

# The effect of common carotid artery occlusion on delayed brain tissue damage in the rat double subarachnoid hemorrhage model

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## Abstract

**Objective** Delayed ischemic brain tissue damage in the time course of cerebral vasospasm in the rat double-subarachnoid hemorrhage (SAH) model has been described before. However, in order to enhance hemodynamic insufficiency during cerebral vasospasm (CVS), we performed—in a modification to the standard double-hemorrhage model—an additional unilateral common carotid artery occlusion (CCAO), expecting aggravation of brain-tissue damage in areas particularly sensitive to hypoxia.

**Methods** CVS was induced by injection of 0.25 ml autologous blood twice in the cisterna magna of Sprague-Dawley rats with and without unilateral CCAO. The animals were examined on days 2, 3, 4 and 5, and compared with the sham-operated control group without SAH. The functional deficits were graded between 0 and 3. Perfusion weighted imaging (PWI) at 3 Tesla magnetic resonance (MR) tomography was performed to assess cerebral blood flow (CBF). The brains were fixed, stained and evaluated for histological changes.

**Results** On day 5, the neurological state was significantly worse in rats with SAH. The relative CBF/muscle blood ratio was significantly decreased by SAH and lowest in rats with CCAO and SAH ( $4.5 \pm 1.1$  vs  $2.7 \pm 0.6$ ) compared with sham ( $7.9 \pm 1.5$ ;  $p < 0.001$ ). Basilar artery (BA) diameter was

$79 \pm 5 \mu\text{m}$  (SAH) vs  $147 \pm 4 \mu\text{m}$  (sham,  $p < 0.001$ ). Neuronal cell count in the hippocampal areas CA1-CA4 was significantly reduced by SAH on day 5 ( $p < 0.001$ ) and lowest in rats with SAH and CCAO.

**Conclusions** CCAO leads to an aggravation of CVS-related delayed brain tissue damage in the modified rat double-SAH model.

**Keywords** Cerebral vasospasm · Rat double-hemorrhage model · Subarachnoid hemorrhage

## Introduction

Delayed cerebral vasospasm (CVS) after aneurysmal subarachnoid hemorrhage (SAH) remains to be a substantial reason for poor clinical outcome [8, 14, 33]. Particularly, because the prevention of delayed ischemic deficits after SAH seems to be more capable for potential treatment or prophylaxis than the initial insult, animal models simulating delayed CVS in humans like the rat double-SAH model are necessary for further improvement of the knowledge about pathophysiological mechanisms and for the development of tailored treatment strategies [41].

We described delayed brain tissue damage in the rat double-SAH model before [12]. Brain tissue damage in the phase of CVS was present in the hippocampus and the inner layer of the cerebral cortex without development of territorial infarctions. Pronounced cortical collateralization, bypassing hypoperfusion during pathological conditions, has been described before and held responsible for the avoidance of territorial infarctions [3].

Unilateral common carotid artery occlusion (CCAO) in the rat results in a moderate reduction of cerebral blood flow (CBF) in both cerebral hemispheres without asym-

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metrical perfusion [6]. However, the reserve capacity of the circulatory system is limited. If the adaptive mechanisms become insufficient, e.g., by CVS, brain tissue may—depending on the severity of hypoperfusion—become hypoxic. In order to enhance hemodynamic insufficiency during CVS, we performed—in a modification to the standard double hemorrhage model—an additional unilateral CCAO at the left side expecting aggravation of brain tissue damage in areas particularly sensitive to hypoxia.

According to recent investigations like the CONSCIOUS-1 trial, the causality between the delayed spasm of proximal cerebral arteries after SAH and the development of delayed ischemic deficits remains to be proven [24]. Accordingly, there is a need for animal models mimicking not only angiographic vasospasm but also neurological deterioration and ischemic brain tissue damage.

The aim of the present investigation was, therefore, to correlate the ischemic brain tissue damage with histological CVS and neurological deterioration in an SAH model with enhanced hemodynamic insufficiency by CCAO. Furthermore, an exact histological characterization of ischemic brain tissue damage after SAH with and without CCAO should be established, for utilization as reference data for the analysis and adjustment of neuroprotective strategies in further studies. Therefore, we analyzed the time course of brain tissue damage in a modified rat double-SAH model using the neuronal cell count method, which was introduced by Soehle et al. [36] and utilized in a former study for the characterization of brain tissue damage in the standard double-SAH model [12]. The neuronal cell count was determined in the cerebral cortex and hippocampus, which are areas differently sensitive to cerebral ischemia.

## Materials and methods

### Animal preparation

Experimental groups are summarized in Table 1. The present investigations were performed with male Sprague-Dawley rats ( $n=70$ ) weighing 280–360 g. Experimental

CVS was induced using a modified rat double-hemorrhage model [37] as described in detail previously [12, 41]. Briefly, the animals were anesthetized on day 1 of the experiment by intraperitoneal application of midazolam (1 mg/kg body weight) and ketamine (100 mg/kg body weight). This anesthesia is commonly used in rat SAH models [5, 35, 41], its feasibility is proven for imaging procedures in rats [7] and significant changes of circulation parameters and of CBF due to this medication were not observed [20, 34]. A tube (Portex polythene tube, luminal diameter 0.96 mm) was inserted in the femoral artery for the measurement of blood gas values, for the control of blood pressure, and for blood sample withdrawal. Another tube of the same size was inserted in the femoral vein to administer additional medications or infusions (crystalloids). Body temperature was maintained at 37°C using a heating pad. In rats assigned for CCAO, the left common carotid artery was exposed through a ventral incision in the neck, separated from the vagosympathetic trunks, and ligated. Thereafter, the animals were positioned in a stereotactic frame and the atlanto-occipital membrane was exposed through a small midline approach. After insertion of a catheter (Portex polythene tube, luminal diameter 0.28 mm) in the cisterna magna, 0.1 ml cerebrospinal fluid (CSF) was withdrawn followed by the injection of 0.25 ml autologous blood to induce the first SAH. The wounds were surgically closed. The animals received 5 ml crystalloid solution with 0.0125 mg fentanyl subcutaneously and were kept in a head down position for 15 min. An identical procedure was performed on day 2 to induce the second SAH. In sham operated rats, 0.25 ml physiological saline was inserted instead of blood. After second SAH, the animals were observed clinically and received 5 ml crystalloid solution with 0.0125 mg fentanyl twice a day.

### Neurological scoring

All animals underwent neurological examination according to the grading system by Bederson et al. [3] on day 2 after induction of a second SAH as well as on days 3, 4, and 5 thereafter. In brief, rats were gently held by the tail above

**Table 1** Experimental groups, and mortality on day 3 and 5

Experimental group				No. of animals	Mortality (%)
Day 3	1	w/o SAH	w/o CCAO	6	0
	2	w/o SAH	with CCAO	6	0
	3	with SAH	w/o CCAO	10	3 (30%)
	4	with SAH	with CCAO	10	4 (40%)
Day 5	1	w/o SAH	w/o CCAO	6	0
	2	w/o SAH	with CCAO	6	0
	3	with SAH	w/o CCAO	12	6 (50%)
	4	with SAH	with CCAO	14	7 (50%)

CCAO permanent left common carotid artery occlusion, SAH subarachnoid hemorrhage, w/o without

the floor. Rats without neurological deficit received the grade 0. Rats with flexion of one forelimb without further deficits were grade 1 (mild dysfunction). After placing the rats on a sheet of plastic coated paper, animals were tested for their resistance to lateral push to one side. Rats with consistently reduced resistance were grade 2 (moderate dysfunction). Rats were then allowed to move freely and observed for circling behavior. Rats with consistent circling to one side or with a reduction of vigilance without any movement were deemed grade 3 (severe dysfunction).

#### Magnetic resonance imaging and cerebral blood flow

The magnetic resonance imaging (MRI) measurements were performed at 3.0 T (Magnetom Trio, Siemens, Germany) with a wrist coil, as described in detail previously [41]. The standardized imaging protocol included axial and sagittal T2-weighted images (WI), and perfusion weighted images (PWI). MRI measurements were performed on day 3 and on day 5—the day of the maximum CVS [41]. In rats with CCAO, the masseter muscles of the contralateral side to the occluded common carotid artery were used for relative regional cerebral blood volume (rrCBV) and rrCBF measurement.

#### Histology

Histological analysis was performed, as prospectively assigned, on day 3 and day 5 after the surgical procedure to evaluate the time course of histological changes after artificial SAH. The number of animals was estimated according to the mortality rates of the double-SAH model in rats described by Vatter et al. [41] (Table 1). Animals were killed in deep anesthesia and perfused with ice-cold heparinized saline from the left ventricle for 10 min. The brains were removed and postfixed in ice-cold phosphate-buffered 4% paraformaldehyde (PFA) for 24 h, and embedded in paraffin to obtain coronal sections of the parietal region as described previously [12]. Sections, 20  $\mu\text{m}$  thick, were cut and stained with hematoxylin-eosin (H & E). Histological evaluation was performed by light microscopy and photographed using a CCD camera (Sony, Tokyo, Japan). Image software was calibrated with a microscope ruler (Ernst Leitz, Wetzlar, Germany). For quantitative assessment of brain tissue damage, vital cells were counted by two independent observers who were blinded to the previous treatment conditions, in seven nonoverlapping regions of interest (ROIs) in the hippocampus (CA1, CA2, CA3, and CA4) and the adjoining cortex (Cortex1 to Cortex3 without relation to cortex layers) as described elsewhere [12, 36]. The investigated hippocampal areas were 0.02  $\text{mm}^2$  and the cortical areas were 0.04  $\text{mm}^2$  each (CA1–CA4 and Cortex1–Cortex3). Neurons were

classified as non-vital when they exhibited pyknosis, karyorrhexis, karyolysis, cytoplasmic eosinophilia, or loss of affinity for hematoxylin [22]. Unilateral CCAO is known to result in a moderate symmetrical reduction of CBF in both cerebral hemispheres [6]. Accordingly, neuronal cell count between both hemispheres did not differ in the current study. Therefore, neuronal cell count is expressed as average values. For the measurement of the basilar artery diameter (BA), cross-sections of the brainstem were performed on days 3 and 5 and stained for H & E. Computer-assisted morphometry was calculated by using three adjusted straight lines inside the basilar lumen. Of each animal, three sections from different levels of the BA were analyzed (proximal, median, distal).

#### Data analysis

Data are expressed as mean  $\pm$  standard deviation (SD). Statistical significance was verified by analysis of variance performed in one-way ANOVA followed by Tukey test for multiple comparisons. Significance of differences between the groups regarding neurologic scores was analyzed by Kruskal-Wallis one-way ANOVA followed by the *t*-test procedure. A probability value of  $p < 0.05$  was considered statistically significant.

## Results

Physiological values of arterial blood pressure, partial pressure of oxygen and carbon dioxide, blood pH, and hemoglobin did not differ among the experimental groups. Experimental groups and mortality are summarized in Table 1.

#### Mortality

Rats without SAH (groups 1 and 2) survived until being killed on day 3 and day 5. In group 3, three of ten SAH rats (30%) died after second SAH until day 3, and six of 12 rats (50%) until day 5. In group 4, four of ten SAH rats (40%) died after the second SAH until day 3, and seven of 14 SAH rats (50%) until day 5.

Parameters of cerebral vasospasm are summarized in Table 2.

#### Neurological and neuroradiological evaluation

In the SAH groups 3 and 4, the clinical state after second SAH was worst on day 5 (median: 2.5 and 3), with a significant worsening compared with day 2 (median: 1.5;  $p < 0.01$ ).

In group 1, rrCBF was 8.1 $\pm$ 1.3-fold higher compared with the perfusion of the masseter muscle on day 3.

**Table 2** Parameters of cerebral vasospasm on day 3 and 5

Experimental group				Neuro assessment	rrCBF ratio	BA diameter ( $\mu\text{m}$ )
Day 3	1	w/o SAH	w/o CCAO	0	$8.1\pm 1.3$	$152\pm 10$
	2	w/o SAH	with CCAO	0	$7.1\pm 1.7$	$167\pm 11$
	3	with SAH	w/o CCAO	2	$5.3\pm 0.9$	$139\pm 9$
	4	with SAH	with CCAO	2	$5.0\pm 1.2$	$153\pm 10$
				$p^a$	$=0.03$	$=0.04$
Day 5	1	w/o SAH	w/o CCAO	0	$7.9\pm 1.5$	$147\pm 4$
	2	w/o SAH	with CCAO	0	$7.7\pm 2.1$	$168\pm 8$
	3	with SAH	w/o CCAO	2.5	$4.5\pm 1.1$	$79\pm 5$
	4	with SAH	with CCAO	3	$2.7\pm 0.6$	$82\pm 7$
				$p^a$	$<0.0001$	$<0.0001$

<sup>a</sup>Compared within the column between group 2 and 4 on day 3 and day 5 (rrCBF, BA diameter: ANOVA; Neuro assessment: Fisher exact test)

Furthermore, rrCBV was  $7.6\pm 1.2$ -fold higher compared with the perfusion of the masseter muscle. In rats after SAH (group 3), these values were reduced to 56% (CBF) and 63% (CBV) on day 5. In group 4, rrCBF was reduced in both hemispheres symmetrically to 70% on day 3 and to 35% on day 5 (Fig. 1).

#### Basilar artery

Basilar artery (BA) diameter on day 3 was  $152\pm 10\ \mu\text{m}$  in sham operated rats (group 1) and discrete but significantly reduced to  $139\pm 9\ \mu\text{m}$  in group 3 ( $p=0.01$ ). On day 5, BA diameter was

$147\pm 4\ \mu\text{m}$  in sham operated rats and significantly reduced to  $79\pm 5\ \mu\text{m}$  in the SAH group 3 ( $p<0.0001$ ).

BA diameter was larger in group 2 compared with group 1 on day 3 ( $167\pm 11\ \mu\text{m}$  vs  $152\pm 10\ \mu\text{m}$ ;  $p=0.03$ ) and on day 5 ( $168\pm 8\ \mu\text{m}$  vs  $147\pm 4$ ;  $p=0.0002$ ) respectively. BA diameter was markedly reduced in group 4 on day 5 compared with day 3 ( $p<0.0001$ ).

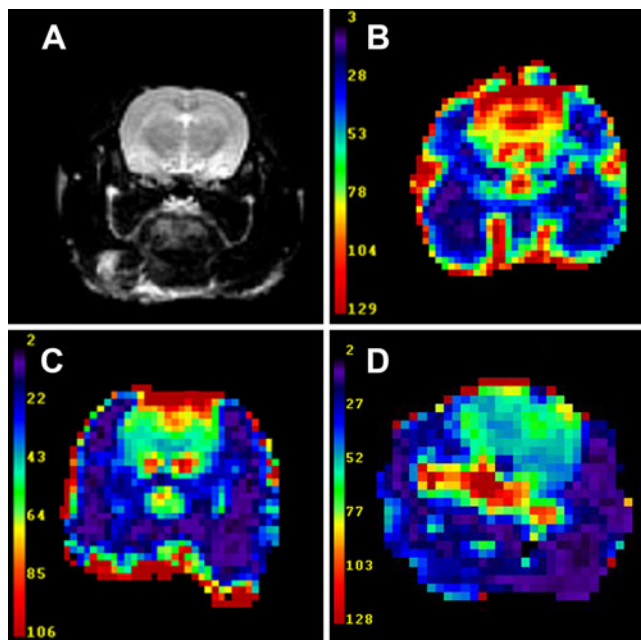
#### Morphological changes

Neuronal cell counts obtained from the defined ROIs in the hippocampal areas and the cerebral cortex are shown in Figs. 2 and 3 and in Tables 3 and 4.

On day 3, the neuronal cell count in the hippocampal areas CA1-CA4 and Cortex1-Cortex3 was not significantly different between the groups 1–4.

On day 5, the neuronal cell count in the hippocampal areas CA1-CA4 was significantly reduced by SAH ( $p<0.001$ ). CCAO without SAH (group 3) was not associated with reduced neuronal cell count on day 5. CCAO with SAH (group 4) was associated with a marked reduction of neuronal cell count in the hippocampal areas CA1-CA4 on day 5 compared with day 3 ( $p<0.001$ ), and in Cortex3 ( $p=0.04$ ), whereas no significant difference could be found for Cortex1 ( $p=0.2$ ) and Cortex2 ( $p=0.5$ ).

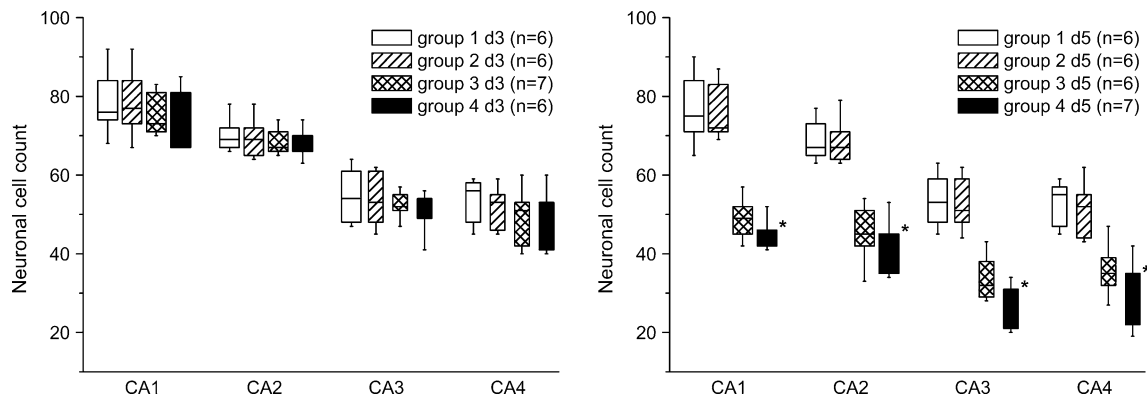
CCAO with SAH was associated with a significantly lower neuronal cell count in the CA1 and CA3 region on day 5 compared with the group of SAH rats without CCAO ( $p=0.03$  and  $p=0.02$ ). Neuronal cell count did not differ between both hemispheres in rats with and without CCAO. Histological changes in the hippocampal layers CA1 and CA3 are exemplified in Fig. 4.



**Fig. 1** MRI axial slices of a sham-operated rat (a, b) and SAH rats without (c) and with CCAO (d) suffering from cerebral vasospasm on day 5. T2-weighted imaging for the demonstration of the anatomic site for rrCBF measurement (a). Blood perfusion (rrCBF) of the central nervous system and the masticatory muscles are plotted on color-coded PWI parameter images indicating reduced rrCBF in the animals suffering from CVS (c, d)

#### Discussion

Delayed cerebral ischemia contributes significantly to morbidity and mortality after aneurysmal SAH [13, 16, 44]. Despite intensive research, the pathogenesis of delayed



**Fig. 2** Quantitative data on neuronal cell count in the rat hippocampal layers (CA1–CA4) of sham operated and SAH rats with and without CCAO on day 3 (left) and day 5 (right). Neuronal cell count is preserved in all hippocampal layers after SAH with and without

CCAO on day 3 and significantly decreased on day 5 (\*). Group 1: without SAH, without CCAO; group 2: without SAH, with CCAO; group 3: with SAH, without CCAO; group 4: with SAH, with CCAO

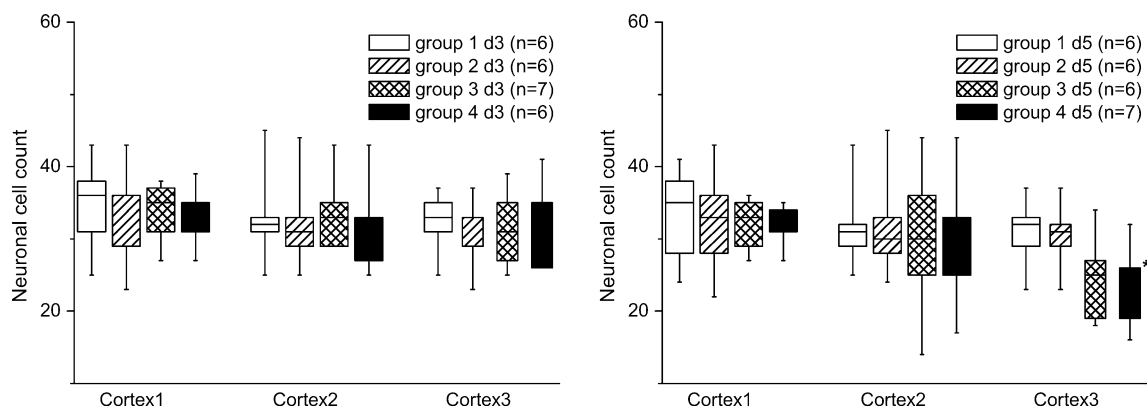
CVS is still not completely understood. Therefore, animal models are needed for further investigation of pathological changes to develop new therapeutic strategies.

The present data demonstrate the development of neurological impairment in rats with cerebral high risk areas created by unilateral CCAO and induced CVS in an aggravated modified double-hemorrhage model.

In the standard double-hemorrhage model, several authors demonstrated an extended distribution of blood in both the supratentorial and infratentorial basal cisterns after artificial SAH by injection of blood into the cisterna magna. Furthermore, these investigations demonstrated that all major cerebral arteries were surrounded by blood [39–41, 45]. Despite the blood degradation in the basal cisterns until day 3 [39, 41], SAH induced CVS led to a maximum reduction of vessel diameter on days 5–7. Furthermore, CBF was decreased by CVS to 30–70% from the control [12, 19, 39, 41]. Determination of the vessel perimeter revealed for the middle cerebral artery a 36% reduction

[17], the posterior communicating artery a 35% reduction, and the BA a 33% reduction [11] compared with the controls. In the measurement of the vessel diameter in histological slices, this reduction amounted to 25–30% for the middle cerebral artery [39, 45], 30% of the posterior cerebral artery [39], and 30–53% for the BA [12, 19, 39, 41]. Therefore, the data from the available literature clearly suggests that a comparable proximal CVS could be induced in all major cerebral arteries in the rat cisterna magna double-hemorrhage model.

In the current study, we analyzed BA diameter on the assumption of the proximal CVS, which is used by the vast majority of such investigations previously [12, 19, 39, 41] and therefore facilitates the comparability of results. Furthermore, reliable measurement of BA diameter at several levels is more reliable to assess by cross-sections of the brainstem compared with the middle cerebral artery, especially when coronal sections of the same animal's parietal region are needed to evaluate the hippocampus.



**Fig. 3** Quantitative data on neuronal cell count in the rat cerebral cortex (Cortex1–Cortex3) of sham-operated and SAH rats with and without CCAO on day 3 (left) and day 5 (right). Neuronal cell count is preserved in all cortical layers after SAH with and without CCAO on

day 3 and significantly decreased in the inner cortical layer (Cortex3) on day 5 (\*). Group 1: without SAH, without CCAO; group 2: without SAH, with CCAO; group 3: with SAH, without CCAO; group 4: with SAH, with CCAO



**Table 3** Histological changes in the hippocampus after SAH

Experimental group			CA1	CA2	CA3	CA4	
Neuronal cell counts (mean±SD) in investigated regions of the hippocampus	Day 3	w/o SAH	w/o CCAO	80±8	70±4	55±6	56±6
		w/o SAH	with CCAO	79±8	70±5	54±6	54±5
		with SAH	w/o CCAO	77±6	68±4	53±4	52±6
		with SAH	with CCAO	76±7	67±4	52±5	52±7
	Day 5	w/o SAH	w/o CCAO	79±9	68±5	54±6	55±5
		w/o SAH	with CCAO	77±7 <sup>a</sup>	69±5 <sup>a</sup>	54±7 <sup>a</sup>	52±7 <sup>a</sup>
		with SAH	w/o CCAO	49±5	46±7	35±6	37±6
		with SAH	with CCAO	43±4 <sup>a</sup>	43±7 <sup>a</sup>	27±5 <sup>a</sup>	31±8 <sup>a</sup>
			<i>p</i> <sup>a</sup>	<0.0001	<0.0001	<0.0001	=0.0004

<sup>a</sup>Compared within the column in rats with CCAO with and without SAH on day 5 (ANOVA)

Therefore, analyzing the BA diameter remains to be the most robust and established measure for estimation of proximal CVS, even if the correlation to neurological impairment and the decreased hippocampal neuronal cell count are not, or only partially, in a brain territory depending on BA supply.

#### Neurological impairment and cerebral blood flow

The mortality rate of rats with CCAO and SAH (50%) was comparable with data of previous studies using the double-SAH model without CCAO [12, 41]. A significant neurological impairment was induced by SAH, not by CCAO alone. The neurological deterioration within the first days after SAH may be an expression of a transient increase of intracranial pressure and reduced CBF [37, 42].

Mechanisms inducing a reduction of CBF in the acute phase after SAH include acute vasoconstriction, brain edema and decreased cerebral perfusion pressure (CPP) [1, 2, 4]. Early changes after SAH have been described in the prechiasmatic and the perforation SAH model [32] that are used to mimic the acute phase of SAH. In the cisterna

magna model used in the present study, early changes do not seem to play a major role. Accordingly, measurements of decreased rrCBF as well as histological confirmation of reduced BA diameter reached their maximum on day 5 in correlation with the severe neurological impairment at that time point in the present investigation, which is consistent with findings in former studies [12, 37, 41]. Therefore the cisterna magna double-hemorrhage model seems to be more suitable to mimic the delayed phase of SAH, including the development of delayed cerebral ischemia.

CCAO was not associated with a significant reduction of neurological impairment compared with SAH rats. Unilateral CCAO is known to result in a moderate reduction of CBF decrease in both cerebral hemispheres without asymmetrical perfusion in animal models of CCAO without SAH [6]. CBF after CCAO is normalized within 24 h after ligation and not different from values found in the control group [6]. In the current study, SAH significantly impaired neurological function of CCAO rats at both day 3 and day 5. Accordingly, rrCBF of CCAO rats was reduced by SAH at day 3 and significantly lower compared with SAH rats without CCAO at day 5. Hence, reflecting the effect of the created high risk area for delayed cerebral ischemia after carotid occlusion and CVS.

MR PWI has been used in the evaluation of rrCBF and rrCBV in the rat double-hemorrhage model before [41]. The correlation of CBF and/or CBV estimation between microsphere technique [43], positron emission tomography (PET) [23, 28, 29], single-photon emission computed tomography (SPECT) [23] and MR PWI has been validated previously. Using radiolabeled microsphere techniques, a stable CBF/masseter muscle blood flow ratio between 6.1 and 7.7 was observed in male Sprague-Dawley rats [10, 31], which is consistent with our data. Therefore, the rrCBF/masseter muscle blood flow ratio may be a good quantitative discriminator between reduced CBF and/or CBV caused by CVS or reduced systemic circulation. In addition, our data demonstrate a significant correlation between the defined ratio (CBF/muscular blood flow and

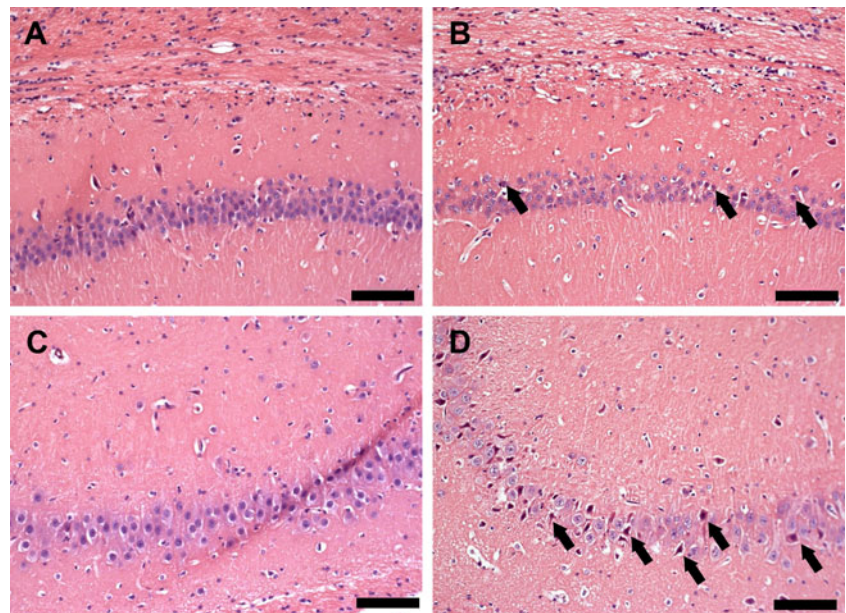
**Table 4** Histological changes in the cortex after SAH

Experimental group			Cortex1	Cortex2	Cortex3
Day 3	w/o SAH	w/o CCAO	37±5	33±6	33±4
	w/o SAH	with CCAO	36±6	32±6	33±4
	with SAH	w/o CCAO	35±4	33±5	32±5
	with SAH	with CCAO	35±4	33±6	32±6
Day 5	w/o SAH	w/o CCAO	35±6	31±6	32±4
	w/o SAH	with CCAO	34±7 <sup>a</sup>	31±7 <sup>a</sup>	31±4 <sup>a</sup>
	with SAH	w/o CCAO	34±3	32±9	26±6
	with SAH	with CCAO	32±3 <sup>a</sup>	30±9 <sup>a</sup>	24±6 <sup>a</sup>
<i>p</i> <sup>*</sup>			=0.5	=0.8	=0.03

Neuronal cell counts (mean ± SD) in investigated regions of the cortex

<sup>a</sup>Compared within the column in rats with CCAO with and without SAH on day 5 (ANOVA)

**Fig. 4** H & E-stained sections of the hippocampal layers CA1 and CA3 of a sham-operated rat (**a, c**) and a SAH rat with CCAO (**b, d**). Non-vital neurons within the hippocampal layers CA1 and CA3 of SAH rats with CCAO (**b, d**) exhibit pyknosis of nuclei and eosinophilia of cytoplasm (arrows) on day 5. Bar 100  $\mu\text{m}$



CBV/muscular blood volume) and the histological reduction of BA diameter, suggesting an adequate noninvasive monitoring of CVS by MR PWI.

The time course of BA diameter reduction by CVS has been characterized in the double-hemorrhage model before [41]. The maximal CVS was described to be on day 5 [25, 26, 41], as well as the consecutive reduction of rCBF and neurological deterioration. The average diameter of the control rat BA was  $152 \pm 10 \mu\text{m}$  in the present study, which is in agreement with previously published values between 150 to 250  $\mu\text{m}$  [4, 12]. Furthermore, the reduction of the BA diameter to  $79 \pm 5 \mu\text{m}$  after SAH on day 5 is in accordance with formerly reported reduction to 47–55% [12, 41]. The increase of the BA diameter of 9.9% after CCAO in control rats is consistent with previously reported dilatation of the BA of  $10 \pm 1\%$  after unilateral CCAO by Fuji et al. [9]. Interestingly, at day 3 the BA diameter in SAH rats with CCAO was also 10% larger compared to SAH rats without CCAO, whereas at day 5 the difference of the BA diameter between SAH rats with and without CCAO was reduced to 4% by CVS. While rCBF was maintained in CCAO rats without SAH at day 3 and day 5 by autoregulatory enlargement of the BA diameter, delayed CVS in CCAO rats led to a significant reduction of BA diameter at day 5 and significantly reduced rCBF compared with SAH rats without CCAO.

The mortality rates for sham rats (0%) and SAH rats (30% until day 3; 50% until day 5) in the current investigation are consistent with findings of our previous studies (24% until day 3; 47% until day 5; 0% sham rats) [12, 41]. Mortality in CCAO rats was 0% without SAH. Mortality in CCAO rats with SAH was 40% until day 3, and 50% until day 5. Thus, CCAO increased mortality at day 3 but not at day 5.

#### Morphological changes

We analyzed neuronal integrity after SAH within the hippocampus because of its known particular sensitivity to cerebral ischemia [15, 18, 21, 38] and within the cerebral cortex. We reported previously a CVS-related significant reduction of neuronal cell count after SAH in the hippocampus (CA1–CA4) and the inner layer of the cortex at day 5 [12].

In the current aggravated double-hemorrhage model with CCAO, neuronal cell count in the hippocampus (CA1–CA4) and the inner layer of the cortex was reduced by CVS at day 5, not at day 3. Furthermore, neuronal cell count in SAH rats with CCAO was significantly lower in the CA1 and the CA3 region at day 5 compared with the group of SAH rats without CCAO ( $p=0.03$  and  $p=0.02$ ). The aggravation of brain tissue damage in areas that are particularly vulnerable to ischemia is attributable to the development of cerebral high risk areas by unilateral CCAO and induced CVS and correlates with the development of neurological impairment and reduction of rCBF at day 5.

Early brain tissue damage after SAH—within 24–72 h after the onset—have already been analyzed qualitatively in the cerebral cortex as well as in the hippocampus of the rat in the endovascular perforation model [4, 30]. Early histological changes included cytoplasmic condensation and nuclear pyknosis as markers of evolving necrosis, as was used for the definition of non-vital neurons in the current study. In contrast to the cisterna magna model used in the current study exhibiting changes of the late SAH phase including CVS [12, 37, 41], the endovascular perforation model is known to exhibit the acute changes

after SAH in a distinct fashion with early reduction of CBF [4, 30, 32].

Quantitative data of brain tissue damage in the phase of CVS in the cisterna magna model has been analyzed before [12]. In the current study we provided quantitative data of significantly decreased neuronal cell count in the hippocampus (CA1–CA4) and the inner layer of the cortex (Cortex3) of SAH rats with and without CCAO on day 5 compared with sham operated rats and demonstrated that on day 3—i.e., the early phase—of SAH, no significant decrease of neuronal cell count was present compared with sham rats. Thus, our data indicate a delayed development of the brain tissue damage in both SAH rats with and without CCAO. Furthermore, brain tissue damage was enhanced in SAH rats by CCAO in the CA1 and CA3 region of the hippocampus, which is known to be vulnerable to ischemia [15, 18, 21, 38].

The occurrence of brain tissue damage in areas particularly sensitive to hypoxia, instead of a global or territorial ischemic infarction, may be explained by CVS that is accentuated in the posterior fossa and cortically bypassed by the pronounced cortical collateralization in the rat [3]. Furthermore, CBF in the rat hippocampus is known to be lower compared with the cerebral cortex [32]. The medially located basilar artery and artificial bleeding is not expected to induce lateralized CVS. Unilateral CCAO is known to result in a moderate symmetrical reduction of CBF decrease of both cerebral hemispheres without asymmetrical perfusion in animal models of CCAO without SAH [6]. Accordingly, the surviving rats are expected to exhibit bilateral brain tissue damage especially in areas with low CBF and vulnerability for hypoxia.

#### Clinical importance

CVS arising after aneurysmal SAH contributes significantly to morbidity and mortality after SAH, leading to brain tissue damage of differing severity [13, 16, 44]. Besides global ischemia, vulnerable brain regions may be affected in humans as well [27]. In contrast to early brain injury after SAH, delayed changes due to ongoing pathophysiological processes are potentially amenable for treatment, e.g., by neuroprotective agents. Therefore the use of animal models simulating not only the acute phase after SAH—i.e., the perforation model—but the time course during CVS are important and have the potential to improve treatment and outcome.

#### Conclusion

The present investigation provides data that CCAO deteriorates the effects of CVS in the rat double-SAH model.

Therefore, our data suggest that the rat double-SAH model in combination with unilateral CCAO is suitable for pharmacological investigations evaluating both the improvement of CBF and neuroprotection during the development of delayed CVS after SAH.

**Conflicts of interest** None.

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