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Ph.D. Dissertation of Medicine

A Rabbit Model for Embolic Infarct  
Potentials of Injectables Using  
Ultrasound-guided Carotid Artery  
Puncture

약물의 혈전생성 위험성 평가를 위한  
초음파 유도하 경동맥주입 가토 뇌경색 모델

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# A Rabbit Model for Embolic Infarct Potentials of Injectables Using Ultrasound-guided Carotid Artery Puncture

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# Abstract

## Objective

To evaluate the *in vivo* thrombogenicity of agents delivered to arteries, it is necessary to use an appropriate animal model. The aim of this study was to establish a minimally invasive technique for inducing non-selective occlusion of the cerebral artery in rabbits via the common carotid artery using real-time ultrasound guidance, which can offer both efficiency and minimizes discomfort to the animals.

## Materials and Methods

This study involved 30 New Zealand white rabbits, with 20 in the experimental group and 10 in the control group. Before the procedure, each rabbit underwent a neurologic examination and gait evaluation. Anesthesia was induced using intramuscular alfaxalone (6 mg/kg) and xylazine (5 mg/kg), and the bilateral common carotid arteries were located using ultrasound. A 23G needle was then inserted into either the right or left common carotid artery, and the control group received 2 mL of normal saline while the experimental group received a 2 mL suspension of PVA embolization particles (225–350 microns in size). The animals were monitored for immediate reactions and underwent MRI scans, followed by a neurologic examination 24 hours later.

## **Results**

In 7 of the 30 rabbits, 2 in the control group and 5 in the experimental group, the administration of either normal saline or the embolic agent failed. Among the successfully injected 15 experimental animals, 14 showed neurologic impairment or deceased, whereas 1 animal did not show significant neurologic deficit. The MRI of 4 experimental animals showed detectable cerebral infarction on diffusion-weighted imaging. None of the 8 control animals showed neurologic abnormality and their brain MRI was normal.

## **Conclusion**

This minimally invasive rabbit model is technically feasible and competent to show thrombogenicity of an injectable agent and consequent in vivo neurologic outcome.

**Keyword:** Thrombogenicity, animal model, rabbit model, embolic infarct, ultrasound guidance.

**Student Number:** 2017-37215

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# I. Introduction

## 1. Study background

Lumbar transforaminal and interlaminar epidural steroid injections are widely used to treat patients with radiculopathy. These therapeutic interventions for lumbar spine have been reported to be clinically effective for short- and long-term pain relief. Although this image-guided treatment is a minimally invasive technique, rare serious adverse events have occurred. Laemmel et al. have performed an *in vivo* study to elucidate the underlying mechanism of vascular obliteration induced by particulate steroids(1). They found that particulate steroids had an immediate and massive effect on microvascular perfusion, which was due to the formation of red blood cell aggregates associated with the alteration of these cells of into spiculated form. In 2011, the U.S. Food and Drug Administration announced a change in the safety labeling for triamcinolone acetonide, warning that triamcinolone acetonide "is not recommended" for epidural use and "spinal cord infarction, paraplegia, quadriplegia, cortical blindness, and stroke (including brainstem) have been reported after epidural administration(2-4)."

In order to evaluate the safety and to assess the

thrombogenicity of certain agents, a preclinical animal model is necessary. Whilst several studies have attempted to generate suitable models, the conducted experiments demonstrated localized changes in vessels or tissues rather than neurologic or pathophysiologic outcomes (1, 5, 6). Although, study of Laemmel et al. demonstrated effect of a particulate steroid in *in vivo* environment by visualizing alteration of microvascular perfusion of mouse cremasteric muscle, it did not attempted to reproduce neurologic or pathologic outcomes in animal(1). Silva et al. performed a study evaluating thrombogenic effect on venous structure of rat model(5). This study was also showed the localized effect on administered vein. Furthermore, these studies required skin incisions and tissue dissections of experimental animals to approach the targeted vessels and to evaluate results. Thus, the development of a minimally invasive animal model that can reproduce neurologic outcomes is key for future investigations. However, to the best of my knowledge, no such animal model is available to date.

## **2. Purpose of Study**

The purpose of this study was to establish a preclinical model of non-selective cerebral artery embolization in rabbit to

evaluate the thrombogenicity of injection agents with minimally invasive procedures.

## II. Materials and Methods

### 1. Animals

Thirty female New Zealand white rabbits (weight range, 3.5–4.0 kg at the beginning of the study) were involved in this study. Rabbits were kept in individual cages in a humidity- and light-controlled (12-h light/12-h dark cycle) environment, temperatures were maintained at  $21 \pm 2$  °C, and animals were provided with standard laboratory chow and had *ad libitum* access to drinking water. All animal experiments were approved by the Regulations of Animal Care and Use Committees of Seoul National University Bundang Hospital (approval number: BA1608–206/050–01). All procedures were performed in accordance with the ARRIVE guidelines and the Guidelines for Animal Care and Use.

### 2. Experimental design

Animals were divided into two groups based on randomization: (1) 20 rabbits were allocated to the experimental group, which received an injection of permanent embolic material, and (2) 10 rabbits were allocated to the control group and received the same volume of normal saline solution.

### **3. Preparation of emboli**

The embolic agent used consisted of 250–355- $\mu\text{m}$  polyvinyl alcohol particles (Contour, Boston Scientific, Marlborough, MA, USA). The agent was suspended in a mixture of 10 mL of normal saline and 10 mL of nonionic contrast agent. A three-way stopcock was attached to the reservoir syringe to agitate the particles thoroughly prior to injection to avoid clumping of the particles.

### **4. Neurologic examination**

Each rabbit received neurologic examination before anesthesia and 24 hours following the procedure, allowing adequate recovery from anesthesia. The four-point neurologic grading system, which was used in the study of Endo et al., was adapted, with the addition of a maximum grade 5 corresponding to convulsive movement and death(7). Neurologic deficits were scored by observing the animals on a smooth rubber mat on the flat floor. Symptoms such as paresis of the legs or abnormal gait (circling movement or difficulty walking) were observed: grade 1: no neurological deficit; grade 2: minimum or suspicious neurological deficit; grade 3: mild neurological deficit without abnormal

movement; grade 4: severe neurological deficit with abnormal movement; grade 5: convulsive movement or death. This method has been successfully used previously in studies using rabbit models(8).

## **5. Anesthesia**

All rabbits were premedicated with an intramuscular injection of 0.5 mg atropine and anesthetized with an intramuscular injection of alfaxalone (6 mg/kg; Alfaxan, Jurox Pty. Ltd., Rutherford, NSW, Australia) and xylazine (5 mg/kg, Rompun, Bayer, Leverkusen, Germany) to the thigh. Anesthesia was maintained with a single intramuscular injection of alfaxalone (3 mg/kg) during transport from the laboratory to the Magnetic resonance (MR) scanner.

## **6. Procedures**

Upon anesthesia, the fur of the anterior neck was removed using an electric clipper, from the sternal notch to the edge of the mandible (Figure 1). The bilateral common carotid arteries were identified using the iU23 ultrasound scanner (Philips Healthcare, Bothell, WA, USA) and linear array transducer (L15–7io). The

larger of the two carotid arteries was punctured using a 23-gauge winged infusion set under ultrasound guidance with a transverse approach. Once the needle was introduced into the arterial lumen, the embolic suspension or normal saline were administered after full regurgitation of blood to avoid an air embolism. Administration of the agent or saline was monitored with ultrasound (Figure 2). In the experimental group, 2 mL of the embolic suspension were slowly injected over 5 seconds followed by 2 mL of normal saline. The control group was administered an injection of 4 mL of normal saline. After adequate compression of the carotid artery (for the prevention of hematoma and significant blood loss), the animals were observed for the immediate neurologic manifestation, such as convulsive movement or abnormal respiration, for 10 min.

In case of a puncture failure, the contralateral carotid artery was used for a second attempt after compression of the initially targeted artery. Puncture failure of both carotid arteries was considered as "puncture failure."

## **7. MR imaging acquisition**

Rabbits were transferred to the MR unit using individual portable cages within 30 min after the injection. Using a 32-channel phased array sensitivity encoding head coil, MR imaging

was performed on the anesthetized rabbits with a 3.0 Tesla Philips MR scanner (Ingenia, Philips Healthcare, Best, The Netherlands). Each rabbit was scanned in dorsal recumbency with the neck and head in an extended position. The rabbits were allowed to breathe spontaneously. The following parameters were used for acquisition of the multi-shot echo-planar imaging diffuse-weighted imaging (DWI), with image reconstruction using image-space sampling (IRIS) functions with b-values of 0 and 1000 s/mm<sup>2</sup>, and 3 orthogonal directions of diffusion gradients: echo time (TE) = 73 ms, repetition time (TR) = 6155 ms, slice thickness = 2 mm, interslice gap = 2.2 mm, number of averaging (NSA) = 2, bandwidth = 936 Hz/pixel, echo train length = 35, field of view (FOV) = 8.0 × 8.0 cm, and matrix = 80 × 80. In addition to DWI, oblique coronal turbo spin echo T2-weighted image (TR = 3000 ms, TE = 80 ms, NSA = 1, FOV = 8.0 × 8.0 cm, matrix = 80 × 80, slice thickness = 1.667 cm) and three-dimensional fluid attenuated inversion recovery images (TR = 4800 ms, TE = 438 ms, NSA = 2, FOV = 8.0 × 8.0 cm, matrix = 80 × 80, slice thickness = 0.8 cm) were obtained.

## 8. Imaging interpretation

Two radiologists (with 8 and 14 years of experience,



respectively) reviewed the MRI. Any of measurable conspicuous parenchymal lesions were assessed for the likelihood of infarction. A lesion with confirmative findings of infarction (hyperintense on DWI with corresponding low value on apparent diffuse coefficient map) was determined as true lesion, through consensus agreement.

## 9. Statistical analyses

The normality of the distribution of the data was evaluated by using the Kolmogorov–Smirnov test. The Student's *t*-test was used to compare the mean weight of the rabbits. P values less than .05 were considered to indicate a statistically significant difference. Data were analyzed with SPSS software (version 22.0; IBM, Armonk, NY, USA).

## III. Results

### 1. Ultrasound–guided carotid artery puncture

In 7 (23.3%) of the 30 rabbits included in this study, puncture of the bilateral common carotid artery failed. In control group, 2 (20%, 2 out of 10) cases were failed to be administered with normal saline. While, 5 (25%, 5 out of 20) cases from experimental group were failed to be administered with embolic agent. Necks of these seven rabbits with failed carotid artery puncture were properly compressed to prevent larger hematoma during procedure. All of them were well awake from anesthesia and showed no neurologic abnormality during 2 weeks of observation in cage.

The mean weight of rabbits in which the puncture failed was  $3.41 \pm 0.18$  kg, whereas that of the successfully punctured counterparts was  $3.75 \pm 0.29$  kg, which was significantly different ( $P = 0.019$ ).

### 2. Control group

All 8 control animals, successfully injected with normal saline, well awake from anesthesia. Their brain MRI did not show any signs of infarction. They did not show any neurologic deficit or

sign of suffering during 2 weeks of observation in cage.

### **3. Experimental group**

The neurologic outcome after 24 h following injection is summarized in Table 1. Among 15 successfully injected experimental animals, 14 rabbits showed grade 4 or greater neurologic deficits and 1 animal showed no deficits. Among the 12 rabbits with grade 5 neurologic deficits, 3 rabbits showed abnormal respiration, similar to Cheyne–Stokes breathing, and died immediately following the injection; another 3 died during the MR scan; the remaining 6 animals showed convulsive seizure–like movements (Figure 3) soon after administration of the embolic agent. All animals with seizure–like movements died within 24 h following the injection. Two rabbits had hemiparesis showing abnormal gait and died 3 and 5 days after the experiment. Excluding the rabbits that died before and during the MRI scan, 9 animals underwent a complete MRI scan. In 4 cases, the area of cerebral infarction was detectable and confirmative on DWI, with corresponding low value on apparent diffuse coefficient (ADC) map (Figure 4 and 5). One rabbit had small hyperintense focus on DWI of the cerebellum, which was questionable for the true infarcted area (Figure 6).

## IV. Discussion

In this study, we developed and tested a new rabbit model to evaluate thrombogenicity of injectable agents under ultrasound guidance. As expected, the majority of rabbits showed severe neurologic deficits, whereas all control animals were unaffected. Rare but serious adverse events, including brainstem, cerebellum, or spinal cord infarction, have been reported following epidural steroid injection, especially with particulate steroids (9–11). There is also some experimental evidence corroborating these observed complications (1, 12, 13). Radiculomedullary arteries are at risk of incidental puncture during epidural injection based on their anatomical proximity to the neural foramen, cervical through sacral. Radiculomedullary arteries along with vertebral arteries and their anastomoses, can provide a direct anatomic pathway to the spinal cord, brainstem, or brain (14).

Some physicians have faith that particulate steroids are still more effective than non-particulate steroid, even though there is no clinical evidence proving their effectiveness. There have been several studies comparing these two types of steroids, although most of them failed to show statistically significant outcomes (15). Nevertheless, patients treated with particulate steroids tend to have

better self-reported outcomes and experience longer pain-free periods (16). It was hypothesized that particulate steroid preparations may provide a local depot effect, with constant release of the active drug from the administration site over a longer time period compared to non-particulate steroids. An animal study performed by Abraham et al. used dogs to compare the duration of action of intramuscularly administered triamcinolone acetonide, a particulate glucocorticoid, to that of intravenously administered triamcinolone acetonide dihydrogen phosphate, a non-particulate steroid. They found that intramuscular administration of the particulate glucocorticoid had a duration of action of up to 4 weeks, which was much longer than a similar intravenous dose of its analogue, the non-particulate steroid(17).

Unfortunately, following an announcement by the U.S Food and Drug Administration in 2011, triamcinolone acetonide, a particulate steroid, has been contraindicated for epidural injection for its serious and permanent complications, including blindness, stroke, paralysis, and death(3). Based on these circumstances, the development of novel forms of injection agents or drug vehicles was suggested, which can deliver steroids and have cumulative effects within the tissue for longer periods than non-particulate steroids, without any adverse effects. Whilst Laemmle et al. demonstrated

the mechanism of arterial obliteration in their experiments, this study aimed to establish a preclinical model that can reproduce the intra-arterial reaction and evaluate the thrombogenicity of the injectable(1).

There are several animal models that have been used to evaluate thrombogenic risks. Silva et al. used femoral veins of rats to elucidate the mechanism by which ultrafine particles interact with the vascular system (5). Both femoral veins were exposed and cannulated, and the ear vein was observed for potential thrombosis following administration of rose bengal. This study used the venous system and required invasive procedures. A mouse carotid artery was used in a study by Kawasaki et al., demonstrating enhanced thrombogenicity of high Factor VIII plasma activity (6). Although their study was aimed to assess intra-arterial reaction, a midline dissection needed for the exposure of the carotid artery of the mouse.

In contrast to these previous studies, proposed thrombogenicity animal model does not require major invasive procedures, including skin incision, tissue dissection or wound closure. Any injection material can be administered by single puncture of carotid artery under ultrasound guidance and manifest its thrombogenic effect in cerebral artery. Since there is no surgical

wound left on animals, these expected thrombogenic effect can be evaluated on immediate imaging or in-life neurologic examination. Immediate MRI scan following the injection would prevent sacrifice of the animal and allow long-term follow-up for the neurologic or functional outcomes.

Despite the intention of this study to develop a minimally invasive animal model, there were several animals died prematurely. However, in the control group where no embolic material was used, there were no cases of mortality and no neurologic deficits observed. Based on this fact, it can be inferred that the procedures utilized in the animal model are not inherently fatal. Instead, it is presumed that the administration of excessive dosage led to neurological outcomes or fatalities that were more severe than intended. Indeed, it is reasonable to think that through dosage adjustment, it may be possible to induce milder neurological outcomes in the future. By carefully calibrating the dosage of the administered substances, researchers can aim to achieve a balance where the desired neurological effects are observed without causing fatal or severe outcomes. This approach allows for fine-tuning the animal model and optimizing it for the intended research objectives, ultimately improving the safety and ethical considerations of the study.

There are several limitations to this study. First, there were puncture failures (23.3%). This was in part due to technical difficulties based on the relatively small size of rabbit carotid arteries compared to the human vascular structure. In fact, 5 cases of failure occurred in initial 15 rabbits and only 2 cases occurred among the subsequent 15 rabbits. It can be assumed that the puncture technique could be improved through repetition, considering the operator's learning curve. Moreover, in retrospective analysis of animal factors, animals with puncture failure weighed significantly less than those in which the procedure was successful. It is assumed that the carotid arteries of lighter rabbits tend to be smaller in diameter and have a vulnerable arterial wall, which can be easily perforated with a slight touch of a needle tip (Figure 7). Accordingly, a lower failure rate is expected in future studies through the utilization of larger animals and the technical improvement of the operator. Second, although neurologic deficits was observed in some animals, not all rabbits showed significant positive findings on MR imaging. There are several possible explanations for these negative MR imaging findings. Most of the symptomatic rabbits underwent MR imaging within 2 hours after administration of the embolic material. In the human brain, DWI is known to be less sensitive for detecting hyperacute



infarction(18, 19). Moreover, some of the rabbits died before or during the MR scan, implying that other brain regions, apart from the infarcted area, could be affected by a lack in normal blood supply to rest of the brain parenchyma. Another potential explanation is the lack of an ideal MR imaging protocol for small animals. Although this study used a protocol for rabbits devised by neuroradiology specialist and MR technician, sometimes diagnosis of infarction was challenged by unexpected artifact and suboptimal image quality. MRI protocol more suitable for small animals should be established in future studies. Further studies will be needed with different doses of embolic material that may be less fatal for the animals. Moreover, subsequent studies with particulate steroids are warranted.

## V. Conclusion

This study proposes a minimally invasive rabbit model for the evaluation of intra-arterial thrombogenicity under ultrasound guidance. This model is competent to demonstrate intra-arterial thrombogenic effect of an injectable agent and consequent in vivo neurologic outcome. Further improvements in the imaging protocol will likely corroborate the usefulness of this new animal model.

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# Table

Table 1. Neurologic Outcome

Grade	Experiment	Control
1	1	8
2	0	0
3	0	0
4	2	0
5	12	0
Failed puncture	5	2
Total	20	10

## Figures

Figure 1. Rabbit prepared for ultrasound-guided carotid artery puncture.





**Figure 2. Puncture of the carotid artery and administration of the embolic agent.**

(A, B) The right common carotid artery (letter "C") of the rabbit before puncture. (C) Puncture of the carotid artery with 23-gauge winged infusion set needle (arrowheads). (D) Monitoring of the embolic agent (arrow) administration

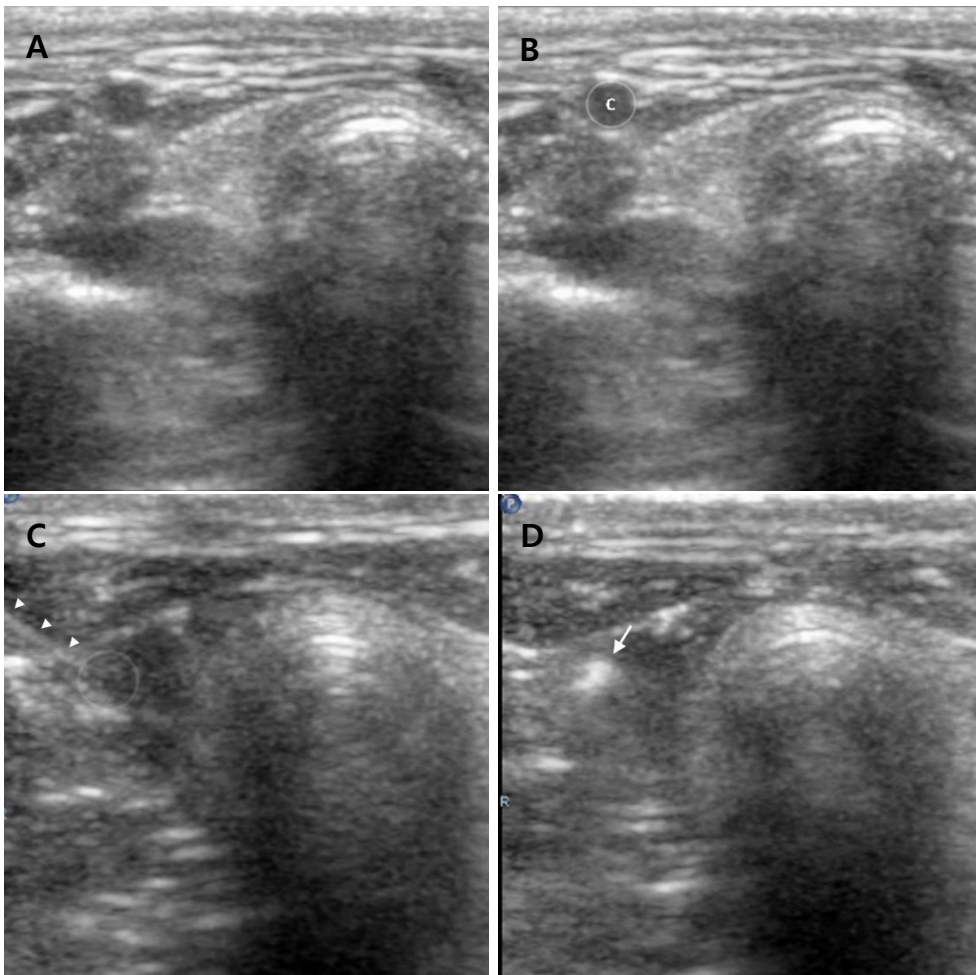


Figure 3. Rabbit showing seizure-like movement after injection of PVA particle on carotid artery.



**Figure 4. MR imaging of a rabbit showing an infarcted lesion.**

(A) Diffusion weighted imaging showing infarction (arrowhead) at the left temporal lobe. (B) Corresponding apparent diffusion coefficient map.

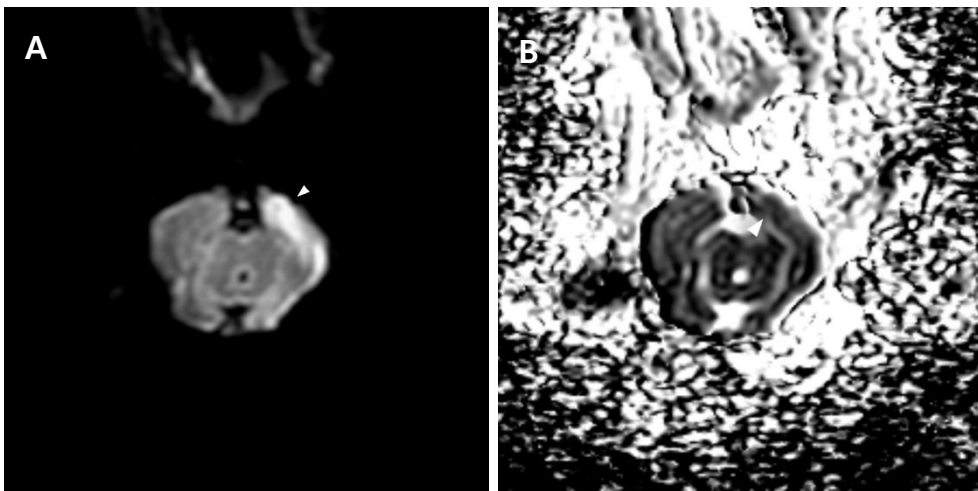


Figure 5. MR imaging of a rabbit showing an infarcted lesion.

(A) Diffusion weighted imaging showing infarction (arrowhead) at the right parietal cortex. (B) Corresponding apparent diffusion coefficient map.

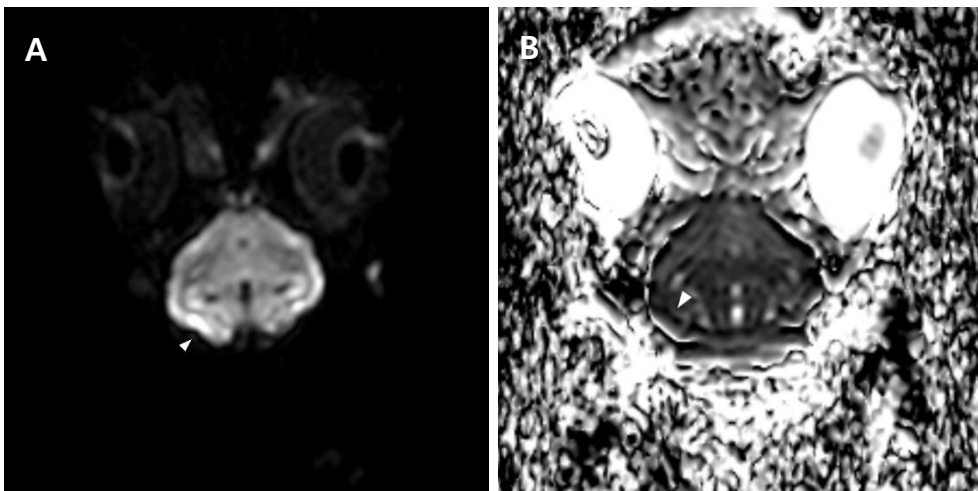


Figure 6. Magnetic resonance imaging of a rabbit showing a questionable infarcted area.

(A) Diffusion weighted imaging showing suspicious infarction (arrowhead) at the right cerebellum. (B) Corresponding apparent diffusion coefficient map.

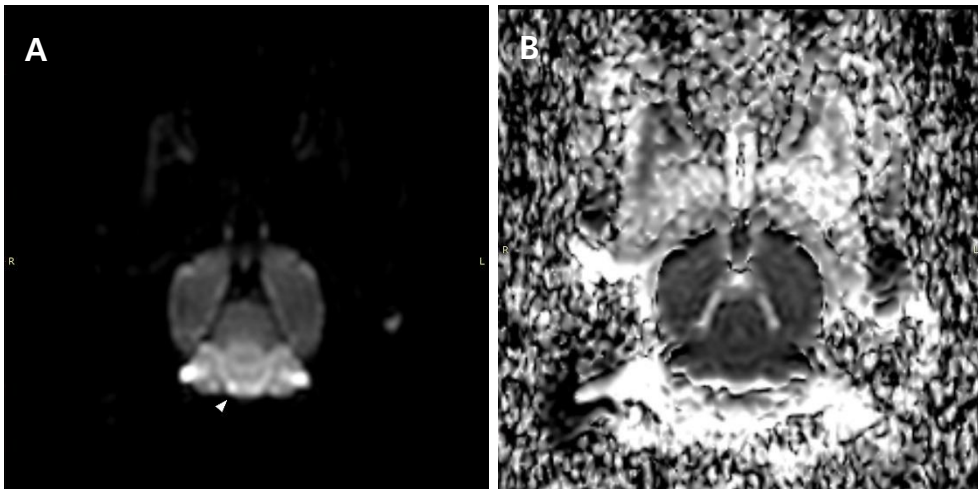
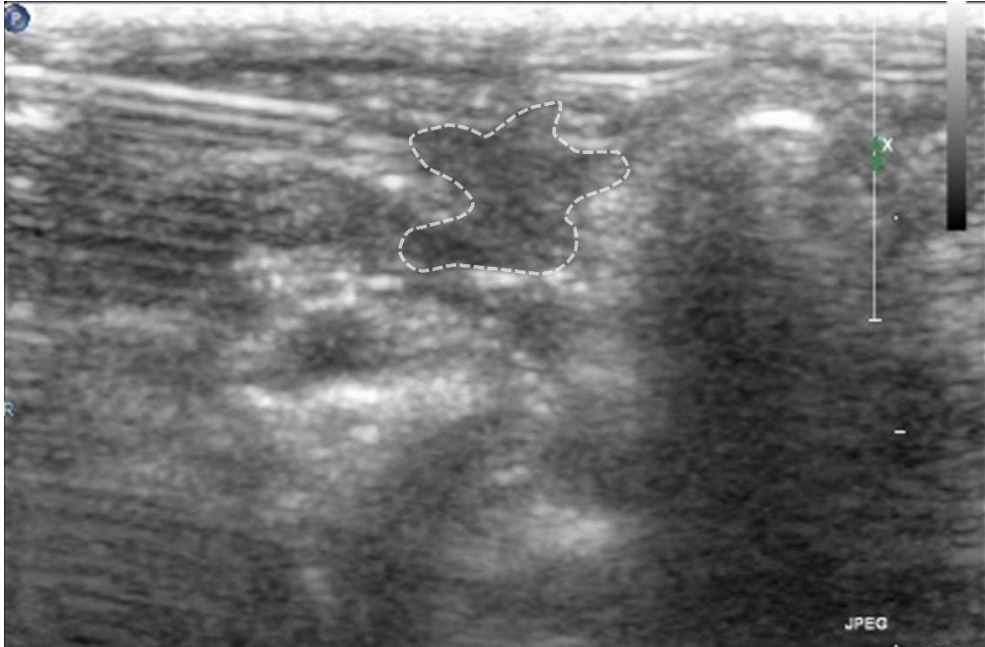


Fig. 6. A large hematoma (dotted area) formation after failure of a puncture attempt.



## 국문 초록

목적: 동맥내 약제의 혈전생성위험성 평가를 위해서는 적절한 전임상 모델이 필요하다. 본 연구의 목적은 최소 침습적인 기술로 가토의 경동맥을 통해 비선택적 색전 또는 뇌경색을 유발시키는 동물 모델을 확립하는 것이다.

재료와 방법: 본 연구는 실험군 20마리와 대조군 10마리의 뉴질랜드 화이트 래빗 30마리를 대상으로 하였다. 모든 가토는 실험에 앞서 신경학적 검사와 보행 평가를 받았다. 마취 후, 우측 또는 좌측 경동맥 중 하나에 23 gauge 주사침을 삽입하고, 대조군에는 생리식염수 2mL을, 실험군에는 PVA 입자 (225-350 마이크론)를 혼합한 2mL의 분산액을 주입하였다. 동물들의 즉각적인 반응을 모니터링하였으며, 이 후 자기공명영상 검사를 시행하였다. 실험 24시간 후, 추적 신경학적 검사로 신경학적 결함을 평가하였다.

결과: 30마리 중 7마리(대조군 2마리, 실험군 5마리)에서는 경동맥 주입에 실패하였다. 성공적으로 주입된 실험군의 15마리 중 14마리는 신경학적 장애가 나타나거나 사망하였으며, 1마리에서는 유의한 신경학적 결손을 보이지 않았다. 실험군 동물 4마리의 자기공명영상 확산강조영상에서 뇌경색 병변이 확인되었다. 생리식염수를 주입한 대조군 8마리에서는 어떠한 신경학적 이상도 보이지 않았으며 뇌 자기공명영상 또한 정상이었다.

결론: 초음파를 이용한 최소 침습성 가토 모델은 기술적으로 실행 가능하였으며, 경색입자 주입에 따른 *in vivo* 신경학적 결함 및 영향을 보여줄 수 있

었다. 향후 약물의 혈전생성가능성을 확인하는 전임상 모델로 이용될 수 있을 것으로 보인다.

**주요어:** 혈전생성가능성, 전임상 모델, 가토 모델, 색전성 경색, 초음파유도

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