Univerzita Karlova v Praze

1. lékařská fakulta

Autoreferát disertační práce

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

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

Abstract:

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.

Abstrakt:

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 Challenge of Traumatic Cardiac Arrest in Resuscitation

The outcomes of resuscitation of exsanguination cardiac arrest (CA) victims are especially dismal. The situation is complicated by the fact that unlike normovolemic CA, the missing volume needs to be repleted to enable resuscitation efforts to be effective. Importantly, both civilian and military trauma CA victims are most likely representing a population characterized by ***"hearts and brains too good to die".***

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.[1] 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.[2] Technically, the injuries were surgically repairable. The conventional CPR is futile because of a volume-depleted and trauma-disrupted circulatory system. Despite the fact that direct cardiac massage via thoracotomy is more effective than external chest compressions, those patients rarely survive.[3,4] The acute mortality from trauma had remained unchanged for decades.[2] More aggressive treatments with thoracotomy and aortic cross clamping did not change the gloomy outcome either.[5]

1.2. 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. They envisioned to put the victim into a state of "suspended animation".[6] Instead of trying to restart circulation in vain, preservation was proposed as the initial intervention that would buy time for transport and damage control surgery. Delayed resuscitation could then be pursued using cardiopulmonary bypass (CPB).

The key component of the Emergency Preservation and Resuscitation (EPR) method, as it was recently renamed, is imposing a state of deep hypothermia (Figure 1).[7,8]

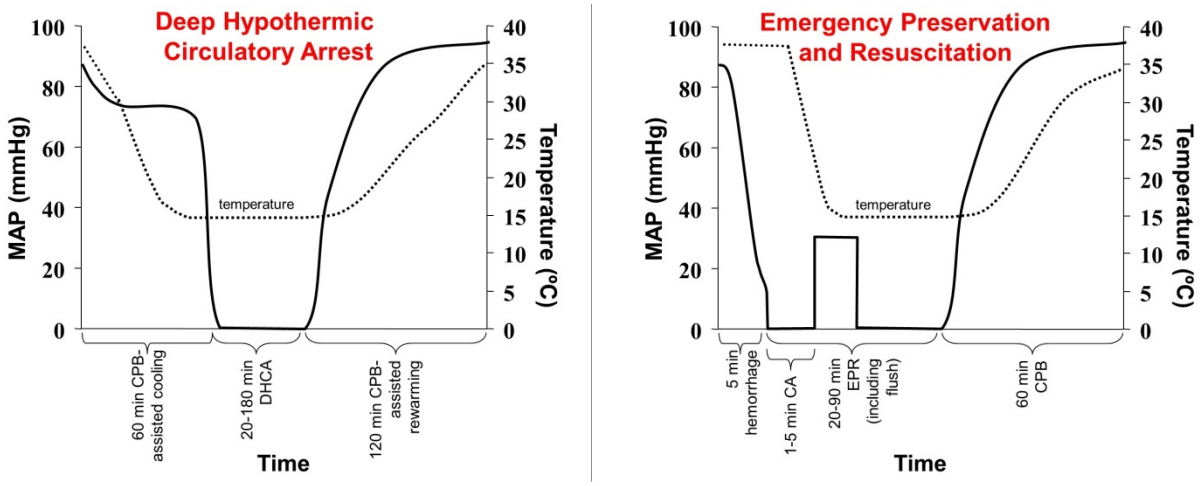

Figure 1. DHCA vs. EPR paradigm. In a traditional DHCA scenario (left panel), the protective cooling precedes the insult, i.e. a period of no-flow under hypothermia. In contrast, a clinically-relevant EPR model (right panel) should have 1) preexistence of insults at normal or near normal body temperatures, including trauma, hemorrhage, and CA; 2) trauma-disrupted circulatory system that makes fluid replacement and chest compressions futile.

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.

1.3. Development of Large Animal 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 large animal models.[2,9]

In a step-wise fashion, the feasibility of the hypothermic approach was tested in hemorrhagic shock (HS) models without CA.[10-13] This series established that the preexisting rapid HS did not obviate the chance for hypothermic preservation. In the second series, EPR was initiated after 2-5 min of CA, to reflect the time that was required for cannulation. A one-way aortic flush was used to mirror the clinical scenario of the disrupted circulatory system. This delay did not prevent favorable outcome. Survival of up to 120 min of hypothermic CA was feasible, with a strong relationship with the level of hypothermia.[14]

The success of EPR paradigm in non-trauma models prompted an exploration of applicability of **EPR in trauma settings**, including thoracotomy, laparotomy, and splenic transection into the EPR model.[15] Despite complex injuries, 60 min of CA plus severe trauma led to intact survival in ~ 50% of animals, while the rest developed multiple organ failure (MOF). However, there was almost no definitive histological damage of the central nervous system (CNS), suggesting that should the extracerebral injuries be ameliorated, the animals could functionally recover.

Alam et al. at the Harvard University independently confirmed the efficacy of EPR method in a pig model with prolonged HS and complex vascular,[16,17] splenic and colon injuries.[18]

1.4. The Exploration of Pharmacological Adjuncts to Hypothermia

We also tested the effects of **14 different promising drugs to augment the protective effects of hypothermia,** divided into **six mechanistic strategies**: (1) delaying energy failure; (2) protecting cell membrane integrity; (3) preventing structural degradation; (4) regulating protein synthesis; (5) preventing reoxygenation injuries; and (6) preserving mitochondria. Unfortunately, none of the 14 tested therapeutical adjuncts granted a breakthrough effect,[6] except the antioxidant tempol.[19] Similarly, novel organ-preservation solutions failed to augment hypothermia induced with normal saline.[9]

1.5. Development of a Small Animal EPR Experimental Model

The dog model was used initially 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 experiments pose a severe obstacle to rapid

screening of the drugs that showed promise in providing additional protection beyond the effects of hypothermia itself.

Regarding the aforementioned limitations of the large animal model, we have decided to establish a rodent model of EPR, using a miniaturized custom-designed CPB.[20,21] 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 in 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.

2. Aims and Hypotheses

In the following series of large and small animal studies we have sequentially pursued several **hypotheses** that fit 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 HS. We hypothesized that EPR will be effective after prolonged HS, while conventional resuscitation will be futile.

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

Adding energy substrates (oxygen and/or glucose) during preservation will allow extending the duration of EPR associated with good outcome.

2.3. Study III – Rat EPR feasibility study

Survival from rapid exsanguination followed by 20 min of deep hypothermic CA including CPB-assisted resuscitation is achievable. In contrast, survival with normothermic EPR and CPB-assisted resuscitation is unlikely.

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

Extending durations of no-flow will result in worse outcome in a time-dependent manner.

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

Extended EPR will be associated with activation of two secondary injury cascades in brain, namely protein nitration and poly (ADP-ribose) polymerase (PARP) activation.

2.6. Study VI – DADLE in EPR in rats

Addition of a delta-opioid agonist would confer additional protection in individual organs including CNS, and improve outcome.

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

Extending the period of normothermic CA prior to preservation, and different levels of intra-arrest hypothermia will result in neurologic deficits and neuroinflammation. Minocycline showed benefits in neuroinflammatory diseases. Deeper level of hypothermia and minocycline will attenuate CNS injuries and improve outcome.

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

The effect of drugs in our EPR model has been limited. 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 could deplete microglia, attenuate neuronal death and improve outcome from EPR.

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

Microglia are a major source of cytokines. We hypothesized 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

3.1. Study I – Prolonged hemorrhagic shock in dogs

Background: Induction of profound hypothermia for EPR of trauma victims who experience exsanguination CA may allow survival from otherwise-lethal injuries. Previously, we achieved intact survival of dogs from 2 hours of EPR after rapid HS. We tested the hypothesis that EPR would achieve good outcome even if prolonged HS preceded CA.

Methods and Results: Two minutes after CA from prolonged HS and splenic transection, dogs were randomized into 3 groups (n=7 each): (1) the 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 10-15 °C. CPR or EPR lasted 60 minutes and was followed in all groups by a 2-h resuscitation by CPB. Splenectomy was then performed. The CPR dogs were maintained at 38 °C. In the EPR groups, mild hypothermia (34 °C) was maintained for either 12 (EPR-I) or 36 (EPR-II) h. Function and brain histology were evaluated 60 h after rewarming in all dogs. CA occurred after 124±16 min of HS. In the CPR group, spontaneous circulation could not be restored without CPB; none survived. Twelve of 14 EPR dogs survived. Compared with the EPR-I group, the EPR-II group had better final OPC, NDS and HDS.

Conclusions: EPR is superior to conventional CPR in facilitating normal recovery after CA from trauma and prolonged HS. Prolonged mild hypothermia after EPR was critical for achieving intact neurological outcomes.

Discussion: 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. Extensive neocortical laminar necrosis was found; preexisting prolonged HS probably set the stage for this delayed neurological deterioration. Prolonged hypothermia ameliorated this phenomenon. We speculate that the mechanism of this delayed neurological deterioration may be cytotoxic brain edema that peaks at ~48 h after reperfusion.[22] Hyperglycemia may have contributed to the delayed neurological deterioration. Hypothermia appears to be effective in protecting against ischemic brain injury during hyperglycemia.[23] The use of mild hypothermia needs to be optimized and targeted to the insult.

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

Background: 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.

Methods and Results: Dogs underwent rapid ExCA. Two min 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 Tty of 8 °C. At 3 h after CA onset, delayed resuscitation was initiated with CPB. At 72 h, all dogs in the O+G+ group regained consciousness, and the group had better OPC and NDS 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 HDS in the O-G+ was worse than the other three groups ($P<0.05$).

Conclusions: EPR induced with a flush solution containing oxygen and glucose allowed satisfactory recovery of neurological function after 3 h of CA, suggesting benefit from substrate delivery during induction or maintenance of a profound hypothermic CA.

Discussion: Previously screened 14 pharmacological adjuncts failed to augment the benefits of hypothermia in EPR paradigm, suggesting a need to consider alternative approaches. 2 h of DHCA in sheep could be achieved with preservation of brain high-energy phosphate levels *via* intermittent infusion of a crystalloid solution containing dissolved oxygen and 2.5%

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

Figure 2 Final overall performance category (OPC) at 72 h after 3 h of CA. See the text for description of groups.

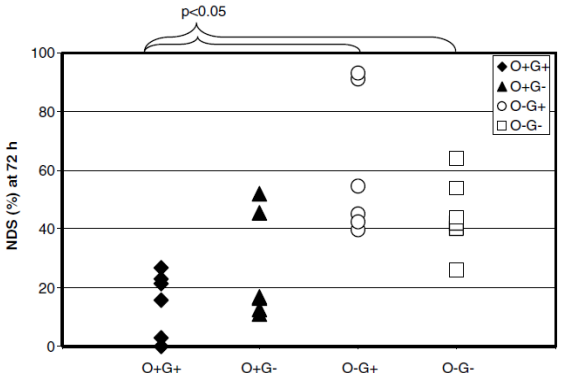

Figure 3 Final Neurologic Deficit Score (NDS) at 72 h after 3 h of CA. See the text for description of groups.

dextrose.[24] The affinity of hemoglobin for oxygen is greatly enhanced in hypothermia, restricting delivery, and increasing the importance of dissolved oxygen.[25] Hyperoxia before DHCA can take advantage of enhanced oxygen solubility and reduced metabolic demands of hypothermia to prevent tissue injury.[26] Deep hypothermia also probably protected against hyperoxic injury that is associated with worse outcome after CA.[27] While we documented an improved outcome, the underpinning mechanism(s) remained to be identified.

3.3. Study III – Rat EPR feasibility study

Background: 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.

Methods and Results: Rats were subjected to rapid HS and 1 min of CA. Three groups (n=6) were studied: (1) hypothermic EPR (H-EPR, 0 °C flush, target Tty 15 °C); (2) normothermic

EPR (N-EPR, 38°C flush); (3) controls. After 20 min of H-EPR or N-EPR, resuscitation was initiated with CPB over 60 min and mechanical ventilation. Controls were subjected to complete experimental preparation and anesthesia without CA followed by 60 min of CPB and mechanical ventilation. Surviving rats were extubated 2 h later. Survival, OPC, NDS, HDS and biochemistry were assessed on day 7. All rats in H-EPR and control groups survived, while none of the rats in N-EPR group had ROSC. All rats in the H-EPR and control groups achieved OPC 1, normal NDS, and normal or near normal HDS and biochemical markers of organ injury.

Conclusions: In this feasibility study in rats, we showed that survival from rapid HS followed by 20 min of deep hypothermic EPR 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. This should facilitate application of molecular tools to study the effects of hypothermic preservation and reperfusion, and to screen novel pharmacological adjuncts.

Discussion: Minimal impairments in NDS in H-EPR and control groups were mostly motor deficits, presumably caused by peripheral nerve injury. Evidence of degeneration within the brains of H-EPR group rats was minimal. No brain lesions were present in control rats.

The use of CPB itself has been reported to produce neurologic deficits. In our study, we did not detect major deficits. 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[28] might have revealed subtle differences between H-EPR and control groups. Extending the duration of EPR may be needed to produce a model with CNS injury.

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

Background: We previously reported that 20 min of EPR was feasible with intact outcome. In this report, we tested the limits for EPR in rats.

Methods and Results: Rats were subjected to rapid HS and 1 min of no-flow. EPR was then induced to decrease body temperature to 15 °C. After 60 min or 75 min (n=7 each) of EPR, resuscitation was attempted with CPB over 60 min. Survival, OPC, NDS and HDS were assessed in survivors on day 3. While all rats after 60 min EPR survived, only 2 out of 7 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 were not statistically different after 60 vs. 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.

Conclusions: We have shown that 60 min of EPR after exsanguination CA is associated with survival and favorable neurologic outcome, while 75 min of EPR results in significant mortality and neurological damage in survivors. Surprisingly, extracerebral lesions predominated at 75 min EPR group.

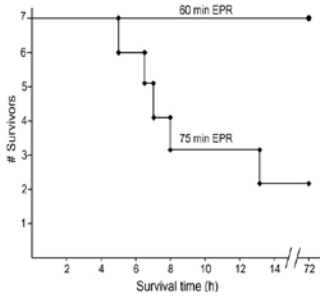

Figure 4. Survival following ExCA treated by 60 min or 75 min EPR. p=0.007, 60 vs. 75 min EPR groups.

Discussion: 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, MOF, high mortality rate and unfavorable neurological outcome in survivors. HDS did not differ between 60 and 75 min groups in any **individual region of the brain** or in the **brain overall**. Further extension of normothermic no-flow before induction of EPR may be needed to induce neuronal injury.

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

Background: We sought to explore what potential secondary injury cascades in brain may play role in the neurologic deficits. We hypothesized that 75 min but not 60 min of EPR would be associated with protein nitration and PARP activation.

Methods and Results: We used our model described in Study IV, with 24 h outcome. 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. 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 64kD band showed that nitration and PARP activation were significantly increased in hippocampus, cortex and striatum in the 75 min EPR group vs. other groups, but not 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.

Discussion: Richards et al.[29] reported an important role for nitration of pyruvate dehydrogenase and other targets after CA, while PARP activation has been shown to have a key role in limiting cerebral recovery across a variety of insults.[30,31] One possibility is that deep hypothermia protects against these mechanisms during EPR, but this protective effect was lost when the ischemic time was extended. 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.

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 was predominantly detected in vascular endothelium** by immunohistochemical staining.

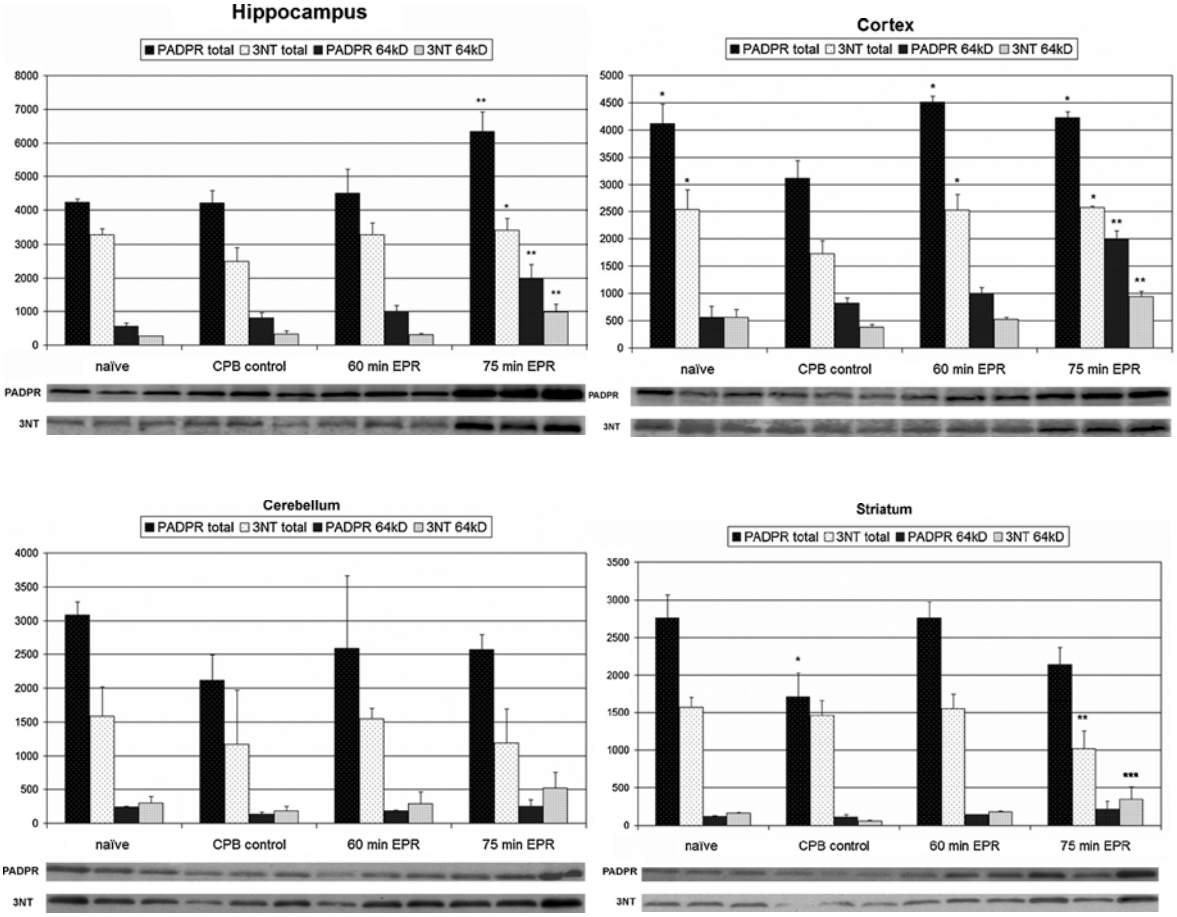

Figure 5 Relative optical densities of Western blotting analyses of nitrotyrosine (3NT) and poly (ADP-ribose) polymers (PADPR) in individual brain regions of each group. **P* < 0.05 vs. naïve and 60 min EPR group; ***P* < 0.05 vs. naïve; ****P* < 0.05 vs. CPB group. Bottom panel shows the Western blot of the 64 kD band. CPB, cardiopulmonary bypass; EPR, emergency preservation and resuscitation.

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

Background: The cause of death after 75 min EPR was likely combination of MOF and CNS injury. Thus, 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 prolonged EPR in rats.

Methods and Results: We used the 75 min EPR model described in Study IV. 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. 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.

Conclusions: DADLE failed to confer substantial benefit on functional or histological outcome in our model of prolonged rat EPR.

Discussion: DADLE, has been implicated as a novel hibernation-inducing trigger (HIT)[32] that also possesses organ-protective capabilities.[33] The underlying mechanisms of action of DADLE are probably complex, mediated via delta 1 and 2 receptors, incl. actions on K_{ATP} channels, protein-kinase C, ERK or p38 MAPK. The dose regimen used in our study was adopted from previous studies using pre-treatment. The timing of DADLE administration in our study might not be optimal in terms of providing sufficient time for the drug to induce protection. However, pre-treatment in the CA models would lack clinical relevance.

We did not determine drugs levels in blood or target tissues. The BBB penetration of DADLE was shown to be limited.

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

Background: 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 and Results: **R**ats were subjected to a lethal HS. After 5 min of no-flow, hypothermia was induced with aortic flush. Three groups were studied: ice-cold (IC) flush, room-temperature (RT) flush, and RT flush followed by minocycline treatment (M). After 20 min of CA, resuscitation was achieved *via* CPB. Survival, OPC, NDS, neuronal death and microglial proliferation in hippocampus were assessed at 72 h. Rats in the IC group had lower Tty during CA vs. other groups (IC, 20.9±1.3 °C; RT 28.4±0.6 °C; M, 28.3±0.7 °C; p<0.001). While survival was similar in all groups (RT, 6/9; IC, 6/7; M, 6/11), neurological outcome was better in the IC group vs. other groups (OPC: IC, 1±1; RT, 3±1; M, 2±1; p<0.05; NDS: IC, 8±9%; RT, 55±19%; 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 vs. other groups (p<0.01).

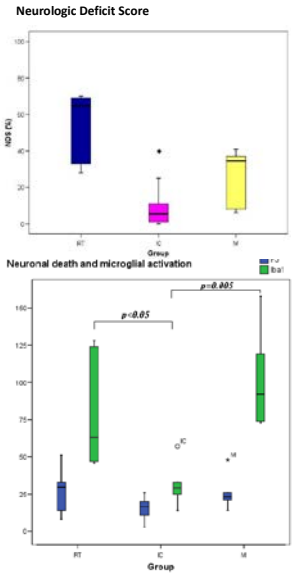

Figure 6. Top: NDS; bottom: neuronal death (blue) and microglial proliferation (green) after 20-min EPR.

Conclusions: Deeper levels of hypothermia induced by the IC vs. 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 results suggest a novel effect of deep hypothermia on microglial proliferation during ExCA.

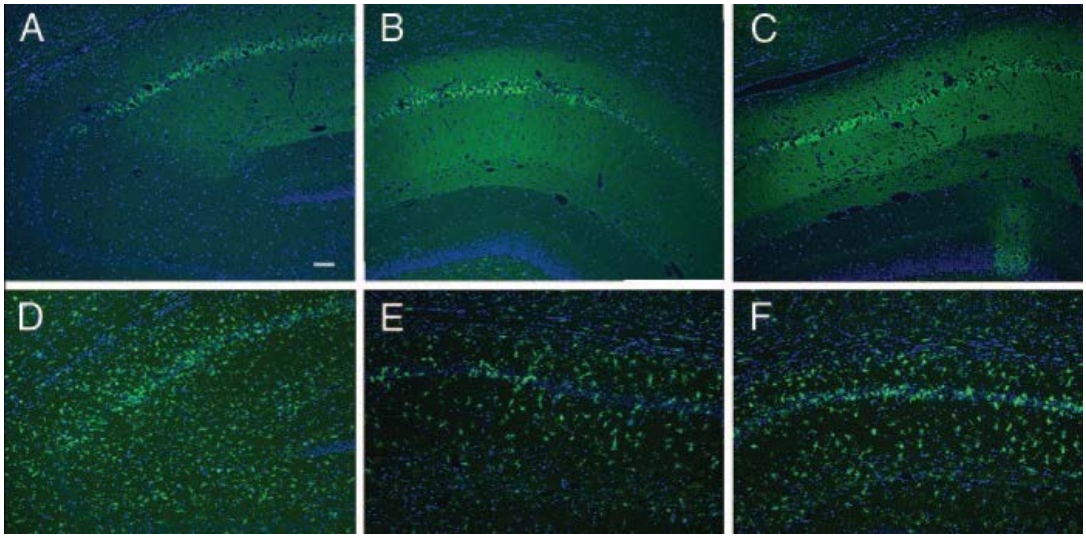

Figure 7. Neuronal death and microglial proliferation after ExCA and EPR with either room-temperature (RT) or ice-cold (IC) flush in the **CA1 region of hippocampus.** Blue staining is DAPI, identifying neurons, and green staining is FJC, Panels A–C, identifying dying neurons, or anti-Iba-1 staining 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 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 M group despite high dose minocycline treatment. Scale bar in Panel A = 80 μm.

Discussion: 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.[34] 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. It is possible that hypothermia-induced attenuation of microglial activation contributes to the improved neurologic outcome in the IC group.

Microglia could also have a protective role,[35-39] possibly in delayed repair after injury via elaboration of growth factors.

We did not see any effect of minocycline on microglia activation or neuronal death. It is possible that minocycline could not add further benefit to hypothermia. Previous studies suggested that the onset of microglial activation starts at 24 h and peaks at 4 to 7 days.[40] 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.

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

Background: 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.

Methods and Results: 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 the TBI group had a controlled cortical impact to the left hemisphere. Naïves were subjected to the same anesthesia and surgery. One h after the insult, rats were injected with EB, a marker of BBB permeability for albumin. EB absorbance was quantified in brain samples harvested after 5 h. 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.

Conclusions: 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.

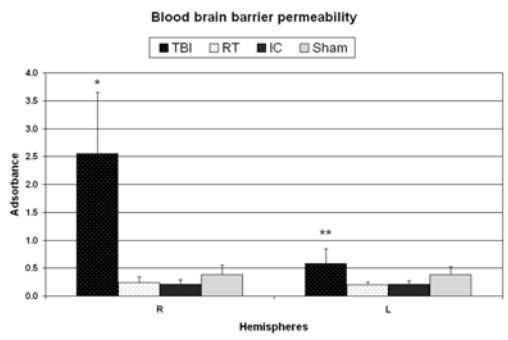

Figure 8. Absorbance (left) and coronal brain sections (top) at the level of the dorsal hippocampus that reveal obvious Evans Blue extravasation after TBI (left panel), but not in EPR-RT flush (central panel) or sham groups (right panel).

Discussion: Remarkably, the status of the BBB in both CA and deep hypothermia remains unclear. In contrast, BBB disruption is well-recognized after TBI. 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.[41-44] However, other smaller molecules, such as amino isobutyric acid,[45] or gadolinium-based agents[45] could be more sensitive to subtle injury.

3.9. Study IX – Clodronate-induced depletion of microglia

Background: Intact survival from EPR 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.

Methods and Results: The model used in Study VII was used. 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.

Conclusions: 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.

Discussion: There is a large body of evidence documenting that microglia are a source of multiple potentially cytotoxic substances.[46] Attenuation of microglial activation has shown benefit in multiple CNS injuries and neuroinflammatory diseases.[47] In contrast, ablation of microglia in stroke models had detrimental effects,[37] and administration of exogenous microglia was neuroprotective,[48] possibly linked to the production of neurotrophic factors.[49] The exact distribution of LEC-induced microglia depletion could not be determined in our model. 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.

3.10. Study X - Cytokines after hypothermic CA in rats

Background: 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.

Methods and Results: The EPR model described in Study VII and IX was used. 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.

Conclusions: 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.

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,[50] representing a valid therapeutic target that needs to be explored.

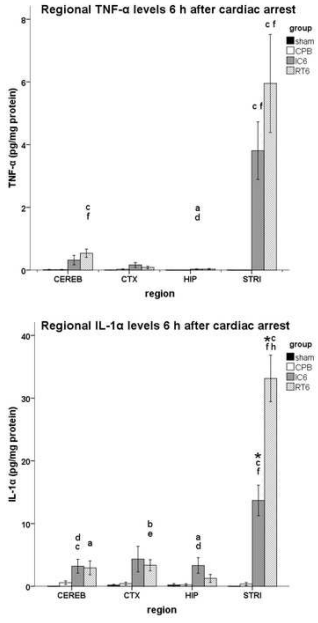

Figure 9. Regional TNF-α and IL-1α levels at 6 h after prolonged cardiac arrest. Marked isolated early increase of TNF-α was observed in striatum in both cardiac arrest groups. This was attenuated by deeper hypothermia. Mean±SEM. CEREB, cerebellum; CTX, cortex; HIP, hippocampus; STRI, striatum. a, p<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.

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.[51] Similarly, region-dependent upregulation of several neurotrophic factors has been seen with hypothermia after CA.[52,53]

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.[49,54,55] Other glia[56-58] and neurons[59] can produce cytokines, depending on the type of insult.[60] The source of cytokines remains to be determined and is a subject of our current work.

4. Conclusions

EPR is a novel method for resuscitation of exsanguination CA victims utilizing ***hypothermia*** to induce ***emergency 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, we have sequentially explored several **hypotheses**, with following **results**:

Study I: ***Extended HS (~ 2 hours) treated by EPR resulted 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: Providing ***energy substrates***, i.e. oxygen and/or glucose ***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: ***20 min EPR at 15 °C in rats is feasible.*** In contrast, identical insult treated with normothermic EPR or normothermic CPB-assisted resuscitation was lethal.

Study IV: Extension of the hypothermic arrest to ***60 min but not 75 min of EPR was associated with favorable outcome***. However, the mortality was most likely due to MOF, since comprehensive neuropathology showed only limited CNS damage.

Study V: Extended EPR was associated with activation of two potential secondary injury cascades in brain, namely ***protein nitration and PARP activation.***

Study VI: Delta-opioid agonist ***DADLE failed to confer benefit*** on functional or histological outcome in our rat model of prolonged EPR.

Study VII: Extending the period of normothermic CA prior to flush, and increasing the intra-arrest temperature from deep to moderate hypothermia resulted in neurologic deficits, neuronal degeneration and neuroinflammation. ***Deeper level of hypothermia was associated***

with attenuated microglial activation independent of extensive hippocampal neuronal death that was similar across groups. ***Minocycline failed*** to confer additional benefits.

Study VIII: ***BBB was not permeable*** to albumin early after the insult. Future studies should focus on therapeutical adjuncts that readily cross the BBB.

Study IX: Intracerebrally administered clodronate induced ~ ***40% microglia depletion*** in the hippocampus. This regional, incomplete microglial ablation ***did not alter the neurologic outcome*** or ***hippocampal neuronal degeneration***, suggesting a limited role of microglia.

Study X: EPR resulted in ***significant temporo-spatial differences in cytokine signatures.*** Surprisingly, ***different cell types co-localized with individual cytokines*** across regions.

5. Summary

Induction of deep hypothermia early after normothermic ExCA seems superior to conventional CPR, which is almost inevitably futile. The drugs pale in comparison with 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. A multi-center clinical trial of EPR is being launched.

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Drabek T, Janata A, Jackson EK, End B, Stezoski J, Vagni VA, Janesko-Feldman K, Wilson CD, van Rooijen N, Tisherman SA, Kochanek PM
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Resuscitation 2012 April; 83(4):517-26. – **IF 4,177**

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