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The Anti-obesity Effect of Natural Vanadium-Containing Jeju Ground Water

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Abstract This study investigated the anti-obesity effects of Jeju ground water containing the vanadium components S1 (8.0±0.9 μg/l) and S3 (26.0±2.09 μg/l) on the differentiation of 3 T3-L1 preadipocytes and obesity in mice that were fed a high-fat diet (HFD). The 3 T3-L1 preadipocyte cells were cultured and differentiated in media consisting of Jeju ground water (S1, S3) or deionized water (DW) containing dexamethasone, isobutylmethylxanthine, and insulin. Oil Red O staining showed that lipid accumulation was attenuated in adipocyte cells treated with Jeju ground water. S3 significantly decreased peroxisome-activated receptor γ and CCAAT-enhancer-binding protein α mRNA expression levels, which play major roles in the transcriptional control of adipogenesis, compared to DW. Furthermore, mRNA expression levels of targeted genes, such as adipocyte fatty acid, lipoprotein lipase, and leptin, were decreased by S3 treatment compared with the control group. In mice with HFD-induced obesity, Jeju ground water decreased HFD-induced body weight gain and reduced total cholesterol, triglyceride, and glucose levels in the plasma compared to control mice. Taken together, Jeju ground water inhibits preadipocyte differentiation and adipogenesis in obesity animal models.

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Introduction

Obesity is a prevalent nutrition-related health risk associated with pathological disorders, type 2 diabetes, hypertension, cancer, and heart disease [1, 2]. Obesity is caused by the accumulation of adipose tissue composed of differentiated adipocyte cells, which leads to an increased amount of fat and an enlarged adipocyte size [3]. Research on adipogenesis has been conducted using in vitro model systems in several adipogenic cell lines. Adipogenesis is unique in that the cells become rounded and is accompanied by an increased expression of transcriptional factors and adipocyte-specific genes such as peroxisome-activated receptor γ (PPARγ) and CCAAT-enhancer-binding protein α (C/EBPα) [4, 5]. Cross-regulation is a key component of the transcriptional control of adipogenesis. These factors direct the final phase of adipogenesis by activating the expression of adipocyte-specific genes and regulating adipocyte differentiation by modulating the expression of their target genes in a coordinated fashion [6–8].

Inhibition of adipocyte differentiation is used as a therapeutic trial for prevention and treatment of obesity. Previous studies have investigated the inhibitory effects of natural products and drinking water on adipocyte differentiation. Natural vanadium-containing Mount Fuji ground water improves hyperglycemia by reducing the liver insulin receptor activity in rats [9]. Recently, several reports have suggested that Jeju ground water stimulates glucose uptake by activating AMP-activated protein kinase in L6 myotubes [10] and enhancing antioxidant systems by scavenging reactive oxygen species (ROS) in human liver cells [11]. This study was performed to investigated the anti-obesity effect of Jeju ground water containing vanadium components on

adipogenesis of 3 T3-L1 preadipocytes and mice fed a high-fat diet (HFD). To better understand the cellular and molecular functioning mechanisms of Jeju ground water on adipocyte differentiation, the mice embryo fibroblast 3 T3-L1 cells were differentiated to adipocytes with adipogenic medium made with Jeju ground water containing insulin, dexamethasone, and isobutylmethylxanthine (IBMX). Quantitative real-time PCR and Western blot demonstrated that Jeju ground water decreased the expression levels of PPARγ and C/EBPα, as well as other transcriptional factor targeted genes. Based on the in vitro results, we performed an in vivo study in a HFD-induced obesity mouse model, and provided the mice with either DW or Jeju ground water and analyzed the concentration of total cholesterol, triglyceride, and glucose in plasma.

Materials and Methods

Materials

Jeju ground water (S1, S3) was provided by the Jeju Special Self-Governing Province Development Corporation (Jeju, Korea). Mice fibroblast preadipocyte cell line 3 T3-L1 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco modified Eagle's medium (DMEM), bovine calf serum (BCS), and fetal bovine serum (FBS) were obtained from Gibco-BRL (Grand Island, NY, USA). Dexamethasone, isobutylmethylxanthine, insulin, vanadyl sulfate hydrate ($VOSO_4 \cdot xH_2O$), and Oil Red O were acquired from Sigma-Aldrich (St. Louis, MO, USA). TRIzol reagent and the SuperScript First-Strand Synthesis System for RT-PCR kit were purchased from Invitrogen (Carlsbad, CA, USA). Brilliant II SYBR Green QPCR Master Mix was obtained from Stratagene (La Jolla, CA, USA). Antibodies were purchased from Cell Signaling (Beverly, MA, USA), and horseradish peroxidase-conjugated goat anti-rabbit IgG was purchased from Sigma-Aldrich. Table 1 shows mineral and vanadium composition of distilled water (DW) and Jeju ground water (S1, S3) used in this study. Cation (Ca, Mg, Na, and K) was measured by ICP-720ES coupled plasma-optical emission spectrometry (Varian, USA). Anion (Cl and SO_4) was measured by ICS-2000 (Ion Chromatography System; Dionex, USA). Vanadium was measured by ICP-820MS coupled plasma-mass spectrometry (Varian).

Cell Culture and Differentiation

3 T3-L1 preadipocyte cells were cultured in DMEM containing 1 % penicillin–streptomycin and 10 % BCS at 37 °C in a humidified atmosphere of 5 % CO_2. For the cell viability assay, 3 T3-L1 preadipocyte cells were cultured with DW and Jeju ground water containing 10 % BCS for 10 passages, then seeded in a 96-well plate at a concentration of 1×10^5 cells/ml, and incubated for an additional 24–48 h at 37 °C. Then 10 μl of MTT solution (5 mg/ml) was added to each well for a reaction volume of 100 μl for 3 h. Formazan crystals were dissolved in isopropanol containing hydrochloric acid and the absorbance was measured at 570 nm. To induce differentiation, 3 T3-L1 cells were grown until reaching confluency and incubated with a mixture of 0.25 μM dexamethasone, 0.5 mM IBMX, and 1 μg/ml insulin in DMEM made with DW or Jeju ground water containing 10 % FBS. After 2–3 days, the induction media was replaced with 10 % FBS/DMEM and supplemented with 1 μg/ml insulin for an additional 2 days. The adipocytes were then kept in media containing 10 % FBS until experimentation.

Table 1 Mineral contents of distilled water (DW), S1, and S3 used in this study

Mineral	DW	S1	S3
Ca (mg/l)	0.035±0.005	3.2±0.3	4.1±0.6
Mg (mg/l)	<0.002	2.7±0.1	2.4±0.2
K (mg/l)	<0.002	2.2±0.1	6.5±0.1
Na (mg/l)	<0.005	5.7±0.5	24.8±1.6
Cl (mg/l)	<0.001	5.9±0.3	8.6±0.7
SO_4 (mg/l)	<0.001	1.6±0.2	3.8±0.4
Vanadium (mg/l)	0	8.0±0.9	26.0±2.1

Measurement of Lipid Accumulation

Adipocyte differentiation was monitored by measuring cellular lipid accumulation using Oil Red O staining. 3 T3-L1 adipocytes differentiated on 12-well plates were washed twice with phosphate-buffered saline (PBS), fixed with 10 % formalin at room temperature for 1 h, washed with 60 % isopropanol, completely dried, and then stained with Oil Red O solution (six parts 0.5 % Oil Red O in isopropanol to four parts water) for 1 h. The images were photographed under a microscope (Nikon, Tokyo, Japan). Stained lipid droplets were dissolved in isopropanol for 10 min and quantified by measuring the absorbance at 500 nm.

Real-Time Quantitative RT-PCR

Total RNA was extracted from differentiated 3 T3-L1 cells using TRIzol reagent according to the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA with the SuperScript First-Strand Synthesis System for RT-PCR kit. The quantitative real-time PCR was performed with Brilliant II SYBR Green QPCR Master Mix using the MX3000P real-time PCR system (Stratagene). The specific

primer sets for adipogenic markers are listed in Table 2. Relative amounts of mRNA levels were calculated using the comparative cycle threshold (C_T) method [12].

Western Blot Analysis

Differentiated 3 T3-L1 cells were washed twice with PBS and lysed in lysis buffer containing 25 mM Tris–HCl (pH 7.2), 0.1 % sodium dodecyl sulfate (SDS), 0.1 % Triton X-100, 1 % sodium deoxycholate, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride (PMSF), 10 μg/ml aprotinin, and 5 μg/ml leupeptin for 20 min on ice. Equal amounts of cell lysate were separated on a 12 % SDS–polyacrylamide gel and transferred to a polyvinylidene fluoride film (Pall Corporation, East Hills, NY, USA). Antibodies against PPARγ, C/EBPα, and adiponectin were used as primary antibodies, and horseradish peroxidase-conjugated goat anti-rabbit IgG was used as the secondary antibody. Proteins were visualized using an enhanced chemiluminescence-based detection system (Amersham-Pharmacia Biotech, Piscataway, NJ, USA). To detect the secreted adiponectin in the culture media, differentiated 3 T3-L1 adipocytes after 10 days of induction were added to serum-free media. The secretion of adiponectin in collected culture media was determined using Western blot analysis.

Animal Experiments

Animal procedures were approved by the ethics committee of Chosun University of Korea. Eight-week-old male C57BL/6 mice were obtained from Orientbio Inc. (Seongnam, South Korea) and housed in a controlled environment at a humidity of 50±5 % and a temperature of 22±2 °C with 12-h light/dark cycles. After adaptation for 1 week, the mice were divided randomly into groups. One group was provided with tap water and another group with Jeju ground water (S1, S3) with HFD. Body weight was monitored and measured once per week. After 9 weeks, mice were fasted overnight and sacrificed. Blood was collected from the abdominal aorta and immediately mixed with EDTA. Serum was isolated from whole blood by centrifugation at 3,000 rpm for 20 min and analyzed the plasma levels of total cholesterol, glucose, and triglycerides.

Statistical Analysis

Student's *t* test and one-way analysis of variance were used to analyze the differences between values obtained in the experimental and control conditions. $P < 0.05$ was considered significant.

Results

Effect of Jeju Ground Water on Intracellular Lipid Accumulation in 3 T3-L1 Adipocytes

Jeju ground water (S1, S3) is composed of various minerals (Table 1). The cell viability and cytotoxicity of 3 T3-L1 preadipocytes were determined using the MTT assay. Cells were cultured with DW or S1, S3 for 24 to 48 h. As shown in Fig. 1a, Jeju ground water had no significant cytotoxicity against preadipocyte cells. To investigate the effect of Jeju ground water on adipocyte differentiation, 3 T3-L1 preadipocyte cells were incubated with culture media made with DW or S1, S3, each containing dexamethasone, IBMX, and insulin, for 10 days. The preadipocytes originally showed a fibroblastic appearance without cellular lipid droplets and then gradually enhanced lipid accumulation during differentiation. S1 and S3 reduced the accumulation of lipid droplets compared to DW (Fig. 1b). To confirm these observations, differentiated cells were stained with Oil Red O solution and then quantified by measuring the absorbance at 500 nm. The staining revealed that S3 significantly decreased cell differentiation (Fig. 1b), which suggests that Jeju ground water inhibits 3 T3-L1 adipocyte differentiation. Because the concentration of vanadium in Jeju ground water (S1, S3) is higher than in other ground water, we next investigated the effects of vanadium ($VOSO_4$) on 3 T3-L1 adipocyte differentiation. As shown in Fig. 1c, $VOSO_4$ decreased 3 T3-L1 adipocyte differentiation, suggesting that suppression of adipocyte differentiation by Jeju ground water might be attributed to vanadium.

Table 2 Gene-specific primers used for real-time PCR

Gene	Forward primer	Reverse primer
PPARγ	GGTGAAACTCTGGGAGATTC	CAACCATTGGGTCAGCTCTT
C/EBPα	AGGTGCTGGAGTTGACCAGT	CAGCCTAGAGATCCAGCGAC
Pref-1	CTAACCCATGCGAGAACGAT	GCTTGCACAGACACTCGAAG
aP2	TCACCTGGAAGACAGCTCCT	AATCCCCATTTACGCTGATG
LPL	TCCAAGGAAGCCTTTGAGAA	CCATCCTCAGTCCCAGAAAA
Leptin	GGATCAGGTTTTGTGGTGCT	TTGTGGCCCATAAAGTCCTC
β-Actin	CCACAGCTGAGAGGGAAATC	AAGGAAGGCTGGAAAAGAGC

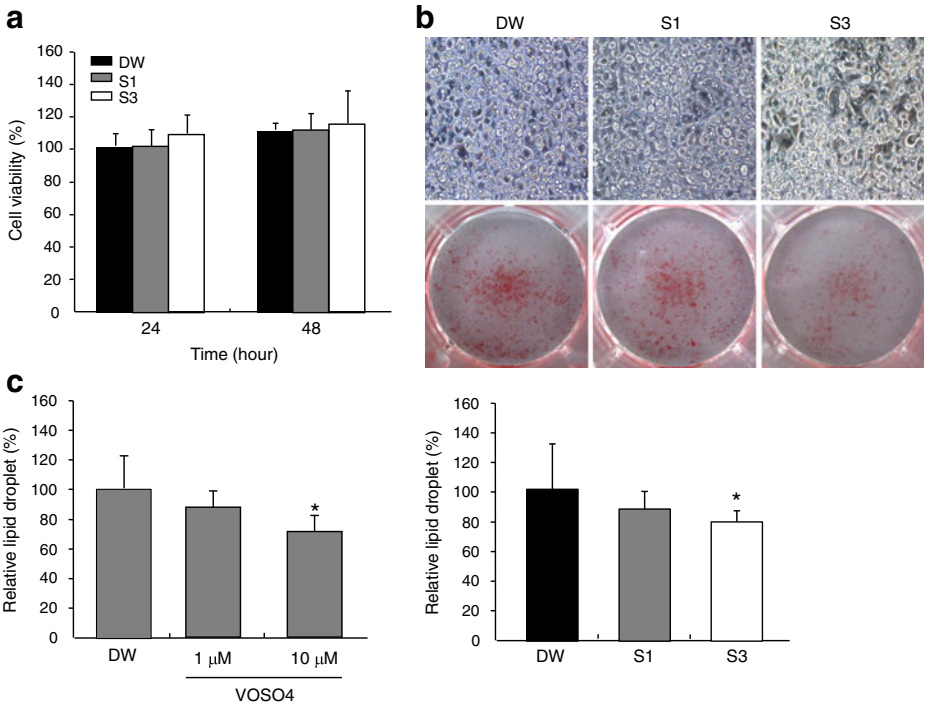

Fig. 1 Effect of Jeju ground water on 3 T3-L1 adipocyte differentiation. **a** Cytotoxicity of JW on 3 T3-L1 preadipocytes. Cells (1×10^4 cells/well) were incubated with DW or S1, S3 for 24 h and 48 h. Cell viability was determined using the MTT assay. **b** Morphological changes in the cell were photographed 10 days after induction of differentiation with DW, S1, and S3. Cells were stained with Oil Red O for intracellular lipid accumulation. The results represent the means ± SD from ten separate experiments. $^{*}P<0.05$ compared with cells treated with DW. **c** $VOSO_4$ (10 μM) treatment decreased intracellular lipid accumulation. The results represent the means ± SD from ten separate experiments. $^{*}P<0.05$ compared with cells treated with DW

Effect of Jeju Ground Water on PPARγ and C/EBPα mRNA Expression

PPARγ and C/EBPα are known to be adipogenic transcriptional factors. Cross-regulation between both genes is a key component of the transcriptional control of adipogenesis [13]. The effect of Jeju ground water on the regulation of PPARγ and C/EBPα mRNA expression was analyzed by quantitative real-time PCR. mRNA expression levels of PPARγ and C/EBPα were reduced in S1- and S3-incubated 3 T3-L1 adipocytes compared to DW-incubated cells (Fig. 2a). We also evaluated PPARγ and C/EBPα protein expression levels by Western blotting. Consistent with our previous results, the expression levels of PPARγ and C/EBPα protein were decreased by S1, S3 (Fig. 2b). Real-time PCR and Western blotting confirmed that Jeju ground water plays a role in 3 T3-L1 preadipocytes to adipocyte differentiation by down-regulating PPARγ and C/EBPα.

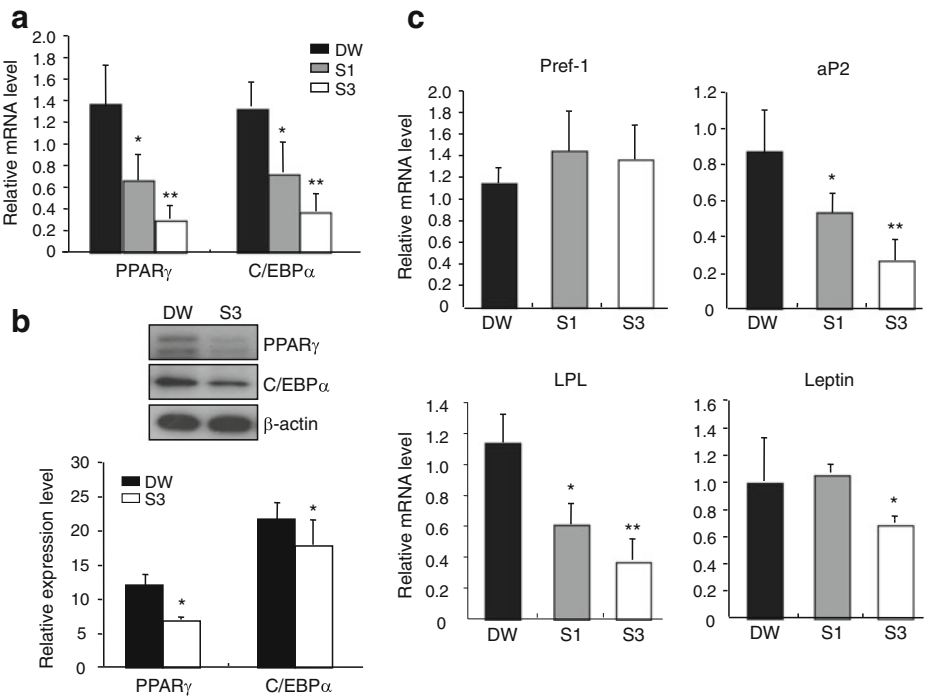

Fig. 2 Effect of Jeju ground water on adipogenic transcription factors during adipocyte differentiation. **a** Expression of PPARγ and C/EBPα mRNA from 3 T3-L1 adipocyte cells were measured by quantitative real-time PCR using specific primers. **b** Western blot analysis was performed with antibodies against PPARγ and C/EBPα. β-Actin was used as a loading control. **c** mRNA expression of adipogenic markers such as Pref-1, aP2, LPL, and leptin were analyzed by quantitative real-time PCR. The results represent the means ±SD from three experiments. $^{*}P<0.05$, $^{**}P<0.01$ compared with cells treated with DW

Effect of Jeju Ground Water on the Expression of Adipogenic Transcription Factor Target Genes

We explored whether Jeju ground water regulates the expression of the adipogenic transcription factor target genes adipocyte fatty acid binding protein (aP2), lipoprotein lipase (LPL), and leptin, as well as the preadipocyte factor-1 (Pref-1) gene. Pref-1 is predominantly expressed in undifferentiated 3 T3-L1 cells and is downregulated by induction agents such as insulin, dexamethasone, and IBMX. The mRNA level of Pref-1, which is predominantly expressed in an undifferentiated state, was upregulated and the mRNA levels of aP2, LPL, and leptin were downregulated by S1, S3 during adipocyte differentiation (Fig. 2c). These results demonstrated that the mRNA expression levels of late adipocyte differentiation makers were also downregulated by Jeju ground water.

Effect of Jeju Ground Water on the Expression and Secretion of Adiponectin

Adiponectin is an adipocytokine secreted by adipose tissue that promotes adipocyte differentiation, insulin sensitivity, and lipid accumulation [14]. To evaluate the expression of adiponectin during adipocyte differentiation induced by Jeju ground water, we assessed the protein expression levels of adiponectin in cell and culture media by Western blot analysis. As shown in Fig. 3, cultivation of 3 T3-L1 cells with S1, S3 decreased adiponectin protein levels during adipocyte differentiation. Consistent with the cellular adiponectin protein expression, secretion of adiponectin into the cell medium also decreased compared to cells incubated in DW.

Effect of Jeju Ground Water on HFD-Induced Obese Mice

To investigate the anti-adipogenic effects of Jeju ground water in vivo, we observed the body weights after administration of Jeju ground water (S1, S3) and DW to HFD-induced obese mice for 9 weeks. As shown in Fig. 4a, S1 and S3 groups relatively decreased the body weight gain compared to the DW groups and final body weight of S3 group was lower by 19 % as compared to the DW group, especially. We next examined the effect of Jeju ground water on the plasma levels of obesity-related markers such as total cholesterol, glucose, and triglyceride in HFD-induced mice blood. Total cholesterol levels in DW administration group and S1, S3 administration group were 348±16.5, 223.5±10.6, and 220.5±15.5 mg/dl, respectively. The Jeju ground water (S1, S3) significantly reduced the level of plasma total cholesterol relative to the DW group. Moreover, S3 also reduced the level of plasma glucose and triglyceride by 7 % and 21.4 % compared to the DW group. These results indicated that administration of Jeju ground water is able to decrease HFD-induced body weight gain and concentration of adipogenic maker in the blood.

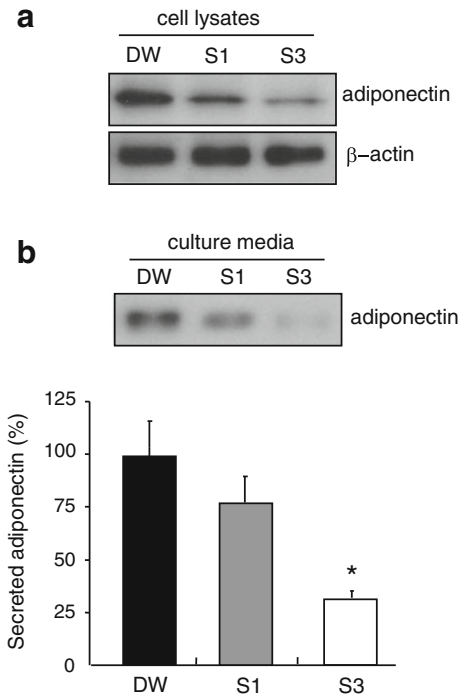

Fig. 3 Effect of Jeju ground water on the expression of adiponectin. The amount of adiponectin protein in cell (**a**) and culture media (**b**) were assessed by Western blot analysis. The graph represents the band intensity of secreted adiponectin. The results represent the means ± SD from three experiments. $^{*}P<0.05$ compared with cells treated with DW

Discussion

Obesity is most commonly caused by a combination of excessive dietary calories, lack of physical activity, and genetic susceptibility. The most effective treatment for obesity is dieting and physical exercise. Research shows that natural products can act as anti-obesity agents without harmful side effects [15–18]. Previously, researchers have investigated the beneficial health effects of drinking water. Kato et al. reported that Mt. Fuji groundwater improves blood glucose, serum hemoglobin A1C levels, and insulin secretion from the pancreas of a genetic animal model of type 2 diabetes [9]. The Mt. Fuji groundwater has relatively high concentrations of the trace element vanadium. Vanadium is a well-known anti-diabetic metal agent that mimics the actions of insulin on mature adipocytes and can exert anti-tumor effects against chemical carcinogenesis in animals, as well as in various types of malignant cell lines [19]. The effect of vanadium in the treatment of adipogenesis as an anti-adipogenic agent has been studied [20, 21]. Vanadium concentration in ground water is lower than the measured effective concentration. However, vanadium exists at relatively high concentrations

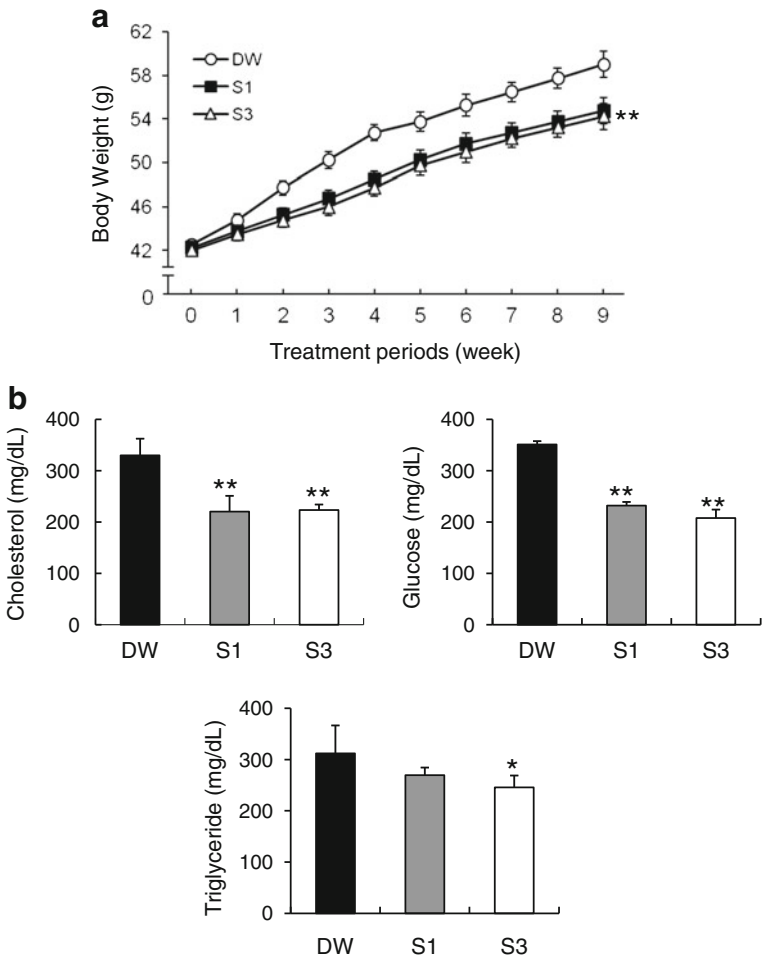

Fig. 4 Effect of Jeju ground water on HFD-induced obese mice. Mice were provided with DW or S1, S3 (n=5 per each group) with HFD for 9 weeks (**a**) recording changes in body weight (**b**). At the end of experiments, blood was collected and serum isolated by centrifugation. The concentration of total cholesterol, triglyceride, and glucose in blood for each group were measured. The results represent the means ±SD from five mice. $^{*}P<0.05$, $^{**}P<0.01$ compared with cells treated with DW

(especially S3, 26.0±2.0 μg/l) in Jeju ground water obtained from Jeju Island in South Korea. Jeju ground water containing vanadium has antioxidant effects via ROS scavenging [22] and stimulates glucose uptake by activating AMP-activated protein kinase (AMPK) in L6 myotubes [23]. AMPK is an intracellular energy sensor that plays a role in regulating food intake, body weight, glucose uptake, and lipid metabolism. However, the anti-adipogenic mechanisms of Jeju ground water as anti-obesity effectors had not yet been studied.

We identified the effect of Jeju ground water containing vanadium components on 3 T3-L1 adipocyte differentiation and obesity of HFD-induced mice. Adipocyte differentiation is a complex process of programmed changes in specific gene expression. C/EBPβ and C/EBPδ are transiently expressed in the early stages of preadipocyte differentiation initiation. C/EBPα is induced relatively late during adipogenesis, and cross-regulation between PPARγ is important to maintain the differentiated state [7, 8]. Activation of PPARγ induces the expression of genes controlling adipocyte fatty acid metabolism, including aP2 and LPL [24, 25]. Jeju ground water, especially S3 treatment, decreased PPARγ and C/EBPα expression and their adipogenic target genes. Adiponectin is expressed by mature adipocytes and is the most abundant circulating adipokine. Adiponectin promotes adipocyte differentiation through autocrine effects, resulting in increased lipid content and insulin sensitivity. Jeju ground water downregulates the expression and secretion of adiponectin during adipocyte differentiation. C57BL/6 mice fed a HFD were generally used as a representative model of obesity-induced diabetes [25]. HFD causes insulin resistance in the skeletal muscle, liver, and adipose tissue, and a HFD-induced increase in the serum concentration of free fatty acids. We investigated whether the ingestion of Jeju ground water prevents HFD-induced obesity in C57BL/6 mice. Consistent with in vitro results, the ingestion of Jeju ground water to mice fed a HFD prevented weight gain. Also, we investigated the plasma levels of total cholesterol, glucose, and triglyceride in the blood obtained from mice fed with the DW and Jeju ground water. A HFD is associated with elevated serum total glucose and triglyceride cause of body weight gain. The mice given the Jeju ground water had lower serum concentrations of total cholesterol, glucose, and triglyceride compared to the mice given DW. Taken together, suppression of adipocyte differentiation by Jeju ground water containing vanadium is mediated through downregulation of the adipogenic transcription factors

PPARγ and C/EBPα, and their target genes in 3 T3-L1 adipocytes. The anti-obesity effect in a HFD-induced mouse model corroborated these observations. Our study suggests that Jeju ground water inhibits adipocyte differentiation and can be used as an anti-obesity agent. Although the vanadium is responsible for the anti-adipogenic effect of Jeju water, we cannot rule out the possibility that other components of Jeju water also contribute to this effect. Thus, further study is needed to determine whether other minerals of Jeju water are involved in suppression of adipogenesis.

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