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Protective effect of a water-soluble polysaccharide from *Salvia miltiorrhiza* Bunge on insulin resistance in rats

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ABSTRACT

Oxidative stress is associated with insulin resistance (IR) and is thought to contribute to the development and progression toward type 2 diabetes (T2DM). This study was undertaken to isolate the bioactive polysaccharide (SMPW1) from *Salvia miltiorrhiza* Bunge and investigated its protective effects on IR model in rats induced by *tert*-butyl hydroperoxide (*t*-BHP). In vivo animal experiments showed that SMPW1 (50 and 100 mg/kg) possessed high antioxidative and protective capacity against the injury induced by *t*-BHP, as reflected in the increased expression or activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and the decreased formation of malondialdehyde (MDA) in serum and liver homogenates. In addition, SMPW1 (50 and 100 mg/kg) also attenuated IR and the morphological injury of liver and pancreas induced by *t*-BHP, and improved insulin sensitivity index. In conclusion, SMPW1 can protect against the development of T2DM and improve IR via reduction of oxidative stress.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a well-known endocrine and metabolic disorder which has reached epidemic proportions worldwide and represents a serious public health concern. It is estimated that cases of T2DM will reach approx. 366 million people by 2030 (Rathmann & Giani, 2004). Insulin resistance (IR), a major causative factor for the early development of T2DM and cardiovascular disease, is even more widespread (DeFronzo, 1997; Reaven, 1988, 2000). Oxidative stress, which is characterized by excessive production of reactive oxygen species (ROS) and reduction of antioxidant defense capacity, has been implicated in the pathogenesis of T2DM and its complications (Ceriello & Motz, 2004; Evans, Goldfine, Maddux, & Grodsky, 2003; Gopaul et al., 2001; Vijayalingam, Parthiban, Shanmugasundaram, & Mohan, 1996). ROS play a major role in the development and progression of diabetes and many kinds of diseases. Under physiological condition, generation and scavenging of ROS maintain dynamic equilibrium (Baynes, 1991). Many studies suggest that β-cell dysfunction is the result of prolonged exposure to high glucose, elevated free fatty acids (FFA) levels, or a combination. There are considerable evidence that chronic hyperglycemia in patients with T2DM contributes to impairing β-cell function (Grodsky, 2000; Robertson et al., 2000). β-Cell is sensitive to ROS and reactive nitrogen species (RNS) because the free-radical quenching (antioxidant) enzymes such as catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) (Tiedge, Lortz, Drinkgern, & Lenzen, 1997) are low in β-cells. It has been shown that ROS are involved in the progression of IR as well as in pancreatic β-cell dysfunction (Evans, Goldfine, Maddux, & Grodsky, 2002).

Salvia miltiorrhiza Bunge (Labiatae) is an herb commonly used in Chinese medicine to treat cardiovascular and cerebrovascular diseases, as well as for hyperlipidemia (Jang et al., 2003; Zhou, Zuo, & Chow, 2005). Recently, clinical trials have also indicated that *S. miltiorrhiza* have beneficial effects in diabetic patients with chronic heart disease. The mechanism may involve the ability of *S. miltiorrhiza* to enhance antioxidant defense enzyme activities and decrease or abolish the production of oxygen-derived free radicals (Jiang, Zhang, & Cai, 1997; Qian, Huo, Wang, & Qian, 2011; Qian, Qian, Fan, Huo, & Wang, 2011). Moreover, tanshinones were reported to have the potential to enhance the activity of insulin on the tyrosine phosphorylation of the insulin receptor as well as the activation of the downstream kinases Akt, ERK1/2, and GSK3β (Jung, Seol, Jeon, Son, & Lee, 2009). Besides, *S. miltiorrhiza* extract has therapeutic effect in the treatment of diabetic nephropathy by suppressing the over-expressions of TGF-β1, connective tissue growth factor, fibronectin and plasminogen activator inhibitor-1 in the renal cortex (Liu et al., 2005). In addition, a study by Qian, Qian, et al. (2011) and Qian, Huo, et al. (2011) showed that

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S. miltiorrhiza hydrophilic extract effectively reversed induction of vascular endothelial growth factor (VEGF) expression by high glucose via ameliorating mitochondrial oxidative stress and can potentially be an effective antioxidant therapy for the treatment of diabetic chronic vascular complication. However, there is no report about the effect and mechanism of *S. miltiorrhiza* polysaccharide on *t*-BHP-induced insulin resistance in rats.

Therefore we establish the IR animal model by high fat and high glucose diet (HGF) quickly and stably, using *t*-BHP as a stimulus of oxidative stress. The objectives of this study were to determine whether *S. miltiorrhiza* polysaccharide could reduce the pancreatic injury and improve IR by *t*-BHP injection in SD rats through modulation of oxidative stress pathways.

2. Experimental

2.1. Plant materials and reagents

The roots of *S. miltiorrhiza* were purchased from local herb drugstore in Xi'an city. DEAE-Sephadex A-50 and Sephadex G-200 were from Amersham (Sweden). Nitroblue tetrazolium (NBT), *t*-BHP, glutathione (GSH), oxidized form of glutathione (GSSG), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. Thiobarbituric acid (TBA) was obtained from Merck (Germany). Serum triglycerides (TG), serum cholesterol (TCH), FFA and T-AOC kits were purchased from Nanjing Jiancheng Company in China. All the chemicals were of high purity and were used without purification.

2.2. Isolation and purification of the polysaccharides from S. miltiorrhiza

The dry roots of *S. miltiorrhiza* (400 g) were ground to powders, and submitted to remove lipids by extracting 3 times with 10 vol% of 95% EtOH under reflux for 3 h each time. After filtration through double gauze, the residue was extracted with 10 vol% of distilled water at 85 °C for three times and 3 h for each time. The combined aqueous extracts were centrifuged (1700 × *g* for 10 min, at 20 °C), concentrated 10-fold, and precipitated with 4 vol% of 95% EtOH at 4 °C for 24 h. The precipitate dissolved in 300 ml of water was deproteinated by Sevag method with mixed solution (chloroform:*n*-butanol = 5:1) until no emulsified layer exists in the interface between Sevag reagent (organic solvent) and water solution, which means the absolute removal of protein (Staub, 1965). The resulting aqueous fraction was extensively dialyzed (cut-off Mw 3500 Da) against tap water for 24 h and distilled water for 24 h, and was further precipitated again by adding EtOH to the concentrated dialysate until the concentration of the EtOH reached 75%. After centrifugation, the resulting precipitate (crude polysaccharides) was washed sequentially with ethanol, acetone, ether, and vacuum-dried.

The crude polysaccharide was applied to a DEAE-Sephadex A-50 column (2.0 cm × 40 cm), eluted stepwise with distilled water, 0.1, 0.2, 0.4, and 1.0 M NaCl, respectively. The eluate was monitored and collected by the phenol–sulfuric acid method. The distilled water elution was concentrated, dialyzed, and lyophilized. The resulting polysaccharide was purified on a Sephadex G-200 column (1.5 cm × 90 cm) and eluted with 0.1 M NaCl at a flow rate of 9 ml/h. Two polysaccharide fractions named as SMPW1 (tube 31–34) and SMPW2 (tube 41–43) were collected, of which the SMPW1 was used in the subsequent studies.

2.3. Analysis of polysaccharide, uronic acid and protein contents

Total neutral sugar content was determined by the reaction with phenol in the presence of sulfuric acid at 486 nm (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) using Glc as standard. Uronic acid content was determined by the m-hydroxybiphenyl colourimetric procedure at 523 nm (Filisetti-Cozzi & Carpita, 1991), with D-glucuronic acid as the standard. The protein content of protein-bound polysaccharide was measured (Lowry, Rosebrough, Farr, & Randall, 1951), using BSA as standard.

2.4. Animals and treatment

Forty male Sprague-Dawley rats weighting 200 ± 20 g were provided by the Experimental Animal Center of the Fourth Military Medical University. They were maintained in a standard environmental condition and fed with water ad libitum. Rats were housed in stainless steel cages in a room kept at 22 ± 1 °C with a 12-h light/12-h dark cycle. Elementary rats' fodders were obtained from the Forage Center at the Fourth Military Medical University. HGF were composed of elementary rats' fodders (67.5%), sugar (20%), refinement pork fat (10%), cholesterol (2%) and sodium deoxycholate (0.5%). The animals were treated according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and their experimental use was approved by the animal Ethics Committee of the University.

Rats were randomly allocated into the following groups: (A) normal + saline group (fed with standard food pellets); (B) normal + saline group (fed with HGF); (C) *t*-BHP (0.2 mmol/kg) + saline group (HGF); (D) *t*-BHP (0.2 mmol/kg) + 50 mg/kg SMPW1 group (HGF); (E) *t*-BHP (0.2 mmol/kg) + 100 mg/kg SMPW1 group (HGF). Eight rats were in each group. Rats were injected intraperitoneally with 0.2 mmol/kg *t*-BHP once everyday for 1 month (saline was used as substitute in the control group). And then intragastric administration of SMPW1 (50 and 100 mg/kg/day) was given to the (D) and (E) group daily for 2 weeks (saline was used as substitute in the (A), (B) and (C) group). On the last day of experiment, the animals were sacrificed. Parts of liver tissues were excised from the animals for the pathological histology by HE stain. Other parts of liver were homogenized and serum were collected for the assays of fasting blood glucose (FGB), fasting serum insulin (FINS), malondialdehyde (MDA), SOD, CAT, TG, TCH, T-AOC, GPx, GSH and GSSG, according to the procedures described below. Samples were stored at −80 °C until assayed.

2.5. Determination of FBG and FINS

FBG was assayed by steady step blood implement (Johnson, USA) and FINS was measured by double antibody radioimmunoassay. The calculation of insulin sensitivity index (ISI): $ISI = \ln(1/FBG \times FINS)$.

2.6. Determination of TG, TCH and FFA

The TG, TCH and FFA of the serum were measured using specific kits purchased from the Nanjing Jianchen Institute (Nanjing, China).

2.7. Determination of lipid peroxidation

The lipid peroxidation products in the serum and livers were determined by measuring MDA. The method of Yagi (1976) was used with some modifications. Briefly, 20 μl of sample was placed in a glass centrifuge tube 4.0 ml of 1/12 N H_2SO_4 was added and mixed gently. Then, 0.5 ml of 10% phosphotungstic acid (Sigma)

was added and mixed. After allowing it to stand at room temperature for 5 min, the mixture was centrifuged at 1600 × *g* for 10 min. The supernatant was discarded and the sediment mixed with 2.0 ml of 1/12 N H_2SO_4 followed by 0.3 ml of 10% phosphotungstic acid. The mixture was centrifuged at 1600 × *g* for 10 min. The sediment was then suspended in 1.0 ml of distilled water and 1.0 ml of 0.67% (w/v) TBA reagent (Merck, German) was added. The reaction mixture was heated at 95 °C for 60 min. After cooling with tap water, 5.0 ml of *n*-butanol (Kemio, China) was added and the mixture was shaken vigorously. After centrifugation at 1600 × *g* for 15 min, the *n*-butanol layer was taken for fluorometric measurement at 553 nm with excitation at 515 nm. 1,1,3,3-Tetraetoxypropane (Sigma) was used as the primary standard.

2.8. Determination of antioxidatant enzymes

The total activity of SOD was examined by using the modified method of Kono (1978). The sample or 50 mM phosphate buffer (blank) was added to the reaction system of 50 mM sodium carbonate buffer (pH 10.2, 0.1 mM EDTA), 24 M NBT and 0.03% (v/v) Triton X-100. The reaction was initiated by 1 mM hydroxylamine in a 37 °C water bath. After 20 min incubation, the reaction was terminated by adding trichloroacetic acid (TCA). The absorbance measurement was carried out with a spectrophotometer at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to inhibit the ratio of hydroxylamine oxidation of the control by 50%.

The activity of CAT was determined by a commercial kit purchased from the Nanjing Jiancheng Institute (Nanjing, China). Ammonium molybdate may end the decomposition reaction of H_2O_2 catalyzed by CAT. The surplus H_2O_2 may have an interaction with ammonium molybdate generating a kind of comoles compound with a distinctive color. The absorbance was measured optically at 405 nm. One unit of enzyme is defined as the amount of enzyme required to breakdown 1 μmol H_2O_2 per second CAT.

The measurement of GPx activity was based on the following principle: GPx catalyzed the reduced glutathione to oxidized glutathione by H_2O_2-induced oxidation (Paglia and Valentine, 1967). A yellow product was produced by reduced glutathione reacting with DTNB and had absorbance at 422 nm. One GPx unit is defined as the amount of the enzyme which lowered the concentration of reduced glutathione 1 μM/min at 37 °C in 1 mg protein of tissue.

2.9. Reduced glutathione (GSH) and oxidized form of glutathione (GSSG) assay

Fluorometric estimation was used to assay GSH and GSSG. Briefly, 200 μl sample was placed in a glass centrifuge tube and 200 μl of 10% TCA was added and mixed, then it was centrifuged at 5000 × *g* for 10 min. 100 μl supernatant was taken to another tube and mixed with 100 μl formaldehyde, 3.6 ml 0.1 M phosphate buffer (pH 8.3, 5 mM EDTA) and 200 μl 0.1% OPT. After being kept at room temperature for 40 min, the mixture was measured with a fluorometric spectrophotometer at 425 nm with activation at 350 nm. For GSSG, fluorescence at 422 nm was determined with the activation at 338 nm. Commercially procured GSH and GSSG were used to determine a standard curve.

2.10. Total antioxidant T-AOC assay

The total antioxidant capacities of the serum were measured using a kit purchased from the Nanjing Jiancheng Institute (Nanjing, China). The results from this kit did not distinguish between lipid- and water-soluble antioxidants but rather provided an estimate of the total antioxidant capacity.

2.11. RNA isolation and reverse transcriptase-polymerase chain reaction analysis (RT-PCR)

Total RNA was extracted from the liver tissue using trizol-reagent (Invitrogen, USA). cDNA was synthesized from 1 μg total RNA using an oligo dT primer in a reaction volume of 20 μl, using a reverse transcription kit according to the manufacturer's instructions. The cDNA was analyzed by polymerase chain reaction (PCR), as described previously. β-Actin was used as a control. The forward and reverse primers were: CAT (forward 5′-CAG CTC CGC AAT CCT ACA CC-3′ and reverse 5′-CAG CGT TGA TTA CAG GTG ATC C-3′); GPx1 (forward 5′-GGG ACT ACA CCG AGA TGA AC-3′ and reverse 5′-TCC GCA GGA AGG TAA AGA-3′); MnSOD (forward 5′-ATC CAC TTC GAG CAG AAG-3′ and reverse 5′-TTC CAC CTT TGC CCA AGT-3′); and β-actin (forward 5′-TCA CCC ACA CTG TGC CCA TCT A-3′ and reverse 5′-CAT CGG AAC CGC TCA TTG CCG ATA G-3′).

2.12. Western blotting

Western blot analysis was performed as follows. Briefly, to obtain total proteins, tissues were lysed for 30 min with a buffer containing 10 mM Tris–Cl, 100 mM NaCl, 1 mM EDTA, 100 μg/ml PMSF and 1 μg/ml Aprotinin. The lysates were centrifuged at 20,000 ×*g* for 20 min, and the supernatants were added with same volume of 2× SDS buffer which contain 100 mM Tris–HCl, 200 mM DTT, 4% SDS, 0.2% Bromphenol Blue and 20% glycerine. Then the mixture was boiled for 10 min and centrifuged at 10,000 × *g* for 10 min. The supernatants were used for immunoblotting. Protein extractions were separated by using SDS-PAGE on 10% polyacrylamide gels, and transferred to nitrocellulose membranes (Millipore, USA). After blocking for 1 h with 8% skimmed milk in TBST buffer (10 mM Tris, 150 mM NaCl, and 0.1% Tween-20), the membrane was incubated with primary antibodies against CAT, MnSOD, GPx1 and β-actin for 1 h at 37 °C. Specific antibody binding was detected by horseradish peroxidase-conjugated secondary antibodies and visualized using enhanced chemiluminescence detection reagent (Santa Cruz). The band densities were quantified from three different observations using an image analyzer Quantity One System (Bio-Rad, USA). All protein quantifications were adjusted for the corresponding β-actin level, which was not consistently changed by the different treatment conditions.

2.13. Statistical analysis

Data were expressed as mean ± S.D. Statistical analysis was performed by analysis of variance (ANOVA) followed by LSD test for multiple comparisons. Data analyses were performed using the SPSS 13.0. software package. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Isolation and purification of SMPW1

In the present work, the ground powder of *S. miltiorrhiza* was defatted with 95% EtOH. Then the residue was extracted with hot water and precipitated with 95% EtOH to yield crude protein–polysaccharide complex. After the Sevag method was used to remove protein components, the remaining solution was then excessively dialyzed for 48 h, precipitated by adding 95% EtOH, collected by centrifugation and washed successively with ethanol, acetone, ether, and vacuum-dried to give a light yellow powder, namely crude polysaccharides. The yield of crude polysaccharides was 6.9% of the dried material.

Table 1
Effect of SMPW1 treatment on the body weight and the levels of FBG and FINS in rats.

Group	Weight (g)	FBG (mmol/l)	FINS (mmol/l)	ISI
A: Control	226 ± 17.28	4.68 ± 0.21	22.25 ± 4.57	−4.57 ± 0.21
B: HGF	227 ± 18.87	5.0 ± 0.23	26.63 ± 2.32	−4.93 ± 0.24
C: *t*-BHP	218 ± 10.06[a]	5.40 ± 0.47[a]	35.46 ± 6.05[a]	−5.16 ± 0.29[a]
D: SMPW1 (50 mg/kg)	221 ± 12.34[b,c]	4.88 ± 0.70[b,c]	23.67 ± 4.68[b,c]	−4.61 ± 0.23[b,c]
E: SMPW1 (100 mg/kg)	220 ± 12.55[b,c]	4.68 ± 0.38[b,c]	22.04 ± 3.21[b,c]	−4.52 ± 0.18[b,c]

[a] Compared with A, $p < 0.05$.
[b] Compared with B, $p < 0.05$.
[c] Compared with C, $p < 0.05$.

Then crude polysaccharides were subjected to a DEAE-Sephadex A-50 chromatography and eluted stepwise with distilled water, 0.1, 0.2, 0.4, and 1.0 M NaCl, respectively. The fraction eluted with distilled water was lyophilized and was purified successively on a Sephadex G-200 column equilibrated and eluted with 0.1 M NaCl, giving two fractions of SMPW1 and SMPW2. The yield of SMPW1 and SMPW2 was about 52.6% and 13.7% from the crude polysaccharide, respectively. Because the large amount of SMPW1 is accessible, it was further analyzed for their physicochemical properties and protective effect against *t*-BHP-induced IR in rats. SMPW1 under investigation consisted for 92.1% (w/w) of carbohydrates. Besides SMPW1 contained 2.3% (w/w) uronic acid and 3.5% (w/w) protein. The detailed structural characterization for this polysaccharide is ongoing to be elucidated by the combination of both chemical and physical analysis.

3.2. Effect of SMPW1 on t-BHP induced IR in rats

Compared with control group, the weight of the *t*-BHP group lost obviously, and the contents of FBG, FINS, TG, TCH and FFA increased respectively ($p < 0.05$) (Table 1 and Fig. 1A–C). On contrary, SMPW1 treatment could decrease the contents of FBG, FINS, TCH, TG and FFA in rats treated with HGF and *t*-BHP, especially at the dose of 100 mg/kg, thus leading to insulin sensitivity obviously ($p < 0.05$).

3.3. Effect of SMPW1 on t-BHP induced lipid peroxidation in rats

The concentration of MDA, an index of lipid peroxidation, was increased in the serum and liver of *t*-BHP group and also in HGF group (Figs. 2A and 3A). Treatment with SMPW1 significantly reduced the formation of MDA both in serum and livers, with respect to that of control group.

3.4. Effects of SMPW1on t-BHP induced toxicity in rats

A single dose of *t*-BHP (0.2 mmol/kg) administered to the rats by i.p. injection decreased their GPx, CAT and SOD activities in serum and livers, This acute hepatotoxicity reaction was significantly ($p < 0.05$) restored in all animals treated with SMPW1 (Figs. 2B–D and 3B–D). GSH is known to play a protective role against *t*-BHP induced toxicity (Joyeux, Rolland, Fleurentin, Mortier, & Dorfman, 1990), and the oxidative stress of tissue generally involves the GSH system. Therefore, we measured the level of GSH for every group of serum and livers. The

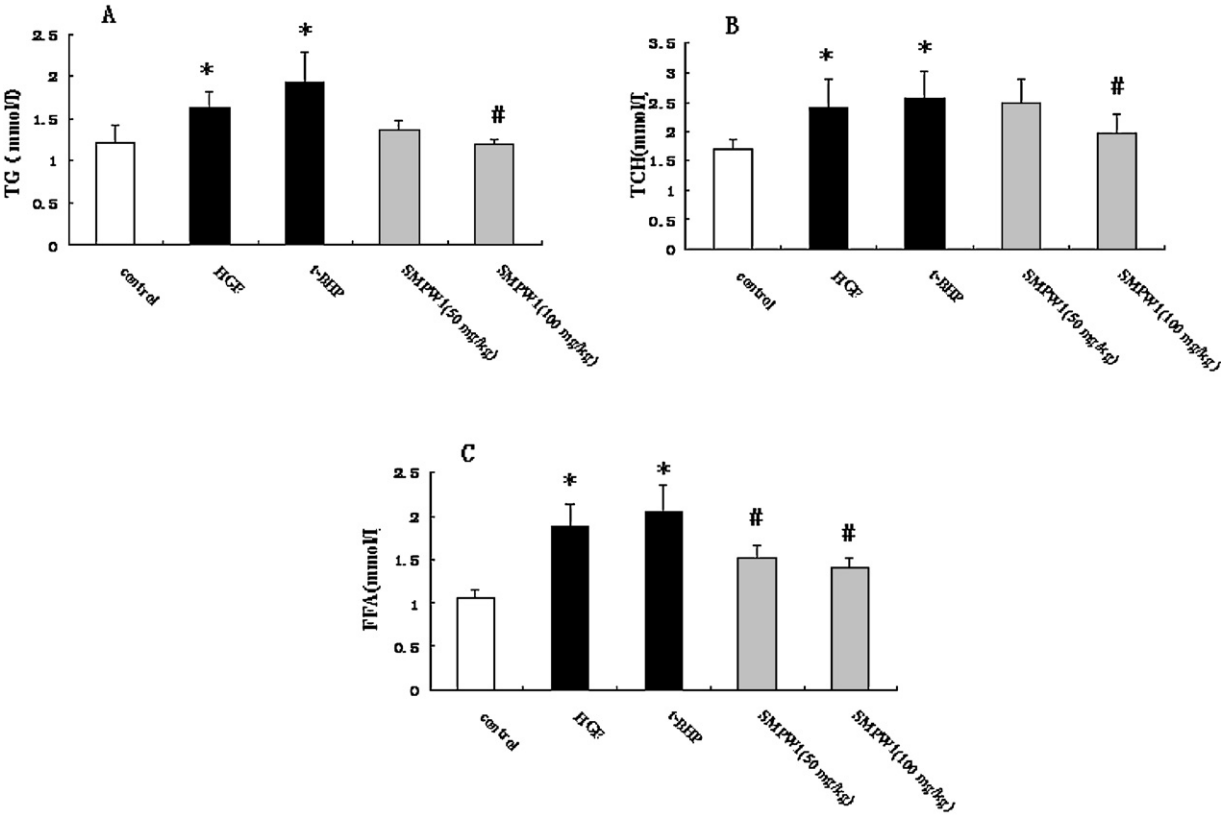

Fig. 1. Effect of SMPW1 treatment on *t*-BHP induced IR in rats. (A) Effects of SMPW1 on TG concentrations; (B) effects of SMPW1 on TCH concentrations; and (C) effects of SMPW1 on FFA concentrations. *Compared with A, $p < 0.05$; #Compared with C, $p < 0.05$.

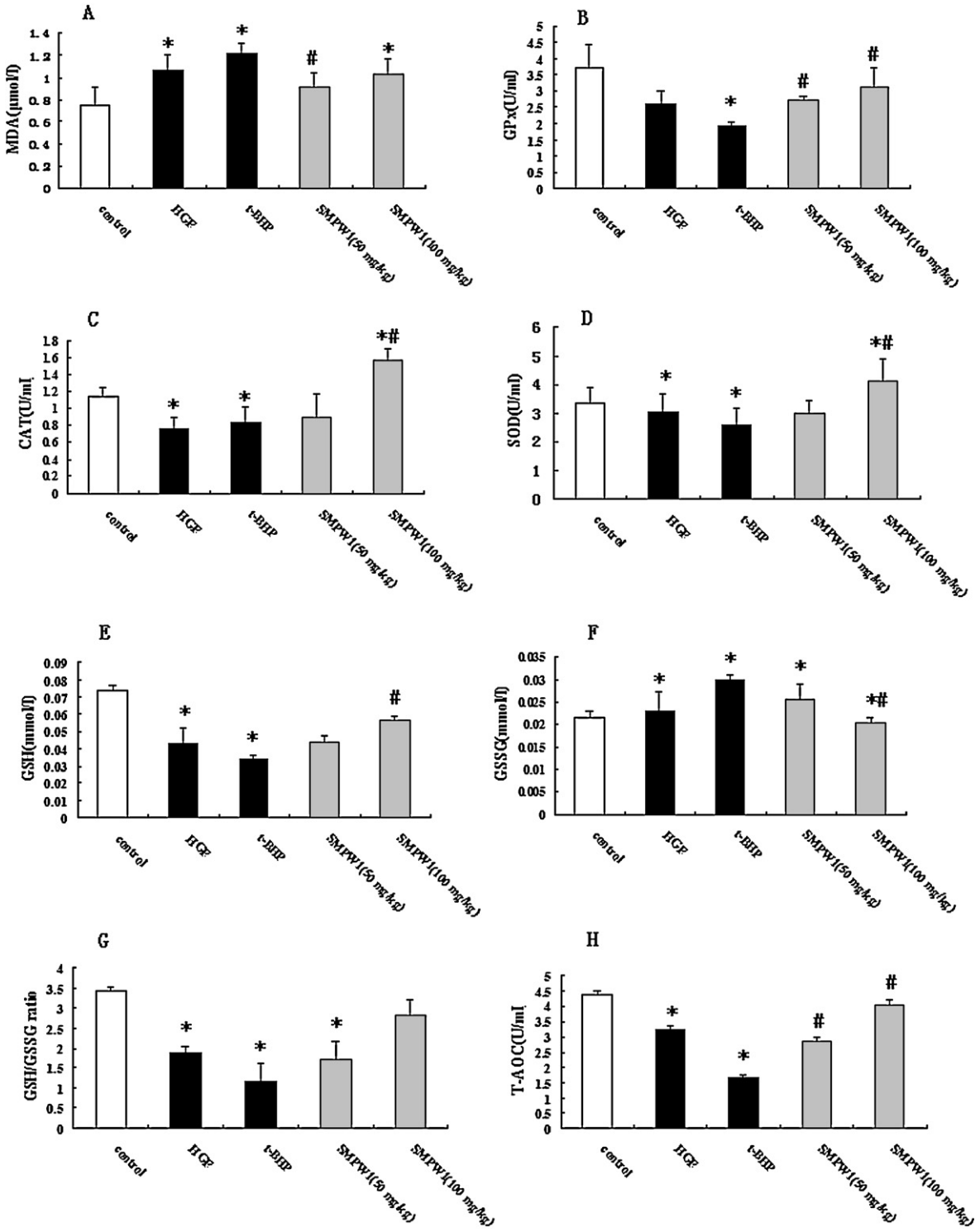

Fig. 2. Effects of SMPW1 treatment on *t*-BHP-induced oxidative stress in serum of rats. (A) Effects of SMPW1 on MDA concentrations; (B) effects of SMPW1 on GPx activity; (C) effects of SMPW1 on CAT concentrations; (D) effects of SMPW1 on SOD activity; (E) effects of SMPW1 on GSH activity; (F) effects of SMPW1on GSSG concentrations; (G) effects of SMPW1 on the ratio of GSH/GSSG; and (H) effects of SMPW1 on total antioxidant competence (T-AOC). *Compared with A, $p < 0.05$; #Compared with C, $p < 0.05$.

administration of *t*-BHP significantly reduced the level of GSH and increased that of GSSG ($p < 0.05$), whereas treatment with SMPW1 significantly protected against GSH reduction and GSSG increment (Figs. 2E, F and 3E, F). As shown in (Figs. 2G and 3G), the GSH/GSSG ratio was significantly decreased in the HGF group and *t*-BHP group, but was increased in the SMPW1 group. The same changes were seen in T-AOC of serum and livers (Figs. 2H and 3H).

3.5. Changes in transcriptional expression of antioxidant enzymes

RT-PCR was used to evaluate the expressions of the antioxidant enzyme transcripts [manganese superoxide dismutase (MnSOD), CAT and GPx1] from livers. Densitometric scan analysis showed that MnSOD, CAT and GPx1 mRNA were decreased significantly in the *t*-BHP treated rats compared to that of the control group (Fig. 4). Surprisingly, SMPW1 (50 and 100 mg/kg) treatment can increase

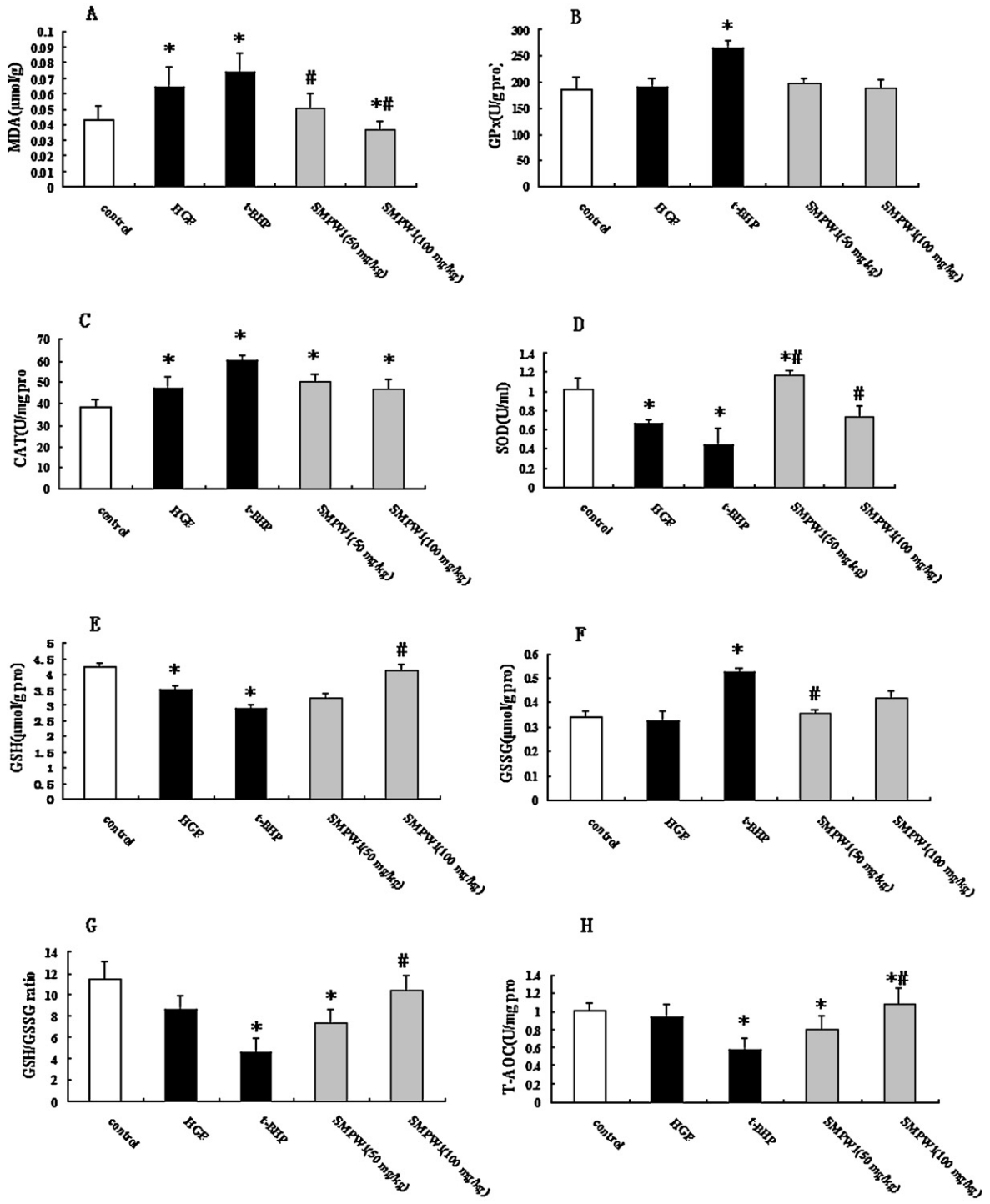

Fig. 3. Effects of SMPW1 treatment on *t*-BHP-induced oxidative stress in liver of rats. (A) Effects of SMPW1 on MDA concentrations; (B) effects of SMPW1 on GPx activity; (C) effects of SMPW1 on CAT concentrations; (D) effects of SMPW1 on SOD activity; (E) effects of SMPW1 on GSH activity; (F) effects of SMPW1on GSSG concentrations; (G) effects of SMPW1 on the ratio of GSH/GSSG; and (H) effects of SMPW1 on total antioxidant competence (T-AOC). *Compared with A, $p < 0.05$; #compared with C, $p < 0.05$.

the expressions of CAT, GPx1 and MnSOD mRNA in comparison to HGF and *t*-BHP group.

3.6. Changes in protein level of antioxidant enzymes

The protein expression of antioxidant enzymes of CAT, GPx1 and MnSOD in the *t*-BHP treated group decreased significantly compared with that of the control group. The expressions of MnSOD, CAT, and GPx1 were also enhanced by SMPW1 (50 and 100 mg/kg), comparing with HGF or *t*-BHP treatment alone (Fig. 5). These results indicate that SMPW1 can obviously decrease the oxidative stress and also clean free radicals by stimulating the activities of antioxidant enzymes, which would be beneficial for preventing from insulin resistant efficiently.

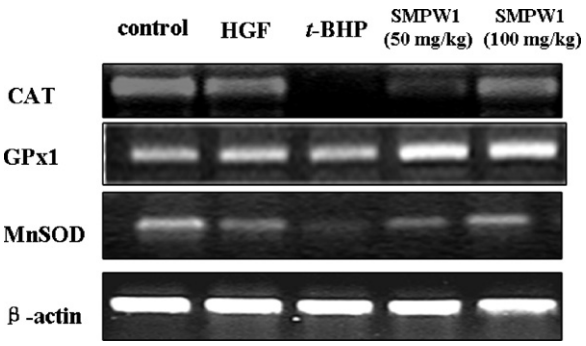

Fig. 4. Effects of SMPW1 treatment on mRNA expression of antioxidant enzymes in liver of rats. Each total of RNA preparation was reverse transcribed and the cDNA products were amplified by PCR using gene-specific primers. The bands correspond to CAT, GPx1, MnSOD, β-actin of control, the double high diet (HGF), *t*-BHP and SMPW1 (50 and 100 mg/kg) treated groups.

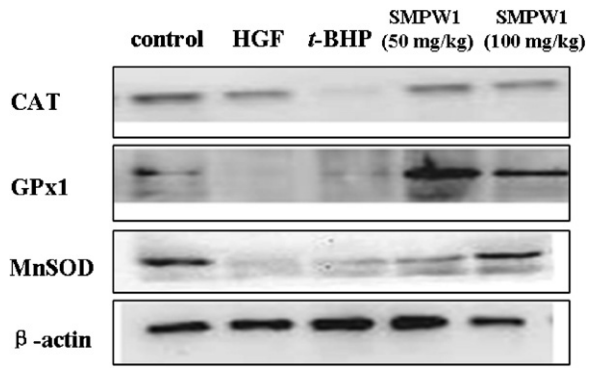

Fig. 5. Effects of SMPW1 treatment on protein expression of antioxidant enzymes in liver of rats. Fifty micrograms of proteins was electrophoresed on a 10% polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membranes. Protein expressions were detected by Western blot analysis. The bands correspond to CAT, GPx1, MnSOD, β-actin of control, the double high diet (HGF), *t*-BHP and SMPW1 (50 and 100 mg/kg) treated groups.

3.7. Changes in histopathology of liver and pancreas

The treatment with *t*-BHP caused degeneration in hepatic cells, such as hepatic cords, focal necrosis, congestion in central vein, and infiltration of lymphocytes (Fig. 6C). *t*-BHP also induced confluent repair necrosis, which is frequently observed in the case of swelling of the pancreas cells, as well as neutrophilic infiltration and several ballooning degenerations (Fig. 7C). However, according to the microscopic examinations, the severe hepatic and pancreatic lesions induced by *t*-BHP were considerably reduced by the administration of SMPW1 at doses of 50 and 100 mg/kg. Necrosis, which

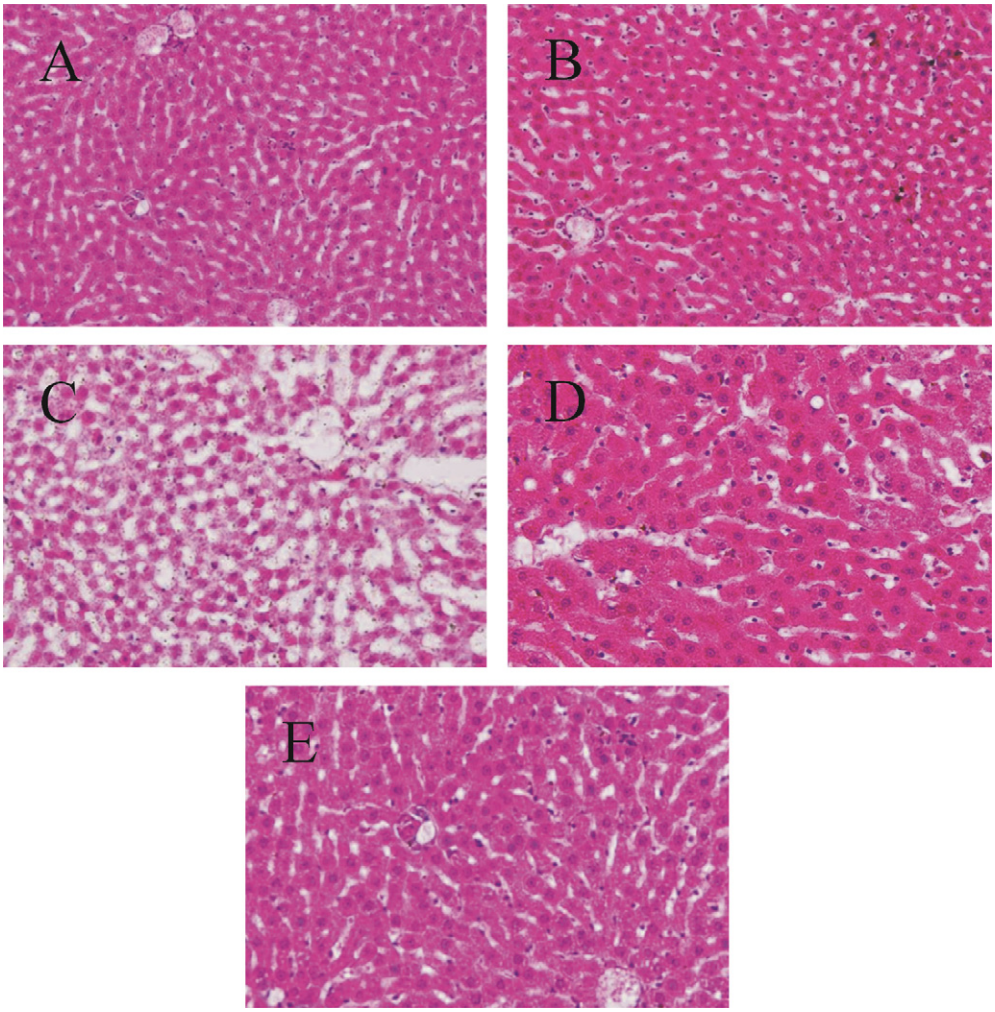

Fig. 6. Effects of SMPW1 treatment on *t*-BHP-induced liver damage in rats. (A) Saline control group; (B) rats fed with high fat and high glucose diet (HGF) and treated with saline; (C) HGF rats treated with *t*-BHP; (D) HGF rats treated with *t*-BHP plus SMPW1 (50 mg/kg); and (E) HGF rats treated with *t*-BHP plus SMPW1 (100 mg/kg). hematoxylin/eosin staining; magnification 400×.

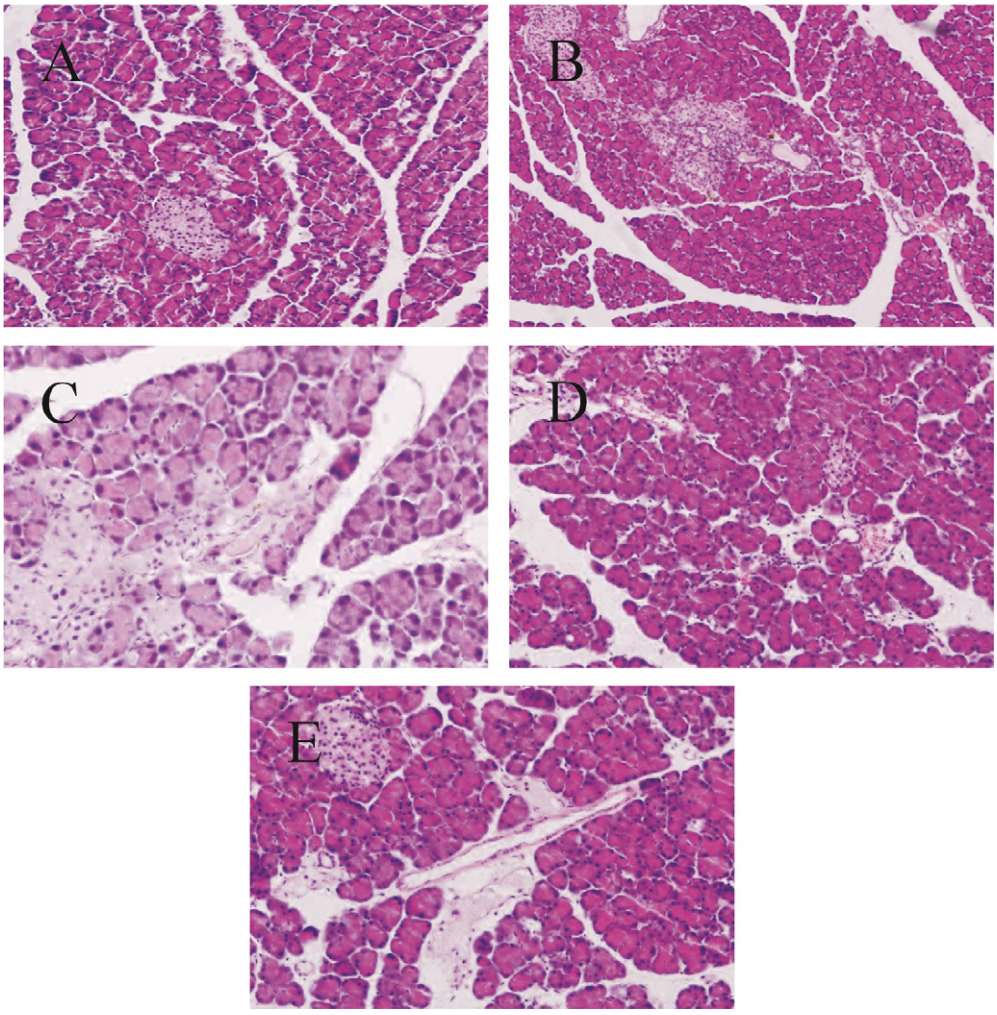

Fig. 7. Effects of SMPW1 treatment on *t*-BHP-induced pancreas damage in rats. (A) Saline control group; (B) rats fed with high fat and high glucose diet (HGF) and treated with saline; (C) HGF rats treated with *t*-BHP; (D) HGF rats treated with *t*-BHP plus SMPW1 (50 mg/kg); and (E) HGF rats treated with *t*-BHP plus SMPW1 (100 mg/kg). hematoxylin/eosin staining; magnification 400×.

is a more severe form of injury, was also markedly prevented and minimized by intervention with SMPW1 (Figs. 6D, E and 7D, E).

4. Discussion

The object of this study was to evaluate the protective effects of SMPW1 on IR model in rats induced by *t*-BHP. The results demonstrated that SMPW1 possessed an important antioxidant activity and could be developed to be a new prophylactic agent of T2DM. Compared with other two intervention groups, the group treated with SMPW1 showed significant beneficial effect on rats injuries induced by oxidant *t*-BHP.

Many studies indicated that the occurrence of IR in experimental animals fed by HGF was related to chronic oxidative stress, which was associated with the development and progression of diabetes and its complications. Antioxidants might be a new strategy for reducing diabetic complications. Oxidative stress, defined as a persistent imbalance between the production and removal of ROS, plays a major role in the pathogenesis of diabetes, cardiovascular disease, cancer, and neurodegenerative diseases. For example, a variety of stimuli, including hyperglycemia-elevated free fatty acids and cytokines, increase ROS production and induce oxidative stress in adipose and muscle tissues, which, if persist, will lead to IR. If pancreatic cells are unable to compensate for this IR by sufficiently increasing insulin secretion, blood glucose levels would increase and T2DM develops (LeRoith, 2002).

t-BHP, a short-chain analog of lipid peroxide, exerts its oxidative role by releasing hydrogen peroxide after the metabolism in vivo. Two distinctive pathways are involved in the metabolism of *t*-BHP in hepatocytes. The first employs the microsomal cytochrome P-450 system leading to the production of ROS, such as peroxyl and alkoxyl radicals, which initiate lipid peroxidation (Hogberg, Orrenius, & O'Brien, 1975); while the second concerns a reaction involving GSH peroxidase and its substrate GSH, which converts *t*-butanol and GSSG. The GSSG is then reduced to GSH by GSH reductase, resulting in NADPH oxidation. Decreased GSH and oxidized NADPH contribute to altering Ca^{2+} homeostasis, which is considered to be a major event in *t*-BHP induced toxicity (Shimizu et al., 1998). So we employed HGF and *t*-BHP to induce oxidative damage and IR in vivo to investigate the protective potential of antioxidant on *t*-BHP induced hepatic damage and IR in rats. Usage of HGF and oxidant *t*-BHP injection produces a large amount of free radicals and reactive oxygen species to cause oxidative stress injury to decrease the antioxidative capability and increase lipid peroxidation capability on the experimental subjects. Compared with those of control group, the contents of FBG and FINS increased respectively, the contents of TG, TCH and FFA also increased. IR was increased in the *t*-BHP group. The results showed that the IR model can be established quickly and stably by HGF in addition of

oxidant *t*-BHP injection and the occurrence of IR in experimental animals fed by HGF was closely related to chronic oxidative stress.

The in vivo study showed that SMPW1 was able to quench reactive free radicals and improve IR. In the hepatotoxicity and lipid peroxidation experiments, SMPW1 not only decreased the contents of FBG, FINS,TG, TCH and FFA, but also attenuated IR and the morphological injury of liver and pancreas induced by *t*-BHP. Meanwhile SMPW1 also improved insulin sensitivity index and increased the activity of SOD and the ratio of GSH/GSSG in serum and liver homogenates. SMPW1 was shown to possess high antioxidative and protective capacity against the injury induced by *t*-BHP, as reflected in the increased activities of CAT, MnSOD, GPx1 and the decreased formation of MDA. The experiment also indicated that *t*-BHP reduced the GSH level and increased the GSSG level in serum and liver, however SMPW1 blocked these phenomena effectively. In addition, the intervention with SMPW1 significantly protected against the GSH depletion induced by *t*-BHP.

In conclusion, the results of this study indicated that the IR model can be established quickly and stably by HGF in addition of *t*-BHP injection and it was a convenient and feasible method with high achievement ratio. The occurrence of IR in experimental animals fed by HGF was related to chronic oxidative stress. SMPW1 can protect against the development of T2DM and improve IR via reduction of oxidative stress. Future studies are ongoing to confirm the effectiveness of this supplement in patients for studying prevention and treatment on T2DM.

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References

Baynes, J. W. (1991). Role of oxidative stress in development of complications in diabetes. *Diabetes*, *40*, 405–412.

Ceriello, A., & Motz, E. (2004). Is oxidative stress the pathogenic mechanism underlying IR, diabetes, and cardiovascular disease? The common soil hypothesis revisited. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *24*, 816–823.

DeFronzo, R. A. (1997). Pathogenesis of type 2 diabetes: Metabolic and molecular implications for identifying diabetes genes. *Diabetes Reviews*, *5*, 177–269.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Evans, J. L., Goldfine, I. D., Maddux, B. A., & Grodsky, G. M. (2002). Oxidative stress and stress-activated signaling pathways: A unifying hypothesis of type 2 diabetes. *Endocrine Reviews*, *23*, 599–622.

Evans, J. L., Goldfine, I. D., Maddux, B. A., & Grodsky, G. M. (2003). Are oxidative stress-activated signaling pathways mediators of IR and beta-cell dysfunction? *Diabetes*, *52*, 1–8.

Filisetti-Cozzi, T. M. C. C., & Carpita, N. C. (1991). Measurement of uronic acids without interference from neutral sugars. *Analytical Biochemistry*, *197*, 157–162.

Gopaul, N. K., Manraj, M. D., Hebe, A., Lee, Kwai., Yan, S., Johnstone, A., et al. (2001). Oxidative stress could precede endothelial dysfunction and IR in Indian Mauritians with impaired glucose metabolism. *Diabetologia*, *44*, 706–712.

Grodsky, G. M. (2000). Kinetics of insulin secretion: Underlying metabolic events in diabetes mellitus. In D. Le Roith, S. I. Taylor, & J. M. Olefsky (Eds.), *Diabetes mellitus: A fundamental and clinical text* (pp. 2–11). Philadelphia: Lippincott Williams & Wilkins.

Hogberg, J., Orrenius, S., & O'Brien, P. J. (1975). Further studies on lipidperoxide formation in isolated hepatocytes. *European Journal of Biochemistry*, *59*, 449–455.

Jang, S. I., Jeong, S. I., Kim, K. J., Kim, H. J., Yu, H. H., Park, R., et al. (2003). Tanshinone IIA from *Salvia miltiorrhiza* inhibits inducible nitric oxide synthase expression and production of TNF-alpha IL-1beta and IL-6 in activated RAW 264.7 cells. *Planta Medica*, *69*, 1057–1059.

Jiang, Z. S., Zhang, S. L., & Cai, X. J. (1997). Effect of *Salvia miltiorrhiza* composita on superoxide dismutase and malonyldialdehyde in treating patients with non-insulin dependent diabetes mellitus (NIDDM). *Zhongguo Zhong Xi Yi Jie He Za Zhi*, *17*, 32–34.

Joyeux, M., Rolland, A., Fleurentin, J., Mortier, F., & Dorfman, P. (1990). *t*-BHP-induced injury in isolated rat hepatocytes: A model for studying anti-hepatotoxic crude drugs. *Planta Medica*, *56*, 171–174.

Jung, S. H., Seol, H. J., Jeon, S. J., Son, K. H., & Lee, J. R. (2009). Insulin-sensitizing activities of tanshinones, diterpene compounds of the root of *Salvia miltiorrhiza* Bunge. *Phytomedicine*, *16*, 327–335.

Kono, Y. (1978). Generation of superoxide radical during autoxidation of hydroxylamine and an assay for superoxide dismutase. *Archives of Biochemistry and Biophysics*, *186*, 189–195.

LeRoith, D. (2002). Beta-cell dysfunction and IR in type 2 diabetes: Role of metabolic and genetic abnormalities. *The American Journal of Medicine*, *113*, 3S–11S.

Liu, G., Guan, G. J., Qi, T. G., Fu, Y. Q., Li, X. G., Sun, Y., et al. (2005). Protective effects of *Salvia miltiorrhiza* on rats with streptozotocin diabetes and its mechanism. *Zhong Xi Yi Jie He Xue Bao*, *3*, 459–462.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry*, *193*, 265–275.

Paglia, D. E., & Valentine, W. N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *The Journal of Laboratory and Clinical Medicine*, *70*, 158–169.

Qian, S., Huo, D., Wang, S., & Qian, Q. (2011). Inhibition of glucose-induced vascular endothelial growth factor expression by *Salvia miltiorrhiza* hydrophilic extract in human microvascular endothelial cells: Evidence for mitochondrial oxidative stress. *Journal of Ethnopharmacology*, *137*, 985–991.

Qian, Q. W., Qian, S. H., Fan, P., Huo, D. X., & Wang, S. J. (2011). Effect of *Salvia miltiorrhiza* hydrophilic extract on antioxidant enzymes in diabetic patients with chronic heart disease: A randomized controlled trial. *Phytotherapy Research*, *26*, 60–66.

Rathmann, W., & Giani, G. (2004). Global prevalence of diabetes: Estimates for the year 2000 and projections for 2030. *Diabetes Care*, *27*, 2568–2569.

Reaven, G. M. (1988). Role of IR in human disease. *Diabetes*, *37*, 1595–1607.

Reaven, G. M. (2000). IR and its consequences: Type 2 diabetes mellitus and coronary heart disease. In D. LeRoith, S. I. Taylor, & J. M. Olefsky (Eds.), *Diabetes mellitus: A fundamental and clinical text* (pp. 604–615). Philadelphia: Lippincott Williams & Wilkins.

Robertson, R. P., Harmon, J. S., Tanaka, Y., Sacchi, G., Tran, P. O., Gleason, C. E., et al. (2000). Glucose toxicity of the β-cell: Cellular and molecular mechanisms. In D. Le Roith, S. I. Taylor, & J. M. Olefsky (Eds.), *Diabetes mellitus: A fundamental and clinical text* (pp. 125–132). Philadelphia: Lippincott Williams & Wilkins.

Staub, A. M. (1965). Removal of protein-Sevag method. *Methods in Carbohydrate Chemistry*, *5*, 5–6.

Shimizu, S., Eguchi, Y., Kamiike, W., Funahashi, Y., Mignon, A., Lacronique, V., et al. (1998). Bcl-2 prevents apoptotic mitochondrial dysfunction by regulating proton flux. *Proceeding of the National Academy Sciences USA*, *95*, 1455–1459.

Tiedge, M., Lortz, S., Drinkgern, J., & Lenzen, S. (1997). Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin producing cells. *Diabetes*, *46*, 1733–1742.

Vijayalingam, S., Parthiban, A., Shanmugasundaram, K. R., & Mohan, V. (1996). Abnormal antioxidant status in impaired glucose tolerance and noninsulin-dependent diabetes mellitus. *Diabetic Medicine*, *13*, 715–719.

Yagi, K. (1976). A simple fluorometric assay for lipoperoxide in blood plasma. *Biochemical Medicine*, *15*, 212–216.

Zhou, L., Zuo, Z., & Chow, M. S. S. (2005). Danshen: An overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. *Journal of Clinical Pharmacology*, *45*, 1345–1359.