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CEREBRAL ANGIOGENESIS AFTER SUBARACHNOID HEMORRHAGE (SAH) AND ENDOTHELIN RECEPTOR BLOCKAGE WITH BQ-123 ANTAGONIST IN RATS

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The aim of the study was to determine the effect of chronic vasospasm after SAH on angiogenesis and the effect of endothelin-1, the main causative factor in vasospasm, on this process. Male Wistar rats, 220—250 g, were examined. Seven days after cannulation of the cisterna magna (CM), a 100 µl dose of non-heparinized blood was administered to induce SAH. Sham SAH (aSAH) was induced by intracisternal injection of 100 µl of artificial cerebrospinal fluid. Endothelin receptor antagonist BQ-123 in a dose of 40 nmol in 50 µl of cerebrospinal fluid was given three times: 20 min. before SAH and aSAH, 60 min and 24 hours after SAH and aSAH. The same pattern of BQ-123 administration was used in the nonSAH group. The brains were removed 48 hours later for histological evaluation. Vascular surface density was measured in cerebral hemisphere sections (at the level of the dorsal part of the hippocampus) and brain stem sections (1/2 of the pons). An increase in angiogenesis was observed after SAH, compared to control values. The administration of BQ-123, a specific endothelin receptor blocker inhibits angiogenesis in cerebral hemispheres after SAH.

Key words: *subarachnoid hemorrhage, endothelin-1, antagonist ET_A receptors — BQ-123, angiogenesis.*

INTRODUCTION

Subarachnoid hemorrhage (SAH) frequently leads to prolonged cerebral vasospasm resulting in vascular pathology due to endothelial cell ischemia and neuronal hypoxia (1). Endothelial damage is manifested by changes such as cellular edema, capillary occlusion and thickening of the muscle layer (2).

As demonstrated in numerous experimental and clinical studies, the spasmogenic activity in SAH is exerted by both the substances released from the thrombus and endothelin-1 (ET_1), a most potent vasoconstrictor produced by hypoxic endothelial cells (3, 4).

Our previous investigations showed a reversal of SAH-induced constriction of the cerebral basilar artery following the administration of BQ-123, a selective ET_A blocker (5). Other authors also reported an increase in ET_1 concentrations in the cerebrospinal fluid and blood of patients after SAH (6, 7). In many cases, plasma ET_1 levels closely correlated with the thrombus size and the magnitude of circulatory disturbances (3).

Both in hypoxia and brain tumor, the affected cells were found to produce increased amounts of vascular endothelial growth factor (VEGF), a major angiogenic factor and enhancer of vascular permeability (8—12).

Angiogenesis has so far been demonstrated in embryogenesis (13), diabetic retinopathy (14, 15), post-infarction myocardium (16), psoriasis (17), lower limb ischemia (18), endometriosis (19, 20), rheumatoid arthritis (21, 22) and, most of all, neoplasms (23, 24). There are no reports in literature concerning angiogenesis after SAH.

The aim of the present study was to determine whether SAH-induced vasospasm and endothelial hypoxia promote angiogenesis in the cerebral hemispheres and brainstem, and whether angiogenesis also occurs after reversing ET_1-induced vasospasm with BQ-123.

MATERIALS AND METHODS

Seventy five male Wistar rats weighing 220—250 g were used. The animals were housed 2 per cage under controlled conditions of lighting (light on from 6 a.m. to 6 p.m.), temperature (20—22°C) and humidity (50—60%), with free access to water and food (Murigran, Motycz). All experiments were performed between 2 p.m. and 5 p.m. on animals anesthetized with i.p. Ketamine ($100\ mg \times kg^{-1}$).

The animals were divided into 5 groups of 10 rats each:

1. control — no surgical intervention
2. nonSAH — cannulation only
3. aSAH — sham SAH by artificial cerebrospinal fluid
4. SAH — subarachnoid hemorrhage
5. SAH + BQ-123

Experiment

The cisterna magna (CM) was cannulated 7 days before SAH or aSAH to allow time for disappearance of any effects of the procedure (25, 26). A technique by Solomon et al., with own modification, was used (27). The procedure was carried out using an operating microscope and a stereotaxic apparatus. A midline parietoöccipital and nape incision was made to expose the parietal and occipital bones, the atlas arch and the apicooccipital membrane. A 0.8-mm hole was drilled at the parietooccipital suture level, through which a cannula (Venocath — 18, Abbot) was inserted into the cisterna magna (CM). Proper positioning of the end of the cannula in CM and free outflow of the cerebrospinal fluid through the cannula were checked.

Subarachnoid hemorrhage

The hemorrhage was induced by intracisternal administration of 100 µl nonheparinized blood drawn through a 0.6 mm Neoflon catheter from the axillary artery prepared in the operating microscope. Sham SAH (aSAH) was induced by intracisternal administration of 100 µl artificial CSF.

The BQ-123 antagonist (40 nmol in 50 µl CSF) was administered three times: 20 min before SAH and aSAH, 60 min after SAH and aSAH, and 24 h after SAH and aSAH (28). The same pattern of BQ-123 administration was used in the nonSAH group.

The results given are those from measurements made at 48 h after SAH, aSAH and nonSAH.

Upon completion of this phase of experiment, the animals were anesthetized with ketamine (100 mg/kg^{-1}, i.p.) and the brains were removed for histologic examination.

Brain removal

After thoracotomy and heart exposure, a catheter was inserted into the left ventricle and 100 ml phosphate-buffered saline (PBS), pH 7.4, was administered under 120 cm H_2O pressure, followed by 200 ml fixative fluid (1% glutaraldehyde and 4% formaldehyde in PBS). The vessels were thus washed and fixed, and the excess fluid was allowed to escape through an incision in the right atrium. Next, the skin in the cerebrocranial region was incised and the brain was removed and immersed in the fixative for 24 h at 4°C. After that time it was placed in cold PBS and subjected to histologic examination.

Histologic examination

Each brain was fixed in the fixative fluid (4% formaldehyde and 1% glutaraldehyde) for 12 h at 4°C and washed in cold PBS, pH 7.4. The brainstem and cerebellum were then dissected and the former was cut transversly at $^1/_2$ pons level. The cerebrum was processed through alcohols and xylens to paraffin (Paraplast X-tra, Sigma) in a histoprocessor. Paraffin sections, 5 µm thick, were cut and stained with cresyl violet.

Vascular density measurements were made using a computerized system of microscope image analysis, equipped with Kontron KS 400 software and connected to a Zeiss Axioplan 2 microscope. A 400 × magnification was used. In each preparation 25 visual fields from the dorsal hippocampal level of the cerebral hemispheres and from $^1/_2$ the pons level of the brainstem (abdominal part) were evaluated. For procedure of capillary counting a macro, working in KS 400 system was designed. The macro was based on the interactive algorithm, discriminating clear spaces, corresponding to cross sections of capillaries (blood cells were washed out during perfusion fixation), seen on the dark background of stained tissue. Spaces smaller than 2,25 µm were excluded from analysis, as they did not have visible endothelial cell nucleus and thus could be not a capillary lumena.

Surface vascular densities (vessels/mm^2), means and standard deviations were calculated.

Statistical analysis

For the statistical analysis nonparametric Wilcoxon test was used. Statistical significance was as $p<0.05$.

RESULTS

Angiogenesis in cerebral hemispheres

In control animals, mean vascular density was 441.2±177.6 (*Tab. 1*). Example of histological picture of rat cerebral vessels in the control group is presented in the *Fig.* 1_A.

Similar densities were observed in the nonSAH and aSAH groups, the values being 370.0±65.9 ($p = 0.4$) and 400.4±265.0 ($p = 0.7$) respectively (*Tab. 1*). After SAH induction, the density increased in relation to the control value, and was 840.2±233.9. The increase was statistically significant, $p = 0.001$ (*Tab. 1*). Example of histological picture of rat cerebral vessels in the SAH group is presented in the *Fig.* 1_B.

Administration of BQ-123 had significant influence on angiogenesis after SAH. In the nonSAH+BQ-123 group the vascular density was 576.4±191.1 ($p = 0.5$) (*Tab. 2*) while in the aSAH+BQ-123 group it was 600.2±279.0 ($p = 0.4$) (*Tab. 2*). In the SAH+BQ-123 group the density was 517.0±199.4, which was statistically significant, $p < 0.05$ (*Tab. 2*). Example of histological picture of rat cerebral vessels in the SAH+BQ-123 group is presented in the *Fig.* 1_C.

Table 1. Mean vascular densities (number of vessels/mm^2) in cerebral hemispheres after SAH in rats.

	Control	nonSAH	aSAH	SAH
Number of vessels/mm^2	441.2±177.6	370.0±65.9	400.4±265.0	840.2±233.9
nonSAH	not statistically significant			
aSAH	not statistically significant	not statistically significant		
SAH	$p<0.001$	$p<0.001$	$p<0.001$	

Table 2. Mean vascular densities (number of vessels/mm^2) in cerebral hemispheres after SAH and BQ-123 administration in rats.

Groups	Mean	p
nonSAH	370.0± 65.9	not statistically significant
nonSAH+BQ-123	576.4±191.1	
aSAH	400.4±265.0	not statistically significant
aSAH+BQ-123	600.2±279.0	
SAH	840.2±.233.9	$p<0.05$
SAH+BQ-123	517.0<199.4	

The nonSAH, aSAH and SAH groups serve as reference for BQ-123 — administered groups.

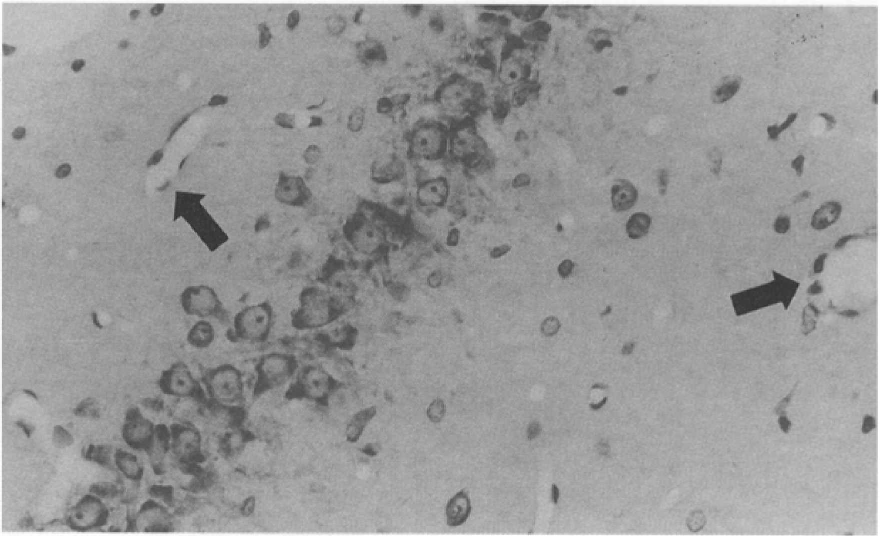

Fig. 1_A. Angiogenesis in cerebral hemispheres — control group. Rat hippocampal cortex. Scarcely distributed capillaries, including larger ones are visible (arrow). The mean vessel density in this specimen was 320 vessels/mm^2. Cresyl violet stain. Magnification 400×.

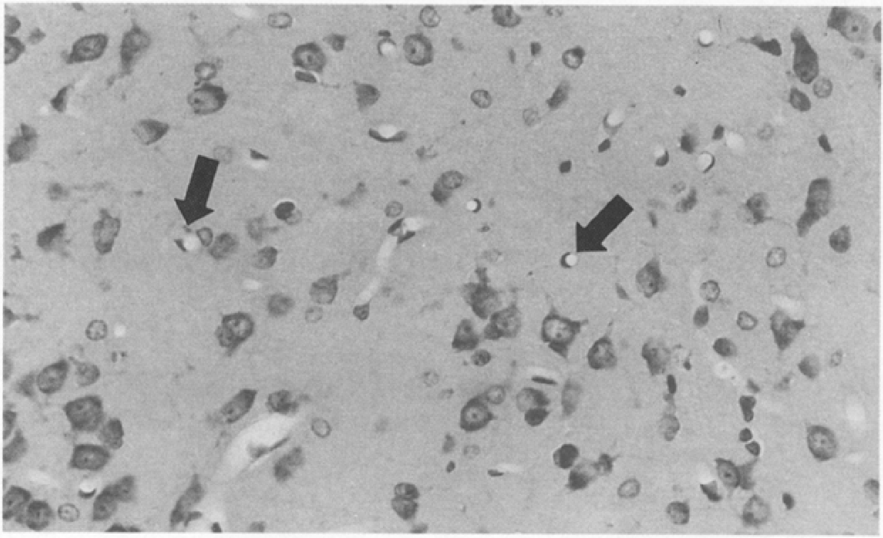

Fig. 1_B. Angiogenesis in cerebral hemispheres — SAH group. Rat hippocampal cortex. Numerous vessels predominantly small capillaries are visible (arrows). The mean vessel density in this specimen was 860 vessels/mm^2. Note empty vessel lumena, resulting from perfusion fixation. Cresyl violet stain. Magnification 400×.

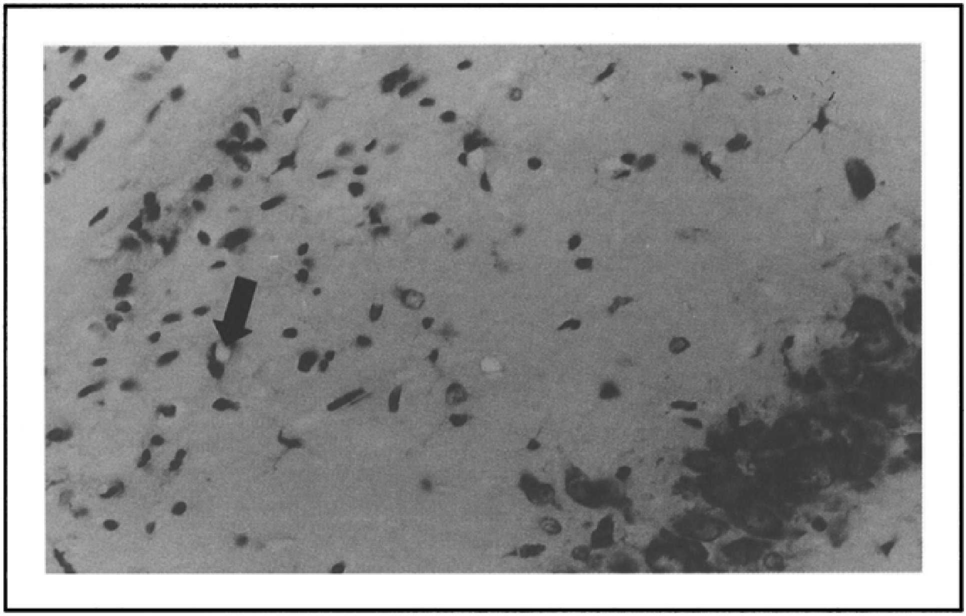

Fig. 1C. Angiogenesis in cerebral hemispheres after SAH and BQ-123 administration. Rat hippocampal cortex. Scarcelly distributed capillaries. The mean vessel density in this specimen was 263 vessels /mm^2. Cresyl violet stain. Magnification 400 ×.

Angiogenesis in brainstem

Example of histological picture of rat cerebral vessels in the control group is presented in the *Fig. 2A* and in the SAH group is presented in the *Fig. 2B*.

No statistically significant differences in vascular density were observed between control and experimental groups. The values obtained in particular groups were : control — 443.0 ± 170.4, nonSAH — 539.5 ± 373.4 ($p = 0.4$), aSAH — 611.4 ± 141.7 ($p = 0.3$), and SAH — 624.6 ± 211.8 ($p = 0.1$) (*Tab. 3*). After BQ-123 administration, the densities in particular groups did not change significantly and were: nonSAH + BQ-123 — 521.2 ± 179.6 ($p = 0.9$) (*Tab. 4*), aSAH + BQ-123 — 471.1 ± 209.9 ($p = 0.5$) (*Tab. 4*), SAH + BQ-123 — 503.7 ± 252.2 ($p = 0.2$) (*Tab. 4*).

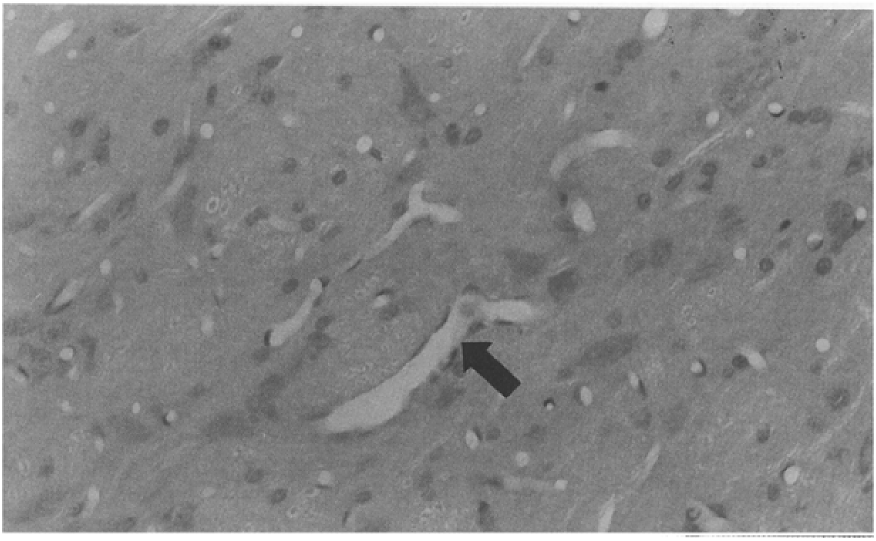

Fig. 2_A. Angiogenesis in brainstem — control group. Ventral surface of the pons. Scarcely distributed capillaries, including larger ones are visible (arrow). The mean vessel density in this specimen was 410 vessels/mm². HE stain. Magnification 400×.

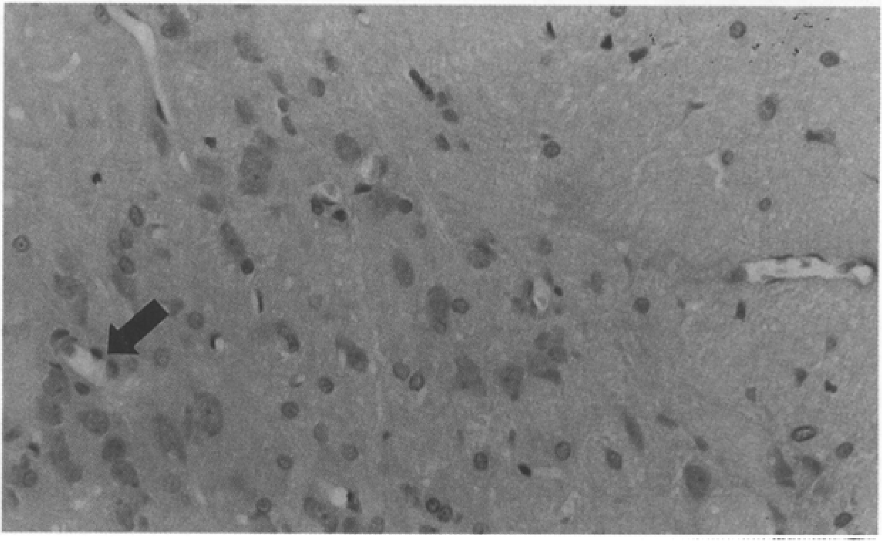

Fig. 2_B. Angiogenesis in brainstem — SAH group. Ventral surface of the pons. Scarcely distributed capillaries. The mean vessel density in this specimen was 350 vessels/mm². HE stain. Magnification 400×.

Table 3. Mean vascular densities (number of vessels/mm^2) in brainstem after SAH in rats.

	Control	nonSAH	aSAH	SAH
Number of vessels/mm^2	443.0±170.4	539.0±373.4	611.4±141.7	624.6±211.8
nonSAH	not statistically significant			
aSAH	not statistically significant	not statistically significant		
SAH	not statistically significant	not statistically significant	not statistically significally	

Table 4. Mean vascular densities (number of vessels/mm^2) in brainstem after SAH and BQ-123 administration in rats.

Groups	Mean	p
nonSAH	539.5±373.4	not statistically significant
nonSAH+BQ-123	521.2±179.6	
aSAH	611.4±141.7	not statistically significant
aSAH+BQ-123	471.1±209.9	
SAH	624.2±.211.8	not statistically significant
SAH+BQ-123	503.7±252.2	

The nonSAH, aSAH and SAH groups serve as reference for BQ-123 — administered groups.

DISCUSSION

The present study has demonstrated enhancement of angiogenesis in the cerebral hemispheres after SAH. The increase in vascular density at 48 h was nearly twofold. The administration of BQ-123, a specific endothelin ET_A receptor blocker, inhibits angiogenesis after SAH in cerebral hemispheres. No significant increase in the density of brainstem capillaries was observed. Studies by other authors have shown that cerebral circulation adapts to hypoxia which lasts more than 4 months in two ways, through angiogenesis and dilatation of microvessels. The reaction to hypoxia depends on the brain area and is not homogenous. In the cerebral cortex, striatum and hippocampus the number of vessels per 1 mm^2 tissue rises significantly, whereas in the cerebellum and medulla oblongata there is only a tendency to an increase (29). These differences, regarding reaction to hypoxia could be explained by special features of celebral circulation, namely its spatial distribution. Cerebral blood flow (CBF) is nearly 2-times higher in cortex in comparison with subcortical areas, it is still subjected to redistribution, depending on actual physiological requirements (30, 31). The cerebral cortex is more susceptible to hypoxia than brainstem. Furthermore, the ratio neuronal cell body/neuronal processes (dendrites and neurites) number is much more higher in cortex than in brainstem, thus explaining its vulnerability during hypoxia (31, 32). In subarachnoid hemorrhage, like in brain or spinal cord injury, acute vasospasm develops within several minutes and late vasospasm within 2 days. Usually after 24 hours 80% of the vessels are involved (33). It should be mentioned that prolonged vasospasm also affects large arteries such as the basilar artery whose diameter becomes 50% smaller (5).

Various factors are involved in the mechanism of chronic vasospasm, from neurogenic to local biochemical. Among them, endothelial factors play a major role, as shown by a long-lasting increase in ET_1 concentration which leads to vascular lumen occlusion and a secondary damage of ischemic endothelial cells. It seems that the products of erythrocyte oxyhemoglobin degradation and neurogenic vasoconstrictors, such as catecholamines and serotonin, do not play a significant role in prolonged vasospasm. Our previous study has clearly shown that the constriction of the cerebral basilar artery can be abolished by ET_A receptor blockade (5) or reduced by inhibition of endothelin converting enzyme (ECE) by phosphoramidone (34).

Occlusion of the vessels leads to vascular injury and ischemia involving endothelial cells as well as neurons, glia and other supporting cells (33). There is growing evidence that microvascular endothelial damage is of ultrastructural character, which may indicate degenerative changes particularly in the mitochondria. The damage is also due to excessive amounts of reactive oxygen forms generated in hypoxia (35). Morphologic examination reveals hypertrophy of the vascular middle layer and accumulation of leukocytes near the vessels (5).

The above findings suggest that endothelial hypoxia is also responsible for disturbed angiogenesis, which is manifested by an increase in the production of VEGF, a potent angiogenic factor, released in abundance from ischemic areas (36, 37). Endothelin-1 is also known to induce production of VEGF in human vessels (38). The expression of VEGF closely correlates with the expression of its receptors in the same hypoxic endothelial cells. Moreover, VEGF modulates the activity of its receptors, particularly Flk-1, which have high binding affinity for VEGF (39). The role of VEGF in cerebral angiogenesis has so far been confirmed mainly in tumors, mostly on the periphery of ischemic tissues, after disruption of the basal membrane and arterial wall, and migration of endothelial cells towards tumor cells (40, 41).

Our study has provided evidence of a markedly enhanced angiogenesis in rat cerebral hemispheres in SAH — induced vasospasm. In the brainstem, only a tendency to angiogenesis was observed, and although this area is anatomically closer to the hemorrhage site, its neurons are less sensitive to hypoxia than the cerebral hemisphere neurons.

Another important finding is that the administration of BQ-123, a selective ET_A blocker, inhibits angiogenesis in the cerebral hemispheres.

Protective role of increased angiogenesis in the compensatory mechanism in vasospasm-induced hypoxia cannot be fully confirmed without examining the behaviour and activity of nerve cells. The increase in the number of capillaries is the result of increased expression of angiogenic factors, VEGF in particular, which in their majority also cause an increase in endothelial permeability, with the resultant nerve cell edema (38). Enhanced angiogenesis after SAH is accompanied by an increase in VEGF expression in small and large cerebral arteries and by VEGF accumulation around endothelial cells (42).

Further investigations are necessary to determine whether cerebral angiogenesis after SAH, like angiogenesis in myocardial infarction, reduces the effects of hypoxia (16, 43), or whether it aggravates the condition by causing cerebral edema (44).

The present study demonstrates that, regardless of possible late effects of cerebral angiogenesis, it is possible to reduce vasospasm by endothelin receptor blockade, thus eliminating hypoxia and but not angiogenesis.

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