I-1. PROJECT RESEARCHES

Project 1

PR1 The effect of boron neutron capture therapy on normal tissues

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In this research project, six research projects were included. One research project (P1-3 could not be reported due to delay of the analysis).

The effect of normal tissues including lung, liver, brain, and bone were investigated in this study. Details of each project is referred to the following contents.

P1-1: "**The effect of boron neutron capture therapy (BNCT) on normal lung in mice**."

The effect of boron neutron capture therapy (BNCT) on normal lung was analyzed with reference to the survival. The whole thorax irradiation (WTI) with thermal neutron beam was carried out at 1 hour after subcutaneous administration of boronophenylalanine (BPA) at the dose of 500mg/kg. The mice were sorted into four treatment groups, 10-minutes (min), 15-min, 20-min, and 25-min irradiation groups. In each group, three to six mice were irradiated. In 25-min irradiation group, all the mice died within 10 days. To compare the result of BNCT-treated group with those of thermal neutron beam irradiation group and X-ray irradiation group, further observation is needed.

P1-2: "**Clarification of the normal cell fractionation as a trigger for radiation-induced liver injury**."

To clarify the mechanism of radiation-induced liver disease, we used the following boron cluster-sugar chain-conjugated albumin.

・Asialo-N-glycan-MID*-albumin (targeting hepatocytes)
・Mannose-N-glycan-MID*-albumin (targeting non-parenchymal cells)
*MID: maleimide combined with *closo*-dodecarborate

These boron compounds were administered subcutaniously at 1.5 hour before sacrifice the mice for sampling their blood and livers. The boron concentrations in the blood and liver were measured and spatial distributions of these compound in the liver were analyzed using α-autoradiography technique. The liver/blood boron concentration ratio for asialo-N-glycan-MID-albumin was higher than that for mannose-N-glycan-MID-albumin. The spatial distribution of these compounds detected on α-autoradiography were visually almost the same.

P1-4: "**Examination of the influence on normal liver tissue by boron neutron capture therapy."**

The purpose of this study is to find the early indicator or surrogate marker which cause fibrosis in the normal liver tissue after BNCT. The degree of the steatosis in the normal liver and the expression level of Sonic Hedgehog protein were investigated at the seven days after thermal beam irradiation with or without boronophenylalanine (BPA). The degree of liver fibrosis was evaluated with Masson trichrome staining at six months after the irradiation. The degree of stenosis, expression of Sonic Hedgehog protein and liver fibrosis in BNCT cohort was higher compared with these in control and thermal neutron beam.

P1-5: "**The Effect of Boron Neutron Capture Therapy to Normal Bones in Mice."**

The purpose of this project is to elucidate the compound biological effectiveness (CBE) factor of normal bone by evaluating the influence on bone strength in mice. In this year, the ^{10}B bio-distribution in the bone was investigated after boronophenylalanine (BPA) administration using α-autoradiography and laser ablation (LA) ICP-NS. The higher accumulation of ^{10}B were seen in the growth plate, the trabecular bone, and the bone marrow.

P1-6: "**The biological behavior of boron compound in normal bone of young mice and the influence of boron neutron capture therapy on bone growth."**

The purpose of this project is to investigate the biological behavior of boron compound in the normal tibia of young mice, including the analysis of the boron concentration and the bio-distribution of administered boron compound. Furthermore, BNCT was performed on the bones of four-week-old C3H/He mice. The results in this study showed that the tibial growth in the young mice was slightly suppressed in the higher doses of BNCT.

PR1-1 The effect of boron neutron capture therapy (BNCT) on normal lung in mice

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INTRODUCTION: We have reported the results of overall survival (OS) following whole thoracic irradiation (WTI) with X-ray and thermal neutron beams derived from Kyoto University Research Reactor (KUR) in previous reports of "Progress Reports.". In this year, we have carried out the experiment of WTI with boron neutron capture irradiation (BNCR) using boronophenyalanine (BPA).

In this report, we reported the result of WTI with BNCR using BPA.

EXPERIMENTS:

Mice

Ten- to twelve-week-old female C3H/He mice were used. The mice were purchased from Japan SLC, Inc.

Treatments

The mice were sorted into four treatment groups, 10-minututs (min), 15 min, 20 min, and 30 min irradiation (IR) groups. Three to six mice were included in each cohort. The thermal neutron flux was measured at the surface of the acryl box in which the mice was contained. The WTI with BNCR were carried out at the 5MW reactor power which was the same condition as that in the WTI with thermal neutron beam. BPA was administered subcutaneously at the dose of 500 mg/kg at 1hour (hr) before WTI.

RESULTS: The acryl box containing mice were irradiated with thermal neutron beam at the thermal neutron flux of 4.8E+09 n/cm^2/s which was measured by analysis of activation of gold foil attached to the surface of the box.

The observation of the cohort irradiated with thermal neutron irradiation was finished since all the mice were dead.

Figures 1 and 2 shows OSs of WTI with thermal neutron beams and with BNCR using BPA.

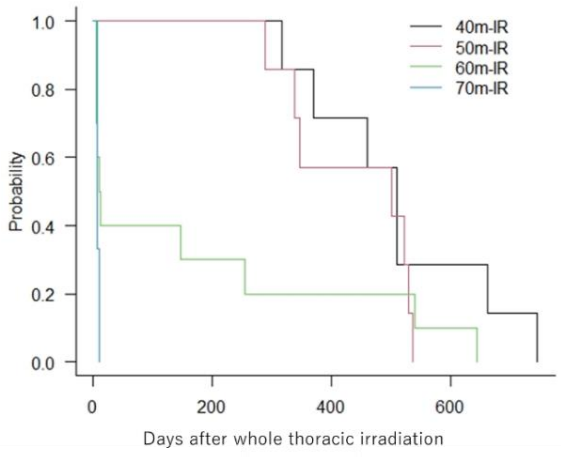

Fig.1 OS of WTI by thermal neutron beam

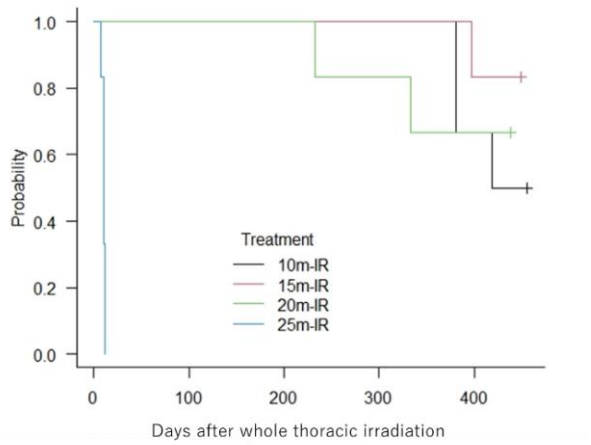

Fig. 2 OS of WTI by BNCR using BPA

Within10 days after WTI with thermal neutron beam, all the mice in the 70 min IR group and 60 % of mice in the 60 min IR group were dead. Within 10 days after WHI with BNCR, all the mice in the 25 min IR groups are dead.

The cause of acute death following WTI within 10 days is supposed to be dysfunction of esophagus since the basal epithelium cells in esophagus decreased substantially in number within 5 days after WTI.

Figure 3 shows the OS of WTI with thermal neutron beam using the data in which the number of the mice were omitted.

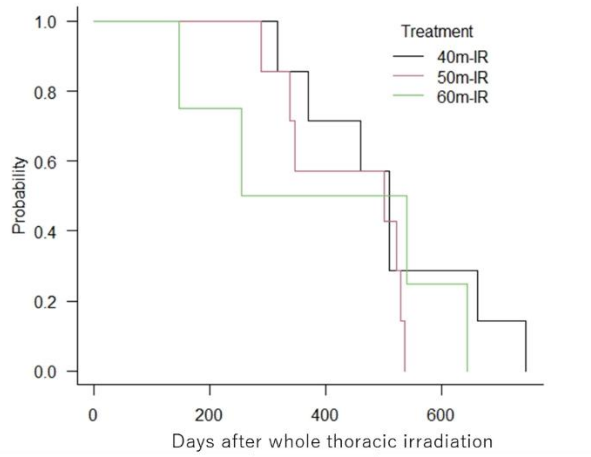

Fig. 3. OS of WTI by thermal neutron irradiation

Further observation is needed to compare the results of WTI with thermal neutron beam and WTI with BNCR.

PR1-2 Clarification of the normal cell fractionation as a trigger for radiation-induced liver injury

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INTRODUCTION:

Radiation-induced liver disease (RILD) is one of the fatal adverse events in radiation therapy. The mechanism of the RILD is still not clear. Boron neutron capture radiation (BNCR) can irradiate the cell fractions in which boron compounds distribute selectively since the path of heavy particles (^{4}He and ^{7}Li atoms) derived from boron neutron capture reaction are no-ticeably short ones less than 10 μm. To inves-tigate the mechanism of the RILD, BNCR is available for detecting the normal cell fractions which triggered to cause RILD. The objective of our study is to clarify the mechanism of radia-tion-induced liver injury using BNCR with bo-ron compounds selectively accumulating in normal cell fractions.

EXPERIMENTS:

Boron compounds

In this experiment, we used two boron compounds. These consist of albumin conjugated with two functionalized portions which are maleimide combined with *closo*-dodecaborate (MID) and sugar chain targeting normal cell fractions in liver. The boron compounds are as follows

Asialo-N-glycan-MID-albumin (distributes in hepatocytes)

- Mannose-N-glycan-MID-albumin (distributes in non-parenchymal cells)

Mice

We used seven-week female BALB/c mice which were purchased from CLEA Japan, Inc.

Treatments

The two boron compounds were administered intravenously via tail vain at the dose of 100 μl. At 1.5 hour (hr) later, the blood was sampled, and the liver were resected. Liver tissue sections were divided into two portions. The one portion was put onto CR-39 (solid state nuclear track detector) and irradiated with thermal neutron to analyze boron spatial distribution using α–autoradiography (ARG) technique described in our previous study [1]. The ^{10}B concentrations of the blood and liver were analyzed with inductively coupled plasma atomic emission spectroscopy.

RESULTS:

The ^{10}B concentrations in the liver and blood

The ^{10}B concentrations in the blood and liver at 1.5 hr after administration of two boron compounds are shown in Table 1.

Table 1. The 10B concentration in the liver and blood

Boron compounds	Liver	Blood	Liver/Blood ratio
	mean ± SD	mean ± SD	
Asialo-N-glycan-MID-albumin	18.4 ± 3.6	20.8 ± 3.7	0.89 ± 0.06
Mannose-N-glycan-MID-albumin	13.3 ± 1.1	20.8 ± 4.0	0.67 ± 0.14

The value of the liver/blood of Asialo-N-glycan-MID-albumin was higher than that of Mannose-N-glycan-MID-albumin.

Distribution of ^{10}B in the liver

The ARGs of each boron compound in liver is shown in Figs. 1 and 2.

Fig.1 ARG of Asialo-N-glycan-MID albumin

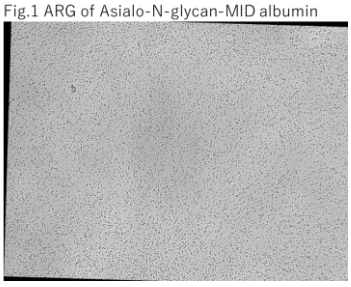
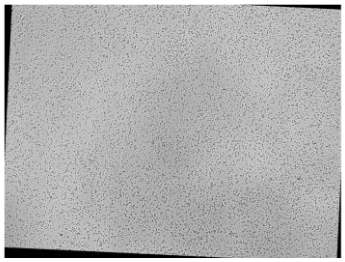


Fig.2 ARG of Mannose-N-glycan-MID albumin



Visually, no apparent difference of ^{10}B distribution was observed. As shown in Table 1, the ^{10}B concentrations in the blood were high, which might obsucure the difference of ^{10}B distribution of each boron compound in normal cell fractions of liver.

The further study is necessary to detect the optimal point in time at which the ^{10}B concentration ratio of liver to blood is higher compared to that in this study.

REFERENCES:

[1] S.Takeno, H.Tanaka, T. Watanabe, T. Mizowaki, M. Suzuki, Quantitative autoradiography in boron neutron capture therapy considering the particle ranges in the samples. Phys Medica 2021;82:306–20. https://doi.org/10.1016/j.ejmp.2021.02.012.

PR1-3 Examination of the influence on normal liver tissue by Boron neutron capture therapy

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INTRODUCTION: Boron neutron capture therapy (BNCT) for liver tumor, which has been conducted up to the present, has used the compound effectiveness factor (CBE) determined by using genotoxicity for hepatocytes as an indicator, which has been clarified by Suzuki et al [1]. But there is a problem whether it is appropriate as a real clinical endpoint. Fundamental researches of liver fibrosis that are the late effect of radiation therapy are necessary. It is necessary to do basic research that uses liver fibrosis, which is a late radiation injury to the liver, as an evaluation index. The hedgehog signaling pathway is one of the important processes involved in animal development, and has been implicated in the maintenance and regeneration of adult tissues. The hedgehog signaling pathway is activated in the damaged liver and affects tissue remodeling. It has also been reported that cell proliferation is promoted and epithelial-mesenchymal transition leading to fibrosis is induced [2]. A purpose of this study is to find the early indicator or surrogate marker which cause fibrosis in the normal liver tissue after BNCT.

EXPERIMENTS: Female C57BL6 mice at 6weeks of age were injected 1,000 mg/kg p-boronophenylalanine (BPA) solution subcutaneously 2 hours before neutron irradiation. The mice were irradiated for 60 minutes at the 1MW output. One week after irradiation, mice were sacrificed and the blood and livers were analyzed. Blood and liver boron concentrations 2 hours and 3 hours after the administration of 1,000 mg/kg BPA were quantified using Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES). In addition, Masson trichrome staining was performed to determine the degree of liver fibrosis six months after neutron irradiation. Hematoxylin Eosin (HE) staining and triglyceride quantification were performed to investigate degree of the steatosis in the mouse normal liver tissue after BNCT. Western blotting was performed to determine the expression level of Sonic Hedgehog protein.

RESULTS: Two hours after the administration of BPA, the liver boron concentration was about 8.1 μg/g, and the blood boron concentration was about 9.2 μg/g. Three hours after BPA administration, the liver boron concentration was about 4.1 μg/g, and the blood boron concentration was about 4.4 μg/g. As shown in Fig. 1, Masson trichrome staining showed a tendency for increased liver fibrosis in the neutron-irradiated group receiving BPA (BNCT group). The result of HE staining demonstrated that the steatosis of the BNCT group was increased (Fig. 2). Triglycerides in mouse normal liver tissue after BNCT tended to be increased compared to control. Furthermore, as a result of Western blotting, the expression of Sonic Hedgehog protein in the BNCT group was higher than in the group only irradiated with neutrons (Fig. 3).

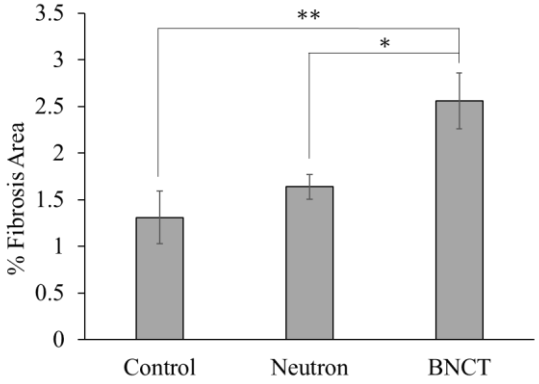

Fig. 1. Fibrosis of mouse liver by BNCT six months after neutron irradiation.

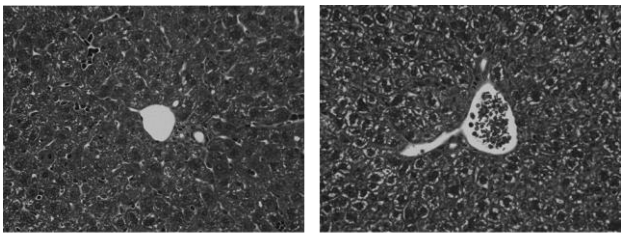

Fig.2 Fatty degeneration of mouse liver by BNCT one week after neutron irradiation.

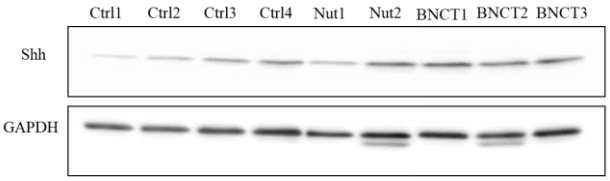

Fig. 3. Shh expression of mouse liver by BNCT. Ctrl: control, Nut: neutron alone, BNCT: BNCT treated.

REFERENCES:

[1] M. Suzuki *et al.*, Jpn. J. Cancer Res., **91** (2000) 1058-1064.
[2] Y. Jung *et al*., Gut, **59** (2010) 655-665.

R3P1-4

PR1-4 The Effect of Boron Neutron Capture Therapy to Normal Bones in Mice

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INTRODUCTION:

There are various tumors in which normal bone is included in the irradiation field, such as bone and soft tissue sarcoma, head and neck cancer, gynecologic cancer, prostate cancer, and tumors that have metastasized to the bone. As a result, radiation-induced bone toxicity, such as fracture, necrosis, and impairment of skeletal growth, can be occurred.

On the other hand, compared with the X-ray irradiation, boron neutron capture therapy (BNCT), a tumor cell -selective particle radiation therapy, is considered to be more effective without any late effects to the normal bone. However, to apply BNCT to the clinical practice, accurate dosimetry is essential. And the compound biological effectiveness (CBE) factor, the value which is necessary when converting to the X-ray equivalent dose, is significant for this purpose.

In this project, we had elucidated the CBE factor of normal bone by evaluating the influence on bone strength in mice. Furthermore, the boron concentrations in whole bones after the administration of the boron compound were analyzed. In this year, we visualized the ^{10}B bio-distribution to investigate the BNCT-specific reduction of bending strength.

EXPERIMENTS: Female eight-week-old C3H/He mice were used for the study (n = 3 in each group). As boron compound, p-boronophenylalanine (BPA) was prepared at a dose of 30 mg/ml.

Bone sample preparation 60 minutes after 125, 250, and 500 mg/kg of BPA were subcutaneously injected into mice, tibias in each mouse were collected. Then, each sample was cut as thin sections of 5 μm-thickness by Kawamoto method, which is a non-demineralized frozen section preparation method.

***α*-autoradiography** The sections were pasted onto CR-39 and irradiated with thermal neutrons at KURNS at a fluence of 6.32×10^{11} n / cm^2 – 1.36×10^{12} n / cm^2 for 7–15 minutes. The irradiated CR-39 was etched with a PEW solution at 50 °C for 8 minutes. The obtained macroscopic pit images were accurately superimposed on the tissue section, and the distribution of α-ray tracks in the tibia was observed with an optical microscope.

Laser ablation (LA) ICP-MS After the sample introduction by LA using the NWR213, analysis by ICP-MS was conducted using the Agilent 8800. The localization of boron was imaged semi-quantitatively based on the previously obtained boron concentration.

RESULTS: Fig. 1 shows the ^{10}B bio-distribution in the tibia evaluated by the *α*-autoradiography. The higher accumulations of ^{10}B were seen in the growth plate (a), the trabecular bone (b), and the bone marrow (c). On the other hand, the lower accumulation was seen in the cortical bone (d), although the ^{10}B was localized a little in the periosteum and endosteum. In each region, the figures clearly show that the boron accumulation is dose dependent. LA-ICP-MS also showed the same results (Fig. 2).

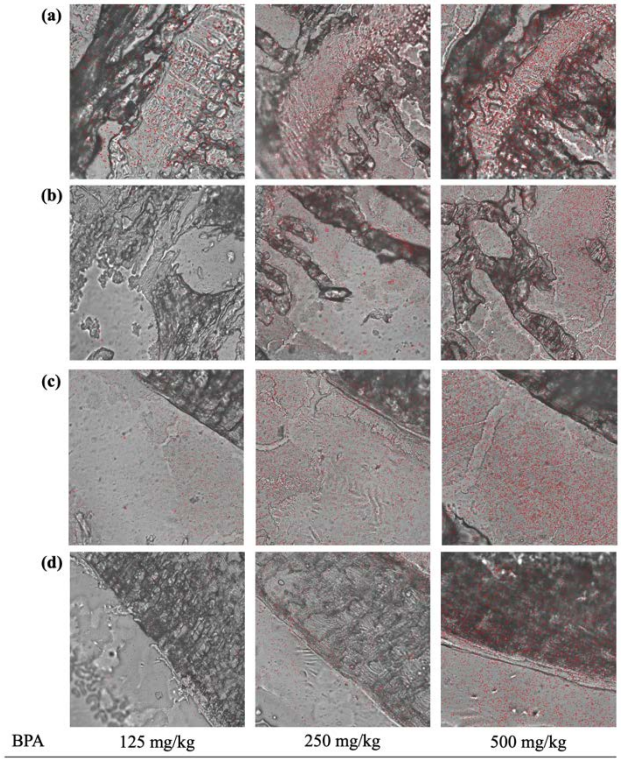

Fig. 1. ^{10}B distribution in the tibia by α-autoradiography. Red dots representing the presence of boron. (a) growth plate, (b) trabecular bone, (c) bone marrow, and (d) cortical bone.

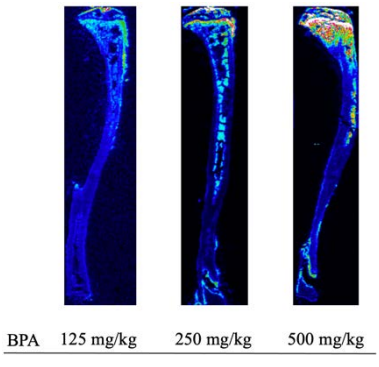

Fig. 2. ^{10}B distribution in the tibia by LA-ICP-MS. Regions of high concentrations of boron are colored white, red, and green.

CONCLUSION: The results showed that ^{10}B was mainly distributed in regions with abundant blood flow, such as cartilage, bone marrow, and trabecular bone. It is necessary to perform the morphological analysis using micro-CT and the histopathological examination to investigate the relationship between the decrease in bone strength by BNCT and the ^{10}B distribution in the normal bone.

PR1-5 The Biological Behavior of Boron Compound in Normal Bone of Young Mice and the Influence of Boron Neutron Capture Therapy on Bone Growth

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INTRODUCTION:

There are various tumors in which normal bone is included in the irradiation field, such as bone and soft tissue sarcoma, head and neck cancer, gynecologic cancer, prostate cancer, and tumors that have metastasized to the bone. In particular, there is a high incidence of bone tumors such as osteosarcoma, chondrosarcoma and Ewing's sarcoma in the adolescent and young adult generation. As a result, radiation-induced bone toxicity, such as fracture, necrosis, and impairment of skeletal growth, can be occurred.

On the other hand, compared with the X-ray irradiation, boron neutron capture therapy (BNCT), a tumor cell-selective particle radiation therapy, is considered to be more effective without any late effects to the normal bone. However, in our previous study using the adult mice, the higher accumulation was seen in the epiphyseal cartilage including the growth plate. This finding indicates that the higher radiation doses might be delivered to the growth plate and may cause the impairment of skeletal growth.

In this project, we investigate the biological behavior of boron compound in the normal tibia of young mice, including the analysis of the boron concentration and the bio-distribution of administered boron compound. Furthermore, BNCT was performed on the bones of young mice to evaluate their influence 3 months after the irradiation.

EXPERIMENTS: Female four-week-old C3H/He mice were used for the study (n = 5 in each group). As boron compound, p-boronophenylalanine (BPA) was prepared at a dose of 30 mg/ml. The X-ray and neutron irradiation was performed at Gifu University and Kyoto University Reactor, respectively.

Boron concentration measurement After subcutaneously injected into mice at doses of 125, 250, 500 mg/kg of BPA, the boron concentrations at each time point (30, 60 and 90 min after administration) in the tibia were measured by ICP-AES.

X-ray irradiation Mice were irradiated at a dose rate of 250 cGy/min to their right hind limb at single doses of 4, 8, 12, 16, 20, 24, 28, 32 and 36 Gy.

Neutron irradiation Each neutron irradiation at a power of 1 MW was carried out as follows; neutron beam only (for 30, and 60 min), neutron beam for 30 and 60 min after subcutaneously injected into mice at doses of 125, 250, and 500 mg/kg of BPA. Based on the results of the biodistribution of BPA, irradiation was started at 30 min after the injection.

Bone growth measurement Tibias were collected at 12 weeks post-irradiation. Subsequently, the length of the tibia in each group was measured using a caliper. The obtained data were shown as relative values to the length of the non-irradiated left tibia.

RESULTS:

Fig. 1 shows the boron concentration in the tibia after BPA administration. The results shows that the boron concentration in the tibia was maintained in a dose-dependent manner at measurement times up to 90 min after BPA administration.

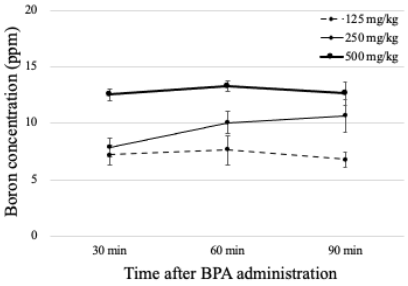

Fig. 1. Boron concentration changes in the tibia after 125, 250 and 500 mg/kg of BPA administration by ICP-AES.

Subsequently, the irradiation experiments to the right hind limb of young mice were performed. Fig. 2 shows the tibias sampled 12 weeks after neutron-irradiation with 500 mg/kg of BPA administration. The growth in the irradiated tibia was slightly suppressed compared with that in the non-irradiated tibia. Fig. 3 shows the changes in the tibial length according to the irradiated dose and the irradiation time. Similar to the X-ray irradiation, higher dose of BNCT mildly suppressed the bone growth.

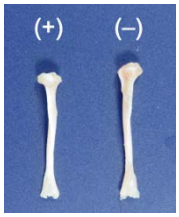

Fig. 2. The length of the tibias 12 weeks after irradiation. Irradiated and non-irradiated tibias are shown as (+) and (-), respectively.

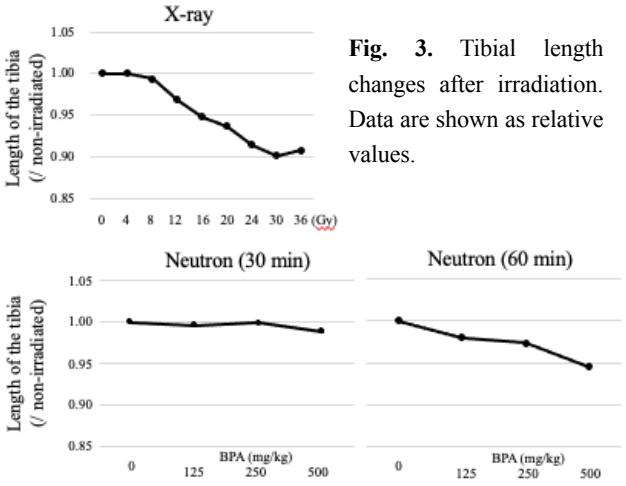

Fig. 3. Tibial length changes after irradiation. Data are shown as relative values.

CONCLUSION: The results showed that the tibial growth in the young mice was slightly suppressed in the higher doses of BNCT. Further investigations such as ^{10}B bio-distribution, the morphological and the pathological analysis are necessary to elucidate its mechanism.