Anti-obesity Effect of Alkaline Reduced Water in High Fat-Fed Obese Mice

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Whether or not alkaline reduced water (ARW) has a positive effect on obesity is unclear. This study aims to prove the positive effect of ARW in high-fat (HF) diet-induced obesity (DIO) in C57BL/6 mice model. Toward this, obesity was induced by feeding the C57BL/6 male mice with high-fat diet (w/w 45% fat) for 12 weeks. Thereafter, the animals were administered with either ARW or tap water. Next, the degree of adiposity and DIO-associated parameters were assessed: clinico-pathological parameters, biochemical measurements, histopathological analysis of liver, the expression of cholesterol metabolism-related genes in the liver, and serum levels of adipokine and cytokine. We found that ARW-fed mice significantly ameliorated adiposity: controlled body weight gain, reduced the accumulation of epididymal fats and decreased liver fats as compared to control mice. Accordingly, ARW coordinated the level of adiponectin and leptin. Further, mRNA expression of cytochrome P450 (CYP)7A1 was upregulated. In summary, our data shows that ARW intake inhibits the progression of HF-DIO in mice. This is the first note on anti-obesity effect of ARW, clinically implying the safer fluid remedy for obesity control.

Key words alkaline reduced water; adiponectin; cytokine; adiposity; epididymal fat; cholesterol 7α-hydroxylase

Obesity has reached global epidemic status. In developed and developing countries, obesity is reported to be the sixth most important risk factor contributing to the overall burden of chronic disease.[1)] Forty three million children, 82% of which from developing countries, are affected with obesity.[2)] The World Health Organization recently reported that there were more than 1 billion overweight adults worldwide, of whom 500 million were obese.[3)] Obesity poses a major public health issue as it is known to be positively associated with increased risk of certain chronic diseases such as cardiovascular disease, hypertension, type 2 diabetes and fatty liver.[3–5)] Excessive intake of dietary fats influences the progression of obesity,[6)] which is characterized by accumulation of body fats marked by abnormal increase in adipose tissue mass and liver fats (adiposity), dysregulated levels of adipokines, and imbalance between pro- and anti-inflammatory cytokine.[7–9)] The complexity of these pathogenetic mechanisms of obesity poses a challenge to the development of effective therapy. In that context, there has been growing concern for the need of newer therapeutic strategies that directly deal with these mechanisms. Of several such candidates, we first introduced the alkaline reduced water (ARW) for obesity control in high-fat (HF) diet-induced obesity (DIO) in mice model.

ARW refers to electrolyzed water produced from minerals such as magnesium and calcium, which is characterized by supersaturated hydrogen, high pH, and a negative oxidation reduction potential. This hydrogen-rich functional water has been introduced as a feasible therapeutic strategy for health promotion and disease prevention.[10–12)] Our previous studies demonstrated that ARW intake reduces the levels of serum triglyceride, total cholesterol and blood glucose in OLETF diabetic rat model.[13,14)] Growing evidences show that hydrogen-rich water acts as a scavenger of active oxygen species, protects DNA from oxidative damage,[10)] and promotes metabolism.[15)] Further, hydrogen-rich water was demonstrated to have salutary effects in prevention of lifestyle diseases such as type 2 diabetes and insulin resistance,[16)] and liver inflammation.[17)] Since hydrogen rich water is generated by the reaction of magnesium metal with water, we postulated that hydrogen-rich ARW produced by the same reaction would be effective against obesity. To address this issue, we adopted an HF DIO mice model mimicking the pathogenesis of human obesity,[18)] to investigate the anti-obesity effect of ARW by way of clinico-pathological parameters, biochemical measurements, histopathological analysis of liver, the expression of cholesterol metabolism-related genes in the liver, and serum levels of adipokine and cytokine.

MATERIALS AND METHODS

Animal and Animal Care Five-week old male C57BL/6 (n=30) weighing 20±2 g mice were purchased from Orient Bio Inc. (Seongnam, South Korea) and were maintained at 22±2°C and 40–60% humidity under a 12 : 12 h light dark cycle. The mice were fed laboratory chow for one week to stabilize metabolic conditions and randomly assigned to one of the three diet groups, normal (NC+TW) which were fed with normal-fat diet (10% fat; Research Diets Inc., New Brunswick, NJ, U.S.A.) and tap water, control (HFD+TW) fed with high-fat diet (45% fat; Research Diets Inc., New Brunswick, NJ, U.S.A.) and tap water, and experimental (HFD+ARW) fed

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with high-fat diet and ARW for 12 weeks. Tap water and ARW were provided daily. The vitamin and mineral ingredients of the two diets were comparable. Food consumption and fluid intake were measured daily and body weight was monitored weekly throughout treatment. At the end of the experimental period, epididymal fat weight was measured. The animal use and care protocol for this animal experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of Wonju College of Medicine, Yonsei University.

Generation and Chemical Properties of Alkaline Reduced Water ARW was produced by the continuously electrolyzing apparatus (HDr Co., Ltd., Korea), wherein tap water was the main water source. ARW was prepared by physical filtering followed by electrolysis and collected in a cell equipped with a cathode platinum-coated titanium electrode (0.9±0.1 A at 2.5±0.3 kilogram force/minute (kgf/min)). ARW was adjusted to pH 9.5±0.3 and oxidative-reduction potential of −325.0±20.5 mV. The electrical conductivity of ARW was 223±5.5 μS, dissolved hydrogen was 0.1 ppm and dissolved oxygen was 4.82±0.11 mg/L. Glass bottles were used for water feeding. ARW was changed two times a day in order to keep the constant water conditions.

Analysis of Oil Droplets Content by Oil Red O Staining Optimal cutting temperature-embedded liver tissues were cut into 10 μm sections, cut sections were then mounted in the cryostat clear slides. Tissues were allowed to dry for 1 h and hydrated in distilled water for 5 min. Tissues were dipped in absolute propylene glycol solution for 2 min. Tissues were then stained with oil red O working solution for 1 h, and were dipped in 85% propylene glycol solution for 1 min and rinsed with distilled water. The oil red O stock solution was prepared by dissolving 500 mg of oil red O powder (Sigma, Ronkonkoma, NY, U.S.A.) in 100 mL of 100% propylene glycol. Harris hematoxylin was used to counter stain the tissues for 2–3 s and was rinsed with tap water. Stained tissues were dipped 2–3 times in 1% HCl solution and rinsed with distilled water, then mounted with glycerin jelly. Sections were visualized under a Motic BA 3000 light microscope (Motic Incorporation Ltd., China) at ×400 magnification, and 10 fields/mouse were used for oil droplet counting. Oil red O-stained sections were quantified and analyzed by a computerized image analyzer (Soft Imaging System 4; Soft Imaging System Corp., Munster, Germany). For oil droplet size measurement, 30 oil droplets were randomly selected from 10 different fields of each mouse liver tissue for a total of 300 oil droplets for each group (n=10).

Quantitative Polymerase Chain Reaction (PCR) Analysis Total RNA was isolated from liver using RNeasy Plus Mini kit (Qiagen, Valencia, CA, U.S.A.), according to the manufacturer's instructions. cDNA was produced using the QuantiTect reverse transcription kit (Qiagen, Valencia, CA, U.S.A.) by reverse transcription of 50 ng/μL total RNA obtained. Real-time PCR analysis was performed with a 7900HT Fast Real-Time PCR System using SYBR Green PCR Master Mix according to manufacturer's instructions (Applied Biosystem Inc., Forest City, CA, U.S.A.). The following primers were used:

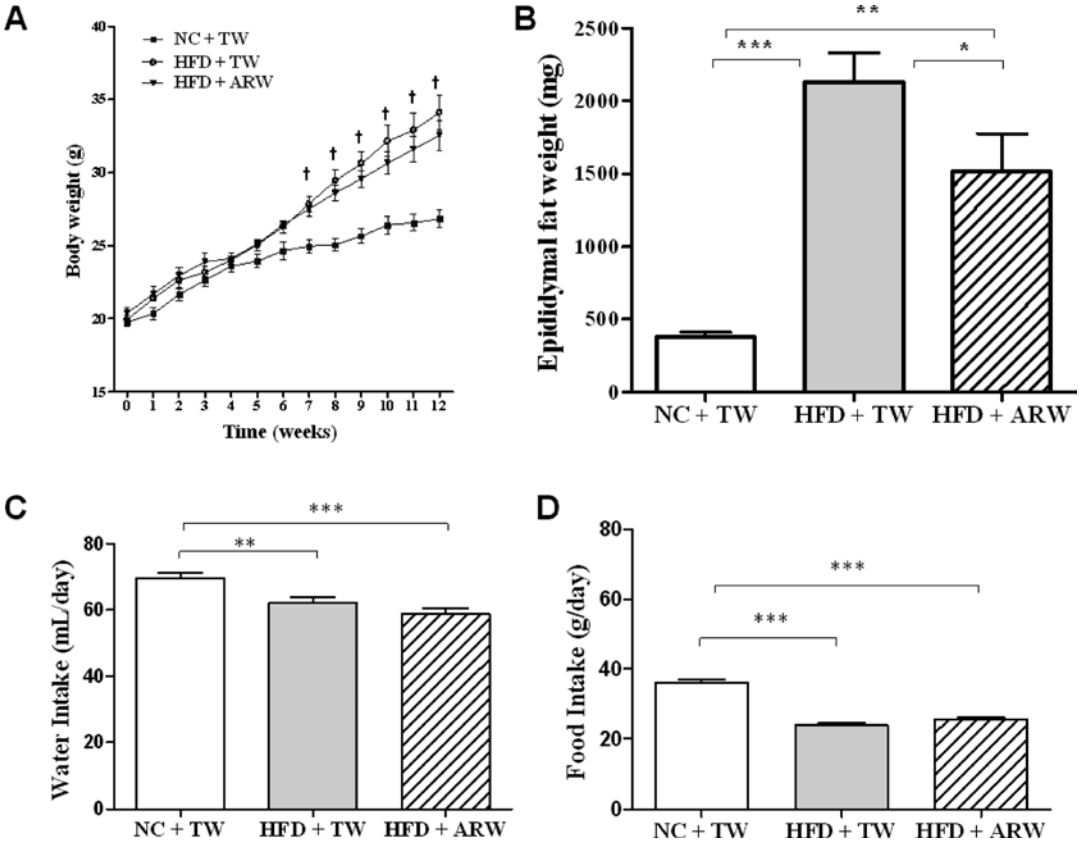

Fig. 1. The Effect of ARW on Body Weight (A) and Epididymal Fat Weight (B)

High-fat diet (HFD) groups were given either tap water (TW) or alkaline reduced water (ARW) and water intake (C) and food intake (D) were measured for 12 weeks. NC+TW group was fed normal chow and TW. Data are mean±S.D., n=10. $^{\dagger}p<0.01$ compared to HFD+TW (ANOVA with repeated measurements followed by Tukey's test), and * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ indicate significant differences when tested with ANOVA followed by Tukey's test.

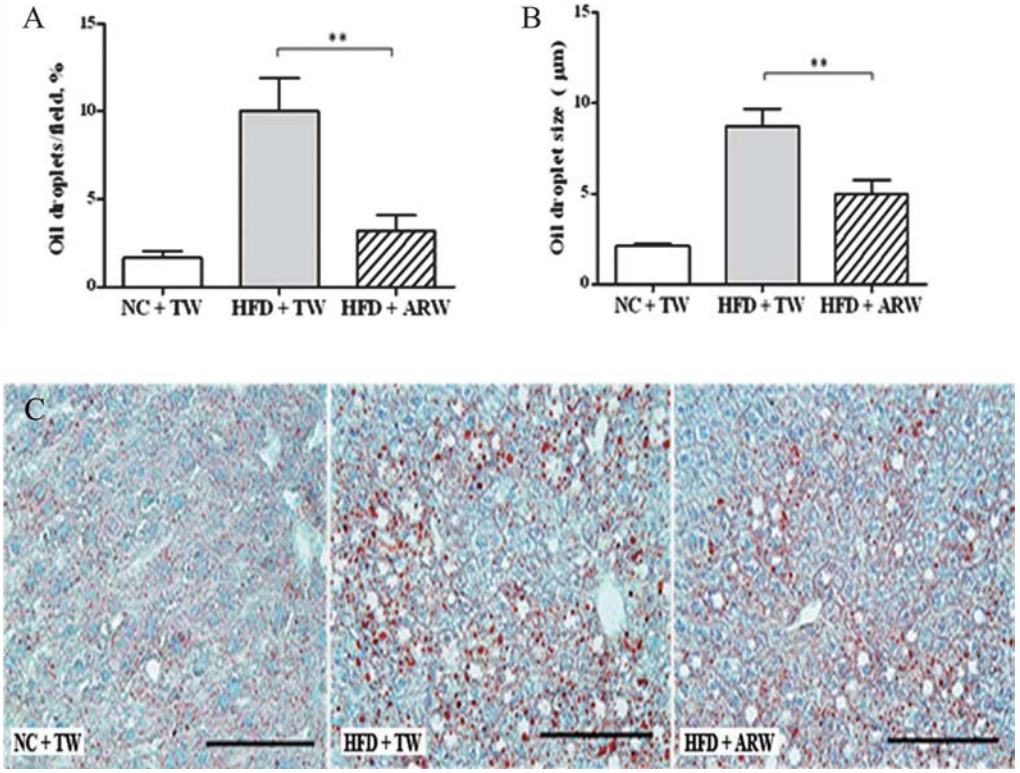

Fig. 2. The Number (A) and Size (B) of Oil Droplet in Liver Tissue of Obesity-Induced Mice Administered with Alkaline Reduced Water (ARW) and Tap Water (TW) for 12 Weeks

NC, normal chow; HFD, high-fat diet. Data are mean±S.D., n=10. ** $p<0.01$ *vs.* HFD+TW tested with ANOVA. Tukey's test was used for *post-hoc* tests. (C) Light micrographs of liver stained with oil red O, ×400 magnification. HFD+TW detected large oil vacuole filling the hepatocyte cytoplasm resulting in peripheral displacement of nucleus; HFD+ARW with small oil droplets on the hepatocyte cytoplasm with a central location of the nucleus. The scale bar represents 100 μm.

cytochrome P450 (CYP)7A1, forward 5′-AGA GCT TGA AGC ACA AGA AC-3′ and reverse 5′-ATG TCA TCA AAG GTG GAG AG-3′ and 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), forward 5′-GGA GCC TCT TAG TGA TTG TG-3′ and reverse 5′-GGT ACT GGC TGA AAA GTC AC-3′. These primers gave final product sizes of 101 and 100 base pairs for CYP7A1 and HMG-CoA reductase respectively. The concentration of each mRNA was normalized using the house keeping gene β-actin, forward 5′-ACG TTG ACA TCC GTA AAG AC-3′ and reverse 5′-GCA GTA ATC TCC TTC TGC AT-3′ with 100 base pairs as an internal standard. All tissues were run in a 384-well reaction plate and all reactions were performed in triplicate. Relative mRNA expressions were calculated as mean expression of mice on normal group, using the $2^{-\Delta\Delta CT}$ method with the β-actin as reference gene.

Analysis of Blood Samples Total white blood cell (WBC) counts and their differential counts such as neutrophil, lymphocytes, monocytes, eosinophil and basophil were determined by an automatic blood analyzer (HEMAVET HV950 FS, Drew Scientific Inc., Dallas, Texas, U.S.A.).

Serum Adipokines and Cytokines Adipokines and cytokines secreted in the blood were investigated. Serum adiponectin, leptin, interleukin (IL)-1β, IL-6, IL-10 and tumor necrosis factor (TNF)-α concentrations were measured using Multiplex kit (Bio-Rad, San Diego, CA, U.S.A.) according to the manufacturer's instruction. Standard curves for each adipokines and cytokines were generated using the reference concentrations provided in the kit. The plate was run on a Luminex 200 Bio-Plex Instrument (Bio-Rad, Hercules, CA, U.S.A.). Raw fluorescence data were analyzed by the software using 5-parameter logistic method.

Statistical Analysis Data values were expressed as the mean±S.D. The mean values among groups were analyzed and compared by one-way analysis of variance (ANOVA) followed by subsequent multiple comparison test (Tukey) with GraphPad Prism version 5.0 software packages (GraphPad Software, La Jolla, CA, U.S.A.). ANOVA with repeated measurements followed by Tukey's test was applied to test the effect of ARW on body weight. Differences were considered statistically significant at $p<0.05$, $p<0.01$, and $p<0.001$.

RESULTS

Body Weight and Epididymal Fat Weight Figures 1A, B showed the changes in body weight and epididymal fat weight among the groups. After 7 weeks of intake of ARW, HFD+ARW group mice significantly reduced ($p<0.01$) body weight compared to the control group (Fig. 1A). In parallel, ARW (HFD+ARW group)-fed mice significantly decreased their epididymal fat weight compared to the control (HFD+TW group) ($p<0.05$) (Fig. 1B). HFD groups showed a similar trend in food and water consumption (Figs. 1C, D).

Analysis of Oil Droplets in Liver Tissue Oil red O staining of the liver showed significant decreased ($p<0.01$) in the percentage of oil droplets deposition, and statistically smaller size ($p<0.01$) of oil droplets in mice administered with ARW compared with the control (HFD+TW) group (Fig. 2A). HFD+TW group showed macrovesicular steatosis with large fat vacuole filling the hepatocyte cytoplasm that result in the displacement of the nucleus towards the periphery (Fig.

2B). However, HFD+ARW group liver tissue has only small oil droplets with a nucleus intact at the center of the hepatocytes (Fig. 2B).

mRNA Expression of Hepatic CYP7A1 and HMG-CoA Reductase To identify the impact of ARW in cholesterol metabolism in liver, we measured the mRNA expression of hepatic CYP7A1 and HMG-CoA reductase involved in cholesterol homeostasis for lipid metabolism. HFD+ARW group significantly increases ($p<0.01$) liver cholesterol 7α-hydroxylase (CYP7A1) gene expression as compared to HFD+TW group (Fig. 3A). Additionally, both HF diet groups showed no significant differences on HMG-CoA reductase expression (Fig. 3B).

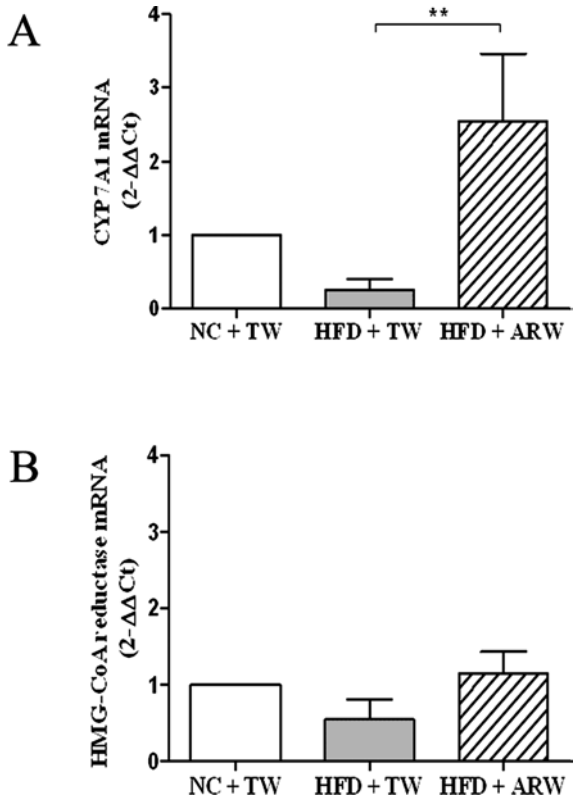

Fig. 3. Effect of Alkaline Reduced Water (ARW) on Cytochrome P450 (CYP)7A1 and 3-Hydroxy-3-methylglutaryl-CoA Reductase (HMG-CoA Reductase) mRNA Expression in Hepatic Tissue

Expression of CYP7A1 (A) and HMG-CoA reductase (B) were measured by real-time polymerase chain reaction, as described in Materials and Methods. Fold changes were calculated by comparison to the mean expression value of normal diet group (NC+TW). All genes were normalized to the endogenous control gene β-actin. Data are mean±S.D., $n=10$. ** $p<0.01$ when compared with high-fat diet+tap water (HFD+TW) group tested with ANOVA. Tukey's test was used for *post-hoc* tests.

White Blood Cells and Their Differential Count The total WBC of HFD+ARW group showed significantly higher in concentration than the mice fed with HFD+TW ($p<0.05$) as evident by the increased in count of neutrophils and lymphocytes (Table 1).

Levels of Adipokines and Cytokines Serum concentration of adipokines secreted mainly by adipose tissue such as adiponectin and leptin were analyzed. We found that the adiponectin level of ARW-fed group was significantly ($p<0.05$) lower than that of the positive control (HFD+TW) group (Fig. 4A). There was a significant increased in leptin concentration on the mice fed with HFD+TW ($p<0.01$) and HFD+ARW ($p<0.05$) compared with mice fed with normal chow (NC+TW). In parallel, we checked pro- and anti-inflammatory cytokines (IL-1β, IL-6, IL-10 and TNF-α). Overall, there is increase in concentration of IL-1β, IL-6, IL-10 and TNF-α in group fed with normal chow compared with HF-fed groups. HFD+TW group showed distinct decreased in the concentration of IL-10 and TNF-α ($p<0.01$) when compared to the HFD+ARW group ($p<0.05$) (Figs. 4C–F).

DISCUSSION

Our results indicate that ARW intake inhibits the progression of HFD-induced obesity through ameliorating adiposity, regulating adipokines and inflammatory cytokine level, and influencing cholesterol homeostasis in the liver. Our hypothesis is that ARW may control diet-induced obesity, fat accumulation, restore cytokine imbalance and ameliorates cholesterol catabolism.

To verify this, we first checked clinico-pathological evidences related to adiposity. Unexpectedly, we found that ARW could reduce abnormal increase in body weight and epididymal fat weight shown in Fig. 1. Subsequently, we examined the hepatic adiposity using oil red O staining. In oil red O staining, greater percentage and bigger size of oil droplets reveals that more fats are present in the high fat-fed control cells. Such an increased percentage of oil droplets could be indicative of increased lipid efflux.[19] Consistent with the decreased body weight and epididymal fat weight, ARW-fed mice significantly decreased the percentage and the size of oil droplets in the liver. This decremented effect strongly suggests a unique property of ARW to counteract the fat accumulation in the liver. Furthermore, the blood pressure in ARW-fed mice was close to normal control (data not shown). This positive ARW may be partly supported by previous results that the treatment with hydrogen rich water with low ORP and high pH, is shown to have significant control over body fats and body weight, as well as decreasing glucose, insulin and triglyceride in diabetic animal.[20]

Table 1. Total White Blood Cell (WBC) and Differential WBC Counts

WBC and members	NC+TW	HFD+TW	HFD+ARW
Total WBC, $\times10^9$/L	8.22±2.18	5.25±1.45	8.02±2.71*
Neutrophil, $\times10^9$/L	2.15±0.82	0.97±0.29	1.80±0.63**
Lymphocyte, $\times10^9$/L	5.82±1.87	4.11±1.15	5.89±2.19*
Monocyte, $\times10^9$/L	0.19±0.07	0.14±0.05	0.24±0.14
Eosinophil, $\times10^9$/L	0.04±0.04	0.03±0.02	0.03±0.02
Basophil, $\times10^9$/L	0.01±0.01	0.01±0.01	0.02±0.02

Abbreviations: NC, normal chow; HFD, high-fat diet; TW, tap water; ARW, alkaline reduced water. Data are means±S.D., $n=10$. *$p<0.05$, **$p<0.01$ *vs.* HFD+TW tested with ANOVA followed by Tukey's test.

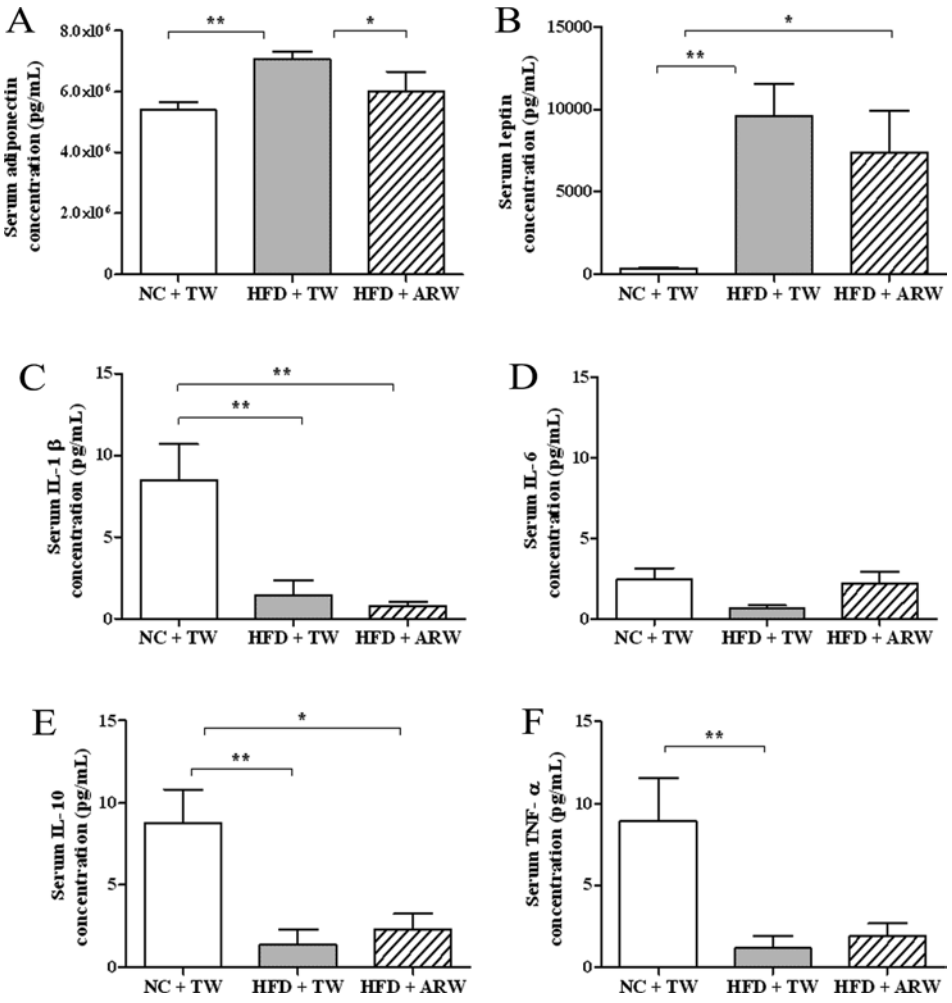

Fig. 4. Effect of ARW on Adiponectin (A), Leptin (B) and selected Cytokines such as IL-1β (C), IL-6 (D), IL-10 (E) and TNF-α (F) Concentration in Serum, C57BL/6 Male Mice Fed Different Diets for 12 Weeks (NC+TW, Normal Chow+Tap Water; HFD+TW, High-Fat Diet+Tap Water; HFD+ARW, High-Fat Diet+Alkaline Reduced Water)

Bio-Plex luminex-bead array system was performed as described in Materials and Methods. Data are mean±S.D., n=10. *p<0.05, **p<0.01 indicate significant differences tested with ANOVA. Tukey's test was used for *post-hoc* tests.

Last, to identify the impact of ARW in cholesterol metabolism in liver, we selected two key enzymes, CYP7A1 and HMG-CoA reductase; and compared their levels to the control mice. Hepatic CYP7A1 was markedly higher in ARW-fed obese mice, suggesting that ARW induces higher cholesterol catabolic rate. It has been well documented that the bile acid synthesized in human liver is highly correlated to signaling molecules that regulate lipid and glucose metabolism implicated in obesity, diabetes and liver disease.[21,22] Conversion of cholesterol to bile acids is one major route in removing excess cholesterol in the body and this occurs exclusively in the liver.[23] Further, CYP7A1 gene is a key regulatory gene in bile acid biosynthesis that encodes the rate-limiting enzyme in the reaction pathway.[24] In that context, we specifically checked hepatic CYP7A1 gene expression as the main player in bile acid biosynthesis. Previous study shows that the increased CYP7A1 expression improves metabolic syndromes, steatosis, and insulin resistance in mice.[25] Through inhibition of gluconeogenesis and triglyceride synthesis, the regulation of bile acid production may relieve the adverse effect of complications that may arise from unregulated glucose and triglyceride.[26] Our data are strongly supported by recent studies that show how the induction of CYP7A1 gene expression prevents high-fat DIO, fatty liver and insulin resistance.[25,27] ARW intake increased HMG-CoA reductase expression although insignificant, we think this is in compatible to previous finding that hepatic HMG-CoA reductase expression is markedly down-regulated in HFD mice.[28,29] Together, we suggest that, ARW intake may influence cholesterol homeostasis in the liver *via* regulation of CYP7A1 activity.

Fat plays significant roles in endocrine and immune functions through release of adipokines such as adiponectin, and leptin, as well as inflammatory cytokines like TNF-α, IL-1β, and IL-6.[30] Specifically, these cytokines are deeply linked in metabolic disturbance-related chronic inflammation. As such, obesity is characterized by its adipokine-cytokine imbalance. Our previous study reveals that ARW ameliorate skin damaged induced by UVB through influencing pro-/ anti-inflammatory cytokine balance in our animal model of skin injury.[31] To further elucidate the effects of ARW on adipokine-cytokine imbalance in HFD mice model, we first checked serum adipokine (adiponectin and leptin) and cyto- kines (IL-1β, TNF-α, IL-6 and IL-10) implicated in obesity. Adipokines are bioactive peptides or proteins secreted mainly

by adipose tissue and their expressions are impaired in obesity such as adiponectin and leptin.[32] We found that both adipokine levels were as high as to other four different cytokine levels, suggesting typical profiles in obesity accompanied by immunologic disturbances. Additionally, ARW intake in HFD mice restored the adiponectin level back to the baseline level of normal control (NC+TW). Adiponectin is abundantly present in the plasma, and it is known to modulate metabolic processes and increase insulin sensitivity through regulation of glucose metabolism, induction of fatty acid oxidation, and down regulation of triglycerides.[33,34] Adiponectin, which is produced mainly by adipose tissue, is down regulated in obese subjects.[35,36] On the contrary, HFD+TW group showed elevated adiponectin level compared to normal control. This is consistent with the fact that the abnormal increase in adiponectin level is highly correlated to the onset of type 1 diabetes in human,[37,38] and to the obesity in HFD mice model. Next, we measured the leptin level, an adipokine that acts as a metabolic regulator. Leptin is deeply involved in the regulation of food intake, energy expenditure and metabolism of glucose and lipid.[39,40] Since serum leptin concentration is elevated in obese subjects in proportion to fat mass and the degree of adiposity,[41] the high leptin level in our HFD mice model confirms the feasibility of our mice model. This is one indication that we have successfully induced obesity in our mice model. Moreover, there is exaggerated increased in leptin in HFD+TW group compared to mild increase in HFD+ARW group. In this regard, the difference in the level of leptin in ARW-fed mice agrees with the decreased adiposity from body weight and epididymal fat weight.

To further delineate the potential effect of ARW on immunological imbalance induced by obesity, we performed serum pro-/anti-inflammatory cytokine profiling using Luminex-bead array system known for its high sensitivity and precision. Lower level of pro-/anti-inflammatory cytokines in normal control may be ascribed to the regulatory action of adiponectin, suggesting that HFD elicits immune dysregulation. However, ARW group displayed increased IL-6, IL-10 and TNF-α levels as well as decreased IL-1β level although insignificant (Figs. 4C–F). Cumulative results show that markers of inflammation such as pro-inflammatory cytokines are increased in expression when fed with high-fat diet.[42] IL-10 is an important immunoregulatory cytokine with multiple biological effects, including downregulation of inflammatory responses and normalization of immune cells such as T and B cells, NK cells and granulocytes.[31] IL-6 can function as anti-inflammatory wherein it inhibits lipopolysaccharide-induced TNF-α and IL-1,[43] and downregulate inflammatory signaling cascades that regulate fatty acid oxidation in obese/diabetic condition.[44] Besides, increased IL-6 secretion in obesity is associated in correcting excess body weight gain.[45] Taken together, we suggest that ARW intake restored pro- and anti-inflammatory cytokine imbalance caused by high fat diet. Consistent with such results, the number of white blood cells, which serve as a source of IL-6, increased in ARW-fed mice compared to the positive control group. On the relationship of the level of white blood cells and obesity,[46] growing evidences revealed that increased level of white blood cells and their differential counts can protect against the adverse effects of obesity. This regulation of leukocytes may contribute to achieving the balance of pro-inflammatory and anti-inflammatory cytokines in adiposity. These cytokine plus hematologic balancing effects may be further supported by our previous results, where electrolyzed water was shown to restore cytokine imbalance in mice model with induced UVB skin injury.[47] Combining these together, our data suggest that ARW intake is effective in maintaining immune homeostasis through adipokine-cytokine network.

Despite these positive evidences of ARW on obesity, how ARW works on a molecular level still awaits an answer. It has been well established that hydrogen-rich water has beneficial effects on different disease models and is mostly dependent on its anti-inflammation,[48] and antioxidative properties.[49] Hydrogen molecules also rapidly diffuse into tissues and cells and it is mild enough to disturb cellular processes. Our conjecture is that the ameliorating effect of ARW in obese mice model at least partially attributable to the existence of the dissolved hydrogen in the ARW. To uncover its profound mechanism on immunological homeostasis, further studies targeting inflammatory signaling pathways and reactive oxygen species production would be necessary, as obesity is a type of oxidative stress-related that involves chronic inflammation.

In summary, our results indicate that ARW intake inhibits the progression of HFD-induced obesity through ameliorating adiposity, regulating adipokines and inflammatory cytokine levels, and influencing cholesterol homeostasis in the liver. This is the first note on anti-obesity effect of ARW, opening opportunity for developing new, safe clinical remedy for obesity control.

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