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Inhibitory Effects of Pre and Post Radon Inhalation on Carbon Tetrachloride-induced Oxidative Damage in Mouse Organs

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Radon inhalation activates antioxidative functions in some organs of mice. We examined the prevention effects of pre radon inhalation and the alleviation effects of post radon inhalation on carbon tetrachloride (CCl_4)-induced oxidative damage in the brain, heart, lung, liver, and kidney of mice. In addition, we compared the effect of pre and post radon inhalation on oxidative damage. Mice inhaled radon at a concentration of 18 000 Bq/m^3 *for 6* hrs *before or after* CCl_4 *administration. As a result, the total glutathione (t-GSH) contents and catalase (CAT) activities in the brain, heart, lung, liver, and kidney and superoxide dismutase (SOD) activities in the heart and lung were significantly higher in pre and post radon-inhaled mice than in mice treated with only* CCl_4. *Pre radon inhalation inhibited and post radon inhalation reduced lipid peroxidation induced by* CCl_4. *In addition, there were no significant differences in lipid peroxide (LPO) levels in the brain, heart, lung, liver, and kidney between pre and post radon-inhaled mice. These findings suggested that post radon inhalation has the same effects as pre radon inhalation against* CCl_4*-induced oxidative damage in the brain, heart, lung, liver, and kidney.*

Key Words : carbon tetrachloride, oxidative damage, pre or post radon inhalation, antioxidative function

1. Introduction

Lifestyle-related diseases such as cancer and diabetes are responsible for oxidative stress occurring by the generation of reactive oxygen species (ROS)[1]. On the other hand, low-dose irradiation induces beneficial effects against ROS in the living body. It has been reported that low-dose X- or γ-irradiation induces or increases endogenous antioxidant substances such as superoxide dismutase (SOD)[2),3)], catalase (CAT)[4)], and glutathione (GSH)[5),6)] in some organs of small laboratory animals. We have reported that pre low-dose X-irradiation inhibits oxidative damage such as carbon tetrachloride (CCl_4)-induced hepatopathy[7)], ischemia-reperfusion injury[8)], and brain edema[9)] in mice. These findings suggested that low-dose irradiation activates defense systems in the living body and therefore contributes to preventing or reducing ROS-related injuries.

Radon (^{222}Rn) hot springs have been used for medical treatment of oxidative stress-related diseases in Misasa, Japan[10),11)] and Badgastein, Austria[12)]. Although several attempts have been made to clarify the mechanism of the

therapy, it is not fully understood ; therefore, we investigated the effects of radon inhalation on mice. For example, radon inhalation increased SOD and CAT activities in some mouse organs[13], suggesting the enhancement of antioxidative functions. In addition, we reported that pre radon inhalation inhibited CCl_4-induced hepatopathy and renal damage[14]. These findings suggested that radon inhalation may contribute to inhibiting oxidative stress-related disease in the liver and kidney. These findings also suggested that radon inhalation has antioxidative effects similar to low-dose X-irradiation.

Radon dissolved in blood entering the gas exchange compartment is transported to many tissues by the blood stream, and launches stimulus effects[15]. We recently reported that radon inhalation activated SOD activity in various organs, such as the brain, thymus, heart, lung, liver, kidney and small intestine, suggesting that pre radon inhalation may inhibit oxidative damage in many organs as well as the liver and kidney[16]. However, there have been no reports of the protective effect of radon inhalation on oxidative damage in the brain, heart, and lung. Moreover, even though radon therapy is performed for treatment rather than preventive purposes, the alleviation effects of post radon inhalation on oxidative damage have never been examined ; therefore, there is no comparative study on the effect of pre or post radon inhalation on oxidative damage.

CCl_4 is a well-established hepatotoxin[17]. A study demonstrated that the liver is not the only target organ of CCl_4 and it causes free radical generation in other organs, such as the brain, heart, lung, and kidney[18]. It has also been reported that CCl_4 administration induces oxidative stress in these organs[19),20]. Considering this background, we performed a comparative study of the effects of pre or post radon inhalation on CCl_4-induced oxidative damage in the brain, heart, lung, liver, and kidney of mice. To assess oxidative damage in these organs, we investigated the following biochemical parameters : SOD and CAT activity, total glutathione content (t-GSH), lipid peroxide (LPO) level.

2. Materials and methods

2・1 Animals

Male BALB/c mice (age, 7 weeks ; body weight, approximately 25 g) were obtained from the Department of Animal Resources Advanced Science Research Center Okayama University. The animals were housed in clear plastic cages with wood chip bedding in a temperature-controlled room (22 ± 3℃). They were fed Oriental MF diet (Oriental Yeast Co., Tokyo) and tap water *ad libitum*. Each group consisted of 5 mice. Ethics approval was obtained from the animal experimental committee of Okayama University.

To clarify the effects of radon therapy, we have co-developed a radon exposure system for small animals (OZ PLAN Co., Ltd. and Radon Medical Treatment Research & Development Co., Ltd., Okayama, Japan). Briefly, air and radon were blown into an exposure box and vented out of the box at a rate of 0.2 L/min. The radon concentration in the box was measured using a radon monitor (PQ 2000 ; Genitron Instruments, Frankfurt, Germany).

Mice were divided into 5 groups : control, Rn-only inhalation, CCl_4-only administration, pre radon inhalation and CCl_4 administration (Rn + CCl_4), and CCl_4 administration and post radon inhalation (CCl_4 + Rn). The Rn-only group was exposed to radon of 18 kBq/m^3 for 6

hrs. The control group was treated with sham radon inhalation. The brain, heart, lung, liver and kidney were quickly excised under ether anesthesia immediately after radon or sham radon inhalation.

The CCl_4-only group received CCl_4 injection (4 mL/kg weight, 5% in olive oil) into the peritoneum. The Rn + CCl_4 group was exposed to radon of 18 kBq/m^3 for 6 hrs. Immediately after radon inhalation, the Rn + CCl_4 group received CCl_4 injection. The CCl_4 + Rn group was exposed to radon of 18 kBq/m^3 for 6 hrs after 18 hrs of CCl_4 injection. Twenty-four hours and 48 hrs after CCl_4 injection, the organs were quickly excised under ether anesthesia. These samples were preserved at −80℃ until biochemical assay.

2・2 Biochemical assay

Mouse brain, heart, lung, liver and kidney 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℃ and the supernatant was used to assay the activity of SOD and catalase. SOD activity was measured by the nitroblue tetrazolium (NBT) reduction method[21] 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 at 560 nm using a spectrophotometer. One unit of enzyme activity was defined as 50% inhibition of NBT reduction.

Catalase activity was measured as the hydrogen peroxide (H_2O_2) reduction rate at 37℃ and was assayed at 240 nm using a spectrophotometer[22]. 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, heart, lung, liver, and kidney supernatants. 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 H_2O_2 split by catalase at 37℃. The reactions were started by addition of the supernatant.

The t-GSH content was measured using the Bioxytech GSH-420™ assay kit (OXIS Health Products, Inc., Portland, OR, USA). Briefly, the brain, heart, lung, liver, and kidney were suspended in 10 mM phosphate-buffered saline (PBS ; 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. The t-GSH content was measured at 420 nm using a spectrophotometer. This assay is based on the formation of a chromophoric thione, the absorbance of which, measured at 420 nm, is directly proportional to the t-GSH concentration.

LPO (malondialdehyde (MDA)) levels were assayed using the Bioxytech LPO-586™ assay kit (OXIS Health Products, Inc.). Briefly, brain, heart, lung, liver, and kidney were homogenized in 20 mM PBS (pH 7.4) on ice. Before homogenization, 10 μL of 0.5 M butylated hydroxytoluene in acetonitrile was added per 1 mL tissue homogenate. After homogenization, the homogenate was centrifuged at 15 000 × g, for 10 min at 4℃ and the supernatant was used for the assay. The MDA assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenylidole, with MDA at 45℃. The optical density of the colored products was read at 586 nm in a spectrophotometer.

The protein content was measured by the Bradford method, using the Protein Quantification Kit-Rapid (Dojindo Molecular Technologies,

Inc., Kumamoto, Japan)[23].

2・3 Statistical analyses

Data 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 Tukey's tests for multiple comparisons where appropriate.

3. Results

3・1 Effect of radon inhalation on antioxidant-associated substances

SOD activities in the heart, lung, and kidney were significantly higher in the Rn-only group than in the control group. CAT activities in the brain, heart, liver, and kidney were significantly higher in the Rn-only group than in the control group. The t-GSH contents in the brain, heart, lung, and kidney were significantly higher in the Rn-only group than in the control group. LPO levels in the brain, lung, liver, and kidney were significantly lower in the Rn-only group than in the control group (Table 1).

3・2 Effect of pre or post radon inhalation on CCl_4-induced oxidative damage in the brain

SOD activity (24 hrs after CCl_4 administration), CAT activity, and the t-GSH content (at 24 and 48 hrs) were significantly lower in the CCl_4-only group than in the control group (Fig. 1 A − C); however, SOD activity (24 hrs) was significantly higher in the CCl_4 + Rn group than in the CCl_4-only and Rn + CCl_4 groups (Fig. 1 A). CAT activity (24 and 48 hrs) and the t-GSH content (24 hrs) were significantly higher in the Rn + CCl_4 and CCl_4 + Rn groups than in the CCl_4-only group (Fig. 1 B, C). The LPO level (24 hrs) was 25% higher in the CCl_4-only group than in the control group, but these differences were not significant; however, the LPO levels (24 hrs) in the Rn + CCl_4 and CCl_4 + Rn groups were closer to the control group (Fig. 1 D).

3・3 Effect of pre or post radon inhalation on CCl_4-induced oxidative damage in the heart

SOD and CAT activities (48 hrs after CCl_4 administration) and the t-GSH content (24 and 48 hrs) were significantly lower in the CCl_4-only

Table 1 Effect of radon inhalation on antioxidant-associated substances

Antioxidat substances		Organs				
		Brain	Heart	Lung	Liver	Kidney
SOD Activity	Control	23.8±1.0	21.3±1.6	16.4±0.1	40.3±1.2	33.2±0.8
[U/mg protein]	Rn	21.6±5.1	29.2±1.5**	18.5±0.6**	41.9±1.9	35.6±0.4*
CAT Activity	Control	1.43±0.07	7.44±0.3	17.6±0.3	83.0±3.7	45.6±1.4
[U/mg protein]	Rn	1.74±0.05**	9.83±1.0*	16.8±1.0	97.4±3.2*	60.2±4.1*
t-GSH Content	Control	44.2±1.2	18.7±0.4	29.7±1.8	65.5±3.4	12.4±0.5
[nmol/mg protein]	Rn	50.0±1.7*	20.9±0.7*	33.8±0.9**	69.4±3.8	15.8±1.3*
LPO Level	Control	0.83±0.05	0.35±0.00	0.63±0.03	0.26±0.02	0.44±0.01
[nmol/mg protein]	Rn	0.64±0.04*	0.33±0.04	0.56±0.02*	0.22±0.01*	0.37±0.01**

Each value is the mean ± SEM. Number of mice per experiment : 5. *P＜0.05, **P＜0.01, Rn (radon inhalation) vs. Control (sham radon inhalation).

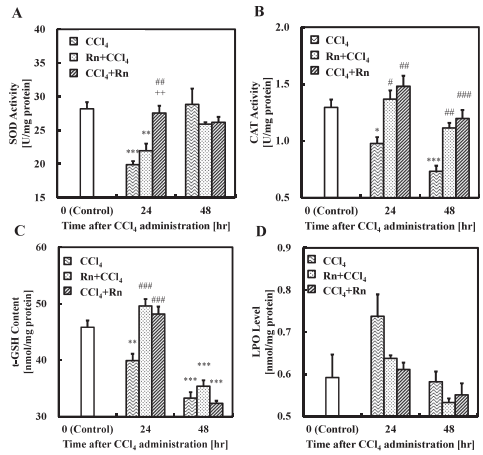

Fig. 1 Effect of pre or post radon inhalation on CCl_4-induced oxidative damage in mouse brain. A : SOD activity, B : CAT activity, C : t-GSH content, D : lipid peroxide level. Each value is the mean ± SEM. Number of mice per experiment : 5. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$: CCl_4 (no inhalation under CCl_4 administration at each time point) vs. Control (sham radon inhalation). $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$: Rn + CCl_4 or CCl_4 + Rn (pre or post radon inhalation under CCl_4 administration) vs. CCl_4 (no inhalation under CCl_4 administration at the same time point). $^{++}P<0.01$ and $^{+++}P<0.001$: CCl_4 + Rn (post radon inhalation under CCl_4 administration) vs. Rn + CCl_4 (pre radon inhalation under CCl_4 administration at the same time point).

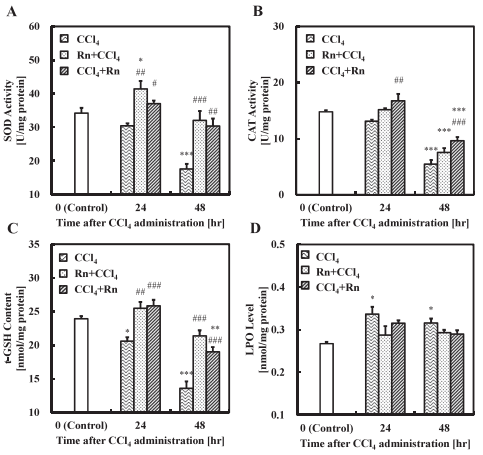

Fig. 2 Effect of pre or post radon inhalation on CCl_4-induced oxidative damage in mouse heart. The number of mice for each experiment and significance are the same as in Fig. 1.

group than in the control group (Fig. 2 A－C) ; however, SOD activities and the t-GSH contents (24 and 48 hrs) were significantly higher in the Rn + CCl_4 and CCl_4 + Rn groups than in the CCl_4-only group (Fig. 2 A, C). CAT activity (24 and 48 hrs) was significantly higher in the CCl_4 + Rn group than in the CCl_4-only group (Fig. 2 B). The LPO levels (24 and 48 hrs) were significantly higher in the CCl_4-only group than in the control group ; however, there was no significant difference in the LPO level (24 and 48 hrs) between the control group and the Rn + CCl_4 or CCl_4 + Rn group (Fig. 2 D).

3・4 Effect of pre or post radon inhalation on CCl_4-induced oxidative damage in the lung

SOD and CAT activities (24 and 48 hrs after CCl_4 administration) and the t-GSH content (24 hrs) were significantly lower in the CCl_4-only group than in the control group (Fig. 3 A－C) ; however, SOD activity and the t-GSH content (at 24 hrs) were significantly higher in the Rn + CCl_4 and CCl_4 + Rn groups than in the CCl_4-only group (Fig. 3 A, C). CAT activities were significantly higher in the Rn + CCl_4 (at 24 hrs) and CCl_4 + Rn groups (48 hrs) than in the CCl_4-only group (Fig. 3 B). The LPO level (24 and 48 hrs) was significantly higher in the CCl_4-only group than in the control group ; however, the LPO levels (24 hrs) were significantly lower in the Rn + CCl_4 and CCl_4 + Rn groups than in the CCl_4-only group (Fig. 3 D).

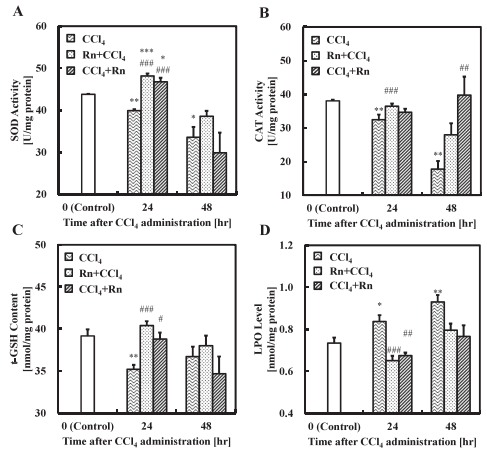

Fig. 3 Effect of pre or post radon inhalation on CCl_4-induced oxidative damage in mouse lung. The number of mice for each experiment and significance are the same as in Fig. 1.

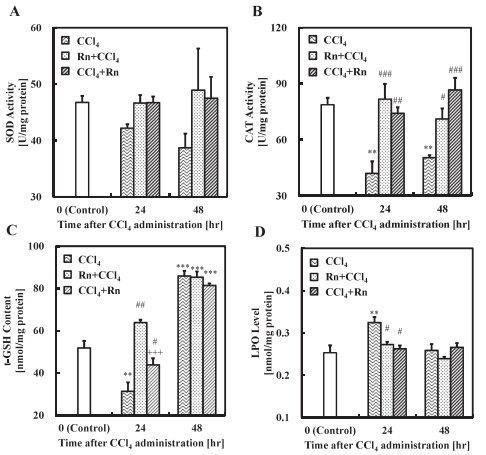

Fig. 4 Effect of pre or post radon inhalation on CCl_4-induced oxidative damage in mouse liver. The number of mice for each experiment and significance are the same as in Fig. 1.

3・5 Effect of pre or post radon inhalation on CCl_4-induced oxidative damage in the liver

CAT activity (24 and 48 hrs after CCl_4 administration) and the t-GSH content (24 hrs) were significantly lower in the CCl_4-only group than in the control group; however, CAT activity (24 and 48 hrs) and the t-GSH content (24 hrs) were significantly higher in the Rn + CCl_4 and CCl_4 + Rn groups than in the CCl_4-only group (Fig. 4 B, C). In addition, the t-GSH contents in the CCl_4-only, Rn + CCl_4, and CCl_4-Rn groups significantly exceeded the control value 48 hrs after CCl_4 administration (Fig. 4 C). The LPO level (24 hrs) was significantly higher in the CCl_4-only group than in the control group; however, the LPO level (24 hrs) was significantly lower in the Rn + CCl_4 and CCl_4 + Rn groups than in the CCl_4-only group (Fig. 4 D).

3・6 Effect of pre or post radon inhalation on CCl_4-induced oxidative damage in the kidney

The activities of SOD and CAT (24 and 48 hrs after CCl_4 administration) and the t-GSH content (24 hrs) were significantly lower in the CCl_4-only group than in the control group (Fig. 5 A－C); however, CAT activity (48 hrs) and the t-GSH content (24 hrs) were significantly higher in the Rn + CCl_4 and CCl_4 + Rn groups than in the CCl_4-only group (Fig. 5 B, C). In addition, the t-GSH content in the CCl_4-only group significantly exceeded the control value 48 hrs after CCl_4 administration. The LPO level (24 and 48 hrs) was significantly higher in the CCl_4-only group than in the control group; however, the LPO levels (24 and 48 hrs) were significantly lower in the Rn + CCl_4 and CCl_4 + Rn groups than in the CCl_4-only group (Fig. 5 D).

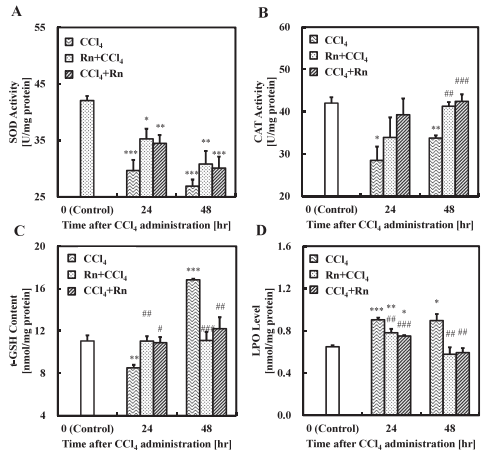

Fig. 5 Effect of pre or post radon inhalation on CCl_4-induced oxidative damage in mouse kidney. The number of mice for each experiment and significance are the same as in Fig. 1.

4. Discussion

We previously demonstrated that radon inhalation of 4 000 Bq/m^3 for 24 or 48 hrs activated antioxidative functions in the liver and kidney of mice[13] ; however, such long-term inhalation is unsuitable for medical treatment. We previously attempted to shorten the inhalation time and demonstrated that radon inhalation of 18 kBq/m^3 for 6 hrs, the same condition as in the present study, significantly activated antioxidative functions in the liver and kidney. These findings suggested that the exposure dose to activate the antioxidative function may be estimated by multiplying the radon concentration and inhalation time[13] ; however, it has not been examined whether the condition of radon inhalation activates antioxidative functions in the brain, heart, and lung.

In the present study, the results showed that antioxidative functions in the brain, heart, lung, liver, and kidney of mice that inhaled radon were significantly higher than in those of control mice. In particular, the brain is a highly fatty organ and has more fat than any other organ[24], and radon dissolves easily in fat ; therefore, antioxidant substances and hormone secretion such as β endorphin[10),25)] in the brain readily increase by radiation, since the brain contains abundant phospholipids that are sensitive to active oxygen[2),16),26)]. In this study, mice inhaled a high concentration of radon ; however, our results indicated the enhancement of antioxidative functions in the brain without causing oxidative damage by radon inhalation.

We next examined the protective effect of radon inhalation on CCl_4-induced oxidative damage in the brain, heart, lung, liver and kidney. Trichloromethyl radical ($\cdot CCl_3$) and trichloromethyl peroxy radical (CCl_3O_2) are generated via the metabolism of CCl_4 in cytochrome P450[27]. These radicals initiate lipid peroxidation chain reactions and cause severe cell damage in many organs[19),20)]. On the other hand, GSH directly reacts with ROS and protects against CCl_4-induced microsomal lipid peroxidation[27]. Our results showed that CCl_4 injection induced oxidative damage, as shown by the decreased t-GSH contents and increased LPO levels in the brain, heart, lung, liver, and kidney. At 48 hrs after CCl_4 administration, the t-GSH content in the liver in the CCl_4-only, Rn + CCl_4, and CCl_4 + Rn groups and in the kidney in the CCl_4-only group was markedly increased compared with the control group. This may be due to homeostasis because the liver and kidney are the main target organs of CCl_4[17),18)]. Our results also showed the decrease of antioxidant enzymes such as SOD and CAT after CCl_4 administration. The scavenging activity of SOD, which catalyzes the conversion of superoxide ($\cdot O_2^-$) into H_2O_2, and CAT, which trans-

forms H_2O_2 into H_2O as well as GSH, is well known, but under the administration of CCl_4, little $\cdot O_2^-$ and H_2O_2 is generated in some organs. The decreased activities of SOD and CAT may be due to cell damage induced by CCl_4; however, pre radon inhalation suppressed the increase of LPO levels and enhanced antioxidative functions in the brain, heart, lung, liver, and kidney induced by CCl_4 administration. These findings suggested that pre radon inhalation reduced oxidative stress in the brain, heart, and lung as well as in the liver and kidney[14].

Furthermore, we examined the alleviation effect of post radon inhalation on oxidative damage in the brain, heart, lung, liver, and kidney. We previously reported that post low-dose γ-irradiation promoted more rapid recovery from CCl_4-induced hepatopathy[28]. This report may indicate that GSH has an important role in the mechanism of accelerated recovery from oxidative damage. The GSH-redox cycle is an important protective process against ROS. GSH directly reacts with ROS, and glutathione peroxidase (GPx) catalyzes the destruction of H_2O_2 and lipid hydroperoxide[29]. This catalysis generates GSSG, and finally GSH; however, glutathione reductase (GR) catalyzes the regeneration of GSH from GSSG[30]. Thus, both GR and GPx are enzymes in the glutathione regenerating pathway, and the changes of both activities occur in a similar fashion. In particular, this cycle is activated immediately after low-dose irradiation of normal mice[4),28]. In the same manner, we previously reported that radon inhalation activated GPx and GR activity in the mouse liver and kidney[14]. Our results showed that the GSH contents in the brain, heart, lung, liver and kidney significantly increased, and the LPO levels reduced closer to the control level after post radon inhalation. These findings may suggest that radon inhalation accelerated recovery through activation of the GSH-redox cycle.

We previously reported that post low-dose X-irradiation promoted more rapid recovery from CCl_4-induced hepatopathy in normal mice than in acatalasemic mice which had one-tenth to half lower the CAT activities in the blood and tissues than in normal mouse[31]. This report may suggest that CAT is also important in the recovery from oxidative damage in the liver. In our previous study, the acatalasemic mouse brain was more damaged than the normal mouse brain by high-dose (5 Gy) irradiation. On the other hand, low-dose (0.5 Gy) irradiation specifically increased the activities of GPx and CAT by 15–50% in the acatalasemic mouse brain, making the activities closer to those in the normal mouse brain. These findings suggested that free radical reactions induced by lack of CAT are more appropriately neutralized by low-dose irradiation[32]. In the present study, post radon inhalation significantly activated CAT activity in the brain, heart, lung, liver, and kidney. Although these results may correlate with the recovery from CCl_4-induced oxidative damage, it was not clear how CAT contributed to the recovery process. Further study is required to clarify this point.

In addition, we compared the effects of pre and post radon inhalation on oxidative damage in the brain, heart, lung, liver, and kidney. It was expected that pre radon inhalation would be more effective than post radon inhalation, because antioxidants reacts with ROS before organs are subjected to oxidative stress; however, there was no significant difference in LPO levels in the brain, heart, lung, liver, and kidney between the Rn + CCl_4 group and the

CCl_4 + Rn group. These findings suggested that post radon inhalation has the same effects as pre radon inhalation against CCl_4-induced oxidative damage in the brain, heart, lung, liver, and kidney. As discussed above, in particular, the GSH-redox cycle may be an important factor in the recovery from CCl_4-induced oxidative damage.

In the present study, our results showed protective and alleviating effects of radon inhalation on oxidative damage in the brain, heart, lung, liver, and kidney in mice. These findings suggested that radon inhalation will contribute to the prevention and treatment of oxidative damage in these organs in humans. The data presented in this study provide a substantial basis for future studies aimed at assessing new radon-based therapies for the treatment of oxidative stress-related diseases in humans.

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