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Extension of the Lifespan of *Caenorhabditis elegans* by the Use of Electrolyzed Reduced Water

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Electrolyzed reduced water (ERW) has attracted much attention because of its therapeutic effects. In the present study, a new culture medium, which we designated Water medium, was developed to elucidate the effects of ERW on the lifespan of *Caenorhabditis elegans*. Wild-type *C. elegans* had a significantly shorter lifespan in Water medium than in conventional S medium. However, worms cultured in ERW-Water medium exhibited a significantly extended lifespan (from 11% to 41%) compared with worms cultured in ultrapure water-Water medium. There was no difference between the lifespans of worms cultured in ERW-S medium and ultrapure water-S medium. Nematodes cultured in ultrapure water-Water medium showed significantly higher levels of reactive oxygen species than those cultured in ultrapure water-S medium. Moreover, ERW-Water medium significantly reduced the ROS accumulation induced in the worms by paraquat, suggesting that ERW-Water medium extends the longevity of nematodes at least partly by scavenging ROS.

Key words: electrolyzed reduced water; *Caenorhabditis elegans*; lifespan; reactive oxygen species

Electrolysis of water typically produces two forms of water: electrolyzed reduced water (ERW) or alkaline ionized water, produced at the cathode site, and electrolyzed anode water (EAW), produced at the anode site. EAW containing hypochlorous acid has been intensively investigated for its oxidizing and disinfectant capacities.[1)] The Ministry of Health, Labor, and Welfare of Japan recognizes ERW as a potable water for the improvement of gastrointestinal symptoms, in the pharmaceutical affairs law of Japan, based on the results of randomized double-blind clinical tests.[2)] Recently, ERW has attracted much attention because of its antioxidative potential. ERW scavenged reactive oxygen species *in vitro* and protected DNA from oxidative damage.[3)] The oxidative damage induced in pancreatic β HIT-T15 cells by alloxan, a type-1 diabetes inducer, was suppressed by ERW.[4)] Kim and Kim *et al.* reported an anti-diabetic effect of ERW in diabetic model mice.[5)] ERW suppressed angiogenesis by tumor cells.[6)] Furthermore, a therapeutic effect of ERW was reported for hemodialysis-induced oxidative stress in end-stage renal disease patients.[7–9)]

The free-radical theory of aging was first suggested by Harman more than 50 years ago,[10)] and has been developing since. It is believed that aging results from an accumulation of molecular damage caused by free radicals.[11)] As the name suggests, antioxidants prevent oxidation and are believed to suppress senescence.

The correlation between delayed aging progression and oxidative stress resistance has been confirmed many times over the years in *Caenorhabditis elegans* and other model organisms. It has been found that vitamin E,[12)] tocotrienols,[13)] EGB761, an extract of *Ginkgo biloba* leaves,[14)] and Pt nanoparticles[15)] can prolong the lifespan of *C. elegans* by increasing resistance to oxidative stress.

Based on these previous studies and the free-radical theory of aging, we hypothesized that since ERW has the ability to alleviate ROS accumulation, it may also have a positive effect on an animal's lifespan. In the present study, we developed a modified culture medium called Water medium and compared the effects of ERW on the lifespan of a *C. elegans* strain in both Water medium and conventional S medium.

Materials and Methods

C. elegans *strain and growth conditions.* The wild-type N2 *C. elegans* strain was kindly provided by Dr. Kazuya Nomura (Department of Biology, Faculty of Sciences, Kyushu University, Fukuoka, Japan). The worms were maintained at 20 °C following the procedures established by Brenner.[16)] Age-synchronous populations were prepared as described by Emmons *et al.*[17)] Briefly, gravid hermaphrodites were washed from NGM agar and decomposed to collect the eggs. The worm eggs were hatched overnight at 20 °C in S-basal buffer, which consisted of 100 mM NaCl, 0.01 mM cholesterol, and 50 mM potassium phosphate (pH 6.0).[18)] The hatched larvae were then transferred to fresh NGM agar seeded with an *E. coli* OP50 lawn as food source, and incubated at 20 °C to the L4 larval stage.

Preparation of ERW. ERW was produced by type TI-200 or type TI-9000 electrolysis devices (Nihon Trim, Osaka, Japan). The TI-200 electrolysis device is a batch-type device consisting of a 4-L

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electrolysis vessel equipped with a pair of Pt-coated titanium electrodes and a semipermeable membrane that bisects the vessel into an anode side and a cathode side.[6] In this study, ultrapure water (MQ water) was produced by a filtration system (Millipore, Billerica, MA). MQ water supplemented with 2 mM NaOH was poured into the electrolysis vessel and electrolyzed for 1 h at a constant voltage of 100 V to produce TI-200 ERW.

In order to examine the effects of a potable ERW, tap water at Kyushu University was first purified with an activated charcoal filter equipped in a commercial electrolysis purifier (Type TI-9000, Nihon Trim) to produce TI-9000 CW water, which was then continuously electrolyzed to produce TI-9000 ERW.

The pH, dissolved hydrogen (DH) and osmolality of the water samples were determined with a pH meter (Model ϕ-32; Beckman-Coulter, Brea, CA), a DH meter (Model DM10; Biott, Tokyo) and a freezing point depression osmometer (Model OM 802-D; Vogel, Giessen, Germany), respectively. The Pt contents of the water samples were measured using an inductively coupled plasma mass spectrometer (Model 7500c; Agilent Technologies, Santa Clara, CA) at the Center for Advanced Instrumental Analysis (Kyushu University, Fukuoka, Japan).

Preparation of Water medium. To evaluate the effects of ERW, two kinds of aqueous media were used. The first medium was S medium (S-basal medium supplemented with 3 mM $CaCl_2$, 3 mM $MgSO_4$, 50 μM EDTA, 25 μM $FeSO_4$, 10 μM $MnCl_2$, 10 μM $ZnSO_4$, 1 μM $CuSO_4$, and 10 mM KH_2PO_4), as described previously.[19] The other medium was named Water medium, which only consisted of water samples with pH adjusted to 7.0 by 4-(2-hydroxyethyl)-1-piperazineehanesulfonic acid (HEPES, Wako Pure Chemical, Tokyo).

To avoid influences from living bacteria, 2×10^9 bacterial cells/ml of heat-killed *E. coli* OP50 (65 °C, 12 h) were used as nematode food. FUdR (50 μM) was also added to the media during the assays to prevent worm death from internal hatching.[20] The dishes were shaken in a gyratory shaker at 120 oscillations/min[20] to supply enough oxygen to the worms.

The control water samples for TI-200 ERW and TI-9000 ERW were 2 mM NaOH neutralized to pH 7.0 with HEPES (TI-200 MQ(NaOH)) and activated charcoal-treated water from tap water (TI-9000 CW) before electrolysis respectively. The control media for the TI-9000 ERW-Water medium and the TI-9000 ERW-S medium were TI-9000 CW-Water medium and TI-9000 CW-S medium, respectively. The final HEPES concentration was about 16 mM, and it varied slightly depending on the pH of the water samples.

Lifespan assay. For lifespan assays, L4 larvae were transferred to the various culture media[19] and cultured at 20 °C. The transfer day was designated day 1. The worms were transferred to fresh culture medium with a platinum wire every second day. In this process, worms were considered to be dead if they did not respond to repeated prodding with the platinum wire, and mortality was scored. Worms that crawled away, had internally hatched larvae, or had eviscerated gonads were excluded. A small proportion of the worms died abnormally during the first transfer, and they too were excluded. The mortality data were derived from Kaplan-Meier survival curves,[20] and statistical comparisons of the mean lifespans between the control and ERW group worms were performed by log-rank test[20] using the StatMate III Excel add-in program (ATMS, Tokyo).

ROS detection. Worms were cultured in ERW-Water medium or MQ(NaOH)-Water medium until day 5. On day 5, the worms were washed extensively with M9 buffer (22 mM Na_2HPO_4, 22 mM KH_2PO_4, 85 mM NaCl, 1 mM $MgSO_4$, and 0.02% gelatin), and then incubated with 0.4 M paraquat (Supelco, Bellefonte, PA), a model for oxidant-initiated toxicity widely used to catalyze the formation of ROS and induce oxidative stress,[21] for 5 h. Subsequently, the worms were washed 3 times with M9 buffer, transferred to 2 ml of Hank's solution (0.44 mM KH_2PO_4, 5.37 mM KCl, 0.34 mM Na_2HPO_4, 136.89 mM NaCl, and 5.55 mM D-glucose) containing 10 μM dichlorofluorescein diacetate (DCFH-DA; Invitrogen, Carlsbad, CA) and incubated for 30 min at 20 °C. Finally, they were fixed with 4% formaldehyde. Fixed cells were subjected to laser scanning confocal microscopy (OLFV-32U2/XE; Olympus, Tokyo) with excitation at 488 nm and emission at 510 nm[15] on the second day. The fluorescence intensities of the worms were determined using FV10-ASW 1.4 software (Olympus). The statistical significance of differences between the control and the treated group was determined by two-tailed T-test.

Table 1. Characteristics of Water Samples

	MQ(NaOH)	TI-200 ERW	TI-9000 CW	TI-9000 ERW
pH	11.3 ± 0.1	11.6 ± 0.1	7.9 ± 0.1	9.6 ± 0.2
DH (ppm)	0	0.4–0.9*	0	0.2–0.5*
Pt (ppb)	0	0.1–2.5*	0	0–0.7*
Osmolality (mOsm/kg)#	14	25	13	15

*DH was determined immediately after the preparation of ERW.
#The osmolalities of the water samples were assayed after adjusting the pH to 7.0 with HEPES. The osmolality of each water sample in S medium was 360 mOsm/kg higher than that in the corresponding Water medium. ERW, electrolyzed reduced water; CW, activated charcoal-treated water; DH, dissolved hydrogen. The pH values were shown as average ± standard deviation (N = 5). The values of DH and Pt were shown the minimum and maximum values after 5 independent measurements.

Results

Characterization of water samples

During electrolysis, ERW exhibits high pH and DH values (Table 1). The pH of ERW prepared by the TI-200 batch type electrolysis device was increased from 11.3 to 11.6 by electrolysis (Table 1). The osmolality of the water samples was determined after pH neutralization to 7.0 with HEPES. The osmolality of TI-200 ERW was 25 mOsm/kg, while MQ(NaOH) exhibited nearly 50% lower osmolality than TI-200 ERW. The conventional S media prepared with the various water sample exhibited 362 Osm/kg higher osmolalities than the corresponding Water media.

Although ERW exhibited high DH values, since gaseous hydrogen molecules are easily lost from water, the DH values of ERW decreased rapidly to less than 50 ppb during the pH adjustment for the preparation of the Water medium. ERW contained Pt, suggesting that during electrolysis, a small amount of Pt was dissolved from the Pt-coated electrodes. Transmittance electron microscope analysis revealed that the Pt in ERW formed Pt nanoparticles of 1–6 nm (data not shown). The value of the Pt content varied depending on the batch of ERW.

Effects of ERW on nematode lifespan

As shown in Table 2, worms cultured in TI-200 ERW-Water medium showed a significantly prolonged lifespan, from 7% to 41%, compared to the control medium ($p < 0.005$ to $p < 0.05$). Furthermore, the TI-9000 ERW-Water medium extended the nematode lifespan to 30% compared to the TI-9000 CW-Water medium ($p < 0.01$) (Table 2, trial 4). The lifespan extension effect of the TI-9000 ERW-Water medium was closely similar to that of the TI-200 ERW-Water medium. Although the composition of TI-9000 ERW derived from tap water is too complicated for scientific research, these results suggest that TI-200 ERW is a good research model for TI-9000 ERW.

On the other hand, no significant differences were found between the worms cultured in TI-200 ERW-S medium and MQ(NaOH)-S medium (Table 2, Fig. 1). There were also no differences between the worms cultured in TI-9000 ERW-S medium and TI-9000 CW-S medium (data not shown).

Table 2. Effects of the TI-200 ERW and TI-9000 ERW Media on the Lifespan of *C. elegans*

Trial	Treatment	MLS (d)	MLS (%)	Max (d)	*p*
1	MQ(NaOH) medium	9.04 ± 0.05	100	27	
	TI-200 ERW medium	12.7 ± 0.05	121	27	<0.001
	MQ(NaOH)-S medium	28.8 ± 0.05	100	43	
	TI-200 ERW-S medium	29.6 ± 0.05	103	43	
2	MQ(NaOH) medium	7.93 ± 0.03	100	19	
	TI-200 ERW medium	8.77 ± 0.10	111	21	<0.05
3	MQ(NaOH) medium	9.04 ± 0.05	100	27	
	TI-200 ERW	12.7 ± 0.05	141	27	<0.001
4	TI-9000 CW	12.8 ± 0.07	100	29	
	TI-9000 ERW	16.7 ± 0.09	130	33	<0.01

MLS, mean lifespan, presented as the mean ± SEM (days); %, change in the mean lifespan compared with the control; Max, maximum lifespan (days); *p*, comparison with untreated animals by the log-rank test; *n*, number of nematodes examined.

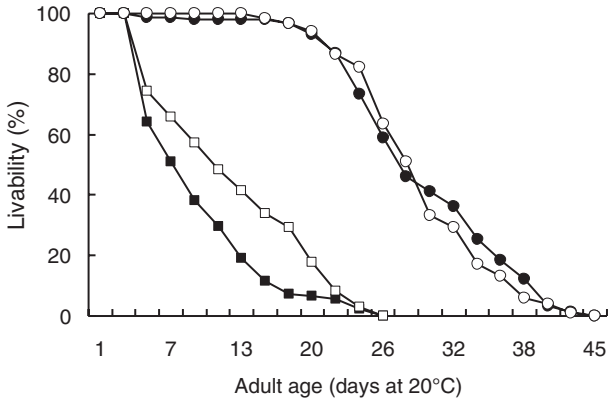

Fig. 1. Survival Curves of N2 Worms Grown in the Various Water Media at 20 °C.

Solid squares, survival curve of worms cultured in MQ(NaOH)-Water medium; open squares, survival curve of worms cultured in TI-200 ERW-Water medium; solid circles, survival curve of worms cultured in MQ(NaOH)-S medium; open circles, survival curve of worms cultured in TI-200 ERW-S medium. The worms used per lifespan assay experiment were $n = 58$–136 and three to six independent experiments were carried out. MQ(NaOH) medium *vs.* ERW medium, $p < 0.001$; MQ(NaOH)-S medium *vs.* ERW-S medium, no significant difference. The data shown are trial 1 (Table 1).

ROS accumulation in C. elegans

On the assumption that ERW extends the lifespan of nematodes by reducing ROS levels in the nematodes, we examined ROS accumulation in *C. elegans* induced by 0.4 M paraquat using ROS detection reagents DCFH-DA. DCFH-DA is a molecular probe that is nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell. Chemically reduced and acetylated forms of 2′,7′-dichlorofluorescein (DCF) and calcein are nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell.[22)]

The photographs in Fig. 2A through Fig. 2D are fluorescent images of *C. elegans* cultured in MQ(NaOH)-Water medium, TI-200 ERW-Water medium, TI-9000 CW-Water medium, and TI-9000 ERW-Water medium respectively. We found that the intracellular ROS accumulation in worms cultured in MQ(NaOH)-Water medium was significantly higher (337%, Fig. 1E) after

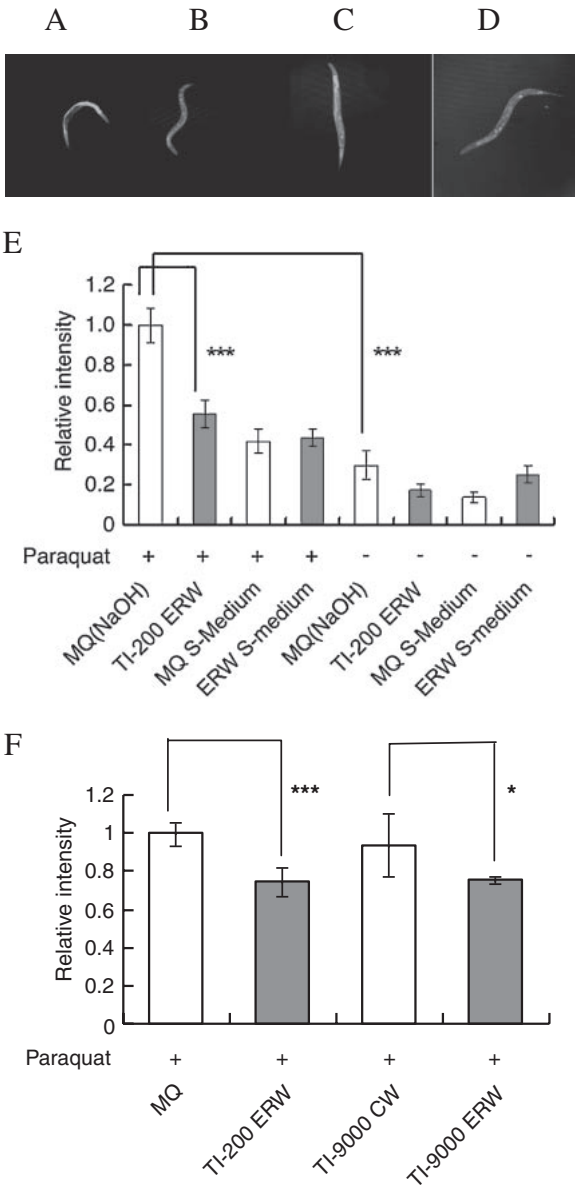

Fig. 2. Effects of ERW Media on ROS Accumulation in *C. elegans*.

Worms were cultured in TI-200 ERW or TI-9000 ERW medium for 5 d ($n = 5$–12). The amounts of peroxides in the nematodes were detected by DCFH-DA staining. A–D, the photographs show dyed worms cultured in MQ(NaOH)-Water medium (A), TI-200 ERW-Water medium (B), TI-9000 CW-Water medium (C), and TI-9000 ERW-Water medium (D). E, The relative fluorescence intensities of the worms tested are presented as means ± SEM in comparison of those of worms cultured in MQ(NaOH)-Water medium. The labels MQ(NaOH), TI-200 ERW, MQ-S Medium, and ERW-S Medium refer to the relative DCFH-DA fluorescence intensities of *C. elegans* cultured in MQ(NaOH)-Water medium, TI-200 ERW-Water medium, MQ(NaOH)-S medium, and TI-200 ERW-S medium respectively, with (+) and without (−) paraquat treatment. F, Relative DCFH-DA fluorescence intensities of paraquat-treated nematodes cultured in MQ(NaOH)-Water medium, TI-200 ERW-Water medium, TI-9000 CW-Water medium, and TI-9000 ERW-Water medium. The average fluorescence intensity of the worms cultured in MQ(NaOH)-Water medium (control) was normalized as 1.0. $^{*}p < 0.05$, $^{***}p < 0.001$.

treatment with 0.4 M paraquat, while the worms cultured in TI-200 ERW-Water medium showed significantly less ROS accumulation (187%) than those cultured in MQ(NaOH)-Water medium. The ROS accumulation in the worms that cultured in S medium was extremely low compared to that in worms cultured in Water medium,

and no significant differences were found between the ROS accumulation levels in the worms cultured in ERW-S medium and MQ(NaOH)-S medium (Fig. 2E). In another trial, the TI-200 ERW-Water and TI-9000 ERW-Water media reduced the levels of paraquat-induced oxidative stress as compared to the corresponding controls ($74.95 \pm 7.8\%$ and $75.8 \pm 2.0\%$ respectively, $p < 0.01$) (Fig. 2F).

Discussion

Considering that ERW is liquid water, we believed the best way to evaluate its effect on nematode lifespan is to use an aqueous medium. Before this research began, we were informed by Dr. Honda of the Tokyo Metropolitan Institute of Gerontology that the antioxidant ability of ERW might not be strong enough to be observed in a traditional aqueous nematode medium like S medium (personal correspondence). For this reason we designed the Water medium, which contains nothing but sample waters, and testified the effects of ERW on nematode lifespan with both S medium and Water medium. We found no significant differences between the worms cultured in ERW-S medium and control-S medium (Table 2, Fig. 1), which accords with Dr. Honda's research.

We found significant differences between the ERW-Water medium and the control-Water medium, which confirmed our expectations as to the antioxidant and lifespan-extending potential of ERW. Since the components of Water medium are much simpler than those of S medium, it is believed that the lifespan-extending capability of ERW is too weak for such a profound background as S medium or that the lifespan-extending capability of ERW can easily be disrupted by certain kinds of ions in S medium.

The worms cultured in Water medium showed a significantly shortened lifespan as compared to those cultured in S medium (Fig. 1). The reason is not clear yet. Solomon *et al.*[23] examined the tolerance of nematodes to high osmotics. However, to the best of our knowledge, there are no previous papers reporting the lifespan of nematodes in a medium with such a low osmotic pressure as Water medium. According to Solomon *et al.*, the isotonic osmotic pressure of *C. elegans* is about 100 mOSM/kg, while, as shown in Table 1, the osmotic pressures of the Water media we used were about 20 mOSM/kg, much lower than the isotonic osmotic pressure of *C. elegans*. A small proportion of worms (about 5%) in each group died like empty capsules during the first 48 h after they were transferred to the Water medium. These worms are believed to have been unable to adapt to the environment of low osmotic pressure, and their lifespan data were excluded from the static calculation. It is possible that the lifespan difference between worms cultured in S medium and Water medium was caused by the extremely low osmotic environment, suggesting that the slightly higher osmotic pressure of ERW-Water medium compared to MQ(NaOH)-Water medium was responsible for the extension of the nematode lifespan. However, when we pre-adjusted the MQ(NaOH)-Water medium to the same osmotic pressure as the ERW-Water medium using NaOH solution and HEPES, the MQ(NaOH)-Water medium still did not lengthen the lifespan of the nematodes (data not shown). These findings indicate that the lengthening effect of ERW on the lifespan of the nematodes was not caused by a change in osmotic pressure.

It has been reported that the longevity of *C. elegans* under more natural conditions (such as heat-treated soil and sand) is much reduced by up to about 10-fold as compared to that of *C. elegans* under standard laboratory culture conditions, suggesting that nematodes receive more severe stress under natural conditions than under laboratory ones.[24] Under more natural conditions, the longevity of the *daf-2* mutant, which exhibits 2-fold longer longevity than wild-type *C. elegans* under conventional laboratory conditions, was shorter than that of the wild-type worms.[24] S medium exhibits an extremely high osmolality, of 360 mOsm/kg, compared to those of Water media, of 14–25 mOsm/kg (Table 2). The osmolalities of tap water and 2 mM NaOH solution, which showed similar conductivities, were 4 mOsm/kg. Oxidative stress in *C. elegans* increased more in the Water media than in the S media, and the lifespans of the nematodes in Water media were a half or one third of those in S media. Taking into consideration that nematodes live in rainwater and soil water with low osmolalities, Water medium may be closer to natural physiological conditions than S medium.

There are many reports indicating that the lifespan of nematodes is shortened by oxidative stress.[25,26] In the present study, it was found that nematodes exhibited higher ROS levels in Water medium than in S medium (Fig. 2), suggesting that higher ROS levels in the Water-medium cultured worms resulted in a shorter lifespan. The ROS level of the worms cultured in ERW Water-medium was significantly lower than that of those cultured in control Water-medium (Fig. 2), which suggests that ERW extends the lifespan of *C. elegans* at least in part by scavenging ROS.

The mechanism of action of ERW on the lifespan of the nematodes remains to be investigated. ERW contains molecular hydrogen and small amounts of Pt nanoparticles (Table 1). Recently, it was reported that molecular hydrogen selectively scavenges hydroxyl radicals and peroxynitrite, and that inhalation of 2% hydrogen gas improves the symptoms of cerebral infarction in model rats.[27] Many papers have reported that molecular hydrogen improves the state of oxidative stress-related disease model animals.[28–32] Sato *et al.*[33] reported that hydrogen molecules suppress superoxide anion radical formation in brain slices of mice.

As shown in Table 1, the ERW used in the present study contained high concentrations of molecular hydrogen, but the concentration rapidly decreased to less than 50 ppb (25 μM) during the pH adjustment. The effects of molecular hydrogen at concentrations below 25 μM on nematodes remain to be investigated.

On the other hand, ERW contains small amounts of Pt nanoparticles. The Pt nanoparticles were assumed to be produced by the Pt-coated electrodes during electrolysis. We have found that synthetic Pt nanoparticles with average sizes of 1–6 nm exhibit superoxide dismutase- and catalase-like activities as well as hydroxyl radical-scavenging activities, and protect cultured cells against oxidative stress.[34] It has also been reported that Pt

nanoparticles are new antioxidants that can extend the lifespan of nematodes.[15] The lifespan of nematodes cultured in S medium was extended by exactly 0.5 mM Pt nanoparticles, but not by either higher or lower concentrations of Pt nanoparticles.

Since the nematodes cultured in Water medium exhibited high oxidative stress and appeared to exhibit high sensitivity to weak antioxidants, further investigation will be carried out to examine whether the ppb levels of Pt nanoparticles in ERW can affect the lifespan of nematodes.

There are many contradictory reports that ROS is or is not responsible for the regulation of the lifespan of nematodes.[35] It has been reported that many antioxidants cannot extend the lifespan of *C. elegans*, or can extend the nematode lifespan, but not because of their intracellular ROS-scavenging activities.[12,36] Because the regulatory mechanisms for lifespan are extremely complicated, it is possible that ERW extends the lifespan of *C. elegans* not only by alleviating ROS accumulation, but also by other mechanisms. It is known that the life-shortening effect of paraquat can be alleviated by EUK-8, a well-known superoxide scavenger,[37] while its lifespan extending capability remains controversial.[36,38] We tested to determine whether ERW Water-medium would protect nematodes from ROS poisoning by counting live nematodes after paraquat treatment, but no significant differences were found between the ERW and the MQ water-medium group (data not shown). Further detailed analysis is necessary to determine the effects of ERW on the lifespan of nematodes when shortened by paraquat.

The findings that TI-200 ERW and TI-9000 ERW exhibited similar activities on lifespan and ROS accumulation suggest that TI-200 ERW is a useful model water for potable ERW. Since the water medium lacked many possibly necessary minerals as compared to the S medium, it might not be a perfect physiological evaluation system for nematodes. However, the high sensitivity for ROS proved the medium to be an applicable model for weak antioxidants such as natural reduced water and natural healthy products.

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