

Electrolyzed-reduced water increases resistance to oxidative stress, fertility, and lifespan via insulin/IGF-1-like signal in *C. elegans*

Seul-Ki Park¹ and Sang-Kyu Park^{1*}

¹ Department of Medical Biotechnology, College of Medical Sciences, Soonchunhyang University, Asan, Chungnam, Republic of Korea.

ABSTRACT

Electrolyzed-reduced water (ERW) scavenges reactive oxygen species and is a powerful anti-oxidant. A positive correlation between oxidative stress and aging has been proved in many model organisms. In *Caenorhabditis elegans*, many long-lived mutants showed reduced fertility as a trade off against longevity phenotype. We aimed to study the effect of ERW on oxidative stress, fertility and lifespan of *C. elegans*. We also investigated the genetic pathway involved in the effect of ERW on resistance to oxidative stress and lifespan. We compared lifespan and fertility of worms in media prepared with distilled water and ERW. ERW significantly extended lifespan and increased the number of progeny produced. Then the effect of ERW on resistance to oxidative stress and lifespan of long-lived mutants was determined. ERW increased resistance to oxidative stress and lifespan of *eat-2*, a genetic model of dietary restriction, but had no effect on those of *age-1*, which is involved in insulin/insulin-like growth factor (IGF)-1-like signal. In addition, knockdown of *daf-16*, the downstream mediator of insulin/IGF-1-like signal, completely prevented the effect of ERW on lifespan. These findings suggest that ERW can extend lifespan without accompanying reduced fertility and modulate resistance to oxidative stress and lifespan via insulin/IGF-1-like signal in *C. elegans*.

Key words: Electrolyzed-reduced water, aging, *daf-16*, fertility, lifespan.

INTRODUCTION

An electric current passed through water results in electrolysis, which produces electrolyzed-reduced water (ERW) near the cathode and electrolyzed-oxidized water near the anode. ERW has a negative redox potential and low oxidation-reduction potential due to increased pH, whereas electrolyzed-oxidized water has a low pH and high oxidation-reduction potential (Shirahata *et al.*, 1997). The most well-known bioactivity of electrolyzed-oxidized water is anti-bacterial activity. Electrolyzed-oxidized water can inactivate numerous microorganisms, including *Salmonella enteritidis*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Campylobacter jejuni* (Kim *et al.*, 2000; Park *et al.*, 2002a; Venkitanarayanan *et al.*, 1999). The effects of electrolyzed-oxidized water on microbial decontamination of food and medical disinfection have also been reported recently (Park *et al.*, 2002b). ERW effectively scavenges reactive oxygen species (ROS) produced as a byproduct of normal cellular metabolism (Shirahata *et al.*, 1997). Cellular ROS include the hydroxyl radical (HO[•]), the superoxide radical anion (O₂^{•-}), nitric oxide (NO[•]), peroxy radicals (ROO[•]), peroxynitrite (ONOO⁻), singlet oxygen (¹O₂), hypochlorous acid (HOCl) and hydrogen peroxide (H₂O₂) (Storz and Imlay, 1999). Since ROS can cause damage to virtually all cellular macromolecules, such as DNA, proteins and lipids, they are considered a major causal factor of many pathophysiological alterations. ROS-induced DNA damage and RNA degradation are significantly inhibited by ERW *in vitro* (Lee *et al.*, 2006). ERW also prevents oxidative cleavage of cellular proteins in human lymphocytes (Lee *et al.*, 2006). Recent studies have shown the therapeutic effect of ERW including the prevention of alloxan-induced type 1 diabetes mellitus and reduced carbon tetrachloride-induced liver

damage (Li *et al.*, 2001). ERW also shows positive effects on the local immune response in mice (Lee *et al.*, 2009).

It has been hypothesized that ROS play a key role in normal aging of tissues that rely heavily on oxidative phosphorylation (Beckman and Ames, 1998; Sohal and Weindruch, 1996). The free radical theory of aging proposes that normal aging results from random deleterious damage to tissues caused by ROS (Sohal and Weindruch, 1996). Due to the potential role of antioxidants in modulating oxidative stress associated with aging, dietary interventions of ROS-scavenging antioxidants have received particular attention. Resveratrol, a polyphenol found in red wine, significantly increases the lifespan of *C. elegans* and *Drosophila melanogaster* (Wood *et al.*, 2004). Gene expression profiling has revealed that vitamin E supplementation can reverse several age-related changes in gene expression in the mouse heart and brain (Park *et al.*, 2008). Curcumin, an Indian spice, protects brain from oxidative stress and scavenges nitric oxide-based radicals (Lakshmanan *et al.*, 2011; Sreejayan and Rao, 1996). One study identified tissue-specific transcriptional biomarkers of aging through genome-wide transcriptome analysis and showed that supplementation of antioxidants significantly prevents age-related transcriptional changes of biomarkers of aging in a tissue-specific way in mice (Park *et al.*, 2009). Interestingly, Yan *et al.* reported that *C. elegans* grown in water medium prepared with ERW live longer than worms grown in distilled water and have increased resistance to oxidative stress due to the ROS scavenging activity of ERW (Yan *et al.*, 2010).

In *C. elegans*, the insulin/IGF-1-like signal activates the cell-surface receptor DAF-2. The signal is transferred to a PI3 kinase AGE-1 and AKT-1/AKT-2. The first lifespan-extending genetic mutant in *C. elegans* was the *age-1* mutant (Johnson, 1990). Mutations in the genes involved in insulin/IGF-1-like signal

Corresponding author: Sang-Kyu Park, 1307 College of Medical Sciences, 220 Soonchunhyang-ro, Asan, Chungnam 336-745, Republic of Korea. E-mail: skpark@sch.ac.kr. Phone: +82-41-530-3094. Fax: +82-41-530-3085

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increase lifespan significantly, suggesting reduced insulin/IGF-1-like signal can extend lifespan in *C. elegans* (Johnson, 1990; Gems *et al.*, 1998; Kenyon *et al.*, 1993). Lifespan extension through reduced insulin/IGF-1-like signal is mediated by DAF-16. DAF-16 is a transcription factor localized in the nucleus by reduced insulin/IGF-1-like signal and regulates the expression of many age-related genes (Tatar *et al.*, 2003).

C. elegans is a free living nematode whose genome was fully sequenced in 1998 (The *C. elegans* Sequencing Consortium, 1998). The life cycle of *C. elegans* is relatively short. After hatching they develop through four larval stages, from L1 to L4. Adult hermaphrodite worms produce 300 progeny on average during their gravid period using self-fertilization. The average lifespan of *C. elegans* is about 15-17 days and a generation is about 4 days, which makes this animal as a good experimental organism for aging research (Wood, 1988).

We have reported that *C. elegans* grown in ERW show increased resistance to various environmental stressors, including oxidative stress, heat stress, and ultraviolet irradiation (Park *et al.*, 2012). The aims of the study are elucidation of the effects of ERW on aging and longevity of *C. elegans* using conventional NGM (nematode growth medium), and investigation of the possible underlying mechanisms of lifespan extension by ERW using various long-lived genetic mutants.

METHODS

C. elegans strains and growth conditions

The N2 CGCb strain, which is the wild type strain provided by the *Caenorhabditis* Genetics Center at University of Minnesota was used as control strain in this study. *age-1* (*hx546*) and *eat-2* (*ad465*) are mutant strains whose lifespan is extended through reduced insulin/IGF-1-like signal and decreased food intake, respectively (Friedman and Johnson, 1988; Lakowski and Hekimi, 1998). Worms were cultured at 20 °C on NGM agar plates (1.7% agar, 2.5 mg / mL peptone, 25 mM NaCl, 50 mM KH₂PO₄ pH6.0, 5 µg / mL cholesterol, 1 mM CaCl₂, and 1 mM MgSO₄) seeded with *Escherichia coli* OP50 as the food source. Age-synchronized hermaphrodites were established by permitting five young adult (L4) worms to lay eggs on a fresh NGM plate for four hours at 20 °C. Liquid NGM was prepared the same as the NGM agar plates without adding agar and sterilized with a 0.2 µm cellulose acetate filter (Advantec, Japan). All adult worms were removed from the plate, and the eggs were incubated at 20 °C until the L4 larval stage.

Preparation of ERW

ERW containing NaCl was produced with an electrolysis device (208X120X90 mm, 650 g, DoDream, Republic of Korea). The NaCl concentration of the ERW was 0.08%, the pH was 10-11, and the oxidation-reduction potential was -700 mV. The pH and oxidation-reduction potential values were measured using a pH / oxidation-reduction potential meter.

Lifespan assay

Sixty age-synchronized three-day-old worms grown in NGM agar plates with OP50 were transferred to liquid NGM to test the effect of ERW on lifespan. 5-Fluoro-2'-deoxyuridine (Sigma-Aldrich, USA) was added to the liquid NGM to block progeny

development. Worms were transferred to fresh liquid NGM every day during the first four days. After four days, worms were transferred to fresh media every two days until all worms died. Live worms were transferred to a fresh plate, and mortality was scored. Worms that did not respond to any mechanical stimuli were regarded as dead. N2 worms grown in NGM prepared with distilled water were the control group. The survival of the control group was compared with that of the experimental group that were worms grown in NGM prepared with ERW.

Fertility assay

Age-synchronized worms were grown in liquid NGM with OP50 for two days at 20 °C. A single young adult worm was transferred to new liquid NGM and allowed to lay eggs at 20 °C for 24 hours. The next day, the adult worm was transferred to fresh liquid NGM, and media containing eggs were incubated at 20 °C for an additional two days. The number of hatched worms was recorded after two days. This process was repeated until no hatched worms were found in the media. The control and experimental groups were worms cultured in NGM prepared with distilled water and ERW, respectively.

Resistance to oxidative stress

To induce oxidative stress in worms, 20 mM paraquat (methyl viologen dichloride hydrate) was added to the liquid NGM. Sixty age-synchronous three-day-old hermaphrodite worms were used in each group. Dead worms were counted three times per day until all worms were dead. N2 and mutant worms grown in NGM prepared with distilled water were the control groups and worms grown in NGM prepared with ERW were the experimental groups.

Statistical analysis

For the statistical analysis of the lifespan and resistance to oxidative stress assays, we performed the log-rank test (Peto and Peto, 1972). The log-rank test is a non-parametric Mantel-Cox test and one of most widely used statistical methods for the comparison of survival and calculation of mean survival time between control and experimental groups. We measured mean and maximum lifespan and tested statistical significance. For statistical significance of the fertility assay we used a standard two-tailed Student t-test.

RNAi assay

The *daf-16* RNAi clone was obtained from the Ahringer RNAi library (Kamath *et al.*, 2003) and verified by sequencing. The Ahringer RNAi library contains genome-wide RNAi clones that can be used for *C. elegans* gene knockdown. PCR-amplified sequences for each gene were cloned in L4440 vector (Kamath *et al.*, 2003). Five L4 / young adult worms were transferred to a fresh NGM plate containing 100 µg / mL ampicillin, 12.5 µg / mL tetracycline, 0.4 mM IPTG and 0.5 mg / mL 5-fluoro-2'-deoxyuridine, and spotted with bacteria expressing double-stranded RNA from an empty vector or *daf-16*. After laying eggs for four hours, all five adult worms were removed. Sixty young adults were selected three days after hatching and were transferred to a fresh plate with double-stranded RNA-expressing bacteria.

RESULTS

Lifespan of C. elegans was increased by ERW

ERW is generally known for its strong anti-oxidant activity. A study showed that worms grown in ERW lived longer than worms grown in distilled water. However, the effect of ERW in conventional NGM had not yet been studied. To examine the effect of ERW on the lifespan of *C. elegans* grown in NGM, we compared the lifespan of wild-type N2 worms grown in liquid NGM prepared with distilled water and ERW. Worms grown in liquid NGM prepared with ERW lived significantly longer than worms grown in liquid NGM prepared with distilled water (Fig. 1). The mean lifespan of worms increased from 18.3 days to 21.5 days (17.3% increase, $p = 0.004$), and maximum lifespan was extended by up to 4 days (29 to 33 days). A replicative experiment showed similar results: mean lifespans were 16.7 and 19.6 days and maximum lifespans were 28 and 31 days in distilled water and ERW, respectively (17.5% increase of mean lifespan; $p = 0.007$).

The effect of ERW on reproduction of C. elegans

Many genetic or environmental interventions that extend lifespan in *C. elegans* accompany a trade-off of reduced reproduction (Hughes *et al.*, 2007; Larsen *et al.*, 1995). To address whether the longevity phenotype by ERW treatment induces reduced progeny production, we examined the time-course distribution of the number of progeny produced and calculated the total number of progeny produced during the gravid period of worms grown in NGM prepared with ERW. As shown in Figure 2A, worms treated with ERW at day five, seven, and eight produced more progeny compared to the control. A comparison of the total number of progeny during the entire gravid period revealed that ERW conferred increased fertility. Total numbers of progeny were 137.1 ± 9.2 in distilled water-medium and 162.7 ± 10.8 in ERW-medium (mean \pm SEM) (Fig 2B).

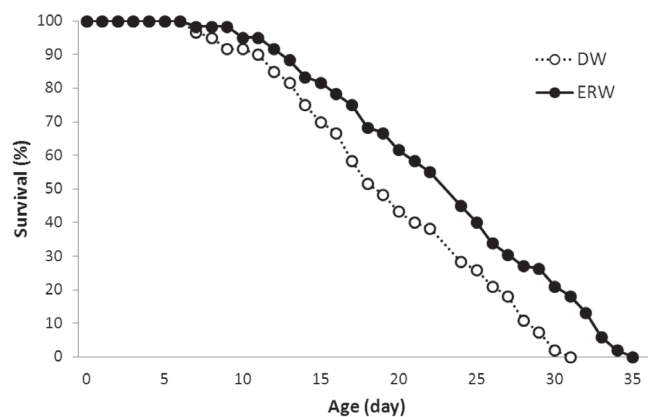


Figure 1: ERW significantly increased both mean and maximum lifespan of *Caenorhabditis elegans*. Worms grown in liquid NGM prepared with ERW lived longer than worms grown in liquid NGM prepared with distilled water. Mean lifespan of worms increased up to 17.3% following ERW treatment. Mean lifespans of animals grown in distilled water- and ERW-medium were 18.3 and 21.5 days, respectively ($p = 0.004$). Y axis indicates % survival, which was calculated as (number of worms survived on each day/total number of worms) \times 100. ERW = electrolyzed reduced water.

Lifespan extension by ERW specifically involves the insulin/IGF-1-like signal

We next examined the effect of ERW on long-lived mutants to identify the possible underlying mechanism of longevity by ERW. The *age-1* mutant shows increased lifespan due to a reduced insulin/IGF-1-like signal and the *eat-2* mutant is a widely-used genetic model of dietary restriction (DR) in *C. elegans* (Friedman and Johnson, 1988; Lakowski and Hekimi, 1998). These two mechanisms have been demonstrated to extend the lifespan in various organisms (Brown-borg *et al.*, 1996; Greer and Brunet, 2009; Kaeberlein *et al.*, 2005; Kapahi *et al.*, 2004; Masoro, 2005). The lifespan assay revealed that the lifespan of *age-1* (*hx546*) was not further extended by ERW, whereas both mean and maximum lifespan of *eat-2* (*ad465*) significantly increased following ERW treatment (Fig. 3). Mean and maximum lifespan of *age-1* (*hx546*) were 23.0 and 32 days in distilled water and 22.3 and 33 days in ERW, respectively ($p = 0.924$). Mean and maximum lifespan in *eat-2* (*ad465*) increased from 21.4 and 31 days to 24.9 and 34 days, respectively, following the ERW treatment ($p = 0.009$). A replicative experiment showed similar results (data not shown).

Resistance to oxidative stress of long-lived mutants grown in ERW-medium

We then asked whether resistance to oxidative stress is altered by ERW in long-lived mutants. Paraquat (20 mM) was used to induce oxidative stress *in vivo*. As shown in Table 1, *age-1* (*hx546*) and *eat-2* (*ad465*) were more resistant to oxidative stress than wild-type N2 ($p < 0.05$). Mean survival times in N2 worms after paraquat treatment were 52.6 and 68.8 hours in distilled water- and ERW-treated worms, respectively ($p = 0.002$). Resistance to oxidative stress also increased by ERW in *eat-2* (*ad465*) from 79.6 hours in distilled water to 92.6 hours with ERW ($p = 0.002$) and from 64.5 to 80.4 hours ($p = 0.018$) in replicative experiments. In contrast, no significant change in resistance to oxidative stress by ERW was observed in *age-1* (*hx546*) (Table I).

TABLE I

The effect of ERW on resistance to oxidative stress in long-lived *C. elegans* mutants

Strain	Medium	Mean survival time (hours)	p-value
N2	Distilled water	52.6	0.002
	ERW	68.8	
<i>age-1</i> (<i>hx546</i>)	Distilled water	66.5	0.169
	ERW	75.3	
	Distilled water	90.4	0.162
	ERW	98.9	
<i>eat-2</i> (<i>ad465</i>)	Distilled water	76.9	0.002
	ERW	92.6	
	Distilled water	64.5	0.018
	ERW	80.4	

Liquid NGM was prepared with distilled water or electrolyzed-reduced water (ERW) as indicated. Data are expressed as mean survival time after treating worms with 20 mM paraquat. The p-value was calculated using the long-rank test

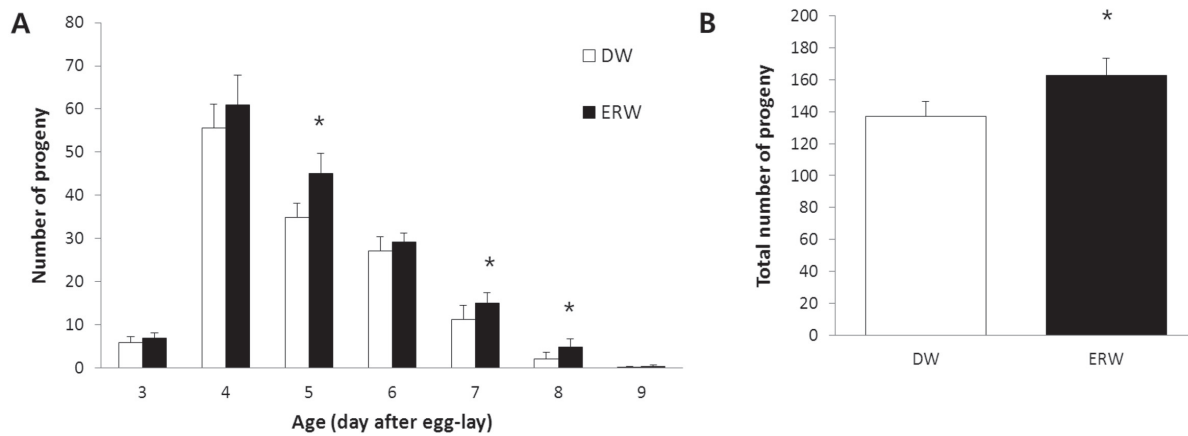


Figure 2: The effect of ERW on *Caenorhabditis elegans* fertility. Age-synchronized wild type N2 worms were transferred to fresh media every day, and the progeny were counted after 2 days. (A) Time-course distribution of the number of progeny produced. Worms grown in ERW produced more progeny compared to those in distilled water. The Y axis indicates the number of progeny produced on each day. (B) The total number of progeny produced also increased significantly following ERW treatment: 137.1 ± 9.2 in distilled water and 162.7 ± 10.8 in ERW. The Y axis indicates the average total number of progeny produced during gravid period. Data are mean \pm SEM of the number of progeny produced by 10 individual worms. * $p < 0.05$. ERW = electrolyzed reduced water.

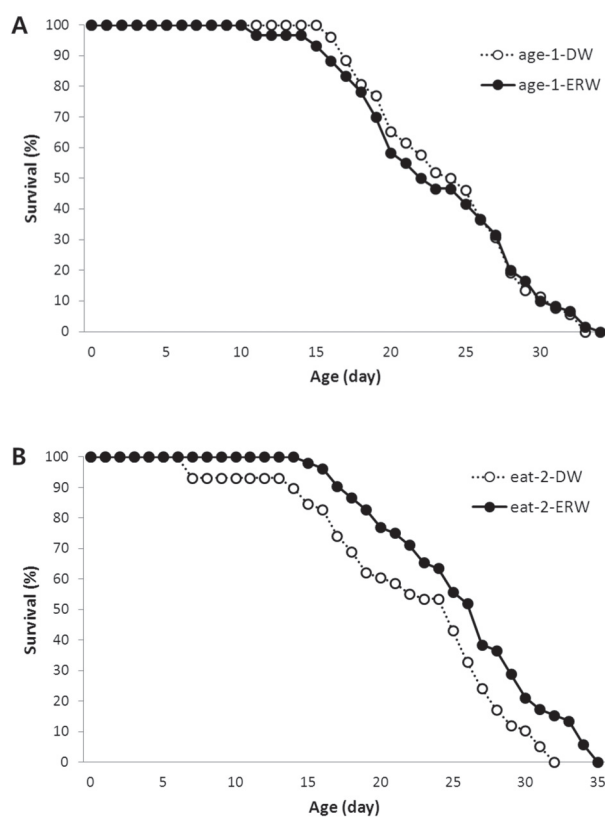


Figure 3: Lifespan-extending effect of ERW overlaps with that of the insulin-IGF-1-like signal. (A) The effect of ERW on the lifespan of *age-1* (*hx546*) was analyzed using a lifespan assay. (B) The lifespans of *eat-2* (*ad465*) grown in NGM prepared with distilled water and ERW were compared. A significant increase in the lifespan of *eat-2* (*ad465*) was observed following ERW treatment. However, ERW failed to extend significantly the lifespan of *age-1* (*hx546*). Y axis indicates % survival, which was calculated as (number of surviving worms each day/total number of worms) $\times 100$. ERW = electrolyzed reduced water.

Lifespan extension by ERW depends on DAF-16

The longevity phenotype with a reduced insulin/IGF-1-like signal requires the transcription factor DAF-16 (Murphy *et al.*, 2003). To elucidate the role of DAF-16 in ERW-induced lifespan extension, we examined the effect of *daf-16* RNAi on lifespan of worms grown in distilled water- or ERW-medium. The increased lifespan conferred by ERW was completely abolished by *daf-16* RNAi in wild-type N2 worms (Fig. 4A). Mean lifespan increased from 18.2 to 23.0 days by ERW ($p < 0.001$). However, the extended lifespan by ERW was prevented by gene knockdown of *daf-16* (mean and maximum lifespans were 15.7 and 21 days, respectively). These findings indicate that the lifespan extension by ERW was dependent on DAF-16, which supports our hypothesis that ERW-induced long lifespan is mediated by a decrease in the insulin/IGF-1 signal. We also observed the same shortened lifespan by *daf-16* RNAi in *age-1* (*hx546*) (Fig. 4B). As previously observed, the lifespan of *age-1* (*hx546*) was not altered by ERW (mean lifespan of distilled water- and ERW-medium was 22.8 and 22.3 days, respectively). RNAi of *daf-16* in *age-1* (*hx546*) significantly reduced both mean and maximum lifespan of worms cultured in distilled water- or ERW-medium.

DISCUSSION

ERW showed a positive impact on longevity of *C. elegans* in conventional NGM. Both mean and maximum lifespan were significantly extended in worms grown in NGM prepared with ERW. To our surprise, the reduced fertility which usually accompanies many long-lived mutants was not observed in lifespan extension by ERW. Genetic analysis using mutants and gene knockdown suggest that the cellular mechanisms involved in lifespan extension by ERW overlap with a decreased insulin/IGF-1-like signal and are independent of DR.

ERW is produced by electrolysis of water and has been extensively studied due to its antioxidant activity. *C. elegans*

grown in ERW accumulate less ROS induced by the oxidative-stress inducer paraquat (Yan *et al.*, 2010). Another study also showed that the conventional NGM *C. elegans* culture medium prepared with ERW confers increased resistance to various environmental stresses compared to NGM prepared with distilled water (Park *et al.*, 2012). A previous study showed that water medium prepared with ERW extended mean lifespan of *C. elegans* (11-41%), but failed to increase maximum lifespan (Yan *et al.*, 2010; Yan *et al.*, 2011). No significant extension of either mean or maximum lifespan by ERW is observed when S-basal medium is used in the lifespan assay (Yan *et al.*, 2010). In the present study, we employed liquid NGM instead of water or S-basal medium to examine the effect of ERW on lifespan, since NGM is the standard culture medium for the *C. elegans* lifespan assay. A comparison of survival between worms in liquid NGM prepared with distilled water and ERW revealed that ERW extended both mean and maximum lifespan of *C. elegans* significantly.

Multicellular organisms allocate their resources between growth and maintenance of soma and reproduction in response to environmental changes, and a reduced total number of progeny is observed in many lifespan-extended mutants of

C. elegans (Hughes *et al.*, 2007; Larsen *et al.*, 1995). A slight extension of the reproductive period is also observed in a few long-lived strains (Gems *et al.*, 1998). In addition, the ablation of germ cells increases lifespan and a DR, which extends lifespan in various organisms, delays the reproductive period in *C. elegans* (Crawford *et al.*, 2007; Hsin and Kenyon, 1999). The number of progeny produced by worms grown in NGM prepared with distilled water and ERW was compared to examine the effect of ERW on reproduction. Unlike other long-lived mutants, the longevity phenotype conferred by ERW did not accompany a reduced reproduction phenotype. To our surprise, the total number of progeny was rather moderately increased. These results indicate that ERW is able to extend lifespan without a cost of reduced fertility and might be beneficial not only to extend lifespan, but also for the health span of *C. elegans*.

The two most studied lifespan-extending mechanisms in *C. elegans* are a reduced insulin/IGF-1-like signal and DR. The first longevity mutant in *C. elegans* was *age-1*, which encodes a PI3 kinase involved in insulin/IGF-1-like signaling. Mutations in other factors mediating the insulin/IGF-1-like signal, such as *daf-2* and *akt-1/akt-2*, also extend survival up to 300% (Gems *et al.*, 1998; Johnson, 1990; Kenyon *et al.*, 1993). DR is the only intervention that increases lifespan in nearly all species tested (Masoro, 2005). In the present study, the lifespan of *eat-2*, the genetic DR mutant, was further extended by ERW. However, no additional increase in lifespan was observed when *age-1* mutants were grown in NGM prepared with ERW. The longevity phenotype with the reduced insulin/IGF-1-like signal is mediated through the FOXO-family transcription factor DAF-16 (Tatar *et al.*, 2003). The lifespan extension by *daf-2* RNAi is completely opposed by *daf-2* and *daf-16* double RNAi (Murphy *et al.*, 2003). Here, we observed that the lifespan-extending effect of ERW was inhibited by *daf-16* RNAi. We also observed increased resistance to oxidative stress in *age-1* and *eat-2* compared to wild type N2. Interestingly, the resistance to oxidative stress in *age-1* was not further increased by ERW, whereas wild-type N2 and *eat-2* worms displayed increased resistance to oxidative stress following ERW treatment. These results suggest that both insulin/IGF-1-like signal and ERW may modulate longevity through increased resistance to oxidative stress. Based on these findings, we conclude that ERW confers the longevity phenotype by reducing the insulin/IGF-1-like signal in *C. elegans* and suggest that there should be overlapping mechanisms between the insulin/IGF-1-like signal and ERW treatment.

It is still unknown whether supplementation of antioxidants can retard the aging process and extend lifespan. Our findings that ERW acted as a strong ROS scavenger and extended lifespan with increased resistance to oxidative stress and no reduction in fertility support the free radical theory of aging. Further studies on the underlying mechanisms involved in ERW, the effect on age-related diseases, and the influence on lifespan of other species will strengthen our understanding of the effect of ERW on aging and provide a possible clinical application for ERW.

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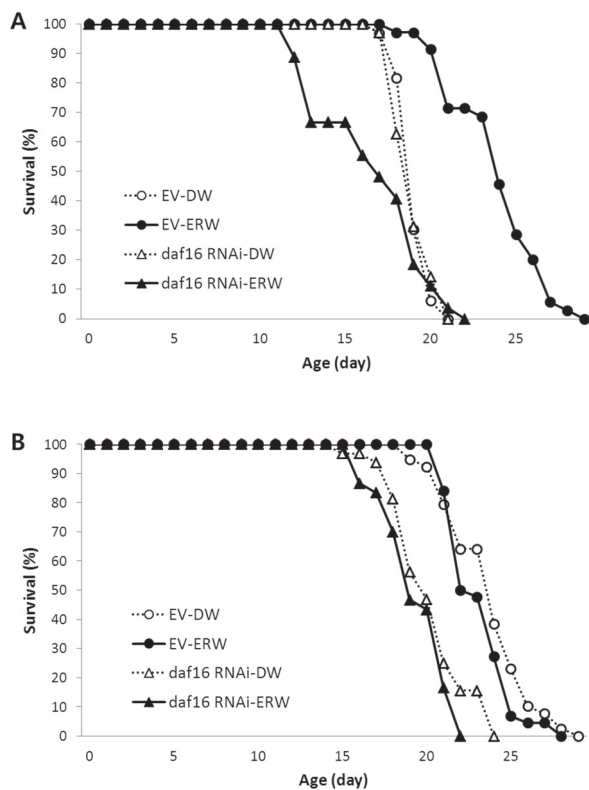


Figure 4: RNAi of *daf-16* abolishes the lifespan-extending effect of ERW. (A) The lifespan of wild-type N2 was markedly extended by ERW. However, extended lifespan was completely prevented by *daf-16* gene knockdown. (B) No additional increase in lifespan following ERW treatment was observed in *age-1* (*hx546*), and *daf-16* RNAi blocked the lifespan-extending effect of the *age-1* mutant or ERW treatment. The Y axis indicates % survival, which was calculated as (number of worms survived on each day/total number of worms) \times 100. ERW = electrolyzed reduced water. *daf-16* = RNAi clone expressing double-stranded RNA of *daf-16* gene.

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