Blood-Brain Barrier Experiments with Clinical Magnetic Resonance Imaging and an Immunohistochemical Study

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Objective: The purpose of study was to evaluate the feasibility of brain magnetic resonance (MR) images of the rat obtained using a 1.5T MR machine in several blood-brain barrier (BBB) experiments.

Methods: Male Sprague-Dawley rats were used. MR images were obtained using a clinical 1.5T MR machine. A microcatheter was introduced via the femoral artery to the carotid artery. Normal saline (group 1, n = 4), clotted autologous blood (group 2, n = 4), triolein emulsion (group 3, n = 4), and oleic acid emulsion (group 4, n = 4) were infused into the carotid artery through a microcatheter. Conventional and diffusion-weighted images, the apparent coefficient map, perfusion-weighted images, and contrast-enhanced MR images were obtained. Brain tissue was obtained and triphenyltetrazolium chloride (TTC) staining was performed in group 2. Fluorescein isothiocyanate (FITC)-labeled dextran images and endothelial barrier antigen (EBA) studies were performed in group 4.

Results: The MR images in group 1 were of good quality. The MR images in group 2 revealed typical findings of acute cerebral infarction. Perfusion defects were noted on the perfusion-weighted images. The MR images in group 3 showed vasogenic edema and contrast enhancement, representing vascular damage. The rats in group 4 had vasogenic edema on the MR images and leakage of dextran on the FITC-labeled dextran image, representing increased vascular permeability. The immune reaction was decreased on the EBA study.

Conclusion: Clinical 1.5T MR images using a rat depicted many informative results in the present study. These results can be used in further researches of the BBB using combined clinical MR machines and immunohistochemical examinations.

KEY WORDS: Animal · Blood-brain barrier · Magnetic resonance image · Immunohistochemical study · Vasogenic edema · Vascular permeability.

INTRODUCTION

The blood-brain barrier (BBB) is a membrane structure that acts primarily to protect the brain from chemicals in the blood, while still allowing essential metabolic function. It is composed of endothelial cells, a basement lamina, and perivascular end feet of astrocytic processes. The higher density restricts the passage of substances from the bloodstream. A number of studies have focused on the BBB from a physical, immunohistochemical, and radiological viewpoint. Technologic developments permit the study of specific functions of the BBB, cellular metabolism, and neurotransmission.

In experimental research involving the BBB, mice or rats (as small animals), rabbits, cats, or dogs (as medium animals), and monkeys (as large animals) have been widely used. The animal model is selected according to the purpose of the research and anatomic characteristics of the animals. We used the femoral arterial approach with the Seldinger technique under angiography. This femoral arterial approach does not give any trauma to the neck and can conserve the normal physiologic status of the head, neck, and brain.

Magnetic resonance (MR) image yields considerable amounts of useful information to researchers in BBB studies. Currently, high-field MR machines for small animals are
able to obtain good quality brain images, even in mice. However, this MR machine is very expensive. On the other hand, clinical MR machines for humans are not suitable for studies of small animals. Owing to the recent development of software and surface coils in MR technology, relatively good quality images of the brain in the medium-size animals can be obtained using clinical 1.5T MR machines. For the inherent physics of MR imaging, image quality depends on the objective volume. Thus, the larger the volume of the animal, the better the MR image quality which can be obtained.

The purpose of the present study was to determine the possibility of BBB studies using the femoral arterial approach under angiography with a clinical 1.5T MR machine and immunohistochemical examinations in rats.

MATERIALS AND METHODS

Animal preparation

All experiments were approved by our institution’s animal care committee and the current institutional regulations for use and care of laboratory animals. Sixteen male Sprague-Dawley rats weighing 250-300 g were anesthetized with an intraperitoneal injection of ketamine HCl (2.5 mg/kg; Korea United Pharm Inc., Seoul, Korea) and xylazine (0.125 mg/kg; Bayer Korea, Seoul, Korea). The rats were allowed to breathe room air spontaneously during the procedure. Their body temperatures were measured using a rectal probe (MGA-III 219; Shibuaura Electronics Co. Ltd., Tokyo, Japan) and were maintained 37 ± 1°C.

Femoral arterial approach using the Seldinger technique under angiography

The rats were placed in the supine position and immobilized by taping of the extremities. After shaving the right inguinal area, an incision was made and the right superficial femoral artery was isolated. After ligation of the distal portion of the exposed artery with 4.0 silk, a 20-gauge intravenous catheter (Insyte; Becton Dickinson, Sandy, UT, USA) was inserted into the artery. After removal of the guiding needle of an intravenous catheter, a microcatheter (Prowler 10; Cordis, Miami, FL, USA) with a guidewire (Agility 14; Cordis) was passed through the intravenous catheter into the lumen of the artery. To prevent leakage of blood through the diameter gap of the angiocatheter and microcatheter, a three-way tube was used prior to the insertion of the microcatheter. Under fluoroscopic guidance and the aid of a guidewire, the right or left common carotid artery was selected and the tip of the microcatheter was positioned at the level of the fourth cervical vertebrae.

Embolization into the carotid artery

Infusion of normal saline (n = 4, group 1)

One mL of normal saline was infused into the selected carotid artery in four rats as a control group.

Embolization with autologous blood (n = 4, group 2)

Clotted autologous blood (0.5 mL) was homogenously crushed using a homogenizer. Then, the crushed blood was infused into the microcatheter and flushed with saline in four rats.

Embolization with triolein emulsion (n = 4, group 3)

A 1 mL syringe containing 100 µL of neutral triglyceride triolein (1, 2, 3-tri [cis-9-octadecenoyl]glycerol, 99% purity, d = 0.91 g/mL; Sigma, St. Louis, MO, USA) and a 25 mL syringe containing 20 mL of saline were connected to a 3-way stopcock. The triolein emulsion was made by mixing the contents of 2 syringes via the 3-way stopcock with vigorous to-and-fro movement of the syringes for 2 minutes. The emulsified triolein (1 mL) was infused manually into the selected carotid artery for 15 seconds, followed by injection of 2 mL of saline in 4 rats.

Embolization with oleic acid emulsion (n = 4, group 4)

Emulsified oleic acid (1 mL, cis-9-octadecenoic acid; Sigma) emulsion was infused using the same technique as the triolein emulsion infusion in four rats.

MR imaging

Thirty minutes after embolization or infusion of the above-described agents into the brain, MR imaging was performed on a clinical 1.5-T MR scanner (Sonata; Siemens, Erlangen, Germany). Each rat was placed in the prone position within a pediatric MR infant restrainer, and a small field of radio-frequency coils (Flexible coil; Siemens) was placed around the rat’s head. In group 1, T1-weighted image (T1WI), T2-weighted image (T2WI), diffusion-weighted image (DWI), the apparent coefficient (ADC) maps, and contrast-enhanced T1WI images were obtained in the coronal plane. In group 2, T1WI, T2WI, DWI, ADC maps, perfusion-weighted image (PWI), and contrast-enhanced T1WI were obtained. In groups 3 and 4, T1WI, T2WI, DWI, ADC maps, and contrast-enhanced T1WI were obtained.

For T1WI, the following scan parameters were used: repetition time (TR) = 320 ms, echo time (TE) = 20 ms, section thickness = 4-5 mm with a 0.1 mm gap; field of view = 50-70 mm; and for excitations, an acquisition matrix = 256 × 166. For T2WI, the following scan parameters were used: TR = 2,000 ms, TE = 65 ms, and for excitations, an
acquisition matrix = 256 × 166. DWI was performed using an echo-planar sequence. The imaging parameters of DWI were as follows: TR/TE = 1600/135, field of view = 100 mm, phase encoding steps = 128, section thickness = 4 mm with a 0.1 mm gap, and acquisition matrix = 96 × 160. The diffusion-sensitizing gradients were oriented in the three axes with b values of 0 and 1000 sec/mm². The ADC map was obtained simultaneously using standard software. For PWI, the imaging parameters were as follows: TR/TE = 1000/89, section thickness = 4 mm, field of view = 90-100, acquisition matrix = 128 × 112, excitation number = 1, acquisition time = 1 sec, and total number of images = 40. Post-processing of PWI was performed in the MR workstation. For contrast-enhanced T1WIs, 0.2 mmol/kg gadopentate dimeglumine (Magnevist; Schering, Germany) was injected intravenously.

Histologic and immunohistochemical examinations

Histologic examination in autologous blood embolization group

After MR imaging, the rats were immediately sacrificed by intravenous injection of sodium thiopental, and the brain was then removed. Brains were sliced with coronal sections (2-mm thick slices from the anterior 3.5 mm to the anterior 13.5 mm) using a homemade brain matrix. One slice was incubated at 37°C in 2% 2, 3, 5-triphenyltetrazolium chloride (TTC; Sigma) solution for 20 minutes. The brain sections were then fixed with 10% formalin and processed with hematoxylin-eosin (H-E) staining for microscopy.

Fluorescein isothiocyanate (FITC)-labeled dextran image in oleic acid emulsion embolization group

For evaluation of vascular permeability, a FITC-labeled dextran image was obtained in the rats of group 4. Heparin (100 units/kg in 1 mL of saline) was injected into the tail vein. Immediately after heparin injection, 3 mL of FITC-labeled dextran (2.5 mg/mL, 71 KDa; Sigma) was slowly infused into the common carotid artery. The right atrium was opened and the descending circulation was clamped off during infusion. Within 2 min, the brains were removed and immediately immersed into the fixative solution (20% sucrose in 4% paraformaldehyde). One day later, brain tissues were frozen at -70°C and sections (40 µm) were cut with a cryostat at -20°C.

Endothelial barrier antigen (EBA) immunoreactivity in oleic acid emulsion embolization group

EBA immunoreactivity was examined in the rats of group 4. The rats were transcardially perfused with normal saline. The brain was removed and fixed in 40 g/L of paraformaldehyde in PBS. The brain was then embedded in paraffin, and cut at a thickness of 6 µm. After deparaffinization and antigen retrieval by boiling in 10 mM citric acid solution (pH 6.0), sections were treated to remove endogenous peroxidase activity with H₂O₂ (0.3 mL/L in PBS) and blocked with normal serum (50 mL/L in PBS) and bovine serum albumin (10 mg/L in PBS). The sections were incubated in the primary antibody against EBA (1 : 1000; Monoclonals, Lutherville, MD, USA). The sections were then incubated in a biotinylated secondary antibody (1 : 200; Vector Labs, Burlingame, CA, USA) and detected using the Vector ABC kit (Elite Vectastain ABC kit; Vector Labs, Burlingame, CA, USA). The reaction was visualized using DAB solution (Vector Labs, Burlingame, CA, USA).

RESULTS

MR images in control group

T1WI, T2WI, DWI, and ADC map images of a normal rat brain were obtained. The T2WI depicted the gray and white matter better than the T1WI. DWI and ADC map images demonstrated moderate signal intensity of the parenchyme. Gray-white matter differentiation was poorer in the present cases than in human brains. DWI and the ADC map images were inferior to those of human brains (Fig. 1).

MR images in autologous blood embolization group

MR and TTC images of rat brains were obtained 1 hour after injection of autologous blood into the right carotid
artery. MR images obtained after injection of clotted autologous blood into the carotid artery revealed typical findings of acute cerebral infarction. Perfusion defects were noted on the PWI. The infarcted hemisphere showed a isointensity on T2WIs, hyperintensity on DWIs, and hypointensity on the ADC map images. On the PWI and a plot of the time-to-signal curve, the lesion showed no descending curve due to input of the contrast media or an ascending curve after signal drop. The mean transit time (MTT) image presented increased transit time in the lesions. The relative cerebral blood volume (rCBV) image revealed a slightly decreased blood volume in the lesion. The TTC photograph showed decreased staining of the lesion (Fig. 2).

MR images in triolein emulsion embolization group
The MR images obtained after infusion of triolein emulsion into the carotid artery presented vasogenic edema and contrast enhancement representing vascular damage. The T1WI showed isointensity of the lesion. On the contrast-enhanced image, the lesion showed mild contrast enhancement. However, the DWI and the ADC map image revealed isointensity of the lesion (Fig. 3).

MR images and immunohistochemical study of oleic acid emulsion embolization group
Injection of oleic acid into the carotid artery caused vasogenic edema on MR images and leakage of dextran on FITC-labeled dextran images, presenting as increased vascular permeability. The immune-reaction decreased on the EBA study. The embolized hemisphere revealed contrast enhancement on the contrast-enhanced image, mild hyperintensity on the T2WI, minimal hyperintensity on the DWI, and isointensity on ADC map image. No leakage was seen in the normal contralateral cortex. FITC-labeled dextran leakage was observed in the lesion hemisphere. EBA immunohistochemical detection of vessels in the rat cortex revealed reductions in the lesion hemisphere compared to the normal hemisphere (Fig. 4).

DISCUSSION
Stroke has been extensively studied in clinical practice and experimental research by means of MR images with ever-emerging new technologies, such as DWI, PWI, and
ADC maps. More recently, different PWI-derived parameters, such as the rCBV, the relative cerebral blood flow (rCBF), the relative MTT, and the time to peak (TTP) have been applied to quantify the perfusion deficit and to evaluate the temporal infarct growth in acute stroke either in patients or in small animals with high field strength MR spectrometers. Using the clinical MR machines, patients with infarction, and middle-sized or small-sized animals have been frequently studied. However, the best methodology for infarction models has been debated and is still under investigation. Recently, the effect of new thrombolytic agents on limiting cerebral infarct size was evaluated with a clinical 1.5T MR scanner. We investigated the feasibility of a clinical 1.5 T MR scanner for the evaluation of cerebral infarction with small animals in the present study, and it is our opinion that various images obtained by clinical MR machines are useful in on-going research.

In the present study, embolization of a small amount (0.5 mL) of crushed autologous blood clot was successful for creating a cerebral infarction in rats. The lesions showed typical MR imaging findings of cerebral infarction; specifically hyperintensity on DWI and hypointensity on the ADC map were noted. On PWI, rCBV, and rCBF decreased and the MTT increased.

There have been several ischemic animal models. Middle cerebral arterial occlusion (MCAO) using rats has been performed since 1957. MCAO using electrocauterization of the middle cerebral artery after craniectomy is invasive and has a weak point of non-reperfusion. MCAO using a clip or phototherapeutic occlusion can be useful; however, these techniques require a craniectomy. Introducing a 4-0 nylon intraluminal suture into the cervical internal carotid artery has been used for 20 years, which is less invasive and does not require a craniectomy. This MCAO technique has been advanced to generate a relatively regular lesion size or site. The technique, however, is not easy to perform and can be disappointing when the same artery is found in the study of the intra-arterial drug infusion after creating the cerebral infarction. In the present study, we used an intraarterial microcatheter insertion technique, which is relatively easy and less invasive. With this technique, we can follow additional studies using the same micro-catheter in the same artery. However, this technique is limited by the requirement of a fluoroscopic unit.

Experimental studies into cerebral fat embolism have revealed that the embolized lesions are demonstrated as the following 2 types because of vasogenic and cytotoxic edema in the hyperacute stage: type 1 lesions contain both vasogenic and cytotoxic edema, whereas type 2 lesions are due to vasogenic edema only. Reversibility of the embolized lesions in the clinical cerebral fat embolism may depend on the type of the lesion.

Animal research for the effect of triolein or oleic acid on the BBB has been performed using cats, and the rat was first used in the present study. A full-grown canine brain is about 4 × 3 × 2.5 cm in size, and is suitable for research of the BBB using clinical 1.5T MR machines. However, MR imaging or histologic studies have not revealed the mechanism by which triolein affects the endothelium resulting in increased vascular permeability. To resolve this problem, an immunohistochemical approach with MR imaging is needed. However, antibodies for canine brain have not been
developed, thus it is not possible to study the mechanism with canine brain. Nevertheless, immunohistochemical studies using rodents are popular and diverse. Among the rodents, the rat is better than the mouse in MR imaging because of the larger brain. In the present study involving embolization with triolein and an oleic acid emulsion, increased vascular permeability was clearly visible on contrast enhanced T1WIs. These results were the same as the results obtained using cat models. In the present study of EBA (known as an endothelial marker representing the characteristics of the BBB), embolization with oleic acid emulsion showed an inflammatory reaction on the endothelium of the brain. These MR and EBA results can be applied to further MR studies and various immunohistochemical studies involving the mechanism of lipids on vessels.

The pathogenesis of the cerebral effects of fat embolism is not well understood. Peliter reported the toxic properties of neutral fat in the lung, suggesting that mechanical obstruction of the lung capillaries by neutral fat caused the disturbed physiology. The classic mechanical theory postulates that triglyceride particles from injured adipose tissue enter the circulation and obstruct the pulmonary vessels. The mechanical theory cannot adequately explain the clinical presentation; additional mechanisms have therefore been suggested. The concept of chemical action has not been substantiated or refuted and is still under investigation. According to this theory, local hydrolysis of emboli occurs by pneumocyte lipase, together with excessive mobilization of free fatty acids. Both triolein and oleic acids are widely used in creating models of fat embolism, especially those in the lung. Considerable evidence shows that oleic acid is more toxic than triolein. Non-esterified oleic acids bind albumin; thus, they are non-toxic. However, free fatty acids are highly toxic to all tissues, especially capillaries, because their binding to albumin is prevented by a combination of inadequate mixing and the size of the globule. In the study of the BBB, oleic acid showed more prominent vascular permeability than triolein. Thus, the mechanism of triolein is thought to be different from that of oleic acid, and immunohistochemical studies may be helpful to evaluate the different mechanisms.

For BBB studies, the external carotid arterial approach is generally used. For medium or large animals, this approach is easy; however, the diameter of the external carotid artery is too small to catheterize for infusion of drugs and the narrow surgical field of the neck, especially in small animals, makes the external carotid arterial approach difficult to perform. The middle cerebral artery is also directly used after removal of the eyeball. These methods are difficult and need a microsurgical apparatus and a significant amount of time for surgery. Thus, we reasoned that the femoral arterial approach with the Seldinger technique using an adequate needle and a microcatheter under angiography is easier than the external carotid arterial approach. However, the femoral arterial approach is not common in studies of the BBB.

### CONCLUSION

In the present study, we performed various MR imaging techniques using a clinical 1.5T MR machine and rats for the combined immunohistochemical study for experiments, including cerebral infarction induced by embolization of autologous blood, and vascular permeability changes caused by embolization with triolein and oleic acid emulsion. The clinical MR machines and immunohistochemical studies may be applicable in combination with further experiments for the BBB.

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