Long-term Observation of Pial Microcirculatory Parameters Using an Implanted Cranial Window Method in the Rat

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Abstract. *The aim of this study was to confirm whether our improved closed cranial window (CCW) method could be used for long-term microscopical observation of pial microcirculation intravitally in the rat. We investigated chronological changes in three microcirculatory parameters: permeability of blood-brain barrier, leukocyte behavior, and plasma velocities in the pial venules, immediately after implantation (control group) and at one and four weeks after implantation in different age-matched rats (implanted group). No extravasation of sodium fluorescein from pial venules was confirmed in any observation period. The number of endothelial-adhering leukocytes in the implanted group kept within the physiological range, being similar to those of the control group. The velocities of fluorescent microspheres flowing in pial venules showed no noticeable changes between the two groups. These findings suggest that our CCW method allows long-term observation of the pial microcirculation without any pathophysiological changes in the evaluated parameters up to four weeks after the implantation.*

Cerebrovascular diseases, such as ischemia, hemorrhage and infection lead to various pathophysiological changes in cerebral microcirculation. For example, experimental ischemia followed by reperfusion increases the number of adhesive leukocytes to endothelial cells of cerebral microvessels in experimental animals (1-3). The process of leukocyte adhesion is accompanied by the release of vasoactive substances or disruption of the blood-brain barrier (BBB) function (4, 5). These responses often cause cerebral edema due to ischemic injury (6). Therefore, studying the changes in the cerebral microcirculatory events is an important approach in studies of inflammatory or pathophysiological mechanisms involved in cerebrovascular diseases.

The closed cranial window (CCW) method (7, 8) is one of the most useful techniques to study the cerebral microcirculation in real time. The advantage of this method is the possibility of direct observation of pial microcirculation through a transparent window attached to the brain surface (9-13). Thus, the CCW method has provided useful information about other microcirculatory parameters, such as BBB function, leukocyte behavior and plasma velocity, under various physiological or pathological conditions (14, 15).

Evaluation of the delayed responses caused by brain disorders and the chronic effectiveness of medication requires long-term observation. The CCW method has the potential for investigating chronic changes in the cerebral microcirculation, as shown in experimental animals. However, only a limited number of studies have used the CCW for this purpose (16-20), because the long-term application of the CCW method is accompanied by two main problems, postoperative bleeding under the CCW and an incompatible with perfusion experiments. Levasseur *et al.* (21) developed a new type of CCW and succeeded in preventing these difficulties. However, they applied the window only for cats and rabbits, but not for small laboratory animals, such as rats and mice. Moreover, it was not clear whether the long-term implantation of CCW influences the cerebral microcirculation by itself.

The first goal of the present study was to design a new type of CWW for the long-term observation of rat pial microcirculation and to verify its applicability for monitoring microcirculatory parameters. Our second goal was to compare several parameters, such as BBB permeability, leukocyte behavior, and plasma velocities obtained using our CCW method with those of previously reported

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methods and then to examine the effects of long-term implantation of the window on these microcirculatory parameters in rat brain.

Materials and Methods

Animals. The twenty-four male Sprague-Dawley rats (10-week-old) used in this experiment were purchased from Tokyo Laboratory Animals Science Co., LTD (Tokyo, Japan). They were fed a standard pellet diet (F-2, Funabashi Farm Co., Ltd., Chiba, Japan) and water *ad libitum*, and were maintained with a 12/12 h light/dark cycle at a temperature of $23.0\pm1°C$ and a relative humidity of $50\pm10\%$. All experimental procedures were conducted in accordance with the ethical guidelines for animal experiments at the National Institute of Public Health, Japan.

Cranial window setup. A closed cranial window (CCW) setup used in present study consisted of two parts (Figure 1). One was an adaptor ring connected with opened parietal region whose outer and inner diameters are 12 mm and 10 mm, respectively. The other was a CCW part for intravital-microscopic observation. Two types of CCW (Type I and Type II) being exchangeable between these were developed based on experimental demands. Type I was developed for long-term observation for pial microcirculation. It had a convex shape which allows the CCW to be inserted into the adaptor ring. The bottom of this window had a 9 mm circular cover-slip. The Type II window was developed for superfusion experiments with pharmacological agents. It has operculiform shape to be able to cover the adaptor ring. A circular cover-glass with 11.5 mm diameter was put on the top of this window. In addition, for perfusion of the fluid, two Teflon tubes with an outer diameter of 1 mm were embedded in the side of ring. Both types of window were fixed to the adaptor with vinyl silicon resin or cyanoacrylate glue.

Preparation of the cranial window. The rats were anesthetized with an intramuscular injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The head of each rat was fixed in a stereotaxic apparatus. After removal of skin and thin tissue from the parietal region, a 12 mm circular skull hole was made using a dental drill with cold saline dripping to prevent heat damage during the drilling. Subsequently, the dura mater and subarachnoid were carefully removed from the cerebrum surface to expose the pia mater. Through the hole in the skull, the adaptor ring was mounted on the pia mater with cyanoacrylate glue. A Type I window was then inserted into the adaptor ring and fixed with silicon resin.

To perform a superfusion study, the Type I window was replaced with the Type II. The Type I window was carefully removed from the adaptor ring. After filling artificial cerebral spinal fluid (CSF: NaCl 133.5 mM, KCl 3.2 mM, $MgCl_2$ 1.33 mM, $NaHCO_3$ 24.5 mM, $CaCl_2$ 1.5 mM, Glucose 3.3 mM, pH 7.34) equilibrated with the mixed gas (10% O_2, 6% CO_2, 74% N_2), the Type II window was inserted into the adaptor ring and fixed with cyanoacrylate glue. The inflow and outflow tubes of the CCW were connected to a superfusion system consisting of a custom made thermostat, an infusion pump (101U/R, Watson-Marlow Ltd., Falmouth, England), and a manometer (AP-611G, Nihon Kohden Co., Tokyo, Japan). The CSF was maintained at $37\pm1°C$. The fluid was perfused over the pial surface through the pump at a speed of 1.0 ml/min. This speed maintained an intra-cranial pressure of 5.0 ± 0.5 mm Hg.
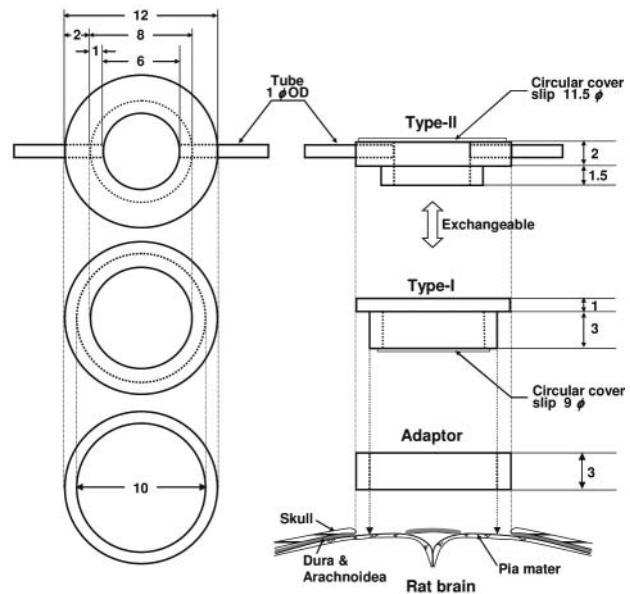

Figure 1. *Schematic drawing of the closed cranial window for long-term implantation. Two types of window, Type I and Type II, were prepared in the present experiment. Each window was inserted through the adaptor ring mounted on the rat skull on experimental demand. The dimensions are given in millimeters.*

Intravital-microscopic observation. The rat pial microcirculation within the CCW was monitored with an intravital-microscopic system while the animal was under anesthesia. The rats were anesthetized with an intramuscular injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and with a subcutaneous injection of pentobarbital (25 mg/kg). The head of each animal was fixed in the stereotaxic apparatus. By placing rats on a custom made heating pad, the rectal temperature of each animal was maintained at $37.0\pm0.5°C$.

Two types of intravital-microscopy were used. One was an intravital-fluorescence microscopic system (IVM), and the other was a confocal laser-scanning microscopic system (CLSM). The IVM consisted of a fluorescent microscope (BX50WI, Olympus Optical Co., Ltd., Tokyo, Japan) and an image intensified camera (C2400-80, Hamamatsu Photonics K.K., Hamamatsu, Japan). The light source was a mercury lamp (U-ULH, Olympus Optical Co., Ltd.). The filter cube had two types of excitation filter, 524 nm and 490 nm. The CLSM consisted of a microscope (BX50WI, Olympus Optical Co., Ltd.), an argon-ion laser (643-YOKO-AO2, Omnichrome, Chino, CA, USA), a confocal scanning unit (CSU-10, Yokogawa Electric Co., Tokyo, Japan), and an image intensified CCD camera (C2400-80, Hamamatsu Photonics K.K.). The light source was an argon laser whose wavelength was 488 nm. The confocal scanning unit obtained real time images through a fluorescence filter (490 nm).

The images of the pial microvascular bed *via* each system were recorded at 30 frames per second to a video cassette recorder (WV-DR7, Sony, Tokyo, Japan) with a video timer (VTG-33, FOR.A Co., Ltd., Tokyo, Japan). All the images were digitized and later analyzed off-line.

BBB permeability. BBB permeability was evaluated using two methods widely used in previous studies. One aimed to examine

Table I. *Experimental protocol followed.*

Group	Measured item	Age of rat			Numbers of rats
		10-weeks	11-weeks	14-weeks	
Implanted	Na+-fluorescein leakage Leukocyte behavior Plasma velocities	Operation (Type I)	→ Measurement	→ Measurement	6
	Extravasation rate of FITC-dx	Operation (Type I)	Window change (Type II) Measurement		3
				Window change (Type II) Measurement	3
Age-matched control	Na+-fluorescein leakage Leukocyte behavior Plasma velocities	Non-treatment	Operation (Type I) Measurement		3
				Operation (Type I) Measurement	3
	Extravasation rate of FITC-dx	Non-treatment	Operation (Type II) Measurement		3
				Operation (Type II) Measurement	3

appearance of BBB disruption, while the other observed the time-dependent changes in BBB permeability.

The transient extravasation of sodium fluorescein (MW: 376, Sigma-Aldrich, Inc., Saint Luis, MO, USA), as an indicator of low molecular leakage, was monitored using the IVM. Sodium fluorescein (2%, 100 μl/kg) was injected intravenously, and the image of the pia mater, including pial venules and the extravascular region, was recorded under the microscope with a fluorescent excitation wavelength of 490 nm. To evaluate the leakage level of fluorescent dye in detail, representative fluorescent images of pial venules under each conditions were observed by the CLSM. The fluorescence intensity profile of the image including pial venule and its surrounding extravascular region was then measured at 5 sec after the dye injection using the Scion Image software (Scion Corp., Frederick, MD, USA). A typical intensity profile in BBB disruption, as a positive control, was obtained by an application of superfusion with mannitol solution (1 M in CSF) using a rat equipped with the Type II CCW.

The extravascular accumulation of FITC-dextran (150 kDa, Sigma-Aldrich, Inc.), as an indicator of large molecular leakage, was monitored through a Type II window using the IVM. Artificial CSF was superfused for 30 min to stabilize physiological conditions inside of the cranial window. FITC-dextran (50 mg/kg) was intravenously injected. Thirty minutes after the dye injection, artificial CSF was changed to mannitol solution (1 M in CSF) to increase the permeability of BBB. An image of the pia mater was then recorded under the microscope with a fluorescent excitation wavelength of 490 nm. The averaged fluorescence intensity in the arbitrary area of the pia mater was measured every 1 min for 25 min.

Leukocyte behavior. The changes in leukocyte behavior were evaluated using the number of leukocytes having interactions with the endothelium in pial venules. The interactions were classified into two states, rolling or sticking aspect. The rolling leukocytes were defined as cells having weak interactions with the endothelium and thereby a rolling behavior on the endothelium. The sticking leukocytes were defined as cells attached to the endothelium for at least 30 sec with strong interactions. The leukocytes were labeled using rhodamine 6G (0.1 mg/kg body weight, Wako Pure Chemical Industries, Ltd., Osaka, Japan) injected intravenously, and were observed under IVM with a fluorescent excitation wavelength of 524 nm. The numbers of leukocytes in the two categories were counted in a 100 μm length of pial venules. In addition, the numbers were compared postcapillary venules (8-30 μm) and collecting venules (31-50 μm), to investigate the detail of changes in each pial venule.

Plasma velocities. Changes in plasma velocities were evaluated using velocities of microspheres flowing in the pial venules. Fluorescence microspheres (2.5% solids-latex, 1.0 μm YG, Polysciences, Inc., Warrington, PA, USA) were intravenously injected, and their motion was measured under the IVM with a fluorescent excitation wavelength of 490 nm. The drifting distance of microspheres flowing at the centerline of a pial venule was measured from the video image frame by frame. The velocity was calculated as the distance for 1/30 sec of the video frame. The plasma velocities expressed by the average of three measurements were compared between postcapillary venules and collecting venules.

Experimental protocol. To compare the previous type of CCW method with our developed method, we divided rats into two groups, an implanted group and a control group (Table I). The rats of the implanted group were equipped with CCW one or four weeks before the observations. Three microcirculatory parameters, sodium fluorescein leakage, leukocyte behavior and plasma velocities, were repeatedly measured one and four weeks after the implantation in the same rats (n=6). Extravasation rate of FITC-dextran was examined one week (n=3) or four weeks (n=3) after the implantation (one of the animals was the same rat used for
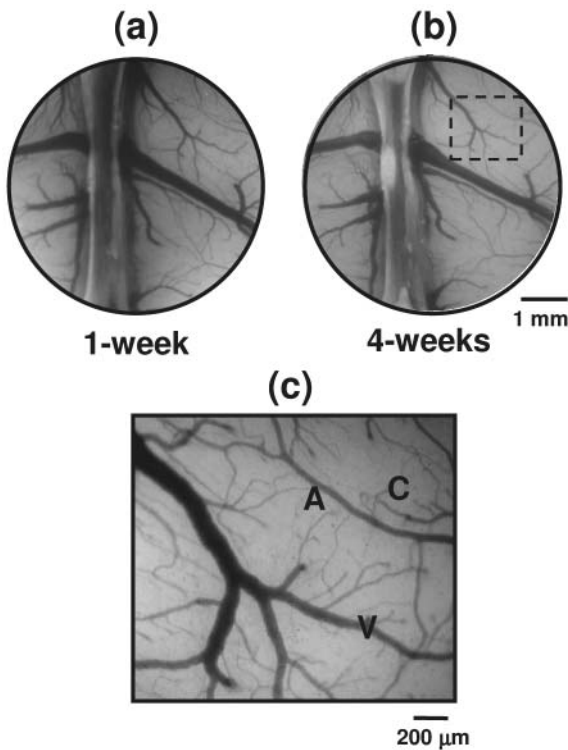
Figure 2. *Overview of the pial microvessels through the long-term implanted cranial window. The architecture of microvessels in one week after window implantation (a) remained until four weeks after window implantation (b). The pial microvessels, arterioles, venules and capillaries, were clearly observed throughout the experimental period (c).*

Figure 3. *Image analysis of sodium fluorescein leakage from pial venules. Upper photographs show the images of pial venules observed by CLSM. The corresponding graphs (bottom) show the fluorescence intensity profiles on the white line indicated in each image. The fluorescence intensity was normalized to the maximum intensity on the pial venule. The BBB under physiological conditions with artificial CSF (a) was disrupted after cortical perfusion with 1 M mannitol solution to induce hyperosmotic shock (b). The BBB disruption was indicated as an increase in the fluorescence intensity outside of the venules. No BBB disruption was seen in the pial venules one week (c) and four weeks (d) after window implantation.*

measurement of the other three parameters four weeks after the implantation). The rats of the control group were kept without any treatments, and the implantation of CCW was performed immediately before the observation. The corresponding parameters of the implanted group were measured using the age-matched rats in the control group (total n=12).

Statistical analysis. All results are presented as means±standard errors. The statistical analysis was carried out using the *t*-test. A value of $p < 0.05$ was considered statistically significant.

Results

Overview of pia mater. To confirm whether the pial microcirculation was visible long-term *via* our CCW, observations were made throughout the experimental period. Our CCW maintained the architecture of the pial vasculature at least for four weeks after the window implantation (Figure 2). There was no infection, inflammation, regrowth of the dura or tortuosity of the blood vessels. The intravital microscopic observation allowed images of pial microvessels including arterioles, venules, and capillaries to be obtained even four weeks after the implantation.
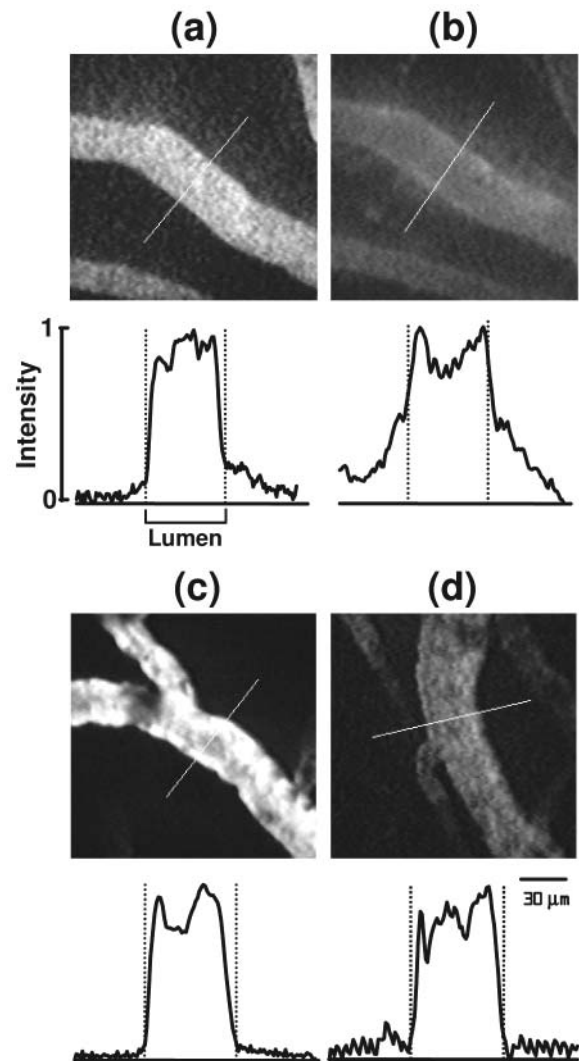
BBB permeability. The BBB permeability in the rat brain after the CCW implantation was examined using two types of fluorescent dyes. No leakage of sodium fluorescein in the pia mater was observed in the rats of either group throughout the four week period after the window implantation. Figure 3 shows detailed quantification of the extravasation of sodium fluorescein around pial venules. The increases in fluorescence intensity outside the pial
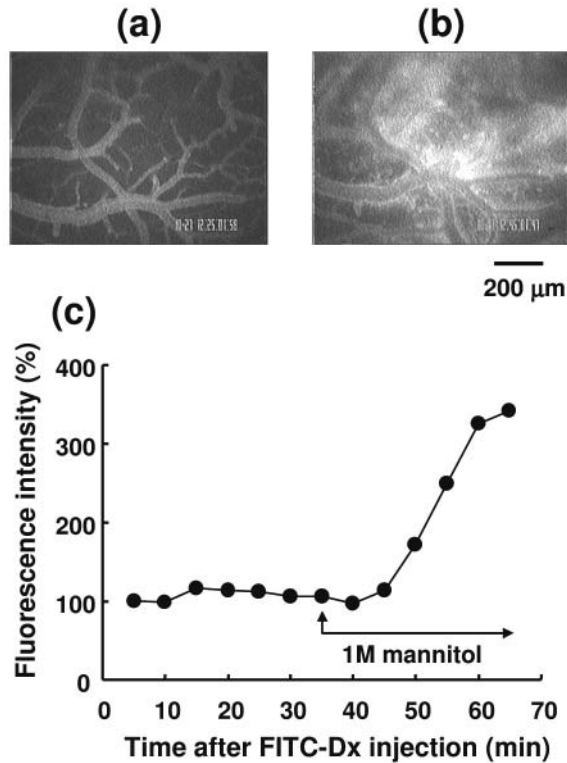
(c)

Figure 4. *Extravasation of FITC-dextran after intentionally induced BBB disruption. Intravenously injected FITC-dextran did not leak from pial microvessels under physiological conditions during cortical perfusion with artificial CSF (a). Twenty minutes after changing the artificial CSF to 1 M mannitol solution, the accumulation of FITC-dextran was observed around pial microvessels (b). (c) shows the time-course of fluorescence intensity in the pia mater region after intravenous injection of FITC-dextran. The application of cortical perfusion with 1 M mannitol solution increased the fluorescence intensity time-dependently.*

venule, which were demonstrated in the positive control of BBB disruption, were not found in the rats one or four weeks after the window implantation.

The pattern of extravascular accumulation of FITC-dextran over time in the pia mater region under hyperosmotic conditions, used as another evaluation of BBB permeability showed no significant difference between control group and window implanted group. The cortical perfusion with mannitol solution increased the fluorescence intensity in the pia mater region time-dependently, whereas the intensity remained at a physiological level with artificial CSF (Figure 4). To compare the changes in BBB permeability between the two groups, we measured the fluorescence intensity in the pia mater region after the application of hyperosmotic solution (Figure 5). The time-dependent increase in the control group showed the same pattern in the implanted group even at four weeks after window implantation.
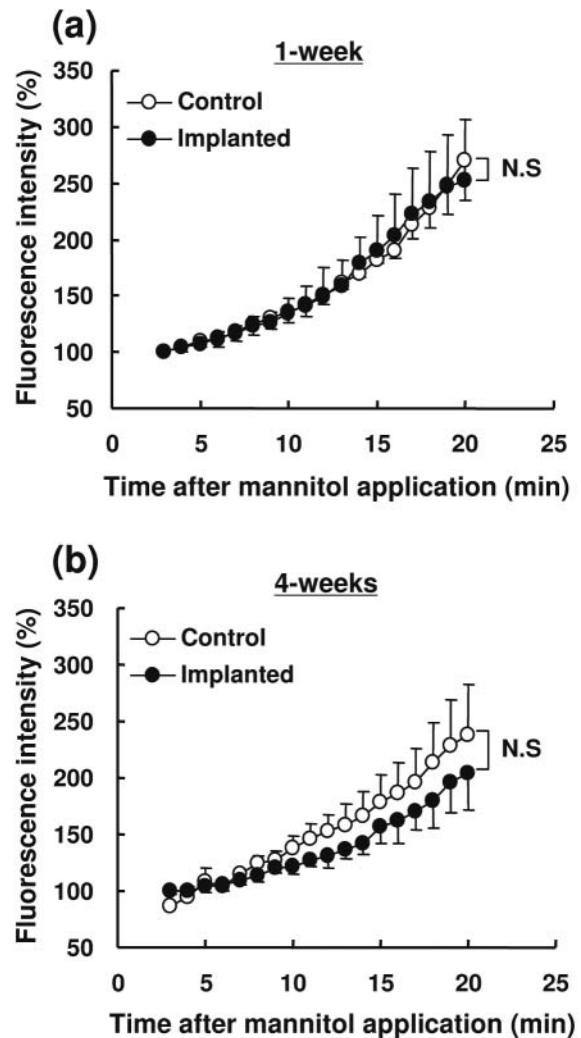

Figure 5. *Time-course of extravascular accumulation of FITC-dextran in the pia mater region under hyperosmotic condition. The time-dependent changes in fluorescence intensity in the pia mater region were measured one (a) or four (b) weeks after window implantation and were compared with those in age-matched control rats. Each value is presented as mean±standard error in three rats.*

Leukocytes behaviors. To evaluate the leukocyte behavior in the pial microcirculation after the CCW implantation, the number of leukocytes having interactions with the endothelium in pial venules was measured. No effect of the window implantation on the number of rolling leukocytes was observed (Figure 6 a). It can be seen that one week after window implantation, there were no significant differences between the control group and the implanted group in the number of rolling leukocytes in postcapillary venules or collecting venules. Moreover, four weeks after the window implantation, the number of rolling leukocytes in both types of venules showed no
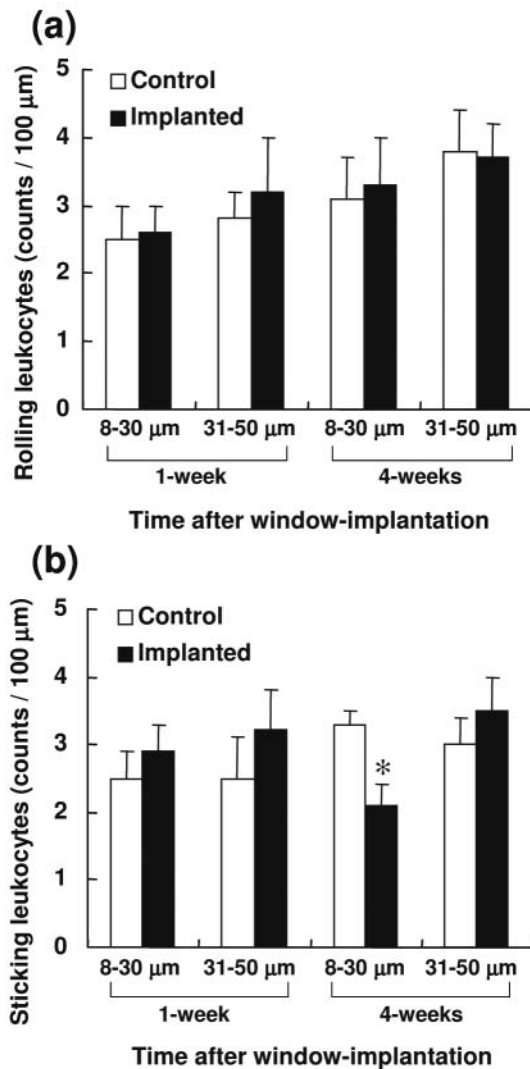
(a)

(b)

Figure 6. *The number of leukocytes having interactions with endothelial cells in pial venules. The interactions were classified into rolling (a) and sticking (b). The numbers of these two types of leukocytes were counted in postcapillary venules (8-30 μm) and collecting venules (31-50 μm) one and four weeks after window implantation and were compared with those in age-matched control rats. Arbitrary venules (1 to 3) were selected in each rat. Each value is presented as mean±standard error in observed venules (7-13 venules). *p<0.05 vs. corresponding value in control group.*

Figure 7. *The changes in plasma velocities in the pial venules. The velocities were measured in postcapillary venules (8-30 μm) and collecting venules (31-50 μm) one and four weeks after window implantation and were compared with those in age-matched control rats. Arbitrary venules (2 to 3) were selected in each rat. Each value is presented as mean±standard error in observed venules (9-18 venules).*

implantation, there were no significant differences between the control and implanted groups in the number of sticking leukocytes in postcapillary venules or collecting venules. Four weeks after the window implantation, the postcapillary venules in the implanted group showed a significant decrease in the number of sticking leukocytes, while the collecting venules showed no significant change. However, it seems that this phenomenon was within physiological limits.

Plasma velocities. To evaluate the blood flow in pial microcirculation after the CCW implantation, the plasma velocities in pial venules were measured. No effect of the window implantation was found on plasma velocities in the pial venules (Figure 7). The plasma velocities in the postcapillary venules or collecting venules showed no significant difference between the control and implanted groups neither one nor four weeks after window implantation.

Discussion

There are two main findings in the present study. First, the closed cranial window (CCW) setup allows the monitoring of the pial microcirculation under intravital-fluorescence microscopy during our experimental period of four weeks. Second, there was no noticeable difference in the three microcirculatory parameters, BBB permeability, leukocyte behavior, and plasma velocities, between control and window-implanted groups. These findings suggest that our CCW

significant difference between the two groups, although the number in the implanted group tended to be higher than that in the control group.

Although the numbers of sticking leukocytes in the implanted group appeared to be lower than that in the control group, we found no noticeable effect of the long-term implantation of the cranial window on the number of sticking leukocytes, except in collecting venules four weeks after window implantation (Figure 6 b). One week after window
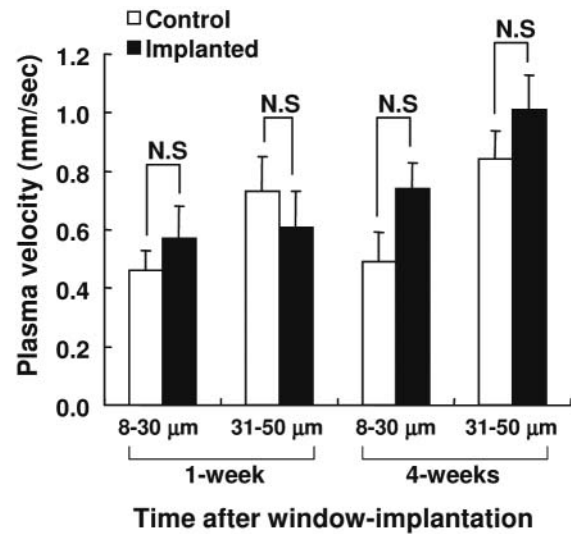
method may be used for long-term observation of the rat pial microcirculation without causing a pathophysiological effect on the examined parameters up to four weeks after window implantation.

The long-term implantation of CCW is useful for evaluation of the delayed responses caused by brain disorders and the chronic effectiveness of medication. Gaber *et al.* (20) used an implanted type of CCW and repeatedly observed changes in BBB permeability and leukocyte adhesion during three to four days after radiation treatment of the rat brain. Yuan *et al.* (22) followed the chronic effect of nimodipine, a calcium channel blocker, on diameters of rat pial arterioles and venules over 13 days using an implanted cranial window. However, only a limited number of studies using the long-term implanted CCW method have been reported (16-20). We believe that there are two main disadvantages that prevent a wider use of these methods. It has been found that bleeding after the CCW implantation obstructed the view of the pial microcirculation *via* the window. Furthermore, it was difficult to perform the pharmacological experiments used in the previous studies, because a perfusion system installed on the brain restricts the free movement of the animals.

To overcome these disadvantages, Levasseur *et al.* (21) developed a new type of CCW. Their window could be opened and closed repeatedly on experimental demand. Thus, blood and clots could be removed while opening the window, if postoperative bleeding took place. In addition, their window had a valve system that made it possible to perfuse artificial CSF over the pia mater. Thus, they succeeded in keeping the view of the pia mater clear without the problem of postoperative bleeding, and also in performing perfusion experiments. However, this cranial window was introduced only for cats and rabbits, not for small laboratory animals like rats, which were widely used in short-term experiments using the CCW method.

In this study, we proposed a small long-term implanted CCW, similar to Levasseur's model, installed in rats. Our CCW window includes an adaptor and two types of windows, Type I and Type II, which have several advantages. The adaptor ring glued to the incision region of the parietal bone helped to arrest the postoperative bleeding from that region. The window installation *via* the adaptor ring made it possible to open and close the window, repeatedly. Furthermore, the replacement of the Type I window with the Type II allowed perfusion experiments on experimental demand. We have already confirmed that the clear observation of the pial microcirculation lasted for 12 weeks (data not shown), although the microcirculatory parameters in this case were not evaluated in detail. These results demonstrate that our CCW method can be used for observation of time-dependent changes in the cerebral microcirculation over a long period.

Whether or not a long-term implantation of CCW affects the cerebral microcirculatory parameters in the rat brain is very important. Although many cerebral microcirculatory parameters have already been studied, this information was mostly obtained in short-term experiments within one day using the CCW method. Thus, a confirmation that the long-term implantation of CCW does not induce abnormal changes in these parameters *via* some pathophysiological effects was needed. In the present study we focused on two microcirculatory parameters, BBB permeability and leukocyte behavior, because both parameters are often simultaneously investigated as important microcirculatory indicators for pathophysiological changes in the brain. For example, increases in BBB permeability and leukocyte adhesion occur under inflammatory conditions in the brain (4, 23).

Changes in the BBB permeability were evaluated by extravasation of intravenously injected fluorescent dyes from cerebral microvessels. In the present study, we used two fluorescent dyes: sodium fluorescein and FITC-dextran. Sodium fluorescein, as small molecules, was applied to confirm the BBB disruption (14, 24). On the other hand, FITC-dextran, as a large molecule, is useful for measurement of the time-dependent changes in BBB permeability (25, 26).

BBB disruption is seen in cerebral microcirculation under inflammatory or pathophysiological conditions. In this case, intravenously injected sodium fluorescein was extravasated from cerebral microvessels, whereas it showed no extravasation under physiological conditions (14). Schilling and Wahl (27) found that cortical superfusion with histamine, one of the inflammatory mediators, induced an extravasation of sodium fluorescein in the cat brain. In addition, Olesen (28) showed that the leakage of sodium fluorescein occurred in venules, but not in arterial vessels or capillaries. Thus, the possible inflammatory response to the long-term implantation of the CCW might appear as a BBB disruption around pial venules. In the present study, we observed extravasation of sodium fluorescein around pial venules under cortical superfusion of hyperosmotic solution. However, no extravasations similar to this were found in any venules that we monitored, even in the long-term implanted group. Moreover, we also confirmed that this absence of extravasation lasted at least 12 weeks after window implantation (data not shown). These findings suggest that no BBB disruption was elicited by the long-term implantation of CCW.

Many investigators have reported that several inflammatory mediators affect the permeability of the BBB (13, 25, 27). This phenomenon is observed as the time-dependent increase in the extravascular accumulation of intravenously injected dye. Mayhan (25, 29) showed that topical application of VEGF or histamine to the pia mater

produced an increase in clearance of FITC-dextran in a time-dependent manner. Furthermore, he found that the pattern of this time-dependent increase was modified by the concentration of VEGF or histamine. Thus, these findings suggest that the level of the inflammatory reaction in the brain is reflected in the time-dependent increase in BBB permeability. Therefore, in the present study, we examined whether the long-term implantation of CCW modified the time-dependent increases in the extravasation of FITC-dextran induced by cortical perfusion with hyperosmotic solution. Our results showed that there were no significant differences in the pattern of the time-dependent increases in extravasation of FITC-dextran between control and implanted groups, even four weeks after the implantation. Therefore, the long-term implantation of CCW induced no inflammatory reaction affecting BBB permeability.

Leukocytes play an important role in the immune system and their behavior changes under inflammatory or pathophysiological conditions, exhibiting an augmented adhesiveness to the endothelial cells in microvessels. The increased leukocyte adhesion was broadly classified into two categories, rolling leukocytes and sticking leukocytes. Many investigators found these two kinds of leukocyte adhesions in the cerebral microcirculation after brain disorders (3, 19, 30) or after experimental application of inflammatory mediators (4, 13, 31). Ritter *et al.* (3) found that the number of rolling and adherent leukocytes increased in the rat brain after focal stroke and reperfusion. Uhl *et al.* (13) reported that local application of platelet activating factor on the brain surface increased the numbers of rolling and adherent leukocyte in venules. We thought that if a long-term implantation of CCW affects the physiological condition around cerebral microvessels, the changes in leukocyte behavior would be found as an increase in the numbers of rolling or sticking leukocytes. We found no significant difference in the number of rolling leukocytes between control and implanted groups. In addition, no significant increase in the number of sticking leukocytes was induced by the long-term implantation of CCW. We also did not see significant differences in plasma velocities between two groups. Thus, there is no concern with respect to blood flow indicated by leukocyte behavior. These findings, therefore, suggest that the long-term implantation of CCW induces no inflammatory or pathophysiological reaction accompanied by an increase in leukocyte adhesion.

Long-term implantation of CCW may cause some effects on the pial microcirculation, because this cranial window method involved excision of a part of the skull and the dura mater. Additionally, the cover-glass in contact with the pia mater was foreign material for animals. Ideally, we should have compared our results in the window-implanted rats with those in intact rats to determine the differences from the normal condition. Unfortunately, there is no method allowing direct observation of microcirculatory parameters in intact rats. Thus, it seems to be reasonable to compare the microcirculatory parameters in the long-term implanted CCW with those in another CCW widely used in short-term experiments.

Conclusion

We developed a new type of CCW for the long-term observation of rat pial microcirculation. The observation of the microcirculatory parameters through this window lasted for four weeks. During the experimental period, we found no inflammatory or pathophysiological reactions in BBB permeability, leukocyte behavior, or plasma velocities in the window-implanted rat brain. Moreover, the replacement of the Type I window with a Type II allowed perfusion experiments on experimental demand. Although many investigators studying cerebral microcirculation have used the CCW method, only a limited number of publications reported the use of the CCW method for long-term observation because of difficulties listed above. Therefore, our CCW may contribute to the investigation of chronic changes in the cerebral microcirculation after brain disorders or drug treatments.

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