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Posters

Clinical commissioning of a cyclotron-based epithermal neutron source at
Southern Tohoku BNCT Research Center

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Purpose/Objective(s): A cyclotron-based epithermal neutron source (C-BENS) was installed at Southern Tohoku BNCT Research Center (STBRC) in April 2014. The C-BENS consists of mainly five parts; a cyclotron accelerator, a beam transport system, a beam shaping assembly (BSA), a collimator assembly, and a patient transport system. A cyclotron accelerator was developed by Kyoto University Research Reactor Institute and Sumitomo Heavy Industries, Ltd. and can produce more than 1 mA proton beam with an energy of 30 MeV. In a beam transport system, the proton beam is transported to the neutron production target made by beryllium plate. Emitted neutrons are moderated by lead, iron, aluminum, and calcium fluoride. The BSA was optimized to obtain a sufficient intensity of epithermal neutrons while reducing fast neutrons and gamma-ray contamination. Concerning a collimator assembly, the aperture diameter of neutron collimator is four types from 100 mm to 250 mm. we evaluated the quality of a beam to check whether a neutron beam to worth using treatment was secured. In this report, an overview of the clinical commissioning of the C-BENS at STBRC will be presented.

Methods and materials: Irradiation tests were performed by using a water phantom. It was difficult to measure a thermal neutron directly, so the foil activation method was used, which is the radio activation method generally used for the measurement of a thermal neutron. In this method, the radioactivity of radiated materials was measured based on the fact that the radioactivity caused by the neutron irradiation depends on a neutron flux and a reaction cross section. On the center of beam axis and off-axis of 5 cm, the depth dose of the thermal neutron flux was measured. The gamma-ray dose was also evaluated by using the thermo luminescent dosimeter (TLD) set in the water phantom along the center of beam axis. In addition, the Monte Carlo simulation (MCNPX) was performed in the same as condition of measurement and compared the calculation results with the measurement results.

Results: The thermal neutron flux in the water phantom at the center of beam axis was confirmed about $1.2 \times 10^{9}$ neutrons/cm$^2$/sec at 20 mm from the surface with 1 mA proton beam. The simulation results were all in good agreement with the measurement results.

Conclusions: Through clinical commissioning, the stability of a neutron beam in the C-BENS at STBRC was confirmed. In the future, it needs to be examined how the beam quality assurance should be carried out.
Optimization of treatment procedure for hospital-installed accelerator-based BNCT: The experience of Southern Tohoku BNCT Research Center


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Introduction: Boron neutron capture therapy (BNCT) delivers the high linear energy transfer particles to tumor cells preferably without severe damage to surrounding normal tissue, and might be beneficial treatment for the inoperable case and patients who have no other treatment options. Therefore the installation of BNCT system into accessible medical institution has been desired. In our institution, the construction of Southern Tohoku BNCT Research Center (STBRC) was completed in September 2014, and the system commissioning including beam commissioning was already finished. From January 2016, a clinical study of hospital-installed accelerator-based BNCT for recurrent malignant glioma has been started. At the beginning of clinical study, we tried to optimize treatment procedure by utilizing our know-how in radiotherapy. In this presentation, I will describe the result of optimized treatment procedure.

Methods and materials: At the preparatory step for the clinical study, we considered the optimization of patient set-up and treatment planning, assuming BNCT protocol with 2-h intravenous infusion with 4-boron-L-phenylalanine (BPA) followed by neutron irradiation while continuing infusion. SERA (Simulation Environment for Radiotherapy Applications) was used for BNCT treatment planning.

Results: The optimized procedures for patient set-up and treatment planning were designed as follows. Preliminary patient set-up condition is provided from simulation by using diagnostic CT/MR image. For treatment of brain tumor, the instrument immobilizer for head fixation was newly developed assuming setup accuracy with 5 to 7 mm. For head and neck cancer, we are now developing a novel immobilizing system with set-up accuracy of 5 to 10 mm (if possible) under sitting position. After checking patient set-up condition by simulation, CT scan is performed with same patient position. Then utilization of these acquired images enables us to evaluate more realistic treatment plan. By using a 3D image analysis system that enables quick and easy access to any slice image, and using a deformable image registration system that enables more accurate fusion and image registration, which are routinely used for photon beam and proton beam radiotherapy in our hospital, any angle slice images are extracted and fitted into planning CT with realistic set-up position. Accordingly the delineation of tumor and organs at risk and the dose evaluation might be achieved with high accuracy.

Conclusions: STBRC experienced the installation of BNCT system as a medical institution for the first time in the world. Now that the cyclotron-based neutron resource is produced, it is expected that introduction of BNCT will be accelerated into other medical institutions with no experience of BNCT. Although the sufficient experience of radiation therapy helps the
Dosimetric Impact Due To Intratreatment Positioning Error in Boron Neutron Capture Therapy for the High-Grade Glioma

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Purpose/Objective(s): Boron neutron-capture therapy (BNCT) needs extremely longer treatment time for neutron irradiation compared with conventional radiotherapy, such as photon, proton, and carbon ion therapy. For feasibility of long-time treatment, it is desirable to perform patient set-up so loosely and comfortably as to permit a bit of patient disposition in consideration of patient’s fatigue. However, to the best of our knowledge, how much we can permit the deviation of irradiation field have not been fully elucidated from the viewpoint of therapeutically effective dose for treatment target. Now that we are eventually starting the first-in-the-world hospital-installed accelerator-based BNCT treatment in our institution, the study aimed to evaluate the dosimetric impact due to positioning error and intratreatment motion error in BNCT for the high-grade Glioma.

Methods and materials: This study was targeted for three patients that has the high-grade Glioma on their temporal lobe. First, based on Gd contrast-enhanced TI-weighted imaging (GdT1WI), tumor (GTV), brain stem, eye-lens, optic-nerve, and optic-chiasm were delineated, and dose calculations were performed with the radiation treatment planning system (SERA). The distance from a collimator to the patient’s surface is different in every cases, but the suitable condition to achieve the best distance was selected. Now, we decide to name the plan of this calculations non-FLAIR plan. After calculations, the maximum, minimum, and mean dose for the GTV and some normal tissues were evaluated. Next, based on FLAIR-weighted imaging (FLAIRWI), the area of high signal intensity on FLAIR (FLAIR-high area) was delineated as FLAIR by additions. The dose calculations for this plan named FLAIR plan was performed, and some doses were evaluated in the same as before. Finally, in condition that the irradiation fields shifted 2, 5, 10 mm to the direction RL and ±2, 5, 10 mm to the direction SI in both non-FLAIR and FLAIR plan, dose calculations were performed and some doses were evaluated, respectively.

Results: For the deviation of irradiation field in the direction RL and SI, The mean dose (D50) and minimum dose for the GTV and the area of high signal intensity on FLAIR (FLAIR-high area) tend to become lower than in original treatment plan. The changes of the minimum dose for the FLAIR-high area were drastic compared to those for the GTV. As for the FLAIR-high area, the degrees of deviation in the direction of RL, AP, and SI that can compensate therapeutically effective dose for the FLAIR-high area were different for each patient. In some cases, even 5-mm deviation did not compensate therapeutically effective dose for the FLAIR-high area.

Conclusions: Intratreatment positioning error due to loose patient set-up has an impact on the dose quality especially for the area of high signal intensity on FLAIR that might include the infiltrated tumor cells, and in some cases makes the possibility of recurrence. Pretreatment evaluation for the degree of deviation that can compensate therapeutically effective dose should be performed in each patient.
SU-I-GPD-T-154

Feasibility of a Newly Developed Water-Equivalent Bolus Technique in Accelerator-Based Boron Neutron Capture Therapy for Skin Tumors

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Purpose: The aim was to evaluate a newly developed technique with a water-equivalent bolus (WEB) in accelerator-based BNCT. Methods: The simulated patient with malignant melanoma lesions localized in a sole, an arch, and a thumb of a unilateral foot was played by a healthy man. A WEB was prepared as follows: Urethane foam cut down into the size of 3-cm larger than the superficial lesion was infiltrated with distilled water with deaeration. The lesions bordered by a catheter were covered with a WEB, and CT scan was performed. The tumor is depicted as a region with 5-mm thickness. A WEB was delineated as water. This was placed into air for calculation in condition with no bolus. For comparison with bolus-like effect of a covered collimator, the outline of an imaginary collimator cover was set as a mass of polycarbonate or a water tank filled with 5–50-mm thickness. For calculation of photon-equivalent dose (Gy-Eq), blood ¹⁰B concentrations, (¹⁰B tumor/blood concentration ratio, and CBE factor for ¹⁰B(n,α)Li reaction were assumed to be 25 ppm, 3.5, and 4.0. Then, irradiation condition was defined as tumor Dmin of 30 Gy-Eq. Results: In condition with no bolus, irradiation time was 185.6 ± 36.4 min, and tumor Dmax and Dmean were 110.7 ± 31.6 Gy-Eq, and 66.2 ± 13.0 Gy-Eq, respectively. Skin Dmax was larger than 18 Gy-Eq in all cases. In condition with WEB technique, irradiation time was 48.4 ± 15.9 min. Tumor Dmax and Dmean were 58.5 ± 5.3 Gy-Eq and 48.2 ± 4.2 Gy-Eq with good dose homogeneity and within skin tolerable dose (12.7 ± 1.6 Gy-Eq). The bolus-like effect of covered collimator with a mass of polycarbonate or water tank was not sufficient. Dose homogeneity and irradiation time were even worse than the condition with a water-equivalent bolus. Conclusion: Our results revealed that the newly developed WEB technique could have a great effectiveness on dose improvement of accelerator-based BNCT for skin tumors.
SU-I-GPD-T-208
Dosimetric Impact of Intratreatment Displacement in Accelerator-Based Boron Neutron Capture Therapy (AB-BNCT) for the High-Grade Glioma

Purpose: To evaluate the dosimetric impact of intratreatment displacement quantitatively in accelerator-based boron neutron capture therapy (AB-BNCT) for the high-grade glioma using Simulation Environment for Radiotherapy Applications (SERA), a currently available BNCT treatment planning. Methods: Newly installed AB-BNCT system (Sumitomo Heavy Industries, Ltd.) was used in this study. 3 patients with the high-grade glioma on their temporal or parietal lobe were selected. First, based on Gd contrast-enhanced T1-weighted imaging, tumor (GTV) and organs at risk were delineated. next, based on FLAIR-weighted imaging, the area of high signal intensity on FLAIR (FLAIR-high area) was delineated. After calculations using SERA, the maximum, minimum, and mean doses for the GTV and the FLAIR-high area were evaluated. To clarify the impact of intratreatment displacement for the doses of the GTV and the FLAIR-high area, simulations were performed in conditions that the irradiation fields shifted 2, 5 and 10 mm to the directions of RL, AP and SI in each respective patient.

Results: For the deviation of irradiation field in the directions of RL and SI, the maximum, minimum and mean doses for the GTV and the FLAIR-high area tended to become lower than that in original plan. A maximum dose reduction of over 25% in GTV minimum dose was noticed for 10 mm shifted to the RL and SI directions. One of 3 patients had unacceptable coverage as defined dose reduction within 10% compared with the original plan for 5 mm shifted to the RL and SI directions. Conclusion: Intratreatment displacement had a significant impact on the dose for the target, so translational positioning error was needed to be maintained below 5 mm even in BNCT. Pretreatment evaluation for the degree of deviation that can compensate therapeutically effective dose should be performed in each patient treatment.
SU-I-GPD-T-206

The Impact of Sitting Positioning On Deviations of Neutron Beam Axis From Condition Pre-Planned with Diagnostic Images in Boron Neutron Capture Therapy

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Purpose: To assess skin marker positioning errors for incidence and emitting points of neutron beam axis due to differences between patient’s supine position for diagnostic CT and sitting position as a treatment position for boron neutron capture therapy (BNCT) of head and neck cancer. Methods: Five patients were assumed to receive BNCT for parotid cancer with sitting position. At first, using treatment planning system SERA, lesions were delineated and beam incidence and emitting points on patient skin were preliminarily decided on diagnostic CT images. These points of interests (POIs) were set on a skin surface of the neck. After the first CT scan was performed in normal supine position, patients were immediately setup with a 45-degree neck rotation toward healthy side, followed by the second CT scan. Furthermore, patients were setup in pseudo-sitting treatment position by adding “bending forward” with a 30-degree waist pad, followed by the third CT scan. After matching three CT datasets along with three standard points, such as apex of bilateral mastoid process and dorsum of nose, deviations of each POI between three CT image datasets were evaluated. Results: The deviations of incidence and emitting points between supine and pseudo-sitting position in the LR or RL (from diseased-to-healthy direction)/SI/AP (mm) were 5.7±4.0/3.7±2.7/4.7±9.7, and 18.7±8.6/15.6±11.6/4.5±6.5 with the differences (mm) of 11.1±7.0 and 28.1±5.6, respectively. Roll and pitch angles (degree) of the neutron beam axis relative to skull structure in supine position were 27.6±3.9, and 16.9±12.5, respectively. The deviations in roll/pitch angles (degree) due to change of position into neck rotation and pseudo-sitting were -7.9±1.2/0.1±1.5 and -10.7±3.2/2.5±2.4, respectively. Conclusion: Our results revealed that it is inevitable to perform re-planning with CT images acquired at sitting position as an actual treatment position because of large beam axis deviations derived from the impact of patient positioning in head and neck BNCT.
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A newly designed water-equivalent bolus technique enables BNCT application to skin tumor.

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Purpose or Objective
The accelerator-based boron neutron capture therapy (ABBNCT) system was developed in order to enable the installation of safe hospital BNCT. An important feature of AB-BNCT system is its capability of delivering great doses to deep-seated tumors under condition in which a beryllium target and neutron-beam-shaping assembly are adjusted for production of epithermal neutron that is applicable for more types of tumor localization. Conversely, AB-BNCT is less suitable for superficial cancers, such as malignant melanoma. In this study, we developed a newly water-equivalent bolus technique that has no production of prompt gamma ray and no influence on complicating dose calculation, and we evaluated the effect of this technique on treatment quality for a case of malignant melanoma patient.

Material and Methods
A water-equivalent bolus was prepared as follows. Urethane foam was cut down into the size of 3-cm larger than the superficial lesion, infiltrated with distilled water with deaeration, and covered with a thin film. The simulated patient was played by a healthy man and simulated condition was originated from a malignant melanoma patient with the lesion of 3-cm diameter localized in a sole of right foot. The superficial lesion was bordered by a catheter and covered with a water-equivalent bolus. Using treatment planning system SERA, the tumor is depicted as a region surrounded by the catheter with 5-mm thickness, and also skin is depicted as the other region except for tumor with 3-mm thickness from body surface. A water-equivalent bolus was delineated as water. This was placed into air in calculation in condition with no bolus. For comparison with bolus-like effect of a covered collimator, the outline of an imaginary collimator cover was set as a mass of polycarbonate or a water tank filled with water with 20-50-mm thickness. For calculation of photon-equivalent dose (Gy-Eq), blood 10B concentrations, 10B tumor/blood concentration ration, and CBE factor for 10B(γ,α)7Li reaction were assumed to be 25 ppm, 3.5, 4.0. Tolerance dose of the skin was regarded as 18 Gy-Eq.

Results
In condition with no bolus, irradiation time was 121.6 min, and tumor Dmax and Dmean were 125 Gy-Eq, and 74.3 Gy-Eq, respectively. In condition with water-equivalent bolus technique, irradiation time was 72.1% decreased (33.9 min) compared with no bolus condition. Also tumor Dmax and Dmean were 54.4 Gy-Eq and 45.0 Gy-Eq, and the dose homogeneity was dramatically improved. Skin Dmax became greatly less than tolerable dose (11.5 Gy-Eq, 59.6% decrease). The bolus-like effect of covered collimator with a mass of polycarbonate or water tank was not sufficient. Dose homogeneity and irradiation time was largely worse than the condition with a water-equivalent bolus.

Conclusion
Although this study was examined for a single case of melanoma patient, our results revealed that water-equivalent bolus technique could have a great effectiveness on dose improvement of AB-BNCT for superficial cancers.
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Investigation of Treatment Indication Restriction Related to Tumor Localization in Boron-Neutron Capture Therapy for Recurrent Head and Neck Cancer


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Purpose: Boron-neutron capture therapy (BNCT) can achieve a cancer cell-selective particle therapy, and damage boron compound-penetrating cells with alpha and lithium particles. We are now conducting BNCT treatment with hospital-installed cyclotron-based BNCT system for recurrent head and neck cancer (HNC). In performing BNCT, there are some limitations of treatment application due to short tracts of neutron beam that restricts the beam settings and patient setup conditions. Therefore, we examined the relationship between tumor localization and treatment indication in BNCT for HNC.

Materials and Methods: For fourteen recurrent HNC patients with a history of radiotherapy, re-planning for BNCT treatment was performed with Simulation Environment for Radiotherapy Applications (SERA) system using the past planning CT. Optimizations of beam alignment and dose calculation were performed with considering patient setup after delineation. All treatment plans were normalized to deliver maximum dose of 12 Gy-Eq to mucosa in oral cavity and pharynx, and we evaluated whether each tumor localization can be indicative for BNCT with adequate dose distribution.

Results: The each size of effective neutron field for treatment was diverse and relied on tumor location. Parotid gland tumor was given extremely higher dose and thought to have good indication for BNCT. Oral cavity tumor had relatively lower dose than parotid gland tumor. Ethmoid sinus, nasopharyngeal, and mesopharyngeal tumors were considered to have no indication for BNCT in this setting.

Conclusion: Tumor localization might become an important clue to judge treatment indication in recurrent HNC.
SU-I-GPD-T-113

Dosimetry of Commercially Available Cyclotron-Based Boron Neutron Capture Therapy System: Commissioning and Quality Assurance for Clinical Use


Purpose: BNCT-30 is a commercially available cyclotron-based boron neutron capture therapy (BNCT) system equipped with beryllium target. This work aims to present our institutional experience-based methodology of commissioning and quality assurance and its optimization for dosimetry of BNCT-30 irradiation field.

Methods: Based on the dosimetric QA items summarized in AAPM TG-142, the dosimetric QA requirements for clinical use of commercial BNCT system were reviewed and rearranged for each dosimetric component such as thermal/epithermal/fast neutrons and gamma-ray constituting the irradiation field.

Results: From the results of tumor dose simulation by MCNPX and acceptance test, collimator surface (Ref0), phantom surface (Ref0), 2-cm and 6-cm depth in phantom (Ref1 and Ref2) were determined as reference depths for measurement of daily, monthly, and annual QA. Each QA procedure consisted of measurements of thermal/epithermal and fast neutron fluence by gold and indium reaction rate, and gamma ray measured by thermoluminescence dosimeter, avoiding the use of real-time neutron detectors because of its unclear durability. However, the measurement of fast neutron fluence with indium foils was limited only to annual QA procedures in view of a high volume of the work despite small contribution to RBE dose. Due to intricacy of the evaluation for all components, daily beam output constancy test was limited to the evaluation for the dominantly contributed thermal neutron at Ref0 with Au activation rate using smaller charge amount. Alternatively, thermal neutron and gamma ray measurement were added as weekly QA. Monthly QA included beam quality profile constancy test, and annual QA included a beam symmetry constancy test, output calibration of proton charge monitor, and evaluation of linearity between charge monitor value and each component dose. Conclusion: This methodology provides an easy and reliable QA method that can be clinically applied with dosimetric validity for the mixed irradiation field of BNCT.
SU-I-GPD-T-116

Design and Construction of Accelerator-Based Boron Neutron Capture Therapy (AB-BNCT) Facility with Multiple Treatment Rooms at Southern Tohoku BNCT Research Center

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Purpose: To describe the design and the construction of accelerator-based boron neutron capture therapy (AB-BNCT) facility with multiple treatment rooms at Southern Tohoku BNCT Research Center (STBRC). Methods: AB-BNCT system at STBRC is equipped with a cyclotron-based epithermal neutron source (C-BENS), which consists of a cyclotron accelerator (HM-30), a beryllium neutron production target, and a beam shaping assembly (BSA). The specifications of the C-BENS at STBRC are same as those at Kyoto University Research Reactor Institute (KURRI) except that STBRC has two beamlines. At first, we considered selecting radiation protection materials around BSA to reduce whole-body exposure of the patients compared to that at KURRI. Next, we developed a patient remote transport system (PRTS) for workers to reduce the work time in the treatment room under the condition of remaining activities just after an irradiation. We studied the feasibility of this system and carefully designed optimum layout to realize patient flow and workflow efficiently. Results: To reduce the activities caused by thermal neutron, BSA is surrounded by LiF-loaded polyethylene blocks and low-activation concrete. The measured out-of-field thermal and fast neutron dose profiles were in good agreement with calculated ones using MCNPX. It was also confirmed that PRTS could be operated up to 9 m apart from PRTS without any problems. We designed the upside-down Y shaped beamline configuration, in which HM-30 and two treatment rooms are assumed to be located on a top and bottoms, respectively. 110 degree of beam deflection angle was thought to be the most desirable from the view point of workflow. Conclusion: We successfully established the environment of BNCT as one of the division of general hospital without sense of incongruity in comparison of environment of conventional radiotherapy. The AB-BNCT system described here can be tolerated enough for practical use for BNCT in a hospital.
Impact of oxygen status on $^{10}$B-BPA uptake into human glioblastoma cells, referring to significance in boron neutron capture therapy

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ABSTRACT

Boron neutron capture therapy (BNCT) can potentially deliver high linear energy transfer particles to tumor cells without causing severe damage to surrounding normal tissue, and may thus be beneficial for cases with characteristics of infiltrative growth, which need a wider irradiation field, such as glioblastoma multiforme. Hypoxia is an important factor contributing to resistance to anticancer therapies such as radiotherapy and chemotherapy. In this study, we investigated the impact of oxygen status on $^{10}$B uptake in glioblastoma cells in vitro in order to evaluate the potential impact of local hypoxia on BNCT. T98G and A172 glioblastoma cells were used in the present study, and we examined the influence of oxygen concentration on cell viability, mRNA expression of L-amin acid transporter 1 (LAT1), and the uptake amount of $^{10}$B-BPA. T98G and A172 glioblastoma cells became quiescent after 72 h under 1% hypoxia but remained viable. Uptake of $^{10}$B-BPA, which is one of the agents for BNCT in clinical use, decreased linearly as oxygen levels were reduced from 20% through to 10%, 3%, and 1%. Hypoxia with <10% O$_2$ significantly decreased mRNA expression of LAT1 in both cell lines, indicating that reduced uptake of $^{10}$B-BPA in glioblastoma in hypoxic conditions may be due to reduced expression of this important transporter protein. Hypoxia inhibits $^{10}$B-BPA uptake in glioblastoma cells in a linear fashion, meaning that approaches to overcoming local tumor hypoxia may be an effective method of improving the success of BNCT treatment.

Keywords: boron neutron capture therapy; boronophenylalanine; hypoxia; glioblastoma

INTRODUCTION

Boron neutron capture therapy (BNCT) can potentially deliver high linear energy transfer (LET) particles to tumor cells without causing severe damage to surrounding normal tissue, and may thus be beneficial for inoperable cancer cases and patients who have no other treatment options. The basic idea of BNCT was first outlined by Locher in 1936 [1]. In BNCT treatment, an intravenously injected boron ($^{10}$B) carrier molecule accumulates preferentially in cancer cells compared with in normal cells. The patient is then irradiated with epithermal or thermal neutrons, which have sufficiently low energy not to harm normal tissues but which stimulate the $^{10}$B carrier molecules to release a substantial amount of localized energy in the form of alpha rays and lithium ions. Alpha rays have very high relative biological effect (RBE) and LET compared with X-rays, and a very short range of 9 μm which is generally equal to or less than the diameter of cells. These characteristics theoretically enable the highly selective killing of cancer cells that are tagged with $^{10}$B, without damage to non-tagged normal cells. However, this revolutionary method depends on the high accumulation in and selective delivery of $^{10}$B into the tumor tissue. To date, only two types of boron carrier are available for use in this context—sodium mercaptoundecahydrododecaborate($^{11}$B) (BSH) and boronophenylalanine (BPA). BSH is a water-soluble drug that is
taken into cancer tissues passively according to the local concentration gradient. On the other hand, BPA which is an amino-acid derivative, is actively taken up by cancer cells via the L-type amine acid transporter 1 (LAT1), with a small amount also being transported by LAT2. Cancer cells are known to overexpress LAT1 compared with normal cells, but LAT2 is expressed by both cancer and non-cancer cells. However, since the amount of $^{10}$B-BPA taken up via LAT2 is less than that via LAT1, there is a preferential accumulation of $^{10}$B in tumor tissues [2]. Several studies have indicated the possibility of a new drug delivery system using liposomes to enhance the density of $^{10}$B in tumor cells [3–8]; however, adverse events associated with this approach have so far proved restrictive.

In the treatment of glioblastoma, surgery followed by radiation therapy and chemotherapy with agents such as temozolomide is standard; however, the prognosis for this disease remains extremely poor. One reason for this is that the typically invasive growth pattern makes complete surgical resection difficult. Because of the infiltrative growth pattern of glioblastoma, the irradiation field of radiation therapy with X-rays needs to be expanded. The large irradiation field also includes normal tissues near the tumor bed and leads to complications; however, selective irradiation with BNCT enables us to damage malignant cells without damaging normal cells, even if the irradiation field is large. More recently, therefore, the targeted approach offered by BNCT has received significant attention; however, the results of BNCT studies are not yet convincing [9–16]. The rapid growth of glioblastoma and abnormal tumor vascularization produces a hypovascular and hypoxic tumor microenvironment, and this increases the fraction of cells resistant to chemotherapy and radiation therapy [17]. Actually, some studies have reported that there is a rich hypoxic area in glioblastoma tumor observed using positron emitting tomography (PET) with hypoxic tracer $^{18}$F-Fluoromisonidazole (FMISO) [18, 20] and 1-[(2-18F)]fluoro-1-(hydroxymethyl)ethyl]methyl-2-nitroimidazole (FET-170) [21, 22], and it is known that increasing the size of the hypoxic area promotes the poor prognosis for conventional therapy [18]; operative resection followed chemoradiotherapy. Successful treatment with BNCT depends on the selective accumulation of $^{10}$B in cancer cells, but it is not yet clear how a hypoxic environment influences this uptake. We investigated the impact of oxygen pressure until the last minute prior to exposure to $^{10}$B-BPA on $^{10}$B uptake in glioblastoma.

**MATERIALS AND METHODS**

**Cell lines**
The human glioblastoma multiforme tumor cell lines T98G and A172 were provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). Cells were cultured in serum-free Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F12, Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, Saint Louis, MO) and 1% penicillin/streptomycin (Gibco Life Technologies), and maintained at 37°C in a 5% CO₂ atmosphere.

**Hypoxic conditions**
Hypoxic conditions were achieved by culturing cells in modular incubator chambers (Billups-Rothenberg Inc., Del Mar, CA, USA).

The chambers, in which cell culture dishes and distilled water were housed, were flushed with mixed gas (95% nitrogen/5% carbon dioxide) to achieve the target oxygen pressure monitored using a JKO-02 Vent, III monitor (JIKKO, Tokyo, Japan), then sealed and incubated at 37°C.

**Trypan blue dye viability assay**
Cells were incubated in 60-mm dishes with 4 ml of culture medium, at $1 \times 10^6$ cells/dish under either normoxic conditions, or hypoxic conditions (1% O₂) for 0, 24, 48 and 72 h. For the trypan blue dye exclusion test, cells were stained using phosphate-buffered saline (PBS, Sigma-Aldrich, Saint Louis, MO) containing 0.5% trypan blue (Nacalai Tesque, Inc., Kyoto, Japan). Cell viability was assessed by counting the number of both stained and unstained cells using cell-count slides.

**Cell cycle analysis**
The cell cycle phase distribution was analyzed with propidium iodide (PI) solution (PI/RI-fluoresceine isothiocyanate, Nippon Boehringer, Tokyo, Japan) staining according to the manufacturer’s instructions. Briefly, cells were harvested following 0, 24, 48 or 72 h incubation in a 60-mm culture dish (Corning) under normoxic/hypoxic, washed with the binding buffer, then suspended in the buffer containing PI. Stained cells were analyzed with a Cytoflexix FS 500 flow cytometer (Beckman Coulter, Tokyo, Japan).

**Boron accumulation study**
$^{10}$B-enriched 1-BPA, D-sorbose, standard boron solution, and yttrium ICP standard solution were kindly supplied by Nippon Pharma Company (Osaka, Japan). 1-BPA was used as a D-sorbose complex as a stock solution containing 30 mg $^{10}$B/ml. The two human glioblastoma cell lines T98G and A172 were cultured in 10 cm culture dishes with either $9 \times 10^6$ cells (T98G) or $3 \times 10^6$ cells (A172) for 72 h under normoxic or hypoxic (10%, 3% or 1% O₂) conditions. After trypan blue staining, cell suspensions were collected in 15 ml centrifuge tubes at $1 \times 10^6$ cells per tube, exposed to BPA at either 10, 20 or 30 mg $^{10}$B/ml in the medium for 2 h under normoxic conditions, washed with PBS, centrifuged, and the supernatant discarded. Cell pellets were digested with 0.67 ml of perchloric acid (HClO₄) and 1.33 ml of hydrogen peroxide (H₂O₂) for 6 h at 70°C, before being mixed with standard yttrium solution (1 ml) and diluted with distilled water to a total volume of 10 ml. The $^{10}$B concentration in each sample was determined with inductively coupled plasma atomic emission spectroscopy (ICP-AES) using an ICP-MS 8500 (Shimadzu, Kyoto, Japan) at a wavelength of 249.772 nm. The calibration curve obtained from dilutions of the standard boron solution was linear in the range of 0.0025–0.35 mg $^{10}$B/ml.

**Total RNA extraction**
T98G and A172 were cultured in 10 cm culture dishes with either $9 \times 10^6$ (T98G) or $3 \times 10^6$ cells (A172) for 72 h under normoxic or hypoxic (10%, 3% or 1% oxygen) conditions at 37°C. After 72 h incubation, total RNA was extracted using the Agenount RNAAdvance Cell v2 system (Beckman Coulter, Danvers, MA) using Agenount's patented SPRI paramagnetic bead technology, according to the
manufacturer's instructions. Briefly, cultured cells were lysed with lysis buffer and proteinase K, and transferred into new 96-multi-well plates (Beckman Coulter). After total RNA was mixed with paramagnetic beads, the beads were washed with wash buffer and 70% ethanol, and separated from contaminants using Agencourt SPRIPlate 96R. Subsequently, DNase I solution (Thermo Fisher Scientific, Waltham, MA, USA) was added into each well to digest the genomic DNA. Total RNA was re-bound to the beads and contaminants removed by washing. Finally, total RNA was eluted from the magnetic particles with nuclelease-free water (Thermo Fisher Scientific).

Quantitative real-time reverse transcription-polymerase chain reaction
First-strand cDNA was synthesized with an iScript RT Supermix for RT-qPCR® (Bio-Rad, Hercules, CA) from extracted total RNA according to the manufacturer's instructions. Gene expression was assessed using real-time reverse transcription-polymerase chain reaction (qRT-PCR) (Bio-Rad), with typical amplification parameters being 95°C for 30 s, followed by 40 cycles at 94°C for 10 s and 60°C for 10 s. Relative differences were determined by the crossing point method with a standard curve. The mRNA expression of each hypoxic condition after 72 h was compared with the expression under normoxia after normalization with the housekeeping gene GAPDH. The oligonucleotide primer sets used for real-time PCR purchased from TAKARA Bio Inc. (Osaka, Shiga, Japan) were as follows: GAPDH, forward 5'-GCACUGTCAAGGTCUGAA C-3' and reverse 5'-TGTGGAAGAGCGCCAGTGA-3'; SLC7A5, forward 5'-GATGCTGGCCTCACCATTTC-3' and reverse 5'-ACCCAG TGGATGAGCCTCTGAC-3'; SLC7A8, forward 5'-TGTATGCTCTT GCCAATGCTGCTTA-3' and reverse 5'-ATGATCCAGGCGTGAC TCCAT-3'.

Statistical analysis
Significance differences were determined using Student's two-tailed t-test or Welch's t-test, depending on the data distribution, with P < 0.05 considered to indicate statistical significance. Excel 2010 software (Microsoft Corporation, Redmond, WA, USA) with the add-in software Statcel 3 was used for statistical analysis.

RESULTS
Influence of hypoxic condition on cell growth and viability
We first investigated the baseline cytotoxicity of hypoxic conditions on T98G and A172. Cells incubated for 0, 24 h, 48 h and 72 h under either normoxic or hypoxic (1% O2) conditions, were assessed by trypan blue viability assay and cell cycle analysis. Hypoxic conditions significantly inhibited T98G cell growth; however, viable cells remained (comparable with the number of cells seen at 0 h under normoxic conditions), even after 72 h in 1% O2 conditions (Fig. 1A). The same trend was found with the A172 cell line (data not shown). With longer incubation periods under hypoxia, more cells were found to have stopped in G0/G1 phase, and there were fewer in S phase. There was no correlation observed, however, between the number of cells in G2/ M phase and the hypoxia incubation time (Fig. 1B).

14C-BPA uptake in human malignant glioblastoma cells under hypoxia
Next, we investigated the influence of oxygen levels on the %B uptake of human glioblastoma cells. Previous work has indicated that 13C-BPA uptake in human head and neck squamous carcinoma cell lines is decreased under hypoxia with 0.2% O2, compared with normoxic conditions [23]; however, it is unknown whether this effect has a critical threshold or whether 13C-BPA uptake gradually decreases according to the oxygen concentration. In the present study, we incubated T98G and A172 under several oxygen conditions (20%, 10%, 2% and 1% O2) for 72 h, exposed them to 13C-BPA at concentrations of 10, 20 and 30 ppm for 2 h, then analyzed
10B-BPA uptake using ICP-AES. We had previously investigated that adding 10B-BPA into culture medium did not cause a cytotoxic effect by preliminary experiment (data not shown). As shown in Fig. 2, the cellular accumulation of 10B increased according to the BPA concentration in the culture medium under all oxygen conditions, while the 10B concentration tended to decrease gradually according to the decrease in oxygen concentration. In the T98G cell line, significant differences in uptake at 30 ppm 10B-BPA could be seen between normoxia and hypoxia at 10% O2 (P = 0.03), 8% O2 (P = 0.02), and 1% O2 (P = 0.02). In the A172 cell line, significant differences could be seen at 10 ppm 10B-BPA between 10% and 8% O2 (P = 0.01), and between 10% and 1% O2 (P = 0.03), and at 20 ppm 10B-BPA between 10% and 1% O2 (P = 0.03). These results demonstrate that 10B-BPA uptake in glioblastoma is affected in a continuous fashion by changes in oxygen concentration.

**Effect of oxygen levels on LAT1/2 expression**

The relationship between oxygen concentration and 10B uptake in the glioblastoma cell lines that we observed above could be due to changes in the mRNA expression level of LAT1/2 or to inactivation of the LAT1/2 transporter. We thus investigated the influence of hypoxia on LAT1 and LAT2 expression in the glioblastoma cell lines. As shown in Fig. 3, relative mRNA expression levels of LAT1 were decreased significantly by both hypoxic conditions tested (10% and 1%). However, LAT2 expression levels in both cell lines were very low, even when incubated under normoxia. The fluorescence from cells under any oxygen conditions could not be detected until after more than 35 cycles of denaturation and annealing/extension processes (data not shown). These results support the concept that hypoxia can reduce 10B-BPA uptake via a reduction in the mRNA expression of LAT1, but do not rule out the possibility that functional inactivation of LAT1/2 at the protein level may also be involved in this response.

**DISCUSSION**

In the present study, we investigated the influence of hypoxia on the ability of human glioblastoma cells to take up 10B-BPA. The findings are consistent with those of other studies [23, 24]; however, ours is the first to assess the effect of several different levels of hypoxia. We showed that the 10B-BPA uptake of human glioblastoma cells decreases gradually, according to the decrease in oxygen concentration, i.e., without a critical threshold. These results suggest that maintaining normoxic oxygen conditions in the cancer cell microenvironment is likely to be very important for successful BNCT.

It has been previously reported that hypoxic conditions can trigger tumor cells to become quiescent (i.e., not actively dividing), and that these quiescent cells take up 10B-BPA or 10B-BSH compared with non-quiescent cells [25]. This negative effect was reported to be stronger with 10B-BPA than with 10B-BSH, an observation thought to be due to the fact that 10B-BSH largely accumulates in cells via diffusion, while 10B-BPA is actively taken up via LAT1/2 transporters [26, 27]. The cell cycle is known to be an important factor affecting 10B absorption in G2/M phase accumulating more 10B than cells in G0/G1 phase [28]. In the present study, we found no impairment of cell growth and a greater proportion of cells in G0/G1 phase after 72 h in hypoxic conditions, consistent with the idea that suppression of the cell cycle and that of growth by hypoxia inhibits the uptake of 10B-BPA.

**Fig. 2. Reduced oxygen concentration results in lower 10B-BPA uptake in glioblastoma cell lines.** Cells were incubated under several oxygen concentrations—20.8% (normoxia), 10%, 8% or 1% oxygen (hypoxia)—for 24 h, exposed to 10B-BPA with a 10B concentration of 10, 20 or 30 ppm for 2 h under normoxic conditions, and analyzed by ICP-AES. (A) In T98G cells, 10B-BPA uptake gradually decreased in parallel with the decrease in oxygen. There was a significant correlation between 10B-BPA uptake and the 10B-BPA concentration of the culture medium. (B) In A172 cells the same trend was observed (i.e. a gradual decrease in 10B-BPA uptake with decrease in oxygen concentration), although this did not reach statistical significance. Values are expressed as the mean ± standard error; one asterisk indicates P < 0.05 and two asterisks indicate P < 0.01, compared with 10 ppm of 10B, at each oxygen concentration.
Fig. 3. Reduced oxygen conditions decrease mRNA expression levels of LAT1. Cells were incubated at oxygen concentrations of 20.8% (normoxia), 10% or 1% oxygen (hypoxia) for 72 h, followed by total RNA extraction and qRT-PCR. (A) In T98G, relative expression of LAT1 was significantly lower than in cells incubated under normoxia. (B) In A172, similar results were obtained as for the T98G cell line. Values are expressed as the mean ± standard error.

largely dependent on the amount of $^{10}$B-BPA taken up by tumor cells. This may suggest a significant challenge, because the tumor microenvironment may be severely hypoxic as the result of rapid tumor growth and a dramatic increase in oxygen consumption with insufficient, or abnormal, vascularization [29]. Recently, several reports [30–32] have indicated that 4-bromono-[18F] fluoro-phenylalanine PET (18F-BPA-PET) could predict approximate accumulation of BPA in a tumor before BNCT; however, this prediction is limited by the spatial resolution of the PET scanner; therefore, the precise accumulation cannot be reflected [33]. Previous studies using a PET image with a hypoxic tracer reported there was a rich hypoxic area in the glioblastoma mass, and in consideration of the present results, the dose distribution in hypoxic areas may be overestimated compared with the actual distribution in BNCT. This may be largely related to poor results of the clinical trial [15]; operative resection followed BNCT for newly diagnosed glioblastoma. For the success of BNCT, techniques are therefore required in order to allow cancer cells under hypoxia to accumulate as much $^{10}$B-BPA as they would under normoxic conditions.

To date, several approaches have been tried for overcoming the hypoxic disadvantage of BNCT. These strategies can be roughly classified into two methods—first, developing improved $^{10}$B carriers, and second, increasing the oxygen concentration at the tumor site. Maruoka et al. tested new $^{10}$B carriers synthesized from a hypoxia-specific cytotoxic bioreductive agent (TN-2100) in vivo, and reported not only that the new chemistry exhibited a radiosensitizing effect in hypoxic tumor cells [34], but that this positive effect was enhanced when combined with mild hyperthermia [35]. Similarly, Lieder et al. evaluated a new $^{10}$B carrier (a bromotetra-2-nitroimidazole derivative) designed to have preferential retention in hypoxic glioma cells, and reported low cytotoxicity in normal cells and higher long-term tumor retention compared with $^{10}$B-BPA [36]. However, these new carriers have not yet been brought into the clinical setting.

Alternatively, since the hypoxic effect is stronger when using $^{10}$B-BPA compared with $^{10}$B-RSH because of the difference in uptake mechanism [23, 24], the combined use with both $^{10}$B-BPA and $^{10}$B-RSH may improve treatment efficacy, but this approach has yet to be formally evaluated. In regard to improving local hypoxia, Maruoka et al. demonstrated the potential benefit of mild hyperthermia in combination with administration of nicotinamide (a vitamin B3 analogue that prevents the development of acute hypoxia) for effective BNCT in vivo [24]. Hyperthermic oxygen therapy prior to $^{10}$B carrier administration may be a further strategy; however, further study of the effects of re-oxygenation, including the timing and duration of this type of intervention is needed.

In conclusion, local hypoxia is likely to have a negative influence on the efficacy of BNCT through reducing the amount of $^{10}$B-BPA taken up by cancer cells, which in turn reduces the cytotoxic effect following neutron irradiation. The present study suggests that this negative influence correlates linearly with oxygen concentration, without a critical threshold, such that maintaining the local oxygen concentration in tumors may be a promising method for decreasing the rate of recurrence of glioblastomas after BNCT.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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