No Different Sensitivity in Terms of Whole-Body Irradiation between Normal and Acatalasemic Mice

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Summary **To elucidate the radiosensitivity of an acatalasemic mouse, we examined the time and dose-dependency in the survival rates, the lymphocytes and the intestinal epithelial cells, and the antioxidant function after 3.0 to 12.0 Gy whole body irradiation. Results showed that no significant differences between acatalasemic mice and normal mice were observed in the survival rates and the histological changes in spleens and small intestine after each irradiation. The catalase activities in livers and spleens of acatalasemic mice were significantly lower than those of normal mice and the glutathione peroxidase activity in livers of acatalasemic mice was significantly higher than that of normal mice. At 10 days after 6.0 Gy irradiation, the catalase activities in livers of acatalasemic and normal mice and that in spleens of normal mice significantly decreased compared with no-irradiation control, and there were no differences between those catalase activities. The total glutathione content in acatalasemic mice was significantly higher than that in normal mice at 10 days after 6.0 Gy irradiation. These findings suggested that the radiosensitivity of acatalasemic mice in terms of whole body irradiation doesn't significantly differ from that of normal mice, probably due to compensated sufficient contents of glutathione peroxidase and total glutathione in acatalasemic mice.**

***Key Words*: acatalasemic mouse, radiosensitivity, catalase, glutathione peroxidase, total glutathione**

Introduction

Irradiation generates reactive oxygen species (ROS) such as hydroxyl radical ($^{\cdot}OH$) and superoxide anion [*1*]. These ROS injure various types of cells [*2*, *3*]. It is well known that scavenging activities of superoxide dismutase (SOD) is a conversion of superoxide anion into hydrogen peroxide (H_2O_2). SOD inhibits radiation-induced oxidative damage such as radioresistance [*4*, *5*]. It has been reported that X-ray dose required to kill 50% of SOD treated mice was higher than that of saline treated mice [*5*].

There has been an awareness that the cellular formation of highly H_2O_2 or $^{\cdot}OH$ probably causes the DNA damage which finally contributes to carcinogenesis [*6*]. Catalase is an important component of the cellular defense system against

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damage induced by ROS. In terms of the regulation of intracellular H_2O_2 in biological systems, both catalase and glutathione peroxidase (GPx) are responsible, the former was suggested to play a major role in H_2O_2 breakdown, particularly when H_2O_2 is overproduced [*7*]. On the other hand, hypocatalasemic mice have higher catalase activity than acatalasemic mice and lower catalase activity than normal mice. For example, the catalase activities of blood and tissues in acatalasemic mice (C3H/AnLCs^{b}Cs^b) are one-tenth to half, and those of hypocatalasemic mice (C3H/AnLCs^{c}Cs^c) are two-thirds those of normal mice (C3H/AnLCs^{a}Cs^a), respectively [*8*]. And no significant differences in the SOD activities and the lipid peroxide levels were observed between brain and liver of acatalasemic mice and those of normal mice, respectively [*9–11*]. Sehaffer discovered a point mutation at amino acid 11 (from glutamine to histidine) of catalase gene in acatalasemic mice, which was suggested to be responsible for the catalase deficiency [*12*].

We have reported that the effects of post low-dose (0.5 Gy) X-irradiation reduced the oxidative damage under carbon tetrachloride-induced hepatopathy in acatalasemic mice or normal mice. Results showed that the histological disorder was improved by 0.5 Gy irradiation. The fat degeneration in normal mice was quickly reduced, in contrast to that in acatalasemic mice. These findings suggest that low-dose irradiation after carbon tetrachloride administration accelerates the rate of recovery and that catalase plays an important role in the recovery from hepatopathy induced by carbon tetrachloride [*13*].

Moreover, we have reported that the histological changes of lymphatic follicles following 0.25, 0.5, or 15 Gy irradiation in the spleens of BALB/c mice, which is sensitive to radiation compared with other strains, and C57BL/6J mice, which is resistant to radiation, using the hematoxylin-eosin staining with the lymphatic follicles or the methylgreen pyronin staining with the plasma cells. Results showed that the lymphatic follicles in the spleens of two mice strains decreased at 24 or 48 h after 15 Gy irradiation. At 4 h after 0.25 Gy irradiation, plasma cells increased in the spleens of two mouse strains. These findings suggest, by histology, that low-dose irradiation activates the plasma cells and enhances the immune function [*14*].

The acatalasemic mice were made by the fractional irradiation with a total dose of 600 R to the offspring of fathers (C3H mice), which were showing a high frequency of specific locus mutation. These mice provide a useful model to study the possible role of H_2O_2 of these mice in carcinogenesis [*15*, *16*]. For example, the C3H mouse spontaneously develops mammary tumors [*17*, *18*], though no mammary tumors are reported in the acatalasemic mouse. It was also reported that the high-dose X-irradiation to hypocatalasemic mice readily induces signet ring cell carcinoma [*19*].

Considering this background, in this study, to elucidate the radiosensitivity of acatalasemic mouse, we examined the time and dose-dependent changes in the survival rates, the weights of whole bodies and spleens, and the lymphocytes and intestinal epithelial cells after each irradiation. We also investigated the time and dose dependent changes in antioxidant function, such as the activities of SOD, catalase and GPx, total glutathione content, and lipid peroxide level, after each irradiation.

Materials and Methods

Animals and X-irradiation

Two strains of C3H mice originally provided by Feinstein *et al.* [*20*], normal and acatalasemic strains, about 6 weeks of age and 20–25 g of body weight were kept under an air-conditioned room (temperature 20°C and humidity 60%) at Department Animal Resources, the Advanced Science Research Center, Okayama University. They were fed on Oriental MF diet (Oriental Yeast Co., Tokyo) and tap water *ad libitum*. The number of mice per experimental group is 5-26. The study protocol was according to the animal experimental guideline of Okayama University.

Each mouse was given whole body irradiation with a single dose of 3.0, 3.6, 4.2, 4.8, 5.4, 6.0, 7.2, 8.4, 9.6, 10.8, or 12.0 Gy at a dose rate of 3.0 Gy/min of X-rays (maximum rated output voltage; 150 kV, ampere; 20 mA, filters; Al:Cu = 0.5:0.2), using an X-ray generator (Hitachi MBR-1505R2). The age-matched control mice were sham-irradiated. Each mouse was weighed at 7, 14, 21, or 28 days after these irradiations. At 5 or 10 days after 6.0 Gy irradiation and at 5 days after 12.0 Gy irradiation, the acatalasemic mice and the normal mice were sacrificed by cervical dislocation. Each experimental group consisted of 5 mice. Livers and spleens were quickly excised for the analyses of the activities of SOD, catalase and GPx, total glutathione content, and lipid peroxide level. The Kaplan-Meier method was used to generate survival curves.

Biochemical assays

Liver and spleen of mouse were homogenized in 1 M Tris- HCl 5 mM ethylendiaminetetraacetic acid buffer (pH 7.4). The homogenate was centrifuged at 15,000 × g, for 45 min at 4°C and the supernatant was used for enzyme assays. SOD activity was measured by nitroblue tetrazolium (NBT) reduction [*21*], using SOD test Wako (Wako Pure Chemical Industry, Co., Ltd., Osaka, Japan). Briefly, the extent of inhibition of the reduction in NBT recorded at 560 nm by a spectrophotometer. One unit of enzyme activity was defined by the 50% inhibition of NBT. The protein content was measured Bradford methods, using Protein Quantification Kit-Rapid (Dojindo Molecular Technologys Inc., Kumamoto, Japan) [*22*].

Liver and spleen of mouse were homogenized in 1 M Tris-HCl 5 mM ethylendiaminetetraacetic acid buffer (pH 7.4). The homogenate of liver was used for enzyme assays. And the homogenate of spleen was centrifuged at 15,000 × g, for 45 min at 4°C and the supernatant was used for enzyme assays. Catalase activity was measured at 240 nm by a spectrophotometer in terms of H_2O_2 reduction rate at 37°C [*23*]. The mixtures for assays consisted of 50 μL of 1 M Tris-HCl buffer containing 5 mM ethylendiaminetetraacetic acid buffer (pH 7.4), 900 μL of 10 mM H_2O_2, 30 μL of deinoized water, and 20 μL of the liver sample. The activity was calculated by using a molar extinction coefficient of 7.1×10^{-3} $M^{-1}cm^{-1}$. Catalase activity was measured the amount of H_2O_2 split by catalase in 20, 40, and 60 s at 37°C. The reactions were started by adding the liver sample.

Total glutathione content was measured by using BIOXYTECH GSH-420™ assay kit (OXIS Health Products, Inc., Oregon, OR). Briefly, livers were suspended in 10 mM phosphate buffer (pH 7.4), mixed with ice-cold 7.5% trichloroacetic acid solution and then homogenized. The homogenates were centrifuged at 3,000 × g for 10 min. The supernatant was used for the assay. Total glutathione content was measured at 420 nm by spectrophotometer. The method is based on the formation of a chromophoric thione. The absorbance measured at 420 nm is directly proportional to the total glutathione concentration.

GPx activity was measured by using BIOXYTECH GPx-340™ assay kit (OXIS Health Products, Inc.). Briefly, liver was homogenized in 1 M Tris-HCl buffer (pH 7.4) containing 5 mM ethylendiaminetetraacetic acid and 1 mM dithiothreitol. The homogenates were centrifuged at 10,000 × g for 20 min at 4°C. The supernatant was used for the assay. The reduced nicotinamide adenine dinucleotide phosphate (NADPH) to oxidized nicotinamide adenine dinucleotide phosphate (NADP+) is accompanied by a decrease in absorbance at 340 nm providing a spectrophotometric means for monitoring GPx enzyme activity. The molar extinction coefficient for NADPH is 6220 $M^{-1}cm^{-1}$ at 340 nm. To assay GPx, the supernatant is added to a solution containing glutathione, glutathione reductase (GR), and NADPH. The enzyme reaction is initiated by adding the substrate, tert-butyl hydroperoxide and absorbance at 340 nm is recorded for 3 min.

Lipid peroxide, malondialdehyde (MDA) was assayed by using BIOXYTECH LPO-586™ assay kit (OXIS Health Products, Inc.). Livers were homogenized in 20 mM phosphate buffer (pH 7.4) on ice. Prior to homogenization, 10 μL of 0.5 M butylated hydroxytoluene in acetonitrile were added per 1 mL of tissue homogenate. After the homogenization, the homogenate was centrifuged at 15,000 × g, for 10 min at 4°C and the supernatant was used for assays. This assay is based on the reaction of a chromogenic regent, N-methyl-2-phenylidole, with MDA at 45°C. The optical density of colored products was read at 586 nm by the spectrophotometer.

Histological examination

We examined histological changes in spleen and small intestine and took spleen weights at 5 and 10 days after 0.2, 5.4, 6.0, or 12.0 Gy irradiation. The animals were killed by a dislocation of cramp. The spleen and the small intestine were excised and divided into small blocks. These blocks were fixed in 10% neutral-buffered formalin, dehydrated by graded ethanol and xylene, and embedded in paraffin. Tissue paraffin sections of the spleen and the small intestine were prepared using a microtome (Department of Radiation Research, Shikata Laboratory Advanced Science Research Center, Okayama University) and stained conventionally with hematoxylin-eosin (HE). Dysplastic cells have a high ratio of nucleus to cytoplasm (N/C ratio) compared with that of normal cells. Therefore we measured the nucleus and the cytoplasm and calculated the ratio in small intestine.

Statistical analysis

The data values are presented as the mean ± standard error of mean (SEM). The statistical significance of differences was determined by Student's *t* test for comparison between two groups. Survival rate was analyzed with the log-rank test.

Results

Time and dose-dependent changes in survival rate after irradiation

At 30 days after 3.0–5.4 Gy irradiation, all of acatalasemic mice and normal mice survived. At 30 days after 7.2–12.0 Gy irradiation, all of acatalasemic mice and normal mice died. Most of mice died from 10 to 20 days after 6.0 Gy irradiation. No significant changes were observed in survival rates between acatalasemic mice and normal mice after each irradiation (Fig. 1A).

Time and dose-dependent changes in death rates and whole body weights after irradiation

The highest frequency period of death rate in acatalasemic mice and normal mice were at 14 (13 to 16) days after 6.0 Gy irradiation. The peak of death rate in normal mice lasted up to 22 (21 to 24) days after irradiation. The peak of death rate after 7.2 Gy irradiation in acatalasemic mice and normal mice was 100% at 14 (13–16) days. Whole body weight at 14 days after 6.0 Gy irradiation dropped by 5–9% was compared to that before irradiation (Fig. 1B).

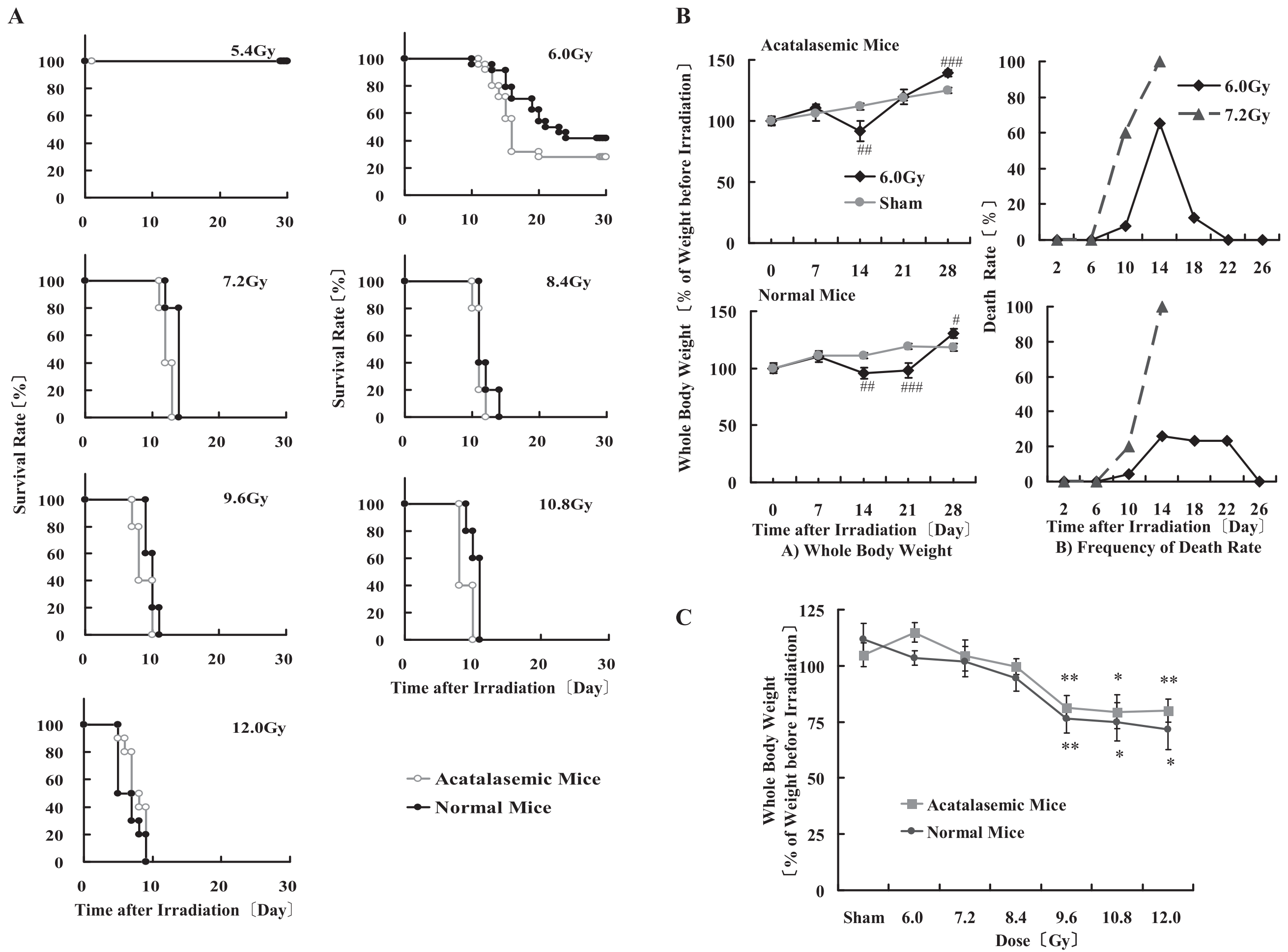


Fig. 1A. Time and dose-dependent changes in survival rates of acatalasemic mice and normal mice after irradiation. The number of mice per experimental point is 5–26.

B. Time and dose-dependent changes in death rates after 6.0 or 7.2 Gy irradiation and whole body weights of mice after 6.0 Gy irradiation. Each value indicates the mean ± SEM. The number of mice per experimental point is 5–26. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs sham irradiation. The death rate for 4 days intervals was calculated by the numerical expression:

$$\frac{\text{the number of dead mice within each period}}{\text{the number of living mice at beginning of each period}} \times 100\ [\%]$$

C. Dose-dependent changes in whole bodies weights after irradiation. Each value indicates the mean ± SEM. The number of mice per experimental point is 5. $^{*}p<0.05$, $^{**}p<0.01$ vs weight before irradiation.

Dose-dependent changes in whole bodies weights after irradiation

Whole body weights at 7 days after 9.6, 10.8, or 12.0 Gy irradiation were significantly decreased in acatalasemic mice and normal mice. On the other hand, no significant differences were observed in the whole body weights at 7 days after sham, 6.0, 7.2, or 8.4 Gy irradiation (Fig. 1C).

Time and dose-dependent changes in weights, antioxidant-associated substances levels, and histological changes in spleen after irradiation

The weights of spleens in acatalasemic mice and normal mice at 5 or 10 days after 5.4, 6.0, or 12.0 Gy irradiation significantly decreased by 55–65% compared with that after sham irradiation, respectively (Fig. 2A).

The spleen weight was significantly decreased by 6.0 or 12.0 Gy irradiation (Fig. 2A). The spleen was too short weight to assay all the antioxidation substances levels. In fact, the spleen weight after 12.0 Gy irradiation is only about 20 mg. We could only assay the activities of SOD and catalase in spleen. Under the condition of non irradiation, the catalase activity in spleens of acatalasemic mice was significantly lower than that of normal mice. The catalase activity in normal mice significantly decreased at 10 days

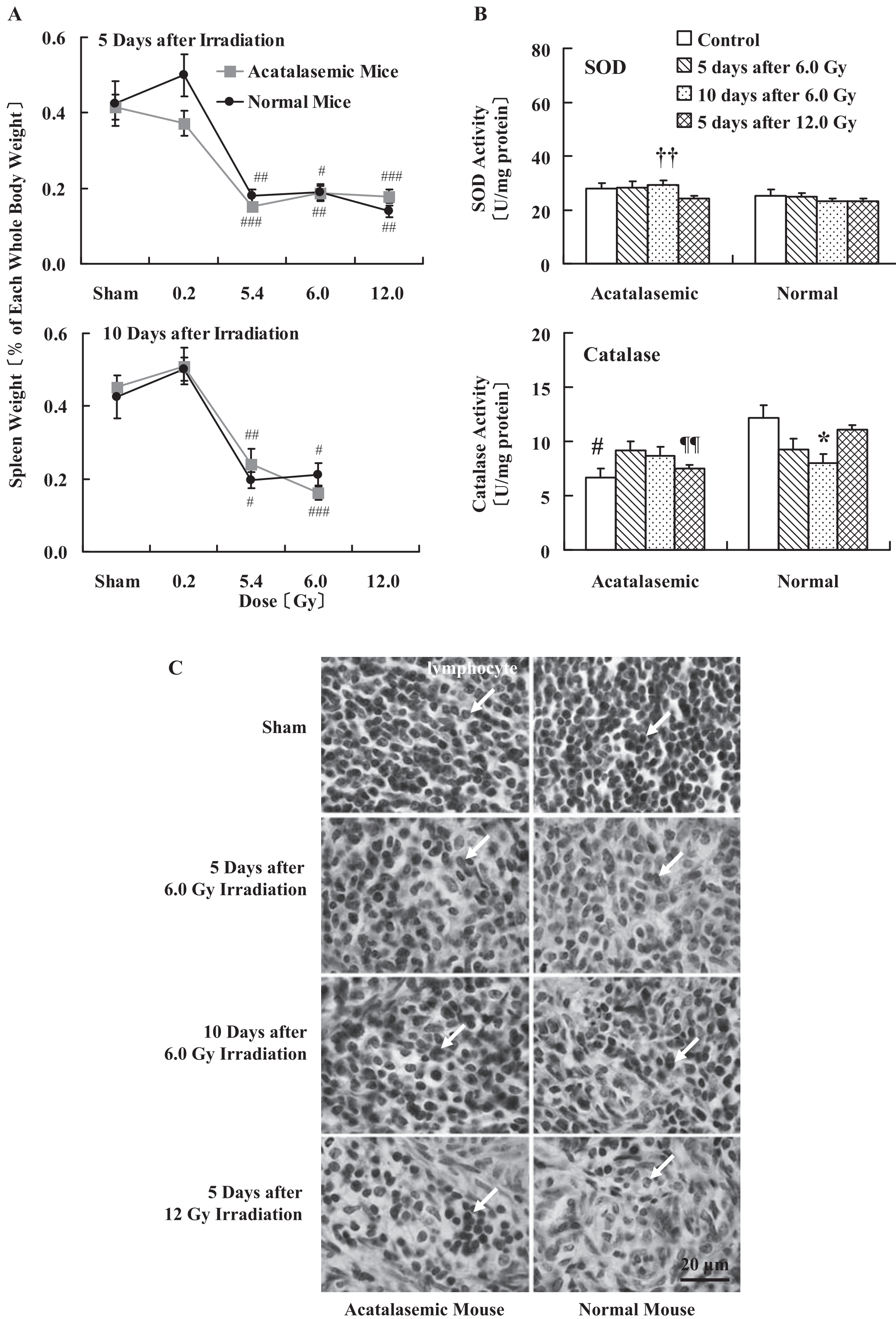


Fig. 2A. Time and dose-dependent changes in spleen weights after irradiation. Each value indicates the mean ± SEM. The number of mice per experimental point is 5. $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 vs sham irradiation.

B. Time and dose-dependent changes in the activities of SOD and catalase in spleen after irradiation. Each value indicates the mean ± SEM. The number of mice per experimental point is 4–5. *p<0.05 vs each control, $^{\#}p$<0.05 vs control in normal mice, $^{\dagger\dagger}p$<0.01 vs 10 days after 6.0 Gy irradiation in normal mice, $^{¶¶}p$<0.01 vs 5 days after 12.0 Gy irradiation in normal mice.

C. Histological changes in mouse spleen after irradiation. The length of scale bar is 20 µm. For all figures HE staining was used.

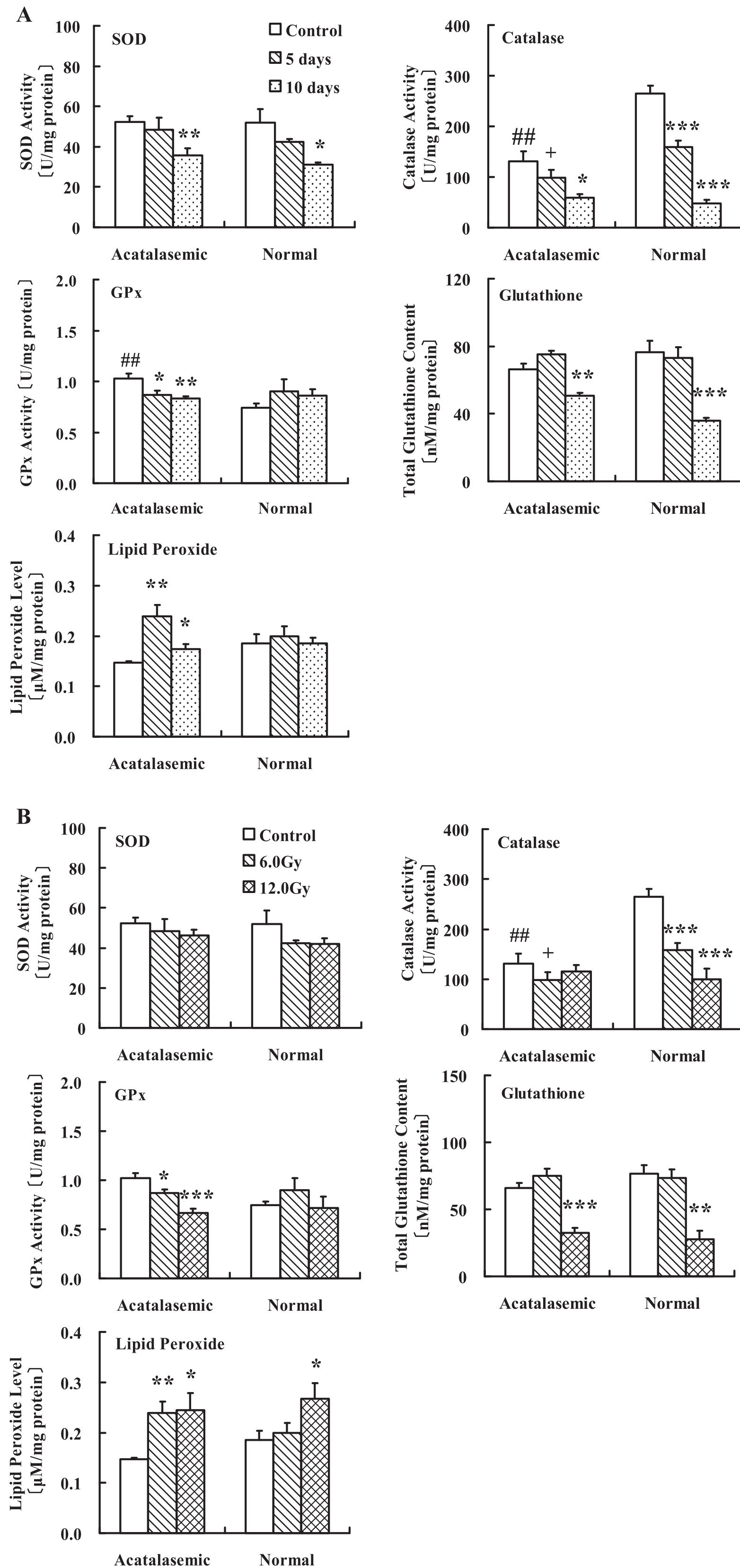


Fig. 3A. Time-dependent changes in the activities of SOD, catalase and GPx, total glutathione contents, and lipid peroxide levels in liver after 6.0 Gy irradiation. Each value indicates the mean ± SEM. The number of mice per experimental point is 5. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs each control, ##$p<0.01$ vs control in normal mice, +$p<0.05$ vs 5 days after 6.0 Gy irradiation in normal mice, ¶¶¶$p<0.001$ vs 10 days after 6.0 Gy irradiation in normal mice.

B. Dose-dependent changes in the activities of SOD, catalase and GPx, total glutathione contents, and lipid peroxide levels in liver after 6.0 or 12.0 Gy irradiation. The number of mice for each experiment and significance are the same as in Fig. 3A.

after 6.0 Gy irradiation. Moreover, the catalase activity in liver of acatalasemic mice was significantly lower than that of normal mice at 5 days after 12.0 Gy irradiation. No significant differences were observed in catalase activity between acatalasemic mice and normal mice at 5 and 10 days after 6.0 Gy irradiation. At 10 days after 6.0 Gy irradiation, the SOD activity in acatalasemic mice was significantly higher than that in normal mice (Fig. 2B).

Lymphocytes in spleens of acatalasemic mice and normal mice were decreased by 6.0 or 12.0 Gy irradiation (Fig. 2C).

Time-dependent changes in antioxidant-associated substances in liver after 6.0 Gy irradiation

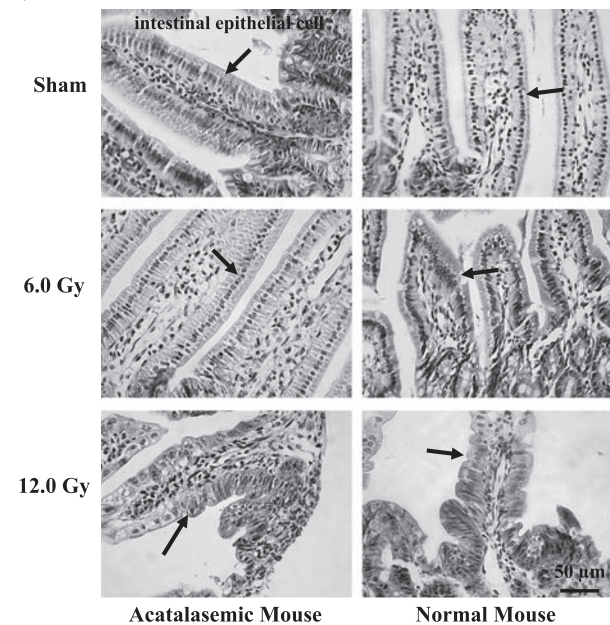
Under the condition of non irradiation, no significant differences were observed in the SOD activity between acatalasemic mice and normal mice. On the other hand, the catalase activity in acatalasemic mice was significantly lower than that of normal mice and the GPx activity in acatalasemic mice was significantly higher than that of normal mice. At 10 days after irradiation, the activities of SOD and catalase significantly decreased in acatalasemic mice and normal mice. Moreover, the catalase activity in acatalasemic mice was significantly lower than that of normal mice at 5 days after 6.0 Gy irradiation. On the other hand, no significant differences were observed in the catalase activity between acatalasemic mice and normal mice at 10 days after 6.0 Gy irradiation. The GPx activity in acatalasemic mice significantly decreased at 5 or 10 days after irradiation. The total glutathione contents in acatalasemic mice and normal mice significantly decreased in at 10 days after 6.0 Gy irradiation. Moreover, the total glutathione content in acatalasemic mice was significantly higher than that in normal mice. The lipid peroxide levels in acatalasemic mice significantly increased at 5 and 10 days after 6.0 Gy irradiation (Fig. 3A).

Dose-dependent changes in antioxidant-associated substances in liver at 5 days after irradiation

The catalase activity in normal mice significantly decreased at 5 days after 12.0 Gy irradiation. The total glutathione contents in acatalasemic mice and normal mice significantly decreased at 5 days after 12.0 Gy irradiation. The lipid peroxide levels in acatalasemic mice and normal mice significantly increased at 10 days after 12.0 Gy irradiation (Fig. 3B).

Dose-dependent changes in small intestine and intestinal epithelial cells after irradiation

The intestinal epithelial cells of acatalasemic mice and normal mice were vacuolated at 5 days after 12.0 Gy irradiation. The N/C ratio of intestinal epithelial cells significantly increased in both acatalasemic mice and normal mice after 6.0 or 12.0 Gy irradiation. There were no significant changes in the N/C ratio between acatalasemic mice and normal mice (Fig. 4).



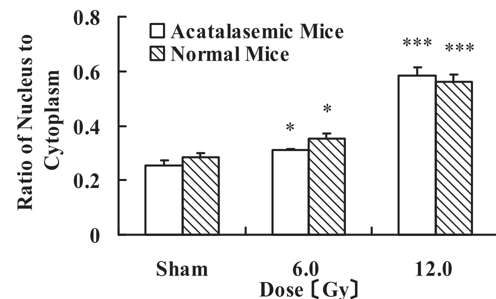


Fig. 4. Dose-dependent changes in 1) small intestine and 2) ratio of nucleus to cytoplasm in the intestinal epithelial cells of acatalasemic mice and normal mice at 5 days after irradiation. The length of scale bar is 50 µm. For all figures HE staining was used. $n = 3$. *$p<0.05$, ***$p<0.001$ vs sham irradiation.

Discussion

In this study, we examined the radiosensitivity in acatalasemic mice. In spite of the deficiency catalase, no significant changes were observed in survival rates between acatalasemic mice and normal mice after each irradiation. The reason may include the antioxidant system in acatalasemic mice.

SOD dismutats superoxide anion to H_2O_2, catalase and GPx detoxicate H_2O_2 into H_2O and O_2. The deficiency of catalase in acatalasemic mice might cause the increase of tissue or the cellular levels of H_2O_2. As a consequence, the

overproduction of ·OH from the undecomposed excessive H_2O_2 in the acatalasemic mice could induce more toxicity effect, for example, on the hepatocytes, leading to enhance the liver damage. Catalase and GPx are the two most important enzymes in the regulation of intracellular H_2O_2 level in biological systems. The catalase activities in brain and liver of the acatalasemic mice showed significantly lower by 80% and by 50% than those of the normal mice, respectively [*10*, *11*]. Moreover, the total glutathione contents in brain of the acatalasemic mouse were significantly higher by 50% than that of normal mouse, and the GPx activity in liver of the acatalasemic mice was significantly higher by 45% than that of the normal mice [*10*, *11*]. Thus the high total glutathione content or the high GPx activity more properly neutralizes a free radical reaction induced by the deficiency of catalase. And we have reported that low-dose irradiation enhanced antioxidant function and reduced oxidative damage [*24–26*]. Catalase detoxifies H_2O_2 into H_2O and O_2. The enzymatic action of GPx on H_2O_2 is stronger or weaker than that of catalase, depending on the concentration of glutathione [*27*]. Glutathione directly reacts with ROS, and GPx catalyzes the destruction of H_2O_2 and ·OH [*28*]. This catalysis generates oxidized glutathione (GSSG), and finally glutathione. However, GR catalyzes the regeneration of glutathione from GSSG. Thus GR and GPx are both the enzymes in the glutathione-regenerating pathway, and the changes of both activities are in a similar fashion.

The SOD administration increases the radioresistance, such as LD_{50} [*29*]. SOD protects radiation-induced oxidative damage [*4*] and overexpression of extracellular-SOD in transgenic mice appears to confer protection against high-dose irradiation-induced lung injury [*5*]. Moreover, overexpression of human Mn-SOD substantially protected cells from radiation injury. Relative to Mn-SOD, GPx could slightly reduce the radiosensitivity while the radioresistance in Cu, Zn-SOD expressing cells did not change significantly [*30*].

Under the condition of non irradiation, there was no significant difference in SOD activities in livers and spleens between acatalasemic mice and normal mice. On the other hand, under the condition of non irradiation, the liver of acatalasemic mice showed a significantly lower catalase activity and a significantly higher GPx activity than that of normal mice. As a consequence, no significant changes were observed in lipid peroxide levels between acatalasemic mice and normal mice. The catalase activities in livers of acatalasemic mice and normal mice significantly decreased after 6.0 Gy irradiation. The GPx activity in acatalasemic mice decreased after 6.0 Gy irradiation and that in normal mice did not decreased. Moreover, the total glutathione contents in acatalasemic mice and normal mice were significantly decreased at 10 days after 6.0 Gy irradiation. However, the total glutathione content in acalatasemic mice was significantly higher than that in normal mice. These findings suggest that the sufficiency of GPx and total glutathione make up for the deficiency of catalase (namely the adjustment function among antioxidant substances [*10*]) in acatalasemic mice. In addition, the catalase activities in liver and spleen of acatalasemic mice and normal mice significantly decreased than control after 6.0 Gy irradiation. However, there were no significant differences in the calatase activities between acatalasemic mice and normal mice. These findings indicate no different radiosensitivity between normal mice and acatalasemic mice in terms of whole body irradiation.

The highest frequency period of death rate after 6.0 Gy irradiation in normal mice was lower than that in acalatasemic mice, but the peak of death rate in normal mice lasted up to 22 (21–24) days after irradiation. On the other hand, the peaks of death rate after 7.2 Gy irradiation in acatalasemic mice and normal mice were 100% at 14 (13–16) days. There were no significant changes in the N/C ratio of intestinal epithelial cells between acatalasemic mice and normal mice after 6.0 or 12.0 Gy irradiation. These findings indicate that the radiosensitive in acatalasemic mice was not significantly different from that in normal mice.

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Abbreviations

ROS, reactive oxygen species; SOD, superoxide dismutase; H_2O_2, hydrogen peroxide; GPx, glutathione peroxidase; NBT, nitroblue tetrazolium; NADPH, reduced nicotinamide adenine dinucleotide phosphate; MDA, malondialdehyde; HE, hematoxylin-eosin; N/C ratio, ratio of nucleus to cytoplasm; SEM, standard error of mean; ·OH, hydroxyl radical; GSSG, oxidized glutathione; GR, glutathione reductase.

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