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ORIGINAL PAPER

# Electrolyzed-reduced water confers increased resistance to environmental stresses

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**Abstract** Electrolysis of water produces reduced water at the cathode and oxidized water at the anode. Electrolyzed-reduced water (ERW) has an extremely negative oxidation-reduction potential. ERW scavenges cellular reactive oxygen species (ROS) and suppresses single-strand breaks of plasmid DNA in bacteria. Here, we examined the effect of ERW on resistance to oxidative stress both *in vitro* and *in vivo*. Oxidative DNA damage in human lymphocytes was significantly alleviated by ERW by reducing cellular ROS levels. *Caenorhabditis elegans* grown in media prepared with ERW had increased resistance to oxidative stress caused by paraquat. We observed a significant effect of ERW on response to other stressors, including heat shock and UV-irradiation in *C. elegans*. These data indicate that the powerful anti-oxidant activity of ERW is due to its radical-scavenging activity and show, for the first time, that ERW could increase thermotolerance and resistance to UV-irradiation. These results suggest that ERW aids resistance to various environmental stresses.

**Keywords** Electrolyzed-reduced water, Oxidative stress, Thermotolerance, Ultraviolet-resistance, *Caenorhabditis elegans*

Oxidative stress causes damage to various cellular macromolecules, including DNA, protein, and lipids and is related with the onset of various diseases and aging[1-4]. Reactive oxygen species (ROS) are highly reactive chemical molecules containing oxygen that induce oxidative damage. ROS are produced as a byproduct of cellular metabolism mainly from the mitochondrial electron transport chain (ETC) reaction. The cellular defense system abolishes ROS through enzymatic reactions or ROS-scavenging antioxidants. The antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), play key roles in the enzymatic cellular defense system. Cellular ROS-scavenging antioxidants include vitamin C, vitamin E, and glutathione. However, the cellular defense system cannot eliminate all cellular ROS, as ~2% of ROS produced by the mitochondrial ETC escape the cellular defense system. These escaped ROS accumulate in cells, increase cellular damage, and eventually cause pathophysiological changes in tissues[1].

Treating *C. elegans* with synthetic SOD/CAT mimetics confers resistance to oxidative stress as well as increased thermotolerance, suggesting the modulation of cellular ROS levels could confer increased resistance to environmental stresses[5]. Previous studies of dietary antioxidants also demonstrated a significant role for the cellular antioxidant defense system to resist the deleterious effect of ROS. Lipid peroxidation, one of the biomarkers of ROS-induced oxidative damages in cells, was markedly decreased by the dietary intervention of vitamin E or lycopene, a major carotenoid found in tomato[6,7]. Resveratrol, an effective antioxidant found in red wine, increases resistance to oxidative stress *C. elegans*, which suggests beneficial role of antioxidants in oxidative damage caused by ROS[8]. ROS causes oxidative damages to DNA and the most well-known biomarkers of DNA oxidative damage is 8-oxo-2′-deoxy-guanosine. Dietary supplementation with α-lipoic acid significantly reduced cellular accumulation of 8-oxo-2′-deoxy-guanosine[9]. Curcumin is a yellow curry spice with potent antioxidant activity, and curcumin supplementation significantly prevents cellular oxidative damage in an Alzheimer's disease

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mouse model[10]. These findings suggest that the modulation of cellular ROS level could affect directly to both cellular oxidative damage and organism's resistance to oxidative stress.

Electrolysis of water triggers the production of $OH^-$ ions from the anode site and $H^+$ ions from the cathode site due to the oxidation-reduction reaction of water molecules. Electrolyzed-oxidized water (EOW) with decreased pH is produced from the anode by electrolysis of water. In contrast, increased pH generates reduced water at the cathode site, which is called electrolyzed-reduced water (ERW). EOW has strong antibacterial activity and can be used as a disinfectant[11-14]. The most well-known bioactivity of ERW is its antioxidant activity. ERW mimics the activity of antioxidant enzymes, such as SOD and CAT by scavenging ROS[15]. Cellular oxidative damage to DNA, RNA, and protein molecules caused by ROS can be markedly opposed by ERW[16]. Additionally, ERW has a therapeutic effect on various diseases, including diabetes, tumors, and renal disease[17-19].

In the present study, we examined the effect of ERW on resistance to oxidative stress both *in vitro* and *in vivo*. We measured the effect of ERW on thermotolerance and ultraviolet (UV)-resistance to test whether ERW can modulate response to other environmental stresses. This study demonstrated that ERW conferred increased resistance to oxidative stress caused by the well-known herbicide paraquat. The response to heat shock and UV-irradiation was also significantly modulated by ERW.

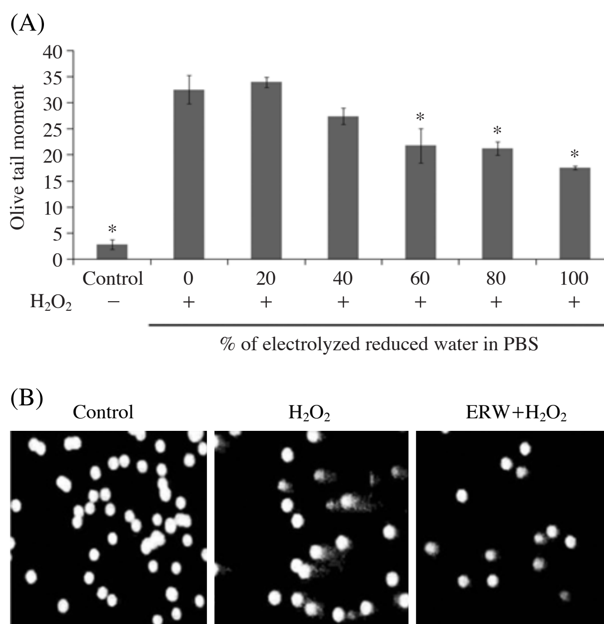


**Figure 1.** Suppressive effect of ERW on $H_2O_2$-induced oxidative DNA damage in lymphocytes. (A) The inhibitory effect of *in vitro* supplementation of different concentrations of ERW on 100 μM $H_2O_2$-induced oxidative DNA damage in lymphocytes was determined by alkaline single-cell gel electrophoresis (the comet assay). (B) Preventive effect of pre-treating lymphocytes with 100% ERW on the oxidative DNA damage by 100 μM $H_2O_2$. Pre-treatment with ERW significantly reduced the oxidative DNA damage caused by $H_2O_2$. *significantly different from $H_2O_2$-alone treated lymphocyte group at $P<0.05$ by Duncan's multiple range test.

### ERW suppresses $H_2O_2$-induced oxidative DNA damage

Hydrogen peroxide is one of strongest ROS found in cells and causes oxidative damage to various cellular macromolecules. For example, hydrogen peroxide causes DNA strand breakage by generating hydroxyl radicals close to DNA molecule. We were interested in whether ERW could prevent oxidative damage occurring in DNA caused by hydrogen peroxide *in vitro*. Human lymphocytes treated with 100 μM $H_2O_2$ exhibited DNA oxidative damage, as determined by the olive tail moment (Figure 1). The olive tail moment increased to 32.5±2.71 in cells treated with 100 μM $H_2O_2$, compared with 2.9±0.94 in the PBS-treated control. These data indicate an approximate 11-fold increase in DNA damage by $H_2O_2$. Adding ERW to the PBS inhibited the oxidative DNA damage caused by $H_2O_2$, as demonstrated by a reduction in the olive tail moment (Figure 1). Additionally, the increases in ERW concentration in PBS enhanced the suppressive effects of ERW against oxidative DNA damage (Figure 1). Pre-treatment of lymphocytes with 60%, 80%, or 100% ERW significantly prevented the oxidative DNA damage caused by $H_2O_2$: the olive tail moment was 21.8±3.30, 21.3±1.24, and 17.6±0.34, respectively. Figure 1B clearly shows the significant decrease in the olive tail moment in lymphocytes pre-treated with 100% ERW.

### ERW reduces ROS generation in oxidatively stressed cells

We measured ROS production in cells to identify the underlying mechanisms of reduced oxidative DNA damage observed in ERW-treated lymphocytes. Cellular ROS level was monitored using DCFH-DA after treating the cells with $H_2O_2$. ROS levels in lymphocytes under an oxidatively-stressed condition increased 6.3-fold compared to those under normal conditions; the fluorescence intensity of oxidatively stressed cells was 18,280±670, whereas that of normal cells was 2,897±56. Pre-treatment with ERW markedly reduced ROS generation by $H_2O_2$ in lymphocytes (Figure 2). ERW (50%) in PBS decreased fluorescence inten-

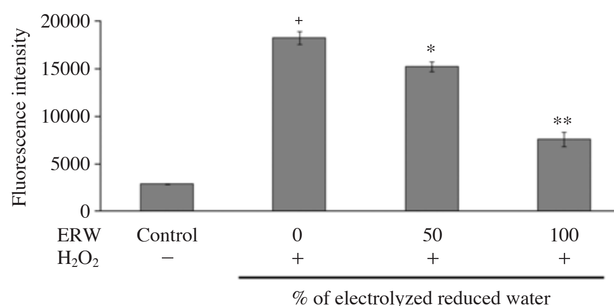


**Figure 2.** Suppressive effect of ERW on ROS generation. The inhibitory effect of *in vitro* supplementation with 50% or 100% ERW on 100 μM $H_2O_2$-induced ROS generation in lymphocytes was measured using 2′,7′-dichlorofluorescein diacetate (DCFH-DA). Pre-treatment with ERW significantly suppressed ROS generation by $H_2O_2$. $^{\dagger}P<0.01$ compared to control lymphocytes; $^{*}P<0.05$ compared to $H_2O_2$-alone treated lymphocytes; $^{**}P<0.01$ compared to $H_2O_2$-alone treated lymphocytes. Values indicate mean ± SEM of three replicate experiments.

sity to 15,232 ± 548, whereas 100% ERW lowered fluorescence intensity down to 7,590 ± 780. The inhibitory effect of ERW was proportional to the amount of ERW added to the PBS. These results suggest that the inhibitory effect of ERW on the $H_2O_2$-induced oxidative DNA damage could be due to the ROS scavenging activity of ERW.

### Resistance to oxidative stress is increased by ERW *in vivo*

Based on the *in vitro* lymphocyte assay, we measured the effect of ERW on resistance to oxidative stress *in vivo* using *C. elegans* as a model system. The susceptibility to oxidative stress in worms grown in NGM medium prepared with ERW was compared to that of worms grown in NGM medium prepared with distilled water (DW). Paraquat (methyl viologen dichloride hydrate), one of most widely used herbicides, was used to induce oxidative stress *in vivo*. Worms grown in ERW medium showed a significant increase in resistance to oxidative stress caused by paraquat (Figure 3). Mean survival time of worms grown in DW medium was 52.6 h, whereas that of worms grown in ERW medium was 68.8 h ($P<0.01$). The percent effect of ERW on resistance to oxidative stress was 30.8%. We observed the same significant positive effect of ERW on resistance to oxidative stress in replicate experiment (mean survival time were 93.2 h and 108.0 h for DW and ERW, respectively ($P=0.02$) and the percent effect was 16.0%). These findings show that ERW suppressed oxidative stress at both the cellular and organismal level.

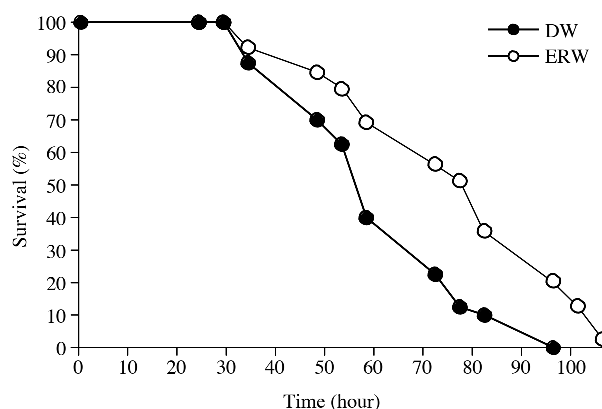


**Figure 3.** The effect of ERW on the response to oxidative stress in *Caenorhabditis elegans*. Worms grown in liquid media prepared with ERW showed increased resistance to oxidative stress compared to that of worms grown in control media prepared with DW. Paraquat was used as the oxidative-stress inducer. Viability under the oxidative-stress condition increased significantly with ERW treatment ($P=0.002$). The X-axis indicates the time exposed to paraquat. Two independent assays showed consistent results.

### ERW confers resistance to heat-stress *in vivo*

Many mutant *C. elegans* strains with increased resistance to oxidative stress are also thermotolerant[20,21]. A previous study showed that adding synthetic SOD/CAT mimetics to NGM medium confers increased resistance to both oxidative stress and heat stress in *C. elegans*[22]. Therefore, we examined whether ERW could confer increased resistance to heat stress in addition to its oxidative-stress-suppressing effect in *C. elegans*. Only 42.2 ± 2.4% of worms survived in DW medium after a 16 h heat shock at 35°C (Figure 4). However, worms grown in ERW medium showed significantly increased survival (75.0 ± 3.5%) compared to worms in DW medium. We conclude that in addition to its ability to induce increased resistance to oxidative stress, ERW is beneficial for thermotolerance in *C. elegans*.

### Susceptibility to UV is also modulated by ERW

Next, we determined whether ERW could modulate the response to UV irradiation in *C. elegans*. Four-day-old adult worms were irradiated with 40 J/cm$^2$/min for 2 h in a 254 nm-UV crosslinker. ERW significantly increased resistance to UV-irradiation (Figure 5). Mean survival times of worms in DW medium and ERW medium were 3.2 and 4.8 days after UV irradiation, respectively. Maximum survival time was also markedly increased in worms maintained in ERW medium; 11 days after UV-irradiation in DW medium

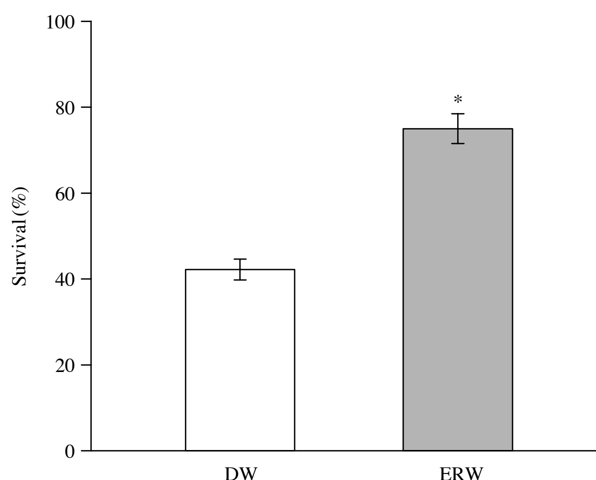


**Figure 4.** The effect of ERW on thermotolerance in *Caenorhabditis elegans*. Age-synchronized wild type N2 worms grown at 20°C were shifted to 35°C for 16 h to measure thermotolerance. Values indicate mean % survival ± SEM of three replicate experiments. *Significantly different ($P < 0.05$).

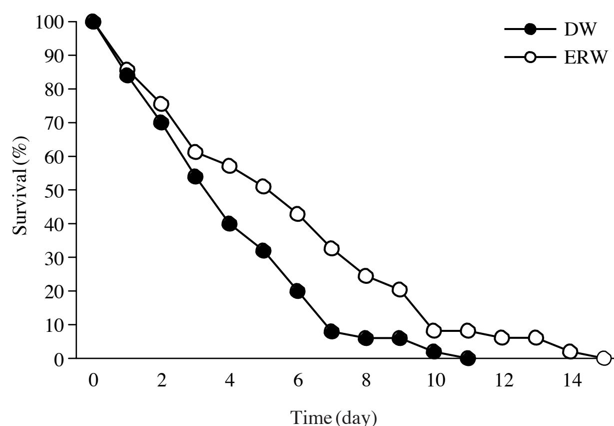


**Figure 5.** Increased survival after UV irradiation following ERW treatment in *Caenorhabditis elegans*. Age-synchronized young adult worms were irradiated with 40 J/cm$^2$/min UV for 2 h to determine the effect of ERW on UV resistance. ERW conferred significantly increased resistance to UV irradiation compared to that of DW. X-axis indicates days after UV irradiation.

and 15 days in ERW medium. A statistical analysis using the log-rank test revealed that ERW significantly altered resistance to UV irradiation in *C. elegans* ($P = 0.02$). Replicate experiment showed similar results: Mean survival time were 2.9 and 3.9 days for DW and ERW, respectively ($P = 0.04$). Our data indicate that ERW decreased susceptibility to UV irradiation in *C. elegans*. However, how ROS-scavenging activity of ERW induces increased resistance to UV irradiation still needs to be determined.

## Discussion

Electrolysis of water produces oxidized water and reduced water at the anode and cathode, respectively. Strong anti-bacterial activity of EOW has been demonstrated in the previous study[11]. The most widely known bioactivity of ERW is ROS-scavenging activity. ERW scavenges superoxide anions ($O_2^-$) and $H_2O_2$ *in vitro* and inhibits single-strand breakage of DNA caused by oxygen radicals[15]. Another study showed the protective effect of ERW on DNA damage, RNA degradation, and peroxidase degradation in human lymphocytes[16]. In this study, we observed the same suppressive effect of ERW on $H_2O_2$-induced DNA damage as previously reported[16]. Then, we measured the ROS levels produced under oxidative-stress conditions. Cells grown in media prepared with ERW produced significantly less ROS compared to cells grown in normal media. These data indicate that anti-oxidant activity of ERW results from its ROS-scavenging activity.

We used the nematode *C. elegans* as a model organism to measure the anti-oxidant activity of ERW *in vivo*. Resistance to oxidative stress caused by the well-known herbicide paraquat significantly increased in animals grown in media made with freshly-prepared ERW. Previous studies have shown that many genetic or environmental interventions that increase resistance to oxidative stress in *C. elegans* also confer increased resistance to other stresses[20-23]. To determine whether ERW also shows an inhibitory effect for other environmental stresses, we compared thermotolerance and UV resistance between worms grown in ERW medium and DW medium. Worms grown in ERW medium survived longer than worms in DW medium after 16 h of heat shock. Interestingly, resistance to UV was also markedly increased by ERW. Taken together, these findings suggest that ERW conferred resistance to various environmental stresses in *C. elegans* in addition to its antioxidant activity. However, the underlying mechanisms of increased resistance to other environmental stresses by ERW are still unknown. Future studies regarding the possible cellular pathways involved in thermotolerance and/or UV resistance by ERW or other bioactivity of ERW will expand our understanding of the effect of ERW.

The free radical theory of aging, suggested by Dr. Harman in 1956, is one of the most widely-studied theories of aging[3]. It suggests that aging is caused by the accumulation of oxidative damage in cellular macromolecules, such as DNA, proteins, and lipids, by free radicals produced as a byproduct of cellular meta-

bolism. A recent study reported that use of ERW can extend lifespan in *C. elegans*[24]. Worms grown in water medium (pH 7.0) prepared with ERW lived longer (11-41%) than worms cultured in water medium made of ultrapure water. However, no lifespan-extending effect of ERW was observed when conventional S-basal medium was used. Another study showed that the nanoparticles contained in ERW, rather than molecular hydrogen, attenuate the cellular oxidative stress and increase the lifespan of *C. elegans*[25]. Further investigations regarding the role of ERW on lifespan extension and the aging process will provide a valuable scientific basis for medical and nutritional applications of ERW as an anti-aging intervention.

## Materials & Methods

### Preparation of ERW

Ultrapure water containing NaCl was subjected to electrolysis (36 VDC/6000 mA) for several minutes at ambient temperature using an electrolysis apparatus equipped with five platinum electrodes (280 × 120 × 90 mm, 650 g, DoDream Co., Ltd, Seoul, South Korea). The pH and oxidation-reduction potential (ORP) of the electrolyzed water were determined with a pH/ORP meter using the appropriate pH or ORP probe. The ERW used in this study had a pH of 9-11, and an ORP of ~300-1,000 mV.

### Treatment of lymphocytes

Fresh whole human blood (400 μL) was added to 600 μL of PBS and layered onto 400 μL of Histopaque 1077 (Sigma-Aldrich, St. Louis, MO). After centrifugation at 1,450 rpm for 5 min at room temperature, the lymphocytes were collected and suspended in PBS. To investigate the ability of ERW to inhibit oxidative DNA damage, the lymphocytes were pre-incubated with ERW for 30 min at 37°C in the dark and then treated with 100 μM $H_2O_2$ for 5 min on ice. The total volume was 1 mL. A PBS-treated sample was used as a negative control.

### Determination of oxidative DNA damage by the comet assay

The alkaline comet assay was performed according to Singh *et al.* with slight modifications[26]. The lymphocytes mixed with 75 μL of 0.7% low-melting-point agarose were added to slides and immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium lauryl sarcosine, 1% Triton X-100, and 10% DMSO) for 1 h at 4°C. The slides were then placed in an electrophoresis tank containing 300 mM NaOH and 10 mM $Na_2EDTA$ (pH 13) for 20 min to allow DNA to unwind. Electrophoresis was performed at 25 V/300 mA for 20 min at 4°C. Images were analyzed using Komet 5.5 software (Kinetic Imaging, Merseyside, UK) and fluorescence microscopy (Leica, Wetzlar, Germany). To quantify DNA damage, the olive tail moment was calculated as (Tail.mean-Head. mean) × tail % DNA/100. In total, 150 randomly captured comets were measured from each slide. The comet assay data were means of three independent measurements and were analyzed using the SPSS package for Windows version 13 (SPSS Inc., Chicago, IL). The mean values of DNA damage (olive tail moment) for each treatment were compared using a one-way analysis of variance followed by Duncan's multiple range test. A $P < 0.05$ was considered significant.

### Intracellular ROS measurement

The intracellular ROS level was determined using the method described by Arai *et al.* with some modifications[27]. The lymphocytes were collected from fresh blood as previously described and incubated with various concentrations of ERW and then stimulated with 100 μM $H_2O_2$ for 5 min on ice. Next, aliquots of $7 \times 10^4$ cells were incubated with 2 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA) for an additional 30 min at 37°C and washed and resuspended in PBS. Intracellular ROS were detected by fluorescence spectrophotometer (GloMax-Multi Detection System; Promega, Madison, WI).

### *Caenorhabditis elegans* culture conditions

The wild-type N2 CGCb strain was used in all experiments. Worms were grown at 20°C on NGM plates (1.7% agar, 2.5 mg/mL peptone, 25 mM NaCl, 50 mM $KH_2PO_4$ (pH 6.0), 5 μg/mL cholesterol, 1 mM $CaCl_2$, and 1 mM $MgSO_4$) with *Escherichia coli* OP50 as a food source. Synchronous populations of hermaphrodites were established by placing five young adults onto a fresh NGM plate and permitting eggs to be laid for 4 h at 20°C.

### Resistance to oxidative stress

Since the oxidation-reduction potential of ERW can be largely diminished at high temperatures, liquid NGM medium (2.5 mg/mL peptone, 25 mM NaCl, 50 mM $KH_2PO_4$ (pH 6.0), 5 μg/mL cholesterol, 1 mM $CaCl_2$, and 1 mM $MgSO_4$), sterilized through filtering, was used for the assay. Age-synchronized young adult worms were transferred to liquid NGM media containing OP50 and 20 mM paraquat (Sigma-Aldrich, St.

Louis, MO). Worm survival was monitored three times per day until all worms were dead. Worms that did not respond to mechanical stimulus were scored as dead. Worms were transferred to fresh NGM media with OP50 and paraquat every 2 days. The log-rank test was used for statistical analysis.

**Thermotolerance assay**

Sixty age-synchronized worms were grown in liquid NGM media with OP50 for 4 days at 20°C. Worms were then shifted to 35°C. After 16 h at 35°C, dead worms were scored as described above. Three independent experiments were performed. The two-tailed, unpaired Student's t-test was used for statistical analysis.

**Measurement of ultraviolet (UV)-resistance**

Age-synchronized 4-day-old young adult worms were picked from liquid NGM media and transferred to NGM agar plates. Worms were irradiated with UV (40 J/cm$^2$/min) for 2 h in a 254 nm-UV crosslinker (BLX-254; Vilber Lourmat, France). After irradiation, worms were transferred back to liquid medium and incubated at 20°C. Dead worms were scored every day as previously described.

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