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3 **Dose-rate effect was observed in T98G glioma cells following BNCT**
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55 Keywords: dose-rate effect, BNCT, DNA-DSBs, glioma
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3 ABSTRACT
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6 Background: It is generally said that low LET radiation produce high dose-rate effect, on the
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9 other hand, no significant dose rate effect is observed in high LET radiation. Although high LET
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12 radiations are produced in BNCT, little is known about dose-rate effect of BNCT.
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16 Materials and Methods: T98G cells, which were tumor cells, were irradiated by neutron mixed
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19 beam with BPA. As normal tissue derived cells, Chinese hamster ovary (CHO-K1) cells and
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22 DNA double strand breaks (DNA-DSBs) repair deficient cells, xrs5 cells were irradiated by the
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25 neutrons (not including BPA). To DNA-DSBs analysis, T98G cells were stained
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28 immunochemically with 53BP1 antibody. The number of DNA-DSBs was determined by
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31 counting 53BP1 foci.
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35 Results: There was no dose-rate effect in xrs5 cells. D_0 difference between 4cGy/min and
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38 20cGy/min irradiation were 0.5 and 5.9 at the neutron and gamma-ray irradiation for CHO-K1,
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41 and 0.3 at the neutron for T98G cells. D_0 difference between 20cGy/min and 80cGy/min
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44 irradiation for T98G cells were 1.2 and 0.6 at neutron irradiation plus BPA and gamma-ray. The
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47 differences between neutron irradiations at the dose rate in T98G cells were supported by not
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50 only the cell viability but also 53BP1 foci assay at 24 hours following irradiation to monitor
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53 DNA-DSBs.
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57 Conclusion: Dose-rate effect of BNCT when T98G cells include 20ppm BPA was greater than
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3 that of gamma-ray irradiation. Moreover, Dose-rate effect of the neutron beam when CHO-K1
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6 cells did not include BPA was less than that of gamma-ray irradiation These present results may
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9 suggest the importance of dose-rate effect for more efficient BNCT and the side effect
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13 reduction.

14 15 16 17 18 19 INTRODUCTION

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22 Boron neutron capture therapy (BNCT) is a treatment that can selectively destroy
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25 tumor tissue by $^{10}\text{B} (n,\alpha) ^7\text{Li}$ reaction without significant damage to normal tissue. In principle,
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28 ^{10}B compounds are selectively delivered to tumor cells and exposed to low-energy thermal
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31 neutrons. The reaction with thermal neutron produces an alpha particle and ^7Li nucleus that have
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34 high linear energy transfer (LET) values and enriched biological effect. Although it depends on
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37 the kinds of ^{10}B compounds, DNA damage or decrease in cell viability produced by BNCT is
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40 greater than neutron alone or gamma-ray (Kinashi et al., 2011, Dagrosa et al., 2011).
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45 It is generally accepted that for cell viability, low LET radiation such as X-ray and
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48 gamma-ray produce high dose-rate effect, on the other hand, no significant dose rate effect is
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51 observed in high LET radiation such as neutrons, alpha particle, and heavy ion (Metting et al.,
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54 1985, Ainsworth et al., 1976, Heiber et al., 1987, Yang et al., 1986, Furusawa et al., 2006,
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57 Fairchild et al., 1985). However, there is no data about dose-rate effect for BNCT. It is also
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3 difficult to estimate dose-rate effect of BNCT from the past studies because the neutron beam
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6 used for BNCT is the mixed beam that contains thermal, epithermal and fast neutrons, and
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9 gamma-ray. A better understanding of dose-rate effect for BNCT will help to improve the
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12 efficiency of this therapy as well as to reduce the side effect in normal tissue.
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16 DNA double strand breaks (DNA-DSBs) are potentially lethal lesions and important
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18 biological effect of ionizing radiation. DNA-DSBs can be repaired by DNA repair proteins, such
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21 as gamma-H2AX, DNA-PKcs, and 53BP1. Therefore, immunochemically stained foci for the
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24 proteins have been used for a sensitive and efficient marker for DNA-DSBs. Several studies
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27 using this method showed that the number of foci may reflect not only the dose but also the
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30 types of radiation. Okayasu et al. reported that the disappearance of phosphorylated DNA-PKcs
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33 foci in 180BR cells irradiated heavy ions were slow (Okayasu et al., 2006).
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38 We investigated dose-rate effect of the neutron mixed radiation beam used for BNCT
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41 in Kyoto University Research Reactor (KUR) at cell survival fraction and DNA-DSBs. We
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44 report here that dose-rate effect of BNCT is observed in T98G cells that are human tumor cell
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47 line at above-mentioned both biological points.
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51 52 53 MATERIALS AND METHODS

54 55 56 57 *Cell culture*

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3 The Chinese hamster ovary-derived, wild-type CHO-K1 cells and the human
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6 glioblastoma cell lines, T98G were purchased from Riken BRC Cell Bank. The radiosensitive
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9 xrs5 mutant cells (Ku80-deficient CHO cell line) kindly supplied by Prof. P Jeggo (Jeggo et al.,
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12 1983, Kemp et al., 1984). CHO-K1 and xrs5 cells were cultured in Memo medium (Invitrogen)
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15 supplemented with 10% heat-inactivated FBS (Biowest). T98G cells were grown in RPMI 1640
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18 (Invitrogen) supplemented 10% heat-inactivated FBS (Biowest). These cells were maintained at
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22 37°C in a humidified atmosphere with 5.0% CO₂.

23 24 25 *¹⁰B compound and Irradiation experiments*

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28 For irradiation, the cells were trypsinized, suspended in the above mentioned medium,
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31 and then aliquoted to each teflon tube. A stock solution of ¹⁰B-para-boronphenylalanine (BPA)
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34 was used for BNCT experiment to T98G cells. T98G cell suspensions were incubated with BPA
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37 at 20ppm concentration 1 hour before neutron irradiation. The Heavy Water Facility of the
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40 Kyoto University Research Reactor (KUR) was used for neutron mixed beam irradiation
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43 (approximately 4cGy/min and 20cGy/min). Neutron fluencies were measured by radioactivation
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46 of gold foil and gamma-ray doses by TLD. The physical dose percentages to total dose of
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49 thermal (<0.5eV), epithermal (0.5eV-10keV), fast (>10keV) neutrons, and gamma-ray were
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52 approximately 30%, 3%, 22% and 45% respectively, when the reactor was operated at
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55 4cGy/min or 20cGy/min (Fig. 1). Gamma-ray irradiation was carried out with a ⁶⁰Co
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3 gamma-ray irradiator at dose rate of about 4cGy/min, 20cGy/min, 80cGy/min and 2Gy/min.
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6 *Cell survival assay*
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9 Survival fraction of CHO-K1, xrs5 and T98G cells were determined by conventional
10 colony formation assay. After irradiation, the cell suspension solutions were properly diluted,
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12 and the known number of cells were seeded in cell culture dishes and incubated for 7-9 days
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14 (CHO-K1 and xrs5) and 2 weeks (T98G). The cells were fixed with 70% ethanol and stained
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16 with crystal violet solution (Wako) for counting colonies.
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25 *Immunofluorescent staining*
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28 Irradiated T98G cells were seeded onto 22 mm × 22 mm cover slips in 6-well micro
29 plates, incubated for 30min, 1, 3, 6 and 24 hours, and washed with cold PBS (Invitrogen). The
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31 cells were fixed with 3.6% formalin/PBS for 15 min, permeabilized with 0.5%
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33 TritonX-100/PBS on ice for 15 min and washed thoroughly with PBS. The cover slips were
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35 incubated for 2 hours at 37°C with primary antibody, rabbit monoclonal anti-53BP1 antibody
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37 (Bethyl Laboratories) in TBS-DT solution (20 mM Tris-HCl, 137 mM NaCl, containing 0.1%
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39 Tween-20, 5% skim milk). After being washed three times with cold PBS, the cells were
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41 incubated for 1 hour at 37°C with secondary antibody, Alexa Fluor 594 goat anti-rabbit IgG
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43 (Invitrogen) in TBS-DT solution. After washing three times with PBS, The cover slips were
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45 mounted on slide glasses with DAPI (4',6-diamidino-2-phenylindole) in 10% glycerol/PBS to
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6 *Image acquisition and Analysis*
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9 Images were acquired with a fluorescence microscope (KEYENCE, BZ-9000). ImageJ
10 (National Institutes of Health) were used for image processing and automated foci counting.
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15 Some foci images were scored by eye.
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22 **RESULTS**
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25 *Dose-rate effect was observed in CHO-K1 cells at cell survival assay*
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28 Fig. 2 shows cell survival fraction of CHO-K1 (referred as K1 in Fig. 2) and xrs5 cells
29 irradiated with the neutron mixed beam. A scatter plot is used because the information of the
30 dose rate was not available in real time so that the total doses were not always same in the
31 mixed beam irradiation. Each point shows the survival fraction, and black and dot lines are used
32 to fit log-linear model with Microsoft Excel software. As expected, xrs5 cells were more
33 sensitive to the mixed beam than CHO-K1 cells. There was no significant difference for the cell
34 survival at xrs5 cells between 4cGy/min and 20cGy/min irradiation (Fig. 2 and Table 1). On the
35 other hand, dose-rate effect between 4cGy/min and 20cGy/min was observed in CHO-K1 cells
36 irradiated with especially gamma-ray (Fig. 2B). The D_0 differences in CHO-K1 cells were 0.5
37 and 5.9 for neutron and gamma-ray irradiation (Table 1). The surviving fraction for CHO-K1
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3 cells after 20cGy/min gamma-ray irradiation was significantly lower than that after 4cGy/min
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6 irradiation.
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10 *Dose-rate effect of BNCT was greater than that of gamma-ray at T98G cell survival assay*

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12 Fig. 3 shows cell survival fraction of T98G cells irradiated with the neutron mixed
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14 beam alone (referred as no BPA in Fig. 3A) or the mix beam plus 20ppm BPA (referred as plus
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16 BPA in Fig. 3B). When T98G cells did not include BPA, D_0 between 4cGy/min and 20cGy/min
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18 irradiation did not show a big difference (D_0 difference was 0.3). 20cGy/min irradiation plus
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20 BPA produced a decrease in cell survival fraction, compared to 4cGy/min plus BPA (Fig. 3B)
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22 and the D_0 difference was 1.2 (Table 2). We also researched dose-rate effect of gamma-ray in
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24 T98G cells because it was thought that gamma-ray in the neutron beam significantly contribute
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26 to dose-rate effect. Assuming that T98G cells include 20ppm BPA, the dose-rate of 4cGy/min
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28 and 20cGy/min neutron irradiation is converted to 20cGy/min and 80cGy/min, due to ^{10}B (n,α)
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30 ^7Li reaction. In Fig. 4, the difference of D_0 between 20cGy/min and 80cGy/min in plus BPA
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32 group was larger than that in gamma-ray group.
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48 *Induction of 53BP1 foci in T98G cells*

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51 Dose-rate effect was greater in T98G cells incubated with BPA (Table 2 and Fig. 4). So,
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53 we researched DNA-DSBs, because it is said that high LET radiation induces more DNA-DSBs
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55 than low LET radiation. The results of 53BP1 foci in T98G cells 30min after neutron irradiation
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3 are shown in Fig. 5. Although induction of 53BP1 foci increased with dose, the number of
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6 53BP1 foci following 20cGy/min irradiation seemed to be larger than 4cGy/min irradiation,
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9 regardless of existence of BPA.
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11 12 *Loss of 53BP1 foci in T98G cells*

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16 Fig. 6 shows time dependence of 53BP1 foci after irradiation. The number of 53BP1
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19 foci in not BPA including cells rapidly decrease to about zero, compared to the cells incubated
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22 BPA. When T98G cells include BPA, a lot of 53BP1 foci had remained even at 24 hours after
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25 neutron irradiation. Moreover, the difference between the cells plus BPA irradiated with
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28 4cGy/min and 20cGy/min was significant at 24hours post irradiation.
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31 32 33 34 35 DISCUSSION

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38 In this report, we showed that dose-rate effect was observed at cell survival rate in
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41 T98G cells following BNCT, and the effect was greater even if it was compared with
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44 gamma-ray irradiation at the same dose-rate (Fig. 4). Little dose-rate effect of gamma-ray or the
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47 neutron mixed beam (not irradiated with BPA) was observed in xrs5 cells. On the other hand,
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50 there was the dose-rate effect in CHO-K1 cells. This means that dose-rate effect is not observed
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53 in DNA damage repair deficient cells, such as xrs5 cells. DNA-DSBs were repaired, when
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56 T98G cells were irradiated with the neutron beams or X-ray (Fig. 6) (Short et al., 2007).
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3 Therefore for T98G cells, the dose-rate effect would be observed.
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6 We treated CHO-K1 and xrs5 cells derived from normal tissues, and T98G cells that
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8 are tumor cells to investigate the dose-rate effect of BNCT. In BNCT, highly ^{10}B distributed
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10 tumor cells are exposed to thermal neutrons. The alpha particles and ^7Li nuclei produced by ^{10}B
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12 $(n,\alpha)^7\text{Li}$ reaction have short paths ($\sim 10\mu\text{m}$), same as a cell size (Liu et al., 2009). Therefore, it is
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14 thought that only tumor cells are affected by BNCT and normal cells are irradiated with the
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16 neutron mixed beam. These present results may suggest that dose-rate effect of the neutron
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18 mixed beam for BNCT would contribute to both higher cytotoxicity reaction for tumor cells
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20 through BNCT and relative decline of biological effect to normal cells.
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31 The kinetics of the number of 53BP1 foci for DNA-DSBs detection was also further
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33 evidence to the dose-rate effect of the neutron mixed beam and BNCT. High-LET radiation
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35 induces DNA-DSBs that are not repaired or more difficult to be repaired compared with
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37 low-LET radiation (Okayasu et al., 2006, Hill et al., 1999, Terato et al., 2008). Although there
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39 did not seem to be the significant difference about induction of DNA-DSBs, we showed that the
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41 DNA-DSBs induced by the neutron mixed beam alone was fast or easy to be repaired compared
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43 to the mixed beam plus BPA. Moreover, we thought that the significant difference between
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45 4cGy/min and 20cGy/min about the number of 53BP1 foci 24 hours after BNCT irradiation
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51 might reflect the difference of the survival rate produced by dose-rate effect in the case of
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3 including BPA (Fig.3B and Fig. 6). We demonstrated the relationship between DNA-DSBs and
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6 the lethal potential, provided that kinetics of 53BP1 foci correlated with DNA-DSBs repair.
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10 There is the hypothesis that residual foci are unable to pass mitosis (Marková et al.,
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12 2007). Although this means that few foci are observed in the cell exceeding the duration of the
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14 cell cycle after irradiation, recent studies show that the residual 53BP1/gamma-H2AX foci may
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16 be available for biological dosimetry. Primary induced foci and residual foci may be different
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18 types of foci (Marková et al., 2011). It is reported that the doubling time of T98G cells are 22-26
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20 hours (Choi et al., 1997, Nakada et al., 2005). We also calculated the doubling time to be
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22 approximately 24 hours from their growth curve analysis. So, we concluded that the difference
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24 of 53BP1 foci between 4cGy/min and 20cGy/min at 24 hours after BNCT irradiation was the
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26 variance of the degree of DNA-DSBs repair.
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38 In this study, using cell survival assay, we found that greater dose-rate effect were
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40 observed in T98G cells underwent BNCT than gamma-ray, and that the effect were less in
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42 CHO-K1 cells irradiated with the neutron beam alone, compared with gamma-ray. Time
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44 dependence of 53BP1 foci was not direct evidence of dose-rate effect of BNCT because the
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46 converted absorbed dose was not same between 4cGy/min and 20c/min irradiation. However,
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48 the difference of 53BP1 foci reflected the difference of the survival rate results. Although we
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51 have thought that dose-rate effect of BNCT was not that of alpha particles or ⁷Li nuclei
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produced by $^{10}\text{B} (n,\alpha) ^7\text{Li}$ reaction, we are interested in this result.

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3 FIGURE LEGENDS
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6 Fig. 1. The physical doses percentage of thermal, epithermal, fast neutrons, and gamma-ray in
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8
9 the neutron mixed beams, when samples were irradiated at KUR operated at 4cGy/min
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12 or 20cGy/min.
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16 Fig. 2. Dose-rate effect on survival fraction for CHO-K1 and xrs5 cells irradiated with neutron
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19 mixed beam (n) (A) or gamma-ray (γ) (B).
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22 Fig. 3 Dose-rate effect at survival fraction for T98G cells irradiated with neutron mixed beam in
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25 case of not including BPA (A) or including BPA (B). In Fig. 3B, the horizontal axis is
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28 the absorbed dose converted assuming that the concentration of BPA in T98G cells is
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31 20ppm.
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35 Fig. 4 Dose-rate dependence of D_0 for T98G cells. The dose rate of plus BPA + neutron
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38 irradiation group is converted assuming that the concentration of BPA in T98G cells is
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41 20ppm.
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44 Fig. 5. The number of 53BP1 foci induced in T98G cells at 30 min after the neutron mixed
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47 beam irradiation in case of not including BPA (A) or including BPA (B). The data are
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50 compiled from two independent experiments. The error bars indicate the standard errors
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53 of the mean.
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3 Fig. 6. Time dependent loss of 53BP1 foci in T98G cells following approximately 50min
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6 neutron mixed beam irradiation at 4cGy/min and 10min at 20cGy/min. Note that the
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8 absorbed dose converted assuming that the concentration of BPA in T98G cells is
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10 20ppm is different between 4cGy/min and 20cGy/min irradiation. The converted dose
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12 was approximately 10Gy in 4cGy/min neutron irradiation plus BPA, and 8Gy in
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14 20cGy/min plus BPA. The data are compiled from two independent experiments at each
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16 point. The error bars indicate the standard errors of the mean.
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Table 1. D_0 for CHO-K1 and xrs5 cells irradiated with neutron mixed beam or gamma-ray

D_0 (Gy)	CHO-K1	xrs5
Neutron mixed beam 4cGy/min	2.2	0.40
Neutron mixed beam 20cGy/min	1.7	0.35
Gamma-ray 4cGy/min	7.4	0.53
Gamma-ray 20cGy/min	1.5	0.55

Table 2. D_0 for T98G cells irradiated with neutron mixed beam in case of including BPA or not.

D_0 (Gy)	no BPA	plus BPA
Neutron mixed beam 4cGy/min	1.8	2.7
Neutron mixed beam 20cGy/min	1.5	1.5

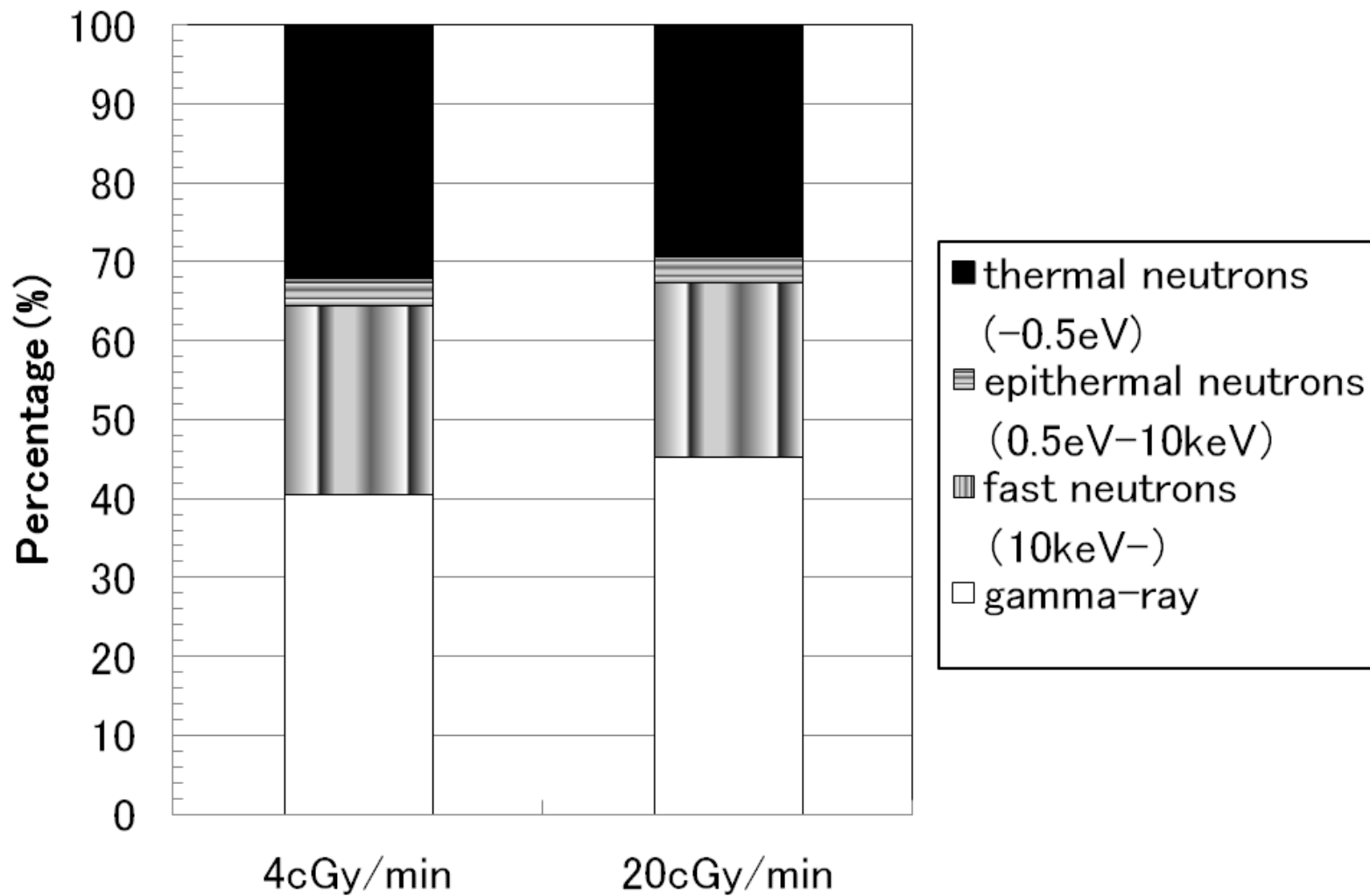


Fig.1

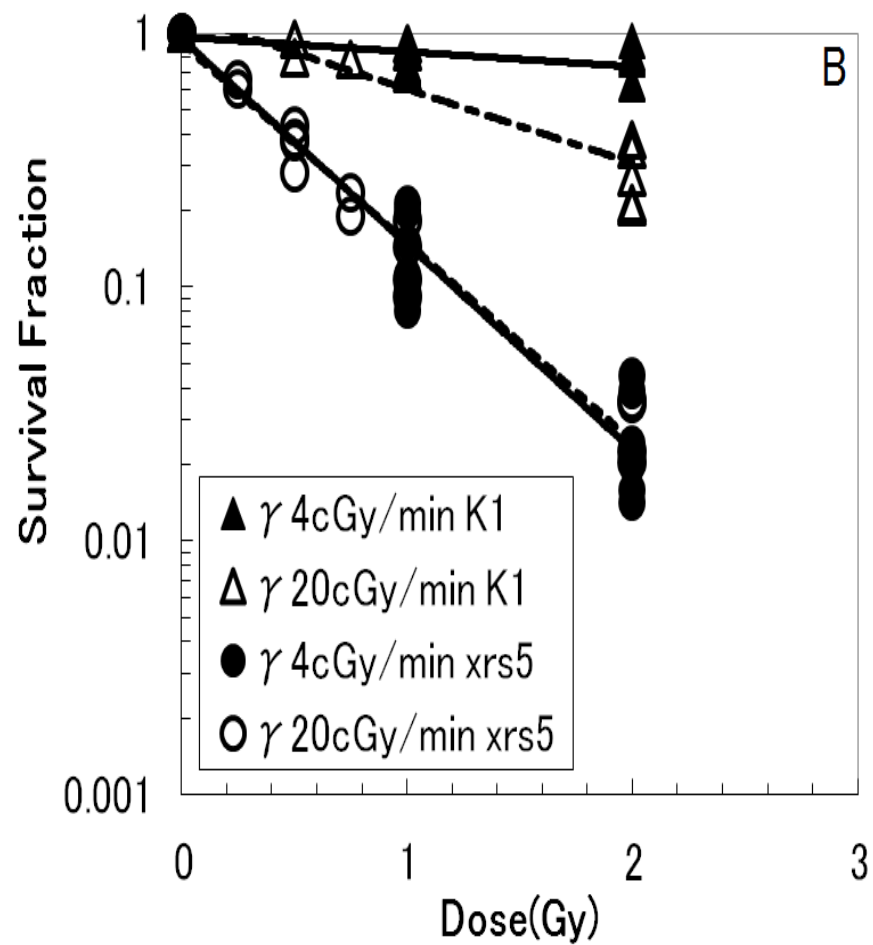
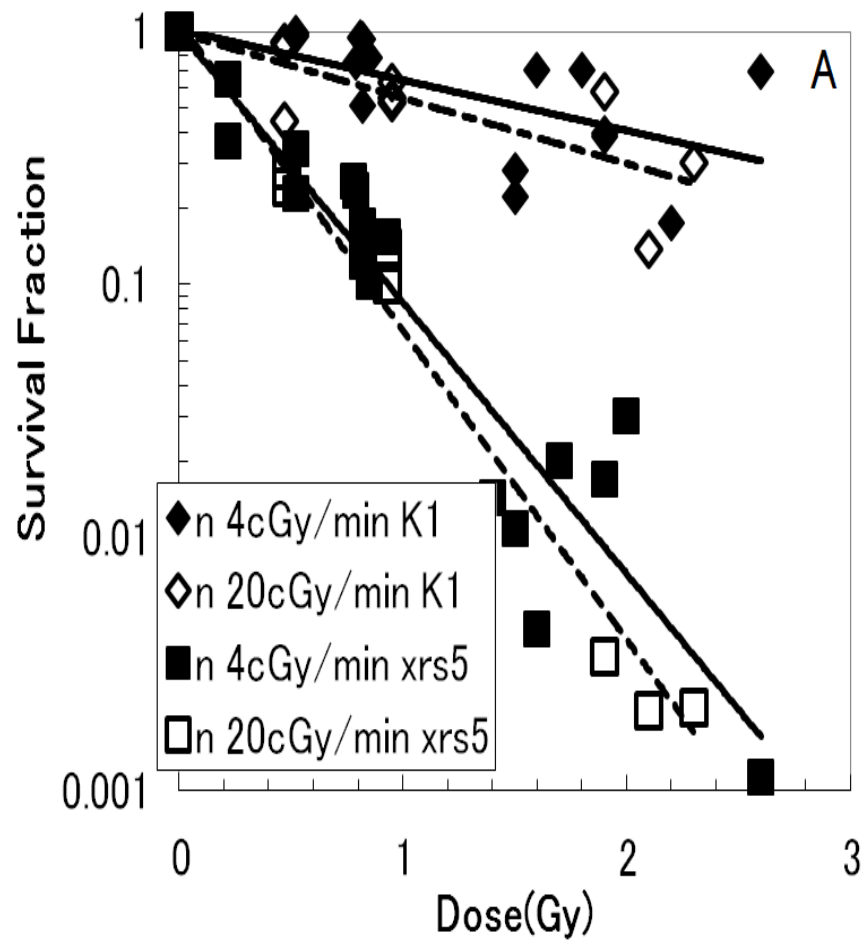


Fig.2

Figure(s)

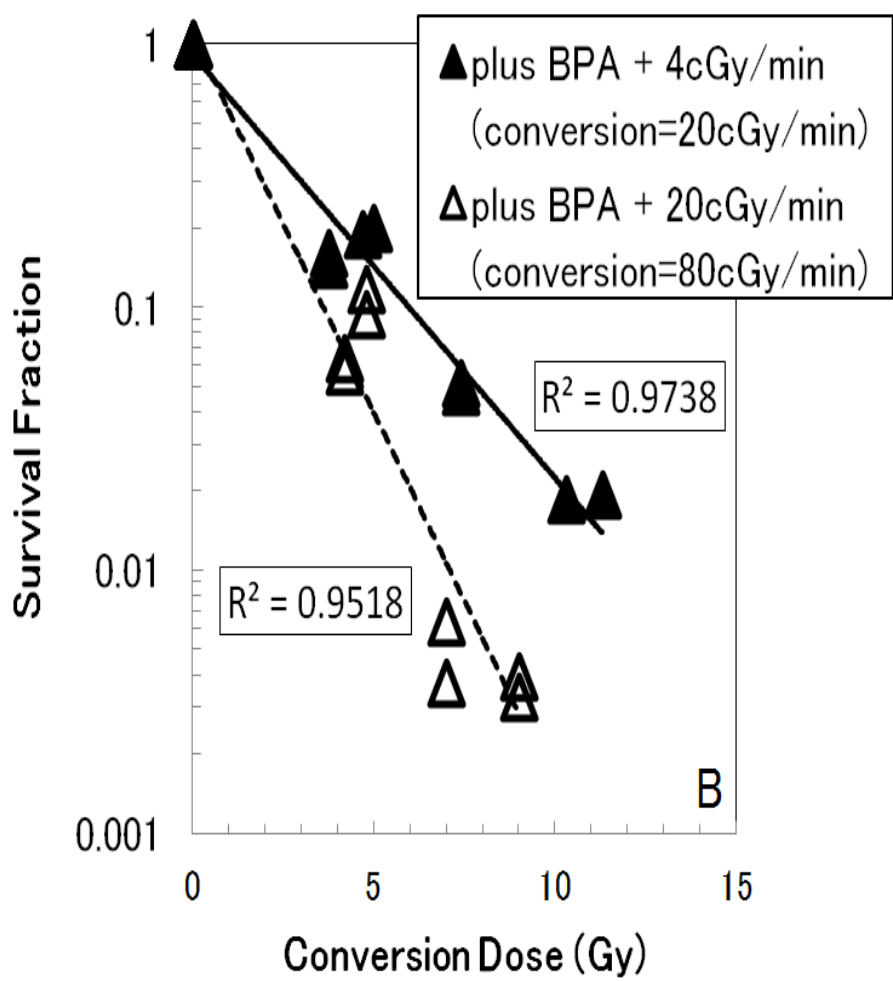
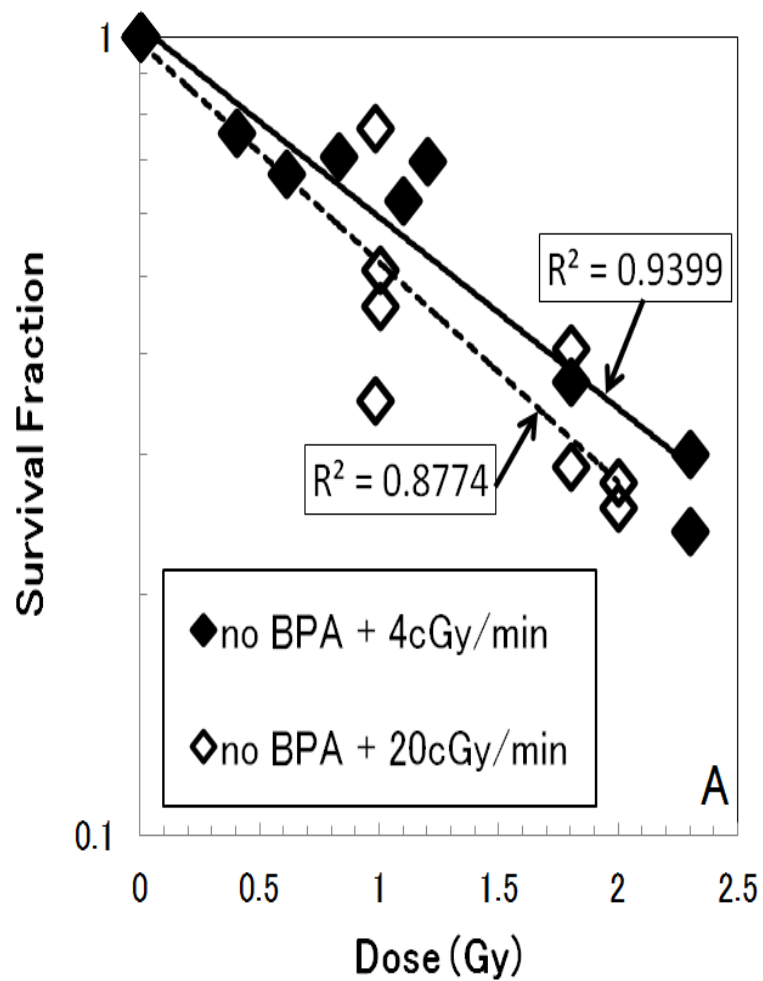


Fig.3

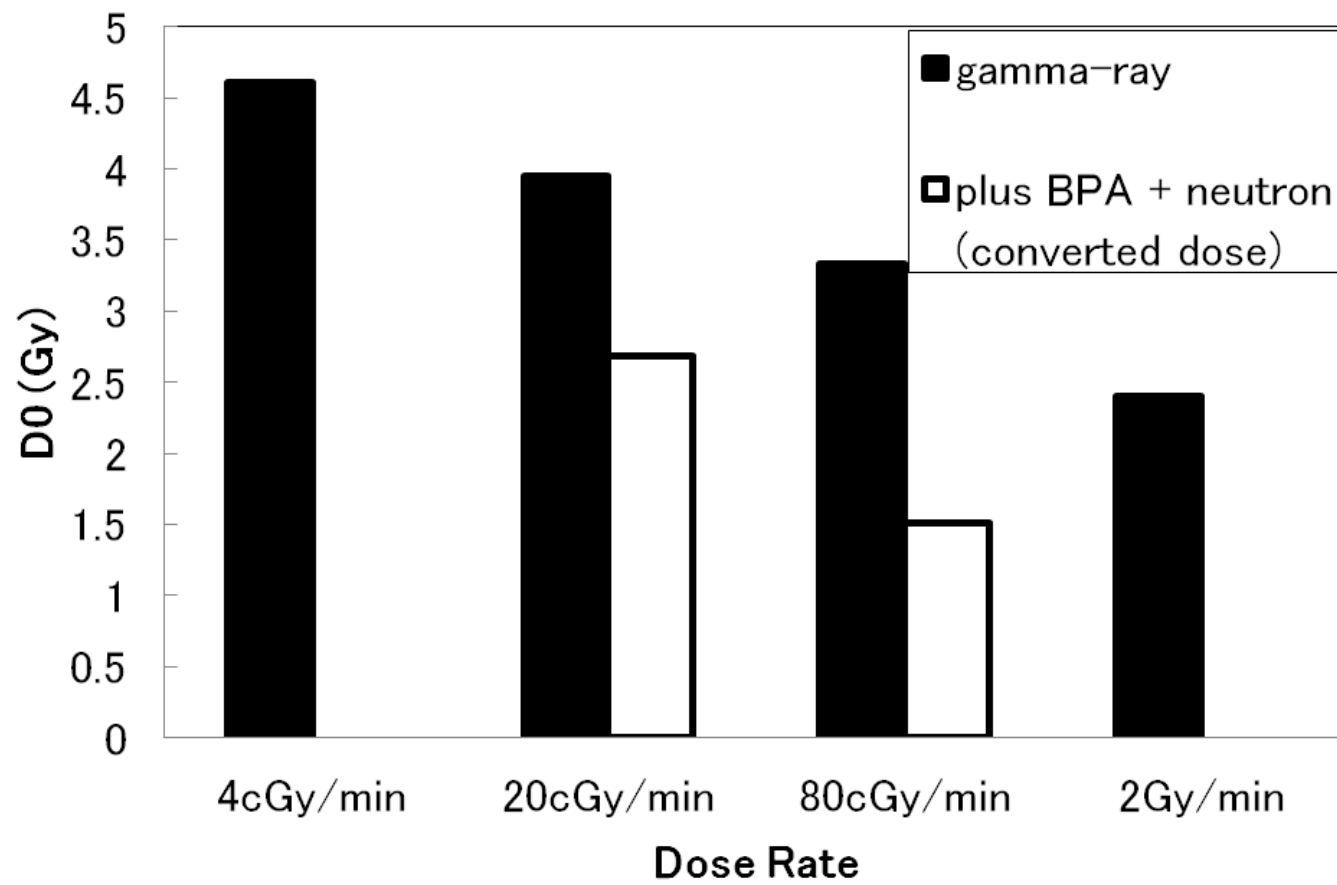


Fig.4

Figure(s)

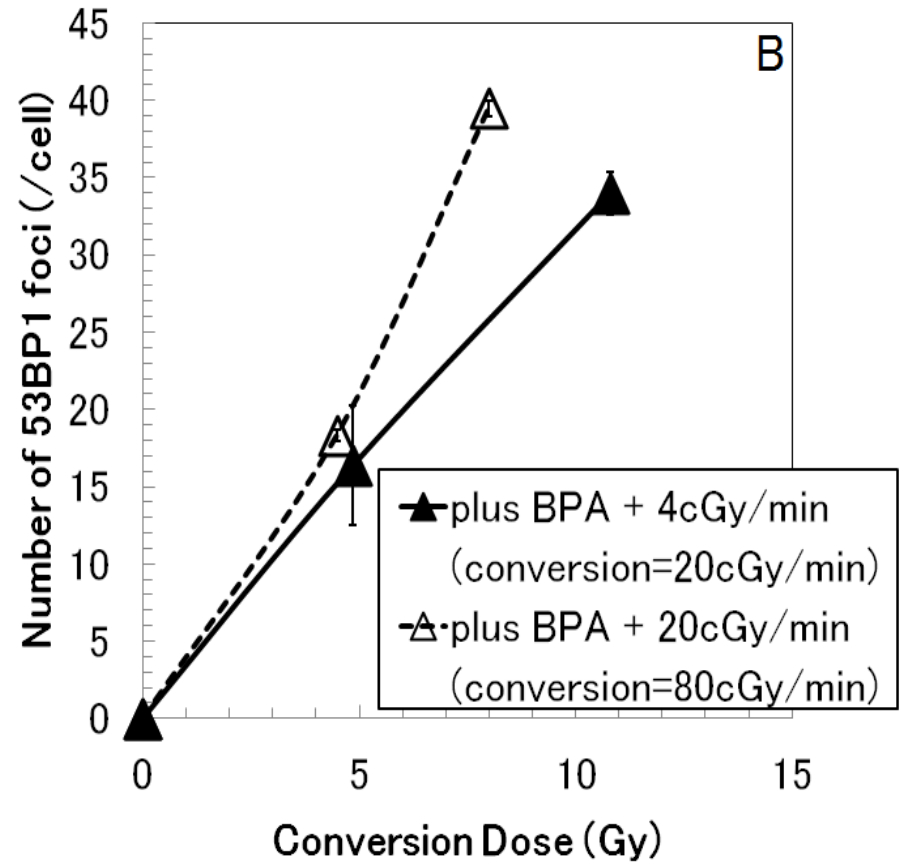
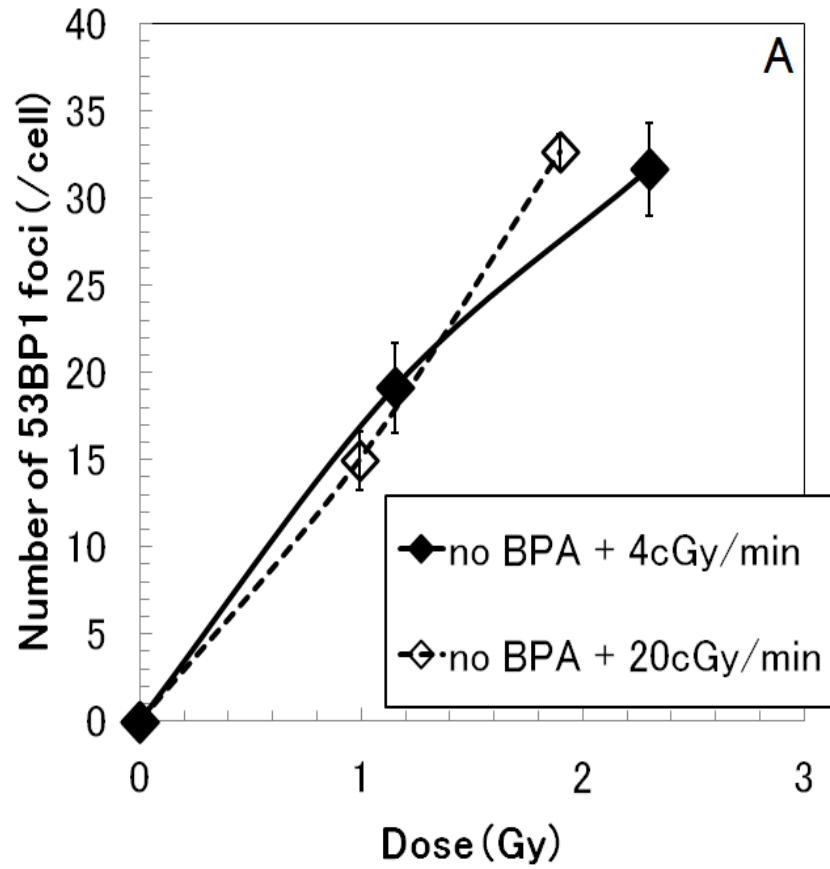


Fig.5

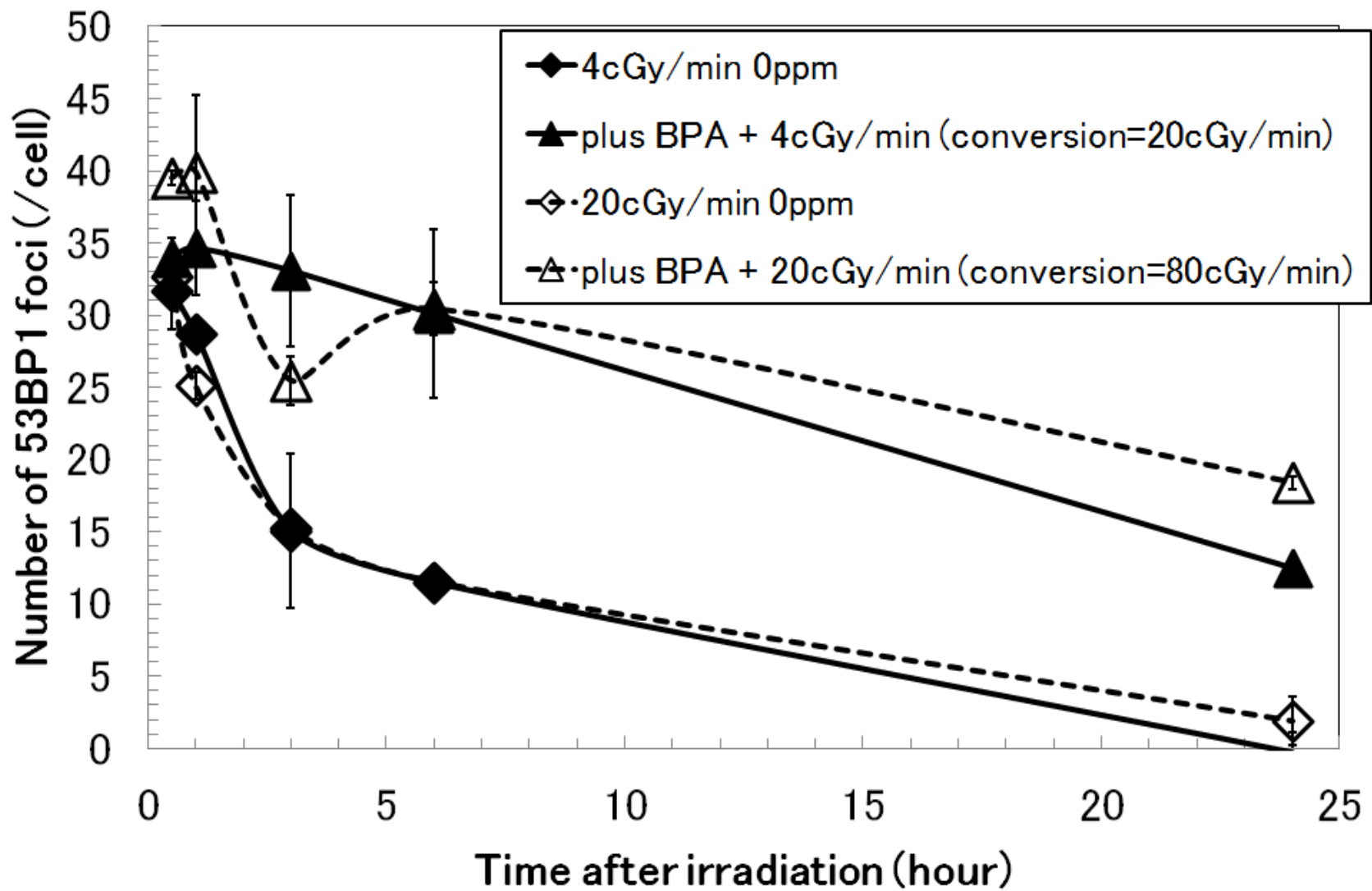


Fig.6