Antioxidacinis poveikis prie laisvuosiu radikalu

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The mechanism of the enhanced antioxidant effects against superoxide anion radicals of reduced water produced by electrolysis

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Abstract

We reported that reduced water produced by electrolysis enhanced the antioxidant effects of proton donors such as ascorbic acid (AsA) in a previous paper. We also demonstrated that reduced water produced by electrolysis of 2 mM NaCl solutions did not show antioxidant effects by itself. We reasoned that the enhancement of antioxidant effects may be due to the increase of the ionic product of water as solvent. The ionic product of water (pK_w) was estimated by measurements of pH and by a neutralization titration method. As an indicator of oxidative damage, Reactive Oxygen Species- (ROS) mediated DNA strand breaks were measured by the conversion of supercoiled ϕX-174 RF I double-strand DNA to open and linear forms. Reduced water had a tendency to suppress single-strand breakage of DNA induced by reactive oxygen species produced by H_2O_2/Cu (II) and HQ/Cu (II) systems. The enhancement of superoxide anion radical dismutation activity can be explained by changes in the ionic product of water in the reduced water.
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Keywords: Reduced water; Antioxidant; Ionic product of water; DNA damage

1. Introduction

Recently, a new technology involving electrolysis of water has been proposed for clinical improvement of various diseases. Reduced water produced by electrolysis of tap water has a higher pH (9.0–10.0), lower oxidation reduction potential (ORP), lower dissolved oxygen (DO) and higher dissolved hydrogen (DH) than non-electrolyzed water. Reduced water [1–3], which has such parameters, is used extensively as drinking water which in addition to its use as a pure filtered drinking water may also act as an antioxidant against oxidative stress. In contrast, the water collected from the anode compartment is oxidized water and has been used extensively as an antiseptic [4–6].

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Oxidative stress [7–12] in the human body is thought to be due to excess reactive oxygen species or free radicals including superoxide anion radicals ($O_2^{-}\bullet$), hydroxyl radicals (•OH), hydroperoxyl radicals (•OOH), nitrogen monoxide radicals (NO•), singlet oxygen ($^1O_2$) and hydrogen peroxide molecules (H_2O_2). Among these, superoxide anion radicals are the best known. It is considered that the dismutation activity for superoxide anion radicals is the most important indicator of antioxidant effects. We have studied the antioxidant effects of reduced water produced by electrolysis [13]. The commonly reported parameters of reduced water which are pH, ORP, DO and DH, do not explain the mechanism of enhanced antioxidant effects but they are useful parameters for determining the energy of electrolysis, if measured immediately after electrolysis. They are the parameters of the solute in the reduced water. We have investigated the parameters of the solvent water. The parameter of the solvent, which is directly related to the enhancement of dismutation activity by reduced water is the ionic product of water. We could obtain the ionic product of water (pK_w) by using pH and the neutralization titration method. We defined the ionic product of water (pK_w) as pIP for electrolyzed water. Water passed through the electrolysis system with no current applied was used as a control. In this study, pIP and the increase of entropy have been estimated from the experimental results. We demonstrate that the reduced water protects DNA from damage by oxygen radicals based on the pIP of the reduced water. The mechanism of the observed antioxidant effects is discussed in relation to these parameters.

2. Materials and methods

2.1. Electrolysis cell

Two electrolysis half-cells, made of acrylonitrile-butadien-styrene mounted in polyvinyl chloride resin, were prepared as shown in Fig. 1. A non-charged membrane, (YUMICRON Y9201-T, YUASA CORPORATION) with an effective area of 100 cm^2 and 0.12 mm thickness was mounted between the two half-cells. Electrolysis was carried out across the non-charged membrane between these two half-cells equipped with platinum-coated titanium electrodes having an effective area of 100 cm^2 on the cathode.

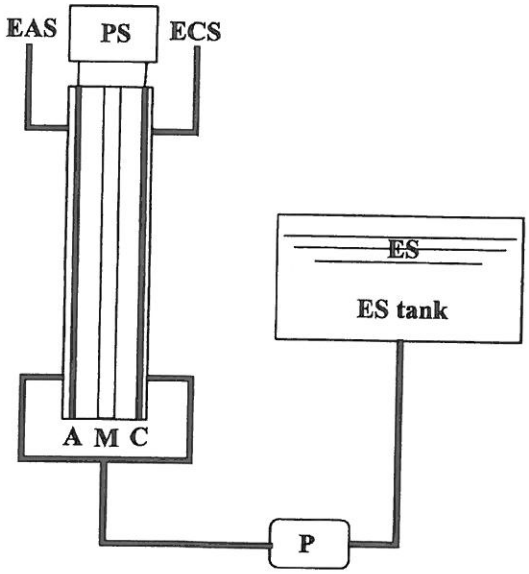


Fig. 1. Electrolysis cells made of an acrylonitrile–butadien–styrene mounted in polyvinyl chloride resin. (A) anode, (C) chathode, (M) non-charged membrane, (ES) electrolyte solutions, (ECS) electrolyzed cathode solutions (the reduced water), (EAS) electrolyzed anode solutions (the oxidized water), (PS) electric power source and (p) pump.

2.2. Chemicals and reagents

The reagents used for this study were special-grade NaCl, KCl, $MgCl_2$, $CaCl_2$, L-ascorbic acid (Wako Pure Chemical Industries Co., Ltd.), and as a solvent, water, which was distilled and deionized with an ion-exchange resin to below 0.3 $\mu s\ cm^{-1}$. To measure the superoxide dismutation activity, a sodium phosphate buffer containing 2 mM hypoxanthine (Sigma Chemical Company), a sodium phosphate buffer containing, 5,5-dimethyl-1-pyrroline-oxide (DMPO, Labotec Co., Ltd.), superoxide dismutase (Boehringer Manheim), 0.4 unit ml^{-1} xanthine oxidase in sodium phosphate buffer (Merck and Co., Inc.) and L-ascorbic acid (AsA) were used. ΦX-174RF1 plasmid DNA (New England Biolabs, Beverly, MA), H_2O_2, $CuSO_4$, hydroquinone, Tris base, sodium acetate, EDTA, and ethidium bromide (Sigma Chemical

Co., St. Louis, MO) and Agarose (Bio-Rad Laboratories, Hercules, CA) were used for the DNA damage measurements.

2.3. Electrolysis of 2 mM NaCl, KCl, 1 mM $MgCl_2$ and $CaCl_2$

20 L sample solutions, containing 2 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$ or 1 mM $CaCl_2$ were prepared. These samples were pumped from the solution container through a pair of electrolysis half-cells with a non-charged membrane mounted between them. The sample solutions were subjected to electrolysis under set conditions of 0 to 0.8 A and 25 °C using a constant electric current source. Electrolysis was carried out with a constant flow rate of 1000 ± 50 ml min^{-1} in the cathode compartment and, 2000 ± 100 ml min^{-1} in the anode compartment. The cathode solution produced by electrolysis, which was called reduced water, was used for the experiments in this study.

2.4. Measurements of pH, oxidation and reduction potential (ORP), and dissolved oxygen (DO)

The parameters that were measured to characterize the reduced water produced by electrolysis of 2 mM NaCl and KCl, and 1 mM $MgCl_2$ and $CaCl_2$ were electrical potential (V) from the power source, electrical conductivity (EC) by an EC meter (TOA EC METER CM-14P), pH by a pH meter (TOA ION METER IM-4OS), oxidation reduction potential (ORP) by an ORP meter (TOA ION METER IM-4OS) and dissolved oxygen (DO) by a DO meter (HORIBA OM-12) at 25 °C.

2.5. Measurement of OH^- ions

The concentration of OH^- ions was obtained from the measurement of pH and by a neutralization titration method using an automatic titrator. In this study, pH was measured using a pH meter (TOA ION METER IM-40S, Toa Electronics Ltd.) in samples produced at each of the following levels of electric current, 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 A. Neutralization titrations with 20 mM HCl were carried out using a COMITE-550 titrator (Hiranuma Industry Co.) for the reduced water produced at each of the same levels of electric current as the pH measurements.

2.6. Measurement of superoxide dismutation activity

1 ml of 10 mM L-ascorbic acid was added to 50 ml samples of the reduced water and to 50 ml of 2 mM NaCl and KCl, and 1 mM $MgCl_2$ and $CaCl_2$ solutions, respectively. Superoxide dismutation activity was measured using an electron spin resonance (ESR) spectrometer (ES-10, Nikkiso Co. Ltd.). Each reaction mixture contained 50 mm^3 of 2 mM hypoxanthine in sodium phosphate buffer, 50 mm^3 of sample, 16 mm^3 of DMPO and 50 mm^3 xanthine oxidase in a sodium phosphate buffer. These reaction mixtures were poured into a special flat cell to conduct ESR measurements. This ESR measurement was carried out using 3.7 mW microwave power, 339.1 ± 5.5 mT magnetic field, 100 kHz frequency, 0.1 mT modulation, 0.12 s response time and 1 min sweep time. As the unit for superoxide dismutation activity, we used the same unit as adopted by Friedovich et al. [14]. The superoxide dismutation activity of the samples was estimated by means of interpolation based on a standard curve of 0 to 30 units per ml of superoxide dismutase.

2.7. The ratio of concentration of cations to that of anions in the cathode compartment

When electrolysis of diluted electrolyte solutions is carried out the concentration of ions in both the cathode and the anode will be changed compared to the initial solutions. The concentration of transported ions in the cathode side was measured by an ion chromatography system (TOA ICA-5000 System) and the ratio of cations and anions for each sample solution was estimated.

2.8. Titration of the reduced water and the control by 10 mM AsA solutions

The amount of 10 mM AsA solutions consumed by the neutralization titration for the reduced water and the control solution was measured. Non-elec-

trolyzed water brought to the same pH as the electrolyzed water by addition of NaOH was used as the control. The pH of the reduced water was changed from 10.1 to 10.94 and that of the control was changed from 10.05 to 10.94.

2.9. The estimation of ionic product $p(IP)_{RW}$ of reduced water and the entropy of reduced water

When electrolysis is carried out with 2 mM NaCl and KCl solutions, and 1 mM $MgCl_2$ and $CaCl_2$ solutions, the following reactions will be observed in the cathode compartment,

$$2H_2O + 2e^- \leftrightharpoons 2OH^- + H_2 \quad (1)$$

and designating monovalent electrolytes as M_A and divalent electrolytes as M_B, for NaCl, or KCl, and $MgCl_2$ or $CaCl_2$, respectively,

$$M_A^+ + OH^- \rightarrow M_AOH \quad (2)$$

$$M_B^{2+} + 2OH^- \rightarrow M_B(OH)_2 \quad (3)$$

In general, even if pure water is used H_2CO_3 (beyond 1.1×10^{-2} mM at 25°) is present in an open system. Therefore, most solutes are dissolved as carbonates as shown in Eqs. (4)–(6).

$$H_2O + CO_2 \leftrightharpoons H_2CO_3 \leftrightharpoons H^+ + HCO_3^- \quad (4)$$

$$M_AOH + H_2CO_3 \rightarrow (M_A)_2CO_3 + 2H_2O \quad (5)$$

$$M_B(OH)_2 + H_2CO_3 \rightarrow M_BCO_3 + 2H_2O \quad (6)$$

The pH of the reduced water is shown as the result of hydrolysis of the carbonate salts. In general, hydrolysis of salts will be represented by the following equations,

$$HA \rightleftharpoons H^+ + A^- \quad (7)$$

Where *HA* is a weak acid.

The dissociation constant in this hydrolysis is given by Eq. (8),

$$Ka = C\alpha^2/(1-a) \quad (8)$$

Where *K*a, α and *C* are dissociation constant, degree of dissociation and initial concentration, respectively. If α is much smaller than 1, Eq. (9) can be obtained from Eq. (8). Eq. (10) can be obtained from Eq. (9).

$$\alpha \cong (Ka/C)^{1/2} \quad (9)$$

Eq. (10) can be obtained from Eq. (8).

$$[H^+] = C\alpha \cong (Ka/C)^{1/2} \quad (10)$$

Thus, pH will be described as follows:

$$pH = (1/2)(pKa - \log C) \quad (11)$$

In the case of $Kw = 10^{-14}$, the used pH will be shown as Eq. (12).

$$pH = 7 + (1/2)(pKa + \log C) \quad (12)$$

However, the dissociation of water will be shown as Eq. (13),

$$H_2O + H_2O \rightleftharpoons H_3O^+ + OH^- \quad (13)$$

Ionic product of water, K_w and IP will be shown as Eq. (14).

$$[H_3O^+][OH^-] = K_w = IP \quad (14)$$

where IP will be described as Eq. (15) under the conditions of 25 °C and 1 atm,

$$(IP)_w = 10^{-14}\ (mol/l)^2 \quad (15)$$

Therefore, $p(IP)_w$ will be shown as Eq. (16),

$$-\log(pK_w) = pK_w = p(IP)_w \quad (16)$$

The titration for 1-1 carbonates with HCl solutions will be carried out as follows,

$$M_2CO_3 + HCl \rightarrow MCl + MHCO_3 \quad (17)$$

$$MHCO_3 + HCl \rightarrow MCl + H_2O + CO_2 \quad (18)$$

$$M_2CO_3 + 2HCl \rightarrow 2MCl + H_2O + CO_2 \quad (19)$$

If the neutralization titration is carried out with 20 mM HCl, two peaks of differential values (dE/dV) are observed, where E and V are potential and volume. The concentration at each neutralization titration point is shown as Eqs. (20) and (21).

$$[OH^-]_{MOH} = [HCl]_{A\ point} - [HCl]_{B\ point} \quad (20)$$

$$[HCl]_{B\ point} = 2[M_2CO_3]_{dissociation} = [MHCO_3]_{total} \quad (21)$$

$$-\log[OH^-]_{M_2CO_3} = 14 - (pH)_{M_2CO_3} \quad (22)$$

$$[OH^-]_{total} = ([OH^-]_{MOH} + [OH^-]_{M_2CO_3} \quad (23)$$

$$p(IP)_{RW} = -\log K_{RW} = 14 + \log([OH^-]_{total}/[OH^-]_{pH}) \quad (24)$$

where $[OH^-]_{pH}$ is the concentration of $[OH^-]$ estimated from pH value of the reduced water.

The free energy change ΔG^0 for a reaction is related to the equilibrium constant K, enthalpy ΔH, and entropy ΔS at equilibrium as shown in following equations.

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 = -RT\ln\ K \quad (25)$$

$$\Delta S^0 = \Delta H^0/T + R\ \ln\ K \quad (26)$$

where R, T and K are the gas constant (1.987 cal mol^{-1} K^{-1}), the absolute temperature and the equilibrium constant which is the ionic product of water, K_{RW} in the reduced water produced by electrolysis. At 25 °C and 1 atm.

$$\Delta S^0 = -68.3/298 + 1.9872 \times \ln\ K_{RW} \quad (27)$$

From Eq. (27), the entropy difference, ΔS^0 of the solvent in the reduced water will be estimated using K_{RW} which is defined as $(IP)_{RW}$.

2.10. Measurement of DNA strand break under oxidative stress

The ROS-mediated DNA strand breaks were measured by the conversion of supercoiled ϕX-174 RF I double-strand DNA to open and linear forms, according to the method described by Li [15] and Win [16]. Briefly, 0.2 μg DNA was incubated with the indicated concentration of H_2O_2 and, 25 μM $CuSO_4$ in 20 μl of 20 mM sodium phosphate buffer (pH 7.5) containing 17 μl of reduced water or, NaOH solution of the same pH as the reduced water at 37 °C for 30 min. For the hydroquinone/copper II (HQ/Cu(II)) induced breaks, 0.2 μg of DNA was incubated with the indicated concentrations of HQ and Cu (II) in PBS at 37 °C at a final volume of 20 μl containing 17 μl of reduced water. Following incubation, the samples were immediately loaded in a 1% agarose gel containing 40 mM Tris, 20 mM sodium acetate and 2 mM EDTA, and subjected to electrophoresis in Tris/acetate gel buffer. After electrophoresis, the gels were stained with a 0.5 μg/ml solution of ethidium bromide for 30 min, followed by another 30 min destaining in water. The gels were then photographed under UV light.

3. Results

When electrolyte solutions are electrolyzed across the membrane, reduction occurs at the cathode and oxidation at the anode. Oxidation of water molecules produces H^+ and O_2 at the anode, and OH^- and H_2 at the cathode. Therefore, cathodic alkaline water (reduced water) is abundant in dissolved hydrogen (DH), whereas anodic acidic water (oxidized water) is abundant in DO. DH and DO produced by electrolysis have particular characteristics [2]. Results of the measurement of electric potential (V), electric conductivity (EC), pH, ORP and DO at different electric currents are shown in Figs. 2–6. The electrolysis potential varies linearly with electric current between 0.1 A and 0.8 A as shown in Fig. 2. The slopes, V/A increased in the order of KCl, $<CaCl_2$, $<MgCl_2$, $<$NaCl solutions. Fig. 3 shows results of electric conductivity measurements. The electrical conductivity for a fixed electric current increased in the order of $MgCl_2$, $<$NaCl, $<CaCl_2$, $<$KCl. The conductivity of KCl solutions was very high compared with the other electrolyte solutions. pH increased from 9.9 to 10.96 in NaCl solutions,

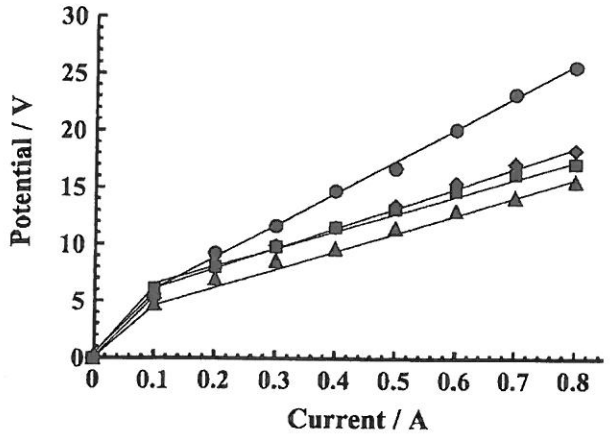


Fig. 2. The relationship between electric potential (V) and electric current (A) using various electrolyte solutions. (●) NaCl, (▲) KCl, (♦) $MgCl_2$ and (■) $CaCl_2$.

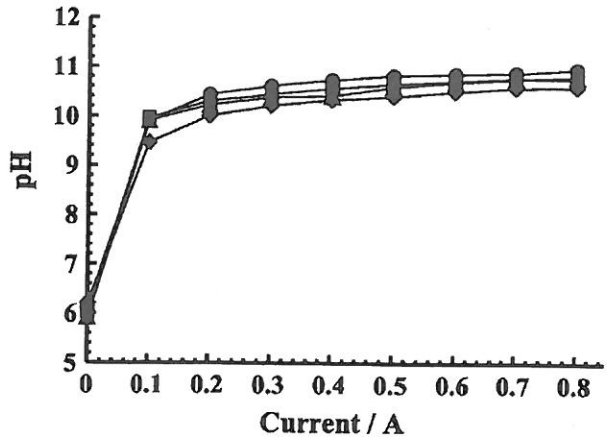


Fig. 4. The relationship between pH and electric current (A) using various electrolyte solutions. (●) NaCl, (▲) KCl, (♦) $MgCl_2$ and (■) $CaCl_2$.

9.91 to 10.77 in KCl solutions, 9.46 to 10.6 in $MgCl_2$ solutions and 9.96 to 10.77 in $CaCl_2$ solutions when the current was increased from 0.1 to 0.8 A. ORP decreased from −50 mV to −170 mV in NaCl solutions, −51 mV to −179 mV in KCl solutions, −70 mV to −176 mV in $MgCl_2$ solutions and −73 mV to −137 mV in $CaCl_2$ solutions when the current was increased from 0.1 to 0.8 A. DO decreased from 6.97 mg l^{-1} to 6.28 mg l^{-1} in NaCl solutions, 6.9 mg l^{-1} to 6.2 mg l^{-1} in KCl solutions, 6.63 mg l^{-1} to 6.03 mg l^{-1} in $MgCl_2$ solutions and 6.61 mg l^{-1} to 6.18 mg l^{-1} in $CaCl_2$ solutions, respectively.

Fig. 7 shows the superoxide dismutation activity of L-ascorbic acid in the reduced water of NaCl, KCl, $MgCl_2$ and $CaCl_2$ solutions, and the control solutions adjusted to the same pH and concentration, respectively. The results were obtained through ESR measurements conducted using 10 mM L-ascorbic acid as proton donor. Electrolysis of 2 mM NaCl and KCl, and 1 mM $MgCl_2$ and

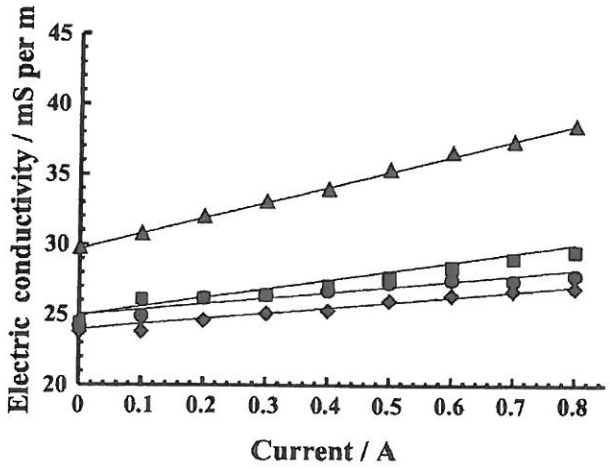


Fig. 3. The relationship between electric conductivity (mS per m) and electric current (A) using various electrolyte solutions. (●) NaCl, (▲) KCl, (♦) $MgCl_2$ and (■) $CaCl_2$.

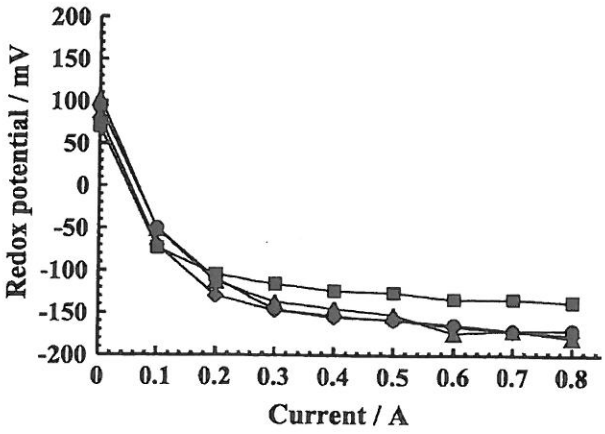


Fig. 5. The relationship between redox potential (mV) and electric current (A) using various electrolyte solutions. (●) NaCl, (▲) KCl, (♦) $MgCl_2$ and (■) $CaCl_2$.

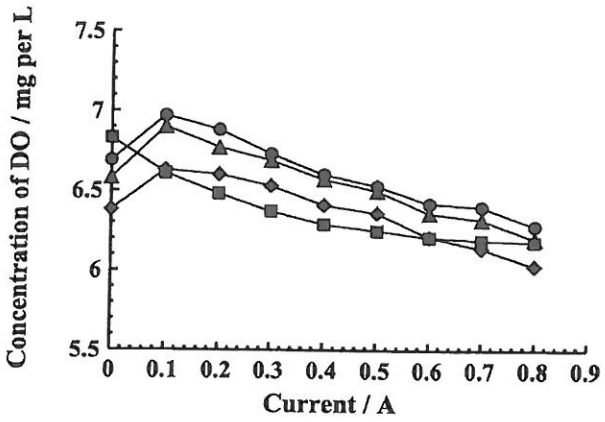


Fig. 6. The relationship between DO and electric current (*A*) using various electrolyte solutions. (●) NaCl, (▲) KCl, (♦) $MgCl_2$ and (■) $CaCl_2$.

$CaCl_2$ solutions increased the superoxide dismutation activity from 119 to 173 units per 20 ml, 115 to 154.3 units per 20 ml, 105 to 140 units per 20 ml, and 128 to 165 units per 20 ml for L-ascorbic acid, respectively, compared to the same solutions without electrolysis adjusted to the same pH as the reduced water,. In the previous paper, we demonstrated that the reduced water did not show any dismutation activity without added ascorbic acid. These results show the enhancement of superoxide dismutation activity by electrolysis compared the control solutions.

Fig. 8 shows the results of the ratio of concentration of cations to that of anions, $[cations]_{cathode}/[anions]_{cathode}$. The ratio of $[cations]_{cathode}/[anions]_{cathode}$ increased in the order of $CaCl_2$, $<MgCl_2$, $<KCl$, $<NaCl$. The value of $CaCl_2$ was negative between 0.1 and 0.8 A of electric current. For KCl and $MgCl_2$ the ratio was nearly 1. The NaCl solution, showed the highest value (between 3.848 and 4.151).

In order to confirm the difference in consumed amount for neutralization titration between the reduced water and the sample solution, the solutions were titrated using a 10 mM AsA solution from pH 10.05 to 10.95. As the result, the consumed amount of 10 mM AsA solution for the reduced water was lower than that of sample solutions at all pH levels as shown in Fig. 9.

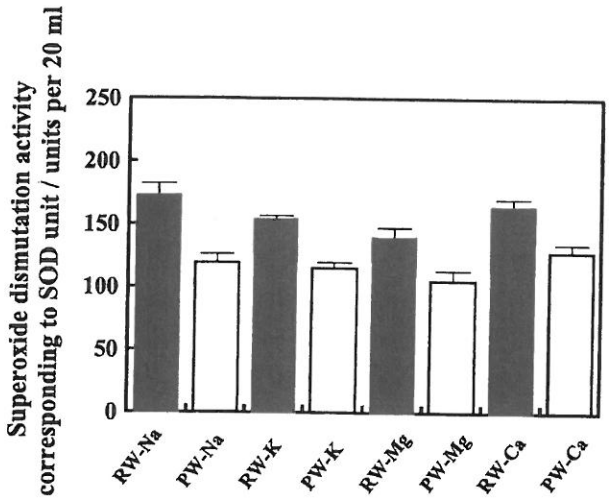


Fig. 7. Superoxide dismutation activity of L-ascorbic acid for the reduced water and control solutions adjusted to the same pH and initial concentration of electrolyte solutions (2 mM NaCl and KCl, and 1 mM $MgCl_2$ and $CaCl_2$), (RW–Na) the reduced water from 2 mM NaCl solutions, (RW–K) the reduced water from 2 mM KCl solutions, (RW–Mg) the reduced water from 1 mM $MgCl_2$ solutions and (RW–Ca) reduced water from 1 mM $CaCl_2$, (PW–Na) the control 2 mM NaCl solutions and (PW–K) the control 2 mM KCl solutions, and (PW–Mg) the control 1 mM $MgCl_2$ solutions and (PW–Ca) the control 1 mM $CaCl_2$ solution.

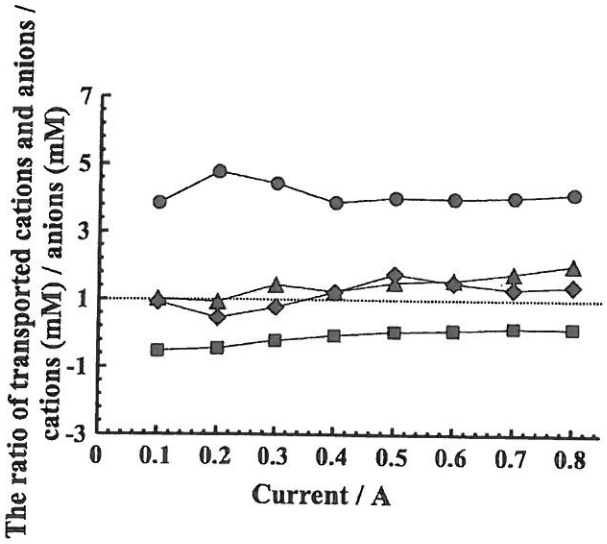


Fig. 8. The ratio of the concentration of cations and anions in the cathode compartment corresponding to electric current (*A*). (●) NaCl, (▲) KCl, (♦) $MgCl_2$ and (■) $CaCl_2$.

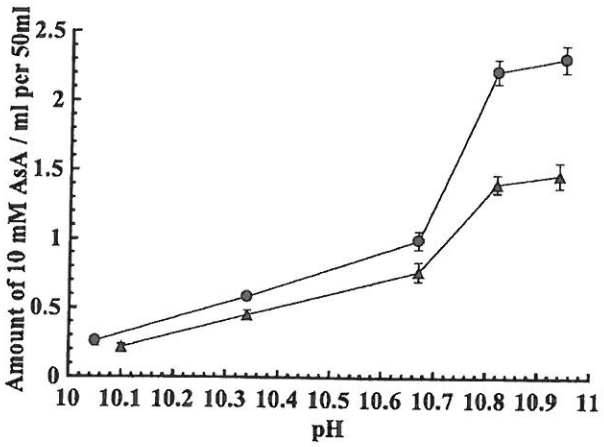


Fig. 9. The amount of consumed AsA for the reduced water and the control solutions by neutralization titration corresponding to pH. (●) the control solutions of NaOH and (▲) the reduced water.

Fig. 10 shows the results of $p(IP)_{RW}$ values for the reduced water of NaCl, KCl, $MgCl_2$ and $CaCl_2$ solutions and control solutions of NaCl and KCl. $p(IP)_{RW}$ decreased in reduced NaCl, KCl, $MgCl_2$ and $CaCl_2$ solutions in proportion to the increase in pH, but control solutions of NaCl and KCl showed no change of $p(IP)_{RW}$ between pH:9.8 and pH:10.96. The $p(IP)_{RW}$ values of the reduced

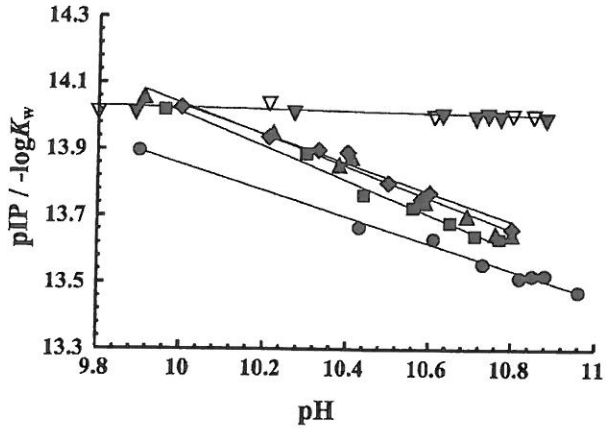


Fig. 10. The relationship between pIP ($-\log K_W$) and pH for the reduced water made from NaCl, KCl, $MgCl_2$ and $CaCl_2$, and control solutions of NaCl and KCl. (●) NaCl, (▲) KCl, (◆) $MgCl_2$ and (■) $CaCl_2$ for the reduced water, and (▼) NaCl and (▽) KCl for the control solutions.

Table 1
The increase, $(\Delta S°)_{A=0.8} - (\Delta S°)_{A=0}$, of entropy between 0 and 0.8 A of electric current for the reduced water made from NaCl, KCl, $MgCl_2$ and $CaCl_2$ solutions

Current A	NaCl 2 mM	KCl 2 mM	$CaCl_2$ 1 mM	$MgCl_2$ 1 mM
0	0	0	0	0
0.8	2.4	1.6	1.6	1.1

water of the electrolyte solutions decreased from 14.058 to 13.474. However, there was almost no change in $p(IP)_{CS}$ between pH:9.8 and pH:10.96 in the control solutions.

Eq. (27) shows the entropy of $p(IP)_{RW}$. We can estimate the increase in entropy by using Eq. (27). Table 1 shows the estimated entropy increase in the reduced water from the sample solutions. The reduced water with 2 mM NaCl solution showed the largest entropy increase (2.4 Kcal/mol). Conversely, the reduced water of $MgCl_2$ showed the lowest entropy increase (1.1 Kcal/mol).

The effects of reduced water on oxidative DNA damage are presented in Figs. 11 and 12. In the present study, oxidative DNA damage is clearly

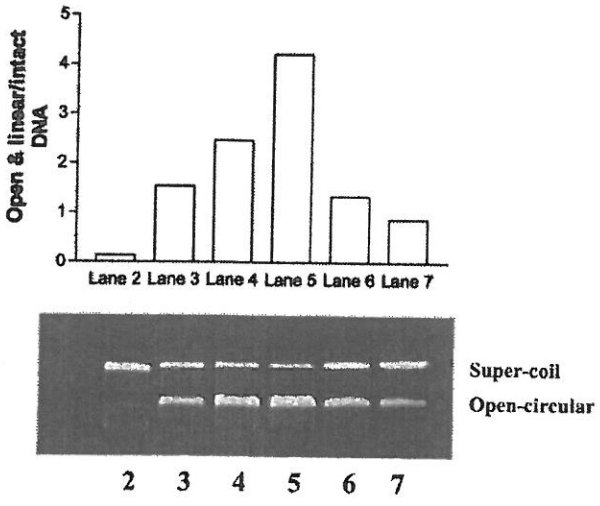


Fig. 11. Effect of electrolyzed water on hydrogen peroxide induced DNA damage. Lane 1: Negative control; Lane 2: Control; Lane 3: $H_2O_2 + Cu(II)$; Lane 4: $H_2O_2 + Cu(II) + KOH$ solution; Lane 5: $H_2O_2 + Cu(II) + 2$ mM KCl solution (0 A), pH 6.30; Lane 6: $H_2O_2 + Cu(II) +$ electrolyzed reduced water (0.4 A), pH 10.47; and Lane 7: $H_2O_2 + Cu(II) +$ electrolyzed reduced water (0.8 A), pH 10.74.

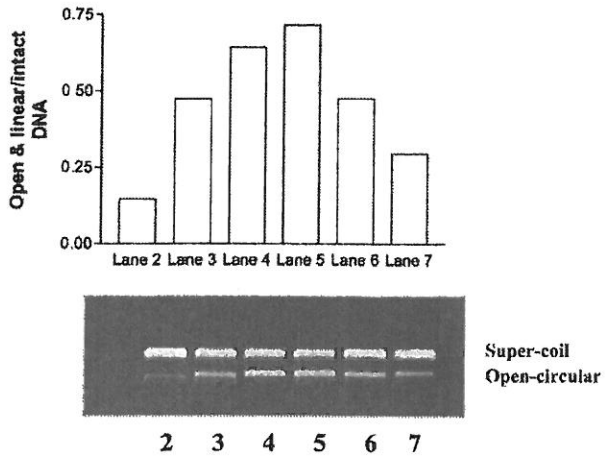


Fig. 12. Effect of electrolyzed water on hydroquinone (HQ) induced DNA damage. Lane 1: Negative control; Lane 2: Control; Lane 3: HQ+Cu(II); Lane 4: HQ+Cu(II)+KOH solution; Lane 5: HQ+Cu(II)+2 mM KCl solution (0 A), pH 6.30; Lane 6: HQ+Cu(II)+electrolyzed reduced water (0.4 A), pH 10.47; and Lane 7: HQ+Cu(II)+electrolyzed reduced water (0.8 A), pH 10.74.

evident in the H_2O_2 /Cu (II) and the HQ/Cu (II) systems (lane 3). Densitometric results, expressed as the fraction of DNA converted to open circular and linear forms, clearly showed that reduced water significantly inhibited oxidative stress induced DNA damage in both H_2O_2/Cu (II) and HQ/Cu (II) systems (lanes 6 and 7) compared to the control solution passed through the apparatus with no voltage applied (lane 5).

4. Discussion

4.1. The characterization of solutes in the reduced water

The parameters related to solutes in water reduced by electrolysis are pH, ORP, DO, DH and EC. pH is the indicator which shows the hydrogen ion concentration. When electrolysis is carried out in electrolyte solutions such as NaCl, KCl, $MgCl_2$ or $CaCl_2$ the pH of the reduced water produced in the cathode compartment will be measured as hydrolysis of carbonates of those electrolytes. Therefore, the pH value depends on the initial concentration of the carbonates and the parameters of hydrolysis. For example, Na_2CO_3 shows pH:9.2 to 10.6 between 10^{-6} mol l^{-1} and 10^{-3} mol l^{-1} and in $NaHCO_3$, pH:7.2 to 8.7 between 10^{-6} mol l^{-1} and 10^{-3} mol l^{-1}, respectively, [17]. At 25 °C, 1.15×10^{-2} mM carbon dioxide is dissolved in pure water. As mentioned above, when sufficiently dilute electrolyte solutions are used for the reduced water, carbonates cannot be neglected. ORP is the oxidation reduction potential and it can be estimated from the Nernst equation as follows,

$$E_V = E_V^0 + 2.303RT/nF\log(\mathrm{ox})/(\mathrm{red}) \quad (28)$$

Where, E_V, E_V^0, R, T, n, F, (ox) and (red) are the oxidation reduction potential, standard potential, gas constant, absolute temperature, number of electrons transferred in the reaction, Faraday constant, product of the activities of the oxidized species and product of activities of reduced species, respectively. Thus, in the case of reduced water, as oxidation and reduction are included for oxygen in the original water and hydrogen in the reduced water, the ORP equation will be shown as Eq. (29)

$$E_V = E_V^0 + (n)\mathrm{pH} + (m)_O\log[O_2] + (m)_H\log[H_2] \quad (29)$$

Where, (n), $(m)_O$ and $(m)_H$ are coefficients relative to pH, O_2 and H_2 [18]. Therefore, ORP is the parameter indicating the concentrations of oxygen and hydrogen dissolved in the reduced water. As DO and DH are the concentrations of oxygen and hydrogen in the reduced water, they are parameters relative to solutes in it. Those solutes will be generated by the process of electrolysis.

The electric potential at each level of electric current was higher in NaCl solution than that in KCl, and $MgCl_2$ solutions, which was higher than in $CaCl_2$ solution as shown in Fig. 2. It is assumed that the difference in ionic mobility between cations and anions will be proportional to the electric potential. For example, the ratio of Cl^- ions to Na^+ ions is 0.767, that of Cl^- ions to K^+ ions is 0.987, and that of Cl ions to Mg^{2+} ions is 0.713 and Cl^- ions to Ca^{2+} is 0.799. It is strongly suggested that the higher the ratio of anions to cations, the lower the electric potential gradient.

Results for electrical conductivity were contrary to those for electrical potential as mentioned above. This is a reasonable result. Furthermore, pH, ORP and DO also changed as expected in proportion to the magnitude of the electrical field energy.

4.2. The enhancement of superoxide dismutation activity in reduced water

Reduced water prepared from all of the electrolyte solutions (NaCl, KCl, $MgCl_2$ and $CaCl_2$) had higher superoxide dismutation activity, when measured using AsA as an antioxidant indicator, than the corresponding control solutions adjusted to the same pH (Fig. 7). AsA has the structure of a 2,3-enediol which is a kind of saccharic acid, and it plays a role in the protection system against oxidative stress in animals and plants [19,20]. The OH at the 2nd functional group has $pKa = 11.79$ and that at the 3rd functional group has $pKa = 4.25$. If AsA is added to reduced water and control solutions which are adjusted to the same pH as the reduced water, the proton on the OH of the 3rd functional group will react with the OH ions of the reduced water and the OH ions in control solutions, and will be neutralized because the OH of the 3rd functional group of AsA is dissociated as shown in Eq. (30),

$$-OH \rightarrow O^- + H^+ \tag{30}$$

Furthermore, it is considered that proton in OH of the third functional group of AsA will be used for the neutralization of the alkaline reduced water and proton in that of the 2nd functional group of AsA which has very low dissociation activity at a $pKa = 11.79$ will be used for the superoxide radical dismutation reaction [21,22]. If AsA is mixed with reduced water, the dissociation activity will increase because of the higher dissociation activity of the reduced water. The higher dissociation activity of the reduced water increases the dissociation activity of substances with lower activity. As shown in Fig. 9, when the neutralization titration was carried out at several different pH levels in reduced water and the control solutions of 2 mM NaOH, there were significant differences at each pH. It is considered that the increase in dissociation of protons at the 3rd functional group of AsA, dissolved in the reduced water accounts for the significant difference between the reduced water and the control solutions. This property is due to water molecules as solvent and also a very stable molecular structure. As described in a previous paper, the increase in dissociation activity of the reduced water is due to the process of electrolysis [10]. When a sufficiently large electric field is applied to the boundary between the electrode and the electrolyte bulk solutions, a very high electric potential will be generated (above 10^7 V cm^{-1}). If a sufficient amount of reduced water is taken into the body, it will increase the dissociation activity for the water-soluble antioxidant substances of relatively lower dissociation activity. As a result, we speculate that their antioxidant capacity will be enhanced. It is quite possible that many useful phenomena related to reduced water will be found to be the result of the increase of dissociation activity of water as solvent by electrolysis. The enhancement of the reduced water will depend on the ionic properties of the solutes such as species and membrane or ionic mobility [23,24]. When a non-charged membrane is used for the production of reduced water a greater difference in ionic mobility between cations and anions will produce higher dissociation activity as shown in Fig. 8. In general, the dissociation activity of water depends on the temperature and pressure. The ionic product of water $p(K_w)$ has been calculated in a wide range of temperatures (0–600) and densities by H. Sato et al. [25].

The difference between $p(IP)_{RW}$ for reduced water and $p(IP)_{CS}$ for control solutions is shown in Eq. (31).

$$\Delta p(IP) = p(IP)_{CS} - p(IP)_{RW} \tag{31}$$

It is considered that the enhancement of antioxidant effects for superoxide radicals will increase in proportion to the increase of $\Delta p(IP)$. Therefore, $\Delta p(IP)$ for reduced water as solvent is the essential parameter.

4.3. The estimation of pIP and entropy produced by electrolysis in NaCl, KCl, $MgCl_2$ and $CaCl_2$ solutions

Fig. 10 shows estimates of $p(IP)_{RW}$ for reduced water made from NaCl, KCl, $MgCl_2$, $CaCl_2$, and control solutions using NaCl and KCl. These results showed significant differences between reduced water and control solutions adjusted to the same pH and initial concentration as the reduced water before electrolysis. In general, ΔG^0 is related to the dissociation constant K and the electric potential $E°$ at the equilibrium state as shown in Eqs. (25) and (27), can be shown as Eq. (32)

$$\Delta G^0 = -nFE° \tag{32}$$

$E°$ and hydrogen ionic concentration can be shown as Eq. (33).

$$E° = (RT/mnF)\ \ln[H^+] \tag{33}$$

Where m is the coefficient of the correction of the relationship between $E°$ and pH at $K_W = 10^{-14}$.

$$\log IP = 2.303(RT/mnF)\ \log[H^+] + C \tag{34}$$

Thus, pIP is described as Eq. (35).

$$p(IP)_{RW} = (0.0591/m)pH + C \tag{35}$$

The plot of $p(IP)_{RW}$ vs pH is a straight line with a slope of (0.0591/m) and a constant. The constant C will depend on the properties of electrolytes and m will depend on the properties of the membrane and the interaction between the membrane and the ions or solvent. The $p(IP)_{CS}$ of the control solutions did not change with a change of pH from 9.8 to 10.88 but that of all sample solutions changed linearly with a change of pH from 9.9 to 10.9. The $p(IP)_{RW}$ of the reduced water decreased in proportion to the increase in pH. This change will depend on the magnitude of the applied electric current. Therefore, the dissociation energy depends on the electric current used during electrolysis.

Entropy is estimated by Eq. (31) using IP instead of K_W. Table 1 shows the entropy difference of each electrolyte solution at 0.8 A $(\Delta S°)_{A=0.8} - (\Delta S°)_{A=0}$. The entropy increased in proportion to the increase of $\Delta p(IP)_w$. The increase of entropy means that the reduced water became more active and offered a higher reaction field for dissolved substances. Thus, if substances of lower dissociation activity are dissolved in the reduced water the dissociation activity will increase compared to non-electrolyzed water.

4.4. The inhibitive effect of reduced water on the single-strand breakage of DNA by using H_2O_2/Cu (II) and HQ/Cu system

In the present study, the effects of reduced water on oxidative DNA damage were investigated by using H_2O_2/Cu (II) and HQ/Cu (II) systems. Induction of single-strand breaks in the supercoiled double-stranded ΦX174 plasmid DNA leads to formation of open circular DNA, while the formation of a linear form of DNA is indicative of double-strand breaks [15]. Cu (II), H_2O_2 is able to cause strand breaks in isolated DNA. As such, H_2O_2/Cu (II) has been widely used as a model system to induce oxidative DNA damage [16,26]. Although the exact reactive species remain to be chemically defined, a bound hydroxyl radical or its equivalent derived from the reaction of H_2O_2 and copper has been suggested to participate in the oxidative DNA damage [15,26]. The addition of reduced water to H_2O_2/Cu (II) resulted in marked inhibition of conversion of supercoiled DNA to open circular forms, suggesting that reduced water is capable of protecting against the H_2O_2/Cu (II)-mediated DNA strand breaks. To further determine the inhibitory effects of reduced water on oxidative DNA damage, HQ+Cu (II) was used in the present study. It has been previously shown that the HQ/Cu (II) system is able to induce DNA breaks, with both Cu (II)/Cu (I) redox cycle and H_2O_2 being critically involved. Similar to what was observed with the H_2O_2/Cu (II), the presence of reduced water also markedly protected against DNA strand breaks induced by the HQ/Cu (II) system. Therefore, the results suggest that reduced water can prevent oxidative DNA damage possibly by enhanced antioxidant effects against superoxide anion radicals as shown

originally by S. Shirahata et al. [27]. Thus, it appears that consumption of electrolyzed reduced drinking water may potentially serve to prevent DNA damage induced by oxygen free radicals produced by the mitochondria due to the rise in oxidative stress.

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