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Metabolic Activity of Desalted Ground Seawater of Jeju in Rat Muscle and Human Liver Cells

Bo Youn Kim[1], Young Ki Lee[1] and Deok-Bae Park[1,2]*

[1]Department of Medicine, School of Medicine, Jeju National University, Jeju 690-756, Korea
[2]Institute of Medical Sciences, Jeju National University, Jeju 690-756, Korea

Abstract

Ground seawater in the east area of the volcanic Jeju Island contains abundant minerals. We investigated the metabolic activity of electrodialyzed, desalted ground seawater (EDSW) from Jeju in both cultured cells and animals. The addition of EDSW to the culture medium (up to 20%, v/v) reduced the leakage of lactate dehydrogenase and increased MTT activity in CHO-IR cells. EDSW (10%) promoted insulin-induced glucose consumption in L6 muscle cells as well as the activities of the liver ethanol-metabolizing enzymes, alcohol dehydrogenase and aldehyde dehydrogenase. Moreover, EDSW suppressed palmitate-induced intracellular fat accumulation in human hepatoma $HepG_2$ cells. Activities of AMP-stimulated protein kinase and acetyl CoA carboxylase, enzymes that modulate fat metabolism, were altered by EDSW in $HepG_2$ cells toward the suppression of intracellular lipid accumulation. EDSW also suppressed hepatic fat accumulation induced by a high-fat diet in mice. Taken together, EDSW showed beneficial metabolic effects, including the enhancement of ethanol metabolism and insulin-induced glucose consumption, and the suppression of intrahepatic fat accumulation.

Key words: Desalted ground seawater, Ethanol-metabolizing enzymes, Glucose consumption, Fat accumulation, Human hepatoblastonema, Chinese hamster ovary cell

Introduction

Water is essential for the maintenance of biological homeostasis and provides a suitable environment for living organisms. Drinking water contains a number of components that are considered to be important to human health. Deep-sea water in Japan and Hawaii has gained attention for its beneficial effects on the cardiovascular system and hyperlipidemia (Ueshima et al., 2003; Yoshioka et al., 2003; Miyamura et al., 2004; Katsuda et al., 2008). However, the mechanisms underlying these effects are not well understood. Deep-sea water is rich in mineral components, such as magnesium, calcium, and potassium, which act as essential cofactors in a number of biochemical processes. An insufficient supply of minerals caused by the civilized lifestyle and an imbalanced diet can cause major health problems, leading to lifestyle-related diseases, such as hyperlipidemia, hypertension, and diabetes (Havelaar and Melse, 2003).

Jeju province in Korea, which is a volcanic island, has characteristic geological features distinct from the mainland. Jeju possesses a clean groundwater resource because the volcanic Halla mountain acts as a water purification system as well as a huge water reservoir. Recently, we discovered a different water resource, ground seawater, which infiltrates to the surface of the east area of Jeju from the Pacific Ocean. The present study found that the mineral composition of this ground seawater is similar to that of the deep-sea water of Muruto Cape (Kochi, Japan) (Miyamura et al., 2004). We prepared desalted water by electrodialysis (electrodialysed seawater [EDSW]) from ground seawater and investigated its biological effects

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***Corresponding Author**
E-mail: parkdb@jejunu.ac.kr

in terms of ethanol metabolism, glucose uptake, and fat accumulation.

Materials and Methods

Materials

Ground seawater was pumped up from a depth of 130 m in Handong-ri, which is located 1.3 km inland from the northeast coast of Jeju (Fig. 1), Korea. Ground seawater was electrodialyzed using an electrodialysis system (CJTTS-2-10; Changjo Techno Co., Paju, Korea) to prepare EDSW deficient in monovalent ion species, such as sodium, potassium, and chloride ions (Table 1). Three EDSWs with electroconductivities of 8, 10, and 12 mS/cm were prepared. However, the data presented herein were obtained using 12 mS/cm EDSW because the majority of significant physiological activities in cells and experimental animals were more pronounced with the use of this fraction (Koh et al., 2008). Mineral content analysis of ground seawater and EDSW was performed by a custom analytical service (Korea Testing and Research Institute, Seoul, Korea)

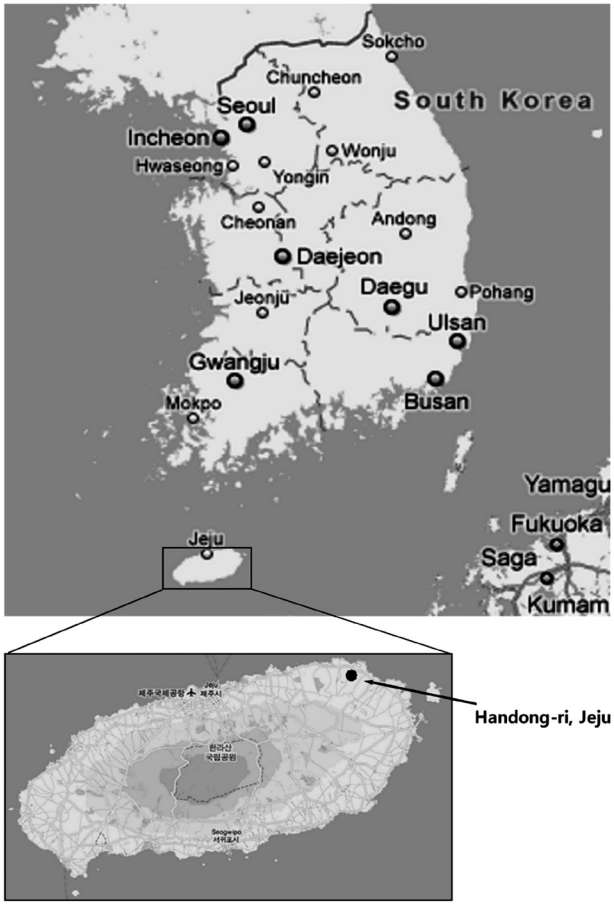

Fig. 1. Ground seawater sample site in Jeju, Korea.

(Table 1). Dulbecco's modified Eagle's medium (DMEM) Ham's F-12 medium, Dulbecco's phosphate-buffered saline (D-PBS), trypsin-EDTA solution, and fetal bovine serum (FBS) were obtained from Life Technologies Inc. (Rockville, MD, USA). Monoclonal and polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Electrophoresis reagents, such as gels, Tris-glycine sodium dodecyl sulfate (SDS) running buffer, and poly(vinylidene difluoride) (PVDF) membranes were from Koma Biotech (Seoul, Korea). Other reagents were from Sigma Chemical Corp. (St. Louis, MO, USA).

Cell culture

$HepG_2$ cells from human hepatoblastonema, Chinese hamster ovary cells which overexpress human insulin receptors (CHO-IR), and differentiated L6 rat skeletal muscle cells (L6 myotubes) were used. Cells were grown in DMEM ($HepG_2$ and L6) or Ham's F-12 medium (CHO-IR) containing 100 units/ml penicillin, 100 mg/mL streptomycin, and 10% FBS, and maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. Cells were grown to confluence (2×10^6 cells/35 mm^2) in 24-well (for lactate dehydrogenase [LDH] and MTT analysis) or 6-well (for oil red staining or Western blot analysis) plates and further treated with reagents.

Table 1. Mineral contents of ground seawater and electrodialyzed, desalted ground seawater (EDSW)

Elements	Deep seawater (koich, Japan)	Ground seawater (Jeju)	EDSW
Na	11,000	10,800	461.4
Mg	1,270	1,329	1,216
Ca	474	407	321
K	403	416	17.32
Cu	0.02	0.014	0.007
Mo	0.00773	0.011	0.003
V	-	0.022	0.013
Ge	-	0.001	<0.001
Se	<0.005	0.008	0.022
Br	80.8	61.2	11.8
Sr	8.03	8.36	7.28
SO_4^{2-}	2,570	1,691	584
$Si(Sio_2)$	-	10.8	11
Zu	<0.005	0.016	0.01
Fe	<0.03	0.01	0.022
Mn	<0.005	0.003	0.004
Cl^-	19,300	19,422	3,630
B	4.69	4.97	5.34
F	1.2	0.82	0.89
Hardness*	6,392	6,466	5,788

Numerical value represents the concentration (mg/L) of each element.
*Hardness: Ca (mg/L) x 2.5 + Mg (mg/L) x 4.1.

Animals

Male three-week-old ICR mice were purchased from Central Laboratory Animal Inc. (Seoul, Korea). Mice were divided into four groups (n = 9 per group) including an experimental group fed a high-fat diet (Rodent diet with 45% kCal fat; Dyets Inc., Bethlehem, PA, USA) and/or 10% EDSW (vol/vol). Filtered EDSW was provided *ad libitum* up to 15 weeks. Animals were maintained on a 12 h light/dark cycle in an air-conditioned facility. Body weight was measured every week and histological analysis of the liver was conducted by standard hematoxylin and eosin staining after 15 weeks.

Analysis of cell viability

MTT analysis was performed as described previously (Mosmann, 1983). Following treatment, media was removed from the wells, and 200 μL MTT reagent (Sigma) at a concentration of 1 mg/mL in RPMI-1640 medium without phenol red was added to each well. After 1 h incubation at 37°C, the cells were lysed by the addition of 1 volume 2-propanol and shaken for 20 min. Absorbance of converted dye was measured at a wavelength of 570 nm. Alternatively, cells were stained with a DNA-specific fluorescent dye (H33342) then observed under a fluorescent microscope equipped with a CoolSNAP-Pro color digital camera (Media Cybernetics, Silver Spring, MD, USA) to examine the degree of nuclear condensation. The quantity of LDH that had leaked from damaged cells into the culture medium was measured using reagents from Takara (Otsu, Shiga, Japan).

Measurement of glucose consumption

Glucose consumption by L6 muscle cells during a 24-h incubation was measured without the use of radiolabelled 2-deoxyglucose. The amount of glucose in the culture medium was measured using the glucose assay reagent (Sigma) before and after treatment, and the differences between groups were regarded as the amount of glucose consumed by muscle cells. Data were normalized to protein levels.

Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) assay

ADH and ALDH activities were measured as described previously (Bostian and Betts, 1978) with minor modifications. Briefly, S9 rat liver post-mitochondrial (cytoplasmic) homogenate (Moltox, Boone, NC, USA) was used as the source of ADH and ALDH fractions. S9 rat liver homogenate was dissolved in 0.1% bovine serum albumin and aseptically filtered. Two commercial hangover drinks (HOD1 and HOD2) were freeze-dried and used as positive test samples stimulating ADH and ALDH activities. The reaction mixture used for the ADH assay consisted of 0.25 M Tris-HCl (pH 8.8), 0.2 mM NAD^+, 1.7 M ethanol, 5% (v/v) ADH fraction, and test samples (3 mg/mL, HOD1 and HOD2) dissolved in distilled water or EDSW. The mixture was incubated at 30°C for 5 min, after which the absorbance at 340 nm was measured to determine the rate of NADH production. The ALDH assay reaction mixture consisted of 0.1 M Tris-HCl (pH 8.0), 0.7 mM NAD^+, 0.03 M acetaldehyde, 0.1 M KCl, 0.01 M 2-mercaptoethanol, 3% (v/v) ALDH fraction, and test samples (3 mg/mL, HOD1 and HOD2) dissolved in distilled water or EDSW. After incubation for 5 min at 30°C, the rate of NADH production from NAD^+ was determined by measuring absorbance at 340 nm.

Oil red staining

Intracellular fat droplets were stained with oil red. Briefly, cells were washed twice in D-PBS, fixed in 10% formalin for 10 min, and then stained with Oil Red O solution (0.3% in 60% isopropanol) for 10 min. Cells were washed in 60% isopropanol and digitally imaged.

Western blot analysis

Unless otherwise indicated, cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl, 1% nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 1 mM leupeptin, 1 mM pepstatin A). Identical amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis on 4-20% polyacrylamide gels and electrotransferred onto PVDF membranes. Membranes were then incubated in blocking buffer (5% nonfat dry milk in Tris-buffered saline [TBS]-0.1% Tween-20 [TBS-T]) for 1 h at room temperature and probed with different primary antibodies (1:1,000-1:5,000). After a series of washes, membranes were further incubated with different horseradish peroxidase-conjugated secondary antibodies (1:2,000-1:10,000). Signals were detected with an enhanced chemiluminescence detection system (Intron, Seoul, Korea).

Statistical analysis

Results are presented as the mean ± SD. Statistical significance was determined by a Student's t-test. Duncan's test was also used to assess the significance of differences between each group. A value of $P < 0.05$ was considered to indicate statistical significant.

Results

Mineral contents

Mineral contents of ground seawater and EDSW were compared to those of the deep-sea water produced in Koich, Japan. There was little difference in the mineral compositions of the

deep-seawater and ground seawater, except for the presence of oxidized silicon (SiO_2) (10.8 mg/L) and negligible vanadium, germanium, and selenium content in ground seawater (Table 1). Hardness of the seawaters was also similar. In the 12 mS/cm EDSW, more than 80% of the monovalent ions sodium (96%), potassium (96%), and chloride (82%) were removed after electrodialysis.

Effect of EDSW on cell viability

CHO-IR cells, which originated from non-tumorous epithelial cells (Tjio and Puck, 1958), were used to investigate any cytotoxic effects of EDSW. When CHO-IR cells were incubated in serum-free medium containing various EDSW doses for 48 h, MTT activity was significantly ($P < 0.01$) increased in cells incubated in 20% EDSW (Fig. 2B). Leakage of LDH from damaged cells was significantly decreased by the addition of EDSW (Fig. 2A). The number of cells with condensed nuclei, which is a marker of apoptotic cell death, was also reduced by EDSW (Fig. 2C). Interestingly, EDSW treatment caused visible cytotoxic insults (decreased MTT activity and increased LDH leakage) in $HepG_2$ cells, a hepatoblastonema cell line (data not shown). These results suggest that EDSW exerts different effects on cell viability, depending on whether the cell-line is normal or cancerous.

Effect of EDSW on glucose consumption in L6 muscle cells

Muscle is a primary target tissue for glucose transport through the actions of insulin receptors in the cell membrane, thereby contributing to the maintenance of normal blood glucose levels. We examined whether EDSW affects basal- or insulin-dependent glucose consumption in L6 muscle cells. The addition of 10% EDSW significantly ($P < 0.05$) stimulated both basal- and insulin-dependent glucose consumption (Fig. 3A). We also determined whether EDSW affected the phosphatidylinositol-3 kinase-protein kinase B (PKB/Akt) pathway, which is a central player in glucose transporter-4 (Glut4)-mediated glucose uptake. Although EDSW failed to stimulate basal PKB activity, it markedly augmented low-dose (1 nM) insulin-induced PKB stimulation (Fig. 3B). Activity of extracellular signal regulated kinase (ERK), a mitogen-activated protein kinase (MapK), was stimulated by EDSW, irrespective of the presence of insulin.

Effect of EDSW on ADH and ALDH

ADH and ALDH activities were measured using S9 rat liver homogenate enzymatic fractions (Fig. 4). EDSW stimulated basal activities of both enzymes by almost two-fold ($P < 0.01$). Solubilization of lyophilized hangover drinks (HOD1 and HOD2) in EDSW also augmented HOD1-stimulated ADH and ALDH activities ($P < 0.05$).

Effect of EDSW on lipid metabolism in $HepG_2$ cells

Incubation of $HepG_2$ cells in medium containing a 0.5 mM palmitate:oleate mixture (1:2) for 24 h induced intracellular fat accumulation (Fig. 5A). However, addition of 10% (v/v) EDSW to the culture medium suppressed the palmitate:oleate mixture-induced fat accumulation. We then determined whether EDSW affects AMP-stimulated protein kinase (AMPK) and acetyl CoA carboxylase (ACC) activities, two key enzymes in intracellular triglyceride biosynthesis in $HepG_2$ cells. Phosphorylation of AMPK and ACC was suppressed after treatment (2 h) with the palmitate:oleate mixture (Fig. 5B).

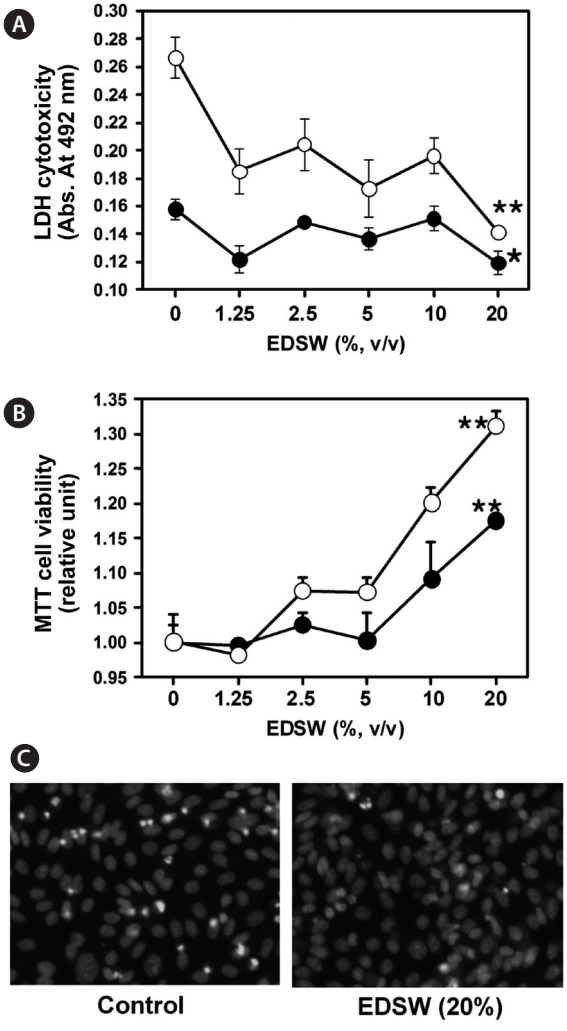

Fig. 2. Effect of electrodialyzed, desalted ground seawater (EDSW) on cellular viability in CHO-IR cells. Cells were serum-starved for 4 h and further incubated in serum-free medium containing different doses of EDSW (0-20%, vol/vol). Activities of lactate dehydrogenase (LDH) leakage (A) and MTT activity (B) were measured at 24 h (●) and 48 h (○) after EDSW treatment. Cells were also stained with H33342 to observe the degree of nuclear condensation after 48 h treatment (C). Data represent the mean ± SE ($n = 4$). $^{**}P < 0.01$, $^{*}P < 0.05$ significantly different from the results of control (non-treated cells).

However, EDSW (10%, v/v) restored the palmitate:oleate mixture-induced suppression of both enzymes, suggesting that EDSW suppresses AMPK and ACC activities, leading to the suppression of intracellular triglyceride biosynthesis.

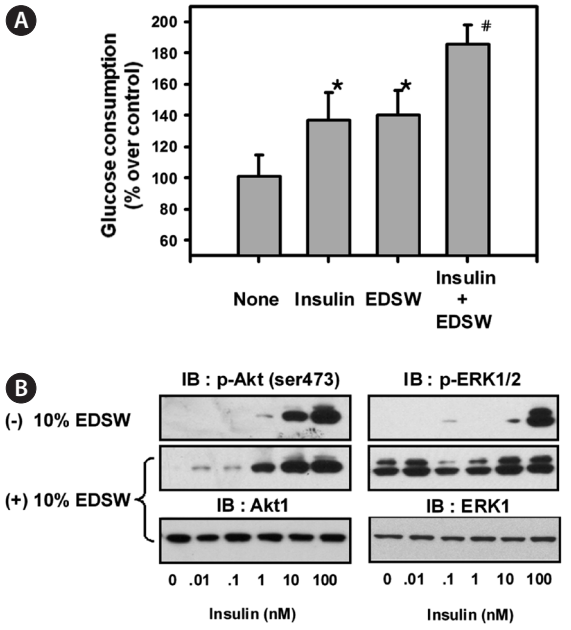

Fig. 3. Effect of electrodialyzed, desalted ground seawater (EDSW) on the glucose consumption and activities of Akt/extracellular signal regulated kinase (ERK) in L6 muscle cells. Serum-starved (4 h) cells were incubated in serum-free medium containing 10% (vol/vol) EDSW for 30 min and then further treated with 10 nM insulin for 10 min (B) or 24 h (A). Data represent the mean ± SE (n = 4) (A). $^{*}P < 0.05$, $^{\#}P < 0.05$, significantly different from the control (non-treated cells) and the insulin-treated group, respectively. Results were representatives of three different experiments (B).

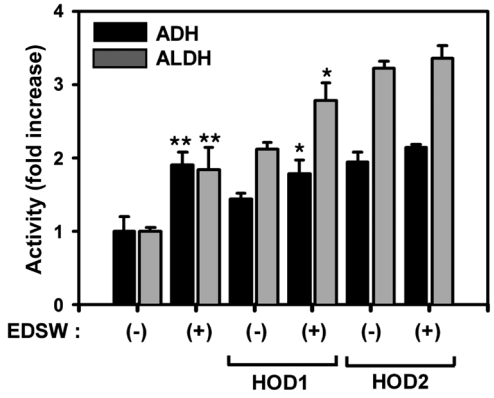

Fig. 4. Effect of electrodialyzed, desalted ground seawater (EDSW) on activities of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). Data represent the mean ± SE (n = 4). $^{**}P < 0.01$, significantly different from the results of control. $^{*}P < 0.05$, significantly different from the results of the HOD1-alone. Two commercialized hangover drinks (HOD1 and HOD2) were freeze-dried and then dissolved in distilled water or 10% EDSW (v/v) for ADH and ALDH assays.

Animal study

We investigated whether the effect of EDSW on fat accumulation might also occur in the liver of experimental animals. ICR mice were allowed free access to a high-fat diet and 10% (v/v) EDSW or tap water (control) for 15 weeks. The high-fat diet lead to a 13.9% increase in body weight (48.9 ± 5.0 g) compared to that of control mice (42.9 ± 1.3 g) after 15 weeks; this was not significantly reduced by EDSW administration (Fig. 6A). However, hepatic fat accumulation was observed in animals fed a high-fat diet and was suppressed by EDSW administration (Fig. 6B).

Discussion

EDSW with 12 mS/cm electroconductivity, which was prepared by electrodialysis of Jeju ground seawater, lacks monovalent ions (sodium, potassium, and chloride), but contains abundant divalent cations including magnesium and calcium. The presence of microelements (vanadium and selenium) in EDSW is also of interest due to their rarity in other water resources. Recently, the physiological importance of mineral components has been stressed for the amelioration of a

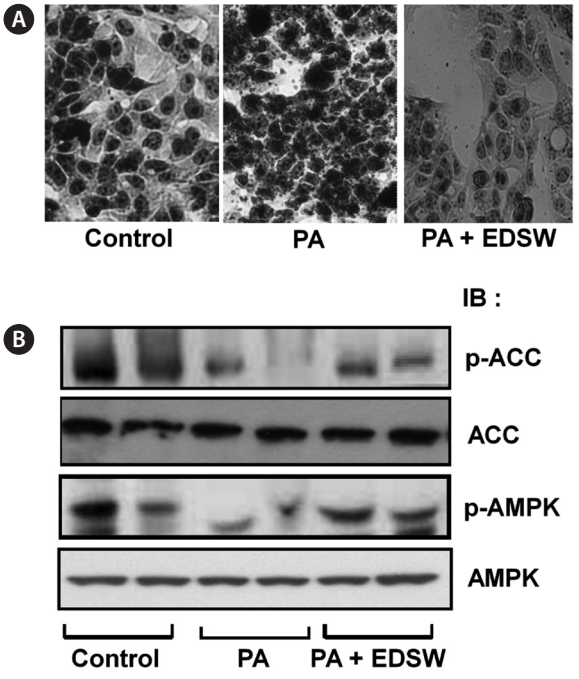

Fig. 5. Effect of electrodialyzed, desalted ground seawater (EDSW) on palmitate (PA)-induced lipid accumulation and the phosphorylation of acetyl CoA carboxylase (ACC) and AMP-stimulated protein kinase (AMPK) in $HepG_2$ cells. Serum-starved (24 h) $HepG_2$ cells were incubated in serum-free medium containing 10% EDSW (vol/vol) for 30 min and further treated with 0.5 mM palmitate:oleate mixture (1:2) for 24 h (A) or 2 h (B). After treatment, cells were stained with Oil Red O (A) or lysed for western blot analysis as described in 'Materials and Methods'.

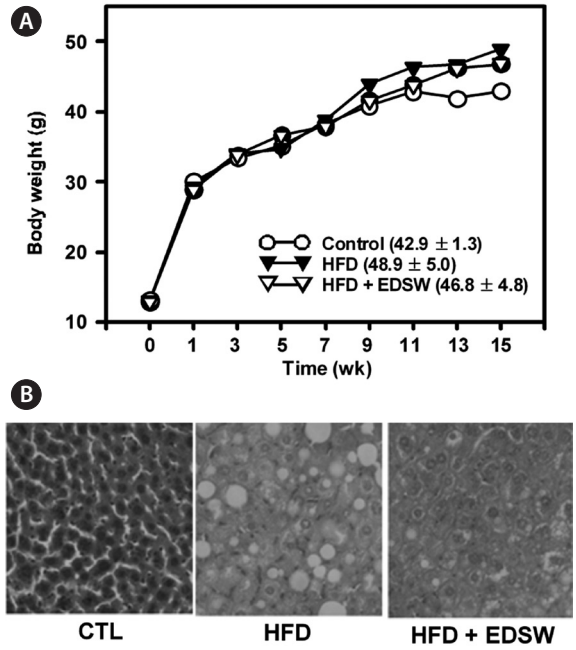

Fig. 6. Effect of electrodialyzed, desalted ground seawater (EDSW) on the body weight change and intrahepatic fat accumulation in ICR mice. Three weeks-aged male ICR mice were allowed to free access to different diets as indicated for 15 weeks. Body weight gains were checked every week (A) and the hematoxylin-eosin staining of liver was conducted after 15 weeks (B). HFD, high-fat diet; CTL, control.

number of chronic metabolic diseases, such as hypertension (Rylander and Arnaud, 2004), hyperlipidemia (Chen and Lin, 2000; Schoppen et al., 2005), and diabetes mellitus (Barbagallo et al., 2007).

Our data show that EDSW promoted the proliferation of non-cancerous CHO-IR cells, but accelerated apoptotic death of hepatoblastonema $HepG_2$ cells. The reason for this discrepancy in modulating cellular viability in different cell types remains unclear. Previous studies have focused on the role of divalent cations, especially Mg^{2+}, as critical cofactors for several enzymes. Although Mg^{2+} promoted citrus flavone tangeretin-induced inhibition of leukemic HL-60 cell growth with less cytotoxicity to normal lymphocytes (Hirano et al., 1995), little is known regarding the direct inhibition of cell growth by Mg^{2+}. Mg^{2+} concentration is critical for phosphorylation of the tyrosine kinase of several protein kinases (Barbagallo and Dominguez, 2007). It was notable that 10% EDSW stimulated basal ERK activity, a mitogenic MapK, in L6 muscle cells. However, it failed to stimulate the basal activity of PKB/Akt, but augmented insulin-stimulation of PKB/Akt. These results suggest that the abundant Mg^{2+} in EDSW stimulates the intrinsic mitogenic enzyme, ERK, thereby promoting CHO-IR cell proliferation. Failure to stimulate the basal activity of PKB/Akt may explain why Mg^{2+} has a limited role in phosphorylating tyrosine residues of insulin receptors, but has an important role in maintaining their tyrosine phosphorylation status. EDSW increased both basal- and insulin-induced glucose consumption of L6 muscle cells. Insulin-induced glucose uptake in muscle is dependent on the localization of Glut4 from the cytosol to the plasma membrane (Antonescu et al., 2009). Thus, stimulation of basal glucose consumption by EDSW suggests that insulin/Glut4-independent pathways are activated by EDSW. Muscle contraction also stimulates glucose uptake independent of insulin receptors through AMPK activation and Glut4 translocation (Santos et al., 2008). In the present study, EDSW stimulated phosphorylation of AMPK and ACC in $HepG_2$ cells. It is likely that insulin-independent glucose consumption by EDSW might arise from AMPK activation and subsequent Glut4 translocation. The additive increase in glucose uptake by insulin and EDSW supports such a possibility. The precise mechanism underlying these effects will be investigated further.

Divalent cations stabilize yeast ADH (De Bolle et al., 1997), and removal of magnesium inactivates ADH completely (Niefind et al., 2003). The binding affinity of *Saccharomyces cerevisiae* cytosolic ALDH for NADP+ is increased by approximately 100-fold in the presence of Mg^{2+} (Dickinson, 1996). Since EDSW contains abundant Mg^{2+}, its effects on ADH and ALDH activities were investigated. Basal ADH and ALDH activities were increased approximately two-fold by EDSW. Moreover, solubilzation with EDSW enhanced enzymatic activities in one (HOD1) of two commercial hangover drinks, suggesting a role for the Mg^{2+} in EDSW.

Pharmacological activities of deep-sea water have been reported in hyperlipemia (Yoshioka et al., 2003), atherosclerosis (Miyamura et al., 2004), cardiovascular hemodynamics (Katsuda et al., 2008), and fibrinolytic activity (Ueshima et al., 2003). The present study determined a role for EDSW on non-alcoholic hepatic steatosis, a feature of metabolic syndrome. Incubation of $HepG_2$ cells with 0.5 mM palmitate induced intracellular fat accumulation within 24 h. Exposure to palmitate over 24 h lead to cell death, due to palmitate-induced lipotoxicity. However, addition of EDSW (10%) to the medium prevented excess cytoplasmic fat accumulation for 24 h. Western blot analyses showed that AMPK and ACC phosphorylation were blocked by palmitate, but restored by EDSW, suggesting a beneficial effect of EDSW in the control of hepatic steatosis. In order to support this notion, the effect of EDSW on hepatic steatosis was examined in a high-fat diet mouse model. A high-fat diet increased body weight, but free access to EDSW for 15 weeks inhibited this effect, although not significantly. As shown in $HepG_2$ cells, EDSW reduced hepatic intracellular fat accumulation.

An association between mortality from ischemic heart disease and drinking water characteristics was first shown in Japan (Kobayashi, 1957). Since then, it has been suggested that a number of diseases are related to a lack of essential minerals, especially cardiovascular and metabolic diseases. Reductions in the global water supply as well as the severe contamination of water resources threaten human health. Oceanic deep-

seawater or ground seawater has been shown to be clean, mineral-rich, and safe. After previous reports on deep-sea water and the physiological roles of minerals, this study developed a new water resource, ground seawater in Jeju, Korea, and investigated the physiological effects of EDSW. EDSW showed a number of beneficial activities in glucose, lipid, and ethanol metabolism. Although the exact mechanisms underlying these effects remain to be elucidated, some mineral ingredients, particularly the abundant Mg^{2+} in EDSW, may play crucial roles in such health-promoting effects.

Acknowledgments

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