Microscopic observation of morphological changes in cerebral arteries and veins in hyperacute phase after experimental subarachnoid hemorrhage: an *in vivo* analysis

Running title: Arterial instability in hyperacute SAH

[+]Kana **Fujimori**[1], [+]Sosho **Kajiwara**[1], *Yu **Hasegawa**[1,2], Hiroki **Uchikawa**[3], Motohiro **Morioka**[1]

[+]These authors contributed equally to this work.

[1]Department of Neurosurgery, Kurume University School of Medicine, Fukuoka, Japan

[2]Department of Pharmaceutical Sciences, School of Pharmacy at Fukuoka, International University of Health and Welfare, Fukuoka, Japan

[3]Department of Neurosurgery, Kumamoto University School of Medicine, Kumamoto, Japan

Address correspondence and reprint requests to:

Yu Hasegawa, MD, PhD

Department of Pharmaceutical Sciences, School of Pharmacy at Fukuoka, International University of Health and Welfare, 137-1, Enokizu, Okawa, Fukuoka, 8318501, Japan

Tel: 81-944-89-2000/Fax: 81-944-89-2001

E-mail: yhasegawa@iuhw.ac.jp

Abstract

This observational study examined morphological changes in superficial cerebral arteries and veins, which were correlated with increased intracranial pressure (ICP)-dependent and -independent hypoperfusion in hyperacute phase after subarachnoid hemorrhage (SAH).

The prechiasmatic injection model was used, and 32 male Sprague–Dawley rats were divided into the sham-operated, saline-injected (V group, ICP increase), and arterial blood-injected (SAH group, subarachnoid blood plus ICP increase) groups. Morphological changes in cortical arteries and veins were observed through the cranial window with a microscope before and up to 10 min after the injection. At 24 h, the stenotic and obstructive cortical arteries and veins were counted. After 6 min, 60% of rats in the V group showed vasodilatation, whereas all rats in the SAH group demonstrated vasodilation and/or vasoconstriction (arterial instability) within 10 min. Similar acute venous congestive changes were observed within 10 min in the V and SAH groups. At 24 h, stenotic and obstructive arteries and veins were observed in the SAH group. Neurological deteriorations were observed at 1 h in the V and SAH groups, and at 23 h in the SAH group. The sham-operated group showed no evident vascular changes and neurological deterioration. The same phenomena, including arterial changes after 6 min and immediate venous changes in the V and SAH groups, may have resulted from ICP increase, whereas subarachnoid blood-related factors produced arterial instability within 5 min after blood injection. Subarachnoid blood plays a significant role in hyperacute SAH pathophysiology in addition to ICP increase.

Key words

subarachnoid hemorrhage, hyperacute phase, arterial instability, venous congestion, vasospasm, neurological finding, rat

Introduction

The prognosis of subarachnoid hemorrhage (SAH) is mainly determined by early brain injury (EBI) and delayed cerebral ischemia. The former results from rapid intracranial pressure (ICP) increase by aneurysm rupture and subsequent reduction of cerebral blood flow (CBF). Moreover, ICP-independent prolonged and profound hypoperfusion are observed in the hyperacute phase after SAH, which result from peripheral constriction of the microvasculature due to subarachnoid blood and bioactive substances [1,2]. As the clinical outcome of SAH depends on patient severity on admission [3], early mechanism of brain damage, particularly in the hyperacute phase, might provide a key role to overcome EBI to achieve favorable outcomes.

Physiological cerebral circulation is controlled by suitable CBF from the major cerebral arteries to veins and maintains brain homeostasis. However, the "vascular network" affects pathological changes at multiple points in SAH [4]. Although morphological changes, such as microvascular constriction, play a role in blood supply and cerebral autoregulation [5], there are limited reports evaluating the morphological changes in the hyperacute phase after SAH [6]. Moreover, it is undetermined whether the morphological changes are independently correlated with subarachnoid blood ICP increase. Therefore, we intended to evaluate the specific pathophysiology, which was provided by subarachnoid blood plus ICP increase in addition to morphological changes of cerebral vessels.

We aimed to observe the morphological changes in cerebral vessels in hyperacute phase after SAH by using a rat model of prechiasmatic saline (ICP increase) and arterial blood (subarachnoid blood plus ICP increase) injection.

Methods

Animals

All experimental procedures were performed in accordance with the Institutional Animal Care and Use Committee of Kurume University. The procedures were conducted according to the National Institutes of Health's Guide for the Care and the Use of Laboratory Animals.

Thirty-two male Sprague–Dawley rats (Japan SLC, Shizuoka, Japan) weighing 310–346 g were randomly divided into the sham-operated (S group, n=9), saline-injected (V group, n=9), and arterial blood-injected (SAH group, n=14) groups.

Surgery

Anesthesia was maintained with 2% isoflurane, and rectal temperature was maintained at 36±0.5°C with a heating pad. SAH was induced using a prechiasmatic single blood injection method [7,8]. In brief, after the tail artery of the rats was canulated with a polyethylene catheter tube, a burr hole at the 7.5 mm anterior to the bregma in the midline and a craniectomy window measuring 5×6 mm on their left hemisphere were made using a drill. The rat was placed in a stereotaxic apparatus (Muromachi Kikai Co., Ltd., Tokyo, Japan). A needle was tilted 30◦ caudally and lowered until the tip reached the skull base and placed at a site 1 mm back from the base (11 mm below the surface). Fresh autologous blood (200 μL) was collected from the tail catheter and infused over 12 s. In the V group, the same amount of saline was injected to the prechiasmatic cistern in the same approach. In the S group, the needle was placed at the same position and no solutions were injected.

Microscopic Observation

Morphological changes in the cortical vessels were observed through the cranial window with a microscope (SMZ800N; Nikon Solutions Co., Ltd., Tokyo, Japan). These changes were recorded using a digital camera (MC170 HD; Leica Microsystems, Tokyo, Japan) before (pre-image) and up to 10 min after the injection. As we intended to observe the vessels on the cortical surface after 24 h, we did not open the dura mater to avoid drying and other mechanical injuries. After the skin was closed, the operative lesion was disinfected with iodine, and a subcutaneous injection of meloxicam (1 mg/kg; Cayman Chemical, Ann Arbor, MI, USA) was used for appropriate analgesia [9].

Image Analysis

To assess the vessel diameter on the cortical surface, we reconstructed the image using ImageJ software (National Institutes of Health, Bethesda, ML, USA) and monitored the dynamic changes for 10 min. Based on the report by Wang et al. [10], we defined the arteries, which were observed on the cortical surface as follows: primary arteries were main branches from the middle cerebral artery, secondary arteries from the primary arteries, and terminal arteries from the secondary arteries (Fig. 1A, right). We selected the primary or secondary arteries with the most evident changes every 2 min for 10 min. We measured the diameter and then calculated the rate of changes in comparison with the vessel before the injection. We defined the vessel changes as follows: none (less than 10% increase and decrease), vasodilatation (more than 10% increase), and vasoconstriction (more than 10% decrease). In addition, we evaluated cortical venous congestion every 2 min for 10 min. We defined venous congestion when cortical vein showed “to and flow changes” or “bluish changes.”

Although the photographs taken 24 h after the injection showed muddy dura mater, reddish changes on the cortical surface and some vessel changes could be obviously found. Therefore, we counted primary/secondary arteries and veins that represented more than 50% vasoconstriction (stenosis) and obstruction, and calculated as follows: number of “the stenotic or obstructive vessels”/all vessel×100% in each primary/secondary artery or vein.

Neurological Findings and Brain Water Content

Neurological scoring tests, including the Modified Garcia scale, beam walking, and rotarod tests were performed in all groups at 1 and 23 h after the injection [9]. Then, anesthesia was maintained with 2% isoflurane, and vessels were photographed on the cortical surface at 24 h. Subsequently, the rats were decapitated, and the brains were quickly divided into the left hemisphere, cerebellum, and brain stem, and weighed. They were incubated at 105°C for 72 h in an oven and measured the brain water content (BWC) [9].

Statistical Analysis

We performed all measurements in a blinded manner. All data are presented as means±SEM, and statistical analyses were evaluated using GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA) and Ekuceru-Tokei 2019 statistical software (Social Survey Research Information Co. Ltd., Tokyo, Japan). Parametric evaluations were performed using a one-way ANOVA with the Tukey–Kramer test in three groups (BWT and RT and Garcia, BWC). Non-parametric analyses were performed using the Kruskal–Wallis test, followed by the Steel–Dwass test among the three groups. Statistical significance was set at $p<0.05$.

Results

Mortality

One rat in the sham group died from anesthesia complications, and the mortality rates in the vehicle and SAH groups were 11.1% (one out of nine rats) and 35.7% (five out 14 rats), respectively.

Image Analysis

Image analysis was performed in six, five, and nine rats in the S, V, and SAH groups, respectively. No changes in the cortical arteries were observed in the S group. After 6 min, three rats (60%) in the V group showed vasodilatation, whereas all the rats in the SAH group demonstrated vasodilation and/or vasoconstriction throughout the 10-min period (Fig. 1A, B). In contrast, acute venous congestive changes were observed throughout the 10-min period in all rats of the V and SAH groups (Fig. 2).

Cerebrovascular appearance was clearly confirmed in seven, five, and six rats in the S, V, and SAH groups, respectively, at 24 h. No stenotic vessels were observed in the S group (0%), whereas injured stenotic and obstructive arteries were noted in the V (17.6±4.2%, $p<0.01$) and SAH (40.7±4.0%, $p<0.01$) groups; the latter showed a significant increase ($p=0.03$, Fig. 3A). Injured vessels were observed in the S (8.7±4.4%), V (28.3±6.1%), and SAH groups (45.8±5.2%), and the SAH group showed significant increase in rate in comparison with the S group ($p=0.01$, Fig. 3B).

Neurological Findings and Brain Edema

Although the values of beam walking and rotatod tests at 1 h in the V (2.5±0.2, p=0.02; and 20.1±7.7 s, p=0.03; n=8, respectively) and SAH groups (1.7±0.3, p<0.01; and 12.5±5.1 s, p<0.01; n=9, respectively) were lower than those in the S group (2.5±0.2 and 47.4±5.3 s, n=8, respectively) (Fig. 4A, B). At 23 h post-injection, no changes in neurological findings were observed in the V group (n=8) in comparison with the S group (n=8) (3.1±0.2 vs. 3.6±0.2 in the beam walking test; 32.9±6.9 vs. 67.7±11.0 s in the rotarod test; 17.1±0.2 vs. 17.4±0.3 in the modified Garcia test). However, the values were significantly lower in the SAH (1.6±0.2, 23.3±8.3 s, and 14.7±1.2, respectively) than in the S (p<0.01, p=0.03, and p=0.04, respectively) and V groups (beam walking; p<0.01) (Fig. 4C–E).

The BWC of the S (n=8), V (n=8), and SAH (n=9) groups in the left hemisphere, cerebellum, and brain stem did not show significant differences (Fig. 4F–H).

Discussion

The strength of this study is the comparison of morphological phenotypes between ICP increase and subarachnoid blood plus ICP increase. This study obtained the following novel findings. First, the SAH group revealed arterial instability, including vasodilation and/or vasoconstriction in the first minute, whereas the rats in the V group showed vasodilation slightly later than the saline-injected group. Second, venous congestion was observed in the V and SAH groups in the first minutes. Finally, the cortical arteries and veins represented significant stenotic and obstructive changes at 24 h after SAH.

Arterial instability, such as vasodilation and vasoconstriction, was immediately observed after SAH. This phenomenon was continued throughout the 10-min period, whereas vasodilation was observed 6 min after the injection. In hyperacute phase after SAH, several factors, such as platelet aggregation, basal lamina degradation, and microvascular permeability, could contribute to the pathophysiology within minutes [11]. Further, exogenous materials in subarachnoid space and cerebrospinal fluid that originate from sudden bleeding enhance EBI in SAH [2]. Therefore, in addition to ICP increase, those subarachnoid blood-related factors may play a significant role on arterial instability in hyperacute SAH pathophysiology. The results

on hyperacute vasodilation were consistent with those of a previous study, which evaluated penetrating and precapillary arterioles using different SAH rat models and reported that vasodilation was correlated with reduction in CBF and velocity [6]. Vasodilation may present the compensatory mechanism for SAH-induced CBF reduction. In contrast, the SAH rats in this study presented vasoconstriction, which was reported to be observed at least 10 min in the experimental SAH model [12]. We did not assess further detailed mechanism of the arterial instability. However, a previous *ex vivo* study showed biphasic responses of vasodilation and vasoconstriction by electrical field stimulation in SAH arterioles [13]. The instability phenotype might depend on the amount of subarachnoid blood, expansion manner, and individual arterial condition.

Venous congestive changes were observed in both the V and SAH groups immediately after the injection. According to papers on prechiasmatic blood injection model by Prunell et al. [7,8], ICP rapidly increases and subsequently decreases within 5 min after blood/saline injection. However, the blood injection showed more than 30% CBF reduction within 15 min, the saline injection instantly recovered within a few minutes. The results suggest that the same phenomenoa, including arterial changes after 6 min and immediate venous changes, in both the V and SAH groups, may have resulted from ICP increase, whereas subarachnoid blood-related factors might produce arterial instability within 5 min after blood injection in this study. As significant neurological deterioration at 1 h after the injection was similar between the V and SAH groups, ICP increase might be more important to determine early neurological condition of patients with SAH. Moreover, significant neurological deterioration at 23 h was observed in the SAH group. Therefore, SAH-induced arterial instability and venous congestion observed in the hyperacute phase could be significantly associated with SAH prognosis.

At 24 h post-injection, arterial stenotic/obstructive changes in the V group increased and were more evident in the SAH group. Conversely, significant venous stenotic/obstructive changes were observed only in the SAH group. Numerous vasoconstrictive factors were observed in SAH pathogenesis, and subarachnoid blood obviously distributes brain parenchyma and participates in microvessel constriction and microcirculatory deterioration [14]. As SAH pathophysiology also includes venous thrombosis [15],

subarachnoid blood-related vasoconstrictive factors in addition to ICP increase provide stenotic/obstructive changes in arteries and veins.

In conclusion, although this observational study did not assess detailed mechanisms related to the abovementioned results, we believe that the findings are helpful in understanding the SAH pathophysiology in the hyperacute phase.

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Figure legends

Fig. 1 Arterial instability in hyperacute phase after SAH.

(A) The left side indicates the changes in vascular diameter of cerebral arteries in each rat of all groups, and the right side shows representative photographs and schematic illustration of the arteries on the cortical surface within the cranial window.

(B) The left side shows temporal morphological changes in the cerebral arteries in each rat, and the right side shows representative photographs of vasoconstriction and vasodilatation. The number of included animals were six, five, and nine in the S, V, and SAH groups, respectively. Arrows and arrowheads indicate vasoconstriction and vasodilatation, respectively.

pa, primary artery; sa, secondary artery; SAH, subarachnoid hemorrhage; ta, tertiary artery.

Fig. 2 Venous congestion in hyperacute phase after SAH. The left side shows the congestive changes in the veins in each rat of all groups, and the right side shows representative photographs of the changes. The number of included animals were six, five, and nine in the S, V, and SAH groups, respectively. Arrowheads indicate bluish veins.

SAH, subarachnoid hemorrhage.

Fig. 3 Stenotic and obstructive changes in arteries and veins at 24 h after SAH.

(A) stenotic and obstructive changes in cerebral arteries at 24 h after the injection in the S (n=7), V (n=5), and SAH (n=6) groups.

(B) Stenotic and obstructive changes in cerebral veins at 24 h after the injection in the S (n=7), V (n=5), and SAH (n=6) groups. Arrows and arrowheads indicate stenotic change in cerebral artery and stenotic and obstructive changes in veins, respectively.

a, artery; SAH, subarachnoid hemorrhage; v, vein.

*, $p<0.05$.

Fig. 4 Neurofunction and brain edema at 24 h after SAH.

Neurofunction of beam walking (A) and rotatod (B) tests at 1 h, and beam walking (C), rotarod (D), and modified Garcia (E) tests at 23 h post-injection. Brain water content in the left hemisphere (F), cerebellum (G), and brainstem (H) at 24 h post-injection. The number of included animals were eight, eight, and nine in the S, V, and SAH groups, respectively.

SAH, subarachnoid hemorrhage.

*, $p<0.05$.

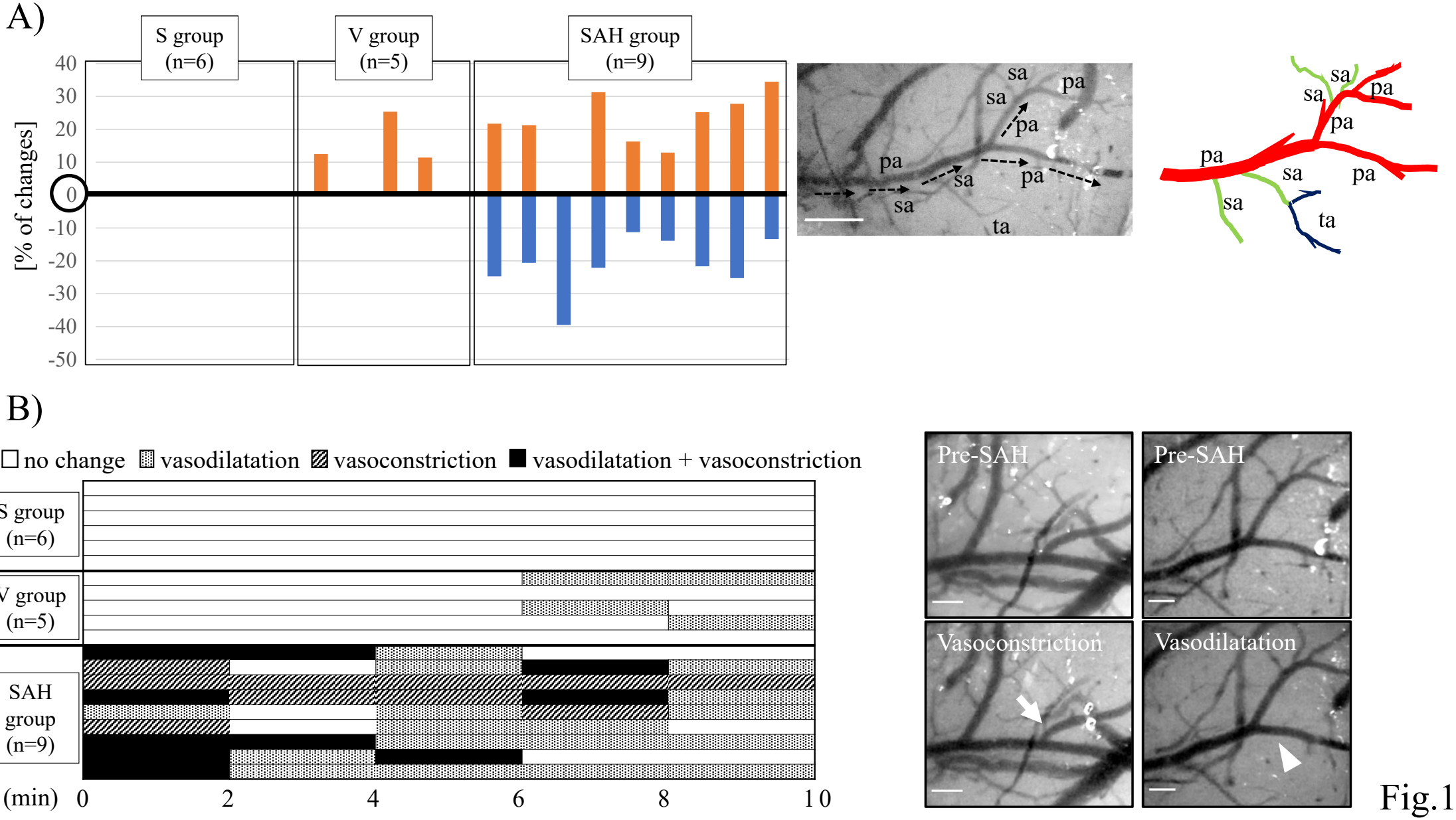
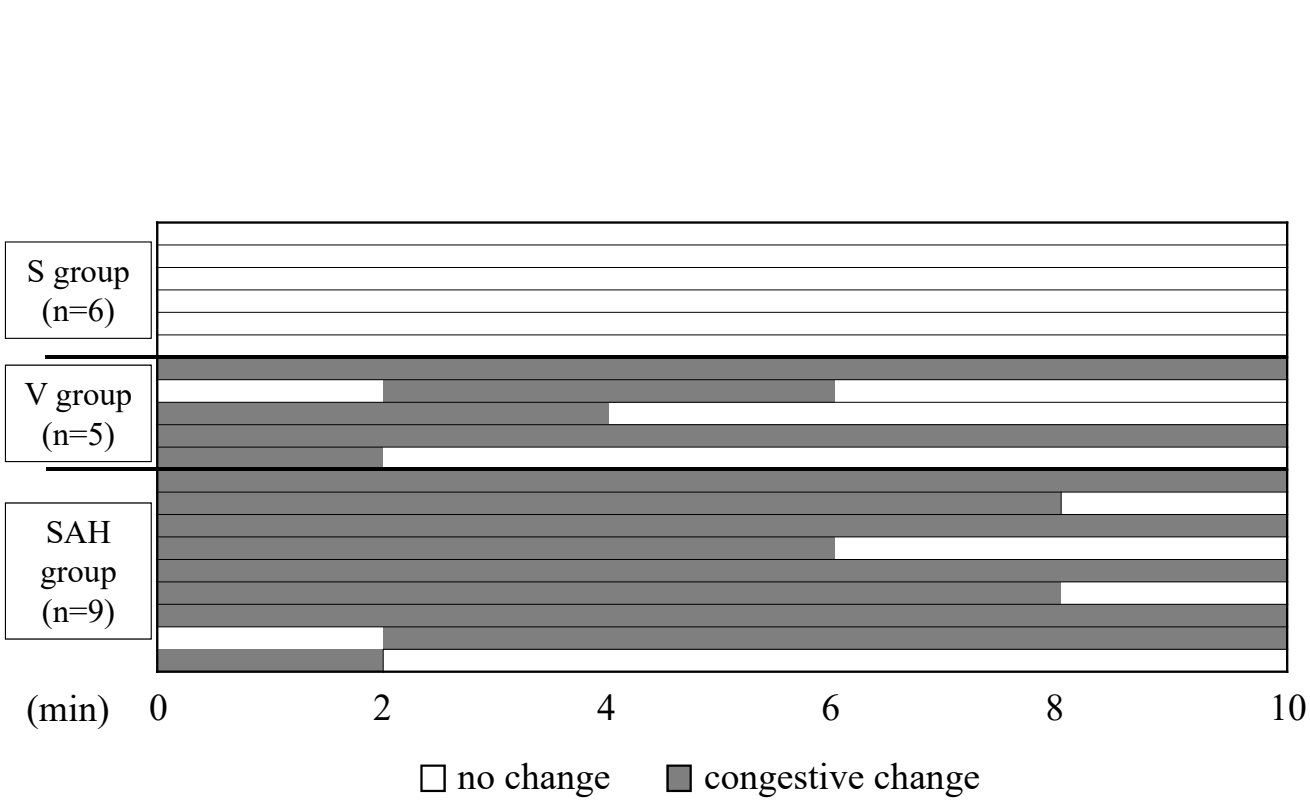

A)
S group (n=6)
V group (n=5)
SAH group (n=9)
[% of changes]
40
30
20
10
0
-10
-20
-30
-40
-50
sa
pa
ta
B)
no change
vasodilatation
vasoconstriction
vasodilatation + vasoconstriction
S group (n=6)
V group (n=5)
SAH group (n=9)
(min) 0 2 4 6 8 10
Pre-SAH
Pre-SAH
Vasoconstriction
Vasodilatation

Fig.1


S group
(n=6)
V group
(n=5)
SAH
group
(n=9)
(min) 0 2 4 6 8 10
no change
congestive change

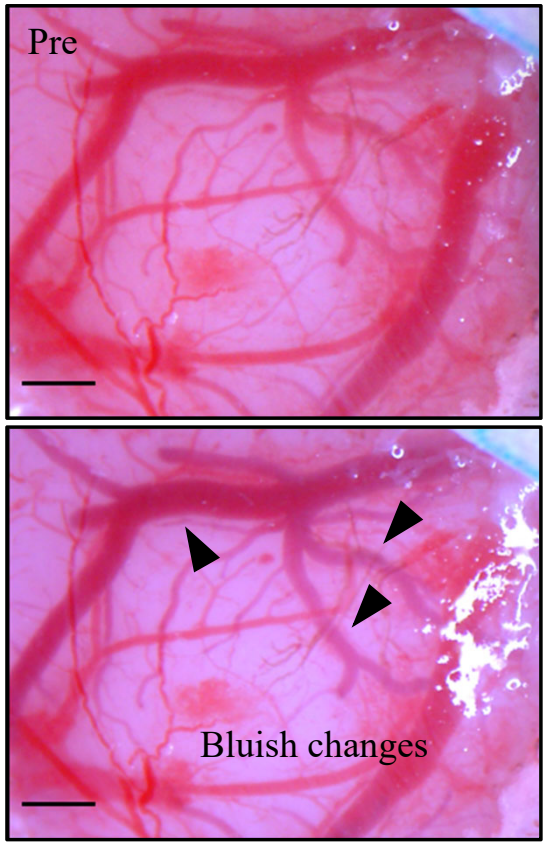
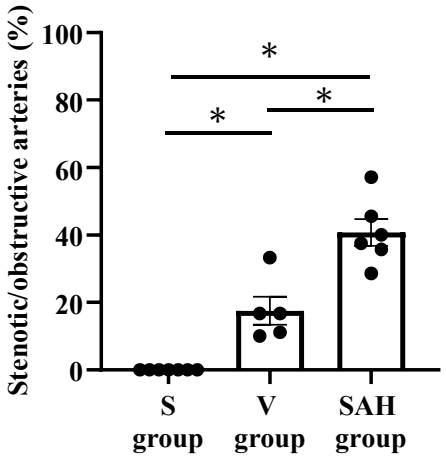
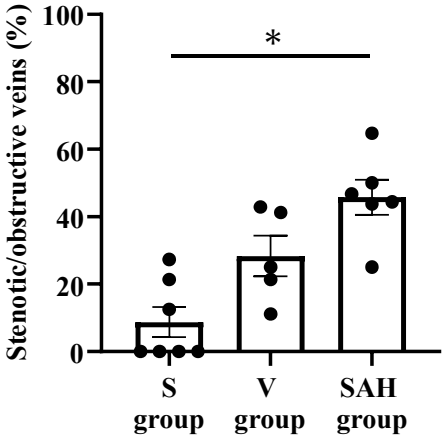

Pre
Bluish changes

Fig.2

A) Arteries



B) Veins



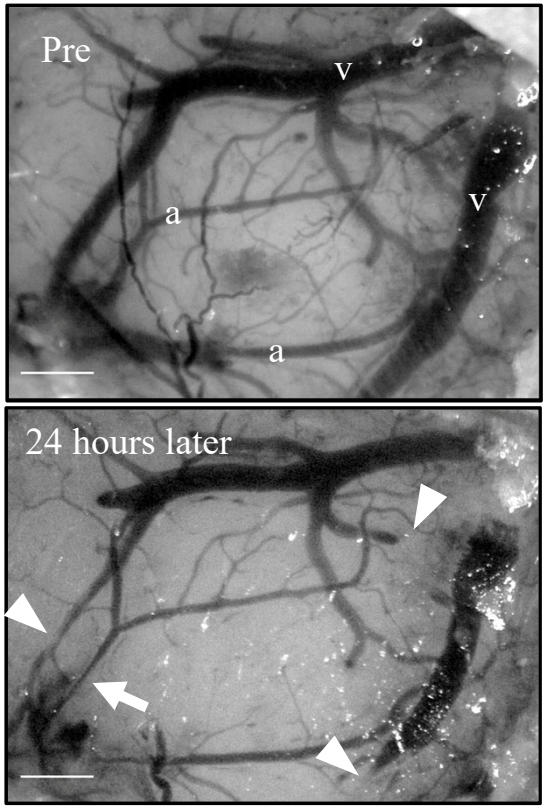


Fig.3

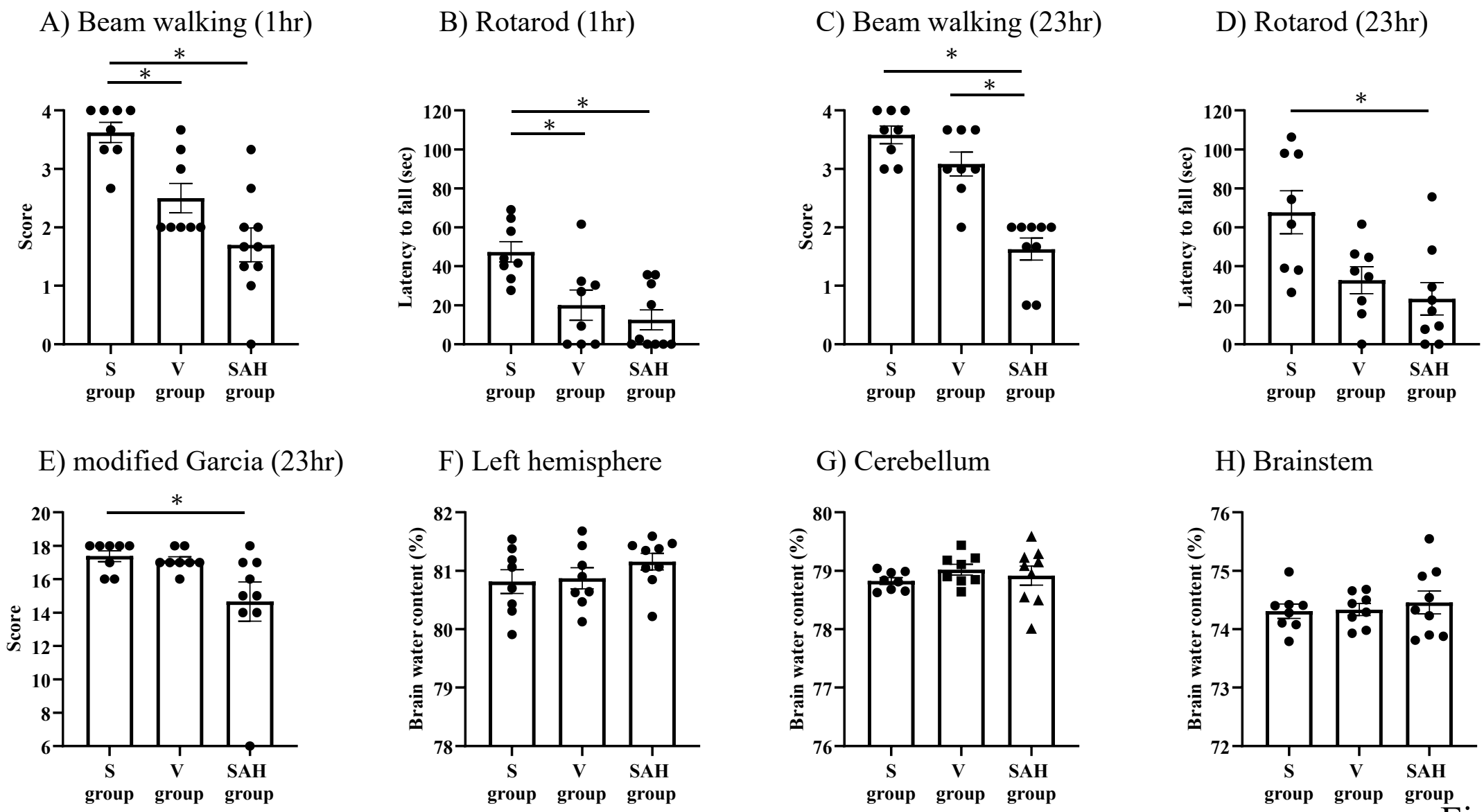

A) Beam walking (1hr)
B) Rotarod (1hr)
C) Beam walking (23hr)
D) Rotarod (23hr)
E) modified Garcia (23hr)
F) Left hemisphere
G) Cerebellum
H) Brainstem
Score
Latency to fall (sec)
Brain water content (%)
S group
V group
SAH group
*

Fig.4