minimal response to pain; positive pupillary light reflex; running movements and opisthotonus common]; and 5=death) and neurological deficit scores. The neurological deficit score is based on assessment of 5 facets of neurological function (level of consciousness, breathing pattern, cranial nerve function, sensory and motor function, and behavior), each with a maximum value of 20% (neurologic deficit score 0% to 10%=normal; 100%=brain death). Evaluations were agreed on by at least 2 team members. Because of the number of team members needed to conduct these experiments and the differences in observation time between groups, there was no practical way for the evaluations to be blinded. In previous experiments, interobserver agreement has been excellent. Results at 60 hours after initiation of rewarming were taken as the final measurements in each group. Blood samples were obtained at baseline and every 24 hours for cardiac (troponin I, creatine phosphokinase MB fraction), and liver (transaminases and bilirubin) enzymes. At 72 hours (EPR-I) or 96 hours (EPR-II), animals were reanesthetized with ketamine and halothane. A left thoracotomy was performed. Perfusion-fixation of the brain was accomplished with aortic infusion of 4% paraformaldehyde. A gross necropsy was performed. The brain was removed ≈1 to 2 hours after perfusion-fixation and retained in 10% neutral buffered formalin until dissection.

Neuropathology

Whole perfusion-fixed brains were divided into multiple coronal slices. Six coronal brain slices plus 3 transverse sections of the medulla oblongata and upper cervical cord were selected for microscopic evaluation. These slices were taken at the following levels: (1) optic chiasm; (2) anterior thalamus; (3) posterior thalamus; (4) midbrain; (5) posterior portions of the occipital lobes; (6) middle of the cerebellum and underlying brain stem; and (7) medulla oblongata and upper cervical cord. Brain slices were processed for paraffin embedding, resulting in 20 tissue blocks per brain. Blocks were sectioned at 5 μm, and the sections were stained with hematoxylin/eosin and Fluoro-Jade B.[16] The examining neuropathologist (R.G.) was blinded to treatment. A total of 25 neuroanatomic regions were examined. Each region with damage on microscopic examination received a pathological grade ranging from 1+ (minimal) to 5+ (severe). Each affected region on each side of the brain received separate scores in hematoxylin/eosin-stained and Fluoro-Jade B–stained sections. In each region, scores for edema were multiplied by 1, and scores for neuronal degeneration were multiplied by 2. Edema was not scored on the Fluoro-Jade B sections. Thus, the total possible scores for each region were 5×1 plus 5×2 (total, 15) for the hematoxylin/eosin stain and 5×2 (total, 10) for the Fluoro-Jade B stain. Total histological damage scores were determined by totaling these individual scores (ie, for each region with each stain). The maximum score was 1250 ([15+10 maximum per region]×25 regions×2 sides of the brain).

Statistical Analysis

Data are presented as mean±SD unless otherwise stated. A repeated-measures ANOVA was performed, followed by Bonferroni post hoc tests to identify differences in hemodynamic parameters, temperature, and neurological deficit and histological damage scores (with ranked data). ANOVA was performed for other physiological variables. The Mann-Whitney *U* test was used for the final neurological deficit score and the total histological damage score. The Fisher exact test was used to assess differences in overall performance category proportions (ie, normal outcome [overall performance category 1] versus abnormal outcome) among groups. A probability value <0.05 was considered significant.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Baseline

Baseline hemodynamics, hematological and biochemical parameters, and acid-base status were similar among groups.

TABLE 1. Physiological Parameters During Cardiac Arrest After Prolonged Hemorrhage

	CPR Group	EPR-I Group	EPR-II Group
Hemorrhage time, min	124.4±10.5	118.4±19.7	126.4±19.8
pH	6.88±0.24	6.99±0.16	6.93±0.12
Pco_2, mm Hg	86±39	58±30	73±28
Po_2, mm Hg	58±8	85±31	79±24
Base deficit, mmol/L	16.6±2	16.4±1.3	15.5±1.8
Potassium, mmol/L	7.6±1.2	6.8±1.3	7.3±0.9
Glucose, mg/dL	449±150	563±110	522±207
Lactate, mmol/L	15.1±1.6	14.6±2.8	14.1±2.3
Blood urea nitrogen, mg/dL	23.4±6.1	27.6±6.5	25.6±8.1
Hematocrit, %	16.2±2.2	18.1±2.3	19.6±1.8

Hemorrhagic Shock and Cardiac Arrest

The hemorrhage time before cardiac arrest was 124±16 minutes and did not differ among groups (Table 1). Samples of arterial blood gases and chemistries taken 1 minute after cardiac arrest were markedly abnormal but did not differ among groups (Table 1).

Resuscitation of the CPR Group

During chest compressions, MAP was maintained at >50 to 60 mm Hg (Figure 2). However, return of spontaneous circulation (ROSC) was not achieved by CPR in any dog.

During CPB, ROSC was achieved with defibrillation (mean total defibrillation energy, 157±181 J) in all dogs 15±16 minutes after initiation of CPB. However, substantial fluid losses from the rectum, orogastric tube, and intraperitoneal drain occurred during the resuscitation phase (Table 2; all $P<0.01$ versus the EPR groups). Progressive hypotension developed despite massive fluid resuscitation and vasoactive support. Lactate levels decreased transiently but increased sharply again until death (Figure 3; $P<0.01$ versus the EPR groups). Tty decreased slightly during CPR to ≈36°C and then increased to 38°C with CPB (Figure 4).

TABLE 2. Fluid Balance During the First 16 Hours of Resuscitation

	CPR Group	EPR-I Group	EPR-II Group
Fluid requirement, L	15.3±2.6*	4.1±0.6	4.2±0.9
Gastrointestinal fluid loss, L†	5.1±1.7*	0.7±0.4	1.5±0.4
Urine output, L	0.03±0.08*	2.0±1.2	2.7±0.9
Abdominal drainage, L	2.4±1.2*	0.3±0.2	0.5±0.4

*$P<0.01$, compared with EPR groups.
†Includes orogastric and rectal fluid losses.

Resuscitation of EPR Groups

The perfusion pressure during aortic flush was ≈20 mm Hg, with no difference between the EPR groups (Figure 2). At the end of the aortic flush, Tty had decreased to similar levels, with little change during circulatory arrest (Figure 4).

EPR dogs were rewarmed to 34°C within 1 hour by CPB. When Tpa reached 32°C, defibrillation yielded ROSC in all dogs at 30±12 minutes in the EPR-I group and at 32±23 minutes in the EPR-II group (P=NS). The total defibrillation energy required was 229±225 J in the EPR-I group and 264±326 J in the EPR-II group (P=NS).

At RT 12 hours, hematocrit was lower in the CPR group (19±9%) compared with the EPR-I (33±7%, $P=0.006$) and EPR-II (28±9%, $P=0.16$) groups. Final hematocrit values were 29±3% at RT 24 hours in the EPR-I group and 30±6% at RT 48 hours in the EPR-II group (P=NS versus the EPR-I group).

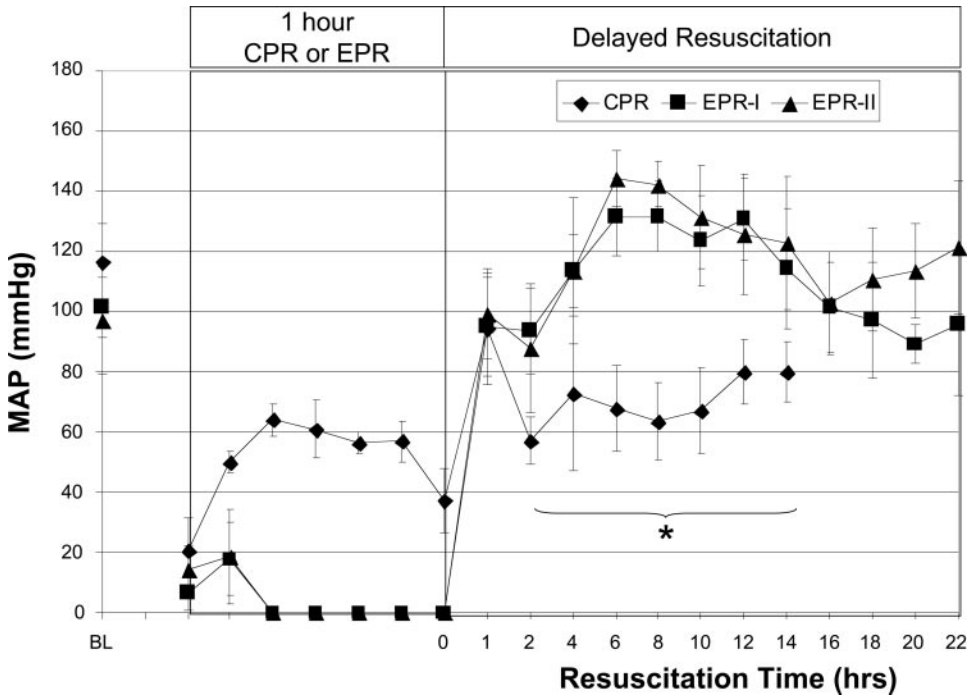

Figure 2. MAP during CPR or EPR, followed by delayed resuscitation. *$P<0.01$, comparing the CPR group with either EPR group.

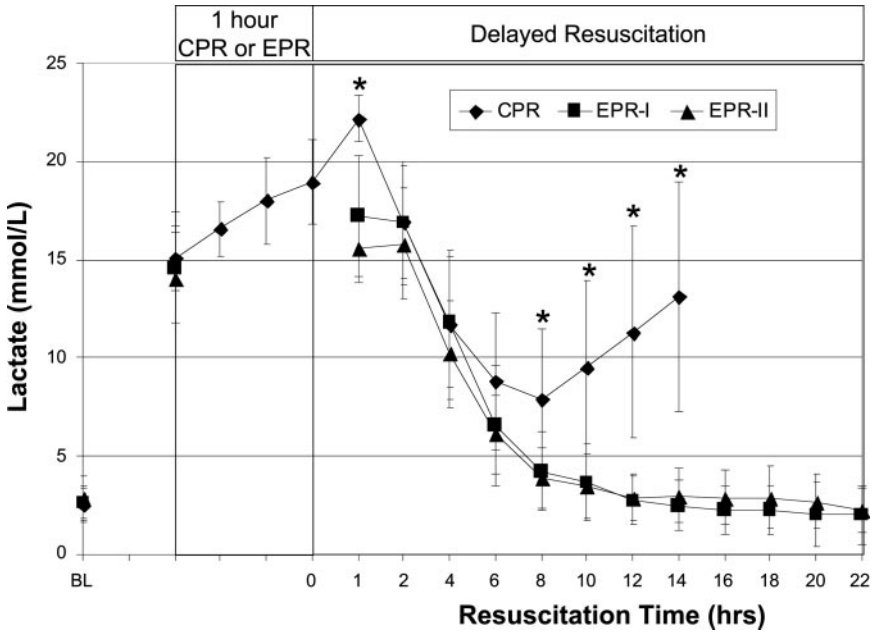

Figure 3. Arterial lactate levels during CPR or EPR followed by delayed resuscitation. *$P<0.01$, comparing the CPR group with either EPR group.

Final Outcome of All Groups

All CPR dogs died, with a median survival time of 14.7 hours (range, 11.5 to 16.5 hours; $P<0.01$ versus the EPR groups). In contrast, 6 of 7 EPR-I dogs survived to 72 hours; 1 died at RT 29.5 hours due to circulatory collapse. Similarly, in the EPR-II group, 6 of 7 dogs survived to 96 hours; 1 dog unexpectedly died at RT 66 hours. This dog was extubated at 48 hours and was in overall performance category 1. At necropsy, gastric contents were found inside the trachea and bronchus, suggesting aspiration.

Thirty-six hours after rewarming (RT 24 hours in the EPR-I group or RT 48 hours in the EPR-II group), 3 of 6 surviving dogs in the EPR-I group and 4 of 6 surviving dogs in the EPR-II group were in overall performance category 1 or 2. In the EPR-I group, however, 2 dogs developed recurrent generalized seizures 24 hours after extubation. Another had seizures shortly after extubation at RT 24 hours. Neurological function deteriorated in these animals. There was only 1 dog that recovered to overall performance category 1. In contrast, none of the EPR-II survivors exhibited seizures, and 5 of 6 survivors continued to improve and regained normal function ($P=0.06$ versus the EPR-I group; Figure 5). The neurological deficit score of the survivors in the EPR-II group was numerically better over time compared

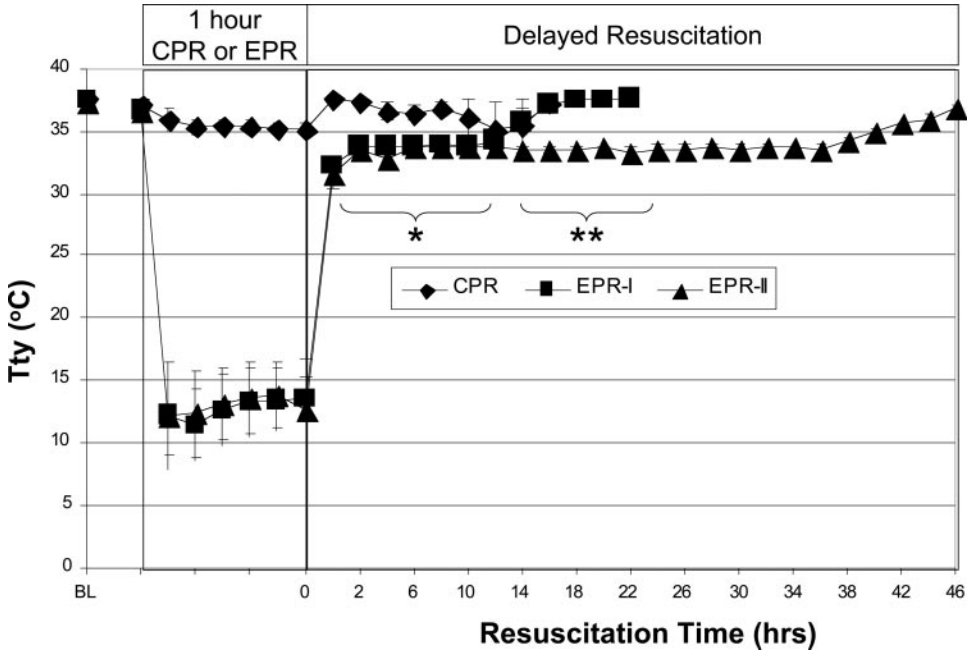

Figure 4. Tty during CPR or EPR, followed by delayed resuscitation. *$P<0.01$, comparing CPR and EPR groups; **$P<0.01$, comparing the CPR and EPR-I groups with the EPR-II group.

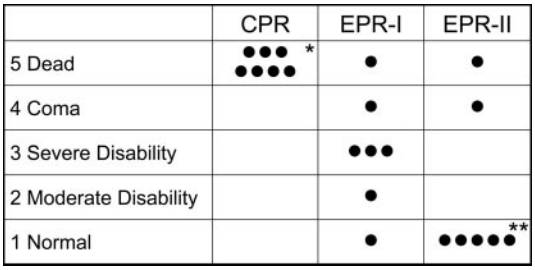

Figure 5. Overall performance categories (OPC; refer to text for details) after prolonged hemorrhagic shock leading to cardiac arrest with resuscitation by CPR or EPR. *$P<0.01$, survival in the CPR group vs the EPR-I and EPR-II groups; **$P=0.06$, normal (OPC 1) vs abnormal (OPC 2 to 5) in the EPR-II vs the EPR-I group.

with the EPR-I group ($P=0.09$), although the final neurological deficit scores were better ($P=0.04$; Figure 6).

Brain Histology

In the EPR-I group, the brains exhibited prominent, acute eosinophilic neuronal degeneration within the frontal, parietal, temporal, and occipital cortices (Figure 7). In the most severely affected regions, a laminar band of necrosis with associated neuropil spongiosis was evident. Marked to severe neuronal degeneration was present in the caudate region, with slightly less degeneration in the putamen. Within the caudate, degeneration was primarily present in the medium spiny neurons. The larger interneurons were relatively unscathed. In the hippocampus, moderate to marked neuronal degeneration was present primarily within the CA1 region, although degeneration often extended to the CA3 and CA4 regions. In the cerebellum of the EPR-I group, Fluoro-Jade B staining revealed small numbers of degenerating Purkinje neuron cell bodies and greater numbers of dendrites. Degrees of neuronal degeneration were scored slightly higher in Fluoro-Jade B–stained sections than in hematoxylin/eosin-stained sections.

In contrast to the EPR-I group, the EPR-II group had only mild damage in the neocortex (Figure 7A; $P<0.05$ versus EPR-I in the frontal and temporal lobes). In the hippocampus of the EPR-II group, damage severity as assessed by Fluoro-Jade B staining was also less (Figure 7B; $P=0.065$). Furthermore, Fluoro-Jade B staining in the hippocampus of EPR-II group dogs was primarily restricted to neuronal processes within CA3 and CA4. Degenerative changes in the caudate and putamen of EPR-I and EPR-II groups were similar (Figure 7). Within the cerebellum of the EPR-II group, Fluoro-Jade B staining revealed scattered Purkinje neurons with degenerative dendrites.

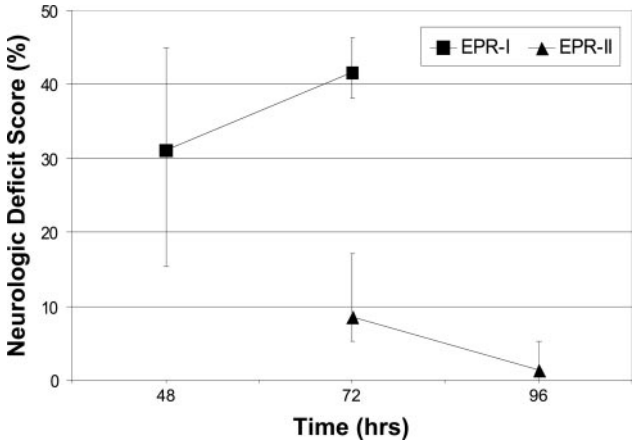

Figure 6. Neurological deficit scores (refer to text for details) after prolonged hemorrhagic shock leading to cardiac arrest with resuscitation by EPR, with or without prolonged resuscitative mild hypothermia. Median and interquartile ranges are shown. *$P=0.09$ for repeated-measures ANOVA; $P=0.04$ for final neurological deficit scores by the Mann-Whitney U test.

Figure 7 includes only those brain regions that had greater than minimal degrees of degenerative changes. All other brain regions, ie, the piriform cortex, entorhinal cortex, septal region, basal forebrain, anterior thalamus, posterior thalamus, amygdala, midbrain, pons, and medulla oblongata, were characterized by no or only minimal degrees of damage, with no differences between groups. By repeated-measures ANOVA for brain regions, the histological damage scores were higher in the EPR-I group than in the EPR-II group ($P=0.006$). The total histological damage scores in the EPR-I group (226 ± 111) were numerically higher than those in EPR-II (102 ± 87; $P=0.15$).

Extracerebral Organ Injury

The heart and liver enzyme levels in the EPR groups were markedly increased after resuscitation. They decreased gradually after 24 hours but remained increased at 72 or 96 hours, with no difference between groups (data available in Data Supplement Table I).

At necropsy, in the CPR group, all dogs had generalized edema, severe endocardial hemorrhage, lung edema, and bloody ascites. Although the serosal side of the intestine had mild hemorrhage, the intestinal mucosa was sloughed over the entire length of the intestine. In the EPR groups, the most consistent findings were mild to moderate endocardial hemorrhage and extensive hemorrhage in the gallbladder wall. Occasionally, hemorrhagic spots were found on the serosal surface of the intestine, but there was no necrosis. Lung edema was found only in 1 EPR-I dog.

Discussion

We established an exsanguination cardiac arrest model that is unsalvageable by contemporary conventional resuscitation. At the time of cardiac arrest, ≈60% to 90% of the estimated blood volume was removed. Arterial blood gases taken at the beginning of cardiac arrest revealed severe acidemia ($pH<7.0$), hyperkalemia, and hyperlactemia. As expected, none of the dogs could be resuscitated with CPR, despite vigorous blood and fluid replacement, standard drug therapy, and chest compressions with an MAP at 50 to 60 mm Hg. Such favorable hemodynamic responses would be difficult to achieve in traumatic, exsanguination cardiac arrest victims. Although all of the dogs could be resuscitated with CPB, all subsequently died of severe multiple-organ failure, including cardiovascular dysfunction, renal failure, and extensive gastrointestinal mucosal necrosis. This pattern is anticipated after prolonged hemorrhagic shock and cardiac arrest.[2] Such trauma victims similarly develop irreversible shock, including vasodilatation unresponsive to vasopressors and massive capillary leak, presumably secondary to the systemic inflammatory response. Thus, it was anticipated that animals in the

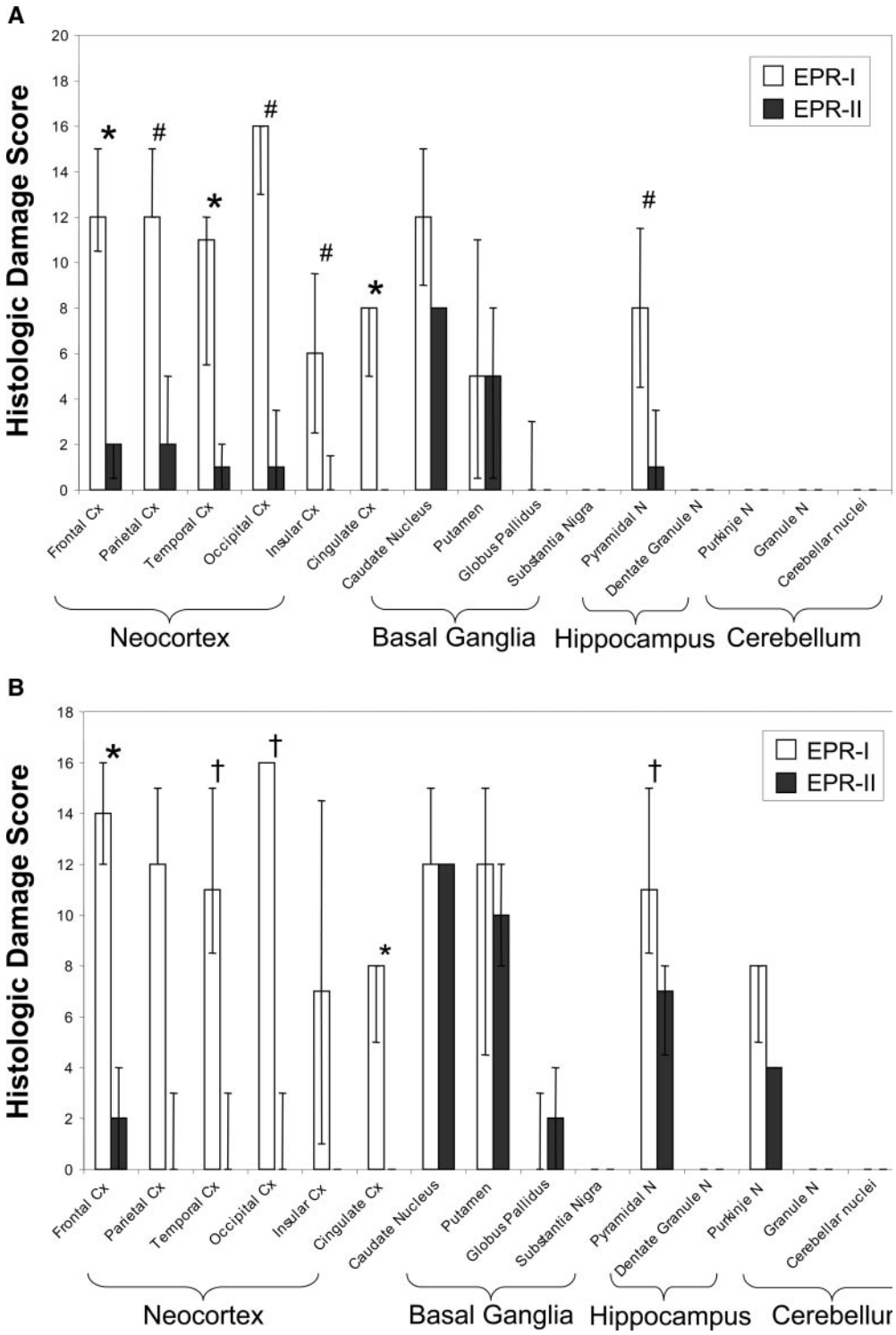

Figure 7. Brain histological damage scores (refer to text for details) after prolonged hemorrhagic shock leading to cardiac arrest with resuscitation by EPR, with or without prolonged postresuscitation mild hypothermia. Brain regions with no or minimal neuronal changes are not included. A, Hematoxylin/eosin staining; B, Fluoro-Jade B staining. Bars represent medians and interquartile ranges. Cx indicates cortex; N, neurons. *$P<0.05$ for comparison between EPR-I and EPR-II; #$P=0.09$, †$P=0.07$.

conventional-resuscitation arm would exhibit considerable gastrointestinal fluid loss and ascites. We suspect that the large amounts of fluids administered in an attempt to sustain these animals led to hemodilution, as reflected in lower hematocrit values at 12 hours; there were no intergroup differences in blood loss. In contrast, EPR with delayed resuscitation was superior to conventional CPR for resuscitation of traumatic, exsanguination cardiac arrest.[1–3] Twelve of 14 dogs survived without severe extracerebral organ damage.

Capone et al[17] also compared EPR with conventional resuscitation. That investigation differed from the current study in that before

induction of EPR, the period of severe hemorrhage was shorter (60 minutes), and there was no period of normothermic cardiac arrest. After profound hemorrhagic shock (MAP of 30 mm Hg for 60 minutes), survival was similar after conventional resuscitation or after addition of 1 hour of EPR at 10°C. The addition of more prolonged hemorrhagic shock and brief cardiac arrest before standard resuscitation in the current study is 100% lethal with standard care, although EPR can yield normal recovery.

Dogs in the EPR-I group had severe neurological deterioration after an initial recovery. Three dogs had generalized seizures, although 2 had regained consciousness initially. This is a unique pattern that we had not previously encountered during years of experience with exsanguination and normovolemic cardiac arrest models. Extensive neocortical laminar necrosis was found, along with cerebellar synaptic injury. This is in sharp contrast to the lack of histological brain damage after rapid exsanguination cardiac arrest in our prior EPR report.[18] Given that the EPR and delayed resuscitation protocols were almost identical between these 2 studies, the preexisting prolonged hemorrhagic shock probably set the stage for this delayed neurological deterioration.

We speculate that the mechanism of this delayed neurological deterioration may be cytotoxic brain edema that peaks at ≈48 hours after reperfusion.[19] Different from rapid exsanguination cardiac arrest, blood glucose levels at the time of cardiac arrest in this model were >500 mg/dL in most dogs, likely related to the stress of prolonged hemorrhagic shock. On the basis of a histological pattern similar to that found after hyperglycemia-associated brain injury in forebrain ischemia models[20] and previous reports that hyperglycemia exacerbates neurological dysfunction after cardiac arrest,[21] we speculate that hyperglycemia may have contributed to the delayed neurological deterioration. Hypothermia appears to be very effective in protecting against ischemic brain injury during hyperglycemia.[22] However, rapid rewarming of the traumatically injured brain can markedly exacerbate injury.[23] This suggests the need to carefully optimize the use of mild hypothermia and rewarming.

The effects of prolonged post-EPR mild hypothermia were impressive. Similar to our finding, Gunn et al[19] documented that delayed brain edema that peaked 48 hours after 30 minutes of cerebral ischemia in fetal lambs was abolished by prolonged (72-hour) hypothermia; 48-hour cooling was associated with rebound seizures. Likewise, Colbourne and Corbett[24] found that to salvage CA1 neurons after 5 minutes of ischemia in gerbils, hypothermia (32°C), induced 1 hour after ischemia, had to be maintained for 24 hours. In our study, with hypothermia for 36 hours and slow rewarming, 5 of 7 dogs regained consciousness, and seizures were not observed. One dog died of probable aspiration. Although mild hypothermia is recommended for comatose survivors of cardiac arrest[25] and laboratory studies suggest the benefit of mild hypothermia during resuscitation from hemorrhagic shock,[26] more research on the optimal timing and rate of rewarming is needed.

This study has some limitations. First, it was not fully randomized. Seven EPR-II dogs were added after 5 experiments had been performed in each of the other groups because we had observed delayed neurological deterioration and we hypothesized that prolonged mild hypothermia with slow rewarming would prevent this deterioration. However, these studies were carried out in sequence, contiguous with those in the previous dogs by the same experienced research team. In addition, outcomes in the CPR and EPR-I experiments performed after the addition of the EPR-II group mirrored the earlier experiments.

Second, final outcome was determined at different times in the EPR-I and EPR-II groups. On the basis of our previous experience,[4–10] we thought it essential to compare functional outcomes after identical postrewarming and extubation periods in the EPR-I and EPR-II groups. This mandated that we compare outcomes at 72 and 96 hours in the EPR-I and EPR-II groups, respectively. We recognize that for histological evaluations, delayed neuronal death may occur 3 days to 1 week after global ischemia,[27] which may have biased the outcome toward worse damage at 96 hours than at 72 hours in the EPR-II group. Despite these differences, we still found significantly better brain histology results in the EPR-II group.

Third, external chest compressions are not standard care for trauma victims who experience cardiac arrest. Emergency department thoracotomy with open chest cardiac massage is indicated because of the potential for treating a surgical cause of cardiac arrest, eg, pericardial tamponade.[3] External chest compressions generated excellent blood pressures with aggressive fluid resuscitation. Despite this, there were no long-term survivors in the CPR group.

Fourth, systemic heparinization was used, because standard CPB equipment was utilized. This precluded studies of coagulation. In the massively traumatized patient, however, one could avoid systemic heparin by using a heparin-bonded CPB system.

Fifth, vessel cannulation for EPR within 2 minutes of cardiac arrest may be difficult in the field, although it is reasonable for the scenario in the Emergency Department by a trauma surgeon. Also, large amounts of fluid are currently required to achieve the desired brain temperature. Despite these limitations, we believe that clinical application of EPR should be studied in trauma victims who have experienced exsanguination cardiac arrest. Ideally, this would be applied in the field. Ambulances could carry large amounts of fluid for the aortic flush. Given the complexity of the procedure with currently available equipment, however, we believe that the first study should be conducted in Emergency Departments of major trauma centers. We are working with companies to develop better vessel cannulation techniques, novel catheters, and cooling systems to facilitate the induction of EPR by civilian emergency medical services and perhaps, on the battlefield. This could also facilitate rapid initiation of CPB for delayed resuscitation in this situation, although this is less of an issue, because preparations for CPB can proceed simultaneously with resuscitative surgery.

EPR is remarkably superior to conventional CPR in facilitating survival and neurological recovery in a model of otherwise unresuscitable prolonged hemorrhage with exsanguination cardiac arrest. Extended application of mild hypothermia with slow rewarming during ICU care after EPR was critical in achieving intact neurological outcomes. Use of mild hypothermia and/or slow rewarming may also have implications for optimal neuroprotection in conventional deep hypothermic circulatory arrest.[28]

Acknowledgments

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Disclosures

Drs Wu, Kochanek, Tisherman and S.W. Stezoski have submitted a provisional patent entitled "Method of Inducing Suspended Animation Following Cardiopulmonary Arrest." The other authors report no conflicts.

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CLINICAL PERSPECTIVE

The success of emergency preservation and resuscitation (EPR) may have important implications. First, the potential for its use may not be limited by the duration of preexisting hemorrhagic shock. This is important clinically, because the exact duration of hemorrhage is usually unknown, and prolonged hemorrhagic shock before cardiac arrest is common in the rural or military trauma situation. Second, EPR is much more reliable in preserving tissue viability during cardiac arrest than is optimized cardiopulmonary resuscitation and may allow intact survival. Third, the impressive benefits of prolonged postischemic mild hypothermia and slow rewarming may have implications for neuroprotection after deep hypothermic circulatory arrest used in cardiac or neurological surgery. Brain damage found in deep hypothermic circulatory arrest is similar to that seen in our study. The development of EPR has focused on the exsanguinating trauma patient. Although most trauma patients are young and have normal coronary arteries, similar to the animals in this study, exsanguination can occur in older patients with coronary artery disease. The effects of inducing EPR in such patients are difficult to predict, although one should keep in mind that the results of current management strategies for trauma patients who experience cardiac arrest are dismal for all victims. Rapid cooling of the heart might help preserve cardiac function, just as brain cooling protects neurological function.

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Emergency preservation and resuscitation with profound hypothermia, oxygen, and glucose allows reliable neurological recovery after 3 h of cardiac arrest from rapid exsanguination in dogs

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We have used a rapid induction of profound hypothermia (< 10°C) with delayed resuscitation using cardiopulmonary bypass (CPB) as a novel approach for resuscitation from exsanguination cardiac arrest (ExCA). We have defined this approach as emergency preservation and resuscitation (EPR). We observed that 2 h but not 3 h of preservation could be achieved with favorable outcome using ice-cold normal saline flush to induce profound hypothermia. We tested the hypothesis that adding energy substrates to saline during induction of EPR would allow intact recovery after 3 h CA. Dogs underwent rapid ExCA. Two minutes after CA, EPR was induced with arterial ice-cold flush. Four treatments (n = 6/group) were defined by a flush solution with or without 2.5% glucose (G+ or G−) and with either oxygen or nitrogen (O+ or O−) rapidly targeting tympanic temperature of 8°C. At 3 h after CA onset, delayed resuscitation was initiated with CPB, followed by intensive care to 72 h. At 72 h, all dogs in the O+G+ group regained consciousness, and the group had better neurological deficit scores and overall performance categories than the O− groups (both P < 0.05). In the O+G− group, four of the six dogs regained consciousness. All but one dog in the O− groups remained comatose. Brain histopathology in the O−G+ was worse than the other three groups (P < 0.05). We conclude that EPR induced with a flush solution containing oxygen and glucose allowed satisfactory recovery of neurological function after a 3 h of CA, suggesting benefit from substrate delivery during induction or maintenance of a profound hypothermic CA.
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Keywords: cardiac arrest; energy metabolism; neuropreservation

Introduction

Traumatic exsanguination cardiac arrest (ExCA) remains a condition with a nearly 100% mortality (Rhee *et al*, 2000). Conventional resuscitation using basic and advanced trauma life support as recommended by the American Heart Association as well as the American College of Surgeons is futile due to profoundly reduced blood volume and ongoing bleeding. In the 1980s, a novel approach to ExCA was proposed by Safar and Bellamy (Bellamy *et al*, 1996), who conceived the idea that emergency preservation with rapid induction of profound hypothermia and/or administration of pharmacological treatments, allowing time for transport, damage control surgery, and delayed resuscitation using cardiopulmonary bypass (CPB) would eventually allow intact survival. This concept of emergency preservation and resuscitation has been given the

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acronym EPR (Wu *et al*, 2006) and its efficacy was demonstrated in dogs (Behringer *et al*, 2001*d*, 2003; Nozari *et al*, 2004*b*), pigs (Alam *et al*, 2006), and rats (Drabek *et al*, 2007). Recently, novel agents have been found to induce a hibernation-like state with carbon monoxide in worms under hypoxia (Nystul and Roth, 2004) or with hydrogen sulfide in mice (Blackstone *et al*, 2005). However, rapid induction of profound hypothermia remains the only approach effective in large animal models and thus the only currently feasible clinical approach.

For induction of EPR in a series of prior reports, we have used ice-cold normal saline. Although pharmacological adjuncts added to the flush solution could have theoretical advantages in preserving vital tissues, long-term neurological outcome in our models was not improved versus normal saline with a number of mechanism-based pharmacological approaches (Behringer *et al*, 2001*a*, *b*) except for the antioxidant tempol, which had a modest effect (Behringer *et al*, 2002). Similarly, the use of conventional or novel alternative flush solutions such as albumin, Unisol (Behringer *et al*, 2001*c*), or the University of Wisconsin solution (Wu *et al*, 2005) did not augment the protection afforded by profound hypothermia. Taking a different approach, Taylor *et al* (1994) demonstrated that continuous perfusion with an asanguineous preservation solution allowed satisfactory recovery over 3 h of ultraprofound hypothermia ($<5^{\circ}$C). Alam *et al* (2006) have had success with a similar approach in pigs with traumatic hemorrhage. However, it is unlikely that in a clinical trauma scenarios continuous perfusion will be an option (Bellamy *et al*, 1996; Wu *et al*, 2005). Using an ice-cold saline flush for induction of EPR, preservation efficacy was improved with the use of either lower temperature or with faster cooling rates; however, efficacy reached its plateau at a core temperature between 7 and 10°C (Behringer *et al*, 2003; Alam *et al*, 2006) or when cooling rate was maximized (Alam *et al*, 2004). However, at the maximal cooling rate that could be achieved, it still took between 12 and 15 mins to achieve a brain temperature of $\sim$10°C in dogs (Behringer *et al*, 2003). This 12–15-min period required to reach target temperatures might represent a key limiting factor in the ultimate success of EPR based on the report that the brain oxygen demand in pigs remains $\sim$50% of baseline at 28°C, $\sim$19% at 18°C, and $\sim$10% at 8°C, respectively (Ehrlich *et al*, 2002).

Logically, we speculated that providing energy substrates during induction of EPR might either avoid further energy depletion, or even restore energy reserves that would probably be reduced during the 5 mins period of shock, 2 mins normothermic CA, and cooling duration—before achieving profound hypothermia. Perfusion of dissolved oxygen at deep hypothermia (without circulatory arrest) can deliver considerable substrate, particularly in a setting of markedly reduced metabolic demands (Dexter *et al*, 1997). Robbins *et al* (1990) reported that intermittent flush of energy substrates into the brain during profound hypothermic CA delayed ATP and creatine phosphate depletion in brain. However, there is no solution that has been convincingly shown to improve neurological outcome in a prolonged CA model, without intermittent perfusion during the arrest.

Using our modified EPR model (Nozari *et al*, 2004*a*), the current study was designed to test if profound hypothermia induced by aortic flush with a solution that was enriched with energy substrates, that is oxygen and glucose, could successfully produce intact long-term neurological outcome despite a prolonged (3 h) CA.

Materials and methods

The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh and the Department of Defense and followed the National Guidelines for Treatment of Animals.

Experimental Design

The model included three phases: (1) exsanguination (5 min) and CA (2 min); (2) EPR (3 h); (3) delayed resuscitation, including CPB (2 h) and intensive care (72 h). At the end of the exsanguination and CA phase, dogs were randomized into four groups based on the specific additives in the ice-cold normal saline flush solution, namely (1) oxygen + glucose (O + G +), (2) oxygen without glucose (O + G−), (3) glucose alone (O−G +), and (4) neither oxygen nor glucose (O−G−).

Anesthesia and Preparation

Custom-bred, male hunting dogs, weighing 19.5 to 24.0 kg, were housed for at least 3 days before the experiment. A total of 24 dogs were used, and no dog was excluded from the protocol once entered. Dogs were fasted with free access to water for 12 h. Ketamine 10 mg/kg and atropine 0.4 mg were administered intramuscularly. A cannula (18 G) was inserted into a peripheral vein and fluid infusion (D5W/0.45 NaCl at 4 mL/kg per h) was started. After anesthesia induction with 4% halothane via face mask, endotracheal intubation (ID 8 to 9 mm) was performed. Continuous anesthesia was provided with halothane titrated during preparation (O_2/N_2: 50%:50%). Controlled ventilation was initiated with tidal volume of 12 to 15 mL/kg, PEEP 2 cm H_2O, and frequency of 20 to 25 per min, titrated to maintain $PaCO_2$ of 35 to 45 mm Hg. Electrocardiogram lead II was continuously monitored. A Foley catheter was placed into the urinary bladder. Temperature probes were inserted for rectal, esophageal, and both tympanic membrane temperatures (Tty). Sterile cut downs were made in both groins and the right side of the neck. A PE 90 catheter was inserted into the left femoral artery for blood pressure monitoring and blood sampling. A

pulmonary artery catheter (7.5 F) was inserted via the left femoral vein into the pulmonary artery to monitor pressure, cardiac output, and core temperature (Tpa). A CPB arterial cannula (7 or 9 G) was inserted into the right femoral artery. A multiple hole, CPB venous cannula (19 F) was inserted 25 cm into the right atrium via the right external jugular vein. The CPB system consisted of a hollow-fiber membrane oxygenator (Medtronic, Grand Rapids, MI, USA) and centrifugal pump (Biomedicus, Eden Prairie, MN, USA). For induction of EPR hypothermia, the CPB system was primed with normal saline at 2°C; for delayed resuscitation after EPR, the system was primed with shed blood (30 mL/kg) and Plasma-Lyte A (Baxter, Deerfield, IL, USA).

Baseline measurements (hemodynamics, arterial and venous blood gases, and body temperatures) were determined when hemodynamics was stable for 15 to 30 mins after surgical preparation and Tpa was controlled at 37.5 to 38.5°C.

Exsanguination and Cardiac Arrest Phase

After two baseline measurements, heating, intravenous fluids, and halothane were discontinued, and the dogs were weaned to spontaneous breathing of air via a T-tube. When the canthal reflex returned, rapid exsanguination was initiated via the right external jugular cannula and the blood was collected in bags with sodium citrate anticoagulant for later reinfusion. Exsanguination was conducted stepwise to a mean arterial pressure of 20 mm Hg at 4 mins. At 5 mins, ventricular fibrillation was induced with transthoracic AC at 95 V to ensure zero blood flow. Ventricular fibrillation was confirmed with electrocardiogram and arterial blood pressure.

Emergency Preservation and Resuscitation Phase

Two minutes after the onset of CA, flush solution (80 mL/kg) at 2°C was infused into the aorta at a rate of 80 mL/kg per min using the CPB pump. Close-chest CPB from the right external jugular vein to the right femoral artery was then initiated for induction of hypothermia until Tty on the right side, which was arbitrarily chosen to represent brain temperatures, reached 8°C. Either 100% oxygen or nitrogen was supplied to the oxygenator throughout the flush interval and induction of hypothermia. The gas flow to the CPB oxygenator was adjusted to maintain $PaCO_2$ 35 to 45 mm Hg. Once Tty of 8°C was reached, the CPB was stopped. The entire body was covered with ice from the onset of flush to the end of 3 h of CA.

Delayed Resuscitation Phase

Cardiopulmonary Bypass: After 3 h of CA, reperfusion was started with CPB that was primed with shed blood plus heparin 1,000 U. Before the start of CPB, sodium bicarbonate (1 mEq/kg) and epinephrine 0.01 mg/kg were injected into the circuit. The temperature of the water bath of the CPB heat exchanger was set to 5°C above Tpa until Tpa reached 34°C. Cardiopulmonary bypass was started with a flow of 50 mL/kg per min when Tpa was <20°C, increased to 75 mL/kg per min when Tpa was 21 to 30°C, and to 100 mL/kg per min when Tpa was >30°C. Repetitive doses of epinephrine (0.01 mg/kg) were given to increase mean arterial pressure to 60 mm Hg at Tpa <20°C, to 80 mm Hg at Tpa 21 to 30°C, and to 100 mm Hg at Tty >30°C. When Tpa reached 32°C, defibrillation was attempted with external DC countershocks of 150 J, increased by 50 J for repeated shocks. Oxygen flow through the oxygenator was adjusted to keep $PaCO_2$ between 35 and 40 mm Hg and $PaO_2 \geq 100$ mm Hg. During CPB, controlled ventilation was given with 100% oxygen at a rate of between 8 and 10 breaths/min. Intravenous fluids were restarted at 4 mL/kg per h. A base deficit of >6.0 mEq/L was corrected with sodium bicarbonate. Mean arterial pressure was maintained at 90 to 150 mm Hg. The CPB flow rate for assisted circulation was sequentially reduced to 75, 50 mL/kg per min, and stopped at 120 mins. During CPB, activated clotting times were maintained at >300 secs with additional heparin.

ICU: The details of life support, including mechanical ventilation, hemodynamic monitoring and support, and correction of acid–base or electrolyte abnormalities, were published previously (Behringer *et al*, 2003). Body temperature was kept at 34°C until 36 h of resuscitation, followed by slow controlled rewarming (0.3°C/h) to 36.5°C, as per our previous study that found the benefit of more prolonged postresuscitation mild hypothermia (Wu *et al*, 2006). Consequently, mechanical ventilation was continued to 48 h with morphine sulfate analgesia, diazepam sedation, and pancuronium. Hemodynamic support included additional fluids and pressors as needed. Acid–base and electrolyte abnormalities were corrected as per typical clinical patient management. At 48 h, neuromuscular blockade was reversed, and sedation and analgesia were discontinued. Dogs were then weaned from mechanical ventilation. After extubation, they were transferred to the step down unit where continuous intravenous fluids and vital sign monitoring were provided until 72 h.

Outcome Evaluation

Functional outcomes were evaluated every 6 h in the step down unit according to the overall performance categories (OPC: 1 = normal or slight disability; 2 = moderate disability; 3 = severe disability; 4 = coma; 5 = death) and the neurological deficit scores (NDS: 0 to 10% = normal; 100% = brain death), which include level of consciousness, breathing pattern, cranial nerve function, sensory and motor function, and behavior. At 72 h, a final functional assessment was performed and animals were then reanesthetized with ketamine and halothane. Perfusion fixation was performed with cephalad infusion of 10% neutral-buffered formalin via the thoracic aorta. The entire brain was removed $\sim$2 h after perfusion fixation and retained in 10% neutral-buffered formalin until dissection.

Neuropathology

Whole perfusion-fixed brains were divided into multiple coronal slices. Six coronal brain slices plus three

transverse sections of the medulla oblongata and upper cervical cord were selected for microscopic evaluation. These represented entire brain slices taken at the following levels: (1) the optic chiasm; (2) the anterior thalamus; (3) the posterior thalamus; (4) the midbrain; (5) posterior portions of the occipital lobes; (6) middle of the cerebellum and underlying brainstem; (7) medulla oblongata and upper cervical cord. These slices were processed for paraffin embedding, resulting in 20 tissue blocks from each brain. The paraffin blocks were sectioned at 5 μm, and the resulting sections stained with hematoxylin and eosin (H&E) and with Fluoro-Jade B. The examining neuropathologist (RG) was masked as to the treatment groups. Each neuroanatomic region with evidence of damage on microscopic examination received a subjective pathological grade ranging from 1+ (minimal) to 5+ (severe). Each affected region on each side of the brain (right and left) received separate scores for the degrees of neuropathological damage detected in H&E- and Fluoro-Jade B-stained sections. The histological damage scores (HDS) in each region were compared with other groups.

Statistical Analysis

Data are presented as mean $\pm$ s.d. unless otherwise stated. Repeated measures analysis of variance followed by Bonferroni *post hoc* tests was performed to identify differences in hemodynamic parameters and temperatures between groups. Overall performance categories, NDS, and HDS scores were analyzed using Kruskal–Wallis and then Mann–Whitney *U*-test with correction of multiple comparisons. The Spearman rank analysis was used to examine the correlation of histological findings from different histological techniques. $P<0.05$ was considered statistically significant.

Results

Induction of Emergency Preservation and Resuscitation

The total EPR induction time to reach Tty 8°C was similar between groups. During the induction of EPR, glucose levels in the G+ groups were approximately five times higher than in the G− groups ($P<0.01$), and the PaO_2 values in the O+ groups were ~20 times higher than in the O− groups ($P<0.01$) (Table 1). $PaCO_2$ did not differ among four groups. Compared with G− groups, the G+ groups had lower levels of plasma sodium ($P<0.05$) and hematocrit (NS) at the end of flush (Table 1). The O+ groups had significantly lower plasma potassium than the other two groups ($P<0.01$) (Table 1). The brain temperatures did not differ among four groups over 3 h of CA (Figure 1).

Resuscitation

After 3 h of CA, rewarming and stable mean arterial pressure (>60 mm Hg without need for vasopressors) were achieved in all dogs with CPB. When Tpa reached 32°C (40 to 50 mins after delayed resuscitation with CPB), restoration of spontaneous circulation was achieved in all dogs with 1 to 2 defibrillation attempts at 150 J. Cardiopulmonary

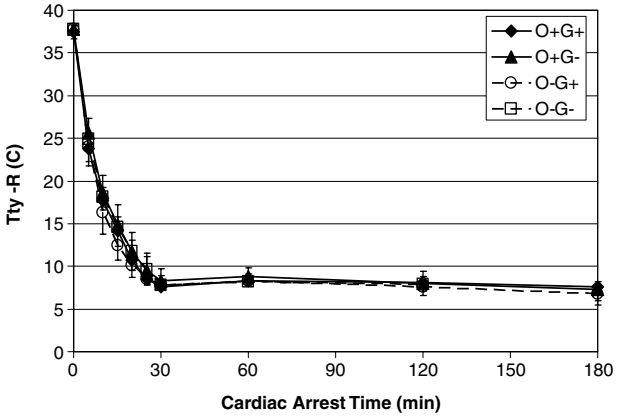

Figure 1 Right side tympanic temperatures during induction of EPR and no-flow cardiac arrest. O + G + : 100% O_2 with 2.5% glucose in normal saline; O + G−:100% O_2 with normal saline; O−G + : 100% N_2 with 2.5% glucose in normal saline; O−G−:100% N_2 with normal saline.

Table 1 Physiological parameters at the end of EPR induction

Groups	*O+G+*	*O+G−*	*O−G+*	*O−G−*
Induction time (mins)	26.2±1.5	26.0±2.3	24.9±2.2	27.1±2.3
Glucose (mg/dl)	975±186**	175±60	957±295**	288±15
PO_2 (mm Hg)	432→800$^+$	410→800$^+$	23.5±7.8$^{\#\#}$	18.6±8.1$^{\#\#}$
pHa	7.2±0.1	7.1±0.0	7.1±0.0	7.1±0.1
PCO_2 (mm Hg)	34.4±3.8	41.6±3.4	35.3±4.4	39.9±4.1
BE (mmol/L)	−13.8±3.0	−13.1±3.5	−15.5±1.0	−15.2±1.9
Lactate (mmol/L)	6.4±1.0	6.7±1.7	7.6±1.0	8.8±0.8
Hematocrit (%)	11.3±2.8	15.5±2.8	9.5±4.3	14.7±3.5
Sodium (mmol/L)	132.9±7.1*	147.3±2.3	136.6±0.8*	145.9±1.4
Potassium (mmol/L)	3.5±0.3	3.1±0.2	4.8±0.6$^{\#}$	5.0±0.6$^{\#}$

EPR, emergency preservation and resuscitation.
*,**$P<0.05$ or 0.01, versus the G− groups; $^{\#,\#\#}P<0.05$ or 0.01, versus the O+ groups; $^+$blood gases were measured at 37°C.

bypass was then weaned off at 2 h in all dogs without any need for pharmacological support. During the delayed resuscitation phase, the lactate levels were significantly higher in the two O− groups ($P<0.05$) (Figure 2). All dogs were maintained with stable vital signs to 72 h.

Functional Outcome

At 72 h, all dogs in the O+G+ group regained consciousness with a significantly better OPC (Figure 3) and better NDS (Figure 4) (both $P<0.05$), compared with the O− groups. In the O+G− group, four of the six dogs regained consciousness (NS versus other groups). In contrast, 11 of the 12 dogs in the O− groups remained comatose.

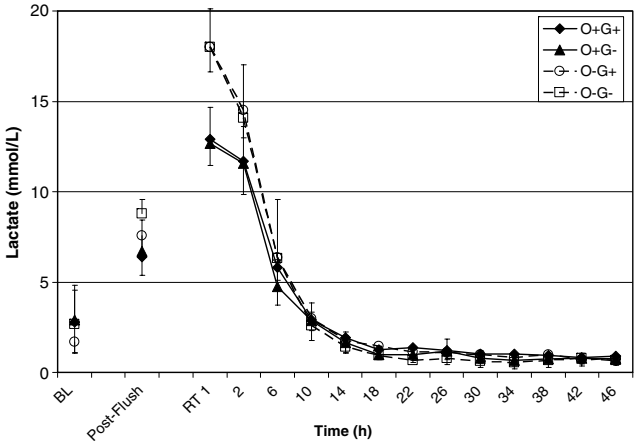

Figure 2 Arterial lactate levels over 3 h of ExCA and 48 h delayed resuscitation. O + G + : 100% O_2 with 2.5% glucose in normal saline; O + G−:100% O_2 with normal saline; O−G + : 100% N_2 with 2.5% glucose in normal saline; O−G−:100% N_2 with normal saline.

	O+G+	O+G-	O-G+	O-G-
5 Dead		p<0.05		
4 Coma		*	***	**
3 Severe Disability		*	***	***
2 Moderate Disability	****	**		*
1 Normal	**	**		

Figure 3 Final overall performance category (OPC) at 72 h after 3 h of cardiac arrest. O + G + : 100% O_2 with 2.5% glucose in normal saline; O + G−:100% O_2 with normal saline; O−G + : 100% N_2 with 2.5% glucose in normal saline; O−G−:100% N_2 with normal saline.

Neuropathology

Total HDS combining scores in all regions on both sides was significantly higher (worse) in the O−G+ group compared with the other groups ($P<0.05$) (H&E staining (median and range): O−G+: 145 (120 to 150), O+G+: 51 (10 to 76), O+G−: 57 (27 to 70), and O−G−: 48 (10 to 112); Fluoro-Jade B staining: O−G+: 132 (106 to 174), O+G+: 56 (38 to 74), O+G−: 36 (0 to 86), and O−G−: 57 (2 to 96)). Of 25 brain regions, there were 11 regions that had substantial brain injury in at least one group (Figures 5A and 5B). Histological damage scores of the O−G+ group was consistently worse than all other groups in all involved brain regions ($P<0.05$) (Figures 5A and 5B). The results with H&E and Fluoro-Jade B staining were significantly correlated in all 25 brain regions ($r=0.52$ to 1.0, all $P<0.01$, Spearman rank test).

Discussion

With the induction of profound hypothermia for EPR with ice-cold saline following rapid exsanguination to CA, we have previously been able to achieve good outcomes in dogs after up to 2 h CA. In the clinical situation of the exsanguinated trauma victim, more time may be needed for transport, resuscitative surgery, and initiation of delayed resuscitation. Thus, there is a clinical need to extend the duration of EPR. In the current study, an energy preservation strategy with oxygen and glucose allowed good recovery after 3 h of CA.

The significance of this finding could be demonstrated when it is placed along our many trials to improve EPR efficacy. In our pursuit of pharmacological preservation (Wu *et al*, 2005) (Behringer *et al*, 2001*a*, *b*), only tempol improved outcome in a 20-min CA EPR model (Behringer *et al*, 2002); in the

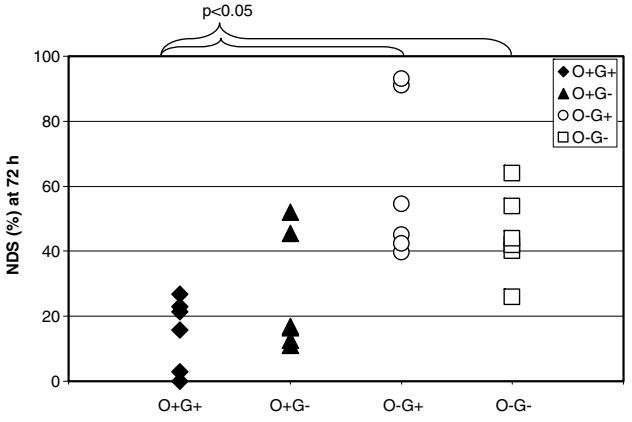

Figure 4 Final neurological deficit scores (NDS) at 72 h after 3 h of cardiac arrest. O + G + : 100% O_2 with 2.5% glucose in normal saline; O + G−:100% O_2 with normal saline; O−G + : 100% N_2 with 2.5% glucose in normal saline; O−G−:100% N_2 with normal saline.

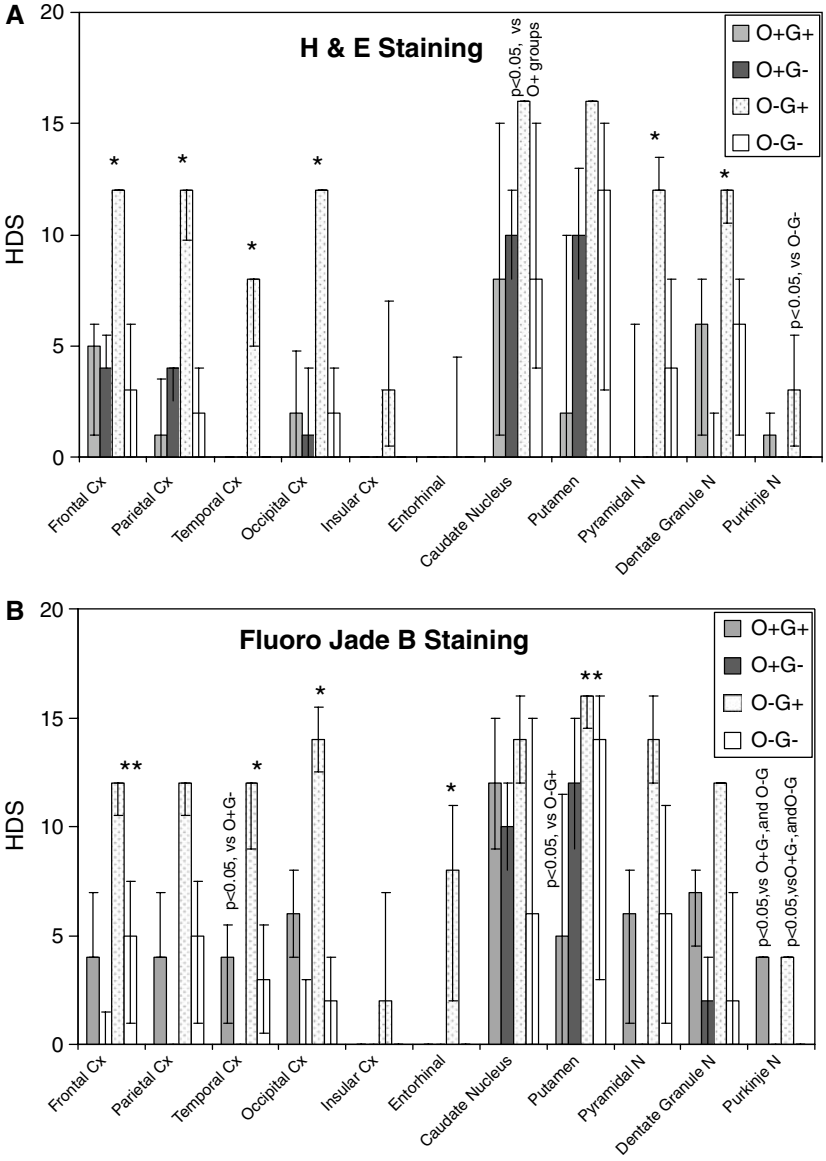

Figure 5 (**A**) Brain histological damage score (HDS) (median, 25–75% range): H&E staining. O + G + : 100% O_2 with 2.5% glucose in normal saline; O + G−:100% O_2 with normal saline; O−G + : 100% N_2 with 2.5% glucose in normal saline; O−G−:100% N_2 with normal saline; N: neurons; Cx: cortex; $^*P < 0.05$ compared with other three groups. (**B**) Brain histological deficit score (HDS): Fluoro-Jade B staining; O + G + : 100% O_2 with 2.5% glucose in normal saline; O + G−:100% O_2 with normal saline; O−G + : 100% N_2 with 2.5% glucose in normal saline; O−G−:100% N_2 with normal saline; N: neurons; Cx: cortex $^*P < 0.05$ compared with other three groups.

pursuit of hypothermic preservation, which was reliable, we came to realize that the maximal effect of profound hypothermia alone could allow consistent intact neurological outcome, but only to 2 h of CA (Behringer *et al*, 2001*d*; Nozari *et al*, 2003). Given this background, our current study, showing consistently good functional outcome after a 3-h ExCA (2.5 h of no flow), represents a significant step of advance in neuropreservation in EPR studies.

Although we have benefited from the earlier explorations in cryobiology and deep hypothermic CA (DHCA) for neuropreservation, EPR studies have distinct challenges and goals. The normothermic hypotension and subsequent normothermic CA *before* induction of hypothermia, modeling ExCA, probably increased the difficulties in achieving successful preservation. In contrast, as early as 1986, successful recovery of neurological functions was achieved after 3 h profound hypothermic *circulatory* arrest in some healthy dogs (Haneda *et al*, 1986). For DHCA, 3 h circulatory arrest is rarely indicated clinically, and thus seldom addressed in labs. While EPR studies target survival with satisfactory neurological functions (OPC: 1 to 2) as an acceptable goal in trauma victims who would otherwise have near 100% mortality after ExCA

(Rhee *et al*, 2000), DHCA studies, however, target the reduction of neurological morbidity after ~60 mins of bloodless surgery (Amir *et al*, 2005). In fact, the 'safe' duration of DHCA appears to be as short as 20 to 30 mins in patients who underwent thoracic cardiac surgery (Immer *et al*, 2004). Neurological recovery after up to 3 to 3.5 h of profound hypothermic CA has been reported using continuous perfusion with an experimental tissue preservation solution (Taylor *et al*, 1994). However, using the same solution significantly worsened NDS and OPC were noticed when intermittent no flow (circulatory arrest) was allowed in a 100-min DHCA model (Miura *et al*, 1996).

The decision to add oxygen and glucose to the perfusate was a logical step in our pursuit of more effective neuropreservation. First, we reported previously (Behringer *et al*, 2003) that despite using an arterial flush catheter of maximal diameter, maximal flush rate, and concurrent surface cooling, target temperature of 7 to 10°C could not be achieved for 12 to 15 mins in dogs. Second, metabolic demands are much greater during cooling than at profound hypothermia (Ehrlich *et al*, 2002). Third, in our EPR model, we previously screened 14 pharmacological strategies covering a broad spectrum of mechanism-based and empiric strategies—including agents targeting apoptosis, mitochondrial failure, anticonvulsants, barbiturates, MK-801 and calcium antagonists, among others (Wu *et al*, 2005). Unfortunately, these drugs targeting secondary injury cascades of neuronal death were disappointing, suggesting the need to consider alternative approaches. Similar disappointing results were observed by Aoki *et al* (1994) with addition of MK-801 to profound hypothermia in DHCA in piglets. Fourth, 2 h of DHCA (12 to 15°C) in sheep could be achieved with preservation of high-energy phosphate levels in brain (to ~60% of baseline) via intermittent infusion of a crystalloid solution containing dissolved oxygen and 2.5% dextrose (Robbins *et al*, 1990). That solution was called 'cerebroplegia' and also contained lidocaine, sodium bicarbonate, nitroglycerine, and mannitol. Fifth, as temperature decreases below 37°C, the affinity of hemoglobin for oxygen is greatly enhanced, restricting delivery, and increasing the importance of the dissolved oxygen component (Dexter *et al*, 1997). Sixth, the solubility of oxygen in saline nearly doubles between 37 and 18°C (Pearl *et al*, 2000). Grist (1996) suggested that the use of hyperoxia before DHCA can take advantage of enhanced oxygen solubility and reduced metabolic demands of hypothermia to prevent tissue injury. Hyperoxic perfusion during induction of hypothermia has been suggested to attenuate tissue acidosis in the clinical use of DHCA (Pearl *et al*, 2000). Finally, our flush rates of 20 L delivered over ~20 mins suggest that substrate delivery during the flush could be substantial, particularly in the setting of reduced metabolic demands. Thus, it was logical to propose that we could meet better metabolic demands with oxygen and glucose added to the flush solution during the induction of hypothermia. Different from DHCA, clinical application of EPR in management of ExCA would only be feasible *after* a normothermic ExCA has occurred. Brain energy reserve is depleted ~5 mins after normothermic CA (Shaffner *et al*, 1999; Eleff *et al*, 1991). Thus, to postpone energy failure, it may be important for preservation strategies to prevent energy depletion in brain and restore energy levels during induction of hypothermia. Based on our favorable outcomes in the dogs flushed with oxygen (with or without glucose) and on the aforementioned study by Robbins *et al* (1990) in which the cerebral ATP depletion was attenuated with the addition of dissolved oxygen and glucose in the perfusate, flush with oxygen and glucose solution after ExCA may have prevented the development of critical energy depletion. Additional studies of ATP or energy charge would be needed to prove that hypothesis.

Recently, important work by Vereczki *et al* (2006) demonstrated deleterious effects of hyperoxic reperfusion after a 10-min ventricular fibrillation cardiac arrest in dogs (Vereczki *et al*, 2006). In contrast, the powerful favorable effect of oxygen in our study probably relates to the fact that it is used to mitigate energy failure during cooling—before it results in cellular disturbances that set the stage for oxidative reperfusion injury. This intriguing hypothesis also needs to be further evaluated.

The best functional outcome was achieved only with the combination of oxygen and glucose in our model. It is possible that added glucose is important in delaying energy depletion during the prolonged hypothermic CA. However, the effects of glucose in cerebral ischemia are complex. On one the hand, high glucose may enhance energy production via glycolysis during anoxia/hypoxia (Tian and Baker, 2002), and/or provide beneficial osmolar effects as did mannitol in a cat middle cerebral artery occlusion model (Little, 1978). However, it is not clear how much glucose was transferred across the blood–brain barrier during hypothermia induction in our model. The G+ groups did not have increased arterial lactate levels either at the end of flush or during early reperfusion. Instead, lactate levels were consistently higher only in both O− groups during early reperfusion, and there was no difference in lactate levels between two O− groups. Cerebral lactate production was not examined in this study. It is certainly possible that cerebral effects were masked by systemic effects. We recognize that it is possible that the choice of 2.5% dextrose does not represent the optimal concentration and could be excessive.

Alternatively, glucose could be detrimental during cerebral ischemia (Vannucci *et al*, 1996), with higher tissue lactate levels, acidosis, oxidative stress, glutamate, DNA fragmentation, and other deleterious effects (Li *et al*, 2001). It is possible that a net

benefit of glucose is seen if energy failure is prevented, while injury exacerbation dominates if frank ischemia occurs. Consistent with a potential dichotomous effect of glucose depending on whether energy failure is prevented, in our EPR model, the combination of oxygen and glucose produced the best functional outcomes, whereas the O−G+ group exhibited the worst histological injury. Additional studies of brain glucose utilization and energy charge would be helpful. Further studies would also be important to define the possible benefits of the osmotic effects of glucose in this model (Shin'oka *et al*, 1998). The findings of hyponatremia and decreased hemoglobin levels in the glucose groups suggest that the systemic osmotic effects were substantial. The effects on the brain, however, are unclear.

Given the dichotomous effects of additional glucose, the ability to successfully achieve 3 h of preservation might be attributed mostly to the addition of oxygen to profound hypothermia. Post-resuscitation lactate was lower in both groups with oxygen added to the flush versus those without oxygen, and there was a trend toward improved functional outcome in the O+G− group. Also, the O+G− and O+G+ groups had similar HDS. Based on the observed OPC and HDS, our study was insufficiently powered to test for differences between groups with and without glucose in the presence of oxygen in the flush. The variable effects of glucose on histology in our model also suggest the possibility that alternative fuels such as β-hydroxybutyric acetate may be worthy of investigation as an adjunct to dissolved oxygen in EPR (Suzuki *et al*, 2001).

Unlike our previous EPR studies where we used one-way flush for induction, we used CPB to induce profound hypothermia in the current study. The hematocrit during induction of EPR was around 5 to 10% in all groups. This deviation from one-way flush with saline could be important. On one hand, higher hematocrit (>10%) during induction of hypothermia before CA was associated with better energy reserve and neurological outcome (Shin'oka *et al*, 1996). If so in the current study, it again suggests the importance of supporting oxygen delivery during induction of hypothermia. However, after cooling to 10 to 12°C, hemodilution to <5% improved neurological function (Sekaran *et al*, 2001). It is therefore difficult for us to predict the impact of the residual hemoglobin without further studies.

Among limitations, the lack of biochemical data (i.e., brain ATP, energy charge, glucose, and lactate levels) suggests the need for caution in making conclusions about a clear relationship between energy metabolism and improved outcome. In addition, it is important to recognize that it would be technically difficult to induce profound hypothermia in the field with CPB—as used in this *proof of concept* investigation. We also recognize that it is impossible to determine in this work whether beneficial effects of oxygen and glucose added to the flush are being produced during the induction of hypothermia, during the period of no flow, during reperfusion, and/or any combination of these time intervals. Biochemical studies of both ATP preservation and osmolar effects during induction and maintenance of hypothermia, and of secondary injury mechanisms during reperfusion are necessary to understand further this intervention and optimize its application. Finally, we recognize that hypothermia can delay the appearance of damage after cerebral ischemia and that assessment of brain histopathology after longer outcome intervals would be valuable.

In summary, EPR using a combination of oxygen and 2.5% glucose plus profound hypothermia allowed satisfactory recovery of neurological function after 3 h of CA. Prevention or reversal of energy failure and other mechanisms may be responsible for the benefit. Adding oxygen and possibly glucose in the cooling solution might augment the efficacy of either resuscitative or elective deep hypothermia.

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Drs Wu, Kochanek, Tisherman, and S.W. Stezoski have submitted a provisional patent entitled 'Method of Inducing Suspended Animation Following Cardiopulmonary Arrest.'

Disclosure/Conflict of Interest

The other authors report no conflicts.

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Emergency preservation and delayed resuscitation allows normal recovery after exsanguination cardiac arrest in rats: A feasibility trial*

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Objective: **Emergency preservation and resuscitation (EPR) comprise a novel approach for resuscitation of exsanguination cardiac arrest victims. EPR uses a cold aortic flush to induce deep hypothermic preservation, followed by resuscitation with cardiopulmonary bypass. Development of a rat EPR model would enable study of the molecular mechanisms of neuronal injury and the screening of novel agents for emergency preservation.**

Design: **A prospective, randomized study.**

Setting: **University research facility.**

Subjects: **Adult male Sprague-Dawley rats.**

Interventions: **Isoflurane-anesthetized rats were subjected to lethal hemorrhage (12.5 mL for 5 mins), followed by KCl-induced cardiac arrest and 1 min of no flow. Three groups (n = 6) were studied: hypothermic EPR (H-EPR; 0°C flush; target temperature, 15°C); normothermic EPR (N-EPR; 38°C flush); and controls. After 20 mins of H-EPR or N-EPR, resuscitation was initiated with cardiopulmonary bypass for 60 mins and mechanical ventilation. Controls were subjected to complete experimental preparation and anesthesia without cardiac arrest, followed by 60 mins of cardiopulmonary bypass and mechanical ventilation. Surviving rats were extubated 2 hrs later. Survival, Overall Performance Category (1, normal; 5, death), Neurologic Deficit Score, Histologic Damage Score, and biochemistry were assessed in survivors on day 7.**

Measurements and Main Results: **All rats in H-EPR and control groups survived, whereas none of the rats in the N-EPR group had restoration of spontaneous circulation. All rats in the H-EPR and control groups achieved Overall Performance Category 1, normal Neurologic Damage Score, and normal or near normal Histologic Damage Score and biochemical markers of organ injury.**

Conclusions: **We have established an EPR model in rats showing no neurologic injury, despite an exsanguination cardiac arrest, followed by 20 mins of EPR using miniaturized cardiopulmonary bypass. Establishment of this model should facilitate application of molecular tools to study the effects of hypothermic preservation and reperfusion and to screen novel pharmacologic adjuncts. (Crit Care Med 2007; 35:532–537)**

KEY WORDS: hypothermia; hemorrhage; shock; cerebral ischemia; cardiopulmonary bypass; suspended animation

Current conventional resuscitation strategies generally fail in improving the outcome from exsanguination cardiac arrest (CA). In both recent military conflicts (1) and civilian settings (2, 3), >50% of deaths resulting from trauma occur at the scene. However, in an appropriate setting, some of those traumatic injuries would be surgically repairable (4).

Emergency preservation and resuscitation (EPR) comprises a novel approach for resuscitation of exsanguination CA victims (5). EPR uses cold aortic flush to induce a deep (15°C) to profound (5–10°C) hypothermic preservation during circulatory arrest, followed by delayed resuscitation using cardiopulmonary bypass (CPB). Similar approaches have been traditionally used, in a protective rather than resuscitative fashion, in cardiac surgery, allowing for repair of complex congenital or acquired pathologies with considerable success (6). In previous studies of EPR, we used a dog model with prolonged intensive care to maximize clinical relevance (7, 8). Because of the lack of molecular tools available for use in dogs, development of a rat EPR model would enable study of the molecular mechanisms of neuronal injury in ischemia-reperfusion injury from exsanguination CA. Understanding the impact of deep hypothermia and reperfusion on these cascades would allow us to define specific targets for future interventions and to assess markers of reversibility. The rodent model would also

***See also p. 660.**

From the Safar Center for Resuscitation Research (TD, JS, XW, SAT, SWS, JAF, LJ, PMK) and the Departments of Anesthesiology (TD, XW), Critical Care Medicine (JS, XW, SAT, SWS, PMK), Surgery (SAT), and Neurological Surgery (LJ), University of Pittsburgh School of Medicine, Pittsburgh, PA; and Charles River Laboratories Pathology Associates, Murrysville, PA (RHG).

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Drs. Wu, Tisherman, and Kochanek and Mr. Stezoski are co-inventors and have a U.S. Provisional Patent on the emergency preservation and resuscitation method.

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allow economic screening of pharmacologic strategies for EPR.

In our current feasibility study, we hypothesized that survival from rapid exsanguination CA followed by 20 mins of hypothermic EPR, including CPB-assisted resuscitation, is achievable and that survival with normothermic EPR and CPB-assisted resuscitation is unlikely. Survival was the primary outcome variable, whereas neurologic function, markers of organ injury, and histology deficit scores served as secondary outcome variables. Because CPB in rats has been reported to induce neurologic injury (9, 10) or even death (11), we also included a CPB-only control group.

MATERIALS AND METHODS

The protocol for the study was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Adult male Sprague-Dawley rats (350–400 g; Hilltop Lab Animals, Scottdale, PA) were housed in the facility for ≥3 days before the experiment with unrestricted access to food and water. On the day of the experiment, rats were anesthetized with 4% isoflurane in oxygen, intubated with a 14-gauge intravenous cannula (Becton Dickinson, Sandy, UT), and mechanically ventilated (Harvard Ventilator 683, Harvard Rodent Apparatus, South Natick, MA). Anesthesia was maintained with 1–1.5% isoflurane in F_{IO_2} 0.5. Using asepsis, the left femoral artery and vein were cannulated. Electrocardiogram, respiratory rate, and arterial and central venous pressure were continuously monitored and recorded (Polygraph, Grass Instruments, Quincy, MA). The right femoral artery was cannulated with a 20-gauge Angiocath (Becton Dickinson, Sandy, UT) that served as an in-flow CPB cannula. The right jugular vein was cannulated with a modified five-hole 14-gauge cannula advanced to the right atrium. That cannula was used for venous drainage during the hemorrhage phase and later as a venous out-flow CPB cannula. Rectal and tympanic probes were used to monitor the temperature. After instrumentation, rats were allowed to breathe spontaneously 2% isoflurane in F_{IO_2} 0.25. Heparin was administered to achieve activated clotting time >400 secs (Haemochron Jr Signature, ITC, Edison, NJ). After a 5-min equilibration period, a rapid exsanguination (12.5 mL of blood for 5 mins) was performed via the internal jugular catheter. After the exsanguination phase, CA was ensured with administration of 1 mL of potassium chloride (2 mEq) intravenously. The no-flow duration was 1 min.

According to the randomization (using Research Randomizer, http://www.randomizer.org), three groups were studied: a) hypothermic EPR (H-EPR; 0°C flush with Plasma-Lyte A; n = 6); b) normothermic EPR (N-EPR; 37°C flush; n = 6); and c) controls (anesthesia and surgical preparation identical to the EPR groups, followed by 60 mins of CPB at 37°C, no CA, n = 6). The flush solution was instilled via the right femoral artery catheter at 50 mL/min using a roller pump and was drained from the jugular vein catheter. For the H-EPR group, a target tympanic temperature of 15°C was achieved with a combination of 270 mL of flush and surface cooling. The same flush volume was used for the N-EPR group. After 20 mins of H-EPR or N-EPR, resuscitation was started with CPB (Fig. 1).

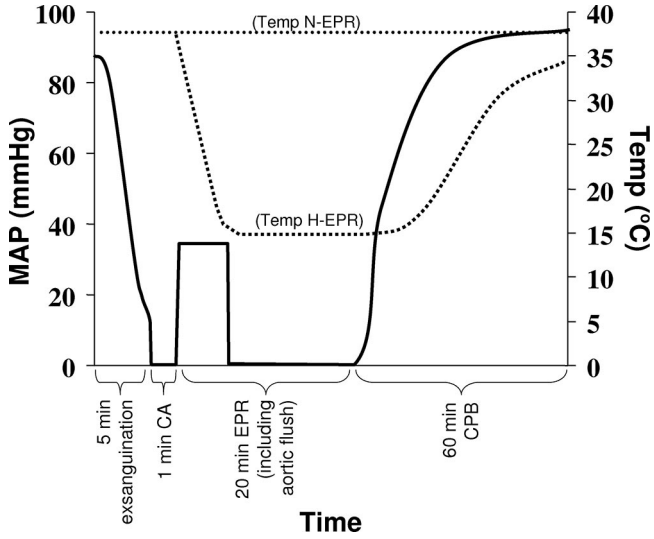

Figure 1. Experimental protocol including rapid hemorrhagic shock followed by 1 min of cardiac arrest (*CA*) and emergency preservation and resuscitation (*EPR*) using cardiopulmonary bypass (*CPB*). *Solid line*, mean arterial pressure (*MAP*) for hypothermic EPR (*H-EPR*) and normothermic EPR (*N-EPR*) groups; *dashed line*, temperature in H-EPR group; *dotted line*, temperature in N-EPR and control groups and MAP for a control group.

In brief, the CPB circuit consisted of a custom-designed oxygenator, an open reservoir (Ing. Martin Humbs, Ingenierburo fur Feinwerktechnik, Munich, Germany), tubing, and a roller pump (Masterflex, Barnant, Barrington, IL). The oxygenator contained a three-layer capillary membrane sufficient to provide oxygen levels >400 mm Hg. Isoflurane was used for maintenance of anesthesia during the CPB phase. Heating and cooling were achieved with a circulating water bath and a forced-air blower (Life-Air 1000, Progressive Dynamics, Marshall, MI). The temperature gradient between the water bath and the body core was not allowed to exceed 10°C.

Immediately before starting resuscitation, the CPB circuit was primed with collected shed blood, and mechanical ventilation with F_{IO_2} 0.3 was restarted to reexpand the lungs and prevent atelectasis. For the H-EPR group, flow rate was gradually increased according to the core temperature (6), targeting attainment of full flow (160–180 mL·kg·min) at 25°C (30 mins CPB time). For the N-EPR and CPB group, full flow was maintained during the entire CPB course. Acid-base management followed alpha-stat principles. pH and electrolyte values outside of the normal range were corrected during CPB and intensive care phases. Additional blood obtained from a donor rat was used, if needed, to maintain the hematocrit level at >25%.

CPB support was discontinued after 60 mins. Mechanical ventilation with an F_{IO_2} of 1.0 was continued while maintaining normocapnia. By using a midline laparotomy incision, a Mini-mitter probe (Mini-Mitter Co., Sunriver, OR) was introduced into the peritoneal cavity to allow postoperative temperature control and continuous monitoring of vital functions. Surviving rats were extubated 2 hrs later after removal of catheters and placed separately in a temperature-controlled cage (34.5°C for 3 hrs) with supplemental oxygen for 18 hrs. Weight and neurologic status were assessed daily, using the Overall Performance Score (OPC; 1, normal; 2, mild disability; 3, moderate disability; 4, severe disability; 5, death or brain death) and a modified Neurologic Deficit Score (12) (NDS; 0–50, normal; 500, maximum deficit). Blood samples were obtained from survivors on day 7, and rats were killed with an isoflurane overdose and perfused with normal saline followed by 10% formalin for histologic evaluation. The brains were divided into multiple coronal slices. Representative slices were also prepared from extracerebral organs. The tissue samples were processed and sectioned at 5 μm. All sections were stained with hematoxylin and eosin. Additional (duplicate) sections of brain were stained with Fluoro-Jade B (13). The tissue sections were examined by a neuropathologist

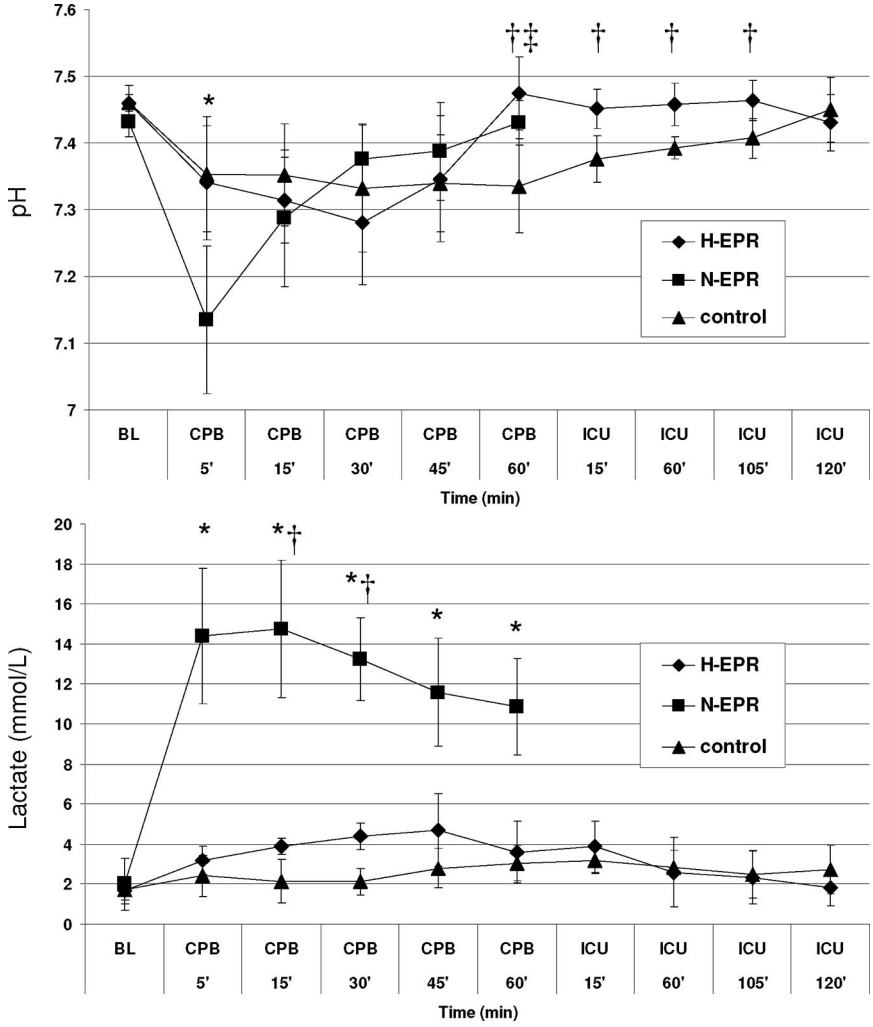

Figure 2. Arterial pH (*top panel*) and lactate (*bottom panel*) levels during cardiopulmonary bypass (*CPB*) and intensive care (*ICU*) phases. *$p < .01$ comparing hypothermic emergency preservation and resuscitation (*H-EPR*) and control groups vs. normothermic emergency preservation and resuscitation (*N-EPR*) group; †$p < .05$ comparing control and H-EPR groups; ‡$p < .05$ comparing control and N-EPR groups. *BL*, baseline.

	H-EPR (0°C)	N-EPR (37°C)	control
OPC 5		●●●●●●	
OPC 4			
OPC 3			
OPC 2			
OPC 1	●●●●●●*		●●●●●●

Figure 3. Survival and Overall Performance Categories (*OPC*) at day 7 after hemorrhagic shock and emergency preservation and resuscitation (*EPR*) with hypothermic flush (*H-EPR*) or normothermic flush (*N-EPR*) and in control groups. OPC 1, normal; OPC 2, mild disability; OPC 3, moderate disability; OPC 4, severe disability; OPC 5, death or brain death. *$p = .002$, OPC in the H-EPR group vs. N-EPR group.

(RHG). Each anatomical region with evidence of damage on microscopic examination received a subjective pathologic grade ranging from 1+ (minimal) to 5+ (severe). A total of 35 regions were evaluated in the brain. Each affected region on each side of the brain (right and left) received separate scores for the degrees of neuropathologic damage detected in hematoxylin and eosin-stained and Fluoro-Jade B-stained sections. A Histologic Damage Score (HDS) was determined by adding up all of these individual scores (i.e., for each neuroanatomic region with each stain).

Statistical Analysis. Data are presented as mean ± SD unless otherwise stated. Repeated measures analyses of variance with *post hoc* Bonferroni correction were used to compare the biochemical data (pH, base excess, lactate). Fisher's exact test was used to assess differences in OPC proportions. To distinguish normal vs. other outcomes, OPC was dichotomized (OPC 1 vs. 2–5). NDS was analyzed using the Mann-Whitney U test. The Kruskal-Wallis test was used to compare baseline values for three groups. The Mann-Whitney U test was used for comparing two groups if the Kruskal-Wallis test indicated that differences between all three groups existed or if data were available only for two groups. The Wilcoxon's signed-rank test was used separately for each group to determine whether values changed from baseline to final. Statistical software SPSS 13.0.1 for Windows was used. A p value $<.05$ was considered statistically significant.

RESULTS

Baseline physiologic variables were similar between groups.

EPR and CPB Phases. Mean arterial pressure observed during the flush was 41 ± 14 mm Hg in the H-EPR group vs. 22 ± 6 mm Hg in the N-EPR group ($p = .011$). During reperfusion, the N-EPR group had significantly lower pH compared with the H-EPR group (Fig. 2, *top panel*). The lactate level in the N-EPR group was significantly elevated compared with the H-EPR group (Fig. 2, *bottom panel*). After correction of the metabolic acidosis, there were no differences in pH between the H-EPR and the N-EPR groups at 60 mins CPB ($p = .22$). To achieve that goal, more bicarbonate was administered in the N-EPR vs. the H-EPR group (4.4 ± 0.8 mL vs. 2.5 ± 0.1 mL, $p = .0003$).

Survival. All rats in the H-EPR and control groups survived. None of the rats in N-EPR group had restoration of spontaneous circulation during 60 mins of CPB, which prevented separation from CPB. After discontinuation of isoflurane and a 10-min wash-out period during continuing CPB support, no spontaneous neurologic activity was observed and no reflexes could be elicited. Electroencephalograph was also isoelectric in each rat. CPB was then stopped and rats were perfused with formalin.

Neurologic Outcome. All rats in the H-EPR and control groups achieved OPC 1 beginning on day 2 (Fig. 3).

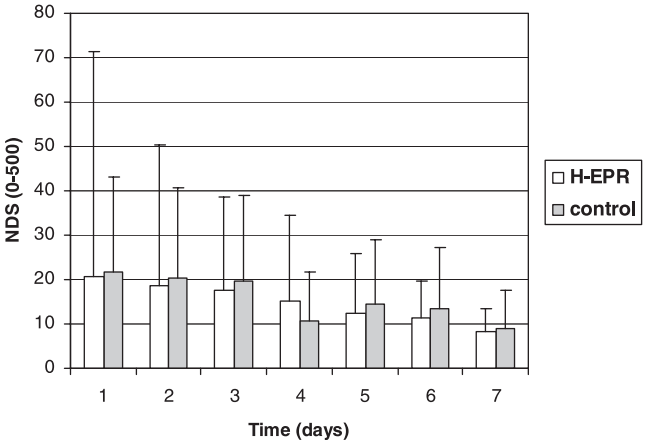

Figure 4. Neurologic deficit score (*NDS*) after hemorrhagic shock and emergency preservation and resuscitation in hypothermic flush (*H-EPR*) and control groups. No differences between groups.

Table 1. Histologic damage score of extracerebral organs

Group	Lung	Heart	Liver	Spleen	Kidney	Small Intestine
Control	0	0	0	0	0	0
	1	0	0	0	2	0
	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	3	0
	0	0	0	0	3	0
Median (range)	0 (0–1)	0	0	0	1 (0–3)	0
H-EPR	0	2	0	0	1	0
	1	0	1	0	0	0
	0	0	0	0	4	0
	1	2	1	2	2	0
	0	2	0	0	2	0
	2	2	2	0	0	0
Median (range)	1 (0–2)	2 (0–2)	1 (0–2)	0 (0–2)	2 (0–4)	0

H-EPR, hypothermic emergency preservation and resuscitation; 0, normal; 1, minimal; 2, mild; 3, moderate; 4, marked; 5, severe.

p not significant for all comparisons.

NDS was also normal or near normal in all rats and did not differ between H-EPR and controls (5 ± 8.4 vs. 7.5 ± 8.8, respectively; $p = .62$) (Fig. 4).

Histologic Evaluation. HDS did not differ between H-EPR and control groups in any region of the brain or extracerebral organs ($p > .05$) (extracerebral organs, Table 1). HDS values in brains of individual animals were 0, 0, 0, 0, 0, 0 (%) and 2.2, 0, 0, 0.2, 0.8, 0.4 (%) in the control and H-EPR groups, respectively.

Brain Lesions. All brains from the rats in the N-EPR group were characterized by multifocal edema, and some had evidence of peracute neuronal necrosis. Brains from the H-EPR group were normal in appearance, except for one rat. However, minimal degrees of Fluoro-Jade staining were present in a minority of rats. No lesions were present in the brains of rats in the control group. Because of the early deaths of rats in the N-EPR group, a brain histology in this group could not be compared with the other groups.

Visceral Lesions. In the N-EPR group, there was evidence of peracute mucosal cell degeneration in some of the intestinal sections. In both the control group and N-EPR group, small amounts of noncellular material could be found within the lumens of vessels in some sections of kidney, and there was often an associated local ischemic nephropathy or infarction. This intravascular material was found to be birefringent when sections were viewed with polarized light and most likely represented fragments from the tips of intravascular catheters. Marked organ congestion, particularly of the liver, was consistently present in the N-EPR group rats.

Biochemistry and Hematology. Enzymatic markers of organ injury (alanine transaminase, aspartate transaminase, creatine phosphokinase, creatine phosphokinase myocardial band, urea, and creatinine) were normal in H-EPR and control rats on day 7. Hemoglobin levels were not significantly different between H-EPR and controls on day 7. Hemoglobin level on day 7 was lower in controls vs. baseline ($p = .028$), and a similar trend was seen for the H-EPR group ($p = .068$). White blood count ($\times 10^9$/L) was lower in the H-EPR vs. control group on day 7 (2.72 ± 1.38 vs. 5.65 ± 1.12; $p = .01$) The platelet counts in both H-EPR and controls were also increased vs. their respective baselines, with borderline significance for the H-EPR group (850 ± 145 vs. 1304 ± 102, $p = .068$) and reaching statistical significance for the control group (771 ± 102 vs. 1290 ± 233, $p = .028$), but the H-EPR and control groups did not significantly differ at any time point (Table 2).

DISCUSSION

To our knowledge, this is the first description of a successful resuscitation from exsanguination CA treated by an emergency preservation with hypothermic flush followed by a delayed resuscitation with CPB in rats. The long (20 mins) duration of the CA prevented successful resuscitation of normothermic animals despite CPB-assisted circulatory support and reestablishing homeostasis with resuscitative drugs and multiple transfusions of the donor whole blood, a measure that is not readily available in large animal models.

Based on this feasibility study, it seems that EPR is achievable in rats and can produce intact neurologic outcome and normal brain histopathology. As expected, after exsanguination CA, the N-EPR protocol did not yield favorable outcome.

Importantly, there were no observed significant differences in any marker of outcome including OPC, NDS, and HDS between survivors in H-EPR and controls. Minimal impairments in NDS were mostly motor deficits, presumably caused by peripheral (femoral) nerve injury during surgery; however, central origin or spinal cord injury could not be ruled out.

Histologically, the brains from the N-EPR group rats were characterized by extensive edema, and peracute neuronal degeneration was suspected in many of the brains from this group based on the contracted morphology of neurons in certain regions. However, it is well recognized

Table 2. Biochemical markers of extracerebral organ injury and hematological values in hypothermic emergency preservation and resuscitation (H-EPR), normothermic emergency preservation and resuscitation (N-EPR), and control groups

Group	Baseline	Final[a]
AST, IU/L		
H-EPR	86 ± 27	88 ± 25
N-EPR	89 ± 18	1001 ± 345[c,d]
Control	98 ± 29	87 ± 35
ALT, IU/L		
H-EPR	63 ± 14	42 ± 8
N-EPR	80 ± 24	728 ± 448[c,d]
Control	73 ± 9	42 ± 6[b]
CPK, $\times 10^3$ IU/L		
H-EPR	213 ± 41	149 ± 72
N-EPR	159 ± 13	3528 ± 1490[c,d]
Control	208 ± 170	168 ± 75
CPK-MB, IU/L		
H-EPR	<0.4	<0.4
N-EPR	<0.4	0.4
Control	<0.4	<0.4
Urea, mg/dL		
H-EPR	23 ± 2	24 ± 5
N-EPR	23 ± 11	23 ± 3
Control	25 ± 2	19 ± 4[b]
Creatinine, mg/dL		
H-EPR	0.6 ± 0.1[c]	0.6 ± 0.3
N-EPR	0.4 ± 0.2	0.7 ± 0.3
Control	0.4 ± 0.1	0.4 ± 0.2
Hgb, g/dL		
H-EPR	14.6 ± 0.7	12.0 ± 1.5
N-EPR	14.1 ± 0.2	11.7 ± 0.3
Control	14.3 ± 0.5	12.2 ± 0.7[b]
Hct, %		
H-EPR	40.7 ± 2.9	35.1 ± 4.8
N-EPR	39.2 ± 1.9	32.8 ± 1.9
Control	40.0 ± 2.5	35.2 ± 3.0[b]
WBC, $\times 10^9$/L		
H-EPR	6.2 ± 1.6	2.7 ± 1.4[c]
N-EPR	5.4 ± 3.1	1.3 ± 1.0[c]
Control	6.9 ± 2.5	5.7 ± 1.1
Plt, $\times 10^9$/L		
H-EPR	850 ± 145	1304 ± 102
N-EPR	764 ± 80	202 ± 77[c,d]
Control	771 ± 102	1290 ± 233[b]

AST, aspartate transaminase; ALT, alanine transaminase; CPK, creatine phosphokinase; CPK-MB, creatine phosphokinase myocardial band; Hgb, hemoglobin; Hct, hematocrit; WBC, white blood cell count; Plt, platelet.

[a]Final samples taken at day 7 in H-EPR and control groups and at 70 mins EPR time in N-EPR group; [b]$p < .05$ vs. baseline; [c]$p < .05$ vs. Control; [d]$p < .05$ vs. H-EPR.

that 70 mins is an extremely early time after CA to appropriately evaluate neuronal death (14). It is expected that the degree of neuronal degeneration/necrosis seen in the rats from the N-EPR group would have been greater if these rats had survived as long as the rats in the other treatment groups. Evidence of degeneration within the brains of H-EPR group rats was minimal. Although one rat in the H-EPR group had a significant degree of neuronal degeneration within multiple brain regions, these lesions were restricted to the right side of the brain and, therefore, probably resulted from vascular occlusion. No thrombi or emboli were found in the brain sections to account for these lesions. However, noncellular intravascular material was found within selected nonnervous system tissues in other H-EPR group rats, and one rat had a small granuloma within its frontal cortex. These lesions are likely to be the result of the CPB. No brain lesions were present in control rats.

Hematologic evaluation yielded two interesting findings: The white blood cell count in the H-EPR group on day 7 was reduced vs. either baseline or day 7 control. It is not clear if this represents white blood cell count consumption or sequestration or impaired production. Also, in both H-EPR and control groups, we observed an almost two-fold increase of the platelet count from baseline. Since the primary outcome variables of the study were survival and neurologic outcome, we did not perform serial measurements of biochemical and hematologic markers. This could only have been accomplished if tunneled catheters had been left *in situ* for serial sampling, additional heparin given, and multiple blood samples taken. Any of those measures could have affected the outcome. Nevertheless, it is well known that both CPB and surgical procedures trigger a prothrombotic state (15, 16).

Biochemical markers did not reveal any major organ injury at day 7. The same limitation of lack of serial sampling applies to those results. However, there was no persistent damage to the extracerebral organs after this substantial 20-min CA. In contrast, data from the N-EPR groups taken after 70 mins of reperfusion (before disconnecting from CPB) suggest acute multiple organ failure with markedly elevated creatine phosphokinase and liver function tests.

The higher perfusion pressures during flush in the H-EPR group could have contributed to the protective effect of hypothermia and better outcome. Recently it has been shown that a sufficient arteriovenous gradient of cold aortic flush allowing decrease of brain temperature can be achieved only when a vasopressor is added to the flush solution (17). It is not clear, however, that increasing a perfusion pressure in the N-EPR group could favorably affect the outcome after this severe insult.

We chose to treat metabolic acidosis with bicarbonate in this study. We recognize that bicarbonate use is controversial; however, we have mirrored our dog model in this regard.

There are certain limitations to this study. Due to the complex nature of the model, the investigators could not be blinded. However, the investigators who assessed OPC and NDS were blinded to the protocol assignment of the tested animal. Histologic evaluation was not blinded in the initial steps of model development. In future studies of EPR of longer duration, where brain injury is anticipated and therapies are tested, it will be critical to mask the neuropathologist to treatment groups.

The use of CPB itself has been reported to produce neurologic deficits. In our study, we did not detect major deficits, and there were no differences between the H-EPR group and CPB controls. However, we did not have a sham-operated group in our study that would expose CPB-induced neurologic deficits. More sophisticated tests that focused on spatial learning memory (18) might have revealed subtle differences between H-EPR and control groups. We also acknowledge that the reperfusion time for rats was different—70 mins for rats in the N-EPR group vs. 7 days for survivors in H-EPR and control groups. However, overwhelming injury in the N-EPR group prevented long-term survival, despite CPB-assisted resuscitation, due to cardiovascular collapse and brain death, as described in similar scenarios elsewhere (19, 20).

In our dog model of EPR, we have been able to achieve favorable outcome after >2 hrs of EPR duration. It will be important to explore the limit of hypothermic preservation after arrest in this newly established rat model. The higher rate of adenosine triphosphate loss (five- to six-fold) in the rat vs. dog heart (21) could be a limiting factor to the extension of EPR time in rats.

CONCLUSIONS

We have established an EPR model in rats that shows no neurologic injury despite exsanguination CA followed by emergency preservation with hypothermic flush, 20 mins of asanguineous CA, and delayed resuscitation using a miniaturized CPB. Successful establishment of

this technically demanding model should facilitate application of molecular tools to study effects of hypothermic preservation and reperfusion on neuronal death and cell death cascades and to screen novel therapies with relevance to resuscitation, cardiac surgery, and transplantation medicine.

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RESUSCITATION

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EXPERIMENTAL PAPER

Exsanguination cardiac arrest in rats treated by 60 min, but not 75 min, emergency preservation and delayed resuscitation is associated with intact outcome☆

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KEYWORDS
Hypothermia;
Hemorrhage;
Shock;
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Cardiopulmonary bypass;
Suspended animation

Summary Emergency preservation and resuscitation (EPR) is a new approach for resuscitation of exsanguination cardiac arrest (CA) victims to buy time for surgical hemostasis. EPR uses a cold aortic flush to induce deep hypothermic preservation, followed by resuscitation with cardiopulmonary bypass (CPB). We previously reported that 20 min of EPR was feasible with intact outcome. In this report, we tested the limits for EPR in rats. Adult male isoflurane-anesthetized rats were subjected to rapid hemorrhage (12.5 ml over 5 min), followed by esmolol/KCl-induced CA and 1 min of no-flow. EPR was then induced by perfusion with 270 ml of ice-cold Plasma-Lyte to decrease body temperature to 15 °C. After 60 min ($n = 7$) or 75 min ($n = 7$) of EPR, resuscitation was attempted with CPB over 60 min, blood transfusion, correction of acid—base balance and electrolyte disturbances, and mechanical ventilation for 2 h. Survival, overall performance category (OPC: 1 = normal, 5 = death), neurological deficit score (NDS), and histological damage score (HDS) were assessed

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in survivors on day 3. While all rats after 60 min EPR survived, only two out of seven rats after 75 min EPR survived ($p < 0.05$). All rats after 60 min EPR achieved OPC 1 and normal NDS by day 3. Survivors after 75 min EPR achieved best OPC 3 ($p < 0.05$ vs. 60 min EPR). HDS of either brain or individual viscera was not statistically different after 60 versus 75 min EPR, except for kidneys (0 ± 0 vs. 1.9 ± 1.3, respectively; $p < 0.05$), with a strong trend toward greater injury in all extracerebral organs in the 75-min EPR group ($p < 0.06$). Histological findings were dominated by cardiac lesions observed in both groups and acute renal tubular and liver necrosis in the 75-min EPR group. In conclusion, we have shown that 60 min of EPR after exsanguination CA is associated with survival and favorable neurological outcome, while 75 min of EPR results in significant mortality and neurological damage in survivors. Surprisingly, extracerebral lesions predominated at 75-min EPR group. This model should serve as a screening model both for testing new pharmacological adjuncts to improve survival after exsanguination CA, and for elucidating the underlying mechanisms of ischemia/reperfusion injury.

Introduction

Currently, the outcomes from traumatic exsanguination cardiac arrest (CA) are dismal. Data from both recent military conflicts[1] and civilian settings[2,3] show that over 50% of deaths due to trauma occur at the scene, where medical care is limited. However, in an appropriate setting, some of those traumatic injuries could be surgically repairable.[4]

Emergency preservation and resuscitation (EPR) is a new approach for resuscitation of exsanguination CA victims.[5] EPR uses a cold aortic flush to induce a deep (15 °C) to profound (<10 °C) hypothermic preservation during circulatory arrest and buys time for transport, damage control surgery, and delayed resuscitation with cardiopulmonary bypass (CPB). Deep hypothermia has been used traditionally for protection rather than resuscitation in cardiac surgery, allowing for repair of complex congenital or acquired pathologies with considerable success.[6] In earlier studies of EPR we used a dog model to maximize clinical relevance. Because of the lack of molecular tools available for use in dogs, development of a rat EPR model would enable study of the molecular mechanisms of neuronal and extracerebral injury. Understanding molecular mechanisms of secondary damage in ischemia—reperfusion injury from exsanguination CA and the impact of deep hypothermia and reperfusion on these cascades would allow us both to define specific targets for future interventions and to assess markers of reversibility. Different mechanisms of cell death are probably attenuated at different hypothermic levels[7,8] and expressed at different timepoints[9]; this model should allow us to rapidly evaluate these mechanisms.

In addition, use of a rat model would obviate the cost and labor-intensiveness of experiments in large animals, enabling rapid screening of promising pharmacological adjuncts to hypothermia.

In our previously published feasibility trial, we established a rat model of EPR using rapid exsanguination CA followed by 20 min of preservation and subsequent delayed resuscitation using CPB with excellent survival and neurological recovery.[10]

We hypothesized that survival and neurological outcome is dependent on the duration of EPR. To test this hypothesis, we assessed survival and neurological outcome after exsanguination CA followed by 60 or 75 min EPR as primary outcome measurements; histological damage score (HDS) served as a secondary outcome measurement.

Materials and methods

The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (0407821A). Adult male Sprague—Dawley rats (350—400 g) were obtained from Hilltop Lab Animals (Scottdale, PA) and housed for at least 3 days before the experiment under 12-h light/dark cycle with unrestricted access to food and water. On the day of the experiment, rats were anesthetized with 4% isoflurane in FiO_2 0.5 in a transparent acrylic jar. After tracheal intubation with a 14G intravenous catheter (Becton Dickinson; Sandy, UT), rats were mechanically ventilated using a piston ventilator (Harvard Ventilator Model 683, Harvard Rodent Apparatus; South Natick, MA) with tidal volume 0.8 ml/100 g, frequency 24—30/min to maintain normocapnia, with a positive end-expiratory pressure (PEEP) of 4 cmH_2O. Anesthesia was maintained with 1—1.5% isoflurane

in FiO_2 0.5. After shaving and preparation with povidone iodine, the left femoral artery and vein were cannulated for blood pressure monitoring and blood sampling. EKG, respiration, arterial, and central venous pressure were continuously monitored and recorded (Polygraph; Grass Instruments, Quincy, MA). The right femoral artery was cannulated with a 20G catheter (Becton Dickinson; Sandy, UT) that served as an arterial CPB cannula. The right jugular vein was cannulated with a modified five-hole 14G intravenous cannula advanced to the right atrium to be used for venous drainage during the hemorrhage phase and later as a venous CPB cannula. Rectal and tympanic probes were used to monitor the temperature. After instrumentation, rats were weaned from the ventilator and allowed to breathe spontaneously 2% isoflurane in FiO_2 0.25 via a nose cone mask over the tracheal tube. Baseline blood samples were obtained and hemodynamic values were recorded. Removed blood volume was replaced with Plasma-Lyte A (Baxter; Deerfield, IL) in a ratio 1:3. Heparin sodium was administered to achieve activated clotting time (ACT) > 400 s (Haemochron Jr. Signature, ITC; Edison, NJ). After a 5 min equilibration period, rapid exsanguination (12.5 ml of blood over 5 min) was performed via the internal jugular catheter. The shed blood was collected in a heparin pre-filled syringe. After the rapid exsanguination phase, CA was ensured with administration of a mixture of 9 mg of esmolol and 0.1 ml of potassium chloride (0.2 mEq) intravenously. After 1 min of no-flow, 270 ml of an ice-cold flush solution (Plasma-Lyte A) was instilled via the right femoral artery catheter at 50 ml/min using the roller pump (Masterflex, Barnant, IL). The flush was drained from the jugular vein catheter. A target rectal temperature of 15 °C was achieved with a combination of 270 ml of flush and surface cooling. Both rectal and tympanic temperatures were maintained at 15 °C during EPR.

After 60 or 75 min of EPR, resuscitation was started with CPB, as described previously[10] (Figure 1). In brief, the CPB circuit consisted of a custom-designed oxygenator made of polymethylmetacrylate, an open reservoir (both made by Ing. Martin Humbs, Ingenierburo fur Feinwerktechnik, Munich, Germany), tubing, and a roller pump (Masterflex, Barnant; Barrington, IL). The oxygenator contained a three-layer capillary membrane sufficient to provide oxygen levels > 400 mmHg. A mixture of 98%O_2/2%CO_2 was used at a flow rate of 50 ml/min through the oxygenator to provide oxygenation and prevent hypocapnia. Isoflurane from a separate vaporizer was used for maintenance of anesthesia during the CPB phase. Heating and cooling were achieved with a circulating water bath

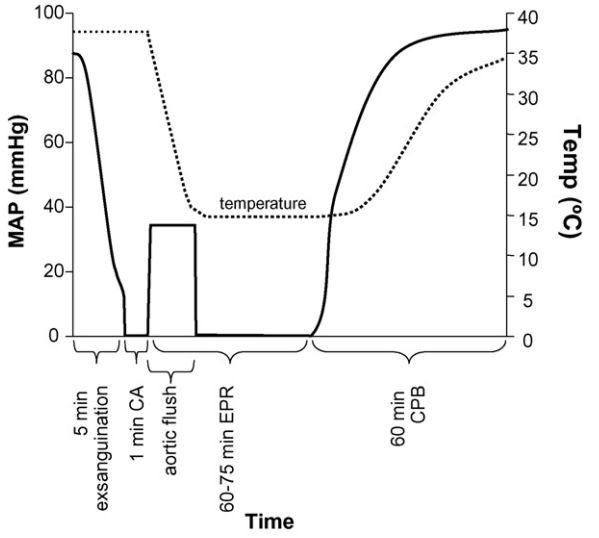

Figure 1 Experimental protocol including rapid hemorrhagic shock followed by 1 min cardiac arrest and emergency preservation and resuscitation (EPR) using cardiopulmonary bypass (CPB). Solid line, mean arterial pressure (MAP); dotted line, temperature.

around the oxygenator, and a forced-air blower (Life-Air 1000, Progressive Dynamics, Marshall, MI).

Immediately before starting resuscitation, the CPB circuit was primed with collected shed blood, and mechanical ventilation with FiO_2 0.3 was restarted to re-expand the lungs and prevent atelectasis. ACT was maintained at >400 s with additional heparin boluses during the CPB course. Blood samples for biochemistry and hematology were obtained at 5, 15, 30, 45, and 60 min CPB time and processed immediately using a point-of-care blood analyzer (Stat Profile, Nova Biomedical; Waltham, MA). Arterial blood gas management followed alpha-stat principles. pH and electrolyte values outside the normal range were corrected during CPB and ICU phases by adjustments in ventilation and/or administration of sodium bicarbonate, calcium chloride, and potassium chloride as needed. Additional blood obtained from an isoflurane-anesthetized donor rat was used to maintain the hematocrit level >25%.

CPB support was gradually discontinued after 60 min. No vasopressor support was used. Mechanical ventilation with a FiO_2 of 1.0 was continued while maintaining normocapnia. Using a midline laparotomy incision, a Mini-mitter probe (Mini-Mitter Co; Sunriver, OR) was introduced into the peritoneal cavity to allow postoperative temperature control and continuous monitoring of heart rate (HR) and movement. The electrodes of the probe were tunnelled subcutaneously and attached to pectoral muscles from separate incisions. Surviv-

ing rats were extubated 2 h later after removal of catheters, and placed separately in a temperature-controlled cage (34.5 °C for 4 h) with supplemental oxygen for 18 h, and free access to food and water. Animals that did not resume normal eating or drinking pattern within 24 h, or lost greater than 10% of their initial body weight, received subcutaneous injections of 0.45NS/D5 twice daily. Weight and neurological status were assessed daily, using overall performance category (OPC; 1 = normal, 2 = mild disability, 3 = moderate disability, 4 = severe disability, 5 = death or brain death) and a modified neurological deficit score[11] (NDS; 0—10% = normal, 100% = maximum deficit). Blood samples were obtained from survivors on day 3, and rats were killed with an isoflurane overdose and perfused via left ventricle with normal saline followed by 10% neutral buffered formalin for histological evaluation (brain, heart and lungs, kidneys, and liver). The brains were divided into multiple coronal slices (typically 11). Representative slices were also prepared from the non-nervous system tissues. The tissue samples were processed for embedding in paraffin. The resulting paraffin blocks were sectioned at 5 μm. All sections were stained with hematoxylin and eosin (H&E). Additional (duplicate) sections of brain were stained with Fluoro-Jade B.[12] The sections were examined by a neuropathologist (RHG) under brightfield or epifluorescent illumination depending upon the stain. Each anatomical region with evidence of damage on microscopic examination received a subjective pathologic grade ranging from 1+ (minimal) to 5+ (severe). A total of 35 regions were evaluated in the brain: olfactory bulb; piriform cortex; frontal cortex; cingulate cortex; septal nuclei; parietal cortex; caudate nucleus/putamen; globus pallidus; basal forebrain; corpus callosum; amygdala; retrosplenial cortex; subiculum; hippocampal pyramidal neuron sectors CA1, CA2, CA3, and CA4; dentate gyrus; thalamus; hypothalamus; temporal cortex; occipital cortex; fornix; midbrain; entorhinal cortex; substantia nigra (pars compacta and pars reticularis); superior colliculus; inferior colliculus; cerebellar cortex; cerebellar nuclei; cerebellar white matter; pons; reticular formation; medulla oblongata; and pyramids. Each affected region on each side of the brain (right and left) received separate scores for the degrees of pathological damage detected in H&E-stained and Fluoro-Jade B-stained sections. Total histological damage score (HDS) was calculated as a sum of the individual scores (i.e., for each anatomic region with each stain). Similarly, in extracerebral organs, each organ received a subjective pathologic grade ranging from 1+ (minimal) to 5+ (severe).

Statistical analysis

Data are presented as mean ± standard deviation, unless otherwise stated. Repeated measures analysis of variance was used to compare serial biochemical data and vital signs. Fisher's exact test was used to assess differences in OPC proportions. To distinguish normal versus other outcomes, OPC was dichotomized (OPC 1 vs. 2—5). NDS and HDS were analyzed using Mann—Whitney *U*-test. The statistical software SPSS 14.0 for Windows was used. A p-value < 0.05 was considered statistically significant.

Results

There were no differences in hemodynamic or biochemical measurements between the groups at the baseline, including mean arterial pressure (MAP), heart rate, temperature, Hct, arterial pH, pO_2, pCO_2, base excess, and serum Na, K, Cl, Ca, Mg, urea, or lactate levels.

EPR, CPB, and ICU phases

During the flush, the temperature decreased in both groups similarly and was maintained at 15 °C for the required duration. During re-warming, the temperature increased to 34.5 °C similarly in both groups (Figure 2). During reperfusion, HR recovered similarly in both groups (Figure 3). Rats in the 75-min EPR group had a higher MAP during the later phase of the CPB course ($p = 0.026$) (Figure 4). The lactate levels were markedly increased compared to baseline in both groups; however, rats in the 75-min EPR group had significantly higher lactate levels compared to 60 min ($p = 0.012$) (Figure 5). There were no changes in Hct between groups ($p = 0.837$). To correct the metabolic acidosis during the CPB phase, more bicarbonate was given to the 75-min EPR group versus the 60-min EPR group (4.0 ± 0.1 ml vs. 3.6 ± 0.4 ml; $p < 0.05$).

Survival

All rats in the 60-min EPR survived, while only two out of seven rats in the 75-min EPR group survived until day 3 (survival time was longer in the 60-min vs. 75-min EPR groups; $p = 0.007$) (Figure 6).

Neurological outcome

All rats in the 60-min EPR group achieved OPC 1 by day 3, while the two surviving rats in

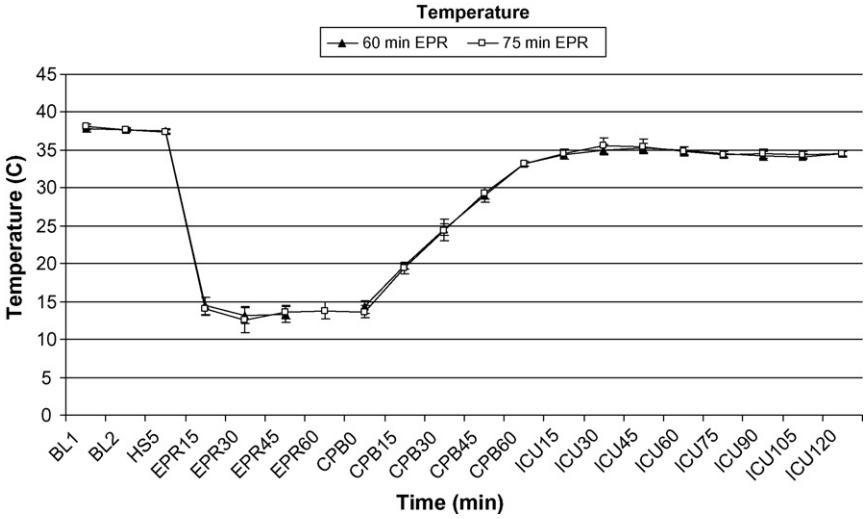

Figure 2 Temperature profile following exsanguination cardiac arrest treated by 60 min or 75 min emergency preservation and resuscitation (EPR) during cooling and rewarming phases of the experiment. No differences between groups.

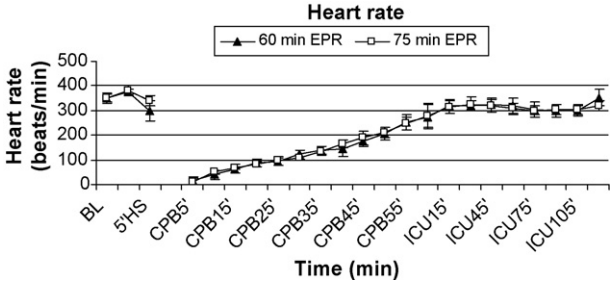

Figure 3 Heart rate following exsanguination cardiac arrest treated by 60 min or 75 min emergency preservation and resuscitation (EPR) during the CPB and ICU phases. No differences between groups.

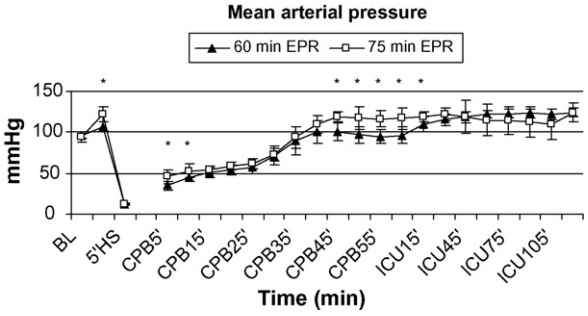

Figure 4 Mean arterial pressure following exsanguination cardiac arrest treated by 60 min or 75 min emergency preservation and resuscitation (EPR) during the CPB and ICU phases. (*) $p < 0.05$, 60 vs. 75-min EPR.

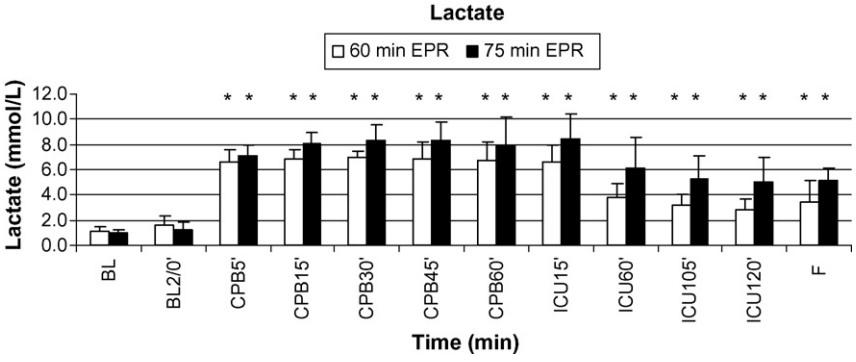

Figure 5 Lactate levels following exsanguination cardiac arrest treated by 60 min or 75 min emergency preservation and resuscitation (EPR) during the CPB and ICU phases. BL, baseline; F, final (72 h); $p = 0.012$ overall between 60-min vs. 75-min EPR; (*) $p < 0.05$ compared to the respective baseline.

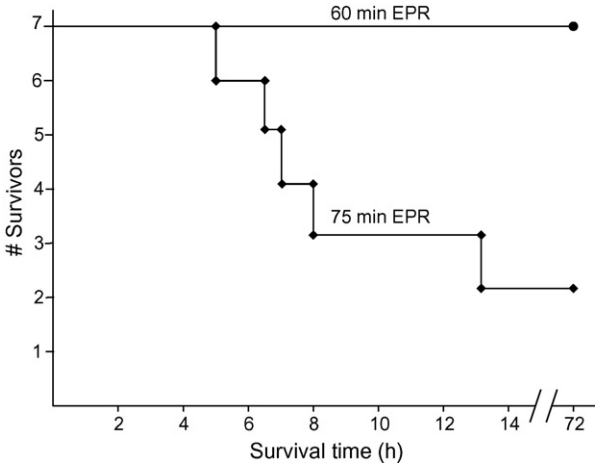

Figure 6 Survival following exsanguination cardiac arrest treated by 60 min or 75 min emergency preservation and resuscitation (EPR); $p = 0.007$, 60-min EPR vs. 75-min EPR groups.

	60 min EPR	75 min EPR
OPC 5 Death / brain death		●●●●●
OPC 4 Severe disability		
OPC 3 Moderate disability		●●
OPC 2 Mild disability		
OPC 1 Normal	●●●●●●●	

Figure 7 Overall performance categories (OPC 1—5) at 72 h after exsanguination cardiac arrest treated by 60 min or 75 min of emergency preservation and resuscitation (EPR). Each dot represents one rat; $p = 0.01$, 60-min EPR vs. 75-min EPR groups.

the 75-min group achieved only OPC 3 ($p = 0.01$) (Figure 7).

NDS was normal in all rats in the 60-min group on day 3 (i.e., NDS < 10%), while both survivors in the 75-min EPR group were neurologically impaired (NDS 40% and 42%, respectively). NDS was significantly better in the 60-min EPR group versus 75-min EPR group (2.4 ± 2.3 vs. 83.1 ± 28.8; $p = 0.001$). (Figure 8)

Histological evaluation

HDS did not differ between 60- and 75-min groups in any individual region of the brain or in the brain overall. HDS between 60- and 75-min EPR groups was not different in the heart, lung, and liver. HDS was significantly worse in kidneys in the 75-min versus the 60-min EPR group ($p < .05$) (Table 1). There was a strong trend toward greater injury in all extracerebral organs — median (range) — total extracerebral organ injury score 6 (3—7) versus 8 (4—13) for 60-min versus 75-min EPR, respectively ($p < 0.06$). The most frequently present microscopic lesion was myocardial degeneration (or frank myocardial fiber necrosis) coupled with a pattern of subacute myocarditis. Some rats also had evidence of either peracute to acute tubular necrosis within the kidney or acute degeneration within the liver. The thymic tissues of some rats were mildly to moderately atrophic.

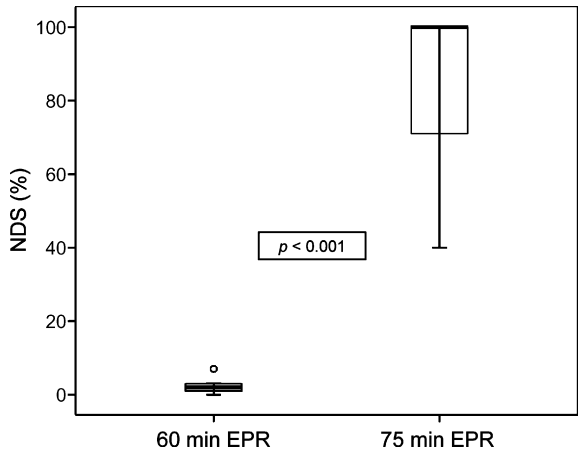

Figure 8 Neurological deficit score (NDS, 0—100%) at 72 h after exsanguination cardiac arrest treated by 60 min or 75 min of emergency preservation and resuscitation (EPR). Boxes represent interquartile ranges. The line across each box indicates the median, and the whiskers are the highest and lowest values. The round marker represents an outlier.

Discussion

Having achieved intact neurological outcome with EPR durations up to 120 min in our well-established canine model,[13—18] we aimed to assess the limits of EPR in rats. Modifying our previously described model of EPR in rats, we have extended the time allowing intact survival from 20 to 60 min. Surprisingly, further extension of EPR duration to 75 min was associated with renal injury, multi-organ failure, high mortality rate, and unfavorable neurological outcome in survivors.

Rapid exsanguination resulted in MAP < 20 mmHg in all rats, suggesting minimal residual cardiac output. Previously, we used highly concentrated KCl to terminate cardiac activity. While a shorter dura-

Table 1 Histological damage score (HDS) at 72 h after exsanguination cardiac arrest treated by 60 min or 75 min of emergency preservation and resuscitation (EPR)

	Brain	Heart	Lung	Liver	Kidney
60 min EPR	15	3	0	0	0
	15	2	1	0	0
	8	2	0	1	0
	14	4	2	1	0
	19	2	2	0	0
	13	2	3	0	0
	29	2	3	0	0
75 min EPR	11	3	0	1	0
	/	3	4	3	3
	/	3	4	2	3
	/	3	2	0	3
	/	2	3	3	2
	22	2	3	0	0
	14	0	2	0	2
p	0.819	0.786	0.187	0.096	0.003

Each line represents one animal. Scale: brain, 0—720; extracerebral organs: 0 = no damage, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, 5 = severe; /, not assessed due to autolysis. *p*-values for individual organ HDS are given. Overall extracerebral HDS 6 (3.7) vs. 8 (4.13) for 60 vs. 75-min EPR, respectively ($p < 0.06$).

tion of EPR (20 min) using this approach allowed survival with intact neurological recovery,[10] pilot studies with longer durations of EPR revealed lung injury that prevented successful weaning from the ventilator (data not shown). One possible explanation for this finding comes from transplantation medicine literature on the optimal preservation of lung tissue. Perfusion with a solution enhanced with either high or low potassium concentrations (K^+: 115 or 10 mmol/L) yielded worse results than perfusion with K^+ (40 mmol/L).[19] This is in agreement with recent studies showing that use of highly concentrated potassium produced tissue damage and hindered post-ischemic recovery.[20,21]

The short acting beta-blocker esmolol was recently tested as a CA-inducing agent in both clinical[22] and experimental settings.[23—26] Esmolol-treated hearts showed better recovery than those receiving potassium in terms of +/— dP/dt, left ventricular systolic pressure, and left ventricular developed pressure.[27] As an additive to cardioplegia in an isolated Langendorff model, esmolol only provided sustained protection when given as a multi-dose or continuous infusion at constant pressure.[25] In light of these findings, in the current study, we used esmolol plus a low dose of KCl to induce CA and minimize additional damage.

Previous parallel studies in a dog model with 60 min of EPR at 15 °C or 90 min at 10 °C showed excellent outcome at 72 h (OPC 1, NDS < 10%).[14] With increased temperature or extended duration of EPR (up to 3 h), neurological outcome worsened although survival was still achieved. After transient multiple organ dysfunction syndrome, the survivors did not show any permanent extracerebral organ damage. In contrast, rats showed high early mortality with multiple organ failure dominated by renal failure (oliguria, anuria) subsequently confirmed by histological findings of acute renal tubular and liver necrosis. In addition, cardiac lesions were consistent with those seen in the brain—heart syndrome[28] (i.e., cardiac degeneration developing as a result of catecholamine release from damaged brain tissue) but could have other pathogenic mechanisms. A recent study in rats revealed variations in responses of individual organs to hemorrhagic shock and resuscitation and that the liver was the most vulnerable organ.[29] The rate of ATP loss in rat hearts was shown to be six times higher compared to that in dogs,[30] possibly limiting the resuscitability of the rats not due to neurological injury after prolonged CA but due to depletion of energy sources, and ensuing cardiovascular failure.

In a pig model of EPR, profound hypothermia (10 °C) prevented neuronal and glial injury observed in normothermic controls[31] and allowed intact neurological recovery after 60 min of low-flow state. Both the rates of cooling[32] and rewarming[33] were critical in order to achieve favorable outcome. However, EPR durations longer than 60 min were not evaluated in that model. Immediately initiated EPR was superior to CPR followed by CPB even after prolonged HS.[32,34] EPR was also shown to be feasible after normovolemic CA.[35]

Biochemical reactions that occur after an ischemic event are complex and are not yet

completely understood. Energy depletion and cell membrane depolarization with ion fluxes occur within minutes after the onset of a global ischemic insult and are followed by formation of free oxygen radicals, altered gene expression,[36] activation of caspases and other apoptotic pathways,[37–39] inflammation,[40] and release of excitatory amino acids in the brain.[41] Deep or profound hypothermia, the key component of EPR, exerts its beneficial effects mainly via reduction of metabolic rate and oxygen consumption,[6] thus preserving the tissue energy state and intracellular pH in both heart and brain.[42] The level of hypothermia that is used is critical to the spectrum of mechanisms of protection that is produced. The time to initiation of hypothermia,[43] hypothermia level,[44] and its duration[34] are all critical factors in limiting injury after CA.

The global effects of hypothermia on metabolism are defined by Q_{10}, which is for the whole human body about 2.0 (i.e., 50% reduction in metabolism per 10 °C decrease in temperature). The Q_{10} for the brain in adults is 2.3,[45] but in neonates, infants, and children it was determined to be 3.65.[46] Also, Q_{10} is not linear. In a canine model, brain Q_{10} was 2.23 between 37 and 27 °C, was doubled to 4.53 between 27 and 14 °C,[47] and returned to 2.19 below 14 °C.[48] In a rat model, overall brain Q_{10} for temperatures 38–28 °C was 5.2, with a two-component response: Q_{10} was 12.1 in 38–30 °C, and 2.1 in 30–28 °C.[49] Those data suggest that Q_{10} is different between species, between age groups, and also between individual organs in one body. Hence, hypothermia may confer different level of protection for the same organ between species, resulting in different outcomes despite similar insults.

Current guidelines endorse extended hypothermia (32–34 °C for 12–24 h) for victims resuscitated from CA.[50] In our model, we have used hypothermia for only 6 h. In pilot studies, we have found that longer durations of postoperative hypothermia are not feasible without sedation or neuromuscular blockade — the rats attempt to rewarm spontaneously by shivering, increasing the oxygen consumption. This could potentially aggravate the preceding insult. After the 6 h of controlled hypothermia, we opted to allow the temperature of the rat to drift spontaneously, avoiding active rewarming.[51]

There are certain limitations to this study. We used esmolol to ensure that profound hemorrhage resulted in CA. Previous studies have showed that a single dose of esmolol followed by 40 min ischemia did not influence myocardial recovery after reperfusion, when compared to ischemia only.[25] In our protocol, cardioplegic solution was likely to be rapidly eliminated from cardiac tissue after 1 min by the large volume of hypothermic flush. Thus, it is likely that our approach provided limited protection that would interfere with the evaluation of new therapeutic adjuncts to EPR.

HDS was assessed in brain only in survivors excepting for one rat from the 75-min EPR group that died 5 h after resuscitation. Except for this rat, the brains from the rats that died prematurely could not be evaluated histologically due to autolysis. However, the histological injury in brain does not appear to be appreciably aggravated when compared to survivors from the 75-min group (data not shown).

After the ICU period, we continued to monitor heart rate, but we did not follow other hemodynamic variables. Thus, we cannot rule out the presence of a period of hypoperfusion in non-survivors that contributed to the aggravated extracerebral organ damage in the 75-min EPR group.

Unlike in our dog model, long-term intensive care was not provided to rats. We cannot rule out that extended intensive care would improve the outcome of the 75-min EPR group, thus allowing assessment of the neurological injury in current non-survivors at the scheduled time of sacrifice. Given this limitation, it would be important to consider testing of promising agents in the definitive dog model.

We acknowledge the fact that starting flush after 5 min HS and 1 min of no-flow would be challenging in most out-of-hospital clinical scenarios. In hospital, arterial cannulation for insertion of a balloon catheter can be accomplished in less than 5 min.[52] To investigate clinical applicability of EPR further, studies focused on longer duration of CA before initiation of EPR are warranted. Also, targeting deep hypothermic levels requires large amount of fluid, which could be a limiting factor for the clinical applicability of the method. Small-volume additives that would enable flush volume to be reduced and/or provide further protection would be highly desirable. When testing potential promising therapeutic adjuncts, we have to consider that pharmacokinetics of many drugs are altered under hypothermia.

Conclusions

Rapid exsanguination CA followed by 1 min of no-flow can be successfully treated by EPR with hypothermic flush, 60 min of asanguineous CA, and delayed resuscitation using a miniaturized CPB allows normal survival. Surprisingly, further exten-

sion of asanguineous CA to 75 min is associated with multi-organ failure, high mortality, and unfavorable neurological outcome in survivors. This model can be used to study effects of hypothermic preservation and reperfusion on cell death cascades underlying multi-organ injury, as well as to test new drugs that may enhance the outcome of EPR strategy.

Conflict of interest statement

Drs. Kochanek and Tisherman and Mr. S. Stezoski are co-inventors and have a U.S. Provisional Patent on the emergency preservation and resuscitation method. Other authors have no conflict of interest to disclose.

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EXPERIMENTAL PAPER

Protein nitration and poly-ADP-ribosylation in brain after rapid exsanguination cardiac arrest in a rat model of emergency preservation and resuscitation☆

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Summary
Background: Emergency preservation and resuscitation (EPR) of 60 min in rats is achievable with favorable outcome, while 75 min is associated with substantial mortality and impaired neurological outcome in survivors. We hypothesized that 75 min but not 60 min of EPR would be associated with activation of two potential secondary injury cascades in brain as reflected by protein nitration and poly (ADP-ribose) polymerase (PARP) activation.
Methods: Rats were rapidly exsanguinated over 5 min. After 1 min of cardiac arrest (CA), rats were cooled to a target tympanic temperature of 15 °C. After either 60 min or 75 min of CA, resuscitation was achieved via cardiopulmonary bypass (CPB). Rats subjected to CPB only served as controls. Overall performance category (OPC) and neurologic deficit score (NDS) were assessed at 24 h. Protein nitration and poly-ADP-ribosylation were assessed by Western blotting and immunohistochemistry for 3-nitrotyrosine and poly-ADP ribose polymers, respectively, in multiple brain regions.
Results: Neurologic outcome was better in the 60 min vs. the 75 min EPR group (OPC, $P < 0.001$; NDS, $P = 0.001$). Densitometric analysis of the major 64 kD band showed that nitration and PARP activation were significantly increased in hippocampus, cortex and striatum in the 75 min EPR

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group vs. other groups. However, there were no differences in cerebellum. Analysis of the full protein spectrum showed significantly increased PARP activation only in hippocampus in the 75 min EPR group vs. other groups.
Conclusions: Extending the duration of EPR beyond the limit that can yield favorable recovery in rats was associated with increased nitration and ribosylation of selected proteins in selectively vulnerable brain regions. The impact of these mechanisms on the outcome remains to be determined.

Introduction

Emergency preservation and resuscitation (EPR) is a novel concept initially developed to treat cardiac arrest (CA) after trauma and rapid exsanguination.[1] It utilizes a strategy to preserve the viability of organs and organism using hypothermia during an otherwise lethal insult to buy time for transport and surgical repair followed by delayed resuscitation with cardiopulmonary bypass (CPB) and intensive care. An extensive series of experiments in dog, swine and rat models have demonstrated its efficacy and clinical feasibility.[2–12] The importance of optimizing EPR in experimental models has taken on greater significance given the recent funding of the first clinical feasibility trial of this strategy. In dogs, the rapid induction of profound cerebral hypothermia by aortic flush of cold saline to tympanic temperature (Tty) 10 °C, immediately after the start of exsanguination CA, can achieve survival without functional or histologic brain damage, after CA of up to 180 min.[5] Although good outcomes were achieved in the studies using a dog model, there are limitations to these large animal experiments including the need for long-term intensive care (72—96 h after resuscitation), lack of molecular tools to study mechanisms of brain injury, and cost. A rat model of EPR has been developed recently by our group.[12] In rats, EPR of 60 min CA is survivable, while 75 min CA is associated with substantial mortality and impaired neurological outcome in survivors.[13] In this report, we initiated studies to explore the possible molecular mechanisms at the threshold of failure of EPR. Two mechanisms of secondary injury that have been suggested to play important roles in cerebral ischemia are nitration and poly (ADP-ribose) polymerase (PARP) activation. Richards et al.[14] recently reported an important role for nitration of pyruvate dehydrogenase (PDH) and other targets in standard resuscitation after CA, while PARP activation has been shown to have a key role in limiting cerebral recovery across a variety of insults.[15,16] We, thus, hypothesized that nitration and PARP activation would be increased after 75 min CA treated by EPR compared to 60 min EPR or controls. We also explored whether CPB alone was associated with nitration or PARP activation.

Methods

The protocol for the study was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Adult male Sprague-Dawley rats (350—400 g; Hilltop Lab Animals; Scottdale, PA) were housed in the facility for at least 3 days before the experiment with unrestricted access to food and water. Four groups were studied in this protocol: naïve, CPB controls, 60 min EPR and 75 min EPR.

Animal protocol

Rats were anesthetized with 4% isoflurane in FiO_2 1.0 for 5 min in a transparent plexiglas chamber, intubated with a 14G intravenous cannula (Becton Dickinson; Sandy, UT) and mechanically ventilated (Harvard Ventilator Model 683, Harvard Rodent Apparatus; South Natick, MA). Anesthesia was maintained with 1—2% isoflurane in FiO_2 0.5. Utilizing asepsis, the left femoral artery and vein were cannulated. EKG, respiration, arterial and central venous pressure were continuously monitored and recorded (Polygraph; Grass Instruments, Quincy, MA). The right femoral artery was cannulated with a 20G catheter (Becton Dickinson; Sandy, UT), that served as an arterial CPB cannula. The right jugular vein was cannulated with a modified five-hole 14G cannula advanced to the right atrium. This cannula was used for venous drainage during the hemorrhage phase and later as a venous CPB cannula. Rectal and tympanic probes were used to monitor the temperature. Heparin was administered to achieve activated clotting time (ACT) >400 s (Haemochron Jr. Signature, ITC; Edison, NJ). After instrumentation, rats were allowed to breathe spontaneously 2% isoflurane in FiO_2 0.25.

After a 5 min equilibration period, a rapid exsanguination (12.5 ml of blood over 5 min) was performed via the external jugular catheter and shed blood was collected for re-transfusion. CA was ensured with administration of a mixture of esmolol (9 mg) and potassium chloride (KCl, 0.2 mEq) intravenously after the exsanguination phase. At CA 1 min, the flush solution (0—2 °C Plasma-Lyte A, Baxter; Deerfield, IL) was administered retrogradely into the aorta via the right femoral artery catheter at 50 ml/min using a roller pump, and was drained from the jugular vein catheter. A target Tty of 15 °C during CA was achieved with a combination of 270 ml of flush and surface cooling started at CA 1 min. After 60 min (60 min EPR group) or 75 min (75 min EPR group) CA, resuscitation was started with CPB. CPB controls (without exsanguination, flush, or no-flow period) were also studied. Identical doses of KCl and esmolol were used to induce asystole, followed immediately by 60 min of CPB at 34 °C.

The CPB circuit consisted of a custom-designed oxygenator, an open reservoir (Ing. Martin Humbs, Ingenierburo fur Feinwerktechnik, Munich, Germany), tubing, and a roller pump (Masterflex, Barnant; Barrington, IL). The oxygenator contained a three-layer capillary membrane sufficient to provide oxygen levels >400 mm Hg. A mixture of $98\%O_2/2\%CO_2$ was used at a flow rate of 50 ml/min via the CPB circuit to provide oxygenation and prevent hypocapnia. Isoflurane from a separate vaporizer was used for maintenance of anesthesia during CPB. Heating and cooling were achieved with a circulating water bath and a forced-air blower (Life-Air 1000, Progressive Dynamics, Marshall, MI) blown over the rat covered with a semi-closed transparent

lid. The temperature gradient between the water bath and the body core was not allowed to exceed 10 °C.

Immediately before starting resuscitation, the CPB circuit was primed with collected shed blood for EPR groups (or Plasma-Lyte A for CPB controls), and mechanical ventilation with FiO_2 0.3 was restarted to re-expand the lungs and prevent atelectasis. In the EPR groups, CPB flow rate was gradually increased according to mean arterial pressure (MAP) and the core temperature[17] targeting a rectal temperature of 34 °C at 60 min. In the CPB controls, CPB flow rate was set at 160—180 ml/kg/min. Acid—base management followed alpha-stat principles. pH and electrolyte values outside of the normal range were corrected during CPB and intensive care unit (ICU) phases. Additional blood obtained from a donor rat was used, if needed, to maintain hematocrit at >25%.

CPB was discontinued after 60 min in control, 60 min EPR and 75 min EPR groups. Mechanical ventilation with FiO_2 of 1.0 was continued while maintaining normocapnia for a 2 h ICU phase. Using a midline laparotomy incision, a Minimitter probe (Mini-Mitter Co; Sunriver, OR) was introduced into the peritoneal cavity to allow post-operative temperature control and continuous monitoring of heart rate, temperature and activity. Surviving rats were extubated at 2 h after removal of catheters, and placed separately in a temperature-controlled cage (34 °C for additional 4 h) with supplemental oxygen for 18 h.

Body weight and neurologic status were assessed at resuscitation time (RT) 24 h, using overall performance category (OPC: 1 = normal; 2 = mild disability; 3 = moderate disability; 4 = severe disability/coma; 5 = death/brain death) and neurologic deficit score (NDS, 0—10%, normal; 100%, brain death).[18] Rats were euthanized with an isoflurane overdose and perfused with either normal saline (for Western blotting) or with normal saline followed by 10% formalin for immunohistochemistry.

Western blotting

Western blots were used to evaluate nitration and ribosylation of proteins. 3-Nitrotyrosine (3NT) is a marker of protein nitration while poly-ADP ribose polymers are a marker of PARP activation. Brain samples were homogenized in lysis buffer containing 0.1 M NaCl, 0.01 M Tris—HCl, 1 mM EDTA, 1 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonylfluoride (PMSF), 1 mM dithiothreitol (DTT) and protease inhibitors, and centrifuged for 30 min at 14,000 g. Supernatants were collected and protein concentration was read using the BCA method (Pierce, IL). Sixty micrograms of protein were run on a 12% SDS—polyacrylamide gel and separated electrophoretically. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, CA) overnight. Membranes were blocked in 5% dry milk and then incubated overnight at 4 °C with the monoclonal primary antibody against 3NT (AB7048-50, AbCam, MA) or antibody against poly (ADP-ribose) (PADPR) polymers (SA-216, Biomol, PA) (1:1000). The activation of PARP was measured by poly-ADP-ribosylation. Membranes were washed for 1 h in 1X PBS containing 0.1% Tween-20 and then incubated in the appropriate secondary antibody (1:3000) for 1 h at room temperature. Membranes were washed repeatedly for 40 min and then incubated with chemiluminescence reagent and exposed to an X-ray film. Relative optical density (ROD) was quantified using MCID Imaging System (Ontario, Canada).

Immunohistochemistry

Brain tissues were processed for paraffin embedding. Five-micrometer sections were deparaffinized in xylene, rehydrated through graded alcohols and rinsed in PBS.

Endogenous peroxide was blocked for 45 min with 0.3% H_2O_2. Slides were rinsed and sections were blocked with 5% normal horse serum for 30 min. Sections were incubated with a 1:200 dilution of a mouse monoclonal antibody against either 3NT (AB7048-50, AbCam, MA) or PADPR polymers (SA-216, Biomol, PA) overnight at 4 °C. Sections were rinsed twice for 10 min each and then incubated in a 1:500 dilution of horse anti-mouse IgG antibody conjugated with fluorescein isothiocyanate for 1 h at room temperature. After three 5 min rinses, coverslips were placed on the slides for microscopic viewing.

Statistical analysis

Data are presented as mean ± standard deviation unless otherwise stated. One-way ANOVA was performed, followed by Student—Newman—Keuls post hoc tests to identify differences between groups in physiological parameters and to evaluate the Western blotting quantification analysis. The chi-square test was used to test the differences in proportions of OPC values between groups (favorable vs. unfavorable outcome, OPC 1—2 vs. OPC 3—5). Kruskal—Wallis test was used to compare NDS between groups. Mann—Whitney U test was used to compare groups if Kruskal-Wallis test indicated an overall effect. Statistical software SPSS for Windows was used. A P value <0.05 was considered statistically significant.

Results

Thirty-four of 41 rats remained in protocol for the duration of the study. One rat from the 60 min EPR group was excluded from the protocol due to failure of the membrane oxygenator. Six rats were excluded from the protocol in the 75 min EPR group due to technical difficulties with either (1) the flush delivery or (2) air embolism.

Physiology

Tty, MAP and blood gas results are shown in Figures 1 and 2 and in Table 1. Baseline hemodynamics, hematological and biochemical parameters, acid—base status and body temperature were all in the normal physiological range in all groups. After cooling, Tty decreased to 12.6 ± 1.24 °C in the 60 min EPR group and 12.6 ± 1.00 °C in the 75 min EPR group as expected during preservation. Lactate levels were significantly increased in both EPR groups after CA (controls, 2.4 ± 1.4; 60 min EPR, 7.9 ± 1.2; 75 min EPR, 9.8 ± 2.4 mmol/l). Cardiac activity was restored after 5—10 min of CPB in controls.

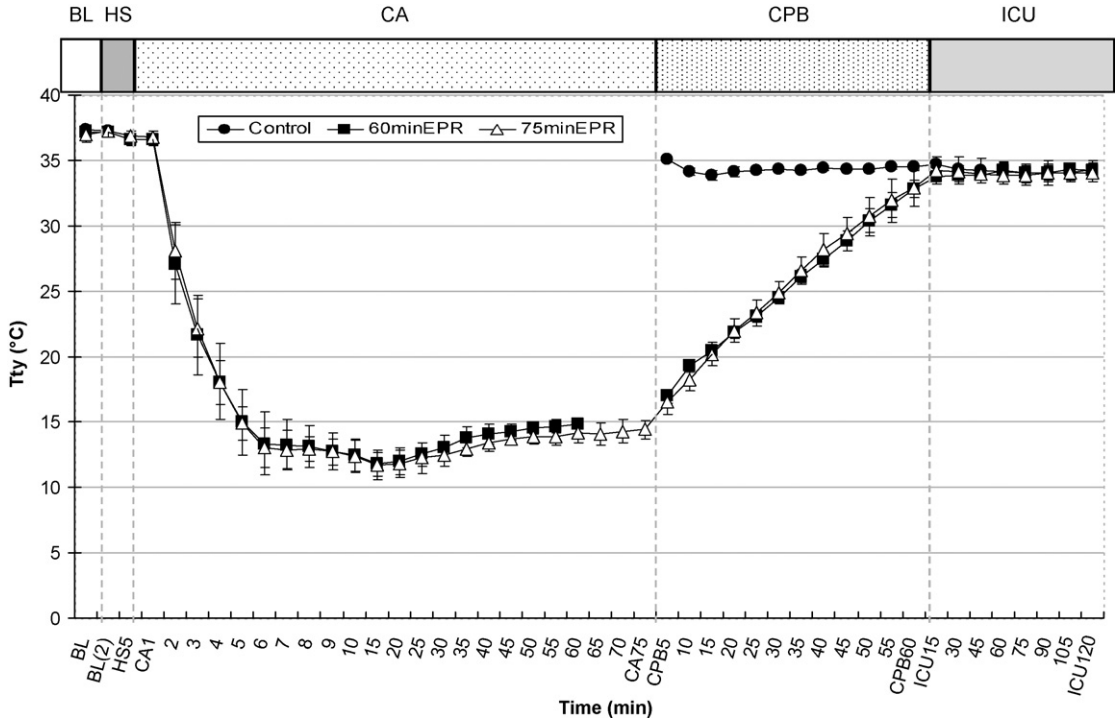

Figure 1 Tympanic temperature (Tty) in control, 60 min EPR and 75 min EPR groups during the experiment. BL, baseline; HS, hemorrhagic shock; CA, cardiac arrest; CPB, cardiopulmonary bypass; ICU, intensive care unit; EPR, emergency preservation and resuscitation.

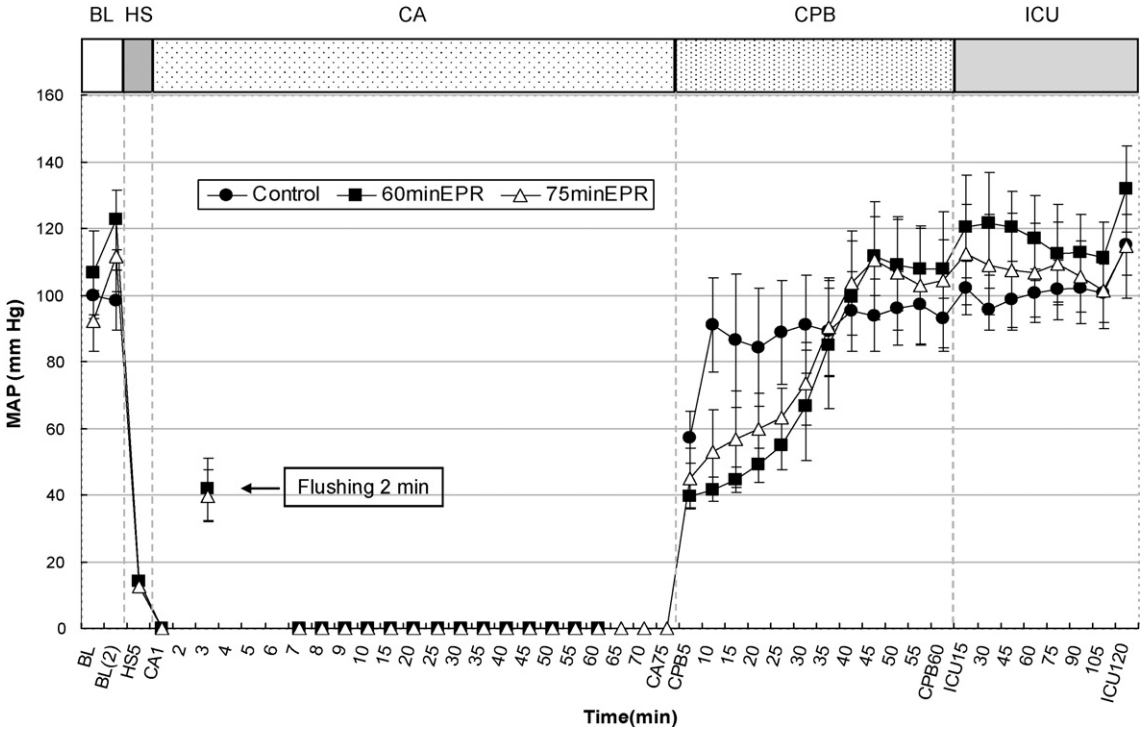

Figure 2 Mean arterial pressure (MAP) in control, 60 min EPR and 75 min EPR groups during the experiment. BL, baseline; HS, hemorrhagic shock; CA, cardiac arrest; CPB, cardiopulmonary bypass; ICU, intensive care unit; EPR, emergency preservation and resuscitation.

Table 1 Hematological, biochemical parameters and acid—base status at baseline (BL), the end of cardiopulmonary bypass (CPB) and the end of intensive care unit (ICU) in control, 60 min EPR and 75 min EPR groups

Blood gas	Control	60 min EPR	75 min EPR	*P*-value
Arterial pH				
BL	7.39 ± 0.03	7.36 ± 0.04	7.39 ± 0.05	0.384
CPB	7.42 ± 0.03	7.41 ± 0.03	7.47 ± 0.07	0.083
ICU	7.43 ± 0.02	7.44 ± 0.07	7.42 ± 0.05	0.724
PaO_2 (mm Hg)				
BL	255.9 ± 21.6	227.5 ± 28.9*	261.1 ± 23.8	0.021
CPB	382.0 ± 35.1	431.6 ± 44.1	418.6 ± 51.9	0.131
ICU	354.4 ± 59.8	201.5 ± 65.2*	305.5 ± 119.9	0.018
Hct (%)				
BL	33.7 ± 1.5	31.7 ± 2.6	33.1 ± 2.0	0.193
CPB	21.6 ± 1.9	27.0 ± 2.7†	28.7 ± 2.4†	<0.001
ICU	24.7 ± 1.7	30.4 ± 3.1†	32.1 ± 3.1†	<0.001
Na^+ (mmol/l)				
BL	141.5 ± 2.5	145.9 ± 2.3†	143.7 ± 3.0	0.021
CPB	141.4 ± 4.0	151.2 ± 2.6†	152.3 ± 3.8†	<0.001
ICU	143.0 ± 2.1	149.4 ± 3.2†	150.5 ± 2.7†	<0.001
Glucose (mg/dl)				
BL	198.0 ± 22.7	206.9 ± 44.1	228.6 ± 67.3	0.434
CPB	289.9 ± 48.7	271.7 ± 55.5	289.7 ± 60.1	0.767
ICU	148.6 ± 28.3	159.6 ± 52.6	171.8 ± 44.8	0.522
Lactate (mmol/l)				
BL	1.0 ± 0.3	1.2 ± 0.6	1.2 ± 0.3	0.462
CPB	2.4 ± 1.4	7.9 ± 1.2†	9.8 ± 2.4†	<0.001
ICU	2.2 ± 1.7	4.2 ± 1.3	7.3 ± 3.4*	0.001

PaO_2: arterial oxygen partial pressure; Hct: hematocrit. Data are presented as mean ± S.D.
* Significant difference compared with other groups in the same time point.
† Significant difference compared with control group in the same time point.

Survival and neurological outcome

One rat died at RT 20 h in the CPB control group. One rat died at RT 22 h in the 60 min EPR group with pulmonary hemorrhage at necropsy. Eight rats died in the 75 min EPR group at RT 4—7 h. Neurologic outcome was significantly better in the 60 min EPR vs. the 75 min EPR group (OPC, $P < 0.001$; NDS, $P = 0.001$) (Figures 3 and 4).

Nitration and PARP activation changes

Semi-quantification of ROD for Western blotting for 3NT and PADPR is shown in Figures 5—8. Evaluating the major 64 kD band, nitration and ribosylation increased significantly in hippocampus and cortex in the 75 min EPR group vs. all other groups. In striatum, there was an increase in nitration of 64 kD band compared to CPB controls only. A more modest decrease in global protein nitration and ribosylation was also found in hippocampus in CPB controls, reaching statistical significance for PADPR in cortex. There were no differences in protein ribosylation or nitration in cerebellum both in total protein spectrum analysis and separate analysis of the 64 kD band.

In general, the pattern of changes observed in cerebellum and striatum were similar to those seen in cortex. Hippocampus showed a unique pattern corresponding to the increasing severity of the insult.

3NT and PADPR expression were also higher in 75 min EPR group than in other groups by immunohistochemistry which corroborated Western blotting results (Figures 9 and 10, online—only supplemental material). 3NT and PADPR was predominantly detected in vascular endothelium by immunohistochemical staining.

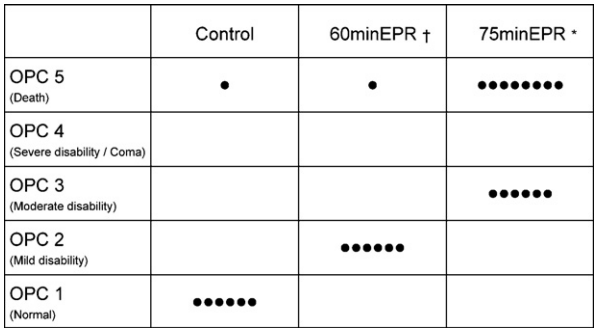

Figure 3 Overall performance categories (OPC, 1—5) at 24 h after resuscitation. *$P < 0.001$, 75 min EPR group vs. other groups; †P = NS, 60 min EPR group vs. control group (favorable vs. unfavorable outcome, OPC 1—2 vs. OPC 3—5). Each dot represents one rat. EPR, emergency preservation and resuscitation.

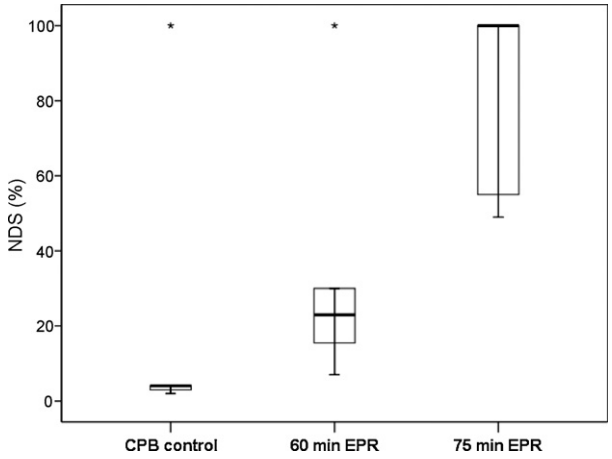

Figure 4 Neurological deficit score (NDS, 0—100%) at 72 h after exsanguination cardiac arrest treated by 60 min or 75 min of emergency preservation and resuscitation (EPR). Boxes represent interquartile ranges. The line across each box indicates the median, and the whiskers are the highest and lowest values. The asterisks represent outliers. $P < 0.01$ 75 min EPR groups vs. 60 min EPR group or control group.

Discussion

EPR is being studied systematically as a strategy to buy time for trauma patients with temporarily unresuscitable CA to allow recovery without brain damage. Attempting to extend this CA duration is being explored to provide sufficient time for transport and surgical repair in patients with traumatic exsanguination leading to CA who would require such prolonged preservation, particularly in military combat scenarios.

In this study, we demonstrated that 75 min of EPR was associated with significant mortality and impaired neurological outcome in rats. Seventy-five minutes EPR was also associated with increases in nitration and ribosylation in brain—consistent with activation of these two secondary injury mechanisms in selected proteins assessed in selectively vulnerable brain regions, namely hippocampus. Our data suggest a possible role for these two important secondary injury mechanisms (nitration and ribosylation) in limiting the successful duration of EPR that can be tolerated. However, the direct cause—effect of these mechanisms on the outcome remains to be determined in the future studies.

One possibility is that deep hypothermia protects against these secondary injury mechanisms during EPR, but this protective effect was lost when the ischemic time was extended to 75 min EPR in our rat model. Given that we did not see neuronal death in our model, we cannot rule out the possibility that nitration and/or ribosylation could be either protective or an epiphenomenon. Surprisingly, CPB alone was associated with a modest decrease in the degree of nitration and ribosylation compared with other groups. CPB alone has been associated with cognitive deficits and possibly neuronal death in rat models of CPB.[19] CPB alone in dogs was recently shown to activate PARP in gut, with impaired vascular reactivity.[20] Most rats in the 75 min EPR group in this study died between 4 and 7 h after resuscitation and necrotic regions in gut were seen on necropsy (data not shown). Complete histological analysis of gut or other extra-cerebral organs in our study was not carried out given our initial focus on brain injury. It seems that extra-cerebral organs are vulnerable to the effects of prolonged EPR in the rat. However, based on the results from our previously published study using the identical model, we can speculate that the cause of death was probably multi-organ failure, with cardiac failure as the terminal event.

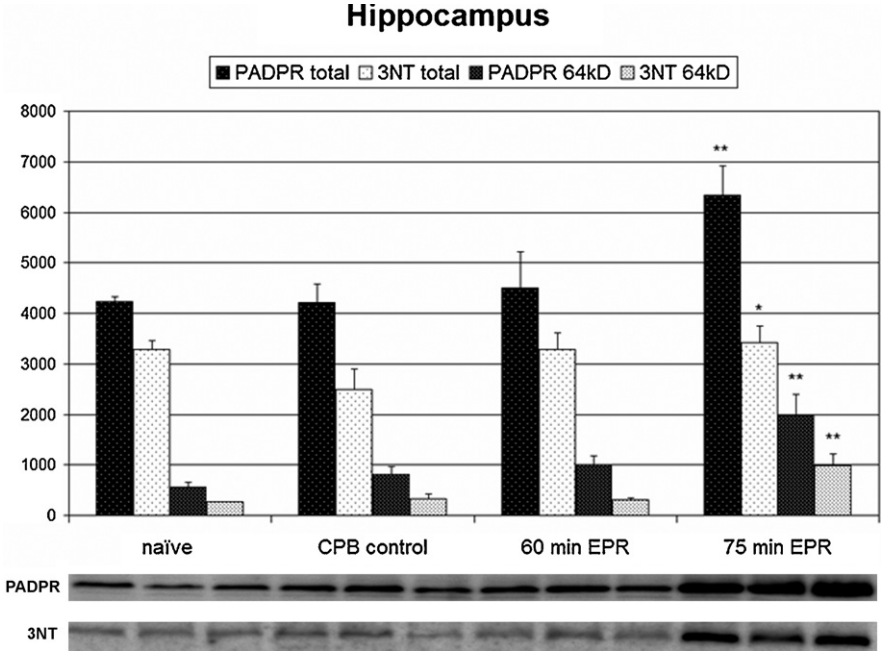

Figure 5 Relative optical densities of Western blotting analyses of nitrotyrosine (3NT) and poly (ADP-ribose) polymers (PADPR) in hippocampus of each group. *$P < 0.05$ vs. CPB; **$P < 0.05$ vs. other groups. Bottom panel shows the Western blot of the 64 kD band. CPB, cardiopulmonary bypass; EPR, emergency preservation and resuscitation.

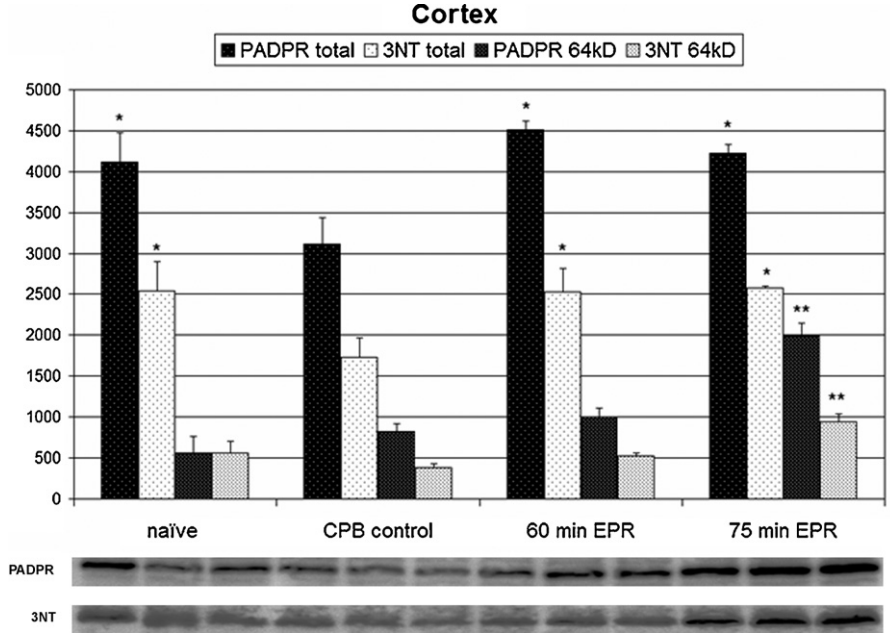

Figure 6 Relative optical densities of Western blotting analyses of nitrotyrosine (3NT) and poly (ADP-ribose) polymers (PADPR) in cortex of each group. *$P < 0.05$ vs. CPB; **$P < 0.05$ vs. other groups. Bottom panel shows the Western blot of the 64 kD band. CPB, cardiopulmonary bypass; EPR, emergency preservation and resuscitation.

Of interest, in an identical model, cardiac lesions were consistent with those seen in the brain—heart syndrome (i.e., cardiac degeneration developing as a result of catecholamine release from damaged brain tissue) but other pathogenic mechanisms may be operating. Immunohistochemistry showed increased 3-NT and PADPR staining mostly in the perivascular regions of the brain. This finding has some support in the literature—Csabo et al. reported reduction of endothelium-dependent vasodilation after 90 min of moderately hypothermic CPB, while PARP inhibitor INO-1001 prevented those effects. Endothelium-independent vasodilatation remained unaffected.[20]

Protective hypothermia, induced and reversed with CPB, is clinically used for some elective operations on heart or brain but has not been performed yet for emergency scenarios such as EPR. Elective therapeutic hypothermia has been

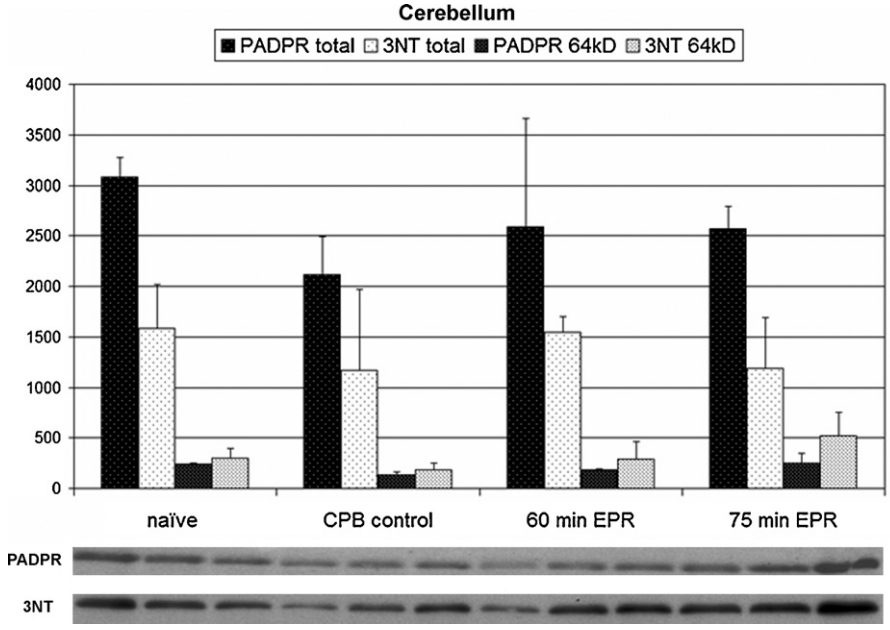

Figure 7 Relative optical densities of Western blotting analyses of nitrotyrosine (3NT) and poly (ADP-ribose) polymers (PADPR) in cerebellum of each group. No differences between groups. Bottom panel shows the Western blot of the 64 kD band. CPB, cardiopulmonary bypass; EPR, emergency preservation and resuscitation.

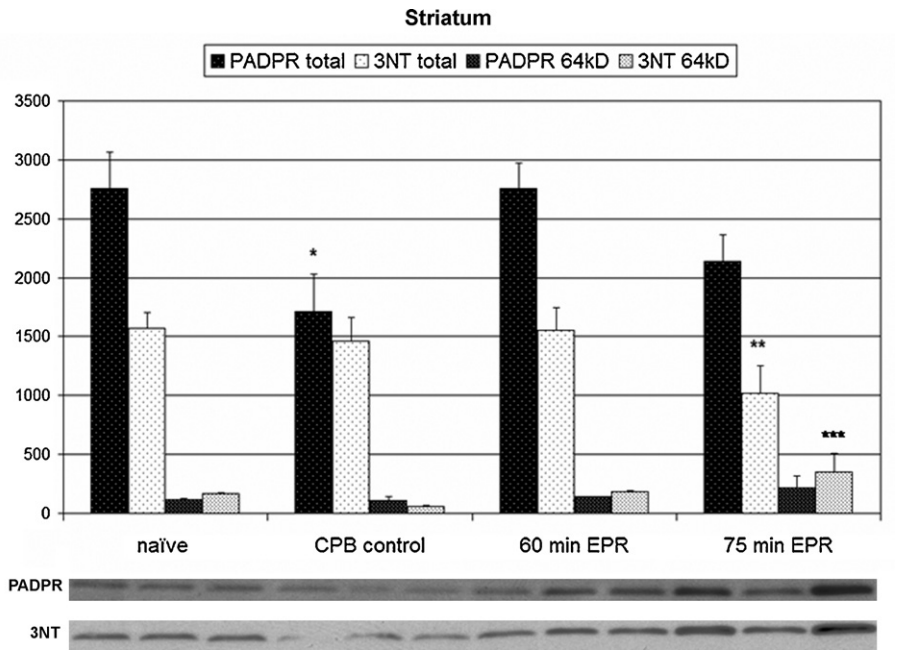

Figure 8 Relative optical densities of Western blotting analyses of nitrotyrosine (3NT) and poly (ADP-ribose) polymers (PADPR) in striatum of each group. *$P < 0.05$ vs. naïve and 60 min EPR group; **$P < 0.05$ vs. naïve; ***$P < 0.05$ vs. CPB group. Bottom panel shows the Western blot of the 64 kD band. CPB, cardiopulmonary bypass; EPR, emergency preservation and resuscitation.

shown to protect the brain and entire organism in animals or patients for up to 15 min of CA at brain temperature of about 35 °C (mild hypothermia),[26,27] for up to 20 min of CA at about 30 °C (moderate hypothermia),[28] for up to 30 min of CA at about 20 °C (deep hypothermia),[29] for up to 60—150 min of CA at 5—10 °C (profound hypothermia),[30—35] and perhaps even for longer CA with ultra-profound hypothermia.[36—38] Treatment induced before arrest (protection) and maintained during arrest (preservation) is more likely to mitigate post-ischemic brain damage than when induced after arrest (resuscitation). Mild hypothermia has been recommended in post-resuscitation treatment of ventricular fibrillation (VF) CA. The benefit derived from mild hypothermia after ROSC for cerebral recovery has been well documented.[21—25] Using a mouse model of CA, Abella et al.[25] reported improved outcome when cooling was induced during CA but not after ROSC. Similarly, in a clinically relevant large animal model of CA, survival with full neurological recovery after 40 min of VF was reported if mild or moderate hypothermia was initiated during ROSC attempts, but not after ROSC.[9]

Many potential mechanisms are believed to be responsible for neuronal damage after prolonged circulatory arrest. Nitration is believed to be an important secondary injury mechanism early after the insult. Nitration of protein tyrosine residues results in diverse pathologies. This reaction decreases the pK_a of the tyrosine hydroxyl group, altering protein structure and function. Protein nitration also results in cytoskeletal damage and disturbances in energy production and signal transduction.[39] Nitration of protein tyrosine residues is a stable modification that can be detected immunochemically. 3NT immunoreactivity has been reported in acute and chronic CNS disease.[40] Several key nitration targets have been suggested including PDH and manganese superoxid dismutase.[14,41] 3NT immunoreactivity is often localized to neurons, infiltrating neutrophils, and microvasculature after CNS injury.[16,39,40,42,43] Our findings are consistent with a possible role for nitration at the threshold between favorable and unfavorable neurological outcome. It is interesting that both protein nitration and poly-ADP-ribosylation were seen in brain in our model despite the paucity of neuronal death in surviving rats in the 75 min EPR group. It is possible that poor outcome reflects either neurological dysfunction or has an important extracerebral component, or is a response to prolonged EPR but has not functional consequence.

A second footprint of oxidative and nitrative injury is activation of the DNA repair enzyme PARP. This enzyme, when activated, adds multiple ribose moieties to proteins which can be detected with antibodies against PADPR. PARP activation may represent a deleterious effect by consuming NAD^+ (the PARP Suicide Hypothesis) or via ribosylation of protein targets.[44] A key role for PARP-1 activation after acute brain injury was established using PARP-1 knockout mice, where deletion of PARP-1 was found to confer significant protective effects in histologic and behavioral outcome in several relevant models.[15,16,45]

Our study has some limitations. First, CPB flow rates were not identical between groups given the wide variance in other physiological conditions between CPB control and EPR insults. A possible influence of CPB flow rate on nitration or ribosylation cannot be excluded. Second, immunohistochemistry results showed increase of 3NT and PADPR especially in the 75 min EPR group in brain but not specifically in hippocampus as shown in Western blots.

In our study, we performed a separate analysis of total protein spectrum and separate analysis of the most

prominent 64 kD band. A similarly prominent 64 kD band was detected by our group after traumatic brain injury. Using a 2D gel electrophoresis to identify the protein, the authors hypothesized that it is a mitochondrial heat-shock protein HSP60.[46] It is evident from our study that hippocampus is selectively vulnerable to increased duration in ischemia as reflected by increased ribosylation and nitration in evaluation both total protein and the most prominent 64 kD band, while other regions show more variable injury pattern.

Finally, fluorescent labelling appeared to be predominantly perivascular in origin—rather than in the vascular lumen. We cannot rule out the possibility of some contribution of autofluorescence by erythrocytes, however, the perfusion-fixation protocol was identical for all groups.

We conclude that protein nitration and PARP activation are seen in brain at the threshold of EPR durations associated with unfavorable neurological outcome. These two mechanisms may represent therapeutic targets to improve outcome in EPR and other CNS insults associated with deep hypothermic circulatory arrest.

Implications statement

Emergency preservation and resuscitation (EPR) is a novel therapeutic approach to traumatic exsanguination cardiac arrest. Its feasibility is supported by a series of studies. In this current report, we suggest a possible role for two secondary injury mechanisms, namely nitration and ribosylation, in limiting further extension of the duration EPR that can be tolerated.

Conflict of interest statement

Drs. Kochanek and Tisherman and Mr. S. William Stezoski are co-inventors and have a U.S. Provisional Patent on the emergency preservation and resuscitation method. Mr. S. William Stezoski is also on the Board of Directors of EPR Technologies.

Other authors have no conflict of interest to disclose.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.resuscitation.2008.06.004.

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EXPERIMENTAL PAPER

Assessment of the delta opioid agonist DADLE in a rat model of lethal hemorrhage treated by emergency preservation and resuscitation☆

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KEYWORDS
Hypothermia;
Hemorrhage;
Shock;
Cerebral ischemia;
Cardiopulmonary bypass;
Suspended animation;
DADLE;
Delta opioid agonist

Summary Emergency preservation and resuscitation (EPR) is a new approach for resuscitation of exsanguination cardiac arrest (CA) victims. EPR uses a cold aortic flush to induce deep hypothermic preservation during no-flow to buy time for transport and damage control surgery, followed by resuscitation with cardiopulmonary bypass (CPB). We reported previously that 20—60 min EPR in rats was associated with intact outcome, while 75 min EPR resulted in high mortality and neurological impairment in survivors. The delta opioid agonist DADLE ([D-Ala(2),D-Leu(5)]-enkephalin) was shown previously to be protective against ischemia-reperfusion injury in multiple organs, including brain. We hypothesized that DADLE could augment neurological outcome after EPR in rats. After rapid lethal hemorrhage, EPR was initiated by perfusion with ice-cold crystalloid to induce hypothermia (15 °C). After 75 min EPR, resuscitation was attempted with CPB. After randomization, three groups were studied ($n = 10$ per group): DADLE 0 mg/kg (D0), 4 mg/kg (D4) or 10 mg/kg (D10) added to the flush and during reperfusion. Survival, overall performance category (OPC; 1 = normal, 5 = death), neurological deficit score (NDS; 0—10% normal, 100% = max deficit), and histological damage score (HDS) were assessed in survivors on day 3. In D0 group, 2/10 rats survived, while in D4 and D10 groups, 4/10 and 5/10 rats survived, respectively (p = NS). Survival time (h) was 26.7 ± 28.2 in D0, 36.3 ± 31.9 in D4 and 47.1 ± 30.3 in D10 groups, respectively ($p = 0.3$). OPC, NDS and HDS were not significantly

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different between groups. In conclusion, DADLE failed to confer benefit on functional or histological outcome in our model of prolonged rat EPR.

Introduction

Currently, the outcomes from traumatic exsanguination cardiac arrest (CA) are poor. Data from both recent military conflicts[1] and civilian settings[2,3] show that over 50% of deaths due to trauma occur at the scene, where medical care is limited. However, in an appropriate setting, some of those injuries could be surgically repairable.[4]

Emergency preservation and resuscitation (EPR) is a new approach for resuscitation of CA victims.[5] EPR uses cold aortic flush to induce hypothermic preservation during circulatory arrest and buys time for transport, damage control surgery, and delayed resuscitation with cardiopulmonary bypass (CPB). Neurological outcome after EPR is dependent on the duration of CA and temperature in both large (dog, swine) and small animal models. Given the logistic challenges of inducing hypothermia and potential risks of hypothermia, testing of potential pharmacological adjuncts that would allow extension of the period of CA is warranted.

The delta receptor agonist ([D-Ala(2),D-Leu(5)]-enkephalin—DADLE) has been recently evaluated as a possible link to hibernation.[6] DADLE possesses organ preservation properties evaluated on liver,[7] heart [8–12] and lungs,[13] or the organs harvested *en bloc*.[14] The vast majority of the experiments revealed positive results, extending ischemic time while preserving post-reperfusion organ function. Neuroprotective properties were also observed in multiple scenarios, including global brain ischemia[15] or pharmacologically induced brain injury.[16,17] Moreover, DADLE effects were exerted even under hypothermia.[8,13]

In our previous studies, we established a rat model of EPR with excellent survival and neurological recovery after 20—60 min CA.[18] Further extension of the EPR duration to 75 min resulted in high mortality resulting from multi-organ failure, and neurological impairment in survivors.[19]

Using this model, we hypothesized that DADLE would augment survival and neurological outcome after exsanguination CA followed by 75 min EPR. To test this hypothesis, we evaluated two doses of DADLE added to the flush and administered during reperfusion. We assessed survival rate, survival time and neurological outcome as primary outcome measurements. Histological damage score (HDS) served as a secondary outcome measurement.

Materials and methods

The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Adult male Sprague—Dawley rats (350—400 g) were obtained from Hilltop Lab Animals (Scottdale, PA) and housed for at least 3 days before the experiment under 12-h light/dark cycle with unrestricted access to food and water. On the day of the experiment, rats were anesthetized with 4% isoflurane in a transparent acrylic jar. After tracheal intubation with a 14G intravenous catheter (Becton Dickinson; Sandy, UT), rats were mechanically ventilated using a piston ventilator (Harvard Ventilator Model 683, Harvard Rodent Apparatus; South Natick, MA) with a tidal volume of 0.8 ml/100 g and a frequency of 20—24 min^{-1} to maintain normocapnia, and a positive end-expiratory pressure (PEEP) of 4 cm H_2O. Anesthesia was maintained with 1.5—2% isoflurane in FiO_2 0.5. After shaving and cleansing with povidone iodine, bilateral femoral and right jugular cutdowns were performed. The left femoral artery and vein were cannulated for blood pressure monitoring and blood sampling. ECG, respiration, arterial and central venous pressure were monitored and recorded continuously (Polygraph; Grass Instruments, Quincy, MA). The right femoral artery was cannulated with a 20G catheter (Becton Dickinson; Sandy, UT) that served as an arterial CPB cannula. The right jugular vein was cannulated with a modified five-hole 14G intravenous cannula advanced to the right atrium to be used for venous drainage during the hemorrhage phase and later as a venous CPB cannula. Rectal and tympanic probes were used to monitor the temperature. After instrumentation, rats were weaned from the ventilator and allowed to spontaneously breathe 2% isoflurane in FiO_2 0.25 via a nose cone mask over the tracheal tube. Baseline blood samples were obtained, and hemodynamic values were recorded. Removed blood volume was replaced with an electrolyte-balanced crystalloid Plasma-Lyte A (Baxter; Deerfield, IL) in a ratio 1:3. Heparin sodium was given to achieve activated clotting time (ACT) >400 s (Haemochron Jr. Signature, ITC; Edison, NJ).

After a 5 min equilibration period, rapid exsanguination (12.5 ml of blood over 5 min) was performed via the internal jugular catheter. The shed blood was collected in a heparin pre-filled syringe. After the rapid exsanguination phase, CA was ensured with a mixture of 9 mg of esmolol and 0.1 ml of potassium chloride (0.2 mequiv.) intravenously. After 1 min of no-flow, 270 ml of an ice-cold flush solution (Plasma-Lyte A) was instilled via the right femoral artery catheter at 50 ml/min using a roller pump (Masterflex, Barnant, IL). The flush was drained from the jugular vein catheter. A target rectal temperature of 15 °C was achieved with a combination of 270 ml of flush and surface cooling. Both rectal and tympanic temperatures were maintained at 15 °C during EPR.

The rats were randomized into three groups ($n = 10$ per group): DADLE 0 mg/kg (D0), 4 mg/kg (D4) or 10 mg/kg (D10) added to the flush and during reperfusion. Rats in the D0 group received the same volume of vehicle (Plasma-lyte A) as rats in the D4 and D10 groups.

After 75 min of CA, resuscitation was started with CPB. (Fig. 1) In brief, the CPB circuit consisted of a custom-designed oxygenator made of polymethylmethacrylate, a reservoir (both made by Ing. Martin Humbs, Ingenierburo fur Feinwerktechnik, Munich, Germany), platinum-cured

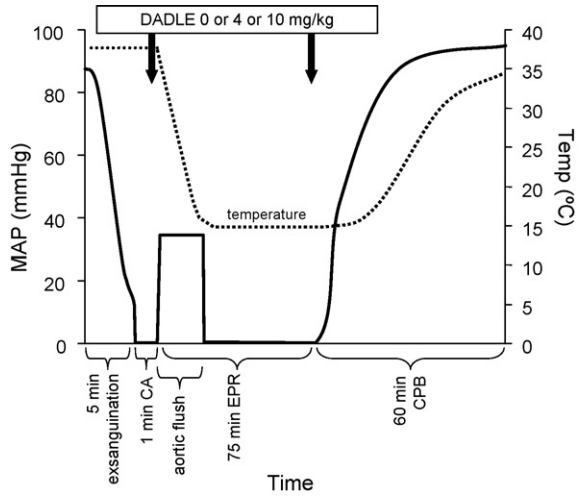

Figure 1 Experimental protocol including rapid hemorrhagic shock followed by 1 min cardiac arrest and emergency preservation and resuscitation (EPR) using cardiopulmonary bypass (CPB). Solid line = mean arterial pressure (MAP); dotted line = temperature.

silicone tubing, and a roller pump (Masterflex, Barnant; Barrington, IL). The oxygenator contained a three-layer capillary membrane sufficient to provide oxygen levels >400 mmHg. A mixture of 98% O_2/2% CO_2 was used at a fresh-gas flow rate of 50 ml/min through the oxygenator to provide oxygenation and prevent hypocapnia. Isoflurane from a separate vaporizer was used for maintenance of anesthesia during the CPB phase. Heating and cooling were achieved with a circulating water bath around the oxygenator and a forced-air blower (Life-Air 1000, Progressive Dynamics, Marshall, MI) blowing heated air over the rat placed under a transparent cover with sealed edges to allow even rewarming.

Immediately before starting resuscitation, the CPB circuit was primed with collected shed blood, and mechanical ventilation with FiO_2 0.3 was restarted to re-expand the lungs and prevent atelectasis. ACT was maintained at >400 s with additional heparin boluses during the CPB course. Blood samples for biochemistry and hematology were obtained at 5 min, 15 min, 30 min, 45 min, and 60 min CPB time and processed immediately using a point-of-care blood analyzer (Stat Profile, Nova Biomedical; Waltham, MA). Arterial blood gas management followed alpha-stat principles. Arterial pH and electrolyte values outside of the normal range were corrected during the CPB and ICU phases by adjustments in ventilation and/or administration of sodium bicarbonate, calcium chloride, and potassium chloride as needed. Additional blood obtained from an isoflurane-anesthetized donor rat was used to maintain hematocrit level >25%.

CPB support was gradually discontinued after 60 min. No pharmacological hemodynamic support was used. Mechanical ventilation with a FiO_2 of 1.0 was continued while maintaining normocapnia. Using a midline laparotomy incision, a Mini-mitter probe (Mini-Mitter Co.; Sunriver, OR) was introduced into the peritoneal cavity to allow post-operative temperature control and continuous monitoring of heart rate and movement. The electrodes of the probe were tunnelled subcutaneously and attached to the pectoral muscles from separate incisions. After removal of catheters, surviving rats were extubated 2 h later and placed separately in a temperature-controlled cage (34.5 °C for 4 h) with supplemental oxygen for 18 h, and provided free access to food and water. Animals that did not resume normal eating or drinking patterns within 24 h or that lost greater than 10% of their initial body weight received subcutaneous injections of 0.45NS/D5 twice daily.

Neurological status was assessed daily using overall performance category (OPC; 1 = normal, 2 = mild disability, 3 = moderate disability, 4 = severe disability/coma, 5 = death or brain death) and a modified neurological deficit score[20] (NDS; 0—10% = normal, 100% = maximum deficit).

Blood samples were obtained from survivors on day 3, and rats were killed with an isoflurane overdose and perfused via the left ventricle with normal saline followed by 10% neutral-buffered formalin for histological evaluation (brain, heart, lungs, kidneys, and liver). The brains were divided into multiple coronal slices (typically 11). Representative slices were also prepared from extracerebral organs. The tissue samples were processed for embedding in paraffin. The resulting paraffin blocks were sectioned at 5 μm. Sections were stained with hematoxylin and eosin. Additional (duplicate) sections of brain were stained with Fluoro-Jade B.[21] The sections were examined by a neuropathologist (RHG) masked to group assignment under brightfield or epifluorescent illumination depending upon the stain. Each neuroanatomical region with evidence of damage on microscopic examination received a subjective pathological grade ranging from 1+ (minimal) to 5+ (severe). Typically, a minimal grade (1+) would be less than 1% of the cells degenerating. Mild (2+) would be approximately 1—10% degenerating cells, moderate (3+) = 10—25%, marked (4+) = 25—50%, and severe (5+) $\geq$ 50%. The neuroanatomical regions that were graded separately included the following: olfactory bulb; piriform cortex; frontal cortex; cingulate cortex; septal nuclei; parietal cortex; caudate nucleus/putamen; globus pallidus; basal forebrain; corpus callosum; amygdala; retrosplenial cortex; subiculum; hippocampal pyramidal neuron sectors CA1, CA2, CA3, and CA4; dentate gyrus; thalamus; hypothalamus; temporal cortex; occipital cortex; fornix; midbrain; entorhinal cortex; substantia nigra (pars compacta and pars reticularis); superior colliculus; inferior colliculus; cerebellar cortex; cerebellar nuclei; cerebellar white matter; pons; reticular formation; medulla oblongata; pyramids. All affected regions on each side of the brain (right and left) received separate scores for the degrees of neuropathological damage detected in H&E-stained and Fluoro-Jade B-stained sections. The total HDS was calculated as a sum of the individual scores (i.e., for each anatomical region with each stain). The hypothetical maximum score was 720. Similarly, in extracerebral organs, each organ received a subjective pathological grade ranging from 1+ (minimal) to 5+ (severe). For organs other than brain, the criteria for grading depended upon the organ and the type of lesion.

HDS was evaluated for all rats included in the study. Additonally, we performed a separate subgroup analysis for those rats that were sacrificed at 72 h and those that died before the planned sacrifice.

Statistical analysis

Data are presented as mean ± S.D. unless otherwise stated. Repeated measures analysis of variance was used to compare serial biochemical data and vital signs. OPC, NDS and HDS were analyzed using Mann—Whitney *U*-test. The statistical software SPSS 14.0 for Windows was used. A p-value < 0.05 was considered statistically significant.

Results

There were no differences in hemodynamic or biochemical measurements between the groups at baseline, including mean arterial pressure (MAP), heart rate (HR), temperature, Hct, arterial pH, pO_2, pCO_2, base excess, and serum Na, K, Cl, Ca, Mg, urea, or lactate levels.

EPR, CPB and ICU phases

During the flush, temperature decreased in all groups similarly and was controlled at 15 °C for 75 min. During rewarming, the temperature was gradually increased to 34.5 °C similarly in all groups. During the CPB phase, HR recovered similarly in all groups. There were no differences in MAP during CPB or ICU phases between groups. The lactate levels were increased markedly versus baseline in all groups after the insult. Rats in the D0 group had slightly higher lactate levels ($p = 0.156$) (Fig. 2). There were no significant differences in pH, Na, K, Ca, Mg, Hct, BUN or glucose levels between groups during resuscitation.

Survival

Two out of 10 rats survived in the D0 group, while 4 out of 10 and 5 out of 10 rats survived in the D4 and D10 groups, respectively (p = NS). Survival time was 26.7 ± 28.2 h in D0, 36.3 ± 31.9 h in D4 and 47.1 ± 30.3 h in D10 groups, respectively ($p = 0.315$) (Fig. 3).

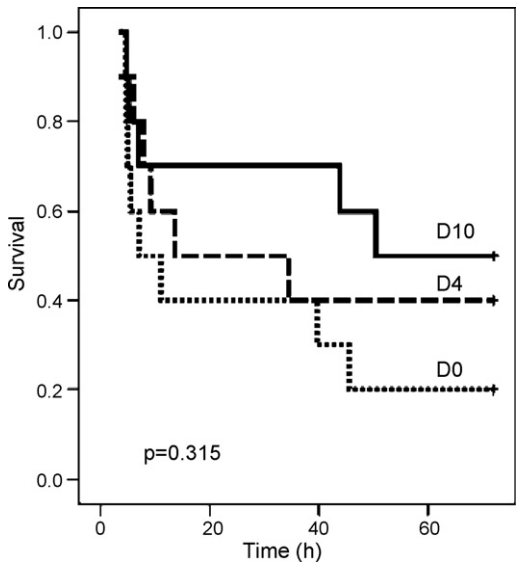

Figure 3 Survival following exsanguination cardiac arrest treated by emergency preservation and resuscitation. No differences between groups.

Neurological outcome

Few of surviving rats achieved OPC 1 in any group (Fig. 4). Six out of eleven survivors remained neurologically impaired (OPC 2—3), and there was no significant difference between groups for either OPC ($p = 0.496$) or NDS ($p = 0.515$) (Fig. 5).

Histological evaluation

Inter-group differences in HDS were not found for the brain or for any of the visceral organs (Table 1). Distribution of degenerative lesions in the brain also did not differ by treatment group. The most frequent microscopic finding in the brain was peracute to acute neuron dege-

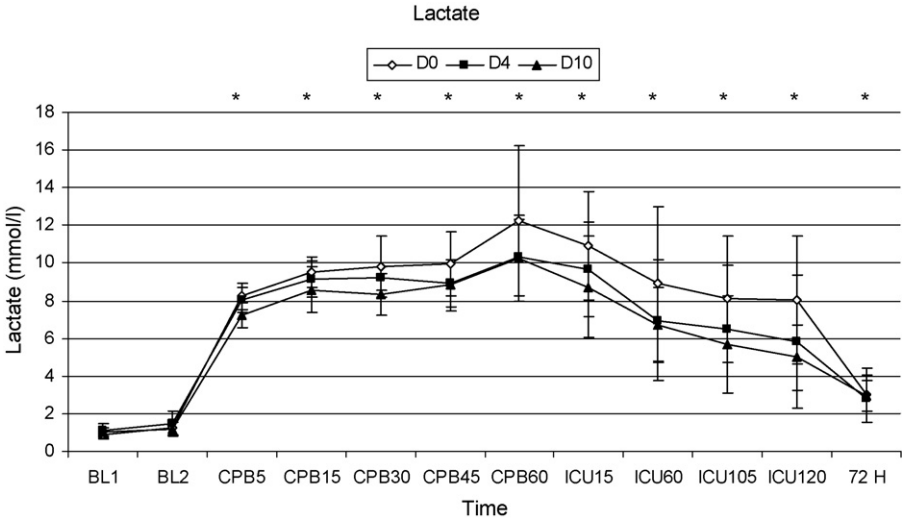

Figure 2 Lactate levels following exsanguination cardiac arrest treated by 75 min emergency preservation and resuscitation during the CPB and ICU phases. BL = baseline, F = final (72 h). $p = 0.156$ overall between groups. *$p < 0.05$ vs. respective baseline.

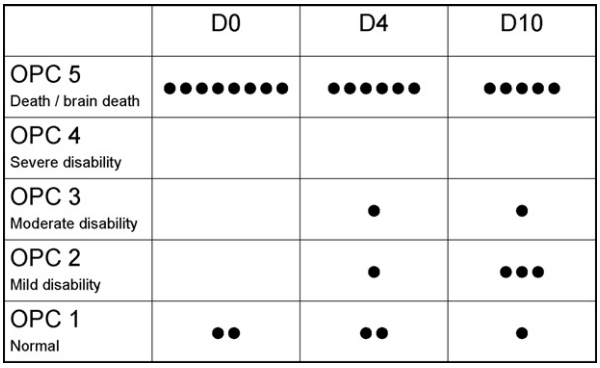

Figure 4 Overall performance categories (OPC 1—5) at 72 h after exsanguination cardiac arrest treated by emergency preservation and resuscitation. Each dot represents one rat. No differences between groups.

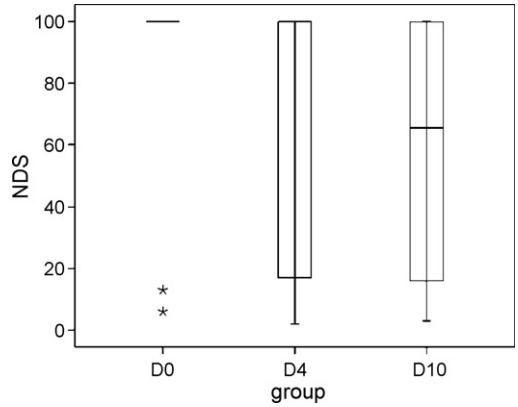

Figure 5 Neurological deficit score (NDS, 0—100%) at 72 h after exsanguination cardiac arrest treated by emergency preservation and resuscitation. Boxes represent interquartile ranges. The line across each box indicates the median, and the whiskers are the highest and lowest values. The asterisk marker represents an outlier. No differences between groups.

neration characterized in H&E-stained sections by neurons with bright eosinophilic cytoplasm and nuclei that were shrunken and darkly stained (Fig. 6A). In sections stained with Fluoro-Jade B, these dying neurons were a bright yellowish-green color (Fig. 6B). Neuron degeneration was most frequently found within various sectors of the hippocampus (especially the CA4 sector) and within the corpus striatum (caudate—putamen and globus pallidus) but, in some rats, also involved portions of the cerebral (especially frontal and piriform) and cerebellar cortices.

Table 1 Histological damage score (HDS) after exsanguination cardiac arrest treated by emergency preservation and resuscitation (EPR)

	Brain	Heart	Lung	Liver	Kidney
D0	17 ± 15	2 ± 1	2 ± 1	1 ± 1	1 ± 1
D4	33 ± 39	1 ± 1	2 ± 2	0 ± 1	1 ± 1
D10	26 ± 16	1 ± 2	1 ± 1	1 ± 1	1 ± 2
p	0.38	0.61	0.5	0.26	0.28

Scale: brain, 0—720; extracerebral organs, 0 = no damage, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked and 5 = severe

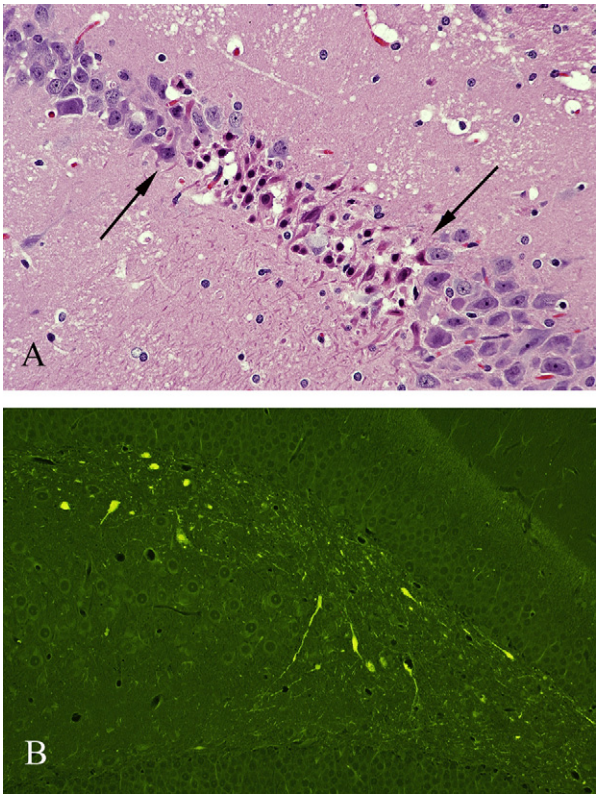

Figure 6 (A) H&E-stained section of the dorsal hippocampus from a rat in the D10 treatment group. (This rat died on study.) Within the CA2 sector of the pyramidal neuron layer (between the arrows), numerous degenerating neurons are present. These neurons have brightly stained eosinophilic cytoplasm and dark condensed nuclei. (B) Fluoro-Jade B-stained section of the dorsal hippocampus from a rat in Group D4. Within the hilar or CA4 sector (i.e. between the blades of the dentate gyrus), numerous dying neurons are stained a bright yellow-green color. This was the most common pattern of hippocampal degeneration seen in this study. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

The principal procedure-related microscopic finding for the heart was peracute to acute myocardial necrosis (Fig. 7A). For the kidneys, the principle finding was peracute proximal tubule degeneration/necrosis (Fig. 7B) suggesting a pathogenesis of ischemia. In a separate analysis of animals that died before the planned sacrifice, cardiac lesions (i.e. acute cardiac degeneration or myocardial necrosis) were more severe in the D0 group versus the other groups (D0, 1 ± 1; D4, 0 ± 0; D10, 0 ± 0; $p = 0.035$). However, in the same subgroup analysis, there was a trend for worse HDS in kidneys (tubular degeneration or necrosis) in both D4 and D10 groups (D0, 1 ± 1; D4, 2 ± 1; D10, 2 ± 1; $p = 0.17$).

Discussion

In this study, this is the first time that we tested a new pharmacological strategy in our EPR model in rats, in

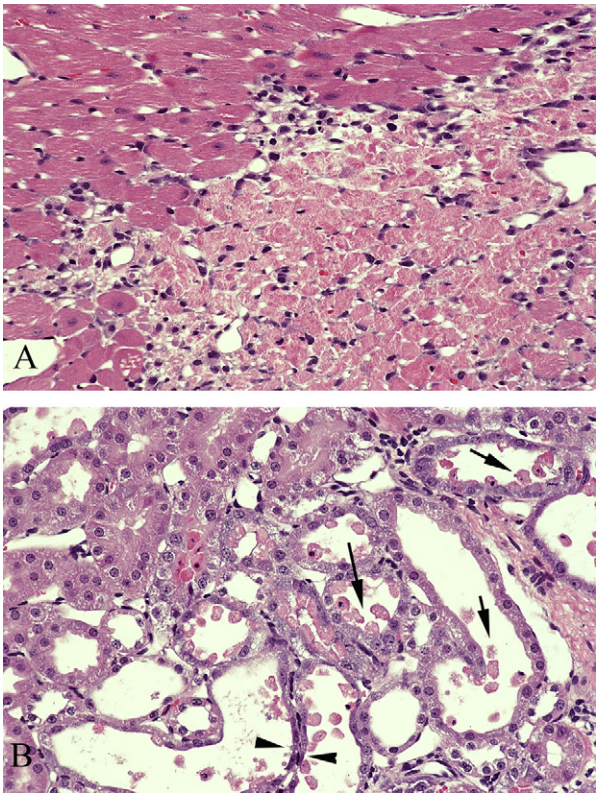

Figure 7 (A) H&E-stained section of the left ventricular wall of the heart of a D4 group rat. The cardiac myocytes in the upper portion of the micrograph are normal in appearance, but a relatively well-circumscribed focus of acute myocardial necrosis occupies the lower portion. The necrotic myocytes have pale granular cytoplasm and are often devoid of nuclei. (B) H&E-stained section of the inner cortical zone of a kidney from a D10 group rat. Many tubules contain necrotic tubular epithelial cells (arrows) characterized by pyknotic or absent nuclei. Some of these necrotic epithelial cells are still attached to the tubular basement membranes, whereas others have exfoliated into the tubular lumena, resulting in a denuded appearance (arrowhead).

which CA durations of up to 60 min were associated with intact outcome, while further extension of CA to 75 min was associated with renal injury, multi-organ failure, high mortality rate and unfavorable neurological outcome in survivors.[19] In our previous studies using a well-established dog EPR model, we have scanned 14 promising pharmacological adjuncts to hypothermic preservation in the EPR paradigm.[5] The only drug that showed benefit was tempol, a blood—brain barrier (BBB) penetrating nitroxide antioxidant.[22] In a similar model, oxygen and 2.5% glucose added as energy substrates during cooling improved neurological outcome.[23]

We considered DADLE as a promising candidate to be tested in our rat EPR model. Despite reports of protection in models of ischemic injury and organ damage, DADLE added during flush and reperfusion in this paradigm yielded no significant benefits to the protective effects of hypothermia in our EPR paradigm.

The delta opioid agonist DADLE, a 44-kDa protein, has been implicated as a novel hibernation-inducing trigger (HIT)[24] that also possesses organ-protective capabilities.[6] The underlying mechanisms of action of DADLE are probably complex. Primarily, DADLE stimulates both delta-1 and delta-2 receptors, and opens potassium-linked ATP (K_{ATP}) channels. Delta receptors activate multiple signaling pathways mediated by various protein kinases, most importantly protein kinase C,[25,26] extracellular signal-regulated kinase (ERK)[27,28] and p38 MAP kinase.[29] Those effects are similar to those elicited by ischemic or anesthetic preconditioning.[8,9,26,30] DADLE also has antioxidant properties.[17,31]

In an isolated rat heart model, DADLE conferred a similar level of protection as that of classic ischemic preconditioning.[8] However, differences between species may exist. In a swine model of cardiac ischemia, DADLE (1 mg/kg i.v.) did not confer cardioprotection and increased the incidence of arrhythmia during ischemia, while other delta opioid agonists showed protective effects.[32] The coadministration of a kappa-antagonist and DADLE was cardioprotective, and arrhythmogenic effects were completely abolished. Studies in an isolated working swine heart model also reported no protection from DADLE.[33]

DADLE added to Euro-Collins solution enhanced hypothermic lung preservation in rats in a living-rat lung transplant model.[13] Similarly, pre-treatment with DADLE protected against ischemia-reperfusion injury in hepatocytes, but not in the sinusoidal endothelial cells of the liver in rats. An anti-oxidative effect was suggested to mediate this effect.[7] In a multi-organ block preparation for long-term organ preservation (heart and lungs, liver, pancreas, kidneys, and a small portion of intestine *en bloc*),[34] DADLE (1 mg/kg q 2 h) extended survival time.[14]

Neuroprotective properties of DADLE have been tested in several models. Pre-treatment with DADLE (2 mg/kg and 4 mg/kg) dose-dependently blocked methamphetamine-induced long-term dopamine transporter loss.[17] In vitro, pre-treatment with DADLE (0.0025 g/ml, 0.005 g/ml and 0.01 g/ml) dose-dependently enhanced cell viability of cultured primary rat fetal mesencephalic cells. In addition, DADLE administration (4 mg/kg i.p. four doses 2 h apart) in adult rats prior to 6-hydroxydopamine lesions of the medial forebrain bundle significantly reduced the severity of neuronal loss.[35] In contrast, DADLE (0—16 mg/kg 30 min before the insult) in a rat model of bilateral carotid occlusion combined with hypotension showed no benefit of any dose on neurologic recovery or hippocampal neuron loss.[36] This could be partially explained by the reportedly poor BBB penetration of DADLE.[37]

Moreover, recent studies showed that another delta opioid agonist pentazocine, acting via delta receptors and K_{ATP} channels, improved outcome and post-resuscitation myocardial performance in a rat CA model.[38,39]

We have observed no, or possibly only a very limited, effect of DADLE on the outcome variables, including post-resuscitation hemodynamic and biochemical data. Previously, DADLE (1 mg/kg) was reported to produce a 50% reduction in the arterial blood pressure in dogs, most likely due to vasodilatation. We did not observe any hemodynamic effects of DADLE in our study. Lactate levels were the lowest in the D10 group at all time points, while the highest levels were observed in the D0 group. However, statistically this was only a trend.

Histologically, the principle treatment-related changes in visceral organs were degeneration and/or necrosis of the cardiac muscle fibers and, similarly, degeneration/necrosis of tubular epithelial cells within the kidney. The renal lesions could represent the results of ischemia (*e.g.* secondary to the cardiac lesions) or could be related to CPB. However, for those rats in which degenerative lesions were present in both the heart and kidneys, the cardiac lesions were considered to be of slightly greater duration. While some rats with renal degeneration did not have evidence of a concurrent cardiac injury, the cardiac lesions were often small in size. Furthermore, since only two sections (one transverse and one longitudinal) were prepared from each heart, some degenerative foci could have been missed.

There are limitations to this study. We did not determine drugs levels in blood or target tissues. The BBB penetration of DADLE was shown to be limited.[37] While the integrity of BBB in our study was not assessed, BBB has been shown to be dysfunctional after normothermic CPB and/or DHCA in infant lambs,[40] suggesting that BBB might be permeable for DADLE in our paradigm. Nevertheless, injury was seen with and without treatment both in and outside of the CNS.

The dose regimen of DADLE used in our study was adopted from previous studies. We chose to use the intravenous route instead of more commonly used intraperitoneal route of administration, to obtain rapid onset of action. Also, we have repeated the same dose only in the immediate post-resuscitation period without extending the therapy further. Unfortunately, no detailed studies on the metabolism of DADLE exist. The only limited information is provided in a study of multi-organ block preservation, which showed rapid accumulation of DADLE in the liver and subsequently in the heart. Two hours later, DADLE was excreted in bile and in urine. No accumulation of DADLE was seen in lungs, where the protective effects were maximally pronounced.[14] Using those limited data, we assumed that DADLE is rapidly eliminated, at least under normothermic conditions.

Most of the studies focused on preconditioning and/or exploring different cell signaling pathways used pre-insult administration of DADLE over variable time periods, up to 48 h before the insult. Continuous infusion of DADLE was shown to induce hibernation in summer-active ground squirrels, in a similar time-dependent manner as a single injection of a known hibernation-induction trigger, plasma from hibernating woodchucks. Typically, it took 4—7 days for the first animals to start hibernating, with the maximal incidence of hibernation at 21 days, mirroring the effect of HIT.[24]

Thus, the timing of DADLE administration in our study might not be optimal in terms of providing sufficient time for the drug to induce protection. On the other hand, pretreatment in the CA models would lack clinical relevance. However, another study testing the delta receptor agonist pentazocine showed benefit even after a single injection of the drug during resuscitation.[38]

A longer duration of CA before starting the flush (representing warm ischemia) might be needed to model the trauma scenario more realistically. It has been shown previously in a clinical setting that cannulation of a femoral artery can be accomplished in less than 5 min.[41] However, the rate of ATP loss in rat hearts was shown to be six times higher compared to that in dogs,[42] possibly limiting the resuscitation potential of the rats not due to neurological injury after prolonged CA but due to a more rapid depletion of energy sources, and ensuing cardiovascular failure than would be anticipated in humans.

In our study, a number of rats died before their scheduled necropsy time points. The lack of prolonged intensive care could play a role here. The cause of death in many rats remained unclear. While some of the brains fixed postmortem were relatively well preserved and showed little evidence of postmortem alteration, other brains were characterized by moderate degrees of postmortem autolysis. This made scoring these brains for neuronal degeneration difficult. Therefore, for some of these brains, the degrees of neuronal degeneration might be higher than actually indicated in the HDS. The model could be producing more extracerebral damage that could mask a neurological effect. Previous EPR studies in large animal models have shown that adding trauma resulted in more frequent and prolonged requirement of intensive care, and worse short term neurological outcome despite normal brain histopathology.[43] In a swine model of vascular, splenic and colon injuries, induction of profound hypothermia preserved the viability of key organs during repair of lethal injuries. After transient neurological decline, long-term neurological outcome was similar despite escalating complexity of injuries and without any considerable increase in post-operative complication rates.[44] The addition of extracerebral trauma to our model could aid in assessing the potential for clinical translation of new therapies.

We did not assess biochemical markers of organ injury that could serve as additional outcome variables to assess organ damage further in our model studying the effects of DADLE. This would have required maintaining intravascular access with tunnelled catheters to allow serial sampling. In order not to influence our primary outcome parameter — i.e. neurological outcome — we chose to perform histological evaluation to assess organ damage. In a separate series of experiments using preventive administration of DADLE (0 mg/kg, 4 mg/kg or 10 mg/kg i.v., $n = 6$ per group) 30 min before deep hypothermic circulatory arrest (90 min at 15 °C), ALT, AST, creatinine, CPK and troponin were increased 2 h after CPB in all groups versus their respective baselines ($p < 0.05$ for all). While no differences in ALT, AST and CPK between groups existed, creatinine and troponin were significantly more increased in the DADLE 10 mg/kg group versus other groups ($p = 0.011$ and 0.039, respectively). It is possible that the higher dose of DADLE exhibits some degree of toxicity.

Using deeper levels of hypothermia during preservation period[45] and/or addition of energy substrates[23] acting via other pathways than opioid receptors could further enhance the protection during CA, and improve outcome.

Conclusions

In our model of prolonged CA treated by EPR, DADLE failed to confer benefit on either functional or histological outcome. Future studies are needed to evaluate other potential adjuncts to hypothermia in the EPR paradigm.

Conflict of interest

Drs. Kochanek and Tisherman and Mr. S. Stezoski are co-inventors and have a U.S. Provisional Patent on the emergency preservation and resuscitation method.

Other authors have no conflict of interest to disclose.

Acknowledgements

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Deep Hypothermia Attenuates Microglial Proliferation Independent of Neuronal Death After Prolonged Cardiac Arrest in Rats

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INTRODUCTION: Conventional resuscitation of exsanguination cardiac arrest (CA) victims is generally unsuccessful. Emergency preservation and resuscitation is a novel approach that uses an aortic flush to induce deep hypothermia during CA, followed by delayed resuscitation with cardiopulmonary bypass. Minocycline has been shown to be neuroprotective across a number of brain injury models via attenuating microglial activation. We hypothesized that deep hypothermia and minocycline would attenuate neuronal death and microglial activation and improve outcome after exsanguination CA in rats.

METHODS: Using isoflurane anesthesia, rats were subjected to a lethal hemorrhagic shock. After 5 min of no flow, hypothermia was induced with an aortic flush. Three groups were studied: ice-cold (IC) flush, room-temperature (RT) flush, and RT flush followed by minocycline treatment (RT-M). After 20 min of CA, resuscitation was achieved via cardiopulmonary bypass. Survival, Overall Performance Category (1 = normal, 5 = death), Neurologic Deficit Score (0%–10% = normal, 100% = max deficit), neuronal death (Fluoro-Jade C), and microglial proliferation (Iba1 immunostaining) in hippocampus were assessed at 72 h.

RESULTS: Rats in the IC group had lower tympanic temperature during CA versus other groups (IC, 20.9°C ± 1.3°C; RT, 28.4°C ± 0.6°C; RT-M, 28.3°C ± 0.7°C; $P < 0.001$). Although survival was similar in all groups (RT, 6/9; IC, 6/7; RT-M, 6/11), neurological outcome was better in the IC group versus other groups (Overall Performance Category: IC, 1 ± 1; RT, 3 ± 1; RT-M, 2 ± 1; $P < 0.05$; Neurologic Deficit Score: IC, 8% ± 9%; RT, 55% ± 19%; RT-M, 27% ± 16%; $P < 0.05$). Histological damage assessed in survivors showed selective neuronal death in CA1 and dentate gyrus, similar in all groups ($P = 0.15$). In contrast, microglial proliferation was attenuated in the IC group versus all other groups ($P < 0.01$).

CONCLUSIONS: Deeper levels of hypothermia induced by the IC versus RT flush resulted in better neurological outcome in survivors. Surprisingly, deep hypothermia attenuated microglial activation but not hippocampal neuronal death. Minocycline had modest benefit on neurologic outcome in survivors but did not attenuate microglial activation in brain. Our findings suggest a novel effect of deep hypothermia on microglial proliferation during exsanguination CA.

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Currently, the outcomes from traumatic exsanguination cardiac arrest (CA) show that more than 50% of deaths caused by trauma occur at the scene[1–3] where medical care is limited. Less than 10% of the patients who become pulseless from trauma survive.[2] However, in an appropriate setting, some of those traumatic injuries could be surgically repairable.[4]

Emergency preservation and resuscitation (EPR) is a novel approach for resuscitation of exsanguination CA victims.[5] EPR uses cold aortic flush to induce deep hypothermic preservation for prolonged CA to buy time for transport, damage control surgery, and delayed resuscitation with cardiopulmonary bypass (CPB). In prior studies of EPR, we used a dog model to maximize clinical relevance. Because of the lack of molecular tools available for use in dogs, we recently developed a rat EPR model to study the cellular and molecular mechanisms underlying deep hypothermic neuroprotection. Understanding cellular and molecular mechanisms of secondary damage in ischemia-reperfusion injury after CA and the impact of deep

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hypothermia on these cascades would allow us to define specific targets for future interventions, assess markers of reversibility, and screen novel therapies.

Although the early brain injury in CA is initiated by energy failure and resultant neuronal death cascades, microglial activation has been suggested to be an additional mechanism of delayed neuronal death, most likely through releasing neurotoxic substances.[6] Pharmacological modulation of microglial proliferation may help to improve outcome after CA. Recently, studies in several central nervous system insults have shown benefit from treatment with minocyline, a drug that attenuated microglial activation and proliferation.[7]

We hypothesized that deeper levels of intraarrest hypothermia would improve functional outcome, attenuate neuronal death, and attenuate microglial proliferation compared with more moderate hypothermia. We also hypothesized that minocycline would further augment the hypothermic protection via attenuating microglial activation, neuronal death, and improve outcome.

METHODS

We used the rat EPR model described in detail previously.[8] All rats received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" (www.nap.edu/catalog/5140.html). The study protocol has been approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Adult male Sprague-Dawley rats (350–375 g) were obtained from Hilltop Lab Animals (Scottdale, PA) and housed for at least 3 days before the experiment under 12-h light/dark cycle with unrestricted access to food and water. On the day of the experiment, rats were anesthetized with 4% isoflurane in a transparent acrylic jar. After tracheal intubation with a 14-gauge IV catheter (Becton Dickinson, Sandy, UT), rats were mechanically ventilated using a piston ventilator (Harvard Ventilator Model 683; Harvard Rodent Apparatus, South Natick, MA) with a tidal volume of 0.8 mL/100 g and a frequency of 20–24/min to maintain normocapnia, and a positive end-expiratory pressure of 4 cm H_2O. Anesthesia was maintained with 1.5%–2% isoflurane in F_{IO_2} 0.5. After shaving and prepping with povidone iodine, bilateral femoral and right jugular cutdowns were performed. The left femoral artery and vein were cannulated for arterial blood pressure monitoring and blood sampling. Electrocardiogram, respiration, and arterial and central venous pressure were continuously monitored and recorded (Polygraph; Grass Instruments, Quincy, MA). The right femoral artery was cannulated with a 20-gauge catheter (Becton Dickinson, Sandy, UT) that served as an arterial CPB cannula. The right jugular vein was cannulated with a modified 5-hole 14-gauge IV cannula advanced to the right atrium to be used for venous drainage during the hemorrhage phase and later as a venous CPB cannula. Rectal and tympanic probes were used to monitor the temperature. Baseline blood samples were obtained, and hemodynamic values were recorded. Removed blood volume was replaced with an electrolyte-balanced crystalloid Plasma-Lyte A (Baxter, Deerfield, IL) in a ratio 1:3. Heparin sodium was administered to achieve activated clotting time >400 s (Hemochron Jr. Signature, ITC, Edison, NJ).

Three groups were studied: 1) ice-cold (IC) flush group ($n = 7$), 2) room-temperature (RT) flush group ($n = 9$), and 3) room-temperature flush group followed by minocycline treatment, 20 mg/kg 1 h after resuscitation and 90 mg/kg IP at 24 and 48 h (RT-M, $n = 11$). Rats in the RT and IC groups received the same volume of vehicle (phosphate buffered saline [PBS]).

After instrumentation, intubated rats were weaned to spontaneous ventilation of isoflurane 2% at F_{IO_2} 0.25 via a nose cone mask. After 5-min equilibration period, rapid exsanguination (12.5 mL of blood over 5 min) was performed via the internal jugular catheter. The shed blood was collected. After the rapid exsanguination phase, CA was ensured with IV administration of 9 mg of esmolol (0.9 mL) and 0.2 mEq of potassium chloride (0.1 mL). After 5 min of CA, 270 mL of either an RT or an IC flush solution (Plasma-Lyte A) was instilled via the right femoral artery catheter at 50 mL/min. The flush was drained from the jugular vein catheter.

After 20 min of CA, resuscitation was started with CPB. Heating and cooling were achieved with a circulating water bath around the oxygenator and a forced-air blower blowing air over the rat covered by a transparent semi-closed lid. Blood samples for biochemistry and hematology were obtained at 5, 15, 30, 45, and 60 min CPB time and processed immediately using a point-of-care blood analyzer (Stat Profile, Nova Biomedical, Waltham, MA).

Arterial blood gas management followed α-stat principles. pH and electrolyte values outside of the normal range were corrected during CPB and intensive care unit phases by adjustments in ventilation and/or administration of sodium bicarbonate, calcium chloride, and potassium chloride. Additional blood obtained from an isoflurane-anesthetized donor rat was used to maintain hematocrit >25%. CPB support was gradually weaned after 60 min. Mechanical ventilation with a F_{IO_2} of 1.0 was continued while maintaining normocapnia for additional 2 h.

Using a midline laparotomy incision, a Mini-mitter probe (Mini-Mitter, Sunriver, OR) was introduced into the peritoneal cavity to allow postoperative temperature control and continuous monitoring of heart rate and movement. Surviving rats were tracheally extubated 2 h later after removal of catheters and placed separately in a temperature-controlled cage (34.5°C for 4 h) with supplemental oxygen for 18 h and free access to food and water. Weight and neurologic status were assessed daily, using Overall Performance Category (OPC; 1 = normal, 2 = mild disability, 3 = moderate

disability, 4 = severe disability, and 5 = death or brain death) and a modified Neurologic Deficit Score[9] (NDS; 0%–10% = normal, 100% = maximum deficit). Postoperatively, the rats that did not resume normal eating and drinking habits received supplemental subcutaneous injections of 0.45NS/D5W (10 mL twice daily).

At 72 h after resuscitation, blood samples were obtained, and the rats were euthanized with an isoflurane overdose and perfused via the left ventricle with normal saline followed by 10% neutral buffered formalin.

Histology

The tissue samples were processed for embedding in paraffin. The resulting paraffin blocks were sequentially sectioned at 5 μm. All sections were stained with Fluoro-Jade C (F-JC).[10] For the Iba-1 staining, sections were washed in PBS, incubated in 0.3% H_2O_2 in methanol for 30 min to inhibit endogenous peroxidase activity, washed in PBS, and blocked in PBS containing 1.5% normal goat serum and 1% bovine serum albumin for 2 h at RT. The sections were then incubated with a rabbit anti-Iba1 polyclonal antibody (1:500, Serotec) overnight at 4°C, washed in PBS, and incubated with a fluorescein isothiocyanate-conjugated goat antirabbit IgG antibody (Invitrogen) for 2 h at RT. For control staining, normal rabbit IgG was used as the primary antibody. After the reaction, the sections were counterstained with 4′,6-diamidino-2-phenylindole, dehydrated in ethanol steps, and mounted.

Adjacent sections obtained at approximately 4.3 mm from bregma were used for assessing neuronal death and microglial activation within the selective brain regions. A photograph of representative sections of dentate gyrus and CA1 region was taken under 10× magnification. F-JC positive neurons and Iba-1-positive activated microglia (characterized by ameboid cell body and retracted processes without thin ramifications)[11] were then counted using National Institutes of Health Image-J software by an observer masked to the treatment group.

Statistical Analysis

Repeated measures analysis of variance was performed, followed by Student–Newman–Keuls *post hoc* tests, to identify differences in hemodynamic and arterial blood gas parameters and temperature among groups. One-way analysis of variance was used to compare histologic damage among groups. The χ^2 test was used to test the differences in proportions of OPC among groups. Kruskal–Wallis H test was used to compare NDS among groups. Mann–Whitney *U*-test was used to compare two groups if Kruskal–Wallis H test indicated there were differences between groups. Pearson and Spearman correlations between variables were determined as appropriate. A P value <0.05 was considered statistically significant.

RESULTS

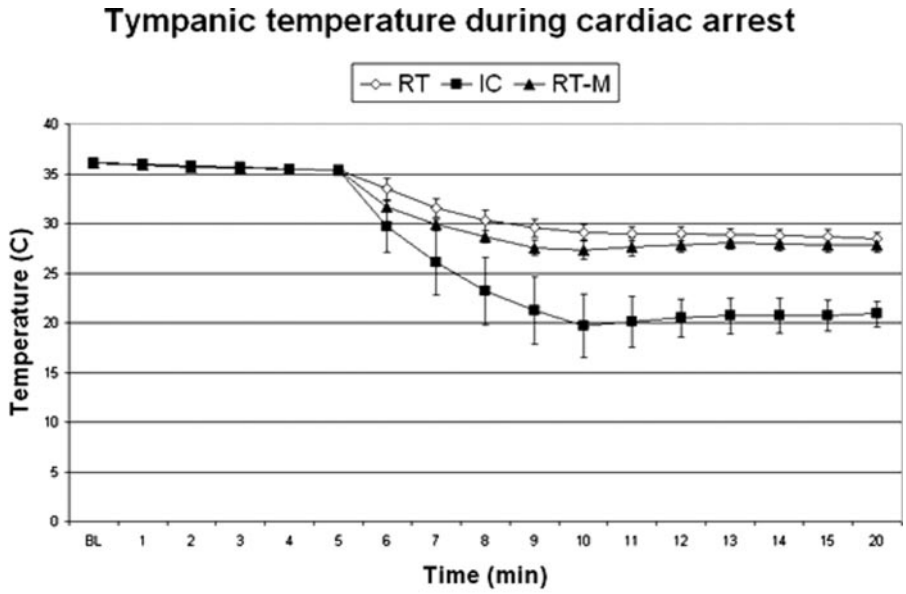
Baseline body weight before the experiment was similar in all groups (RT, 387 ± 3 g; IC, 385 ± 1 g, RT-M, 386 ± 10 g; $P > 0.05$). The surgical time did not differ among groups (RT, 98 ± 7 min; IC, 99 ± 19 min; RT-M 99 ± 12 min, $P > 0.05$).

After cooling, rats in the IC group had significantly lower temperature during CA versus other groups (tympanic, 21°C vs 28°C; rectal, 20°C-25°C vs 27°C-30°C, $P < 0.001$) (Fig. 1). Rats in the IC group had higher mean arterial blood pressure during flush (35 ± 5 vs 27 ± 6 mm Hg, $P < 0.05$). Retained volume, i.e., the amount of flush that was not drained from the jugular catheter, was also lower in the IC group versus RT and RT-M groups combined (29 ± 7 vs 42 ± 4 mL, $P < 0.001$). Heart rate increased more slowly during resuscitation in the IC versus RT group ($P < 0.01$) (Fig. 2, Panel A). Mean arterial pressure was higher in the IC group versus RT group over time ($P < 0.05$; Fig. 2, Panel B). After discontinuation of temperature control, body core temperature increased more rapidly in the IC group. This steady increase reached statistical significance versus the RT group but not the RT-M group ($P < 0.05$ IC versus RT group, Fig. 3). However, all groups achieved normothermia at 24 h after resuscitation. While pH and base excess were similar at 5 min after the start of resuscitation, lactate was lower in the IC group versus other groups ($P < 0.05$) (Table 1). The survival rate was not different among groups. Neurological outcome was significantly better in the IC versus other groups ($P < 0.05$) (Table 2, Fig. 4). The markers of microglial activation but not neuronal death were attenuated in the IC versus other groups (Figs. 5–7). Selective vulnerability of CA1 neurons and hilar neurons in the dentate gyrus was observed (Figs. 6 and 7), with proliferated microglial cells with ameboid-shaped cell bodies and shortened, retracted processes. Microglial activation was attenuated in the IC group (Figs. 6 and 7, Panel E). There was a positive correlation between OPC and neuronal death ($r = 0.566$, $P = 0.018$). Similarly, there was a correlation between F-JC and Iba-1 ($r = 0.513$, $P = 0.035$). In contrast, there was only a trend for a correlation between Iba-1 and neurologic outcome (Iba-1 versus OPC, $r = 0.345$, $P = 0.176$; Iba-1 versus NDS, $r = 0.393$, $P = 0.119$).

We performed a formal necropsy on all rats that died before completion of the study. However, we were not able to determine the cause of death in all rats. The common findings were pulmonary edema and/or pulmonary hemorrhage. The time of death was between 15 and 45 h of resuscitation in all the groups (RT, 29.5 ± 14.1 h; IC, 13.1 h; RT-M, 24.0 ± 7.5 h).

DISCUSSION

Exsanguination CA is a relatively unexplored form of CA. Resuscitation of exsanguination CA victims with conventional cardiopulmonary resuscitation technique has a poor prognosis because of a volume-depleted and



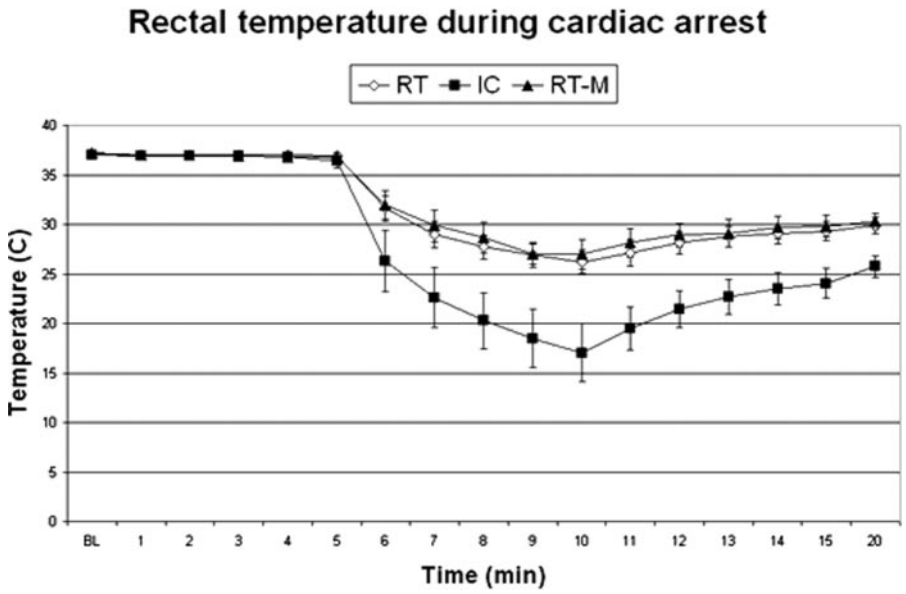


Figure 1. Tympanic and rectal temperatures during cardiac arrest (CA). $P < 0.001$ ice-cold (IC) flush group versus other groups.

trauma-disrupted circulatory system. In the civilian setting, 50% of deaths caused by trauma occur at the scene and another 30% within hours from injury.[12] More aggressive treatments with thoracotomy and aortic cross-clamping have also not improved the poor outcome in these patients.[2] The surgeon cannot obtain hemostasis and resuscitation before vital organs (particularly brain and heart) have suffered irreversible ischemic damage. However, in an appropriate setting, many of those injuries would be technically repairable.

Traditionally, deep hypothermic circulatory arrest (DHCA) has been used in cardiac surgery to provide a bloodless field and enable repair of the congenital cardiac malformations or acquired pathologies with considerable success. Hypothermia for deep hypothermic circulatory arrest (DHCA) is used in a protective rather than a therapeutic fashion; the use of hypothermia in EPR is a much more challenging situation. In resuscitation of exsanguination CA, including EPR, the rapid onset of cooling can only be initiated after a period of normothermic CA. Adjuncts to hypothermia would thus be of great potential benefit. Recently, we modified the rat EPR model to produce a screening tool to study mechanisms of neuronal death and evaluate novel therapeutic adjuncts to hypothermia. Also it should be recognized that the use of a normothermic control group is not feasible because the rats would not survive the insult if maintained normothermic throughout the period of emergency preservation.

In our paradigm, we use 5 min of hemorrhagic shock followed by ice-cold or room-temperature flush initiated 5 min after CA. This is a clinically relevant delay that would allow cannulation of a large vessel.[13] Flushing with either IC or RT saline resulted in a brain temperature of 21°C or 28°C, respectively. Better protection achieved after IC flush was likely reflected by

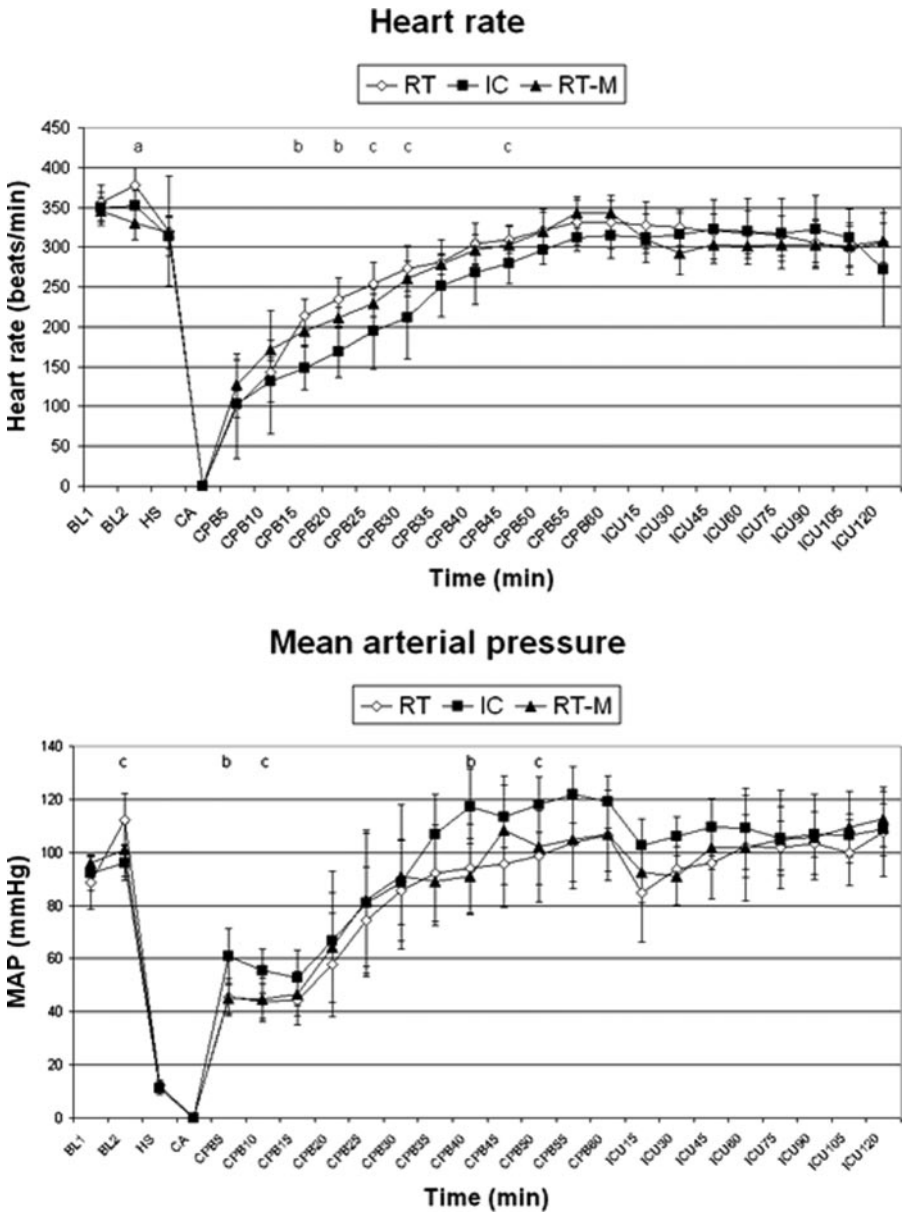


Figure 2. Heart rate (top panel) and mean arterial blood pressure (bottom panel). a = $P < 0.05$ room-temperature (RT) flush versus room-temperature flush followed by minocycline (RT-M) group; b = $P < 0.05$ ice-cold (IC) versus RT and RT-M groups; c = $P < 0.05$ RT versus IC group. BL = baseline; HS = end of hemorrhagic shock; CA = cardiac arrest; CPB = cardiopulmonary bypass; ICU = intensive care.

lower lactate levels at 5 min after reperfusion and better neurologic function in survivors at 72 h. The latter was not affected by minocycline treatment. Despite the functional benefit with IC flush, neuronal death seen in traditionally selectively vulnerable brain regions did not differ among groups. We also noted robust microglial activation surrounding the dying neurons. Surprisingly, deep hypothermia (21°C) was able to attenuate microglial activation but not neuronal damage. It is possible that hypothermia-induced attenuation of microglial activation contributed to the improved neurologic outcome in the IC group.

After discontinuation of the postoperative hypothermia, rats in the IC group spontaneously rewarmed more quickly than rats in other groups. The attenuation of microglial activation in the IC group thus could not be explained by unintentional prolonged postoperative hypothermia.

While the early brain injury in CA is believed to result from release of excitatory mediators, energy failure, oxidative stress, damage to mitochondria and endoplasmic reticulum, and cell signaling pathway disturbances in neurons, secondary damage could also be triggered by microglia, that transform into phagocytes. Microglial activation starts immediately after ischemia and thus precedes the morphologically detectable neuronal damage.

Microglial activation has been suggested to contribute to delayed neuronal death, most likely through releasing neurotoxic substances, including reactive oxygen radicals, nitric oxide, and proinflammatory cytokines.[6] Microglial activation could contribute to neuronal death or microglial-mediated synaptic injury and/or neuronal dysfunction, which could mediate cognitive deficits even in the absence of overt neuronal death. Additional studies focused on these secondary injury mechanisms in our model are warranted. Microglia could also have a protective role,[14–18] possibly in delayed repair after injury via elaboration of growth factors. Thus, there may be a specific time window for benefit from inhibition of

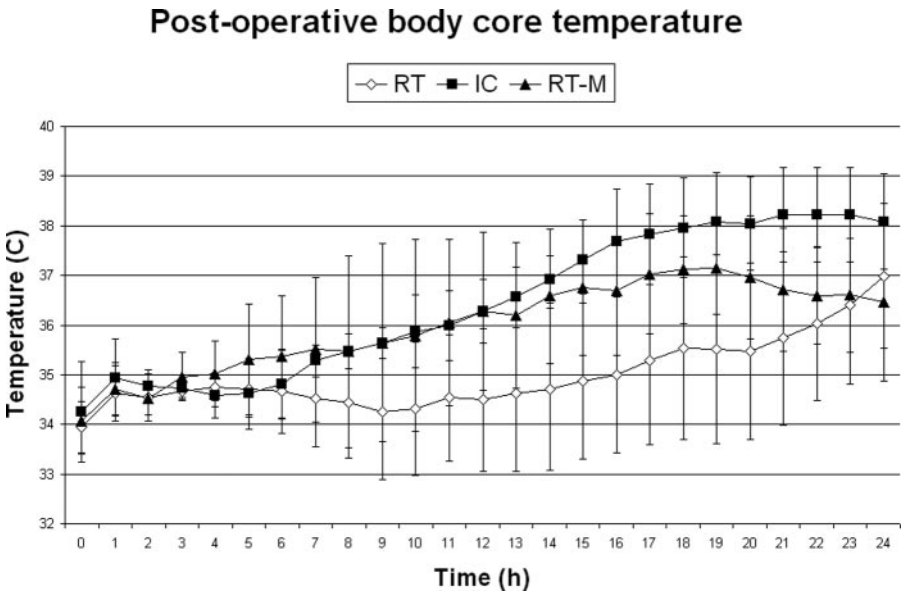


Figure 3. Postoperative body core temperature within the first 24 h. $P < 0.05$ room-temperature (RT) versus ice-cold (IC) group.

Table 1. Biochemical and Hematological Values After 20 min Cardiac Arrest Treated by Emergency Preservation and Resuscitation

	BL1	BL2	CPB5	CPB60	ICU120	FINAL
pHa						
RT	7.37 ± 0.04	7.45 ± 0.03*	6.99 ± 0.05	7.41 ± 0.05	7.40 ± 0.04	7.46 ± 0.06
IC	7.40 ± 0.04	7.40 ± 0.05	7.00 ± 0.08	7.36 ± 0.12	7.45 ± 0.04	7.49 ± 0.06
RT-M	7.39 ± 0.04	7.36 ± 0.06	6.95 ± 0.07	7.42 ± 0.03	7.40 ± 0.04	7.48 ± 0.03
Pao_2						
RT	246 ± 29	119 ± 17†	454 ± 32	386 ± 31	273 ± 97	497 ± 96
IC	328 ± 111	271 ± 52	536 ± 49†	382 ± 51	351 ± 126	379 ± 138
RT-M	262 ± 83	244 ± 85	474 ± 33	374 ± 33	349 ± 154	529 ± 51
$Paco_2$						
RT	47 ± 6	30 ± 4	34 ± 3	42 ± 7	48 ± 7	33 ± 6
IC	42 ± 6	37 ± 6	37 ± 9	40 ± 9	44 ± 1	33 ± 8
RT-M	44 ± 3	43 ± 6†	38 ± 4	44 ± 7	48 ± 7	27 ± 3
BE						
RT	1 ± 2	−1.9 ± 2.4	−21 ± 1.4	2.7 ± 3.2	4.9 ± 2.7	0.6 ± 3.0
IC	1.0 ± 2.8	−0.7 ± 2.1	−20.7 ± 2.0	0.9 ± 2.4	6.9 ± 2.2	−0.6 ± 1.6
RT-M	1.1 ± 2.2	−0.6 ± 1.8	−21.8 ± 1.5	2.2 ± 2.6	6.3 ± 3.3	−1.1 ± 3.0
Lactate						
RT	1.1 ± 0.9	1.6 ± 0.6	7.0 ± 0.9	6.7 ± 1.2	2.5 ± 0.7	2.5 ± 1.4
IC	1.1 ± 0.6	1.7 ± 0.8	5.3 ± 1.0†	5.7 ± 2.0	4.2 ± 2.6	3.6 ± 1.4
RT-M	1.2 ± 1.1	1.8 ± 1.0	6.9 ± 0.8	6.5 ± 1.6	3.1 ± 2.1	4.3 ± 1.5
Hct						
RT	38 ± 3	32 ± 3	25 ± 2	29 ± 3	30 ± 3	28 ± 3
IC	37 ± 3	34 ± 2	26 ± 4	29 ± 3	32 ± 4	28 ± 5
RT-M	37 ± 3	34 ± 1	26 ± 1	30 ± 2	32 ± 2	26 ± 3
Glucose						
RT	227 ± 51	224 ± 62	210 ± 20	225 ± 52	152 ± 29	149 ± 33
IC	208 ± 42	232 ± 49	213 ± 27	255 ± 75	177 ± 51	207 ± 38
RT-M	228 ± 18	241 ± 54	223 ± 35	248 ± 84	175 ± 64	132 ± 33

IC = ice-cold flush group; RT = room-temperature flush group; RT-M = RT flush followed by minocycline treatment group; BL = baseline; CPB = cardiopulmonary bypass; CPB5 = 5 min after start of CPB; CPB60 = at the end of CPB; ICU = intensive care unit; ICU120 = 2 h after CPB; FINAL = at 72 h; pHa = arterial pH; BE = base excess; Hct = hematocrit.

* $P < 0.05$ RT versus RT-M group.

† $P < 0.05$ IC versus RT and RT-M groups.

the early microglial contribution to damage. Recent studies also suggested that the severity of neuronal injury determines microglial release of "toxic" versus "protective" effectors.[15] To visualize microglia, we chose to use anti-Iba-1 staining. Iba-1 is a calcium-binding protein expressed specifically in activated microglia,[19] with its peak occurring at 4–7 days after injury.[20] While resident microglia exist in a ramified state, after brain injury they migrate toward the lesion, their cell body becomes ameboid-shaped, the processes shorten, and become virtually indistinguishable from macrophages.

Minocycline is a widely used antibiotic with anti-inflammatory and antiapoptotic properties which has been tested in several models of neurologic injury, including global[21–23] and focal brain ischemia,[24–27] traumatic brain injury,[28,29] spinal cord injury,[30,31] and

intracerebral hemorrhage.[32] Most recently, minocycline showed favorable results in a clinical trial in acute stroke patients.[33] It penetrates the blood-brain barrier,[34] reduces tissue injury, and improves functional recovery.[21,35,36] The primary effect of minocycline is probably inhibition of activation of microglia.[21,22,24,30,37] Surprisingly, minocycline was also reported to be more protective than brief hypothermia after focal cerebral ischemia.[26,27] Specifically, inhibition of p38 mitogen-activated protein kinase activation in microglia has been suggested as a key mechanism underlying minocycline antiinflammatory effects, although other mechanisms may also be involved.

In preliminary studies, we did not observe a beneficial effect with a lower dose of minocycline (3 mg/kg IV followed by 45 mg/kg IP; data not shown). Thus, we chose to use the high-dose minocycline (20 mg/kg IV followed by 90 mg/kg IP), which was previously used by others in similar settings.

Table 2. Overall Performance Categories (OPC) After 20-min Cardiac Arrest Treated by Emergency Preservation and Resuscitation

	No. of rats[a]		
OPC	RT	IC	RT-M
5 = Death	•••	•	•••••
4 = Severe disability			
3 = Moderate disability	••••		••
2 = Mild disability	••	••	•••
1 = Normal		••••	•

[a] Each dot represents one rat.

No differences among groups in survival rate ($P > 0.05$). Favorable neurological outcome (assessed by OPC) was significantly better in the IC group versus other groups ($P < 0.05$). IC = ice-cold flush group; RT = room-temperature flush group; RT-M = RT flush followed by minocycline treatment group.

In our study, hypothermia attenuated microglial activation. Temperatures used in our study (21°C-28°C) were generally lower than those used in other studies of mild-to-moderate hypothermia. Postischemic hypothermia (32°C for 24 h) suppressed microglial activation after hypoxic-ischemic injury in the developing brain.[38] Even a brief period of hypothermia (33°C for 2 h) attenuated neuroinflammation after experimental stroke and brain inflammation induced by IV injection of lipopolysaccharide.[39] A similar effect of hypothermia was observed in microglial cell cultures stimulated by lipopolysaccharide.[40]

Although many studies used minocycline as a drug suppressing microglial activation, we did not see any effect of minocycline on microglia activation or neuronal death. This striking lack of effect could be potentially explained by the fact that minocycline was added to augment the protective effects of preexisting moderate hypothermia (28°C). It is possible that minocycline could not add further benefit to hypothermia. Moderate hypothermia in the 28°C group was limited to the intraischemic time, followed by mild hypothermia for 6 h. Previous studies suggested that the onset of microglial activation starts at 24 h and peaks at 4–7 days.[20] In our study, we administered minocycline up to 72 h. However, we cannot rule out that hypothermia delayed or modified the course of microglial activation, and therefore the dosing regimen or assessment time were not optimal.

The lack of effect of minocycline in our EPR paradigm is not entirely surprising. Previously, we tested 14 pharmacological adjuncts to hypothermia. Using our similar moderate hypothermia canine model with 20-min CA, only the antioxidant tempol showed some benefit.[41]

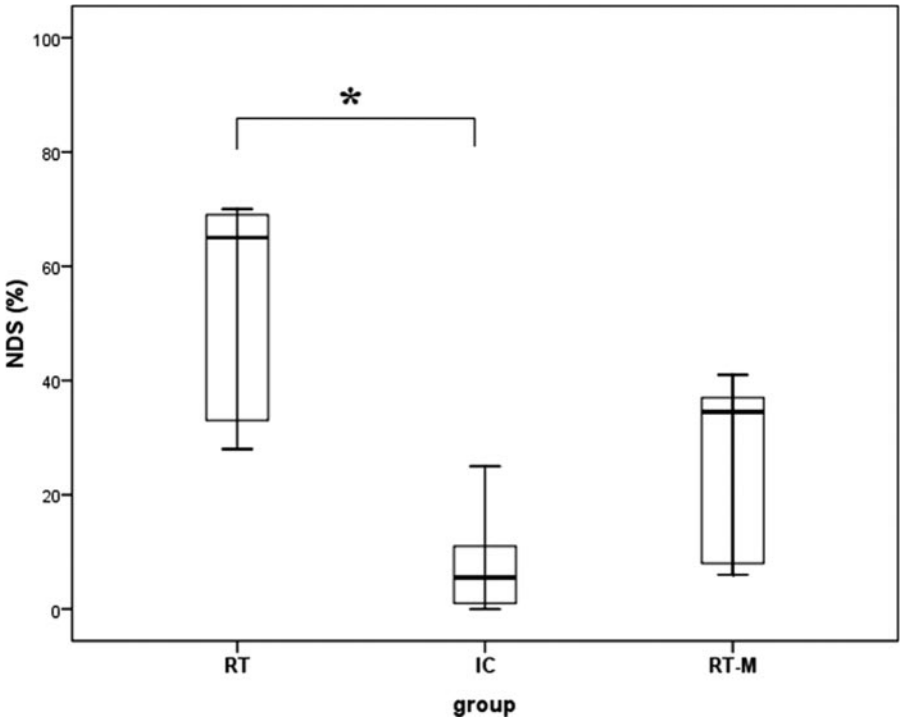


Figure 4. Neurologic Deficit Score after 20-min cardiac arrest treated by emergency preservation and resuscitation. Boxes represent interquartile ranges. The line across each box indicates the median, and the whiskers are the highest and lowest values. *P <0.05, room-temperature (RT) versus ice-cold (IC) group.

Recently, there has been increasing evidence suggesting the neuroprotective role of microglia in central nervous system pathologies. Selective ablation of microglial cells before cerebral ischemia *in vivo* revealed a marked neuroprotective potential of proliferating microglia, serving as an endogenous pool of neurotrophic molecules such as IGF-1.[16] Microglia cells were also shown to protect neurons by direct engulfment of invading neutrophil granulocytes that infiltrate ischemic lesions in an *in vitro* model.[18] Despite robust microglial proliferation, we have not, however, observed neutrophil accumulation in our model.

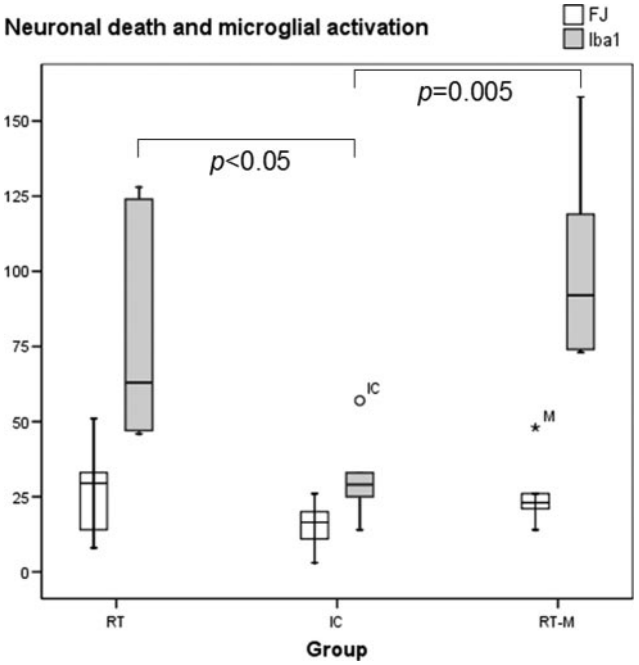


Figure 5. Neuronal death and microglial activation after 20-min cardiac arrest (CA) treated by emergency preservation and resuscitation (EPR) with either room-temperature (RT) flush, ice-cold (IC) flush, or room-temperature flush followed by minocycline treatment (RT-M) in the dentate gyrus region of hippocampus. The line across each box indicates the median, and the whiskers are the highest and lowest values. The round marker and the asterisk represent outliers of the respective groups.

In our study, NDS assessments were not very tightly coupled to hippocampal cell loss. Rats in the IC group that achieved favorable OPC and NDS scores still had substantial neuronal injury. Advanced neurobehavioral testing will be needed in a future study to define the association between hippocampal neuronal loss, microglial activation, and neurocognitive outcome. Our exploratory study was focused on the histological markers of injury. Previously we have shown that motor deficits observed in this complex model persist up to Day 7.[42] This would require delaying the period of water maze tests until after Day 7. The time of completion of water maze tasks would then occur outside of the peak microglial activity.

We have observed a significant correlation between neuronal death and neurological outcome. A larger number of animals would be necessary to appropriately test the hypothesis that microglial activation contributes to neurologic deficits. We cannot exclude that injuries in other brain regions or extracerebral injuries played a role and influenced the neurologic outcome.

In conclusion, deeper levels of hypothermia compared with moderate hypothermia (21°C vs 28°C) induced by aortic flush resulted in better neurologic outcome in survivors. Surprisingly, hypothermia attenuated microglial activation but not hippocampal neuronal death. Minocycline did not improve either neurologic outcome or attenuate microglial activation in brain. Our preliminary findings suggest a potentially novel effect of

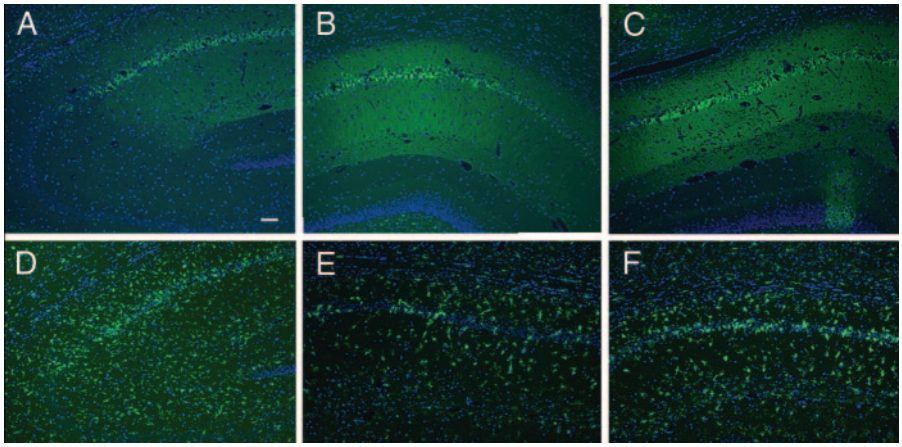


Figure 6. Neuronal death and microglial proliferation after exsanguination cardiac arrest and emergency preservation and resuscitation with either room-temperature (RT) or ice-cold (IC) flush in the CA1 region of hippocampus. Blue staining is 4′,6-diamidino-2-phenylindole, identifying neurons, and green staining is Fluoro-Jade C, Panels A–C, identifying dying neurons, or anti-Iba-1 staining visualizing microglia (Panels D–F). Microglial activation is attenuated in the IC group. Representative samples from each group are shown. A, 10×: Hippocampal neuronal loss in a rat from the RT group. Full CA1 loss. B, 10×: CA1 region in a rat from the IC group. Intensive neuropil staining between CA1 and dentate gyrus (DG). C, 10×: Hippocampal neuronal loss in a rat from RT-M group. D, 10×: Microglial activation in CA1-CA2 regions of hippocampus in a rat from the RT group. E, 10×: Microglial activation was attenuated in a rat from the IC group. F, 10×: Microglial activation was marked in a rat from RT-M group despite high dose minocycline treatment. Scale bar in Panel A = 80 μm.

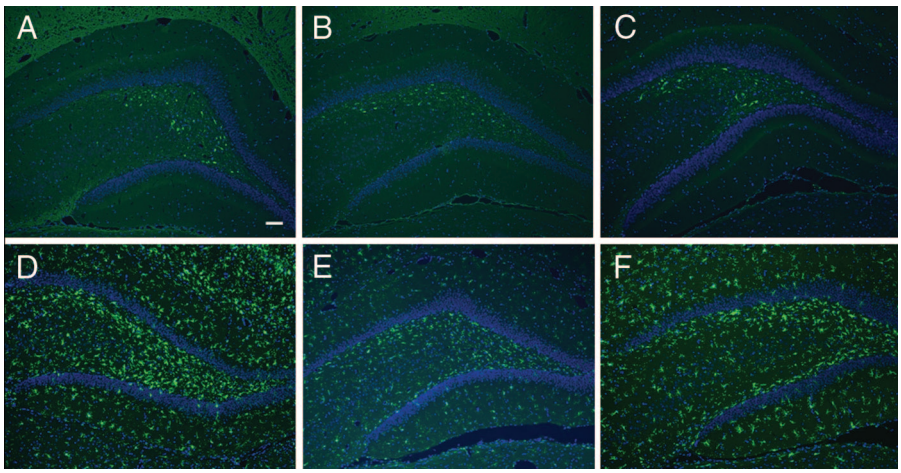


Figure 7. Neuronal death and microglial activation after exsanguination cardiac arrest and emergency preservation and resuscitation with either room-temperature (RT) or ice-cold (IC) flush in the dentate gyrus (DG) region of hippocampus. Blue staining is 4′,6-diamidino-2-phenylindole, identifying neurons, and green staining is Fluoro-Jade C, identifying dying neurons (Panels A–C), or anti-Iba-1 staining visualizing microglia (Panels D–F). Microglial activation (Panels D–F) is attenuated in the IC group (Panel E). Representative samples from each group are shown. Hippocampal neuronal loss in DG. Hilar neurons are selectively injured in all groups (A, 10×, RT group; B, 10×, IC group; C, 10×, RT-M group, respectively). D, 10×: Microglial activation in hippocampal DG in a rat from the RT group. E, 10×: Microglial activation was attenuated in a rat from the IC group. F, 10×: Microglial activation was marked in a rat from RT-M group despite high dose minocycline treatment. Scale bar in Panel A = 80 μm.

hypothermia on microglial activation during deep hypothermia. Further studies with comprehensive neurobehavioral testing will be needed to further elucidate the role of microglia on functional outcome.

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Experimental paper

Blood–brain barrier integrity in a rat model of emergency preservation and resuscitation☆

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ABSTRACT

Emergency Preservation and Resuscitation (EPR) represents a novel approach to treat exsanguination cardiac arrest (CA) victims, using an aortic flush to induce hypothermia during circulatory arrest, followed by delayed resuscitation with cardiopulmonary bypass (CPB). The status of the blood–brain barrier (BBB) integrity after prolonged hypothermic CA is unclear. The objective of this study was to assess BBB permeability in two EPR models in rats, associated with poor outcome. Rats subjected to traumatic brain injury (TBI) and naïve rats served as positive and negative controls, respectively.

Hypothesis: The BBB will be disrupted after TBI, but intact after prolonged hypothermic CA.

Methods: Four groups were studied: (1) EPR-IC (ice cold)-75 min CA at 15 °C; (2) EPR-RT (room temperature)-20 min CA at 28 °C; (3) TBI; (4) sham. Rats in EPR groups were subjected to rapid hemorrhage, followed by CA. Rats in the TBI group had a controlled cortical impact to the left hemisphere. Naïves were subjected to the same anesthesia and surgery. 1 h after insult, rats were injected with Evans Blue (EB), a marker of BBB permeability for albumin. Rats were sacrificed after 5 h and EB absorbance was quantified in brain samples.

Results: TBI produced an approximately 10-fold increase in EB absorbance in the left (injured) hemisphere vs. left hemisphere for all other groups ($p = 0.001$). In contrast, EB absorbance in either EPR group did not differ from sham.

Conclusion: BBB integrity to albumin is not disrupted early after resuscitation from prolonged CA treated with EPR. Neuroprotective adjuncts to hypothermia in this setting should focus on agents that penetrate the BBB. These findings also have implications for deep hypothermic circulatory arrest.

1. Introduction

Emergency preservation and resuscitation (EPR) is a novel concept created to resuscitate traumatic cardiac arrest (CA) victims, using a cold aortic flush to induce deep hypothermia during circulatory arrest, thus buying time for transport and damage control surgery, followed by delayed resuscitation with cardiopulmonary bypass (CPB).[1,2]

The most vulnerable organ during CA is the brain. Although hypothermia is a potent neuroprotective intervention and the key platform for EPR, current research is focused on the development of adjuncts to hypothermia, toward additional neuroprotection. It is unclear, however, whether blood brain barrier (BBB) penetration is a requisite for therapies added during the induction of hypothermic preservation in EPR. The BBB is a complex system with transport and metabolic functions. Barrier breakdown occurs with stroke, head trauma, sepsis, exposure to inflammatory mediators, among many other causes. Remarkably, the status of the BBB in both CA and deep hypothermia remains unclear.[3–7] In this study, we sought to investigate the BBB status in our EPR rat model, and we chose specifically two EPR paradigms that were associated with mortality and neurologic impairment in survivors.[8–10] One includes prolonged CA under deep hypothermia (15 °C) and one includes brief CA under only moderate hypothermia (28 °C). These paradigms thus represent scenarios where adjuncts to hypothermia are needed. In addition, we chose a traumatic brain injury (TBI) model with known disruption of the BBB as a control. We hypothesized that the BBB would

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be disrupted after TBI, but intact after prolonged hypothermic circulatory arrest.

2. Materials and methods

The protocol for the study was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Adult male Sprague–Dawley rats (350–400 g; Hilltop Lab Animals, Scottdale, PA) were housed in the facility for ≥3 days before the experiment with unrestricted access to food and water.

Four groups were studied:

(1) EPR-RT (room temperature) flush ($n = 4$), 5 min of no flow, hypothermia (28 °C) initiated using room temperature flush. The total duration of CA was 20 min.
(2) EPR-IC (ice-cold) flush ($n = 3$), 1 min of no flow, hypothermia initiated using ice-cold flush to target a temperature of 15 °C. The total duration of CA was 75 min.
(3) TBI ($n = 3$), controlled cortical impact (CCI) at normothermia.
(4) Sham ($n = 3$), subjected to the same anesthesia and surgery, and maintained normothermic.

On the day of the experiment, rats were anesthetized with 4% isoflurane in oxygen, intubated with a 14-gauge intravenous cannula (Becton Dickinson, Sandy, UT), and mechanically ventilated (Harvard Ventilator 683, Harvard Rodent Apparatus, South Natick, MA). Anesthesia was maintained with 1–1.5% isoflurane in FiO_2 0.5. Using sterile preparation, the left femoral artery and vein were cannulated. Electrocardiogram, respiratory rate, and arterial and central venous pressure were continuously monitored and recorded (Polygraph, Grass Instruments, Quincy, MA). The right femoral artery was cannulated with a 20-gauge Angiocath (Becton Dickinson, Sandy UT) that served as an arterial CPB cannula. The right jugular vein was cannulated with a modified five-hole 14-gauge cannula advanced to the right atrium. That cannula was used for venous drainage during the hemorrhage phase and later as a venous CPB cannula. Rectal and tympanic probes were used to monitor the temperature. After instrumentation, rats were allowed to breathe spontaneously 2% isoflurane in FiO_2 0.25.

2.1. EPR protocol

Heparin was administered to achieve activated clotting time >400 s (Haemochron Jr Signature, ITC, Edison, NJ). After a 5 min equilibration period, a rapid exsanguination (12.5 mL of blood over 5 min) was performed via the internal jugular catheter. After the exsanguination phase, CA was ensured with administration of 9 mg (0.9 ml) of esmolol intravenously. Aortic flush (270 ml of Plasma-Lyte A – Baxter; Deerfield, IL) at different temperatures was initiated, via the right femoral artery at 50 ml/min using a roller pump and drained via the internal jugular catheter.

Resuscitation was initiated with full-flow CPB for 60 min. In brief, the CPB circuit consisted of a custom-designed oxygenator, an open reservoir (Ing. Martin Humbs, Ingenierburo fur Feinwerktechnik, Munich, Germany), tubing and a roller pump (Masterflex, Barnant, Barrington, IL). The oxygenator contained a three-layer capillary membrane sufficient to provide oxygen levels >400 mm Hg. Isoflurane was used for maintenance of anesthesia during the CPB phase. Heating and cooling were achieved with a circulating water bath and a forced-air blower (Life-Air 1000, Progressive Dynamics, Marshall, MI).

Immediately before starting resuscitation, the CPB circuit was primed with collected shed blood, and mechanical ventilation with FiO_2 0.3 was restarted to re-expand the lungs and prevent atelectasis. Full flow (160–180 mL/kg min) was maintained during the entire CPB course. Acid–base management followed alpha-stat principles. pH and electrolyte values outside of the normal range were corrected during the CPB phase. Additional blood obtained from a donor rat was used to maintain the hematocrit level at >25%.

After weaning off bypass, isoflurane anesthesia, mechanical ventilation and controlled hypothermia at 34.5 °C were continued for 5 h.

2.2. TBI protocol

TBI ($n = 3$): isoflurane anesthetized rats were endotracheally intubated and mechanically ventilated similarly to the EPR groups, then positioned in a stereotaxic frame (David Kopf, Tujunga, CA), and a 6 × 6-mm left parietal craniotomy was made by using a high-speed dental drill. They were then subjected to controlled cortical impact (CCI) using a pneumatically driven 3-mm metal impactor tip at a velocity of 6 m/s and a depth of penetration of 1.2 mm.[11] The rats were kept normothermic.

2.3. BBB studies

To determine BBB integrity, after CPB or 1 h after TBI or 1 h after cannulation in shams, rats were injected intravenously with 2% Evans Blue (EB) at 4 ml/kg (Sigma).[12] EB was allowed to circulate for 5 h. The animals were then perfused with 200 ml of ice cold normal saline 0.9%, through the left cardiac ventricle at a pressure of 40 mmHg until colorless fluid was obtained from the right atrium. Afterwards, the brains were removed and dissected. Each hemisphere was weighed and the samples were placed in 1 ml of formamide and stored for 72 h in the dark. The supernatant was spun for 10 min at 7000 rpm. The absorbance of the supernatants for EB dye was measured at 620 nm with a spectrophotometer (DU 800 UV/Beckman Coulter). EB dye content is expressed as ng/g of brain tissue against a standard curve.

In addition, we performed separate experiments in sham, EPR-IC and TBI group ($n = 1$ per group) to assess macroscopic penetration of EB into the brain tissue. After completion of the respective protocol, the rats were sacrificed and perfusion-fixed with 200 ml of 10% formalin. After 72 h, the brains were removed from the skull and 1 mm thick coronal slices were prepared.

Comparison of groups was performed using one-way ANOVA with post-hoc Tukey's test. Values of $p < 0.05$ were considered statistically significant.

3. Results

Results are summarized in Fig. 1. EB absorbance in either EPR group did not differ from sham. In contrast, TBI resulted in obvious EB extravasation as visualized in a photograph of coronal brain sections taken through the dorsal hippocampus (Fig. 2). TBI produced an approximately 10-fold increase in EB absorbance in the left (injured) hemisphere at 5 h after injury vs. left hemispheres of all other groups ($p = 0.001$), and a more modest albeit readily detectable increase in the right (uninjured) hemisphere vs. EPR-IC or EPR-RT groups ($p < 0.05$).

4. Discussion

The blood vessels of the brain have a unique barrier function that ensures an optimally controlled homeostasis of the brain's internal environment. Disruption of the BBB is seen in many conditions such as sepsis, TBI and stroke, among other conditions.[13] This allows passage of neurotransmitters,[14] hormones and large proteins that might be a contributing factor to the evolution of secondary injury process – such as inflammation and edema.[15] Our EPR model is

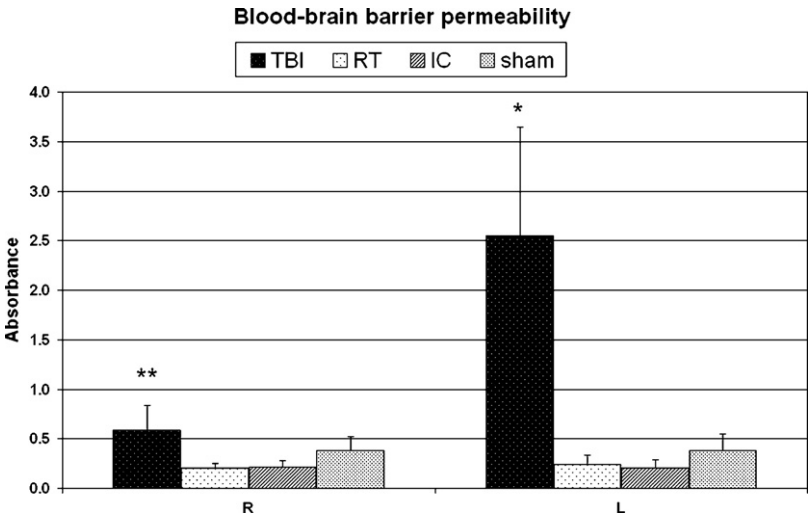

Fig. 1. Absorbance, reflecting Evans Blue concentration in rat brain in studied groups: traumatic brain injury (TBI), EPR – room temperature flush (RT), EPR-ice cold flush (IC) and sham rats. TBI produced a significant increase in EB absorbance in the left hemisphere 5 h after injury vs. left hemispheres of all other groups (*$p = 0.001$), and a more modest albeit readily detectable increase in the right (uninjured) hemisphere vs EPR-IC or EPR-RT groups (**$p < 0.05$). Y bars represent 1 standard deviation of the measurements.

complex, since it includes exsanguination, CA, deep hypothermic circulatory arrest (DHCA) and CPB. Review of the literature revealed conflicting reports of the status of the BBB in large animals and in rodents who underwent insults that represent one or more of the interventions that constitute our EPR model.[3–5,16–18]

Remarkably few studies have addressed the BBB disruption in hemorrhagic shock, CA, DHCA, or even CPB – and much of that work is in large animal models. Initial studies in pigs showed no changes in BBB permeability after 2 h of normothermic CPB,[4] or 3 h of normothermic non-pulsatile CPB.[18] Using pulsatile CPB, minute foci of extravasated serum proteins appeared.[18] In a follow-up study comparing 2 h of hypothermic vs. normothermic CPB in a pig model, neither technique induced significant changes in BBB permeability.[5] The extent of brain damage is also CPB flow- and pressure-dependent – either modest decreases in CPB flow (from 70 to 50 ml/kg min) or even high flow at lower MAP were associated with increased BBB permeability to serum proteins.[19] On the contrary, Cavaglia et al. showed universal BBB dysfunction after 15–120 min normothermic CPB in infant lambs, further aggravated by superimposing 2 h of DHCA.[3] In humans, cerebral swelling was observed after both normothermic and hypothermic (28 °C) CPB using magnetic resonance imaging.[20,21] Thus, the anticipated findings with regard to BBB permeability from CPB alone are controversial.

The surrogate marker of BBB integrity, S-100β protein, an astroglial cell marker, has been recently monitored in CPB and DHCA. Increased serum concentrations of S-100β were found immediately after reperfusion and termination of CPB following 60 min DHCA in a rabbit model, suggested a reperfusion injury to the astroglial cell complex that forms the BBB.[22] Serum S-100β also correlated well with the histopathologic outcome after prolonged DHCA in pigs.[23] This technique potentially allows one to advance the assessment of BBB function after CPB or DHCA to the bedside. How increases in S-100β compare to albumin/EB permeability, however, are not clear. It is certainly possible that release of S-100β could occur at a level of insult below the threshold for detection of albumin permeability.

However, it has been shown that other extracerebral organs release S-100β when subjected to ischemia. In a Langendorff isolated heart model, both S-100β and troponin increased after cardiac ischemia.[24] Previously we have documented in our hypothermic CA model a marked increase in cardiac troponin at 3 h after reperfusion (baseline, 0.45 ± 0.71 ng/mL, post-reperfusion 11.43 ± 5.10 ng/mL; $p < 0.01$).[25] While S-100β could be an additional end-point in our study, it would be difficult to precisely interpret the data in our complex model.

In experimental CA, BBB permeability was not observed at 4 h after a 10-min normothermic CA in dogs.[26] Surprisingly, studies in a pig model showed preserved BBB integrity in the initial post-CA phase, but delayed BBB permeability.[27] In a mouse model, a brief CA did not result in BBB permeability to albumin.[6] In a study using rat global ischemia model, BBB permeability was disrupted early after reperfusion (up to 6 h) but restored at 24 h.[28] However, germane to our findings, the BBB disruption could only be detected with a small-molecule tracer (amino-isobutyric acid), but not with EB. Studies in a global cerebral ischemia model in a rat showed no

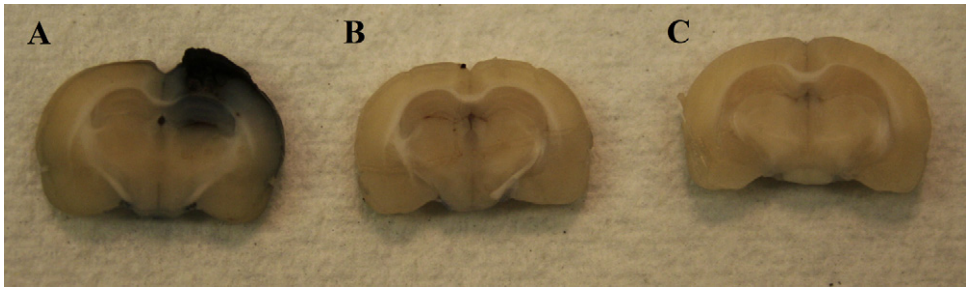

Fig. 2. Photograph of coronal brain sections at the level of the dorsal hippocampus that reveal obvious Evans Blue extravasation after TBI (panel A), but not in EPR-RT flush (panel B) or sham groups (panel C). TBI; traumatic brain injury; EPR, emergency preservation and resuscitation; RT, room temperature.

difference of EB extravasation vs. sham at 40 min, but significant differences could be found at 24 and 48 h.[12] This was associated with poly (ADP-ribose) polymerase activation, which was also shown to be increased in our 75 min EPR model.[29]

The few studies addressing the integrity of BBB during hemorrhagic shock revealed that the BBB is disrupted during "decompensated" phase of hemorrhagic shock, i.e., mean arterial pressure 40 mm Hg. A significant increase in BBB permeability was observed using sodium fluorescein, but not EB.[17]

In contrast, BBB disruption is well-recognized after TBI, and this was confirmed in our current work.[30,31]

In our rat EPR model, we have previously shown that a 60-min DHCA is well tolerated with CPB resuscitation.[10] However, intact neurological outcome is not achievable for insults of 75 min or beyond. Thus we chose to explore a 75-min insult in this initial work to examine the BBB given that it is beyond the threshold for good outcome. Our data suggest that BBB permeability does not contribute to the poor outcomes after 75 min of EPR.

We also did not assess BBB permeability at after longer follow-up periods (beyond 5 h). While some studies in DHCA show immediate disruption of BBB, we may speculate that BBB disruption could be delayed in the setting of prolonged post-resuscitation hypothermia. Increased BBB dysfunction could be detected in rats subjected to 10 min CA even after 2 months,[16,32] or 1 year.[33,34] Given the conflicting data in CA previously discussed, assessment at more delayed time points after DHCA is warranted. However, if resuscitative therapies are needed to be added to the flush and used for neuropreservation, agents that are permeable to an intact BBB are likely to be required. Using our canine model, we have previously tested fourteen of the most promising agents based on their potential role in preventing or ameliorating neuronal damage after ischemic injury. In accordance with our current results, only the BBB penetrating antioxidant tempol showed benefit.[35,36]

To study the status of BBB integrity, a variety of BBB markers have been described. In circulation, EB is tightly bound to albumin, thus serving as a readily identifiable tool that has been used in hundreds of studies of BBB.[5,12,18,31,33,37] Given the track record for use of Evans Blue as a BBB marker and the inflammation known to be associated with use of CPB,[5,18] we believe that albumin was the appropriate first approach to assess BBB in our model. However, other smaller molecules, such as amino isobutyric acid,[38] or gadolinium-based agents[38] could be more sensitive to subtle injury. In our study we found that BBB integrity to albumin is not disrupted early after resuscitation from prolonged CA treated with moderate or deep hypothermic EPR.

The positive control used in this study is represented by the traumatic brain injury (TBI) model, that shows extensive disruption of BBB, as shown in Fig. 1, that quantifies the disruption, and in Fig. 2, that allows visual comparison of the injured vs. non-injured hemisphere vs. representative samples of brains in the EPR models. Although the number of the animals per group was small, we were able to demonstrate dramatic differences between groups and germane to the question of our ability to detect subtle BBB injury with the sample size used, we detected a modest increase in BBB permeability in the hemisphere contralateral to the injury in the TBI group vs. EPR groups with an $n = 3–4$ per group. Of note, in the hemisphere contralateral to impact in our TBI model there is no neuronal death and routine neuropathology reveals a normal appearance.[39] Thus, we were able to detect BBB permeability with statistical significance in our study even in brain regions with rather subtle damage. We believe that this strongly supports our findings even with a modest sample size.

Drugs administered to rats varied between groups. Specifically, esmolol was shown to decrease TNFα in a rat model of sepsis.[40] Similarly, heparin improved clinical outcome in animal models of sepsis with endotoxemia.[41,42] We cannot rule out a protective effect of esmolol and/or heparin on inflammation in rats in the EPR groups.

We acknowledge the fact that this model may not represent the typical pre-hospital setting in which prolonged hemorrhage and/or no-flow time before intervention could be anticipated. However, data from military setting suggest that two-thirds of wounded soldiers died within 5 min from the injury.[1] Previously, we have shown that prolonged hemorrhagic shock (over 120 min) before CA does not prevent successful resuscitation using EPR.[43]

If needed, cannulation of a femoral artery could be established in less than 5 min,[44] and cannulation of a saphenous vein in less than 1 min,[45] allowing rapid initiation of hypothermia. Alternative approach via thoracotomy could also be considered. Obviously, the overall success of resuscitation is related to the delay between CA and onset of hypothermia.[46,47] Other factors like depth of hypothermia, duration and reperfusion strategies may also play a role.

Our results could also be applied to the cardiac surgery setting, in which deep hypothermic circulatory arrest is used to facilitate complex repair of aortic pathologies without preceding ischemic insult.

5. Conclusion

Resuscitation from exsanguination CA with EPR using deep or moderate hypothermia does not increase BBB permeability to albumin. Our data suggest that BBB damage does not appear to contribute to the impaired outcome in these models. In addition, the development of neuroprotective adjuncts to hypothermia that are designed to enhance the outcome of EPR and/or DHCA should focus on agents that penetrate the BBB.

Conflict of interest

Drs. Kochanek and Tisherman are co-patent holders with the University of Pittsburgh on "Method of Inducing Suspended Animation Following Cardiopulmonary Arrest". Patent pending, #60/692,722.

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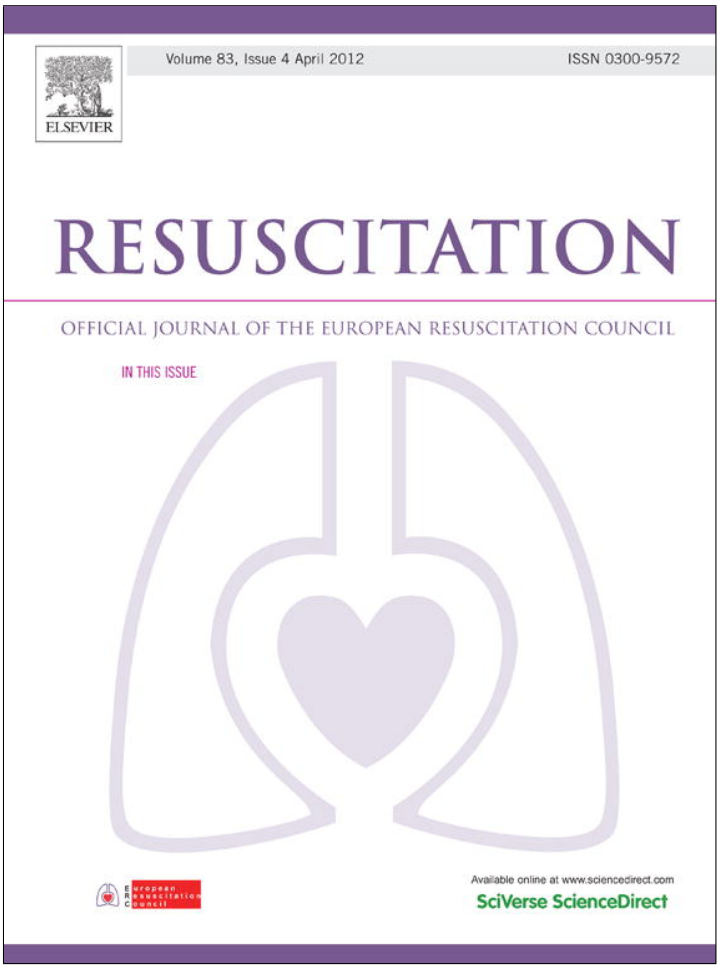

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Experimental paper

Microglial depletion using intrahippocampal injection of liposome-encapsulated clodronate in prolonged hypothermic cardiac arrest in rats☆

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ABSTRACT

Trauma patients who suffer cardiac arrest (CA) from exsanguination rarely survive. Emergency preservation and resuscitation using hypothermia was developed to buy time for resuscitative surgery and delayed resuscitation with cardiopulmonary bypass (CPB), but intact survival is limited by neuronal death associated with microglial proliferation and activation. Pharmacological modulation of microglia may improve outcome following CA. Systemic injection of liposome-encapsulated clodronate (LEC) depletes macrophages. To test the hypothesis that intrahippocampal injection of LEC would attenuate local microglial proliferation after CA in rats, we administered LEC or PBS into the right or left hippocampus, respectively. After rapid exsanguination and 6 min no-flow, hypothermia was induced by ice-cold (IC) or room-temperature (RT) flush. Total duration of CA was 20 min. Pre-treatment (IC, RTpre) and post-treatment (RTpost) groups were studied, along with shams (cannulation only) and CPB controls. On day 7, shams and CPB groups showed neither neuronal death nor microglial activation. In contrast, the number of microglia in hippocampus in each individual group (IC, RTpre, RTpost) was decreased with LEC vs. PBS by ~34–46% ($P < 0.05$). Microglial proliferation was attenuated in the IC vs. RT groups ($P < 0.05$). Neuronal death did not differ between hemispheres or IC vs. RT groups. Thus, intrahippocampal injection of LEC attenuated microglial proliferation by ~40%, but did not alter neuronal death. This suggests that microglia may not play a pivotal role in mediating neuronal death in prolonged hypothermic CA. This novel strategy provides us with a tool to study the specific effects of microglia in hypothermic CA.

1. Introduction

Patients who suffer a cardiac arrest (CA) from trauma, often from exsanguination, have <10% chance of survival, even with aggressive fluid resuscitation and an emergency department thoracotomy. Emergency preservation and resuscitation (EPR) is a novel approach for resuscitation of exsanguination CA victims.[1] EPR uses cold aortic flush to induce deep hypothermic preservation for prolonged CA to buy time for transport, damage control surgery, and delayed resuscitation with cardiopulmonary bypass (CPB).

Prolonged CA results in neuronal death and a reactive glial response. Specifically, microglial activation and proliferation has been linked to delayed neuronal death, presumably via releasing neurotoxic substances, including reactive oxygen radicals, nitric oxide (NO), and pro-inflammatory cytokines.[2] Microglial activation could contribute to neuronal death or microglial-mediated synaptic injury and/or neuronal dysfunction – which could mediate cognitive deficits even in the absence of overt neuronal death.

Microglia could also have beneficial effects, contributing to delayed repair after injury via elaboration of growth factors,[3] or their presence could represent an epiphenomenon. The effect of microglia could also depend on the severity of the primary insult, resulting in neurotoxicity vs. neuroprotection. Thus, there may be

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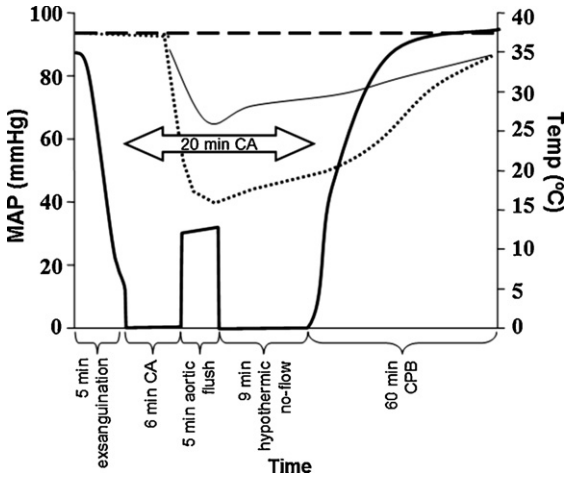

Fig. 1. Experimental protocol including rapid hemorrhagic shock and 6 min normothermic cardiac arrest (CA), followed by emergency preservation and resuscitation (EPR) using ice-cold (IC) or room-temperature (RT) aortic flush and resuscitation with cardiopulmonary bypass (CPB). Bold solid line, mean arterial pressure (MAP) in IC and RT groups; dotted line, temperature in IC group; thin solid line, temperature in RT groups; dashed line; MAP and temperature in sham and CPB control groups.

a specific time window for benefit from inhibition of the microglial contribution to damage, as well as specific scenario in which inhibiting microglia could be helpful. Therapeutical modulation of the microglial response for insults even less than the threshold for neuronal death may help to improve outcome following global brain ischemia.[4]

Pharmacological modulation of microglial proliferation may help to improve outcome following CA. Recently, studies in several CNS insults have shown benefit from treatment with minocycline, an agent that attenuated microglial activation and proliferation.[5,6]

Liposome-encapsulated clodronate (LEC) is an agent that – when used systemically – depletes macrophages[7] and has been shown to deplete microglia *in vitro*, including brain slices.[8] In brain ischemia, however, the local inflammatory response is predominated by microglial rather than macrophage accumulation.[9] Since LEC does not cross the blood–brain barrier (BBB), we hypothesized that intraparenchymal injection of LEC into the brain would selectively deplete microglia and attenuate hippocampal neuronal degeneration.

2. Methods

We used the rat EPR model described in detail previously (Fig. 1).[10] All rats received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" (www.nap.edu/catalog/5140.html). The study protocol has been approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Five groups were studied: (1) rats pre-treated 24 h prior to CA and subjected to deep hypothermia during CA using ice-cold (IC) flush (IC, $n = 6$); (2) rats pre-treated 24 h prior to CA and subjected to moderate hypothermia during CA using room-temperature (RT) flush (RTpre, $n = 3$); (3) rats injected 24 h after CA and subjected to moderate hypothermia during CA using RT flush (RTpost, $n = 3$); (4) shams ($n = 4$), subjected to the same cannulations and duration of anesthesia; (5) CPB controls ($n = 3$), subjected to the same cannulations, anesthesia and 60 min of normothermic CPB.

2.1. Intrahippocampal injections

Adult male Sprague-Dawley rats (350–375 g) were obtained from Hilltop Lab Animals (Scottdale, PA) and housed for at least three days before the experiment under 12-h light/dark cycle with unrestricted access to food and water. Rats were anesthetized with 4% isoflurane in a transparent acrylic jar. After tracheal intubation with a 14 gauge (G) intravenous catheter (Becton Dickinson; Sandy, UT), rats were mechanically ventilated using a piston ventilator (Harvard Ventilator Model 683, Harvard Rodent Apparatus; South Natick, MA) with a tidal volume of 0.8 ml/100 g and a frequency 36–42/min to maintain normocapnia, and a positive end-expiratory pressure (PEEP) of 4 cm H_2O. Anesthesia was maintained with 1.5–2% isoflurane in FiO_2 0.5.

Using a stereotaxic frame, burr holes (diameter 0.45 mm) were created bilaterally (−4.3 mm dorsoventral, −2.0 mm lateral from bregma). A 27G needle was then inserted 3.5 mm deep into the hippocampus. Each rat received simultaneous intrahippocampal injections of either 5 μL of liposome-encapsulated phosphate-buffered saline (PBS) (left hemisphere) or 5 μL of LEC (right hemisphere) over 10 min via a 27G needle connected by a polyethenylene tubing to a 10 μL Hamilton syringe (Hamilton, 701N) and an infusion pump (Harvard Apparatus; South Natick, MA). Clodronate was a gift of Roche Diagnostics GmbH (Mannheim, Germany). It was encapsulated in liposomes as described previously.[11] Using a different treatment in each hemisphere, each rat served as its own control. After a 3 min additional period with the needle in place to allow distribution of the compound, the needle was withdrawn at the rate of 1 mm/min to prevent leakage through the burr hole.

In addition, we tested if intrahippocampal injections of 10 μl would cause an increase in intracranial pressure (ICP), and thus potentially alter our model. ICP was monitored in selected rats ($n = 4$) via a 1 French intraparenchymal ICP probe (SPR-1000; Millar Instruments, Houston, TX) inserted from a separate burr hole in the frontal lobe. The ICP monitoring was discontinued and the probe was withdrawn after the completion of the injections.

After completion of the injections and ICP monitoring, the burr holes were sealed with a bone wax and skin was closed by layers using 2.0 silk. Anesthesia was discontinued, rats were extubated and allowed to recover in the cage.

2.2. Cardiac arrest

Rats were anesthetized, intubated and mechanically ventilated as mentioned above. After shaving and prepping with povidone iodine, bilateral femoral and right jugular cutdowns were performed. The left femoral artery and vein were cannulated for blood pressure monitoring and blood sampling. EKG, respiration, arterial and central venous pressure were continuously monitored and recorded (Polygraph; Grass Instruments, Quincy, MA). The right femoral artery was cannulated with a 20G catheter (Becton Dickinson; Sandy, UT) that served as an arterial CPB cannula. The right jugular vein was cannulated with a modified five-hole 14G intravenous cannula advanced to the right atrium to be used for venous drainage during the hemorrhage phase and later as a venous CPB cannula. Rectal and tympanic probes were used to monitor the temperature. Baseline blood samples were obtained, and hemodynamic values were recorded. Removed blood volume was replaced with an electrolyte-balanced crystalloid (Plasma-Lyte A Injection pH 7.4; Baxter; Deerfield, IL) in a ratio 1:3 (blood:crystalloid). Heparin sodium was administered to achieve activated clotting time (ACT) > 400 s (Haemochron Jr. Signature, ITC; Edison, NJ) to prevent clotting of the CPB circuit; the effects of heparin were not reversed.

After instrumentation, intubated rats were weaned to spontaneous ventilation of isoflurane 2% at FiO_2 0.25% via a nose

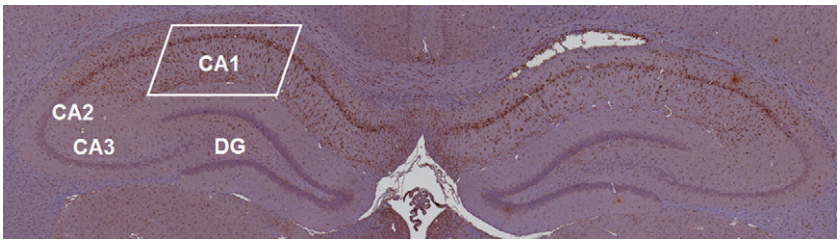

Fig. 2. A microscopic pan-scan of both left and right hippocampi with identification of its main regions. A rectangle in the CA1 area shows the area of interest where cell counts were performed. The DAB staining used in this slide shows activated microglia. CA, cornu ammonis; DG, dentate gyrus.

cone mask. After 5 min equilibration period, rapid exsanguination (12.5 ml of blood over 5 min) was performed via the internal jugular catheter. The shed blood was collected. After the rapid exsanguination phase, asystole was induced with intravenous administration of 9 mg (0.9 ml) of esmolol (Baxter; Deerfield, IL) and 0.2 mEq (0.1 ml) of potassium chloride (Hospira; Lake Forest, IL). After 5 min of CA, 270 ml of either an IC or RT flush solution (Plasma-Lyte A Injection pH 7.4) was instilled via the right femoral artery catheter at 50 ml/min. The flush was drained from the jugular vein catheter.

After 20 min of CA, resuscitation was started with CPB. Heating and cooling were achieved with a circulating water bath around the oxygenator. Blood samples for biochemistry and hematology were obtained at 5, 15, 30, 45, and 60 min CPB time and processed immediately using a point-of-care blood analyzer (Stat Profile, Nova Biomedical; Waltham, MA).

Arterial blood gas management followed alpha-stat principles. pH and electrolyte values outside of the normal range were corrected during CPB and ICU phases by adjustments in ventilation and/or administration of sodium bicarbonate, calcium chloride, or potassium chloride. Neuromuscular blockade was induced with cisatracurium (Nimbex; Abbott, North Chicago, IL) to prevent gasping during CPB. Additional blood obtained from an isoflurane-anesthetized donor rat was used to maintain hematocrit > 25%. CPB support was gradually weaned after 60 min. Mechanical ventilation with a FiO_2 of 1.0 was continued while maintaining normocapnia for additional 2 h.

Using a midline laparotomy incision, a Mini-Mitter telemetric probe (Mini-Mitter Co; Sunriver, OR) was introduced into the peritoneal cavity to allow post-operative temperature control and continuous monitoring of heart rate and movement. Surviving rats were extubated 2 h later after removal of catheters, and placed separately in a temperature-controlled cage (34.5 °C for 6 h) with supplemental oxygen for 18 h, and free access to food and water. Neurological outcome was assessed daily by determining overall performance categories (OPC; 1 = normal, 5 = death) and neurologic deficit score (NDS; 0–10% = normal, 100% = maximum deficit).[12] At 7 days after resuscitation, rats were euthanized with an isoflurane overdose and perfused via left ventricle with normal saline followed by 10% neutral-buffered formalin.

2.3. Histology

The tissue samples were processed for embedding in paraffin. The resulting paraffin blocks were sequentially sectioned at 5 μm. All sections were stained with Fluoro-Jade C (FJC) to indicate neuronal degeneration[13] and with anti-Iba-1 staining visualizing microglia. Iba-1 is a calcium-binding protein expressed specifically in activated microglia,[14] with its peak occurring at 4–7 days in experimental stroke.[15] For the Iba-1 staining, sections were washed in TBST, incubated in 0.3% H_2O_2 in TBST for 30 min to inhibit endogenase peroxidase activity, washed in TBST, and blocked in TBST containing 3% normal goat serum for 30 min. The sections were incubated with a rabbit anti-Iba1 polyclonal antibody (1:250, Wako, Richmond, VA) overnight at 4 °C. The sections were then washed in TBST and incubated with a FITC-conjugated goat anti-rabbit IgG antibody (Invitrogen, Carlsbad, CA) for 1 h at room temperature. After the reactions, sections were washed and coverslipped with Vectashield Mounting media containing 4′,6-diamidino-2-phenylindole (DAPI) counterstain.

In addition, colorimetric visualization of Iba-1 immunostaining using diaminobenzamide (DAB) (Vector, CA) was used as a secondary confirmatory method to visualize microglia. In short, sections were processed the same as for fluorescent labeling on day 1, using a 1:250 dilution of anti-rabbit Iba-1 overnight at 4 °C. Sections were washed with TBST, incubated at RT for 1 h with a biotinylated anti-rabbit IgG, followed by 1 h of avidin-biotin complex binding using an ABC kit (Vector, CA). Sections were washed and incubated for 10 min with DAB followed by hematoxylin counterstaining. Tissue was dehydrated, cleared and coverslipped for microscopic analysis. For control staining, normal rabbit IgG was used as the primary antibody.

Adjacent sections obtained at approximately 4.3 mm from bregma were used to assess neuronal degeneration and microglial proliferation within the CA1 region of the hippocampus (Fig. 2). A photograph of the representative section of the CA1 region was taken under 10× magnification. FJC positive neurons and Iba-1 positive activated microglia (characterized by ameboid cell body and retracted processes without thin ramifications)[16] were then quantitated morphometrically by two independent researchers (KJ, CDW) in a CA1 region of the hippocampus marked in Fig. 2 using the National Institutes of Health Image-J software. No automated features of the software were used. Image-J was used solely to track the cell counts and provide a controlled feedback between the independent evaluators.

2.4. Statistical analysis

Repeated measures ANOVA was performed, followed by Tukey post hoc tests, to identify differences in hemodynamic parameters and temperature between groups. For the aforementioned comparisons, data from RTpre and RTpost groups were pooled since they did not differ. One-way ANOVA was used to compare histologic damage, biochemical and hematologic data between groups. Kruskal–Wallis H test was used to compare NDS among groups. Mann–Whitney U test was used to compare two groups if Kruskal–Wallis H test indicated differences between groups existed. A P value < 0.05 was considered statistically significant.

3. Results

There were no differences in baseline characteristics between individual study groups. Induction of hypothermia IC flush resulted in lower intra-arrest temperature compared to the RT groups ($P < 0.05$ IC vs. RT). Physiologic parameters (heart rate, blood pressure) and temperature profiles during resuscitation are shown in Figs. 3–6. Prolonged CA in IC and RT flush groups resulted in marked

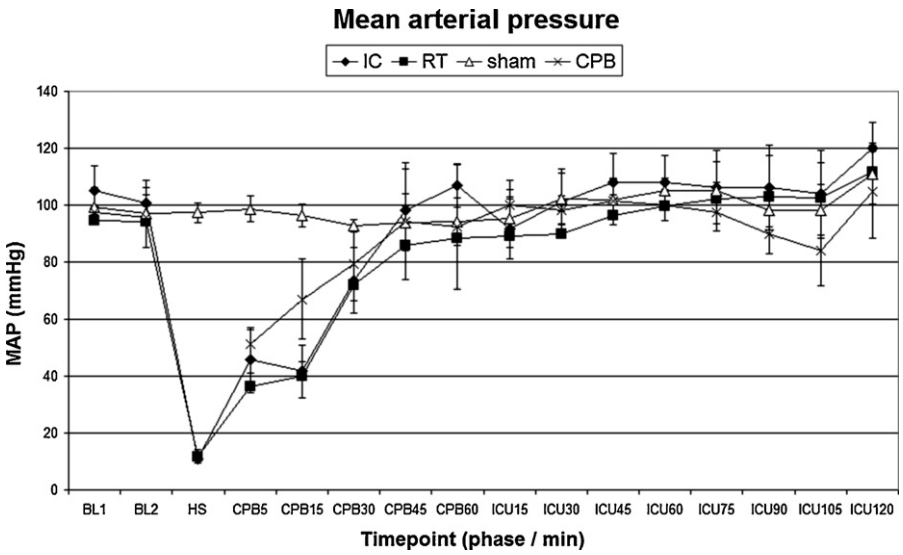

Fig. 3. Mean arterial pressure. IC, ice-cold flush; RT, room-temperature flush; CPB, cardiopulmonary bypass control group. $P < 0.05$ RT vs. sham.

physiologic derangements in the acid–base status with extremely low pH, BE up to −20, and increased lactate up to 6 mmol/L. These changes were gradually improved during resuscitation and were largely ameliorated by the end of the ICU phase. Minimal changes between groups were observed after 7 d (Table 1).

All rats improved in neurologic status over time but only rats from both RTpre and RTpost groups exhibited persistent neurologic deficits ($P < 0.01$ vs. IC, shams or CPB group, respectively; OPC, Table 2; NDS, Fig. 7).

At 7 d after the insult a robust microglial response was seen in the hippocampus in groups subjected to CA. Minimal microglial proliferation was observed in the CPB group. No activated microglia were visualized in shams. The number of microglia in hippocampus was decreased in the hemisphere injected with LEC vs. PBS in all individual groups and when pooled together ($P < 0.05$). The number of FJC-positive neurons, however, did not differ between hemispheres in individual groups ($P = 1.0$) (Figs. 8 and 9, and Table 3). ICP did not significantly differ before (6 ± 1 mmHg) and after (5 ± 2 mmHg) LEC injection into hippocampus. Continuous ICP monitoring for 20 min during the LEC injection is shown (Fig. 10).

4. Discussion

Using our established, clinically relevant model of EPR to study prolonged hypothermic CA in trauma resuscitation we found that both pre- and post-treatment with direct injection of LEC into the brain attenuates local microglial proliferation in hippocampus. This effect was not associated with a decrease in neuronal loss or a change in ICP.

The role of microglia in neuroinflammation in prolonged CA remains poorly defined. There is a large body of evidence documenting that microglia are a source of multiple potentially cytotoxic substances including NO, free radicals and pro-inflammatory cytokines, especially tumor necrosis factor α and interleukin-1β.[17] Attenuation of microglial activation has shown benefit in multiple CNS injuries and neuroinflammatory diseases.[18] In contrast, ablation of microglia in transgenic mice in stroke models showed detrimental effects,[3] and administration of exogenous microglia had neuroprotective effects after ischemia,[19,20] possibly linked to the production of neurotrophic factors like insulin-growth factor-1 or brain-derived neurotrophic factor.[21] It has been

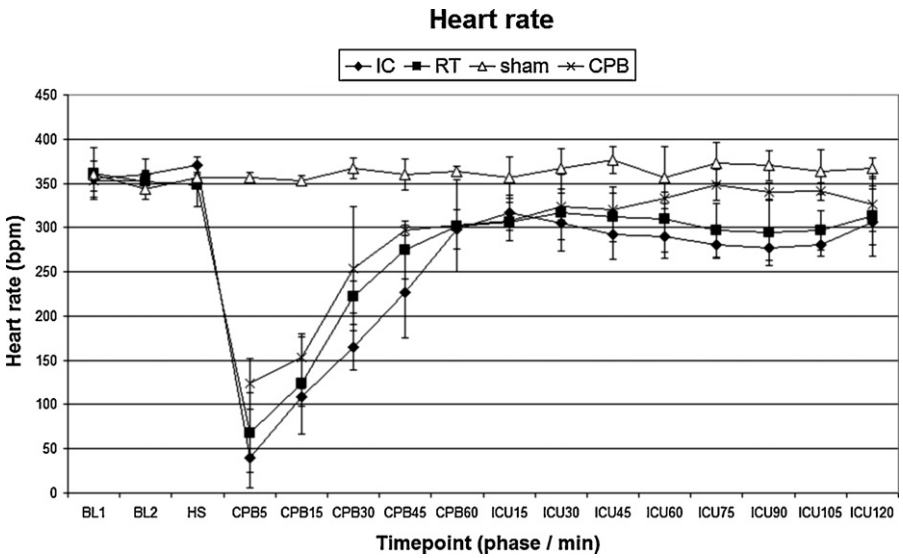

Fig. 4. Heart rate. IC, ice-cold flush; RT, room-temperature flush; CPB, cardiopulmonary bypass control group. $P < 0.05$ IC vs. RT, sham or CPB; RT vs. IC or sham; sham vs. IC, RT or CPB.

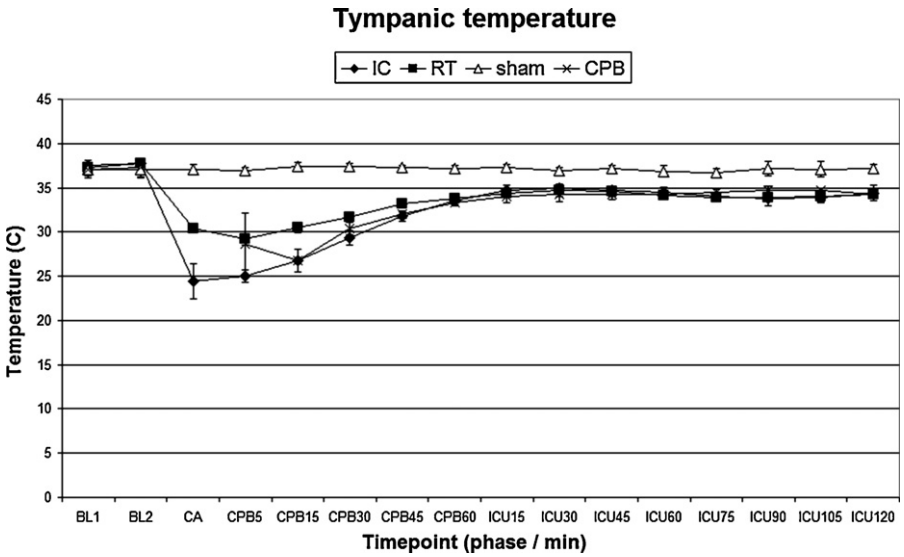

Fig. 5. Tympanic temperature. IC, ice-cold flush; RT, room-temperature flush; CPB, cardiopulmonary bypass control group. $P < 0.05$ IC vs. RT or sham; RT vs. IC, sham or CPB; sham vs. IC, RT or CPB; CPB vs. RT or sham.

postulated that the microglial reaction is dependent on the severity of the insult, and their role could be either "toxic" or "protective".[22,23] The tetracycline derivative minocycline has been used traditionally to attenuate microglial activity in multiple studies and showed potential as a neuroprotective agent.[5] However, minocycline is non-specific.[24] We specifically targeted microglia with local LEC administration.

Clodronate (dichloromethylene bisphosphonate – Cl_2MDP) has been developed to eliminate macrophages in order to permit "in vivo" studies of their function. Clinically, it has been used in the treatment of osteoporosis,[25] including prevention of skeletal events in patients with breast cancer.[26] Prolonged administration of clodronate (oral, intravenous or intramuscular) seems to be safe.[25]

The exact mechanism of effects of clodronate is not yet fully elucidated. Systemically injected liposomes including encapsulated clodronate are ingested by macrophages which are then destroyed following phospholipase-mediated disruption of the liposomal bilayers and release of clodronate via a so-called macrophage "suicide" technique.[27] Clodronate released from the liposomes has a short half-life that allows prompt removal from the circulation.[28] Depletion of macrophages occurs rapidly (within 24 h after intravenous administration) and lymphocytes are not depleted. The effect persists for as long as one month after a single injection.[29] A second dose was used to deplete bone-marrow residing macrophages and optimize the depletion.[30] Despite profound effects, an increase in infectious complications has not been reported in long-term outcome models. Microglia remain unaffected because liposomes do not cross the BBB.[31] We previously showed that our model of EPR is not associated with BBB disruption.[32] Intraventricular injection of LEC resulted in a selective depletion only of perivascular and meningeal macrophages in the CNS. The macrophages started to repopulate in the given areas 14 d after the LEC depletion.[33] Thus, we chose to target microglia in hippocampus with a direct intrahippocampal injection of LEC.

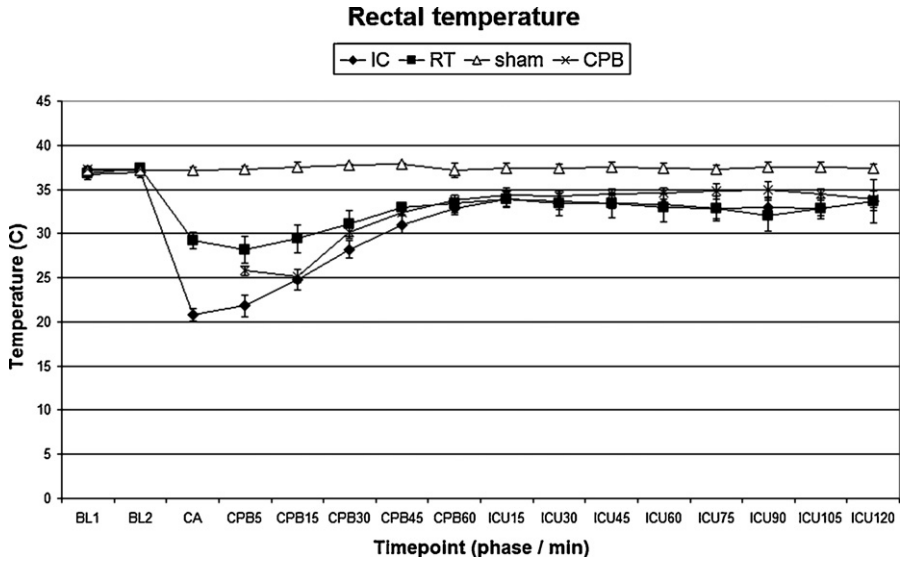

Fig. 6. Rectal temperature. IC, ice-cold flush; RT, room-temperature flush; CPB, cardiopulmonary bypass control group. $P < 0.05$ sham vs. IC, RT or CPB groups.

Table 1
Selected biochemical and hematological values after 20 min hypothermic cardiac arrest.

	BL	CPB5	CPB60	ICU120	Final
pHa					
IC	7.36 ± 0.03	7.09 ± 0.07[c,d]	7.41 ± 0.08	7.45 ± 0.01[c,d]	7.45 ± 0.03
RT	7.35 ± 0.02	7.02 ± 0.04[c,d]	7.40 ± 0.04	7.41 ± 0.03[c,d]	7.48 ± 0.06
sham	7.42 ± 0.04	7.39 ± 0.06[a,b,d]	7.37 ± 0.04	7.41 ± 0.02[a,b]	7.44 ± 0.08
CPB	7.38 ± 0.03	7.22 ± 0.06	7.28 ± 0.04	7.42 ± 0.07[a,b]	7.49 ± 0.06
paO2					
IC	231 ± 32	600 ± 46[b,c,d]	417 ± 34[b,c,d]	233 ± 65	412 ± 194
RT	214 ± 24	351 ± 109[a]	305 ± 75[a]	194 ± 57	491 ± 164
sham	224 ± 27	200 ± 20[a]	213 ± 19[a]	163 ± 54	384 ± 73
CPB	233 ± 47	387 ± 182[a]	246 ± 100[a]	163 ± 114	452 ± 79
paCO2					
IC	42 ± 4	32 ± 12	41 ± 9	42 ± 2	34 ± 4
RT	39 ± 5	30 ± 3	44 ± 7	40 ± 2	26 ± 5
sham	36 ± 3	37 ± 2	38 ± 4	34 ± 3	38 ± 7
CPB	39 ± 2	36 ± 8	42 ± 10	33 ± 3	37 ± 10
BE					
IC	−1.0 ± 1.7	−17.5 ± 6.9[c,d]	1.8 ± 2.3[c]	5.1 ± 1.4[b,c,d]	−0.3 ± 2.0
RT	−2.7 ± 0.5	−20.7 ± 1.0[c,d]	2.0 ± 2.1[c]	1.5 ± 1.7[a,c,d]	−2.1 ± 0.6[d]
sham	−1.0 ± 1.0	−1.7 ± 2.6[a,b]	−3.1 ± 1.0[a,b]	−2.4 ± 1.0[a,b]	1.4 ± 2.8
CPB	−2.0 ± 1.1	−4.7 ± 0.3[a,b]	−2.0 ± 2.2	−3.9 ± 3.3[a,b]	3.8 ± 2.9
Lactate					
IC	1.5 ± 0.5	4.2 ± 1.2[b]	4.3 ± 1.2[b]	2.7 ± 1.1	1.4 ± 0.4[b,c]
RT	1.4 ± 1.2	5.9 ± 0.8[a,c,d]	6.4 ± 1.0[a,c,d]	3.3 ± 1.6	3.1 ± 1.4[a]
sham	1.9 ± 0.4	2.5 ± 0.4[b]	3.2 ± 0.9[b]	3.4 ± 1.2	3.2 ± 0.8[a]
CPB	1.4 ± 0.4	2.1 ± 0.6[a,b]	3.5 ± 0.6[b]	4.9 ± 1.9	2.0 ± 0.6
Hct					
IC	36 ± 2	24 ± 2	29 ± 2	30 ± 2	35 ± 4
RT	33 ± 2	25 ± 2	29 ± 2	32 ± 1	35 ± 1
sham	40 ± 3	41 ± 4	39 ± 1	37 ± 4	42 ± 2
CPB	40 ± 2	25 ± 2	25 ± 2	31 ± 1	35 ± 2
Glucose					
IC	228 ± 46	179 ± 45	229 ± 44	163 ± 16	224 ± 56
RT	196 ± 68	183 ± 13	206 ± 16	130 ± 30	251 ± 113
sham	322 ± 15	333 ± 25	316 ± 81	198 ± 89	232 ± 17
CPB	227 ± 6	247 ± 10	325 ± 102	152 ± 71	241 ± 26

IC, ice-cold flush; RT, room-temperature flush; CPB; cardiopulmonary bypass control group; BE, base excess; Hct, hematocrit.
[a] $P < 0.05$ vs. IC.
[b] $P < 0.05$ vs. RT.
[c] $P < 0.05$ vs. sham.
[d] $P < 0.05$ vs. CPB.

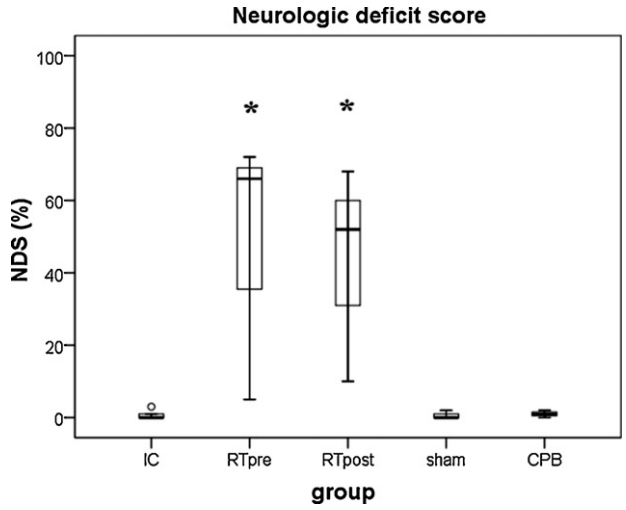

Fig. 7. Neurologic deficit score between groups. Boxes represent interquartile ranges. The line across each box indicates the median, and the whiskers are the highest and lowest values. The round marker represents an outlier. IC, ice-cold flush; RTpre, pre-treatment, room-temperature flush; RTpost, post-treatment, room-temperature flush; CPB, cardiopulmonary bypass control group. *$P < 0.05$ vs. IC, sham or CPB group, respectively.

Table 2
Overall performance categories (OPC 1–5) at 7 d after prolonged hypothermic cardiac arrest and in control groups.

	IC	RTpre	RTpost	sham	CPB
OPC 5 Death/brain death					
OPC 4 Severe disability					
OPC 3 Moderate disability		••	••		
OPC 2 Mild disability			•		
OPC 1 Normal	••••••	•		••••	•••

Each dot represents one rat. IC, ice-cold flush; RTpre, pre-treatment, room-temperature flush; RTpost, post-treatment, room-temperature flush; CPB, cardiopulmonary bypass control group.

On a subcellular level, after its phagocytosis by macrophages, clodronate causes collapse of the mitochondrial membrane potential via inhibition of the ADP/ATP translocase by its metabolite AppCCl2p, resulting in delayed apoptosis.[34]

The effects of direct injection of LEC into tissues have not yet been fully explored. An intraparenchymal injection of LEC was used to induce macrophage depletion in rat testes. After local injection, the number of residing macrophages in testes were reduced at least by 90–97% at 14 d after injection, with repopulation observed at 60 d.[35] These results suggest that the local spread of the liposomes and

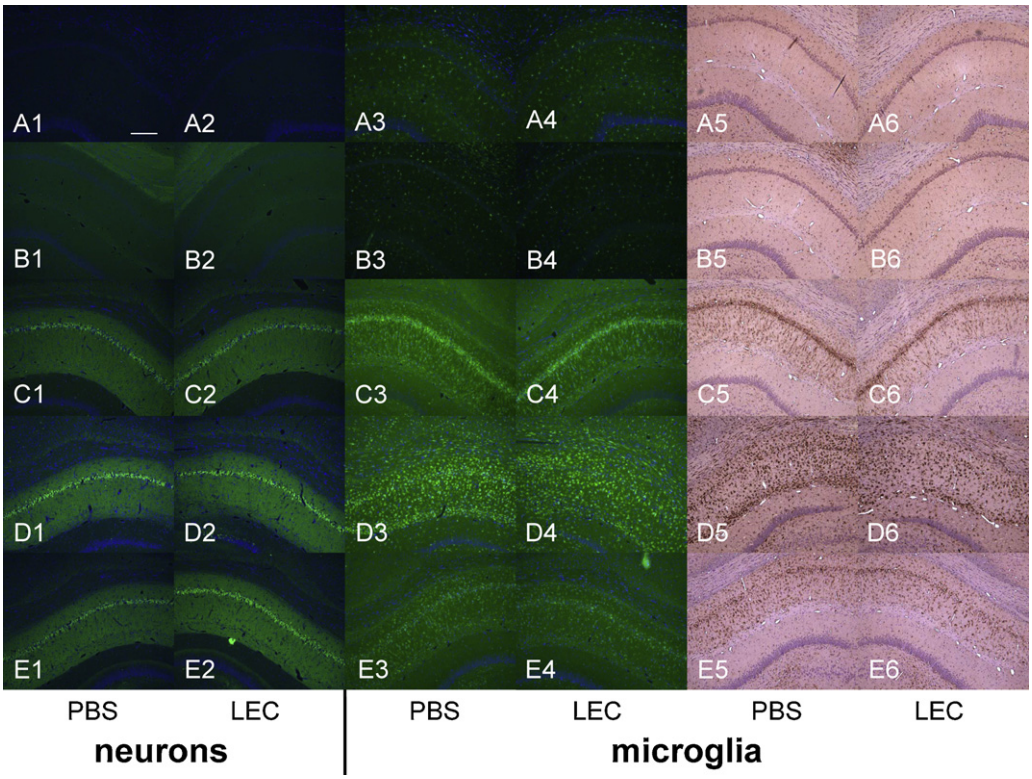

Fig. 8. Histological outcome after prolonged hypothermic cardiac arrest. Representative samples of five animals are displayed, each row showing one animal from each individual group (A–E): A, shams; B, CPB controls; C, IC group; D, RTpre group; E, RTpost group. The two left columns (1–2) show degenerating neurons as visualized by the Fluoro-Jade C staining, in the left and right CA1 regions of hippocampus, respectively. The four right columns show microglial proliferation and activation detected by anti-Iba-1 FITC staining and visualized by fluorescence (columns 3 and 4) or with DAB and hematoxylin counterstaining (columns 5 and 6). Both shams and CPB controls showed no neuronal death and minimal microglial staining. All groups subjected to cardiac arrest showed extensive neuronal degeneration with no differences between groups and between left and right hemispheres in the individual groups. The microglial proliferation was significantly attenuated in all groups subjected to cardiac arrest in the right hippocampus, the site of LEC injection, vs. the corresponding left hippocampus, where PBS was injected. Blue staining in columns 1–4 is DAPI, identifying nuclei of intact neurons. Scale bar in panel A1 = 200 μm.

the depletion of tissue macrophages could be slower compared to rather rapid uptake of liposomes by circulating macrophages.

Stereotactic administration of LEC into hippocampus allowed us to selectively deplete resident microglia either before or after the insult. However, although successful, microglial depletion did not attenuate neuronal degeneration in CA1 region of hippocampus – a site of selective vulnerability that is accompanied by robust microglial activation and proliferation in our model.[10] While we achieved significant microglial depletion at the area of injection, the exact distribution of LEC-induced microglia depletion could not be determined in our model.

It is plausible that microglia could mediate bi-phasic effects – initially contributing to secondary injury, while later being neuroprotective. In an *in vitro* study, LEC depleted microglia and inhibited microglial secretion of pro-inflammatory cytokines and NO in excitotoxically injured organotypic hippocampal slice cultures.[36] Our newly developed method will enable us to study in the future the effects of microglia in temporal sequence.

Other methods of microglial depletion have been tested previously. Ganciclovir-treated transgenic mice that express a mutant form of herpes simplex virus type I thymidine kinase driven by a myeloid-specific CD11b promoter show 75% reduction in

Table 3
Cell counts showing neuronal degeneration and microglial proliferation after prolonged cardiac arrest and in control groups. Control groups did not receive intrahippocampal injection. The numbers represent the sum of all counted cells from all animals in individual groups.

Staining	Neuronal death FJC			Microglial proliferation FITC			DAB		
Treatment/ratio	PBS	LEC	Ratio	PBS	LEC	Ratio	PBS	LEC	Ratio
Treated groups									
IC, $n=6$	892	921	1.03	1520	1011	0.67	1731	993	0.57
RTpre, $n=3$	453	405	0.89	677	406	0.6	1474	838	0.57
RTpost, $n=3$	312	327	1.04	346	187	0.54	560	362	0.65
Sum of all treated groups	1657	1653	1	2543	1604	0.63*	3765	2193	0.58*
	0% difference in FJC			37% attenuation per FITC			42% attenuation per DAB		
Control groups	–	–		–	–		–	–	
sham, $n=4$	0	0		22	26		43	35	
CPB, $n=3$	0	0		67	54		186	193	

IC, ice-cold flush; RTpre, pre-treatment, room-temperature flush; RTpost, post-treatment, room-temperature flush; CPB, cardiopulmonary bypass control group.
* $P<0.05$ PBS vs. LEC.

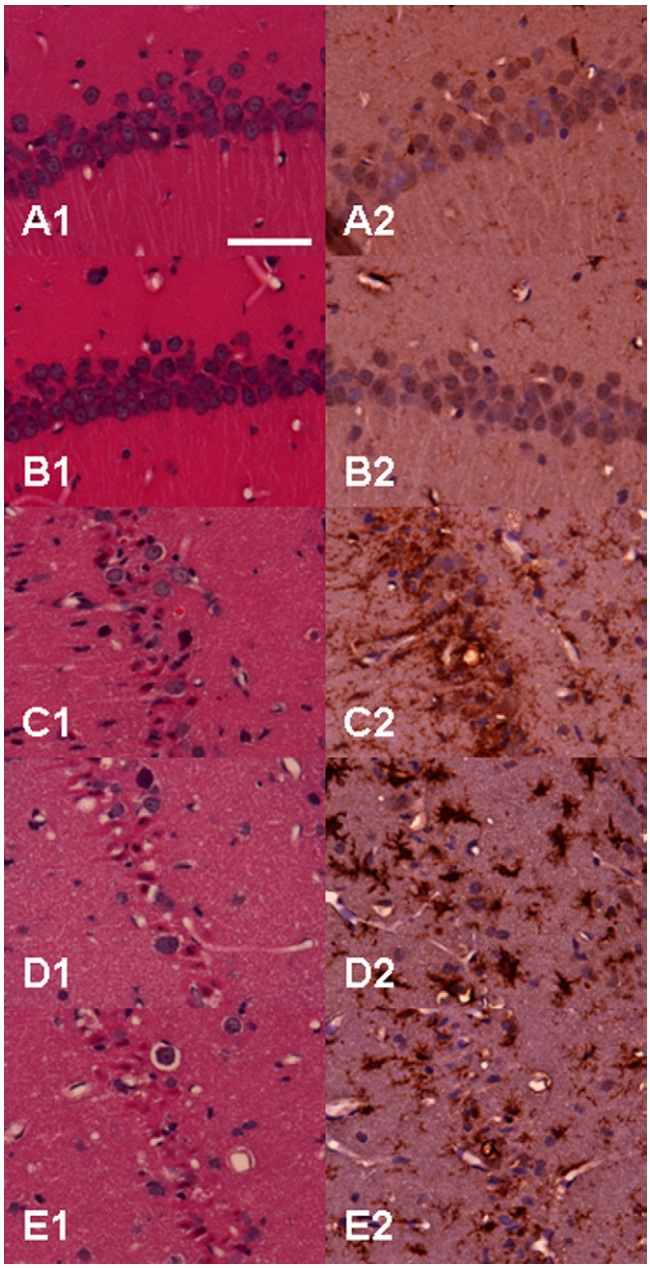

Fig. 9. Histological outcome after prolonged hypothermic cardiac arrest. Same animals and groups as in Fig. 8 are shown here under high-power magnification (40×). No neuronal degeneration is seen in shams (A1) and CPB controls (B1), while large number of injured neurons with shrunken, acidophilic cytoplasm and pyknotic nuclei are present in the groups subjected to cardiac arrest (C1–E1). None or only a few scattered quiescent microglia are seen in shams (A2) or CPB controls (B2). In contrast, proliferated microglia with bushy appearance surround the degenerating neurons in the CA1 layer of hippocampus. Panels A1–E1, hematoxylin and eosin; panels A2–E2, DAB and hematoxylin. Scale bar in panel A1 = 50 μm.

proliferating microglia after nerve injury.[37] Using this technique in a brain ischemia model, a ~40% reduction of Iba-1 immunoreactivity was noted at 72 h, which is similar to the ~40% reduction observed with our technique. Mac-2, an alternative marker of activated/proliferating microglia, showed even higher reduction rate, up to 65%.[3] However, technical limitations of currently available experimental CPB techniques do not allow CPB-assisted resuscitation, an integral part of our paradigm, in a mouse model. Rats are currently the smallest animal that can be resuscitated with CPB. Thus, it was necessary for us to explore alternative methods of microglial depletion to study the effects of microglia after CA in our rat model.

Minocycline has been widely used to deplete microglia in models of neuroinflammatory diseases and brain ischemia in rats.[5] While the effects of minocycline in neuroinflammation have been generally positive, the outcomes of studies in brain ischemia models have been controversial. We and others showed minocycline-induced neuroprotection in both experimental[6,38,39] and clinical settings.[40] In contrast, others reported that selective ablation of proliferating microglial cells exacerbates ischemic brain injury.[3] Minocycline effects are also non-specific. In a spinal cord injury model, minocycline reduced delayed oligodendrocyte death and attenuated axonal dieback, thus improving functional outcome.[41] However, the effect of minocycline on oligodendrocytes could affect the neurological outcome by mechanisms other than microglial depletion.[42,43] We have previously reported that our model of hypothermic CA is associated with persistent neurological and motor deficits.[10,44] Minocycline was only marginally beneficial.[10] These controversies underscore the need for a treatment paradigm that would enable us to study selective microglial depletion in a rat model.

Brain ischemia can also result in an early post-insult of the ischemic lesion by polymorphonuclear neutrophils (PMN) that could aggravate the injury. Microglia were previously shown to be neuroprotective against invading PMNs via their engulfment in an *in vitro* model.[45] It is not clear whether LEC had any effect on PMNs in our model, but it seems unlikely given the small dose of LEC used in our study. Studies aimed at systemic depletion of PMN used repeated doses of LEC to achieve complete depletion of PMN.

We explored both pre-treatment and post-treatment with LEC in a brain region that previously showed an extensive damage in our model, using a proof-of-concept approach in which each animal served as its own control (only one hemisphere was treated with LEC). Only the intra-arrest temperature, but not the timing of the microglial depletion, had an effect on outcome. There may exist a certain window of opportunity for post-insult treatment given the fact that even delayed treatment with minocycline improved outcome after focal brain ischemia.[43,46,47]

Consistent with the lack of a role for microglia in affecting neuronal death in hypothermic CA, we reported that attenuation of microglial proliferation with deep hypothermia during CA in our model vs. moderate hypothermia resulted in improved neurological outcome, despite not preventing neuronal loss.[10] We were not able to demonstrate this effect in the current study. It is possible that the intrahippocampal injections, with either clodronate or PBS containing liposomes, affected the microglial activation and proliferation in all groups, and the net effect of different levels of hypothermia were not as marked as they were in intact tissues. We did not use intrahippocampal injections in our control groups. We cannot exclude the possibility that intrahippocampal injections alone would produce a certain degree of gliosis and/or neuronal degeneration.

We did not explore the possibility of neurogenesis in our model. It has been postulated that delayed neuronal death is complete within 5–7 days. Several studies in both global and focal cerebral ischemia reported neurogenesis and/or synaptogenesis after an ischemic insult.[48,49] However, neurogenesis starts at the subgranular zone of the dentate gyrus, and becomes maximal at two weeks after the insult.[50] Based on these studies, we chose a 7 day outcome as a timepoint when neuronal loss is complete but repopulation of CA1 is unlikely. However, the role of neurogenesis in our model and the impact of hypothermia and microglia on this process remain to be determined in future studies, using bilateral LEC-induced depletion and complex neurobehavioral assessment with Morris water maze tasks.

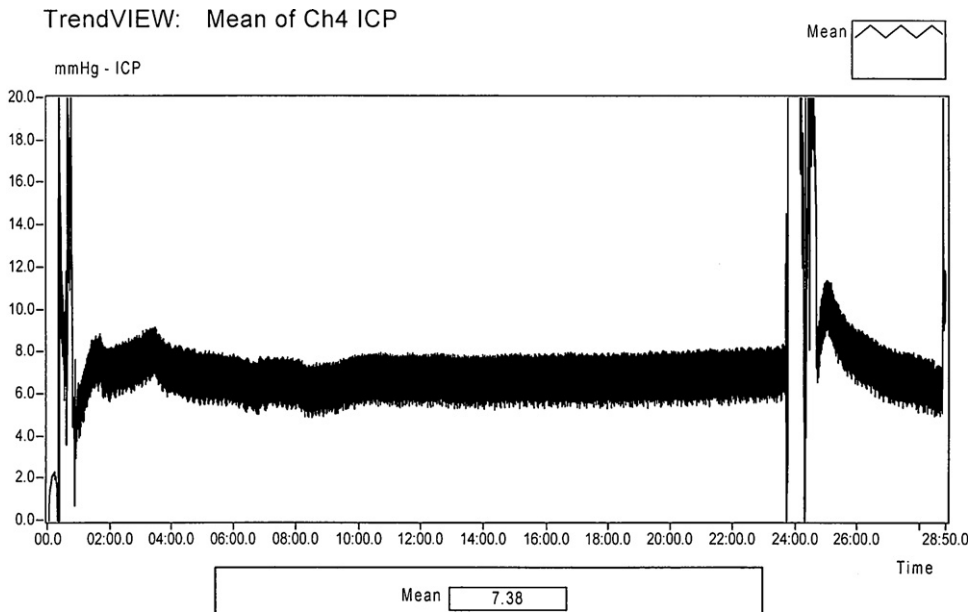

Fig. 10. Intracranial pressure during intrahippocampal injection. The tracing shows a representative sample from one rat during the simultaneous bilateral intrahippocampal injection of clodronate and PBS. No change in the intracranial pressure was observed. The tracing fluctuation seen at timepoints 0:00–1:00 and 22:00–25:00 min represent interference during the needle insertion and withdrawal, respectively.

In this feasibility trial, we showed that either pre- or post-treatment with direct injection of LEC into the hippocampus (1) attenuated local microglial proliferation in hippocampus by ~40%, and (2) did not acutely increase ICP. However, depletion did not alter neuronal degeneration in the hippocampus in our model of hypothermic CA. This suggests that microglia do not play a pivotal role in mediating neuronal death. However, LEC at the dose and treatment time chosen did not cause total ablation of microglial activity. An optimized dose and a timing of pretreatment may be needed to achieve higher depletion rate. Also, our insult produces marked loss of neurons and thus we cannot rule out the possibility that depletion of microglia could further exacerbate neuronal loss. It is also possible that the insult was too severe for LEC to have a robust impact. A detailed topographical map of microglial depletion needs to be characterized in future experiments. This novel strategy provides us with a tool to study the effects of microglia comprehensively in hypothermic CA, and in other models of neuroinflammation.

Conflict of interest statement

The authors declare that there is no conflict of interests.

Acknowledgments

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