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Attenuated Effects of Deep-Sea Water on Hepatic Apoptosis in STZ-Induced Diabetic Rats

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Abstract

Diabetes mellitus (DM) is a metabolic disorder and increasing evidences have indicated a connection between DM and hepatic abnormality. Deep-sea water (DSW) has been applied in many fields, especially in medicine; herein, we investigated the influence of DSW on hepatic apoptosis in streptozocin (STZ)-induced diabetes rats. Our experimental results firstly demonstrated the beneficial effects of 1×DSW, 2×DSW and 3×DSW in alleviating hepatic apoptosis in STZ-induced diabetic rats. We demonstrated that 1×DSW, 2×DSW and 3×DSW significantly suppressed the caspase-3 activity and TUNEL-positive cells in livers of STZ-induced diabetic rats. Significant reductions of both Fas-dependent and mitochondrial-dependent apoptotic molecules were also detected in livers of STZ-induced diabetic rats receiving DSW. Additionally, apoptotic signaling molecules such as phosphorylated IκB-α and NF-κB were significantly reduced in livers of DSW-treated STZ-induced diabetic rats. These findings indicate hepatic protective effects of DSW on DM and suggest DSW as a possible ingredient for health food.

Key Words: apoptosis, deep-sea-water (DSW), diabetes mellitus (DM), liver, magnesium (Mg), signaling

Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia and is one of the most common chronic diseases worldwide. DM is caused either by impaired insulin secretion and insulin action or by defects in both (5). Increasing evidences have indicated a connection between DM and hepatic abnormality. A previous study reported that the levels of alanine aminotransferase (ALT) and gamma glutamyl transpeptidase (γ-GT) were significantly higher in the diabetic group as compared to the controls (2). In an-

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other 4-year longitudinal study, nonalcoholic fatty liver disease subjects with both elevated serum ALT and ultrasonographic steatosis have significantly increased risk for subsequent diabetes development (4). Recent data also suggest a role of endoplasmic reticulum (ER) stress-induced apoptosis in the liver and adipose tissue in relation to diabetes. Indeed, ER stress-induced apoptosis may be an important mechanism in the development of DM, not only for beta-cell impairment but also for insulin resistance (37). Accordingly, severe liver injuries such as visible fatty degeneration, inflammatory cell infiltration and Fas-dependent hepatocyte apoptosis were also detected in streptozocin (STZ)-treated Sprague Dawley rats (24).

Magnesium (Mg), the second and fourth abundant cation in the cell and in the body, respectively, plays an important role of metabolic functions in physiological and pathophysiological conditions (18). Evidences have indicated a relation between Mg deficiency and enhanced risk of metabolic syndrome and type 2 DM (10, 34). The association between hypomagnesemia and insulin resistance (IR) in DM has been documented earlier (10, 17). Indeed, low Mg levels and high triglyceride levels in association with enhanced Hemoglobin A1c (HbA1c) levels could thus serve as a reliable biochemical indicator in patients with type 2 DM (35). In contrast, oral Mg supplementation of insulin-requiring patients with Type 2 DM increased plasma Mg concentration and urinary Mg excretion, which is associated with a tendency to decreases in diastolic pressure (6, 19). These findings suggested the protective and therapeutic significance of Mg administration in type 2 DM patients.

Characterized by its multiple benefits such as clarity, sanitary quality, plentiful nutrients, especially abundances in ionic magnesium, calcium and potassium, Deep-Sea water (DSW) has received attention for its utilization in treatments of many diseases (28, 40). Recently, DSW has been widely investigated for its therapeutic or preventive effects in hypertension (26), dermatitis syndrome (13), hyperlipidemia and atherosclerosis (8). Our recent studies also revealed that DSW alleviated abnormal cardiac architecture and apoptosis, and enhanced insulin-like growth factor-1 receptor cardiac survival signaling in mice on high-cholesterol diet (32). However, little is known about the effects of DSW on hepatic abnormality in DM, especially on apoptosis. The purpose of this study was to investigate the effects of DSW on hepatic apoptosis in STZ-induced diabetic rats.

Materials and Methods

DSW

DSW (LC-90K Do-Minerals), supplied by Taiwan Yes Deep Ocean Water Co., Ltd (Hualien, Taiwan), was obtained from seawater below 662 m in the outer sea of Hua-Lien County, Taiwan. DSW was subjected to a series of procedures, including filtration, reverse osmosis and concentration as previously described (8, 33). The concentrated DSW has a hardness of 400,000 mg/l, and the content of ionic magnesium (Mg^{2+}) was 96,000 mg/l. The DSW used in this experiment was pasteurized, bottled, and provided by Taiwan Yes Deep Ocean Water Co. (Table 1).

Table 1. Compositional analysis of DSW.

Element	Concentration (mg/l)
Mg	96,200
Na	9,010
K	10,800
Ca	39
Fe	0.0033
Zn	0.0038
Mn	0.0016
Se	2.48

Experimental Design for Animal Studies

Referring to the recommended human daily allowance (RDA) of magnesium, the mineral concentrate was diluted to three different dosages: 1×DSW (equivalent to 37.5 mg Mg^{2+}/kg DSW); 2×DSW (75 mg Mg^{2+}/kg DSW); and 3×DSW (112.5 mg Mg^{2+}/kg DSW). Eight week-old male Sprague Dawley (SD) rats were randomly divided into five groups. Group I (Control) were the control rats (n = 10), group II (DM) were STZ-induced DM rats (n = 10), groups III (1×DSW), IV (2×DSW) and V (3×DSW) were STZ-induced rats treated respectively with 1×, 2× and 3× DSW (n = 10). After fasting the rats for 24 h, DM was induced by giving a single intraperitoneal injection (IP) of STZ (65 mg/kg body weight) dissolved in 10 mM sodium citrate, pH 7.0. Rats of each experimental group were fed with different multiples (1×, 2× and 3×) of 37 mg DSW/kg/day through gavage administration for a period of 4 weeks, and the blood glucose levels were checked on a weekly basis. All the rats were fed with normal feed (Lab Diet 5001; PMI Nutrition International, Brentwood, MO, USA). All protocols were reviewed and approved by the Institutional Review Board, and the animal care and use committee of the China Medical University, Taichung, Taiwan, ROC.

Caspase 3 Activity Assay

A caspase-3 ELISA kit (BD Pharmingen, San

Diego, CA, USA) was used for *in vitro* determination of caspase-3 enzymatic activity in 20 μg liver lysates derived from normal rats, STZ-induced DM rats and STZ-induced DM rats treated with 1×DSW, 2×DSW and 3×DSW according to the manufacturer's instructions.

DAPI Staining and TUNEL Assay

Apoptotic cells were identified by TUNEL (TdTmediated dUTP nick end-labeling) (22, 23). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed treating the tissue sections with proteinase K, and incubated with permeabilization solution followed by blocking buffer; intermittent washing was done twice with PBS. The sections were then incubated at 37°C in the presence of terminal deoxynucleotidyl transferase and fluorescein isothiocyanate-dUTP for 60 min by using an apoptosis detection kit (Roche Applied Science, Indianapolis, IN, USA) according to manufacturer's instructions. Under florescence (excitation wavelength of 460 nm and detection in the range of 515-565 nm), TUNEL-positive nuclei with fragmented DNA were illuminated in bright green. To visualize the nuclei, the tissue sections were stained with 0.1 μg/ml 4, 6-diamidino-2-phenylindole (DAPI), and the nuclei were detected and photographed at 454 nm using a Zeiss Axiophot microscope.

Protein Extraction and Western Blotting

Tissue extracts were obtained by homogenizing the livers in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM b-glycerophosphate, 1 mM Na_3VO4 and 1 μg/ml leupeptin). The homogenates were then centrifuged at 12,000 × *g* for 40 min. The supernatants were collected and stored at -80°C for further experiments. Western blotting was performed as described elsewhere (3, 14, 15, 21, 41). Briefly, the loading sample for each lane of western blot was a pool of three randomly selected rats of the same group. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using 12.5% acrylamide gel, was performed as previously described (14, 15). Protein samples were denatured for 5 min in boiling water with sample buffer (0.0625 M Tris-HCl buffer, pH 6.8, containing 2.3% SDS, 5% 2-mercaptoethanol, and 10% glycerol). Samples applied to the gel were run at 100-150 V for 1.5 h and electrophoretically transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). The membrane was then soaked in PBS with 5% nonfat dry milk for 30 min at room temperature. Antibodies against Fas ligand, Fas, activated-caspase-8, t-Bid, Bax, activated-caspase-9, activated-caspase-3, cytochrome *c*, Apaf-1, phosphorylated IκB-α(p-IκB-α) and NF-κB (p-p65) and β-actin (Upstates, Charlottesville, VA; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted in PBS with 2.5% BSA and incubated for 1.5 h with gentle agitation at room temperature. The membranes were washed twice with PBS-Tween for 1 h, and a secondary antibody conjugated with horseradish peroxidase (HRP) was added. Pierce's Supersignal West Dura HRP Detection Kit (Pierce Biotechnology Inc., Rockford, IL, USA) was used to detect antigen-antibody complexes. The blots were scanned and quantified by densitometry (Appraise, Beckman-Coulter, Brea, CA, USA).

Statistical Analysis

All statistical analyses were performed using SPSS 10.0 software (SPSS Inc., Chicago, IL, USA). Three independent experiments were repeated. Statistical analyses were performed using the analysis of variance plus posterior multiple comparison test to determine the difference. $P < 0.05$ was considered statistically significant. The significant differences were stressed with symbols as shown in figures.

Results

DSW Attenuates Hepatic Apoptosis in STZ-Treated Rats

To investigate the effect of DSW on hepatic apoptosis in DM rats, liver samples from DM rats were obtained and detected by TUNEL and caspase-3 activity assays. Significant increase of caspases-3 activity was detected in livers of rats from the DM groups as compared to those from the control group (Fig. 1A). Conversely, significant reduction of caspase-3 activity was detected in livers of rats from the 1×DSW, 2×DSW and 3×DSW groups as compared to those from the DM group (Fig. 1A). Significant amount of nicked-DNA was also observed in livers of DM rats as compared to those from the Control group whereas the significant reduction of nicked-DNA was observed in livers of rats the 1×DSW, 2×DSW and 3×DSW groups as compared to those from the DM group (Fig. 1, B and C).

Down-Regulatory Effects of DSW on Fas-Dependent Apoptosis in STZ-Treated Rats

Fas-dependent apoptotic signaling was next investigated to examine the effects of DSW on hepatic apoptosis. The expressions of Fas ligand (FasL) and Fas proteins were examined by western blot (Fig. 2A). Significant increases of both FasL and Fas proteins were observed in livers of DM rats as compared to those from the control group whereas significant re-

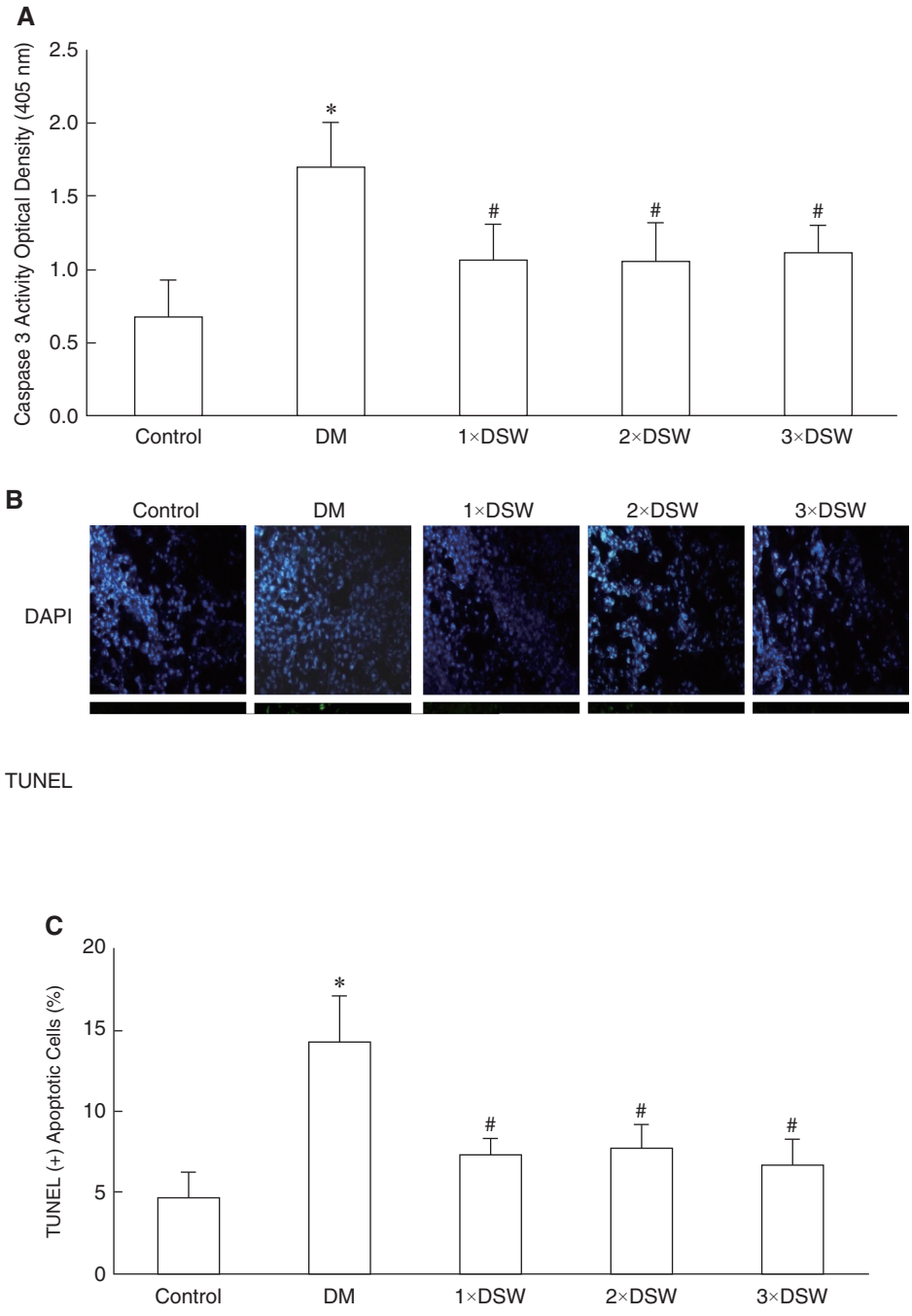

Fig. 1. Detection of caspase-3 activity and TUNEL-positive cells. (A) Activity of caspase-3 was measured in 20 μg liver lysates from normal rats, DM rats and DM rats treated with 1×DSW, 2×DSW or 3×DSW. The UV-induced apoptotic U937 lysate included in the kit was used as a positive control. (B) TUNEL assay was performed with liver sections from normal rats, DM rats and DM rats treated with 1×DSW, 2×DSW or 3×DSW. FITC-labeled terminal deoxy-transferase was bound to nicked end of DNA. as indicated by the arrows. DAPI staining was used as a control. (C) The percentage of TUNEL-positive cells in liver sections was calculated. Three independent experiments were performed. * and # indicate significant difference as compared to control or the DM rats, respectively.

ductions of FasL and Fas proteins were detected in livers of DSW-treated rats of all three concentrations (Fig. 2, B and C). Additionally, the expression of caspase 8, a downstream molecule of the Fas protein, and its cleaved form were also investigated. The presence of procaspase-8 and its cleaved form with a molecular weight at 23 kDa is shown in Fig. 3A. Significant increase of cleaved caspase-8 was detected in DM rats as compared to those from the control group, whereas significant reduction of cleaved

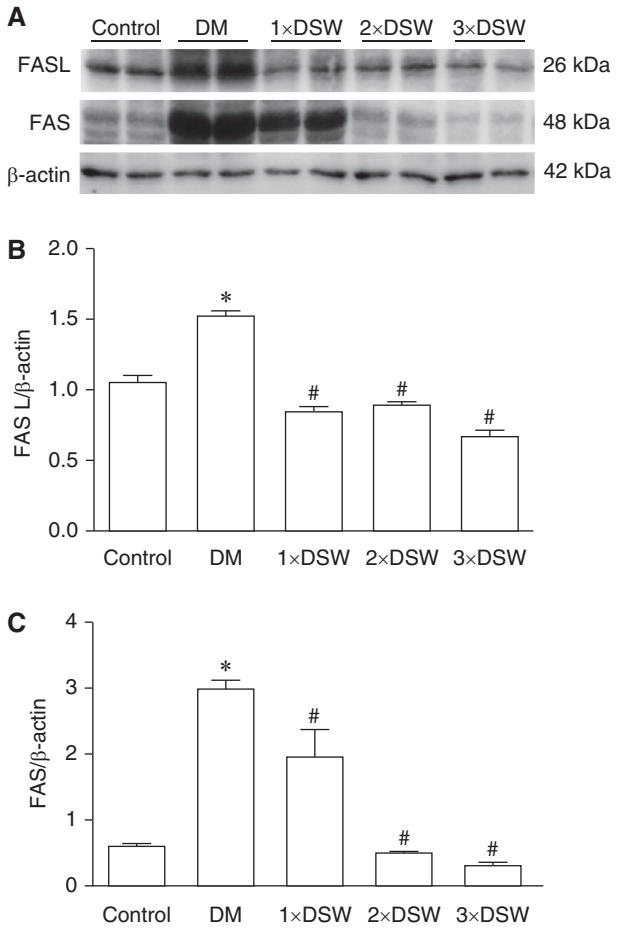

Fig. 2. Expression of FasL and Fas. Liver lysates obtained from normal rats, DM rats and DM rats treated with 1×DSW, 2×DSW or 3×DSW were probed with antibodies against (A) FasL and Fas. Bars represent the relative densitometric quantification of (B) FasL and (C) Fas on the basis of β-actin. Similar results were obtained in three independent experiments. * and # indicate significant difference as compared to control or DM rats, respectively.

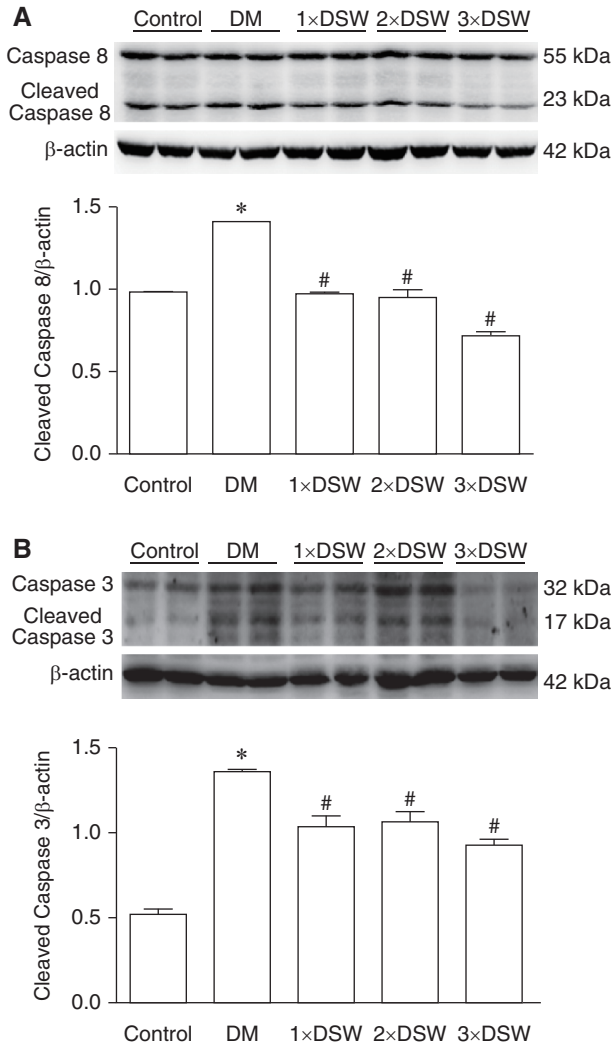

Fig. 3. Expression of caspase-8 and caspase-3. Liver lysates obtained from normal rats, DM rats and DM rats treated with 1×DSW, 2×DSW or 3×DSW were probed with antibodies against (A) caspase-8 and (B) caspase-3. Densitometric analyses are shown in the lower panel. Similar results were obtained in three independent experiments. * and # indicate significant difference as compared to control or DM, respectively.

caspase 8 was observed in the livers of rats from the 1×DSW, 2×DSW and 3×DSW groups as compared to those from the DM group (Fig. 3A). Similar result was observed in the presence of cleaved caspase 3. Significant increase of cleaved caspase 3 was detected in rats from the DM group as compared to those from the Control group whereas significant reduction of cleaved caspase 3 was observed in livers of DSW-treated rats as compared to those from the DM group (Fig. 3B).

Down-Regulatory Effects of DSW on Mitochondria-Dependent Apoptosis in STZ-Treated Rats

The effects of DSW on mitochondria-dependent apoptotic molecules in livers from the DM rats treated with different concentrations of DSW were also studied. Significant increases of both the Bax and tBid proteins were detected in livers of DM rats as compared to those from the Control group, whereas significant reductions of both the Bax and tBid proteins were observed in livers of DSW-treated rats as compared to those from the DM group (Fig. 4, A and B). Similar results were also observed in the expression of cytochrome *c* and Apaf-1. Significant increases of cytochrome *c* and Apaf-1 were detected in livers of DM rats. Conversely, significant reduction of cytochrome c and Apaf-1 was observed in livers of DSW-treated rats (Fig. 5, A and B). Additionally, cleaved

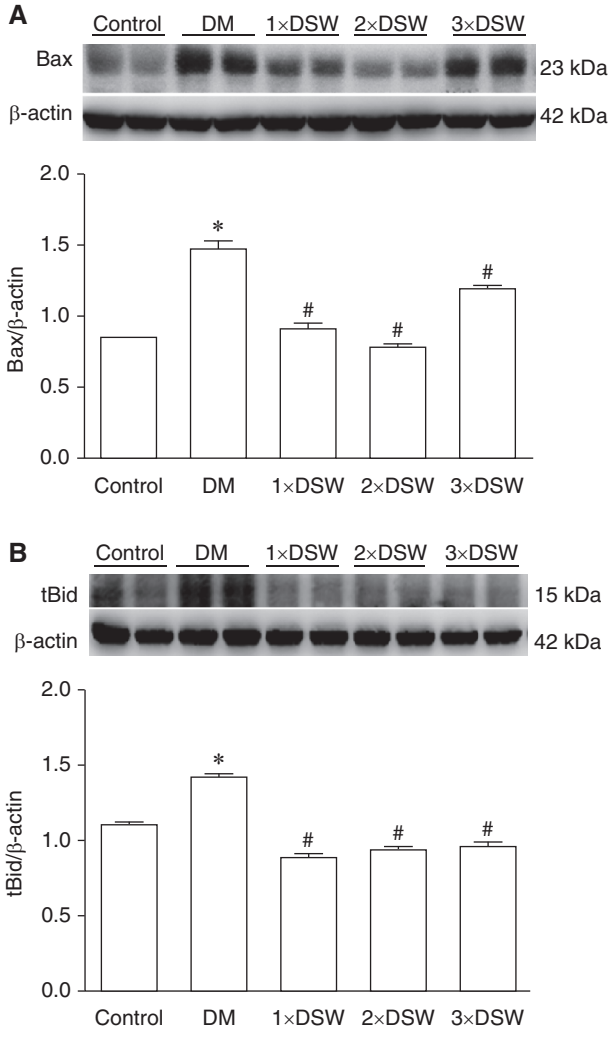

Fig. 4. Expression of Bax and tBid. Liver lysates obtained from normal rats, DM rats and DM rats treated with 1×DSW, 2×DSW or 3×DSW were probed with antibodies against (A) Bax and (B) tBid. Densitometric analyses are shown in the lower panel. Similar results were obtained in three independent experiments. * and # indicate significant difference as compared to control or DM, respectively.

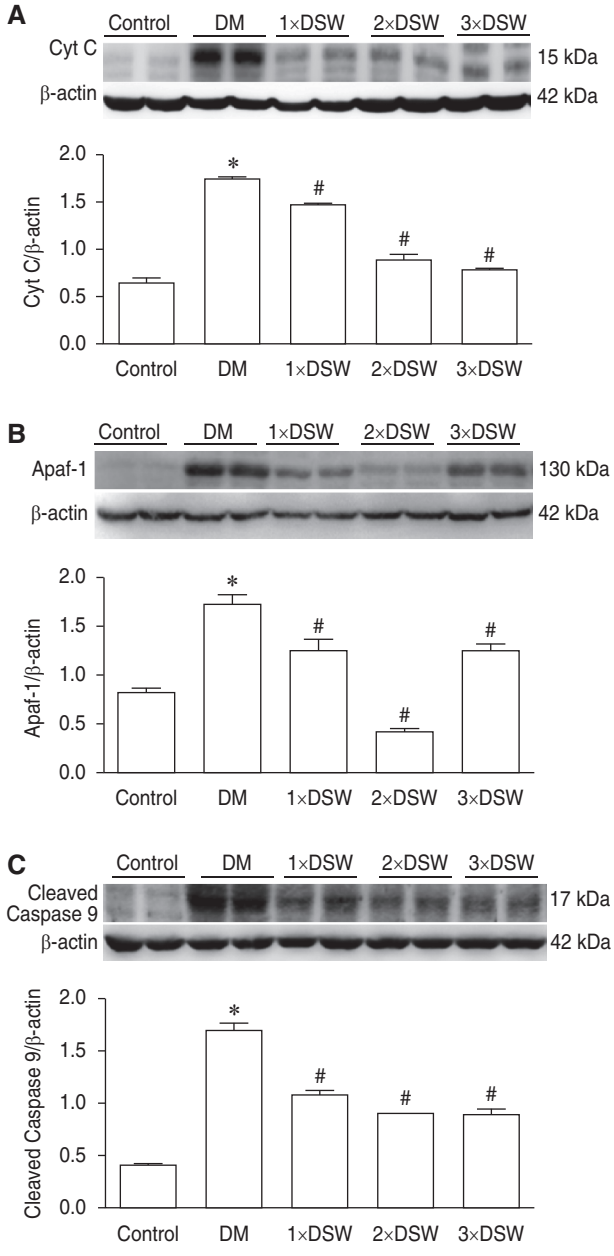

Fig. 5. Expression of cytochrome *c* and Apaf-1. Liver lysates obtained from normal rats, DM rats and DM rats treated with 1×DSW, 2×DSW or 3×DSW were probed with antibody against (A) cytochrome *c*, (B) Apaf-1 and (C) caspase 9. Densitometric analyses are shown in the lower panel. Similar results were obtained in three independent experiments. * and # indicate significant difference as compared to control or DM, respectively.

caspase 9, a downstream molecule of Apaf-1, was also significantly increased in livers of DM rats (Fig. 5C). Conversely, significant reduction of cleaved caspase 9 was observed in livers of rats from the 1×DSW, 2×DSW and 3×DSW groups compared to those from the DM group (Fig. 5C).

Signaling Molecules Involved in the DSW-Reduced Hepatic Apoptosis in STZ-Treated Rats

To clarify the possible signaling pathways involved in the effects of DSW on liver of rats treated with different concentrations of DSW, the presence of phosphorylated IκB-alpha (p-IκB-α) and NF-κB (p65-p) was examined. The expression of both p-IκB-α and NF-κB (p65-p) proteins was significantly increased in livers of rats from the DM group as compared to those from the Control group (Fig. 6, A and B). Conversely, significant reduction of p-IκB-αand NF-κB (p65-p) proteins was observed in livers of DSW-treated as compared to those from the DM group (Fig. 6).

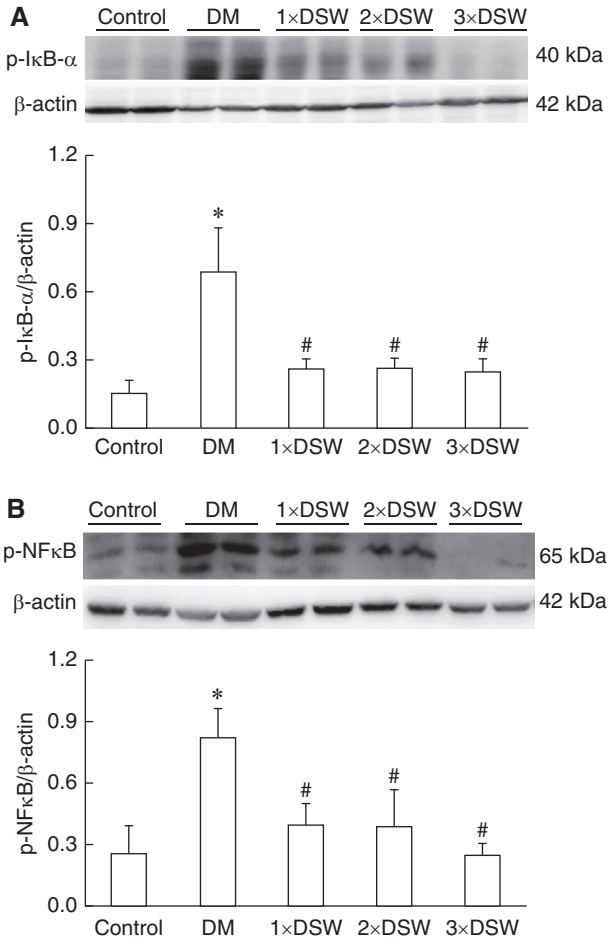

Fig. 6. Expression of IkB-α and NF-κB. Liver lysates obtained from normal rats, DM rats and DM rats treated with 1×DSW, 2×DSW or 3×DSW were probed with antibody against (A) IkB-α and (B) NF-κB. Densitometric analyses are shown in the lower panel. Similar results were obtained in three independent experiments. * and # indicate significant difference as compared to control or DM, respectively.

Discussion

Evidences have been documented to show a connection between DM and a variety of liver abnormalities (2, 4, 24). However, little is known about the effects of DSW on hepatic apoptosis induced by DM. In the present study, we firstly reported the alleviative effects of different concentrations of DSW on hepatic apoptosis in STZ-induced diabetic rats by reducing both extrinsic and intrinsic apoptotic signaling.

Magnesium is recognized as an important fundamental mineral acting as the co-factor of nearly 300 enzymes, and plays crucial roles in both physiological and pathological processes (1). Evidences have documented the association between hypomagnesaemia and DM (10). Significant hypomagnesemia was observed in diabetic patients as compared to controls (20). In an 18-year follow-up study of 2,504 patients with type 2 DM and hypertension, high blood glucose and low serum magnesium in both men and women are significantly associated with all-cause mortality (11). Correspondingly, many studies have also indicated that hypomagnesemia is strongly associated with the induction of hepatic oxidative stress and subsequent hepatic apoptosis (25, 37). Since insulin is known to regulate intracellular Mg *via* membrane-bound ATPase, Mg deficiency may be the consequence of insulin resistance (30, 31). Besides, Mg deficiency also leads to decreased synthesis of glutathione and subsequently increased lipid peroxidation, which may contribute to the pathogenesis of DM mainly through the role of magnesium in mediating the effects of glutathione on peripheral insulin action (16). Therefore, oxidative stress-induced apoptosis might be an important cause in the development of diabetes, not only for β-cell impairment but also for insulin resistance (12, 25, 37). Conversely, magnesium supplementation revealed a beneficial effect on lipid profile in patients with metabolic syndrome, type 2 diabetes, heart failure and chronic hemodialysis (19, 20). Since magnesium is the most abundant component in DSW and is known for its therapeutic effects in a variety of diseases, the alleviative effects of DSW on hepatic apoptosis in STZ-induced diabetic rats might be due to the high level of magnesium in DSW. However, further investigations are still needed to verify the precise mechanism.

The cytoprotective function of nuclear factor-kappaB (NF-κB) has been shown by numerous studies in various cell types (18). In general, the cytoprotective factors induced by NF-κB were thought to be fully responsible for the cell-death inhibiting effect of NF-κB activation. These factors include the Bcl-2 family members Bcl-xL and A1/Bfl-1, X-linked inhibitor of apoptosis (XIAP), and cellular inhibitor of apoptosis (c-IAP) 1 and c-IAP2 (39). Conversely, other study reported that the induction of apoptosis in leukemic cell-bearing mice by beta2-microglobulin is through the activation of caspase-3 and NF-κB (27). In a rat model of cord injury, administration of tamoxifen significantly attenuated the expression of active caspase-3 resulting in the reduction of apoptosis, and infiltration of leukocytes, as well as the reduced expressions of NF-κB p65 and phosphorylated I-κBα (38). These findings suggested that NF-κB affect a diverse array of cellular processes including apoptosis and cell survival (7).

Since DSW has a high level of Mg and been applied in many fields, especially in medicine (9, 28, 29, 36), herein we investigated the influence of DSW on hepatic apoptosis in STZ-induced diabetes rats. Notably, our experimental results firstly demonstrated

the beneficial effects of different concentrations of DSWs, including 1×DSW, 2×DSW and 3×DSW, on alleviating hepatic apoptosis in STZ-induced diabetic rats by repressing the Fas- and mitochondria-dependent apoptotic cascades and the related apoptotic signaling molecules, p-IκB-α and p-NF-κB. Altogether, these findings suggest the protective effects of DSW on hepatic apoptosis in DM rats. Furthermore, DSW could be used as a possible ingredient for hepatic health food.

Acknowledgments

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Conflict of Interest

There was an involvement of financial or non-financial interests with Taiwan Yes Deep Ocean Water Co., Ltd., (Hualien, Taiwan) regarding the materials discussed in this manuscript, and the nature of the interest is Funding 0994267S (Financial support: Materials). The funder had no role in data collection and analysis, decision to publish, or preparation of the manuscript.

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