Comparison of antioxidative effects between radon and thoron inhalation in mouse organs

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Background: Radon therapy has been traditionally performed globally for oxidative stress-related diseases. Many researchers have studied the beneficial effects of radon exposure in living organisms. However, the effects of thoron, a radioisotope of radon, have not been fully examined. Objective: In this study, we aimed to compare the biological effects of radon and thoron inhalation on mouse organs with a focus on oxidative stress. Methods: Male BALB/c mice were randomly divided into 15 groups: sham inhalation, radon inhalation at a dose of 500 Bq/m^3 or 2000 Bq/m^3, and thoron inhalation at a dose of 500 Bq/m^3 or 2000 Bq/m^3 were carried out. Immediately after inhalation, mouse tissues were excised for biochemical assays. Results: The results showed a significant increase in superoxide dismutase and total glutathione, and a significant decrease in lipid peroxide following thoron inhalation under several conditions. Additionally, similar effects were observed for different doses and inhalation times between radon and thoron. Conclusion: Our results suggest that thoron inhalation also exerts antioxidative effects against oxidative stress in organs. However, the inhalation conditions should be carefully analyzed because of the differences in physical characteristics between radon and thoron.

Keywords: radon; thoron; oxidative stress; antioxidative function

Introduction

The inert gas, radon (^{222}Rn), is a natural alpha-emitter emanated from soil and dwellings and is a dominant source of exposure to ionizing radiation. Exposure to radon is known to cause lung cancer because of the deposition of its progenies in the lung, bronchi, and alveoli. This is supported by experimental evidences from cohort studies and biological experiments using cell culture and laboratory animals (Al-Zoughool and Krewski 2009; Pershagen et al. 1994; World Health Organization 2009).

Inhaled radon and its progenies are transported to organs through blood circulation and expose them to ionizing radiation. Reactive oxygen species (ROS) such as hydroxyl radicals (OH^-) isolated from biomolecules damage DNA, proteins, and lipids in organs or tissues. This is called oxidative stress and is known to be related to various diseases. Cells exposed to oxidative stress also acquire resistance to the stress. Antioxidants like superoxide dismutase (SOD) and glutathione are involved in this effect as radical scavengers. Oxidative stress is known to be induced by many factors including physical exercise, psychological stress, and radiation. Organs have antioxidative functions to mitigate oxidative stress, and radon therapy activates them. This phenomenon is called radio-adaptive response and has been used for

therapy (Otsuka et al. 2006; Yamaoka et al. 1991). According to the previous studies, it could be induced by low dose irradiation at doses of approximately 0.01 to 0.5 Gy (Tapio S et al. 2007). Using this phenomenon, radon has been traditionally applied for treatment against oxidative stress-related diseases such as rheumatic diseases (Falkenbach et al. 2005; Franke et al. 2000), osteoarthritis (Yamaoka et al. 2004), and bronchial asthma (Mitsunobu et al. 2003) in some regions including Badgastein (Austria) and Misasa (Japan). The evidence and mechanism of this therapy remained unclear for a long time. However, ROS related pathways in cells were recently examined using laboratory animals. SOD can be synthesized via nuclear factor-kappa B (NF-κB)-related pathways. A previous study reported the possibility that radon inhalation activates this pathway, thus increasing SOD activity (Kataoka et al. 2017).

Radon has a radioactive isotope, ^{220}Rn, called thoron. Because of its short half-life (55.6 sec), the influence of thoron on the human body has been underestimated. However, after detecting high-thoron background regions, researchers have focused on the effects of inhaled thoron (Ramola et al. 2012). Menon et al. (2014) examined changes in antioxidative functions including expression of SOD and reduced glutathione (GSH) in mouse organs by thoron inhalation at a high concentration of approximately 500 kBq/m^3 within 30 days of inhalation. The results showed a significant increase in DNA damage of lung tissue, and significant decrease in some antioxidants. In contrast, according to the data from a clinical trial on thoron and thermal therapy, mitigation effects on oxidative stress in humans were observed. The therapy was performed at a concentration of 5 kBq/m^3 for 40 min per day, every 2 days for 3 weeks (Kataoka et al. 2006). From the above results, we hypothesized that if the exposure conditions including concentration and inhalation times are adjusted, thoron inhalation also has mitigation effects against oxidative stress. It can be considered that due to the significant difference of physical half-lives of thoron (55.6 sec) and radon (3.8 days), the distribution of radon, thoron and their progenies in the body was different following radon and thoron inhalation even under the same conditions of concentration and inhalation time. This might influence absorbed doses in organs, and consequently - radical production.

In this study, we exposed mice to radon and thoron using a small thoron inhalation system as well as our ready-made radon inhalation system (Ishimori et al. 2010) and compared the changes in antioxidants and ROS-related biomarkers. In addition, we varied the inhalation conditions by varying the concentrations and inhalation times for each nuclide and aimed to identify the optimal conditions for antioxidative effects.

Our results provide not only new insights into the influence of internal exposure of low-dose irradiation by radon and thoron but is also relevant to thoron therapy which has been conventionally performed in some regions without any scientific evidence.

Materials and Methods

Thoron inhalation system

The thoron source was provided by SanteCrear CO., LTD (Aichi, Japan) and included natural minerals rich in thorium-series nuclides. Before the animal experiment, we empirically confirmed that the radon emanation from this source was negligibly small compared to the thoron emanation.

A small thoron inhalation system was assembled and composed as shown in Fig. 1. Thoron source containers, air pumps (MPΣ30, SIBATA, Japan), Lucas scintillation cells (300A, PYLON, Canada), counting assemblies (AB-5, PYLON, Canada), and breeding cages were properly connected by tubes. Indoor air and thoron gas were mixed in thoron source containers and were continuously flowed into the scintillation cells and breeding cages through air pumps at a flow rate of 2 L/min. To eliminate the influence of thoron progenies, breeding cages and scintillation cells were equipped with HEPA filters and membrane filters, respectively, and glass filters were placed on the inlet side. The analysis of thoron concentration was calculated based on previous studies (Sakoda et al. 2015, 2016). Thoron has a short half-life, as mentioned above, and its decay correction in the system was performed accordingly.

Radon gas inhalation system

Radon inhalation was carried out by the radon exposure system previously developed by our group and was composed as shown in Fig. 2. Because the source of radon is natural soil, thoron gas was also released into the atmosphere. To remove coexisting thoron in radon atmosphere, a decay chamber (0.2 m^3) was installed. The radon concentration in the breeding cages was measured by radon monitors (AlphaGUARD PQ200 PRO, SAPHYMO, Germany). To eliminate the influence of radon progenies, breeding cages and scintillation cells were equipped with HEPA filters and membrane filters, respectively, and glass filters were placed on the inlet side (Ishimori et al. 2010).

Animals

Male BALB/c mice (8 weeks of age, body weight 25–30 g) were purchased from CLEA Japan Inc. (Tokyo, Japan). The mice were housed in plastic cages under controlled conditions of temperature (average 21.6°), humidity (average 79.0%), and light (12 h light, 12 h dark) and were fed food and water ad libitum. Ethics approval for all protocols and experiments was obtained from the Animal Experimental Committee of Okayama University.

Radon and thoron inhalation

Mice were randomly divided into 15 groups (n = 6 for each group): (1) sham inhalation only (Control), radon inhalation at a doses of (2) 500 Bq/m^3 (Rn500) or (3) 2000 Bq/m^3 (Rn2000), and thoron inhalation at a doses of (4) 500 Bq/m^3 (Tn500) or (5) 2000 Bq/m^3 (Tn2000). The mice were exposed to air only, radon, or thoron for 1, 2, or 4 days and were given free access to food and water during the inhalation. Immediately after inhalation, mice were euthanized by excessive carbon dioxide inhalation, and blood was drawn from the heart for serum analysis. The brain, lung, liver, pancreas, and kidney were quickly excised for use in biochemical assays.

Biochemical assay

SOD activity was assayed by the nitroblue tetrazolium (NBT) reduction method using the Wako-SOD test (Wako Pure Chemical Industry, Co., Ltd., Osaka, Japan), according to the manufacturer's recommendations (Baehner et al. 1975). The brain, lung, liver, pancreas, and kidney were homogenized on ice in 10-mM phosphate buffer (PBS; pH 7.4). Briefly, the homogenates were centrifuged at 12,000×g for 45 min at 4°C, and supernatants were used to assay SOD activity. 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.

Lipid peroxide (LPO) levels were measured using the Bioxytech LPO-586TM assay kit (OXIS Health Products, Inc., OR, USA) according to the manufacture's recommendations. Briefly, samples were homogenized on ice in 10-mM phosphate buffered saline (PBS; pH 7.4). Prior to homogenization, 10 μL of 0.5-M butylated hydroxytoluene in acetonitrile was added per 1 mL buffer-tissue mixture. The homogenate was centrifuged at 15,000×g for 10 min at 4°, and the supernatant was then used for assay. This assay is based on the reaction between a chromogenic reagent, N-methyl-2-phenylindole, and malondialdehyde and 4-hydroxyalkenals at 45°. The data are derived from the optical density of colored

products at 586 nm.

The total glutathione (t-GSH) content was measured using the Bioxytech GSH-420 assay kit (OXIS Health Products, Inc., Portland, OR, USA). Briefly, tissue samples were suspended in 10-mM 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. 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 t-GSH concentration.

The protein content in each sample measured by the Bradford method (Bradford 1976) using the Protein Quantification Kit-Rapid (Dojindo Molecular Technologies, Inc., Kumamoto, Japan).

Statistical analyses

The data are presented as the mean ± standard deviations (SD). Each experimental group consisted of samples of six animals. The one-way ANOVA test was used to evaluate the dose dependency of biological effects. Dunnet's test was used for multiple comparisons. P values were considered significant at $P < 0.05$.

Results

Measurement of radon and thoron in the inhalation systems

Fig. 3 shows time-dependent changes in radon and thoron concentrations in breeding cages during the experiment. The mean concentrations were 501 Bq/m^3 in the Rn500 group, 1951 Bq/m^3 in the Rn2000 group, 599 Bq/m^3 in the Tn500 group, and 2045 Bq/m^3 in the Tn2000 group. The relative standard deviation was approximately 10% in the Rn500 and Rn2000 groups, approximately 20% in the Tn500 group, and approximately 5% in the Tn2000 group.

Fig. 4 shows the comparison between measurements and calculated alpha-ray counts by radon, thoron, and their progenies in scintillation cells after the gas flow was shut off. This data indicates that there was no contamination of thoron atmosphere by radon gas.

Changes in SOD activities in mouse organs following radon and thoron inhalation

SOD is a catalytic substance that catalyzes superoxide anions (O_2-.) generated in organs to form hydrogen peroxide (H_2O_2) and oxygen (O_2). Our group has already shown the possibility that radon inhalation can increase SOD activity at therapeutic concentrations (Kataoka et al. 2014; Nishiyama et al. 2012, 2016).

Therefore, by adjusting the inhalation conditions we tested the hypothesis that thoron also has similar effects on antioxidative functions. We assayed the SOD activity in mouse organs following thoron inhalation and explored the optimal therapeutic conditions. Results showed significant increases in SOD activity in mouse brain ($P < 0.05$), pancreas ($P < 0.05$), and kidney ($P < 0.05$) compared with control mice under specific conditions of 500 or 2000 Bq/m^3 within 2 days of inhalation. In contrast, SOD activity in mouse organs in radon inhalation groups was not significantly altered compared with control mice (Fig. 5).

Changes in t-GSH content in mouse organs following radon and thoron inhalation

Following disproportionation by SOD, hydrogen peroxide remains to produce hydroxyl radical (OH-.), the most reactive free radical. Glutathione peroxidase (GPx) plays an important role in the removal of hydroxyl radical by t-GSH. Therefore, we next evaluated changes in t-GSH contents in mouse organs. Results showed a significant increase in t-GSH contents in the livers of mice from the Tn500 ($P < 0.01$) and Tn2000 ($P < 0.01$) groups. T-GSH contents in Tn2000 mice also showed an increasing trend, although no significant difference was observed. These trends were observed within 1 day of inhalation. In contrast, t-GSH contents in the liver ($P<0.01$) and plasma ($P<0.05$) were significantly decreased after 2 or 4 days of thoron inhalation. These dose- and inhalation time-dependent trends were not significantly different between radon and thoron (Fig. 6).

Changes in LPO levels in mouse organs following radon and thoron inhalation

LPO is generated by the reaction between lipid and ROS and is used as a biomarker of oxidative stress. In contrast, LPO could be directly removed by t-GSH. To evaluate the mitigation effects by thoron inhalation against oxidative stress, we assayed LPO levels in mouse organs. Results showed a significant decrease of LPO level in mouse liver ($P < 0.01$), pancreas ($P < 0.05$), and kidney ($P < 0.01$) compared with control mice under specific conditions, i.e., in Tn500 or Tn2000 mice within 2 days of inhalation. LPO levels in Tn500 mouse brain also showed a decreasing trend, although there was no significant difference. These time-dependent changes in LPO levels were similar to those caused by radon inhalation. In contrast, under specific conditions, significant increases in LPO levels were observed, i.e., in Tn2000 mouse livers (1 day, $P < 0.01$) and Tn500 mouse pancreas (4 days, $P < 0.05$) (Fig. 7).

Discussion

As described above, multiple conditions for each inhalation group could be regulated by new- and previously developed systems. Coexistence of radon and thoron might not influence the biological effects because the concentrations were at background level. In some conditions and organs, significant increases in antioxidant (SOD and t-GSH) levels and significant decreases in indicators of oxidative stress (LPO) were shown after thoron inhalation. And these effects were mainly observed within 1-2 days of inhalation. In contrast, significant decreases in t-GSH content in the liver, pancreas and plasma and a significant increase in LPO level in the liver were also shown in thoron inhalation groups. According to the previous report of radon inhalation, antioxidative effects tend to be elevated especially 1-2 days after the inhalation. They are temporarily decreased and gradually returned to the original level after several days (Kataoka et al. 2011). These trends were also observed in the results of this study. Additionally, dose- and time-dependent changes of these indicators were different between radon and thoron inhalation.

These findings suggest that thoron may have antioxidative effects on organs and tissues similar to those observed for radon inhalation. However, these effects were induced by different exposure conditions between radon and thoron. From the perspective of intracellular pathways, it has been reported that radon and its progenies exert oxidative stress on cells and may activate pathways related to one of the nuclear transcription factors, NF-κB. These pathways can be activated by intracellular ROS generation and DNA damage and are thought to contribute to the induction of Mn-SOD (Kataoka et al. 2017). Thoron inhalation is also a factor that exerts oxidative stress to cells and activation of antioxidative function may occur via the same pathways under radon inhalation.

There are some possibilities regarding how these gaps in changing characteristics of oxidative stress and antioxidants were observed between radon and thoron. First, dose conversion factors described in the UNSCEAR report 2000 have different values between radon (6-15 nSv (Bq h m^{-3})$^{-1}$) and thoron (40 nSv (Bq h m^{-3})$^{-1}$). This gap might be induced by different biological effects on organs (United Nations 2000). In this study, Tn500 mice showed similar but insignificant changes in SOD, t-GSH, and LPO levels when compared with the levels in several organs of Rn2000 mice. Especially in the brain, the time-dependent changes in SOD and LPO levels were not comparable between Tn500 and Rn2000 mice. In contrast, the changes exhibited by the Tn2000 mice were not consistent with any other inhalation groups. It can be speculated that the inhalation conditions of the Tn2000 group induced higher oxidative stress in organs. Furthermore, the equilibrium between antioxidative effects and oxidative stress might have been

temporarily disturbed. These results suggest that similar antioxidative effects may be induced by thoron inhalation at lower concentrations than radon in specific organs. Second, there might be differences between radon and thoron inhalation in the pharmacokinetics of the progenies and in the energy provided to tissues. The effective half-life of radon has been reported to be about 30 minutes, and it is thought that unattached radon and its progeny is absorbed at a faster rate into blood than the attached radon progeny (Butterweck et al. 2002). Absorbed dose by radon inhalation has been computed by Sakoda et al. (2010). In this calculation, it was assumed that radon and the short half-life progenies simultaneously decayed, and alpha-ray energy of ^{222}Rn (5.490 MeV), ^{218}Po (6.003 MeV), and ^{214}Po (7.687 MeV) was considered. In contrast, following alpha decay of thoron, ^{216}Po rapidly decays and releases an alpha particle (6.78 MeV) with a half-life of 0.145 s to become ^{212}Pb which has a half-life of 10 hours. Subsequently, alpha particles are emitted by the decaying of ^{212}Bi (6.051 MeV) and ^{212}Po (8.784 MeV). Thus, it could be necessary to consider the pharmacokinetics of long-lived nuclides such as ^{212}Pb in thoron inhalation. From the above discussion, it is speculated that because of the higher alpha-ray energy and differences in pharmacokinetics of their progenies, thoron inhalation might provide equivalent biological effects at lower concentrations compared with radon.

Focusing on the changes in antioxidants and LPO levels in each organ, the results showed a tendency to mitigate oxidative stress, especially in the brain, liver, pancreas, and kidney. Significant changes in t-GSH were observed only in the liver, and an increase in SOD was observed in the brain, pancreas, and kidney. Although t-GSH mainly has a direct reducing effect on LPO, other antioxidants are considered to indirectly suppress the chained generation of radicals. In these organs, beneficial effects were observed mainly in the inhalation group within 2 days, and tendencies to decrease antioxidants and increase oxidative stress were observed in several organs in the 4-day inhalation groups. As shown in the previous studies on radon therapy in mice, radon inhalation is performed for approximately 24 hours at the concentration of 500 to 2000 Bq/m^3. The concentration used for actual treatment for humans is about 44 kBq/m^3 in Badgastein and about 2000 Bq/m^3 in Misasa, and inhalation is performed for about 40 minutes a day for 3 to 4 weeks on alternate days. As mentioned above, conditions for obtaining beneficial effects have been set empirically for radon therapy. Similarly, in the case of thoron inhalation, it is necessary to identify the appropriate inhalation conditions. The results of this study, in which concentration-time conditions were set in a matrix, may be helpful for appropriate thoron administration for therapy.

There were several difficulties in clarifying the mechanisms on how these antioxidative effects are induced. To investigate these mechanisms in more detail, the pharmacokinetics of each nuclide should be analyzed by measurement or calculation. However, the mechanisms may have been undetectable under our experimental conditions and oxidation stimulation to organs might have been too small to detect significant changes. This may be caused by the low-dose of radon irradiation of 0.04–1.4 nGy $(Bqm^{-3})^{-1}day^{-1}$ (Sakoda et al. 2010). Additionally, there are various factors that affect oxidative stress, including radiation dose, alcohol intake, and smoking. To separate these factors appropriately, cell-culture assays must be performed in biological experiments. In this study, because of the characteristics of the experiment for radon inhalation, it was necessary to use laboratory animals. In addition, to consider the stress derived from the living environment, we used control groups for each inhalation day.

In conclusion, this is the first report to compare the changes in antioxidant function and oxidative stress by radon and thoron inhalation at the concentration used for therapeutic purposes. Our results suggest that thoron inhalation also exerts antioxidative effects in organs. However, some organs did not show a decrease in LPO, and other antioxidants like catalase also need to be examined. In further studies, the inhibitory effect on oxidative stress-related diseases should be examined similar to radon therapy. Furthermore, we emphasize that the pharmacokinetics of each radionuclide by thoron inhalation remain unclear. Thus, it must be confirmed that organs are not exposed to unnecessary irradiation beyond the dose required for obtaining beneficial effects.

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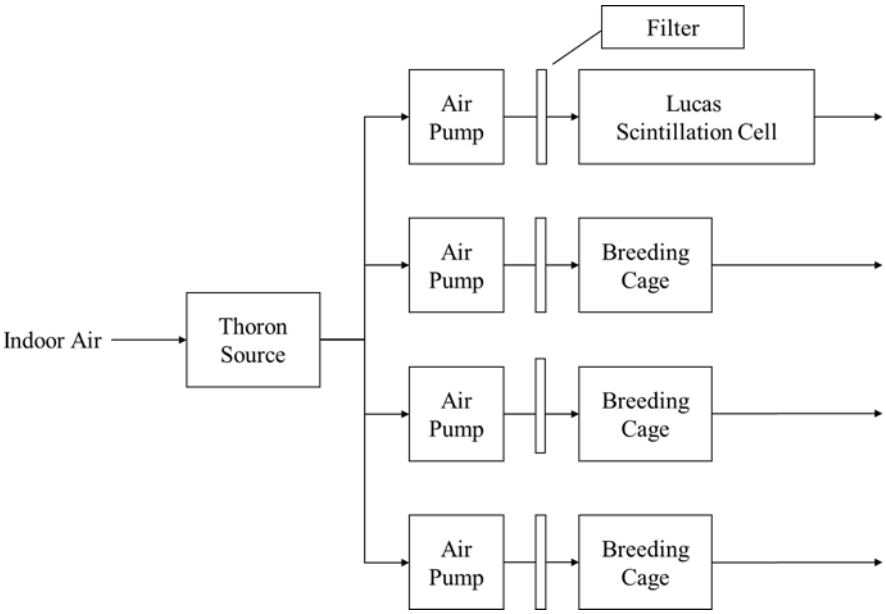

Fig. 1 The composition of the thoron gas inhalation system.

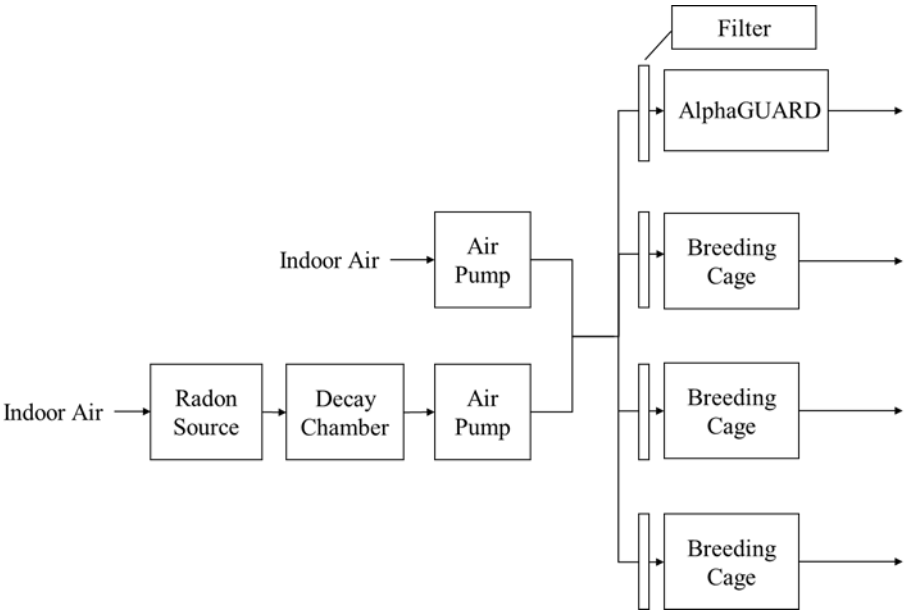

Fig. 2 The composition of the radon gas inhalation system.

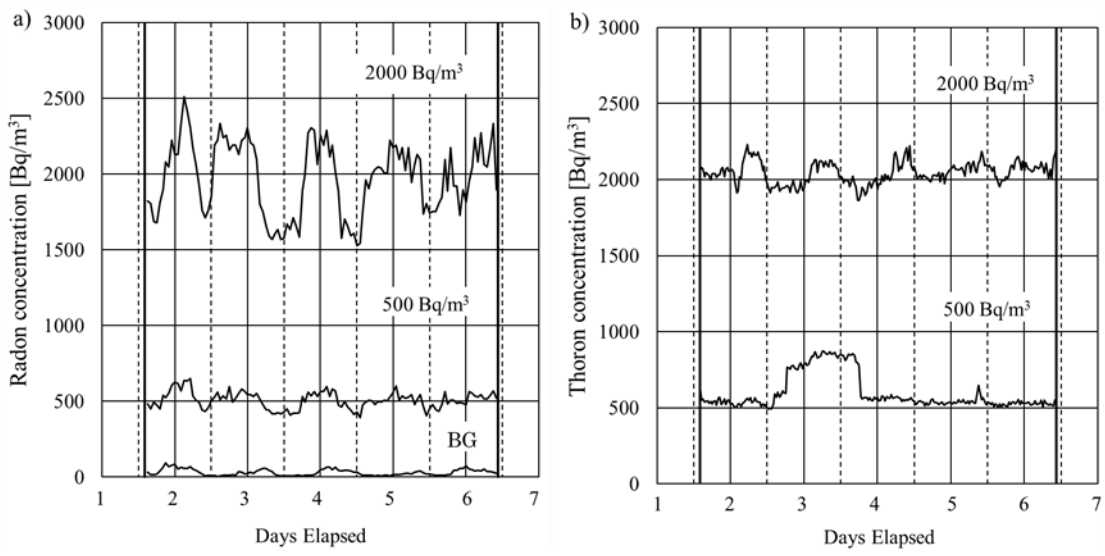

Fig. 3 Changes of radon and thoron concentrations during the experiment. (a) Radon group, (b) Thoron group.

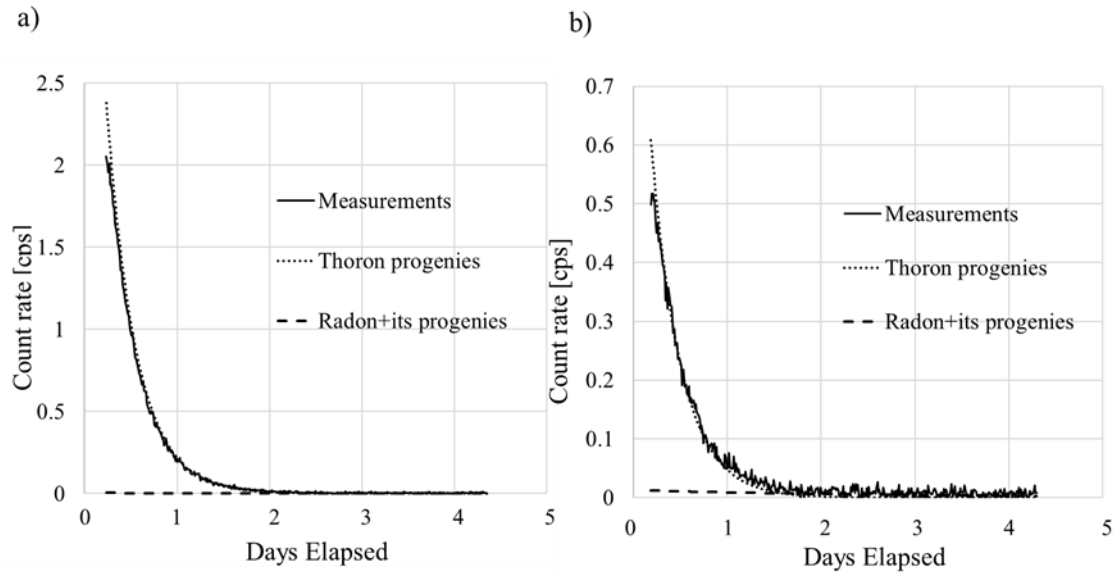

Fig. 4 Measurements and calculated alpha-ray counts from ^{220}Rn or ^{222}Rn and their progenies in scintillation cells after the gas flow was shut off. (a) Tn500 group, (b) Tn2000 group.

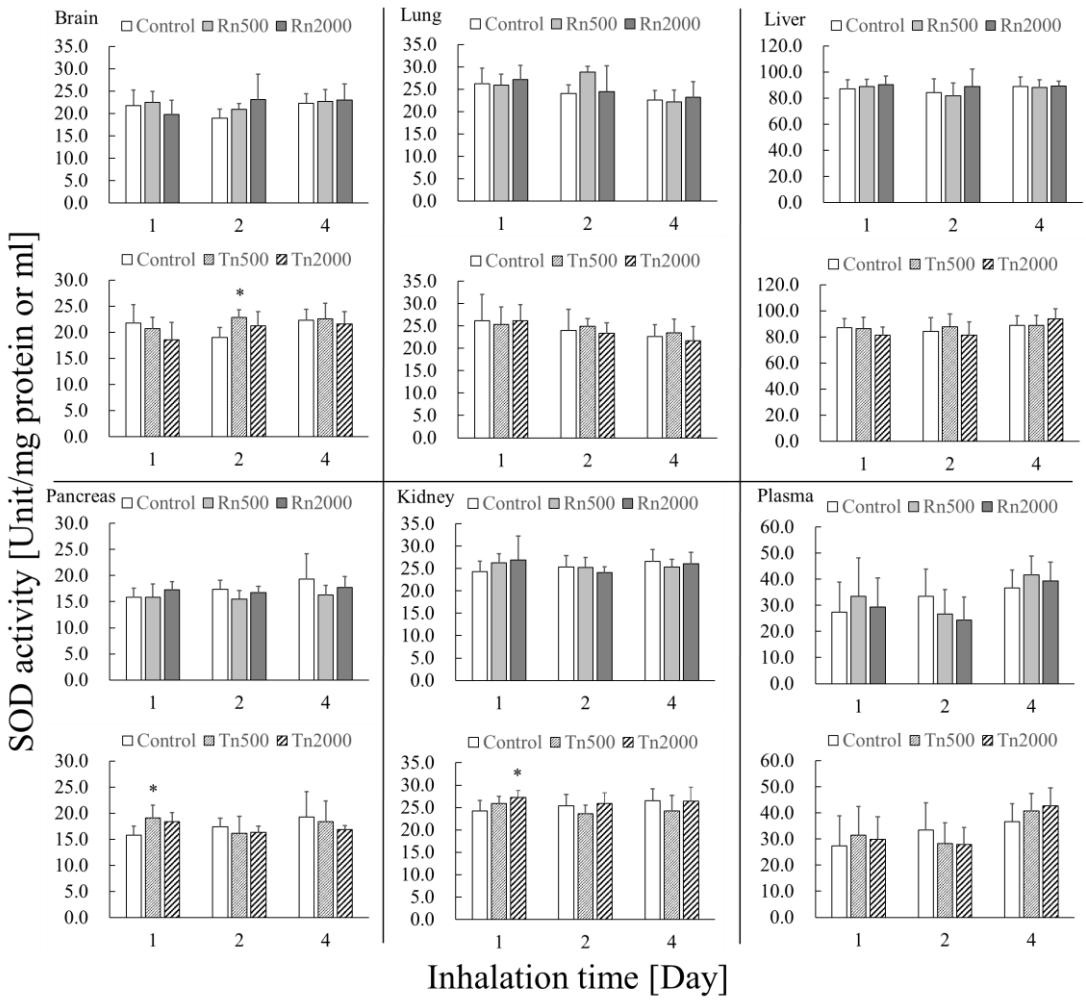

Fig. 5 Dose- and time- dependent changes in SOD activities in mouse organs following radon or thoron inhalation. Mean ±SD, N=6, *P<0.05 vs Control

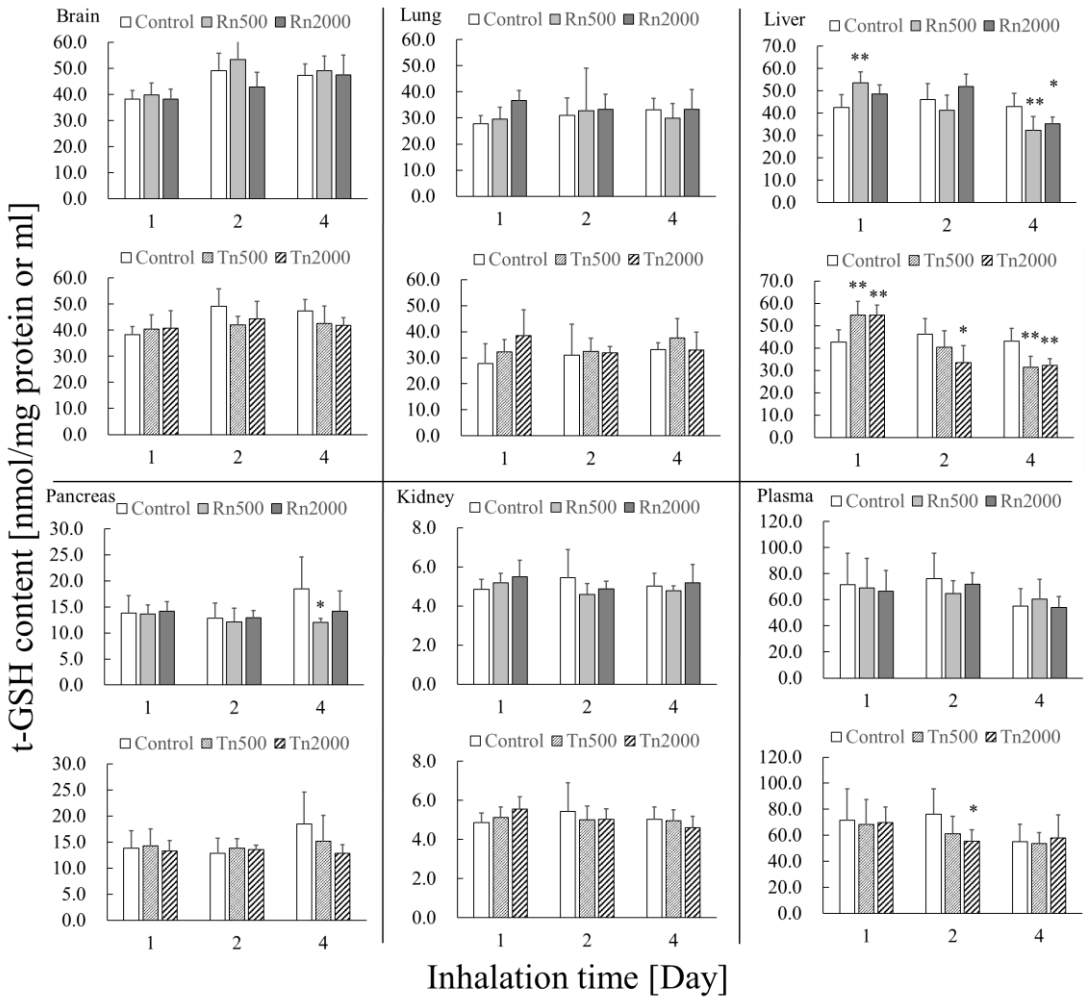

Fig. 6 Dose- and time- dependent changes in t-GSH contents in mouse organs following radon or thoron inhalation. Mean ±SD, N=6, *P<0.05, **P<0.01 vs Control

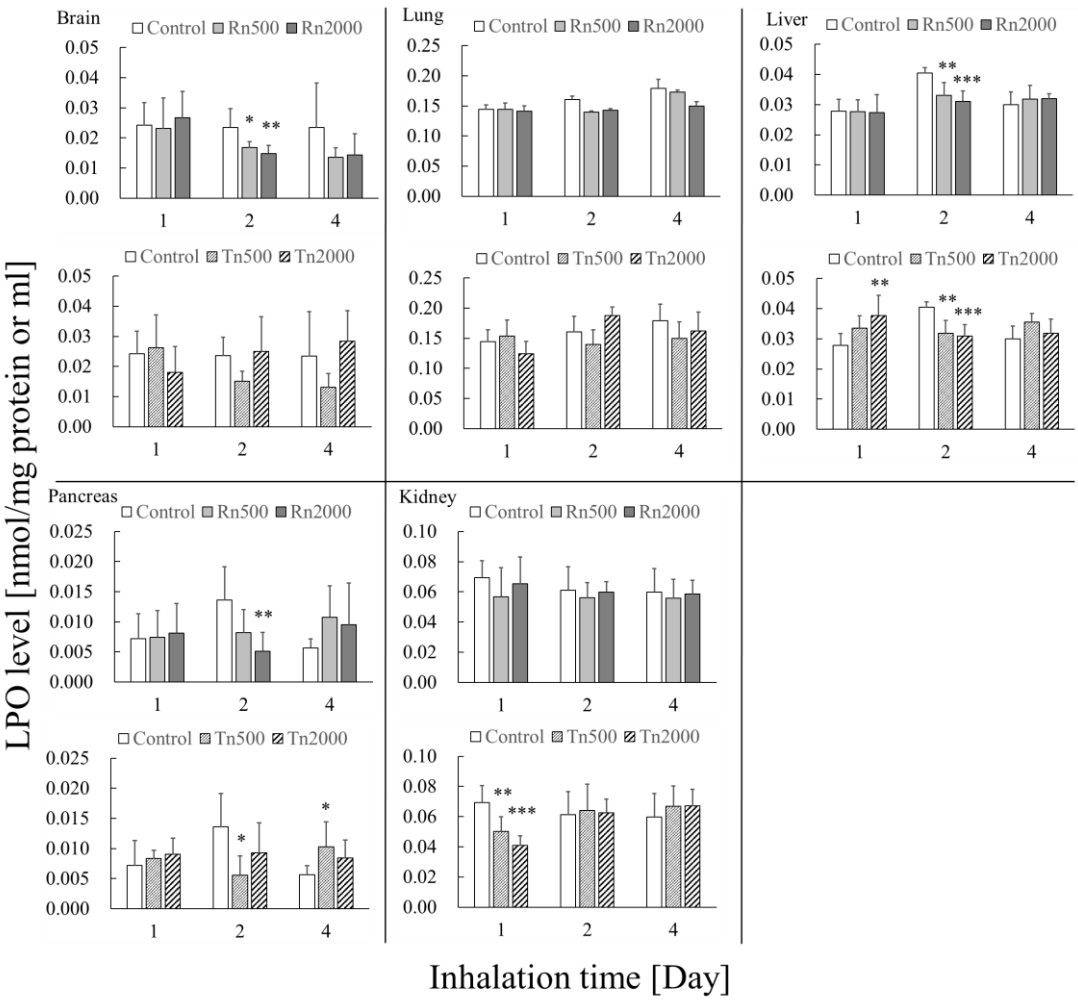

Fig. 7 Dose- and time- dependent changes in LPO levels in mouse organs following radon or thoron inhalation. Mean ±SD, N=6, *P<0.05, **P<0.01, ***P<0.001 vs Control