行政院國家科學委員會專題研究計畫 成果報告

電解還原水對糖尿病控制作用評估
研究成果報告(精簡版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 95-2320-B-040-032-
執 行 期 間 ：95 年 08 月 01 日至 96 年 07 月 31 日
執 行 單 位 ：中山醫學大學應用化學系

計 畫 主 持 人：呂鋒洲
共 同 主 持 人：劉興華、巖正傑
計畫參與人員：博士班研究生-兼任助理：許又文
碩士班研究生-兼任助理：孔令詔

處 理 方 式 ：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 96 年 10 月 31 日

行政院國家科學委員會補助專題研究計畫 ■成果報告 □期中進度報告

電解還原水對糖尿病控制作用評估

計畫類別：■ 個別型計畫　　□ 整合型計畫
計畫編號：NSC　95-2320-B-040 -032
執行期間：　95　年　8　月　1　日至　96　年　7　月　31　日

計畫主持人：呂鋒洲
共同主持人：劉興華、巖正傑
計畫參與人員：　許又文、孔令詔兼任助理

成果報告類型(依經費核定清單規定繳交)：□精簡報告　□完整報告

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：中山醫學大學

中　華　民　國　96　年　10　月　25　日

Abstract

Electrolyzed-reducing water (ERW) exhibits high pH, low dissolved oxygen, extremely high dissolved active hydrogen, and negative oxidation-reduction potention (ORP) values. In this study, we proved that ERW scavenged hydrogen peroxide and hydroxyl radical in cheminescence analyzing system and also scavenged superoxide anion in the xanthine-xanthine oxidase system. Pancreatic β-cell death induced by oxidative stress plays an important role in diabetes mellitus. Alloxan produces reactive oxygen species (ROS) in a cyclic reaction between this substance and its reduction product, dialuric acid. The autoxidation of dialuric acid were produces superoxide radicals and hydrogen peroxide, then finally hydroxyl radicals. We also proved that ERW could scavenge intracellular ROS which protect human pancreatic β-cell lines from alloxan-induced cells damage. Alloxan-treated RINm5F and HIT-T15 cells revealed lower viability and high ROS level, but ERW can decrease ROS and prevented pancreatic β-cell lines to death. Moreover, GSH can reduce the content of ROS, whether the high or low concentration GSH, and the ERW can enhance the ability of GSH to decrease the intracellular ROS.

Keyword: Electrolyzed-reducing water, reactive oxygen species, alloxan, pancreatic beta cells

Introduction

Reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) play an important role in oxidative damage related to the chronic diseases and inflammatory diseases, such as cardiovascular diseases, cancer, cataract, and inflammation [1-4], and antioxidants have been found to have some preventive and therapeutic effects on these diseases [5]. Chemiluminescence has become a very widely used method for the determination of superoxide anion, hydrogen peroxide, and hydroxyl radical. There are many free radicals reactions that are known to produce light. A typical reaction would be: $A + B \rightarrow C^* \xrightarrow{D} DC^* \rightarrow DC + Light$ where (*) indicates an excited state [6]. For determination of superoxide anion, the xanthine/xanthine oxidase dependent chemiluminescence was enhanced by lucigenin to create a sensitive, specific, and rapid chemiluminescent method for determination of superoxide dismutase (SOD) activity [7]. Okubo et al. proposed that the reactive oxygen species-hydrogen donor-mediator system was a new chemiluminescence system for the measurement of scavenging activity of reactive oxygen species [8-10]. Ferrous iron induced luminol chemiluminescence has been used for monitoring hydroxyl radical [11].

Electrolyzed-reduced water (ERW) has a higher pH, lower oxidation-reduction potential (ORP), lower dissolved oxygen (DO), and higher dissolved hydrogen (DH) than tap water or distilled water. In Taiwan, more and more people are using electrolyzed-reduced water as drinking water, but a few studies concerning free radicals scavenging activity of electrolyzed-reduced water have been reported. In 1997, Shirahata et al. reported that electrolyzed-reduced water could scavenge active oxygen species [12]. But the results of Hanaoka's study suggest the electrolyzed-reduced water increased the activities of some antioxidants such as L-ascorbic acid, d-catechin and quercetin dehydrate, however it does not show superoxide dismutation activity itself [13]. In order to further understand the properties of antioxidant action of electrolyzed- reduced water, we applied ultra-weak chemiluminscence analyzer to monitor free radical scavenging effects of electrolyzed-reduced water.

Diabetes is grouped into type 1 diabetes and type 2 diabetes. Type 1 diabetes, insulin-dependent diabetes mellitus, is caused by a deficiency in insulin secretion from pancreatic β cells. Type 2 diabetes, insulin-independent diabetes mellitus, is related to damage in the insulin signaling pathway. Alloxan, a cyclic urea derivate of pyridimdine, which was reduced to dialuric acid by biological reducing agent. It has been widely used for the induction of type 1 diabetes [14]. Alloxan induced pancreatic β cell damage which mediated by generation of cytotoxic reactive oxygen species (ROS) [15]. Therefore, the aim of the present work was to investigate the influence of ERW on antioxidant action in alloxan-treated pancreatic β cells.

Materials and methods

Chemicals

Xanthine, xanthine oxidase (EC 1.1.3.22; grade I, from buttermilk), lucigenin (bis-N-methylacridinium), L (+)-ascorbic acid, superoxide dismutase (EC 1.15.1.1, from bovine erythrocytes), luminol sodium, gallic acid, ferrous sulfate heptahydrate, Proteinase K, RNase A, catalase and 3-(4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2) (30%) and acetaldehyde (MeCHO) were from Aldrich. Dimethyl sulfoxide (DMSO) was purchased from Merck (Schuchardt, Germany).

Apparatus of the electrolyzed-reduced water

Apparatus (Antioxidant Water System ET-3) for producing the electrolyzed-reduced water was supplied commercially. The apparatus consisted of two parts. One was used for the purification of water while the other was used for the electrolysis of water. The equipment for the electrolysis of water would control the pH regulator from 8.10 to 9.50, ORP values from –160mV to –400mV, water flow rate, alkalinity, acidity, and purity of water. The entire apparatus was connected to a water tap. After opening the switch, the tap water was first purified and then electrolyzed to produce

both the reduced water and oxidized water. The electrolyzed-reduced water was collected and used in this experiment.

Scavenging activity of hydrogen peroxide (H_2O_2) by electrolyzed-reduced water
Reduction of hydrogen peroxide was determined according to Okubo [10] with some modifications. For scavenging of hydrogen peroxide, reaction mixture (total volume of 2000µl) consisting of 100 µl of 3.75% H_2O_2 and 500µl of 10% MeCHO were added to distilled water in stainless steel cell in chemiluminescence analyzing system (CLA 2100, Tokoyo Electronic Indust. Co., Ltd., Japan) and measurement of chemiluminescence intensity was started. At the 60-second mark, various volumes of electrolyzed-reduced water were injected into the cell and the chemiluminescence intensity was measured continuously for a further 180 seconds. The total amount of chemiluminescence intensity was calculated by integrating the area under the curve and subtracting the background level. Gallic acid was used as reference compound.

Scavenging activity of superoxide anion ($O_2^{\bullet -}$) by electrolyzed-reduced water
Superoxide radicals were generated by the xanthine-xanthine oxidase system as previously described [30-32] with some modifications. In brief, 100 µl xanthine oxidase grade I (0.25U; one unit converts 1µmol of xanthine to uric acid per min at pH 7.5 at 25ºC), 100µl of 300uM lucigenin and various volume of electrolyzed-reduced water (range from 100µl to 400µl) were added to phosphate-buffer saline (PBS), pH 7.4 in a special chamber unit that included a stainless steel cell with a magnetic stirrer and stirrer bar in a dark chamber of the chemiluminescence analyzing system. At the 60-second mark, 100µl of 2.5mM xanthine was injected into the cell and the superoxide radical-induced lucigenin chemiluminescence was measured continuously for a total of 180seconds. L (+)-ascorbic acid and superoxide dismutase (SOD) were used as reference compounds.

Scavenging activity of hydroxyl radical ($^{\bullet}OH$) by electrolyzed-reduced water
Hydroxyl radical was generated by the addition of ferrous iron to the buffer solution [11, 33]. Freshly prepared $FeSO_4$ (200 µM, in 0.9%NaCl) and various volume of electrolyzed-reduced water (range from 100µl to 400µl) were added to phosphate– buffered saline, pH 7.4 in the stainless steel cell and measurement of chemiluminescence intensity began. At the 60-second mark, luminol was injected to the cell and the chemiluminescence system was measured continuously for a further 180 seconds. The total amount of the chemiluminescence intensity was calculated by integrating the area under the curve and subtracting the background level. DMSO and quercetin were used as reference compounds.

Cell culture
A hamster pancreatic β cell line, HIT-T15 and RINm5F insulin-producing tissue culture cell were supplied by Bioresource Collection and Research Center. HIT-T15 cells were cultured in a RPMI 1640 medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 25 mM HEPES, 100 IU ml^{-1} penicillin-G and 100 µg ml^{-1} streptomycin at 37℃ in a humidified atmosphere of 5% CO_2. RINm5F insulin-producing tissue culture cells were cultured in a RPMI 1640 medium containing 10 mmol/L glucose, 10% fetal bovine serum (FBS), 100 IU ml^{-1} penicillin-G and 100 µg ml^{-1} streptomycin at 37℃ in a humidified atmosphere of 5% CO_2. The medium was exchanged every 2 days.

Measurement of cell viability
HIT-T15 cells and RINm5F (1×10^5 cells ml^{-1}) were seeded onto 24 well plates and pre-incubated in a 10%FBS/RPMI 1640 medium containing ERW with a 0.22 µm filter for 24 h. Alloxan dissolved in cold PBS was added to the cells that were then incubated for 1 h. After incubation, the medium was aspirated and fresh medium containing 30µl of 2 mg/ml

3-(4,5-dimethylthiazol-s-yl-)-2,5-diphenyl tetrazolium bromide (MTT) was added. After 4 h, the medium was removed and replaced with blue formazan crystal dissolved in 10％ SDS solution. The absorbance at 450 nm was measured with a spectrophotometer.

Measurement of intracellular ROS

Intracellular ROS generation was monitored by flow cytometry using the peroxide-sensitive fluorescent probe [2', 7'-dichlorofluorescin diacetate (DCFH-DA)]. HIT-T15 cells and RINm5F cells were coincubated with 20μM DCFH-DA for 15 min a 37℃. DCFH-DA was converted by intracellular esterases to 2',7'-dichlorofluorescin (DCFH). In the presence of a proper oxidant, DCFH was oxidized into the highly fluorescent 2', 7'-dichloroflurescein (DCF). After incubation with the dye, cells were resuspended in ice-cold PBS and placed on ice in a dark environment for flow cytometry analysis with excitation and emission wavelengths of 495 and 525 nm, respectively.

Result

Scavenging activity of hydrogen peroxide (H_2O_2) by electrolyzed-reduced water

We adopted Okubo's theory that the reactive oxygen species/hydrogen donor/ mediator system was a chemiluminescence system for the measurement of hydrogen peroxide scavenging activity [7, 8]. Based on this evidence, we measured that scavenging activity of H_2O_2 by electrolyzed-reduced water (Figure 1A). At the volumes of 0 μl, 100 μl, 200 μl, and 400 μl of electrolyzed-reduced water, chemiluminescence intensity markedly increased from baseline to 30000 chemiluminescence counts, respectively. The more the chemiluminescence intensity increased, the stronger the scavenging activity of hydrogen peroxide of the electrolyzed-reduced water reached. The hydrogen peroxide scavenging effects of gallic acid have been studied by several researchers [33, 34] and we used gallic acid as positive control. Figure 1B showed the hydrogen peroxide scavenging activities of gallic acid were in a dose-dependent manner.

Scavenging activity of superoxide anion ($O_2^{\bullet -}$) by electrolyzed-reduced water

Superoxide anion scavenging activity of electrolyzed-reduced water was evaluated using the xanthine-xanthine oxidase system. This system was one of potential sources of free radicals. Xanthine oxidase catalyzed the oxidation of hypoxanthine to xanthine and xanthine to uric acid and superoxide anions. The electrolyzed-reduced water proved to be an effective scavenger for the superoxide anions with an IC_{50} value of 254 μl (Figure 2A). L (+)-ascorbic acid was used as reference compound and IC_{50} was 22.5 μM and SOD was used as positive control (Figure 2B, 2C). The tap water, distilled water, and sodium hydroxide at the same pH value of the electrolyzed-reduced water had no scavenging activity in the xanthine-xanthine oxidase system.

Scavenging activity of hydroxyl radical ($^{\bullet}OH$) by electrolyzed-reduced water

Ferrous iron–induced chemiluminescence was depressed by electrolyzed- reducing water (100μl to 400 μl) in a dose-dependent manner (Figure 3A). One-hundred μl of electrolyzed-reduced water could scavenge 41.1 ± 4.89 % of hydroxyl radical and 400μl of electrolyzed-reduced water exhibited the maximum hydroxyl radical scavenging activity (79.9 ± 1.58 %) (Figure 3B). And quercetin dehydrate (49.0 ± 1.49 %, at 5 μM; 96.0 ± 0.22 %, at 500μM) and DMSO (70.5 ± 4.43 %, at 3 M) showed a similar dose-dependent manner of inhibition of hydroxyl radical (Figure 3C).

Increasing free radical scavenging activities of antioxidants by electrolyzed-reduced water

The activity of scavenging hydrogen peroxide was significantly different ($p < 0.05$) between gaillic acid dissolved in electrolyzed-reduced water and gaillic acid dissolved in distilled water (Figure 4A). Superoxide dismutation activity of L (+)-ascorbic acid in electrolyzed-reduced water had significant increases than that of L (+)-ascorbic acid in distilled water ($p < 0.05$) (Figure 4B). The scavenging activity of hydroxyl radical by quercetin dehydrate in electrolyzed-reduced water had significant increases than that of quercetin dehydrate in distilled water ($p < 0.05$) (Figure 4C).

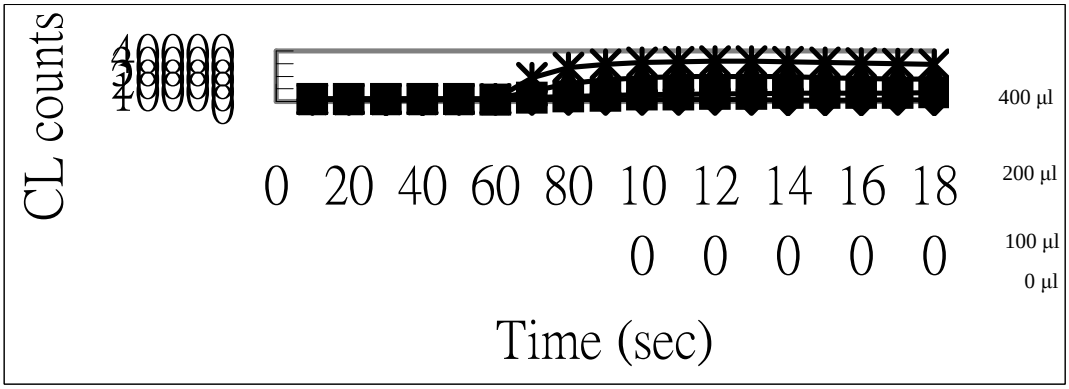
Effect of ERW on cytotoxicity of alloxan in HIT-T15 cells and RIN cells

Alloxan is selectively toxic to pancreatic β cells. RIN cells were grown in the presence of concentrations of alloxan ranging from 20mM to 60mM. Pancreatic β cells were pre-cultured for 24 h in media and then exposed to different dose of alloxan for time series scanning. As shown in figure 5, the cells treated with ERW showed protective effect, after alloxan treatment.

Effects of ERW on intracellular redox state

Free radicals could be involved in the cytotoxic effect of alloxan on pancreatic β cells. We observed the fluorescent intensity of intracellular redox state on HIT-T15 cells treated with alloxan using an ROS-sensitive probe DCFHDA. As shown figure 6, ERW decreased the ROS level of HIT-T15 cells. ERW-treated cells sustained 40-60% lower intracellular ROS level than that of non-treated cells, even after alloxan and GSH-treatment(Fig 7). These results clearly show that ERW has antioxidative capability that can scavenge intracellular ROS, enhance GSH antioxidativity and protect β cells from alloxan-induced ROS damage.

(A)



(B)

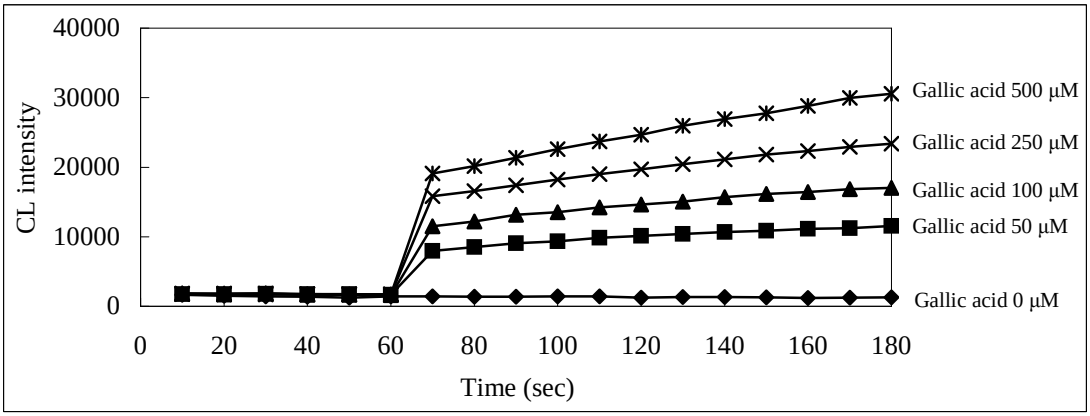
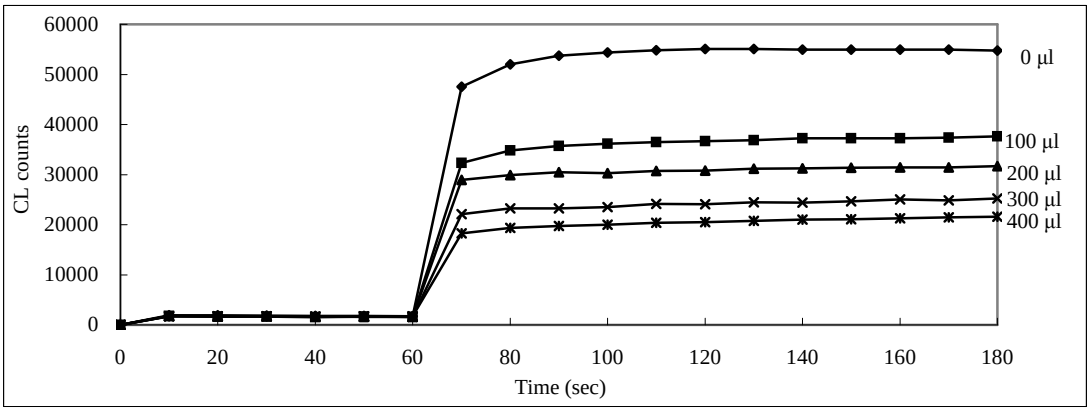
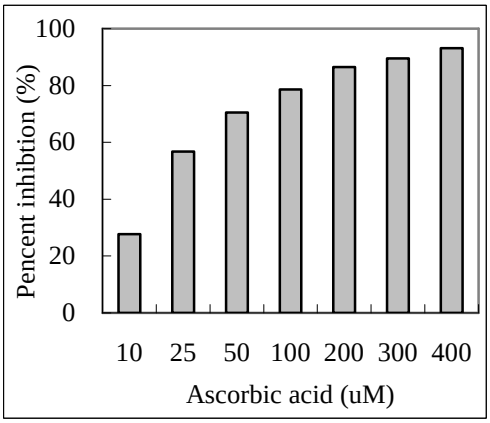


Figure 1. Chemiluminscence intensity properties from reactive oxygen species/ hydrogen donor/ mediator system (P = *k*[X][Y][Z]). X was 3.75% hydrogen peroxide and Z was 10% acetaldehyde. (A) Y was hydrogen donor of electrolyzed-reduced water (ORP=-344±13.62; pH=8.99±0.04) and test volume between 0µl to 400µl. (B) Y was reference compound as gallic acid at concentration from 0µM to 500µM.

(A)



(B)



(C)

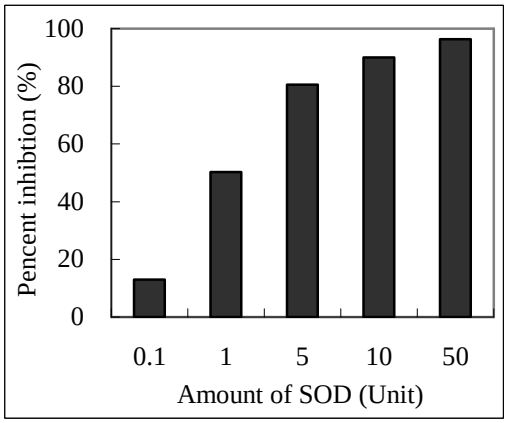
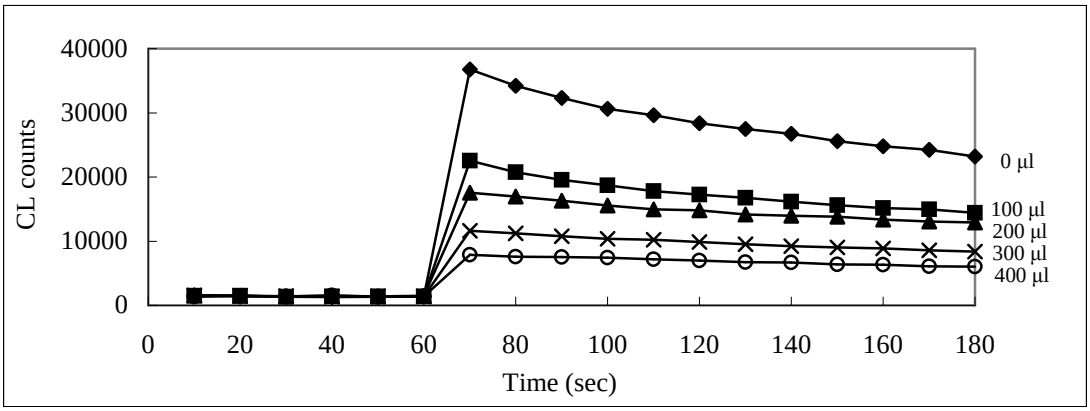
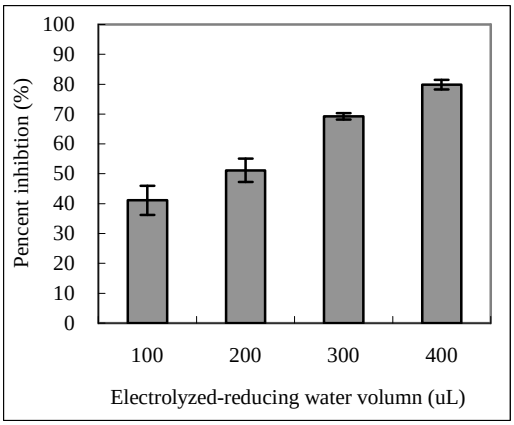


Figure 2. (A) Superoxide anion scavenging ability of electrolyzed-reduced water (ORP=-344±13.62; pH=8.99±0.04) was evaluated using the xanthine-xanthine oxidase system. (B) Superoxide anion scavenging ability of L (+)-ascorbic acid. (C) Superoxide anion scavenging ability of superoxide dismutase (SOD).

(A)



(B)



(C)

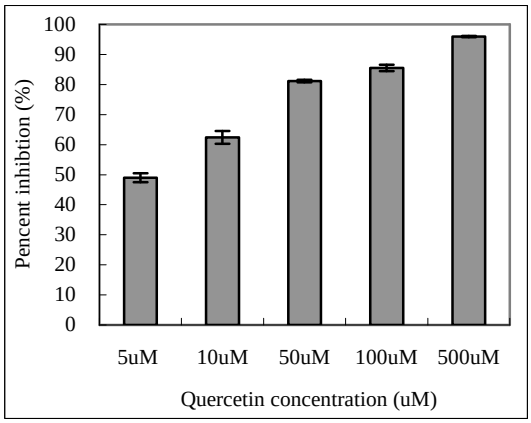
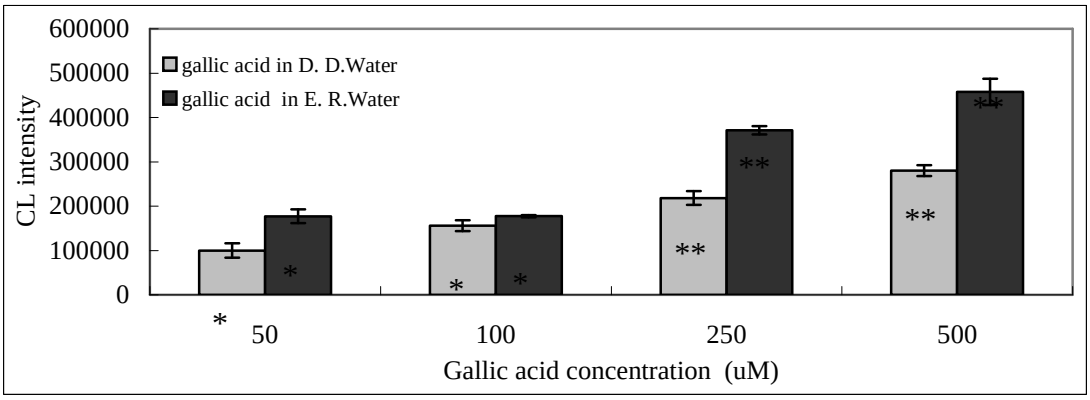
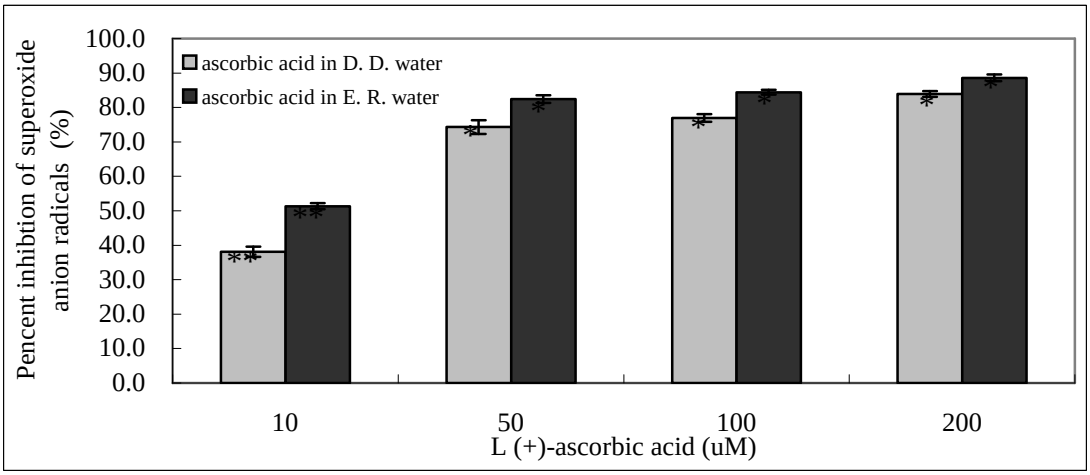


Figure 3. (A) Hydroxyl radicals scavenging ability of electrolyzed-reduced water (ORP=-344±13.62; pH=8.99±0.04) was evaluated using the ferrous iron–induced chemiluminescence system. (B) Percent inhibition of hydroxyl radicals by electrolyzed-reduced water. (ORP=-344±13.62; pH=8.99±0.04) (C) Percent inhibition of hydroxyl radicals by quercetin dehydrate.

(A)



(B)



(C)

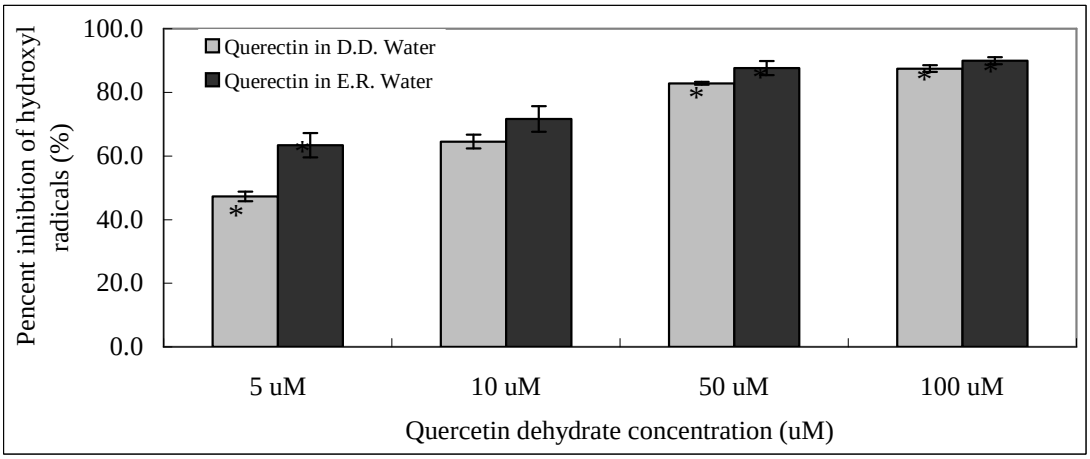


Figure 4. Reactive oxygen species scavenging activities of some antioxidants with or without electrolyzed-reduced water. (ORP=-344±13.62; pH=8.99±0.04) (A) Hydrogen peroxide scavenging activity. (B) Superoxide anion scavenging activity. (C) Hydroxyl radicals scavenging activity. Data are shown as mean ± SD. * $p < 0.05$, had statistically significantly difference (Student t-test). ** $p < 0.001$, had statistically significantly difference (Student t-test).

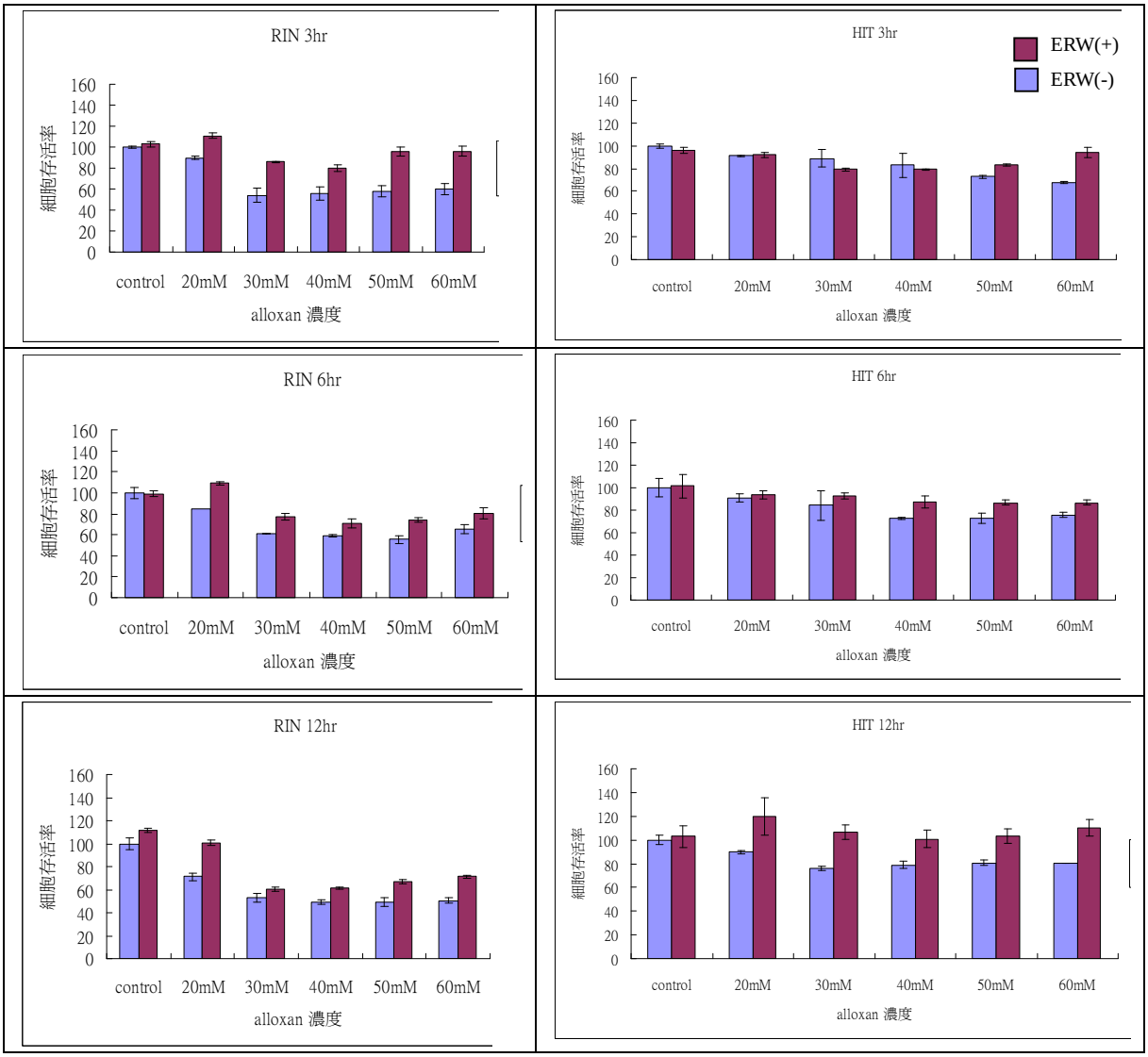


Figure 5. Effect of ERW on the pancreatic RIN cells and HIT-T15 cells viability induced by alloxan.

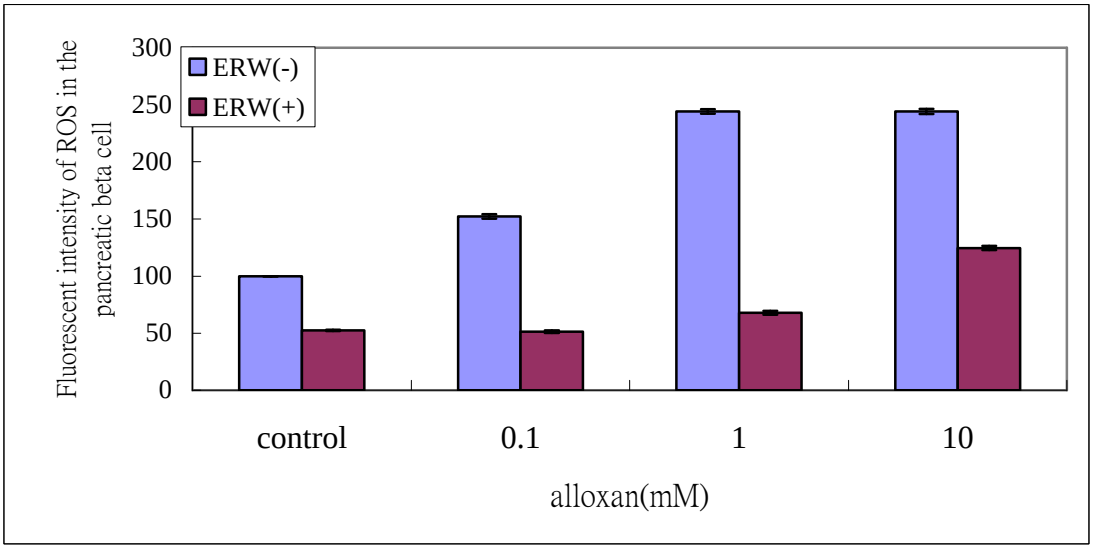


Figure 6. Effects of ERW on intracellular redox state of HIT-T15 cells induced by alloxan

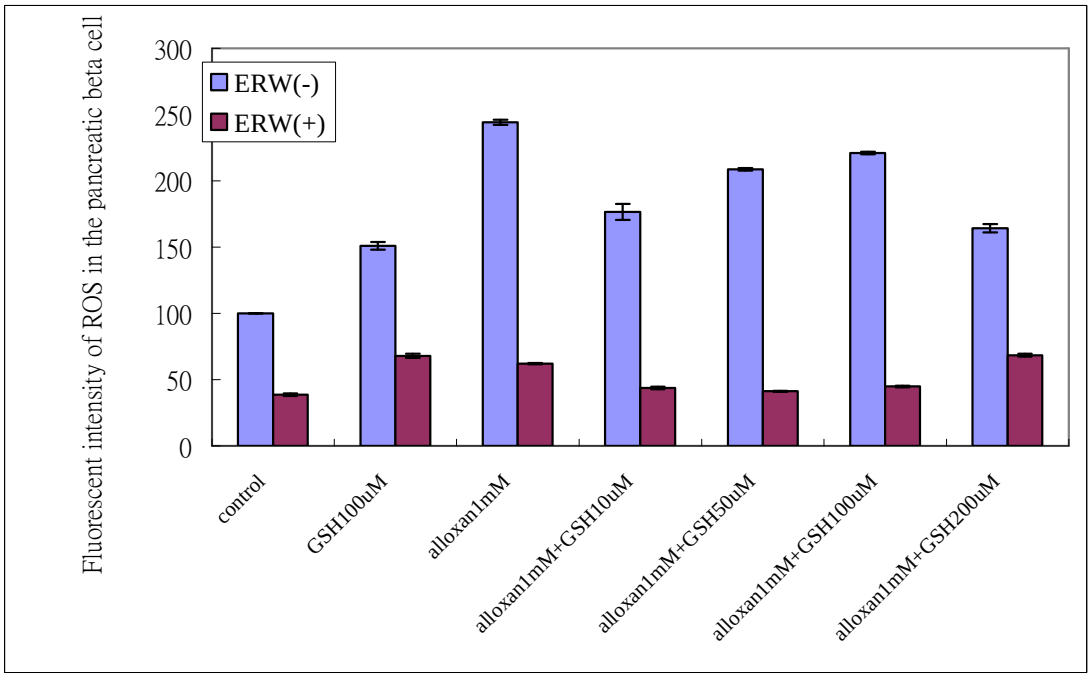


Figure 7. Effect of ERW and GSH on intracellular redox state of HIT-T15 cells induced by alloxan.

References

[1] Cheryl LF, Lisa MS, Tim DO. Extracellular superoxide dismutase in biology and medicine. Free Radic Bio. Med 2003;35:236–256.

[2] Fukai T, Folz RJ, Landmesser U, Harrison DG. Extracellular superoxide dismutase and cardiovascular disease. Cardiovasc Res 2002;55:239–249.

[3] Bowler RP, Crapo JD. Oxidative stress in airways: is there a role for extracellular superoxide dismutase? Am J Respir Crit Care Med 2002;166:S38–S43.

[4] Halliwell B, Gutteridge JM. Free radicals in biology and medicine. 3rd ed. New-York: Oxford Science Publication; 2000

[5] Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci USA 1993;90:7915–7922.

[6] Townshend A. Solution chemiluminescence-some recent analytical developments. Analyst 1990;115:495–500.

[7] Jarmo KL, Christer T, Jansen MA. Lucigenin and linoleate enhanced chemiluminescent assay for superoxide dismutase activity. Free radic Biol Med 1993;14:457–461.

[8] Yoshiki Y, Yuan H, Iida T, Kawane M, Okubo K, Ishizawa T, Kawabata S. Chemiluminescence of horseradish peroxidase and acetaldehyde related with gallic acid and hydrogen peroxide interaction. Photochem Photobiol 1998;68:802–808.

[9] Yohsiki Y, Kawane M, Iida T, Yuan H, Okubo K, Ishizawa T, Kawabata S. Photon emission by heme-protein in the presence of reactive oxygen species and phenolic compounds In: Sibamoto T, Terao J, Osawa T, editors ACS Symposium Series 702, Functional foods for disease prevention II. American Chemical Society, Washington DC, 1998; 256–265.

[10] Yoshiki Y, Yamanaka T, Satake K, Okubo K. Chemiluminescence properties of soybean protein fraction in the hydroperoxide and hydrogen donor system. Luminescence 1999;14:315–319.

[11] Yıldız G, Demiryürek AT. Ferrous iron-induced luminol chemiluminescence: a method for hydroxyl radical study. J Pharmacol Toxicol Methods 1998;39:179–184.

[12] Shirahata S, Kabayama S, Nakano M, Miura T, Kusumoto K, Gotoh M, Hayashi H, Otsubo K, Morisawa S, Katakura Y. Electrolyzed–reduced water scavenges active oxygen species and protects DNA from oxidative damage. Biochem Biophys Res Commun 1997;234:269–274.

[13] Hanaoka K. Antioxidant Effects of reduced Water produced by electrolysis of sodium chloride solutions. J Appl Electrochem 2001;31:1301–1313.

[14] Rho H, Lee J, Kim H, Park B and Park J. Protective mechanism of glucose against alloxan-induced β cell damage: Pivotal role of ATP. Exp Mol Med 2000; 32:12-17.

[15] Malaisse WJ and Lea MA. Alloxan toxicity to the pancreatic β cells. A new hypothesis. Biochem Pharmacol 1982; 31:3527-3534.

[16]