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Inhibitory Effects of Prior Low-dose X-irradiation on Ischemia-reperfusion Injury in Mouse Paw

Takahiro KATAOKA, Yuko MIZUGUCHI, Masaaki YOSHIMOTO, Takehito TAGUCHI and Kiyonori YAMAOKA*

Edema/Ischemia-reperfusion injury/Low-dose irradiation/Reactive oxygen species/Antioxidation function.

We have reported that low-dose, unlike high-dose, irradiation enhanced antioxidation function and reduced oxidative damage. On the other hand, ischemia-reperfusion injury is induced by reactive oxygen species. In this study, we examined the inhibitory effects of prior low-dose X-irradiation on ischemia-reperfusion injury in mouse paw. BALB/c mice were irradiated by sham or 0.5 Gy of X-ray. At 4 hrs after irradiation, the left hind leg was bound 10 times with a rubber ring for 0.5, 1, or 2 hrs and the paw thickness was measured. Results show that the paw swelling thickness by ischemia for 0.5 hr was lower than that for 2 hrs. At 1 hr after reperfusion from ischemia for 1 hr, superoxide dismutase activity in serum was increased in those mice which received 0.5 Gy irradiation and in the case of the ischemia for 0.5 or 1 hr, the paw swelling thicknesses were inhibited by 0.5 Gy irradiation. In addition, interstitial edema in those mice which received 0.5 Gy irradiation was less than that in the mice which underwent by sham irradiation. These findings suggest that the ischemia-reperfusion injury is inhibited by the enhancement of antioxidation function by 0.5 Gy irradiation.

INTRODUCTION

Low-dose irradiation induces various stimulating effects on living organs,[1,2] such as radio-adaptive response,[3–6] and increases life span.[7] With respect to the efficacy of low-dose irradiation on its *in vivo* antioxidant potential, it was reported that low-dose irradiation with X-ray increased the activities of superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx), and the total glutathione contents in various organs of rat, mouse, and rabbit.[8–11] Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), hydroxyl radical, and superoxide anion radicals (O_2^-), are readily generated in many cells by metabolic processes such as respiration, ischemia-reperfusion, and oxidation of fatty acids. These ROS damage DNA, lipids, and enzymes, and are highly toxic. Cells can be injured or killed when the ROS level exceeds the cellular antioxidant capacity.[12]

Toxic O_2^- metabolites from xanthine oxidase (XOD) contribute to the development of injury seen during reperfusion of a variety of ischemic tissues.[13,14] This impression is primarily based on observations that XOD appears located within endothelial cells,[15–18] that XOD generates O_2^- metabolites *in vitro,*[7] that endothelial cells make O_2^- metabolites *in vitro,*[19,20] and that treatment with SOD or allopurinol reduces reperfusion injury in some models of intestinal and cardiac ischemia.[21–24] ROS is induced in these cells by increased oxygen concentration and increases the fluidity of endothelial plasma membrane and damages membrane permeability and receptor function.[25] Thereby, ischemia-reperfusion produces edema *in vivo* by disrupting endothelial cell junctional integrity. Histological changes in interstitial edema formation were used as indicator of ischemia-reperfusion injury in skeletal muscle. It was reported that ischemia-reperfusion injury increased the interstitial edema formation.[26]

On the other hand, it is possible that low-dose irradiation inhibits the ischemia-reperfusion injury, such as cardiac surgery or organ transplantation. Therefore, in this study, we examined the edema changes in mouse paw by ischemia-reperfusion after low-dose X-irradiation.

MATERIALS AND METHODS

Animals

Female BALB/c mice which were seven to eight weeks of

*Corresponding author: Phone: +81-86-235-6852,
Fax: +81-86-235-6852,
E-mail: yamaoka@md.okayama-u.ac.jp
Graduate School of Health Sciences, Okayama University, 5-1 Shikata-cho, 2-chome, Okayama 700-8558, Japan.
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age (about 20 g of body weight) were kept under an air-conditioned room (temperature 20°C and humidity 60%) at the Animal Center for Medical Research, Okayama University Medical School. They were fed on Oriental MF diet (Oriental Yeast Co.,Tokyo) and tap water *ad libitum*. The study protocol was performed according to the animal experimental guideline of Okayama University.

Irradiation

These mice received whole body irradiation at a dose of 0.5 Gy (3.0 Gy/min) of X-rays (voltage: 150 keV, ampere: 20 mA, filters: Cu:Al = 0.5 mm:0.2 mm). The age matched control mice were sham-irradiated. All the animals were sacrificed by cervical dislocation. Each experimental group consisted of 5–15 mice.

Measurement of ischemic paw edema

Always the same commercial rubber ring (1 × 1 mm, d = 4.45 mm, Pocket, No.18) was used for making ischemia in BALB/c mice. At 4 hrs after irradiation, the left hind leg was bound 10 times with a rubber ring at just above articulation. The rubber ring was scissor after 0.5, 1, or 2 hrs and the paw thickness was measured with micrometer caliper at 0.5, 1, 2, 3, or 24 hrs after the recirculation.[27)]

Biochemical assays

Ischemia-reperfusion injury in paw of mouse or rat was inhibits by parenteral infusion with SOD originated from mouse or rat.[27)] This inhibitory effect of ischemia-reperfusion injury may be caused by the increase in the SOD activity in blood. Therefore we assayed SOD in serum after ischemia-reperfusion. Blood was collected from the heart after the dislocation of cramp, and serum was obtained by centrifugation at 3,000 × g for 15 min under 4°C. Total SOD activity was measured by nitroblue tetrazolium (NBT) reduction[28)] using SOD test Wako (Wako Pure Chemical Industry, Co., Ltd.). Briefly, the extent of inhibition of 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 by the Bradford method using Protein Quantification Kit-Rapid (Dojindo Molecular Technologys Inc.).[29)]

Histological observation

At 24 hrs after reperfusion from ischemia for 0.5, 1, or 2 hrs and at 0.5, 2, or 24 hrs after reperfusion from ischemia for 1 hr, the left hind leg was excised, and divided into small blocks. These blocks were fixed in 10% neutral-buffered formalin, and decalcified with Plank-Rychlo solution. And then, those were dehydrated by graded ethanol and xylene, and embedded in paraffin. Tissue paraffin sections of the left hind leg were stained conventionally with hematoxylin-eosin (HE). Ischemia-reperfusion increases interstitial fluid and thereby increases interstitial edema. Therefore we calculated the percentage of the cell spacing part using the image-editing software.

Statistical analysis

The data values are presented as the mean ± the standard error of mean (SEM). The statistical significance of differences was determined by Student's t-test for comparison between two groups or two-way repeated-measures analysis of variance (ANOVA) and Dunnett's tests for multiple comparisons, where appropriate.

RESULTS

Ischemic interval time dependent morphological changes in paw edema

The paw swelling thicknesses after reperfusion for 0.5, 1, or 3 hrs from ischemia for 1 or 2 hrs were significantly bigger than that from ischemia for 0.5 hr. The paw swelling thickness from ischemia for 2 hrs was four times bigger than that from ischemia for 0.5 hr. The peak time of paw swelling thickness at every ischemia interval time was appeared at 1 hr after reperfusion (Fig. 1).

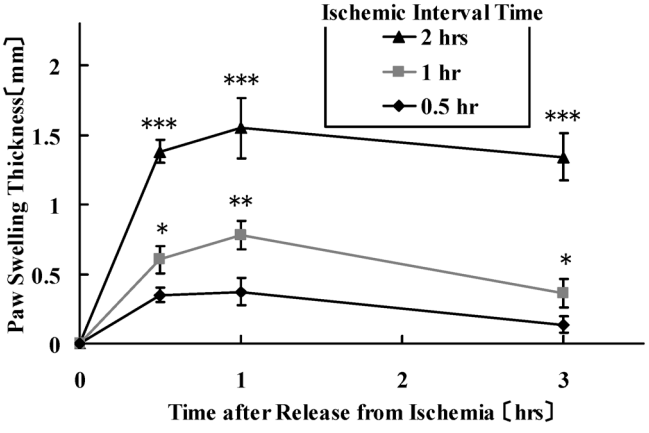

Fig. 1. Ischemic interval time dependent morphological changes in mouse paw edema. Each value indicates the mean ± SEM. The number of mice per experimental point is 5–15. *P < 0.05, **P < 0.01, ***P < 0.001 ischemia for 1 hr or 2 hrs vs. ischemia for 0.5 hr, respectively.

Time dependent changes in paw edema after ischemia-reperfusion by prior irradiation

In case of ischemia for 0.5 hr, 0.5 Gy prior irradiation significantly inhibited the paw swelling at 1 hr after reperfusion compared with that of sham irradiation. In case of ischemia for 1 hr, 0.5 Gy prior irradiation significantly inhibited the paw swelling in a similar fashion and accelerated the rate of recovery. In case of ischemia for 2 hrs, there was no significant inhibition in the paw swelling in those mice which received 0.5 Gy irradiation compared with that of sham irradiation (Fig. 2).

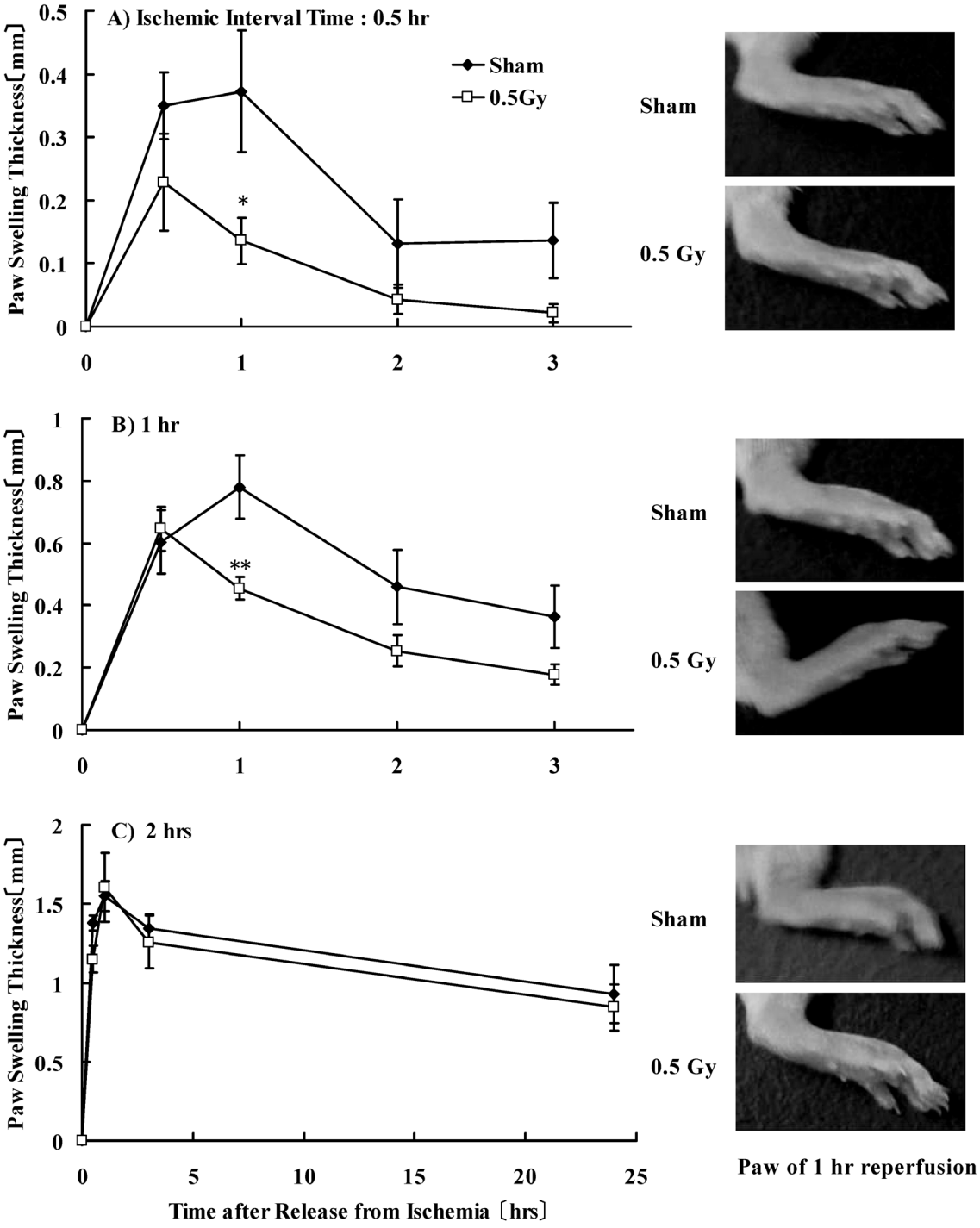

Fig. 2. Time dependent changes in mouse paw edema after ischemia-reperfusion by prior irradiation. Each value indicates the mean ± SEM. The number of mice per experimental point is 5–15. *P < 0.05, **P < 0.01 vs. sham irradiation.

Changes in SOD activity in serum after irradiation

At 6 hrs after 0.5 Gy irradiation, SOD activity in serum was significantly increased compared with that of sham irradiation (Fig. 3).

Time dependent changes in SOD activity in serum after ischemia-reperfusion by prior irradiation

At 1 hr after reperfusion from ischemia for 1 hr, SOD activity in serum was significantly increased in those mice which received 0.5 Gy irradiation compared with that of sham irradiation (Fig. 4).

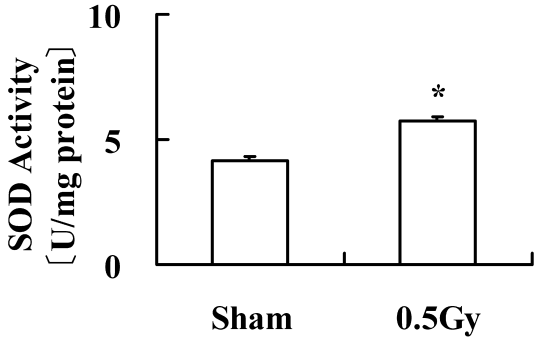

Fig. 3. Changes in SOD activity in serum of mouse at 6 hrs after irradiation. Each value indicates the mean ± SEM. The number of mice per experimental point is 5–7. *P < 0.05 vs. sham.

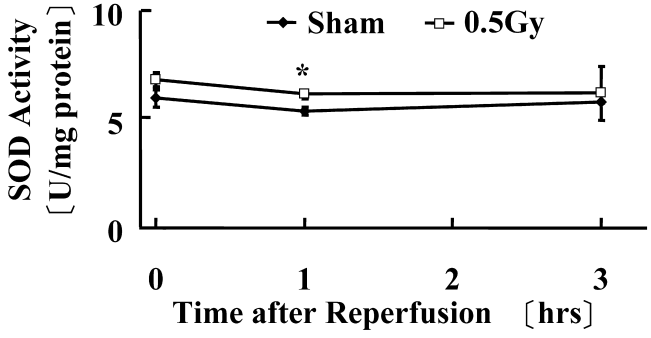

Fig. 4. Time dependent changes in SOD activity in serum of mouse after reperfusion under 1 hr ischemia by prior irradiation. Each value indicates the mean ± SEM. The number of mice per experimental point is 5–7. *P < 0.05 vs. sham irradiation after 1 hr reperfusion.

Ischemic interval time dependent changes in SOD activity in serum after reperfusion by prior irradiation

At 1 hr after reperfusion from ischemia for 1 hr, SOD activity in serum was significantly increased in those mice which received 0.5 Gy irradiation compared with that of sham irradiation. No significant changes were observed after 1 hr reperfusion from 0.5 or 2 hrs ischemia (Fig. 5).

Time dependent changes in muscle cells and the ratio of cell spacing in paw after ischemia-reperfusion by prior irradiation

Interstitial edema (ratio of cell spacing) exposed to sham irradiation at 0.5, 2, or 24 hrs after reperfusion from ischemia for 1 hr was significantly bigger than that of no treatment. However, no significant differences were observed in the interstitial edema exposed to 0.5 Gy irradiation after 0.5, 2, or 24 hrs reperfusion from 1 hr ischemia compared with that of no treatment. Moreover 0.5 Gy irradiation inhibited the interstitial edema after reperfusion for 0.5, 2, or 24 hrs from ischemia for 1 hr compared with that of sham irradiation (Fig. 6).

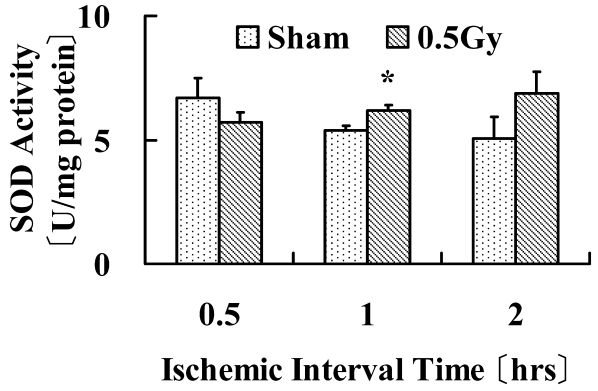

Fig. 5. Ischemic interval time dependent changes in SOD activity in serum of mouse after 1 hr reperfusion by prior irradiation. Each value indicates the mean ± SEM. The number of mice per experimental point is 5–7. *P < 0.05 vs. sham irradiation after 1 hr ischemia.

Ischemic interval time dependent changes in muscle cells and the ratio of cell spacing in paw after ischemia-reperfusion by prior irradiation

At 24 hrs after reperfusion from ischemia for 1 or 2 hrs, interstitial edema exposed to sham irradiation was significantly bigger than that of no treatment. However, no significant differences were observed in the interstitial edema exposed to 0.5 Gy irradiation at 24 hrs reperfusion from ischemia for 0.5, 1, or 2 hrs compared with that of no treatment. Moreover 0.5 Gy irradiation inhibited the interstitial edema after reperfusion for 1 hr from ischemia for 0.5 or 1 hr compared with that of sham irradiation (Fig. 7).

DISCUSSION

It is well known that scavenging activity of SOD is transformation from superoxide anion radical into H_2O_2. Catalase transforms H_2O_2 into H_2O as well as glutathione. Low-dose irradiation and radon inhalation produce adequate oxygen stress, and antioxidant enzymes were increased and the lipid peroxide level was decreased.[30–33] SOD-like substances are compounds that eliminate superoxide anion, which is a free radical and one of the ROS. Low-dose irradiation induced SOD in various organs, such as brain, liver, thymus, spleen, or bone marrow.[8] It may indicate that the induction of SOD occurs throughout the body by low-dose irradiation. We have reported that low-dose irradiation inhibit CCl_4-induced hepatopathy in mice.[34–36] The hepatopathy is thought to be induced by the trichloromethyl radical. These results suggested that antioxidant enzymes, which were elevated by 0.5 Gy irradiation, reduced the fat liver caused by CCl_4 administration and antioxidant substances play an important role in the recovery of CCl_4-induced hepatopathy. In this study, the paw swelling thickness after 0.5 Gy irradiation was lower than that of sham irradiation and 0.5 Gy irradiation

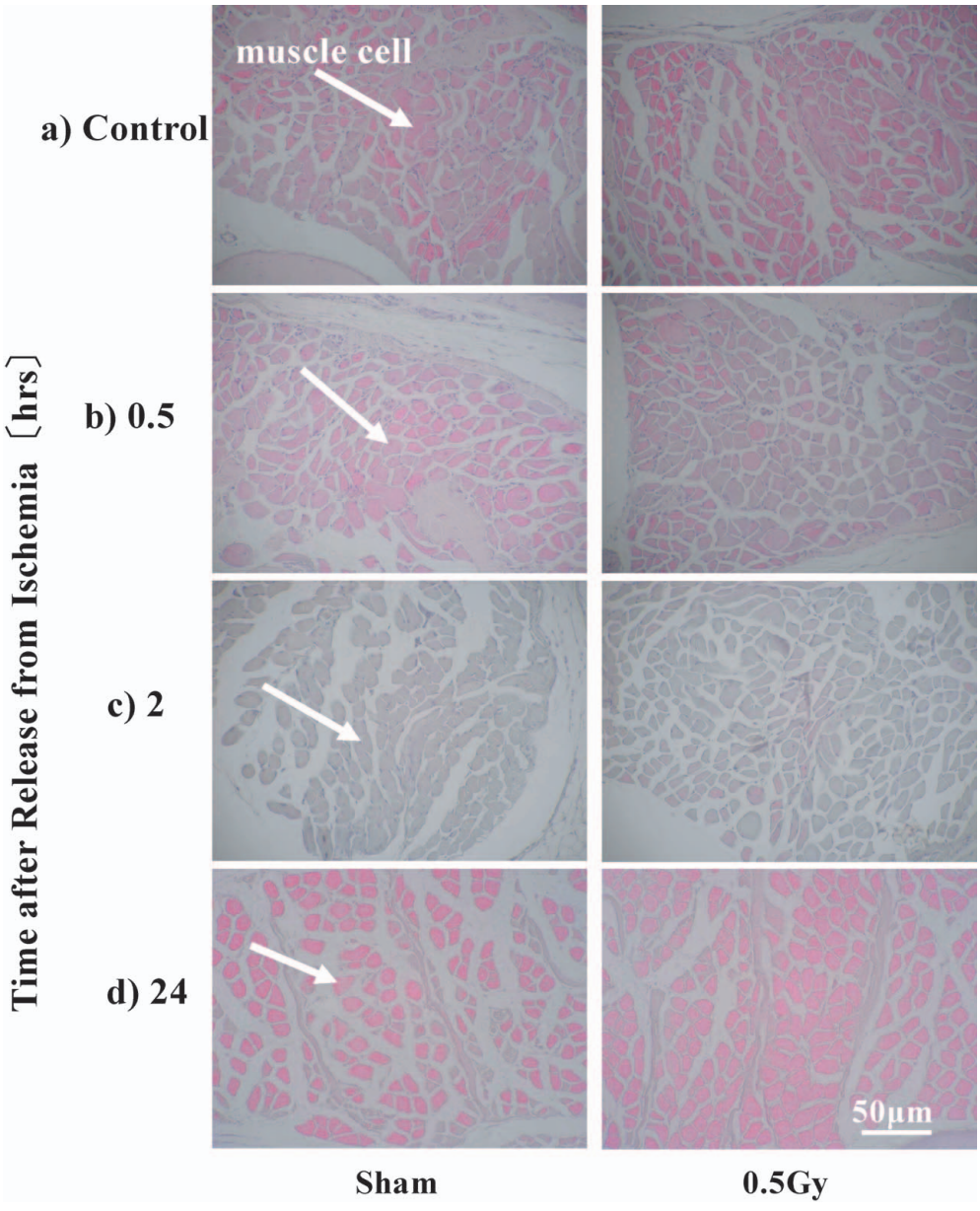

Fig. 6-1. Time dependent changes in muscle cells in mouse paw after reperfusion from ischemia for 1 hr by prior irradiation. The length of scale bar is 50 μm. For all Figs. HE staining was used.

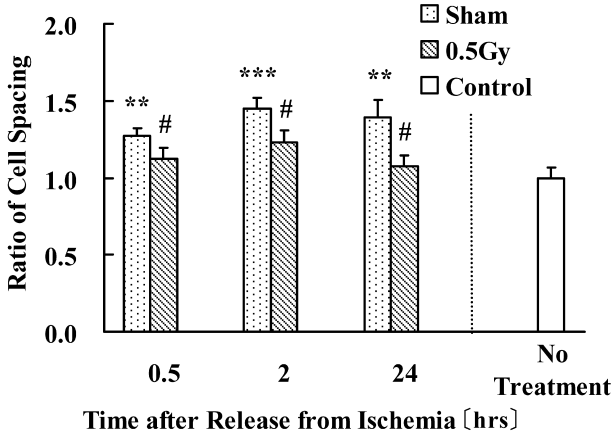

Fig. 6-2. Time dependent changes in the ratio of cell spacing in mouse paw after reperfusion from ischemia for 1 hr by prior irradiation. Each value indicates the mean ± SEM. The number of mice per experimental point is 5. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. no treatment group, $^{\#}P < 0.05$ vs. sham irradiation.

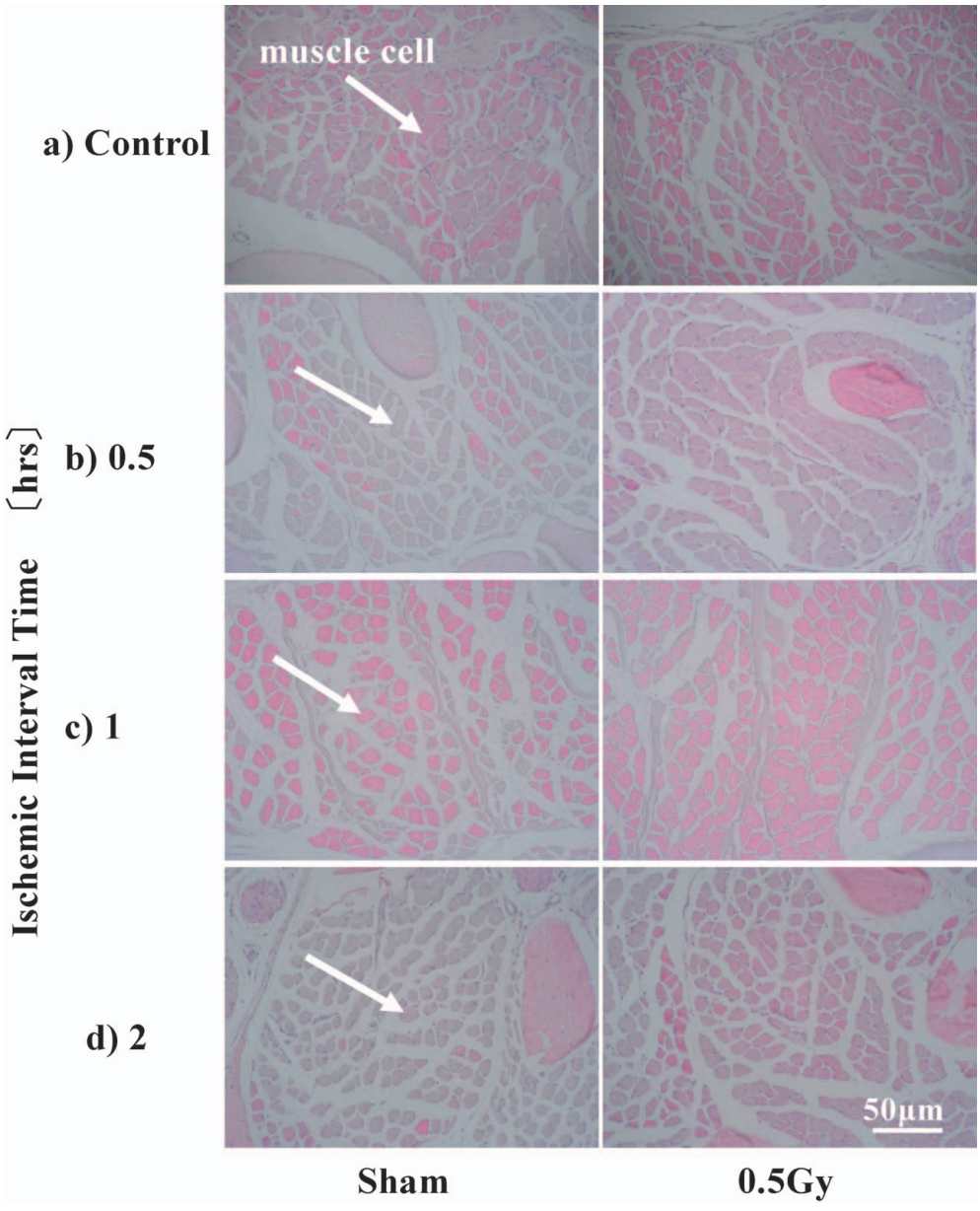

Fig. 7-1. Ischemic interval time dependent changes in muscle cells in mouse paw at 24 hrs after reperfusion from ischemia by prior irradiation. The length of scale bar is 50 μm. For all Figs. HE staining was used.

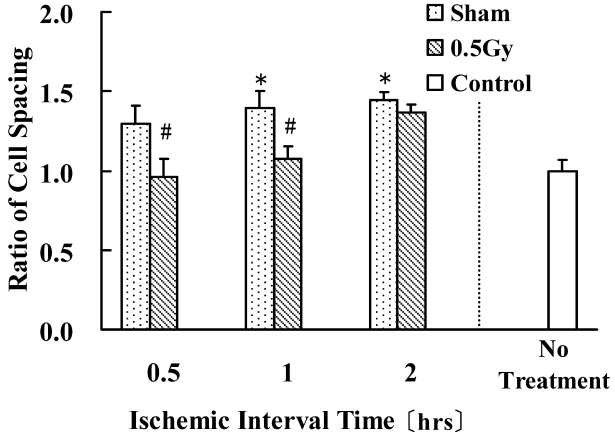

Fig. 7-2. Ischemic interval time dependent changes in the ratio of cell spacing of mouse paw at 24 hrs after reperfusion from ischemia by prior irradiation. Each value indicates the mean ± SEM. The number of mice per experimental point is 5. *$P < 0.05$, vs. no treatment group, $^{\#}P < 0.05$ vs. sham irradiation.

accelerated the recovery from ischemia- reperfusion injury. These results may indicate that prior 0.5 Gy irradiation suppresses ischemia-reperfusion injury.

There are various mechanisms known to increase superoxide generation by XOD during ischemia-reperfusion. Vascular endothelial cells contain XOD. When the paw becomes ischemia, XOD is increased in the vascular endothelial cells. Cell-bound XOD has been reported to produce radical; finally, during ischemia, adenosine triphosphate (ATP) is degenerated to xanthine and hypoxanthine, thereby increasing the XOD substrate levels, which leads to increased superoxide production.[37] Production of superoxide and H_2O_2 are enhanced due to increased conversion of xanthine dehydrogenase to XOD. In present study, we examined ischemic interval dependent changes in mouse paw edema. The mouse paw for 2 hrs ischemia was four times bigger than that of 0.5 hr ischemia. 0.5 Gy irradiation before ischemia for 2 hrs did not inhibit the paw swelling. These findings suggest that low-dose irradiation inhibit this injury up to for 1 hr ischemia. It means that low-dose irradiation cannot inhibit excessive injury.

The edema which is the condition of increased interstitial fluid is induced by ischemia-reperfusion. The edema is caused by excess leakage of blood element into capillary vessel, dysfunctional lymphatic vessel, or change in anatomy of gel structure. Vascular endothelial cells are adhered by such cytadherence protein as platelet endothelial cell adhesion molecule-1 or vascular endothelial cadherin,[38,39] which are an important role of permeability alteration. The capillary vessel forms by one layer endothelial cells and passes easily electrolytes. In this case, one layer endothelial cells have no osmotic pressure and albumin, globulin, or fibrinogen has a greater osmotic pressure. Moreover it was reported that hypoxia-reoxygenation caused the functional deletion of gap junctional communication in cultured human umbilical vein endothelial cells.[40]

The fact that SOD and catalase together were as effective when administered just before reoxygenation as when administered before anoxia confirms that the injury occurs primarily at reoxygenation and that the injury is mediated by process dependent upon superoxide formation. The ability of allopurinol to substantially reduce the injury suggests that xanthine is major source of the free radical generation within the endothelial cell at reoxygenation.[27] Selenium supplementation increased the endogenous activity of thioredoxin reductase and glutathione peroxide, and resulted in improved recovery of cardiac function post ischemia-reperfusion.[41] In this study, SOD activity in serum was increased by 0.5 Gy irradiation at 1 hr after reperfusion from ischemia for 1 hr compared with that of sham irradiation. These findings suggest that oxidant-antioxidant balance might play an important part in free radical induced injuries.

Ischemia-reperfusion increases interstitial edema and interstitial fluid in interstitial edema. It was also reported that reperfusion after 2.5 hrs ischemia led to severe interstitial edema formation and significant damage to muscle fibers.[42] Microvessel size was significantly reduced in the 2.5 hrs ischemia and was associated with a no-reflow phenomenon, ie, a persistent decrease in blood flow after the initial hyperfusion phase.[43] In addition, the 2.5 hrs ischemia was characterized by mitochondrial swelling, loss of mitochondrial cristae, and dilatation of sarcoplasmic reticula. It is described in a previous design involving 2 to 3 hrs of ischemia and 30 min of reflow.[44] It was reported that the ultrastructural damage to skeletal muscles after 2 hrs ischemia, but not after 1 hr reperfusion from 1 hr ischemia.[45] In this study, ischemia-reperfusion increased interstitial space and longer ischemic interval time was further worsening interstitial edema. However, interstitial edema exposed to 0.5 Gy irradiation was less than that in the mice which underwent sham irradiation at any ischemic interval time. These findings suggest that prior low dose irradiation inhibits interstitial edema.

In future clarification, this study is required to elucidate the possibility of remission of cardiac surgery or organ transplantation, which is mainly caused by ischemia-reperfusion injury, by low- dose irradiation.

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