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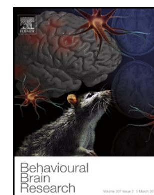

Research report

Behavioral characterization of the anterior injection model of subarachnoid hemorrhage

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HIGHLIGHTS

- Flex field analysis of SAH animals showed a significant reduction of locomotor activity compared to controls in correlation with severity.
- SHIRPA score revealed a significant reduction in motor behavior in SAH animals two days after surgery.
- SAH animals show a significant increase of GFAP expression, Fluoro Jade C and TUNEL positive cells as well as microthrombi.
- Significant negative correlation between flex field righting and the number of degenerative neurons or microthrombi.

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ABSTRACT

Background: The applicability of various neurological scores has not been sufficiently characterized in the anterior injection model of subarachnoid hemorrhage (SAH). Therefore this study was performed to evaluate different behavioral tests for quantifying disease severity.
Methods: Different volumes of autologous blood were injected stereotaxically into the prechiasmatic cistern of mice. Sham controls underwent the same procedure without blood injection. The following seven days after surgery, mice were evaluated for behavioral deficits by the SHIRPA score, beam balance and flex field analyses. Brains were further processed for histological analyses.
Results: Flex field analysis of SAH animals showed a significant reduction of locomotor activity compared to controls in the first two days after SAH. This reduction was more intense in animals with a higher amount of injected blood. The SHIRPA score revealed a significant reduction in motor behavior in SAH animals two days after surgery. A significant increase of GFAP expression, Fluoro Jade C and TUNEL positive cells as well as microthrombi was observed in SAH animals compared to sham controls in the early phase of SAH. There was a significant negative correlation between flex field righting and the number of degenerative neurons or microthrombi in the first two days after SAH.
Conclusion: The results of flex field analysis and SHIRPA single test show behavioral and functional deficits in the first two days after SAH in parallel to histological alterations indicating neuronal damage. In summary these tests can be used as functional outcome parameters in the anterior injection model of SAH.

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1. Introduction

Aneurysmal subarachnoid hemorrhage (SAH) is a neurologic emergency accounting for approximately 5% of all strokes. Its overall incidence is estimated to range between 10 and 20 per 100 000 per year [1,2]. Despite recent advances in techniques for securing aneurysms and neurocritical care, mortality remains high [3]. If the initial bleeding is survived and the aneurysm can be secured patients are at risk to experience a variety of potentially devastating secondary complications. The injurious consequences of SAH consist of defined early and delayed events. In the early phase the aneurysmal rupture may cause an increase of intracranial pressure (ICP) and reduces perfusion pressure as well as cerebral blood flow. Ischemic deficits due to hypo-perfusion and intracranial hypertension due to brain edema or acute hydrocephalus are typically observed in the first three days after the bleeding and are commonly referred to as early brain injury (EBI) [4]. Delayed cerebral ischemia (DCI) and cerebral vasospasm (CVS) occur in the major-

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ity of patients between day 4 to 14 after SAH [5] Importantly, the pathophysiology of these events after SAH is still poorly understood [6]. Therefore, animal models that mimic SAH are indispensable to study early and delayed brain injury. Numerous experimental models to examine SAH exist, each having advantages and disadvantages [7]. An ideal SAH model would be characterized by consistency of experimental SAH and close resemblance of the human pathophysiology. The advantage of the prechiasmatic injection technique in mice is the high reproducibility due to a defined amount of injected blood. Especially common complications such as neuronal cell death triggered by SAH are preferably investigated in the anterior injection model [8]. Numerous studies have shown that the injection model can be used to investigate early and late complications of SAH [9–11]. However, an important limitation of this model is the absence of mechanical disruption to the cerebral vasculature upon aneurysm rupture [12].

Further, this model has not well been characterized regarding the applicability in neuro-behavioral scores in the acute and post-acute phase after SAH. Therefore, this study was performed to evaluate the influence of SAH on the behavior of rodents. Mice underwent different behavioral tests to figure out if SAH mice show behavioral and physiological impairments compared to control groups after surgery. We show that flex field analysis and some single tests of the SHIRPA score battery are markedly altered in SAH animals compared to controls. Moreover, the grade of SAH defined by different volumes of injected blood correlates with the severity of the impairment.

2. Material and methods

2.1. Ethics statements

All animal studies conformed to the Austrian guidelines for the care and use of laboratory animals and were approved by the Austrian Ministry for Education, Science and Culture with the reference number: 535233. All surgery was performed under anesthesia and animals received analgesic treatment as described below. Importantly, anesthetic and analgesic regimens were carefully designed to minimize any opportunity for pain and distress.

2.2. Animals and surgery

A total of 65 10–12 weeks old C57BL/6 mice weighing 22–28 g were used for the study. The anterior injection model of SAH was performed as previously described [8]. Mice were randomly assigned to each of the experimental groups (sham, CSF, SAH). Briefly, mice were anesthetized with 3% isoflurane in 70%/30% medical-air/oxygen, animals received 3 mg/kg hydal subcutaneously immediately before surgery and 24/48 h after surgery. Experimental SAH was induced in mice by stereotaxic injection of autologous arterial blood (80, 100, 120 μl) or artificial CSF (120 μl) (controls) into the prechiasmatic cistern in an angle of 30° via a 0,6 mm skull borehole. Sham operated mice (controls) underwent the same procedure without blood or CSF injection. After surgery the incision was closed with skull wax, and mice were individually housed in heated cages until recovery. The overall mortality of 9% was calculated at 48 h after SAH; mice that did not survive the process were excluded from further histological assessment.

2.3. Neurological scores

On the day before and daily until day seven after surgery a battery of three different behavioral tests was performed. For the test 16 sham animals, 10 CSF controls and 33 SAH animals with 3 severity levels induced by the injection of different blood volume of 80 μl (mild SAH n = 16), 100 μl (moderate SAH, n = 5) and 120 μl (severe SAH, n = 12) were tested daily over a time period of two days. 5 SHAM, 5 CSF controls and 6 SAH animals were tested daily over a time period of seven days. Animals were investigated in 3 independent experimental trials.

The SHIRPA score comprises 40 single tests which can be summarized in 5 different functional categories giving a good overview about vegetative functions, neuropsychology, reflex and sensory, muscle tone strength and motor behavior of each animal [13,14].

The beam balance test is an assay for fine motor coordination and balance. Animals must traverse the length of a 100 cm elevated, exposed beam. Time and number of slips are measured on a broad and narrow beam [15,16]. Each traverse was repeated for three times. Two days before surgery mice underwent a training session. These measurements were done one day before and on day one to seven after surgery.

The flex field analysis system consists of clear cages which are connected to a computer running the PASF Photobeam Activity System w/Flex Field (San Diego, CA, USA). Every cage contains two horizontal grids of infrared light barriers at heights of two and five centimeters. The infrared light barriers measure beam breaks and discriminate between center, periphery and rearing of the animals over a timeframe of 15 min and are counted by the software [17]. Flex field analyses were performed daily at the same time to avoid circadian variability beginning with the day before surgery.

2.4. Histology

Mice were fatally anesthetized at two time points (2 and 7 days post SAH) with 500 μl sodium thiopental followed by cardiovascular perfusion with ice-cold PBS and 4% paraformaldehyde. Brains were removed and post fixed for 24 h in 4% paraformaldehyde. After fixation, brains were transferred into 30% sucrose in PBS for 3 days. Brains were embedded in mounting medium, frozen with −20 °C/−40 °C methylbutane and stored at −80 °C. For all neuropathological analysis 20 μm thick coronal sections were cut on a cryostat (Leica Microsystems LM3050S), mounted on SuperFrost® plus slides (OTS, Hartenstein). Brains were sectioned according to a previously published cutting protocol [18]. Brain pathology was assessed on selected slides at bregma −2 (±1,5 μm) and morphometric analysis of stained slides was performed by a blinded observer (Nikon E-800 microscope, Nikon digital camera DXM 1200; Stereo Investigator Software, Micro Bright Field, Magdeburg, Germany). The optical fractionator was applied for cell number estimations in the cortex (TUNEL, Fluoro Jade C, NeuN and fibrinogen) and the whole brain slide (GFAP). A grid size of X = 535 μm and Y = 670 μm and a counting frame of X = 120 μm and Y = 110 μm was used for the stereological investigations.

The monoclonal antibody anti-Glial Fibrillary Acidic Protein GFAP (clone GA5, Merck) was used to detect activated astrocytes in brain sections of SAH, CSF and sham animals. The monoclonal anti fibrinogen antibody (5A6, Abcam) was used to stain small infarcts in the cortex of SAH, CSF and sham control mice. Counterstaining was done with Hematoxylin II (Ventana, Roche). The monoclonal antibody anti-NeuN (clone A60, Merck) was used to detect neuronal nuclei in brain sections of SAH and sham animals.

For cell death analyses Fluoro Jade C (AG325, Merck) was used to mark degenerating neurons. Additionally, TUNEL staining (Click It Plus TUNEL 488, Life Technologies) was performed to label apoptotic cells in SAH, CSF control and sham animals. Both stainings were performed following user standard protocol provided from the company. Counterstaining was performed with 4′,6-diamidino-2-phenylindole (DAPI).

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