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13 **Cortical microcirculatory disturbance in the super acute phase of subarachnoid hemorrhage**
14 **- In vivo analysis using two-photon laser scanning microscopy -**
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ABSTRACT

Objective: Subarachnoid hemorrhage (SAH) causes cerebral ischemia and drastically worsens the clinical status at onset. However, the arterial flow is surprisingly well maintained on the cerebral surface. We investigated cortical microcirculatory changes in the super acute phase of SAH using two-photon laser scanning microscopy (TPLSM).

Methods: SAH was induced at the skull base in 10 mice using a prone endovascular perforation model. Before SAH, and 1, 2, 5, 10, 20, 30 and 60 minutes after SAH, the cortical microcirculation was observed with TPLSM through a cranial window. Diameters of penetrating and precapillary arterioles were measured and red blood cell (RBC) velocities in precapillary arterioles were analyzed using a line-scan method after administration of Q-dot 655 nanocrystals.

Results: One minute after SAH, RBC velocity and flow in precapillary arterioles drastically decreased to less than 20% of the pre-SAHA values, while penetrating and precapillary arterioles dilated significantly. Subsequently, the arterioles either dilated or constricted inconsistently for 60 minutes with continual decreases in RBC velocity and flow in the arterioles, suggesting neurovascular dysfunction.

Conclusion: SAH caused sudden worsening of the cortical arteriolar velocity and flow at onset. The neurovascular unit cannot function sufficiently to maintain cortical microcirculatory flow in the super acute phase of SAH.

Keywords: subarachnoid hemorrhage, ischemia, vasodilation, neurovascular function, microcirculation

1. Introduction

Subarachnoid hemorrhage (SAH) causes acute brain injury, which results from global cerebral ischemia and determines the acute phase mortality and the long-term prognosis. It was reported that intracranial pressure (ICP) increased and cerebral blood flow (CBF) decreased about one minute after SAH induced by cerebral artery puncture or injury in mammalian models [1-5]. These direct vascular injury models represent the actual SAH and show the acute flow dynamics after SAH. Using mice or rats, it has been also demonstrated that ICP increases substantially at 1-5 minutes after experimental SAH induced using an endovascular perforation model, while CBF decreases and is maintained at a low level for 30-60 min [6-13]. These endovascular perforation models in mice and rats are representative mammalian models regarding the effects of bleeding on ICP and CBF. We previously reported on the sudden decrease in CBF after SAH using the endovascular perforation model in mice [8]. Furthermore, SAH at the skull base results in an acute inflammatory response with platelet-leukocyte-endothelial cell interactions, which was observed *in vivo* in venules on the cerebral surface 2 hours after SAH induction [8]. Using serial coronal brain sections following fluorescent tracer injection, substantial reductions in vascular filling at 10 minutes after SAH was observed *ex vivo* [14]. Microvascular constriction 3 hours after SAH was also observed *in vivo* using fluorescence microscopy [15]. The *in vivo* observation of vessels has been performed at the cerebral surface, where CBF is maintained, perhaps by extensive cortical anastomosis. In these microcirculatory experiments using a cranial window and fluorescence microscopy, observation 1-3 hours after SAH at the cerebral surface has been made, however cortical microcirculation has not been observed immediately after SAH. Acute changes in flow dynamics may be one of the pathophysiological mechanisms underlying post-SAH brain injury. Therefore, we have designed

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7 a microcirculatory experiment, using both two-photon laser scanning microscopy (TPLSM) and a
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9 prone endovascular perforation model, in order to record and analyze cortical flow dynamics
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11 immediately after SAH. The TPLSM offers the advantage that cortical microcirculation,
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13 astrocytes, and neurons can be visualized [16-18].
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16 Recently, neurovascular coupling has been the focus of discussion, suggesting the
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18 combined importance of neurons, astrocytes, and endothelial cells as a CBF-regulatory unit
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20 [17-21]. It is believed that neurovascular coupling is disrupted in pathological conditions [16]
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22 such as SAH [22]. To examine the regulatory responses of precapillary arterioles as a part of this
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24 unit, we employed GFAP-GFP mice to identify precapillary arterioles branching from a
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26 penetrating arteriole, where the GFAP-GFP-labeled astrocytic feet were attached. We analyzed
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28 changes of the diameter and velocity of penetrating and precapillary arterioles in this study.
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33 In order to elucidate the acute brain injury immediately after SAH, the cortical
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35 microcirculation was first observed *in vivo* using TPLSM with line-scan analysis at the time of
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37 SAH induction at the skull base and compared to that before SAH.
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43 **2. Methods**

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45 The experimental procedures were reviewed and approved by the Keio University
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47 Animal Care and Use Committee (12070-2).
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53 *2.1. Animal Preparations*

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55 Experiments were performed using male mice (FVB/N-Tg (GFAPGFP) 14Mes/J),
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57 weighing 25-28 g, to identify astrocytes and their endfeet around precapillary arterioles. The
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59 animals were anesthetized with an intraperitoneal injection of α -chloralose (60 mg/kg) and
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7 urethane (600 mg/kg). All mice were tracheostomized with a polyethylene catheter (PE-90,
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9 Intramedic, Clay Adams) and allowed to breathe spontaneously. The femoral artery and vein were
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11 cannulated with polyethylene catheters (PE-10, Intramedic, Clay Adams) to monitor mean
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13 arterial blood pressure, sample arterial blood for blood gas analysis, and for intravenous
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15 administration of Qdot[®] 655 nanocrystal (Q21021MP; Invitrogen, Carlsbad, USA). Rectal
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17 temperature was monitored and maintained at 36.0-37.0°C using a heating pad throughout the
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19 experimental procedure.
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26 *2.2. Cranial Window*

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28 The head of each mouse was fixed in a metallic frame in the sphinx position. The skull
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30 bone was exposed by a midline skin incision followed by a craniectomy (diameter, 2 mm) using a
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32 drill at the parietal bone. The dura mater was not cut. A 7-mm glass coverslip was placed over the
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34 craniectomy field and the space between the glass and dura mater was filled with sterile
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36 phosphate buffered saline.
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43 *2.3. SAH Model*

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45 SAH was induced using the prone endovascular perforation model to observe cortical
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47 microcirculation immediately after SAH. After a midline neck incision, the left external carotid
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49 artery (ECA) and the pterygopalatine artery were isolated and ligated. After the left internal
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51 carotid artery (ICA) was exposed from the common carotid artery (CCA) to the basal cranium,
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53 the ECA and CCA were ligated with silk sutures. After occlusion of the ICA with a
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55 microvascular clip (#15911, World Precision Instruments, Sarasota, FL) at the pterygopalatine
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57 bifurcation, a guide sheath made from a 7-cm-long polyethylene (PE) tube (SP 8; ID, 0.2 mm;
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OD, 0.5 mm; Natsume, Tokyo, Japan) was inserted from the CCA to the pterygopalatine bifurcation. A 6-0 nylon monofilament had been previously inserted into the PE tube. The nylon monofilament was then advanced 3 mm from the pterygopalatine bifurcation and clamped with a Kocher clamp to keep the nylon in the polyethylene catheter without movement before inducing SAH. The tip of the 6-0 nylon monofilament was blunted to 0.21 mm in diameter with a coagulator before experiments. The prone thread middle cerebral artery occlusion model [23] was applied to the mouse SAH model [8,24] for examination of the vascular responses and arteriolar flow immediately after SAH, because it was not feasible to place the mouse in the supine position with a fixed skull and enable immediate observation of cortical microcirculation. After the 6-0 nylon monofilament was placed in the ICA and a cranial window was made, the mouse was placed on the stage of the TPLSM and a precapillary arteriole was chosen for observation with the TPLSM. Sixty minutes after inserting the 6-0 nylon monofilament in the ICA, SAH was induced at the prone position on the stage by inserting a further 6 mm of the nylon thread through the PE tube. Then, we initiated timed measurements after SAH. The 6 mm length was sufficient to perforate the middle cerebral artery and induce SAH. The nylon monofilament was advanced into the left internal carotid artery 9 mm in total from the pterygopalatine bifurcation. A few seconds later, the thread was pulled into the PE tube. The targeted precapillary arteriole was observed to move to the surface side very slightly when SAH occurred. We refocused on the precapillary arteriole, which required about 1 minute to refocus, and recorded it. Arteriolar diameter and velocity were measured at 1 minute after SAH and for 60 minutes. The changes of diameter and velocity were examined and compared between pre- and post-SAH in the SAH group, and between the SAH group and the sham group (experimental protocol), because ICA occlusion occurred when the 6-0 nylon monofilament was set in the PE tube.

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7 At the end of the experiments, the skull base was opened and the SAH spread was
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9 observed. In this study, the SAH was observed to spread within the skull base, while SAH was
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11 not observed on the cerebral surface in all mice (Video, Fig. 1A).
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14 The cerebral surface was observed and recorded using a visible light microscope
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16 equipped with a 3CCD camera (JK-TU52H; Toshiba, Tokyo, Japan) during SAH induction.
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21 *2.4. Intra-vital Two-photon Laser Scanning Microscopy (TPLSM)*

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23 The TPLSM consisted of an upright microscope (BX61WI, Olympus, Tokyo, Japan)
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25 attached to a mode-locked Titanium-Sapphire laser system (Chameleon Vision II, Coherent,
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27 Tokyo, Japan) that could achieve 140 fs pulse width and 80 MHz repetition rate with 17-W pump
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29 power at a 920 nm laser. Images were acquired with a scanning unit (FV1000, Olympus) using
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31 Fluoview™ software (FV10-ASW, Version 2.0, Olympus) and a 25x objective (XLPLN25 x WMP,
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33 NA 1.05). Fluorescence emission was detected by an external photomultiplier tube (R3896,
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35 Hamamatsu Photonics, Hamamatsu, Japan) after passing through an infrared blocking filter
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37 (685-nm cut off) and an emission filter (495-540 nm for GFP, 641-675 nm for Qdot®655). RBC
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39 velocities in precapillary arterioles were measured with a line-scan method [25] using Fluoview™
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41 software.
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50 *2.5. Experimental Protocol*

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52 The purpose of the experiment was to measure penetrating and precapillary arteriolar
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54 responses and RBC velocities in precapillary arterioles immediately after SAH. A small bolus of
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56 15 µL of Qdot® 655 nanocrystals and 85 µL of saline was injected slowly in order to visualize the
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58 cerebral microvasculature. We identified penetrating arterioles from the arteries on the cerebral
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7 surface and traced along a penetrating arteriole to locate branched precapillary arterioles at a
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9 depth of $\approx 100 \mu\text{m}$. We focused on the branching point to concomitantly record images of both a
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11 penetrating arteriole and a precapillary arteriole (Fig. 1B), around which the astrocyte endfeet
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13 were recognized in all GFAP-GFP transgenic mice in this study.
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16 Mice were assigned to one of three experimental groups: (1) control group (n=5), (2)
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18 sham group (n=5), and (3) SAH group (n=10). The PE tube with the nylon monofilament was
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20 inserted into the ICA, and the left ICA was occluded in the SAH group. The left ICA was
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22 exposed and occluded in the sham group. The left ICA was exposed but not occluded in the
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24 control group.
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29 The diameters of the penetrating and precapillary arterioles were measured. RBC
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31 velocities and blood flow ($\pi \times [\text{diameter}/2]^2 \times [\text{velocity}]$) in precapillary arterioles were
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33 calculated using the line-scan method (Fig. 1B) before SAH and at 1, 2, 5, 10, 30 and 60 minutes
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35 after SAH. Each post-SAH value was compared to the pre-SAH values of control and sham
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37 groups.
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43 *2.6. Image Analysis*

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46 After the experiments, we measured the diameters of the penetrating and precapillary
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48 arterioles using recorded images and the velocities of precapillary arterioles with the line-scan
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50 method, using FluoviewTM software.
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55 *2.7. Statistical Analysis*

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58 Data were analyzed using analysis of variance (ANOVA) and Fisher's post hoc test or
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60 paired t-test as appropriate. The data are reported as mean $\pm$ SD. Statistical significance was set at
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7 $P < 0.05$.
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14 15 16 *3.1. In Vivo Images using Visible Light Microscopy and TPLSM* 17

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19 The cerebral surface was observed and recorded with a 3CCD camera. When SAH was
20 induced at the skull base with the prone endovascular perforation model (video timer: 19:11:09),
21 slight brain swelling was observed and venous flow was slightly decreased, but bleeding was not
22 observed at the cerebral surface (Fig. 1A, Video). Superimposed TPLSM images show the
23 cortical microvasculature consisting of arterioles, capillaries and venules. Each image is the sum
24 of 13 slices at depths of 90-114 μm from the cerebral surface (slice-interval, 2 μm) before SAH
25 and after SAH. The superimposed images showed the capillary networks, and the cross sections
26 represent either a penetrating arteriole or venule. Immediately after SAH, comparison of the
27 video images to the cerebral surface indicated that the cortical capillary networks were broken,
28 and discontinuous capillaries showed the loss of flow (observed in most longitudinal sections).
29 Identified capillaries were narrower than those before SAH. At 30 minutes after SAH induction,
30 capillary flow was slightly improved, but not to the pre-SAH level (Fig. 2A).
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34 Penetrating arterioles first branched into precapillary arterioles at the depth of $96.8 \pm$
35 $12.2 \mu\text{m}$ (n=16, Fig. 1B, left) from the cerebral surface. Around the branching point, GFAP-GFP
36 astrocytes surrounded the arterioles in all mice (Fig. 1B, right). The velocity of RBCs was
37 measured in a precapillary arteriole using the line-scan method using FluoviewTM software (Fig.
38 1B, right).
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42 The diameter of vessels at the cerebral surface and at a depth of about 100 μm
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(branching depth of the penetrating arteriole) was compared, as well as between pre- and post-SAH (Fig. 2B). At the surface, the arterioles were dilated, while the venules were not altered. At a depth of $\approx 100 \mu\text{m}$, penetrating arterioles were either dilated or constricted, while the venules and capillaries were narrowed, occluded and not detected (Fig. 2B). There was a difference between vascular responses in the cortex versus on the cerebral surface, where the cerebral microcirculation might be modified by the collateral circulation. We next investigated the arteriolar changes after SAH at $\approx 100 \mu\text{m}$ depth in the cortex as described below.

3.2. Arteriolar Responses and Velocities Immediately after SAH

Before SAH induction, there were no significant differences in the diameters of penetrating arterioles or precapillary arterioles and velocities of precapillary arterioles between the control, sham and SAH groups (Table 1).

One minute after SAH, RBC velocity and blood flow ($\pi \times [\text{diameter}/2]^2 \times [\text{velocity}]$) drastically decreased, while penetrating and precapillary arterioles dilated significantly. The arterioles then either dilated or constricted inconsistently for 60 minutes with continual decreases in blood flow (Fig. 2C, Fig. 3). The velocity and blood flow of the precapillary arterioles decreased significantly after SAH compared to those before SAH. The decreased velocity and blood flow gradually recovered significantly compared to 1 minute after SAH. This tendency was observed in 6 out of 10 mice after SAH (Fig. 2C). In 2 out of 10 mice in the SAH group, the arteriolar flow disappeared when SAH was induced. Afterwards, arteriolar flow improved slightly, but the arterioles did not dilate and arteriolar flow disappeared again. The velocity and diameter could not be measured (Fig. 2D). Another two mice died of respiratory failure after SAH, perhaps due to brain damage, and their parameters were not recorded.

3.3. Physiological Parameters

Blood pressure (BP) at each time point and blood gas analysis data after experimental measurements are shown in Table 2. The BP and blood gas analysis data did not differ significantly between groups.

4. Discussion

When a sudden increase in ICP occurs immediately after SAH at the skull base, what are the effects on the cortical microvasculature? The increased ICP induces cerebral ischemic damage to the whole brain [1-13], which is followed by inflammatory responses, such as increased vascular permeability [9, 26-29] or platelet-leukocyte-endothelial cell interactions [8]. We first directly observed changes in the cortical microcirculation of mice in the super acute phase of SAH, using TPLSM and the prone endovascular perforation model. TPLSM has been applied to neurovascular coupling experiments to observe astrocytes, neurons and microcirculation in the cortex. TPLSM has shown high utility for observing cortical microcirculation, but not the cerebral surface, and has enabled scientific advances in brain *in vivo* experiments [16-18]. We focused on changes in cortical arteriolar diameter and velocity in the super acute phase of SAH, in order to investigate whether lethal damage is induced by severe brain ischemia immediately after SAH.

In the super acute phase of SAH, three phenomena were observed during the 60-minute period after SAH in this study. First, when SAH was induced, the capillary networks were attenuated and broken in the cortex. The velocity and blood flow drastically decreased in the precapillary arterioles. Second, the autoregulatory dilatory response was observed in arterioles 1

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7 minute after SAH (neurovascular response). Third, the dilation of the arterioles did not continue,
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9 although the velocity and blood flow did not recover (neurovascular dysfunction). The first
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11 phenomenon could be induced by the mechanical collapse of capillaries following the sudden
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13 increase in ICP induced by SAH, as capillaries are more delicate and easily compressed in the
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15 skull. At almost the same time as the attenuation of capillary networks, the velocity and blood
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17 flow in the precapillary arterioles decreased drastically. The velocity and blood flow gradually
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19 but significantly increased in the precapillary arterioles compared to those 1 minute after SAH;
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21 however, neurovascular function could not recover the capillary network and the vasodilatory
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23 response disappeared. At this point post-SAH, autoregulation has already been disrupted
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25 (neurovascular dysfunction), although changes in the arteriolar responses after SAH have been
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27 reported *ex vivo* using TPLSM and an autologous blood injection model [30-31], which differs
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29 from the endovascular perforation model [32].
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36 In our experiment, we used the prone endovascular perforation model, which was
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38 essential for observation of the cortical microcirculation at the time of SAH. The SAH group was
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40 compared to the sham group (IC occlusion on one side) and the control group (no IC occlusion)
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42 to examine the effect of IC occlusion on cortical microcirculation, because the IC was occluded
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44 in this SAH model. In the sham and control groups, the velocity and blood flow in the
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46 precapillary arteriole did not decrease. Cerebral infarction is not induced in rats and mice by
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48 bilateral common carotid artery occlusions, still less by unilateral IC occlusion.
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53 After the drastic decrease in blood flow of the cortical precapillary arteriole following
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55 SAH, the cortical blood flow tended to recover in some mice but not in others over the next hour.
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57 These changes are similar to the improvement in the clinical consciousness state observed in the
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59 super acute phase of SAH patients. Even in circumstances where the patient is unconscious at
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onset, consciousness may return 30 to 60 minutes later in the emergency room, depending on the patient. If a clot from SAH worsens the patient's state of consciousness, consciousness cannot return, because the clot cannot be washed out within a few hours. Thus, the increased ICP and cortical microcirculatory disturbance can be considered related to the disturbance of consciousness, and their improvement is associated with a return of consciousness in patients with SAH. Experimentally, the most severe cortical microcirculatory disruption occurs in the super acute phase of SAH; however, in some mice the precapillary arterioles dilated and cortical blood flow recovered, whereas in other mice the precapillary arterioles did not dilate and cortical blood flow did not recover. Of the 10 mice in our experiment, two died, the arteriolar flow disappeared in another two, and the arteriolar flow decreased but could be measured in the remaining six. Furthermore, the sudden increase in ICP after SAH is pathophysiologically important; it is followed by a decrease in CBF, and induces inflammatory responses, and platelet-leukocyte-endothelial cell interactions in the cerebral venules [8]. The slow injection of blood into the cisterna magna without an increase of ICP does not induce inflammatory responses. Further experimental and clinical studies focusing on the development of clinical treatments during the acute phase of SAH are required to ensure better clinical outcomes. When the level of consciousness is severely diminished due to increases in ICP, amelioration of ICP may be an important treatment for resuscitating patients and allowing surgical treatment to proceed in order to reduce mortality and morbidity.

In conclusion, we observed the cortical microcirculation in the super acute phase of SAH using TPLSM, and reported cortical microcirculatory disturbance, autoregulatory dilatory response and neurovascular dysfunction after SAH.

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7 **Potential conflict of interest disclosure**
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9 The authors have no conflicts of interest.
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Figure Legends

Figure 1

A. Cerebral surface and skull base after SAH. SAH was not observed on the cerebral surface but was observed in the skull base.

B. Illustration of vessels from the cerebral surface into the cortex. A penetrating arteriole (red arrowheads) branched into a precapillary arteriole (red arrow) (left). The velocity of the precapillary arteriole was calculated using the line-scan method (right). Green arrowheads: an astrocyte (GFAP labeled with GFP) around an arteriole. Green arrow: branching point.

Figure 2 Cortical microvasculature consisting of arterioles, capillaries, and venules.

A. Representative microvasculature in the cortex before and after SAH. Each picture is the sum of 13 slices at depths of 90-114 μm from the cerebral surface (slice-interval, 2 μm) before SAH, at 1 minute, and at 30 minutes after SAH. Qdot[®] was administered once 10 minutes before SAH induction. There was no additional administration during the experiment. Red arrow: penetrating arteriole, blue arrow: venule, yellow arrow: representative capillary.

B. Microvasculature at the surface and in the cortex before and after SAH. Pial arteries and penetrating arterioles (red arrows) on the surface dilated at 30 and 60 minutes after SAH, but no changes were observed in the veins. Arterioles (red arrows) in the cortex dilated or constricted at 30 and 60 minutes after SAH, but venules (blue arrows) and capillary networks gradually attenuated 60 minutes after SAH (yellow arrows).

C. In six of 10 mice, the penetrating arteriole and the precapillary arteriole dilated 1 minute after SAH and the velocity of the precapillary arteriole decreased, as demonstrated by the slope on the

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7 line-scan. Two minutes after SAH, the flow in the precapillary arteriole continued to be at a low
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9 level, and the diameters of the penetrating arteriole and the precapillary arteriole did not dilate
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11 compared to those before SAH. Five minutes after SAH, the precapillary flow recovered,
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13 regardless of the lack of diameter change. Ten minutes after SAH, the precapillary velocity
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15 increased further, and it was observed to dilate. Thirty and 60 minutes after SAH, the diameter
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17 and velocity were also unstable.
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21 D. In two of 10 mice, the flow in the precapillary arteriole disappeared after SAH and could not
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23 be analyzed. The diameters of the penetrating and precapillary arterioles were smaller than those
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25 before SAH. In this mouse, the precapillary arteriole flow disappeared 1 minute after SAH, and a
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27 slower flow was observed again with a line scan at 4 minutes after SAH but the diameter did not
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29 dilate, compared to that before SAH. The flow disappeared again at 40 minutes after SAH.
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36 **Figure 3** Changes in the diameters of the penetrating arteriole (A), the precapillary arteriole (B),
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38 blood cell flow in the precapillary arteriole measured using the line-scan method (C), and flow (D,
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40 $[\pi \times (\text{diameter}/2)^2 \times (\text{velocity})]$) before and at 1, 2, 5, 10, 30, and 60 minutes after SAH. Values
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42 are expressed as a percentage compared to pre-SAH values. SAH: subarachnoid hemorrhage,
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44 Control: sham surgery group, Sham: sham surgery group with internal carotid artery occlusion
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46 (ICAO). *: $p < 0.05$ vs before SAH. #: $p < 0.05$ vs 1 min. after SAH.
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Table 1 Diameter, velocity and flow of arterioles before SAH.

	Diameter (μm)		Velocity (mm/s)
	Penetrating arteriole	Precapillary arteriole	Precapillary arteriole
Control	13.4 ± 2.5	6.6 ± 2.1	1.6 ± 0.2
Sham	12.6 ± 2.2	5.9 ± 1.0	1.4 ± 0.2
SAH	14.8 ± 3.4	6.7 ± 2.0	1.6 ± 0.3

No significant differences in diameters of penetrating or precapillary arterioles and velocities of precapillary arterioles between groups were observed.

Table 2 Blood pressure and blood gas analysis.

	Blood pressure (mmHg)				Blood gas analysis		
	Before SAH	Immediate	30 min	60 min	pH	pO ₂ (mmHg)	pCO ₂ (mmHg)
Control	84.9 ± 6.6	84.8 ± 6.1	93.5 ± 11.8	93.5 ± 11.4	7.259 ± 0.078	83.0 ± 28.1	36.3 ± 2.7
Sham	95.8 ± 8.0	96.3 ± 7.6	98.3 ± 9.5	99.8 ± 8.3	7.279 ± 0.063	80.7 ± 5.9	44.8 ± 4.3
SAH	89.6 ± 11.2	84.5 ± 8.0	86.5 ± 14.9	91.2 ± 17.4	7.286 ± 0.054	94.6 ± 16.3	39.9 ± 6.6

The BP and blood gas analysis data did not differ significantly between groups.

Figure
Fig. 1A

Fig. 1B

Fig. 2A

before SAH

1 min. after SAH

30 min. after SAH

100μm

Fig. 2B

Surface

before SAH

30 min. after SAH

60 min. after SAH

Cortex (100μm deep)

Fig. 2C

Fig. 2D

Fig. 3

Fig. 3