Acta Neurochir (Wien) (2000) 142: 575–581

Acta Neurochirurgica
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Printed in Austria

Time-Course of Blood-Brain Barrier Permeability Changes After Experimental Subarachnoid Haemorrhage

A. Germanò[1], **D. d'Avella**[1], **C. Imperatore**[2], **G. Caruso**[1], and **F. Tomasello**[1]

[1] Neurosurgical Clinic, University of Messina School of Medicine, Messina, Italy
[2] Institute of Pharmacology – University of Messina School of Medicine, Messina, Italy

Summary

An increase in blood-brain barrier (BBB) permeability after subarachnoid haemorrhage (SAH) has been described in humans and has been correlated with delayed cerebral ischemia and poor clinical outcome. Few studies examined in the laboratory the relationship between SAH and BBB, with contrasting results due to limitations in experimental probes adopted and in timing of observation. The aim of this study was to quantify the time-course of BBB changes after experimental SAH. Groups of eight rats received injections of 400 µl of autologous arterial blood into the cisterna magna. BBB was assessed 6, 12, 24, 36, 48, 60, and 72 hours after SAH and in sham-operated animals separately for cerebral cortex, i.e. frontal, temporal, parietal, occipital, subcortical gray matter (Caudate-Putamen-Thalamus), cerebellar cortex and nuclei, and brain stem by a spectrophotofluorimetric evaluation of Evans Blue dye extravasation. As compared to sham-operated controls, SAH determined a significant BBB permeability change beginning 36 hours after SAH, peaking at 48 hours, and normalizing on day 3. This study provides a quantitative description of the temporal progression and recovery of BBB dysfunction after SAH. These results have implications for the management of aneurysm patients and for assessing the rationale and the therapeutic window of new pharmacological approaches.

Keywords: Blood-brain barrier; evans blue; rat; subarachnoid haemorrhage.

Introduction

Considerable clinical [1, 8, 14, 15, 22, 23] and experimental [2–5, 12, 16, 18, 19] studies have demonstrated that since its acute stage aneurysmal subarachnoid haemorrhage (SAH) induces focal and generalized disturbances of several brain functions. A significant increase in the permeability of the blood-brain barrier (BBB) after SAH has been described both in humans [8] and in different experimental settings [4, 6, 9–12, 16, 18–21, 26, 27, 30–32, 34, 36, 40] and has been correlated with the development of delayed cerebral ischemia (DCI) and with a poor clinical outcome [4, 8, 9, 12–16, 18]. The disturbance in the BBB function develops since the acute stage of SAH [8, 9]. This phenomenon is independent of raised intracranial pressure (ICP), hypertension, brain oedema and cerebral swelling, reduced cerebral blood flow (CBF), and disrupted brain metabolism, which, also, in turn, may disrupt the BBB itself [2–5, 8, 9, 11–13, 16–21, 26, 27, 30–34, 36, 40].

There are only relatively few laboratory studies examining the relationship between SAH and changes in BBB functions. Results of these investigations have not been uniform, with BBB permeability reported to be decreased [6, 26, 27], increased [4, 8–10, 12, 16, 18, 20] or unchanged [35]. Discrepancies in these findings may result from limitations inherent in the experimental probes adopted to assess the BBB status or the timing chosen for observations. Most importantly there is a lack of detailed quantitative data as related to the time-course of the BBB dysfunction after SAH and to the eventual reversibility of these permeability changes.

The present study was designed to assess the time-course of BBB permeability changes induced by experimental SAH. To this purpose we utilized an experimental model of SAH in the rat developed in our laboratory, which has already provided extensive information about changes in hemodynamic [2–5, 16, 18], angiographic and biochemical [2, 16], pathophysiological and acute and chronic behavioural parameters [13, 16, 18], paralleling those seen in humans after SAH [14, 15].

Material and Methods

Experimental Design and Induction of SAH

Studies were conducted using 64 male Albino Sprague-Dawley rats (Charles River Italia SpA, Como, Italy; Crl: CD (SD) BR), weighting approximately 250 g. Animals were housed at a constant temperature of 22 °C (four per 580 × 385 × 200-mm cage), under a 12h-hour light/dark cycle (light switched on at 6 am), with free access to food and water. The procedures used in this study were based on the guidelines of the ethical committee on the care and use of laboratory animals at our institution.

All surgical procedures were performed in these animals after induction of ketalar anesthesia (Ketamine, Parke-Davis Italia SpA, Milan, Italy) (150 mg/kg in a volume of 3 ml/kg) via the intraperitoneal route.

SAH was induced in 56 rats by injection of blood into the subarachnoid space via the cisterna magna. Details of the procedure have been published previously [2–5, 12, 13, 16, 18]. Briefly, the atlantooccipital membrane was exposed through a midline occipital incision. For the simulated SAH, 400 μl of autologous arterial nonheparinized blood was injected into the cisterna magna over a period of approximately 30 seconds via a 30-gauge needle fitted to a 500-μl Hamilton syringe. Sham-operated group consisted of 8 rats in which the atlantooccipital membrane was exposed through a midline occipital incision and punctured as described previously. No intracisternal injection was given, because in previous studies performed in which this rodent model was used, we demonstrated that in mock cerebrospinal fluid-injected animals there was no appreciable change in BBB assessment as compared with uninjected control rats [4, 12].

Blood-Brain Barrier Evaluation Protocol

The BBB assessment protocol was conducted on eight animals per group 6, 12, 24, 36, 48, 60, and 72 hours after the SAH or sham procedures.

The jugular vein was exposed through a midline linear 2-cm ventral neck incision, and a PE50 catheter (Clay Adams, Parsippany, NJ) filled with saline was inserted and exteriorized at the nape of the neck. The wounds were then infiltrated with bupivacaine (0.25 mg/kg/500 μl, Marcaina 0.5%, Pierrel SpA, Capua, Italy), and sutured.

The method we used involved the quantitative evaluation of the vascular permeation of Evans Blue by means of a fluorescence spectrophotometer technique, according to the measurement protocol of Uyama, *et al.* [37], and the extraction technique of Rössner and Tempel [29] and already employed in our laboratory [16, 18]. Briefly, 2% Evans Blue in saline (5 ml/kg) was administered intravenously through the cannulated jugular vein as blood-brain permeability tracer and was allowed to circulation for 60 minutes. To remove the intravascularly localized dye, the rats' chests were opened and the animals were perfused with saline through the left ventricle at a pressure of 110 mm Hg until colorless perfusion fluid was obtained from the right atrium. The whole brain was removed, and measurement of vascular permeability was made by comparing its weight with preweighed loci in the frontal, temporal, parietal, occipital, and cerebellar cortices, and subcortical and cerebellar gray matter and brain stem nuclei, as defined by the Paxinos and Watson's atlas [25]. Each brain area was homogenized in 1 ml of 50% trichloroacetic acid (weight/volume), and centrifuged (10,000 rpm, 20 minutes). One milliliter of the supernatant was added to 1.5 ml of the solvent (50% trichloroacetic acid/ethanol, 1 : 3). A fluorescence detector (model FP-920, Jasco Corp., Tokyo, Japan) was used at an excitation wavelength of 620 nm (bandwidth 10 nm) and an emission wavelength of 680 nm (bandwidth 10 nm). Calculations were based on external standards in the solvent (10–500 ng/ml). Data are expressed as mean ± SD (in micrograms) of extravasated Evans Blue dye per gram of tissue.

Statistical Analysis

Data were statistically analyzed using a one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test. Differences were accepted as being significant at the probability of less than 0.05.

Results

General observations and systemic physiological evaluations concerning this model (including mean arterial blood pressure, arterial blood gas levels, blood pH, plasma glucose levels, and body temperature as monitored throughout the experimental procedure) have been described in detail elsewhere [2–5, 12, 13, 16, 18]. Briefly, rats tolerated the procedure well and no signs of acute neurological dysfunction were noted. After SAH, a blood clot was clearly identifiable in the cisterna magna and in the basal cisterns in the animals sacrificed between 6 and 48 hours. No extradural haemorrhages were found, whereas the presence of blood in the ventricles was demonstrated in 25% of cases at 48 hours after SAH.

Table 1 summarizes the mean concentration ± SD of extravasated Evans Blue dye expressed as micrograms per gram of brain tissue for all loci examined in the eight experimental groups. In sham-operated rats, baseline levels of Evans Blue, ranged from 1.959 ± 0.32 to 4.182 ± 0.46. Values of extravasated Evans Blue dye, obtained from various brain loci in control and SAH animals were consistent with those described in previous experiments performed in our laboratory [16, 18]. In SAH rats Evans Blue dye extravasation was significantly increased ($p < 0.01$) as compared with sham-operated animals in the frontal, temporal, occipital cortices, cerebellar nuclei, and brain stem, and ($p < 0.05$) in the parietal and cerebellar cortices, and subcortical gray matter at 36 hours examination. At 48 hours post-SAH evaluation Evans Blue dye extravasation was significantly increased ($p < 0.01$) in the frontal, temporal, parietal, occipital, and cerebellar cortices, and in subcortical gray matter, cerebellar and brain stem nuclei. Conversely, 6, 12, 24, 60, and 72 hours examinations did not evidence a significant difference in Evans Blue dye as compared with sham-operated animals.

Table 1. *Changes in BBB Permeability as Evidenced by Extravasated Evans Blue Dye*

	Evans Blue Dye (μg/g tissue)							
Area	Sham	6 h	12 h	24 h	36 h	48 h	60 h	72 h
Frontal cortex	4.182 ± 0.46	4.556 ± 0.24	4.559 ± 0.35	4.840 ± 0.30	8.667 ± 0.42**	9.141 ± 1.69**	4.470 ± 0.14	4.410 ± 0.26
Temporal cortex	3.667 ± 0.40	3.421 ± 0.21	3.844 ± 0.15	4.215 ± 0.15	7.921 ± 1.65**	8.772 ± 1.73**	4.832 ± 0.33	4.213 ± 0.21
Parietal cortex	3.675 ± 0.39	3.569 ± 0.53	3.955 ± 0.24	4.220 ± 0.23	7.372 ± 1.14*	8.405 ± 0.06**	4.258 ± 0.41	4.263 ± 0.24
Occipital cortex	3.839 ± 0.29	3.743 ± 0.15	3.899 ± 0.11	3.967 ± 0.12	7.387 ± 1.09**	8.207 ± 0.27**	3.746 ± 0.24	3.646 ± 0.31
Subcortical GM	2.365 ± 0.43	2.292 ± 0.12	2.470 ± 0.14	2.563 ± 0.22	3.492 ± 0.90*	4.261 ± 0.11**	2.643 ± 0.31	2.482 ± 0.24
Cerebellar cortex	4.156 ± 0.36	4.240 ± 0.16	4.599 ± 0.38	4.241 ± 0.20	5.340 ± 0.77*	6.974 ± 0.28**	4.250 ± 0.26	4.291 ± 0.25
Cerebellar nuclei	1.959 ± 0.32	2.195 ± 0.28	2.084 ± 0.33	2.195 ± 0.34	3.204 ± 1.17**	3.772 ± 0.43**	2.262 ± 0.35	2.193 ± 0.32
Brain stem	3.009 ± 0.68	3.449 ± 0.22	3.389 ± 0.12	3.353 ± 0.13	5.342 ± 0.64**	6.047 ± 0.07**	3.257 ± 0.21	3.369 ± 0.43

The BBB permeability changes were measured in sham-operated animals and 6, 12, 24, 36, 48, 60, and 72 hours after SAH in groups of eight rats each. Values are expressed as means ± SD for 8 rats in each group.
CPT Caudate-putamen-thalamus; *GM* gray matter (caudate-putamen-thalamus).
$^{*}p < 0.05$; $^{**}p < 0.01$ SAH groups vs Sham group. Statistical analysis was carried out by one-way ANOVA followed by post-hoc Dunnett's test.

Discussion

The purpose of this investigation was to a) quantify the time-course of BBB permeability changes after experimental SAH and b) verify if these changes exhibit a reversible pattern over the observational period. Regional BBB permeability changes were analyzed separately for cerebral cortex, i.e. frontal, temporal, parietal, occipital, subcortical gray matter (Caudate-Putamen-Thalamus), cerebellar cortex and nuclei, and brain stem nuclei. As compared to sham-operated control animals, SAH determined a significant increase in Evans Blue dye extravasation beginning at 36 hours observation, peaking 48 hours after SAH, and significantly declined at later observations normalizing on day 3 after blood injection. These observations indicate that the post-SAH BBB breakdown is a self-repairable phenomenon and a functional recovery may ensue. This is the first study which provides a comprehensive quantitative description of the temporal progression and recovery of BBB dysfunction after SAH. These results possess relevant clinical implications for the management of SAH patients and for assessing the rationale and the optimal therapeutic windows of new pharmacological approaches.

The integrity of the BBB is essential for maintaining a constant environment for the nervous system. Numerous pathological conditions have been found to alter the BBB, including hypertension, infarct, seizures, trauma, and increase in ICP [26]. The disturbance in the BBB function develops from the acute stage of aneurysmal SAH onwards [8, 9]. A significant increase in the permeability of the BBB after SAH has been described both in humans [8] and in different experimental settings [4, 6, 9–12, 16, 18–21, 26, 27, 30–32, 34, 36, 40]. This phenomenon is independent of such factors as raised intracranial pressure (ICP), hypertension, brain oedema and cerebral swelling, reduced cerebral blood flow, and disrupted brain metabolism, which, also, in turn, may disrupt the BBB [2–5, 8, 9, 11–13, 16–21, 26, 27, 30–34, 36, 40]. In the clinical setting an impairment of the BBB was found in nearly two fifths of patients within 5 days of SAH, and the majority of these patients developed vasospasm and ischemic complications in the late phase of SAH and had a poor prognosis [8]. However, the state of the capillary system after experimental SAH remains controversial, and studies concerning post-SAH BBB alterations are relatively few. Results have been confounded by differences in experimental design, such as animal species, timing of experiments, probes of BBB function employed, and, most importantly, there is, surprisingly a lack of information with regard to the time-course of BBB alterations (Table 2). In addition, findings of these investigations have not been uniform with BBB permeability reported to be decreased [6, 26, 27], increased [4, 8–10, 12, 16, 18, 20] or unchanged [35].

A simple and inexpensive animal model of SAH was used in this study, which has already been extensively studied in our laboratory [2–5, 12, 13, 16, 18]. Using this model we described the spectrum and time course of changes of intracisternal thromboxane, prostaglandin$_2$, and prostaglandin$_{2\alpha}$ concentrations after SAH [2], the occurrence of angiographically confirmed arterial spasm in both the vertebrobasilar and internal carotid system [2], the induction of quantitative and qualitative marked regional alterations in

Table 2. *Review of Tracer Studies of the BBB After Experimental SAH*

Author, year (ref.)	Animal model of SAH	Tracer	Timing of observation	Pattern of BBB status
Trojanowski, 1982 (36)	rat, cortical	EB (L)	4 h	significant leakage
Peterson, 1983 (26)	cat, cisternal injection + hypertension	EB (L)	<1 h	preservation of BBB integrity
Peterson, 1983 (27)	cat, cisternal injection + $HgCl_2$ intracarotid	EB (L)	<1 h	preservation of BBB integrity vs damaging agents
Sasaki, 1985 (30)	dog, double cisterna magna injection	EB (L) HRP (S)	72 h	leakage, increased with time
Doczi, 1985 (8)	rat, cortical	EB (L) Sucrose (S)	0–48 h	leakage, increased with time
Doczi, 1986 (9)	normal and SHR rats, cortical	EB (L)	6 h	leakage, significant
Doczi, 1986 (10)	rat, intraventricular and cortical	EB (L)	0–48 h	leakage, significant, increased with time
Davis, 1986 (6)	rat, cisternal injection + Na dehydrocolate intracarotid	EB (L)	180 min	maintenance of BBB integrity vs damaging agents
Joshita, 1990 (20)	rabbit, single cisterna magna injection	HRP (S)	0–6 days	leakage, multifactorial in time course and location
Germanò, 1992 (12)	rat, single cisterna magna injection	AIB (S)	48 h	leakage, significant
d'Avella, 1994 (4)	rat, single cisterna magna injection	IgG (L) Albumin (L)	48 h	leakage, significant
Germanò, 1998 (16)	rat, single cisterna magna injection	EB (L)	48 h	leakage, significant
Imperatore, 1999 (18)	rat, single cisterna magna injection	EB (L)	48 h	leakage, significant

EB Evans Blue; *HRP* horseradish peroxidase; *SHR* spontaneous hypertensive rats; *L* large molecule; *S* small molecule.

BBB permeability [4, 12], the occurrence of a widespread depression of brain metabolism in the acute stage after SAH [5] together with the induction of lasting behavioural deficits over a 5-day observation period [13], and its usefulness to evaluate the efficacy of new pharmacological agents [3, 16, 18]. The specificity of extravasated blood for causing these pathophysiological changes was demonstrated [14, 15]. These findings substantiated previous observations reported by others that used the same investigational parameters and a similar experimental model [7, 8–10, 19, 20, 30–32, 34, 38, 40] with which they demonstrated the occurrence of pathophysiological changes that parallel those seen in humans after SAH [22, 23]. The results of these studies have led to the hypothesis that the blood itself and/or active substances derived in part from the degradation of the extravasated blood are centrally involved in the pathogenesis of these phenomena.

There is accumulating clinical and experimental evidence that free radical reactions, deriving from blood-breakdown products, play a pivotal role in the pathophysiology of secondary brain damage, particularly following SAH [1, 4, 12, 16, 18]. Clot lysis generates activated oxygen species [39] and vasoactive substances [40], which, in turn, directly act on brain microvessels [9, 12, 15], initiate lipid peroxidation phenomena and free radicals reaction cascades [2, 20, 30–32, 38] and induce macro- [2, 7, 17, 28] and microvascular changes [40]. Two models of barrier opening to water-soluble materials are known. For one model it is postulated that capillary wall deformation, caused by endothelial shrinkage and/or capillary vasodilatation, stretches and opens the interendothelial tight junctions. For the other model it is hypothesized that increased vesicular activity augments transfer of material blood and brain either by shuttling of microvesicles across the endothelium or by coalescing of vesicles to form continuous intracellular channels through the endothelium [11]. Recent experimental investigations consistently demonstrated that a marked capillary permeability increase occurs at the very acute stage after experimental SAH [28]. Doczi *et al.* [8–10] demonstrated that cortical SAH determined a marked quantitative Evans Blue dye extravasation in the ipsilateral hemisphere as early as 3 hours after SAH, with a maximum reached at 48 hours observation. More recently, Joshita *et al.* [20] presented evidences of a difference in time peak and regional localization of barrier disruption of the intraparenchymal microvessels located proximal or distal to cisternal SAH clots. In the present study the maxi-

mal degree of BBB disruption occurred between 36 and 48 hours after SAH, a time when, in this model, the blood clot is still clearly identifiable in the cisternal subarachnoid spaces. Interestingly, microvascular permeability returned to baseline values by 60 hours after SAH, paralleling the process of cisternal clot lysis which, in this model, is completed within 72 hours after SAH. In line with these observations, in previous experiments conducted in our laboratory employing this rodent model of experimental SAH, we have demonstrated that the maximal degree of angiographically confirmed arterial spasm in both the vertebrobasilar and internal carotid systems, the raised vasoactive eicosanoids cerebrospinal fluid levels, and the increase of Ki for [^{14}C]-α-aminoisobutyric acid transport across the BBB occurred on the 2nd day after SAH [4]. In addition, qualitative electron microscopy observations disclosed a functional response of the microvascular endothelium, occurring 48 hours after experimental SAH without opening of the tight junctions, and resulting in a conspicuous transport of endogenous proteins, albumin and IgG across the intact endothelia [12]. The widespread distribution of regions in which the capillary permeability was increased is evidence that this functional change does not represent a specific event related to focal vascular alterations but reflects the occurrence of a global brain dysfunction that diffusely involves the cerebral microvascular system. Authors of reports demonstrating alterations in BBB after SAH have proposed a number of possible additional pathomechanisms, including arterial hypertension, massive increases in ICP with a consequent decrease in cerebral perfusion pressure (CPP) and tissue ischemia [19], local increases in tissue pressure with microvascular compression [20]. Trojanowski found and early marked capillary permeability increase in cats suffering from SAH produced by puncturing the internal carotid artery. The greatest leakage was seen in animals with a high mean arterial blood pressure [36]. Jackowski *et al.* depicted the time-course of different mechanisms of pathology occurring after SAH, including blood pressure, arterial blood gases, ICP, CBF, mean arterial, CPP, and BBB [19]. It is unlikely, however, that acute increases in ICP or blood pressure alone could play a critical role in determining the observed BBB changes, because these changes were evident as late as 2 days after SAH and ICP changes have been reported to cause only transitory and self-limiting changes in CPP in this model [19].

There is still a need for precise understanding of basic mechanisms underlying the post-SAH global brain dysfunction, and pathophysiological and experimental data may provide significant clinical implications for the management of patients with SAH and for assessing the rationale of new pharmacological approaches. It should be emphasized that because of significant interspecies differences, a direct extrapolation to humans may be considered inappropriate. However, it could be speculated that the permeability changes in the BBB observed in the present study are possibly involved in the pathogenesis of the post-SAH cerebral dysfunction visible in humans.

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Comments

The authors report on the time course of blood brain barrier permeability changes after experimental subarachnoid haemorrhage. Experimental subarachnoid haemorrhage was induced in rats by single cisternal injection of 0.4 ml of autologous arterial blood. Maximum blood brain barrier disruption was observed at 48 hours. The changes normalised by day 3. The time course of the disruption is interesting and it appears that the blood brain barrier changes are not in synchrony with the development of vasospasm which appears to be peaking somewhat later in such SAH models [1, 2]. However, it remains questionable whether the temporary profile of the blood brain barrier changes as well as the vasospasm can be transferred directly to the situation in humans.

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H. J. Steiger

The authors have studied the sequential changes in BBB permeability induced by experimental subarachnoid hemorrhage (SAH) in

a rat model which has been extensively studied at their laboratory. In the present experiments they assessed BBB disruption by using a spectrophotofluorimetric detection of Evans Blue dye extravasation and replicated their previously published findings in the same animal model when using a quantitative alpha-amino isobutyric acid technique. On this occasion they found that Evans blue extravasation started by 36 hours after SAH, peaking at 48 hours and returning to base line values by 60 hours thus paralleling the course of cistern clot lysis. The authors conclude that SAH produces a global dysfunction of the cerebral microvasculature system which may be responsible for disturbances of brain function.

The authors do not provide a tentative explanation for the discordances between the time course of BBB disruption observed in the present experiments and those reported by other groups working with similar animal models in which increased capillary permeability with passage not only of tracers but also of sodium and water to the extracellular space was observed as early as 2–3 hours after SAH.

On the other hand, regarding the mechanism for global dysfunction of BBB they hypothesize that it is caused by the blood itself and/or active products derived from extravasated blood. Though it is widely accepted that blood degradation may be involved in the production of vasospasm of major cerebral arteries crossing the subarachnoid spaces, the basic mechanism underlying early global dysfunction of cerebral microvasculature occurring after SAH remains to be determined.

R. D. Lobato

Correspondence: Antonino Germanò, M.D., Neurosurgical Clinic, Policlinico Universitario, Via Consolare Valeria, 1, 98125 Messina, Italy.