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Early Changes of Brain Perfusion After Subarachnoid Hemorrhage – the Effect of Sodium Nitroprusside

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Summary

Causes of early hypoperfusion after subarachnoid hemorrhage (SAH) include intracranial hypertension as well as vasoconstriction. The aim of the study was to assess the effect of intracerebroventricular (ICV) administration of sodium nitroprusside (SNP) on early hypoperfusion after SAH. Male Wistar rats (220-240 g) were used, SAH group received 250 µl of fresh autologous arterial blood into the prechiasmatic cistern; sham-operated animals received 250 µl of isotonic solution. Therapeutic intervention: ICV administration of 10 µg SNP; 5 µl 5 % glucose (SNP vehicle) and untreated control. Brain perfusion and invasive blood pressure were monitored for 30 min during and after induction of SAH. Despite SNP caused increase of perfusion in sham-operated animals, no response was observed in half of SAH animals. The other half developed hypotension accompanied by brain hypoperfusion. There was no difference between brain perfusion in SNP-treated, glucose-treated and untreated SAH animals during the monitored period. We did not observe expected beneficial effect of ICV administration of SNP after SAH. Moreover, half of the SNP-treated animals developed serious hypotension which led to brain hypoperfusion. This is the important finding showing that this is not the option for early management in patient after SAH.

Key words

Subarachnoid hemorrhage • Sodium nitroprusside • Nitric oxide • Early brain injury • Rat

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Introduction

Non-traumatic aneurysmal subarachnoid hemorrhage (SAH) causes both early and delayed changes of brain perfusion. The early brain injury (EBI) develops within first 72 h after aneurysm rupture (Cahill *et al.* 2006) and it is a major cause of mortality in such cases (Broderick *et al.* 1994). The arterial wall rupture causes extravasation of arterial blood into subarachnoid space under high pressure, leading to rapidly increased intracranial pressure (ICP) and reduced cerebral perfusion pressure (CPP) and cerebral blood flow (CBF) (Cahill *et al.* 2006, Sehba *et al.* 2012). Even animal models show that the early hypoperfusion is related not only to intracranial hypertension, but also to vasoconstriction associated with arterial blood present in subarachnoid space; reduction of CBF independent from increased ICP or decreased CPP was observed (Bederson *et al.* 1998, Schubert *et al.* 2009).

One of the mechanisms of early vasoconstriction is impairment of nitric oxide (NO)-mediated vasodilation. Decreased levels of NO metabolites were observed as early as 10 min after SAH induction (Sehba *et al.* 2000). The NO deficiency is probably caused by scavenging of NO (Sehba *et al.* 2012, Schwartz *et al.* 2000a,b). In physiologic conditions, the activity of NO is terminated by reaction with oxyhemoglobin which forms methemoglobin and nitrate (Helms and Kim-Shapiro 2013). Oxyhemoglobin in subarachnoid space is

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considered to be one of the major NO scavengers after SAH and thus it contributes to the early vasoconstriction (Li *et al.* 2016, Sehba *et al.* 2012).

Due to this mechanism, it is reasonable to consider NO donors for treatment of early vasoconstriction. In previous studies the cerebral vessels did respond to external administration of NO (Sehba *et al.* 1999, Sehba *et al.* 2007).

One of the NO donors studied in conditions of delayed cerebral vasospasm is sodium nitroprusside (SNP). The desired effect of SNP in the treatment of vasospasm is reached by release of NO at the adventitial side of brain circulation from the SNP molecule. Such a replacement of NO in the vascular wall attenuates the vasospasm after intrathecal SNP administration (Thomas *et al.* 1999). Beneficial effect of SNP was observed in several experimental (Egemen *et al.* 1993, Vatter *et al.* 2007) and clinical studies (Pachl *et al.* 2005, Raabe *et al.* 2002).

As far as we are aware, there were no studies with administration of sodium nitroprusside conducted under conditions of early brain injury. We hypothesized that intracerebroventricular (ICV) administration of SNP could be used as an effective treatment of the early vasospasm immediately after SAH. Because SNP is a potent vasodilator, the ICV route could help to reduce systemic side effects seen after intravenous administration, preserve its vasodilating effect on brain circulation and increase availability of NO in brain tissue.

Materials and Methods

All procedures were performed in accordance with the Ethical Guidelines of the Third Faculty of Medicine, Charles University, Prague, Czech Republic. They were in accordance with the Guidelines of the Animal Protection Law of the Czech Republic, which correspond with European Guidelines on Laboratory Animal Care. Special care was taken to minimize animal suffering.

Adult male Wistar rats (AnLab, Czech Republic; 220-240 g) were used. The animals were housed in cages by four under a 12-h light/dark cycle, with food and water *ad libitum*.

Surgical preparation of animals and induction of non-traumatic SAH

All procedures were conducted in deep general anesthesia (ketamine 100 mg/kg and midazolam 1.2 mg/kg, intraperitoneally) with additional local anesthesia of the soft tissues of the head (trimecaine 1 % 0.3 ml).

Femoral artery was cannulated to obtain blood sample for SAH induction as well as to allow invasive monitoring of arterial blood pressure. Mean arterial blood pressure (MAP) was calculated as 2/3 of diastolic pressure + 1/3 of systolic pressure. Rectal temperature was monitored and maintained at 37 °C during the whole procedure.

The skull of the animals was exposed from soft tissues. Left lateral ventricle was cannulated for ICV administration access (Brain Infusion Kit 2, Alzet, USA). The catheter was inserted -2 mm and 2 mm left from bregma and 3 mm deep from the skull surface into the cerebral ventricle (Paxinos and Watson 2013).

The skull above right hemisphere was mechanically thinned to allow the measurement of brain perfusion. Prechiasmatic cistern injection model of subarachnoid hemorrhage was used (Prunell *et al.* 2002). Briefly, a small borehole was drilled 7 mm rostral from the bregma. A needle with 26G cannula was inserted into prechiasmatic cistern at the 30° angle anteriorly. In the SAH groups, 250 µl of fresh non-heparinized autologous arterial blood (aBLD) was injected during 15 s immediately after withdrawal from femoral artery; sham animals received 250 µl of isotonic solution (ISOSOL) of body temperature.

Experimental groups

Sixty four animals were divided into six groups: 1) SAH-induced animals (SAH; n=12); 2) SAH + ICV administration of sodium nitroprusside (SAH-SNP; n=18); 3) SAH + ICV glucose 5 % (SAH-glc; n=10); 4) sham-operated animals (sham, n=8); 5) sham + ICV SNP (sham-SNP; n=8); and 6) sham + ICV glucose 5 % (sham-glc; n=8).

The SAH-SNP animals received 10 µg of sodium nitroprusside (Sigma-Aldrich) in 5 µl of 5 % glucose solution 3 min after SAH induction. If secondary decrease of perfusion occurred after previous increase (decrease >15 % of perfusion after reaching peak level), second dose of SNP solution was administered. The solution of SNP was meticulously protected from light during preparation as well as during the whole experiment.

The SAH-glc group received 5 µl of 5 % glucose solution according to the same time pattern, the SAH group remained untreated. The corresponding sham-operated groups received the treatment in the same pattern.

Evaluation of changes in the perfusion of cerebral cortex after SAH

Changes in the perfusion of cerebral cortex were assessed using Laser speckle-contrast analysis (LASCA). For detection and evaluation of changes, PeriCam PSI HR with PimSoft software (Perimed, Sweden) was used. The region of interest (ROI) for perfusion measurement was placed above the right hemisphere and it was limited by sagittal suture and lateral ridge, caudally by lambdoid suture and exceeded coronal suture for 3 mm in rostral direction. The tissue perfusion was measured in perfusion units (PU) standardized by manufacturer. Because the PUs cannot be converted to ml/min/100 g, changes in perfusion were described in percentage of PU deviation from baseline record. The baseline levels were obtained from measurement performed in each animal during 1 min before induction of SAH. Framing speed was set as 1 image/s and the mean perfusion in the designated ROI was used for evaluation.

Statistical analysis

GraphPad Prism 6 (GraphPad Software, Inc., USA) was used for statistical evaluation. Changes in perfusion between groups were compared using two-way ANOVA for repeated measures with Bonferroni's post-test. Following changes in the perfusion of animals with SAH were analyzed using t-test: maximum values of perfusion which followed an initial drop after application of blood into prechiasmatic cistern; minimum values of perfusion decreased secondary after reaching maximum; as well as changes in mean arterial blood pressure and perfusion in groups treated with SNP.

Level of statistical significance was set to $p<0.05$. All data are presented as mean ± SEM.

Results

Changes of brain perfusion

Intracisternal administration of both aBLD and ISOSOL was associated with significant decrease of brain perfusion.

The perfusion in the SAH group decreased to 23.3±4.5 % (mean ± SE) of baseline values during the injection of aBLD. Three of 12 studied animals developed profound hypoperfusion leading to death within 5 min after the injection. In the remaining 9 animals, the perfusion recovered slowly after initial drop. Compared to control, the SAH group showed prolonged return towards baseline values and significantly reduced perfusion within first 5 min after SAH induction (Fig. 1).

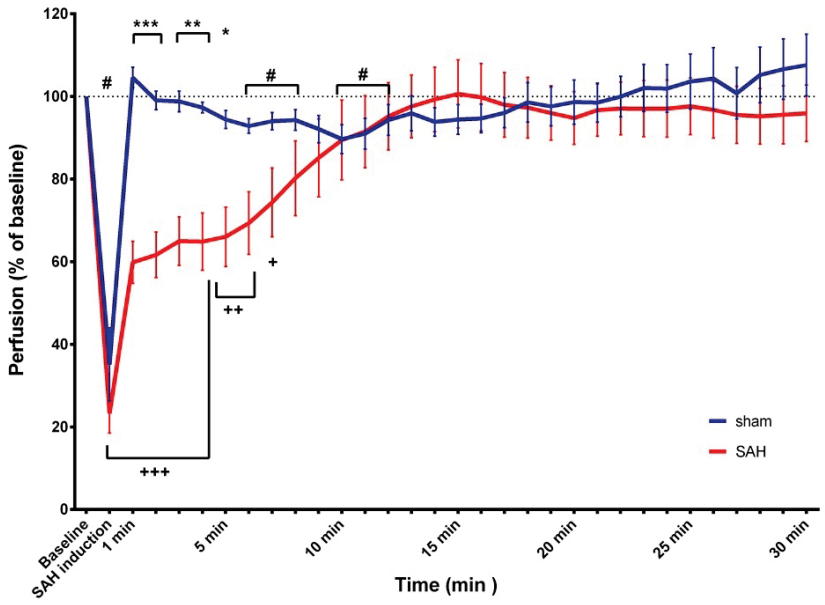

Fig. 1. Perfusion changes in SAH and sham-operated group. The return of perfusion towards baseline levels was prolonged in SAH group. The perfusion was significantly reduced during and first 7 min after SAH induction in SAH animals and at the time of intracisternal injection and 6-8 min and 10-12 min in sham-operated animals (+, # $p<0.05$, ++ $p<0.01$, +++ $p<0.001$, compared to baseline values). Compared to sham-operated animals, the perfusion was significantly lower first 5 min after SAH induction (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

The perfusion changes in SAH animals showed biphasic time-course in 8 of 9 animals. After the injection, the perfusion recovered slowly reaching its peak in 14 min 22 s ± 2 min 10 s and culminated at the level of 113±5.5 % of baseline values. This was followed by another significant decrease ($p<0.001$ and $p<0.01$, compared to peak and baseline values, respectively) in 24 min 52 s ± 1 min 36 s at the level 86.4±3 % of baseline after which the perfusion increased gradually. Similar pattern was observed in SAH-glc group, but only

in half SAH-SNP animals.

In the sham group, the initial drop in perfusion was followed by short period of hyperperfusion after which the perfusion returned toward baseline level. The perfusion was significantly reduced during the time of intracisternal injection and 6-8 min and 10-12 min in sham-operated animals, compared to baseline values (Fig. 1).

The effect of intracerebroventricular administration of sodium nitroprusside

In the study group assigned for SNP treatment after SAH, one of 18 animals died before administration of SNP and 2 died after SNP administration. The response to SNP showed two different patterns. Eight animals showed no change of MAP and the time-course of brain perfusion did not differ significantly from SAH and SAH-glc groups. The other 7 animals developed serious systemic hypotension (drop in MAP up to 50 % of pre-dosing values) which was accompanied with decrease of brain perfusion (Fig. 2). In this subgroup, the brain perfusion was significantly lower 1 min after the SNP administration, compared to non-hypotensive animals (Fig. 3).

Both normotensive and hypotensive animals showed increase of perfusion above the pre-dosing levels 3 min after the first SNP injection. Comparison with SAH-glc and SAH groups nevertheless shows that this increase of brain perfusion occurs in this time period independently from the SNP treatment.

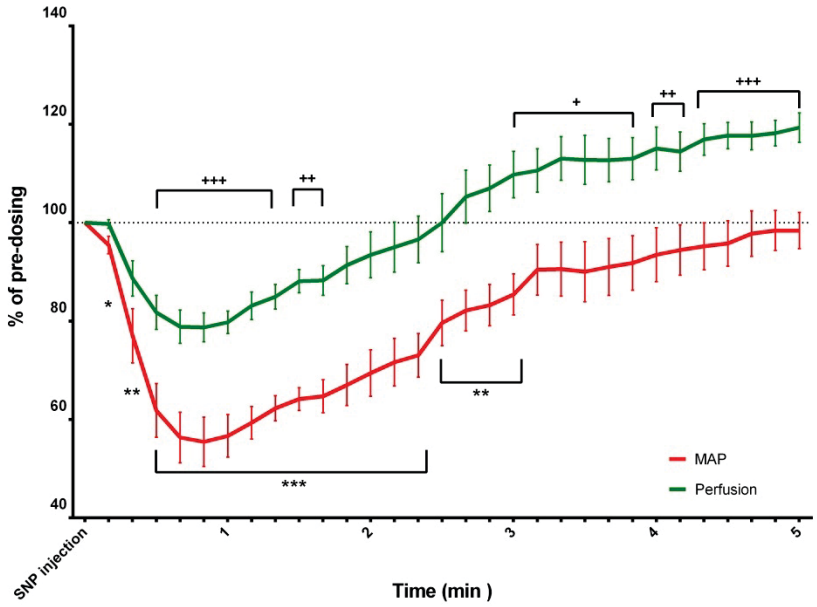

Fig. 2. Perfusion and MAP changes in SAH-SNP group after the first SNP dose. In 7 of 15 animals, SNP administration 3 min after SAH induction caused decrease of MAP, which corresponded with significant decrease of brain perfusion. Significant increase of brain perfusion above pre-dosing levels was observed 3 min after the injection; nevertheless this increase followed trends observed even in SAH and SAH-glc groups (*, + $p<0.05$, **, ++ $p<0.01$, ***, +++ $p<0.001$, compared to pre-dosing levels). In contrast to other figures, baseline is set as pre-dosing level.

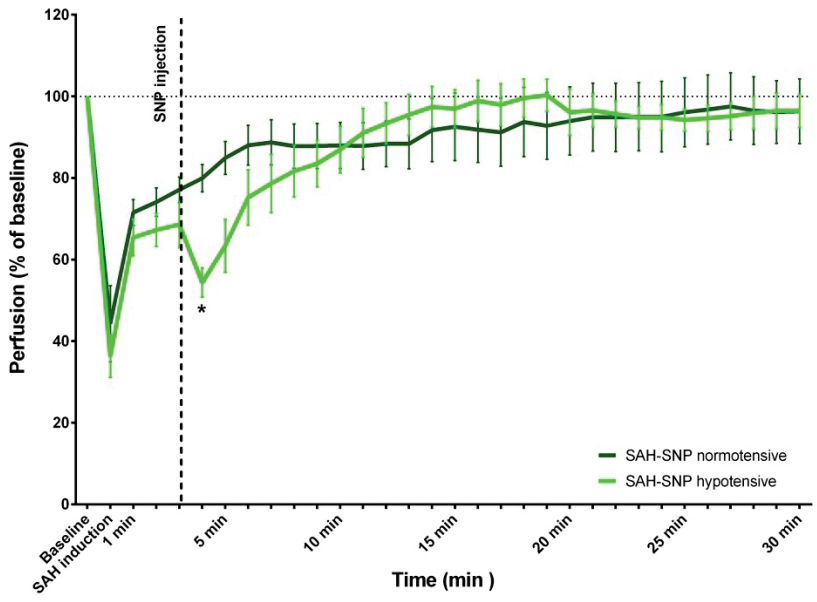

Fig. 3. Perfusion changes after SNP administration. Administration of SNP 3 min after SAH induction was followed by arterial hypotension and brain hypoperfusion in 7 of 15 animals. Brain perfusion was significantly lower 1 min after SNP injection in those animals which developed hypotension, compared to normotensive animals (* $p<0.05$).

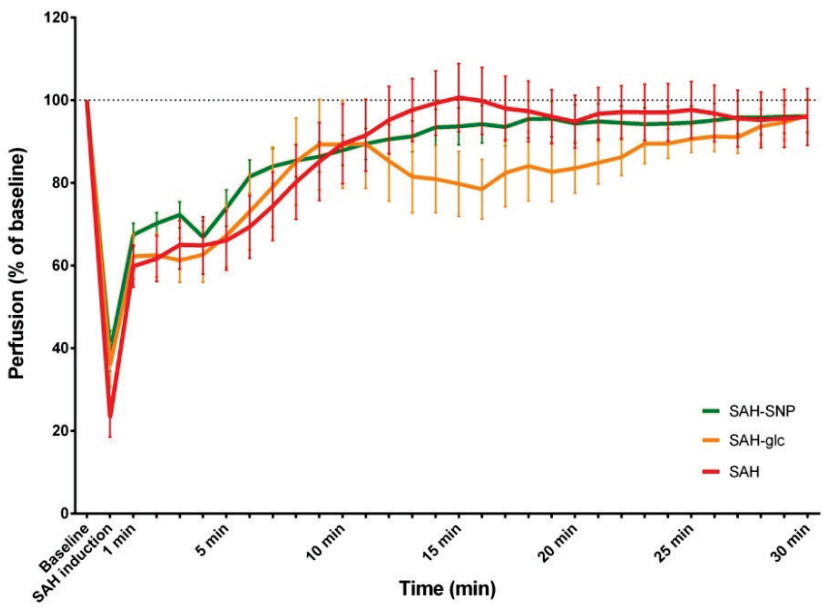

Fig. 4. Perfusion changes in groups with SAH. No statistical significance was observed between SAH, SAH-SNP and SAH-glucose groups.

Eight SNP-treated animals developed secondary decrease of perfusion after previous reaching of peak values. This decrease corresponded with biphasic time-course of changes in perfusion seen in SAH group. These animals received another ICV dose of 10 μg of SNP after secondary decrease between 11 and 25 min. Sequentially a mild but significant decrease of MAP developed with insignificant decrease of perfusion. The perfusion increased 2 min after the second SNP injection and the raise above the baseline was significant 3 min 20 s after the injection (compared to pre-dosing values). This increase of perfusion however did not reach the values observed in sham-SNP animals.

Despite mild increase of the perfusion after the second dose of SNP, we did not observe any statistically significant increase of brain perfusion in SNP-treated animals, compared to SAH and SAH-glc group during the monitored period (Fig. 4). Also no difference was observed between animals which received 1 or 2 doses (data not shown).

The effect of intracerebroventricular administration of 5 % glucose

One of 10 SAH-glc group animals died before and 2 died after the glucose injection. In 7 animals, the first dose of glucose did not cause any change of either mean arterial pressure or brain perfusion, compared to SAH group.

All 7 animals received second dose of glucose due to decrease of the perfusion after reaching the peak. The MAP remained unaffected after the injection. The injection of glucose caused transient increase of brain perfusion which was significant at 1.5 min (compared to pre-dosing values). In contrast to the SNP treated animals, the increase was observed within first 2 min after the injection. The perfusion in SAH-glc was significantly ($p<0.05$) higher between 40 s and 1 min, compared to SAH-SNP group.

Sham-operated animals

Animals in the sham-operated groups received ISOSOL instead of aBLD into the prechiasmatic cistern. Three minutes later, ICV injection of 5 μl of either SNP or G5 % solution was administered; one group received no treatment. Intracerebroventricular injection of SNP led to significant increase in brain perfusion above pre-dosing levels in 7 of 8 animals; this effect persisted till the end of the monitored period. In 1 animal, the increase of brain perfusion was achieved after repeated dose of SNP. The ICV injection of SNP was followed by mild decrease of MAP, nevertheless the brain perfusion increased in the same time. ICV injection of 5 % glucose solution did not lead to any change of the perfusion (Fig. 5).

Discussion

Our study is the first to test the effect of intracerebroventricular administration of SNP in conditions of early hypoperfusion after subarachnoid hemorrhage.

Markedly reduced cortical perfusion is one of the initial consequences of SAH. The primary decrease of perfusion after intracisternal injection of both arterial

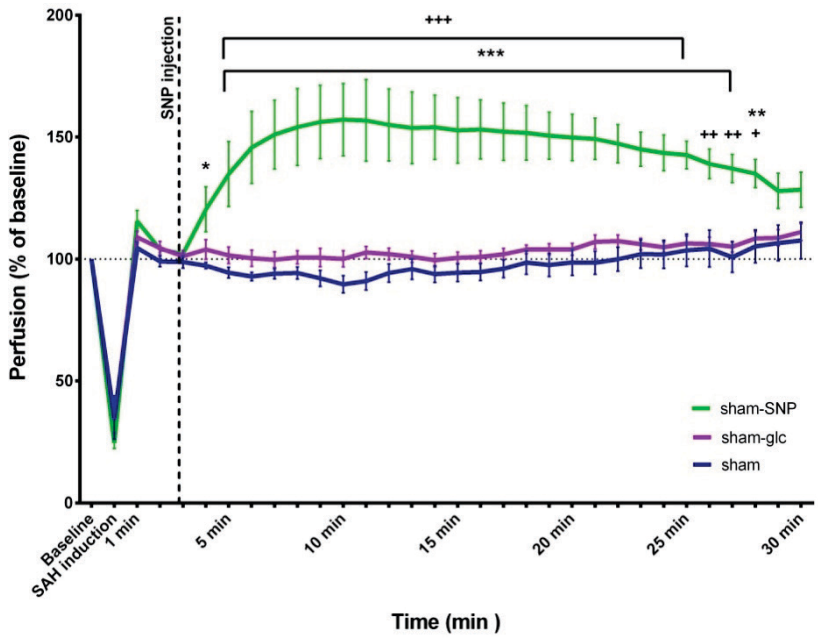

Fig. 5. Perfusion changes in sham-operated groups. Intracerebroventricular administration of SNP to the sham-operated animals significantly increased the brain perfusion (*, + p<0.05, **, ++ p<0.01, ***, +++ p<0.001; sham-SNP versus sham and sham-glc). Administration of 5 % glucose solution caused no change of perfusion.

blood and isotonic solution is caused by elevated ICP and decreased CPP (Bederson *et al.* 1995, Sehba *et al.* 2012). Nevertheless, animal studies show that the perfusion remains reduced even after CPP restore (Bederson *et al.* 1995). In clinical setting, measurement of CBF in patients suffering from aneurysmal SAH showed its reduction within the first 12 h after aneurysm rupture. That correlated with Hunt-Hess grade but not with ICP or CPP (Schubert *et al.* 2009). The protracted hypoperfusion after SAH has been described (Bederson *et al.* 1995, Schubert *et al.* 2008) and it can be attributed to early vasoconstriction occurring in presence of arterial blood in subarachnoid space.

It was suggested that hypoperfusion after SAH is caused both by intracranial hypertension and by disorders of brain macro- and microcirculation (Buhler *et al.* 2015, Sehba *et al.* 2007). Deficiency of NO in brain circulation due to its scavenging is considered to be a reason of early vasoconstriction (Sehba *et al.* 2012, Schwartz *et al.* 2000a,b). This is supported by finding of reduced levels of NO and its metabolites in the early phase after SAH (Sehba *et al.* 2000). In addition, brain vessels remain responsive to exogenous administration of NO (Sehba *et al.* 1999, Sehba *et al.* 2007). The supposed NO scavengers are hemoglobin (Sehba *et al.* 2000), superoxide anion (Kajita *et al.* 1994) or myeloperoxidase produced by neutrophils (Friedrich *et al.* 2011). Hemoglobin, a potent NO scavenger, is able to diffuse into the brain parenchyma, contact with microvessel pericytes and cause microvessel constriction (Li *et al.* 2016).

Nitric oxide donors treatment was studied in several experimental (Egemen *et al.* 1993, Vatter *et al.* 2007) and clinical (Pachl *et al.* 2005, Raabe *et al.* 2002) trials in conditions of delayed cerebral vasospasm.

Nitric oxide donors for prevention or treatment of EBI were used only in experimental settings: administration of an NO donor S-nitrosoglutathion (GSNO) was successfully used to increase CBF and preserved vascular wall integrity briefly after SAH induction (Sehba *et al.* 1999, Sehba *et al.* 2007). Another more common and affordable NO donor is sodium nitroprusside. The dose used in the present study was calculated according to the doses used in previous laboratory and clinical experiments and was adjusted on rats' body weight and volume of cerebrospinal fluid. The dose 10 μg of SNP for bolus administration was proven as most effective in preliminary experiments.

In the present study, no favorable effect was observed after administration of SNP 3 min after SAH simulation. Half of the SNP-treated animals developed brain hypoperfusion instead. This can be related to either arterial hypotension or intracranial hypertension. Disturbance of autoregulation of brain perfusion is a frequent feature of intracranial pathologies. The brain perfusion depends on CPP and MAP in conditions of impaired autoregulation (Lang *et al.* 2003) and thus arterial hypotension after SNP injection can lower the brain perfusion substantially. Intracranial hypertension can also contribute to further reduction of perfusion. Different methods of SAH induction can produce

different ICP profiles (Schwartz *et al.* 2000a,b); according to our previous yet unpublished findings, intracranial pressure 3 min after SAH induction is approx. 15-20 mm Hg in this model; similar results were obtained in another studies (Prunell *et al.* 2003). Injection of additional fluid volume (e.g. SNP) can increase ICP significantly in conditions of reduced brain compliance (Marmarou *et al.* 1975). Nevertheless this is doubted by fact that we observed no further brain perfusion decrease immediately after ICV injection of glucose solution.

Biphasic time course of the perfusion changes was observed in SAH animals. Reason for this pattern is uncertain. It can be related to the method of measurement of perfusion which includes both microcirculation and large pial vessels. Effect of SAH on vasoconstriction can differ between macro- and microcirculation (Sehba *et al.* 2012). The decrease of perfusion after previous peak can also reflect the changes in NO concentration in brain circulation (Sehba *et al.* 2000).

Mild increase of perfusion was observed after the second dose of SNP, which was administered after the second drop of perfusion. This increase did not reach the levels observed in sham-SNP group and there was no significant difference in perfusion between SAH-SNP group and SAH or SAH-glc groups. In contrast, in sham-SNP group, the ICV administration of SNP led to prompt increase of brain perfusion which persisted till the end of the monitored period. This discrepancy in the effect of SNP between SAH and sham-operated group can have more reasons. Lack of effect of ICV administration of SNP can be caused by impaired distribution of the drug in subarachnoid space. The arteries of the circle of Willis are coated with the coagulum which can prevent from diffusion of the vasodilator and thus the concentration of NO in the vessel wall does not reach adequate level (Pluta *et al.* 2009).

Another possible explanation is that the dose leading to perfusion increase in sham-SNP animals can be insufficient in situation of NO depletion and scavenging which occurs after SAH. This can be supported by the finding of mild increase of brain perfusion after the second dose of SNP, which did not reach the levels of perfusion observed in sham-SNP animals. Variable response on SNP administration, mainly after the first dose of SNP, is consistent with marked interindividual variability which was observed also in clinical studies (Agrawal *et al.* 2009, Pachl *et al.* 2005).

So far, SNP was successfully used in experiments with delayed vasospasm – dilation of spastic vessels after SNP administration was observed in experiments on isolated arteries (Vatter *et al.* 2007) as well as in animal (Egemen *et al.* 1993) and clinical studies (Pachl *et al.* 2005, Raabe *et al.* 2002). The observed effect of intrathecal SNP administration in conditions of delayed cerebral vasospasm can be explained by the degradation of blood clot and improvement of SNP penetration into the target structures. The velocity of clot resolution was estimated as 10.8 % of clot volume per day (Naff *et al.* 2001) and significant proportion of the hematoma is degraded at the time of occurrence of delayed cerebral vasospasm. Hemoglobin degradation products attribute to delayed vasospasm (Clark and Sharp 2006). Therefore, difference in mechanisms of vasoconstriction in the early and delayed phase after SAH can explain different finding in our model after SNP administration.

Hypotension after ICV injection of SNP was observed in significant proportion of study animals. This side effect was reported also in clinical studies (Agrawal *et al.* 2009). It can be attributed to direct effect on brain structures responsible for arterial blood pressure regulation – e.g. *organum vasculosum laminae terminalis* (Chang *et al.* 2001, Lin *et al.* 1999). Previous study demonstrated decrease of MAP after ICV injection of SNP (Lin *et al.* 1999), nevertheless the drop of MAP was less striking after dose equal to the dose used in our study.

The present study has some limitations, as well. Concentration of oxyhemoglobin in arterial blood can potentially affect the extent of the vasospasm, however neither hematocrit, nor SpO_2 were measured in our study. The brain perfusion might also be influenced by anesthesia. Increase of CBF was described after ketamine; nevertheless the effect of ketamine on CBF was prevented by co-medication with midazolam (Strebel *et al.* 1995).

Conclusions

Intracerebroventricular administration of sodium nitroprusside did not produce expected effects in the conditions of early brain hypoperfusion in prechiasmatic cistern injection model of subarachnoid hemorrhage. It was also complicated with serious side effects – systemic hypotension that was connected with further decrease of cerebral perfusion pressure. The important outcome of the present study is that intracerebroventricular

administration of sodium nitroprusside is not the option for early management in patient after subarachnoid hemorrhage.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Changes of Cortical Perfusion in the Early Phase of Subarachnoid Bleeding in a Rat Model and the Role of Intracranial Hypertension

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Summary

Brain perfusion is reduced early after subarachnoid hemorrhage (SAH) due to intracranial hypertension and early vasospasm. The contribution of these two mechanisms is unknown. By performing a prophylactic decompressive craniectomy (DC) in a rat model of SAH we aimed to study brain perfusion after the component of intracranial hypertension has been eliminated. We used 2x2 factorial design, where rats received either decompressive craniectomy or sham operation followed by injection of 250 µl of blood or normal saline into prechiasmatic cistern. The cortical perfusion has been continually measured by laser speckle-contrast analysis for 30 min. Injection of blood caused a sudden increase of intracranial pressure (ICP) and drop of cerebral perfusion, which returned to baseline within 6 min. DC effectively prevented the rise of ICP, but brain perfusion after SAH was significantly lower and took longer to normalize compared to non-DC animals due to increased cerebral vascular resistance, which lasted throughout 30 min experimental period. Our findings suggest that intracranial hypertension plays dominant role in the very early hypoperfusion after SAH whilst the role of early vasospasm is only minor. Prophylactic DC effectively maintained cerebral perfusion pressure, but worsened cerebral perfusion by increased vascular resistance.

Key words

Subarachnoid hemorrhage • Decompressive craniectomy • Early brain injury • Laser speckle-contrast analysis • Rat

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Introduction

Aneurysmal subarachnoid hemorrhage (SAH) is a cerebrovascular accident with high mortality, which causes both early and delayed changes of brain perfusion. Development of early brain injury (EBI) after subarachnoid aneurysm rupture has a complex pathogenesis (Sehba *et al.* 2012). Vascular wall rupture leads to immediate inflow of arterial blood into subarachnoid space under pressure equal to arterial blood pressure. This causes a rapid increase of intracranial pressure (ICP), which in turn reduces cerebral perfusion pressure (CPP) and cerebral blood flow (CBF), resulting in EBI (Bederson *et al.* 1995, Prunell *et al.* 2003, Prunell *et al.* 2004). In addition, early vasoconstriction of both large and small cerebral parenchymal vessels, which occurs within minutes after hemorrhage, further reduces CBF (Bederson *et al.* 1998, Sehba *et al.* 2012). The proportion of these two mechanisms on the development of EBI remains uncertain. In order to elucidate the relative contribution of raised ICP and early vasospasms on EBI, we measured changes of CBF after SAH in rats with and without decompressive craniectomies performed before the induction of SAH. We hypothesized that elimination of intracranial hypertension achieved by

decompressive craniectomy (DC) can improve the early hypoperfusion.

Methods

All procedures were performed in accordance with the Guidelines of the Animal Protection Law of the Czech Republic, which comply with the respective EU regulations. Special care was taken to minimize animal suffering.

Young adult male Wistar rats (AnLab, Czech Republic; 220 – 240 g) were used. The animals were housed in cages by four under a 12-hour light/dark cycle, with free access to food and water.

Surgical preparation of animals and induction of non-traumatic SAH

Thirty six animals were divided into four groups. There were two experimental groups: 1. animals with induced non-traumatic subarachnoid hemorrhage (SAH, n=12); 2. animals with decompressive craniectomy and SAH (DC-SAH, n=8); and two control groups: 3. sham-operated animals that receive normal saline solution of body temperature into the subarrachnoidal space instead of blood (Sham, n=8); and 4. sham-operated animals with decompressive craniectomy and normal saline injection (DC-Sham, n=8).

All procedures were conducted under deep general anaesthesia (ketamine 100 mg/kg and midazolam 1.2 mg/kg, i.p.) with additional local anaesthesia of the skin and subcutaneous tissues of the head (trimecaine 1 % 0.3 ml).

The skull of the animals was exposed from soft tissues. In DC groups (DC-SAH and DC-Sham), bilateral fronto-temporo-parietal craniectomy and durotomy were performed. The craniectomy extended to the lambdoid suture caudally and 2 mm ahead from the coronal suture rostrally. The medial border was the sagittal suture; the lateral border approached the floor of the middle fossa. The dura was opened by a large cruciate incision. In non-DC groups (SAH and Sham), the skull above right hemisphere was mechanically thinned to allow the measurement of brain perfusion.

Femoral artery was cannulated for blood pressure measurement and also for obtaining blood to be used for SAH induction.

An ICP probe (Codman Microsensor ICP transducer, Johnson & Johnson Health Care Systems, USA) was inserted into left cerebellar hemisphere as previously described (Rooker *et al.* 2002) in order to avoid changes of CBF caused by supratentorial ICP monitoring (Verhaegen *et al.* 1992). ICP and arterial blood pressure were measured continuously (ICP Express, Johnson & Johnson Health Care Systems, USA) during the whole 30-minute monitored period. CPP was calculated as MAP – ICP.

The prechiasmatic cistern injection model of subarachnoid hemorrhage was induced as previously described (Prunell *et al.* 2002). Briefly, a small borehole was drilled 7 mm rostrally from the bregma. A needle with 26G cannula was inserted into the prechiasmatic cistern at the 30° angle anteriorly. The cannula was fixed to the skull and the needle was removed. In the SAH groups (SAH and DC-SAH), 250 µl of fresh non-heparinized autologous arterial blood was injected during 15 s, whilst sham animals (Sham and DC-Sham) received 250 µl of normal saline solution of body temperature. The amount of both blood and normal saline solution was derived from previous studies (Prunell *et al.* 2003) as well as previous experiments in our laboratory which showed significant hypoperfusion with acceptable mortality rate (approx. 25 %).

Rectal temperature was monitored and maintained at 37 °C throughout the experiment.

Measurement of cerebral perfusion

Changes in the perfusion of cerebral cortex were assessed using Laser speckle-contrast analysis (LASCA). This method is based on the detection of moving particles; in case of tissue perfusion these are erythrocytes (Dunn *et al.* 2001). High resolution apparatus PeriCam PSI HR using PimSoft software (Perimed, Sweden) was used for measurement. The cerebral perfusion was measured in arbitrary perfusion units (PU) and reported as percent of deviation from the baseline (i.e. perfusion during 1 min before induction of SAH). The device allows to measure cerebral perfusion in designated regions of interest (ROIs). In non-DC animals, the ROI was placed over the right hemisphere where skull was thinned; in DC animals, the perfusion was measured over the craniectomies.

Statistical analysis

Cerebral perfusion pressure was determined as MAP-ICP. As a measure of the degree of vasoconstriction, we calculated resistance of cerebral

vascular bed as CPP divided by a change of perfusion

$$R = CPP / \Delta \text{ perfusion},$$

calculated as the percentage of the baseline values. GraphPad Prism 6 (GraphPad Software, Inc., USA) was used for statistical evaluation. Changes in ICP, CPP, perfusion and cerebral vascular resistance between groups were compared using two-way ANOVA for repeated measures with Bonferroni's *post hoc* test. Data are shown as mean ± SEM. Differences were considered significant at $p<0.05$.

Results

Determinants of cerebral perfusion. Immediate effects of subarachnoidal injection

DC ameliorated the early rise of ICP after subarachnoidal injection of both blood (74.8±11.5 vs. 18.4±4.6 mm Hg, $p<0.001$) and normal saline (76.2±6.9 vs. 20.7±1.7 mm Hg, $p<0.001$; Fig. 1A), and because there were no significant differences among groups in the dynamics of MAP, CPP followed the trends observed for ICP (Fig. 1B). In all groups ICP and CPP returned to physiologic levels within 4 min. (Fig. 1A, B).

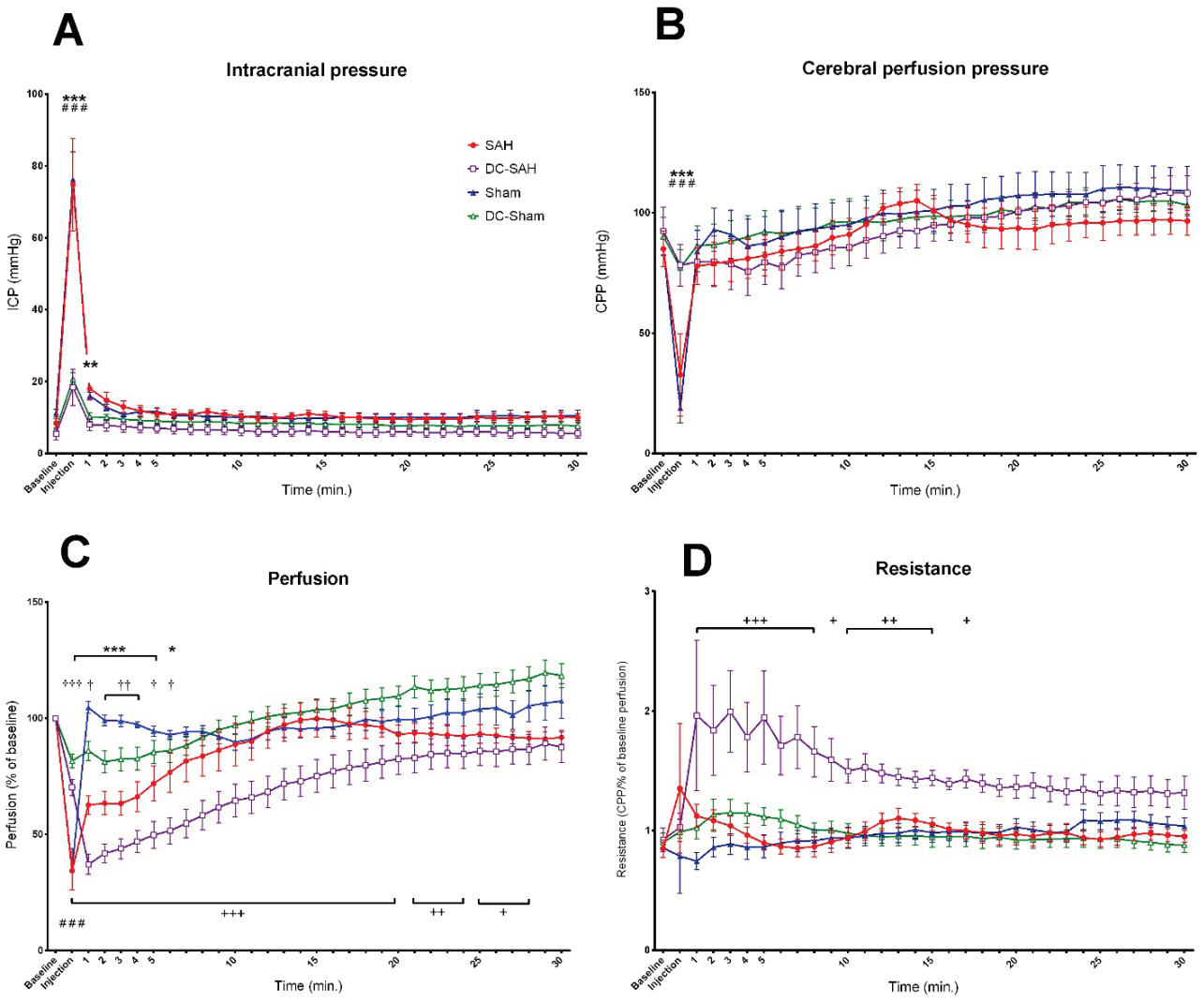

Fig. 1. (**A**) Preemptive decompressive craniectomy significantly reduced ICP after injection in both DC-SAH (** $p<0.01$, *** $p<0.001$) and DC-Sham groups ($^{###}$ $p<0.001$), compared to the respective non-DC groups. (**B**) The rise of ICP was accompanied by the drop of CPP in both SAH and Sham groups (***, $^{###}$ $p<0.001$). (**C**) Compared to baseline, brain perfusion was significantly reduced in all groups at the time of subarachnoid injection ($p<0.001$ for all groups). SAH animals showed significantly reduced perfusion within first 6 min (* $p<0.05$, *** $p<0.001$), whilst in DC-SAH group the perfusion was reduced till 28th minute ($^{+}$ $p<0.05$, $^{++}$ $p<0.01$, $^{+++}$ $p<0.001$). Hypoperfusion was observed even in DC-Sham group († $p<0.05$, †† $p<0.01$). (**D**) The increase of resistance was observed only in DC-SAH group and it persisted till 16th minute after SAH induction ($^{+}$ $p<0.05$, $^{++}$ $p<0.01$, $^{+++}$ $p<0.001$). Data are shown as mean ± SEM.

Increased ICP at the time of intracisternal injection caused a severe reduction of perfusion in both SAH and Sham groups (25.7±5 % of baseline, $p<0.001$, and 35.1±8.3 %, $p<0.001$, respectively). Reduced perfusion at the time of the injection was observed also in both DC-SAH and DC-Sham groups (70.2±3.9 % of baseline, $p<0.001$, and 80.9±3 %, $p<0.001$, respectively), nevertheless the hypoperfusion was less severe in comparison with respective non-DC groups ($p<0.001$). In Sham group, the cerebral perfusion returned back to baseline within 1 min whilst it remained significantly reduced up to minute 6 after injection in SAH group ($p<0.05$, compared to baseline; Fig. 1C). Surprisingly, in DC-SAH group, cerebral perfusion remained impaired up until minute 28 ($p<0.05$, compared to baseline), which was caused by increased resistance (Fig. 1C, D). Mild but significant ($p<0.05$) hypoperfusion lasting for 6 min after the injection was observed even in DC-Sham animals (Fig. 1C).

Changes of cerebral vascular resistance and the effect of DC

Resistance of vascular bed did not change neither in SAH, Sham or DC-Sham group, whilst increased in DC-SAH group shortly after injection (at 20 s) and remained above baseline level till minute 16 (Fig. 1D).

Mortality

Four animals (25 %) in the SAH group died within 2 min since the intracisternal injection. All animals in SAH-DC and both sham groups survived till the end of experiment.

Discussion

Perfusion of the brain (i.e. volume/time) is determined by cerebral perfusion pressure (i.e. MAP-ICP) and the global resistance of cerebral vessels to flow (calculated in Ohm's law analogy as CPP/flow). In the early phase after subarachnoidal hemorrhage, both determinants of cerebral perfusion change dynamically and contribute to impaired cerebral perfusion. The initial reduction of perfusion is attributed to increased volume of subarachnoid space and thus elevated ICP and decreased CPP (Bederson *et al.* 1995, Prunell *et al.* 2003, Sehba *et al.* 2012). Nevertheless, animal studies show that the perfusion remains reduced after CPP increase (Bederson *et al.* 1995, Prunell *et al.* 2003, Prunell *et al.* 2004, Schubert *et al.* 2008) and the hypoperfusion is not even related to development of brain edema and amount of brain water content (Westermaier *et al.* 2012). In the clinical setting, a significant reduction of CBF was observed within the first 12 h after aneurysm rupture, which correlated with Hunt-Hess grade, but not with ICP or CPP (Schubert *et al.* 2009). This hypoperfusion can be attributed to early vasoconstriction (and thus increased resistance to the blood flow) caused by nitric oxide (NO) scavenging and an impairment of endothelium – dependent vasodilation (Sehba *et al.* 2005). In this study we aimed to experimentally separate the impairment of CPP from changes of cerebral vascular resistance and analyze their respective contribution to the perfusion abnormalities.

The main finding of our study is that although preemptive DC prevented 25 % of deaths after injecting blood into the subarachnoid space, it led to a protracted impairment of cerebral perfusion, which was mainly caused by increase resistance of cerebral vessels. The reasons why DC performed before SAH increased cerebral vascular resistance remain unclear. Reduced brain perfusion with DC preceding SAH has been seen before in a study (Buhler *et al.* 2015), where SAH was induced by endovascular puncture model (EPM). Because the intracranial hypertension contributes to the cessation of bleeding after aneurysm rupture (Nornes 1973), the DC before SAH could have led to a larger volume of the hematoma in EPM and thus made these results difficult to interpret. We avoided this bias by choosing to induce SAH by injection of a controlled volume of blood. Nonetheless, the volume was chosen as per described protocol (Prunell *et al.* 2002) and the results might have been different with different volumes of blood used (Bederson *et al.* 1998).

In our study, both groups with DC showed brain edema with protrusion of brain tissue after subarachnoid injection. Decreased perfusion in DC-SAH group could be explained by brain herniation, which is a frequent complication of decompressive craniectomy in humans (Yang *et al.* 2008). The protrusion of edematous brain tissue above the skull surface may lead to compression of vessels at the edge of the craniectomy and subsequent venous congestion and arterial occlusion (Huang and Wen 2010, Mitchell *et al.* 2004), together with a degree of an impairment of cerebral microvascular regulation and metabolic deterioration (Bor-Seng-Shu *et al.* 2013). The herniation was observed mostly in inappropriately small craniectomies and performing large fronto-

temporo-parietal craniectomy appeared to prevent this complication (Forsting *et al.* 1995, Yang *et al.* 2008) and this is the surgical approach we used in our experiment. Nevertheless, the increased resistance of cerebral vessels together with mild reduction of perfusion in DC-Sham group suggests that DC itself could have increased cerebral vascular resistance to some extent. The expansion of edematous brain and the presence of blood cells in subarachnoid space in DC-SAH group could have exacerbated these mechanisms, together with causing axonal stretch and subsequent neuronal injury (Cooper *et al.* 2011).

Our experimental design was effective in terms of DC disturbing Monro-Kellie doctrine. In animals without DC, inducing a SAH caused a sharp rise in ICP, which resulted in a drop of CPP with a nadir close to 0. This was the main factor impairing cerebral perfusion during this short period after injection, 4 out of 12 animals (25 %) died at this stage. DC allowed the intracranial volume to expand enough so that there was only a minor raise in ICP following induction of SAH. All animals with DC survived SAH. The reductions of CBF in both SAH and DC-SAH groups in our experiments were less marked compared to previous studies (Bederson *et al.* 1995, Prunell *et al.* 2003, Prunell *et al.* 2004, Schubert *et al.* 2008). This can be explained by both technique of SAH induction (Prunell *et al.* 2003) and the method of measurement of CBF. Especially in endovascular puncture model (EPM) of SAH, the presence of blood above brain convexities can induce bias into the perfusion measurement (Prunell *et al.* 2003). Also this model produces heterogeneity in the volume of blood in subarachnoid space, risks rebleeding and may induce vasospasms by a direct injury to vessel wall (Buhler *et al.* 2015, Prunell *et al.* 2003). Our model avoided all these disadvantages. On the other hand, LASCA method of brain perfusion measurement integrates the signal from both microcirculation and large pial vessels. In previous studies (Bederson *et al.* 1995, Prunell *et al.* 2003, Schubert *et al.* 2008) the CBF was measured by laser-Doppler flowmetry probes, which allow measurement in only approx. 1 mm^3 of tissue (Dirnagl *et al.* 1989) and the probes were placed away from large pial vessels, which may increase the sensitivity to detecting perfusion changes at the level of microcirculation, which are indeed functionally more important (Herz *et al.* 1975, Park *et al.* 2001, Schubert *et al.* 2009).

Although putting our data into clinical context should be performed with caution, there are some interesting analogies. In SAH patients, the indication, timing and extent of DC have not been fully established yet (Buschmann *et al.* 2007, Otani *et al.* 2008). Even though prompt decrease of ICP and increase of partial pressure of O_2 in brain tissue was described (Jaeger *et al.* 2003), the correlation with patient's outcome is rather weak (Uozumi *et al.* 2014). DC can be beneficial in some subgroups of patients, e.g. SAH with intracerebral hemorrhage or large Sylvian hematoma and our model did not involve these subgroups. The expansion of edematous brain can also cause axonal stretch and subsequent neuronal injury (Cooper *et al.* 2011). In our animal model, DC decreased mortality of SAH from 25 % to 0 %, but worsened brain perfusion. In line, in high quality randomized controlled trial DC reduced mortality but increased proportion of severely disabled patients in brain trauma (Hutchinson *et al.* 2016) and similar results were observed in patients SAH (D'Ambrosio *et al.* 2005).

In conclusion, cerebral perfusion imminently after SAH was mainly impaired by increased intracranial pressure in this study. Then ICP, CPP and cerebral perfusion normalize within 6 min. Pre-emptive DC effectively prevented the early alteration of cerebral perfusion pressure, but led to a protracted impairment of cerebral perfusion, almost solely attributed to increased cerebral vascular resistance. This phenomenon is likely caused by deleterious interaction between effects of DC itself and the blood in the subarrachnoidal space.

Conflict of Interest

There is no conflict of interest.

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Role oxidu dusného a NO-syntázy v patofyziologii poškození mozku po subarachnoidálním krvácení; laboratorní modely subarachnoidálního krvácení

The role of nitric oxide and NO-synthase in the pathogenesis of cerebral damage after subarachnoid hemorrhage; laboratory models of subarachnoid hemorrhage

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SOUHRN

Subarachnoidální krvácení (SAK) je akutní život ohrožující stav. Kromě vzestupu nitrolebního tlaku a poklesu mozkového perfuzního tlaku dochází k poškození mozku časnou a pozdní vazokonstrikcí. Mechanismus vazokonstrikce je komplexní a jednu z hlavních rolí při jejím rozvoji hraje oxid dusnatý (NO).
Tato práce přináší přehled patogeneze netraumatického SAK, s důrazem na regulaci mozkové perfuze zprostředkovanou NO a její změny po SAK. Dále jsou popsány mechanismy časného a pozdního poškození mozku po subarachnoidálním krvácení. Diskutovány jsou i možnosti farmakologické prevence poškození mozku a laboratorní modely netraumatického SAK.
Klíčová slova: subarachnoidální krvácení, oxid dusnatý, NO-syntáza, patogeneze, laboratorní modely, potkan

SUMMARY

Subarachnoid hemorrhage (SAH) of CNS is acute life-threating condition. In addition to its well understood sequential increase in intracranial pressure and decreased cerebral perfusion pressure, there is also early and late vasoconstriction. Mechanism of vasoconstriction is complex and one of important roles play changes in the amount of nitric oxide (NO).
Present work overviews known pathogenesis of non-traumatic SAH, with stress on NO regulation of cerebral blood flow and its changes during SAH. It also describes mechanisms of early and late brain damage following subarachnoid hemorrhage. We discuss possible pharmacological prevention of the damage and laboratory models of non-traumatic SAH.
Key words: subarachnoid hemorrhage, nitric oxide, NO-synthase, pathogenesis, laboratory models, rat

EPIDEMIOLOGIE SUBARACHNOIDÁLNÍHO KRVÁCENÍ

Subarachnoidální krvácení (SAK) představuje pouhých 5 % všech cévních mozkových příhod; protože však postihuje mladší pacienty a má závažné neurologické následky, má zároveň značný socioekonomický dopad (de Rooij et al., 2007). Z tohoto důvodu je subarachnoidální krvácení v centru zájmu jak kliniků, tak i experimentální medicíny, jak dosvědčuje i počet publikací na toto téma – do současnosti (leden 2014) bylo publikováno více než 22 000 článků a počet publikací na toto téma má setrvale rostoucí trend (zdroj: PubMed).

V klinické praxi se objevuje SAK při úrazech mozku nebo netraumatické etiologie. Nejčastější příčinou netraumatického SAK je krvácení z aneurysmatu mozkových tepen (85 %), v 10 % se jedná o tzv. perimesencefalické krvácení neznámé etiologie, zbylých 5 % je způsobeno vrozeným nebo získaným poškozením mozkových arterií nebo systémovými poruchami (např. srpkovitá anémie) (Sehba et al., 2012). Netraumatickým SAK je postiženo každoročně průměrně 9/100 000 osob, s určitými regionálními rozdíly (Finsko – 19,7, Japonsko 22,7, Střední a Jižní Amerika – 4,2/100 000) (de Rooij et al., 2007). Z těchto postižených cca 65 % zemře do 1 roku od krvácení (Steiner et al., 2013), 12 % pacientů umírá před dosažením lékařské pomoci (Huang a van Gelder, 2002). Asi 50 % přeživších trpí dlouhodobým kognitivním deficitem a 30 % přeživších zůstává odkázáno na pomoc okolí (Ciurea et al., 2013). Incidence SAK stoupá s věkem, nejčastěji je postižena věková kategorie 40–60 let. Asi 1,6krát častěji jsou postiženy ženy (Bederson et al., 2009).

PATOFYZIOLOGIE POŠKOZENÍ MOZKU PO SAK

Důsledky subarachnoidálního krvácení jsou akutní i dlouhodobé – vznikající v rámci tzv. pozdního ischemického neurologického deficitu (delayed cerebral ischemia, DCI). Zatímco dříve byla většina pozornosti soustředěna na pozdní vasospasmy, v posledních několika letech se množí publikace věnující se důležitosti a mechanismům poškození mozku časně po SAK.

ROLE OXIDU DUSNATÉHO A NO-SYNTÁZY

Dosavadní výzkumy ukazují, že stěžejní roli v patofyziologických změnách následujících bezprostředně i dlouhodobě po SAK hraje oxid dusnatý, NO, a oxidem dusnatým zprostředkovaná vazodilatace. NO se za fyziologických podmínek podílí na regulaci průtoku krve mozkem − působí vazodilataci, zvyšuje krevní průtok, inhibuje adhezi a agregaci trombocytů, adhezi leukocytů a apoptózu endoteliálních buněk, snižuje proliferaci hladkého svalstva cévní stěny a je účinný jako scavenger volných radikálů (Andrew a Mayer, 1999; Toda et al., 2009; Tousoulis et al., 2012).

NO je syntetizován z L-argininu účinkem enzymu NO-syntázy (NOS), který se vyskytuje v podobě homodimeru (Andrew a Mayer, 1999). Byly popsány 3 izoformy NO-syntázy – 2 konstitutivně exprimované (endoteliální – eNOS a neuronální − nNOS) a inducibilní NOS (iNOS), která je exprimována v endotelu (i v dalších buňkách − hladká svalovina cév, makrofágy, glie, fibroblasty, neurony) (Suzuki et al., 1999) pouze po stimulaci zánětlivými mediátory (Andrew a Mayer, 1999; Tousoulis et al., 2012). Účinkem eNOS dochází ke kontinuální syntéze NO v bazální dávce (Toda et al., 2009; Tousoulis et al., 2012).

V experimentech na zvířatech byl pozorován trifazický průběh hladin NO v mozku po SAK. V prvních 10 minutách dochází k poklesu hladiny NO, během dalších 3 hodin následuje vzestup na bazální hodnoty a po 24 hodinách elevace nad normální hodnoty (Sehba et al., 2011; Sabri et al., 2013). Trifazický průběh hladin NO po SAK může mít dvojí efekt – nedostatek NO v iniciální fázi způsobí vazokonstrikci a pokles CBF, nadbytek NO v pozdější fázi přispívá k radikálovému poškození (Sehba et al., 2011; Suzuki et al., 1995).

ČASNÉ POŠKOZENÍ MOZKU

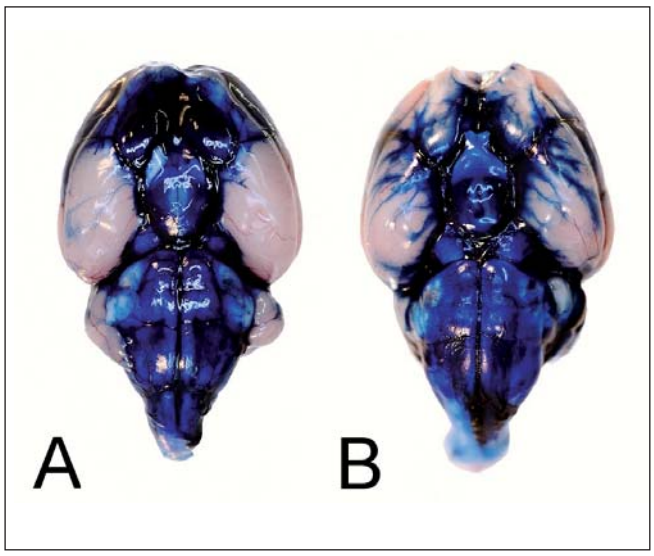
Časným poškozením mozku (early brain injury, EBI) se rozumí poškození mozku vzniklé v prvních 72 hodinách po SAK a považuje se za hlavní příčinu mortality a morbidity po SAK (Cahill et al., 2006). Vzhledem k akutní povaze onemocnění pochází informace o dějích bezprostředně následujících krvácení především ze studií na zvířatech.

V okamžiku ruptury aneurysmatu dochází k vniknutí krve do subarachnoidálního prostoru pod tlakem rovnajícím se arteriálnímu krevnímu tlaku. To způsobí prudký vzestup nitrolebního tlaku (intracranial pressure, ICP) (Voldby a Enevoldsen, 1982), v jehož důsledku dochází k poklesu mozkového perfuzního tlaku (cerebral perfusion pressure, CPP) a průtoku krve mozkem (cerebral blood flow, CBF) (Cai et al., 2012) a tím k rozvoji časného ischemického poškození (Bederson et al., 1995). Část pacientů postižených SAK v tomto okamžiku umírá v důsledku zástavy dechu a/nebo oběhu (Hijdra a van Gijn, 1982).

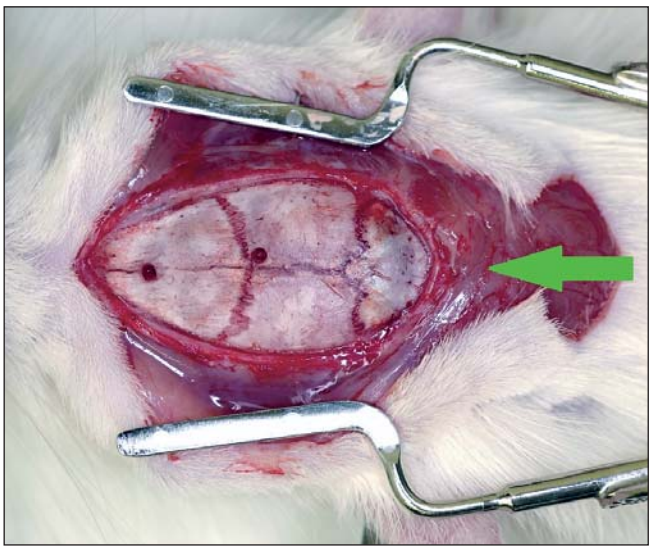
Během dalších minut až desítek minut po SAK dochází k poklesu ICP (který zůstává nad fyziologickou hodnotou) a vzestupu CPP. Trvá však pokles CBF, který je do jisté míry nezávislý na hodnotě ICP nebo CPP a je způsoben časnou vazokonstrikcí (Bederson et al., 1995; Bederson et al., 1998; Schubert et al., 2009; Tateyama et al., 2013). U pacientů postižených SAK trvá tento pokles CBF až několik desítek hodin po zakrvácení (Hayashi et al., 2000) a koreluje s neurologickým stavem a kvalitou přežití (Schubert et al., 2009, Tateyama et al., 2013).

MECHANISMY ČASNÉHO POŠKOZENÍ MOZKU

Dosud popsaných mechanismů podílejících se na EBI je celá řada – porucha permeability hematoencefalické bariéry, vazogenní a cytotoxický mozkový edém, apoptóza a nekróza neuronů, endotelií i gliových buněk, šířící se kortikální depolarizace (cortical spreading depolarization), porucha



Obr. 1: *Při navození subarachnoidálního krvácení aplikací krve (zde znázorněno Evansovou modří, 100 μl) do prechiasmatické cisterny (A) se větší množství podané krve nachází v oblasti Willisova okruhu než v oblasti bazilárního řečiště, což je bližší situaci u člověka při ruptuře aneurysmatu mozkových cév. Při aplikaci do cisterna magna (B) se krev nachází z větší části v zadní jámě lební.*



Obr. 2: *Přístup do prechiasmatické cisterny (+7,5 mm, sklon hrotu jehly 30° kaudálně), postranní komory (+1 mm, +1 mm od bregmatu, hloubka 3 mm) a cisterna magna (po rozpreparování nuchálních svalů, naznačeno šipkou)*

homeostázy kalcia a magnézia a další (Sehba et al., 2012). Z hlediska významu NO a NOS se v současnosti uvažuje o dvou mechanismech – nedostatek NO způsobený jeho vychytáváním, a dysfunkce endotelu. V experimentu na laboratorním potkanovi byl bezprostředně po SAK pozorován pokles hladiny metabolitů NO v mozku (Sehba et al., 2004; Sehba et al., 2000), je však zachována schopnost mozkové cirkulace reagovat na externí přívod NO vazodilatací (Sehba et al., 1999). Tento jev je vysvětlitelný vychytáváním (scavenging) oxidu dusnatého (Sehba et al., 2012; Schwartz et al., 2000). Za scavenger NO považují někteří autoři hemoglobin (Hb), který se za fyziologických podmínek podílí na metabolismu NO (Beckman a Koppenol, 1996) a který je po SAK přítomen v subarachnoidálním prostoru (Clark a harp, 2006; Sehba et al., 2000). NO volně prostupuje buněčnými membránami a může tudíž vstupovat do erytrocytů a reagovat s oxyhemoglobinem za vzniku methemoglobinu a dusičnanového aniontu (Beckman a Koppenol, 1996). Vazokonstrikční efekt hemoglobinu byl opakovaně demonstrován – v experimentu na izolované a. basilaris byla pozorována inhibice vazodilatačních mechanismů za přítomnosti hemoglobinu a průnik hemoglobinu do hladké svaloviny cévní stěny během 10 minut po extraluminální aplikaci hemoglobinu (Hongo et al., 1988), *in vivo* byla angiograficky potvrzena vazokonstrikce 15 minut po intratekální aplikaci hemoglobinu (Byrne et al., 1989). Zda je však přítomnost hemoglobinu v subarachnoidálním prostoru zodpovědná za rozvoj časného poškození mozku v prvních minutách až desítkách minut po vzniku SAK, je sporné. V případě SAK je hemoglobin v subarachnoidálním prostoru zprvu přítomen v erytrocytech, jejichž hemolýza začíná až za 16–32 hod. po SAK a vrcholí 7. den (Pluta et al., 1998). Bylo pozorováno, že NO reaguje s hemoglobinem obsaženým v erytrocytech 500–1000× pomaleji než s volným Hb (Liu et al., 1998; Vaughn et al., 2000), v experimentu byl vazokonstrikční efekt na izolované mozkové tepny pozorován pouze po podání hemolyzované krve, a nikoliv po podání plné krve s intaktními erytrocyty (Vecchione et al., 2009).

Dalším známým scavengerem NO, který může hrát roli v rozvoji EBI, je superoxidový aniont (O^{2-}), volný kyslíkový radikál, k jehož tvorbě dochází po SAK (Kajita et al., 1994; Shishido et al., 1994; Schwartz et al., 2000) a jehož přítomnost byla v experimentu na zvířecím modelu v mozku pozorována 1 hodinu po navození SAK (Endo et al., 2007). Za podmínek ischemie během vzestupu ICP a poklesu CPP dochází k poruše elektronového transportního řetězce v mitochondriích, která vede k nadprodukci superoxidových aniontů (Marzatico et al., 1988). Oxid dusantý se slučuje se superoxidovým aniontem za vzniku prudce reaktivního a toxického peroxynitritu (ONOO-), který dále reaguje s nenasycenými mastnými kyselinami, tyrosylovými skupinami proteinů nebo DNA (Beckman a Koppenol, 1996; Kalyanaraman, 2013). Popsaným mechanismem dojde za přítomnosti O^{2-} jednak k poklesu hladiny NO a jednak k dalšímu poškození buněk toxickým efektem peroxynitritu.

Druhým uvažovaným mechanismem je endoteliální dysfunkce, která je charakterizována morfologickými i funkčními změnami endotelu a vede k poruše NO-zprostředkované vazodilatace (Friedrich et al., 2011; Sehba et al., 2012). Spouštěcím mechanismem tohoto procesu je pravděpodobně ischémie při iniciálním vzestupu ICP a poklesu CBF (Sehba et al., 2005). Už 10 minut po SAK lze pozorovat agregaci trombocytů v luminu cév, adhezi neutrofilů k cévní stěně, aktivaci kolagenáz a degradaci kolagenu IV – základní komponenty bazální membrány endotelu (Friedrich et al., 2011; Sehba et al., 2007; Sehba et al., 2005). Destrukce bazální

membrány vede k odloučení endotelu od lamina media (Friedrich et al., 2010). Dysfunkce poškozeného endotelu v cerebrovaskulárním řečišti následně způsobí poruchu vazodilatace zprostředkované endotelem (Park et al., 2001), zničení bazální membrány vede k poruše hematoencefalické bariéry (Scholler et al., 2007).

POZDNÍ ISCHEMIE MOZKU

Častou komplikací netraumatického SAK, která se vyskytuje v prvních 2 týdnech po inzultu, je tzv. pozdní ischemie mozku (delayed cerebral ischemia, DCI). Tento termín byl zaveden kvůli nejasnostem v patofyziologii pozdního ischemického poškození mozku a vztahu DCI k pozdním vazospasmům. Pozdní ischemií mozku se rozumí zhoršení fokálního neurologického nálezu (např. hemiparéza, afázie) nebo zhoršení Glasgow Coma Score o aspoň 2 body, které se pomocí klinického vyšetření nebo zobrazovacích metod nedá vysvětlit jinou příčinou (Vergouwen et al., 2010).

Dlouhou dobu byly za hlavní příčinu zhoršení neurologického stavu pacientů po SAK považovány pozdní vazospasmy. K vzniku pozdních vazospasmů dochází od 3. dne po SAK, jejich závažnost vrcholí 7. den a odeznívají obvykle během 3. týdne (Pluta, 2005). Zobrazovacími metodami jsou vazospasmy prokazatelné u cca 50–70 % pacientů (Kassell et al., 1985). Kauzální souvislost mezi vazospasmy a DCI není zcela přesvědčivá (Sehba et al., 2011; Vergouwen et al., 2010). Klinické příznaky jsou pozorovatelné jen u zhruba poloviny pacientů postižených vazospasmem (Bederson et al., 2009), terapeutické intervence zvyšující regionální CBF nevedou k ovlivnění samotného vazospasmu (Pluta et al., 2000), naopak vazospasmus sám o sobě nevede k rozvoji DCI a u některých pacientů se hypoperfundovaná oblast mozku nenachází v povodí spastické tepny (Dankbaar et al., 2009). Samotné zmírnění vazospasmu neovlivňuje další patologické procesy probíhající v mozkové cirkulaci (Sabri et al., 2011b). To vyplývá i ze studie CONSCIOUS-2, ve které clazosentan, antagonista endotelinového receptoru, vedl ke zmírnění vazospasmů a DCI, nicméně nesnížil mortalitu pacientů po SAK ani nezlepšil jejich kvalitu přežití (Wang et al., 2012).

Z těchto důvodů je v současnosti uvažováno i o jiných příčinách rozvoje DCI – spasmus nebo trombóza na úrovni mikrocirkulace, zánětlivá reakce, Leaova šířící se deprese (Vergouwen et al., 2008).

MECHANISMY POZDNÍHO ISCHEMICKÉHO POŠKOZENÍ

Rozvoj pozdních vazospasmů a pozdní mozkové ischémie je pravděpodobně multifaktoriální proces. Jako hlavní faktor v patogenezi CVS a DCI se uvažuje zejména o hemoglobinu a jeho metabolitech (Mayberg et al., 1990); jeho role v rozvoji pozdních změn perfuze je zdokumentovaná lépe než v případě časného poškození mozku a souvisí s vazodilatací zprostředkovanou NO i s radikálovým poškozením.

Po SAK dochází k hemolýze erytrocytů v subarachnoidálním prostoru a tedy k uvolnění hemoglobinu do likvoru (Pluta, 2005; Pluta et al., 1998). Protože vazebný systém haptoglobin-hemoglobin v SA prostoru má výrazně menší kapacitu než v krvi a je záhy vyčerpán, dosahuje hemoglobin v mozkomíšním moku vysokých koncentrací (Galea et al., 2012). Za fagocytózu erytrocytů a fibrinu jsou zodpovědné makrofágy a T-lymfocyty, které infiltrují subarachnoidální prostor; maximum této infiltrace bylo u pokusných zvířat pozorováno 2. a 3. den po SAK (Jackowski et al., 1990), kdy byly pozorovány i největší strukturální změny na mozkových arteriích (ztluštění medie a adventicie, zvrásnění intimy) (Kubota et al., 1993) i nejvýraznější změny angiografické (Delgado et al., 1985).

Mechanismů, kterými může hemoglobin působit vazokonstrikci, je několik. Po hemolýze erytrocytů dochází k penetraci hemoglobinu do cévní stěny, jeho přítomnost po experimentálním SAK byla pozorována v adventicii, hladké svalovině a endotelu a závažnost vazoskonstrikce korelovala s množstvím Hb v cévní stěně (Foley et al., 1993). Zde může Hb výše popsaným mechanismem fungovat jako scavenger NO.

Dalším uvažovaným mechanismem je autooxidace hemoglobinu, která vede ke vzniku volných kyslíkových radikálů. Oxyhemoglobin (obsahující atom železa ve formě Fe2+) podléhá spontánní oxidaci na methemoglobin (obsahující železo ve formě Fe3+) za vzniku superoxidového aniontu O2- a sekundárně dalších kyslíkových radikálů, což v experimentu *ex vivo* vedlo ke kontrakci buněk hladké svaloviny cévní stěny (Steele et al., 1991). Výše popsaným mechanismem pak může docházet k dalšímu poškození účinkem peroxynitritu.

Přítomnost oxyhemoglobinu může mít i vliv na funkci endoteliální NO-syntázy. Za četných patologických okolností (např. hypertenze, diabetes mellitus, hypercholesterolémie, kouření) dochází k dysfunkci eNOS a ke zvýšené tvorbě kyslíkových radikálů na úkor tvorby NO (Forstermann a Munzel, 2006). Tento jev byl popsán i na zvířecím modelu SAK; snížená syntéza NO a zvýšená produkce kyslíkových radikálů pak mohou přispět k pozdnímu neurologickému poškození (Sabri et al., 2011a). Statiny, inhibitory 3-hydroxy-3-methylglutaryl koenzym A reduktázy běžně používané jako hypolipidemika, zlepšují tuto dysfunkci endoteliální NOS a posouvají rovnováhu zpět k produkci NO – za experimentálních podmínek vedlo podávání simvastatinu ke zvýšení produkce NO, zmírnění vazospasmů a agregace trombocytů v mikrocirkulaci a snížení produkce volných kyslíkových radikálů (Sabri et al., 2011a).

MOŽNOSTI FARMAKOLOGICKÉHO OVLIVNĚNÍ PERFUZNÍCH PORUCH

Základem léčby pozdních vazospasmů v klinické medicíně jsou v současnosti kalciové blokátory, především nimodipin (Bederson et al., 2009), nicméně za experimentálních podmínek je testována řada dalších látek. Z uvedeného vyplývá, že procesy probíhající po SAK mohou být příznivě

ovlivněny podáváním látek odštěpujících NO. Experimentální data skutečně ukazují, že podávání donorů NO je efektivní v rámci časných i pozdních perfuzních změn, účinek NO je v tomto případě multifaktoriální – má nejen vazodilatační efekt, ale i brání agregaci trombocytů (Sehba et al., 1999) a inhibuje degradaci kolagenu IV matrix-metalloproteinázou 9 (Sehba et al., 2007). Při exogenním podávání NO jsou tepny i v případě vazospazmu schopny reagovat vazodilatací (Kim et al., 1988; Vatter et al., 2007).

L-ARGININ

Jednou z účinných látek, úspěšně použitou v experimentech, byl L-arginin, substrát NO-syntázy. Podávání L-argininu pokusným zvířatům zlepšilo CBF v prvních 24 hodinách po SAK (Sun et al., 2003), což může být způsobeno vyšší syntézou NO inducibilní NO-syntázou, jejíž exprese je v prvních 24 hodinách po SAK rovněž zvýšena (Sehba et al., 2004). Aplikace L-argininu byla s úspěchem použita i k ovlivnění pozdního vazospasmu, vazodilatační efekt na spastické tepny i zvýšení CBF bylo pozorováno již během prvních 10 minut od zahájení intracisternální a intrakarotické aplikace (Goksel et al., 2001; Hirose et al., 1995; Ozum et al., 2007). Naproti tomu Pluta et al. (2000) pozorovali po intraarteriální aplikaci L-argininu zvýšení regionálního CBF, avšak bez ovlivnění samotného angiograficky prokázaného vazospasmu.

DONORY NO

Donory NO, sloučeniny odštěpující ze své molekuly NO, nevyžadují na rozdíl od L-argininu enzymatickou reakci, jejich efekt je tudíž nezávislý na aktivitě NO-syntázy.

Jednou z látek použitých v experimentu je S-nitrosoglutathion. Jeho podávání v rámci časného poškození zmírnilo vazokonstrikci mozkových tepen, zvýšilo CBF a snížilo rozsah degradace kolagenu IV (Sehba et al., 1999; Sehba et al., 2007).

Další ze studovaných látek je nitroprussid sodný. Studie klinické (Agrawal et al., 2009; Kumar et al., 2003; Raabe et al., 2002; Thomas a McGinnis, 2002) i experimentální (Dizdarevic, 2008; Egemen et al., 1993; Macdonald et al., 2002; Vatter et al., 2007) poskytují protichůdné výsledky. V experimentech ex vivo byla pozorována relaxace spastických tepen po aplikaci nitroprussidu (Dizdarevic, 2008; Vatter et al., 2007). Zatímco studie Egemen et al. (1993) ukazuje dobrý efekt intratekálně podávaného nitroprussidu pokusným zvířatům, Macdonald (2002) tento efekt nepozoroval.

V klinických studiích Agrawal (2009) popisuje zlepšení parametrů perfuze mozku u všech pacientů po bolusovém podání nitroprussidu, Thomas (2002) a Pachl (2005) pozorovali zlepšení klinického stavu i perfuze u části pacientů, Raabe (2002) popisuje zlepšení jen u necelé poloviny pacientů léčených bolusovým nitroprussidem. Lepšího výsledku bylo dosaženo při kontinuálním podávání, výsledky však nejsou reprezentativní pro nízký počet (n = 2) studovaných pacientů (Raabe et al., 2002).

MODELY SAK A VAZOSPASMŮ NA POTKANECH

Vzhledem k minimální možnosti pozorovat časné perfuzní změny přímo na pacientech postižených SAK pochází většina informací o časném poškození mozku z experimentů na zvířatech. Z pohledu přínosu pro kliniku jsou sice preferovány modely na opicích nebo na jiných větších zvířatech (kočky, psi, králíci) (Marbacher et al., 2010), ale během posledních let z důvodů ceny i snazší opakovatelnosti experimentů v dalších laboratořích výrazně přibývá pokusů prováděných na laboratorním potkanovi, jak plyne i z počtu prací (více než 900 od r. 1979, z toho 69 v roce 2013). Vzhledem k časovému průběhu perfuzních poruch po SAK lze potkany použít ke studiu časného poškození mozku i pozdních vasospasmů, v závislosti na použitém modelu.

Rozvoj perfuzních poruch po navození SAK pokusným zvířatům má bifazický průběh. K časnému vazospazmu dochází během cca 10 minut od aplikace autologní krve (Delgado et al., 1985; Ono et al., 2003; Suzuki et al., 1999), tato časná fáze trvá cca 60 minut (Marbacher et al., 2010; Solomon et al., 1985; Suzuki et al., 1999). Druhá fáze vazospasmu byla pozorována podle použité metodiky ve 2. (Delgado et al., 1985) –7. dni (Suzuki et al., 1999) s normalizací průměru bazilárního řečiště do 5.–12. dne (Delgado et al., 1985; Suzuki et al., 1999).

Experimentální techniky na potkanech zahrnují buď poranění přímo mozkových tepen, a to jak mikrochirurgicky z adventiciálního povrchu tepny, tak transluminálně (endovascular puncture model, EPM); aplikaci autologní arteriální krve do subarachnoidálního prostoru; aplikaci koagula do blízkosti mozkových tepen. Pro studium pozdního vazospasmu je možno použít extrakraniální tepny – obvykle a. femoralis (Dizdarevic, 2008).

K hodnocení farmakologických intervencí je u potkana možno použít nejen podání farmak do cévního řečiště, ale i do postranní komory. Tu lze snadno punktovat stereotakticky, místo punkce se u dospělého potkana nachází 1 mm kaudálně a 1 mm laterálně od bregmatu a postranní komora je zastižena v hloubce 3 mm (Paxinos a Watson, 2006).

Historicky první model SAK na potkanovi spočíval v mikrochirurgické vizualizaci a. basilaris a její perforaci (Barry et al., 1979). V současnosti nejpoužívanější metody navození SAK je injekční aplikace krve do subarachnoidálního prostoru a model endovaskulární punkce (endovascular puncture model, EPM). V dalším textu bude popsána metodika a diskutovány klady a zápory jednotlivých metod.

MODEL ENDOVASKULÁRNÍ PUNKCE (EPM)

Principem tohoto modelu je perforace Willisova okruhu vláknem zavedeným cestou a. carotis interna do oblasti bifurkace s následným krvácením do subarachnoidálního prostoru (Bederson et al., 1995; Veelken et al., 1995). Pokusnému zvířeti je provedena incize ve střední čáře ventrální části

krku a vypreparována a. carotis externa. Ta je přetnuta tak, aby byl zachován pahýl o délce cca 3–4 mm. Luminem tohoto pahýlu je zavedeno nylonové vlákno se šikmo sestřiženým koncem do a. carotis int. Po dosažení lehkého odporu v místě bifurkace a. carotis int. je pro navození krvácení vlákno zavedeno dále tak, aby došlo k perforaci cévní stěny. Ta je zpravidla perforována v oblasti a. carotis interna, a. cerebri anterior nebo a. cerebri media. Poté je vlákno extrahováno, aby došlo k reperfuzi řečiště a. carotis interna a k rozvoji SAK.

Výhodou tohoto modelu je vznik SAK mechanismem bližším vzniku aneurysmatického SAK u člověka – dochází k poškození cévní stěny v oblasti Willisova okruhu, hematom se nachází na bazi mozku, krev do subarachnoidálního prostoru proniká pod tlakem stejným, jako je arteriální krevní tlak.

Hlavní nevýhodou EPM je obtížně kvantifikovatelný rozsah krvácení. Ve studii Prunell et al. (2003) se objem krve v subarachnoidálním prostoru pohyboval v rozmezí 50–480 µl. U zvířat s menším rozsahem krvácení byla krev lokalizována na bazi mozku, zejména kolem Willisova okruhu, mozkového kmene a cerebella, u větších krvácení byla krev nalezena i nad a mezi hemisférami a v komorovém systému (Prunell et al., 2003), popsán byl i výskyt subdurálního a intracerebrálního krvácení (Gules et al., 2002). Tento model je rovněž zatížen vysokou mortalitou pokusných zvířat, která se může blížit až 50 % (Gules et al., 2002; Prunell et al., 2003).

MODELY ZALOŽENÉ NA INJEKČNÍ APLIKACI ARTERIÁLNÍ KRVE DO SA PROSTORU

Tyto modely jsou používané častěji než EPM – ve více než 80 % (Marbacher et al., 2010). Oproti předchozímu modelu má injekční aplikace krve výhodu v lepší predikovatelnosti objemu krve v subarachnoidální prostoru i její lokalizace. Další výhodou oproti EPM je možno použít kontrolní skupinu s aplikací fyziologického roztoku nebo umělého likvoru. Nevýhodou je absence poškození cévní stěny, ke kterému při ruptuře aneurysmatu dochází vždy. Objem injikované krve se pro dospělého potkana pohybuje zpravidla mezi 200–300 mikrolitrů (Cai et al., 2012; Marbacher et al., 2010; Solomon et al., 1985). Pro nácvik operační techniky je výhodné použít aplikaci umělého likvoru zbarveného Evansovou modří (obr. 1).

INJEKCE DO CISTERNA MAGNA

Aplikace do cisterna magna se provádí ze zadního kraniocervikálního přístupu. Pokusnému zvířeti je provedena incize subokcipitálně a tupou preparací ozřejměna okcipitální kost, atlas a atlantookcipitální membrána, která je punktována. Po aspiraci likvoru je aplikována čerstvá autologní arteriální krev. K dosažení optimální distribuce krve je pokusné zvíře nakloněno hlavou dolů v úhlu 20° po dobu 30 minut (Gules et al., 2002).

Minimálně invazivní modifikace, které můžou snížit rizika rané infekce nebo rozvoje likvorové píštěle, umožňují perkutánní punkci cisterna magna bez nutnosti preparace měkkých tkání, pouze změnou sklonu punkční jehly nebo hlavy pokusného zvířete (Dusick et al., 2011; Munoz-Sanchez et al., 2012).

Pro studium pozdních vazospasmů je vhodnější tzv. „double hemorrhage model“ (DHM). Při navození SAK metodou dvojí hemoragie je stejným způsobem aplikována druhá dávka krve v odstupu 48 hodin. Při DHM dosahuje pozdní fáze vazospasmu maxima v 7. dni od první aplikace krve a spasmus je výraznější (Gules et al., 2002; Marbacher et al., 2010; Suzuki et al., 1999).

INJEKCE DO PRECHIASMATICKÉ CISTERNY

Alternativou předchozího modelu je aplikace krve do prechiasmatické cisterny (Prunell et al., 2002). V tomto modelu je spinální jehla stereotakticky zavedena do prechiasmatické cisterny ve střední čáře 7,5 mm rostrálně od bregmatu (obr. 2). Jehla je skloněna o 30° a zavedena intracisternálně, dokud se hrot jehly nedotkne lebeční baze. Následně je aplikována krev.

Výhodou tohoto modelu je, že distribuce krve v subarachnoidálním prostoru se více blíží distribuci krve při ruptuře aneurysmatu Willisova okruhu, zatímco při aplikaci do cisterna magna je krev lokalizována převážně v zadní jámě lebeční (Prunell et al., 2003). Aplikace krve do prechiasmatické cisterny způsobuje výraznější vazospasmus na a. cerebri anterior, ve srovnání s aplikací do cisterna magna (Cai et al., 2012). Při použití této metody nehrozí riziko poškození mozkové tkáně (Prunell et al., 2003). U modelu používajícího aplikaci do cisterna magna byla popsána větší variabilita intrakraniálního objemu podané krve, která může částečně unikat do páteřního kanálu (Prunell et al., 2003).

ZÁVĚR

Jednoduchá manipulace, dobrá reprodukovatelnost i nízké náklady jsou příčiny rostoucí popularity laboratorního potkana pro modelování subarachnoidálního krvácení. Obzvlášť modely založené na injekční aplikaci krve umožňují dobrou predikovatelnost rozsahu krvácení, která je jedním z faktorů ovlivňujících rozvoj pozdních vazospasmů a DCI (Claassen et al., 2001; Kistler et al., 1983).

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