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Protective mechanism of reduced water against alloxan-induced pancreatic β-cell damage: Scavenging effect against reactive oxygen species

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Abstract

Reactive oxygen species (ROS) cause irreversible damage to biological macromolecules, resulting in many diseases. Reduced water (RW) such as hydrogen-rich electrolyzed reduced water and natural reduced waters like Hita Tenryosui water in Japan and Nordenau water in Germany that are known to improve various diseases, could protect a hamster pancreatic β cell line, HIT-T15 from alloxan-induced cell damage. Alloxan, a diabetogenic compound, is used to induce type 1 diabetes mellitus in animals. Its diabetogenic effect is exerted via the production of ROS. Alloxan-treated HIT-T15 cells exhibited lowered viability, increased intracellular ROS levels, elevated cytosolic free Ca^{2+} concentration, DNA fragmentation, decreased intracellular ATP levels and lowering of glucose-stimulated release of insulin. RW completely prevented the generation of alloxan-induced ROS, increase of cytosolic Ca^{2+} concentration, decrease of intracellular ATP level, and lowering of glucose-stimulated insulin release, and strongly blocked DNA fragmentation, partially suppressing the lowering of viability of alloxan-treated cells. Intracellular ATP levels and glucose-stimulated insulin secretion were increased by RW to 2–3.5 times and 2–4 times, respectively, suggesting that RW enhances the glucose-sensitivity and glucose response of β-cells. The protective activity of RW was stable at 4 °C for over a month, but was lost by autoclaving. These results suggest that RW protects pancreatic β-cells from alloxan-induced cell damage by preventing alloxan-derived ROS generation. RW may be useful in preventing alloxan-induced type 1-diabetes mellitus.

Abbreviations: BSA, bovine serum albumin; DCF, dichloroflurescein; DCFH-DA, 2′,7′-dichlorofluorescin-diacetate; ELISA, enzyme-linked immunosorbent assay; ERW, electrolyzed-reduced water; HBSS, Hank's balanced salt solution; IDDM, insulin-dependent diabetes mellitus; IIDM, insulin-independent diabetes mellitus; KRB, Krebs Ringer bicarbonate buffer; NRW, nature reduced water; ROS, reactive oxygen species; RW, reduced water.

Introduction

Diabetes is mainly grouped into insulin-dependent diabetes mellitus (IDDM) (Type 1-diabetes) and insulin-independent diabetes mellitus (IIDM) (Type 2-diabetes). Type 1-diabetes is caused by a deficiency in insulin secretion from pancreatic β cells. Type 2-diabetes mellitus is related to damage in the insulin

signaling pathway. Chemical compounds that selectively damage pancreatic β-cells constitute a class of diabetogenic drugs. Alloxan, a cyclic urea derivative, is a potent diabetogenic agent that has been widely used for the induction of experimental type 1 diabetes (Rho et al., 2000). It has been reported that alloxan rapidly and selectively accumulates in β-cells in comparison with non-β cells (Gorus et al., 1982). Although the precise diabetogenic mechanism of alloxan is not fully understood, evidence indicates that pancreatic β-cell damage induced by alloxan is mediated through the generation of cytotoxic reactive oxygen species (ROS) (Yamamoto et al., 1981; Malaisse and Lea, 1982; Takasu et al., 1991). Okamoto (1985) proposed that the primary target of ROS produced by alloxan is the DNA of the pancreatic β-cells and causes DNA strand breaks. Increase of cytosolic Ca^{2+} also plays an important role in the diabetogenesis of alloxan, in relation to radical generation and DNA fragmentation (Park et al., 1995).

The clinical effect of electrolyzed-reduced water (ERW) was recognized by the Ministry of Health and Welfare of Japan in 1965. Double blind clinical tests demonstrated that ERW was safe and effective for intestinal abnormal fermentation, acid indigestion, chronic diarrhea, chronic constipation, dyspepsia, and antacid (Tashiro, 1999). However, the mechanism of the clinical effect of ERW has not been clarified. Several natural mineral waters in the world are called miracle waters because of their curative powers against various diseases. Tracote water in Mexico was found in 1991. Nordenau water in Germany was found in 1992. The authors found Hita Tenryosui water in Japan in 1997. It has been reported by many people that daily consumption of these waters results in improvement in various diseases including diabetes, cancer, arteriosclerosis and allergies. However, strict double-blind clinical tests have not yet been performed.

ERW contains a lot of hydrogen, scavenges ROS, and protects DNA from oxidative damage, suggesting that stable active hydrogen in ERW might be a reducing agent responsible for scavenging against ROS (Shirahata et al., 1997). We found that Hita Tenryousui water, Nordenau water, and Tracote water were all antioxidative waters that could scavenge intracellular ROS and called those waters natural reduced waters (NRW) (Shirahata, 2002). Based on extensive evidence, Shirahata (2000, 2002) proposed the active hydrogen theory of reduced water as follows: (1) Water is a good supplier of active hydrogen. (2) Active hydrogen can be easily produced by weak electrolysis and stabilized in the form of hydrogenated or reduced metal colloids in reduced water (RW) like ERW and NRW. (3) The reducing activity of RW is lost by heating or autoclaving due to dehydrogenation of metal colloids or instability of metal colloids. (4) Active hydrogen in RW may be an ideal scavenger against ROS because it does not produce oxidized molecules after reduction like other organic antioxidants (vitamin C, vitamin E, and polyphenols). Recently, Hanaoka (2001) reported the scavenging activity of ERW against ROS. RW stimulates glucose uptake in muscle cells and adipocytes, suggesting that RW might be effective for preventing type 2-diabetes (Oda et al., 1999; Shirahata et al., 2001). However, the effect of RW on pancreatic β-cells in relation to type 1 diabetes is not understood. Here we report that RW strongly protects pancreatic β cells from damage induced by the diabetogenic agent alloxan, suggesting that RW may be effective in preventing alloxan-induced type-1 diabetes.

Materials and methods

Regents

Bovine serum albumin (BSA), an ATP Bioluminescence Assay Kit CLS II and a cellular DNA fragmentation ELISA Kit for the detection of apoptosis were obtained from Roche Diagnostics GmbH (Mannheim, Germany). Krebs Ringer bicarbonate (KRB) buffer (pH 7.4), alloxan and Fluo 3-AM were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). WST-8 Assay Kit was from Promega Co. (Madison, WI, U.S.A.). RPMI 1640 medium was from Nissui Pharmaceutical Co. (Tokyo, Japan). A rat insulin enzyme immunoassay (EIA) kit, D(+)-glucose, HEPES ((4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) and all other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Reduced water and preparation of medium

Electrolyzed-reduced water (ERW) was prepared by the electrolysis of ultra pure water containing 0.002 N NaOH using an electrolyzing device equipped with platinum-coated titanium electrodes (Type TI-200s, Nihon Trim Co., Osaka, Japan). The electrolyzing device used was a batch type one and consisted of a vessel of 4 l (190 mm length × 210 mm wide × 140 mm height) divided by a semi-permeable membrane (190 mm wide × 130 mm

height). Two platinum-coated titanium electrodes (70 mm × 110 mm square) were placed at a distance of 55 mm from each side of the semi-permeable membrane for electrolysis with a direct current of 100 V for 60 min. The alkaline ERW was stored in a closed glass bottle at 4 °C and neutralized with HEPES buffer in medium before use. Nordenau Water was kindly supplied by Mr. Theo Tommes from Nordenau in Germany. Hita Tenryosui water was obtained from Hita Tenryosui Co. in Japan. Natural mineral water A and B were purchased from a local market in Japan. In order to inactivate its reducing activity, the RW was autoclaved in an open bottle at 121 °C for 20 min. In order to investigate the protective effect of RW on alloxan-induced cytotoxicity in HIT-T15 cells, a medium was prepared using RW instead of ultra pure water.

Cell culture

A hamster pancreatic β cell line, HIT-T15, was supplied by Dainippon Pharmaceutical Co. (Osaka, Japan). The cells were cultured in a RPMI 1640 medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 25 mM HEPES, 100 IU ml^{-1} penicillin-G and 100 μg ml^{-1} streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. The medium was exchanged every 2 days.

Measurement of cell viability

HIT-T15 cells (1×10^5 cells ml^{-1}) were seeded onto 24 well plates and pre-incubated in a 10% FBS/RPMI 1640 medium containing RW (sterilized with a 0.22 μm filter or autoclaved at 121 °C for 20 min) for 18 h. Alloxan dissolved in 0.2 mM citrate buffer (pH 4.0) was added to the cells that were then incubated for 1 h. The medium was exchanged for fresh medium containing RW and the cells incubated for 24 h. Cell viability was determined by the WST-8 Reduction Assay (Mossman et al., 1983). The absorbance at 450 nm was measured with a spectrophotometer.

Measurement of intracellular ROS

(A) Con-focal laser microscopic analysis
The amount of intracellular ROS, especially the intracellular H_2O_2 produced by alloxan was determined by using a fluorescent dye, 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) (LeBel et al., 1992). HIT-T15 cells were pre-cultured for 24 h in RPMI 1640 with or without RW. The cells were then incubated for 30 min in a KRB buffer with or without RW. After the addition of 1mM alloxan, the cells were incubated for a further 30 min. After removal of the supernatant, 5 μM DCFH-DA in a Ca^{2+}, Mg^{2+}-free HBSS buffer was added and the cells incubated for 60 min. The cytoplasmic fluorescence intensity was measured using a con-focal laser scanning microscope (Molecular Dynamics, U.S.A.) with a FITC barrier filter.

(B) DCFH-DA flow cytometric analysis
Cells treated as described in (A) were harvested by trypsinization, washed with PBS, resuspended in PBS and analyzed immediately using a flow cytometer (Coulter Elite FACSCAN) with excitation and emission wavelengths of 495 and 525 nm, respectively. Gating was performed to remove apoptotic cells and cellular debris before data were collected.

Measurement of cytosolic free Ca^{2+} concentration

The change of intracellular free Ca^{2+} concentration was analyzed using Fluo 3-AM by flow cytometric analysis. HIT-T15 cells were cultured for 24 h in a RPMI 1640 medium with or without RW, and then incubated for 30 min in a KRB buffer with or without RW. After the addition of 1 mM alloxan or 0.5 mM H_2O_2, the cells were incubated for 60 min. The medium was then removed and an HBSS buffer containing 4 μM Fluo 3-AM (Sigma) added. The cells were incubated for 60 min and then washed twice. The cytoplasmic fluorescence intensity of the cells was monitored, according to the manufacturer's instructions.

Measurement of DNA fragmentation

The cellular DNA fragmentation of HIT-T15 cells was determined according to the manufacturer instructions using a cellular DNA fragmentation ELISA Kit (Roche). Briefly, HIT-T15 cells (2×10^5 cells ml^{-1}) were pre-incubated in a RPMI 1640 medium containing RW (sterilized with a 0.22 μm filter or autoclaved at 121 °C for 20 min in a open bottle) for 24 h. The cells were then incubated with 10 mM BrdU overnight at 37 °C, centrifuged at 250 × g for 10 min, adjusted to 1×10^5 cells ml^{-1} in a KRB buffer containing RW, plated in a 96-multiwell plate. After treatment with 1 mM alloxan for 2 h, DNA fragmentation was determined with the ELISA Kit using a peroxidase-conjugated anti-BrdU antibody solution. The absorbance was measured at 450 nm against a substrate solution as a blank.

Measurement of intracellular ATP and insulin release

The concentration of intracellular ATP in HIT-T15 cells was determined by the luciferin-firefly luciferase method (Ludin, 1978). Briefly, HIT-T15 cells (2×10^5 cells ml^{-1}) were pre-incubated for 24 h in a RPMI 1640 medium with or without RW. The cells were then incubated for 30 min with a KRB buffer with or without 1mM alloxan. The alloxan-treated cells were washed and incubated for 60 min with a KRB buffer containing 10 mM glucose The spent medium was collected to examine the insulin release. The cells were trypsinized and resuspended in PBS. The cell suspension (0.1 ml) was immediately mixed with 0.9 ml of 100 mM Tris-HCl buffer (pH = 7.8) containing 4 mM EDTA. The relative amount of intracellular ATP was determined according to the manufacturer's instructions using an ATP assay Kit. For the measurement of insulin release, the amount of hamster insulin in the spent medium was determined by a competitive ELISA technique using a rat insulin assay Kit because of high homology between hamster and rat insulin.

Results and discussion

Effect of RW on cytotoxicity of alloxan in HIT-T15 cells

Alloxan itself is non-toxic. It is reduced extracellularly to dialuric acid in the presence of a reducing agent (e.g. cysteine). Oxidation of dialuric acid in the presence of oxygen results in the production of both superoxide anion radicals and hydrogen peroxide, the latter of which can diffuse across the plasma membrane and into the cell interior (Zhang et al., 1995; Szkudelski, 2001). Neither of these molecules is overly reactive, but they, in turn, lead to the production of the highly reactive hydroxyl radical (Halliwell et al., 1990), that is rapidly taken up by the pancreatic β-cells with consequent possible damage (Tomita et al., 1994). Alloxan is selectively toxic to pancreatic β-cells. HIT-T15 cells were grown in the presence of concentrations of alloxan ranging from 0.25 to 5 mM. The addition of alloxan to the culture medium caused a dose-dependent decrease in cell viability (data not shown). At a concentration of 1 mM, alloxan was able to kill about 48% of the cells compared with the untreated control. Hence, all further studies were conducted using this concentration of alloxan.

In order to evaluate the effect of RW on alloxan-induced cytotoxicity, HIT-T15 cells were pre-cultured for 18 h in media containing various waters and then exposed to 1 mM alloxan for 1 h. The cells were further cultured for 24 h. As shown in Figure 1a, 52.3% of the control cells treated with alloxan exhibited viability. On the other hand, the cells treated with Nordenau water, ERW and Hita Tenryosui water showed significantly increased viability of 88.7, 82.2 and 66.7%, respectively, after alloxan treatment. However, in contrast, two commercial natural mineral waters lowered the cell viability to 22.7 and 15.8% after alloxan treatment (Figure 1a). Since natural mineral water usually contains various kinds of metal ions and organic compounds, the cytotoxicity of alloxan might be potentiated by such substances in the commercial natural mineral waters examined. These results indicate that RW can partially protect HIT-T15 cells from alloxan-induced cytotoxicity. The protective activity was stable in a closed glass bottle at 4 °C for over a month. The activity was not lost even after neutralization and repeated filtration with a 0.22 μm filter. However, autoclaving of RW at 121 °C for 20 min resulted in the loss of the protective activity (Figure 1b). No significant difference between alloxan-treated HIT-T15 cells and alloxan/RW-treated cells was found, suggesting that active agents in RWs were lost by autoclaving. It has been reported that the ROS-scavenging activity of ERW is stable at 4 °C for more than one month and the activity is not lost by neutralization and filtration, but lost by autoclaving (Shirahata et al., 1997). These results support the active hydrogen theory that the reducing activity of RW like ERW and NRW is lost due to dehydrogenation of metal colloids or instability of metal colloids by autoclaving (Shirahata, 2002).

Effect of RW on intracellular redox state

Free radicals are believed to be involved in the cytotoxic action of alloxan on pancreatic β-cells. Hence, we observed the intracellular redox state of HIT-T15 cells treated with alloxan using an ROS-sensitive probe DCFH-DA. The membrane-permeable non-fluorescent fluorescein derivative, DCFH-DA (reduced 2′,7′-dichlorofluorescein diacetate) is cleaved by intracellular esterases to membrane-impermeable DCFH and oxidized by ROS like H_2O_2 in cells to 2′,7′-dichlorofluorescein (DCF), which is highly fluorescent. We monitored DCF fluorescence by using both a con-focal laser microscope and a flow cytometer. As shown Figure 2a, the con-focal microscopic analysis revealed that RW scavenged intracellular ROS

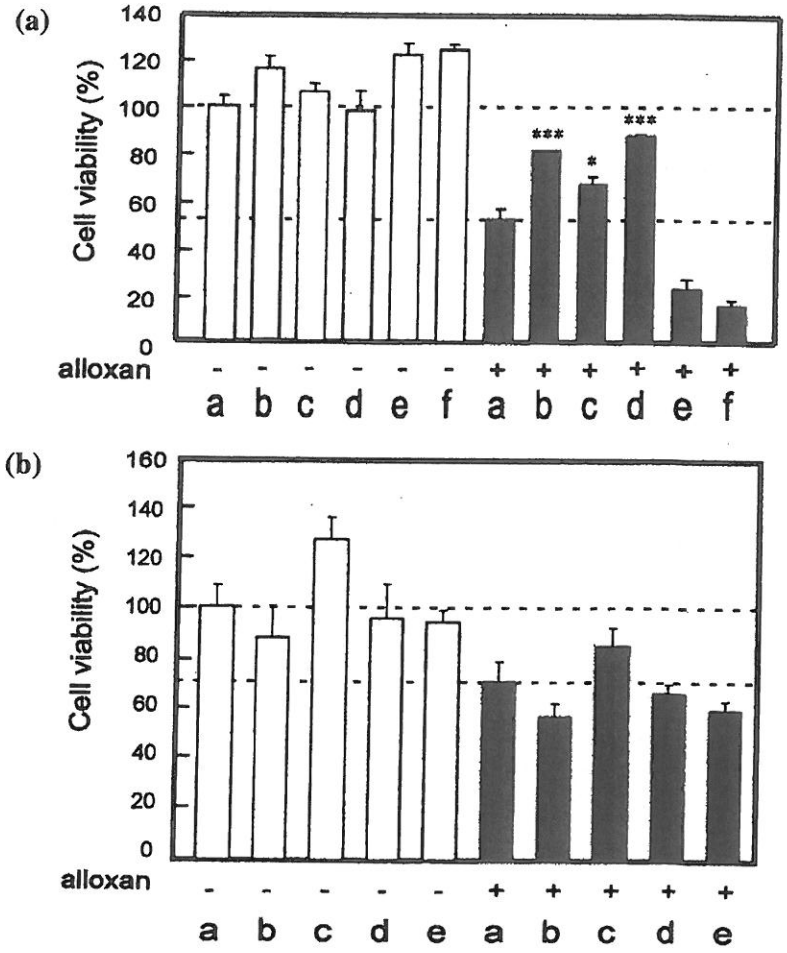


Figure 1. Effects of pretreatment with various waters on alloxan-induced cytotoxicity of HIT-T15 cells. (a) Effect of RW filtrated with 0.22 μm filter on alloxan-induced cytotoxicity. (b) Effect of RW treated with autoclaving at 121 °C for 20 min in an opened condition on alloxan-induced cytotoxicity. After cells were pre-incubated with various waters for 18 h and treated with 1mM alloxan for 1 h, the medium was removed. Incubation was continued in RPMI 1640 medium with or without various waters for 24 h. The WST-8 reduction assay was used for determining viability. The alloxan-untreatment and alloxan-treatment were shown by – and +, respectively. Each value denotes the mean ± S.D. of three separate experiments. Statistical analysis was done using the Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$, compared to alloxan untreated control. a, ultrapure water (control); b, ERW; c, Hita Tenryosui water; d, Nordenau water; e, commercial natural mineral water A; f, commercial natural mineral water B.

both in the absence and presence of alloxan. The commercial natural mineral waters did not exhibit the ROS-scavenging effect. Figure 2b shows the results of the DCFH-DA flow cytometric analysis. The RWs decreased the ROS level by about 35%. Alloxan elevated the ROS level of HIT-T15 cells by about 30%. RW-treated cells sustained 10–30% lower intracellular ROS level than that of non-treated cells, even after alloxan-treatment. Whereas, the commercial natural mineral waters did not suppress the intracellular ROS level and the cells treated with the natural mineral waters exhibited 40% higher ROS levels than non-treated cells after alloxan-treatment. These results clearly show that RW is anti-oxidative water that can scavenge intracellular ROS and protect β-cells from alloxan-induced ROS stress.

Effect of RW on cytosolic free Ca^{2+} concentration

It has become apparent that Ca^{2+} is important in both physiological and toxicological processes (Kim et al., 1994). The association of cytotoxicity with a sustained

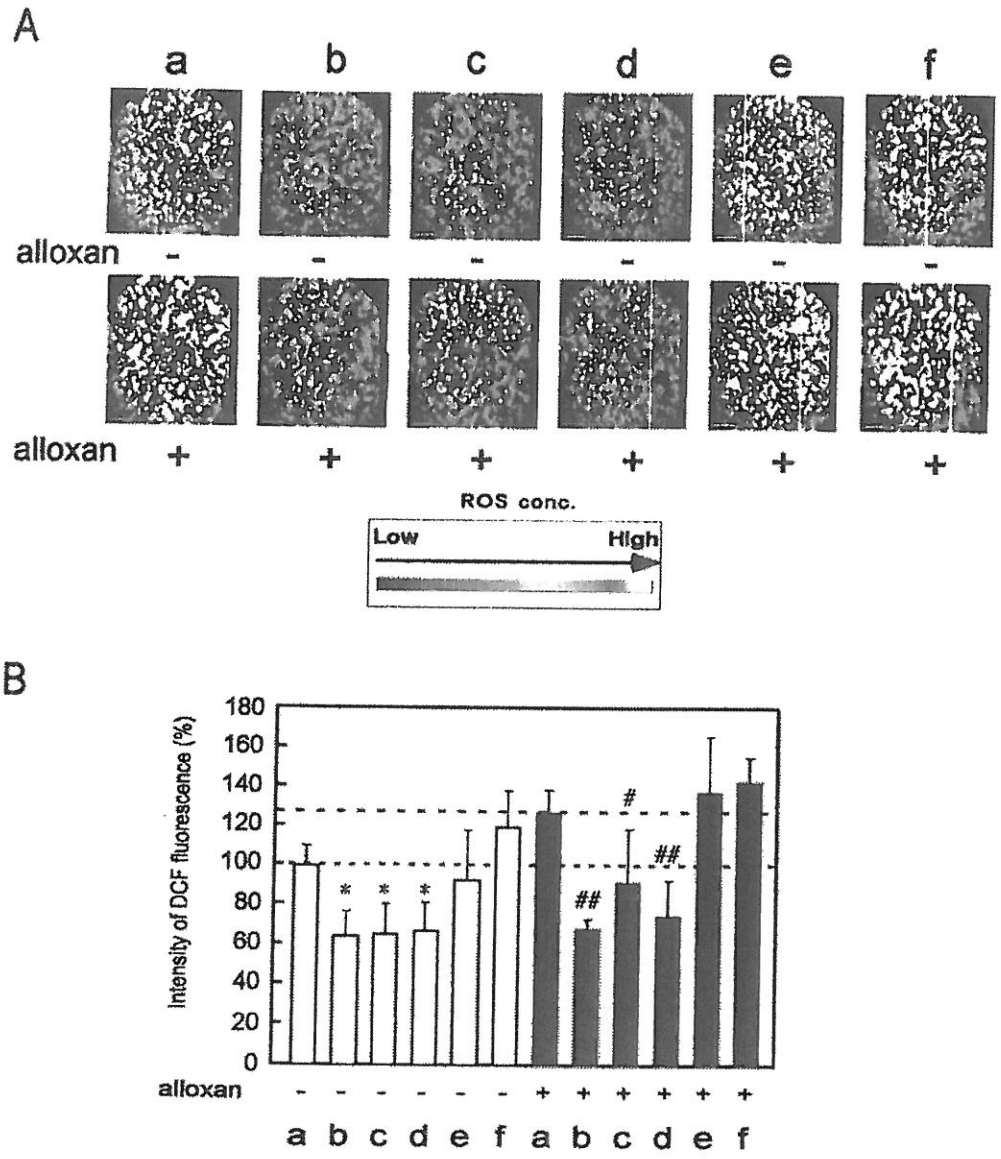


Figure 2. Effect of pretreatment with various waters on intracellular redox state of HIT-T15 cells treated with alloxan. (a) Con-focal laser microscopic analysis. After cells were pre-incubated with various waters for 24 h and treated with or without 1 mM alloxan for 30 min, the medium was removed. Incubation was continued with 5 μM DCFH-DA in HBSS buffer for 60 min. The change of cytoplasmic fluorescence intensity was measured by a con-focal laser microscope (a) and flow cytometer (b). Each value in (b) denotes the mean ± S.D. of three separate experiments. The alloxan-non-treatment and alloxan-treatment were shown by – and +, respectively. Statistical analysis was done using the T-test. * $P \leq 0.05$; ** $P < 0.01$, compared to non-treated control. # $P \leq 0.05$; ## $P \leq 0.01$, compared to alloxan-treated control. a, ultrapure water (control); b, ERW; c, Hita Tenryosui water; d, Nordenau water; e, natural mineral water A; f, natural mineral water B.

increase of cytosolic Ca^{2+}levels has been reported in many different cell types (Orrenius et al., 1989). The oxidative stress is accompanied by an increase in cytosolic free Ca^{2+} (Masumoto et al., 1990; Kim et al., 1994; Janciauskiene and Ahren, 1998). Alloxan-derived ROS may disturb intracellular Ca^{2+} homeostasis. This results in secondary reactions ultimately leading to DNA strand breaks and cytotoxicity of pancreatic β-cells (Kim et al., 1994; Szkudelski, 2001), suggesting the importance of the Ca^{2+}-dependent

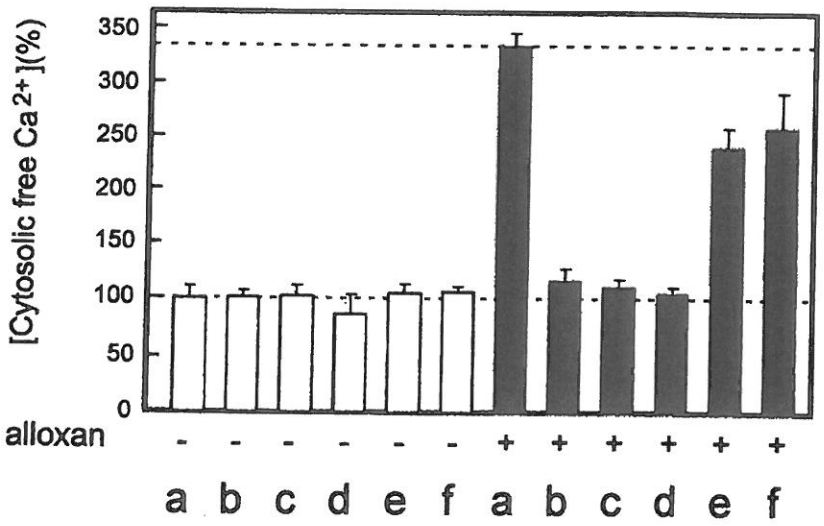


Figure 3. Effects of pretreatment with various waters on cytosolic free Ca^{2+} in alloxan-treated HIT-T15 cells. After cells were pre-incubated with various waters for 24 h and treated with 1 mM alloxan for 60 min, the medium was removed, and treated with 4 μM Fluo 3-AM (Sigma) in HBSS buffer at 37 °C for 60 min and then washed twice. The change of cytoplasmic fluorescence intensity of selected cells was monitored by flow cytometry. The alloxan-non-treatment and alloxan-treatment were shown by – and +, respectively. Each value denotes the mean ± S.D. of three separate experiments. a, ultrapure water (control); b, ERW; c, Hita Tenryosui water; d, Nordenau water; e, natural mineral water A; f, natural mineral water B.

pathway in cell damage. To verify the effect of RW on Ca^{2+}-influx, the change of cytosolic free Ca^{2+} concentration was traced using Fluo 3-AM in HIT-T15 cells. As shown in Figure 3, alloxan-treated cells exhibited 3.4 times higher concentration of cytosolic free Ca^{2+} than non-treated cells. The three kinds of RWs completely inhibited the alloxan-induced increase of cytosolic free Ca^{2+}. While, the two commercial natural mineral waters could not strongly suppress the alloxan-induced increase of cytosolic free Ca^{2+}. Similar results were obtained by using 0.5 mM H_2O_2 instead of alloxan (data not shown). These results suggest that the protective action of RW against alloxan-induced pancreatic β-cell damage is exerted primarily by inhibiting ROS generation before the change of Ca^{2+} influx.

Effect of RW on DNA fragmentation

It is known that alloxan can cause accumulation of enough ROS to induce an increase of Ca^{2+} influx, which results in secondary reactions ultimately leading to the fragmentation of DNA of β-cells (Yamamoto et al., 1981). Okamoto (1985) has proposed that the primary target of ROS produced from alloxan is the DNA of pancreatic β-cells, and that causes alloxan DNA strand breaks. The DNA fragmentation by alloxan is a critical step in the induction of alloxan-diabetes. In order to investigate the effect of RW on DNA strand break, the DNA fragmentation was measured using the ELISA method. As shown in Figure 4a, alloxan increased approximately 8-fold the extent of DNA fragmentation when HIT-T15 cells were treated with 1 mM alloxan for 2 h. However, the DNA fragmentation induced by alloxan was almost perfectly inhibited in HIT-T15 cells by RW. The two commercial natural mineral waters did not inhibit this DNA fragmentation. Autoclaving of RW resulted in almost complete loss of the protective activity of RW against the alloxan-induced DNA fragmentation (Figure 4b), because no significant difference was found between the values of alloxan-treated control and alloxan/RW-treated cells. These results suggest that the protective activity of RW against the alloxan-induced DNA fragmentation is lost by heating.

The DNA fragmentation induced by alloxan may be mediated by Ca^{2+}-dependent endonuclease, the activity of which is affected by ATP. This initiates the repair process involving the activation of poly (ADP-ribose) synthetase and the associated NAD utilization. Alloxan causes the depletion of cellular ATP and this is believed to be a result of a lack of NAD^+. It is believed that NAD depletion is so precipitous that it

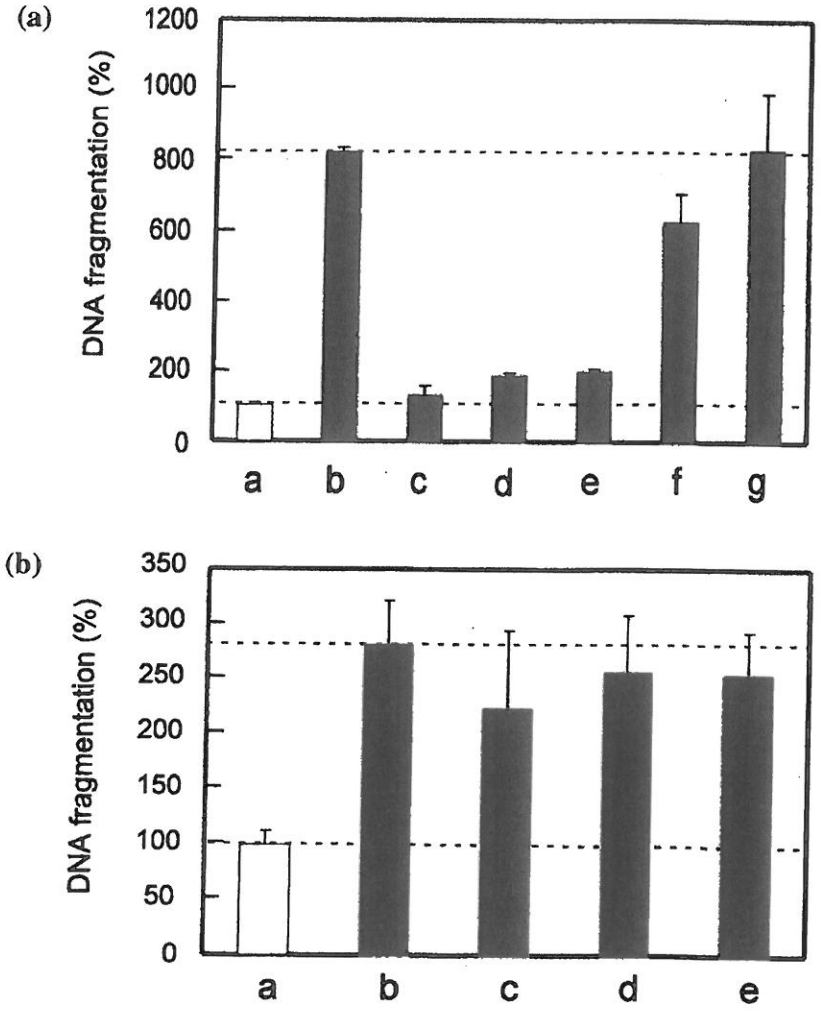


Figure 4. Effects of pretreatment with various waters on alloxan-induced DNA fragmentation in HIT-T15 cells. (a) Effect of RW on alloxan-induced DNA fragmentation. (b) Effect of autoclaved RW on alloxan-induced DNA fragmentation. Cells were pre-incubated with various waters for 24 h and treated with 1mM alloxan for 2 h. The amount of DNA fragmentation in 1×10^5 cells ml^{-1} was measured by ELISA. Each value denotes the mean ± S.D. of three separate experiments. a, ultrapure water (control); b, Control + alloxan; c, ERW + alloxan; d, Hita Tenryosui water + alloxan; e, Nordenau water + alloxan; f, natural mineral water A + alloxan; g, natural mineral water B + alloxan.

becomes irreversible and results in a virtual cessation of NAD-dependent metabolism leading to cell death. This is supported by the fact that nicotinamide supplementation and free radical quenchers can prevent alloxan-induced diabetes (Suresh et al., 2001).

Effect of RW on intracellular ATP and insulin release

ATP plays an important role in many cellular functions and the depletion of cellular ATP is a known hallmark for the deterioration of cell metabolism. Alloxan has a direct effect on islet cell permeability and acts at the site of hexose transport. It also interferes with the generation of glucose-derived energy by inhibiting glycolytic flux and pyruvate oxidation, and decreases ATP production (Borg et al., 1979). Alloxan depletion of the cellular ATP, leads to the opening of K^+_{ATP} channels and to cell membrane hyper-polarization. The latter event closes voltage-dependent Ca^{2+} channels, decreases $[Ca^{2+}]_I$ and suppresses Ca^{2+} oscillations, eventually leading to the inhibition of insulin secretion. Thus, we investigated the effect of RW on glucose-stimulated cellular ATP levels (Figure 5)

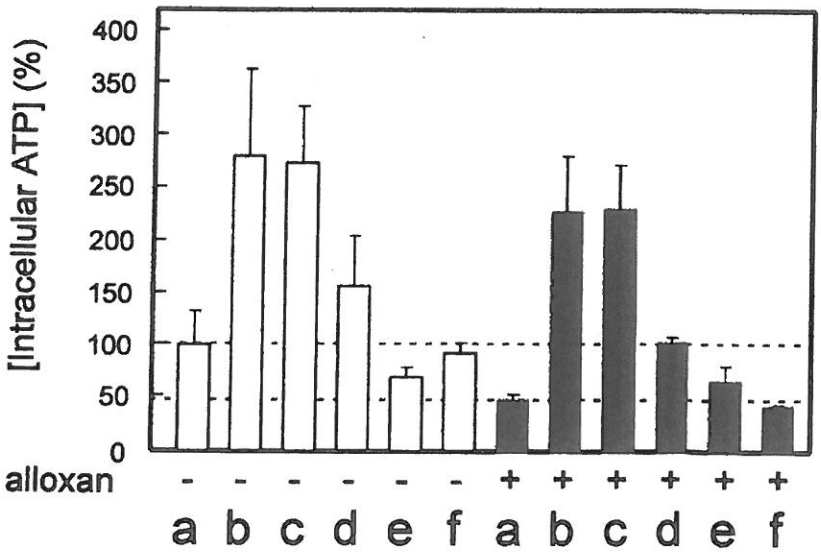


Figure 5. Effects of pretreatment with various waters on glucose-stimulated intracellular ATP level in HIT-T15 cells treated with alloxan. After cells were pre-incubated with various waters for 24 h and treated with 1 mM alloxan for 30 min, the medium was removed. After washing, the cells were incubated with KRB buffer containing 10 mM glucose for 60 min. The relative amount of intracellular ATP was determined using an ATP assay Kit by chemiluminescence method. The alloxan-non-treatment and alloxan-treatment were shown by – and +, respectively. Each value denotes the mean ± S.D. of three separate experiments. a, ultrapure water (control); b, ERW; c, Hita Tenryosui water; d, Nordenau water; e, natural mineral water A; f, natural mineral water B.

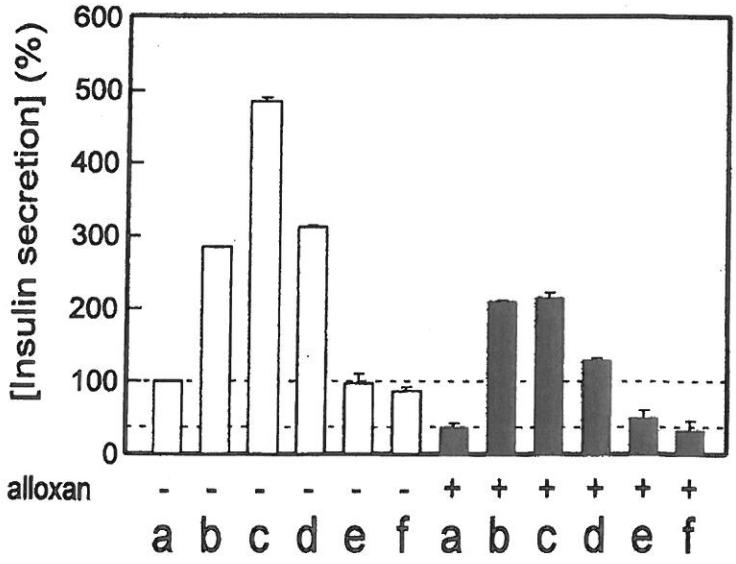


Figure 6. Effects of pretreatment with various waters on the glucose-stimulated insulin release in HIT-T15 cells treated with alloxan. Cells were pre-incubated with various waters for 24 h and treated with 1mM alloxan for 30 min. After washing, the cells were incubated with KRB buffer containing 10 mM glucose for 60 min. The released insulin in the spent medium was determined by radioimmunoassay with rat insulin as standard. The secreted insulin concentration of 100% was 2.38 ng ml^{-1}. The alloxan-non-treatment and alloxan-treatment were shown by – and +, respectively. Each value denotes the mean ± S.D. of three separate experiments. a, ultrapure water (control); b, ERW; c, Hita Tenryosui water; d, Nordenau water; e, natural mineral water A; f, natural mineral water B.

and the glucose-stimulated increase of insulin release (Figure 6). ERW and Hita Tenryosui water increased glucose-stimulated ATP levels to 270% and Nordenau water 150%. Alloxan decreased glucose-stimulated cellular ATP levels to 47% of the level of non-treated HIT-T15 cells. Even after alloxan treatment, ERW and Hita Tenryosui-treated cells sustained the high ATP level of 220% and Nordenau water-treated cells 100%. The commercial natural mineral waters did not exhibit such an enhancing effect on glucose-stimulated ATP level or protective effects against alloxan-induced damage. It is suggested that RW increased glucose-stimulated ATP production, leading to the closing of K^+_{ATP} channels and to cell membrane depolarization. The latter event may open voltage-dependent Ca^{2+} channels, increase $[Ca^{2+}]_I$ and eventually lead to the increase of insulin secretion.

As shown in Figure 6, Hita Tenryousui water, Nordeanu water and ERW enhanced glucose-stimulated insulin release to 480, 310, and 280% compared to that of non-treated cells. On the other hand, 1 mM alloxan decreased the glucose-stimulated insulin release of HIT-T15 cells to 36% of that of non-treated cells. Even after alloxan treatment, Hita Tenryosui water, ERW, and Nordenau water enhanced glucose-stimulated insulin release to 220, 210 and 130%. The commercial natural mineral water did not exhibit an enhancing effect on the glucose-stimulated insulin release and exhibited low insulin release level of 48 and 33% of that of non-treated cells after alloxan treatment. Because RW neither increased cellular ATP levels nor enhanced insulin release without glucose stimulation (data not shown), it is suggested that RW might activate the function of β-cells by increasing the glucose sensitivity and response of pancreatic β-cells against glucose. These results suggest that RW is effective for prevention of alloxan-induced IDDM.

Environmental agents modulate the incidence of IDDM, possibly by inducing the initial β-cell lesions. Numerous immunological and genetic studies including those with non-obese diabetic mice (Wogensen et al., 1994) have established that cellular and humoral autoimmunity against pancreatic β-cells is important in the pathogenesis of IDDM (Eisenbarth et al., 1986), presumably subsequent to β-cell injury by viruses and/or chemotoxins (Krowlewski et al., 1987). Cellular responses to such factors often involve the generation of ROS. Oxidative stress such as the production of ROS, especially the generation of H_2O_2 and hydroxyl radicals in cells have been implicated in numerous reports as a mechanism of cell death in a number of disease states.

This is the first paper reporting the protective effect of RW against pancreatic β-cells. We confirmed that RW could scavenge intracellular ROS and prevent alloxan-induced β-cell damage by ROS. Anti-oxidative water like RW would be beneficial in assisting the treatment of diabetes mellitus as well as prevention of diabetes, because water can rapidly permeate all the body, it has no calorie and the uptake of large quantities of water is possible. In clinical tests of Nordenau water on 139 diabetes patients, a downward trend of average blood sugar, HbA1c, cholesterol, triglyceride and LDL values was exhibited (Shirahata et al., 2001). Daily comsumption of RW may prevent not only environmental agent-induced cytotoxicity on β-cells and IDDM, but also many other diseases caused by ROS including cancer, arteriosclerosis, neural disease and allergies. The detailed action mechanism of the reducing agents in RW responsible for the ROS scavenging activity will be reported elsewhere.

Conclusion

(1) RWs like ERW, Hita Tenryosui water and Nordenau water were all anti-oxidative waters that could scavenge intracellular ROS in hamster pancreatic β-cells, HIT-T15.

(2) Alloxan induced lowering of viability, increase of cytosolic free Ca^{2+}, increase of DNA fragmentation, decrease of intracellular ATP, and decrease of glucose-stimulated insulin release. RW could inhibit all those cytotoxic effects of alloxan, increasing markedly the glucose-stimulated increase of ATP levels and insulin release.

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