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Enhanced Induction of Mitochondrial Damage and Apoptosis in Human Leukemia HL-60 Cells Due to Electrolyzed-Reduced Water and Glutathione

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Electrolzyed-reduced water (ERW) is a higher pH and lower oxidation-reduction potential water. In the present study, we examined the enhanced effect of ERW in the apoptosis of leukemia cells (HL-60) induced by glutathione (GSH). An enhanced inhibitory effect on the viability of the HL-60 cells was observed after treatment with a combination of ERW with various concentrations of GSH, whereas no cytotoxic effect in normal peripheral blood mononuclear cells was observed. The results of apoptotic related protein indicated that the induction of HL-60 cell death was caused by the induction of apoptosis through upregulation of Bax and downregulation of Bcl-2. The results of further investigation showed a diminution of intracellular GSH levels in ERW, and combination with GSH groups. These results suggest that ERW is an antioxidant, and that ERW, in combination with GSH, has an enhanced apoptosis-inducing effect on HL-60 cells, which might be mediated through the mitochondria-dependent pathway.

Key words: electrolyzed-reduced water (ERW); reactive oxygen species (ROS); HL-60 cells; apoptosis; Bcl-2

Reactive oxygen species (ROS), *viz.*, the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\bullet}OH$), might play an underlying role in the oxidative damage process that is closely related to many chronic and inflammatory diseases, such as cardiovascular diseases, cancer, cataract, and inflammation,[1,2] but antioxidants have been found to have some preventive and therapeutic effects on the above-mentioned diseases.[3]

Mitochondria is known as a primary source of intracellular ROS production, and ROS have implicated as second messengers that participate in physiological processes, *e.g.*, apoptosis and proliferation. Some studies have reported that proliferation induced by epidermal growth factor can be mediated through ROS production, and ROS have been found to induce proliferation in tumor progression.[4,5] Therefore, antioxidants might possess an effective inhibitory effect on the promotion of tumors by reducing ROS production.

Electrolyzed-reduced water (ERW) is characterized by higher pH, lower oxidation-reduction potential, lower dissolved oxygen, and higher dissolved hydrogen than any form of tap water or distilled water. In Taiwan, more and more people are using ERW as a fashionable source of drinking water, but only a few studies of the free radical scavenging activity of ERW have been reported. Shirahata *et al.* reported that ERW can scavenge ROS such as superoxide anions and H_2O_2[6] and the results of Kim's study suggest that ERW with ROS scavenging ability might have a potential effect in diabetic animals.[7] Huang *et al.* (2003) also reported that ERW with high reducing ability and/or direct ROS scavenging activity can be used in clinical studies of hemodialysis patients due to strong antioxidant activity.[8]

The tripeptide glutathione (GSH) is a low-molecular-weight thiol reductant present in most cells. It is assumed to play a pivotal role in numerous cell functions, such as protection of cells from toxic oxygen species and detoxification of various xenobiotics.[9–11] Aside from the role of GSH in defense mechanisms, programmed cell death and apoptosis is also closely correlated with this tripeptide.[12] Furthermore, some of the causes of apoptosis, such as increases in the generation of reactive oxygen species, can be accompanied by depletion of GSH.[9]

Since oxidative damage has been implicated in the etiology of cancer complications, ERW, a potent ROS scavenger, might have a therapeutic role in cancer mellitus. There have been very few studies of the effects of ERW on the formation of cancer cells and growth patterns, although limited evidence suggests that the use of electrolyzed hydrogen-saturated water as a source of drinking water in the feeding test on rats elicited an antioxidative effect.[13] The goal of the present study was to evaluate growth inhibition of HL-60 cells by the use of ERW, and to elucidate the molecular mechanism of ERW in enhancing the effects of GSH-induced apoptosis in HL-60 cells.

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Abbreviations: ERW, electrolyzed-reduced water; GSH, glutathione; H_2O_2, hydrogen peroxide; PBMCs, peripheral blood mononuclear cells; ROS, reactive oxygen species; SOD, superoxidase dismutase

Materials and Methods

Apparatus producing ERW. The apparatus, Antioxidant Water System Health Control (H. C.-1400), for producing the ERW is supplied commercially (Health Control, Taichung, Taiwan). The apparatus consists of two parts; one is used for water purification, while the other is used for the electrolysis of water. The equipment for the electrolysis of water can control the pH regulator from pH 8.10 to 9.50, ORP values from −160 mV to −400 mV, and the water flow rate at 2.40 liter per min. When the switch is turned on, tap water is purified and then electrolyzed through the system to produce the ERW needed. ERW was collected and utilized in this experiment.

Characterization of ERW. Characterization of ERW was conducted by various apparatuses: (i) a pH meter (Mettler Toledo MP220, Zurich, Switzerland); (ii) an ORP meter (Mettler Toledo MP220, Zurich, Switzerland); (iii) a nuclear magnetic resonance (NMR) Spectrometer (Bruker, DMX-600, Rheinstetten, Germany); (iv) an ion chromatograph (Dionex DX-120, Boston, MA).

Scavenging activity of hydrogen peroxide by ERW. Reduction of hydrogen peroxide was determined according to Yoshiki *et al.*[14] with some modifications. For scavenging of hydrogen peroxide, a reaction mixture consisting of H_2O_2, ERW, and acetaldehyde was added to phosphate-buffered saline (PBS) in the stainless steel container of a chemiluminescence analyzing system (CLA 2100, Tokoyo Electronic Industries, Tokoyo, Japan), and measurement of chemiluminescence intensity was recorded accordingly. The total amount of chemiluminescence intensity was calculated on the integration of the area under the curve and subtracted from the background level. Gallic acid was used as the reference compound.

Scavenging activity of superoxide anions by ERW. Superoxide radicals were generated by the xanthine-xanthine oxidase system, as previously described[15,16] with some modifications. In brief, xanthine oxidase (EC 1.1.3.22; grade I, from buttermilk, 0.25 U; one unit converts 1 μmol of xanthine to uric acid per min at pH 7.5 at 25 °C), lucigenin, xanthine, and various volumes of ERW were added to PBS, pH 7.4, in the stainless steel container of the chemiluminescence analysis system, and measurement of chemiluminescence intensity was started. Superoxidase dismutase (SOD) was used as the reference compound.

Scavenging activity of hydroxyl radicals by ERW. Hydroxyl radicals were generated by the addition of ferrous iron to the buffer solution.[17] Freshly prepared $FeSO_4$ (in 0.9% NaCl), luminol, and various volumes of ERW were added to PBS, pH 7.4, in the stainless steel container, and measurement of chemiluminescence intensity was started. The total chemiluminescence intensity was calculated on the integrating area under the curve and subtracted from the background level. Quercetin dehydrate was used as the reference compound.

Cell culture. Human promyelocytic leukemia HL-60 cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 with RPMI 1640 medium supplemented with 10% heat-inactive fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 mg/ml). In order to investigate the effects of ERW on GSH-induced cytotoxicity in HL-60 cells, a medium was prepared using ERW instead of ultra-pure water, with the pH of the medium adjusted to neutral. Unless otherwise specified, the HL-60 cells were seeded at a density of 1×10^5 cells/ml in RPMI 1640 medium with and without ERW and treated with GSH for the periods indicated. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by Ficoll-Hypaque density gradient centrifugation. The PBMCs were cultivated in RPMI 1640 medium containing GSH.

Measurement of intracellular ROS. Intracellular ROS levels were measured using a fluorescent probe, H_2DCF-DA. Briefly, HL-60 cells were incubated with TPA (20 nM) for 30 min and treated with various concentrations of GSH with or without ERW for the indicated period. Then, HL-60 cells were washed twice with PBS to remove the extracellular compounds and treated with H_2DCF-DA (20 μM) for 30 min. The fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission of 530 nm using a fluorescent Mutilabel Counter (Perkin Elmer, Boston, MA). The relative amounts of intracellular hydrogen peroxide production by GSH with and without ERW was expressed as the fluorescence ratio of treatment to control.

Cell viability and cytotoxicity. The viability of cells was determined by the trypan blue dye exclusion method, and cytotoxicity was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) reduction assay. Exponentially growing cells (1×10^5) were seeded onto plates and incubated in a 10% FBS/RPMI 1640 medium containing ERW. During the incubation period, from 24 h to 72 h at 37 °C, the HL-60 cells and PBMCs were exposed to growth treatment by a series of concentrations of GSH. The HL-60 cells and PBMCs exposed to 0.2% trypan blue were counted in a Hemocytometer. MTT solution was added to each well (1.2 mg/ml) and the plates incubated for 4 h at 37 °C. The resulting MTT-formazan product was dissolved by the addition of SDS solutions. Absorbance was measured at 595 nm with an ELISA plate reader.

DNA fragmentation. The HL-60 cells and PBMCs (2×10^5 cells/ml) were treated with various concentrations of GSH for the indicated periods. The cells were collected, washed with PBS, and then lysed with digestion buffer containing 0.5% sarkosyl, 0.5 mg/ml proteinase K, 50 mM Tris (hydroxymethyl) aminomethane (pH 8.0), and 10 mM EDTA at 50 °C for 6 h and treated with RNase A (0.5 ug/ml) for another 3 h at 50 °C. DNA was extracted with phenol/chloroform/isoamyl alcohol (25/24/1) before loading and analyzed by 2% agarose gel electrophoresis. The agarose gels were applied to 50 V for 120 min in TBE (Tris-borate/EDTA electrophoresis buffer). Approximately 20 μg of DNA was loaded in each well, and visualized under UV light and separately photographed.

Western blot analysis. The HL-60 cells (2×10^5 cells/ml) were treated with various concentrations of GSH for the indicated periods. Cells were harvested and homogenized in a gold lysis buffer (20 mM Tris–HCl, pH 8.0, 5 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanodate, 10 mM NaF, 1 mM sodium pyrophosphate, 137 mM NaCl, and 100 mM β-glycerophosphate) for 30 min on ice. The cell debris was collected by centrifugation at 12,000 rpm at 4 °C for 30 min. The supernatant proteins were measured by Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). Fifty μg of total cellular proteins was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), transferred to polyvinylidene difluoride membranes (Amersham, Arlington, IL), and then probed with primary antibody, followed by adding horseradish peroxidase-labeled secondary antibody. The immunocomplexes were visualized by the ECL system (Amersham Life Science, Amersham, UK).

Data analysis. All of the experiments mentioned were repeated at least 3 times and the means ± SD were calculated. Comparisons between groups were performed using one-way analysis of variance (ANOVA), followed by Dunnet's *t*-test. Statistically significant differences between groups were defined as $p < 0.05$.

Results

Characteristics of ERW

The characteristics of tap water, distilled-deionized (DD) water, ERW, and electrolyzed-oxidized water (EOW) are shown as Table 1. The ORP and pH values (the two most important properties of electrolyzed water) of the parameters obtained were 690 mV and 5.89 for EOW and −344 mV and 9.0 for ERW. Table 1 also presents the results of ^{17}O NMR relaxation measurements of electrolyzed water of various sources that were considered different. The means of full widths at half amplitude of all fresh ERW specimens were

Table 1. Characteristics of Tap Water, DD Water, ERW, and EOW

	Tap water	DD water	ERW	EOW
pH	7.37	7.39	9.0	5.89
ORP (mV)	579	385	−344	690
Full width at half amplitude of NMR Absorption signal of ^{17}O (Hz)	103.2	92.73	45.61	44.51
Na^+ (mg/l)	6.45	ND*	7.91	4.68
NH_4^+ (mg/l)	ND	ND	ND	ND
K^+ (mg/l)	1.71	ND	2.25	1.02
Mg^{2+} (mg/l)	11.2	ND	11.9	8.40
Cl^- (mg/l)	6.80	ND	4.00	10.2
NO_3^- (mg/l)	7.60	ND	4.80	13.6
SO_4^{2-} (mg/l)	99.4	ND	69.9	142

The above properties were determined in this study. The values for ERW and ROW, obtained with an electrolytic water, varied as the electric field strength varied. *ND, no detect.
DD water, double distilled-deionized water
ERW, electrolyzed-reduced water
EOW, electrolyzed-oxidized water

45.61 Hz, as compared with 103.2 Hz for tap water and 92.73 Hz for DD water. Some of additional water quality items determined for ERW were as follows: (i) Na^+, 7.91 mg/l; (ii) NH_4^+, <0.05 mg/l; (iii) K^+, 2.25 mg/l; (iv) Mg^{2+}, 11.9 mg/l; (v) Cl^-, 4.00 mg/l (vi) NO_3^-, 4.80 mg/l, and (vii) SO_4^{2-}, 69.9 mg/l.

Scavenging activity of ROS by ERW

ERW proved to be an effective scavenger of H_2O_2, superoxide anion radicals and hydroxyl radicals with IC_{50} values of 267.6 µl, 254.0 µl, and 173.0 µl respectively (Fig. 1A). Gallic acid, SOD, and quercetin dehydrate were used as positive controls of scavengers for ROS, and they scavenged 33.8–78.4%, 13.1–90.0%, and 49.0–85.5% of the H_2O_2, superoxide anion radicals, and hydroxyl radicals respectively (Fig. 1B). The tap water, distilled water, and sodium hydroxide solution at the same pH value as the ERW showed no significant scavenging activity in the chemiluminescence system of

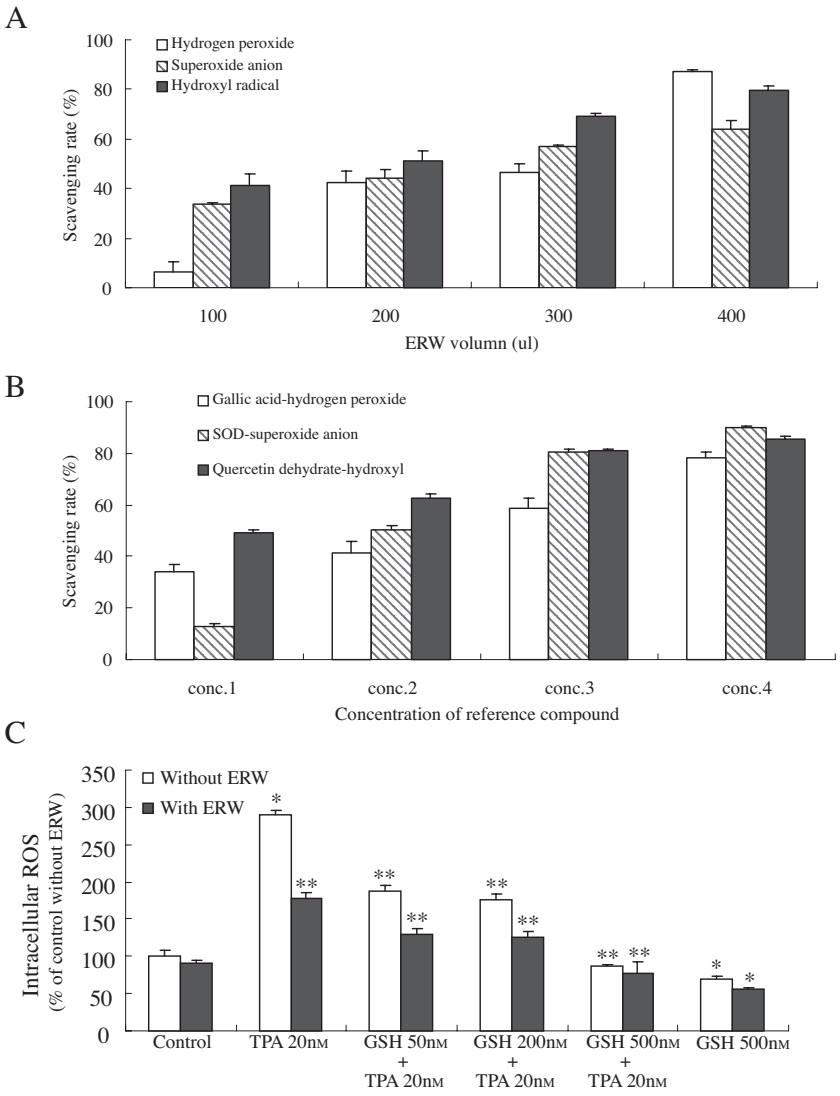

Fig. 1. Reactive Oxygen Species Scavenging Activities of ERW and Some Reference Compounds of Antioxidants.
A, Scavenging rate of reactive oxygen species by ERW (pH = 9.0, ORP = −344 mV) in chemiluminescence system. B, Scavenging rate of reactive oxygen species by reference compounds of antioxidants in chemiluminescence system. The concentrations of H_2O_2 scavenging activities by gallic acid were 25, 50, 100, and 500 µM. The concentrations of superoxide anion scavenging activities by SOD were 0.1, 1, 5, and 10 units. The concentration of hydroxyl radical scavenging activities by quercetin dehydrate were 5, 10, 50, and 100 µM. C, HL-60 cells were treated at increasing GSH concentrations with and without ERW. TPA treatment induced ROS production in HL-60 cells, and ROS production was measured using DCFH-DA, as described in "Materials and Methods." (*$p < 0.05$ compared with control group; **$p < 0.05$ compared with TPA group)

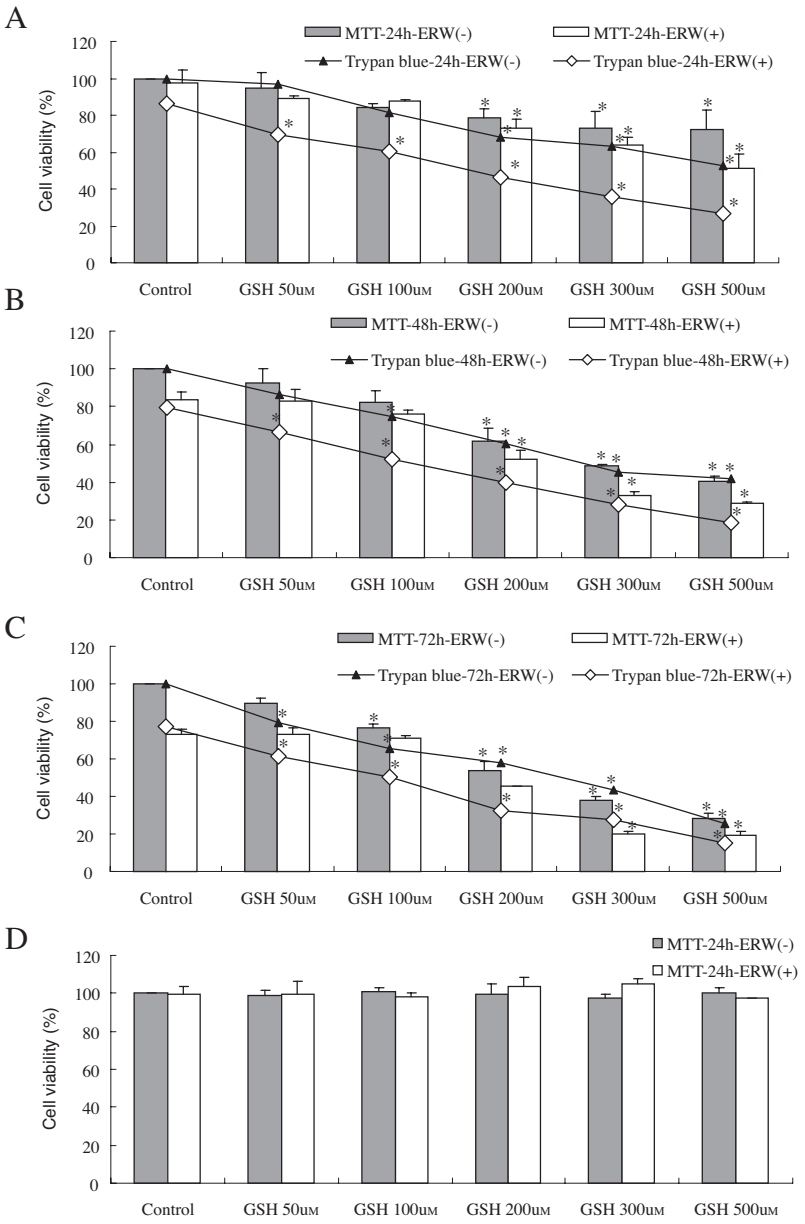

Fig. 2. Effects of GSH with and without ERW on the Cell Viability of HL-60 Cells.
Cell viability was determined by the trypan blue dye exclusion method (line) and MTT assay (bar) exclusion at the times indicated. Cells were treated for 24 h (A), 48 h (B), and 72 h (C). D, Effects of GSH with and without ERW on cell viability at the 24 h of PBMCs by MTT assay. ($^*p < 0.05$ compared with control group)

this study (data not shown). We further evaluated the effects of GSH with and without ERW treatment on the production of intracellular of ROS by the DCFH-DA staining technique. Figure 1C shows the intracellular ROS percentages of control without ERW in HL-60 cells after treatment with 20 nM TPA. The HL-60 cells treated with 500 μM GSH alone and ERW with decreased in DCF fluorescence intensity as compared with the control group ($p < 0.05$). Significantly, TPA treatment caused approximately 3-fold increases in DCF fluorescence intensity as compared to the normal controls ($p < 0.05$), indicating that TPA treatment was more effective in inducing intracellular ROS of the HL-60 cells. There were a significant decreased ($p < 0.05$) in intracellular ROS in the GSH-treated groups with and without ERW at doses of 50, 200, and 500 μM compared to the TPA treated group. Intracellular ROS levels in the GSH treated group with ERW (at doses of 0, 50, and 200 μM, respectively) were significantly lower than in the GSH treated group without ERW ($p < 0.05$). Based on these results, we confirmed that the GSH treated group with ERW very effectively inhibited TPA-induced ROS generation.

ERW decreased the survival of HL-60 cells

To test the effect of ERW on the viability in HL-60 cells, the cells were treated with different concentrations of GSH with and without ERW for 24 h to 72 h and with GSH in a concentration range of 50 μM to 500 μM. As Fig. 2 indicates GSH with and without ERW exhibited dose-dependent and time-dependent cytotoxicity in the HL-60 cells, and ERW significantly decreased the survival rate of the HL-60 cells at 72 h ($p < 0.05$). These results indicate that ERW increased the cytotoxicity of GSH in HL-60 cells. The IC_{50} of GSH with and without ERW at 48 h were 141 mM and 348 mM by the

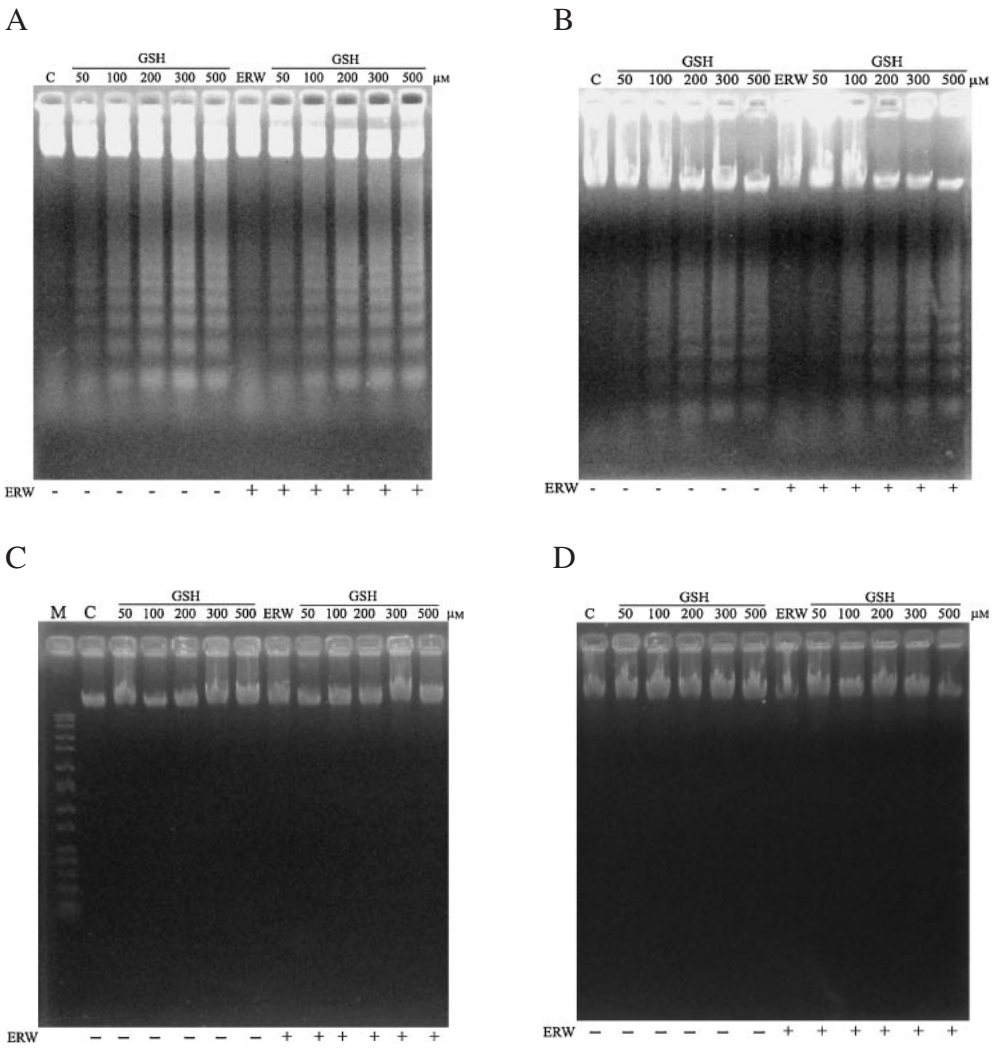

Fig. 3. Induction of DNA Fragmentation by GSH Treated of HL-60 Cells and PBMCs in RPMI 1640 Medium with and without ERW. Internucleosomal DNA fragmentation was analyzed by agarose electrophoresis. M, 100 base pair DNA ladder size. HL-60 cells in RPMI 1640 medium with and without ERW were treated at various concentrations of GSH, as indicated for 3 h (A), and 6 h (B). PBMCs in RPMI 1640 medium with and without ERW were treated at various concentrations of GSH as indicated for 3 h (C) and 6 h (D).

trypan blue dye exclusion method, respectively ($p < 0.05$, Fig. 2B). Using the same conditions for determination by incubating PBMCs at different concentrations of GSH with and without ERW for 24 h, the results showed that GSH with and without ERW had no effect of PBMCs on cell viability (Fig. 2D).

DNA fragmentation of HL-60 cells

The effects of GSH with and without ERW treatment on DNA fragmentation, a hallmark of apoptosis, were further examined. After GSH treatment with different concentrations for 3 h and 6 h, DNA fragmentation was observed (Fig. 3A and B). DNA fragmentation appeared at 50 μM of GSH with and without ERW treatment at 3 h, and the DNA ladder response was dose-dependent from 50 to 500 μM of GSH with and without ERW treatment. We also found the DNA ladder in the ERW group at 6 h (Fig. 3B). Using the same conditions for determination and incubating PBMCs at different concentrations of GSH with and without ERW for 3 h and 6 h, as the results shown in Fig. 3C and D indicate, GSH with or without ERW had no effect on DNA fragmentation.

Enhanced effects of combining ERW and GSH on Bcl-2, Bax, and Caspase-3 expression

To analyze further the possible mechanism underlying the enhanced effect of combining ERW and GSH induced apoptosis, we examined the expression of pro-apoptotic proteins Bax, anti-apoptotic proteins Bcl-2, and Caspase-3 (proform) at various concentrations of GSH with and without ERW treatment after 24 h of incubation. After being normalized and verified with GAPDH, expression of Bax increased remarkably in a dose-dependent manner, especially in the GSH and ERW combination group. Moreover, there was an obvious decrease in the Bcl-2 protein level in the combination group as compared to the control group (Fig. 4A). The expression of pro-caspase-3 was significantly decreased after treatment in the GSH and ERW combination group and ERW-alone group as compared with the control group (Fig. 4C). Quantitative Bcl-2 and Bax expression after being standardized to GAPDH ($n = 2$) is shown in Fig. 4B. Expression of Bcl-2 decreased significantly after treatment in the GSH and ERW combination group, whereas the Bax protein level increased significantly after GSH and ERW combination

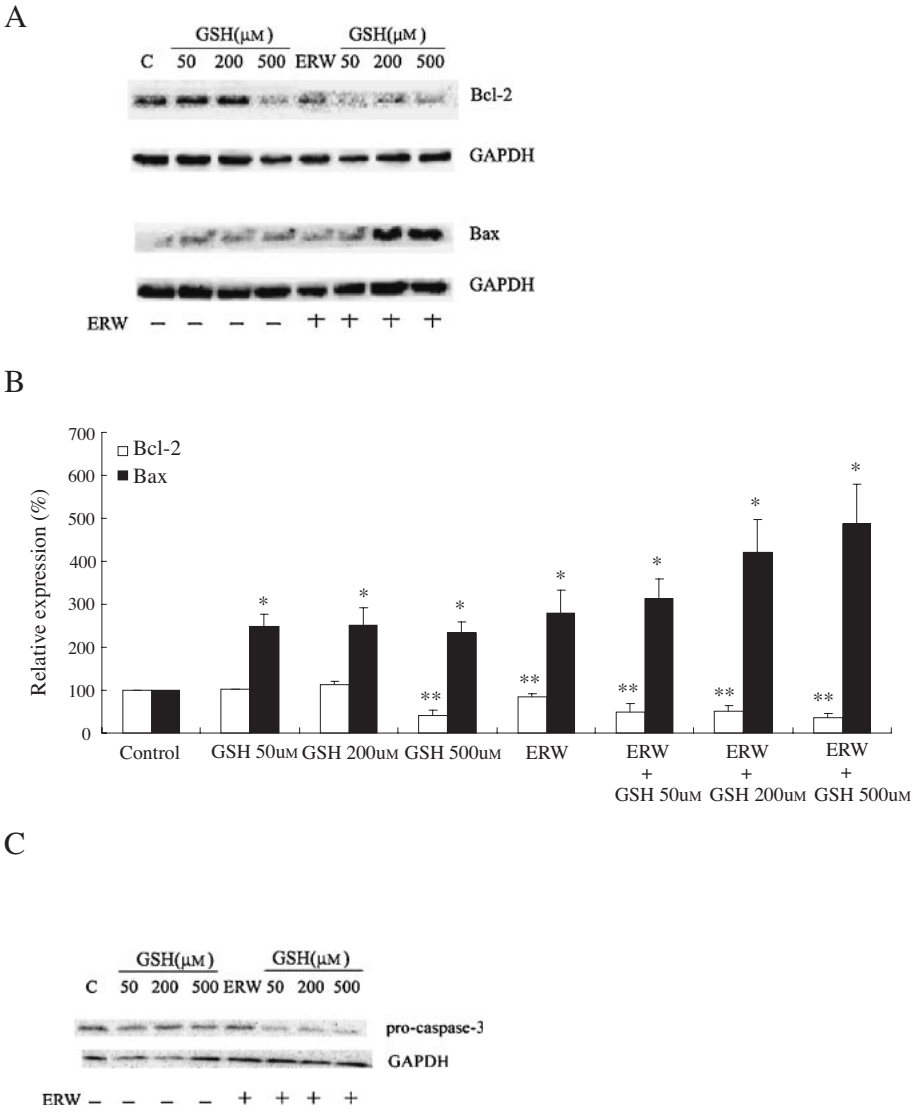

Fig. 4. Expression of Bcl-2, Bax, and Pro-Caspase-3 in HL-60 Cells in RPMI 1640 Medium with and without ERW Treated at Different Concentrations of GSH for 24 h.

Western blot analysis to determine the protein levels of Bcl-2, Bax (A), and peo-caspase-3 (C). GAPDH was used as the protein loading control. The protein levels of Bcl-2, and Bax (B) were quantitatively expressed after being standardized to GADPH (n = 2 at each concentration point). (*$p < 0.05$ as compared with Bax control group; **$p < 0.05$ compared with Bcl-2 control group)

treatment induction, statistically ($p < 0.05$ as compared with the control group). ERW treatment not only increased Bax protein expression and decreased Bcl-2 and pro-caspase-3 protein expression, but also enhanced GSH-induced Bax up-regulation, and Bcl-2 down-regulation, and reduced pro-caspase-3 expression.

Discussion

Several lines of evidence indicate that ERW had the ability of a scavenger free radical, such as H_2O_2, the hydroxyl radical, and the superoxide radical, to protect DNA, RNA, proteins, cells, and tissues against strong oxidative stress.[6,18] As shown in Fig. 1A and B, ERW inhibited chemiluminiescence intensity, indicating the scavenging activity of hydrogen peroxide, the superoxide anion, and the hydroxyl radical. The chemiluminiescence measurement was specific to hydrogen peroxide, the superoxide anion, and the hydroxyl radical, which were determined according to the method of Shirahata *et al.*, with some modifications. The scavenging activity of ERW as to hydrogen peroxide, the superoxide anion, and the hydroxyl radical increased in a dose-dependent manner, as shown in Fig. 1A, and as to the various concentrations of antioxidant. The addition of gallic acid, SOD, and quercetin decreased the levels of hydrogen peroxide, the superoxide anion, and the hydroxyl radical respectively, as judged by chemiluminiescence. Hence we consider the effect of ERW to have been due to the antioxidative activity of scavenging ROS, and conclude that this antioxidative activity of ERW was like that gallic acid, SOD, and quercetin. Kim *et al.*[19] suggested that the possible anti-diabetic effect of ERW in the genetically diabetic mouse strain C57BL/6J-*db*/*db* (*db*/*db*) and ERW with ROS scavenging ability might reduce the blood glucose concentration, increase the blood insulin level, and preserve the β-cell mass in *db*/*db* mice.[20] A clinical study reported that ERW treatment was effective in palliating chronic hemodialysis-evoked oxidative stress in chronic hemodialysis patients.[21] In addition to high pH and extremely negative redox potential values, ERW

also exhibits smaller full widths at half amplitude than tap water or DD water (Table 1). In general, there is a direct correlation between the value of the full width at half amplitude and the size of the water-molecule clusters. In other words, the size of the water molecule cluster of each fresh ERW specimen obtained in this work was smaller than that of tap water or DD water. A smaller water molecule cluster gives rise to greater penetration ability.[22] Although ERW has been applied to animal models and in clinical research, the mechanism of the bioactivity of ERW in cell culture has not been clearly determined. Hence we considered that ERW should be useful in the prevention of various cancer diseases induced by oxidative stress. The findings of the present study indicate that ERW and GSH possessed growth inhibition on HL-60 cells in a dose-dependent, time-dependent manner. The cell viability of the GSH and ERW combination group was significantly lower than that of the group treated with GSH alone at various concentrations. On the other hand, we also examined the cell viability of HL-60 cells using water containing sodium hydroxide at the same pH value as ERW at 24 h. Treatment with sodium hydroxide caused a marked decrease in viability at 36%, which is to be attributed to its pH value. The ERW group and the ERW with adjusted pH group, we did not observe significant differences in cell viability. We also observed that the pH value of ERW returned to the pH value of the RPMI medium after treatment with ERW with incubation for 30 min. Hence these results suggest that ERW alone can attenuate the cell viability of HL-60 cause by active atomic hydrogen or negative redox potential values as opposed to the pH value. Treatment with ERW enhanced the cell viability of GSH-induced apoptosis in HL-60 cells, perhaps due to a reaction of ERW with the GSH, possibly by increasing the ratio of thiolate anions of GSH or a change in its reactivity, but the other possible mechanisms should be further investigated.

Lower cytotoxicity in normal cells as compared to cancer cells is a prerequisite for any chemopreventive agent. Sakamoto *et al.* showed that diallyl trisulfide effectively inhibited cell proliferation and induced apoptosis in human lung cancer A549 cells, but not in non-neoplastic lung MRC-5 cells.[23] Dirsch *et al.* also reported that ajoene induced apoptosis in human acute myeloid leukemia cell line HL-60 as well as in PBMCs isolated from a chronic leukemia patient, but quiescent and proliferating PBMCs isolated from healthy donors remained unaffected.[24] In this study, we used normal cells of PBMCs isolated from healthy donors as compared to HL-60 cells to determine cytotoxicity effects and DNA fragmentation. Our results suggest that treatment of GSH and ERW combination significantly decreased the survival rate of HL-60 cells and induced DNA fragmentation of HL-60 cells (Fig. 2 and Fig. 3A, B). DNA fragmentation is one of major characteristics of apoptosis. However, ERW and GSH had no effect on cell viability (data not show) or DNA fragmentation of PBMCs (Fig. 3C, D). On the other hand, ERW and GSH protected normal cells in PBMCs, but inhibited cancer cells in HL-60 cells growth and the induction of DNA fragmentation.

Other reports indicated that decreased Bcl-2 levels are associated with concomitant decreases in the intracellular GSH content.[25,26] Bcl-2 is an anti-apoptotic protein, and over-expression has been linked to cancer development, metastatic growth, and chemotherapy resistance.[27,28] Expression of pro-apoptotic protein Bax is often reduced in cancer cells, and Bax is translocated to the mitochondria after induction of apoptosis.[29] Apoptosis is associated with activation of a number of caspases, and caspase-3 is considered essential to propagation of the apoptotic signal by several types of anticancer drugs. Our present studies indicate that the expression of Bax increased remarkably, in a dose-dependent manner, especially in the combination group. Furthermore, a significant decrease in the ratio of Bcl-2/Bax was observed when ERW was treated in combination with GSH, which correlated with the incidence of apoptosis. Our results suggest that HL-60 cells incubated with GSH in RPMI 1640 medium with ERW exhibited intracellular GSH depletion, Bax translocation, Bcl-2 down-regulation, and pro-caspase-3 inactivation (Fig. 4). The present findings indicate that combining ERW and GSH induced apoptosis is associated with Bcl-2 down-regulation, Bax translocation, and pro-caspase-3 inactivation, suggesting that apoptosis due to this combination occurs through a mitochondria-dependent pathway.

In conclusion, the results of this study indicate that ERW possesses potent antioxidant activity, and that ERW with GSH induced HL-60 apoptotic cell death. Our results indicate that induction of apoptosis in HL-60 cells combining ERW and GSH is due to Bcl-2 down-regulation and Bax translocation. Hence we suggest that active atomic hydrogen and negative redox potential values participated in the redox regulation of cellular functions and the apoptosis process. Further studies are necessary in order to assess roles and to elucidate the action mechanism.

Acknowledgments

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