Brief Report

Comparative study on the inhibitory effects of α-tocopherol and radon on carbon tetrachloride-induced renal damage

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ABSTRACT

Since the 2011 nuclear accident in Fukushima, the effects of low-dose irradiation, especially internal exposure, are at the forefront of everyone’s attention. However, low dose radiation induced various stimulating effects such as activation of antioxidative and immune functions. In this study, we attempted to evaluate the quantitative effects of the activation of antioxidative activities in kidney induced by radon inhalation on carbon tetrachloride (CCl₄)-induced renal damage. Mice were subjected to intraperitoneal (i.p.) injection of CCl₄ after inhaling approximately 1000 or 2000 Bq/m³ radon for 24 h, or immediately after i.p. injection of α-tocopherol (100, 300, or 500 mg/kg bodyweight). In case of renal function, radon inhalation at a concentration of 2000 Bq/m³ has the inhibitory effects similar to α-tocopherol treatment at a dose of 300 - 500 mg/kg bodyweight. The activities of superoxide dismutase and catalase in kidneys were significantly higher in mice exposed to radon as compared to mice treated with CCl₄ alone. These findings suggest that radon inhalation has an anti-oxidative effect against CCl₄-induced renal damage similar to the anti-oxidative effects of α-tocopherol due to induction of anti-oxidative functions.

Keywords: radon; carbon tetrachloride; oxidative damage; α-tocopherol; kidney
INTRODUCTION

Carbon tetrachloride (CCl₄) is a well-established hepatotoxin [1]. A study demonstrated that liver is not the only target organ of CCl₄ and it causes free radical generation in other organs, such as the brain, heart, lung, and kidney [2]. It has also been reported that CCl₄ administration induces oxidative stress in these organs and that vitamin E (α-tocopherol), which is an antioxidant vitamin, inhibits CCl₄-induced renal damage [3].

A large number of patients are treated in various countries with traditional spa therapy (Japan [4-6], and central Europe [7]), and Misasa town is especially famous for radon hot spring in Japan. Therapy involving radon gas volatilized from radon-enriched water is performed for treating various diseases at the Misasa Medical Center, Okayama University Hospital. Most conditions treated with radon therapy are pain- or respiratory-related diseases such as arteriosclerosis, osteoarthritis [4], and bronchial asthma [5]. Recently, we demonstrated that radon inhalation inhibits CCl₄-induced liver and renal damage in mice, indicating that radon inhalation has anti-oxidative effects in liver and kidney [8]. In addition, we demonstrated that radon inhalation has anti-inflammatory effects and inhibits carrageenan-induced inflammatory paw edema [9]. Furthermore, in a search for more new indications for radon therapy, we reported the responsiveness of superoxide dismutase (SOD) in mouse organs to radon [10]. In that study, we examined the changes in SOD activity in many mouse organs including plasma, brain, lung, thymus, heart, liver, stomach, pancreas, kidney, and small intestine. The results suggest that radon inhalation increases SOD activities in most organs.

Since the 2011 nuclear accident in Fukushima, many reports have been published on the radioactive contaminations in foods and water. Therefore, the effects of low-dose irradiation, especially internal exposure, are at the forefront of everyone’s attention. In contrast, many reports suggest that low-dose irradiation induces various stimulating effects on living organs, especially the activation of biological defense system such as antioxidative and immune
functions [11-16]. However, there have been no quantitative reports on the anti-oxidative effects of low dose irradiation. Therefore, it is difficult for everyone to understand the effects of low dose irradiation.

The purpose of this study was to compare the anti-oxidative effects of radon and α-tocopherol. To assess the anti-oxidative effects of radon, we used the CCl₄-induced renal damage model. We examined the following biochemical and histological parameters to assess the effects of radon inhalation on α-tocopherol: creatinine (CRE) level, SOD activity, catalase activity, total glutathione content (t-GSH), lipid peroxide levels and kidney histology.

MATERIALS AND METHODS

Animals

Female ICR mice (age, 8 weeks; body weight approximately 28 g) were obtained from Charles River Laboratories Japan Inc. (Yokohama, Japan). Ethical approval for all protocols and experiments was obtained from the animal experimental committee of Okayama University. Mice inhaled radon at a concentration of 1000 or 2000 Bq/m³ for 24 h. The radon concentration in the mouse cage was measured using a radon monitor (CMR-510, Femto-Tech Inc., OH, USA). Mice had free access to food and water during radon inhalation and sham treatment. A total of 4 ml/kg bodyweight of CCl₄ (5% in olive oil; Wako Pure Chemical Industries, Ltd. Osaka Japan) was injected into the peritoneum of the mice immediately after radon inhalation or immediately after (i.p.) injection of DL-α-tocopherol in olive oil (100, 300, or 500 mg/kg weight; Nacalai Tesque Inc. Kyoto Japan). Twenty-four hours after CCl₄ administration, blood was drawn from the heart for serum analysis and kidneys were quickly excised to analyze the levels of SOD, catalase, t-GSH, and lipid peroxide. Serum was separated by centrifugation at 3000 × g for 5 min for assay of CRE levels. These samples were preserved at -80 °C until biochemical assay. Kidney tissue samples were fixed in 10% neutral-buffered formalin for histological
examinations.

Biochemical Assays

The CRE level in serum was measured using CRE-EN kainosu (Kainosu Co., Ltd., Tokyo, Japan) according to the manufacturer’s recommendations.

Lipid peroxide levels were assayed using the Bioxytech LPO-586™ assay kit (OXIS Health Products, Inc., Portland, OR, USA) according to the manufacturer’s recommendations. The lipid peroxide assay is based on the reaction between a chromogenic reagent, N-methyl-2-phenylidole, and malondialdehyde and 4-hydroxyalkenals at 45°C. Data were derived from the optical density of the colored products at 586 nm. Briefly, kidney samples were homogenized in 10 mM PBS (pH 7.4) on ice. Prior to homogenization, 10 µL 0.5 M butylated hydroxytoluene in acetonitrile was added per 1 mL of buffer-tissue mixture. After homogenization, the homogenate was centrifuged at 15,000 × g for 10 min at 4 °C and the supernatant was used for the assay.

Mouse kidneys were homogenized on ice in 10 mM PBS (pH 7.4). The homogenates were centrifuged at 12,000 × g for 45 min at 4 °C and the supernatants were used to assay the activity of SOD and catalase. SOD activity was measured by the nitroblue tetrazolium (NBT) reduction method [17] using the Wako-SOD test (Wako Pure Chemical Industry, Co., Ltd., Osaka, Japan) according to the manufacturer’s recommendations. Briefly, the extent of inhibition of 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 rate of hydrogen peroxide (H₂O₂) reduction at 37 °C at 240 nm wavelength [18]. The assay mixture consisted of 50 µL 1 M Tris-HCl buffer containing 5 mM ethylenediaminetetraacetic acid (pH 7.4), 900 µL 10 mM H₂O₂, 30 µL deionized water, and 20 µL kidney supernatant. Activity was calculated using a molar extinction coefficient of
7.1×10^{-3} \text{M}^{-1}\text{cm}^{-1}.

Total glutathione content was measured using the Bioxytech GSH-420™ assay kit (OXIS Health Products, Inc.) according to the manufacturer’s recommendations. This assay is based on the formation of a chromophoric thione, whose absorbance is at 420 nm and is directly proportional to the total glutathione concentration. Briefly, kidney samples were suspended in 10 mM PBS (pH 7.4), mixed with ice-cold 7.5% trichloroacetic acid solution and homogenized. The homogenates were centrifuged at 3000 × g for 10 min. The supernatants were used for the assay.

The protein content in each sample was measured by the Bradford method, using the Protein Quantification Kit-Rapid (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer’s recommendations [19].

Histological Examination

Kidney samples were fixed in 10% formalin, processed through a graded ethanol series and finally xylene, and embedded in paraffin. Six-micrometer-thick tissue sections were prepared and stained with hematoxylin-eosin (H&E). The ratio of Bowman’s capsule in kidney was calculated.

Statistical Analyses

The data values are presented as tmean ± 95% confidence intervals. Each experimental group consisted of samples from 5 to 8 animals. The statistical significance of differences was determined by Student's t-test for comparisons between the control group and CCl₄-administrated group. Dunnett's test was used for multiple comparisons.
RESULTS

Effects of Radon or α-tocopherol on Renal Function Following CCl₄ Administration

To assess the effects of radon inhalation or α-tocopherol treatment on the inhibitory effects of CCl₄-induced renal damage, the CRE levels in serum were examined.

In mice injected with CCl₄ in the absence of α-tocopherol or radon pretreatment, the CRE level in serum was significantly higher \((p<0.001)\) than in control animals. The CRE level in serum of radon-treated mice (2000 Bq/m³; \(p<0.05\)) or α-tocopherol-treated mice (300 or 500 mg/kg weight; \(p<0.05\)) was significantly lower than that of CCl₄-administrated mice. Precisely, the CRE levels in serum of radon (1000 or 2000 Bq/m³) or α-tocopherol (100, 300, or 500 mg/kg weight) decreased from 1.63 ± 0.20 to 1.36 ± 0.17, 1.20 ± 0.36, 1.31 ± 0.38, 1.20 ± 0.12, or 1.25 ± 0.06, respectively (Fig. 1).

Effects of Radon or α-Tocopherol on Oxidative Damage Following CCl₄ Administration

To assess the inhibitory effects of radon inhalation or α-tocopherol treatment on CCl₄-induced renal oxidative damage, the lipid peroxide level in kidney was examined.

In mice injected with CCl₄ in the absence of α-tocopherol or radon pretreatment, the lipid peroxide level in kidney was significantly higher \((p<0.001)\) than in control animals. However, the lipid peroxide level in the kidney of radon-inhaled mice (1000 Bq/m³) was significantly lower \((p<0.05)\) in CCl₄-administrated mice. In addition, the lipid peroxide level in the kidney of α-tocopherol (100, 300, or 500 mg/kg weight) treated mice were significantly lower \((p<0.05, p<0.01, p<0.01, \text{respectively})\) than that of CCl₄-administrated mice. Precisely, the lipid peroxide levels in kidney of radon (1000 or 2000 Bq/m³) or α-tocopherol (100, 300, or 500 mg/kg weight) decreased from 0.56 ± 0.05 to 0.47 ± 0.06, 0.49 ± 0.08, 0.45 ± 0.05, 0.42 ± 0.12, or 0.42 ± 0.07, respectively (Fig. 2).
Histological Observation in Kidney Following CCl₄ Administration

The effects of radon inhalation on the histology of kidneys subjected to CCl₄ administration were examined. CCl₄ administration resulted in dilatation of Bowman’s space and glomerular atrophy. However, α-tocopherol treatment (100, 300, or 500 mg/kg weight) significantly decreased ($p<0.05$, $p<0.05$, $p<0.01$, respectively) the dilatation of Bowman’s space and glomerular atrophy. Radon inhalation at a concentration of 2000 Bq/m³ inhibited the dilatation of Bowman’s space and glomerular atrophy, but these differences were not statistically significant. Precisely, the Bowman’s space of radon (1000 or 2000 Bq/m³) or α-tocopherol (100, 300, or 500 mg/kg weight) decreased from 0.28 ± 0.02 to 0.28 ± 0.02, 0.25 ± 0.03, 0.23 ± 0.06, 0.22 ± 0.04, or 0.20 ± 0.03, respectively (Fig. 3).

Effects of Radon or α-Tocopherol on Antioxidative Functions Following CCl₄ Administration

To assess the protective effects of radon inhalation or α-tocopherol treatment on CCl₄-induced renal damage, various parameters of oxidative damage were assayed in kidney. In mice injected with CCl₄ in the absence of α-tocopherol or radon pretreatment, the activities of SOD and catalase in kidney were significantly lower ($p<0.01$ or $p<0.001$, respectively) than in control animals. However, the SOD activities in the kidney of radon-inhaled mice (2000 Bq/m³) and catalase activity in the kidney of radon-inhaled mice (1000 or 2000 Bq/m³) were significantly higher in CCl₄-administrated mice. In addition, pre-treatment with α-tocopherol did not result in an increase in SOD or catalase in kidney. Moreover, there were no significant differences in the t-GSH content in kidneys among all groups (Fig. 4).

DISCUSSION

Radon is a radioactive gaseous element that mainly emits α-rays and is colourless,
tasteless and odourless gas. Therefore, mice are exposed to radon without any stress. In addition, it is easy to estimate the absorbed doses in all organs [20].

A report suggested that \( \alpha \)-tocopherol administration inhibits \( \text{CCl}_4 \)-induced renal damage [3]. In contrast, we previously demonstrated that radon inhalation inhibits \( \text{CCl}_4 \)-induced renal damage [8]. Generally, it is assumed that radiation is harmful to humans. However, we have reported that low-dose irradiation induced various stimulating effects such as activation of antioxidative functions [21-28]. In this study, we attempt to compare these inhibitory effects of \( \text{CCl}_4 \)-induced renal damage since it is difficult for everyone to understand these radioadaptive responses that we have already demonstrated [21-28]. After the 2011 nuclear accident in Fukushima, the effects of low-dose irradiation are at the forefront of everyone's attention. Therefore, we conducted this research to give information to the public about the effects of low dose irradiation.

The results of this study show that \( \text{CCl}_4 \) administration significantly increases the CRE levels in serum. These findings indicate that \( \text{CCl}_4 \) administration depresses renal function. However, radon inhalation at a concentration of 2000 Bq/m\(^3\) and \( \alpha \)-tocopherol administration at a dose of 300 and 500 mg/kg weight significantly decreased the CRE levels in serum. These findings suggested that radon inhalation and \( \alpha \)-tocopherol administration inhibit \( \text{CCl}_4 \)-induced renal damage. Furthermore, this inhibitory effect tended to depend on the dosage of radon or \( \alpha \)-tocopherol. In case of renal function, radon inhalation at a concentration of 2000 Bq/m\(^3\) has the inhibitory effects similar to \( \alpha \)-tocopherol treatment at a dose of 300 - 500 mg/kg bodyweight.

Our results showed that \( \text{CCl}_4 \) administration significantly increases the lipid peroxide levels in kidney. These findings indicate that \( \text{CCl}_4 \) administration induced oxidative damage in kidney. However, radon inhalation at a concentration of 1000 Bq/m\(^3\) and \( \alpha \)-tocopherol administration at a dose of 100, 300, and 500 mg/kg weight significantly decreased the lipid peroxide levels in
kidney. This inhibitory effect did not depend on the dosage of radon or α-tocopherol unlike the CRE levels. In addition, the protective effect of α-tocopherol on CCl₄-induced renal damage was larger than that of radon.

It has been reported that CCl₄ administration induced mild dilatation of Bowman’s space with glomerular atrophy [29]. In addition, we previously reported that radon inhalation inhibited the dilatation of Bowman’s space and glomerular atrophy. In the present study, radon inhalation at a concentration of 2000 Bq/m³ slightly inhibited the dilatation of Bowman’s space. However, our results showed that the inhibitory effects of α-tocopherol are larger than that of radon inhalation.

It is well known that free radicals are one of the major causes of CCl₄-induced renal damage [2, 30-31]. To clarify the mechanisms underlying the differences between radon and α-tocopherol, we examined antioxidant-associated substances such as SOD, catalase, and t-GSH. Results showed that CCl₄ administration significantly decreases the activities of SOD and catalase in kidney. These findings indicate that CCl₄ administration depresses antioxidative function. However, the activities of SOD (2000 Bq/m³) and catalase (1000 or 2000 Bq/m³) in kidney of radon inhaled mice were significantly higher than that of CCl₄ treated mice. These findings indicate that radon inhalation activated antioxidative functions. In contrast, there were no significant differences in the activities of SOD and catalase in kidneys between CCl₄ administrated group and α-tocopherol treated groups. These findings suggest that activation of antioxidative function induced by radon inhalation has the same effects of α-tocopherol administration.

In conclusion, radon inhalation has an anti-oxidative effect against α-tocopherol that is comparable to treatment with α-tocopherol at a dose of 300 - 500 mg/kg weight, due to activation of anti-oxidative functions. In case of lipid peroxidation and tissue damage in kidney, radon inhalation was less effective than α-tocopherol administration. However, our data suggest that radon inhalation has a similar effect to α-tocopherol against α-tocopherol.
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Figure Legends

Figure 1 Effects of radon (A) and α-tocopherol (B) on renal function-associated parameters in the serum of CCl₄ administrated mice. Each value indicates the mean ± 95% confidence intervals. The number of mice per experimental point is six to eight. *p < 0.05 vs CCl₄, ###p < 0.01 vs Control.

Figure 2 Effects of radon (A) and α-tocopherol (B) on oxidative damage-associated parameters in the serum of CCl₄ administrated mice. Each value indicates the mean ± 95% confidence intervals. The number of mice per experimental point is five to seven. * p < 0.05, ** p < 0.01 versus CCl₄, ### p < 0.001 versus Control.

Figure 3 Effect of radon (H) and α-tocopherol (I) on CCl₄-induced renal damage in mouse: (A) control, (B) CCl₄, (C) α-tocopherol 100 mg/kg + CCl₄, (D) α-tocopherol 300 mg/kg + CCl₄, (E) α-tocopherol 500 mg/kg + CCl₄, (F) radon 1000 Bq/m³ + CCl₄, (G) radon 2000 Bq/m³ + CCl₄. Mouse kidneys were examined histologically. The length of the scale bar is 100 μm. All samples were stained with H&E. The arrow indicates dilatation of Bowman’s space with glomerular atrophy. Each value indicates the mean 95% confidence intervals. * p < 0.05, ** p < 0.01 versus CCl₄, ### p < 0.001 versus Control.

Figure 4 Effects of radon and α-tocopherol on antioxidative-associated parameters in the serum of CCl₄ administrated mice. Each value indicates the mean ± 95% confidence intervals. The number of mice per experimental point is six to eight. * p < 0.05, ** p < 0.01 vs CCl₄, ### p < 0.001 versus Control.
Fig. 1
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