

Original Article

**Radon Inhalations Protects Mice from Carbon-Tetrachloride-induced Hepatic and Renal
Damage.**

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Running title: Radon inhalation protects CCl₄-induced damage

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Abstract

Keywords: radon inhalation; ascorbic acid; carbon tetrachloride; antioxidative function; liver

INTRODUCTION

Carbon tetrachloride (CCl_4) is a well-established hepatotoxin, and metabolism of CCl_4 via the cytochrome P450 system produces free radicals, including trichloromethyl radical and trichloromethyl peroxy radical [1,2]. Overproduction of these radicals initiates lipid peroxidation of polyunsaturated fatty acid in membrane and eventually leads to cell necrosis. These free radicals also react with antioxidants such as glutathione [3] and cause the depletion of glutathione in liver [4]. Additionally, CCl_4 causes free toxic radical generation in kidneys [5,6]. Various studies demonstrate that some antioxidants protect the liver and kidney from the oxidative damage induced by CCl_4 [6-8].

Low-dose irradiation induces various stimulating effects on living organs, especially the activation of biological defense system such as antioxidative [9-13] and immune functions [14,15]. For example, low-dose irradiation increases endogenous antioxidants in animal tissue. It has been reported that antioxidants such as superoxide dismutase (SOD) [11,16], glutathione peroxidase (GPx) [16], glutathione reductase (GR) [10], glutathione [10,16], catalase [16], and thioredoxin [16] are activated and/or induced by low-dose irradiation. Low-dose X- or γ -irradiation activate antioxidative functions in some organs and inhibit oxidative injury [17-22]. We have reported that pretreatment with low-dose X-irradiation inhibited CCl_4 -induced hepatopathy in mice with a catalase deficiency (acatalasemic mice). However, there were no significant differences in CCl_4 -induced hepatopathy between normal mice pretreated with low-dose radiation and acatalasemic mice pretreated with low-dose radiation because GPx activity in the liver of acatalasemic mice is significantly higher than that of normal mice. These findings indicate that the free radical accumulation induced by the lack of catalase and the administration of CCl_4 is more completely neutralized by high GPx activity and low-dose irradiation in acatalasemic mice [17]. We also reported that the effects of post-treatment with low-dose (0.5 Gy) X-irradiation reduced the oxidative damage

associated with CCl₄-induced hepatopathy in acatalasemic mice. These findings suggest that low-dose irradiation after CCl₄ administration accelerates the rate of recovery and that catalase plays an important role in the recovery from hepatopathy induced by CCl₄ [18]. Moreover, we reported that low-dose X-irradiation inhibited ischemia-reperfusion injury in mouse paw [20] and that low-dose irradiation of the extirpated mouse liver (not whole body) increased the activity of SOD and catalase [9]. It is highly possible that low-dose X-irradiation activates the defensive systems in the living body and, therefore, contributes to preventing or reducing reactive oxygen species (ROS)-related injuries, which are thought to involve peroxidation.

Therapy involving radon gas volatilized from radon-enriched water is performed for various diseases at Misasa Medical Center, Okayama University Hospital. Most conditions treated with radon therapy are lifestyle-related diseases such as arteriosclerosis, osteoarthritis [23], and bronchial asthma [24]. To assess the effects of radon, we have co-developed a radon exposure system (OZ PLAN Co., Ltd. Okayama, Japan); radon inhalation using this system activated antioxidative functions in the liver, kidney, lung, and brain of mice, including suggesting the possibility of a new therapy to treat liver, kidney, lung, and brain damage [25]. Although hepatic and renal damage are not the main indication for radon therapy, radon inhalation therapy may mitigate liver and kidney damage. However, there have been no reports of the protective effect of radon inhalation on liver and kidney damage.

The purpose of this study was to assess the effects of radon inhalation on CCl₄-induced hepatic and renal damage. We examined the following biochemical and histological parameters to assess the effects of radon treatment on antioxidative functions: total glutathione content (t-GSH), GPx and GR activity, and lipid peroxide levels in livers and kidneys; glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and alkaline phosphatase (ALP) activity, and creatinine (CRE) and total cholesterol (T-CHO) in

serum; triglyceride level (TG) in liver; and histological examination of liver and kidney tissue.

MATERIALS AND METHODS

Conditions of radon inhalation

The radon exposure system is shown in Fig. 1. To generate conditions for inhalation of radon concentration, we used a radon source that is in a tank (Radon Medical Treatment Research and Development Mechanism Ltd., Okayama, Japan). Air with radon was blown into the box from the tank (Fig. 1) at a rate of 0.2 L/min and blown out the box at a rate of 0.2 L/min. Mice had free access to food and water during radon inhalation and the sham treatment. The radon concentration in the box was measured using a radon monitor (PQ2000, Genitron Instruments, Frankfurt, Germany). The mean concentration of the background and the radon-treatment concentration were approximately 20 Bq/m³ and 18 kBq/m³, respectively.

Animals

Male BALB/c mice (age, 7 weeks; body weight, approximately 25 g), which are sensitive to radiation compared with other strains, were obtained from the Department of Animal Resources Advanced Science Research Center Okayama University. Ethical approval was obtained from the animal experimental committee of Okayama University. Mice inhaled radon at a concentration of 18 kBq/m³ for 6 h. 4 ml/kg of CCl₄ (5% in olive oil) was injected into the peritoneum of the mice immediately after radon inhalation. Control and CCl₄-injected mice were treated with sham radon inhalation. Twenty-four hours after CCl₄ administration, blood was drawn from the heart for serum analysis under ether anesthesia, and livers and kidneys were quickly excised to analyze t-GSH content, lipid peroxide levels, and GPx and GR activity. Serum was separated by centrifugation at 3,000 × g for 5 min for the assays of

GOT, GPT, and ALP activity and the levels of T-CHO and CRE. These samples were preserved at -80 °C until biochemical assay. Liver and kidney tissue samples were fixed in 10% neutral-buffered formalin for histological examinations.

Biochemical assays

Total glutathione content was measured using the Bioxytech GSH-420TM assay kit (OXIS Health Products, Inc., Portland, OR, USA). Briefly, tissue samples from liver or kidney were suspended in 10 mM phosphate buffer (pH 7.4), mixed with ice-cold 7.5% trichloroacetic acid solution, and homogenized. The homogenates were centrifuged at $3,000 \times g$ for 10 min. The supernatants were used for the assay. Total glutathione 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 total glutathione concentration. The protein content was measured by the Bradford method, using Protein Quantification Kit-Rapid (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) [26].

GPx activity was measured by using the BIOXYTECH GPx-340TM assay kit (OXIS Health Products, Inc.). Briefly, liver or kidney samples were homogenized in 1 M Tris-HCl buffer (pH 7.4) containing 5 mM ethylenediaminetetraacetic acid and 1 mM dithiothreitol. The homogenates were centrifuged at $10,000 \times g$ for 20 min at 4 °C. The supernatants were used for the assay. The reduction of nicotinamide adenine dinucleotide phosphate (NADPH) to nicotinamide adenine dinucleotide phosphate (NADP⁺) is accompanied by a decrease in absorbance, measured at 340 nm, providing a spectrophotometric means for monitoring GPx enzyme activity. The molar extinction coefficient for NADPH is $6220 \text{ M}^{-1} \text{ cm}^{-1}$ at 340 nm. To assay GPx, the supernatant is added to a solution containing glutathione, GR, and NADPH. The enzyme reaction is initiated by adding a substrate, tert-butyl hydroperoxide, and absorbance at 340 nm is recorded for 3 min.

GR activity was assayed using the Bioxytech GR-340TM assay kit (OXIS Health Products, Inc.). Briefly, liver or kidney samples were homogenized in 1 M Tris-HCl buffer with 5 mM ethylenediaminetetraacetic acid (pH 7.4) on ice. The homogenates were centrifuged at $8,500 \times g$ for 10 min. The supernatants were used for the assay. The assay is based on the oxidation of NADPH to NADP⁺ catalyzed by a limiting concentration of GR. One GR activity unit is defined as the amount of enzyme that catalyzes the reduction of 1 μmol of oxidized glutathione (GSSG) per minute at pH 7.4 and 25 °C. The reduction of GSSG is determined indirectly by the measurement of the consumption of NADPH, as demonstrated by a decrease in absorbance at 340 nm as a function of time.

Lipid peroxide (malondialdehyde (MDA)) levels were assayed using the Bioxytech LPO-586TM assay kit (OXIS Health Products, Inc.). Briefly, liver or kidney samples were homogenized in 20 mM phosphate buffer (PBS; pH 7.4) on ice. Prior to homogenization, 10 μL of 0.5 M butylated hydroxytoluene in acetonitrile were added per 1 mL of the buffer-tissue mixture. After homogenization, the homogenate was centrifuged at $15,000 \times g$, for 10 min at 4 °C, 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 °C. The optical density of the colored products was read at 586 nm in a spectrophotometer.

The serum activity of GOT, GPT, or ALP; the serum level of T-CHO and CRE; and the level of TG in liver were measured using TA-LN kinosu, TA-LN kinosu, ALP kinosu, T-CHO kinosu, TG-EN kinosu, and CRE-EN kinosu, respectively (Kainosu Co., Ltd., Tokyo, Japan). Briefly, blood was collected from the heart under ether anesthesia and serum was obtained by centrifugation at $3,000 \times g$ for 5 min under 4 °C for the analyses of the activities of GOT, GPT, and ALP, and the levels of T-CHO and CRE. Liver samples were suspended in 10 mM phosphate buffer (pH 7.4) and homogenized for the analyses of TG level in liver. Whole homogenate was used for the assay. GOT and GPT activity and the level of TG were

measured at 550 nm, ALP activity was measured at 498 nm, and the CRE level was measured at 515 nm using a spectrophotometer.

Histological examination

Liver and kidney samples were fixed in 10% formalin, processed with a graded mixture of ethanol and xylene and embedded in paraffin, and embedded in paraffin. Six-micrometer-thick tissue sections were prepared and stained with hematoxylin-eosin (HE).

Statistical analyses

The data values are presented as the mean \pm 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.

RESULTS

Effects of radon inhalation on hepatic or renal function

The differences of hepatic or renal functions following radon inhalation for 6 h were examined. No significant differences were observed in GOT, GPT, or ALP activity and the level of T-CHO and CRE in serum between mice exposed to radon and control mice (Fig. 2A, C); similarly, the TG level in liver was not significantly different (Fig. 2B).

Effects of radon inhalation on antioxidative function in liver and kidney

The antioxidative function of liver and kidney in mice exposed to radon inhalation for 6 h were examined. The t-GSH content and the GPx and GR activity in liver were significantly higher, and the lipid peroxide level in liver significantly was significantly lower (Fig. 3A) in mice exposed to radon as compared to control mice. The t-GSH content and the activity of

GPx and GR in kidney were significantly higher, and the lipid peroxide level was significantly lower in the mice exposed to radon inhalation for 6 h (Fig. 3B).

Effects of radon inhalation on hepatic or renal function following CCl₄ administration

The effects of CCl₄ administration on hepatic or renal function of mice pretreated with radon were examined. In mice injected with CCl₄ in the absence of radon pretreatment, the activities of GOT, GPT, and ALP in serum were significantly higher and the T-CHO level in serum was significantly lower than in control animals (Fig. 4A, Table 1). The TG level in liver was significantly higher in the CCl₄ group when compared to the control animals (Fig. 4B). However, the activities of GOT and ALP in serum and TG level in liver of CCl₄ administrated only group were significantly higher than those of the radon inhalation pretreatment group (Fig. 4A, B, Table 1). The T-CHO level in serum of CCl₄ administrated group was significantly lower than that of radon inhalation pretreatment group (Fig. 4A). In addition, CRE level in serum of CCl₄ administrated group were significantly higher than those of radon inhaled group before CCl₄ administration (Fig. 4C).

Effects of radon inhalation on oxidative damage levels in mice liver and kidney following CCl₄ administration

To determine the protective effects of CCl₄-induced hepatic and renal damage, various parameters of oxidative damage were assayed in livers and kidneys following radon inhalation. In mice injected with CCl₄ in the absence of radon pretreatment, the GPx activity and the t-GSH content in liver were significantly lower and the lipid peroxide level in liver was significantly higher than in control animals. However, the activities of GPx and GR and the t-GSH content in liver of group pretreated with radon were significantly higher than those of CCl₄ administrated group. Lipid peroxide level in liver of radon inhaled group before CCl₄

administration was significantly lower than that of CCl₄ administrated group (Fig. 5A).

The activities of GPx and GR and the t-GSH content in kidney were significantly lower and the lipid peroxide level in kidney was significantly higher than in control animals. However, the t-GSH content and the activity of GPx and GR in kidney of the group pretreated with radon were significantly higher and the lipid peroxide level in kidney was significantly lower than those of CCl₄ administrated mice (Fig. 5B)

Histological observation in liver and kidney following CCl₄ administration

The effects of radon inhalation on the histology of livers and kidneys subjected to CCl₄ administration were examined. CCl₄ administration resulted in centrilobular necrosis of the liver, and the necrosis in the CCl₄ group was significantly greater than that in the group pretreated with radon (Fig. 6A, B).

In case of kidney, CCl₄ administration resulted in dilatation of Bowman's space and glomerular atrophy, and radon inhalation inhibited the dilatation of Bowman's space and glomerular atrophy (Fig. 7).

DISCUSSION

Radon is an inert gas and as such does not react with any chemical component of the body. On entry through lungs, radon reaches the blood stream and is then distributed throughout the body. Therefore, many organs are subjected to the actions of free radicals created by the radiation. Radon is a source of α -rays, and these α -rays can only travel a distance of about 20 μ m through body tissues. The relatively large transfer of energy that is associated with the absorption of α -particles causes a series of complicated reactions within tissues. Radon inhalation has been reported to have therapeutic effects on senile brain disorder and hypertension [27]. Another known effect of a radon spring is to promote the effects of such

tissue perfusion agents as adrenaline in plasma; specifically, the level of plasma adrenaline is increased by radon inhalation [28,29]. We previously reported that radon inhalation of a concentration of 4000 Bq/m³ for 1 or 2 days significantly increases the antioxidative function of mouse liver and kidney [25]. In the current study, we attempted to shorten the inhalation time. Therefore, we needed to maintain as high a concentration of radon as possible. Although it is difficult to keep radon concentration levels up in the radon exposure box, our exposure system can maintain a radon concentration of 18 kBq/m³ over the time frame used in this experiment. The exposure dose of activation of antioxidative function may be estimated by multiplication of radon concentration and inhalation time. In fact, our results showed that t-GSH contents and the activities of GPx and GR in liver and kidney were significantly increased and lipid peroxide levels in liver and kidney were significantly decreased by radon inhalation, suggesting that radon inhalation activated antioxidative function.

The oxidative stress induced by CCl₄ is an established model for hepatotoxicity. The toxic effect of CCl₄ is due to the peroxidation of the membrane lipids by the trichloromethyl radical or trichloromethyl peroxy radical [1,2]. These radicals initiate lipid peroxidation chain reactions and cause severe cell damage. This damage induces an increase in fat accumulation in liver. In addition, severe liver damage interferes with cholesterol synthesis in liver. The activities of GOT, GPT, and ALP and the level of T-CHO and TG are used as indicators of liver damage. In this study, CCl₄ administration significantly increased the activities of GOT, GPT, and ALP in serum, and significantly decreased the serum T-CHO level. Moreover, TG level in liver significantly increased, suggesting that CCl₄ induced hepatopathy. On the other hand, CRE level is used as an indicator of kidney damage. Our results show that CCl₄ significantly increased the CRE level in serum, suggesting that CCl₄ administration also induced kidney damage.

Glutathione reacts directly with ROS, and GPx catalyzes the destruction of hydrogen

peroxide and hydroxyl radical [30]. This catalysis generates GSSG and finally glutathione. However, GR catalyzes the regeneration of glutathione from GSSG. Thus, GR and GPx are both the enzymes in the glutathione-regenerating pathway, and the activity of these enzymes responded to the treatments used in our experiments in a similar fashion. Glutathione protects against CCl₄-induced microsomal lipid peroxidation [31] and can scavenge trichloromethyl peroxy radical but not trichloromethyl radical [31]. Our results showed that CCl₄ significantly decreased t-GSH contents and the activities of GPx and GR and significantly increased lipid peroxide level in liver and kidney. These results clearly indicated that CCl₄ injection is associated with a weakening or failure of the antioxidative function in the liver or kidney. However, radon inhalation significantly activated antioxidative functions in liver and kidney. These results were consistent with the results in our previous reports [17,18]. Taken together, these findings suggest that radon inhalation has a similar biological effect to that of low-dose X-irradiation.

It has been reported that pathological changes induced by CCl₄ are mediated by induction of oxidative stress [32]. Lipid peroxidation induced by CCl₄ causes membrane disruption, resulting in the loss of membrane integrity. Weakened cellular membranes allow sufficient leakage of calcium into the cytosol to disrupt intracellular calcium homeostasis. The increase in intracellular calcium can activate endonucleases that can cause chromosomal damage and also contribute to cell death [32]. In this study, livers subjected to CCl₄ administration exhibited centrilobular necrosis, and the extent of centrilobular necrosis in CCl₄-treated mice was greater than that of control mice. However, radon inhalation inhibited the centrilobular necrosis induced by CCl₄ treatment. Moreover, our results indicated that the activation of antioxidative function inhibited lipid peroxidation in cellular membranes. This conclusion is also supported by the increased hepatic function (e.g., GOT, GPT, and ALP activity) associated with radon inhalation.

It has been reported that CCl₄ administration induced mild dilatation of Bowman's space with glomerular atrophy [33]. Our results also revealed a mild dilatation of Bowman's space with glomerular atrophy. However, antioxidants such as caffeic acid phenethyl ester protect against kidney damage induced by CCl₄ [33]. In this study, radon inhalation significantly increased t-GSH contents and GPx and GR activity and mitigated the mild dilatation of Bowman's space with glomerular atrophy, suggesting that glutathione protected the kidney from damage induced by CCl₄. This conclusion was also supported by an increase in serum CRE level associated with radon treatment.

The radon therapy is performed for various diseases, such as ankylosing spondylitis, chronic polyarthritis, spondylosis deformans, osteoarthritis, and bronchial asthma, at Misasa Medical Center, Okayama University Hospital. Hepatic and renal damage are not the main indications for radon therapy. However, our results demonstrated that radon inhalation clearly inhibited oxidative damage of liver and kidney. The data presented in this study provide a substantial basis for future studies aimed at assessing new radon-based therapies for treatment of hepatic and renal damage in humans.

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Figure Legends

Fig. 1 Schematic diagram of the radon exposure system.

Fig. 2 Changes in hepatic function-associated parameters in the serum (A), TG level in liver (B) and renal function-associated parameters in the serum (C) of radon inhalation and control mice. Each value indicates the mean \pm SEM. The number of mice per experimental point was 5-6.

Fig. 3 Changes in antioxidant-associated parameters in the liver (A) and kidney (B) of radon inhalation and control mice. Each value indicates the mean \pm SEM. The number of mice per experimental point was 5-6. *P < 0.05, ***P < 0.001 vs Control.

Fig. 4 Effects of radon inhalation on hepatic function-associated parameters in the serum (A), TG level in liver (B) and renal function-associated parameters in the serum (C) of CCl₄ administrated mice. Each value indicates the mean \pm SEM. The number of mice per experimental point is 4-6. **P < 0.01, ***P < 0.001 vs Control, #P < 0.05, ##P < 0.01 vs CCl₄.

Fig. 5 Effects of radon inhalation on antioxidative-associated parameters in the serum (A) and kidney (B) in mice injected with CCl₄. Each value indicates the mean \pm SEM. The number of mice per experimental point was 4-6. *P < 0.05, **P < 0.01 vs Control, #P < 0.05, ##P < 0.01, ###P < 0.001 vs CCl₄.

Fig. 6 Effect of radon inhalation on CCl₄-induced liver damage in mouse. Mouse livers were examined histologically. (A) Control, (B) CCl₄ administration, (C) radon inhalation before CCl₄ administration. The length of the scale bar is 50 μm. All samples were stained with HE. The area of cell necrosis surrounding the central vein (cv), but not portal vein (pv), in the CCl₄-administrated mice was measured (D). Less area was damaged in the mice pretreated with radon relative to those treated with only CCl₄ administration. Each value indicates the mean ± SEM. The number of mice per experimental point was 6. **P < 0.01 vs CCl₄.

Fig. 7 Effect of radon inhalation on CCl₄-induced kidney damage in mouse. Mouse kidneys were examined histologically. (A) Control, (B) CCl₄ administration, (C) radon inhalation before CCl₄ administration. The length of the scale bar is 50 μm. All samples were stained with HE. The arrow indicates dilatation of Bowman's space with glomerular atrophy.

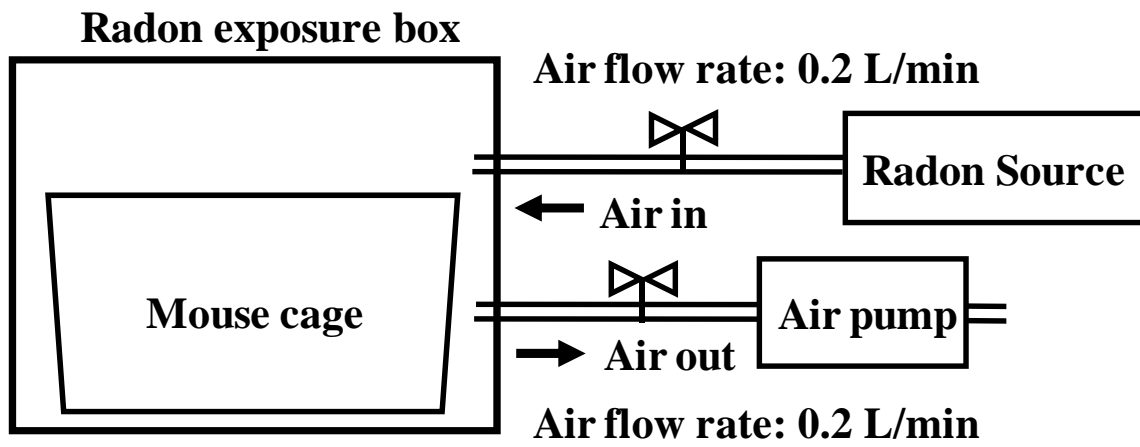


Fig.1

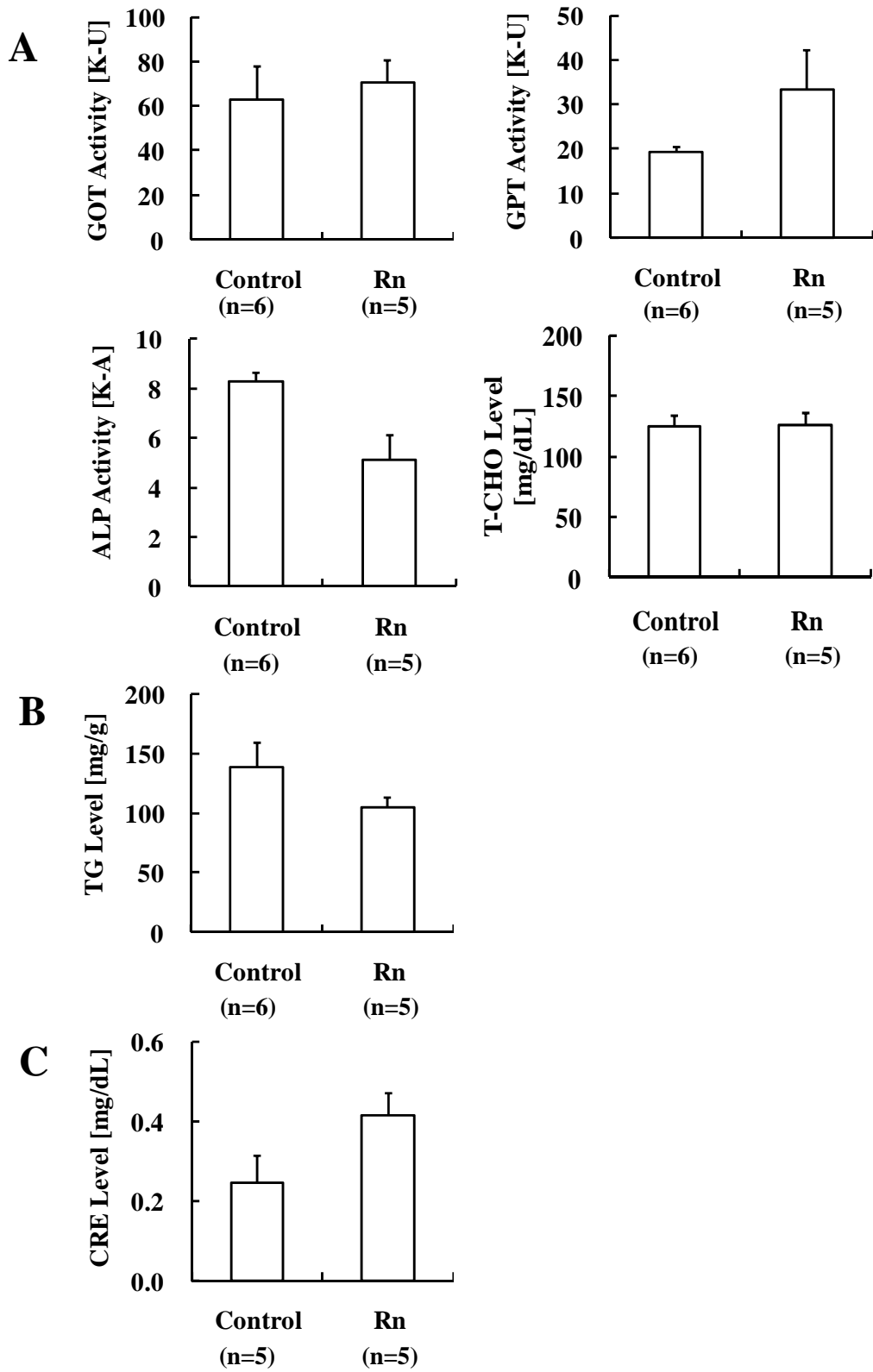
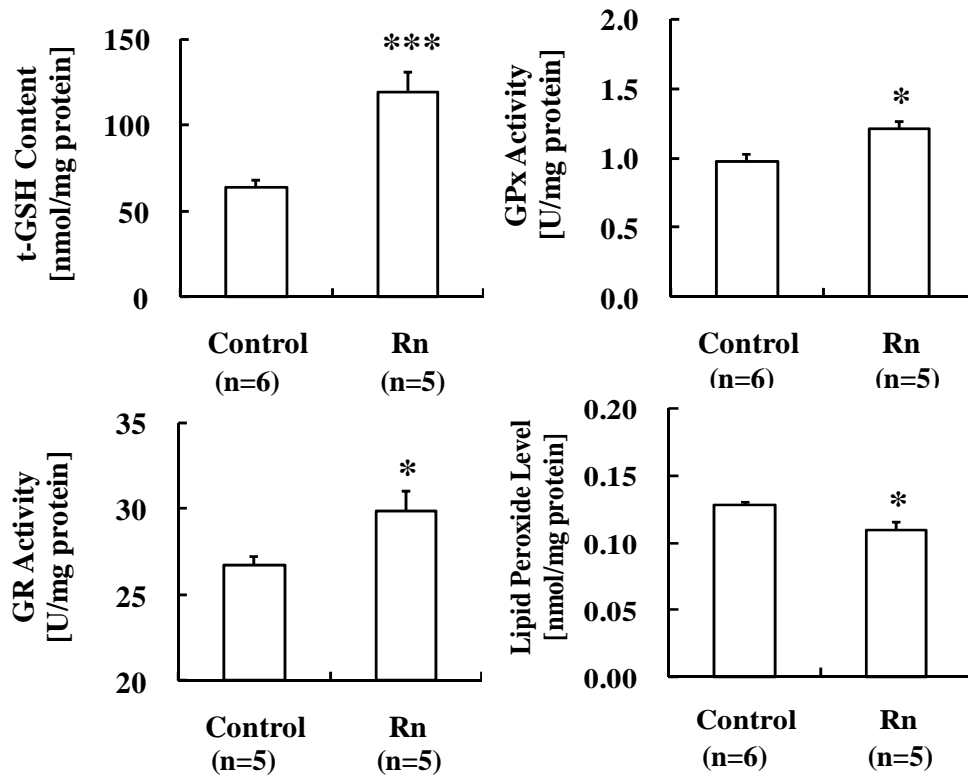
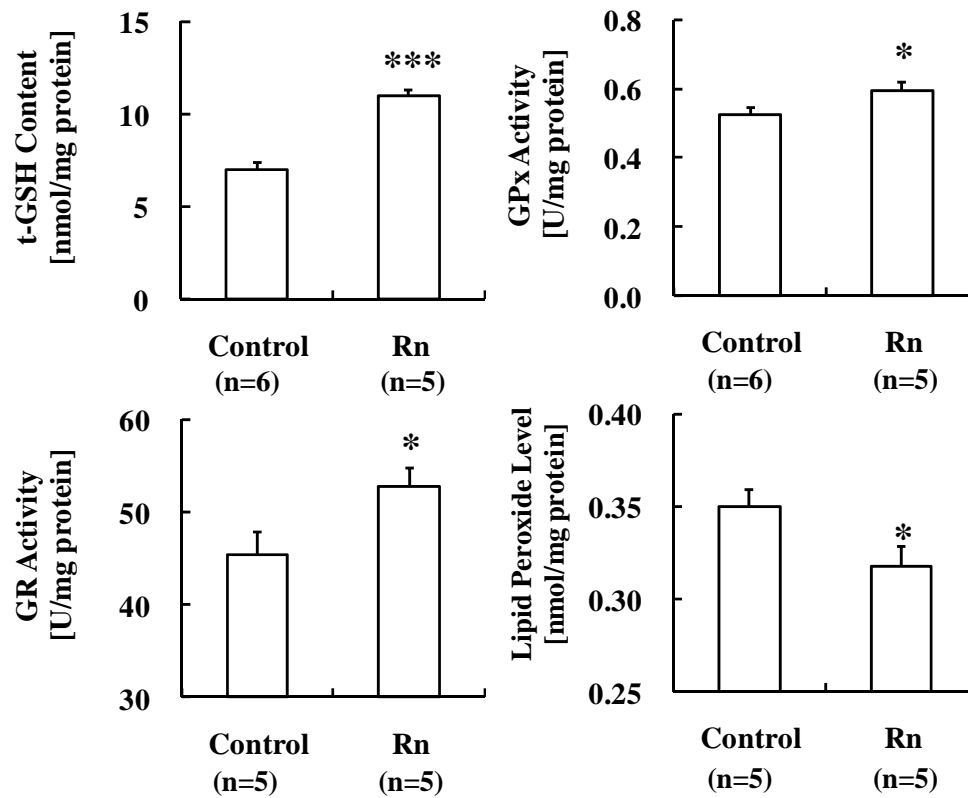


Fig.2

A**B***Fig.3*

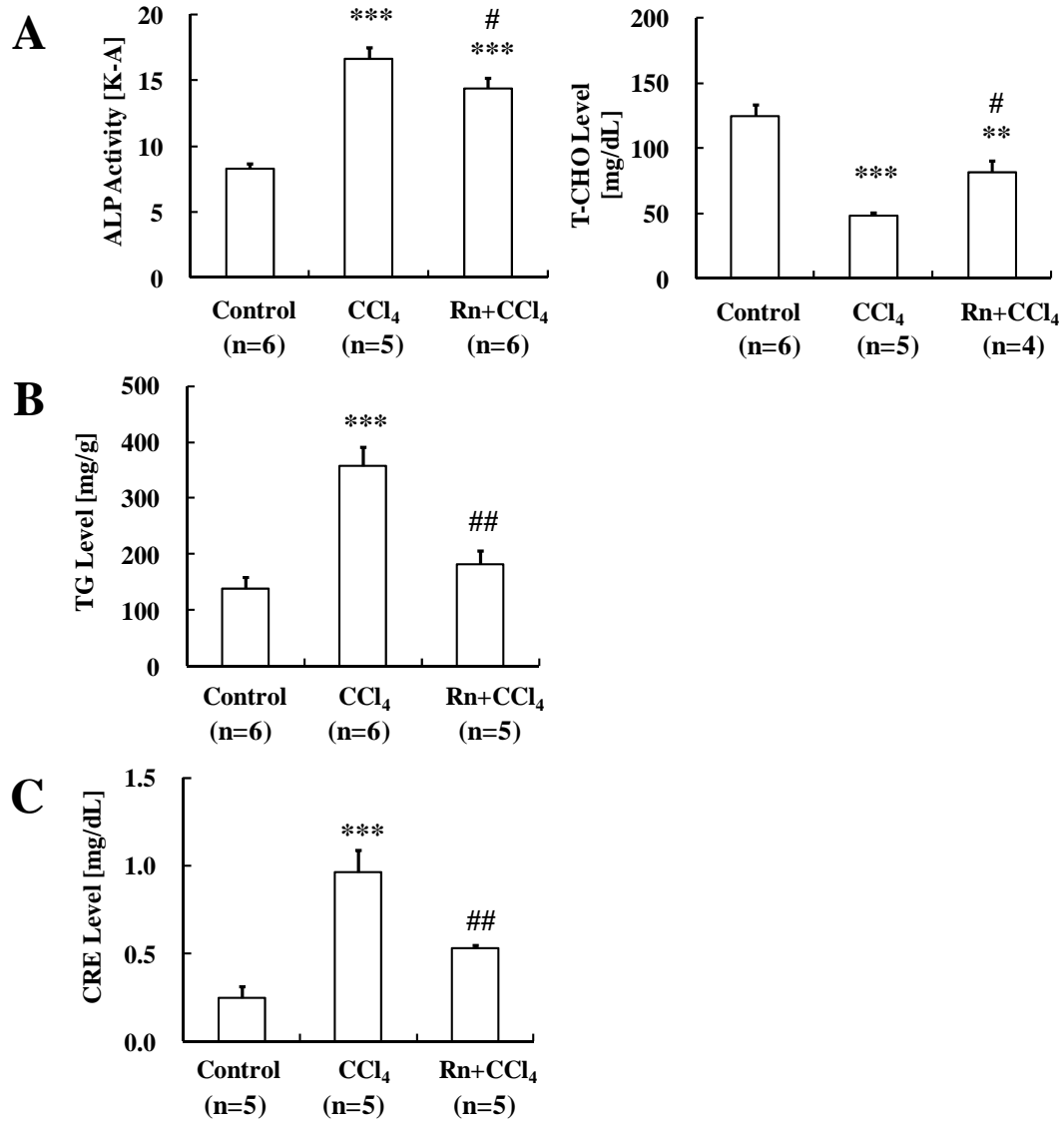


Fig.4

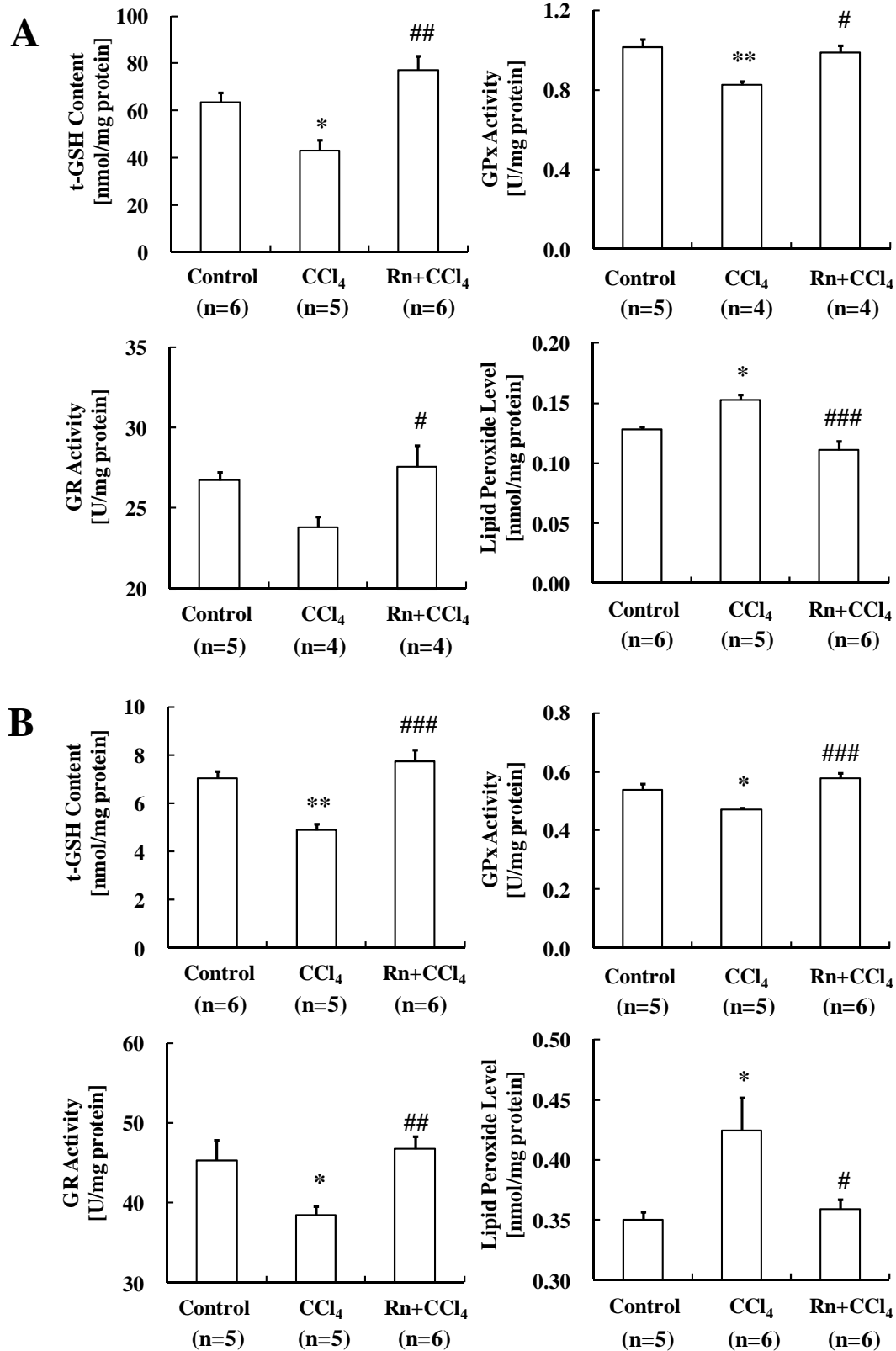


Fig.5

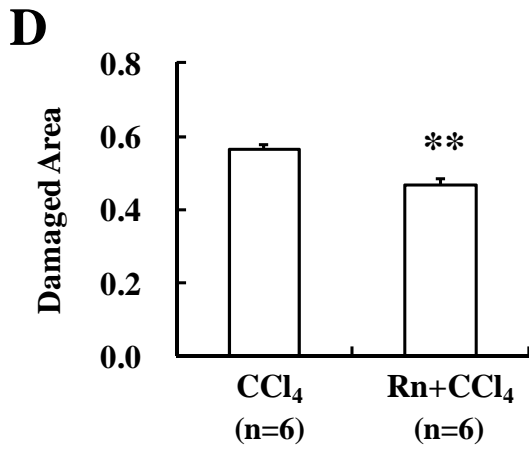
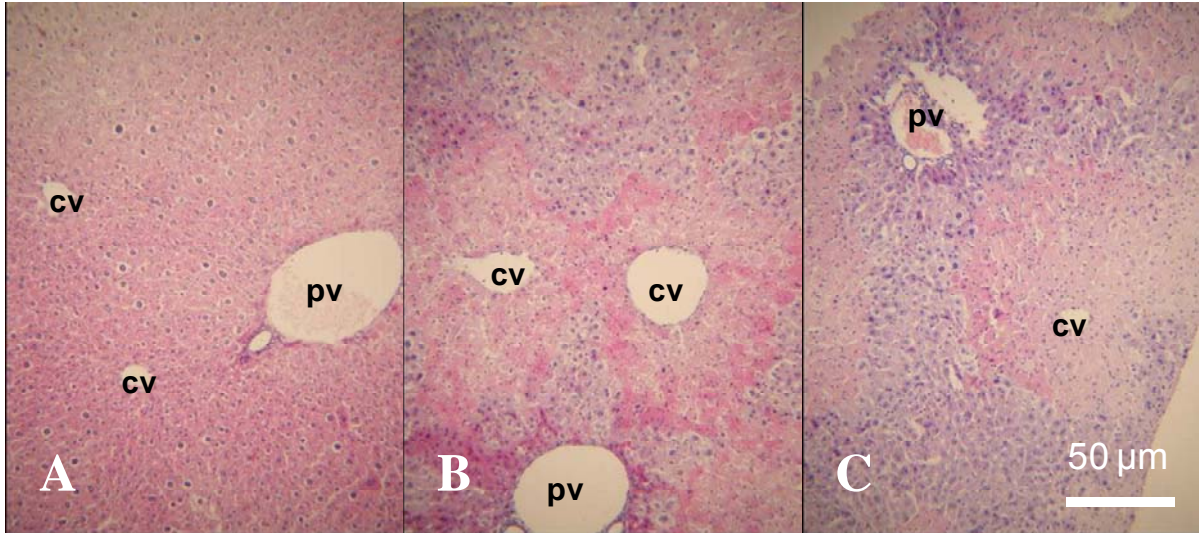


Fig.6

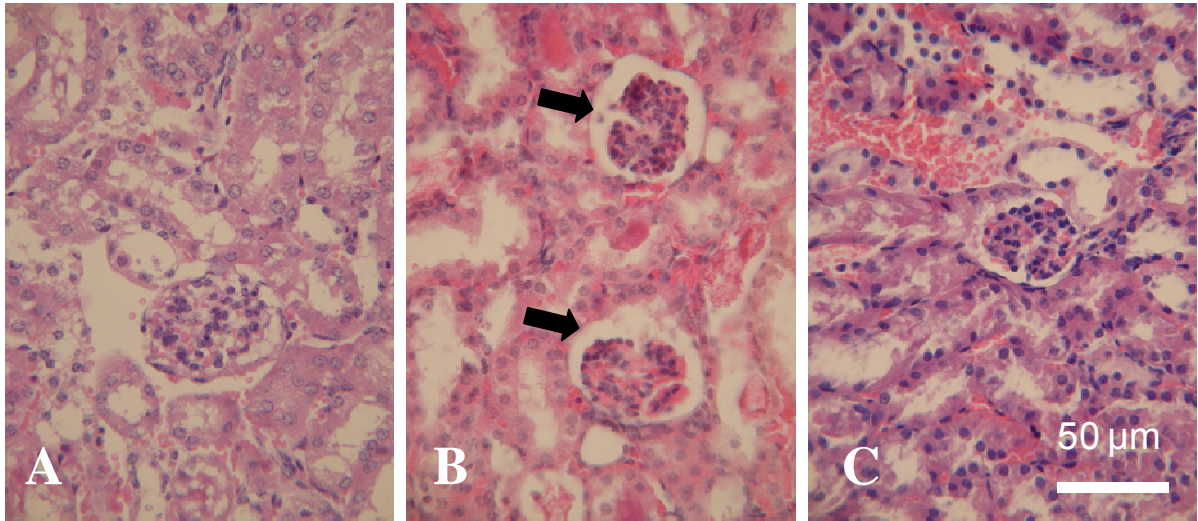


Fig.7

Table.1 Effects of radon inhalation on hepatic function-associated parameters in the serum of CCl₄ administrated mice. Each value indicates the mean \pm SEM. The number of mice per experimental point is 5-6. ***P < 0.001 vs Control, ##P < 0.01 vs CCl₄.

	Control (n=6)	CCl₄ (n=5)	Rn+CCl₄ (n=5)
GOT Activity [K-U]	63 \pm 15	22121 \pm 1314***	15055 \pm 359***,##
GPT Activity [K-U]	19 \pm 1	16170 \pm 1364***	14965 \pm 1409***

Table 1