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Electrolyzed-Reduced Water Protects Against Oxidative Damage to DNA, RNA, and Protein

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Abstract

The generation of reactive oxygen species is thought to cause extensive oxidative damage to various biomolecules such as DNA, RNA, and protein. In this study, the preventive, suppressive, and protective effects of in vitro supplementation with electrolyzed-reduced water on H_2O_2-induced DNA damage in human lymphocytes were examined using a comet assay. Pretreatment, cotreatment, and posttreatment with electrolyzed-reduced water enhanced human lymphocyte resistance to the DNA strand breaks induced by H_2O_2 in vitro. Moreover, electrolyzed-reduced water was much more effective than diethylpyrocarbonate-treated water in preventing total RNA degradation at 4 and 25°C. In addition, electrolyzed-reduced water completely prevented the oxidative cleavage of horseradish peroxidase, as determined using sodium dodecyl sulfate-polyacrylamide gels. Enhancement of the antioxidant activity of ascorbic acid dissolved in electrolyzed-reduced water was about threefold that of ascorbic acid dissolved in nonelectrolyzed deionized water, as measured by a xanthine–xanthine oxidase superoxide scavenging assay system, suggesting an inhibitory effect of electrolyzed-reduced water on the oxidation of ascorbic acid.

Index Entries: Electrolyzed-reduced water; oxidative damage; DNA; RNA; protein.

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Introduction

Electrolysis of water produces oxidized water near the anode and reduced water near the cathode *(1)*. The bactericidal activities of anodic oxidized water prepared by the electrolysis of water containing a small amount of electrolytes have been widely demonstrated *(2)*. Electrolyzed-oxidized water has been shown to effectively inactivate various bacteria, such as *Salmonella enteritidis (2)*, *Listeria monocytogenes (2)*, *Escherichia coli* K12 *(3)*, *E. coli* O157:H7 *(4)*, and methicillin-resistant *Staphylococcus aureus (5)*. The efficacies of electrolyzed-oxidized water for the microbial decontamination of vegetables *(6,7)* and poultry *(8,9)* have been extensively examined. Moreover, oxidized water is considered a safe and strong disinfectant in the medical *(5,10)* and dental fields *(11)*. Electrolyzed-oxidized water was as efficient as glutaraldehyde in eliminating bacteria from patient-used endoscopes *(10)* and was effective in the microbial decontamination of dental unit waterlines *(11)*. Furthermore, it has been shown that electrolyzed-oxidized water is not harmful to the human body *(12)*.

Electrolyzed-reduced water has recently generated much interest as an ideal and powerful radical scavenger *(13)*. Shirahata et al. *(13)* showed that electrolyzed-reduced water could scavenge active oxygen species such as superoxide anions (O_2-) and H_2O_2 *(13)*. Thus, the superoxide dismutase-like and catalase-like activity of the electrolyzed-reduced water suppressed single-strand breakage of bacterial plasmid DNA caused by the mixture of ascorbic acid and Cu. Moreover, it has been suggested that the reduced water is useful in preventing alloxan-induced type 1 diabetes mellitus in hamster cells *(14)*. Electrolyzed-reduced water has been shown to have a much stronger permeability and much higher dissolution rate than nonelectrolyzed water *(15)*. Reduced water has previously been shown to protect human lymphocyte DNA against paraquat-induced oxidative damage *(16)*. However, other properties and efficacies of reduced water remain to be clarified.

In the present study, the protective effects of electrolyzed-reduced water against oxidative damage to total RNA and protein were investigated. Moreover, the suppressive effect of electrolyzed-reduced water on H_2O_2-induced DNA damage of human lymphocytes was examined using alkaline single-cell gel electrophoresis (the comet assay).

Materials and Methods

Preparation of Electrolyzed-Reduced Water

The electrolyzed water-generating apparatus consisted of an anode, a cathode, and middle chambers. Ultrapure water was supplied to each chamber, and the diluted electrolyte (NH_4Cl) was supplied to the middle chamber. Electrolyzed-reduced water was generated by electrolysis of the water and the diluted electrolyte with a current of 9 A and a voltage of

10.5 V near the cathode using a Redox-water generator (Microbank). The reduced water used had a pH of 11.0 and an oxidation-reduction potential of –850 mV.

Alkaline Single-Cell Gel Electrophoresis (Comet) Assay

Alkaline single-cell gel electrophoresis (comet) assay was conducted according to Singh et al. *(17)* with little modification. Human blood samples were obtained from two healthy male volunteers (nonsmokers, 24 and 25 yr old, respectively), and 5 mL of fresh whole blood was added to 5 mL of phosphate-buffered saline (PBS). The samples were layered onto 5 mL of Histopaque 1077. After centrifuging for 30 min at 4000*g* at room temperature, the lymphocytes were collected from just above the boundary with the Histopaque 1077 and washed with 5 mL of PBS. The cells were then used for the comet assay or were resuspended in freezing medium (90% fetal calf serum, 10% dimethylsulfoxide [DMSO]) at 6×10^6 cells/mL. The cells were frozen at –80°C, and the lymphocytes were thawed rapidly in a water bath at 37°C prior to each experiment. PBS based on electrolyzed-reduced water instead of deionized water was prepared at concentrations of 1000, 800, 500, 200, and 0 µL of electrolyzed-reduced water/mL of PBS. The diluted reduced water was applied to the lymphocytes under four different treatment conditions. First, lymphocytes (2×10^4 cells/mL) were incubated in each concentration of diluted electrolyzed-reduced water for 30 min at 37°C in the dark. Second, lymphocytes were oxidatively damaged with 200 m*M* H_2O_2 for 5 min on ice and then incubated in each concentration of electrolyzed-reduced water for 30 min at 37°C. Third, lymphocytes were incubated in the diluted electrolyzed-reduced water for 30 min at 37°C in the dark and then oxidatively damaged with 200 m*M* H_2O_2 for 5 min on ice. Fourth, lymphocytes were simultaneously incubated with electrolyzed-reduced water and 200 m*M* H_2O_2 for 5 min on ice. After each treatment, the samples were centrifuged at 1450*g* for 5 min and washed with PBS. All the experiments were repeated three times with lymphocytes from both donors on separate days. The cell suspension was mixed with 75 mL of 0.5% low-melting agarose and added to slides precoated with 1.0% normal melting agarose. After solidification of the agarose, the slides were covered with another 75 µL of 0.5% low-melting agarose and then immersed in lysis solution (2.5 *M* NaCl, 100 m*M* EDTA, 10 m*M* Tris, 1% sodium lauryl-sarcosine, 1% Triton X-100, and 10% DMSO) for 1 h at 4°C. The slides were placed in an electrophoresis tank containing 300 m*M* NaOH and 10 m*M* Na_2EDTA (pH 13.0) for 40 min for DNA unwinding. For electrophoresis of the DNA, an electric current of 25 V and 300 mA was applied for 20 min at 4°C. The slides were washed three times with a neutralizing buffer (0.4 *M* Tris, pH 7.5) for 5 min at 4°C and then treated with ethanol for another 5 min before being stained with 50 µL of ethidium bromide (20 µg/mL). Measurements were made by image analysis (Kinetic Imaging, Komet 5.0, Liverpool, UK) and fluorescence microscopy (LEICA DMLB, Bensheim, Germany) to

determine the percentage of fluorescence in the tail (tail intensity, 50 cells from each of three replicate slides). The data were analyzed using an SPSS package for Windows (Version 10). Values were expressed as mean ± SE. The mean values of the tail intensity from each treatment were compared using one-way analysis of variance followed by Duncan's test. A p value of less than 0.05 was considered significant.

Isolation of Total RNA and Agarose Gel Electrophoresis

Total RNA from plant seedlings (*Zea mays* L.) was isolated using TRI reagent® (Molecular Research Center) according to the manufacturer's instructions. Total RNA (2 μg) was incubated with diethylpyrocarbonate (DEPC)-treated water or electrolyzed-reduced water at 4 or 25°C, and then electrophoretic separation on formaldehyde agarose gels was performed for 30 min at 50 V.

Preparation of Protein and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using horseradish peroxidase (HRP) was carried out as described by Laemmli *(18)*. Myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), fructose-6-phosphate kinase (84 kDa), albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), and a-lactalbumin (14.2 kDa) served as molecular weight markers. Twenty-five micrograms of HRP protein was treated with 1% SDS and 2-mercaptoethanol for 5 min at 100°C and then subjected to SDS-PAGE on a 12% gel. The gels were stained with Coomassie Blue R-250.

Determination of Antioxidant Activity

The antioxidant activity of electrolyzed-reduced water was indirectly determined by comparing the antioxidant activity of ascorbic acid dissolved in electrolyzed-reduced water with that in deionized water. The antioxidant activity of ascorbic acid dissolved in 50 m*M* potassium phosphate buffer (pH 7.8) based on electrolyzed-reduced water or deionized water was measured using a xanthine–xanthine oxidase superoxide scavenging assay. The assay mixture contained, in a final volume of 1 mL, 50 m*M* potassium phosphate buffer (pH 7.8), 0.75 m*M* EDTA, and 15 m*M* xanthine in a 0.3 m*M* nitroblue tetrazolium (NBT) solution. Different concentrations of ascorbic acid (250, 500, and 1000 ppm) were added before the addition of xanthine oxidase. Superoxide anion was generated by xanthine–xanthine oxidase and measured spectrophotometrically at 550 nm by the NBT reduction method *(19)*.

Results and Discussion

Protective Effect of Electrolyzed-Reduced Water Against Oxidative Damage of Human Lymphocyte DNA Determined by Comet Assay

Electrolyzed-reduced water has been reported to scavenge active oxygen species and to protect against oxygen radical–induced DNA damage of bacterial plasmids *(13)*. Moreover, electrolyzed-reduced water protected hamster pancreatic b-cell lines from alloxan-induced cell damage *(14)*. However, the protective effects of reduced water on human lymphocyte DNA damage have not been considered until now. A comet assay was used to examine whether the electrolyzed-reduced water suppressed H_2O_2-induced oxidative damage of DNA in human lymphocytes. Comet assays have been proven to be sensitive systems for investigating the genotoxicity of chemicals or complex mixtures, and numerous studies have used comet assays to investigate levels of DNA damage in individual cells *(20–23)*. The application of comet assays for monitoring the in vivo effects of chemicals on every type of eukaryotic cell has been explored. Comet assays are able to detect DNA damage induced by alkylating and intercalating agents, as well as oxidative damage caused by the generation of free radicals *(24,25)*. To evaluate the cytotoxicity of electrolyzed-reduced water on human lymphocyte, the effect of supplementation in vitro with different concentrations of electrolyzed-reduced water was quantified as percent tail fluorescence in the comet assay, as shown in Fig. 1. Increasing the amount of electrolyzed-reduced water did not affect the percent tail fluorescence of lymphocyte DNA compared with the percent fluorescence of the control, suggesting that electrolyzed-reduced water was not cytotoxic to human lymphocytes under the test conditions. Figure 2 shows the protective effect of the reduced water against H_2O_2-induced DNA strand breakage in human lymphocytes. Lymphocytes were treated with 200 m*M* H_2O_2 for 5 min and then incubated with each dilution of electrolyzed-reduced water for 30 min. DNA oxidatively damaged by H_2O_2 showed about 30% tail fluorescence, indicating marked DNA damage. On treatment with increasing concentrations of electrolyzed-reduced water, however, the percent fluorescence in the tail was reduced to the control level. Following treatment with 800 and 1000 µL of electrolyzed-reduced water/mL of PBS, the DNA damage was reduced to close to the control level, as evaluated by percent fluorescence of the tail. This result indicated that electrolyzed-reduced water protected human lymphocyte DNA against oxidative damage from reactive oxygen species (ROS).

Figure 3 shows that the DNA damage was prevented by pretreatment with electrolyzed-reduced water for 30 min, whereas human lymphocytes treated with 200 m*M* H_2O_2 without the addition of reduced water had marked DNA strand breakage. This result suggested the preventive effect of in vitro supplementation with electrolyzed-reduced water on oxidative

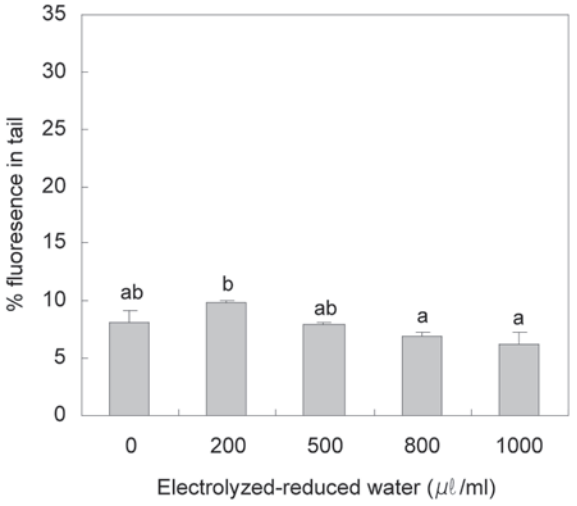

Fig. 1. Effect of in vitro supplementation with various concentrations of electrolyzed-reduced water in human lymphocytes. The values presented are means with SEs for experiments performed in triplicate with lymphocytes from two donors. Bars with different superscript letters are significantly different at $p < 0.05$ by Duncan's test.

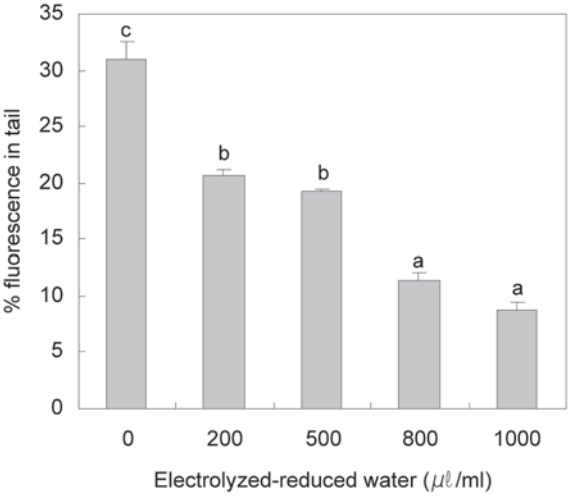

Fig. 2. Protective effect of in vitro supplementation with different concentrations of electrolyzed-reduced water on 200 m*M* H_2O_2-induced human lymphocyte DNA damage. The values are means with SEs from triplicate experiments with lymphocytes from two donors. Bars with different superscript letters are significantly different at $p < 0.05$ by Duncan's test.

DNA damage in human lymphocytes. Figure 4 shows the suppressive effect of in vitro supplementation with electrolyzed water on H_2O_2-induced human DNA damage. Simultaneous treatment with the reduced water and H_2O_2 also suppressed the H_2O_2-induced DNA damage in human lympho-

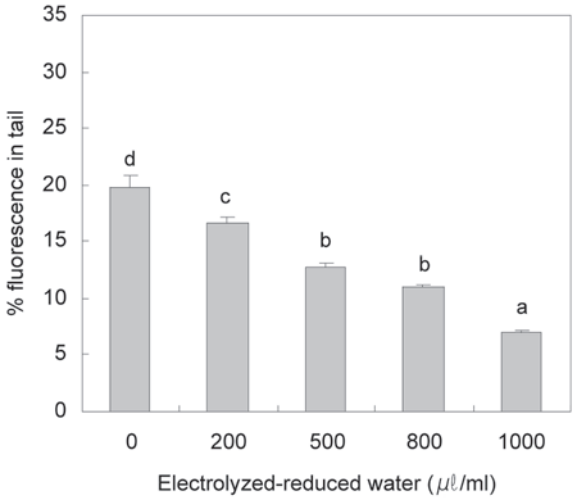

Fig. 3. Preventive effect of in vitro supplementation with different concentrations of electrolyzed-reduced water on 200 m*M* H_2O_2-induced human lymphocyte DNA damage. The values are means with SEs from triplicate experiments with lymphocytes from two donors. Bars with different superscript letters are significantly different at $p < 0.05$ by Duncan's test.

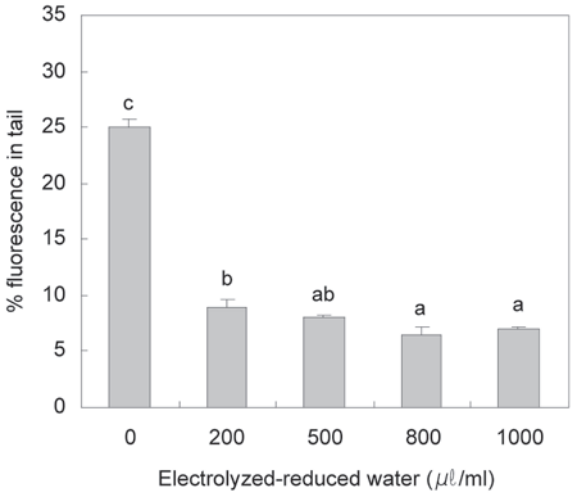

Fig. 4. Effect of simultaneous in vitro supplementation with different concentrations of electrolyzed-reduced water and 200 m*M* H_2O_2 in human lymphocytes. Values are means with SEs from triplicate experiments with lymphocytes from two donors. Bars with different superscript letters are significantly different at $p < 0.05$ by Duncan's test.

cytes, as quantified by percent tail fluorescence. H_2O_2, a known mutagen, can cause obvious damage to the DNA of mammalian cells, and H_2O_2-induced DNA damage in cells has been thought to occur through the Fenton reaction *(26)*. This oxidative DNA damage was significantly eliminated in

human lymphocytes in the present study by pretreatment, cotreatment, and posttreatment with electrolyzed-reduced water. Oxidative DNA damage can be repaired by a number of different enzymes *(22,27)*, and some phytochemicals, such as tannic acid, curcumin, resveratrol, indole-3-carbinol, and ellagic acid, have been shown to help in DNA recovery by accelerating the DNA repair efficiency in the damaged cells *(22,27)*.

Suppressive Effect of Electrolyzed-Reduced Water on Total RNA Degradation

RNA is known to be very unstable under alkaline conditions and vulnerable to RNase contamination. Consequently, it is important to avoid the accidental introduction of trace amounts of RNase from other potential sources. DEPC, which is a strong inhibitor of RNase, is a suspected carcinogen, and traces of DEPC may modify purine residues in RNA by carboxymethylation *(28)*. Total RNA was dissolved in electrolyzed-reduced water at 4 or 25°C for 1, 3, and 7 d, and the degradation of the total RNA was analyzed on formaldehyde agarose gels. The stability of the total RNA in DEPC-treated water and sterilized ultrapure water was also analyzed. As shown in Fig. 5, the electrolyzed-reduced water almost completely prevented the degradation of total RNA during the test period. However, total RNA dissolved in DEPC-treated water began to break down after 24 h at 4°C and was completely degraded after 7 d at 4°C. Total RNA dissolved in DEPC-treated water was almost completely degraded after 24 h at 25°C (Fig. 5, lanes 1 and 2). However, total RNA dissolved in various reduced waters remained intact, even at 25°C, showing that the preventive effect of the electrolyzed-reduced water was not lost by freezing, melting, or several days of storage at 4 and 25°C. The total RNA dissolved in sterilized ultrapure water showed degradation similar to that in DEPC-treated water. The oxidation-reduction potential of the electrolyzed-reduced water (pH 11.0) used here was –850 mV, whereas that of alkaline solution having the same pH of 11.0 was reported to be +20 mV *(1)*, showing much stronger reducing power of electrolyzed-reduced water than that of alkaline solution having the same pH. Actually, several alkaline buffers did not prevent RNA degradation at all (data not shown), indicating that the protective effect of electrolyzed-reduced water against biomolecule is not just a pH effect. The mechanism for the stability enhancement of total RNA in the presence of electrolyzed-reduced water is still unclear. The environmental factors causing the instability of total RNA under alkaline conditions and RNase attack may be altered by the presence of electrolyzed-reduced water. It is noteworthy that electrolyzed-reduced water prevented the degradation of total RNA and protein in the absence of radical scavengers in the aerobic reaction system. Aerobic organisms use oxygen, yet oxygen is principally known as a toxic substance that undergoes successive one-electron reductions to ROS.

The DNA sequences were amplified accurately when electrolyzed-reduced water was applied to reverse transcriptase-polymerase chain reac-

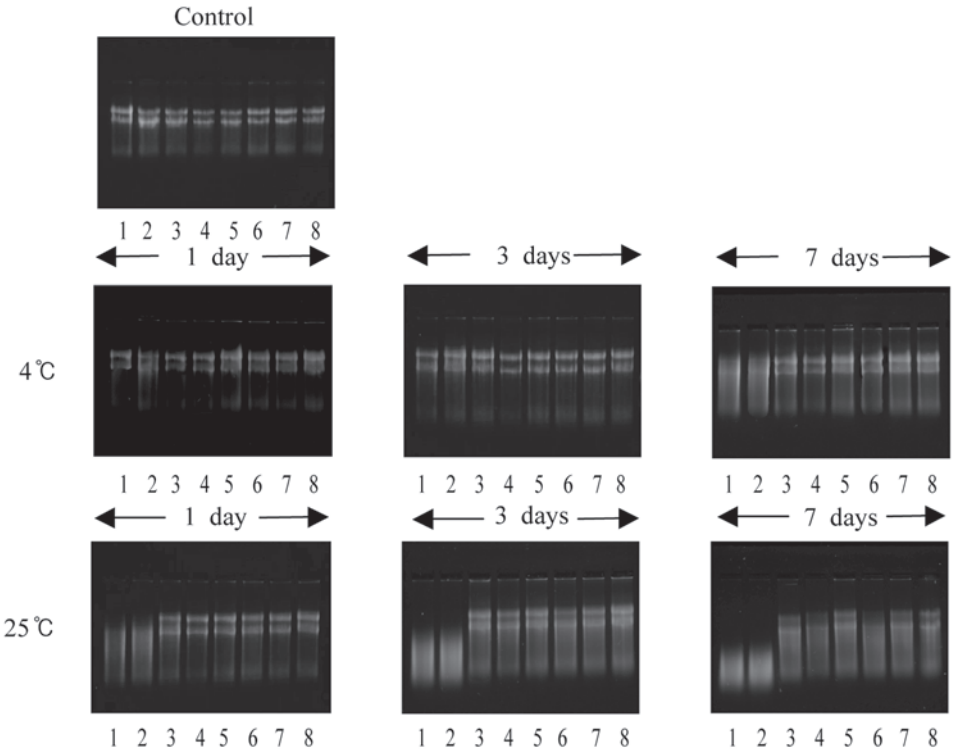

Fig. 5. Suppressive effect of electrolyzed-reduced water on total RNA degradation. Total RNA was dissolved in sterilized ultrapure water (lane 1) and DEPC-treated water (lane 2) at 4 and 25°C for the indicated number of days and was then analyzed on formaldehyde agarose gels. Total RNA was also dissolved in electrolyzed water and stored under different conditions: fresh electrolyzed-reduced water (lane 3) and electrolyzed-reduced water stored at 4°C for 2 d (lane 4), at 4°C for 4 d (lane 5), at –20°C for 4 d (lane 6), at 25°C for 2 d (lane 7), and at 25°C for 4 d (lane 8).

tion, showing that there is no difference between the DNA amplification in the electrolyzed-reduced water and DEPC-treated water (data not shown). The data taken together show that electrolyzed-reduced water has no effect on enzymatic reaction with RNA and is highly superior in RNA preservation.

Suppressive Effect of Electrolyzed-Reduced Water on Protein Degradation

As shown in Fig. 6, electrolyzed-reduced water prevented the oxidative cleavage of proteins. HRP dissolved in the electrolyzed-reduced water, deionized water, or electrolyzed-oxidized water for 10 min to 10 d at 4 and 25°C was analyzed on SDS-polyacrylamide gels. No cleaved protein fragments could be seen in the enzyme dissolved in the reduced water for 10 min, 7 d, and 10 d (Fig. 6), showing that electrolyzed-reduced water protected the protein against oxidative degradation. However, several cleaved peptide fragments were found in the enzyme dissolved in deion-

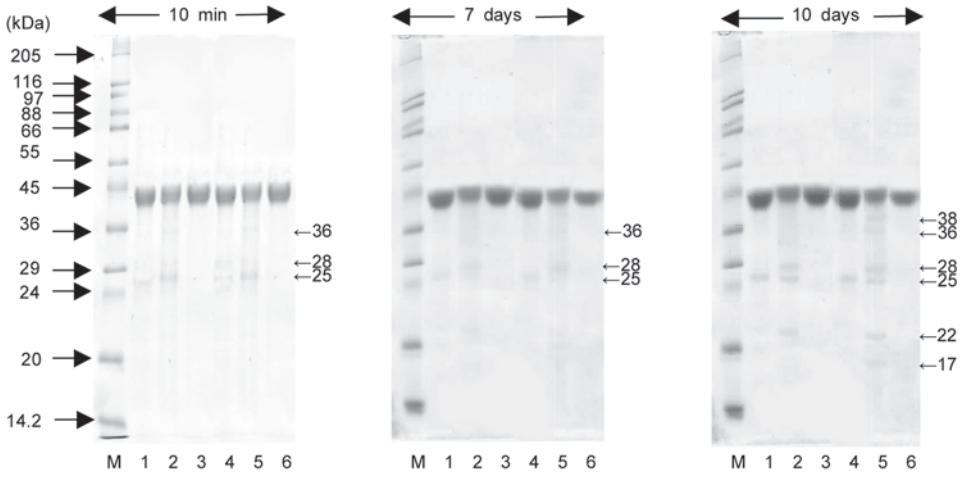

Fig. 6. Protective effect of electrolyzed-reduced water on peroxidase degradation. Peroxidase protein was dissolved in deionized water (lane 1), electrolyzed-oxidized water (lane 2), and electrolyzed-reduced water (lane 3) at 4°C for the indicated number of days and was then analyzed on SDS-polyacrylamide gels. Peroxidase protein was also dissolved in deionized water (lane 4), electrolyzed-oxidized water (lane 5), and electrolyzed-reduced water (lane 6) at 25°C. M, molecular mass marker.

ized water and in electrolyzed-oxidized water. As shown in Fig. 6, the main protein band of 43 kDa was degraded and cleaved into several discrete smaller peptides of 38, 36, 28, 25, 22, and 17 kDa at 25°C for 10 d in the presence of electrolyzed-oxidized water. The remaining intensity of the main band was about 81% owing to degradation and cleavage of the protein in the oxidized water compared with that of the enzyme in the reduced water. Hemoproteins such as peroxidase are easily cleaved without the addition of other metal ions, because the presence of heme iron may make the enzyme more susceptible to oxidative modification in the presence of oxygen *(29)*. The oxidative cleavage of Korean radish peroxidase was also inhibited by treatment with radical scavengers owing in part to the complexation of the Fe^{+2} ions necessary for hydroxyl radical formation *(30)*. Thus, electrolyzed-reduced water seems to inhibit hemoprotein degradation by scavenging ROS as well as iron ions.

Stimulation of Antioxidant Activity of Ascorbic Acid

Figure 7 shows the stimulating effect of electrolyzed-reduced water on the radical scavenger activity of ascorbic acid. Ascorbic acid is known for its prooxidant properties as well as an antioxidant effect in vivo *(31,32)*. The stimulating effect of electrolyzed-reduced water on the radical scavenging of ascorbic acid is an indirect evidence of the antioxidant activity of the reduced water. The antioxidant activity of ascorbic acid dissolved in electrolyzed-reduced water was compared with that in deionized water using a xanthine–xanthine oxidase superoxide scavenging assay. The maxi-

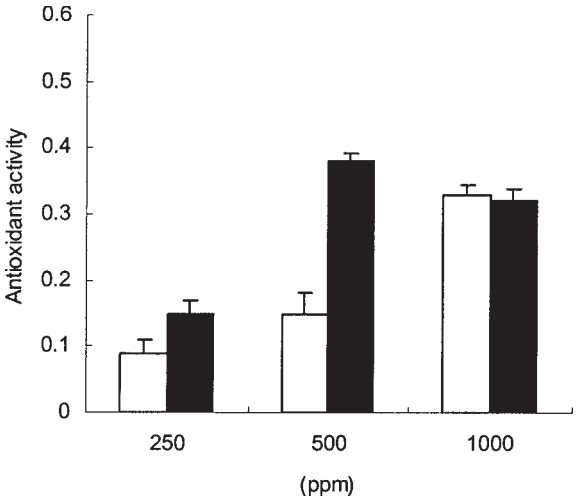

Fig. 7. Stimulation of electrolyzed-reduced water on antioxidant activity of ascorbic acid. The antioxidant activities of various amounts of ascorbic acid dissolved in electrolyzed-reduced water (solid bars) were compared with that in deionized water (open bars) using a xanthine–xanthine oxidase superoxide scavenging system.

mum antioxidant activity enhancement of 500 ppm of ascorbic acid dissolved in electrolyzed-reduced water was about three times that of ascorbic acid dissolved in nonelectrolyzed deionized water. The effect was less at 1000 ppm of ascorbic acid, suggesting that the reducing power of electrolyzed-reduced water was not sufficient to prevent 1000 ppm of ascorbic acid oxidation under these conditions. The enhanced antioxidant activity caused by the electrolyzed-reduced water may be attributable to the preventive effect of the reduced water on ascorbic acid oxidation, indirectly suggesting the presence of active hydrogen in the reduced water *(13)*. Further intensive characterization of electrolyzed-reduced water is needed to identify the actual reducing power of the reduced water. Moreover, the effects of electrolyzed-reduced water on human physiology should be investigated in detail, given that approx 70% of the human body is water and that water can penetrate everywhere in the body.

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