7-*O*-Galloyl-D-sedoheptulose Attenuates Oxidative Stress-Induced Diabetic Injury *via* Decreasing Expression of Nuclear Factor- κ B- and Apoptosis-Related Protein in the Liver

Jeong Sook Noh,^{a,b} Chan Hum Park,^a Takashi Tanaka,^c and Takako Yokozawa^{*a,d}

^aInstitute of Natural Medicine, University of Toyama; 2630 Sugitani, Toyama 930–0194, Japan; ^bDepartment of Food Science and Nutrition, Pusan National University; 30 Jangjeon Dong, Kumjeong Ku, Busan 609–735, Korea; ^cGraduate School of Biomedical Sciences, Nagasaki University; 1–14 Bunkyo-cho, Nagasaki 852–8521, Japan; and ^dOrganization for Promotion of Regional Collaboration, University of Toyama; 3190 Gofuku, Toyama 930–8555, Japan.

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The present study was conducted to examine whether 7-*O*-galloyl-D-sedoheptulose (GS) has an ameliorative effect on diabetic alterations such as oxidative stress, inflammation, and apoptosis in the liver of type 2 diabetic *db/db* mice. GS was administered at 20 or 100 mg/kg body weight per day for 6 weeks to *db/db* mice, and its effect was compared with vehicle-treated *db/db* and *m/m* mice. In the serum and hepatic tissue, biochemical factors and protein expressions associated with nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, inflammation, and apoptosis were examined. As a result, GS administration to type 2 diabetic mice lowered serum and hepatic oxidative stress through the reduction of reactive oxygen species and lipid peroxidation. These results were derived, at least in part, from attenuating the expression of NADPH oxidase subunit proteins, Nox-4 and p22^{phox}. In the diabetic condition, augmented nuclear factor (NF)-E2-related factor 2 and heme oxygenase-1 were reduced with a decrease in oxidative stress on GS treatment. Furthermore, in the GS-treated group, NF- κ B-related pro-inflammatory factors and pro-apoptotic protein expressions were alleviated in the hepatic tissue. Taking these into consideration, our findings support the therapeutic evidence for GS ameliorating the development of diabetic complications *via* regulating oxidative stress, inflammation, and apoptosis.

Key words 7-*O*-galloyl-D-sedoheptulose; *db/db* mouse; hepatic injury; oxidative stress; apoptosis

Increased oxidative stress induced by hyperglycemia is associated with type 2 diabetes. Reactive oxygen species (ROS) activate stress-sensitive intracellular signaling pathways such as the transcription of nuclear factor- κ B (NF- κ B), which plays a central role in inflammation-related disease¹⁾ and also stimulates intracellular protein kinase cascades such as mitogen-activated protein kinase (MAPK), which mediate the induction of NF-E2-related factor 2 (Nrf2).^{2,3)} Several researchers have demonstrated that ROS generation induced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and the mitochondrial electron transport chain is an early event in diabetic development.^{4,5)} In addition, oxidative stress, in turn, activates different processes involving protein kinase C, cytokines, and others.¹⁾ Therefore, novel approaches are necessary to identify therapeutic agents which can act with a pleiotropic effect including antioxidant properties to prevent and/or treat diabetic development.

The continual activation of NF- κ B results in increased levels of pro-inflammatory cytokines, especially tumor necrosis factor- α (TNF- α) and interleukin-6, which are involved in hepatocellular injury from apoptotic cell death.^{6,7)} These cytokines also aggravate insulin resistance and hyperglycemia, represented as glucotoxicity in various tissues. It is known that high-level glucose induces endothelial apoptosis through activation of the Bax-caspase protease pathway.⁸⁾ The effectors of apoptosis are now well-known to be represented by a family of intracellular cysteine proteases known as caspases.⁹⁾ A feature of apoptosis is the serial activation of caspases which alters mitochondrial function, characterized by a reduction in the electrochemical gradient across the mitochondrial

membrane and release of mitochondrial cytochrome *c* into the cytoplasm,¹⁰⁾ and it is inhibited by the presence of B-cell lymphoma (Bcl)-xL in these organelles.^{11,12)} Translocation of pro-apoptotic Bax protein into the mitochondrial membrane is accompanied by a significant increase in caspase-3 and -9 activities.⁸⁾

7-*O*-Galloyl-D-sedoheptulose (GS, Fig. 1) is the bioactive polyphenol isolated from the low-molecular-weight fraction of Corni Fructus (*Cornus officinalis* SIEB. *et* ZUCC.) which was reported in our previous study to exhibit an attenuating effect on hyperglycemia-induced metabolic disorders in streptozotocin-induced diabetic rats.¹³⁾ GS showed the inhibition of lipid accumulation in the liver of hypertriglyceridemic rats, and exhibited regulatory activity on lipid-related metabolism by down-regulating the expression of sterol regulatory element binding protein-1 (SREBP-1) in the liver and kidney of a type 2 diabetic animal model.^{14,15)} Furthermore, GS administration exerted its hepato- and reno-protective effects against ROS-related formation of advanced glycation endproducts (AGE) and inflammation in types 1 and 2 diabetes.^{15,16)} In the present study, we investigated the effect of GS to ameliorate oxidative stress by decreasing ROS-sensitive protein expression and

Fig. 1. Chemical Structure of 7-*O*-Galloyl-D-sedoheptulose

The authors declare no conflict of interest.

*To whom correspondence should be addressed. e-mail: yokozawa@inm.u-toyama.ac.jp

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protect against hepatic injury *via* the inhibition of mitochondrial stress-apoptosis.

MATERIALS AND METHODS

Materials GS was isolated from Corni Fructus, as described previously.¹⁶ Protease inhibitor mixture, 4,6-dihydroxy-2-mercaptopyrimidine (2-thiobarbituric acid, TBA), and ethylenediaminetetraacetic acid (EDTA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 2',7'-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR, U.S.A.). The Bio-Rad protein assay kit and pure nitrocellulose membrane were purchased from Bio-Rad Laboratories (Tokyo, Japan). β -Actin and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Rabbit polyclonal antibodies against p22^{phox}, Nrf2, heme-oxygenase-1 (HO-1), NF- κ Bp65, Bax, cytochrome *c*, and mouse monoclonal antibody against cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), Bcl-2, and histone were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Poly anti-Nox-4 (LifeSpan BioSciences, Seattle, WA, U.S.A.) and caspase-3 (BioVision, CA, U.S.A.) were also used. Goat anti-rabbit and goat anti-mouse immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. ECL Western Blotting Detection Reagents were purchased from GE Healthcare (Piscataway, NJ, U.S.A.).

Animals and Treatment Animal experiments were performed according to the 'Guidelines for Animal Experimentation' approved by the Ethics Committee of the University of Toyama (Registration No.: S-2006 INM-22). Six-week-old male C57BLKS/J *db/db* and age-matched non-diabetic *m/m* mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). Mice were maintained under a 12-h light/dark cycle, and housed at a controlled temperature ($23 \pm 3^\circ\text{C}$) and humidity (about 60%). The mice were allowed free access to laboratory pellet chow (CLEA Japan Inc., Tokyo, Japan, comprising 24.0% protein, 3.5% lipids, and 60.5% carbohydrate) and water *ad libitum*. After adaptation (at 9 weeks of age), the glucose level of blood taken from the tail vein was measured, and then *db/db* mice were divided into three groups ($n=10/\text{group}$). GS was orally administered every day at a dose of 20 or 100 mg/kg body weight, respectively, while vehicle-treated *db/db* mice were orally given water. The non-diabetic *m/m* mice ($n=6$) as a normal control group were used for comparisons with the diabetic groups. The body weight, food intake, and water intake were measured every day during the administration period. After 6 weeks of administration, blood samples were collected by cardiac puncture from anesthetized mice. The serum was separated immediately from blood samples by centrifugation. Subsequently, mice were perfused with ice-cold physiological saline after cardiac puncture, and the liver was harvested, snap-frozen in liquid nitrogen, and stored at -80°C until analyses.

Measurement of Serum Parameters Serum glucose was measured using a commercial kit (Glucose CII-Test from Wako Pure Chemical Industries, Ltd., Osaka, Japan). Serum leptin and insulin (Morinaga Institute of Biological Science, Yokohama, Japan) levels were measured based on enzyme-linked immunosorbent assays. The serum ROS

generation level was determined using the method of Ali *et al.*,¹⁷ and the TBA-reactive substance (TBARS) concentration was examined employing the method of Naito and Yamanaka.¹⁸ Hepatic functional parameters (alanine aminotransferase, ALT; aspartate aminotransferase, AST) were determined using a commercial kit (Transaminase CII-Test, Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Assessment of Hepatic ROS Generation and TBARS Levels ROS generation was measured employing the method of Ali *et al.*¹⁷ Hepatic tissues were homogenized on ice with 1 mM EDTA-50 mM sodium phosphate buffer (pH 7.4), and then 25 mM DCFH-DA was added to homogenates. After incubation for 30 min, the changes in fluorescence values were determined at an excitation wavelength of 486 nm and emission wavelength of 530 nm. The hepatic TBARS content was determined using the method of Uchiyama and Mihara.¹⁹

Preparation of Nuclear and Post-Nuclear Fractions Nuclear protein extraction was performed according to the method of Komatsu.²⁰ In brief, hepatic tissues were homogenized with ice-cold lysis buffer containing 5 mM Tris-HCl (pH 7.5), 2 mM MgCl_2 , 15 mM CaCl_2 , and 1.5 M sucrose, and then 0.1 M dithiothreitol (DTT) and protease inhibitor mixture were added. After centrifugation ($10500 \times g$ for 20 min at 4°C), the pellet was suspended with extraction buffer containing 20 mM 2-[4-(2-hydroxyethyl)-1-piperazyl]ethanesulfonic acid (pH 7.9), 1.5 mM MgCl_2 , 0.42 M NaCl, 0.2 mM EDTA, and 25% (v/v) glycerol, and then 0.1 M DTT and protease inhibitor mixture were added. The mixture was placed on ice for 30 min. The nuclear fraction was prepared by centrifugation at $20500 \times g$ for 5 min at 4°C . The post-nuclear fraction was extracted from the liver of each mouse as described below. In brief, hepatic tissue was homogenized with ice-cold lysis buffer (pH 7.4) containing 137 mM NaCl, 20 mM Tris-HCl, 1% Tween 20, 10% glycerol, 1 mM PMSF, and protease inhibitor mixture. The homogenate was then centrifuged at $2000 \times g$ for 10 min at 4°C . The protein concentration in each fraction was determined using a Bio-Rad protein kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Immunoblotting Analyses For the determination of Nrf2 and NF- κ Bp65, 10 μg of protein from each nuclear fraction was electrophoresed through a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated protein was transferred to a nitrocellulose membrane, blocked with 5% (w/v) skim milk solution for 1 h, and then incubated with primary antibody to Nrf2 and NF- κ Bp65 overnight at 4°C . After the blots were washed, they were incubated with anti-rabbit or anti-mouse IgG HRP-conjugated secondary antibody for 1.5 h at room temperature. Also, 10 μg of protein of each post-nuclear fraction of Nox-4, p22^{phox}, HO-1, COX-2, iNOS, Bax, Bcl-2, cytochrome *c*, and caspase-3 was electrophoresed through 8–15% SDS-PAGE. Each antigen-antibody complex was visualized using ECL Western Blotting Detection Reagents and detected by chemiluminescence with LAS-4000 (FUJIFILM, Tokyo, Japan). Band densities were determined using ATTO Densitograph Software (ATTO Corporation, Tokyo, Japan) and quantified as the ratio to histone or β -actin. The protein levels of groups are expressed relative to those of *m/m* mice (represented as 1).

Histological Examination The excised parts of livers were immediately fixed in 10% neutral-buffered formalin and, after embedding in paraffin, they were cut into 5- μm thick

sections. After hematoxylin-eosin (HE) staining, these sections were examined with a light microscope.

Statistical Analysis Data are expressed as means±standard error of mean (S.E.M.). Significance was assessed employing one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test (SPSS 11.5.1 for Windows, 2002, SPSS Inc., U.S.A.). Values of $p < 0.05$ were considered significant.

RESULTS

Body Weight, Food Intake, Water Intake, and Liver Weight In *db/db* mice, their weight gain after 6-week experimental periods was greater than in age-matched *m/m* mice, which was accompanied by a significant increase in food and water intake. This augmentation in *db/db* mice was significantly alleviated by GS treatment except for weight gain. Furthermore, the liver weight in *db/db* mice was significantly raised compared to that in *m/m* mice; however, it was slightly reduced by GS treatment (Table 1).

Hematological Analyses As shown in Table 2, *db/db* vehicle mice showed diabetic characteristics such as hyperglycemia, hyperleptinemia, and hyperinsulinemia compared with *m/m* mice. GS administration lowered the serum glucose

concentration and significantly attenuated leptin and insulin concentrations in the serum of *db/db* mice. Furthermore, the type 2 diabetic state exhibited rising oxidative stress due to increased ROS and TBARS in the serum of *db/db* mice; however, GS administration markedly reduced these levels in a dose-dependent manner. Concerning hepatic functional parameters, serum ALT and AST levels in *db/db* mice were increased compared to in *m/m* mice, whereas these augmented levels showed a significant decrease at a dose of 100 mg/kg.

Hepatic ROS and TBARS Levels As shown in Table 3, the levels of ROS and TBARS in the liver of vehicle-treated *db/db* mice were both markedly increased compared to those in *m/m* mice, whereas these elevated levels were significantly reduced in GS-treated *db/db* mice dose-dependently. Especially, hepatic ROS and TBARS levels were lowered nearly to those of *m/m* mice by 100 mg/kg GS treatment.

Hepatic NADPH Oxidase Subunit-Related Protein Expressions The expression levels of NADPH oxidase subunit proteins, Nox-4 and p22^{phox}, were enhanced in the livers of *db/db* mice at the age of 15 weeks, with the results presented in Fig. 2. These increased protein expressions in the liver of *db/db* mice were significantly attenuated in a dose-dependent manner by GS treatment.

Hepatic Oxidative Stress-Related Protein Expressions

Table 1. Body Weight, Food Intake, and Water Intake

Group	Body weight			Food intake (g/d)	Water intake (mL/d)	Liver weight (g/100 g body weight)
	Initial (g)	Final (g)	Gain (g/6 weeks)			
<i>m/m</i>	21.7±0.4**	25.1±0.6**	3.4±0.1**	3.0±0.2**	3.6±0.2**	5.67±0.39**
<i>db/db</i>						
Veh	40.3±0.7	47.1±0.9	6.8±0.6	7.1±0.1	18.9±3.8	7.58±0.33
GS20	40.2±0.7	45.4±1.4	5.4±0.7	6.0±0.1*	14.1±0.2*	7.37±0.44
GS100	40.1±0.6	44.1±1.2	4.0±0.8	5.4±0.1*	13.3±0.2*	7.12±0.35

m/m, Misty; Veh, vehicle-treated *db/db* mice; GS20, 7-O-galloyl-D-sedoheptulose 20 mg/kg body weight-treated *db/db* mice; GS100, 7-O-galloyl-D-sedoheptulose 100 mg/kg body weight-treated *db/db* mice. The results are presented as the means±S.E.M. * $p < 0.05$, ** $p < 0.001$ vs. vehicle-treated *db/db* mouse values.

Table 2. Hematological Analyses

Item	<i>m/m</i>	<i>db/db</i>		
		Veh	GS20	GS100
Glucose (mg/dL)	194±8***	746±33	725±28	683±41
Leptin (ng/dL)	2.0±0.3***	20.2±0.3	19.5±0.7	17.8±1.1*
Insulin (ng/mL)	1.82±0.06*	3.25±0.41	2.49±0.09	2.40±0.04*
ROS (fluorescence/min/mL serum)	577±79***	1334±128	790±85**	632±97***
TBARS (nmol/mL)	10.6±0.5**	19.7±1.4	12.6±1.6**	8.7±2.7***
ALT (IU/L)	57.4±3.5***	145.8±6.6	126.6±8.6	122.4±8.1*
AST (IU/L)	7.7±0.7***	51.4±5.3	45.0±6.1	38.8±2.7*

m/m, Misty; Veh, vehicle-treated *db/db* mice; GS20, 7-O-galloyl-D-sedoheptulose 20 mg/kg body weight-treated *db/db* mice; GS100, 7-O-galloyl-D-sedoheptulose 100 mg/kg body weight-treated *db/db* mice. The results are presented as the means±S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. vehicle-treated *db/db* mouse values.

Table 3. Biomarkers Associated with Oxidative Stress in the Liver

Item	<i>m/m</i>	<i>db/db</i>		
		Veh	GS20	GS100
ROS (fluorescence/min/mg protein)	2710±282**	4611±385	3285±244*	2705±220***
TBARS (MDA nmol/mg protein)	3.3±0.4**	5.6±0.5	3.9±0.4*	3.1±0.2***

m/m, Misty; Veh, vehicle-treated *db/db* mice; GS20, 7-O-galloyl-D-sedoheptulose 20 mg/kg body weight-treated *db/db* mice; GS100, 7-O-galloyl-D-sedoheptulose 100 mg/kg body weight-treated *db/db* mice. The results are presented as the means±S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. vehicle-treated *db/db* mouse values.

Fig. 2. Nox-4 and p22^{phox} Protein Expressions in the Liver

m/m, Misty; Veh, vehicle-treated *db/db* mice; GS20, 7-*O*-galloyl-*D*-sedoheptulose 20 mg/kg body weight-treated *db/db* mice; GS100, 7-*O*-galloyl-*D*-sedoheptulose 100 mg/kg body weight-treated *db/db* mice. The results are presented as the mean $\pm$ S.E.M. * p < 0.05, ** p < 0.01 vs. vehicle-treated *db/db* mouse values.

Compared to *m/m* mice, hepatic expressions of Nrf2 and HO-1 were markedly raised in *db/db* vehicle mice (Fig. 3). However, GS administration adversely regulated the nuclear Nrf2 and cytosolic HO-1 expressions in the liver of *db/db* mice.

Hepatic Inflammation-Related Protein Expressions

The hepatic expressions of inflammatory protein, NF- κ Bp65, COX-2, and iNOS, were all enhanced in *db/db* mice; however, these increased expressions were significantly reduced by GS treatment (Fig. 4).

Hepatic Apoptosis-Related Protein Expressions In *db/db* vehicle mice, proapoptotic protein, Bax, cytochrome *c*, and caspase-3 expressions were significantly enhanced compared with those in *m/m* mice (Fig. 5). GS-treated *db/db* mice showed a reduction of all pro-apoptotic protein expressions in the liver, and especially caspase-3 expression decreased to the normal level in the GS 100 mg/kg treatment group. On the contrary, hepatic Bcl-2 expression was decreased in the type 2 diabetic group, and the GS 100 mg/kg-treated group exhibited increased expression.

Hepatic Histological Examination Figure 6 shows the results of histological examinations using HE staining, which detects hepatocellular damage. The level of hepatocellular damage was higher in the livers of *db/db* vehicle mice compared with *m/m* mice. However, GS-treated *db/db* mice clearly showed decreased hepatocellular damage.

DISCUSSION

Increased oxidative stress due to the up-regulation of NADPH oxidase activity likely exacerbates ROS-induced cellular injury in the liver of type 2 diabetic *db/db* mice. As a major source of ROS generation, the Nox family of NADPH oxidase strongly contributes to the initial step and/or further aggravates the development of oxidative stress. Nox-derived ROS play a physiological role in stimulating various growth

Fig. 3. Nrf2 and HO-1 Protein Expressions in the Liver

m/m, Misty; Veh, vehicle-treated *db/db* mice; GS20, 7-*O*-galloyl-*D*-sedoheptulose 20 mg/kg body weight-treated *db/db* mice; GS100, 7-*O*-galloyl-*D*-sedoheptulose 100 mg/kg body weight-treated *db/db* mice. The results are presented as the mean $\pm$ S.E.M. * p < 0.05, ** p < 0.01 vs. vehicle-treated *db/db* mouse values.

Fig. 4. NF- κ Bp65, COX-2, and iNOS Protein Expressions in the Liver

m/m, Misty; Veh, vehicle-treated *db/db* mice; GS20, 7-*O*-galloyl-*D*-sedoheptulose 20 mg/kg body weight-treated *db/db* mice; GS100, 7-*O*-galloyl-*D*-sedoheptulose 100 mg/kg body weight-treated *db/db* mice. The results are presented as the mean $\pm$ S.E.M. * p < 0.05, ** p < 0.001 vs. vehicle-treated *db/db* mouse values.

Fig. 5. Bax, Bcl-2, Cytochrome *c*, and Caspase-3 Protein Expressions in the Liver

m/m, Misty; Veh, vehicle-treated *db/db* mice; GS20, 7-*O*-galloyl-*D*-sedoheptulose 20mg/kg body weight-treated *db/db* mice; GS100, 7-*O*-galloyl-*D*-sedoheptulose 100mg/kg body weight-treated *db/db* mice. The results are presented as the mean $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. vehicle-treated *db/db* mouse values.

factors, cytokines, and hormones including insulin,²¹⁾ and have pathophysiological roles in endothelial dysfunction, inflammation, apoptosis, fibrosis, angiogenesis, and important processes underlying diabetes and liver injury.²²⁾ Structurally, NADPH oxidase comprises a membrane-associated cytochrome *b558*, composed of one p22^{phox} (where phox stands for phagocyte oxidase) and one gp91^{phox} subunit and at least four cytosolic subunits (p47^{phox}, p67^{phox}, p40^{phox}, and the small GTPase Rac1).²³⁾ In obesity-induced type 2 diabetes, such as the *db/db* mice model which is associated with nonalcoholic steatohepatitis, enhanced NADPH oxidase activity is involved in steatosis and insulin resistance.²⁴⁾ Nox-4, gp91^{phox} (known as Nox-2) and p22^{phox} may activate the c-Jun N-terminal kinase pathway, serine/threonine phosphorylation of insulin receptor substrate 1, and expression of transcriptional factors such as activator protein-1, leading to steatosis and insulin resistance.^{24,25)} In addition, increased Nox-4 promotes endoplasmic reticulum

stress and induces hepatocyte apoptosis.^{26,27)} Furthermore, gp91^{phox} and Nox-4 increase fibrosis/inflammation-related protein expression and thus stimulate the proliferation of stellate cells.²⁸⁾ It has also been reported that the expression of p22^{phox} is increased in parallel with elevation of the levels of lipid peroxidation.²⁹⁾ Accordingly, we have performed immunoblotting analyses of p22^{phox} and Nox-4 in hepatic tissue of *db/db* mice.

In the present study, GS administration to *db/db* mice significantly attenuated oxidative stress by reducing ROS and TBARS levels in serum and hepatic tissue, showing similar levels with those of normal *m/m* mice. These results could suggest that the effect of GS effectively involved the control of oxidative stress-induced hepatic injury, without serum glucose adjustment. Additionally, the increased expressions of hepatic p22^{phox} and Nox-4 were significantly decreased by the administration of GS, which is also related to the reduction of hepatic ROS and TBARS levels. Therefore, the efficacy of GS may be related to the suppression of ROS-generating NADPH oxidase triggered by hyperglycemia, which is a potential source of oxidative stress in diabetes.

Otherwise, oxidative stress induces alterations in the Nrf2 complex and its gene transcription, such as that of HO-1, is enhanced.³⁰⁾ Under physiological conditions, Nrf2 is sequestered in the cytoplasm by Keap1, which facilitates its ubiquitination and proteasomic degradation.³¹⁾ Upon exposure to oxidative stress, the sequestration complex breaks down and the dissociated Nrf2 translocates into the nucleus, where it binds to cis-acting antioxidant response elements and promotes the transcription of a large number of cytoprotective genes.^{32,33)} NADPH oxidase-derived superoxide and the consequently induced activation of intracellular protein kinase cascades such as MAPK can mediate the induction of Nrf2 and HO-1 expression.^{2,3)} Besides ROS, AGE, oxidized low-density lipoprotein, and TNF- α also activate Nrf2 transcription and increase HO-1 expression in various cell types.^{3,34,35)} Therefore, increased Nrf2–HO-1 pathway activation could be a biomarker of oxidative stress and an adaptive response under pathological conditions. In our results, type 2 diabetic *db/db* mice showed enhanced expressions of Nrf2 and HO-1 in the liver compared with normal *m/m* mice; however, GS treatment significantly reduced these expressions. These results suggest that GS administration effectively alleviates oxidative stress, and results in the down-regulation of Nrf2 and HO-1.

Fig. 6. HE Staining of Hepatic Tissue

(A) Misty, (B) vehicle-treated *db/db* mice, (C) 7-*O*-galloyl-*D*-sedoheptulose 20mg/kg body weight-treated *db/db* mice, and (D) 7-*O*-galloyl-*D*-sedoheptulose 100mg/kg body weight-treated *db/db* mice. $\times 200$.

Chronic hyperglycemia also favors the increased expression of COX-2 and iNOS mediated by the activation of NF- κ B, which is also involved in ROS generation and inflammatory responses.³⁶⁾ Following inflammatory stimuli, both COX-2 and iNOS have been reported to induce deleterious effects on the liver.³⁷⁾ Among them, iNOS can be induced by infectious and pro-inflammatory stimuli, assumed to be one of the candidates that mediate inflammation-involved insulin resistance.³⁸⁾ According to a recent study, iNOS inhibitor improved hepatic insulin signaling at the levels of insulin receptor substrate-1 and -2 and protein kinase B in the liver of genetically obese diabetic mice.³⁹⁾ Excess nitric oxide (NO) generation, most of which is attributable to iNOS expression, often occurs under pathogenic conditions. High NO production by iNOS in macrophages and some other cells is an inflammatory mediator.⁴⁰⁾ In *db/db* mice, NADPH oxidase-derived ROS and iNOS-produced NO were augmented together, suggesting that the type 2 diabetic condition augmented oxidative and nitrosative stress in the liver. In the present study, GS significantly suppressed hepatic NF- κ B, COX-2, and iNOS protein expressions in a type 2 diabetic *db/db* mouse model, which was probably the result of a reduction of ROS and TBARS in the hepatic tissue. These results suggest that GS could effectively prevent oxidative/nitrosative stress and its related inflammatory response *via* attenuating the expression of NADPH oxidase subunits and NF- κ B-related protein.

Liver homeostasis is achieved by a tightly regulated steady state in cell turnover involving the proliferation and apoptosis of hepatocytes.⁶⁾ A disturbance of this balance can result in apparent liver disease. Compensatory activation of repair mechanisms in the liver following liver injury involves the activation of hepatic stellate cells and promotes the development of liver fibrosis, potentially resulting in chronic liver injury.⁴¹⁾ The chronic activation of NF- κ B in these mice resulted in increased levels of pro-inflammatory cytokines, especially TNF- α and interleukin-6,⁴²⁾ which are predominant cytokines triggering an apoptotic response *via* the receptor/ligand system. The sources of the pro-inflammatory cytokines in the liver are residential macrophages, the Kupffer cells—importantly, Kupffer cells responded to exposure to free fatty acids with an increased expression of pro-inflammatory cytokines induced by NF- κ B, and, thus, the blockade of NF- κ B activation in these cells prevented the secretion of cytokines and apoptotic cell death.⁴³⁾ Additionally, in diabetes, hyperglycemia increases the production of ROS in the liver, leading to the translocation of the pro-apoptotic protein Bax from the cytoplasm to mitochondria, increasing the release of cytochrome *c* from mitochondria to the cytosol.¹⁰⁾ This event leads to the activation of caspase-3, which, coupled with the decline of anti-apoptotic protein X-linked inhibitor of apoptosis protein, induces apoptotic cell death. Insulin, through the reduction of hyperglycemia, helps to decrease the production of ROS, which causes a reduction in the translocation of Bax from the cytosol to mitochondria and cytochrome *c* release. In this study, GS administration to *db/db* mice significantly suppressed hepatic expression of Bax, cytochrome *c*, and caspase-3; however, Bcl-2 expression was augmented by GS treatment. These data suggest that GS attenuated apoptotic cell death and improved oxidative stress-induced mitochondrial dysfunction in type 2 diabetes.

In summary, the present study showed that type 2 diabetes

increased hyperglycemia and oxidative stress, as well as enhanced the expression of NADPH oxidase, which aggravated NF- κ B-induced pro-inflammatory factors and apoptosis in the liver. On the other hand, GS administration effectively alleviated these adverse pathways in diabetic injury of the liver. Therefore, the current study suggests that GS could exert its hepato-protective potential through the inhibition of oxidative stress-sensitive mechanisms of the pro-inflammatory response and apoptosis in the liver of type 2 diabetics.

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