Regular Paper

Inhibitory effects of prior low-dose X-irradiation on cold-induced brain injury in mouse

Masaaki YOSHIMOTO,[1] Takahiro KATAOKA,[1] Teruaki TOYOTA,[1] Takehito TAGUCHI,[1] and Kiyonori YAMAOKA[1, 2]

[1]Graduate School of Health Sciences, Okayama University, 5-1 Shikata-cho 2-chome, Kita-ku, Okayama-shi, Okayama, 700-8558, Japan

[2]To whom correspondence should be addressed at Graduate School of Health Sciences, Okayama University, 5-1 Shikata-cho 2-chome, Kita-ku, Okayama-shi, Okayama, 700-8558, Japan. E-mail: yamaoka@md.okayama-u.ac.jp

Running title: Low-dose X-irradiation inhibits brain injury

ABBREVIATIONS: BBB, blood-brain barrier; ROS, reactive oxygen species; SOD, superoxide dismtase; GPx, glutathione peroxidase; GR, glutathione reductase; total GSH, total glutathione; EDTA, ethylendiaminetetraacetic acid; NBT, nitroblue tetrazolium; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; HE, hematoxylin-eosin; KB, Kluver-Barrera; TUNEL, Terminal dUTP *in situ* nick-end labeling; PBS, phosphate-buffered saline; DAB, 3,39-diaminobenzidine; SEM, standard error of the mean; MCA, middle cerebral artery

Abstract

We examined the inhibitory effects of low-dose X-irradiation on mouse brain tissue with cold-induced injury by comparing tissue samples from three groups of mice: control, sham-irradiated cold-exposed, and X-ray-irradiated (0.5 Gy) cold-exposed mice. The water content in brain increased significantly in the sham-irradiated group following the cold-induced injury relative to the control group. However, water content in brain tissue from the X-ray-irradiated group was significantly lower than that from the sham-irradiated group. Levels of antioxidants, such as superoxide dismutase and glutathione, in brain tissue from the X-ray-irradiated group were higher than those from the sham-irradiated group. Moreover, the cold injury induced cell death, particularly apoptosis, while low-dose irradiation inhibited cell death, especially among glial cells, but not numeral cells. These findings suggest that prior low-dose X-irradiation activated antioxidant function and inhibited cold-induced brain injury.

Keywords: cold injury; brain edema; 0.5 Gy irradiation; antioxidative function

INTRODUCTION

Brain edema is a pathophysiological condition characterized by increased water content in brain tissue due to a variety of coexisting brain injuries, and it has been classified into several subtypes based on the pathogenesis of edema development. These subtypes include cytotoxic, vasogenic, and interstitial brain edema. Vasogenic brain edema is caused by disruption of the blood-brain barrier (BBB) and develops in association with several types of cerebral insults, including ischemia, trauma, tumor growth, or infection. Moreover, neuronal death resulting from necrosis or apoptosis occurs following these disease states. Recent studies have reported that secondary neuronal damage occurred not only from necrotic neuronal death but that apoptotic neuronal death may result also from the development of vasogenic edema and that apoptosis may contribute to the expansion of the lesion in both the acute and secondary phase after cold injury [1].

Cold injury has been used as a model for vasogenic brain edema and delayed infarction. Among the various biochemical events associated with cold injury, experimental evidence points to reactive oxygen species (ROS) formation as one of the key events leading to breakdown of the BBB and neuronal death. ROS, such as superoxide anion and hydroxyl radical, contribute significantly to the development of vasogenic brain edema [2, 3]. There are several reports that show that protection against this kind of edema can be obtained by the antioxidant enzyme superoxide dismutase (SOD) [4-10].

Low-dose irradiation increases endogenous antioxidants in animal tissues. Antioxidants, such as SOD [11-14], glutathione peroxidase (GPx) [12, 14], glutathione reductase (GR) [15], glutathione [14, 15], catalase [14], and thioredoxin [14], are activated and/or induced by low-dose irradiation, and they contributed to the inhibition of oxidative injury. Moreover, our previous studies have demonstrated that low-dose irradiation activated antioxidative function and inhibited edema in mouse paws induced by ischemia-reperfusion injury [16].

Severe brain edema increases intracranial pressure resulting in brain herniation and a decrease in cerebral blood perfusion resulting in secondary ischemia. Therefore, prevention and treatment of brain edema is of clinical importance. Vasogenic brain edema is of considerable clinical importance because it develops in association with several brain disease states. Conventional treatments, including drug-mediated dehydration, are used clinically to reduce the brain edema, but the effect is incomplete. Development of more effective medical treatments has been expected. Barinaga [17] suggested that

when the etiology and pathology of cerebral infarction is clear, scavengers of free radicals that target ROS may be used to treat acute stroke patients. Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a novel, potent free radical scavenger that has been used clinically in Japan since 2001 to prevent oxidation injury to neuronal cells and vascular endothelial cells following ischemic stroke.

To assess whether prior low-dose X-irradiation inhibits cold injury in mouse brain, we examined changes in water content, antioxidative function, and neuron viability induced by cold injury following low-dose X-irradiation. Specifically, we investigated water content, biochemical parameters (i.e., SOD, catalase, total glutathione (total GSH; GSH + GSSG), and lipid peroxide levels), and histological changes in brain tissue after cold injury.

MATERIALS AND METHODS

Animals

Male BALB/c mice (age: eight weeks; body weight: approximately 25 g) were obtained from the Department of Animal Resources Advanced Science Research Center Okayama University. Ethics approval was obtained from the animal experimental committee of Okayama University. Each experimental group consisted of 4–8 mice.

Irradiation and Cold Injury

The mice received whole body irradiation at a dose of 0.5 Gy (3.0 Gy/min) using an X-ray generator (Hitachi MBR-1505R2; Hitachi Medico, Ibaraki, Japan, voltage; 150 kV, ampere; 20 mA, filters; Cu:Al = 0.5 mm:0.2 mm) at room temperature. The age matched control mice were sham-irradiated. Mice were injected intraperitoneally with 50 mg/kg pentobarbital (Nembutal, Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) 4 hr after irradiation, and body temperature was maintained at 37 °C with a hot plate. The scalp was cut in the right hemisphere, and the skull was exposed. A metal probe (4 mm in diameter) and cooled with liquid nitrogen was applied to the skull with a force of 100 g for 30 sec. This cold injury model resulted in reproducible time-dependent vasogenic brain edema [18]. At 1, 4, 24, or 48 hr after cold injury, blood was collected from the heart under ether anesthesia and serum was obtained by centrifugation at 3,000×g for 5 min at 4 °C. Mice were sacrificed by decapitation under anesthesia. The scalp and the skull were conducted a midline incision, and the brain was exposed. Brains were carefully removed to assess water content,

biochemical activity, and histology. Brains were immediately assayed for water content, preserved at -80 °C until use in the biochemical assays or fixed in 10% neutral-buffered formalin for histological observation.

Measurement of Water Content

At 0, 1, 4, 24, or 48 hr after the cold injury, the brains were rapidly removed after decapitation, and each cerebral hemisphere was weighed. The samples were dried at 105 °C for 24 hr and reweighed. Water content was calculated as (wet weight – dry weight) / wet weight × 100% [18].

Biochemical Assays

Mouse 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. Mouse brains were homogenized in a 1 M Tris-HCl buffer containing 5 mM ethylendiaminetetraacetic acid (EDTA) (pH 7.4) on ice. The homogenate was centrifuged at 12,000 × g for 45 min at 4 °C, serum and supernatant was used to assay the activity of SOD and catalase.

SOD activity was measured by the nitroblue tetrazolium (NBT) reduction method [19] using the Wako-SOD test (Wako Pure Chemical Industry, Co., Ltd., Osaka, Japan). Briefly, the extent of inhibition of the reduction in NBT was measured by determining NBT content at 560 nm using a spectrophotometer. One unit of enzyme activity was defined as 50% inhibition of NBT reduction. The protein content was measured by the Bradford method, using Protein Quantification Kit-Rapid (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) [20].

Catalase activity was measured as the hydrogen peroxide (H_2O_2) reduction rate at 37 °C and was assayed at 240 nm using a spectrophotometer [21]. The assay mixture consisted of 50 μl of 1 M Tris-HCl buffer containing 5 mM EDTA (pH 7.4), 900 μl of 10 mM H_2O_2, 30 μl deionized water, and 20 μl brain supernatant. Activity was calculated using a molar extinction coefficient of 7.1×10^{-3} $M^{-1}cm^{-1}$. Catalase activity was measured by the amount of hydrogen peroxide split by catalase at 37 °C. The reactions were started by addition of the brain supernatant.

Total glutathione content was measured using the Bioxytech GSH-420^{TM} assay kit (OXIS Health Products). Briefly, brains 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. This assay is based on the formation of a chromophoric thione whose absorbance, measured at 420 nm using a spectrophotometer, is directly proportional to the total glutathione concentration.

Lipid peroxide (malondialdehyde (MDA)) levels were assayed using Bioxytech LPO-586 ^{TM} assay kit (OXIS Health Products, Inc., OR, USA). Briefly, brains 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. The homogenate was centrifuged at 15,000 × g, for 10 min at 4 °C, and the supernatant was used for assay. The MDA assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenylidole, with MDA at 45 °C. The optical density of the colored products was read at 586 nm in a spectrophotometer.

Histological Observation

Brains were fixed in 10% formalin, processed with a graded mixture of ethanol and xylene and embedded in paraffin. The 6-μm-thick tissue sections were prepared and stained with hematoxylin-eosin (HE) or Kluver-Barrera (KB). We calculated number of cells and the percentage of non-structural area in the lesion area using the image-editing software. Non-structural area was defined as regions that had lost structure, including ‘halo’, vacuolization, and perivascular space.

TUNEL Stains

Terminal dUTP *in situ* nickend labeling (TUNEL) was performed, utilizing the apoptosis *in situ* detection kit Wako (Wako Pure Chemical Industry, Co., Ltd., Osaka, Japan) following the manufacturer’s protocol. Briefly, slides were deparaffinized and sections were stripped of proteins by incubation with proteinase K for 5 min at 37 °C. Endogenous peroxidase was inactivated by immersion in phosphate-buffered saline (PBS) containing 3% H_2O_2 for 5 min at room temperature. Residues of digoxigenin-nucleotide were catalytically added to the DNA by TdT, an enzyme that catalyzes a template-independent addition of deoxyribonucleotide triphosphate to the 3’-OH ends of DNA. The reaction was conducted at 37 °C for 10 min. The color reaction was visualized by peroxidase-conjugated anti-digoxigenin antibody and 3,39-diaminobenzidine (DAB). The sections were counterstained by 0.5% methyl green. Distilled water was substituted for TdT enzyme for the negative controls.

Statistical Analyses

All values are presented as the mean ± standard error of the mean (SEM). The statistical significance of differences was determined by Student's *t*-test for comparison between two groups and Dunnet's tests for multiple comparisons where appropriate.

RESULTS

Changes in water content in brains after cold injury

Changes in water content of sham-irradiated group relative to the control group are shown in Fig.1A. The water content of right hemisphere was significantly elevated at 1, 4, 24, and 48 hr following cold injury, and it reached a maximum at 24 hr. Water content of left hemisphere was significantly evaluated at 4 and 24 hr following cold injury (Fig.1A). At 1, 4, 24, and 48 hr, the water content of the right brain hemisphere of mice in the 0.5 Gy-irradiated group was significantly lower than that of mice in the sham-irradiated group (Fig.1B). In addition, there were no significant differences in water content of right hemisphere between the control group and 0.5 Gy irradiated group (Fig.1B).

Histological observation

There were characteristic changes in cells and cytoplasm at the core lesion. In this lesion, cells were shrunken and decreased, and others cells had a 'halo' surrounding the cytoplasm. There was marked edema with tissue vacuolization. Bleeding and expanded perivascular spaces in the cortical blood vessels were apparent in this lesion (Fig.2, 3). Changes in the non-structural area at this lesion after 0.5 Gy irradiation were observed: non-structural area was defined as regions that had lost structure, including 'halo', vacuolization, and perivascular space. Non-structural area in lesion tissue of the sham and 0.5 Gy-irradiated groups were significantly increased at 4, 24, and 48 hr relative to the same area in the control group. At 4 hr, those of the 0.5 Gy-irradiated group were lower than those of the sham-irradiated group (Table 1). The number of cells in area of the lesions from the sham-irradiated group significantly decreased at 4, 24, or 48 hr. At 4 hr, those of the 0.5 Gy-irradiated group were lower than those of the sham-irradiated group (Table 2).

In addition, the number of neurons in lesion tissue had decreased significantly 24 or 48 hr after

application of the cold injury in the sham- and 0.5 Gy-irradiation groups (Table 3).

Changes in apoptotic cell death in brain after 0.5 Gy irradiation

Some cells were labeled by the TUNEL staining after cold injury. These TUNEL positive cells showed shrunken (Fig.4). The number cells undergoing apoptosis in the cold-injured lesions were significantly lower in the 0.5 Gy-irradiated group than in the sham-irradiated group 1 hr after cold injury (Table 4).

Changes in antioxidative function in brain after 0.5 Gy irradiation

SOD activity in serum from the 0.5 Gy-irradiated group was significantly higher at than that of sham-irradiated group at 0, 4, 24, and 48 hr after cold injury (Fig.5). At 0 (i.e., 4 hr after sham or 0.5 Gy irradiation) and 1 hr after cold injury, SOD activity in the right brain hemisphere was higher significantly in the 0.5 Gy-irradiated group than in the sham-irradiated group (Fig.6A). At 1 hr after cold injury, total glutathione content in the right brain hemisphere was significantly higher in the 0.5 Gy-irradiated group than in the sham-irradiated group (Fig.6C). At 0 (at 4 hr after sham or 0.5 Gy irradiation), 1, and 4 hr after cold injury, lipid peroxide levels in right hemisphere were significantly lower in the 0.5 Gy-irradiated group than the sham-irradiated group. Moreover, lipid peroxide levels in right hemisphere of sham-irradiated group were significantly increased at 1 and 4 hr after cold injury (Fig.6D).

DISCUSSION

In the present study, we used a model of cold injury in mice to investigate the potential protective effect of prior 0.5 Gy irradiation against brain edema because this model produces reliable vasogenic brain edema and secondary brain lesion in mice without a craniotomy, which may damage the cortical surface. We evaluated the severity of brain edema by measuring the water content of mouse brain. The water content was measured using the wet-dry weight method, which is accurate because the samples used in this study, mouse brain hemispheres, are lightweight (approximately 220 mg). In this study, we confirmed that cold injury induces edema and increase water content. Increases in water content of right hemisphere were first observed 1 hr after cold injury, and the water content reached a maximum at 24 hr after cold injury and decreased thereafter. Nevertheless, the water content was higher at all

time points after cold injury than the control level. Meanwhile, water content in left hemisphere, which was not directly injured, increased more slowly and less severely than water content in right hemisphere that was directly subjected to cold injury. This phenomenon has been observed before and it is due to extravasated fluids from blood vessel in the right hemisphere being transmitted through callosum to the left hemisphere [22]. This finding indicates that, even if localized, brain edema can affect remote regions over time.

The results of this study show that pre-treatment with 0.5 Gy irradiation partially inhibited the increases of water content in the mouse brain after cold injury. The present results suggest that 0.5 Gy irradiation may be useful in the treatment of vasogenic brain edema following brain injury. Vasogenic brain edema is associated with the disruption of endothelial cells and a resultant increase in vascular permeability. It is well known that ROS, including the superoxide anion, initiate lipid peroxidation chain reactions and severe cell damage, and ROS contribute significantly to the development of vasogenic brain edema. In a previous study, the lipid peroxide levels in injured brain tissue increased within 1 min after cold injury and reached maximal values at 2 hr [29]. Our results also show that lipid peroxide levels in brain increase immediately after cold injury. These findings indicated that the oxidative damage induced by ROS contribute to the pathogenesis of vasogenic brain edema and that the protection against oxidative damage is a potential treatment for vasogenic brain edema. Low-dose X-irradiation and radon inhalation produce adequate oxygen stress to induce antioxidant enzymes and suppress lipid peroxide levels [16, 23-28]. We have reported that prior 0.5 Gy-irradiation activated antioxidative function and inhibited edema in mouse paws induced by ischemia-reperfusion injury [16]. In fact, the present study also showed that total-GSH content in the brain and the activity of SOD in serum and brain tissue were significantly increased and lipid peroxide levels in brain were significantly decreased by 0.5 Gy irradiation. These findings suggested that low-dose irradiation activated antioxidative function and that ROS generated in brain following cold injury was reduced by these activated antioxidative functions.

Histological observation in the present study also indicated that 0.5 Gy irradiation inhibited increases in the water content of the brain following cold injury. Histological changes in the area of the lesions following cold injury described non-structual area including vacuolization, and expanded perivascular space. At 4 hr after cold injury, the percentage of non-structural area was significantly lower in brain sections from the 0.5 Gy-irradiated group than those from the sham-irradiated group.

This result was similar to the results of the water content analyses. These findings indicates the possibility that 0.5 Gy irradiation inhibited vasogenic brain edema following cold injury. Furthermore, the other characteristic histological changes associated with cold injury, such as the number of cells in the lesion area, were significantly reduced and more cell death was observed in the sham-irradiated group than in the 0.5 Gy-irradiated group. The nature of cell death in the lesion area in tissue samples from both the sham- and 0.5 Gy-irradiated groups was mainly apoptotic, as evidenced by occurrence of apoptotic bodies as shown by TUNEL staining. Murakami *et al*. also reported that apoptotic cell death contributes to cell depletion after cold injury [1]. Arachidonic acid metabolism, which may produce ROS, contributes to apoptotic cell death after cold injury [3]. In cold injury, a glutamate-mediated increase in intracellular calcium releases arachidonic acid, which leads to the formation of ROS from membrane phospholipids of cell. ROS are generated through this pathway, activate caspase, and lead to the apoptotic cell death. Our results showed that 0.5 Gy irradiation inhibited apoptotic cell death after cold injury and indicated that activated antioxidative function by 0.5 Gy irradiation reduced ROS generated through arachidonic acid metabolism following cold injury. However, these effects are only apparent 1 hr after cold injury.

Edaravone is a novel and potent free-radical scavenger that has been used clinically to reduce neuronal damage following ischemic stroke. It was reported that treatment with edaravone diminished water content of the core of middle cerebral artery (MCA) occlusion [30]. In addition, neuronal death in hippocampal CA1 region of rats subjected to 10 min ischemia with 3 days reperfusion has been reduced by edaravone [31]. Our results showed that prior 0.5 Gy irradiation was effective in reducing the water content of the mouse brain after cold injury to control (non-injury) level. These findings suggested that 0.5 Gy irradiation may be as potent as edaravone.

Transgenic mice that expressed 5-fold more extracellular SOD activity in their brains were significantly protected against edema formation relative to non-transgenic mice [9]. Our results show that SOD activity in serum (extracellular SOD) was significantly higher in mice from the 0.5 Gy-irradiated group than those from the sham-irradiated group more than 48 hr after cold injury. These results suggest that SOD in serum activated by 0.5 Gy irradiation protected the vascular endothelial cells from ROS generated outside the cell for more than 48 hr after cold injury.

Chan has reported that transgenic mice overexpressing copper-zinc SOD show significant attenuation of brain edema and neurological deficits following traumatic brain injury [32]. In our

current study, the SOD activity in brain of mice from the 0.5 Gy-irradiated group was high for 1 hr after cold injury. The activated SOD in brain detoxified ROS generated in the cell, and inhibited neuronal damage. In the 0.5 Gy-irradiated group 4, 24, and 48 hr after cold injury, SOD activity in brain was at control levels, and neuronal damage progressed to the sham level. These findings indicated that ROS contributed significantly to neuronal damage and apoptosis. The brain readily suffers from oxidant stress because it contains many unsaturated fatty acids, and the antioxidative enzymes, including catalases, are less abundant than in other internal organs such as the liver [33]. The oxidant stress such as livers is controlled by 0.5 Gy irradiation [24]. The effects of 0.5 Gy irradiation may be relatively weak protective effects in neuronal damage and apoptosis. However, the protective effects of 0.5 Gy irradiation against edema immediately following cold injury are strong.

In conclusion, our results indicate that ROS are important mediators of vasogenic brain edema after cold injury. In addition, we suggested that 0.5 Gy irradiation activated antioxidative function and inhibited vasogenic brain edema induced by cold injury. These results indicate that inhibitory effect of 0.5 Gy irradiation was weak against cell damage and was strong against brain edema. The data presented in this study provide an essential baseline study aimed at assessing the possibility of inducing remission of cerebrovasucular disease.

REFERENCES

1. Murakami, K., Kondo, T., Sato, S., Li, Y., and Chan, PH. 1997. Occurrence of apoptosis following cold injury-induced brain edema in mice. *Neuroscience* 81: 231-237.

2. Chan, PH. 2001. Reactive oxygen free radicals in signaling and damage in the ischemic brain. *Journal of Cerebral Blood Flow and Metabolism* 21: 2-14.

3. Lewen, A., Matz, P., and Chan, PH. 2000. Free radical pathway CNS injury. *Journal of Neurotrauma* 17: 871-890.

4. Ikeda, Y., Anderson. JH., and Long, DM. 1989. Oxygen free radicals in the genesis of traumatic and peritumoral brain edema. *Neurosurgery* 24: 679-685.

5. Chan, PH., Fishman, RA., Wesley, MA., and Longar., S. 1990. Pathogenesis of vasogenic edema in focal cerebral ischemia. Role of superoxide radicals. *Advances in Neurology* 52: 177-183.

6. Erlansson, M., Bergqvist, D., Marklund, SL., Persson, NH., and Svensjö, E. 1990. Superoxide dismutase as an inhibitor of postischemic microvascular permeability increase in the hamster. *Free Radical Biology & Medicine* 9: 59-65.

7. Ikeda, Y., Brelsford, KL., Ikeda, K., Bulkley, GB., and Long, DM. 1990. Effect of superoxide dismutase in cats with cold-induced edema. *Advances in Neurology* 52: 203-210.

8. Inoue, M., Watanabe, N., Morino, Y., Tanaka, Y., Amachi, T., and Sasaki, J. 1990. Inhibition of oxygen toxicity by targeting superoxide dismutase to endothelial cell surface. *FEBS letters* 269: 89-92.

9. Chan, PH., Yang, GY., Chen, SF., Carlson, E., and Epstein, CJ. 1991. Cold-induced brain edema and infarction are reduced in transgenic mice overexpressing CuZn-superoxide dismutase. *Annals of Neurology* 29: 482-486.

10. Chan, PH. 1992. Antioxidant-dependent amelioration of brain injury: role of CuZn-superoxide dismutase. *Journal of Neurotrauma* 9: S417-S423.

11. Kojima, S., Matsuki, O., Nomura, T., Kubodera, A., Honda, Y., Honda, S., Tanooka, H., Wakasugi, H., and Yamaoka, K. 1998. Induction of mRNAs for glutathione synthesis-related proteins in mouse liver by low doses of γ-rays. *Biochimica et Biophysica Acta* 1381: 312-318.

12. Kojima, S., Matsuki, O., Nomura, T., Shimura, N., Kubodera, A., Yamaoka, K., Tanooka, H., Wakasugi, H., Honda, Y., Honda, S., and Sasaki, T. 1998. Localization of glutathione and induction of glutathione synthesis-related proteins in mouse brain by low-doses γ-rays. *Brain*

Research 808: 262-269.

13. Jain, A., Mårtensson, J., Stole, E., Auld, PA., and Meister, A. 1991. Glutathione deficiency leads to Mitochondrial damage in the brain. *Proceedings of the National Academy of Sciences of the United States of America* 88: 1913-1917.
14. Mårtensson, J., Jain, A., Stole, E., Frayer, W., Auld, PA., and Meister, A. 1991. Induction of glutathione synthesis in the new born rat: a model of endogenously produced oxidative stress. *Proceedings of the National Academy of Sciences of the United States of America* 88: 9360-9364.
15. Kojima, S., Matsuki, O., Kinoshita, I., Gonzalez, TV., Shimura, N., and Kubodera, A. 1997. Dose small-dose γ-ray radiation induce endogenous antioxidant potential in vivo? Biological & Pharmaceutical Bulletin 20: 601-604.
16. Kataoka, T., Mizuguchi, Y., Yoshimoto, M., Taguchi, T., and Yamaoka, K. 2007. Inhibitory effects of prior low-dose X-irradiation on ischemia-reperfusion injury in mouse paw. Journal of Radiation Research 48: 505-513.
17. Barinaga, M. 1996. Finding new drugs to treat stroke. Science 272: 664-666.
18. Murakami, K., Kondo, T., Yang, G., Chen, SF., Morita, Y., Fujimura, Y., and Chan, PH. 1999. Cold injury in mice: a model to study mechanisms of brain edema and neuronal apoptosis. *Progress in Neurobiology* 57: 289-299.
19. Baehner, RL., Murrmann, SK., Davis, J., and Johnston, RB. 1975. The role of superoxide anion and hydrogen peroxide in phagocytosis-associated oxidative metabolic reactions. *The Journal of Clinical Investigation* 56: 571-576.
20. Bradford, MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248-254.
21. Aebi, H., Wyss, SR., Scherz, B., and Gross, J. 1976. Properties of erythrocyte catalase from homozygotes and heterozygotes for Swiss-type acatalasemia. *Biochemical Genetics* 14: 791–807.
22. Kempinsky, WH., Boniface, WR., Keating, JB., and Morgan ,PP. 1961. Serial hemodynamic study of cerebral infarction in man. Circulation Research 9: 1051-1058.
23. Yamaoka, K. 2006. Activation of antioxidant system by low dose radiation and its applicable possibility for treatment of reactive oxygen species-related diseases. *Journal of Clinical Biochemistry and Nutrition* 39: 114-133.
24. Yamaoka, K., Kataoka, T., Nomura, T., Taguchi, T., Wang, DH., Mori, S., Hanamoto, K., and

Kira, S. 2004. Inhibitory effects of prior low-dose irradiation on carbon tetrachloride-induced hepatopathy in acatalasemic mice. *Journal of Radiation Research* 45: 89-95.

25. Kataoka, T., Nomura, T., Wang, DH., Taguchi, T., and Yamaoka, K. 2005. Effects of post low-dose X-ray irradiation on carbon tetrachloride-induced acatalasemic mice liver damage. *Physiological Chemistry and Physics and Medical NMR* 37: 109-126.
26. Kataoka, T., Mizuguchi, Y., Notohara, K., Taguchi, T., and Yamaoka, K. 2006. Histological changes in spleens of radio-sensitive and radio-resistant mice exposed to low-dosce X-ray irradiation. *Physiological Chemistry and Physics and Medical NMR* 38: 21-29.
27. Kataoka, T., Aoyama, Y., Sakoda, A., Nakagawa, S., and K. Yamaoka. 2006. Basic study on biologic effects of thoron and thermal therapy. *Physiological Chemistry and Physics and Medical NMR* 38: 85-92.
28. Kataoka, T., Yoshimoto, M., Nakagawa, S., Mizuguchi, Y., Taguchi, T., and Yamaoka, K. 2009. Basic study on active changes in biological function of mouse liver graft in cold storage after low-dose x-irradiation. *Journal of Clinical Biochemistry and Nutrition* 45: 219-226.
29. Vagnozzi, R., Marmarou, A., Tavazzi, B., Signoretti, S., Di Pierro, D., del Bolgia, F., Amorini, AM., Fazzina, G., Sherkat, S., and Lazzarino, G. 1999. Changes of cerebral energy metabolism and lipid peroxidation in rats leading to mitochondrial dysfunction after diffuse brain injury. *Journal of Neurotrauma* 16: 903-913.
30. Abe, K., Yuki, S., and Kogure, K. 1988. Strong Attenuation of Ischemic and Postischemic Brain Edema in Rats by a Novel Free Radical Scavenger. *Stroke* 19: 480-485
31. Yamamoto, T., Yuki, S., Watanabe, T., Mitsuka, M., Saito, KI., and Kogure, K. 1997. Delayed neuronal death prevented by inhibition of increased hydroxyl radical formation in a transient cerebral ischemia. *Brain Research* 762: 240-242.
32. Chan, PH., Yang, GY., Chen, SF., Carlson, E., and Epstein, CJ. 1991. Cold-induced brain edema and infarction are reduced in transgenic mice overexpressing CuZn-superoxide dismutase. *Annals of Neurology* 29: 482-486.
33. Rueff-Barroso, CR., Trajano, ET., Alves, JN., Paiva, RO., Lanzetti, M., Pires, KM., Bezerra, FS., Pinho, RA., Valenca, SS., and Porto, LC. 2010. Organ-related cigarette smoke-induced oxidative stress is strain-dependent. *Medical Science Monitor* 16: 218-226.

Figure Legends

Fig. 1. Water content (A) of right and left hemisphere after cold injury. Water content (B) in the right brain hemisphere after cold injury in mice pre-treated with irradiation. The number of mice per experimental point was 5 - 8. *P<0.05, **P<0.01, ***P<0.001 vs. Control, $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. Sham.

Fig. 2. Histological changes in lesion area 4 hr after cold injury in mice pre-treated with irradiation: (A) control, (B) sham irradiation, (C) 0.5 Gy irradiation. Black arrows indicate cells in the lesion area. Some cells were shrunken. The black arrowheads indicate bleeding and white arrows indicate vacuolization in the lesion area. The length of scale bar is 100 μm. For all panels HE staining was used.

Fig. 3. Histological changes in lesion area 4 hr after cold injury in mice pre-treated with irradiation: (A) control, (B) sham irradiation, (C) 0.5 Gy irradiation. The black arrows indicate neurons in the lesion area. The black arrowheads indicate bleeding and the white arrows indicate expanded perivascular space in the lesion area. The length of scale bar is 100 μm. For all panels KB staining was used.

Fig. 4. Histological changes in lesion area 1 hr after cold injury in mice pre-treated with irradiation: (A) control, (B) sham irradiation, (C) 0.5 Gy irradiation. The black arrows indicate TUNEL-positive cells in the lesion area. The length of scale bar is 100 μm. For all panels TUNEL staining was used.

Fig. 5 Activities of SOD in serum after cold injury in mice pre-treated with irradiation. The number of mice per experimental point was 5 - 8. $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. sham.

Fig. 6 (A) SOD activity, (B) catalase activity, (C) t-GSH content, and (D) lipid peroxide level in the right brain hemisphere of mice pre-treated with irradiation and after cold injury. The number of mice per time point was 5 - 8. *P<0.05, **P<0.01 vs. control, $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. sham.

Table 1. Percentage of non-structural area in lesion area after cold injury in mice pre-treated with irradiation. The number of mice per experimental point was 4 - 6. ***P<0.001 vs. control, $^{\#}$P<0.05 vs. sham.

Table 2. Number of cells in lesion area after cold injury in mice pre-treated with irradiation. The number of mice per experimental point was 4 - 6. *P<0.05, **P<0.01, ***P<0.001 vs. control.

Table 3. Number of neurons in lesion area after cold injury in mice pre-treated with irradiation. The number of mice per experimental point was 4 - 6. **P<0.01, ***P<0.001 vs. control.

Table 4. Number of TUNEL-positive cells in the lesion area after cold injury in mice pre-treated with irradiation. The number of mice per experimental point was 4 - 6. $^{\#}$P<0.05 vs. sham.

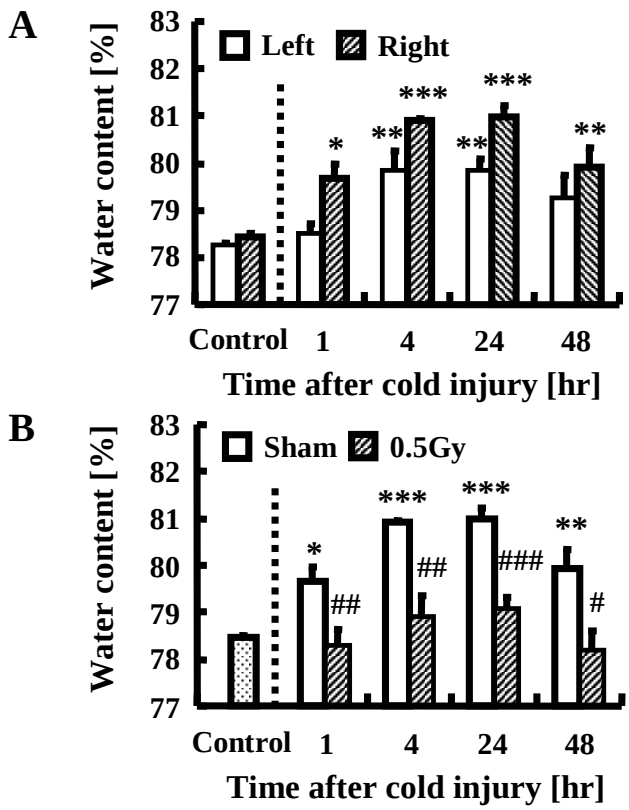
A
Left
Right
Water content [%]
83
82
81
80
79
78
77
*
**
***
**
***
**
Control
1
4
24
48
Time after cold injury [hr]
B
Sham
0.5Gy
Water content [%]
*
##
***
##
***
###
**
#
Control
1
4
24
48
Time after cold injury [hr]

Fig.1

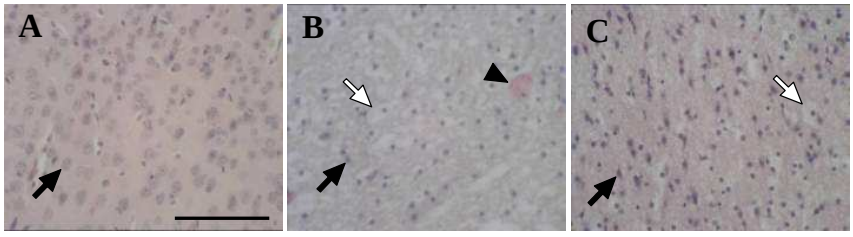
A
B
C

Fig.2

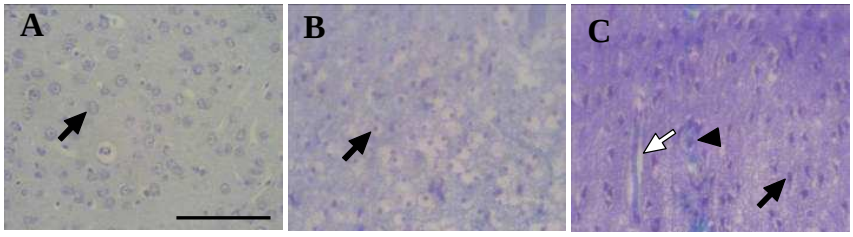

Fig.3

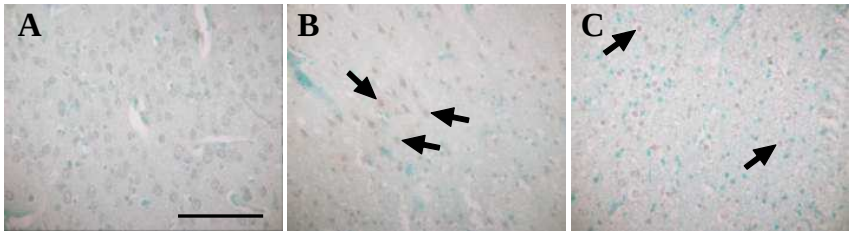

Fig.4

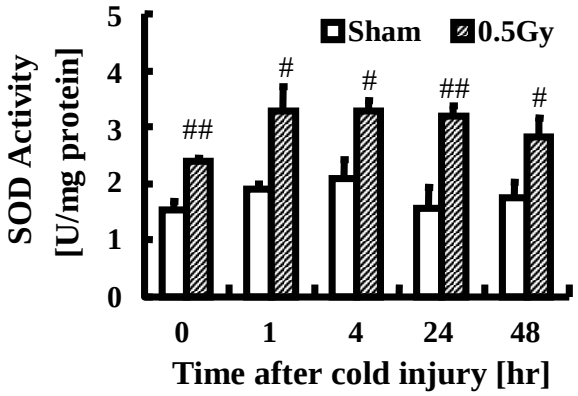
SOD Activity
[U/mg protein]
Sham
0.5Gy
##
#
#
##
#
0
1
2
3
4
5
0
1
4
24
48
Time after cold injury [hr]

Fig.5

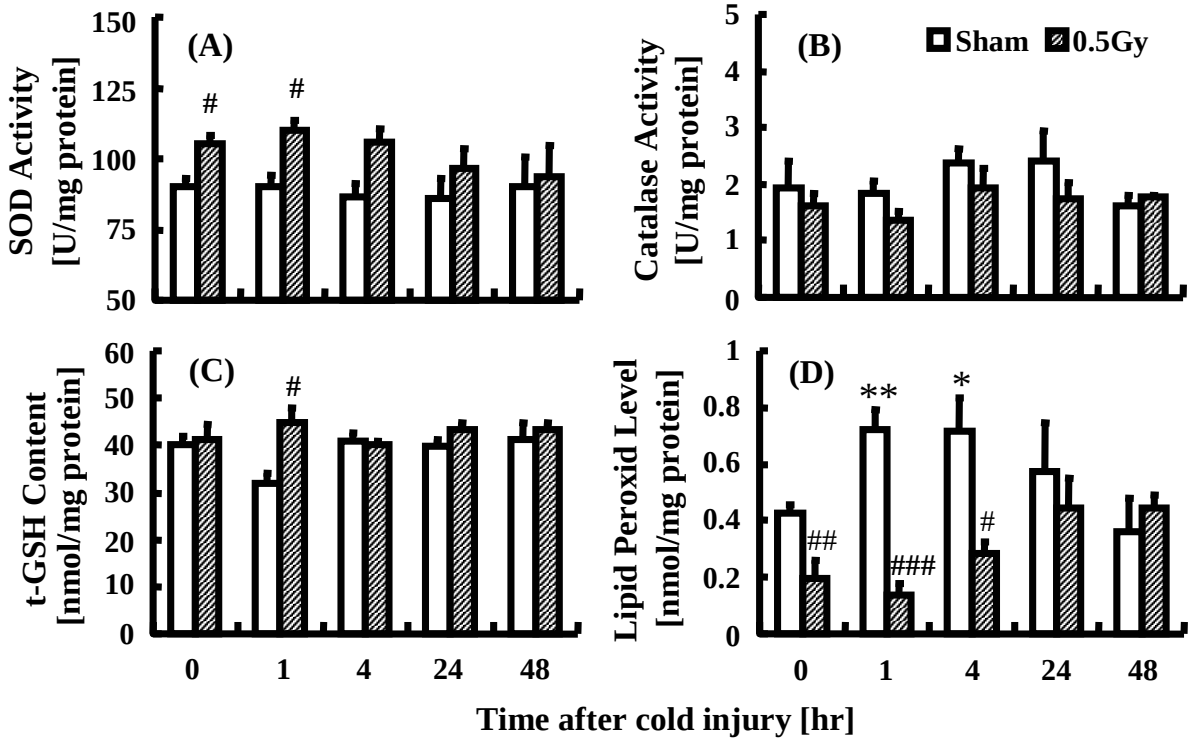
(A)
SOD Activity
[U/mg protein]
(B)
Catalase Activity
[U/mg protein]
Sham
0.5Gy
(C)
t-GSH Content
[nmol/mg protein]
(D)
Lipid Peroxid Level
[nmol/mg protein]
0
1
4
24
48
Time after cold injury [hr]

Fig.6

	Ratio of non-structural area [%]	
	Sham	0.5Gy
Control	2.55±0.50	
1 hr	4.65±1.12	6.00±1.92
4 hr	25.3±3.41***	15.8±2.79***, #
24 hr	34.0±4.17***	34.5±5.83***
48 hr	35.4±1.26***	36.5±3.01***

Table.1

	Number of cells [cells/mm^2]	
	Sham	0.5Gy
Control	7452±612	
1 hr	6487±982	5313±1056
4 hr	4457±498*	5837±588
24 hr	2478±271***	2722±849**
48 hr	2120±506***	2365±687***

Table.2

	Number of neurons [cells/mm^2]	
	Sham	**0.5Gy**
Control	**4417±389**	
1 hr	**3991±345**	**3496±379**
4 hr	**3663±400**	**3891±176**
24 hr	**2191±495****	**1511±325****
48 hr	**1402±125*****	**1557±276*****

Table.3

	Number of TUNEL positive cells [cells/mm^2]	
	Sham	**0.5Gy**
Control	**33±22**	
1 hr	**489±115**	**165±57$^{\#}$**
4 hr	**285±45**	**269±90**
24 hr	**162±62**	**286±63**
48 hr	**85±40**	**123±43**

Table.4