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Protein kinase C inhibition reduces critical weight loss and improves functional outcome after experimental subarachnoid haemorrhage

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ABSTRACT

Objectives: Subarachnoid haemorrhage (SAH) carries a high burden of morbidity and mortality. One in three patients develop vasospasm, which is associated with Delayed Cerebral Ischemia. The pathophysiology includes vasoconstrictr receptor upregulation in cerebral arteries. The protein kinase C – inhibitor RO-31-7549 reduces the expression of several vasoconstrictor receptors and normalizes cerebral blood flow in experimental SAH but functional and behavioural effects are unknown. This study was undertaken to analyse functional outcomes up to 14 days after experimental SAH.

Materials and methods: 54 male rats were randomised to experimental SAH or sham, using the pre-chiasmatic, single injection model, and subsequent treatment or vehicle. 42 remained for final analysis. The animals were euthanized on day 14 or when reaching a humane endpoint. The primary endpoint was overall survival, defined as either spontaneous mortality or when reaching a predefined humane endpoint. The secondary outcomes were differences in the rotating pole test, weight, open field test, novel object recognition and qPCR of selected inflammatory markers.

Results: In the vehicle group 6/15 rats reached the humane endpoint of >20 % weight loss compared to 1/14 in the treatment group. This resulted in a significant reduced risk of early euthanasia due to >20 % weight loss of HR 0.15 (0.03-0.66, p = 0.04). Furthermore, the treatment group did significantly better on the rotating pole test, RR 0.64 (0.47-0.91, p = 0.02).

Conclusion: RO-31-7549 improved outcomes in terms >20 % weight loss and rotating pole performance after experimental SAH and could be investigated.

Introduction

Non-traumatic subarachnoid haemorrhage (SAH) carries a high morbidity and mortality rate. Among causes for a bad outcome delayed cerebral ischemia (DCI) is a particularly enigmatic cause of morbidity or death. DCI describes a delayed deterioration of neurological function or consciousness which typically develops 4–14 days after ictus. The pathogenesis of DCI is incompletely understood, although an association with arterial narrowing is common, although DCI is possible in the absence of angiographic spasm. Moreover, amelioration of angiographic vasospasm with endothelin receptor antagonists does not necessarily improve outcome or reduce mortality, but there is remaining agreement that narrowing of large and small cerebral vessels is an important contributor. Moreover, recent data from our group suggest redundancy of signalling. Hence, a single specific ETα-receptor antagonist, as in the CONSCIOUS-1 and 2 trials, was not necessarily sufficient to prevent all relevant pathogenetic cascades. There is consensus that initial hypoperfusion and extravasation of blood are two important initiators of the pathogenic cascades causing early brain injury. Flow reduction in this phase activates the
MAPK/ERK/1/2 pathway. This activation shows redundancy since both Focal Adhesion Kinase and Protein Kinase C (PKC) activate the MAPK/ERK/1/2 pathway. Activation of MAPK/ERK/1/2 leads to upregulation of not only Endothelin-A (ET\textsubscript{A}), but also of the vasoconstrictory 5-hydroxytryptamine-1B- (5-HT\textsubscript{1B}), Angiotensin II-1 (AT\textsubscript{1}), and thromboxane A\textsubscript{2} (TX\textsubscript{A}2) receptors in cerebrovascular smooth muscle cells. ET\textsubscript{A} are constrictor, but MAPK/ERK activation also reverts the normally relaxant ET\textsubscript{B} receptors on the vascular endothelial cells into contractile ET\textsubscript{B} receptors due to phenotypical changes. Taken together, previous experience suggests that pharmacological treatments should address redundancy and that mechanisms should be targeted upstream of ET\textsubscript{A} expression. Our group showed that upstream inhibition of the MAPK/ERK pathway, with the MEK inhibitor U0126 reduced upregulation of contractile receptors and improved outcomes in experimental SAH.

The PKC family is implicated in cellular processes such as inflammation, proliferation and death, upregulation of membrane receptors, cytokines and gene regulation/translation and in pathogenesis of reperfusion injury. It is increasingly clear that SAH induces inflammatory cascades in brain parenchyma and vessels. Moreover, protein C activation remains a potential pharmacological target as an alternative activator of the MAPK/ERK pathway. Protein C activation is implicated in vasoinconstriction in organ culture and experimental SAH. Specifically, we found that intraventricular administration of the PKC-inhibitor RO-31-7549 (RO-31) inhibited molecular changes after experimental SAH while functional outcomes of PKC inhibition after experimental SAH have not been studied.

Despite extensive studies on molecular pathways of PKC-inhibition as a potential target for vasospasm during more than 30 years, behavioural effects and outcomes are unknown. These studies were conducted to fill the knowledge gap on functional outcomes after PKC inhibition following experimental SAH. The study was conducted in two steps. First a dose finding study in vitro to identify a concentration at which RO-31 inhibited vasoconstriction was performed and next an experimental study of functional outcome with RO-31 dosage to reach the desired concentration in vivo was performed.

We hypothesized that the inhibition of PKC via intrathecal administration of RO-31 in a rat model of SAH would improve long-term outcomes in a 14-day perspective. We therefore performed a randomised, blinded interventional study with RO-31 in a rat model of SAH. The study involved selection of a physiologically relevant dose of RO-31 and the primary outcome was 14-day survival defined by either experimental mortality or reaching a pre-defined humane endpoint. The sample size was calculated to detect a 40 % decrease in high performers in the rotating pole test. Using an alpha value of 0.05 and a power of 80 %, the group sizes should be twelve.

**Preparation of RO-31 solution**

1 mg RO-31 (Sigma-Aldrich, USA) was dissolved in 5 mL DMSO (Sigma-Aldrich, USA) and diluted in 96 mL Elliot’s B solution (Lukare medical, USA) resulting in a concentration of 10 mM. Vehicle was made by adding DMSO to Elliot’s B in the same ratio.

**Dose finding study**

For dose finding, basilar arteries in organ culture were analysed with a myograph as previously described. The rats were sedated with CO\textsubscript{2} gas and decapitated. Brains were removed quickly and chilled in cold bicarbonate buffer solution. Basilar arteries were dissected from the brain and cut into approximately 1.5 mm-long segments. The segments were incubated for 48 h in DMEM altering ET\textsubscript{A} receptor functionality into a contractile phenotype localized in smooth muscle cells. Segments were co-cultured with varying concentrations of RO-31 to reduce the transcriptional upregulation. After the 48 h in culture, the functional ET\textsubscript{B} contractility was measured in the myograph (Danish Myograph Technology, Denmark). Artery segments were mounted on two 40 μm-diameter wires in a wire myograph setup. The segments were immersed in a bicarbonate buffer solution (pH 7.4) of the following composition (mmol/L): NaCl 119, NaHCO\textsubscript{3} 15, KCl 4.6, MgCl\textsubscript{2} 1.2, NaHPO\textsubscript{4} 1.2, CaCl\textsubscript{2} 1.5, and glucose 5.5 aerated with carbogen to maintain a pH of 7.4. Vessel segments were stretched to an optimal pretension (~ 2 mN) in a process previously described. Concentration-response curves were obtained by the cumulative application of Sarafotoxin 6c (S6c) (Bachem, Switzerland), a specific ET\textsubscript{A} receptor agonist, in the concentration range of 10\textsuperscript{-13} to 10\textsuperscript{-7} M.

**Study design**

We did a 2 × 2 split plot study with animals randomised to SAH or sham surgery. After surgery, both groups were randomised to intrathecal injection with RO-31 or vehicle. To minimise inter-group variations, each cage consisted of one Sham-vehicle/treatment-, one SAH-vehicle- and one SAH-treatment group animal. The animals were treated intrathecally three times, at 2-, 8- and 24 h following induction of SAH. On day two, seven and 14 the animals underwent rotating pole- and open field testing. On day three and eight, the animals underwent novel object recognition testing. The animals were sacrificed on day 14 (Fig. 1).

**Sample size**

The sample size was calculated to detect a 40 % decrease in high performers in the rotating pole test. Using an alpha value of 0.05 and a power of 80 %, the group sizes should be twelve.

**Blinding**

The experimenter responsible for all surgeries and injections, was blinded to the content of the vials. The animal caretaker responsible for behavioural tests was blinded for surgery and treatment.

**Surgery**

The surgical procedure was approved by the Danish Animal Experimentation Inspectorate (no. 2016-15-0201-00940). We employed the pre-chiasmatic single injection model developed by Prunell and colleagues. Briefly, following anaesthesia, a catheter was placed in the tail artery to monitor blood pressure and blood gas parameters. A catheter was placed in the cisterna magna to measure ICP and serve as a site for intrathecal administration. Two holes were drilled in the skull, one in the midline and one lateral. In the first a catheter was introduced to the level of the pre-chiasmatic cistern and in the latter a laser-Doppler
is placed epidurally to monitor the cerebral blood flow (CBF). 0.30 mL autologous blood was drawn from the tail catheter and introduced into the pre-chiasmatic cistern while confirming an ICP rise and CBF drop on the monitor. Following surgery, 0.1 mL/100 g body weight of 5 mg/mL carprofen and 3 mL saline was administered subcutaneously, and the animal was placed in a single cage and allowed food and water ad libitum. In sham animals the pre-chiasmatic catheter was not inserted.

The following parameters were measured during surgery: Weight, surgical time, ICP (before-, during- and after SAH induction), change in ICP ($\Delta$ICP = ICP$_{15 \text{min post-SAH}}$ – ICP$_{\text{Pre-SAH}}$) mean arterial blood pressure (MABP), Cerebral perfusion pressure (CPP), calculated as $\text{CPP} = \text{MABP} – \text{ICP}$, and blood gas analysis (pH, pCO$_2$ and pO$_2$).

After surgery, all animals received carprofen at 24 and 48 h after surgery. Six hours after the final treatment the animal was sedated and the cisternal catheter was removed. Three days after surgery, following the first round of behaviour testing, animals were reintroduced to their original cage mates. We followed a “best care” policy for the animals. This included frequent observations and weighing, varied food, and reunification with cage mates. In case of continued weight-loss, animals received up to 6 mL saline subcutaneously daily and the animal caretaker facilitated eating behaviour.

Exclusion criteria were failure of technical aspects of surgery; failure to regain spontaneous respiration; hemiparesis or hemiplegia following surgery. Humane endpoints resulting in euthanasia were hemiparesis or hemiplegia, >20 % weight loss (according to Danish legislation) or if an animal’s overall condition required euthanasia after assessment by a veterinarian.

12 animals were excluded from the study: Nine animals were excluded due to technical aspect of the surgery, two were excluded due to lack of spontaneous respiration following surgery and one, belonging to the SAH-vehicle group, was found deceased within 24 h of surgery.

**Rotating Pole**

The rotating pole tests sensorimotor skills, balance, and motivation. The animals were trained 3 or 4 days before surgery and tests were performed on day 2, 7, and 14. The test consisted of the animal being placed at one end of a wooden beam, 150 cm in length and 45 mm in diameter. At the other end, there is an entrance to a cage filled with bedding material from the home cage. The test was done rotating right and left twice in each direction with 10 rpm. The animals were scored dichotomously on their ability to traverse the rotating pole and reaching the cage without falling off or not. Immediately before testing, the animal was trained by letting it cross the beam without rotation and then at 3 rpm in both directions, as in line with former studies. Four days before surgery the animals were tested and if they were not able to cross the bar with less than two-foot slips they were excluded. This ensured the baseline did not differ between groups. The animals were tested three times following surgery. Due to apparent paralysis of the left, anterior paw, one animal in the SAH-treatment group could not complete the tests. The animal was otherwise well and performed average in other tests.

**Open Field Test**

The open field test (OFT) examines behaviour in an environment without shelter which is assumed to be perceived risky by rodents. The test was performed on day 2, 7, and 14. Distance and rearing are exploratory behaviour. Time mobile reflects exploratory behaviour and the intention to perform exploratory behaviour as it does not take distance travelled into consideration. Rodents have a tendency to avoid the open area in the central zone and stay along the walls, called thigmotaxis, as open areas are thought to be perceived risky. Increased thigmotaxis and grooming is possible markers for anxiety. (Self-)grooming is the most common activity of the awake rat. It has been hypothesised to be a de-stressor.

The test box is 100 cm x 100 cm with 40 cm high, black walls. The central zone was defined as a centred quadrant covering 50 % of the surface area. The remaining area was defined as the peripheral zone. The test was performed in 35 lux and lasted 10 min. The Any-Maze recording software (Stoelting Europe, Ireland) recorded all parameters. Thigmotaxis was measured using the Thigmotaxis Index, $T_I = (T_{\text{Peripheral Zone}} / T_{\text{Total}}) \times 100$. Rearing data was retrieved by counting each rearing as one action. On every test day, rearing from all groups was compiled and the median served as a binary cut-off point for “high”- or “low”-performers. All data were continuous except rearing which were discrete, numerical data. A statistical outlier was removed from the Sham group on day 14 regarding time mobile. Eight statistical outliers of ~135 tests were removed from the $T_I$. From the SAH-vehicle group three were removed from day two and one from day seven and 14. From the Sham group, two were removed from day two and one each from day seven and 14.

**Novel object recognition**

The novel object recognition (NOR) test examines the ability to discriminate between familiar and novel objects as a memory marker. The test was performed in the same box as the OFT on day 3 and 8. Rodents have a natural tendency to focus on novel objects in their environment and an amelioration of this might be a marker of reduced memory. The NOR consists of two phases, the sample phase and the test phase, both lasting five minutes. In the sample phase, two similar objects (A1+A2) were placed 10 centimetres away from adjacent corners, allowing the animal to move around the objects. The test phase began after a two-hour intertrial interval. In the test phase, each object was replaced, one with a similar object and one with a new object (A3+B1). Exploration was defined in line with Ennaceur as “directing the nose at a distance ≤ 2 cm to the object and/or touching it with the nose. Turning around or sitting on the object was not considered as an exploration.” Exploration time of each object was recorded and a discrimination index, DI, was calculated using $\text{DI} = (T_{\text{Novel + Familiar}})/(T_{\text{Novel}} + T_{\text{Familiar}})$. In the second test, B objects were replaced with C objects to avoid memory bias. The objects were a yellow, rectangular bottle; a red-white cylindrical canister and a grey spray bottle. The sizes of the objects were 20 $\times$ 8 cm and impossible for the animals to sit on. One animal in the SAH-treatment group was not tested on day 14.

Fig. 1. Timeline overview from pre-training on day −3/−4 to sacrifice on day 14.
Weight

Changes in weight were calculated as the fraction of the pre-operative weight, \( W_{\text{Weight}} = \left( \frac{\text{Weight}_{\text{Actual}}}{\text{Weight}_{\text{Pre-Operative}}} \right) \times 100. \) The weights were measured on day 1, 2, 3, 4, 7, 9, 10, 11, and 14 and the intermittent days if deemed necessary for monitoring wellbeing.

Removal of cerebral arteries

On day 14 the animals were sedated using in 30 % O\(_2\)/70 % CO\(_2\) and decapitated. The brain was removed as fast as possible and placed in a chilled buffer solution of 119 mM NaCl, 4.6 mM KCl, 1.5 mM CaCl\(_2\), 1.2 mM MgCl\(_2\), 1.2 mM NaH\(_2\)PO\(_4\), 15 mM NaHCO\(_3\) and 5.5 mM glucose, pH 7.4. The basilar arteries, middle cerebral arteries and the circle of Willis were dissected under magnification ensuring no removal of cerebral tissue. The samples were frozen at −80 °C until RNA analyses.

RNA extraction

Total RNA, from animals euthanised on day 14, was extracted from the circle of Willis, the MCA and theACA bilaterally using spin columns (Nucleospin miRNA, Mini kit for total RNA, MACHERY-NAGEL) in combination with QIAzol (Qiagen, Germany) and chloroform (Sigma Aldrich, Denmark). The samples were homogenised using QIAzol lysis buffer (Qiagen, Germany) and 1.4 mm ceramic beads (Lysing Matrix D, MP Biomedicals, USA) for 40 s at max speed using a FastPrep-24TM 5G instrument (MP Biomedicals, USA). The RNA concentration was measured using a Nanodrop 2000c (Thermofisher, USA) at 260 nm.

QuantiNova LNA PCR focus panels

Four samples from SAH-vehicle and four from SAH-treatment were included in the screen and gene expression of 84 inflammamome related genes and house-keeping genes were quantified and analysed with QuantiNova LNA PCR Panels (GeneGlobe ID SBRN-097ZU) using a one-step protocol (Qiagen, Germany).

The maximum recommended concentration of RNA per reaction (200 ng RNA) was loaded to ensure detection of low abundance transcripts. The temperature profile for the One-step reactions were: 2 min 95 °C PCR initial heat activation, followed by a 2-step cycling program for 50 cycles (Denaturation 5 s 95 °C, Combined annealing/extension 10 s 60 °C). Data analysis was performed using the free, online software from Qiagen GeneGlobe.

For the initial screening, using the online software from Qiagen GeneGlobe, candidates were selected by fulfilment of the criteria of a significant difference in the fold change between the SAH-vehicle and SAH-treatment groups to detect a possible treatment effect.

cDNA and qRT-PCR

For validation, 1 μg RNA was reversely transcribed using the iScript cDNA Synthesis Kit (Biorad, USA) according to the manufacturer’s protocol. qRT-PCR was performed using 20x pre-designed TaqMan rat specific gene expression assay (MEVF: Rn.PT.58.36070627 and IL12i: Rn.PT.58.9242218, IDT, USA), and analysed using the Quant-Studio 6 Pro Real-Time PCR system (Applied Biosystems, USA). The thermal cycling condition included an initial denaturation step at 50 °C for 2 min and 95 °C for 10 min followed by 45 PCR cycles at 95°C for 15 s and 60 °C for 1 min. Relative mRNA expression levels were normalised to B2M and β-actin and determined by calculating \( 2^{-\Delta\Delta C_T} \).

Statistical analyses

The two sham groups were compared on all parameters including pre-operative weight, ICP, surgical time, blood gas analysis, weight difference over time and all test parameters. They were pooled into one group, as no statistically significant differences were found between the groups. Therefore, we compared three groups: SAH-vehicle, SAH-treatment, and Sham.

Continuous data were calculated using one-way ANOVA and post-hoc testing using Tukey’s multiple comparison tests. Unpaired, categorical data were tested by the Chi-square test and post-hoc testing using Fisher’s exact test. A fixed, mixed effects model employing repeated measures, was fitted to analyze weight changes, as missing values made repeated measures ANOVA unusable. Both factors, group and day, were fixed, the model was adjusted using the Greenhouse-Geisser correction followed by Tukey’s multiple comparison test.

Difference in time each animal spent exploring different objects in NOR was analysed with paired t-tests. The hazard ratio (HR) for death was calculated using the log-rank test and post-hoc testing controlling for false positive rates was done using the two-stage step-up method of Benjamini, Krieger and Yekutieli with a False Discovery Rate of 5 %.\(^6\) ROUT (Q=1 %) was used to identify statistical outliers. Data is presented as mean ± SD unless otherwise specified.

GraphPad Prism 9.0 (GraphPad, USA) was used for statistical analysis. Results was deemed statistically significant if \( P < 0.05 \), with the exception of the log-rank test for survival which is corrected used the above-mentioned method.

Results

Dose finding

At concentrations of 10\(^{-6}\) and 10\(^{-5}\) M, RO-31 significantly reduced the S6c induced contraction (Fig. 2). Based on this, 0.065 μg/g body weight of 10 μM RO-31 was considered the optimal concentration for in vivo experiments, resulting in a final concentration between 10\(^{-6}\) M and 10\(^{-5}\) M in the CSF.

Surgery

54 rats underwent surgery and a total of 42 rats were included in the study after exclusion of ineligible animals (Fig. 3). The peak ICP during injection of autologous blood was 257 ± 141 mmHg in the SAH-vehicle group and 361 ± 114 mmHg in the SAH-treatment group (\( P = 0.038 \)). ΔICP was 7.1 ± 3.9 mmHg in the SAH-vehicle group and 11.5 ± 3.6 mmHg in the SAH-treatment group (\( P = 0.005 \)). CPP during blood injection was significantly reduced in the SAH-treatment group, –282 ± 110 mmHg compared to SAH-vehicle, –180 ± 139 mmHg (\( P = 0.039 \)). CPP post SAH was equal between SAH-treatment, 66 ± 14 mmHg and SAH-vehicle 65 ± 24 mmHg (\( P = 0.93 \)). Remaining parameters were similar between groups (Supplementary table 1).

Survival

No animals died in the Sham group. One rat of 14 in the SAH-treatment group and six of 15 in the SAH-vehicle group were euthanised due to >20 % weight loss (humane endpoint according to Danish legislation). The median time to euthanasia in the SAH-vehicle group was 5.5 days (3–8, 95 % CI). The animal in the SAH-treatment group was euthanised on day 6. Comparing each group, SAH-vehicle had an increased risk of death due to >20 % weight loss of HR 7.9 (1.6–40.9 95 % CI, \( P = 0.01 \)) compared to the Sham group. SAH-treatment had a reduced risk of 0.15 (0.03–0.66, \( P = 0.04 \)) compared to the SAH-vehicle group. No statistical difference between SAH-treatment and Sham groups were observed, HR 6.9 (0.14–348.0, \( P = 0.34 \)). Post-hoc analysis showed \( P < 0.0476 \) as significant discoveries (Fig. 4).

Weight loss

All animals lost weight following surgery. Mixed-effects model showed significant differences between the groups, \( P = 0.008 \).
Compared to the Sham group, the weight loss was significantly larger on day 1 in both the SAH-vehicle, $-3.2\% \text{ (} -4.8 \text{ to } -1.5 \text{ CI, } p < 0.001 \text{)}$, and the SAH-treatment groups, $-3.6\% \text{ (} -5.8 \text{ to } -1.5, p = 0.001 \text{)}$ as well as on day two, respectively $-4.4\% \text{ (} -7.4 \text{ to } -1.3, p = 0.005 \text{)}$ and $-5.0\% \text{ (} -7.7 \text{ to } -2.2, p < 0.001 \text{)}$. There was a tendency to a lower weight in the SAH-treatment group throughout the study (Fig. 5).

**Rotating pole**

There were significant differences between the three groups on day two using the chi-square test, $p = 0.018$. Post-hoc testing showed there was an increased relative risk of falling off the pole in the SAH-vehicle group compared to the Sham group, $1.49 \text{ (} 1.05-2.05 \text{ CI, } p = 0.037 \text{)}$. The relative risk was reduced in the SAH-treatment group, $0.64 \text{ (} 0.47-0.91, p = 0.02 \text{)}$ compared to the SAH-vehicle group. There was no
difference between the SAH-treatment and Sham groups, 0.94 (0.52–1.46, \( p > 0.99 \)).

Post-subgroup analysis dividing the SAH-vehicle group into the animals which would survive at day 14 (SAH-vehicle_{alive}) or the ones that would reach the humane endpoint (SAH-vehicle_{dead}) and comparing them with SAH-treatment showed significant difference between groups, \( p = 0.004 \). The risk of falling off was reduced in the SAH-vehicle_{alive} compared to SAH-vehicle_{dead}, 0.53 (0.28–0.97, \( p = 0.058 \)), but the difference was not statistically significant. We observed a significant reduction in the SAH-treatment group compared to the SAH-vehicle_{dead} group of 0.51 (0.29–0.80, \( p = 0.002 \)). There was no significant difference between the SAH-vehicle_{alive} and SAH-treatment groups, 0.76 (0.43–1.12, \( p = 0.30 \)).

The results indicated that vehicle-treated animals that survived past day seven performed equally well as animals that received RO-31. There were no significant differences on day seven (\( p = 0.80 \)) and 14 (\( p = 0.29 \)) (Fig. 6).

Open field field

Distance covered on day two was 28.8 m (20.9–36.8, 95 % CI) in the Sham group. The numerical value, although not significant (\( p = 0.072 \)) was shorter in the SAH-vehicle group, 18.0 m (12.6–23.3), and in the SAH-treatment group, 22.3 m (14.6–30.0). Subgroup analysis comparing SAH-treatment, SAH-vehicle_{Alive} 20.6 m (13.7–27.5) and SAH-vehicle_{dead} 14.0 m (3.4–24.7) showed no difference, \( p = 0.35 \). There were no significant differences on day seven (\( p = 0.55 \)) and day 14 (\( p = 0.51 \)) between groups (Fig. 7). Regarding rearing, the number of high performers on day two were 7/15 animals in the SAH-vehicle group, 7/14 in the SAH-treatment group and 9/13 in the Sham group, (\( p = 0.44 \)). There were no significant differences on day seven (\( p = 0.27 \)) or day 14 (\( p = 0.59 \)). “High performer” was defined as an amount of rearing equal or above the median of rearing on the day of the test.

Time mobile on day two showed significant differences (\( p = 0.035 \)), where the Sham group moved for 225 s (172–278, 95 % CI), the SAH-vehicle group only 145 s (100–190) and the SAH-treatment group 162 s (119–204). Post-hoc testing showed a significant reduction in the SAH-vehicle group compared to the Sham group of –79 s (–154 to –5, \( p = 0.035 \)) (Fig. 7). There were no significant differences on day seven (\( p = 0.53 \)) or day 14 (\( p = 0.37 \)).

The thigmotaxic indexes on day two were 99.0 % (98.4–99.7, 95 % CI) in the Sham group. 99.6 % (99.3–99.9) in the SAH-vehicle group and 99.4 % (99.0–99.7) in the SAH-treatment group, \( p = 0.19 \). All TI was above 95 % and there were no significant differences on day seven (\( p = 0.18 \)) or day 14 (\( p = 0.63 \)).

Grooming time showed no significant differences (\( p = 0.59 \)) on day two; where the grooming time was 69 s (36–10, 95 % CI) in the Sham group, 48 s (20–77) in the SAH-vehicle group and 60 s (30–89) in the SAH-treatment group. Subgroup analysis comparing SAH-treatment, SAH-vehicle_{Alive} and SAH-vehicle_{dead} showed no difference, \( p = 0.59 \). There were no significant differences on day seven (\( p = 0.97 \)) and 14 (\( p = 0.99 \)). All results were calculated using 1-way ANOVA, except rearing, which was calculated using the Chi-square test.

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**Fig. 4.** Survival curve. The SAH-treatment (\( n = 14 \)) group had a reduced risk of 0.15 (0.03–0.66, \( p = 0.039 \)) compared to the SAH-vehicle group (\( n = 15 \)). The SAH-vehicle group had an increased risk compared to the Sham group (\( n = 13 \)), 7.9 (1.6–40.0, \( p = 0.01 \)). No difference between SAH-treatment and Sham group, 6.9 (0.14–348.0, \( p = 0.34 \)) Data are shown as percent ± SE. Statistics are generated by calculating the Hazard Ratio.

**Fig. 5.** Percental changes in weight. The weight loss in both the SAH-vehicle group (\( n = 15 \)) and SAH-treatment group (\( n = 14 \)) were significant lower on day 1 and 2 compared to the Sham group (\( n = 13 \)). Day 1: SAH-vehicle, −3.2 % (−4.8 to −1.5, \( p < 0.001 \)), and SAH-treatment group −3.6 % (−5.8 to −1.5, \( p = 0.001 \)). Day 2: SAH-vehicle −4.4 % (−7.4 to −1.3, \( p = 0.005 \)) and SAH-treatment −5.0 % (−7.7 to −2.2, \( p < 0.001 \)). Data are shown as mean ± SEM. Statistics are generated using a fixed, mixed effects model followed by Tukey’s multiple comparison test.
**Fig. 6.** Rotating Pole test. (a) On day two the relative risk of falling of the pole in the SAH-vehicle \( (n = 15) \) compared to the Sham group \( (n = 13) \) was increased, 1.50 (1.05–2.05, \( p = 0.037 \)). The relative risk of falling of the pole was reduced in the SAH-treatment group \( (n = 13) \) compared to SAH-vehicle, 0.64 (0.47–0.91, \( p = 0.021 \)). (b) When dividing SAH-vehicle into animals who was alive after 14 days \( (n = 9) \) or met the humane endpoint \( (n = 6) \), there was a reduced relative risk of falling off in the SAH-treatment group compared to the SAH-vehicle \( \text{Dead} \) group of 0.51 (0.29–0.80, \( p = 0.002 \)). Sample size of the groups were: SAH-vehicle \( (n = 15 \text{ on day 2, } n = 10 \text{ on day 7, } n = 9 \text{ on day 14}) \), SAH-treatment \( (n = 13 \text{ on day 2, } n = 12 \text{ on day 7 and 14}) \) and Sham \( (n = 13 \text{ all days}) \). Data are shown using percentage ± upper/lower limit.

**Fig. 7.** Open Field Test and Novel Object Recognition. (a) Distance in Open Field Test, \( -79 \text{ s (–154 to –5, } p = 0.035 \). (b) Discrimination Index of Novel Object Recognition. No preference of the novel object on day three. Significant difference in all three groups on day eight, no differences between groups. Sample size of the groups were: SAH-vehicle \( (n = 15 \text{ on day 2, } n = 10 \text{ on day 7, } n = 9 \text{ on day 14}) \), SAH-treatment \( (n = 14 \text{ on day 2, } n = 13 \text{ on day 7 and 14}) \) and Sham \( (n = 13 \text{ all days}) \). Data are shown as mean ± SD. Statistics is generated using one-way ANOVA followed by Tukey’s multiple comparison test.
Novel object recognition test

On day three, the discrimination indexes showed no significant differences in exploration time between the novel and familiar object. $-0.24 (-0.52-0.05, 95\% \text{ CI})$ in the Sham group, $0.18 (-0.21-0.56)$ in the SAH-vehicle group, and $-0.06 (-0.50-0.37)$ in the SAH-treatment group (Fig. 7). No significant differences were observed between groups ($p = 0.24$). On day eight all discrimination indexes were significant in favour of the novel object, $0.24 (0.06-0.47)$ in the Sham group, $0.18 (0.21-0.56)$ in the SAH-vehicle group, and $0.06 (0.50-0.37)$ in the SAH-treatment group. There were no significant differences between the groups ($p = 0.84$).

Screening of inflammasome related genes

The screening was performed on cerebral arteries from four animals in both the SAH-vehicle group and the SAH-treatment group, using one-step qRT-PCR on inflammasome PCR panels. When normalised to Beta-2-Microglobulin (B2M) and $\beta$-actin, significant differences in fold change were found for the Interleukin (IL)-12 $\beta$ gene, SAH-treatment ($0.005 \pm 0.003$ SEM) vs SAH-vehicle ($1.0 \pm 0.36, p = 0.002$) and the Mediterranean fever (MEFV) gene, SAH-treatment ($0.006 \pm 0.003$) vs SAH-vehicle ($1.0 \pm 0.29, p = 0.004$). In the SAH-treatment group, the maximum cycle threshold of 45 was reached in three animals regarding IL-12$\beta$ and in one animal in MEFV indicating no expression of the gene. C-C motif chemokine ligand 2 (CCL2) ($p = 0.38$), C-X-C motif chemokine ligand 1 (CXCL1) ($p = 0.91$), IL-1$\beta$ ($p = 0.96$), and IL-6 ($p = 0.21$) were not significantly different between groups. 

Validation of gene expression changes in cerebral arteries

The validation of IL-12$\beta$ and MEFV were performed on the same samples, normalised to B2M and $\beta$-actin (Fig. 8). Both genes were detectable in the samples. T-test identified no differences in the fold change in either gene between the two groups. IL-12 $\beta$: SAH-vehicle ($1.046 \pm 0.17$) vs SAH-treatment ($1.079 \pm 0.29, p = 0.93$). MEFV: SAH-vehicle ($1.053 \pm 0.20$) vs SAH-treatment ($1.303 \pm 0.16, p = 0.36$).

Discussion

RO-31 is known to ameliorate upregulation of vasoconstrictor mediators, but effects on functional outcomes after inhibition of PKC in experimental SAH were unknown. We found that treatment with RO-31 significantly improved outcome in terms of performance in the rotating pole test and weight-loss. Weight-loss is a proxy parameter of animal well-being and Danish legislation requires euthanasia for animals that reach >20 % weight-loss; this is considered a humane endpoint. This endpoint was reached by 40 % of the animals in the SAH-vehicle group compared to 7 % in the SAH-treatment group. Relevance of the model for clinical application depends on interpretation of animal data and behaviour. For animal well-being, weight-loss is a common and necessary parameter of health and significant weight loss necessitates euthanasia. Compared to clinical SAH, weight-loss receives an unproportionally large influence on outcomes in experimental SAH. Still, weight loss reflects lack of well-being or failure to feed optimally and is a robust, readily quantifiable parameter. Moreover, lack of wellbeing and failure to feed are features also of clinical SAH and may thereby have translational relevance. The 30-day mortality in clinical SAH is around 1/3, while mortality is an uncommon parameter in animal studies of SAH; experimental SAH parameters are typically titrated to minimize peri-ictal mortality, and there is no consensus on what could comprise an experimental model of DCI.

Vasospasm is common in DCI and delayed deterioration is a clinical diagnostic criterion for DCI, we therefore suggest that our...
Importantly, inflammation is also involved in SAH pathophysiology and ameliorates vasospasm in vitro and that ameliorates delayed deteriorations of cognition and well-being, could be interpreted to model DCI. Since our group and others have already shown that PKC inhibition restores contractile receptor expression and functional vasoconstriction. Upregulation of vasconstrictory receptors is associated with reduction of CBF early after experimental SAH. Vessel wall vasconstrictor has a maximal upregulation at 48 h in this model. Hence, the pharmacologically effective concentrations of RO-31 were determined in vitro at 48 h. Phosphorylated PKC-δ is increased at 1- and 48 h after SAH and PKC-α is increased at 48 h after SAH, in brain vessels with increased activation of all three MAPK-paths, and PKC-α is increased at 48 h after SAH, in brain vessels with increased activation of all three MAPK-paths. In the same model, mRNA expression and protein levels of the ET₃ and 5-HT₁B receptors were increased in SAH animals compared to sham at 48 h. The enhanced expression of contractile cerebrovascular receptors has been verified by qRT-PCR, protein expression analyses with Western blot, immunohistochemistry and flow cytometry, and increased contractility was confirmed by myography studies. These changes were reversed by cisternal administration of the PKC blocker RO-31 for up to seven days. Our findings thus corroborate previous SAH studies where the vehicle Cremophor EL unexposedly showed a beneficial effect. Nevertheless, the vasculature is not the only target for PKC. Yang and colleagues described that PKC-δ stimulates inflammation and neutrophil migration by activating NF-κB. Moreover, neutrophil migration through brain microvascular endothelial cells imbued with TNF-α is restricted by a PKC-δ inhibitor. Importantly, inflammation is also involved in SAH pathophysiology and is a significant component of DCL. The inflammatory mediators TNFα, IL-1β, and IL-6 are expressed in cerebral blood vessels early following experimental SAH. IL-23 is built in part of the IL-12β subunit and known to be increased in serum of patients on day 7 after SAH. Our inflammatory markers could only detect long-term changes at 14 days. At this time point mRNA expression of IL-12β was similar between the SAH-treatment and SAH-vehicle groups and PKC-inhibition did not affect IL-12β at this time point. CCL2, CXCL1, IL-1β and IL-6 which are increased in the first days following SAH were not significantly different between groups at 14 days. Pharmacological inhibition of PKC should not be expected to affect the inflammasome of the vessels in a long-term perspective. Surprisingly, the exploratory analyses: the open field test and the novel object recognition did not display convincing differences between the three groups despite clear differences in weight-loss and rotating pole performance. The open field test would agree with benefit of treatment, but the groups had high variability and results remained statistically inconclusive. Our experiment was designed to use the open field test as an exploratory outcome as other groups have detected more outspoken differences between groups at later time points and not as early as we used in our study. We also failed to detect differences between groups in novel object exploration. There is currently no consensus on how to perform the novel recognition test in rodent trauma- or SAH-models, and there are large variations in object shape and size, and exploration definitions and in inter-trial intervals. The exploratory tests remained largely inconclusive. Main reasons for an inconclusive result could again be survival bias, the fact that experiments were designed to detect group differences at earlier time points than those that might be most relevant for the open field and novel object recognition tests and that RO-31 might have affected some aspects of memory generation. In this context, inhibition of the ERK1/2 pathway has been suggested to inhibit object memory generation. Moreover, albino rats have impaired colour vision and may not have differentiated the colours of our objects sufficiently. Scientific conclusions from the open field- and novel object recognition tests appear to require a targeted protocol, larger follow-up and larger groups to compensate for variability in our model and compensation for survival bias. Limitations This is an experimental study with several limitations inherent in the methodology. Although randomization, blinding and power-calculation were strengths, experimental modelling necessarily limits validity. Internal validity is high for male 12-week-old Sprague-Dawley rats and for the rotating-pole and weight loss endpoints subjected to pre-chiasmatic experimental SAH. Motor-behaviour with rotating pole is a well-established robust test, while the two other behavioural tests are still insufficiently established in the context of experimental SAH. Male rats were selected to avoid confounding neuropathological effects by variation of sex-hormone levels during the estrus cycle, but external validity can probably be extended to female rats. Sex difference is a potential variable but previous studies did not detect sex-differences in similar experimental settings. The relative mortality in human SAH is comparable between the sexes, but females have an increased risk of being afflicted by SAH. Thus, confirmatory studies should include both males and females. External validation includes experiments in different strains or species and with other experimental models. For SAH, the main models are the MCA perforation model and the pre-chiasmatic injection. We developed the pre-chiasmatic model to minimize variability and mortality. The model is based on titration and monitoring of ICP and CBF during ictus. Thereby, early brain injury is highly reproducible and variability and experimental mortality minimized. Still, peak ICP and ΔICP were higher in the SAH-treatment group compared to SAH-vehicle and represent confounders that could diminish the observed treatment effect since higher ICP correlates with worse neurological outcomes. Compared to the MCA perforation model, potential effects of arterial perforation are foregone. Yet, the pre-chiasmatic model is in general use and is considered relevant for translational SAH research. Moreover, experimental SAH does not incorporate risk factors such as hypertension or smoking. Lastly, although the molecular effects on contractile receptors and the MAPK/ERK-pathway at the relevant early time-points have been studied in extenso and are well known, the study design did not allow tissue harvest for confirmation. Vasoconstriction and receptor upregulation were the targets of PKC inhibition and drug dosage was based on myographic experiments to optimize RO-31 cisternal concentration. Conclusion This study meets the need to study functional benefit of PKC inhibition after experimental SAH. Intrathecal treatment with RO-31
reduced the risk of reaching a >20 % weight loss in a rat model of SAH and improved outcome in the rotating pole test at day 2. Our data suggest functional benefit from PKC inhibition with RO-31 could be further explored to improve outcomes after SAH.

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CRediT authorship contribution statement

Jesper P Bømers: Writing – review & editing, Writing – original draft, Visualization, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

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Tiit I Mathiesen: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Kristian A Haanes: Writing – review & editing, Supervision, Project administration, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

None.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jstrokecerebrovascas.2024.107728.

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