Original Article

Suppression of Streptozotocin-induced Type-1 Diabetes in Mice by Radon Inhalation

Yuichi Nishiyama¹, Takahiro Kataoka¹, Junichi Teraoka¹, Akihiro Sakoda²,
Hiroshi Tanaka², Yuu Ishimori², Fumihiro Mitsunobu³, Takehito Taguchi¹
and Kiyonori Yamaoka¹

¹Graduate School of Health Sciences, Okayama University, 5-1 Shikata-cho 2-chome,
Kita-ku, Okayama-shi, Okayama 700-8558, Japan
²Ningyo-toge Environmental Engineering Center, Japan Atomic Energy Agency, 1550
Kagamino-cho, Tomata-gun, Okayama 708-0698, Japan
³Misasa Medical Center, Okayama University Hospital, 827 Yamada, Misasa-cho,
Tohaku-gun, Tottori 682-0192, Japan

Address for correspondence: Kiyonori Yamaoka, Professor
Graduate School of Health Sciences, Okayama University
5-1 Shikata-cho 2-chome, Kita-ku, Okayama-shi, Okayama 700-8558, Japan,
Phone/Fax: +81-86-235-6852
E-mail:

Short Title: Suppression of Type-1 diabetes by Radon Inhalation
Summary

We examined the protective effect of radon inhalation on streptozotocin (STZ)-induced type-1 diabetes in mice. Mice inhaled radon at concentrations of 1000, 2500, and 5500 Bq/m$^3$ for 24 hours before STZ administration. STZ administration induced characteristics of type-1 diabetes such as hyperglycemia and hypoinsulinemia; however, radon inhalation at doses of 1000 and 5500 Bq/m$^3$ significantly suppressed the elevation of blood glucose in diabetic mice. Serum insulin was significantly higher in mice pre-treated with radon at a dose of 1000 Bq/m$^3$ than in mice treated with a sham. In addition, superoxide dismutase activities and total glutathione contents were significantly higher and lipid peroxide was significantly lower in mice pre-treated with radon at doses of 1000 and 5500 Bq/m$^3$ than in mice treated with a sham. These results were consistent with the result that radon inhalation at 1000 and 5500 Bq/m$^3$ suppressed hyperglycemia. These findings suggested that radon inhalation suppressed STZ-induced type-1 diabetes through the enhancement of antioxidative functions in the pancreas.

Keywords: radon inhalation, antioxidative function, streptozotocin, type-1 diabetes
Introduction

Free radicals and other reactive oxygen species (ROS) are constantly formed in the human body. Free-radical mechanisms have been implicated in the pathology of several human diseases, such as cancer, atherosclerosis, and malaria (Aruoma 1998). On the other hand, it has been reported that low-dose irradiation induces various biological effects, such as increased resistance to ROS (Kojima et al. 1997) and enhanced immune function (Ishii et al. 1995, Kojima et al. 2004). We have reported that low-dose X- or γ-irradiation increases or induces antioxidant substances such as superoxide dismutase (SOD) (Yamaoka et al. 1991, Yamaoka et al. 1999), catalase (CAT) (Kojima et al. 1999), and glutathione (GSH) (Kojima et al. 2004) in some organs of small animals. These findings indicate that low-dose irradiation may contribute to preventing or alleviating ROS-related injury (Yamaoka 2006). In fact, we previously reported that low-dose X-irradiation inhibited oxidative damage, such as brain edema (Yoshimoto et al. 2012), ischemia-reperfusion injury (Kataoka et al. 2007), and carbon tetrachloride (CCl₄)-induced hepatopathy in mice (Kataoka et al. 2005, Yamaoka et al. 2004a).

Therapy using radon (²²²Rn), which is volatilized from radon-enriched water and mainly emits α-rays, is performed for ROS-related diseases, such as arteriosclerosis, osteoarthritis (Yamaoka et al. 2004b), and bronchial asthma (Mitsunobu et al. 2003) at Misasa Medical Center, Okayama University Hospital; however, the mechanisms of the therapy have not been fully understood. To clarify the effects of the therapy, we investigated the effects of radon inhalation on mice. We previously reported that radon inhalation increased antioxidant substances and inhibited CCl₄-induced hepatopathy (Kataoka et al. accepted by J Radiat Res) and renal damage (Kataoka et al. 2011a, Nishiyama et al. 2012). These findings suggested that radon inhalation has antioxidative effects similar to low-dose X- or γ-irradiation.

Diabetes mellitus is now a worldwide disease and, in particular, the number of young patients is increasing (Harjutsato et al. 2008, Mayer-Davis et al. 2009). The β cells in the pancreas are susceptible to ROS because they express very low levels of antioxidants (Lenzen et al. 1996);
therefore, it is considered that β cells are easily subject to oxidative stress. It is well known that oxidative stress caused by ROS contributes to β cell death or dysfunction of the pancreas in type-1 diabetes (Cnop et al. 2005). On the other hand, we recently reported that radon inhalation activated SOD activity in various organs, including the pancreas of mice (Kataoka et al. 2011b), suggesting that radon inhalation may prevent type-1 diabetes; however, there has been no report of a protective effect of radon inhalation on type-1 diabetes.

Streptozotocin (STZ) is widely used in studies of experimental type-1 diabetes because it selectively destroys pancreatic β cells through the generation of ROS and alkylation of deoxyribonucleic acid (DNA) (Lenzen 2008, Szkudelski 2001). To assess the protective effect of radon inhalation on STZ-induced type-1 diabetes, we examined the following antioxidant- and diabetes-associated parameters and histological changes of the pancreas: SOD activity, CAT activity, total glutathione (t-GSH) content, lipid peroxide (LPO), blood glucose, serum insulin, and body weight.

Methods

Animals

Male C57BL/6J mice (9 weeks of age, body weight 25-28 g) were purchased from CLEA Japan Inc. (Tokyo, Japan). The animals were housed in clear plastic cages with wood-chip bedding in a temperature-controlled room (20 ± 1 °C). They were fed Oriental MF diet (Oriental Yeast Co., Tokyo, Japan) and tap water ad libitum. Each group consisted of 5-8 mice. Ethics approval was obtained from the animal experimental committee of Okayama University.

Radon inhalation

Mice were exposed to radon using a large-scale facility developed at Misasa Medical Center, Okayama University Hospital (Ishimori et al. 2010). Briefly, it was designed to examine a number of animals at various radon concentrations at the same time, and the facility has adopted
a whole-body exposure system. Air with radon was blown into an exposure box and then blown out of the box. The radon concentration was measured using a radon monitor (Genitron Co., Ltd., Germany).

Experimental procedure

Mice were divided into eight groups: control (Control), radon inhalation only (1000, 2500, and 5500 Bq/m$^3$), sham inhalation with STZ administration (Sham + STZ), and radon inhalation with STZ administration (1000 Bq/m$^3$ + STZ, 2500 Bq/m$^3$ + STZ, and 5500 Bq/m$^3$ + STZ). Mice were exposed to radon at doses of 1000, 2500, and 5500 Bq/m$^3$ for 24 hours. A single high-dose of STZ (200 mg/kg weight, 50 g/L in saline solution) was administrated into the peritoneum immediately after radon inhalation. A single dose of STZ causes mild to severe types of diabetes according to the dosage used. A single high dose of STZ (200 mg/kg weight) destroys most of the β cells present in the islets and induces a rapid and permanent insulin-dependent diabetic condition in C57BL strain mice (Shertzer et al. 2009). On the other hand, it has been reported that a single low dose of STZ (approximately 100 mg/kg weight) sometimes causes a non-insulin-dependent diabetic condition (such as type 2 diabetes) in some strains of mice because the dose level is not sufficient to destroy most of the β cells (Ito et al. 2001); therefore, we injected a single high dose of STZ into C57BL strain mice, which are sensitive to the toxicity of STZ and develop complete insulin-dependent type-1 diabetes. Changes in blood glucose and body weight were monitored during the experiment. Blood glucose was measured by Glucose Pilot (Aventir Biotech, LLC, CA, USA) using tail tip blood. Four days after STZ administration, mice were killed by an overdose of ether anesthesia and blood was collected from the heart for analysis of insulin in serum. Serum was obtained by centrifugation at 3,000 × g for 5 min at 4 °C. The pancreas was quickly excised for SOD, CAT, t-GSH, and LPO analyses. Part of each pancreas was fixed in 10% formalin for histological examination.
Biochemical assays

Mouse pancreas was homogenized in 1 M Tris-HCl buffer containing 5 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.4) on ice. The homogenate was centrifuged at 12,000 × g for 45 min at 4 °C and the supernatant was used for assay of the activity of SOD and CAT. SOD activity was measured by the nitroblue tetrazolium (NBT) reduction method (Braehner et al. 1975) 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.

CAT activity was measured as the hydrogen peroxide (H$_2$O$_2$) reduction rate at 37 °C and was assayed at 240 nm using a spectrophotometer (Aebi et al. 1976). 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$_2$O$_2$, 30 μl deionized water, and 20 μl pancreas supernatant. Activity was calculated using a molar extinction coefficient of 7.1 × 10$^{-3}$ M$^{-1}$cm$^{-1}$. CAT activity was measured by the amount of H$_2$O$_2$ split by CAT at 37 °C. 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 pancreas was suspended in 10 mM phosphate buffer 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)) was assayed using the Bioxytech LPO-586 assay kit (OXIS Health Products, Inc.). Briefly, the pancreas was 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 °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 using a spectrophotometer.

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

Serum insulin was measured by the enzyme linked immunosorbent assay (ELISA) using an
insulin assay kit (Morinaga Institute of Biological Science, Inc., Yokohama, Japan).

Histological examination

Tissue samples fixed in 10 % formalin were embedded in paraffin. Six micrometer-thick tissue
sections were prepared and stained with hematoxylin-eosin (HE). The size of pancreatic islets
was measured using image-editing software.

Statistical analyses

Data are presented as the mean ± standard error of the mean (SEM). The statistical
significance of differences was determined by Dunnett’s tests and Tukey’s tests for multiple
comparisons where appropriate. P < 0.05 was considered significant.

Results

Effects of radon inhalation on antioxidant-associated substances in the pancreas

SOD activity and t-GSH content in the pancreas were significantly higher and the LPO level
was significantly lower in the 1000 Bq/m³ group than in the control group. CAT activity was
significantly higher in the 5500 Bq/m³ group than in the control group (Figure 1A-D)
Effects of radon inhalation on body weight following STZ administration

Significant decreases were observed in body weight 4 days after STZ administration in Sham + STZ, 2500 Bq/m$^3$ + STZ, and 5500 Bq/m$^3$ + STZ groups compared with the control group; however, there were no significant differences in body weight throughout the experiment between the 1000 Bq/m$^3$ + STZ group and control group (Figure 2).

Effects of radon inhalation on blood glucose and serum insulin following STZ administration

Four days after STZ administration, blood glucose was significantly higher in all groups pre-treated in the presence or absence of radon inhalation than in the control group; however, blood glucose was significantly lower in 1000 Bq/m$^3$ + STZ and 5500 Bq/m$^3$ + STZ groups than in the Sham + STZ group (Figure 3A).

STZ administration significantly decreased serum insulin in all groups pre-treated in the presence or absence of radon inhalation compared with the control group; however, serum insulin was significantly higher in the 1000 Bq/m$^3$ + STZ group than in the Sham + STZ group (Figure 3B).

Histological observation

A significant decrease in the mean size of pancreatic islets was observed in all groups pre-treated in the presence or absence of radon inhalation compared with the control group (Figure 4A-E); however, the mean sizes of pancreatic islets were significantly larger in 1000 Bq/m$^3$ + STZ and 5500 Bq/m$^3$ + STZ groups than in the Sham + STZ group (Figure 4F).

Effects of radon inhalation on antioxidant-associated substances following STZ administration

To assess the protective effect of radon inhalation on STZ-induced type-1 diabetes, antioxidant substances in pancreas were assayed (Figure 5A-D). SOD activity and the t-GSH content were significantly lower and the LPO level was significantly higher in the Sham + STZ group than in
the control group. CAT activity was 51% lower in the sham group than in the control group, but these differences were not significant; however, SOD activities were significantly higher in 1000 Bq/m³ + STZ, 2500 Bq/m³ + STZ, and 5500 Bq/m³ + STZ groups than in the Sham + STZ group. The t-GSH contents were significantly higher and the LPO levels were significantly lower in 1000 Bq/m³ + STZ and 5500 Bq/m³ + STZ groups than in the Sham + STZ group.

Discussion

We have reported that radon inhalation activates antioxidative functions in various organs of BALB/c strain mice (Kataoka et al. 2011b). In this study, we used C57BL/6J strain mice, which have low sensitivity to radiation compared with BALB/c (Kallman and Kohn 1956) because in this strain of mice it is especially easy to induce insulin-dependent type-1 diabetes by STZ administration (Cardinal et al. 1998); however, the effects of radon inhalation on antioxidative functions in the pancreas of C57BL/6J strain mice have never been examined and were assessed here for the first time. Our results showed that antioxidative functions in the pancreas were significantly higher in mice that inhaled radon at doses of 1000 and 5500 Bq/m³ than in control mice. These findings suggested that radon inhalation may contribute to preventing oxidative stress-related disease in the pancreas.

We examined diabetic conditions 4 days after STZ administration, considering that this is an adequate interval to induce type-1 diabetes when STZ is used. STZ administration induces permanent hyperglycemia within about 7 days and complete degranulation of β cells is seen within 12 to 48 hrs (Lenzen 2008). In addition, metabolic alterations are usually found in animals 3 to 5 days after STZ administration (Catanzaro et al. 1994). STZ administration induces certain typical characteristics of type-1 diabetes, such as hyperglycemia, hypoinsulinemia and body weight loss (Tomlinson et al. 1992). In the present study, all groups pre-treated in the presence or absence of radon inhalation finally showed hyperglycemia and hypoinsulinemia; however, pretreatment with radon inhalation at doses of
1000 and 5500 Bq/m$^3$ significantly suppressed blood glucose elevation and body weight decrease compared with the Sham + STZ group. In addition, serum insulin was significantly higher in the 1000 Bq/m$^3$ + STZ group than in the Sham + STZ group. These results indicated that radon inhalation partially suppressed type-1 diabetes induced by STZ administration.

Histological observation of the pancreatic tissue further substantiated the claim that radon inhalation has protective effects on pancreatic tissue. STZ administration induced severe injury to the pancreas, such as a decrease of the islet size, which was probably due to the reduction in the number of β cells; however, mice that inhaled radon at doses of 1000 and 5500 Bq/m$^3$ showed slight pancreatic islet damage compared with sham-treated mice.

To clarify the mechanism of radon inhalation suppressing STZ-induced type-1 diabetes, we examined antioxidative functions in the pancreas. STZ is a nitric oxide (NO) donor and NO partially restricts adenosine triphosphate (ATP) generation in mitochondria and increases xanthine oxidase (XOD) (Lenzen 2008, Szkudelski 2001). XOD catalyzes the synthesis of superoxide anion radical (O$_2^-$) and, as a result, H$_2$O$_2$ and hydroxyl radical (OH$^-$) are formed (Szkudelski 2001). In the case of SOD deficiency or increased O$_2^-$ production, it reacts with NO to produce peroxynitrite (ONOO), which is a highly toxic agent that can cause direct damage to proteins, lipids and DNA (Szkudelski 2001). The scavenging activity of SOD, which catalyzes the conversion of O$_2^-$ into H$_2$O$_2$, and CAT, which transforms H$_2$O$_2$ into H$_2$O as well as GSH, is well known. ROS scavengers such as SOD protect β cells against ROS attack induced by STZ administration (Kubisch et al. 1994, Robbins et al. 1980). It was reported that low-dose γ-irradiation increased SOD activity in the pancreas and suppressed β cell apoptosis and diabetes incidence in non-obese diabetic mice (Takahashi et al. 2000). A similar result was reported in alloxan-induced type-1 diabetic rats, in which single γ-irradiation at 0.5 Gy prevented the elevation of pancreatic lipid peroxidation and blood glucose (Takehara et al. 1995). Our results showed that STZ administration caused oxidative damage, represented by decreased SOD activity and t-GSH content and increased LPO in the pancreas; however, SOD
activities and t-GSH contents were significantly higher and the LPO was significantly lower in 1000 Bq/m$^3$ + STZ and 5500 Bq/m$^3$ + STZ groups than in the Sham + STZ group. These results are consistent with the result that radon inhalation at doses of 1000 and 5500 Bq/m$^3$ suppressed hyperglycemia. These findings suggested that radon inhalation suppressed STZ-induced type-1 diabetes through the enhancement of antioxidative functions in the pancreas.

It is well known that insulin is the main hormone to lower blood glucose. Although hypoinsulinemia in the 5500 Bq/m$^3$ + STZ group was not improved, hyperglycemia was significantly suppressed. These findings may suggest that radon inhalation has a blood-glucose-lowering effect which is non-insulin dependent; however, no report has shown that low-dose irradiation, including radon, has such effects. It was reported that low-dose X-irradiation enhanced the ability to regulate energy metabolism and the membrane transport mechanism, as reflected by the increase in adenosine triphosphatase (ATPase) activity (Yamaoka et al. 1994). Low-dose irradiation may contribute to the suppression of hyperglycemia through the enhancement of glycolytic metabolism in cells. Further study is required to clarify this point.

Radon inhalation did not prevent type-1 diabetes in a dose-related fashion. We previously reported that SOD activity changes in some organs, such as the pancreas and liver, had a complex response to radon (Kataoka et al. 2011b). Briefly, radon inhalation of low (500 Bq/m$^3$) or high (4000 Bq/m$^3$) concentrations for 24 hrs increased SOD activity; however, there was little change in SOD activity following the inhalation of intermediate (2000 Bq/m$^3$) radon concentration for 24 hrs. That is, the distinctive feature was that it had two activation points of SOD activity, although it was not clear whether the total antioxidative function, including CAT and t-GSH, changed in a similar manner with SOD, which may correlate with poor efficacy in suppressing type-1 diabetes by radon inhalation at a dose of 2500 Bq/m$^3$. Further study is required to understand the effects of radon inhalation on antioxidant systems in the living body.
High-level radon has been found to cause lung cancer (Lubin et al. 1995); however, based on
the recommendations of the International Commission on Radiological Protection (ICRP), the
risks associated with exposure to radon therapy are low. In fact, adverse effects and negative
effects of radon therapy have not been reported in the past. The dose of radon absorbed under
our experimental conditions was also very low according to previous reports (Franke et al. 2000,
Sakoda et al. 2010), and radon inhalation has only a small risk compared to lifestyle influences,
such as smoking (Sethi et al. 2012).

In conclusion, radon inhalation activated antioxidative functions in the pancreas and partially
suppressed STZ-induced type-1 diabetes. The data presented in this study provide a substantial
basis for future studies aimed at assessing new radon-based therapies for the treatment of type-1
diabetes in humans.

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Abbreviations

ATP – adenosine triphosphate; ATPase – adenosine triphosphatase; CAT – catalase; CCl₄ –
carbon tetrachloride; DNA – deoxyribonucleic acid; EDTA – ethylenediaminetetraacetic acid;
ELISA – enzyme linked immunosorbent assay; GSH – glutathione; HE – hematoxylin-eosin;
H₂O₂ – hydrogen peroxide; ICRP – International Commission on Radiological Protection; LPO
– lipid peroxide; MDA – malondialdehyde; NBT – nitroblue tetrazolium; NO – nitric oxide; O₂⁻
– superoxide anion radical; OH⁻ – hydroxyl radical; ONOO⁻ – peroxynitrite; PBS – phosphate
buffer saline; ROS – reactive oxygen species; SEM – standard error of mean; SOD – superoxide
dismutase; STZ – streptozotocin; t-GSH – total glutathione; XOD – xanthine oxidase.
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Figure Legends

Figure 1
Effects of radon inhalation on antioxidant-associated substances in the pancreas. (A) SOD activity, (B) CAT activity, (C) t-GSH content, and (D) LPO level in the pancreas. Each value indicates the mean ± SEM. The number of mice per experimental point was 5-6. *P<0.05, radon inhalation vs. no inhalation.

Figure 2
Effects of radon inhalation on body weight following STZ administration. Each value indicates the mean ± SEM. The number of mice per experimental point was 5-8. *P<0.05, **P<0.01, ***P<0.001, radon or sham inhalation before STZ administration vs. no inhalation at the same time point. *P<0.05, **P<0.01, ***P<0.001, radon inhalation before STZ administration vs. sham inhalation before STZ administration at the same time point.

Figure 3
Effects of radon inhalation on blood glucose and serum insulin following STZ administration. (A) Blood glucose and (B) serum insulin. Each value is the mean ± SEM. The number of mice for each experiment and significance are the same as in Figure 2.
Figure 4
Histological changes in the pancreas after STZ administration. (A) Control, (B) sham inhalation under STZ administration, (C) radon inhalation of 1000 Bq/m$^3$ under STZ administration, (D) radon inhalation of 2500 Bq/m$^3$ under STZ administration, and (E) radon inhalation of 5500 Bq/m$^3$ under STZ administration. Arrow indicates pancreatic islets. Scale bar = 50 μm. All samples were stained with H.E. (F) Larger pancreatic islets of mice pre-treated with radon inhalation than those of mice treated with sham inhalation. Each value is the mean ± SEM. The number of mice for each experiment and significance are the same as in Figure 2.

Figure 5
Effects of radon inhalation on antioxidant-associated substances following STZ administration. (A) SOD activity, (B) CAT activity, (C) t-GSH content, and (D) LPO in the pancreas. Each value is mean ± SEM. The number of mice for each experiment and significance are the same as in Figure 2.
Figure 1

A) SOD Activity [U/mg protein]

B) CAT Activity [U/mg protein]

C) t-GSH Content [nmol/mg protein]

D) LPO Level [nmol/mg protein]
Figure 2
Figure 3
Figure 4
Figure 5