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Effect of Mineral-induced Alkaline Reduced Water on Sprague-Dawley Rats Fed on High-fat Diet

Dan Jin[1,5], Seung Kyu Park[2], Young Mi Lee[4], Yang Suk Yoon[4],
Dong Heui Kim[3], Young Kun Deung[3] and Kyu Jae Lee[2†]

[1]Department of Microbiology, [2]Department of Parasitology and Institutes for Basic Medical Science, and
[3]Department of Basic Science, Wonju College of Medicine, Yonsei University, Wonju, Gangwon 220-701, Korea.
[4]Biotech Research Institute, HDr Co., Ltd., 1214-6, Heung Yang-Ri, Socho-myun, Wonju, Gangwon 220-836, Korea.
[5]Department of Microbiology and Immunology, Yanbian University College of Medicine, Yanji 133000, China

Mineral-induced alkaline-reduced water (MRW) is generated by the chemical reaction of water with alkaline earth metals and characterized by high pH and low oxidation-reduction potential. As ROS are believed to have a role in the pathogenesis of obesity, we attempted to determine the effect of MRW on obesity in Sprague-Dawley (SD) rats fed on a high-fat diet. The body weight of the MRW group was significantly lower than that of the control group in most periods of the examination ($P<0.05$). Serum level of triglycerides ($P<0.05$) and fat deposition in the livers of the MRW group were found to have been significantly reduced. This suggests that MRW down-regulates lipid metabolism, thereby suppressing obesity. Possible mechanisms of MRW related to reactive oxygen species were also discussed. Our results suggest that MRW is effective in the alleviation of obesity in SD rats fed on high-fat diet.

Key Words: Mineral-induced alkaline-reduced water (MRW), High-fat diet, Obesity

INTRODUCTION

Alkaline-reduced water (ARW) is produced at the cathode during electrolysis of water. The ARW is characterized by high pH, low oxidation-reduction potential (ORP), high dissolved hydrogen and low dissolved oxygen (Shirahata et al., 1997). The ARW includes supersaturated colloidal hydrogen (Kikuchi et al., 2001), which is believed to be related to low ORP as well as the scavenging of reactive oxygen species (ROS). In previous reports, the ARW is effective on the promotion of growth in fetal and neonatal SD rats (Watanabe, 1995), suppression of metastasis of B16 melanoma cells in C57BL/6 mice (Lee et al., 2003), and reduction of oxidative stress in renal disease patients during hemodialysis (Huang et al., 2003).

The ARW is also generated by chemical reactions of alkaline earth metal such as magnesium. Exposure to granulated magnesium metal gives the water characteristics of high pH and low ORP (Park et al., 2004). In order to distinguish the ARW according to its generating method, we define that "electrolyzed reduced water (ERW)" is the water generated at cathode by electrolysis of water, and that "mineral-induced alkaline-reduced water (MRW)" is the water generated by reaction of alkaline earth metal such as magnesium.

We have previously reported that the MRW is effective on the reduction of body fat rates in human (Lee et al., 2004). We have also found that oral administration of the MRW significantly reduces body fat rate in broiler chickens and downregulates lipid parameters in blood in OLETF rats (unpublished data). Although the MRW is very alkaline in the pH, pH-retaining potential of the MRW is so low that titration of the MRW readily neutralize the pH, which impose little or no affect on the acidic gastric environment (Park et al., 2005).

In this study, we attempted to determine the effects of the MRW on the obesity induced by a high-fat diet. Growing

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†Corresponding author: Kyu Jae Lee, Department of Parasitology and Institutes for Basic Medical Science, Wonju College of Medicine, Yonsei University, Wonju, Gangwon, 220-701, Korea.
Tel: +82-33-741-0331, Fax: +82-33-731-6953
e-mail: kjlee@wonju.yonsei.ac.kr

Sprague-Dawley (SD) rats were permitted to the high-fat diet and the MRW, and the changes in the body weight of the rats, changes in the weight of inguinal white adipose tissue (IWAT), epididymal adipose tissue (EDAT) and blood parameters representing obesity were also measured. Lipid depositions in the liver were examined by histostaining.

MATERIALS AND METHODS

1. Animals

Three week-old male SD rats were purchased from Folas International (Korea), and kept at Animal Facility at Wonju College of Medicine, Yonsei University according to the provided Animal Care Regulations. The rats were then randomly divided to control (n=10) or MRW (n=10) groups. Twelve hour light and dark cycle was maintained throughout this study. Temperature was controlled between 22~24℃. Humidity was controlled at 40%.

2. MRW

MRW was generated by dipping Alkalogen® Stick (HDr, Korea) in 240 ml water bottle containing 2 mM NaCl (control water) for more than 30 min. Alkalogen® Stick is cylindrical polyprostyrene housing (Φ17 mm in inside) containing magnesium granules (1.5 mm in mean diameter). While the control water was supplied to the rats in the control group, the MRW was supplied to SD rats in the MRW group after confirmation that the pH is higher than 9.5 and the ORP lower than -100 mV. The water bottles containing the MRW were changed every 24 hrs, and ICP-MS analysis of retrieved MRW revealed that the concentration of magnesium was lower than 0.3 ppm.

3. Administration of high-fat diet

SD rats in the control and MRW groups were *ad libitum* permitted to control or high-fat diet, respectively (Table 1). Body weights were measured every week. On day 83, the rats were sacrificed, and the bloods were collected from their hearts for serological examination. The weights of inguinal white adipose tissue (IWAT) and epididymal adipose tissue (EDAT) were also measured.

4. Serological examination

Sera from the SD rats of both groups were assayed for GOT, GPT, glucose, triglyceride (TG), total cholesterol, HDL and LDL using Hitachi 7150 analyzer (Japan) as instructed in user's manual.

Table 1. Composition of normal and high-fat diet

Composition	Control diet[1)]		High-fat diet[2)]	
	Weight (%)	Calorie (%)	Weight (%)	Calorie (%)
Total protein	20	38.6%	19.0%	26.2%
Total fat	4.5	8.7%	34.9%	48.1%
Total fiber	6		3.3%	
Carbohydrate	61.5	52.7%	42.0%	25.7%
Total	93%	100.0%	100.1%	100.0%

[1)] Control diet (Samyang Oil & Feed Co., Ltd, Inchon, Korea) was composed of 7.0% animal products, 86% cereals, 1.0% vitamins and minerals and 6.0% other nutrients.

[2)] High-fat diet was mixture of the normal diet and butter (Seoul Milk Co., Seoul, Korea) that is composed of 1.0% carbohydrate, 1.0% protein, 83% fat and 0.5% sodium

5. Histostaining

Sections of frozen liver from the control and MRW groups were cut to a thickness of 3 m and stained with oil red O and hematoxylin to assess for the presence of lipid droplets.

6. Statistics

Differences between the two groups were assessed by Student's *t*-tests, using Prism version 3.0 (GraphPad Software, USA). All data is expressed as the mean ± SD.

RESULTS

1. Effects of MRW on body weight and adipose tissues

Until day 35, the body weights of the control and MRW groups increased steeply, then slowed down until the end of the study, on day 83 (Fig. 2). From day 14 to day 63, the body weights of the rats in the MRW group were significantly lower than those in the control group ($P<0.05$), except for on day 28. However, in the last two examinations on days 77 and 83, no significant differences were found. The weights of the IWAT of the control and MRW groups were 6.50±0.14 g and 6.79±1.05 g, respectively. The weights of the EDAT of the control and MRW groups were 5.77±0.11 g and 5.36±0.69 g, respectively. There were no significant differences in the weights of IWAT and EDAT between the two groups (Fig. 3).

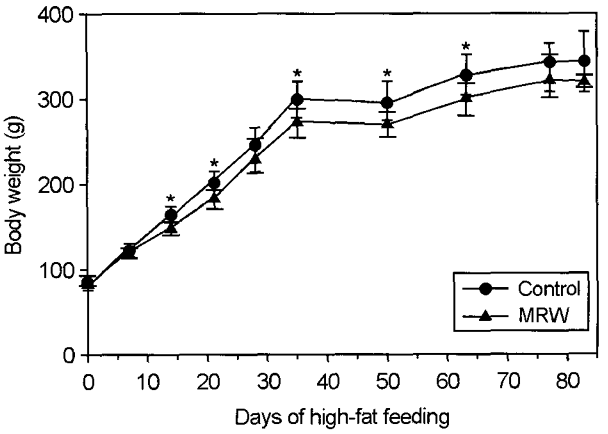

Fig. 1. ROS scavenging effect of MRW. 0 or 50% MRW was assayed in the HX/XOD system. The experiment was performed in triplicate, and the data was expressed as mean ± SEM.

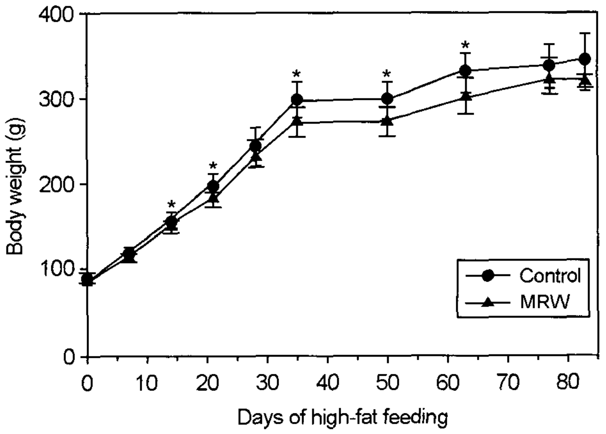

Fig. 2. Change in body weight. Three week-old SD rats were fed on the control water (n=10) or MRW (n=10) for 83 days. Data was expressed as the means ± SEM. ($*P<0.05$)

2. Serological examination

The GOT and GPT levels in the MRW group were 84.5 ±19.5 IU/l and 44.3±5.8 IU/l, respectively, significantly lower than those of the control group, which were 147.9± 64.7 IU/l ($P<0.01$) and 62.3±12.1 IU/l ($P<0.001$), respectively. Glucose levels of the control and MRW groups were 153.0±14.2 mg/dl and 146.6±9.9 mg/dl, respectively, and this was not statistically significant. With regard to lipid parameters, the level of TG in the MRW group was 52.2± 9.8 mg/dl, which was significantly lower than that seen in the control group, 60.6±7.1 mg/dl ($P<0.05$; Table 2). Levels of other parameters, including total cholesterol, HDL and LDL, were not significantly different, as shown in Table 2.

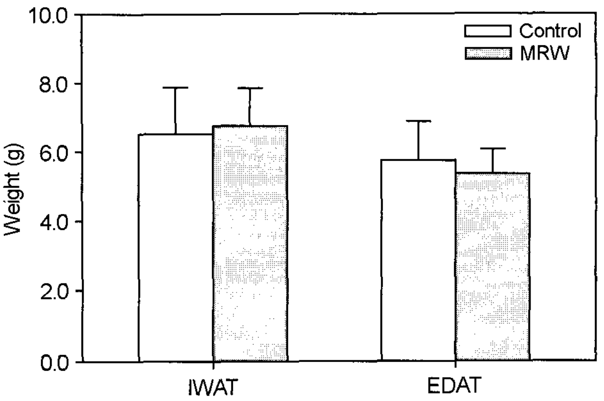

Fig. 3. Changes in the weight of adipose tissues. On day 83, the weight of the IWAT and EDAT of SD rats were measured. Data are expressed as means ± SEM.

Table 2. Change of serological parameters

Group	Unit	Control	MRW	Significance
n		10	14	
GOT	IU/L	147.9±64.7	84.5±19.5	$P<0.01$
GPT	IU/L	61.3±12.1	44.3±5.8	$P<0.005$
Serum glucose	mg/dl	153.0±14.2	146.6±9.9	n.s.*
TG	mg/dl	60.6±7.1	52.2±9.8	$P<0.05$
Total cholesterol	mg/dl	124.9±10.7	123.7±17.1	n.s.*
HDL	mg/dl	92.0±8.0	88.6±10.2	n.s.*
LDL	mg/dl	29.1±5.2	28.6±5.2	n.s.*

* n.s.: not significant

3. Histological changes in liver tissue

Lipid droplets in the liver parenchyma were stained by oil red O as seen in Fig. 4. In the control groups, the liver showed microvesicular steatosis of densely scattered droplets (Fig. 4A), In the MRW group, the liver showed macrovesicular steatosis of relatively rare droplets compared with that of the control group (Fig. 4B).

DISCUSSION

In present study, we demonstrated for the first time that the MRW suppresses increases in body weight and liver fat deposition in SD rats fed on high-fat diet. At the beginning of present study, the body weights of the three-week-old SD rats in the control and MRW groups increased rapidly until day 35, because the rats were in their growing stage. With the exception of day 28, the body weights of the rats

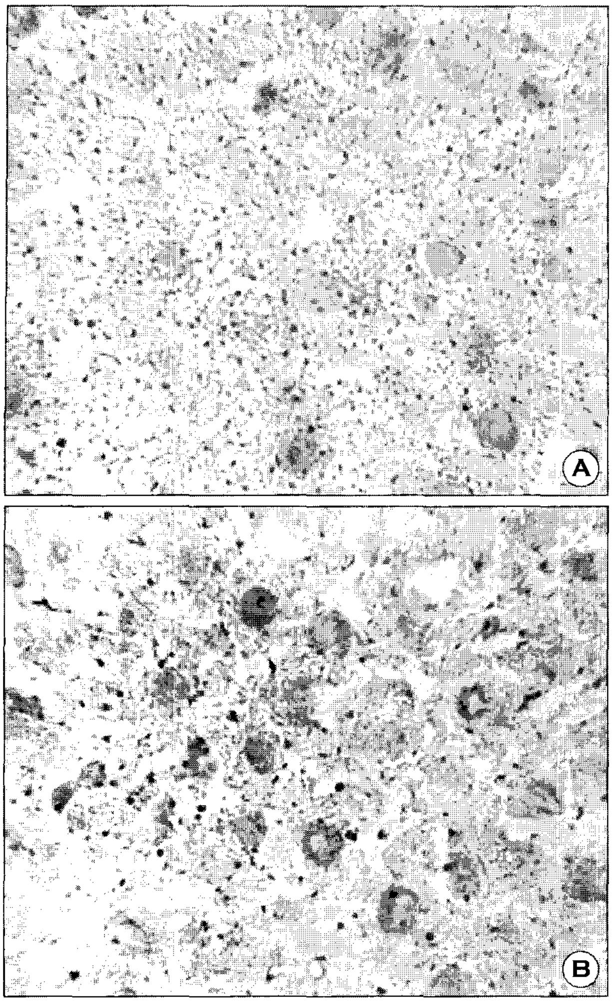

Fig. 4. Lipid deposition in liver. Five μm liver sections from the control **(A)** and MRW **(B)** groups were stained with hematoxylin and oil red O.

in the MRW group between day 14 and day 63 were found to be significantly lower than those of the control group (Fig. 2). This difference in body weight was so marginal that, in the last two measurements, on days 77 and 83, the differences were not statistically significant. However, this result strongly suggests that the MRW can exert *in vivo* biological effects on the reduction of body weight of SD rats fed on the high-fat diet.

Microvesicular steatosis in the control group and macrovesicular steatosis in the MRW group shows the effect of the MRW on suppression of obese progression. Physiopathology of microvesicular and macrovesicular steatosis can be summarized as followed (Fromenty et al., 1997; Fromenty and Pessayre, 1995). Severe impairment of mitochondrial fatty acid β-oxidation accumulates poorly oxidized nonesterified fatty acid (NEFA). Amphiphilic NEFA is supposed to form microvesicules by capsuling hepatic TG, resulting in microvesicular steatosis. Meanwhile, NEFA and its metabolites exert detrimental effect on mitochondrial function, resulting in reduction of local energy output and reduction of ketone body and glucose delivery to pheripheral tissues. As the result, progress of microvesicular steatosis in diverse organs including liver causes hepatic failure, coma and death. In the other hand, macrovesicular steatosis is mainly occurred in alcohol abuse, obesity, diabetes and some dyslipidemia, and is supposed to be resulted by increased mobilization of fat from adipose tissue, increased hepatic synthesis of fatty acid, increased esterification of fatty acid into TG and/or decreased egress of TG from the liver (Fromenty and Pessayre, 1995). Macrovesicular steatosis without other liver lesion is classified as benign condition.

However, continuous ingestion of high-fat diet increases incomplete β-oxidation in the mitochondria (Koves et al., 2005) and increased amount NEFA in the cells, resulting in microvesicular steatosis (Fig. 4). In the present study, the control group shows microvesicular hepatic steatosis with many small lipid droplets, which is compared with that of the MRW group showing macrovesicular hepatic steatosis with little large lipid droplets. This difference is supposed by that activated lipid metabolism in hepatic mitochondria by MRW administration is sufficient for esterification of the over-ingested lipid. However, it is to be elucidated how the MRW activates the lipid metabolism in the rats.

It is suggested as a clue that obesity is intensified by oxidative stress, which can be neutralized by administration of the MRW. Oxidative stress is increased in accumulated fat in obese subjects, which is one of the important cause of systemic oxidative stress (Furukawa et al., 2004). Adipose tissue under oxidative stress downregulates the expression of peroxisome proliferators-activated receptor (PPAR)-γ and resulting downregulation of adiponectin expression (Furukawa et al., 2004; Saltiel, 2001). Adiponectin is an adipocytokine expressed only in adipose tissue, and expected to suppress insulin resistance and dyslipidemia (Saltiel, 2001). Authors had shown that the MRW scavenges ROS generated by *in vitro* HX/XOD system, and reduces lung metastasis of B1BL6 melanoma cells in mouse model (Lee et al., 2003; Park et al., 2004). Huang *et al.* (2003) have reported that electrolyzed reduced water that has similar characteristics in pH and ORP with MRW significantly reduced hemodialysis-induced oxidative stress of end-stage renal disease patients. These facts support that the reduction of oxidative stress in SD rats fed on high-fat diet by administration of the MRW increases the expression of adiponectin that is effective on suppression of obesity.

The MRW is basic water with the pH of over 9.0. However, its pH-retaining potential is so weak that it is readily neutralized by the addition of acidic agents. This ensures that administered MRW is quickly neutralized in the acidic environment of stomach. This explanation is supported by the fact that nine volumes of the MRW did not result in more than 0.1 unit increase in the pH of RPMI 1640 medium (pH 7.4) (unpublished data). Also, the administration of the MRW resulted in no increases in the serum levels of GOT and GPT, both of which are parameters for liver damage (Table 2). Although the levels of GOT and GPT were found to significantly decreased, we give less importance on the results because the lowered values were within normal range. It is rather important that the liver was not found to have been significantly damaged after administration of the MRW.

Among the serum lipid parameters examined in this study, the TG levels of the MRW group were significantly reduced. TG is known to be related to obesity, steatosis and insulin resistance, and TG accumulation in the liver have been shown in many cases (Browning and Horton, 2004; Marceau et al., 1999; Wanless and Lentz, 1990). In fatty individuals, the robust activity of lipogenic enzymes is expected. This tends to increase the biosynthesis of fatty acids in the liver, thereby stimulating the biosynthesis of TG, and increasing the concentration of TG in the blood (Coppack et al., 1994). Therefore, decrease in TG in this result implies that oral administration of the MRW downregulates lipid metabolism in the liver, resulting in the decrease of TG levels in the blood. This assumption is proven by the oil red O staining of the liver section. Red spots, oil red O-stained lipid droplets, in the control group were found to be very densely scattered around the hepatocytes, whereas those of the MRW group were fewer in the number (Fig. 4).

We had previously investigated on the effects of the ERW on the reduction of the body weights in OLETF rats, and found that the mean TG and total cholesterol levels in the sera were reduced in the ERW-fed rats (Kim et al., 2003). However, it is still remained to be elucidated what MRW mechanism biologically affects on the suppression of steatosis. The chemical and electrochemical properties of the MRW are similar to those of the ERW (Fig. 1), thus the effect of the MRW can also be explained by the effects of the ERW. Actually, almost all of the investigations regarding the ERW have been conducted at the viewpoint of chemistry or electrochemistry. Only one clinical study has focused on reduction of oxidative stress of an end stage renal disease patients during hemodialysis (Huang et al., 2003). Hemodialysis by-products such as hydrogen peroxide, hypochlorite (HOCl) and the resulting oxidized products of lipids and proteins were significantly reduced by using ERW, resulting in less oxidative stress on the patients. The authors have explained the result by noting that ERW exhibits activities of ROS-neutralizing enzyme such as superoxide dismutase and catalase against superoxide and hydrogen peroxide, which originate from the 'hydrogen atoms' generated by electrolysis. Although the 'hydrogen atom theory' has a matter of controversy, the ROS scavenging effects of the ERW are evident in the results of other investigations (Hanaoka, 2001; Shirahata et al., 1997).

Relatively little is known regarding the role of ROS in obesity. Fenster *et al.* (Fenster et al., 2002) have proposed a hypothesis that explains the relation of ROS and obesity as follows. Oxidative stress in obesity may result, in part, from the accumulation of intracellular TG, which has been proposed to elevate superoxide production in the electron transport chain via the inhibition of the adenosine nucleotide transporter (Bakker et al., 2000). Inhibition of the adenosine nucleotide transporter results in the reduction of the levels of mitochondrial ADP, which is the proton acceptor in the ATP synthesis reaction (Fenster et al., 2002). As the result, electrons transported from the electron transport chain react with oxygen to form superoxides (Fenster et al., 2002). In this study, rats were fed on high-fat diet for 83 days. Excess calorie supplementation may serve to promote increased TG synthesis that is the causative agent of insulin resistance (Chen and Farese, 2004). However, the MRW administration resulted in a significant reduction in serum TG levels (Table 2), which were positively proportional to liver TG levels (Coppack et al., 1994). As the result, the reduction of TG in the sera may represent the reduction not only of insulin resistance and obesity but also of superoxide generation in the mitochondria. Reduction of body weight of the SD rats in the MRW group in this study was consistent with the results of our previous study, in which significant reductions in body weights were observed in volunteers to whom one thirty volume of ERW against their body weights was administered everyday (Lee et al., 2004). We have also reported that mean ROS levels in the livers of B16BL6 melanoma-metastased C57BL6 mice fed on the MRW were decreased compared with control mice (Lee, et al., 2003). In fatty individuals, the activity of superoxide dismutase decreased significantly, although the body weight of the rats did not change significantly (Beltowski et al., 2000).

In this study, we evaluated, for the first time, the effects of the MRW on obesity in SD rats fed on a high-fat diet. The body weights of the rats decreased significantly, although not until the end of present study. Reduced levels of serum TG suggest further investigations on the effect of the MRW on fatty acid metabolism and obesity. Steatosis was greatly reduced in the rats of the MRW group. We expect that these results are attributable to uncover the ROS scavenging properties of the MRW.

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