

# CORRELATION BETWEEN ENDOTHELIAL INJURY AND CEREBRAL VASOSPASM FOLLOWING A DOUBLE SUBARACHNOID HEMORRHAGE IN THE RAT

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## ABSTRACT

While a wide array of pathological changes occur in cerebral arteries following subarachnoid hemorrhage (SAH), the most consistent is endothelial damage. Since the endothelium normally modulates reflexes that influence vascular tone, any damage to it may represent a significant contributor to cerebral vasospasm following SAH.

This experimental study investigates the correlation between endothelial injury of rat basilar (BA) and middle cerebral arteries (MCA) and vasospasm following a double SAH.

Animals were divided into three groups of control (nonsurgical-noninjected), saline-injected, and blood-injected rats. Rats in the blood-injected group were injected with two 0.3 mL doses of autologous blood into the subarachnoid space at intervals of 72 hours. Rats were killed at different time intervals to study the time course of endothelial injury along with vasospasm following the second SAH with scanning electron microscopy and image analysis system, respectively. Cerebral arteries exposed to blood demonstrated severe pathological alterations during acute (30 min. to 2 hrs.) and chronic (48 hrs.) periods of time post second SAH. Concurrent with endothelial injury, there was widening of inter-endothelial tight junctions. Morphometric evaluation revealed severe arterial constriction starting at 30 min. ( $p < 0.01$ ) and again at 48 hrs. ( $p < 0.05$ ) post second SAH.

The correlation between the time course of ultrastructural alteration of endothelial cells with arterial constriction provides further morphological contribution to the major complication of SAH-cerebral vasospasm.

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## INTRODUCTION

Subarachnoid hemorrhage (SAH), a major public

health problem, usually occurs when an intracranial berry aneurysm ruptures and bleeds into the subarachnoid space. As blood enters the posterior cranial fossa and intracranial pressure increases, the patient experiences neck rigidity and vomiting, respectively.<sup>1</sup> Subarachnoid bleeding usually stops when the arterial extraluminal and intraluminal pressures are equalized.<sup>1,2</sup> The most complicated and disastrous outcome of SAH is cerebral ar-

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terial spasm, which is the leading cause of morbidity and mortality in patients who survive SAH.<sup>2,4</sup> In man, vasospasm is time-dependent, rarely occurs before the third day, and reaches its peak around seven to ten days after the bleed.<sup>1,5</sup> Despite extensive clinical and experimental investigations, the exact pathologic mechanism of cerebral vasospasm still remains obscure, which inhibits the development of more effective therapy.

Since Crompton's first necropsy description in 1964, a number of studies, both in human cerebral arteries at autopsy and upon a variety of laboratory animal species, demonstrated that the earliest arterial injury following SAH occurs in the endothelial layer.<sup>6,12</sup> Since endothelia not only occupy a strategic anatomical location between blood and the arterial wall, but also undertake several important functional roles to maintain blood circulation, any damage to them may contribute to the development of cerebral vasospasm. Therefore, this study was undertaken for the purpose of elucidating: 1) The time course of endothelial ultrastructural changes in BA as well as MCA sequentially from 30 min. to 10 days following two episodes of hemorrhage in rats; and 2) The correlation between the time course of endothelial changes with vasospasm.

### MATERIAL AND METHODS

A total of 23 male Sprague-Dawley rats (300-425 g) were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.). Then, the head and neck were shaved and wiped with alcohol. Mineral oil was dropped onto each eye to prevent drying, and lidocaine was injected into both ears before the head positioning in the stereotaxic apparatus (Kopf Instruments, Tujunga, CA) at a 45° angle. In addition, 0.1 mL atropine (60 µg, i.m.) was given to help control respiration. The animal breathed room air and body temperature was maintained close to 37°C.

#### Surgical procedure

Under aseptic conditions, a dorsal midline incision was performed from eye level extending caudally to the level of the fourth cervical vertebra. The subcutaneous fascia along with cervicocutularis, cervicoauricularis, superficialis, semispinalis capitis, and occipitalis muscles were all elevated to expose the atlas, the occipital bone, and the atlanto-occipital (OA) membrane. By using the operating OME microscope (Olympus Co., Japan), electrocautery along with sponge and avitene were used to control bleeding. Detachment of the OA was performed to expose the dura mater and the underlying arachnoid membrane.

A microhook was used to pierce through the dura to gain entry to the subarachnoid space. Exactly 5 mm of a

6 cm polyethylene tube (PE-10, Clay Adams Co., NJ), attached distally to a 27-gauge needle was inserted into the opening perpendicular to the lateral side of the medulla. Therefore, the tip of the injection tube was positioned near the distal end of BA. The tube was sutured subcutaneously to the external skull muscle. No surgical procedures were performed on the non-injected control rats.

#### Induction of SAH

Donor rats were anesthetized with sodium pentobarbital (50 mg/kg, IP) and their femoral arteries were isolated for blood withdrawal. Using a 27-gauge needle, 0.3 mL of unheparinized blood was withdrawn from the donor rat and injected through the free end of the calibrated tube into the subarachnoid space of the experimental rat over approximately 30 seconds. The free end of the tube was sealed off and reopened after 72 hours for the second blood injection to create a double-hemorrhage model of SAH. Rats were given a subcutaneous injection of 1.5 mg/kg butorphanol three times a day on the first postoperative day. The muscle layers were used to cover the tube and the wound was closed with the skin sutured around the injection tube.

Mock SAH was created by injection of 0.3 mL mock CSF (buffered saline, pH 7.2) into the subarachnoid space. The second saline injection was introduced 72 hours after the first one. The mortality rate for all the animals was under 10%.

#### Experimental protocol

For the study of endothelia and vasospasm, scanning electron microscopy and image analysis (morphometric techniques) were used, respectively. The animals were divided into three groups of control, mock SAH, and experimental SAH.

**Group I: Control.** 2 rats served as non-surgical non-injected controls. The rats were sacrificed after administration of anesthesia.

**Group II: Mock SAH.** 7 rats were injected with 0.3 mL of isotonic saline (pH 7.2) into the subarachnoid space 72 hours after the first saline injection. One rat was sacrificed at each of the second injection time intervals of 1/2, 2, 4, 24, 48, 72, and 240 hours.

**Group III: Experimental SAH.** 14 rats were subjected to SAH. Two blood injections of 0.3 mL each were performed at intervals of 72 hours to induce vasospasm. Two rats were sacrificed at each of the second injection time intervals of 1/2, 2, 4, 24, 48, 72, and 240 hours.

Statistical analysis was achieved using unpaired Student's t-test. A p-value of 0.05 and/or less was ac-

cepted as the level of significance.

### Perfusion and fixation

At sacrifice, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Perfusion-fixation was performed transcardially with 200 mL of 0.1 M phosphate buffer (pH 7.2, osmolarity 400-425), followed by 400 mL of 1% glutaraldehyde with 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The brains were placed in fresh primary fixative for at least 2 hours at room temperature prior to being stored in 0.1 M phosphate buffer at 5°C.

### Tissue preparation

The BA and both MCA's of each brain were removed using a dissecting microscope (Leica Wild M8). For this study, the portion of the BA which extends from its origin to where it terminates into the PCA was examined. The M1 segment of both right and left MCA's which extends from its origin to its division were removed for further examination.

**Scanning electron microscopy procedure:** Segments of BA and MCA's were cut in half longitudinally under the Olympus IMT-2 inverted microscope (Olympus Corp, Cherry Hill, NJ) to expose the luminal surface. They were rinsed in 0.1 M phosphate buffer, and post-fixed in 1% aqueous osmium tetroxide in 0.1 phosphate buffer (pH 7.2) for 2 hours. After several washes in deionized water, dehydration in a graded series of acetones was performed. After two changes, 60 minutes each, in 100% peldri (oven 35°C), the arterial segments were left in cool peldri for another 15 minutes. They were finally dried in a desiccator vacuum apparatus for 12 minutes. The arterial segments were mounted on stubs and grounded with silver paint. They were coated with 700 Angstroms of gold (Au) for 6 minutes using an E6100 vacuum evaporator sputtering system. They were examined with a JSM-T300 SEM (JEOL USA) at an accelerating voltage of 10 or 15 KV.

**Image analysis system:** Morphometric analysis was performed on 4 microscopic fields for each blood vessel. Light microscopy sections were projected as digitized video images using image analysis (video interactive image analysis system, Carl Zeiss, Germany). By computer-aided tracing of the circumference of the digitized image of the vessel, planimetric measurements were made for the diameter of the BA and MCA's. The measurements were made from 5 consecutive arterial sections of both control and SAH rats.

## RESULTS

The light and electron micrographs are presented in

**Table I.** Diameter changes ( $\mu\text{m}$ ) in basilar (BA) and middle cerebral (MCA) arteries at different time intervals after subarachnoid blood injections.

Time (hrs.)	BA	MCA
Control	366 $\pm$ 23	332 $\pm$ 12
1/2	202 $\pm$ 21**	208 $\pm$ 19**
2	209 $\pm$ 09**	227 $\pm$ 13**
4	241 $\pm$ 09	253 $\pm$ 18
24	298 $\pm$ 27	277 $\pm$ 16
48	272 $\pm$ 19*	253 $\pm$ 16*
72	294 $\pm$ 21	278 $\pm$ 18
240	367 $\pm$ 23	320 $\pm$ 18

Values are means  $\pm$ SEM, n= 5.

\* $p$ <0.05; \*\* $p$ <0.01; other values not significant.

Figures 1-8. The results are summarized in Table I.

### General and gross examination

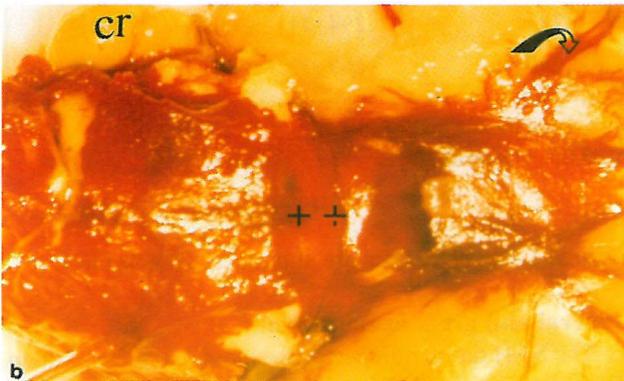
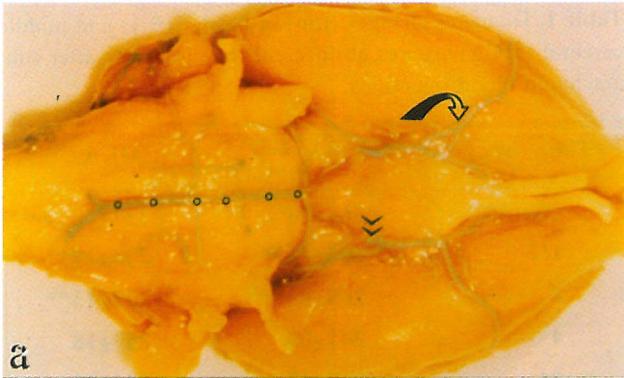
The SAH rats developed drowsiness, but none of them showed any noticeable paralysis. The saline-injected rats, on the other hand, were normal and continued their regular activities. The basal vessels of groups I and II showed no evidence of swelling or hemorrhage (Figure 1a). In group III, on the other hand, extensive clot accumulation was observed over the ventral surface of the brains from 1/2 to 4 hours post second hemorrhage. The clot accumulation usually extended from the medulla to the superior border of the pons in contact with BA. Smaller clots were scattered in areas around the origin of MCA's (Figures 1b). In rats killed at 24, 48, and 72 hours, the clots were partly absorbed and remained predominantly on the rostral brain stem. By 10 days, the hemorrhage was resolved with no sign of clot accumulation.

### Morphological studies

**Group I (Control):** The diameter of the BA was 366 $\pm$ 23  $\mu\text{m}$  (n= 5), and of the MCA 332 $\pm$ 12  $\mu\text{m}$ , n= 5 (Table I). The endothelium consisted of a single layer of polygonal and elongated cells with their long axes oriented in the direction of blood flow. The endothelia with ovoid protruding nuclei were tightly attached to each other with their marginal folds situated around the border of the cells. Finger-like microvilli projections were randomly scattered over the luminal surface (Figure 2).

**Group II (Saline-injected):** The arterial surface was indistinguishable from the control group (Figure 3). There were also no areas devoid of endothelial cells. The arte-

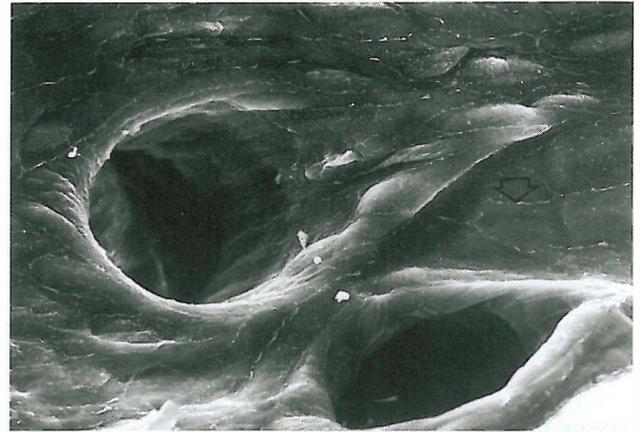
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**Fig. 1.** Whole brains from control (a) and blood-injected rat (b). **a)** Ventral aspect of the control rat brain. Open circles and curved arrow mark the section of the BA and MCA's, respectively. Internal carotid artery (arrowheads). **b)** Ventral aspect of the brain from rat killed 30 min after second hemorrhage. Note large blood clot along the lateral and ventral aspect of the medulla and pons. General position of BA is indicated by ++. Curved arrow indicates the position of left MCA. Cerebellum (cr).

rial diameters were slightly increased, but insignificant when compared with control values; the BA diameter was  $381 \pm 17 \mu\text{m}$  ( $n=5$ ), and MCA diameter  $344 \pm 14 \mu\text{m}$  ( $n=5$ ).

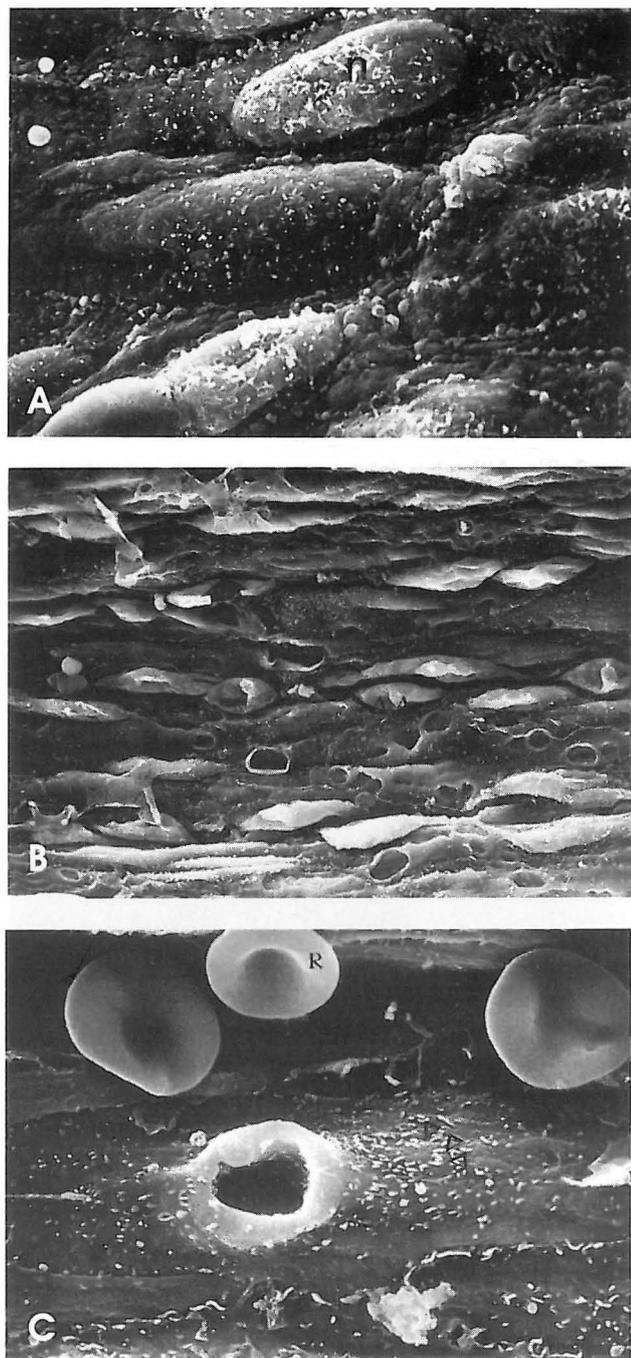
**Group III (Blood-injected):** Arterial diameter was observed to be significantly reduced ( $p < 0.01$ ) at 1/2 hour after the second blood-injection by an average of 45% (BA) and 37% (MCA), as compared with control values. It was also reduced again ( $p < 0.05$ ) at 48 hours by an average of 23% (BA) and 20% (MCA) (Table I). By 30 min. following double hemorrhage, the arterial surface showed prominent structural damage. A spiral configuration of the endothelial cells along with severe folding and corrugation was revealed in arterial segments. The endothelia were pulled toward each other and their nuclei protruded into the lumen. In addition, numerous



**Fig. 2.** Luminal surface of BA from control rat. Normal endothelia with distinct cell margins (arrow) are apparent. The orifices of arterial branches are also clear of thrombus.  $\times 1,000$ .



**Fig. 3.** Cerebral arteries from saline injected rats. **a)** A layer of endothelial cells lining the lumen of BA, joined by intact cell margins.  $\times 5,600$ . **b)** Intact luminal lining of MCA.  $\times 3,000$ .



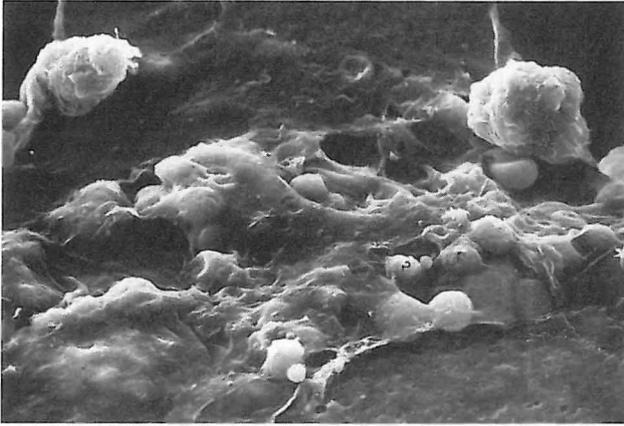
**Fig. 4.** Cerebral arteries of rats killed 30 min after second SAH. **A)** The irregular surface of the luminal side of MCA. Endothelial nuclei (n) are severely invaginated  $\times 3,200$ . **B)** A crater on the endothelial surface of MCA. Compare the size and shape of erythrocytes (R) with that of the crater. Microvilli (arrowheads).  $\times 3,660$ . **C)** Spiral configuration and corrugation of endothelia of BA. Endothelial balloons (arrowheads) are scattered over the luminal surface. Inter-endothelial tight junctional disruption (arrow).  $\times 2,500$ .

outpouchings or luminal balloons were seen protruding into the lumen (Figure 4a). These balloon-like protrusions were present in some areas, while adjoining areas appeared normal. The presence of holes or "craters" which varied considerably in size, shape, and distribution were seen on the luminal surface (Figure 4b). Irregular and thickened marginal folds at the border of the endothelial cells were another feature of the arterial lumen at this stage. Another alteration was the disruption of the inter-endothelial tight junctions with a consequent breakdown of the blood-arterial wall barrier (Figure 4c). At 2 and 4 hours, the number of luminal craters was significantly increased when compared to specimens from 30 min. In addition, endothelial cell detachment and necrosis, resulting in exposure of the cell nucleus and the underlying subendothelium, was apparent in most sections. This type of endothelial injury appeared to have attracted blood cells and fibrin (Figure 5). By 24 hours, the intima showed moderate corrugation with occasional presence of endothelial craters. The corrugated intima showed no significant accumulation of blood elements (Figure 6). After 48 hours, severe endothelial corrugation and detachment was evident again. The endothelial cells were elevated with their nuclei severely protruding (Figure 7). By 72 hours, occasional endothelial necrosis with degenerative changes were found. However, the intima returned to normal, and there was no sign of crater formation 10 days post second hemorrhage (Figure 8).

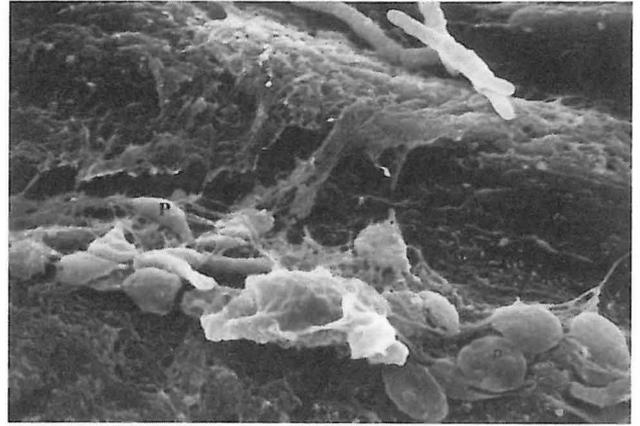
## DISCUSSION

In this study, rats were used for the induction of SAH which offered several advantages including: 1) no respirator was needed and the survival rate was relatively high; 2) the entire surgical procedure could be mastered within a short period of time; 3) by stabilizing the injection tube, two injections was performed in rats; 4) the rats were easy to handle, easily accessible, and cheaper than other species commonly used for the study of SAH. In addition, as Tamura et al. (1981) pointed out, the anatomy of the rat cerebral arteries are similar to that in humans—each hemisphere.<sup>13</sup> To induce delayed-vasospasm and examine the time course of endothelial changes, the new method of SAH, namely "double hemorrhage model" was applied. Since it is well documented in humans that a direct correlation exists between the severity of arterial spasm and the amount of blood in the subarachnoid space,<sup>8,10</sup> it became necessary for 2 episodes of blood-injections to develop delayed vasculopathy. Cain in 1989 induced SAH in rats by single cisternal-injections of 0.3 mL of blood, and killed the animals at different time intervals—up to 72 hours post-hemorrhage.<sup>9</sup> She, however, observed pathological changes only during the early periods—up to 4 hours. The vascular alterations were re-

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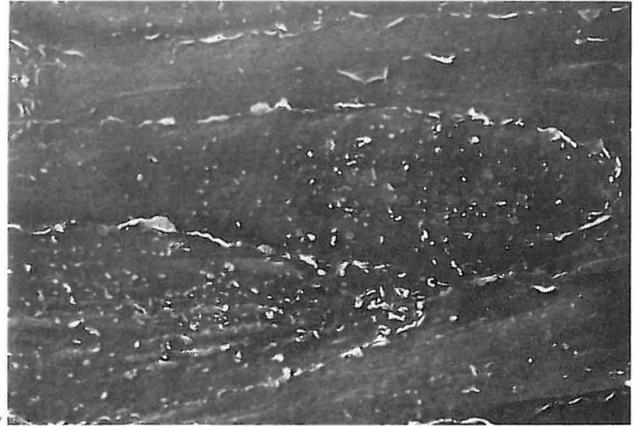
**Fig. 5.** MCA from rat killed 2 hrs. after SAH. Necrotic endothelia attracted platelets (p) with fibrin and thrombus formation (t). Note severe irregularity of the luminal surface.  $\times 5,000$ .



**Fig. 7.** Blood platelets (p) are attracted to injured endothelial cells of MCA 48h post SAH. The activated and aggregated platelets are interconnected by their pseudopods. Also, note the severe irregularity of the luminal surface.  $\times 5,000$ .



**Fig. 6.** Clear, but corrugated endothelial surface is apparent in 24h post second hemorrhage. Note increased number of luminal microvilli (arrowheads).  $\times 2,600$ .



**Fig. 8.** Normal appearing endothelia with distinct cell margins of BA 10 days after SAH.  $\times 5,000$ .

versed by 24 hours. Therefore, it seemed that multiple episodes of hemorrhage would be necessary to produce results that “semi-mimic” the human condition. Mecklenburg et al. (1990) also applied a triple-blood injection model of SAH in dogs to produce spastic arterial walls.<sup>14</sup> Despite their findings, delayed vasospasm with pathological consequences similar to that of man has rarely been successfully produced in small animal models such as rats.

Only the BA and MCA's were studied, since these not only were in direct contact with blood, but also supply vital brain areas such as the cerebellum (basilar) and the primary motor and sensory areas (middle cerebral). In addition, their size is large and always susceptible to rupture. It is also well-documented that only large cere-

bral arteries undergo spasm after SAH insult, while small pial vessels remain intact. In another study, it was noticed that all the BA after SAH showed significant constriction (reduced to 46%).<sup>15</sup> On the other hand, lack of constriction in pial arteries was noted in rabbits, even though two blood-injections were introduced into the cisterna magna. In the present experiment, the degree of arterial spasm was in direct correlation with endothelial injury. A biphasic vasospasm was confirmed as spasm was observed 30 min. after the bleed (acute phase), and again at 48 hours (chronic phase). After 30 min. post-SAH, all rats showed marked spasm with 45% and 37% decrease in the diameters of the BA and MCA's, respectively. Also, about 23% and 20% reduction was observed in BA and MCA 48 hours post second SAH. This bi-

phasic phenomenon which has previously been demonstrated in dogs<sup>16</sup> as well as primates<sup>15</sup> possesses two distinctive characteristics of human SAH induced vasospasm, and its correlation to the amount of subarachnoid blood as well as endothelial injury.

In the Macaque monkey, Peerless and associates (1982) also produced a biphasic vasospasm which was observed after 1 hour and again 1 week post-hemorrhage.<sup>15</sup> Delgado et al. (1985) reported the first biphasic pattern of spasm in rats, with an acute phase at 10 minutes (maximal spasm of 40%), and a late phase at 48 hours (maximal spasm of 27%) post-SAH.<sup>16</sup> In contrast, an earlier study by Barry and colleagues (1979) documented only an immediate onset of spasm in BA's of rats, which steadily recovered to normal by day 3 and followed by vasodilatation from day 5 onward.<sup>17</sup> The shortcoming of their investigation was the use of direct arterial puncture without knowing the amount of blood released into the subarachnoid space. Our results, which demonstrated pronounced spasm may be explained by the method of SAH induction (blood-injection versus arterial-puncture), and also by the amount of blood which was twice the amount used in most of the previously described rat models of SAH. Therefore, delayed spasm is a reproducible consequence of SAH in rats by either massive hemorrhage or multiple subarachnoid blood-injections.

The time-course of the development of the pathological changes affecting the cerebral arteries of the rat after SAH has not been previously reported. In the present study, however, the arterial lumen was studied at different time periods (30 min. to 10 days) following the second blood or saline injections. In particular, endothelial cells were studied with electron microscopy, since in both man and experimental animals, the earliest and most consistent vascular injury after SAH appears to occur in the endothelia which may contribute to vasospasm.<sup>7,8</sup> Upon induction of SAH, pathological changes occurred within the endothelia during acute (1/2 to 4 hours) and chronic (48 to 72 hours) stages of vasospasm. The most common changes during the acute period included endothelial corrugation and necrosis, intercellular disruption of tight-junctions, and cellular attraction to injured-endothelia. The results agree with those reported by Cain (1989) and Clower et al. (1988) who studied the effect of SAH upon endothelial cells during the acute phase.<sup>9</sup> During the chronic stage, similar endothelial damage as found in the acute period was seen, e.g., endothelial detachment and platelet attachment were some of the findings. Similar electron microscopic changes have also been described by other investigators in humans,<sup>18</sup> in monkeys,<sup>14,19</sup> in pigs,<sup>20</sup> in cats,<sup>7</sup> and in rats.<sup>7,8-11,12</sup> However, Pickard et al. (1985) did not observe any changes in the cerebral arteries of dogs.<sup>22</sup>

The events triggering endothelial damage still remain a matter of discussion. Although endothelial damage which almost always occurs following SAH is thought to be one of the main factors in the pathogenesis of vasospasm,<sup>23</sup> it is assumed that vasoactive substances released during blood clot lysis are responsible for the endothelial damage. One likely candidate, oxyhemoglobin, is known to be released after erythrocyte breakdown. Among the actions of oxyhemoglobin which contribute to endothelial injury as investigated are the initiation of lipid peroxidation and the release of scavenger free radicals.<sup>23,24</sup>

Another important patho-morphological finding, the disruption of inter-endothelial tight junctions, was also observed 30 min. after hemorrhage. This agrees with the SAH experiment on rats by Sasaki et al. (1986).<sup>25</sup> In addition, Zuccarello et al. (1987) observed the ultrastructural disruption of endothelial tight-junctions in cerebral arteries of rats 30 min, and 72 hours post-hemorrhage.<sup>26</sup> They proposed three pathogenic factors responsible for the barrier-damage: 1) a sudden increase in intracranial pressure, 2) acute arterial hypertension, and 3) effect of subarachnoid clots around the arteries. Later, Nakagomi and co-workers (1989) demonstrated that disruption of the blood-arterial wall barrier following SAH in rabbits may last up to 3 weeks.<sup>27</sup> Breakdown of this barrier would allow penetration of blood cell contents into the vessel wall to stimulate both smooth muscle and fibroblast proliferation. Furthermore, vasoactive substances such as prostaglandins, catecholamines, and serotonin, may also penetrate the smooth muscle vasodilator prostacyclin.<sup>25,27</sup> Under normal conditions, as Vanhoutte and Houston (1985) pointed out, the endothelial cells play three major functions.<sup>28</sup> 1) Physical and metabolic barrier properties; 2) Regulation of vascular tone; and 3) Anti-thrombogenic properties. However, following endothelial injury, prostacyclin production is reduced, thereby allowing platelets to adhere to the injured endothelia and release their  $\alpha$ -granule contents (platelet derived growth factor-PDGF) which stimulates smooth muscle cells.<sup>7</sup>

In this study, the abnormal presence of plasmalemmal pits, balloons, and crater defects were demonstrated on the injured endothelia. These pathological changes were reported by other investigators; though, the precise mechanism is still obscure. Hughes (1987) observed the presence of plasmalemmal pits, blebs, and craters on the arterial lumen of experimental SAH in baboons.<sup>19</sup> In addition, in the current experiments, blood cells adhered to the luminal surface starting from 2 up to 48 hours post-SAH. It seemed that the degree of blood-cell accumulation was directly related to the severity of the endothelial damage. The possible role of plasmalemmal pits in the development of endothelial degeneration was pointed out by Hazama et al. (1979) who also observed similar

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changes in cerebral arteries of hypertensive rats.<sup>21</sup> These changes may participate in increasing the permeability of the arterial intima as well as the adhesion of blood elements upon the arterial lumen.<sup>7,8,21</sup> In addition, blood platelets contain serotonin which is a vasoconstrictor. Injured endothelial cells facilitate the penetration of serotonin into the vascular wall.<sup>28</sup> Therefore, endothelial injury which generally occurs after SAH may contribute to the pathogenesis of vasospasm.

The remarkable ability of injured endothelium to repair itself was observed by 10 days following SAH. As Cain (1989) and Clower et al. (1994) reported, the pathological changes in cerebral arteries of experimental SAH were also reversed within 1 day.<sup>8,9</sup> Clower et al. (1988) noticed endothelial repair by 48 hours post-SAH in cerebral arteries of cats.<sup>7</sup> The endothelial regeneration after injury in the aorta of rats was found to increase 10% over normal.<sup>9</sup> The speed of regeneration is directly related to the extent and severity of injury.

In summary, the time course of biphasic vasospasm following SAH along with endothelial pathomorphology may indicate the unique role of endothelial injury in the pathogenesis of vasospasm. However, it is emphasized that the belief in a singular vasospasm-causing factor is not popular, and the pathogenesis is considered to be a complicated and multifactorial process.

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