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Severity Assessment in Rats Undergoing Subarachnoid Hemorrhage Induction by Endovascular Perforation or Corresponding Sham Surgery

Annika Bach-Hagemann[a] Ekaterina Harder[a] Laura Warner[a]
Catharina Conzen-Dilger[a, b] Tobias Philip Schmidt[a, b] Sarah Pinkernell[a]
Rupert Palme[c] Ute Lindauer[a]

[a]Translational Neurosurgery and Neurobiology, Department of Neurosurgery, Medical Faculty, RWTH Aachen University, Aachen, Germany; [b]Department of Neurosurgery, Medical Faculty, RWTH Aachen University, Aachen, Germany; [c]Unit of Physiology, Pathophysiology and Experimental Endocrinology, Department of Biomedical Sciences, University of Veterinary Medicine, Vienna, Austria

Keywords
Rat · Subarachnoid hemorrhage · Severity assessment · Behavior

Abstract
Introduction: Animal models for preclinical research of subarachnoid hemorrhage (SAH) are widely used as much of the pathophysiology remains unknown. However, the burden of these models inflicted on the animals is not well characterized. The European directive requires severity assessment-based allocation to categories. Up to now, the classification into predefined categories is rather subjective and often without underlying scientific knowledge. We therefore aimed at assessing the burden of rats after SAH or the corresponding sham surgery to provide a scientific assessment. ***Methods:*** We performed a multimodal approach, using different behavior tests, clinical and neurological scoring, and biochemical markers using the common model for SAH of intracranial endovascular filament perforation in male Wistar rats. Up to 7 days after surgery, animals with SAH were compared to sham surgery and to a group receiving only anesthesia and analgesia. ***Results:*** Sham surgery ($n = 15$) and SAH ($n = 16$) animals showed an increase in the clinical score the first days after surgery, indicating clinical deterioration, while animals receiving only anesthesia without surgery ($n = 5$) remained unaffected. Body weight loss occurred in all groups but was more pronounced and statistically significant only after surgery. The analysis of burrowing, open field (total distance, erections), balance beam, and neuroscore showed primarily an effect of the surgery itself in sham surgery and SAH animals. Only concerning balance beam and neuroscore, a difference was visible between sham surgery and SAH. The outcome of the analysis of systemic and local inflammatory parameters and of corticosterone in blood and its metabolites in feces was only robust in animals suffering from larger bleedings. Application of principal component analysis resulted in a clear separation of sham surgery and SAH animals from their respective baseline as well as from the anesthesia-only group at days 1 and 3, with the difference between sham surgery and SAH being not significant. ***Discussion/Conclusion:*** To our knowledge, we are the first to publish detailed clinical score sheet data combined with advanced behavioral assessment in the endovascular perfo-

Karger@karger.com
www.karger.com/esr

Correspondence to:
Ute Lindauer, ulindauer@ukaachen.de

ration model for SAH in rats. The tests chosen here clearly depict an impairment of the animals within the first days after surgery and are consequently well suited for assessment of the animals' suffering in the model. A definitive classification into one of the severity categories named by the EU directive is yet pending and has to be performed in the future by including the assessment data from different neurological and nonneurological disease models.

Introduction

Stroke is a devastating disease being the third most common cause of disability and the second most common cause of death worldwide. Although ischemic strokes have a higher prevalence, the hemorrhagic ones, including subarachnoid hemorrhage (SAH), have a higher mortality and individuals are affected at younger age [1]. Up to now, many aspects of the pathophysiology of SAH, in both the early brain injury and the delayed cerebral ischemia phase, remain unknown. Therefore, preclinical models are widely used and necessary to gain further insight into the complex pathophysiology. The endovascular perforation model is, besides the blood injection model, the most common used model for SAH in rodents [2, 3].

Since the implementation of the EU directive 2010/63/EU, prospective and retrospective severity assessments are required. Furthermore, severity experienced by the animals is differentiated as mild, moderate, and severe [4]. However, less or no validated objective methods to assess the actual severity of a procedure are available up to now. As proposed by Keubler et al. [5], a multimodal approach for severity assessment is useful, as severity is composed of many aspects, like pain, suffering, harm, discomfort, or stress and all those aspects need to be considered. Koska et al. [6] for example showed an impaired well-being in their multimodal approach after status epilepticus, based on analysis for burrowing behavior and of corticosterone levels among other parameters. Recently, burrowing-analysis has been frequently used in rodents to evaluate animal well-being as it is postulated to be a natural behavior and rewarding activity [7]. Several authors were able to show reduced burrowing behavior after various interventions in mice and rats and concluded that this was due to reduced well-being [8–10]. Another parameter often mentioned in the context of well-being and severity assessment is nest building, shown to be especially suitable in mice [11, 12]. However, rats rather show poor nest building with high day-to-day fluctuations [13]. Particularly in neurological models, the associated neurological deficit complicates the severity assessment of the animals by using tests that rely on a normal moving ability, rendering an analysis of the motor deficits necessary. Beam walk or balance beam tests are commonly used, albeit showing different results [14, 15]. To further analyze motor behavior, open field (OF) can be used. Zieglowksi et al. [16] reported OF to be a reliable test for severity assessment after laparotomy in rats, leaving it open whether additional aspects beside motor activity may be reflected by OF behavior analysis, even when the animals are accustomed to the OF. Besides behavioral tests, Keubler et al. [5] addressed biochemical parameters being useful for severity assessment. Especially, corticosterone is postulated to be a valuable indicator of stress in rodents [17]. However, for brain injury models, the inflammatory aspect of the pathophysiology as well as a possible activation of the hypothalamic-pituitary-adrenal axis need to be kept in mind. Yet, in an acute model of SAH, we were not able to show increased corticosterone concentrations up to 6 h after SAH [18].

With the work presented here, we aimed at testing a multidimensional approach to assess the burden of rats after endovascular perforation of an intracranial artery or the corresponding sham surgery and in a control group only receiving anesthesia and analgesia to provide more objective parameters and therefore facilitate future severity assessment. Furthermore, finding criteria to detect animals at risk of a severe burden before dying spontaneously would be beneficial. Severity evaluation consisted of clinical score sheet assessment, analysis of body weight, execution of behavioral tests (assessment of neurological score [neuroscore] and performance on a balance beam [beam walk], in OF and while burrowing) and measure of biochemical parameters (fecal corticosterone metabolites [FCMs], serum corticosterone, serum and tissue IL6 and IL10). Behavioral tests were chosen based on being commonly used, simple to conduct, non- or minimally invasive, and easy to implement in a lab. To our knowledge, this is the first study analyzing the aspect of burden after SAH in the context of severity assessment required by the EU.

Materials and Methods

All experiments were conducted in accordance with the German Federal Law regarding the protection of animals and the DIRECTIVE 2010/63/EU on the protection of animals used for scientific purpose. The governmental care and use committee (LANUV, Recklinghausen, Germany) granted official permission

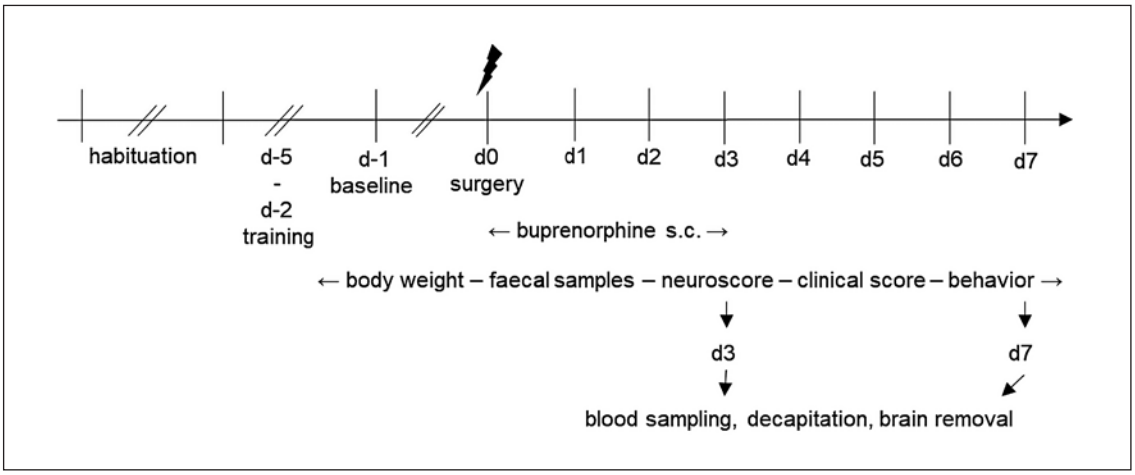


Fig. 1. Experimental design. After habituation for 10 days, animals were trained in the behavioral tests for 4 days with baseline recording occurring on the following day (= d-1). Two to 3 days after baseline recording. animals were allocated to surgery/anesthesia only (= d0). After surgery body weight, behavioral tests and neuroscore were assessed once daily and clinical score at least twice daily for the first 3 days after surgery and once daily thereafter. Analgesia was administered twice per day for the first 3 days after surgery. Fecal samples were collected, and on the final day, blood was withdrawn in deep isoflurane anesthesia, and after decapitation, the brain was removed.

(file reference: 81-02.04.2017.A457). Animals' health monitoring was carried out according to FELASA recommendations [19].

A total of 37 male Wistar rats (Janvier Labs, Le Genest-Saint-Isle, France) weighing between 299 ± 17 g (range 267–350 g) at the day of surgery were used. Animals were divided in groups either receiving surgery with SAH elicitation (SAH) or sham surgery without bleeding induction (sham surgery), with survival time of 3 days or 7 days after surgery. An additional group with only applying the anesthesia and analgesia regime without any surgery (anesthesia only) with 7-day survival was added as control. The animals were kept in Type 2000P rat filtertop cages (Tecniplast, Hohenpeißenberg, Germany) in the central facility within the Institute for Laboratory Animal Science (University Hospital Aachen; certified according to DIN ISO 9001/2015 QM). Room temperature (22 ± 2°C) and humidity (55 ± 5%) were controlled, and the light-dark cycle was set at 12 h (lights on from 7:00 a.m. to 7:00 p.m.) The cages were changed weekly and contained bedding material (Holzgranulat ¾ S; Rettenmeier, Rosenberg, Germany) and nestlets for enrichment (Plexx, Elst, Netherlands). Food (V1534-300, Ssniff, Soest, Germany) and water were given ad libitum. During the acclimation period after delivery and the following training for handling and behavioral testing, the animals were group-housed, which was changed to single housing after surgery or anesthesia only, respectively. No exact data on the variance of the parameters to be measured (behavioral tests, clinical scoring, humoral parameters) existed beforehand from own experiments; therefore, a marker-specific a priori sample size calculation was not possible. Thus, a number of 6–8 animals per surgery group per survival day was considered adequate for this study, and 5 animals received anesthesia only.

Experimental Design

After habituation to the new surrounding for 10 days (d), animals were trained for 4 days in behavior tests (OF, burrowing, beam walk). On the fifth day, baseline (bl) was recorded for the behavior tests and for the neuroscore. Two to 3 days after baseline recording, animals were allocated to either sham surgery or SAH surgery (day of surgery = d0). One additional group received only anesthesia and analgesia. This group was performed in a row and was not included in the surgery schedule of sham and SAH. After surgery, buprenorphine analgesia was given subcutaneously (s.c.) for the first 3 days every 12 h (0.03 mg/kg). Additionally, food pellets and soaked food in a petri dish were provided on the cage floor for the first 3 postoperative days. A clinical score, covering body weight loss, outside appearance, breathing, spontaneous behavior, reaction to handling, and a simple neurological evaluation (shown in online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000524432) was assessed at least twice daily. Based on the score sheet, humane endpoint (HE) criteria were defined beforehand as follows: body weight loss ≥20%, snap breathing in agony, long lasting seizures, coma, complete paralysis, most of the time motionless in the same place when checked repetitively over 6 h, combination of several parameters reaching an overall score ≥30. An additional supportive measure of oral feeding of jelly food (as suggested by [20]) was planned for animals reaching clinical score points of >10 within the first 3 days. Behavioral tests were conducted every morning, approximately 2 h after analgesia administration. At the end of the experiment (d3 or d7 after surgery), blood samples were withdrawn via heart puncture in deep isoflurane anesthesia (no additional analgesic was applied while unconsciousness assured [21]). The least invasive method was used with piercing a needle cannula through the skin perpendicular to the body surface from lateral or ventral to the sternum [22]. Toe pinch reflex was absent, the animals did not react to the needle prick, and death was induced shortly thereafter by decapitation. The brain was removed and frozen at −80°C, and blood and tissue samples of the brain were prepared for further analysis using ELISA (shown in Fig. 1). Exclusion criteria were defined before-

hand as HE not being SAH-related and intraoperative deaths [23]. One sham-surgery animal reached the HE in the evening after surgery and is not included in any analysis.

Surgery

A detailed description of the surgery is provided in the online supplemental Material. Briefly, surgery was carried out under isoflurane anesthesia, and fentanyl and buprenorphine were injected subcutaneously for analgesia. Body temperature was constantly monitored and regulated via a heating plate and rectal temperature probe. After orotracheal intubation, the head was prepared to place a microprobe pressure sensor measuring intracranial pressure (ICP; Codman Integra, Germany) through a small craniotomy. Additionally, the skull left of bregma over the supply area of the middle cerebral artery was thinned to allow regional cerebral blood flow (CBF) measurement via a laser Doppler flowmetry probe (Moor instruments, Great Britain). The tail artery was cannulated for blood pressure (BP) monitoring and blood gas analyses. The neck was prepared to introduce a 3-0 Prolene filament into the right common carotid artery after proximal permanent ligation. After 5 min of baseline recording of ICP, CBF, and BP, the filament was advanced via the internal carotid artery until the vessel was perforated at the circle of Willis in the SAH group. For sham surgery, the filament was advanced without perforation. After 30 min of recording after filament withdrawal, vessels were ligated, probes removed, and all wounds were closed. Animals were allowed to recover for up to 2 h in a preheated warming chamber. Animals receiving only anesthesia and analgesia (= anesthesia-only group) were not intubated. Anesthesia, analgesia, and length of anesthesia were comparable to those of animals from the sham surgery or SAH group.

Behavioral Tests

OF tests were performed on d-5, -3, and -1 and on postoperative d1, 3, 4, and 7. Animals were placed in a gray box open at the top (72 × 72 × 50 cm) for 10 min and recorded with a video camera from top-down. Analyses were performed offline regarding number of erections and total distance moved (EthoVision XT; Noldus, Wageningen, Netherlands). After each animal, the OF was cleaned with disinfectant.

The burrowing test was performed as previously reported [24]. Briefly, animals were put in a Typ2000P cage for 15–30 min, which contained an underlay for patients and some food pellets at the floor. The cage was closed by the filtertop cover without a metal lid. After this acclimatization period, a tube (32 cm long, diameter 10 cm, entrance raised by 6 cm, closed at the back) filled with 2.5 kg gravel (2–4 mm diameter aquarium gravel, Jens Rosnerski, Königslutter, Germany) was placed inside the cage for 60 min. After 60 min, the gravel remaining inside the tube was weighted, and the difference was calculated as burrowed material. Only on the first day of training, an empty tube was placed in the cage. After completion of the test, the animals were transferred back into their homecages, and the test cage was cleaned. Each animal kept its own tube and gravel during the whole observation period, and only after euthanasia, the gravel was put in 0.1% acetic acid for 30 min, and the tubes were cleaned with water and disinfectant.

The beam walk test consisted of a starting platform (12 × 12 cm) and an end platform (20 × 20 cm, prepared with food and bedding), connected by a wooden beam (18 mm wide, 150 cm long, square). The home cage was put next to the end platform in approximately the same height. Training started with the animal being placed on the end platform, and then, gradually, the distance to the end platform was increased. When the animal reached the end platform, it was transferred back into its home cage. For actual analysis, the animal was placed on the starting platform, and the time to reach the end platform was measured, with the maximum time limited to 60 s. According to their variable performance, animals were allocated the one of the following four categories: "successful": reaching end platform <60 s; "too slow": starting from platform but not reaching end platform within 60 s or starting but turning back to start platform within the time limit), "no start": not starting within 60 s, and "drop": dropping from the beam or from the start platform.

The neuroscore consists of tests for hemiparesis, proprioception, and reflexes, occurrence of seizures or myoclonus, and the way of balancing on the beam, summing up to a maximum of 20 points for worst performance. To assess the ability to balance on the beam, after testing for beam walk performance, the animal was placed in the middle of the beam, and its performance was evaluated over 1 min and graded according to a rating scheme (online suppl. Table 2). After the balance beam test, the other subtests of the neuroscore were assessed. Hemiparesis was evaluated by lifting the animal carefully upwards on its tail and checking the posture of the head and limbs. While putting the animal back down on a straight surface, first, the ability to actively putting the paws down and afterward the gait were assessed. For proprioception, the correction reaction of the animal was tested. Reflex tests consisted of checking the pinna and the corneal reflexes as well as the startle response. Overall, neurological impairment was graded according to the summed points gathered within all tests (1–6 points: mild, 7–12 points; moderate, 13–20 points; severe, neurological impairment).

Blood Samples

To measure corticosterone, the blood samples were kept at room temperature for 60 min until clotting, followed by centrifugation (3,500 rpm, 10 min, 4°C; Eppendorf 5424R; Eppendorf AG, Hamburg, Germany). For analysis of IL6 and IL10, blood was withdrawn into an EDTA tube and centrifuged right after withdrawal (1,000 *g*, 20 min, room temperature; Eppendorf 5424R; Eppendorf AG). Serum and plasma were stored at −80°C for further analysis. For analysis, commercial ELISA kits were used (corticosterone: DEV9922; Demeditec diagnostics, Kiel, Germany; IL6: R6000B; R&D, Minneapolis, MN, USA; IL10: R1000; R&D, Minneapolis, MN, USA), and the procedures were performed in duplicates according to the manual. The final absorbance of the colorimetric reactions was determined by a microplate reader (Synergy HT Multi-Mode Microplate Reader; BioTek, Winooski, VT, USA) at 450 nm (with wavelength correction for IL6 and IL10 at 540 nm). Concentrations were calculated after blank correction using the simultaneously assessed concentration curve from standard samples.

Fecal Corticosterone Metabolites

The aim was to collect fecal samples at baseline and on each postoperative day during the behavioral tests. However, not all animals defecate at all timepoints, resulting in a lower sample size at some days. Fecal samples were stored at −80°C until further preparation. At the end of the study, fecal samples were freeze-dried overnight (freeze dryer Alpha 1-2 LD plus; Martin Christ freeze

dryer, Osterode am Harz, Germany) and extracted with 80% methanol (1 mL methanol per 0.05 g sample). The sample supernatant was stored at −20°C until shipment to the Vetmeduni Vienna (Austria) and analyzed via a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay [25], which has been well validated for use in rats [26]. FCM levels are presented in μg/g.

Brain Tissue

For analysis of brain samples, the brain regions of interest (basal and parietal cortex, left hemisphere) were removed and mixed with 350 μL RIPA + buffer. After homogenization (disperser, IKA® T10 basic – Ultra Turrax®, level 1.5) for one min, the homogenate was transferred (QIAshredder; QIAGEN inc. Hilden, Germany) and centrifuged (2 min, 4°C, 2,000 rpm, Eppendorf Centrifuge 5424R; Eppendorf AG). Supernatant was collected, aliquotized, and stored at −80°C. Protein determination was performed using the Pierce™ BCA Protein Assay Kit from Thermo Fisher. Briefly, the supernatant was diluted 1:10, pipetted in duplicates to a 96-well plate, and after 30 min of incubation in a heating oven, measured at 562 nm (Synergy HT Multi-Mode Microplate Reader; BioTek®, Winooski, VT, USA). Total protein count was calculated (μg/μL) and samples normalized using RIPA to 1–2 mg/mL. Normalized samples were stored in aliquots at −80°C. For IL6 and IL10 analysis, commercially available ELISA kits were used (Rat IL-6 ELISA [for lysates] Kit #ERA32RB; Rat IL-10 [for lysates] Kit #ERA24RB; Thermo Fisher, Carlsbad, CA, USA). Analysis was performed according to the manual. Briefly, normalized samples were diluted 1:5. Samples, standard, and a blank were applied in duplicates to a precoated 96-well plate. After incubation for 2.5 h, absorbance was measured at a wavelength of 450 nm, including wavelength correction for IL6 at 550 nm. Concentrations were calculated using "MyAssay" [27].

Statistical Analysis

The analyses as well as figure design were performed using GraphPad Prism 7.04® and 8.4.1. ® (GraphPad Software, Inc., La Jolla, CA, USA). Normal distribution was tested using Shapiro-Wilk normality test. Results are described as median [25% percentile–75% percentile] and p value. The data are presented as boxplots with minimum to maximum values, and sometimes, additionally, all data points are shown. Animals were included, according to their predetermined survival time. As some SAH animals reached the HE or died overnight (= severe SAH), those were included in the final analysis as long as data were available. Therefore, depending on the comparison (predefined comparisons: within-group comparison of corresponding baseline vs. postoperative d1, 3, 4, 7; between-group comparisons of sham surgery vs. SAH at d1, 3, 4, 7), analysis results in varying sample sizes.

For ICP and CBF, mean and standard deviation were calculated (Excel 2016; Microsoft Cooperation, Washington, DC, USA), and mean values were used for graphical presentation using GraphPad Prism. Animals within the SAH group dying overnight or being euthanized fulfilling HE criteria were regarded as suffering from severe SAH. For SAH subgroup analysis, comparison of ICP and CBF values of severe versus surviving SAH animals was done by repeated measures two-way ANOVA with Sidak's multiple comparison test. Additionally, a correlation analysis of maximum score sheet points against peak ICP or lowest CBF values was performed using Pearson correlation. To determine a possible ICP or CBF cutoff value for risk of high clinical scoring, a receiver operating characteristic (ROC) analysis was used with maximum ICP or drop of CBF values from surviving SAH animals and those with severe SAH, respectively.

The time needed for filament positioning and the total surgery time in minutes was taken from the detailed surgery protocols of each animal. For comparison of time needed for filament positioning between sham surgery and SAH, Mann-Whitney test was applied. Total surgery time comparisons between sham surgery, SAH, and anesthesia-only groups were performed by one-way ANOVA followed by Sidak's multiple comparison tests.

For clinical score analysis, the maximum score from each day was used. For statistics of clinical score, body weight loss and all behavioral tests, within-group comparisons to the corresponding baseline (time as dependent factor), and between-group comparisons (treatment as independent factor: SAH vs. sham surgery vs. anesthesia only) were performed using repeated measure two-way ANOVA with Sidak's multiple comparison test. Subgroup analysis of body weight and clinical score on d1 was performed using a Kruskal-Wallis test with Dunn's multiple comparisons test.

Principal component analysis (PCA) was conducted using the factoextra [28] package in base R [29]. PCA analysis can only be performed on a complete dataset; therefore, missing data were completed as follows: for animals not being repetitively tested in the OF, mean values from the other animals were calculated. For those animals reaching the HE at d1, missing values were replaced by the worst values of the other SAH animals.

Results

A total of 37 animals was assigned to the different procedures and survival times. A randomization was intended. However, it was not possible to advance the filament sufficiently deep to the perforation site in every case when SAH was assigned by lot. As our goal was to assess the burden of SAH as well as sham surgery animals, we decided to include those animals with an unsuccessful advancement as sham surgery animals. Therefore, randomization was suspended early after starting the study, and each of the following surgeries were intended as SAH aiming at a total number of 8 in each survival group. When filament advancement for vessel perforation was unsuccessful after a maximum try of 15 min, the animal was allocated to the sham surgery group. Successful perforation was defined by an increase of ICP and a drop in CBF. In 4 cases with ICP course clearly indicating SAH, CBF measurement failed. Group allocations and failure rate are shown in Figure 2. While analyzing the time needed for filament positioning as well as for the total surgery, the time for filament positioning was significantly longer for sham surgery animals compared with SAH surgery animals (in minutes: sham surgery: 15 [11–15]; SAH: 2 [1–3.75]; $p = 0.0002$). However, this prolonged

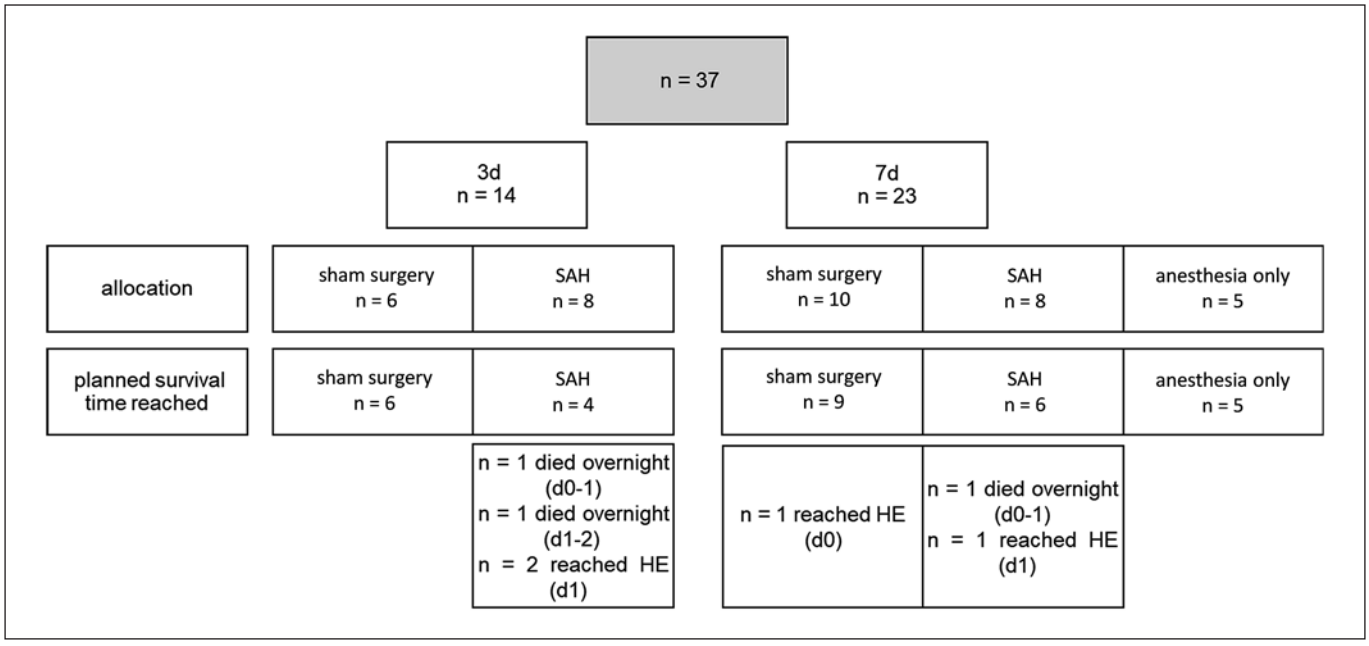


Fig. 2. Sample size and failure rate. Depicted is the allocation to the different groups according to survival time and procedure (SAH, sham surgery, anesthesia only) and the reasons for failure. HE, humane endpoint; d, day(s).

Table 1. Physiological parameters

	Sham surgery (n = 15)	SAH (n = 16)	Anesthesia only (n = 5)
Measurement at baseline			
BT (°C)	37.4 [37.2–37.6]	37.2 [37.1–37.7]	38 [37.3–38.3]
Bpm	350 [321–400]	383 [360–395]	325 [297–350]
SpO_2	99.4 [99.3–99.6]	99.2 [99–99.4]	99.5 [97.5–99.7]
Measurement after 30 min recording			
BT (°C)	37.9 [37.6–38.1]	37.7 [37.1–38]	37.6 [37.4–37.7]
Bpm	369 [326–405]	378 [359–405]	324 [297–346]
SpO_2	99.4 [99.2–99.5]	99.3 [99–99.4]	99.5 [98.5–99.6]

Values are expressed as median (1st quartile–3rd quartile). BT, body temperature; bpm, beats per minute = heart rate; SpO_2, arterial oxygen saturation.

filament positioning time did not have an impact on the total surgery time (in minutes: sham surgery: 156 [142–164]; SAH: 154 [138–173]; anesthesia only: 147 [142–147]; p = 0.935 for comparing sham surgery with SAH). In a detailed subgroup analysis, outcome of animals from sham surgery conducted as planned at the beginning of the study (sham surgery (as planned); n = 3) was compared with the outcome of animals from sham surgery after switching to allocation as sham surgery with unsuccessful filament positioning (sham surgery*, n = 12). Besides a significantly prolonged time needed for filament positioning in sham surgery* compared with sham surgery (as planned) or SAH (shown in online suppl. Fig. S6a), no significant difference was detectable in all other parameters and behavioral outcome measures between both sham surgery subgroups (shown in online suppl. Fig. S6b–i).

Physiological Monitoring and SAH Verification

Recording of BP and blood gas parameters was not possible in all animals or at each time point due to several reasons (catheter blocked or slipped out, no placement possible). Additional vital parameters of heart rate and systemic arterial oxygenation were monitored by pulse oximetry and were stable during the surgery (shown in Table 1). ICP increased and CBF decreased after successful perforation (shown in Fig. 3 and online suppl. Fig. S1). SAH animals reaching the HE or dying overnight (= severe SAH) showed significantly higher ICP values right after perforation (0.5 min: surviving SAH 29 [24–34], severe SAH 37 [33–55], p = 0.0009; 1 min: surviving SAH 23 [15–25], severe SAH 30 [26–35], p = 0.0154) (shown in Fig. 3b). No significant difference was found for CBF (data not shown).

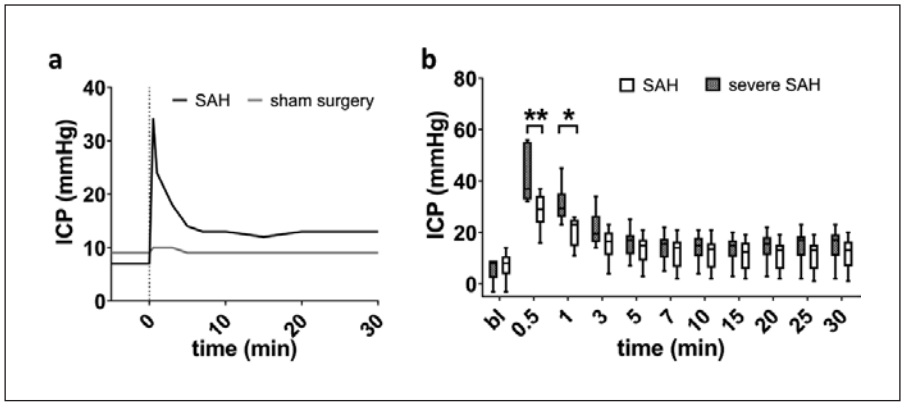


Fig. 3. Overview of ICP (**a**, sham surgery n = 15, SAH n = 16) with the dotted line indicating the start of 30 min recording period. Comparison of ICP between surviving and severe SAH cases (**b**, severe SAH n = 6, surviving SAH n = 10, $^{**}p < 0.01$, $^{*}p < 0.05$).

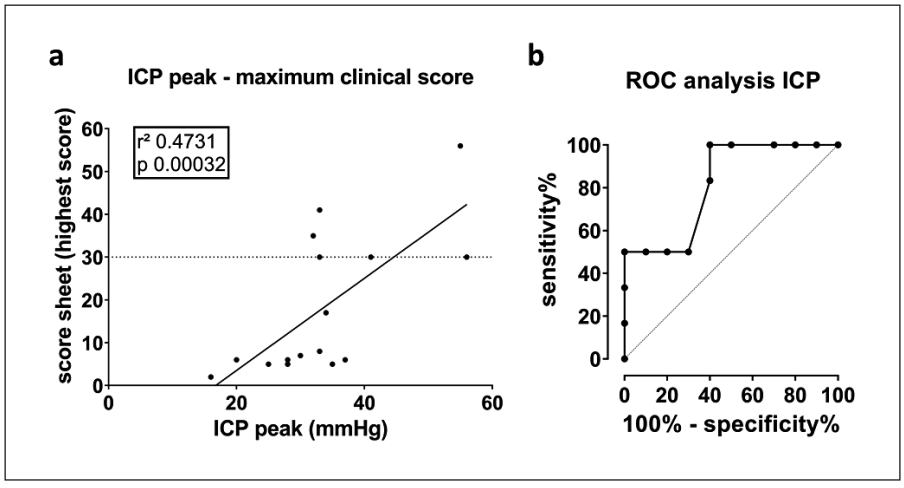


Fig. 4. Pearson correlation and ROC analysis of ICP. Depicted is the Pearson correlation of ICP (**a**) values and the maximum score of the SAH animals; furthermore, the ROC curve of ICP (**b**) of surviving versus severe SAH rats is shown.

Pearson Correlation and ROC Analyses of ICP and CBF Data

Pearson correlations were performed using ICP (peak) and CBF (drop) values correlated to the respective maximum clinical scoring points of each SAH animal. Both parameters showed significant correlation (ICP peak and maximum clinical score: $r = 0.6868$, $r^2 = 0.4731$, 95% CI: 0.4256–1.729, $p = 0.00032$, $n = 16$). For detailed results concerning CBF data, please refer to the online supplemental material (Pearson correlation and ROC analysis of CBF data, online suppl. Fig. S2a). Additionally, ROC analysis using the highest ICP value of each surviving SAH animal ($n = 10$), and of those which died overnight or had to be euthanized (severe SAH, $n = 6$), revealed an area under the curve of 0.8167 with a p value of 0.03937. The sensitivity and specificity were best at an ICP >36 mm Hg (sensitivity 50%, 95% CI: 11.81%–88.19%; specificity 90%, 95% CI: 55.5%–99.75%, likelihood ratio 5; shown in Fig. 4). The corresponding results of ROC analysis of CBF are shown in the online supplemental material (Pearson correlation and ROC analysis of CBF data, online suppl. Fig. S2b).

Clinical Score and Body Weight Loss

Animals dying overnight were assigned a clinical score of 30 the following day ($n = 2$ at d1, $n = 1$ at d2). For analysis, always, the highest score reached each day was used. In-group comparison (postoperative time point vs. corresponding baseline) showed significant differences for sham surgery and SAH but not for the anesthesia-only group (shown in Fig. 5a; corresponding bl for any comparison and group 0 [0–0]; sham surgery d0: 2 [1–5], $p = 0.0025$, $n = 15$; sham surgery d3: 1 [0–1], $p = 0.0454$, $n = 15$; sham surgery d4: 1 [0–4], $p = 0.0299$, $n = 9$; SAH d0: 5 [2–6], $p < 0.0001$, $n = 16$; SAH d1: 6 [2–30], $p < 0.0001$, $n = 16$; SAH d3: 1 [0.8–2], $p = 0.0113$, $n = 10$; for details see online suppl. Table 3). Comparison between sham surgery and SAH revealed a significant difference at d0 (p

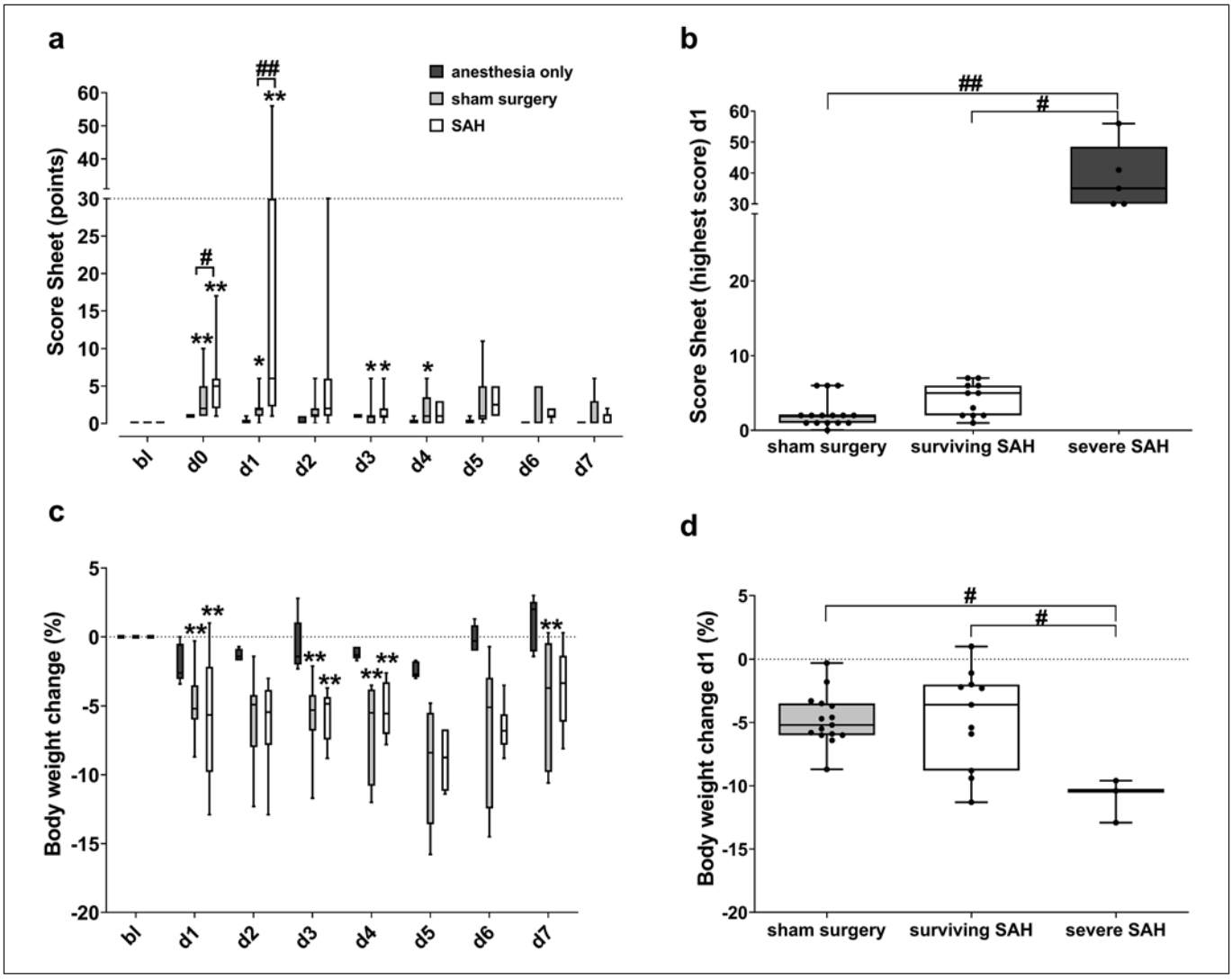


Fig. 5. Clinical score sheet and body weight data. Score sheet data shows the highest score per day (**a**), body weight change (**c**), score sheet (**b**), and body weight change (**d**) at d1 with the SAH group split in surviving and severe SAH (reaching the HE on d1); statistical analysis was only performed at d0 (only a), 1, 3, 4, and 7; asterisk indicates in-group comparison to the corresponding baseline; hashtag indicates significant between-group comparisons; $^{**/\#\#}p < 0.01$; $^{*/\#}p < 0.05$; dotted line: HE (**a**), no weight loss (**c**).

= 0.0317) and at d1 ($p = 0.0005$). Animals receiving only anesthesia and analgesia remained unaffected throughout the whole observation period (no significantly different score values compared to baseline). Separating SAH animals in severe and surviving ones at d1 revealed significant higher clinical scores of severe animals when compared to sham surgery or surviving rats (sham surgery: 2 [1, 2], $n = 15$; surviving SAH: 5 [2–6], $n = 11$; severe SAH: 35 [30–49], $n = 5$; sham surgery vs. severe SAH $p = 0.0002$; surviving SAH vs. severe SAH $p = 0.0436$) (shown in Fig. 5b).

Animals from the surgery groups significantly lost body weight after surgery. Body weight loss was most pronounced at d5, but as defined beforehand, statistical analysis was only performed for d1, 3, 4, and 7. The anesthesia-only group did not show a significant change of body weight throughout the observation period. For sham surgery, the significant weight loss lasted up to d7, whereas SAH animals statistically regained their starting weight at the end of the observation period (shown in Fig. 5c; corresponding bl for any comparison and group 0 [0–0]; sham surgery d1: −5 [−6 to −4], $p < 0.0001$, $n =$

15; sham surgery d3: −5 [−7 to −4], $p < 0.0001$, $n = 15$; sham surgery d4: −6 [−11 to −4], $p < 0.0001$, $n = 9$; sham surgery d7: −4 [−10 to −0.5], $p = 0.0039$, $n = 9$; SAH d1: −6 [−10 to −2], $p < 0.0001$, $n = 14$; SAH d3: −5 [−7 to −4], $p < 0.0001$, $n = 10$; SAH d4: −6 [−7 to −3], $p = 0.0003$, $n = 6$; for details see online suppl. Table 4). No significant differences were found between sham surgery and SAH. As 3 SAH animals reached the HE during the first day, a subgroup analysis was performed with separating data from SAH animals in surviving SAH and severe SAH on d1. This analysis showed a significant body weight loss of the subgroup of severe SAH in comparison to sham surgery as well as the subgroup of surviving SAH (sham surgery: −5 [−6 to −4], $n = 15$; surviving SAH: −4 [−9 to −2], $n = 11$; severe SAH: −10 [−13 to −10]; sham surgery vs. severe SAH $p = 0.0406$; surviving vs. severe SAH $p = 0.0286$; shown in Fig. 5d).

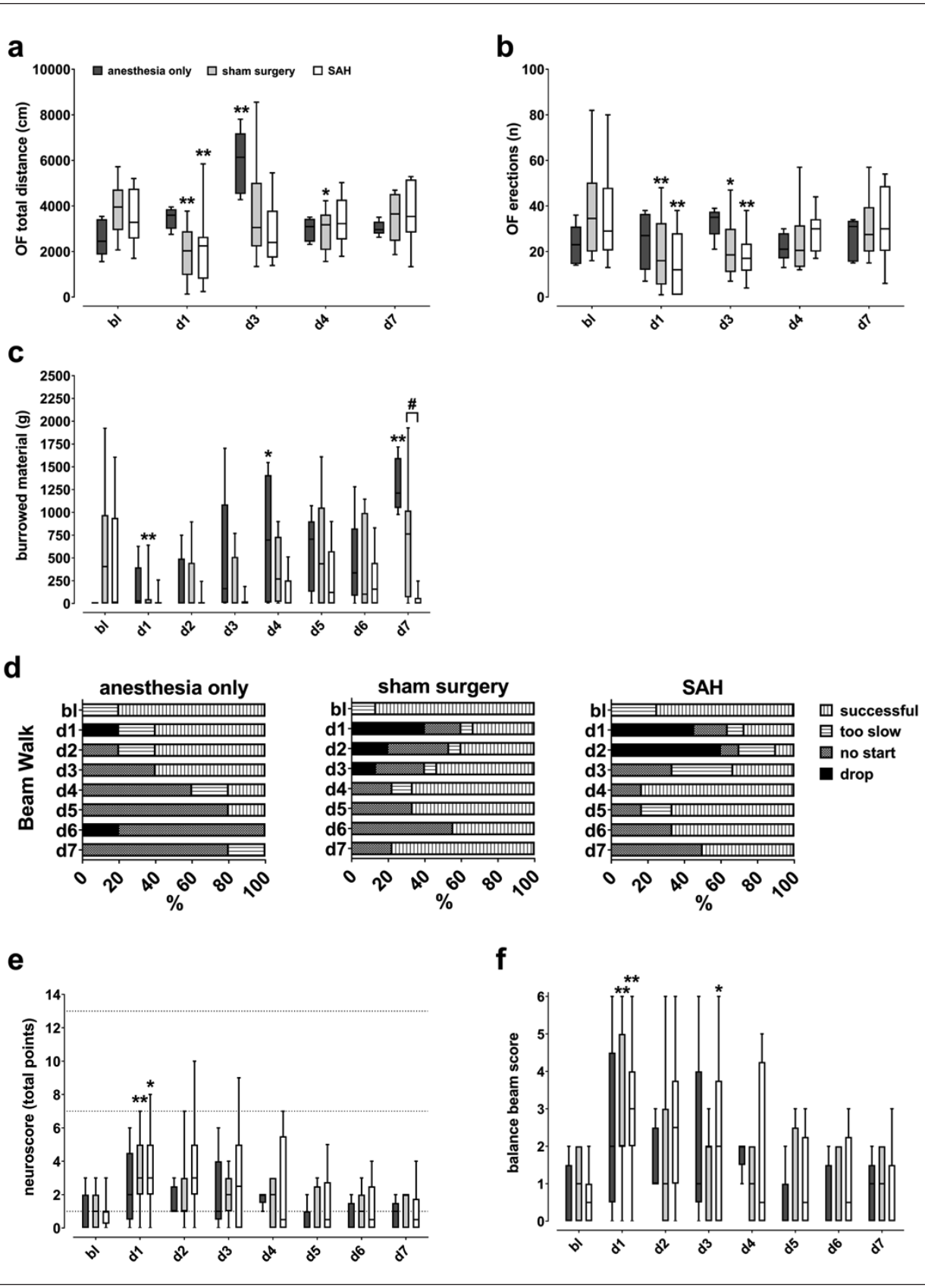
Behavior Tests

OF: The total distance covered in the OF showed a significant decrease in sham surgery and SAH on d1 (sham surgery: $n = 12$, corresponding bl 3,954 [2,940–4,722], d1 2,031 [971–2,894], $p < 0.0001$; SAH: $n = 11$, corresponding bl 4,116 [2,805–4,908], d1 2,248 [805–2,647], $p = 0.0006$). In addition, the sham surgery animals showed a (marginally) significant decrease on d4 ($n = 8$, corresponding bl 3,796 [2,940–4,317], d4 3,173 [2,067–3,626], $p = 0.0487$). On d3, the anesthesia-only group covered significantly more distance compared to their baseline ($n = 5$, corresponding bl 2,451 [1,871–3,421], d3 6,134 [4,525–7,189], $p = 0.0003$) (shown in Fig. 6a; for statistical details, see online suppl. Table 5). The number of erections during OF testing, including supported (with stabilizing by the forepaw at the cage wall) and unsupported (free erection) once, decreased significantly in sham surgery and SAH on d1 and d3 (sham surgery d1: $n = 12$, corresponding bl 35 [20–50], d1 16 [6–33], $p = 0.0008$, sham surgery d3: $n = 12$, corresponding bl 35 [20–50], d3 19 [11–30], $p = 0.0265$; SAH d1: $n = 11$, corresponding bl 29 [24–48], d1 12 [1–28), $p < 0.0001$; d3: $n = 9$, corresponding bl 44 [23–49], d3 17 [12–24], $p = 0.0050$). The animals only receiving anesthesia and analgesia showed minor changes without significant differences (shown in Fig. 6b; for statistical details, see online suppl. Table 6). Comparisons of sham surgery with SAH on the postoperative days did not reveal any significant differences, neither for the total distance nor for the number of erections.

Burrowing: When analyzing the burrowing behavior, comparisons to the corresponding baselines revealed a significant decrease in burrowed material for sham surgery on d1 ($n = 15$, bl 406 [0–971], d1 0 [0–48], $p = 0.0081$). Between-group comparison of sham surgery and SAH revealed a significant difference only at d7 (sham surgery d7: $n = 9$, 762 [68.5–1,020], SAH d7: $n = 6$, 0 [0–61.8], $p = 0.0331$). The anesthesia-only group did not burrow at baseline, yet they started burrowing at d1 and further improved during the 7-day observation period with a significant increase in burrowing at d4 and 7 (d4: $n = 5$, corresponding bl 0 [0–0], d4 697 [11.5–1,409], $p = 0.0333$; d7: $n = 5$, corresponding bl 0 [0–0], d7 1,211 [1,048–1,596], $p = 0.0005$) (shown in Fig. 6c, online suppl. Table 7). As some researchers exclude animals burrowing less than 500 g at baseline [9, 30, 31], we performed a subgroup analysis with only animals burrowing >500 g at baseline. Sham surgery as well as SAH animals with good burrowing performance showed a clear decline of burrowed material on the first postoperative days with a slight hint toward faster recovery of sham surgery compared with SAH (online suppl. Fig. S3; Table 8).

Beam walk: Due to the variable behavior, the data are analyzed as percentage of animals within each group allocated to the predefined four categories (see Materials and Methods section). At bl, all animals started from the platform and were able to walk on the beam, albeit some showed insufficient running behavior, not reaching the end platform within 60 s. After surgery, the percentage of animals dropping from the beam or from the platform increased. Additionally, over time, the percentage of animals not starting from the start platform at all increased, which was especially remarkable in the anesthesia-only group (shown in Fig. 6d). Statistical analysis was not possible as group sizes were too small for the χ^2 test.

Neuroscore: Points in the neuroscore primarily resulted from a poor performance in the balance beam (analysis see below). Additionally, pinna reflex was often negative already at baseline as well as postoperatively in all groups. Mainly due to the regular failure of the pinna reflex and the tight threshold of "0 points" for normal performance, already at baseline, many animals are categorized as mildly neurologically impaired (≥1 point). Postoperatively, SAH animals were classified as moderately impaired, but even some sham surgery animals gained points ≥7. Significant differences to the respective bl were found on d1 for SAH and for sham surgery (SAH: $n = 11$, corresponding bl 1 [1–1], d1 3 [2–5], $p = 0.0279$; sham surgery d1: $n = 15$, corresponding bl 1 [0–2], d1 3 [2–5], $p = 0.0098$). Animals from the anesthesia-only group did not show significant differences in their neurological behavior. No significant differences were found between sham surgery and SAH at any time point (shown in Fig. 6e



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and online suppl. Table 9). The analysis on the beam represents an important part of the neuroscore; therefore, a subanalysis of only this part seems reasonable. Balance beam analysis showed an increase in the number of animals not being able to balance on the beam after surgery (≥4 points) with significant differences found for SAH animals up to d3 and for sham surgery only at d1 (SAH d1: n = 11, corresponding bl 0 [0–1], d1 3 [2–4], p = 0.0082; SAH d3: n = 10, corresponding bl 0 [0–1], d3 2 [0–4], p = 0.0302; sham surgery d1: n = 15, corresponding bl 1 [0–2], d1 2 [2–5], p = 0.0027). Albeit even few animals from the anesthesia-only group dropped, no significant differences were found in that group (shown in Fig. 6f; online suppl. Table. 10). Between-group comparison of sham surgery and SAH did not show significant differences.

Blood Samples

Data from regular blood sampling on final d3 and d7 showed consistently low values for corticosterone, IL6 and IL10 within the physiological range with no differences between the groups (for details see online suppl. Fig. S4). Blood samples from 2 animals, which had to be euthanized on d1 by reaching the HE criteria, showed higher values for IL6 and corticosterone as compared with the results from final blood sampling obtained on d3 or d7 from surviving animals.

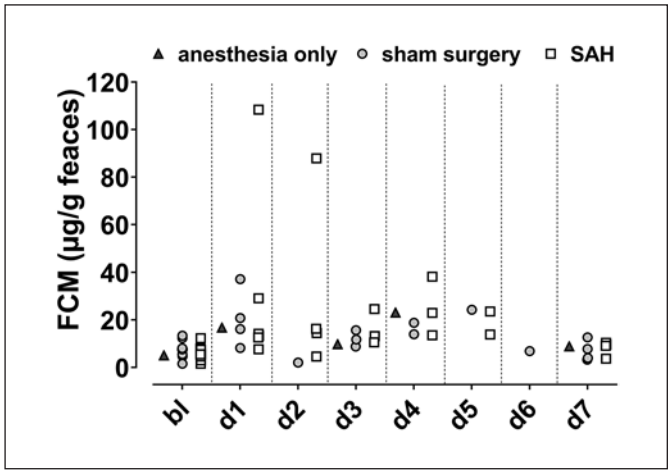


Fig. 7. Fecal corticosterone metabolites (FCM) concentrations.

Fig. 6. Behavioral tests. Depicted are the results for OF (**a**: total distance, **b**: erections), burrowing (**c**), beam walk (**d**), neuroscore (**e**), and balance beam (**f**); statistical analysis was only performed for d1, 3, 4, 7; within-group comparisons are depicted with asterisk, between-group comparisons with hashtag; $^{**}p < 0.01$, $^{*/\#}p < 0.05$; dotted line in E indicates the different neurological impairments (≥1: mild; ≥7: moderate; ≥13: severe impairment). Beam walk shows percentages of different performances (**d**).

Fecal Corticosterone Metabolites

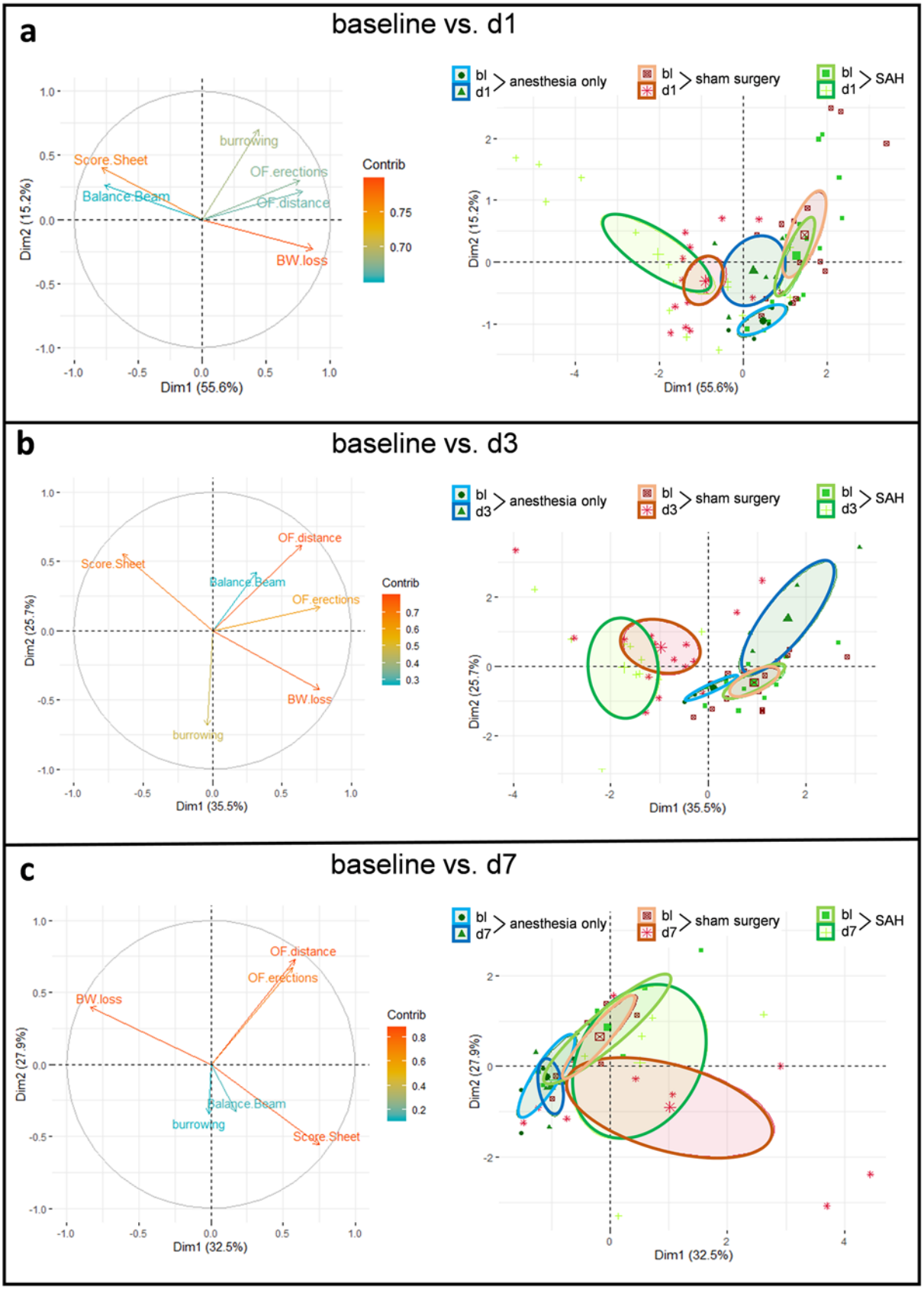
It was planned to collect the fecal samples each morning during behavioral testing, starting at baseline recording. However, not all animals defecated, so sample collection from all animals on all days was not possible. Thus, due to variable and on some days very low sample numbers, no statistical analysis was performed. Except for one SAH animal, postoperative FCM concentrations only marginally increased in all groups. The one SAH animal showing marked higher concentrations than all other animals (108.4 µg/g at d1, 87.9 µg/g at d2, no sample at d3) also gained a comparably high score in the clinical score sheet evaluation at d0 (score sheet points: 17). At d7, FCM concentrations were back at baseline values for all animals (shown in Fig. 7).

Brain Tissue

Parietal and basal cortical brain tissue was analyzed via IL6 and IL10 ELISA. Both markers are significantly higher in the parietal regions when compared to the basal region of the same group ($p < 0.01$, for details see online suppl. Table 11; Fig. S5). No significant differences were detected for within-region comparisons between groups.

Principle Component Analysis

For comparison of bl versus d1, the first two principal components captured 70.8% of the variance in the data (PC1: 55.6%, PC2: 15.2%), with body weight loss showing the highest contribution, followed by clinical score sheet evaluation (which includes change of body weight). Furthermore, while displaying the confidence ellipses (95%) around group clusters (sham surgery, SAH, anesthesia only), a clear separation along the x-axis between the baseline and first postoperative day is visible for sham surgery as well as SAH, whereas both anesthesia-only group clusters were separated from both postoperative clusters of sham surgery and SAH. This separation is even more clearly visible at d3 where still 61.2% of the variance in the data are captured (PC1: 35.5%, PC2: 25.7%). The parameters mostly contributing seem to change with total distance in the OF gaining a higher priority here. At d7, the confidence ellipses of the surgery groups (sham surgery and SAH) approach their baseline values, and the principal components capture 60.4% of the variance in the data (PC1: 32.5%, PC2: 27.9%). Overall, the contribution of each parameter is not highly different with the most important contributing factors varying between the days (shown in Fig. 8).



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Discussion

In a multimodal approach, we evaluated the burden induced by SAH after endovascular perforation or the corresponding sham surgery by using behavioral tests and biochemical markers. For comparison, additionally a group of animals receiving only anesthesia and analgesia was included. This multimodal approach used here showed an impairment of SAH as well as sham surgery animals after surgery but none of the anesthesia-only group.

Usefulness of ICP as a Cutoff Value for Severity

The filament perforation model results in different bleeding grades even when an identical filament size is used in all animals. The severity of the SAH can be modeled by the size and material of the filament [32, 33], with small filaments typically inducing smaller blood covering with milder disease courses. In our model with a commonly used filament size (3-0), 38% of the SAH animals did not reach the planned survival time, with half of them dying overnight. This is in line with reports from other groups or reviews [3, 34] and also reflects the mortality in patients. Nevertheless, since only half of the animals with a fatal course could be euthanized according to HE definition before reaching the level of severe suffering or before spontaneously dying, a criterion to detect those animals at risk at a very early time point would be an important refinement strategy for preclinical SAH research. Very early measurable and thus useful parameters might be ICP and CBF changes already assessable during surgery. As a link between increased ICP values and worse outcome is already published for human patients, we took a closer look at those parameters [35]. The comparison of ICP values from surviving animals compared to those dying or reaching HE showed a significant difference with higher level in fatal cases. Although the CBF did not show significant differences between surviving and severe SAH, in a correlation analysis and ROC analysis, both ICP and CBF showed significant results, rendering those parameters useful as indicators. Thus, we were able to confirm the findings from human patients. Based on the data from this study group and according to the ROC analysis, animals with an ICP peak of more than 36 mm Hg or a CBF drop to less than 54% of baseline were at higher risk for a fatal course. In further studies using our model, animals exceeding these values should be monitored more closely. Under normal laboratory conditions, it is easily possible to increase the scoring frequency during daytime; however, due to typical restrictions on available personnel, especially in small research groups, the adequate observation frequency during nighttime very often poses a challenge. Therefore, further attempts should be undertaken to develop an algorithm to identify animals that will most probably deteriorate toward the level of HE scoring or even die before the next regular scoring the following morning. From our results, it may be suggested that a risk calculation considering the ICP peak value during the surgery (most important for animals exceeding a specific ICP cutoff value) combined with the progression of the clinical scoring data (see next paragraph) achieved during daytime assessment may be most promising. Based on such an algorithm, animals may be euthanized beforehand at the last scoring time point in the evening even when the clinical scoring alone does not reach the HE level. For a more generalized approach and for the proof of the transferability to other rat models of SAH and to the other often used rodent species mouse, the validity of the here defined cutoff values and of a (still to be developed) risk assessment algorithm has to be controlled in prospective studies in the future. In addition, it might be useful to further combine CBF and ICP measurements, for example, with an implanted transmitter to detect changes in heart rate or body temperature and combine those parameters to develop an even more precise risk assessment instrument.

Score Sheet, Body Weight, and Behavioral Tests Show Primarily an Influence of the Surgery

To our knowledge, this is the first study to report data of the clinical score sheet assessment from animals after endovascular perforation. Animals that received identical anesthesia and analgesia but did not undergo surgery showed no evidence of suffering in any parameters measured. As there was no indication for stress or burden in the specifically stress-sensitive (fecal corticosterone) or well-being-associated (burrowing) measures in this group, we postulate that our chosen behavioral tests themselves do not affect the animals, rendering them suitable to assess burden and suffering in the surgery groups.

Fig. 8. PCA. Depicted are postoperative d1 (**a**), 3 (**b**), and 7 (**c**), each compared to the corresponding baseline (bl); PC1 and PC2 are expressed in %; on the left the contributions of the single parameters are expressed by using color, direction, and length of the arrows; the ellipses on the right depict the 95% confidence intervals. **a** Sham surgery (n = 15), SAH (n = 16), anesthesia only (n = 5). **b** Sham surgery (n = 15), SAH (n = 10), anesthesia only (n = 5). **c** Sham surgery (n = 9), SAH (n = 6), anesthesia only (n = 5).

In animals receiving surgery, we detected an increase of clinical score points within the first days for SAH as well as sham surgery procedure. Comparing sham surgery with SAH revealed a significant difference at d0 and d1, with the difference on d1 mainly caused by the fatal cases and surviving animals rapidly approaching the situation of sham surgery thereafter. HE criteria seem to be well chosen and severe suffering in the fatal course was preventable by euthanasia. While leaving the fatal cases out, it may be considered whether there is, beside the phase directly after surgery, indeed no difference between sham-operated animals and animals surviving SAH or whether our clinical score just does not detect an albeit existing difference. For the surviving animals with SAH, the summed points are at the lower range and thus would point to mild severity. Thus, with the chosen clinical score, we were mainly able to detect either mild or severe burden, but scoring for moderate burden rarely occurred. This was also mentioned by Keubler et al. [5] as a drawback of many score sheets in use. Therefore, it may be considered to revise the chosen score sheet to either better detect a currently hidden but existing moderate burden or to ensure that animals surviving the first one to 2 days after SAH are correctly rated as mildly – and thus from sham surgery animals not differently – affected.

The pattern of body weight loss was comparable in both sham surgery and SAH animals. Additionally, the anesthesia-only group also showed a tendency toward minor albeit not significant body weight loss within the first 5 days. As we used buprenorphine for analgesia, this might be the reason for at least part of the body weight loss. It is described in the literature that buprenorphine is associated with side effects as pica behavior and body weight loss [36–38]. In our study, we saw only little pica behavior, but the body weight loss in the anesthesia-only group probably occurred due to buprenorphine. As it is also reported that body weight loss might occur still after the drug is no longer administered [39], this could be a reason for the prolonged and pronounced weight loss at day five in all groups. As the weight loss is more pronounced in the postoperative groups, the surgery seems to have an important additional impact. SAH animals do not seem worse affected than sham surgery, probably showing some success of our supportive measures (soaked food pellets, food pellets on the floor). Further support by oral feeding of jelly food was not applied because none of the surviving animals reached the beforehand defined clinical scoring criteria for this additional measure. However, as the body weight loss is still visible after d3, it might be necessary to provide the food supplements not only up to d3 but for a longer time period until regain of body weight can be detected. As stated by Liles and Flecknell [40], suffering from pain due to insufficient analgesia may lead to body weight loss. As the summed clinical score was rather in the lower range, as discussed above, we have no evidence that our analgesia was under dosed. However, a sustained release drug would be beneficial [41, 42] as it presents a continuous drug level and requires less handling and fewer injections of the animals, making it a refinement [43]. Besides an effect on body weight, possible behavioral changes due to buprenorphine have to be considered. Various reports are available describing divers effects of buprenorphine on the behavior, ranging from no effect [44] to hyperactivity [45], probably depending on the respective dosage or application interval used in these studies. With the dosage (0.03 mg/kg) and the application interval (every 12 h) used here, no obvious behavioral impairment was detectable in the anesthesia-only group on the days the animals received buprenorphine compared to those without it. Merely, the increase of total distance run of the anesthesia-only group in the OF test on d3 might be a hint for buprenorphine-induced hyperactivity, albeit not detected in any other test. Thus, any alteration in the behavioral outcome can mainly be considered as surgery and/or SAH dependent. To sum up, we therefore assume that the aftereffects of the surgical procedures are mainly responsible for a reduced food uptake, resulting in a slight to moderate weight loss in both sham surgery and SAH animals, with a significantly higher loss in SAH animals that reached HE criteria or died overnight. Prolonged offer of soaked food pellets beyond the limit of 3 days after surgery may be recommended. Further supportive measures like oral feeding of jelly food may also be suggested however carefully considering the restraint stress applied for oral feeding against the benefit for keeping appropriate body weight.

The OF shows a similar pattern for sham surgery as well as for SAH animals with a reduction in total distance run and number of erections. The reduction in erection needs to be considered as the animals also normally need to rise if they want to get food from the feeder, making the application of food at the floor even more meaningful. Concerning the test for burrowing, a decrease of burrowed material was visible for sham surgery as well as SAH animals up to d2–3. Although burrowing is described as a good parameter to assess well-being [7] and was postulated to be decreased and thereby detecting pain in many animal models (e.g., inflammation [9], neuropathic pain [46], epilepsy [6]), it needs to be kept in mind that many other aspects beside pain might have

an impact on the burrowing behavior. There seem to be differences on the individual burrowing behavior, and training or the presence of other animals can also influence burrowing behavior [9, 30]. Finally, models of hippocampal damage were able to show a reduction in burrowing in mice without being necessarily accompanied by pain [47, 48]. It is worth to notice here that by chance, all animals of the anesthesia-only group did not burrow at baseline but nicely started and improved burrowing within the days after anesthesia, demonstrating the reported individual variability of burrowing performance. Thus, in our setting, the burrowing tests seems to be applicable at group level to evaluate the model but not meaningful at an individual level to detect the actual burden experienced by a single animal. The neuroscore was originally designed as a specific assessment of the hemiparesis-induced neurological deficit after ischemic stroke. Due to the absence of hemiparesis in most animals after SAH, the outcome of the neuroscore in the here described study is dominated by the performance on the balance beam. Both sham surgery and SAH animals showed a reduced performance on the beam and thus seem to be affected by the surgery, with a longer lasting significant impairment up to d3 for SAH animals, pointing toward an additional and extended effect of the bleeding itself. What is striking is that not all animals delivered a totally accurate run at baseline, resulting in a balance beam score ranging from 0 to 2 points in the still healthy animals. This observed individual ability of the rats to perform this test was also reported by Germano et al. [15]. The variable performance of the animals on the balance beam also influences the neuroscore, thus leading to points >1 already at baseline, which – according to our predefined classification – would already be interpreted as a mild neurological impairment. As stated above, the neuroscore used here was originally designed to mainly uncover a functional impairment based on primarily one-sided motor and sensory deficits. A revision of the neuroscore for application after SAH therefore seems to be reasonable, explicitly addressing the neurological symptoms often observed in animals after SAH in our study, like lethargic appearance and rather slow movements. This is in line with the literature reporting that only few animals after SAH show hemiparesis [34, 49] but instead seem to be lethargic after the insult [50]. During the balance beam test, the animals are somehow forced to move on the beam due to being positioned directly on the beam, whereas during the beam walk test, the animals are positioned on a start platform and are allured to cross the beam by presenting an award (some food, the home cage) at the end platform. At baseline, all animals started to walk and reached the end platform, most of them successfully within the time limit. At the first days after surgery, the proportion of animals with a successful run decreased and the number of animals dropping from the platform or from the beam increased. A striking feature of the outcome of the beam walk test however was the increase in the number of animals that did not start walking from the start platform at all. In sham surgery as well as SAH, this changed performance may be interpreted as reduced motivation to move, increased anxiety or overall weakness due to the surgery. However, the increasing portion of animals not starting from the platform at all was most obvious and even larger in the group that received only anesthesia and analgesia without any surgery. An increasing behavior of not starting from the platform after a training period may therefore reflect learning behavior rather than impaired beam crossing ability. The animals may have learned that if they do not start walking, they are nevertheless awarded by returning them to their cage after the 60 s without having to walk over the beam. Since mainly the animals from the anesthesia-only group showed this behavior, it may be assumed that the operated animals (sham surgery and SAH) show poorer learning behavior after the surgery. In older mice, a reduced cognitive performance has been shown up to 7 days after a surgical intervention [51], which has also been described for older patients after major surgery [52]. Beside an unspecific effect of surgical stress on learning behavior, cognitive deficits are also often described in patients suffering from SAH [53].

Systemic and Local Pro-Inflammatory Processes after SAH

Inflammation is an important aspect in the pathophysiology of SAH with pro-inflammatory [54] and anti-inflammatory reactions being reported [55]. Beside a possible inflammatory local as well as systemic response to the bleeding, the perforation model requires a soft tissue surgery at the neck for filament placement, which might in addition lead to systemic inflammatory reactions. Liu et al. [56] showed increased IL6 after SAH in rat brain tissue. In the present study, we were not able to show differences between sham surgery and SAH animals or animals of the anesthesia-only group neither for local IL6 nor IL10. In the systemic blood circulation, IL6 was only elevated in animals reaching the HE on d1, thus pointing to an early pro-inflammatory reaction mainly induced by a severe bleeding, with the concentrations in these animals being comparable to those reported previously after

acute SAH (6 h after blood injection) [18]. We conclude that the inflammatory reaction shown here is not as pronounced as to inflict burden to the animals. But it needs to be kept in mind that the *n*-numbers are rather small, thus data should be viewed primarily as a trend.

Corticosterone and FCMs as Useful Parameters in SAH Models

The results from the analyses of serum corticosterone and FCMs need to be viewed as a trend due to the low sample size. As blood sampling may itself induce changes in corticosterone values, we decided against a repetitive blood withdrawal [57]. Except for one HE animal showing higher concentration of serum corticosterone, all other measured samples showed concentrations within the same range, being rather low and within the range of our previously performed acute study [18]. For a repetitive stress assessment, we used the FCM analysis because feces collection is described as an easy, noninvasive method [58, 59]. Unfortunately, in our study, animals did not defecate regularly during the behavior sessions, and thus, sampling did not cover all timepoints in all animals. FCM concentrations only marginally increased at the first days after baseline in all groups, and at d7, FCM concentrations were back at baseline values for all animals. Only one animal from the SAH group showed a pronounced increase after SAH, which in view with the comparably high clinical score probably indeed points toward an increased stress-response. From d3 onward, all animals showed approximately the same range. We therefore conclude that only more severe bleedings may be reflected in elevated FCM concentrations early after surgery. Beside this, our results support the notion that corticosterone values can vary a lot on the individual basis [60, 61]. A disadvantage of both the analysis of corticosterone from blood as well as of its metabolites in feces is that they cannot be used in everyday life and only provide a retrospective assessment of the animals.

The Multimodal Approach Shows a Separation of Postoperative Days Compared to Baseline in PCA

The PCA showed a clear separation of sham surgery as well as SAH animals from baseline at d1 and 3 with an approach back to baseline at d7. The difference between sham surgery and SAH is rather small, which confirms our results of the individual analyses that the primary influence on the animals seems to come from the surgery. Furthermore, the tests chosen here seem to depict an impairment of the animals and are consequently well suited for severity assessment. Beside body weight and clinical scoring, the total distance in OF seems to explain the data quiet well. However, according to the PCA, the balance beam test contributed to a much lesser extent than the other tests. This is especially remarkable because when analyzing it separately, it was one of few tests where a difference between sham surgery and SAH was obvious. Thus, PCA is helpful to get an overview over the combination of tests; however, the single analyses of the tests should also be kept in mind.

Limitations

Albeit careful planning and conduction, the study has some limitations. First, the group size is overall small, rendering especially the biochemical assessments (blood ELISA and FCM data) less robust. Regarding FCM samples, a change in the setup toward a longer collection interval would be beneficial, so, in case of single housing, a new cage can be offered daily, from which the fecal samples can then be obtained. After starting the surgeries, it emerged that the intracranial advancement of the filament necessary for the perforation of the cerebral vessel was not possible in all cases, making the originally planned randomization impossible. A comparably high failure rate of SAH induction with 52% of unsuccessful elicitation of bleeding into the subarachnoid space in rats has also been described by Park et al. [62], and problems with SAH induction using the filament perforation model in rats have also been reported by others, albeit at lower incidence [50, 63]. It may therefore be recommended to identify the best way of filament induction in a pilot study beforehand to avoid high failure rates in advance. We could have tried a different size of the filament or could have used a wire within a tubing for perforation, but as the study had already started when this problem became evident, we decided not to change the method during the study. As the total surgery was standardized, the groups did not differ noticeably in the length of the surgery, even while the time for filament positioning was prolonged in the sham surgery group. Allocation of animals without successful perforation within the limit of 15 min can thus be considered as an adequate control and consequently implemented in the group of sham surgery animals. Furthermore, we only used male animals here, so we cannot give any suggestions on whether female rats might be affected differently by this procedure. Furthermore, the single housing after surgery needs to be considered. It is known that housing conditions can have different impacts on lesion size and recovery. Group housing for example can improve functional recovery [64], whereas after filament-induced middle cerebral artery occlusion, an increased lesion size has been reported

with single housed animals [65]. Lastly, we cannot exactly define whether a specific part of the surgery was mainly responsible for the impairment in sham-operated animals. Also in sham surgery, the right common carotid artery was permanently occluded. Although it has been shown that collateral blood flow via the circle of Willis is well developed in rodents, the unilateral occlusion of the common carotid artery has been shown to induce a transient reduction of CBF by up to approximately 25% [66, 67]. However, an even longer lasting mild hypoperfusion (25–50%, up to 10 weeks) did not induce neuronal dysfunction [68], rendering the soft tissue lesion at the neck region most likely accountable for the reduced performance in sham surgery.

Summary, Conclusion, and Outlook

With the study performed here, we aimed at characterizing and grading the extent of suffering experienced by rats after cerebral endovascular perforation for SAH or its related sham surgery more closely. Additionally, we wanted to find objective criteria as a scientifically sound basis to better classify this model in one of the predefined severity grades according to the EU directive in the future. The results achieved from the applied methods form a solid basis and an important step toward a final severity grading. With the tests used, we were able to differentiate the burden of postoperative days from the unaffected state at baseline. However, the differentiation between sham surgery and SAH was not clearly determined and needs further analysis. For early risk assessment, the peak value of the ICP at bleeding initiation seems to be a promising factor for an easy identification of animals that might develop higher severity further on. An adaptation of the clinical score sheet and the neuroscore turned out reasonable to better decide upon a possible moderate suffering and to better reflect the SAH specific neurological impairment. The test for burrowing seems to be a sensitive method, albeit more on the group rather than on the individual level and mainly when analyzing only animals that are effectively burrowing at baseline. In addition, the OF test turned out to be another useful test, especially as the number of erections might give a hint to a possible impairment of food uptake. All these parameters are furthermore easily assessable in unexceptionally equipped research laboratories in everyday use. Balance beam and neuroscore showed a differentiation between sham surgery and SAH, yet they are at least partially based on subjective criteria, demanding for more experienced personnel to achieve reasonable results. FCMs seem to be promising for repetitive stress assessment. Even though individual differences and variations need to be considered, FCMs are well suited for longitudinal studies as each animal represents its own control [59]. The multimodal approach turned out as very promising as all parameters are set in a context with each other. A definitive classification into one of the severity categories named by the EU directive is yet pending and has to be performed in the future by including the assessment data from different neurological and nonneurological disease models. An overall and careful interpretation of the here described results points toward a mild to moderate burden to the animals mainly caused by the surgery, with animals undergoing a large and severe bleeding experiencing a higher suffering that should be terminated by euthanasia according to well-defined HE criteria to avoid a severe level of burden. Finally, it is highly recommended to test whether the results obtained here are transferable to other models of SAH such as the blood injection model. A direct comparison of both most-often used models would add to the scientific criteria for a decision of which model would be best-suited for a specific question the aspect of the level of burden inflicted on the animal by the model. Because the bleeding grade in the blood injection model can be better standardized by the amount and velocity of the blood injection, an ICP analysis in this model may help to validate the proposed ICP cutoff value for early risk stratification of animals suffering from more severe SAH.

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Statement of Ethics

All experiments were conducted in accordance with the German Federal Law regarding the protection of animals and the DIRECTIVE 2010/63/EU on the protection of animals used for scientific purpose. The governmental care and use committee

(LANUV, Recklinghausen, Germany) granted official permission (file reference: 81-02.04.2017.A457). The study planning and conduction followed the ARRIVE guidelines.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

A.B.H. performed the surgeries, clinical scoring, postoperative care, and data analysis and drafted the first version of the manuscript; E.H. performed the behavioral tests and helped with data analysis; L.W. and S.P. helped with the surgeries and postoperative care; C.C.D. and T.S. made substantial contributions to the interpretation of the data; A.B.H. and U.L. designed the study; R.P. performed the FCMs in his laboratory; U.L. was responsible for overall conduct of the study, contributed to data analysis, and mainly contributed to the final draft of the manuscript; all the authors corrected and agreed on the final version.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material files. Further inquiries can be directed to the corresponding author.

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