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Inhibitory Effects of Post Low Dose γ-Ray Irradiation on Ferric-Nitrilotriacetate-Induced Mice Liver Damage

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We studied the effects of a single post whole-body low-dose irradiation (50 cGy of γ-ray) on mice with ferric nitrilotriacetate (Fe^{3+}-NTA)-induced transient hepatopathy. As a result, low-dose irradiation accelerated the rate of recovery. Based on the changes in glutamic oxaloacetic transaminase (GOT) activities, glutamic pyruvic transaminase (GPT) activities and lipid peroxide levels, it was shown that hepatopathy was improved by low-dose irradiation 3 h after Fe^{3+}-NTA administration. This may be because of the enhancement of antioxidant agents such as total glutathione (GSH + GSSG), glutathione peroxidase (GPX), glutathione reductase (GR) and γ-glutamylcysteine synthetase (γ-GCS) by low-dose irradiation. These findings suggest that low-dose irradiation relieved functional disorders at least in the livers of mice with active oxygen species related diseases.

Keywords: Fe^{3+}-NTA, hepatopathy, low-dose γ-irradiation, lipid peroxide, antioxidant

Abbreviations: Fe^{3+}-NTA, ferric nitrilotriacetate; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; GSH, reduced form glutathione; GSSG, oxidized form glutathione; GPX, glutathione peroxidase; GR, glutathione reductase; γ-GCS, γ-glutamylcysteine synthetase; MDA, malondialdehyde

INTRODUCTION

It was recently shown that low-dose ionizing irradiation induced various stimulating effects on living organs,[1,2] such as a radio-adaptive response,[3–7] and increased life span.[8] With respect to the efficacy of low-dose ionizing irradiation on its *in vivo* antioxidant potential, we previously reported that low-dose irradiation with radon or X-ray increased superoxide dismutase activity in various organs of the rat, mouse and rabbit.[9–12] It was also reported that the levels of metallothionein,[13] catalase[14] and of non-enzymatic antioxidants including reduced form

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glutathione (GSH)[15] and thioredoxin[16,17] were increased by low-dose irradiation, suggesting that this irradiation activates the antioxidative defense mechanism. It was also shown that pretreatment with low-dose irradiation inhibited drug-related functional disorders of the liver and brain in mice treated with active oxygen species-inducing agents such as carbon tetrachloride (CCl_4)[18] and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.[19] Moreover, it was reported that 50 cGy γ-ray irradiation (from ^{60}Co) before alloxan administration suppressed the development of alloxan-induced diabetes in rats,[20] and that inhalation of radon suppressed the development of diabetes or hypertension in rabbits.[21] It was also found that when the iron-complex, Fe^{3+}-NTA was administered to rats, transient hepatopathy occurred, a response resembling excessive iron disease in humans.[22,23] Oxidative stress due to Fe^{3+}-NTA administration was considered to have induced this condition. Therefore, we previously investigated whether or not X-ray irradiation at a dose of 50 cGy before Fe^{3+}-NTA administration suppresses transient hepatopathy in rats.[24] It is of interest whether 50 cGy γ-ray irradiation after Fe^{3+}-NTA administration affects the oxidative mouse liver damage. In this study, to investigate whether active oxygen related diseases can be treated with low-dose irradiation, 50 cGy of γ-ray was irradiated to mice with Fe^{3+}-NTA-induced transient hepatopathy. Moreover, to elucidate the mechanism, we examined changes in antioxidant agents such as total glutathione (GSH + GSSG), GPX, GR and γ-GCS.

MATERIALS AND METHODS

Animal Model

Female C57BL/6 mice, 8-week-old, were obtained from CLEA Co., Ltd. (Tokyo, Japan). They were housed in standard polycarbonate cages with sterilized wood chip bedding and were acclimated to the animal facility environment for one week in a light (lights on 08.00 h and off 20.00 h; 12 h light/12 h dark) and temperature controlled (22 ± 2°C). Mice were allowed free access to water and sterilized normal diet (CE-2, CLEA Co., Ltd., Tokyo, Japan). At these experiments, the whole body of mice were irradiated with γ-rays from a ^{137}Cs source (Gammacell 40, Nordin International Inc., Canada) at a single dose of 50 cGy (117 cGy/min). A sham procedure without γ-ray irradiation (sham irradiation) was performed on control mice. Three hours before γ-ray irradiation, a single dose of Fe^{3+}-NTA (7.5 mg Fe/kg body weight) was administered intraperitoneally to mice. One milligram per milliliter of Fe^{3+} was used to prepare Fe^{3+}-NTA with the ratio between Fe^{3+} and NTA of 3 to 1.[25] Mice were cut off from food for 18 h before Fe^{3+}-NTA administration, and then allowed to eat adlibitum after administration. The animals were killed by exsanguination from the abdominal aorta under anesthesia 6, 24 or 48 h after irradiation when a hepatectomy was performed to provide liver tissues for study described below.

Assay

(1) GOT and GPT Activities

Blood was collected from above treatments, and plasma was obtained by centrifugation at $1500 \times g$ for 10 min under 4°C. The activities of GOT and GPT were measured by UV-rate method[26] using GOT and GPT-test kits Wako (Wako Pure Chemical Industries, Co., Ltd., Osaka, Japan).

(2) Lipid Peroxide Level

Lipid peroxide, malondialdehyde (MDA), was assessed by thiobarbituric acid (TBA)-reactive substances according to the method of Uchiyama and Mihara.[27] Liver was homogenized in 154 mM KCl on ice and reflexed for 60 min at 95°C in the presence of 0.3% (w/v) TBA and 7.5% (v/v) acetic acid at pH 3.5. The optical density of colored product was read at 532 nm. 1,1,3,3-tetraethoxypropane was used as the standard curve and the results were expressed as nmol of

MDA per milligram of protein. The protein content was measured according to method of Lowry *et al.*[28]

(3) Glutathione Assay

Total glutathione content in the liver were measured using a modified spectrophotometric technique.[29] Briefly, tissues were suspended in 10 mM phosphate buffer (pH 7.4), mixed with ice-cold 10% trichloroacetic acid (TCA) solution and then homogenized in a teflon-glass homogenizer. The homogenates were centrifuged at 14,000× *g* for 15 min. Subsequently 0.5 ml of the supernatant was treated with 3 ml of ice-cooled diethylether and the diethylether layer was removed with a pipette. This procedure was repeated 5 times to remove excess TCA. The final supernatant was assayed for GSH content. 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB) was obtained from Wako Pure Chemical Industries, Co., Ltd. (Osaka, Japan). GSH and GR (from yeast) were from Boehringer Mannheim (Germany). β-Reduced nicotinamide adenine dinucleotide phosphate (β-NADPH) (Type II) was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan).

The sample solution (25 ml) was mixed with 1 mM DTNB, 733 ml of 0.3 mM β-NADPH and 10 ml of GR (2 U/ml). The rate of change in absorbance was measured at 412 nm. GSH standards (1–20 mg/ml) were analyzed in the same manner. Glutathione concentrations of the sample were calculated as mmol/mg protein.

(4) Activities of GPX, γ-GCS, and GR

Each liver was homogenized in chilled 10 mM Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose and 1 mM EDTA using a teflon-glass potter homogenizer. The homogenate was centrifuged at 36,000× *g* for 30 min at 4°C and the supernatant was used for enzyme assays. The activity of GPX was assayed by coupling the reduction of tert-butyl hydroperoxide to the oxidation of β-NADPH by GR.[30] The assay mixture consisted of 100 µl of 1 M Tris-HCl (pH 8.0) containing 5 mM EDTA, 20 µl of 0.1 M GSH, 100 µl of GSH reductase solution (10 U/ml), 100 µl of 2 mM NADPH, 650 µl of distilled water, 10 µl of 7 mM tert-butyl hydroperoxide, and 10 µl of the brain supernatant. Oxidation of NADPH at 37°C was followed spectrophotometrically at 340 nm. One unit of activity is defined as the amount of GPX required to oxidize 1 µmol of NADPH per min.

The activity of γ-GCS, the rate-limiting enzyme for *de novo* GSH biosynthesis was measured by means of a coupled enzyme assay that evaluates nicotinamide adenine dinucleotide phosphate (NADH) oxidation.[31] The reaction mixture consisted of 600 µl of pre-mixture solution containing 100 mM Tris-HCl (pH 8.2), 150 mM KCl, 20 mM $MgCl_2$, 50 mM EDTA, and 0.2 mM NADH, 100 µl of 0.1 M ATP, 100 µl of 0.1 M phosphoenol pyruvate, 50 µl of 0.1 M L-glutamate, 5 µl of pyruvate kinase solution (5 U), 5 µl of lactate dehydrogenase solution (10 U), 50 µl of 0.1 M L-α-aminobutyric acid, and 90 µl of the liver supernatant. The NADH oxidation at 37°C was followed by measuring the absorbance at 340 nm. Activity was calculated using a molar extinction coefficient of $6.22 \times 10^3\,M^{-1}\,cm^{-1}$ and expressed as µmol of NADH oxidized/min/mg protein.

The activity of GR was measured spectrophotometrically at 340 nm in terms of β-NADPH oxidation at 30°C.[32] Briefly, the reaction mixture consisted of 840 µl of 50 mM phosphate buffer (pH 7.6), 10 µl of 1 mM GSSG, 10 µl of 0.1% (w/v) bovine serum albumin, and 20 µl of the liver supernatant. The activity was expressed as nmol of β-NADPH oxidized/min/mg protein.

Statistical Analysis

Data were presented as mean ± SEM. Significance was used to determine the significance of the difference between Students' *t*-test, each Fe^{3+}-NTA administrated group value and control group value, or between each group value at various intervals after irradiation and the values at the same intervals after sham-irradiation. Each experimental group consisted of 4–6 mice.

RESULTS

Transaminase Activities in Serum

Three hours after Fe^{3+}-NTA administration, the serum GOT and GPT activities in the sham-irradiated and 50 cGy irradiated groups reached a peak of about 3–4 fold that before administration. In the irradiated groups, increases in the activities were significantly suppressed between 24 and 48 h after irradiation and returned to normal values earlier than those in the sham-irradiated groups (Figure 1).

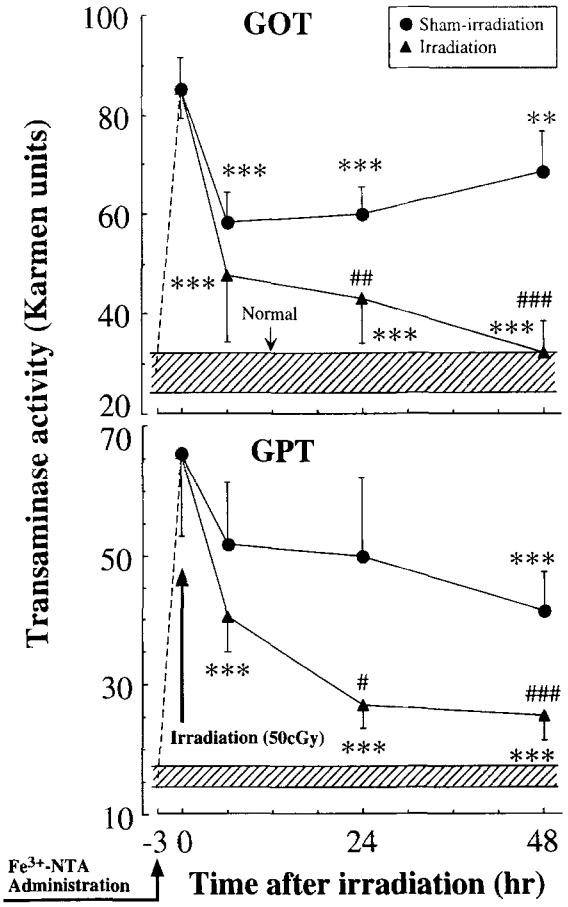

FIGURE 1 Time dependent changes in GOT and GPT activities in mice serum treated with Fe^{3+}-NTA after 50 cGy γ-ray irradiation. Each value indicates the mean ± SEM. The number of mice per experimental point is 4–6. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by *t*-test, each sham-irradiated or irradiated group value vs the control group value (3 h after Fe^{3+}-NTA administration). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ by *t*-test, each group value at various intervals after irradiation vs the values at the same intervals after sham-irradiation.

Lipid Peroxide Level in the Liver

The MDA level was expressed as the lipid peroxide level. Within 48 h after irradiation, the lipid peroxide levels in the livers of the sham-irradiated groups significantly increased to about 4–7 fold that before Fe^{3+}-NTA administration. Thereafter, it decreased, but did not reach the level before administration. In the irradiated groups, the relative increase in the lipid peroxide levels was smaller than those in the sham-irradiated groups, but both groups showed a similar time course. The increases in the lipid peroxide levels were significantly suppressed between 6 and 48 h after irradiation and each point value was significantly lower than the sham-irradiated group (Figure 2).

Total GSH Content in the Liver

Within 48 h after irradiation, the total glutathione content in the livers of the sham-irradiated groups significantly decreased compared with that before Fe^{3+}-NTA administration. In the irradiated group, the relative decrease in the glutathione content was smaller than that in the sham-irradiated group, both groups showed a similar time course. The decrease in the glutathione content was significantly suppressed between 6 and 48 h after irradiation (Figure 3).

GPX Activity in the Liver

Within 6 h after irradiation, the GPX activities in the livers of the sham-irradiated groups significantly decreased compared with those before Fe^{3+}-NTA administration. In the irradiated groups, the relative decrease in the GPX activities was smaller than that in the sham-irradiated groups. Both groups showed a similar time course. The decrease in the GPX activities was

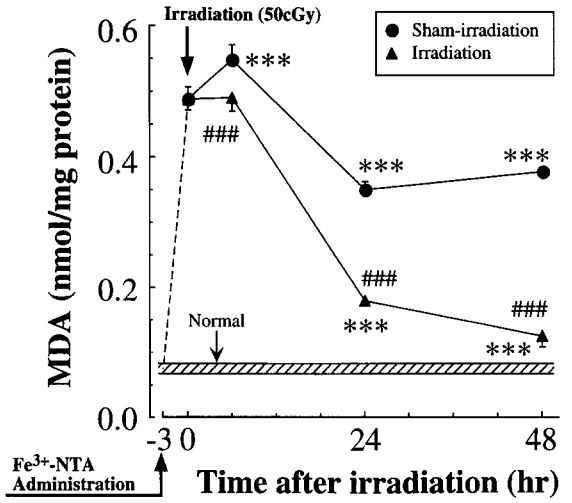

FIGURE 2 Time dependent changes in lipid peroxide levels in mice livers treated with Fe^{3+}-NTA after 50 cGy γ-ray irradiation. The data, numbers of mice and significance are as described in Figure 1.

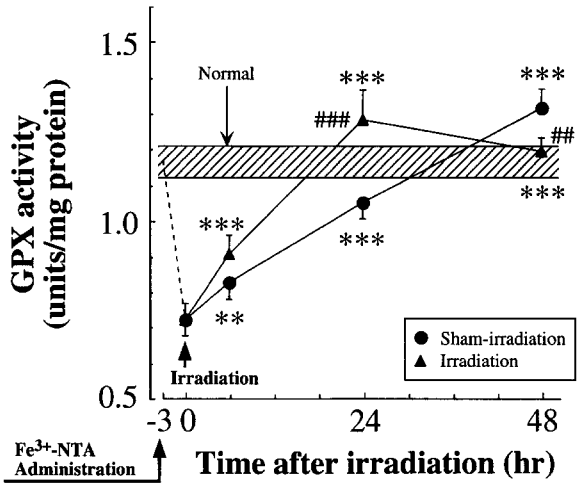

FIGURE 4 Time dependent changes in GPX activities in mice livers treated with Fe^{3+}-NTA after 50 cGy γ-ray irradiation. The data, numbers of mice and significance are as described in Figure 1.

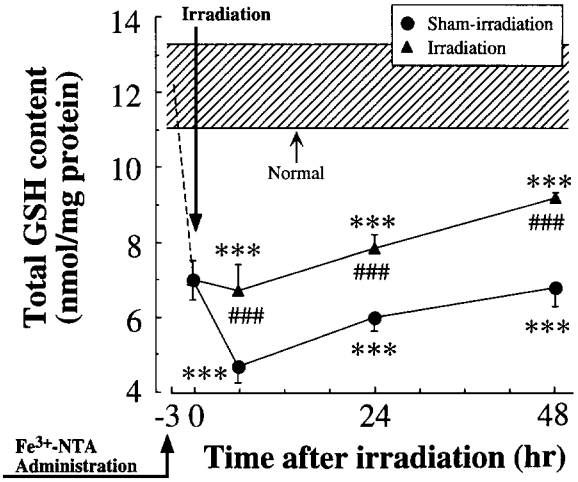

FIGURE 3 Time dependent changes in total glutathione content in mice livers treated with Fe^{3+}-NTA after 50 cGy γ-ray irradiation. The data, numbers of mice and significance are as described in Figure 1.

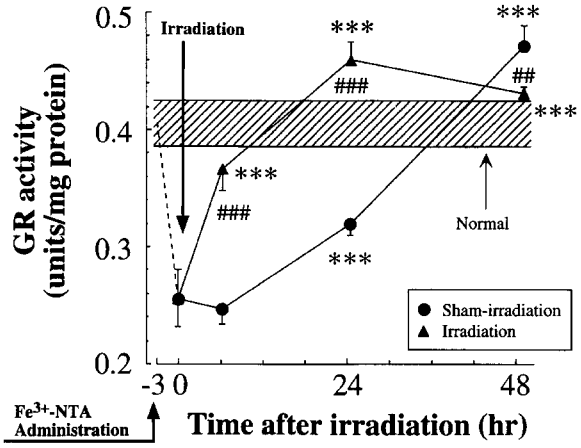

FIGURE 5 Time dependent changes in GR activities in mice livers treated with Fe^{3+}-NTA after 50 cGy γ-ray irradiation. The data, numbers of mice and significance are as described in Figure 1.

significantly suppressed 24 h after irradiation (Figure 4).

GR Activity in the Liver

Within 24 h after irradiation, the GR activities in the livers of the sham-irradiated groups significantly decreased compared with those before Fe^{3+}-NTA administration. In the irradiated groups, the relative decrease in the GR activity was smaller than that in the sham-irradiated group. The decrease in the GR activities was significantly suppressed between 6 and 24 h after irradiation (Figure 5).

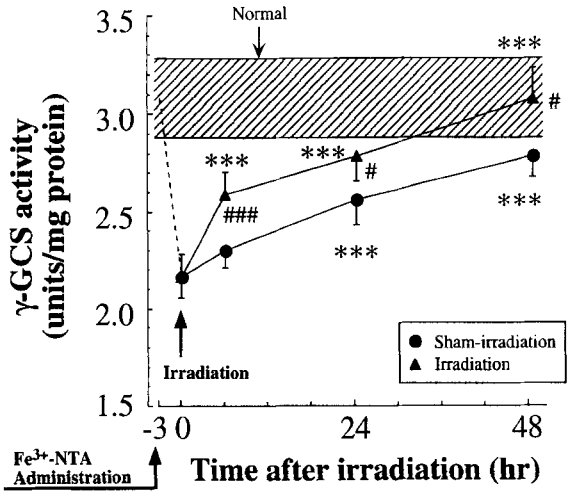

FIGURE 6 Time dependent changes in γ-GCS activities in mice livers treated with Fe^{3+}-NTA after 50 cGy γ-ray irradiation. The data, numbers of mice and significance are as described in Figure 1.

γ-GCS Activity in the Liver

Within 48 h after irradiation, the γ-GCS activities in the livers of the sham-irradiated groups significantly decreased compared with those before irradiation. In the irradiated group, the relative decrease in the γ-GCS activities was smaller than that in the sham-irradiated group, both groups showed a similar time course. The decrease in the γ-GCS activities was significantly suppressed between 6 and 48 h after irradiation (Figure 6).

DISCUSSION

Transient liver cells disorder after Fe^{3+}-NTA administration is considered to be induced by free radicals produced by iron-complexes. Awai *et al.* reported that free radicals were produced 1 and 3 h after Fe^{3+}-NTA administration, but had almost disappeared after 6 h.[33] They proposed that iron-complexes induced an adverse reaction by forming free radicals after its administration in the early stage between intracellular uptake and transformation into storage types. For example, many biological substances such as membrane lipids, proteins and nucleic acids are injured by $^{\bullet}OH$ produced by catalytic reaction of iron including Haber–Weiss reaction or Fenton reaction.[34] As clarified in this study, changes in liver lipid peroxide levels suggested a free radical reaction induced by iron-complexes. Moreover, Awai *et al.* suggested that peroxidation of membranes induced by free radical reaction gradually destroys the localization of GPT or GOT. Although it was interesting that there was a shift in the peak of enzyme release into the blood depending on the kind of enzymes, the details of the mechanism should be clarified in future investigations.

In this study, we clarified that both the increase in liver lipid peroxide level and the release of enzymes derived from liver cells such as GPT reached their peaks 3 h after Fe^{3+}-NTA administration. On the other hand, the increase in the lipid peroxide level of the liver was suppressed and significantly decreased by 50 cGy irradiation. Release of GPT and GOT from liver cells into the blood was also suppressed by 50 cGy irradiation. Furthermore, the decrease in the GPX, GR and γ-GCS activities and total glutathione content in the liver was significantly suppressed by irradiation. The 50 cGy irradiation accelerated the rate of recovery more than the sham-irradiation.

We previously reported that pre-irradiation with 50 cGy of γ-ray inhibited the toxicity of subsequent CCl_4 administration.[18] In addition, our experiment involving irradiation with 50 cGy of γ-ray in normal mice demonstrated that irradiation promoted production of a small volume of active oxygen *in vivo*, producing antioxidant substances such as catalase and GPX in various organs and decreasing lipid peroxide levels.[14,15]

On the other hand, the function of glutathione includes various roles in metabolism, transport, catalysis as coenzymes, maintenance of the thiol moieties and reduced form of other molecules such as cysteine, coenzyme A. The role in the detoxification of electrophilic metabolites of xenobiotics and active oxygen species has also been well documented. Glutathione protects cells from the toxicity of these compounds, serving as a

substrate for glutathione transferase, and is utilized as a reducing agent. Of these, the protection against oxidative damage caused by active oxygen species is the most important function. GSH directly reacts with active oxygen species, and GPX catalyzes the destruction of hydrogen peroxide and hydroperoxide.[35] Our previous studies showed that antioxidant potency of various tissues such as the liver, pancreas and brain in mice, was elevated by irradiation with low doses of γ-rays, and that a higher total glutathione content was observed in parallel with the potency of these tissues.[18,36] The effect of low dose γ-ray pre-irradiation after Fe^{3+}-NTA administration was already examined in mice. Elevations of serum transaminase activity and lipid peroxides induced by Fe^{3+}-NTA were significantly suppressed by low dose pre-irradiation.[37] Low-dose irradiation induces intracellular antioxidants, leading to scavenge active oxygen species generated in liver cells at early time after treatment with Fe^{3+}-NTA. In addition to the roles described above, another important function of glutathione in the regulation of DNA synthesis, cell division/proliferation was recently suggested.[38–40] The detailed mechanism that regulates the onset of cell proliferation remains unclear. Terradez *et al.*[41] suggested that glutathione is involved in the control of protein kinase C (PKC) activity, one of the major signal-transducing factors for cell proliferation in various cells. PKC is a cysteine-rich enzyme, and modification of the thiol redox status of these residues may affect its activity. Furthermore, Ibuki *et al.*[7] examined the adaptive response in Chinese hamster V79 cells using the cell survival (colony formation assay) and DNA synthesis ([^{3}H]-thymidine incorporation) as endpoints. They found that when V79 cells were pre-irradiated with an adapting dose (5 cGy), their survival after irradiation with the challenging dose (400 cGy) was about 120% of the control without such pre-irradiation. Moreover, following irradiation with the challenging dose, the DNA synthesis of pre-irradiated cells was less reduced than that of controls. They suggested that the adaptive responses were associated with the signal transduction via PKC based on the results that this response was not observed when the cells were pre-irradiated with the adapting dose in the presence of the PKC inhibitor, H-7. Glutathione causes a more reduced redox state, resulting in increased proliferative activity.[42,43] Based on this point of view, the post-treatment with low dose irradiation likely causes some beneficial effects on the recovering processes from the tissue damage as well. Thus, the effect of post-irradiation with low dose γ-ray on acute hepatopathy induced by Fe^{3+}-NTA was examined. This acute damage is considered to be induced by active oxygen species generated by iron-complexes. It was found that active oxygen species are produced 1–3 h after Fe^{3+}-NTA administration, and almost disappeared after 6 h. As shown in the results section, low dose γ-ray irradiation at 3 h after treatment with Fe^{3+}-NTA clearly accelerated the rate of the recovery from this transient hepatopathy. The low dose irradiation increased intracellular glutathione contents, leading to control of the onset of liver cell proliferation. Furthermore, the contributions of irradiation-induced antioxidants such as glutathione should be considered in scavenging the remaining active oxygen species 3 h after post-treatment with Fe^{3+}-NTA.

As a result, low-dose irradiation accelerated the rate of recovery. The decrease in the transaminase activities and lipid peroxide levels showed that hepatopathy was recoverd at 24–48 h after irradiation. This may be because of the enhancement of antioxidant agents such as glutathione by low-dose irradiation. These findings suggest that post-treatment with low dose of γ-ray is useful for clinical prevention and/or therapy of various active oxygen species related diseases.

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