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Electrolyzed-reduced water inhibits acute ethanol-induced hangovers in Sprague-Dawley rats

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ABSTRACT

Ethanol consumption disturbs the balance between the pro- and anti-oxidant systems of the organism, leading to oxidative stress. Electrolyzed-reduced water (ERW) is widely used by people in East Asia for drinking purposes because of its therapeutic properties including scavenging effect of reactive oxygen species. This study was performed to investigate the effect of ERW on acute ethanol-induced hangovers in Sprague-Dawley rats. Alcohol concentration in serum of ERW-treated rats showed significant difference at 1 h, 3 h and 5 h respectively as compared with the rats treated with distilled water. Both alcohol dehydrogenase type 1 and acetaldehyde dehydrogenase related with oxidation of alcohol were significantly increased in liver tissue while the level of aspartate aminotransferase and alanine aminotransferase in serum was markedly decreased 24 h after pre-oral administration of ERW. Moreover, oral administration of ERW significantly activated non-ezymatic (glutathione) and enzymatic (glutathione peroxidase, glutathione-S-transferase, Cu/Zn-superoxide dismutase and catalase) antioxidants in liver tissues compared with the control group. These results suggest that drinking ERW has an effect of alcohol detoxification by antioxidant mechanism and has potentiality for relief of ethanol-induced hangover symptoms.

Large quantities of ethanol consumption leads to liver damage, pancreatitis, myocardial infarction and neuropathy and exacerbates the symptoms of tuberculosis and other diseases (15). Ethanol consumption and associated medical disorders continue to grow in most western countries (12, 15). Heavy drink of ethanol induces increased serum ethanol concentration and levels of liver marker enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT) (1, 16, 18). Moreover, ethanol administration causes more damage to the liver than to any other organ (14). The activities of antioxidants, such as reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), or glutathione-S-transferase (GST), were greatly decreased in ethanol-treated mice or rats (3, 13, 16, 18). Therefore, the efficient detoxification of ethanol is crucial for the preservation of proper liver function as well as relief from ethanol-induced hangover.

Upon consumption, ethanol is rapidly oxidized in liver tissue to acetaldehyde and acetate by alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH), respectively (4, 14). Furthermore, ethanol consumption disturbs the balance between

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the pro- and anti-oxidant systems of the organism, thereby leading to oxidative stress by generating free radicals or reactive oxygen species (ROS) (18), which results in liver injury. To scavenge these oxidants, cells possess an antioxidant system including non-enzymatic (GSH) and enzymatic (SOD, CAT, GPx and GST) antioxidants.

Electrolyzed-reduced water (ERW), generated by electrolysis of water, has been shown to exert a suppressive effect on the free radical level in living organisms, thereby resulting in disease prevention (6). Various biological effects of ERW have been reported such as anti-diabetic effect (9, 10), antioxidant effect (6), DNA protecting effect from oxidative damage by ROS (19), growth-stimulating effect of fetus (24), and growth-stimulating effect of anaerobic microflora in the human intestine (23). Huang *et al*. (8) demonstrated the potential therapeutic effects of ERW on end-stage renal disease patients, in whom the combined use of ERW during hemodialysis caused a reduction in oxidative stress. Although a variety of bioactive functions have been reported, the effect of ERW on alcoholic disorders *in vivo* is still unknown. Therefore, we performed a study to examine the effects of ERW on acute ethanol-induced hangover in Sprague-Dawley rats.

MATERIALS AND METHODS

Animals and experimental design. Male Sprague-Dawley rats (8 weeks, 350 ± 20 g) purchased from Orient Bio Inc. (Seongnam, Korea) were housed in stainless steel cages in a controlled environment of a 12 h light and dark cycle, 55 ± 5% humidity and 22 ± 2°C. After 1 week of acclimatization, the rats were randomly assigned to control (Con, n = 10) and experimental (Exp, n = 10) groups. After fasting for 12 h, the Exp and Con groups were given electrolyzed-reduced water (ERW; 10 mL/kg b.w.) and distilled water (DW) (10 mL/kg b.w.) respectively by gavage. After 30 min, 40% ethanol (5 g/kg b.w.) was given to each rat by gavage, neither food nor water was supplied for 5 h. Blood sample was taken from the tail vein of each rat after 1 h, 3 h, and 5 h, respectively for alcohol concentration assay, and from the retro-orbital plexus after 24 h for detection of liver marker enzymes: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma glutamyl transpeptidase (GGT). The blood samples were centrifuged at 3000 × *g* for 10 min at 4°C. Liver tissues were separated from each rat of the Exp and Con groups after anesthesia with ether and liver perfusion for detection of alcohol dehydrogenase (ADH), acetaldehyde dehydrogenase (ALDH), Cu/Zn-superoxide dismutase (Cu/Zn-SOD) and catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GPx), and glutathione S-transferase (GST) 24 h after oral-treatment of ethanol. The animal use and care protocols for these experiments were approved by Yonsei University at Wonju Campus IACUC, Gangwon, Korea.

Preparation of electrolyzed-reduced water. ERW was generated from tap water using a continuously electrolyzing apparatus (DOD-003; HDr, Inc., Korea). The ERW used in this study has the following physical properties: pH 10.05 ± 0.05, oxidation reduction potential (ORP) of −395.0 ± 16.5 mV, dissolved oxygen (DO) of 4.82 ± 0.11 mg/L, and electrical conductivity (EC) of 223 ± 5.5 μS.

Alcohol concentration assay in serum. The serum alcohol concentration was measured using an ethanol assay kit (BioVision, Mountain View, CA) according to the manufacturer's instructions. Briefly, 50 μL of diluted standard or sample was incubated with reaction mix (46 μL assay buffer, 2 μL ethanol probe, and 2 μL ethanol enzyme mixture) at room temperature for 60 min, protected from light. Alcohol oxidase oxidized ethanol to generate H_2O_2 that reacts with ethanol probe to generate a colored product which can easily be quantified by colorimetric detection. The absorbance at 570 nm was detected with an automated microplate reader (Beckman Coulter, Fullerton, CA). The serum ethanol concentrations of the test samples were calculated from the standard curve, multiplied by the dilution factor.

Estimation of liver marker enzyme levels. Twenty four hours after treatment with ethanol, the serum levels of ALT, AST, ALP, and GGT were determined enzymatically using the FUJI DRI-CHEM 3500i (Fuji Photo Film Co., Tokyo, Japan), and expressed in U/L.

Reduced glutathione (GSH) concentration assay. The GSH concentration in liver tissue was measured using a glutathione assay kit (Cayman Chemical Company, Michigan, USA) according to the manufacturer's instructions. Briefly, samples were prepared at appropriate dilutions using MES buffer (0.4 M 2-(N-morpholino) ethanesulphonic acid, 0.1 M phosphate, and 2 mM EDTA, pH 6.0). Then, 50 μL of diluted samples and standards were incubated with 150 μL assay cocktail [MES buffer (11.25 mL),

reconstituted cofactor mixture (0.45 mL), enzyme mixture (2.1 mL), HPLC grade water (2.3 mL), and reconstituted 5,5'-Dithiobis (2-nitrobenzoic acid) (0.45 mL)] in the dark on an orbital shaker. The absorbance at 405 nm was measured using an automated microplate reader (Beckman Coulter) at 25 min. The GSH concentrations of the samples were calculated from the standard curve.

Glutathione peroxidase (GPx) activity assay. The GPx activity of liver tissue was measured using a glutathione peroxidase assay kit (Cayman Chemical Company) according to the manufacturer's instructions. In brief, samples were prepared at appropriate dilutions using sample buffer (50 mM Tris-HCl, 5 mM EDTA, and 1 mg/mL BSA, pH 7.6). The reaction between samples and co-substrate mixture (which consists of NADPH, glutathione, and glutathione reductase) was initiated by cumene hydroperoxide. The absorbance at 340 nm was measured at least 5 times using an automated microplate reader (Beckman Coulter) at one minute intervals. The GPx activity was calculated using the following formula:

$$\text{GST activity (nmol/min/mL)} = \frac{\Delta A340/\text{min.}}{0.00373/\mu M} \times \frac{0.19\ \text{mL}}{0.02\ \text{mL}} \times \text{sample dilution.}$$

Glutathione S-Transferase (GST) activity assay. The GST activity of liver tissue was measured using a glutathione S-Transferase assay kit (Cayman Chemical Company) according to the manufacturer's instructions. Briefly, samples were prepared at appropriate dilutions using sample buffer (100 mM potassium phosphate, 0.1% triton X-100, 1 mM glutathione, and 1 mg/mL BSA, pH 6.5). The reaction between samples and glutathione was initiated by adding 1-chloro-2, 4-dinitrobenzene (CDNB). The absorbance at 340 nm was measured at least 5 times using an automated microplate reader at one minute intervals. GST activity was calculated using the following formula:

$$\text{GST activity (nmol/min/mL)} = \frac{\Delta A340/\text{min.}}{0.00503/\mu M} \times \frac{0.2\ \text{mL}}{0.02\ \text{mL}} \times \text{sample dilution.}$$

Real time-PCR analysis. To detect ADH1, ALDH, Cu/Zn-SOD and CAT one gram of liver was homogenized with 5 mL of cold homogenization solution (100 mM potassium phosphate, 2 mM EDTA, pH 7.0) by a homogenizer (MICCRA D-1; ART-moderne Labortechnik e.K., Germany). The homogenates were centrifuged at 10,000 × *g* for 15 min at 4°C, and the supernatants were removed for evaluation and stored at −80°C until use. Total RNA was isolated from liver tissue using the TRI reagent (Molecular Research Center Inc., Cincinnati, USA). The quantity and purity of RNA were verified by measuring A260 and A280. cDNA was synthesized from total RNA (2 μg) and oligo (dT)18 primers (0.5 μg) using Prime RT premix (GeNet Bio, Korea) according to the manufacturer's instructions. cDNA was added to QuantiTect SYBR green PCR master mix (Qiagen GmbH, Germany) and was then subjected to quantitative real-time PCR analysis using Rotor-Gene™ 3000 (Corbett Life Science). The primers used in this study are as follows: ADH1 (forward) 5'-CTG TAA AGC AGC AGG AGC AG-3', (reverse) 5'- TCC TAC GAC GAC GCT TAC AC-3'; ALDH (forward) 5'-GAT CAA CAC AGG TTG GCA AG-3', (reverse) 5'-TGA GGG CCT TGA TTT ATT CC-3'; Cu/Zn SOD (forward) 5'-ACC AGT GCA GGA CCT CAT TTT AA-3', (reverse) 5'-TCT CCA ACA TGC CTC TCT TCA TC-3'; CAT (forward) 5'-ACC AGG GCA TCA AAA ACT TG-3', (reverse) 5'-GCC CTG AAG CTT TTT GTC AG-3'; and β-actin (forward) 5'-TCA CCC ACA CTG TGC CCA TCT ACG A-3', (reverse) 5'-GGA TGC CAC AGG ATT CCA TAC CCA-3'. β-actin was used as an internal control. After a 10 min denaturation at 95°C, the reactions were cycled for 45 cycles at 95°C for 30 s and at 60°C for 1 min. To verify that only the specific product was amplified, a melting point analysis was done after the last cycle by cooling samples to 55°C and then increasing the temperature to 95°C at 0.2°C/s. Results were calculated using the ΔCt method normalizing to β-actin expression for each sample.

Statistical analysis. All data are presented as mean ± SD (n = 10). Comparisons between two groups were analyzed by using the Student's *t* test. A *P* value less than 0.05 was considered to be statistically significant.

RESULTS

Ethanol concentration in serum

To examine the effect of ERW on acute ethanol-induced hangovers, the serum ethanol concentration of rat was measured at 1, 3, and 5 h after oral admin-

istration of 40% ethanol. As shown in Table 1, the alcohol concentration of the control (Con) and the experimental (Exp) groups peaked at 1 h and decreased at 3 h and 5 h in order. The ethanol concentration of the Exp group treated with ERW was significantly reduced at 1 h ($P < 0.05$), 3 h ($P < 0.05$) and especially 5 h ($P < 0.01$) compared with that of the Con group.

Effect of ERW on the expression of ADH and ALDH
The relative expression of ADH1 in serum of the ERW-treated group was increased by about 3.3-fold compared with that in the Con group ($P < 0.01$). In addition, the expression of ALDH of the Exp group increased by approximately 3.5-fold compared with the Con group ($P < 0.05$) (Fig. 1).

Effect of ERW on the levels of liver marker enzymes (ALT, AST, ALP and GGT)
Oral administration of ERW led to a significant reduction of AST level in the Exp group (117.1 ± 8.005 U/L) compared with the Con group (173.2 ± 29.15 U/L) ($P < 0.05$). ALT level was also slightly decreased in the Exp group (35.13 ± 2.467 U/L) compared to the Con group (42.17 ± 6.215 U/L), although there was no statistical significance. However, ALP and GGT levels did not show any differences between two groups (Fig. 2).

Effect of ERW on GSH, GPx and GST in liver
The GSH level of the Exp group (2397 ± 145.9 μM) was significantly increased 24 h after ERW and 40% ethanol administration orally when compared with the control group (1807 ± 185.1 μM) ($P < 0.05$) (Fig. 3). Pre-oral administration of ERW significantly increased hepatic GPx activity (1655 ± 226.6 nmol/min/mL) compared to the control group (817.6 ± 158.1 nmol/min/mL) 24 h after treatment with ethanol ($P < 0.05$) (Fig. 4). On the other hand, as shown in Fig. 4, the activity of GST in the ERW treated

Table 1 *Serum alcohol concentration in rats treated with 40% ethanol*

Time	Serum alcohol concentration (mM)	
	Control group	Experimental group
1 h	89.70 ± 19.172	40.67 ± 3.865*
3 h	81.98 ± 17.454	33.61 ± 3.564*
5 h	68.42 ± 9.420	32.77 ± 2.946**

Values are expressed as mean ± SD for 10 rats in each group.
*$P < 0.05$ vs. control group, ** $P < 0.01$ vs. control group

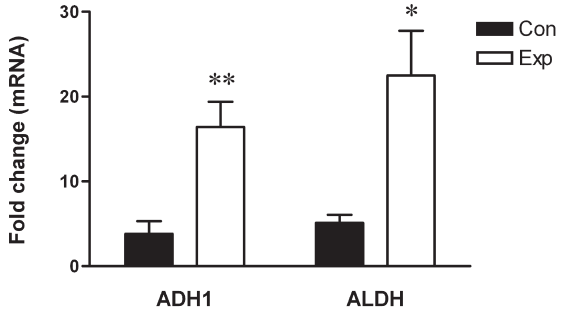

Fig. 1 The mRNA expression levels of alcohol dehydrogenase1 (ADH1) and acetaldehyde dehydrogenase (ALDH) in liver tissue 24 h after administration with 40% ethanol by quantitative real-time PCR. Values represent mean ± SD (n = 10). Exp: ERW-treated group, Con: DW-treated group, *$P < 0.05$, *$P < 0.01$

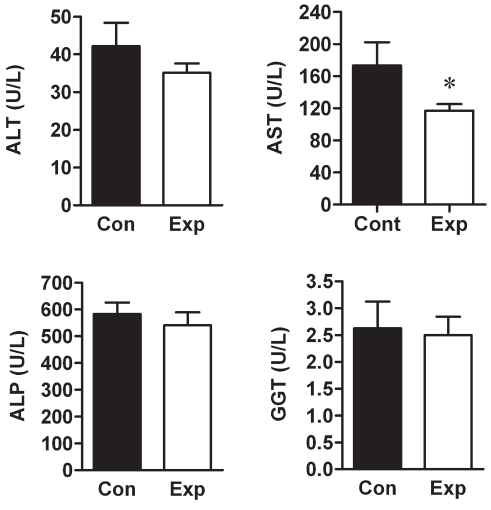

Fig. 2 The level of liver marker enzymes (ALT, AST, ALP, and GGT) in the serum 24 h after 40% ethanol treatment. Values represent mean ± SD (n = 10). ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, and GGT: gamma glutamyl transpeptidase, Exp: ERW-treated group, Con: DW-treated group, *$P < 0.05$

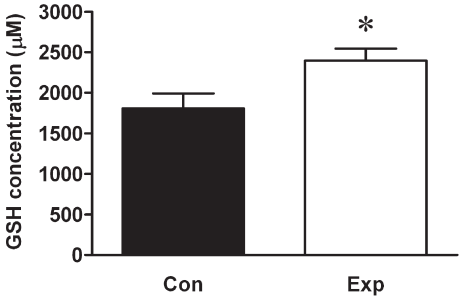

Fig. 3 Glutathione (GSH) concentration in liver tissue of rats 24 h after 40% ethanol. Values represent mean ± SD (n = 10). Exp: ERW-treated group, Con: DW-treated group, *$P < 0.05$

experimental group (1984 ± 323.5 nmol/min/mL) was increased by approximately 50% compared to the control group (987.6 ± 268.2 nmol/min/mL) ($P < 0.05$).

Effect of ERW on the expression of Cu/Zn-SOD and CAT

The activities of Cu/Zn-SOD and CAT were analyzed by measuring the mRNA expression levels of these enzymes in liver tissue by quantitative real-time PCR. The mRNA expression levels of Cu/Zn-SOD was increased by approximately 3.1-fold in the ERW-treated group compared to the Con group ($P < 0.05$). Similarly, CAT mRNA expression levels were also significantly higher in the Exp group than that in the Con group ($P < 0.05$) (Fig. 5).

DISCUSSION

Hangovers are induced by ethanol and its metabolites such as acetaldehyde and acetate (3). Therefore, ethanol metabolism in the liver is involved in recovery of hangover. It is known that ethanol is oxidized to acetaldehyde and acetate by ADH and ALDH. These enzymes are directly related with ethanol metabolism (4, 14). The activities of ADH and ALDH have been shown to be greatly reduced in liver tissue of ethanol-loaded mice (3). In this study, relative expression of ADH and ALDH in the ERW-administered group was significantly increased 24 h after 40% ethanol intake compared with the control group administrated with distilled water (Fig. 1). Increased ADH and ALDH expression explains the result that serum alcohol concentration of the ERW-administrated group was significantly lower than that of control group at 1 h, 3 h and 5 h after alcohol intake (Table 1).

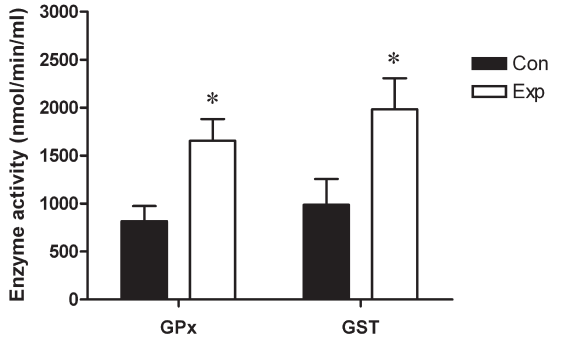

Fig. 4 The activities of glutathione peroxidase (GPx) and glutathione S-Transferase (GST) in liver tissue of rats 24 h after 40% ethanol treatment by quantitative real-time PCR. Values represent mean ± SD (n = 10). Exp: ERW-treated group, Con: DW-treated group, $^*P < 0.05$

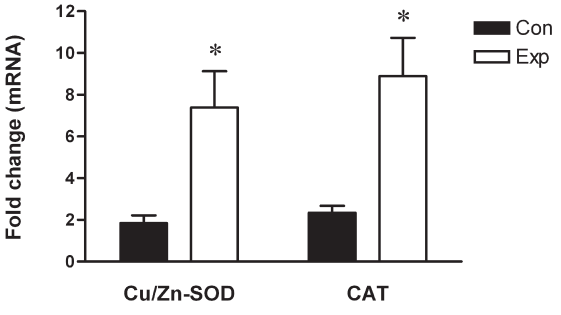

Fig. 5 The mRNA expression of Cu/Zn-superoxide dismutase (Cu/Zn-SOD) and catalase (CAT) in liver tissue of rats 24 h after 40% ethanol treatment by quantitative real-time PCR. Values represent mean ± SD (n = 10). Exp: ERW-treated group, Con: DW-treated group, $^*P < 0.05$

We further explored the potential mechanism involved in the inhibition of ethanol-induced hangovers by ERW. Heavy intake of ethanol disturbs the delicate balance between the pro- and anti-oxidant systems of the organism, therefore leading to oxidative stress (26). Elevated generation of reactive oxygen species (ROS) or free radicals causes auto-oxidation of hepatic cells, resulting in marked hepatic lesions (21), therefore inducing leakage of cellular enzymes into the serum (2). Kumar Rajagopal *et al.* (11) demonstrated that serum levels of AST or ALT increased in ethanol-administered rats by damage of both hepatic cellular and mitochondrial membranes. The increased activities of liver marker enzymes such as ALT, AST, ALP, and GGT have previously been detected in alcohol-administered mice (13, 16), implying the increased permeability, damage and necrosis of hepatocytes. However, in this study, the levels of ALT and AST, but not those of ALP and GGT, were decreased by pre-administration of ERW compared with control group 24 h after 40% ethanol oral-administration. This indicates that ERW could induce some mechanism to preserve the structural integrity of liver cells and hepatic mitochondria from the adverse effects of ethanol.

The potential harmful effects of ROS and/or free radicals are controlled by the cellular antioxidant defense system (22). The inhibition of the antioxidant system may cause the accumulation of H_2O_2 or products of its decomposition (5). Enzymatic antioxidants such as SOD, CAT, GPx and GST are the first line of defense against oxidative injury. SOD and CAT are the enzymes that help to scavenge superoxide ions and hydroxyl ions respectively. It is known that the activities of SOD and CAT are sig-

nificantly decreased in ethanol-administered mice (3, 13, 16, 18). On the other hand, GPx and GST play a crucial role in scavenging ROS and/or free radicals, and acute or chronic ethanol oral administration causes a decrease in the activities of GPx and GST in liver tissue of rats (3, 15, 16, 18). Ethanol or its metabolites might specifically target GST isoenzymes, and the reduction in enzyme activity or expression may contribute to ethanol hepatotoxicity (16). In addition, GSH (the major non-enzymatic antioxidant) plays a central role in coordinating the antioxidant defense processes, which is involved in the maintenance of normal cell structure and function because of its involvement in redox and detoxification reactions (17). In the present study, the oral administration of ERW showed to increase the concentration and activities of several non-enzymatic and enzymatic antioxidants such as GSH, GPx, GST, SOD, and CAT in liver tissue of rats 24 h after 40% ethanol intake. This result indicates that ERW potentially has a positive effect on the alcohol metabolism through the elevation of antioxidant-related enzyme activity.

ERW produced by electrolysis from tap water has the properties of a high pH (9.0–10.0), significantly low oxidation reduction potential (ORP) values, low dissolved oxygen (DO) and high dissolved hydrogen (DH) compared to non-electrolyzed water. According to other researchers, ERW exhibits various bioactive functions. Jin *et al.* (9) and Kim *et al.* (10) demonstrated anti-diabetic effect of ERW through the results of reduced blood glucose concentration, increased blood insulin level, improved glucose tolerance and preserved pancreatic β-cell mass using diabetic animal model. Huang *et al*. (7, 8) demonstrated the potential therapeutic effects of ERW through reduction of hemodialysis-induced oxidative stress and erythrocyte impairment in end-stage renal disease patients. ERW also has a tendency to suppress single-strand breakage of DNA induced by ROS *in vitro* (19) and selectively stimulates the growth of anaerobic microflora in the human intestinal tract (23). The mechanism of these researches has been partly explained by anti-oxidant effect of ERW and reduced oxidative stress. Recent increasing evidences suggest that ERW has an antioxidant effect by scavenging ROS or free radicals including superoxide anion radicals ($O_2^{-}\cdot$), hydrogen peroxide molecules (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radicals (·OH) (20, 25). Moreover Hanaoka *et al.* (6) reported that the enhancement of antioxidant effects of ERW was due to the increase of the ionic product of water (pK_w) as solvent, therefore enhancing the superoxide anion radical dismutation activity in ERW. ERW also exhibits strong and stable SOD- and CAT-like activities because of extremely high concentration of dissolved molecular hydrogen (DH) produced in ERW during electrolysis of water (19). On the basis of these mechanisms, oral administration of ERW is supposed to elicit detoxification effect against alcohol-induced toxicity as seen from the lower level of liver marker enzymes in serum and higher expression of antioxidant enzymes in liver tissue in the SD rats treated with 40% ethanol.

In conclusion, pre-oral administration of ERW was more effective in increasing ADH and ALDH expression in liver tissue 24 h after ethanol administration and in reducing alcohol concentration in serum at 1 h, 3 h and 5 h compared with DW-treated control group. In addition, it decreased the activity of liver marker enzymes in serum (AST and ALT) and antioxidant enzymes in liver tissue (GPx, GST, Cu/Zn-SOD and CAT) compared with control group. These results indicate that ERW protects anti-oxidative system from excess ROS and/or free radicals induced by ethanol administration, and enhances ethanol detoxification by increase in the activities of enzymes related to the alcohol metabolism *in vivo*. Hence it is considered that drinking ERW has a potential relieving effect in acute ethanol-induced hangovers.

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