PR2

Preclinical studies for applying BNCT to veterinary medicine

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In this research project, six research projects were included. The four researches were planned by veterinarians. Details of six projects are referred to each progress report.

P2-1. The Basic Study Aimed at Performing the Boron Neutron Capture Therapy for Canine Hemangiosarcoma

In this study project, the possibility of applying BNCT to canine hemangiosarcoma (HAS) using the three canine HSA cell lines were investigated. In conclusion, BNCT can be applied to canine HSA because enough ^{10}B uptake and the anti-tumor effect of BNCT were confirmed in this study.

P2-2. Preparation of Rabbit-Dog Chimeric Anti-BSH Antibody and Comparison with Caninized Anti-BSH Antibody

In this study, since the yield and purity of caninized anti-BSH antibodies were low, creating a rabbit-dog chimeric anti-BSH antibody in which only the constant region was canonized were attempted. Since chimeric anti-BSH antibody (chBSH IgG) has high binding ability and excellent production efficiency, usage of chBSH IgG to produce a bispecific antibody against Her2 and BSH will be attempted to estimate an antitumor effect on canine cancer cells.

P2-3. Preparation of immunoliposomes comprising hydrophobic boron cluster via exchanging reaction.

In this study, applicability of immunoliposome comprising highly concentrated carborane that is prepared by exchanging reaction as a boron agent for BNCT was investigated. The developed im-munoliposome exhibited higher BNCT activity *in vitro* study and high accumulation in the tumor tissue *in vivo* study. The immunoliposome system are promising candidate for BNCT.

P2-4. Investigation of the relationship between the therapeutic efficacy of boron neutron cap-ture therapy and the residence of boron drugs in tumors

In this study, the effects of tumor tissue diversity (stromal volume and blood flow distribution) on BPA retention and response to BNCT were investigated. It was observed that BNCT inhibited tumor growth regardless of the amount of stroma, suggesting that macroscopic BPA uptake alone does not predict therapeutic efficacy.

P2-5. The basic study of the effect of BNCT for squamous cell carcinoma of the head and neck in dogs

In this study, the efficacy of boron neutron therapy using a tumor-bearing model of canine tonsillar squamous cell carcinoma (TSCC) was investigated. Tumor growth curves for each group indicates that BNCT has the potential to be a treatment for NTSCC.

P2-6. Boron Delivery to Brain Cells via Cerebrospinal Fluid (CSF) Circulation for BNCT in a Rat Glioma Model

In the previous study, use of the boron CSF administration method resulted in the same amount of BPA accumulation in the brain tumor as that achieved using the intravenous (IV) administration method. In this study, the therapeutic effect by the CSF administration method was almost equiva-lent to that following the IV administration method, even though the dose of BPA in the CSF ad-ministration method was quite low.

The Basic Study Aimed at Performing the Boron Neutron Capture Therapy for Canine Hemangiosarcoma

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INTRODUCTION: Hemangiosarcoma (HSA) is one of the most major malignant cancers in dogs. Canine HSA occurs frequently in the spleen, liver, heart and skin and can easily rupture, subsequently causing serious local problem. Furthermore, HSA is known to be highly metastatic. Thus, it is important to prevent the progression of metastatic lesions as well as local control of primary lesion. Although doxorubicin-based chemotherapy has been shown to prolong prognosis, long-term survival in not expected, with a median survival time of 4–7 months. Therefore, new treatment strategies are needed.

Boron neutron capture therapy (BNCT) is a therapeutic method that selectively destroys the tumor while leaving normal tissues almost unharmed by utilizing the nuclear reaction with neutron and boron, which tends to accumulate in the cancer cells. In human, LAT1, an amino acid transporter that has been found to be particularly involved in the intracellular transport of boron compound, is shown to be overexpressed in many malignant tumor cells.

In this study project, we investigated the possibility of applying BNCT to canine HSA by examining the LAT1 expression, the intracellular boron concentration, and the survival after the neutron irradiation using the canine HSA cell lines.

EXPERIMENTS: Three cell lines derived from canine HSA, JuB2, Ud6 and Re21, were used for this study. As boron compound, p-boronophenylalanine (BPA) was prepared at a dose of 30 mg/ml.

RT-PCR Total RNA was isolated from each cell line using the RNA extraction solution (NucleoSpin RNA, Macherey-nagel, Germany). RT-PCR amplification was performed using the One-step RT-PCR kit (QIAGEN, Germany). A mixture of RT-PCR products and loading buffer (Takara Bio Inc., Japan) was electrophoresed in a 2% agarose gel. RP19 primer was used as a housekeeping gene.

Inductively coupled plasma atomic emission spectrometry (ICP-AES) 1×10^6 cells of JuB2, Ud6 and Re21 were co-incubated with BPA for 0.5, 1 and 2 h. The boron-10 (^{10}B) concentrations in BPA solution were adjusted with culture medium to 28 ppm. Each sample was digested by heating overnight in nitric acid (60%), then diluted with distilled water and divided into three test tubes. After measuring the boron concentration in these tubes using ULTIMA2 (HORIBA, Ltd., Kyoto, Japan), their average was taken as the amount of the sample and expressed in "ng ^{10}B / 10^6 cells."

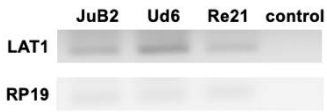

Fig. 1. Expression of LAT1 mRNA by RT-PCR.

Irradiation In the neutron group, 1×10^5 cells of JuB2, Ud6 and Re21 were irradiated with thermal neutron at a power of 1 MW for 15, 30, and 45 minutes (fluence: $7.35–23.5 \times 10^{11}$ n / cm^2) at KURNS. In the BNCT group, cells were co-incubated with 28 ppm ^{10}B of BPA for 8 hours before irradiation. In the X-ray group, cells were irradiated with 1, 2, 4, 8, and 12 Gy of X-ray at a dose rate of 250 cGy / min at Gifu University. After irradiation, each survival rate was evaluated by colony formation assay.

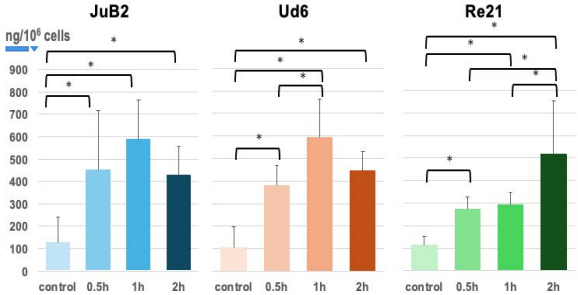

Fig. 2. Intracellular ^{10}B concentrations by BPA co-incubation time using ICP-AES.

RESULTS: The mRNA expressions of LAT1 were seen in all three cell lines (Fig. 1). Fig. 2 shows the intracellular ^{10}B uptake measured by the ICP-AES. In JuB2 and Ud6, there were peaks in ^{10}B uptake for 1-h co-incubation, whereas the peak was seen for 2-h co-incubation in Re21. The amount of ^{10}B uptake at the peak was highest in Ud6, followed by JuB2 and Re21. The colony formation assay showed that the cell survival rate was significantly lower in the BNCT group than in the neutron group in all three cell lines (Fig. 3). Furthermore, the effect of BNCT among cell lines was dependent on the amount of ^{10}B uptake.

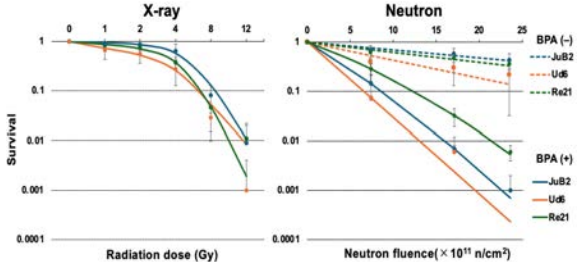

Fig. 3. Survival curves for each cell line after X-ray or neutron irradiation by colony forming assay.

CONCLUSION: Our results indicate that BNCT can be applied to canine HSA because enough ^{10}B uptake and the anti-tumor effect of BNCT were confirmed in this study.

Preparation of Rabbit-Dog Chimeric Anti-BSH Antibody and Comparison with Caninized Anti-BSH Antibody

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INTRODUCTION: It has been reported that 39% of deaths in long-lived dogs were due to age-related diseases, especially cancer [1]. Boron neutron capture therapy (BNCT) is expected to be a new treatment strategy for canine cancer [2]. In our laboratory, bispecific antibodies against BSH and Her2 have previously prepared as boron delivery tool [3]. It is composed of rabbit-derived IgG that bins BSH and llama VHH antibody that binds Her2, typical tumor antigen. In order to use this bispecific antibody against BSH and Her2 for canine BNCT, we have successfully produced a caninized anti-BSH IgG antibody. Unfortunately, it was found that the efficiency of the antibody production was too low to apply *in vivo* estimation [4]. Since the yield and purity of caninized anti-BSH antibodies were low, we herein attempted to create a rabbit-dog chimeric anti-BSH antibody in which only the constant region was caninized.
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EXPERIMENTS: The heavy chain expression vector of a bispecific antibody against BSH and Her2 and the heavy chain expression vector of canine anti-PD-1 IgG antibody were digested with restriction enzymes Eco RI and Nhe I and ligated. In this way, a rabbit-dog chimeric anti-BSH heavy chain expression vector (pCAGEN chBSH HC) was constructed. On the other hand, PCR was performed using the rabbit anti-BSH light chain expression vector (pCAGEN caBSH LC) and the light chain expression vector of canine anti-PD-1 IgG antibody as templates to produce 1st PCR products. Using the 1st PCR product as a template, 2nd PCR was performed to produce a 2nd PCR product. The 2nd PCR product was digested with restriction enzymes Eco RI and Not I and ligated to pCAGEN caBSH LC, which had been previously digested with the same enzymes, to create a rabbit-dog chimeric anti-BSH light chain expression vector (pCAGEN chBSH LC). Using ExpiCHO cells as a host, rabbit-dog chimeric anti-BSH heavy chain and light chain expression vectors (pCAGEN chBSH HC and LC) were cotransfected to produce rabbit-dog chimeric anti-BSH antibody (chBSH IgG). ELISA was performed using an HRP-labeled secondary antibody to evaluate the BSH-binding ability of the produced antibody (Fig.1).
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RESULTS: The amount of antibody produced per a liter of culture medium was 0.94 mg for the caninized antibody, whereas it was 10.1 mg for the chimeric antibody. The ELISA results are shown in Fig.1. As the concentration of antibody increased, the absorbance value at 450 nm also increased. Therefore, it was confirmed that both the caninized antibody (caBSH IgG) and the chimeric antibody (chBSH IgG) had the ability to bind to BSH. Furthermore, when comparing caBSH IgG and chBSH IgG, chBSH IgG had higher absorbance at any concentration, and chBSH IgG was found to have higher antigen binding than caBSH IgG.
Since chimeric anti-BSH antibody (chBSH IgG) has high binding ability and excellent production efficiency, we will use chBSH IgG to produce a bispecific antibody against Her2 and BSH, and estimate an antitumor effect on canine cancer cells.
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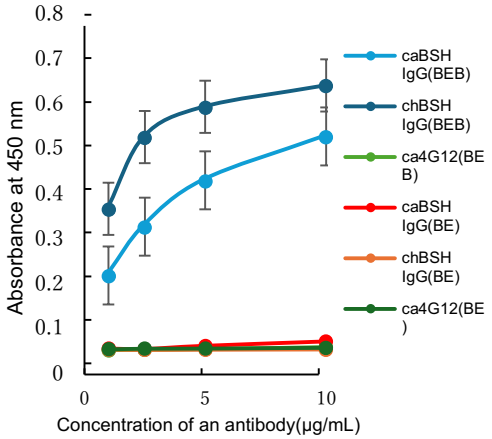

Fig. 1. Evaluation of antigen binding ability of canonized and chimeric anti-BSH IgG.

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PR2-3

Preparation of immunoliposomes comprising hydrophobic boron cluster via exchanging reaction

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INTRODUCTION: Liposomes has been widely used nanoplatform for drug delivery system since the system can encapsulate both hydrophilic and hydrophobic pharmaceuticals in internal water phase and lipid bilayer, respectively. Moreover, the functionalization such as tumor targeting and controlled release properties can be achieved by surface modification. For these fascinating properties of liposomes, this nanomaterial is the first example which was approved in clinical. In our group, drug loading technique based on supramolecular chemistry for hydrophobic compounds was developed, that is exchanging reaction. The strategy consists of two steps. Initially, liposomes are mixed with interests dispersed in water using natural products such as cyclodextrins, polysaccharides, and polypeptides. Next, the complex of hydrophobic compounds with natural products were decomposed by inducing heat stress and released cargo molecules were trapped by liposomes. The system could introduce much larger amount of pharmaceuticals to lipid bilayer compared with conventional drug loading method for liposomes. In this work, we demonstrated applicability of immunoliposome comprising highly concentrated carborane that is prepared by exchanging reaction as a boron agent for BNCT.
EXPERIMENTS: Lipids comprising maleimide conjugated polyethylene glycol were used as antibody presenting units and PEGylated liposomes were prepared by extrusion method. Carboranes were introduced to the PEGylated liposomes via exchanging reaction and the proceeding of the reaction was monitored by ^{1}H-NMR. Antibody was introduced to the PEGylated liposome loading carborane and the conjugated amount of antibody was quantified by protein detection reagents after purification with ultracentrifugation. We demonstrated BNCT activity *in vitro* using HER-2 targeting antibody. Here, we employed human cervical cancer cell (SK-OV-3) which overexpress HER-2 on the cellular membrane. Finally, we demonstrated deliverability of current system *in vivo* using tumor xenograft model mice.
RESULTS: After mixing of PEGylated liposomes and carborane-cyclodextrin complex, H-NMR measurement was carried out and representative proton peak of carborane was gradually broadened with time, indicating the kinetics of carborane including molecular rotation got decreased. Moreover, the exchanging reaction could load larger amount of carborane in each liposomes.Then, carborane was trapped by PEGylated liposomes by just heating dispersion. Afterward, antibody was introduced to the system. After 24 h incubation, almost all the maleimide groups were consumed and over 80% of antibody was successfully conjugated with liposomes. During these procedures, changes in size and formulation of aggregates were not found. When HER-2 targeted antibody was introduced to the immunoliposomes, the immunoliposomes exhibited higher BNCT activity toward SK-OV-3 cells than L-BPA-fructose complex. This is why the cellular uptake amount of boron was significantly boosted by antibody conjugation. We next investigated deliverability of current system *in vivo* using tumor xenograft model mice established by transplantation of SK-OV-3 to nude mouse. After administration, our system could accumulate to the tumor tissue with high selectivity. For these results, our immunoliposome systems are promising candidate for BNCT.
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PR2-4

Investigation of the relationship between the therapeutic efficacy of boron neutron capture therapy and the residence of boron drugs in tumors

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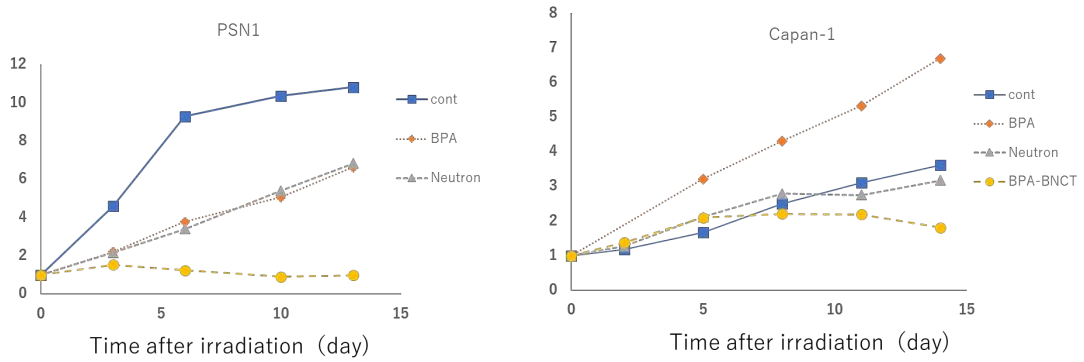
INTRODUCTION: The decision to use BNCT depends on the ratio of tumor uptake of 18FBPA to normal tissue (T/N ratio) on 18F-BPA-PET scans. However, it has recently been reported that prolonged residence time of BPA in tumors contributes to improved therapeutic efficacy of BNCT.[1] Therefore, BPA uptake alone may not be sufficient to predict therapeutic efficacy. In this study, we investigate the effects of tumor tissue diversity (stromal volume and blood flow distribution) on BPA retention and response to BNCT.

EXPERIMENTS: Capan-1cells and PSN1 cells(5.0 ×106/100 μL in 0.1 ml PBS) were injected into the subcutaneous femoral of mice (six-week-old female BALB/c nude mice), and the mice were divided into the cold control (no treatment, no neutron irradiation), hot control (neutron irradiation only), BPA administration only and BNCT (intraperitoneal BPA administration and neutron irradiation) groups. . Irradiation was performed 48 days after the injection of Capan-1cells and PSN1 cells at the heavy water facility of Kyoto University Research Reactor for 12 min at a power of 5 MW. BPA was injected subcutaneously at a dose of 250 mg/kg 1 h before irradiation. Tumor size in the four groups was investigated.

RESULTS: Tumor growth curves for each group are shown in Fig1, where X-axis indicates the number of days after irradiation and Y-axis indicates the tumor growth rate. The results showed that both of the two cell types showed tumor growth inhibition in the BNCT group.

Conclusion: It was observed that BNCT inhibited tumor growth regardless of the amount of stroma, suggesting that macroscopic BPA uptake alone does not predict therapeutic efficacy.

Fig. 1.
Tumor growth curves for Capan-1 and PSN1 treated with the combination of BPA and radiation.



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PR2-5

The basic study of the effect of BNCT for squamous cell carcinoma of the head and neck in dogs

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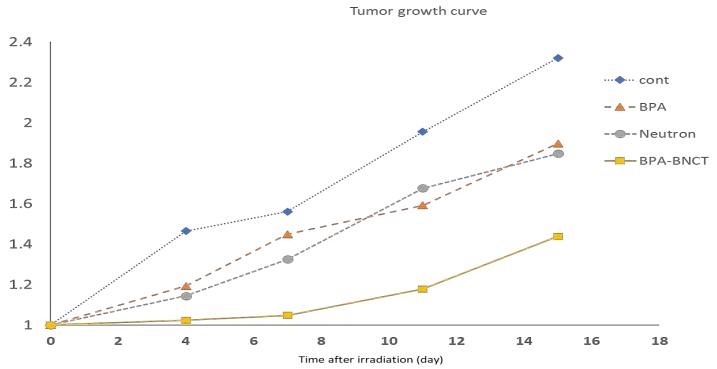
INTRODUCTION: The disadvantage of boron neutron capture therapy is that the neutron beam cannot be absorbed deeply into the body. However, for companion animals, almost all malignant tumors in dogs and cats are applicable because of their size. In this study, we focused on canine tonsillar squamous cell carcinoma. Canine oral squamous cell carcinoma is the second most frequently occurring tumor in the canine oral cavity. Canine tonsillar squamous cell carcinoma (TSCC) has a high rate of metastasis and a short prognosis even with surgical treatment, radiation therapy, and chemotherapy. In a report of a patient treated with radiotherapy and chemotherapy, CR was temporarily observed, but most of the patients died due to local recurrence.[1] Therefore, there is a need for a treatment method that can provide strong local control of TSCC. If BNCT is an effective treatment for canine TSCC, it may be effective against both localized and metastatic lesions and improve prognosis. This study is designed to investigate the efficacy of boron neutron therapy in a tumor-bearing model of TSCC.

EXPERIMENTS: TSCCLN-5 cells (5.0×10^6/100 μL in 0.1 ml PBS) were injected into the subcutaneous femoral of seven-week-old female BALB/c nude mice, and the mice were divided into the cold control (no treatment, no neutron irradiation), hot control (neutron irradiation only), BPA administration only and BNCT (intraperitoneal BPA administration and neutron irradiation) groups. Irradiation was performed 48 days after the injection of TSCCLN-5 cells. at the heavy water facility of Kyoto University Research Reactor for 12 min at a power of 5 MW. BPA was injected subcutaneously at a dose of 250 mg/kg 1 h before irradiation. Tumor size in the four groups was investigated.

RESULTS: Tumor growth curves for each group are shown in fig1, where X-axis indicates the number of days after irradiation and Y-axis indicates the tumor growth rate. The results show proliferation inhibition in the BNCT group. The results indicate that BNCT has the potential to be a treatment for NTSCC.

Fig.1.
Tumor growth curves for TSCCLN-5 treated with the combination of BPA and radiation.



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PR2-6

Boron Delivery to Brain Cells via Cerebrospinal Fluid (CSF) Circulation for BNCT in a Rat Glioma Model

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INTRODUCTION: Recently, some drug delivery systems to bypass blood brain barrier (BBB) have been developed for brain tumor therapy. Our laboratory has been developing a system for boron delivery to brain cells using cerebrospinal fluid (CSF) circulation in boron neutron capture therapy (BNCT), which we call the "boron CSF administration method"[1][2][3]. The understanding of CSF circulation has changed since meningeal lymphatic vessels were first described in 2015. We have obtained important results that use of the boron CSF administration method resulted in a level of a boron accumulation in the brain cells of normal rats that was equal to that achieved using the intravenous (IV) administration method, even though the dose of BPA was quite low (around 1/90 of the BPA dose used in the IV administration method) [4].Based on the results, we designed a CSF-based administration protocol of BNCT for rat glioma models and a thermal neutron irradiation experiment was conducted in Kyoto University Reactor.

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EXPERIMENTS: The C6 rat glioma models were supplied for the present experiment 10 days after implantation. Sixteen models were randomly divided into the following four groups: untreated control group (non- irradiated), neutron-irradiated control group (irradiation only), the group subjected to thermal neutron irradiation after the end of the infusion of BPA via IV route and the group subjected to thermal neutron irradiation after the end of the infusion of BPA via CSF route. In the IV administration group, 350 mg/kg of BPA was administered to four rats via the tail vein for 1.5 h. In the CSF administration group, BPA was administered to four rats via the intracisterna magna at a rate of 8.0 mg/kg/h for 2 h. The C6 glioma rats were irradiated at a 5 MW reactor with a heavy water irradiation facility for 20 min (3.8×10^{12} neutrons/cm^2 on average). After the thermal neutron irradiation, all rats remained under the same experimental conditions as the control groups for seven days. The therapeutic effects of BNCT were evaluated using MRI.

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RESULTS: As shown in Fig. 1, we demonstrated that the therapeutic effect by the CSF administration method was almost equivalent to that following the IV administration method, even though the dose of BPA in the CSF administration method was quite low.

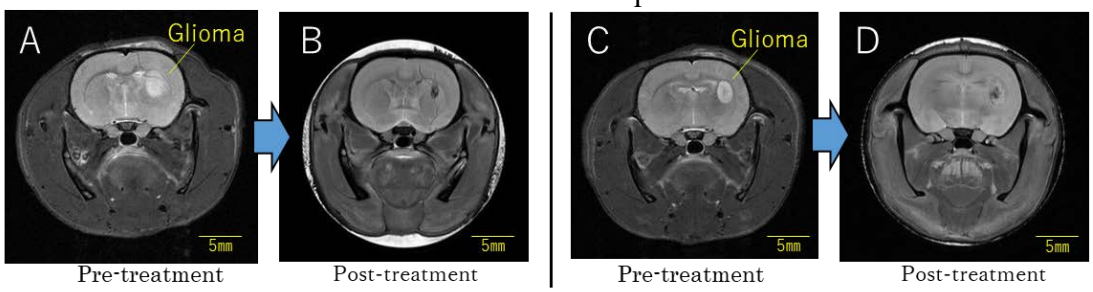

Fig. 1. Representative T2 images of rat brain in IV administration group (A, B) and CSF administration group (C, D).

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