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Electrolyzed–Reduced Water Scavenges Active Oxygen Species and Protects DNA from Oxidative Damage

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Active oxygen species or free radicals are considered to cause extensive oxidative damage to biological macromolecules, which brings about a variety of diseases as well as aging. The ideal scavenger for active oxygen should be 'active hydrogen'. 'Active hydrogen' can be produced in reduced water near the cathode during electrolysis of water. Reduced water exhibits high pH, low dissolved oxygen (DO), extremely high dissolved molecular hydrogen (DH), and extremely negative redox potential (RP) values. Strongly electrolyzed–reduced water, as well as ascorbic acid, (+)-catechin and tannic acid, completely scavenged $O_2^{\cdot-}$ produced by the hypoxanthine-xanthine oxidase (HX-XOD) system in sodium phosphate buffer (pH 7.0). The superoxide dismutase (SOD)-like activity of reduced water is stable at 4°C for over a month and was not lost even after neutralization, repeated freezing and melting, deflation with sonication, vigorous mixing, boiling, repeated filtration, or closed autoclaving, but was lost by opened autoclaving or by closed autoclaving in the presence of tungsten trioxide which efficiently adsorbs active atomic hydrogen. Water bubbled with hydrogen gas exhibited low DO, extremely high DH and extremely low RP values, as does reduced water, but it has no SOD-like activity. These results suggest that the SOD-like activity of reduced water is not due to the dissolved molecular hydrogen but due to the dissolved atomic hydrogen (active hydrogen). Although SOD accumulated H_2O_2 when added to the HX-XOD system, reduced water decreased the amount of H_2O_2 produced by XOD. Reduced water, as well as catalase and ascorbic acid, could directly scavenge H_2O_2. Reduced water suppresses single-strand breakage of DNA by active oxygen species produced by the Cu(II)-catalyzed oxidation of ascorbic acid in a dose-dependent manner, suggesting that reduced water can scavenge not only $O_2^{\cdot-}$ and H_2O_2, but also $^1O_2$ and $^{\cdot}OH$. © 1997 Academic Press

Active oxygen species or free radicals, such as singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) are considered to cause extensive oxidative damage to biological macromolecules (DNA, membrane polyunsaturated fatty acid chains, enzymes and so on), which bring about a variety of diseases, as well as aging (1, 2). We believe that the ideal countermeasure against active oxygen is 'active hydrogen'. Electrolysis of water produces reduced and oxidized water near the cathode and anode, respectively. Reduced water exhibits high pH, low dissolved oxygen (DO), high dissolved hydrogen (DH) and significant negative redox potential (RP) values. Soft water in Japan made it possible to develop domestic devices to reform water by electrolysis about half a century ago.

So far, the characteristics of neither reduced water nor oxidized water have been well clarified. Based upon the interesting clinical improvement of a variety of diseases by intake of reduced water since 1985, Hayashi proposed the hypothesis 'Water Regulating Theory' (3). Here, based on his theory we first demonstrate that reduced water scavenges active oxygen species and protects DNA from damage by oxygen radicals.

MATERIALS AND METHODS

Electrolysis of water. Ultrapure water produced by an ultrapure system (ULTRAPUR LV-10T, TORAY, Tokyo) was added 0.1 g/l NaCl

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Abbreviations: AET, 2-(aminoethyl)isothiuronium; AsA, ascorbic acid; CL, chemiluminescence; CLA, *Cypridina* luciferin analog; DO, dissolved oxygen; DH, dissolved hydrogen, EC, electrical conductance; HX, hypoxanthine; RP, redox potential; SOD, superoxide dismutase; XOD, xanthine oxidase.

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to elevate electrical conductance (EC) to about 20 ms/m. The water was then electrolyzed with various voltages by an electrolyzing device (Type TI-7000S and TI-7000SL, Nihon Trim Co., Osaka) equipped with a platinum-coated titanium electrode to produce reduced water which exhibited various RP. RP, EC, DO and DH were measured using a RP meter (type, HM-14P), a EC meter (CM-14P), a DO meter (DO-14P) and a DH meter (DHDI-1) from Toa Electronics Ltd. (Tokyo) at 25°C. pH was measured using a pH meter (Beckman, Type pHI32) at 25°C.

Assay of the SOD-like activity of reduced water, ascorbic acid (AsA), (+)-catechin, and tannic acid. The reaction mixture (1 ml) for measuring chemiluminiescence (CL) intensity specific to $O_2^{\cdot -}$ contained 500 mM hypoxanthine (HX), 200 mM EDTA, 40 mM sodium phosphate buffer (pH 7.0), 0.6 ml of the reduced water or NaOH solution of the same pH as reduced water, 2.5 mM 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a] pyrazin-3-one (*Cypridina* luciferin analog (CLA), Tokyo Kasei Industrial Co., Tokyo) and 0.5 U/l of xanthine oxidase (XOD) (Wako Purechemical Industries, Tokyo). The CL intensity of the reaction mixture (0.85 ml) except for XOD solution was measured in a glass-tube in a CL reader (Aloka, type BLR-301) at 26 °C. At 18 seconds time point, 0.15 ml of XOD solution was injected into the tube and the CL intensity was continuously measured for 120 sec. The change of the CL intensity in the presence of reduced water, SOD derived from bovine erythrocytes (Sigma), AsA (Wako), (+)-catechin (Wako) or tannic acid (Wako) was measured with the HX-XOD system to evaluate the scavenging activity of $O_2^{\cdot -}$. The strongly reduced water (RP, −820 mV; pH 11.0) was diluted with a NaOH solution (pH 11.0) to assure the same final pH of the reaction mixture (pH 7.3) among the test samples. The extinguishing rate of $O_2^{\cdot -}$ (%) was calculated by dividing the total CL intensity of the test sample by that of the control.

Analysis of catalase-like activity of reduced water and AsA. The reaction mixture (100 μl) of the HX-XOD system described above in the presence or absence of SOD or reduced water was put into a 96-well microplate and incubated at 37 °C. Time course of the change in the H_2O_2 concentration was measured by the addition of 200 μl of substrate containing peroxidase (0.3 mg/ml 2,2′-azino-di-(3-ethyl benzthiazolin sulfonic acid), 0.1 M citrate buffer, pH 4.0, streptavidine-horseradish peroxidase conjugate (Amersham) diluted to 1000 times). Absorbency of the reaction product was measured at 405 nm by an ELISA reader. In order to examine the catalase-like activity, the H_2O_2 solution was directly incubated with control NaOH solution (pH 11.0), reduced water (pH 11.0), AsA or catalase (Wako) in 40 mM sodium phosphate buffer (pH 7.0) at 37 °C and the H_2O_2 concentrations were determined.

Analysis of single-strand breakage of DNA caused by active oxygen species produced by the Cu(II)-catalyzed oxidation of AsA. Time-course of the single-strand DNA breaking reaction caused by Cu(II)-catalyzed oxidation of AsA was examined by using super-coil plasmid DNA. One μg of pBluescript II plasmid DNA (Stratagene, La Jolla, CA) was incubated with the mixture of 25 μM AsA and 25 μM $CuSO_4$ in 20 μl of 20 mM sodium phosphate buffer (pH 7.5) containing 17 μl of reduced water, NaOH solution of the same pH as the reduced water, SOD, catalase, 2-(Aminoethyl)isothiuronium (AET)(Wako), KI (Wako), or NaN_3 (Wako) at 37°C for various periods of time. The reaction was stopped by adding 4 μl of 15 mM EDTA (pH 7.0). Super-coil DNA, open-circular DNA and linear DNA were separated by electrophoresis with 1% agarose gel and any changes of the amount of super-coil DNA were determined from the photo images by a NIHimage computer software.

RESULTS AND DISCUSSION

Characteristics of electrolyzed–reduced water. The principle of electrolysis was founded by Michael Faraday (1791-1867). In this process, reduction occurs at the cathode and oxidation at the anode. Dissociation of H_2O produces H^+ and OH^- ions. At the cathode, H^+ ions gain electrons to change into active atomic hydrogen (H). Active atomic hydrogen exhibits high reducing potential. It is then changed to hydrogen molecules (H_2) which are chemically inert at room temperature. At the anode, OH^- ions lose electrons to form OH, which results in the production of O_2 and H_2O. Cathodic alkaline water (reduced water) is abundant in DH, whereas anodic acidic water (oxidized water) is abundant in DO. The relationships of RP with pH, DO, and DH in reduced water were shown in FIG. 1. Marked changes in these values occur in water after electrolysis. It should

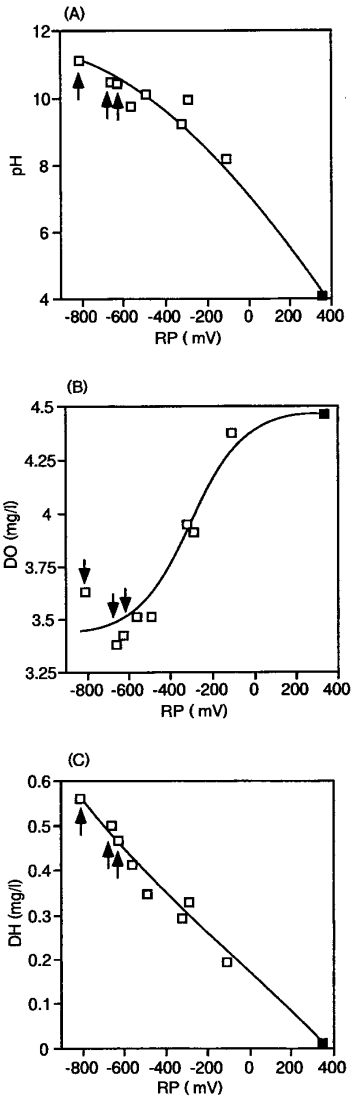

FIG. 1. Relationships of RP with pH (A), DO (B) and DH (C) in electrolyzed-reduced water. Ultrapure water was electrolyzed by an electrolyzing device and RP, pH, DO and DH values were immediately measured. The data of water before electrolysis was shown by dark squares. Arrows show the reduced water which exhibited the strong SOD-like activity.

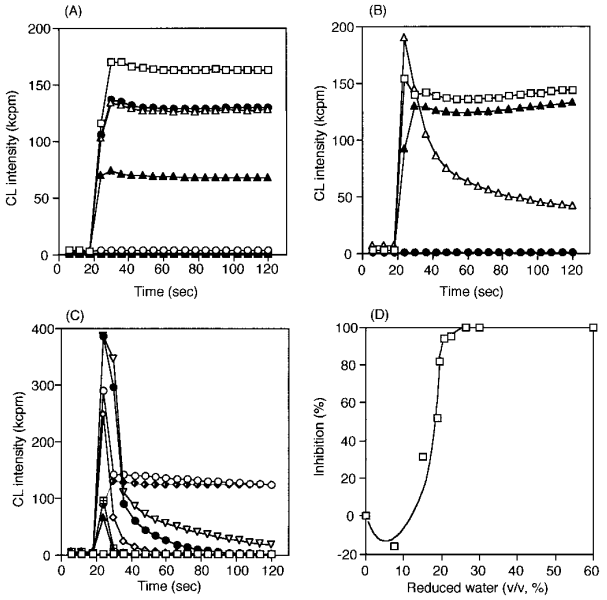

FIG. 2. Analysis of the SOD-like activity of reduced water. (A) Effects of reduced water, SOD, H_2-water and N_2-water on the accumulation of $O_2^{\cdot-}$ generated by the HX-XOD system. The CL intensity was determined in phosphate buffer (pH 7.0) in the presence of reduced water (RP −820 mV, pH 11.0; IC_{50}SO(18%)), a NaOH solution of the same pH as reduced water, SOD, H_2-water, or N_2-water. □, control NaOH solution; ■, reduced water; ▲, SOD (0.13 U/ml); ○, SOD (66.7 U/ml); ●, H_2-water; △, N_2-water. (B) Stability of the SOD-like activity of reduced water. □, control NaOH solution. ●, reduced water and reduced water treated with repeated freezing and melting, deflation with sonication, vigorous mixing for 10 minutes, boiling for 10 minutes, repeated filtration with 0.22μm filter, or closed autoclaving at 121°C for 20 minutes. ▲, reduced water treated with opened autoclaving at 121°C for 20 minutes. △, reduced water treated with closed autoclaving in the presence of tungsten trioxide (0.5 g/70ml reduced water) at 121°C for 20 minutes. (C) The SOD-like activity of diluted reduced water. Amount of reduced water (%): □, 60%; +, 30%; *, 26%; ▲, 23%; □, 21%; ◇, 20%; ●, 19%; ▽, 15%; ○, 7.5%; ◆, 0% (control). (D) Inhibition of the accumulation of $O_2^{\cdot-}$ by variously diluted reduced water. All experiments were triplicated and the average values were shown in the figures. The SD errors were within 5%.

be noticed that the DH value is higher in reduced water than in the original water by two orders of magnitude.

The SOD-like activity of reduced water. XOD oxidizes HX to xanthine, coupling to generation of $O_2^{\cdot-}$ from O_2. CLA specifically reacts with $O_2^{\cdot-}$ and $^1O_2$ and emits a CL (4). As shown in FIG. 2A, reduced water completely inhibited the CL, demonstrating the SOD-like activity of reduced water. Since the CL was completely inhibited by SOD, the CL was specific to $O_2^{\cdot-}$.

Bubbling of hydrogen gas into a NaOH solution (pH 10.5) for 5 minutes resulted in remarkable changes of RP from +116 mV to −842 mV; DO from 7.66 mg/l to 1.98 mg/l; DH from 0.0002 mg/l to 0.938 mg/l; but no change of pH. Bubbling of nitrogen gas into a NaOH solution (pH 10.5) resulted in changes of RP from +116 mV to +83 mV; DO from 7.66 mg/l to 1.82 mg/l; DH from 0.0002 mg/l to 0.0001 mg/l; but no change of pH. As shown in FIG. 2A, water bubbled with hydrogen gas (H_2-water) and nitrogen gas (N_2-water) showed decreased CL intensity by about 20% as compared to that of the control in the steady state, suggesting that the decreased CL intensity in N_2-water and H_2-water was due to low DO and H_2-water had no SOD-like activity. The SOD-like activity of reduced water is retained during storage, although the RP and DH values exhibit decay. These results clearly indicate that dissolved molecular hydrogen gas in reduced water was responsible for the negative RP value, but not for the SOD-like activity.

Stability of the SOD-like activity of reduced water. The SOD-like activity of reduced water was very stable in a closed glass bottle at 4°C for over a month. The activity was not lost even after neutralization, repeated freezing and melting, deflation with sonication for 10 minutes, repeated filtration with a 0.22 μm filter, boiling for 10 minutes, or autoclaving in a closed glass bottle at 121°C for 20 minutes (FIG. 2B). However, 90% the SOD-like activity was lost by autoclaving in an opened glass bottle, suggesting that the active substance in the reduced water is volatile. Atomic hydrogen is volatile and can reduce metallic oxide such as tungsten trioxide, though molecular hydrogen cannot easily do this (5). A sensitive detection method of atomic hydrogen is based on the color change of tungsten trioxide by reduction (6). The closed autoclaving of reduced water with tungsten trioxide at 121°C for 20 minutes resulted in the loss of 52% of the SOD-like activity (FIG. 2B). These results strongly suggest that the substance responsible for the SOD-like activity of reduced water is active atomic hydrogen. Active hydrogen in gas phase is rather stable taking into consideration of the collision rate (7). Since two atoms of hydrogen in the ground state have larger energy than molecular hydrogen in any stable state, the extra energy is required to be eliminated by a third substance to produce a hydrogen molecule from two atoms of hydrogen by collision (8). Atomic hydrogen can stably exist avoiding the attack by oxygen in solution and crystals at room temperature for a period longer than a year (9). Water vapor is known to prevent the recombination of atomic hydrogen on a tungsten surface (8). The characteristics in aqueous solution of active atomic hydrogen produced by electronic discharge is under investigation.

When XOD solution was injected into the reaction mixture containing the diluted reduced water, strong CL was emitted immediately after injection (FIG. 2C), but the intensity of CL rapidly dropped below the control value. The initial strong transient emission of CL in diluted reduced water could be inhibited by SOD, indicating that a large amount of $O_2^{\cdot-}$ was transiently generated just after the addition of XOD. AsA, (+)-

catechin and tannic acid, as well as SOD, did not show such a strong initial transient emission of CL (data not shown). Injection of the solvent without enzyme did not result in an emission of CL. More detailed experiments will be needed to clarify the mechanism of this phenomenon. The inhibitory effect of reduced water on the accumulation of $O_2^{\cdot -}$ was increased in a dose-dependent manner as shown in FIG. 2D, suggesting the stoichiometric action of active substance in reduced water.

Definition of the SOD-like activity of reduced water. Since this paper first reports the $O_2^{\cdot -}$ scavenging activity of electrolyzed-reduced water, the standardization of this activity is needed to compare the reducing potency of the reduced water prepared each time. In order to standardize the reducing potency of the reduced water, we defined a $IC_{50}SO$ unit as a reducing potency of which reduced water can scavenge 50% of $O_2^{\cdot -}$ generated by the HX-XOD system under the conditions described in the MATERIALS AND METHODS section and a $IC_{50}SO$ (%) as the concentration (%) of reduced water in which the 50% of $O_2^{\cdot -}$ generated by the HX-XOD system is scavenged. The $IC_{50}SO$ values of SOD, AsA, (+)-catechin, and tannic acid were 0.05 U/ml, 3 μM (0.53 μg/ml), 130 μM (38 μg/ml), and 33 μg/ml in the HX-XOD system used here.

Catalase-like activity of reduced water. Although reduced water could scavenge $O_2^{\cdot -}$, there was a possibility that reduced water inhibited the enzyme activity of XOD or inhibited the reaction between $O_2^{\cdot -}$ and the luciferin analog reagent. To eliminate this possibility, the production of H_2O_2 was determined in the HX-XOD system. XOD can produce not only $O_2^{\cdot -}$ but also H_2O_2 in this HX-XOD system. As shown in FIG. 3A, XOD produced H_2O_2 even in the presence of reduced water, demonstrating no inhibition of the enzyme activity by reduced water. As expected, the addition of SOD to the HX-XOD system resulted in the accumulation of H_2O_2, indicating that $O_2^{\cdot -}$ produced by XOD was changed to H_2O_2 by SOD. Reduced water first accumulated H_2O_2 and then gradually lowered the concentration of H_2O_2, suggesting that reduced water exhibits not only SOD-like activity but also catalase-like activity. The fact that reduced water stimulated the accumulation of H_2O_2 in the HX-XOD system in the first 5 minutes indicated that the decreased CL intensity in the presence of reduced water is not due to the inhibition of the reaction between $O_2^{\cdot -}$ and luciferin analog by reduced water, but due to the conversion of $O_2^{\cdot -}$ into H_2O_2 by reduced water. To demonstrate the catalase-like activity of reduced water, H_2O_2 was directly incubated with reduced water. As shown in FIG. 3B, reduced water scavenged H_2O_2 as well as AsA and catalase.

Suppressive effect of reduced water on the single-strand breakage of DNA caused by the Cu(II)-catalyzed oxidation of AsA. The DNA strand breakage is caused by the mixture of AsA and Cu(II) (10, 11). The Cu(II)-catalyzed oxidation of AsA is known to produce $O_2^{\cdot -}$ and H_2O_2 which react to produce $^{\cdot}OH$ (12). In order to demonstrate that reduced water can scavenge not only $O_2^{\cdot -}$ and H_2O_2 but also other active oxygen species, we examined the effect of reduced water on DNA breakage caused by the mixture of AsA and Cu(II). Super-coil plasmid DNA (Form I) changes to open-circular DNA (Form II) by a single-strand breakage. Open-circular DNA changes to linear DNA (Form III) by a double-strand DNA breakage. When plasmid DNA was incu-

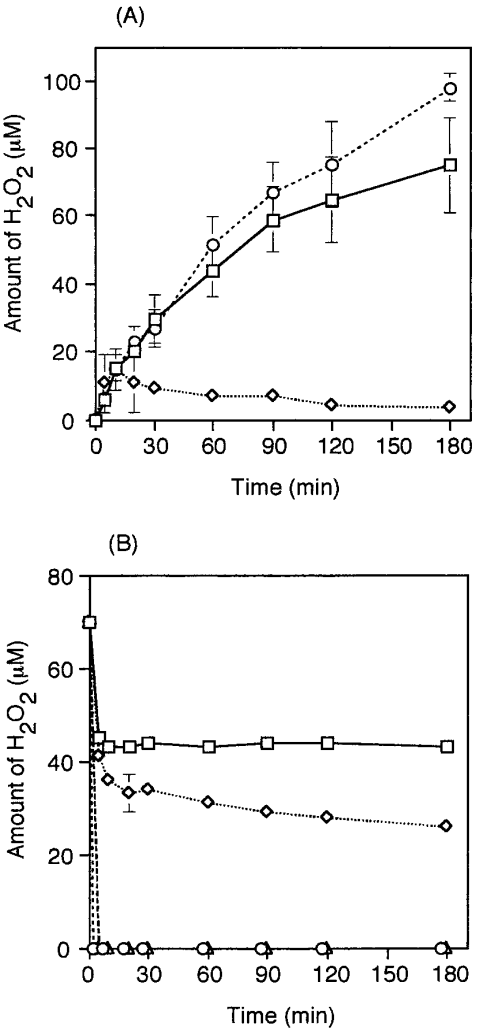

FIG. 3. The SOD-like and catalase-like activity of reduced water and AsA. (A) Change of the amount of H_2O_2 in the HX-XOD system in the presence of SOD or reduced water. SOD (70 U/ml) or reduced water (12 $IC_{50}SO$ units) were incubated in the HX-XOD system at 37 °C. □, control NaOH; ◇, reduced water; ○, SOD. (B) Degradation of H_2O_2 by reduced water, AsA and catalase. Reduced water (12 $IC_{50}SO$ units), AsA (100 μM) or catalase (20 U/ml) was incubated with H_2O_2 (70 μM) in 20 mM sodium phosphate buffer (pH 7.5) at 37 °C. □, control; ◇, reduced water; △, AsA; ○, catalase. All experiments were triplicated and the average values were shown. The standard errors were shown by vertical bars. In (B) most of the SD error bars are embedded in the symbols.

bated with the mixture of AsA and Cu(II), the amount of super-coil DNA gradually decreased (FIG. 4). However, reduced water significantly inhibited the single-strand breakage by the mixture of AsA and Cu(II). As shown in Table 1, reduced water inhibited the DNA breaking reaction in a dose-dependent manner. Catalase also inhibited the breaking reaction but SOD did not, indicating that H_2O_2 participated in the breaking reaction, but $O_2^{\cdot -}$ did not. Both radical scavengers specific to $^{\cdot}OH$ or $^1O_2$ inhibited the breaking reaction. These results indicated that reduced water can prevent DNA damage caused by active oxygen species such as H_2O_2, $^{\cdot}OH$, and $^1O_2$ produced by the Cu(II)-catalyzed oxidation of AsA. Significant synergistic effect between reduced water and the radical scavengers used here was not observed (data not shown). It is noteworthy that reduced water can prevent DNA damage even in the presence of metallic ions which catalyze the autooxidation of most established antioxidants and reverse their protective effect against active oxygen species. However, the water must have a higher reducing ability for this purpose than scavenging $O_2^{\cdot -}$.

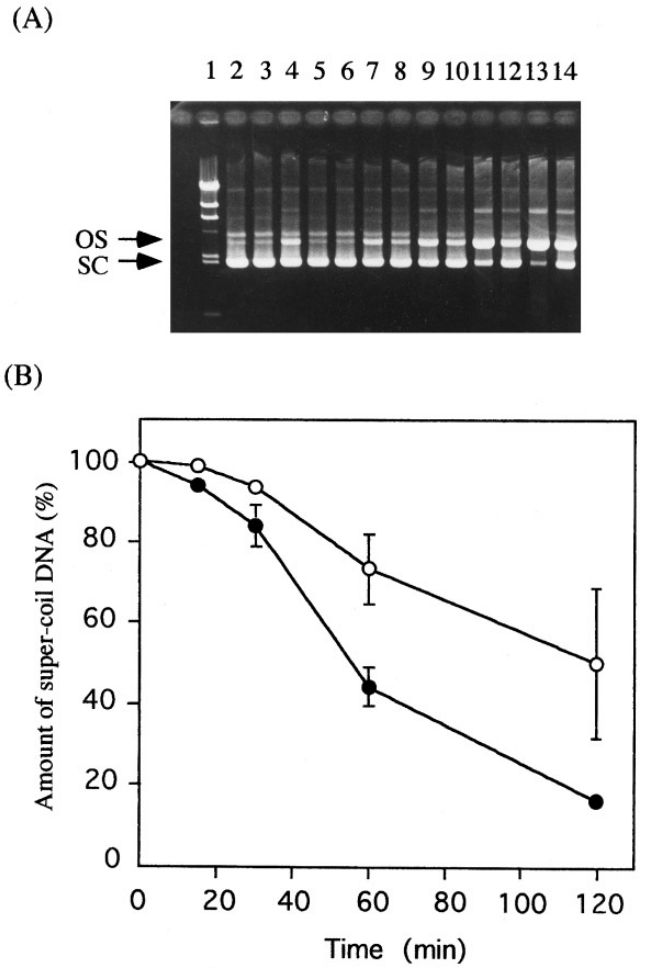

FIG. 4. Inhibitory effect of reduced water on single-strand breakage of DNA caused by oxygen radicals produced by the Cu(II)-catalyzed oxidation of AsA. (A) Electrophoresis of plasmid DNA treated with the mixture of AsA and Cu(II) in the presence of 4.1 $IC_{50}SO$ units of reduced water (RP, −659 mV; pH 10.5; $IC_{50}SO$(18%)) or NaOH solution (pH 10.5). SC, super-coil DNA. OC, open-circular DNA. Lane 1, marker (λ DNA-HindIII); lane 2, control DNA (2 hours); lane 3, DNA + Cu(II) (2 hours); lane 4, DNA + AsA (2 hours). Lanes 5, 7, 9, 11 and 13 show the DNA breaking reaction by the mixture of AsA and Cu(II) in the presence of NaOH solution for 0, 15, 30, 60 and 120 minutes, respectively. Lanes 6, 8, 10, 12 and 14 show the DNA breaking reaction by the mixture of AsA and Cu(II) in the presence of reduced water for 0, 15, 30, 60 and 120 minutes, respectively. (B) Decrease of the relative amount (%) of super-coil DNA during the incubation of plasmid DNA with the mixture of AsA and Cu(II) in the presence of reduced water (○) or NaOH solution (●). Average values of two independent experiments (n=2, each) were shown. The vertical bars show the SD errors.

TABLE 1

Effect of Reduced Water, SOD, Catalase, and Various Radical Scavengers on Single-Strand Breakage of DNA by the Cu(II)-Catalyzed Oxidation of AsA

Addition	Concn	Specificity	Inhibin, %
Reduced water	3.7 $IC_{50}SO$ units		19
	7.5 $IC_{50}SO$ units		38
	15 $IC_{50}SO$ units		49
SOD	150 U/ml	$O_2^{\cdot -}$	2
Catalase	0.8 U/ml	H_2O_2	6
	4 U/ml	H_2O_2	36
	20 U/ml	H_2O_2	90
AET[a]	4×10^{-5} M	gereral	44
KI	1×10^{-2} M	$^{\cdot}OH$	18
	5×10^{-2} M	$^{\cdot}OH$	53
NaN_3	1×10^{-2} M	$^1O_2$	14
	5×10^{-2} M	$^1O_2$	43

[a] AET = 2-(aminoethyl)isothiuroium.

Although aerobic organisms have evolved by acquisition of the ability to utilize oxygen, oxygen is principally a toxic substance. Recently biological activation of hydrogen by hydrogenases was reported (13). Hydrogenases, which are among in the oldest enzymes (3.8 billion years old), can reversibly split molecular hydrogen to produce active atomic hydrogen. Active atomic hydrogen may have participated in the redox regulation of cellular functions. Water can permeate everywhere in the body and penetrates every membrane including the blood-brain barrier. To neutralize the toxic action of active oxygen species, electrolyzed-reduced water may be an ideal and very powerful antioxidant. Further intensive investigation on the effect of reduced water on cell biology, immunology and oncology should be promoted.

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