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ORIGINAL ARTICLE

Early Inflammatory Response in the Brain and Anesthesia Recovery Time Evaluation After Experimental Subarachnoid Hemorrhage

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Abstract

The main objective was to evaluate, whether the subarachnoid hemorrhage (SAH)-associated early inflammatory response has focal or global character, i.e., whether areas distant to hematoma may be affected by an early inflammatory response. The second objective was to evaluate the association of anesthesia recovery time for basic reflexes/neurological functions with severity of SAH. SAH was induced in rats using an endovascular perforation model. Anesthesia recovery time was evaluated for pain reaction recovery time (spinal level), spontaneous ventilation recovery time (brain stem level), and consciousness recovery time (neocortical level). mRNA expressions of TNFα, IL-1β, IL-6, ICAM-1, and VCAM-1 in areas adjacent and distant to hematoma were evaluated between 2 and 8 h after SAH. Serum levels of TNFα, IL-1β, and IL-6 were assessed at 4 and 8 h after SAH. Anesthesia recovery time of all selected parameters was associated with severity of SAH. The consciousness recovery time test had the best predictive value, while the spontaneous ventilation recovery time test was able to bring information in the shortest time. The mRNA expressions of pro-inflammatory cytokines were significantly increased in severe SAH groups in both adjacent and distant areas. The inflammatory response in mild/moderate SAH groups was less strong, peaking at 4 h after SAH. Serum levels of pro-inflammatory cytokines were ambiguous. Anesthesia recovery time may be useful for bleeding severity prediction in the SAH model; however, further validation is needed. Severe subarachnoid hemorrhage is associated with the strong early inflammatory response, which has a global character, while mild subarachnoid hemorrhage is accompanied by a weaker inflammation.

Keywords Subarachnoid hemorrhage · Inflammation · Early brain injury · Anesthesia recovery time

Introduction

Early brain injury (EBI) is a complex of pathophysiological processes, which are associated with subarachnoid hemorrhage (SAH), and which may significantly contribute to SAH-related morbidity and mortality [1, 2]. The most important contributors to early brain injury are transient global cerebral ischemia, oxidative stress, neuronal/endothelial apoptosis, blood-brain barrier disruption, and inflammation [1, 3].

Inflammation plays a major role in EBI; inflammation may be considered as a cross-link among above-mentioned pathologies [4–8]. Activation of the immune system occurs immediately after onset of bleeding and inflammation after SAH may be considered as a two-step process: it is initially a local reaction which is later generalized to systemic response [9, 10]. Local response is represented by the activation of microglia and endothelial cells, which produce pro-inflammatory cytokines and adhesion molecules [11, 12]. Pro-inflammatory cytokines released into blood activate leukocytes in the periphery; adhesive molecules attract them to the affected area, resulting in leukocyte infiltration and subsequent promotion of inflammatory response [11, 13, 14].

Local response, an initial step of inflammatory response after SAH, is a minor topic of current research. Previous studies demonstrated that the local inflammatory response in the brain may be detected within hours after onset of bleeding,

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peaking between 4 and 6 h after SAH [11]. However, it is still not clear whether the local inflammatory response, which reflects activation of the immune system in the brain after SAH, has focal or global character, i.e., whether an early inflammatory response occurs only in areas affected by bleeding or whether it may occur also in distant areas. Information about the spatial context of early inflammatory response SAH may help us to understand whether the early brain injury has global impact on the brain from the very early beginning or whether it develops over time. The main goal of this work was to find out, whether the early inflammatory response after SAH occurs only in areas adjacent to hematoma or whether it may occur also in distant areas of the brain.

Considering experimental SAH, there is currently no test designed for evaluation of initial neurological status. Standard behavioral tests are not suitable for this purpose; these tests are substantially influenced by anesthesia, because the tests are partially based on spontaneous activity (psychological functions) [15, 16]. However, we may assume that basic reflexes/neurological functions, which are crucial for the organism, may be affected by anesthesia in a more consistent way and similar exposures to anesthesia may be associated with similar recovery time unless there is an influence of pathology. Evaluation of recovery time for basic reflexes/neurological functions could be potentially used for evaluation of consistency of the model and prediction of SAH severity. Potential candidates for such evaluation are pain reflex (spinal level), respiratory reflex (brain stem level), and consciousness regaining (cortical level). The second goal of this study was to evaluate whether recovery time for the above-mentioned reflexes/neurological functions may be associated with severity of SAH.

Material and Methods

Animal Experiments

All the experiments were approved by the Masaryk University Institutional Animal Care and by the Ministry of Education, Youth and Sports of the Czech Republic. Adult male Sprague Dawley rats (260–300 g) purchased from the Animal Facility of Masaryk University were used for the purpose of this study. The animals were assigned to sham or SAH group and to one of the following timepoint groups: 2, 4, 6, or 8 h. The animals were anesthetized using isoflurane in 60%/40% medical gas/oxygen mixture. Five percent concentration of isoflurane was used for induction of anesthesia and after the intubation, the anesthesia was maintained using 3% isoflurane concentration. SAH was induced by sharpened 4–0 nylon suture through the left internal carotid artery as described previously [17]. In the case of sham animals, the suture was inserted into the artery, but no perforation was performed. The anesthesia was terminated 5 min after induction of SAH/suture insertion. At the end of surgery, 1 ml of saline was injected intraperitoneally to prevent dehydration.

After the surgery, animals were kept on mechanical ventilation until spontaneous ventilation was sufficient. If spontaneous ventilation did not recover, the animals were kept on mechanical ventilation during the whole period of monitoring and basic vital signs (heart action, color of eyes) were observed. When basic vital signs were not present in unconscious animals, these were excluded, generating mortality group. In animals which recovered from anesthesia, the following neurological parameters were evaluated: spontaneous ventilation recovery time, pain reaction recovery time, and consciousness recovery time. Respiratory reflex recovery was considered as a criterion for spontaneous ventilation recovery, no matter whether spontaneous ventilation was sufficient. A pain stimulus represented pinch to tail and any escape/defensive reaction was considered as a positive result of the pain reaction recovery test. The criterion for consciousness regaining was a spontaneous awake reaction or awake reaction in response to pain stimulus, no matter whether animal later fell asleep again. These parameters were evaluated every 5 min until full recovery or up to 1 and half hour after surgery. All the evaluations were performed in a blind manner by one person. A heating lamp was used during the whole period of unconsciousness to prevent hypothermia. Detailed protocol of anesthesia and anesthesia recovery time evaluation may be found as a supplementary file.

Animals were sacrificed one of the following timepoints: 2, 4, 6, or 8 h, the picture of the basal part of the brain was taken to evaluate SAH grade, and the samples of the brain and serum were collected. Severity of SAH was evaluated using the SAH grading system [17]. According to the SAH grade, the animals were assigned to mild/moderate SAH group (SAH grade 6–12; below labeled as mild SAH) and severe SAH group (SAH grade 13–18), while animals with SAH grade lower than 6 were excluded for insufficient SAH, as it is usual [17].

Samples

Brain samples were collected from the basal part of the left hemisphere (the area adjacent to hematoma—directly affected by hematoma; below labeled as A samples) and from the right dorsal hemisphere (area distant from hematoma—not affected by hematoma; below labeled as D samples). The samples were homogenized using the glass bead beating technique, frozen in RNA later, and stored at − 80 °C until processed. Isolation of total RNA was performed with Tripure reagent (Qiagen) according to the manufacturer's instructions. RNA samples were suspended in RNAse-free water and quantified spectrophotometrically. Genomic DNA contamination was removed by DNAse I treatment (Invitrogen). Single-stranded cDNA was synthesized from 1 μg total RNA in a volume of 50 μl using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland)

(using random hexamers). Gene expression was evaluated by real-time reverse transcription polymerase chain reaction (RT-PCR) analysis using TaqMan chemistry (Applied Biosystems). TaqMan assays were run for TNFα, IL-1β, IL-6, ICAM-1, VCAM-1, and two housekeeping genes—β-actin and hypoxanthine phosphoribosyltransferase (HPRT1) to determine which housekeeping genes were most suitable for subsequent normalization of data. Amplification reactions were performed on ABI PRISM 7000 Sequence Detection System (Applied Biosystems) with the following thermal cycling conditions: 95 °C for 10 min and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. An average was calculated for Ct of control after normalization with HPRT1. The calculation of fold change was according to the comparative Ct method (2–ΔΔCt method). Serum cytokines TNFα, IL-1β, and IL-6 were measured in samples frozen immediately after sampling and then stored in − 80 °C awaiting analysis. The samples were analyzed using commercial ELISA provided by Invitrogen (IL-6, 0–1500 pg/ml; TNFα, 0–750 pg/ml) and USCN Life Science (IL-1β, 0–800 pg/ml). Intra-assay CV and inter-assay CV for all analysis were < 10%.

Statistical Analyses

The distribution of the data of SAH grade and neurological parameters met the criteria of normal distribution (D'Agostino and Pearson normality test). Ordinary one-way ANOVA followed by Bonferroni's multiple comparison test was used to evaluate these data. ROC analysis was subsequently performed in mild and severe SAH groups. The results of laboratory analyses were log-normally distributed; these data were logarithmically transformed, and the statistical analyses were performed on transformed data using ordinary one-way ANOVA followed by Bonferroni's multiple comparison test. A p value of < 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism version 7.03 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com). Data are presented as mean and standard deviation (SAH grade and neurological parameters) or geometric mean and geometric SD factor (the rest of the data). The data representing mRNA expressions are presented as a fold change normalized to the absolute control group. In figures, $p < 0.05$ is labeled as *, $p < 0.01$ is labeled as **, and $p < 0.001$ is labeled as ***.

Results

Basic Characteristics of the Study Group

In total, 108 animals were used for purpose of this study, 104 surgeries were performed in this project and another four animals were used as an absolute control (no surgery) for mRNA standardization. From the total amount of operated animals, 16 animals were excluded due to mortality of the model (14.6%) and another 4 animals were excluded because of the insufficient SAH grade. The final laboratory analyses were performed on the group of 84 animals divided into 12 groups according to SAH grade (sham, mild SAH, severe SAH) and timepoint (2, 4, 6, and 8 h), $n = 7$ per group. The evaluation of the initial neurological status was performed in sham, mild, and severe SAH groups with no regard to timepoints used for laboratory analyses.

Three animals did not fully recover from anesthesia and were kept on mechanical ventilation for the whole period of monitoring (2-h timepoint, 1 animal; 4-h timepoint, 1 animal; and 8-h timepoint, 1 animal). Vital signs (color of the eye and heart action) were present in all animals for the whole period of monitoring and reaction to pain recovered in two cases. These animals were not used for evaluation of neurological parameters (infinite recovery time), but all the laboratory parameters were in the range typical for severe SAH groups and these samples were included in the evaluation of inflammatory response.

Basic characteristics of the study group, according to the SAH grade, are summarized in Table 1. In mild SAH groups, SAH grades are comparable in all the timepoints; the mean value of SAH grade is around 9. In severe SAH groups, the SAH grades are also similar in all the timepoints; the mean value of SAH grade is around 16 in these animals.

The Initial Neurological Status

The aim of this assessment was to evaluate, whether recovery time of basic neurological parameters is suitable for evaluation of initial neurological status of experimental animals. We evaluated spontaneous ventilation recovery time, reaction to pain recovery time, and consciousness recovery time. An important prerequisite for such an evaluation was similar exposure to anesthesia in all the groups.

Figure 1 and Table S1 (in supplementary file) demonstrate that the duration of anesthesia was comparable in all the three

Table 1 Basic characteristics of the study group according to SAH grade expressed as a mean (M) and standard deviation (SD); $n = 7$ per group

	Mild 2 h	Mild 4 h	Mild 6 h	Mild 8 h	Severe 2 h	Severe 4 h	Severe 6 h	Severe 8 h
M	9.29	8.57	9.00	8.71	16.43	16.13	15.57	16.14
SD	1.89	1.51	2.10	2.14	1.51	1.89	1.62	1.57

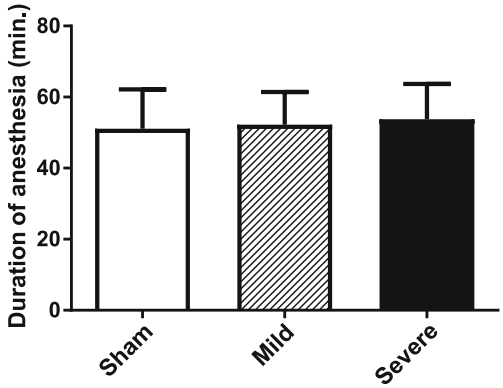

Fig. 1 Duration of anesthesia groups in sham, mild, and severe SAH groups. The data are plotted as a mean and SD; $n = 28$ per group

groups. A total time of anesthesia (including induction of anesthesia) was, in average, around 52 min.

Figure 2 and Table S2 (in supplementary file) show the recovery time of basic vital parameters (A, spontaneous ventilation recovery; B, pain reaction recovery; and C, consciousness recovery). The recovery time was significantly longer in the severe SAH group compared to that in both the sham and mild SAH groups in all three parameters. The highest contrast was observed in the case of consciousness recovery; an average time in the sham group was around 15 min, in the mild SAH group around 20 min while in the severe SAH group, regaining of consciousness took around 45 min on average (Fig. 2c, Tab. S2).

Table 2 summarizes the results of ROC analysis of mild and severe SAH groups for a spontaneous ventilation recovery time, pain reaction recovery time, and consciousness recovery time. In this case, sensitivity represents the ability of assessment to correctly detect absence of particular reflex in the severe SAH group, while specificity represents an ability of assessment to correctly detect presence of the reflex in the mild SAH group. The best predictive value has consciousness recovery time (AUC, 0.95; AUC SE, 0.026); the suitable time for this assessment is around 32nd minute after SAH induction: if the counsciousness is recovered, there is a 92% chance that SAH will be mild and if the cousciousness is not recovered, there is an 83% chance that SAH will be severe. Finally, if the consciousness will not recover within approximately 38 min after SAH induction, severe SAH may be expected with almost 100% chance and around 67% of severe SAH animals in our study group did not recover within this period. Spontaneous ventilation recovery time has lower predictive value (AUC, 0.87; AUC SE, 0.053) than previous test, but this test may bring us an information earlier. The suitable time for this assessment is around 12th minute after SAH induction: if an animal breathes, there is a 93% chance that SAH will be mild and if an animal does not breathe, there is a 76% chance that SAH is severe. If ventilation will not recover within approx. 18 min, the severe SAH may be expected with almost 100% chance and around 56% of animals in our study group with severe SAH did not recover ventilation reflex within this period. Pain reaction recovery time does not bring us better information than previous tests, predictive value is lower (AUC, 0.83; AUC SE, 0.060), and the suitable time for this assessment is around an 18th minute after SAH induction.

Inflammatory Response in the Brain Following SAH

The inflammatory response in the brain was evaluated on the level of mRNA expressions of pro-inflammatory cytokines (TNFα, IL-1β, IL-6) and adhesive molecules (ICAM-1, VCAM-1). Expressions of the above-mentioned parameters

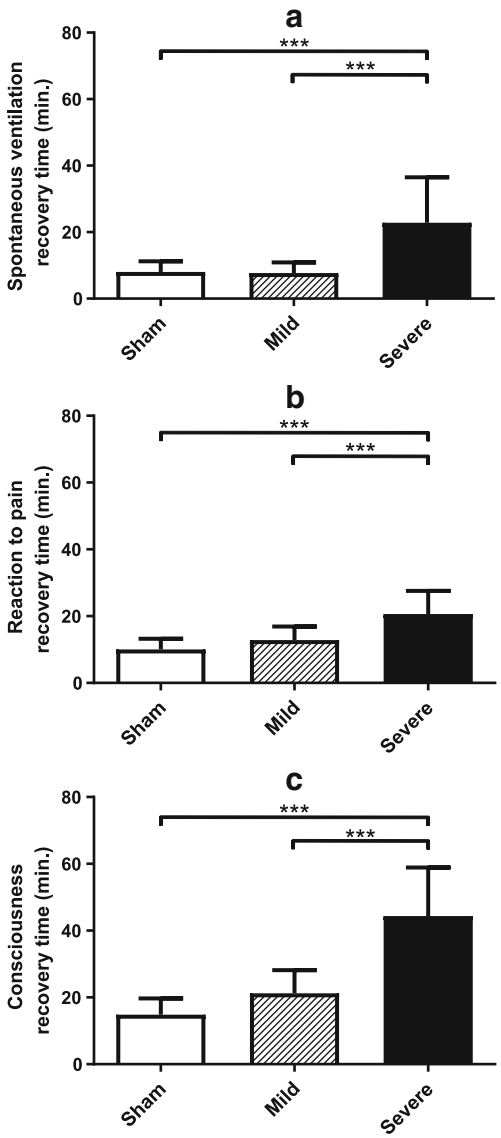

Fig. 2 Spontaneous ventilation recovery time (**a**), reaction to pain recovery time (**b**), and consciousness recovery time (**c**) in sham ($n = 28$), mild ($n = 28$), and severe ($n = 25$) SAH groups. The data are plotted as a mean and SD. $p < 0.001$ is labeled as ***

Table 2 Selected results of ROC analysis expressed in percent and 95% confidence interval range in mild ($n = 28$) and severe ($n = 25$) SAH groups for ventilation recovery time (ventilation), pain recovery time (pain), and consciousness regaining time (consciousness)

	Time (min)	Sensitivity (%)	95% CI	Specificity (%)	95% CI
Ventilation	> 7.5	88	69 to 97%	54	33 to 73%
	> 12.5	76	55 to 91%	93	75 to 99%
	> 17.5	56	35 to 76%	100	87 to 100%
Pain	> 12.5	88	69 to 97%	48	28 to 69%
	> 17.5	72	51 to 88%	88	69 to 97%
	> 22.5	36	18 to 58%	100	86 to 100%
Consciousness	> 22.5	100	86 to 100%	68	47 to 85%
	> 27.5	88	68 to 97%	76	55 to 91%
	> 32.5	83	63 to 95%	92	74 to 99%
	> 37.5	67	45 to 84%	100	86 to 100%

were evaluated 2, 4, 6, and 8 h after induction of SAH in the areas adjacent to hematoma (ipsilateral basal hemisphere) and in the areas distant to hematoma (contralateral dorsal hemisphere).

Figure 3 and Table S3 (in supplementary file) show TNFα mRNA expression 2, 4, 6, and 8 h after induction of SAH. There was a statistically significant difference between sham and severe SAH groups in all the timepoints both in areas adjacent to hematoma (A) and distant areas (D). The difference between sham and mild SAH groups was statistically significant 4 h after induction of SAH in both areas. The difference between mild and severe SAH groups was statistically significant in 2- and 6-h timepoints in both adjacent and distant areas. Finally, the statistically significant difference in TNFα expression between adjacent and distant areas was observed in sham groups 2 h after surgery.

Figure 4 and Table S4 (in supplementary file) show IL-1β mRNA expression 2, 4, 6, and 8 h after induction of SAH. The difference between sham and severe SAH groups was statistically significant in all the timepoints in both areas, while the difference between sham and mild SAH groups was statistically significant at 4 h after surgery in both areas. The difference between mild and severe SAH groups was statistically significant in 2-, 4-, and 6-h timepoints in the adjacent area and in 2- and 6-h timepoints in distant areas. Finally, the statistically significant difference in IL-1β expression between adjacent and distant areas was observed in severe SAH groups at 2 and 6 h after surgery.

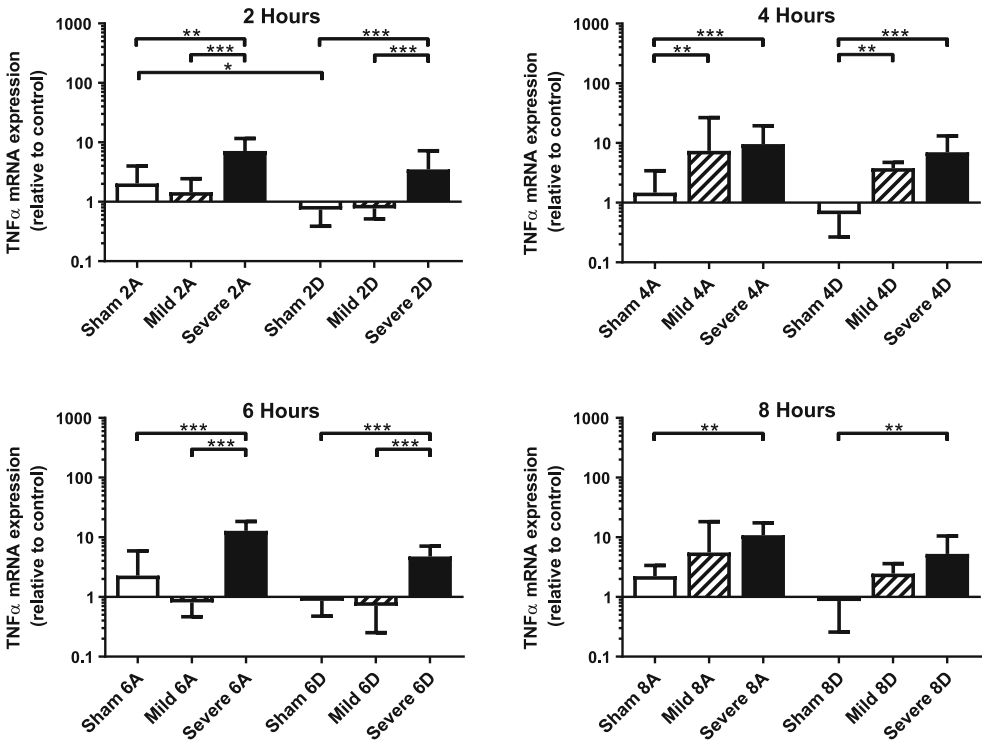

Fig. 3 TNFα expression in areas adjacent (A) and distant (D) to hematoma. The data are normalized to absolute control group and plotted as a geometric mean and geometric SD factor. $p < 0.05$ is labeled as *, $p < 0.01$ is labeled as **, and $p < 0.001$ is labeled as ***; $n = 7$ per group

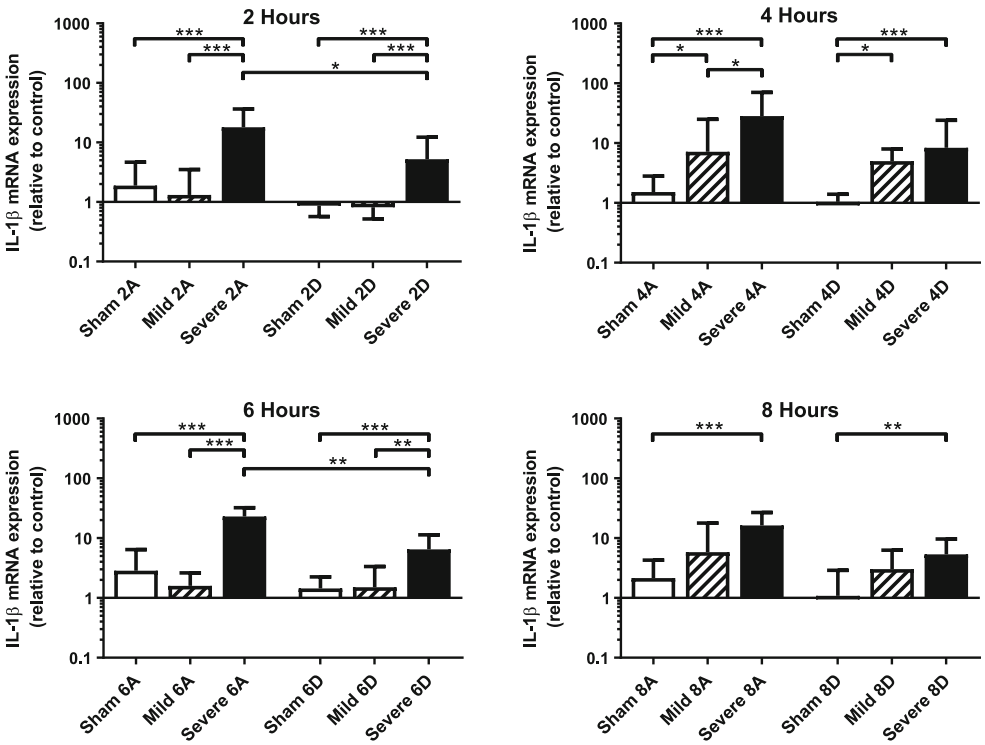

Fig. 4 IL-1β expression in areas adjacent (A) and distant (D) to hematoma. The data are normalized to absolute control group and plotted as a geometric mean and geometric SD factor. $p < 0.05$ is labeled as *, $p < 0.01$ is labeled as **, and $p < 0.001$ is labeled as ***; $n = 7$ per group

Figure 5 and Table S5 (in supplementary file) show IL-6 mRNA expression 2, 4, 6, and 8 h after induction of SAH. The difference between sham and severe SAH groups was statistically significant in all the timepoints in adjacent areas and in 2-, 4-, and 8-h timepoints in distant areas. The difference between sham and mild SAH groups was statistically significant in none of timepoints in any areas. The difference between mild and severe SAH groups was statistically significant in all the timepoints in the adjacent area and in 2- and 4-h timepoints in distant areas. Finally, the statistically significant difference between adjacent and distant areas was observed in severe SAH groups at 4, 6, and 8 h after the surgery.

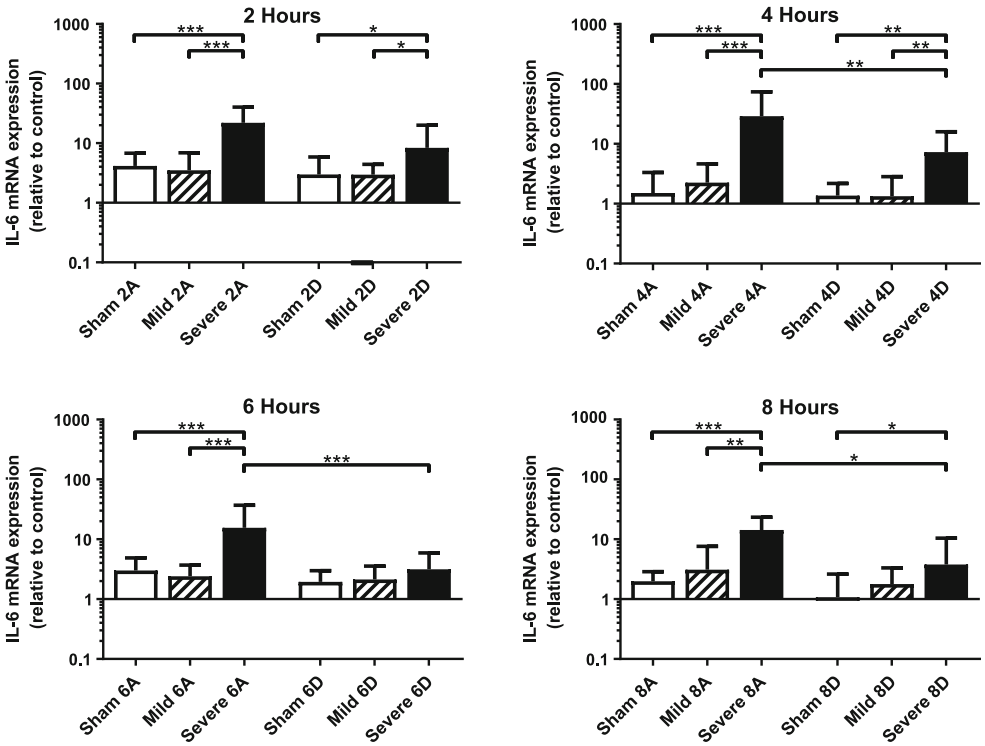

Fig. 5 IL-6 expression in areas adjacent (A) and distant (D) to hematoma. The data are normalized to absolute control group and plotted as a geometric mean and geometric SD factor. $p < 0.05$ is labeled as *, $p < 0.01$ is labeled as **, and $p < 0.001$ is labeled as ***; $n = 7$ per group

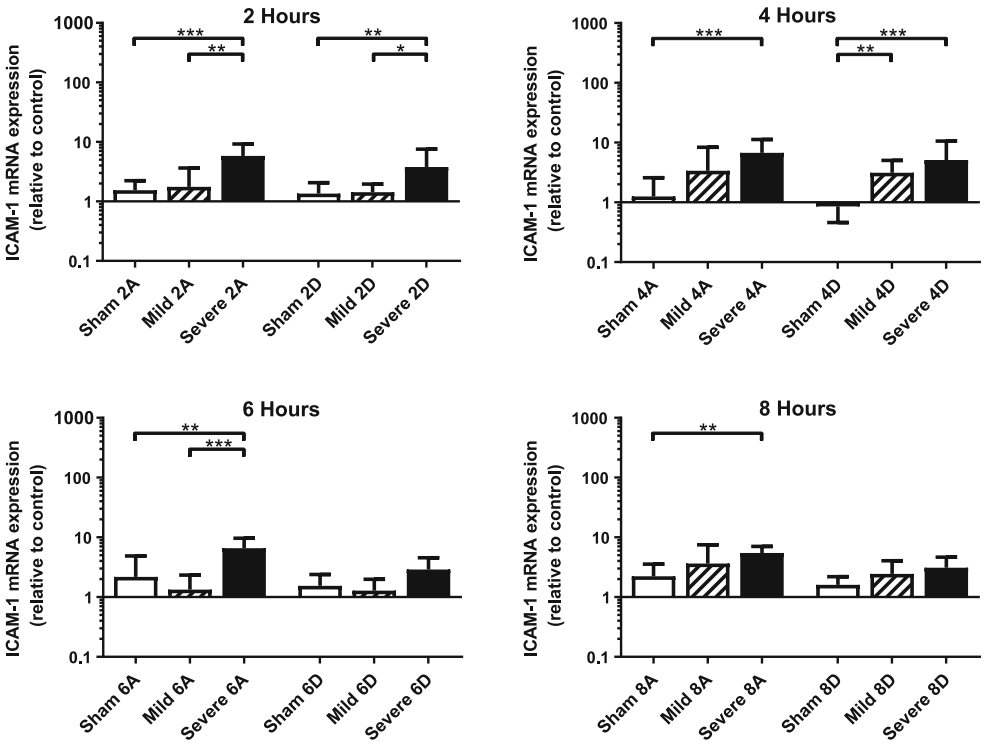

Fig. 6 ICAM-1 expression in areas adjacent (A) and distant (D) to hematoma. The data are normalized to absolute control group and plotted as a geometric mean and geometric SD factor. $p < 0.05$ is labeled as *, $p < 0.01$ is labeled as **, and $p < 0.001$ is labeled as ***; $n = 7$ per group

Figure 6 and Table S6 (in supplementary file) show ICAM-1 mRNA expression 2, 4, 6, and 8 h after induction of SAH. There was a statistically significant difference between sham and severe SAH groups in all the timepoints in adjacent areas and at 2 and 4 h in distant areas. Statistically significant difference between mild and severe SAH groups was observed in 2-h timepoint in both areas and in 6-h timepoint in adjacent areas. Finally, there was statistically significant difference between sham and mild SAH groups in 4-h timepoint in distant areas.

Figure 7 and Table S7 (in supplementary file) show VCAM-1 mRNA expression 2, 4, 6, and 8 h after induction of SAH. In contrast to ICAM-1, no significant difference was

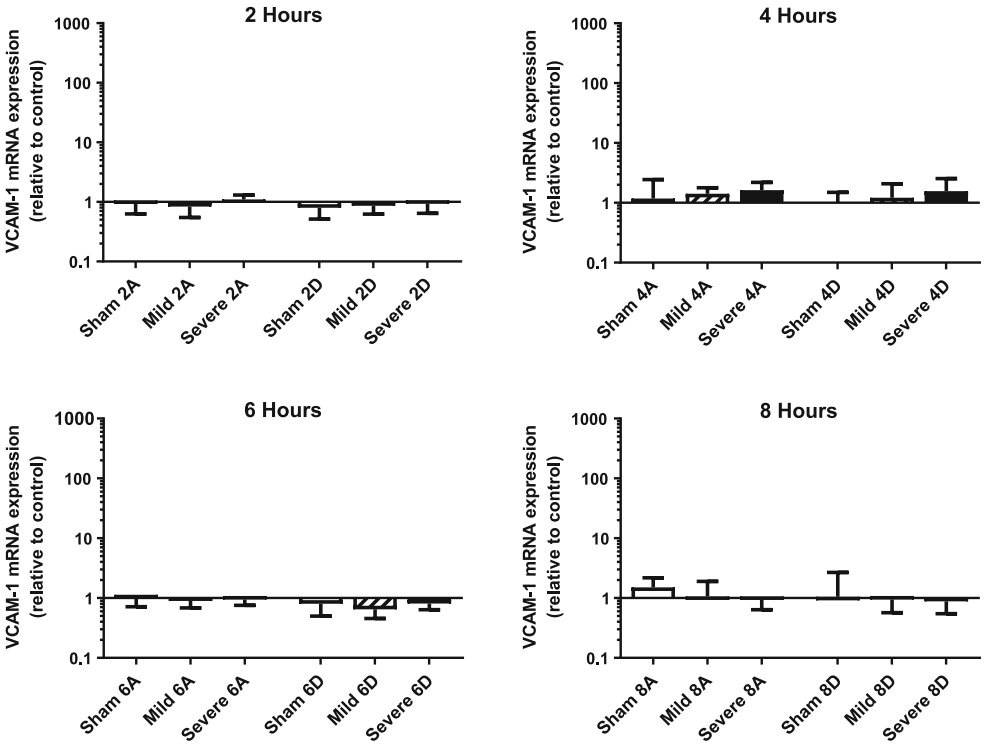

Fig. 7 VCAM-1 expression in areas adjacent (A) and distant (D) to hematoma. The data are normalized to absolute control group and plotted as a geometric mean and geometric SD factor. $p < 0.05$ is labeled as *, $p < 0.01$ is labeled as **, and $p < 0.001$ is labeled as ***; $n = 7$ per group

observed between the groups in any timepoint in case of VCAM-1.

Serum Levels of Pro-inflammatory Cytokines After SAH

Figure 8a, b and Table S8 (in supplementary file) show serum levels of TNFα and IL-1β in sham and severe SAH groups 4 and 8 h after surgery. No significant differences between sham and severe SAH groups were observed in the case of TNFα and IL-1β neither 4 nor 8 h after induction of SAH. Overall levels of TNFα were within physiological range, while levels of IL-1β were increased above physiological levels in a similar way in all the groups.

Figure 8c and Table S8 (in supplementary file) show serum levels of IL-6 in sham, mild, and severe SAH groups 4 and 8 h after surgery. There was significant difference between sham and severe groups as well as between mild and severe SAH groups 4 h after surgery. No significant difference was observed 8 h after surgery.

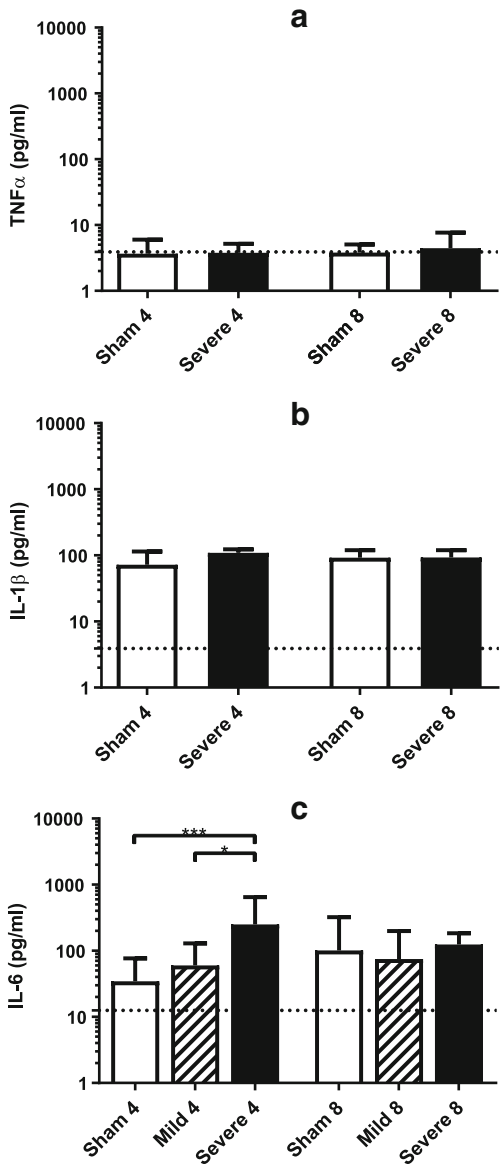

Fig. 8 Serum levels of TNFα (**a**), IL-1β (**b**), and IL-6 (**c**) at the 4- and 8-h timepoints. The data are plotted as a geometric mean and geometric SD. The dotted line represents the threshold value of physiological levels. $p < 0.05$ is labeled as * and $p < 0.001$ is labeled as ***; $n = 7$ per group

Discussion

The main goal of this study was to evaluate whether the early inflammatory response after SAH has in brain focal or global character. In this study, we used timepoints of 2, 4, 6, and 8 h after induction of bleeding and since there is no neurological evaluation designed for such short timepoints, we have also tested whether the anesthesia recovery time of basic reflexes/neurological parameters may be associated with severity of SAH.

The mortality of the model was 14.6% that is lower than typical data published in a perforation model of SAH [18–20]. We attribute it firstly to short timepoints, because we may assume that some of the severe SAH animals, which survived for a short period of time, would not survive for 24 h or longer. Additionally, severe SAH animals with respiration failure may be kept on mechanical ventilation for several hours. It is well known that severe stroke is associated with respiratory failure and the need of mechanical ventilation is often in patients suffering from severe stroke [21]. Keeping animals on mechanical ventilation for limited period allowed us to evaluate severe SAH animals which would die due to respiratory failure. We had three animals which were kept on mechanical ventilation for the whole period of monitoring (2-h timepoint, 1 animal; 4-h timepoint, 1 animal; and 8-h timepoint, 1 animal). The vital signs were monitored in these animals—the heart action and the color of the eyes as a sign of cerebral circulation—because an ophthalmic artery is a branch of the internal carotid artery. These animals were not used for basic reflexes recovery time evaluation, because the recovery time of some of the parameters would be nonsense (infinite). Interestingly, parameters of inflammatory response were within a standard range for severe SAH. Based on these observations (no statistical analysis was performed because of the sample size), we may assume that these animals may be potentially used for evaluation of inflammatory together with all other severe SAH animals.

Basic neurological parameters used for evaluation of anesthesia recovery time were respiratory reflex (spontaneous ventilation recovery), pain reflex, and consciousness recovery time. These parameters reflect activity on the spinal level (pain reflex), brain stem level (respiratory reflex), and neocortical level (consciousness). We used these parameters because standard neurological tests are not designed (and suitable) for

early timepoints [15, 16]. An effect of anesthesia was discussed in the "Introduction." Additionally, the standard neurological tests are designed as a sort of outcome tests and it has no meaning to perform the tests early after SAH. We used anesthesia recovery time-based tests to evaluate the influence of SAH on the initial neurological status. The prerequisite for this evaluation is similar exposure to anesthesia in all the groups and this criterion was fulfilled in our study groups. We have also paid attention to consistency in the interval between SAH induction and termination of anesthesia; the anesthesia was terminated 5 min after SAH induction/suture insertion. The results of all the evaluated parameters were quite uniform: there was a significant difference in recovery time between severe SAH groups and mild SAH groups while there was no difference between sham and mild SAH groups. The highest contrast was observed in the consciousness recovery time test and this test had the best predictive value. The spontaneous ventilation recovery time was able to bring us relatively accurate information in the shortest time, while the pain reaction recovery test did not bring us better information than previous tests. We think that the most interesting result of ROC analysis is the finding that an absence of respiratory response for more than 18 min after induction of bleeding (or 13 after termination of anesthesia) may indicate severe SAH with almost 100% probability. However, since the above-proposed tests represent different levels of neural activity, we think that it is useful to perform all the tests, even though some of them do not bring valuable information. These tests may be theoretically used in two ways. Firstly, these tests may be used for prediction of SAH severity and for evaluation of consistency of the model. Secondly, these tests may be potentially used for evaluation of the preconditioning treatment effect. Hypothetically, if preconditioning treatment protects against impact of primary injury (for example, by the decrease of oxygen/glucose consumption in neurons), recovery time may be shorter than in cases of untreated animals. And if the recovery time is similar in groups with preconditioning treatment and without treatment, while later evaluated, neurological status is improved in treated groups; this may be indirect evidence that preconditioning targets secondary brain injury. We may thus assume that anesthesia recovery tests may be potentially used in addition to standard neurologic tests and may possibly bring us useful information. However, we are aware that further independent validation is needed before reaching conclusion about the usefulness of anesthesia recovery time tests.

The major goal of this study was to answer the question, whether the early inflammatory response in the brain after SAH has a focal or global character. We evaluated mRNA expressions of widely used pro-inflammatory cytokines (TNFα, IL-1β, IL-6) and adhesive molecules (ICAM-1, VCAM-1) in areas adjacent to hematoma and the areas distant to the hematoma. In general, the mRNA expressions of selected pro-inflammatory cytokines were mainly increased in severe SAH groups and this was clear in both adjacent and distant areas in majority of the timepoints. The inflammatory response in mild SAH groups was less strong with a peak at 4 h after SAH. Significant changes were also found in ICAM-1 mRNA expression, while VCAM-1 mRNA expression was not changed. These results indicate that mainly severe SAH is associated with strong activation of the immune system in the brain and the early inflammatory response has a global character. Areas adjacent to hematoma are in contact with blood and possibly damaged. The inflammatory response in these areas may be considered as a "physiological" reaction to the presence of blood and potential tissue damage. The activation of an immune response in adjacent areas has been previously described and our results are in accordance with those of previously published data [11]. Areas distant to hematoma are not primarily affected by hematoma and activation of the immune system in these areas indicates global activation of the immune system in the brain. This suggests that pathophysiological processes associated with early brain injury are complex and may affect all the brain from the very early beginning, especially in the case of severe SAH. A possible reason for activation of the inflammatory response in distant areas may be transient global cerebral ischemia, ischemia caused by acute vasospasms after SAH, or with deregulation of inflammatory response which may occur after stroke [22–24]. ICAM-1 mRNA expression indicates that the inflammatory response may be in severe cases directed to systemic inflammatory response from very early beginning and this reaction may have also global character. Early upregulation of ICAM-1 mRNA expression is known from the ischemic stroke model as well as the fact that VCAM-1 expression may be unaffected [25].

Finally, we have evaluated serum levels of pro-inflammatory cytokines (TNFα, IL-1β, IL-6) in selected groups and timepoints to find out whether we may, early after SAH, detect an effect of cerebral immune system activation in periphery. Serum levels of pro-inflammatory cytokines were ambiguous. TNFα was physiological in all the groups, while IL-1β was increased in a similar way in both sham and severe SAH groups in these timepoints. Serum levels of IL-6 were increased in the severe SAH group at 4-h timepoint, while at 8 h after SAH, IL-6 levels were increased in all three groups in a similar way. These results may indicate that the surgical procedure may mask the effect of SAH and make hard to evaluate levels of pro-inflammatory cytokines in periphery early after SAH.

There are several limitations of this study. Firstly, the short timepoints are good to answer the questions about the pathophysiology of early brain injury, but short timepoints cannot give us information about the outcome. We did not use any standard timepoints and this is the limitation. We were, however, focused on evaluation of the initial step of inflammatory response (local reaction in the brain). If we use later timepoints, we would need to take into account inflammation mediated by leukocytes from periphery, while in timepoints

we used, we may assume that inflammatory response represents local reaction [11, 13]. Secondly, we did not evaluate the potential reason for global activation of the inflammatory response. However, in this study, we were focused only on description of the spatial context of the inflammatory response, because the exaggerated inflammatory response itself may cause significant tissue damage [14, 26]. Thirdly, regarding anesthesia recovery time evaluation, we did not evaluate inter-rater reproducibility, because all the evaluations were performed by one person. We assume that inter-rater reproducibility may be potentially consistent, because all the parameters may be considered as relatively objective (presence/absence of reaction). However, final conclusion has to be drawn from the future studies.

Conclusion

Severe subarachnoid hemorrhage is associated with the strong early inflammatory response, which has a global character, while mild subarachnoid hemorrhage is accompanied by a weaker inflammatory response. This indicates that inflammation may affect the whole brain from the very early beginning of SAH pathophysiology, especially in severe cases. The serum levels of pro-inflammatory cytokines do not reflect the early inflammatory response in the brain, possibly because of the effect of surgery on peripheral inflammation. Anesthesia recovery time may be useful for prediction of severity of bleeding in SAH model. Consciousness recovery time has the best predictive value, while spontaneous ventilation recovery time may indicate severe SAH as early as 18 min after induction of SAH. Further validation is needed to evaluate usefulness of anesthesia recovery time tests.

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Compliance with Ethical Standards

All procedures performed in this study involving animals were in accordance with the ethical standards of Masaryk University Institutional Animal Care.

Conflict of Interest The authors declare that they have no conflict of interest.

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