Food and Chemical Toxicology 129 (2019) 97–107

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Aqueous extract of *Salvia miltiorrhiza* Bunge-*Radix Puerariae* herb pair ameliorates diabetic vascular injury by inhibiting oxidative stress in streptozotocin-induced diabetic rats

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ARTICLE INFO

Keywords:
Diabetic vascular injury
Salvia miltiorrhiza bunge
Radix puerariae
Oxidative stress
Cell adhesion molecules

ABSTRACT

Vascular diabetic complications are the leading cause of mortality and morbidity for diabetes. The present study was designed to investigate the protective effect of herb pair *Salvia miltiorrhiza* Bunge-*Radix Puerariae* (DG) on diabetic vascular injury induced by streptozotocin. The protective effect of DG was determined by oral administration of DG (50 and 200 mg/kg) in rats and on high glucose (HG)-induced endothelial injury. DG showed no effect on body weight, fasting blood glucose (FBG) but decreased the serum levels of insulin, nitric oxide (NO), hydrogen peroxide (H_2O_2), malondialdehyde (MDA), soluble intercellular cell adhesion molecule-1 (s-ICAM-1) and vascular cell adhesion molecule-1 (s-VCAM-1), and increased superoxide dismutase (SOD) and catalase (CAT) levels. The pathological alterations of aorta was improved by DG. Furthermore, the increased expression of ICAM-1,VCAM-1, NOX2, and NOX4 in aorta were inhibited by DG. HG-induced endothelial ROS formation, ICAM-1,VCAM-1, NOX4 expression and monocyte-endothelial adhesion were dramatically suppressed by DG as well. In addition, both GKT137831, a NOX4 inhibitor, and PDTC, a NF-κB inhibitor, could significantly inhibited HG-induced ICAM-1, VCAM-1 expression and monocyte-endothelial adhesion. These results suggested that DG improved diabetic vascular injury possibly by reducing oxidative stress, which provides scientific evidence for the application of DG for diabetic vascular therapy.

1. Introduction

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, and/or both. Persistent high glucose leads to chronic damages and dysfunctions of various tissues, especially in the heart, blood vessel, eye, and kidney, causing a series of chronic complications. These complications (microvascular and macrovascular) are the major cause of disability in diabetic patients and has already been a serious social health problem (Wang et al., 2015). Thus, prevention and alleviation of the vascular complications has become a major challenge in diabetes therapy.

Mechanisms leading to the vascular complications in diabetes are complicated and still largely unclear, which results in the absence of effective preventive strategies and therapeutic drugs. However, vascular injury caused by hyperglycaemia appears to play a key role. Endothelial dysfunction (Shi and Vanhoutte, 2017), oxidative stress (Ceriello et al., 2016), inflammation (Domingueti et al., 2016), insulin resistance (IR) (Groop et al., 2005), the non-enzymatic protein glycation (Yamagishi et al., 2012), and among others, have been considered as the main pathological factors for vascular injury in diabetes. Among which, oxidative stress might be the “final common pathway” mediating the deleterious effects of others. Therefore, the oxidative stress pathways provide strategies for prevention and therapy.

Salvia miltiorrhiza Bunge and *Radix Puerariae* are two frequently prescribed herbs in traditional Chinese medicine (TCM). The former is widely investigated for it cadio-protective effects while the latter is a nutrition food widely consumed in Asia. Interestingly, they are generally used in combination in clinical practice to comprise a herb pair (1:1, w/w). Previous studies showed that this pair extract (7:3, w/w)

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https://doi.org/10.1016/j.fct.2019.04.018
Received 8 January 2019; Received in revised form 12 April 2019; Accepted 14 April 2019
Available online 29 April 2019
0278-6915/

protect ischemia/reperfusion (Chiu et al., 2011). Furthermore, it promotes the proliferation and protects hypoxia/reoxygenation-induced apoptosis in heart myocardium H9c2 cells (Chiu et al., 2012; Fong et al., 2011). In addition, the two herbs demonstrated synergistic, additive, and antagonistic effect in anti-inflammation, anti-foam cell formation and anti-vascular smooth muscle cells proliferation, respectively (Wing-Shing Cheung et al., 2012). Although the anti-diabetic effect of extracts and pure compounds isolated from them alone has been accumulated (Hsu et al., 2003; Huang et al., 2012; Qiang et al., 2015; Wong et al., 2011), their protective effect as a pair on diabetic vascular injury remains to be clear. In this study, aqueous extract was prepared following clinical practice, its effect on diabetic vascular injury was evaluated and the underlying mechanisms were explored.

2. Materials and methods

2.1. Reagents

Salvia miltiorrhiza Bunge and *Radix Puerariae* were purchased from Changda Prepared Chinese Medicinal Herbs Co. Ltd. (Anguo, China). D-(+)-Glucose, N-acetylcysteine (NAC) acetylcholine chloride (Ach) and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Solarbio Science & Technology Co., Ltd (Beijing, China). Noradrenaline bitartrate was obtained from Shanghai Hefeng Pharmaceutical Co., Ltd. (Shanghai, China). BCA protein kit, 5-(6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (CM-DCFH$_2$-DA), pyrrolidine dithiocarbamate (PDTC), and RIPA lysis buffer were purchased from Beyotime (Haimen, China). GKT137831 (GKT) was obtained from BioChemPartner (Shanghai, China). Streptozotocin (STZ) was obtained from Sigma (USA). Antibodies for intercellular adhesion molecule-1 (ICAM-1) and GAPDH were purchased from Proteintech (Wuhan, China). Antibodies for vascular cell adhesion protein 1 (VCAM-1) and NF-κB p65 and phosphorylated NF-κB p65 were purchased from Santa Cruz (USA). Antibodies for NOX1, NOX2, and NOX4 were purchased from Abcam (UK). ELISA kits for s-ICAM-1, s-VCAM-1 and insulin (INS) were purchased from R&D system (USA). Kits for glycohemoglobin (GHb), catalase (CAT), superoxide dismutase (SOD), hydrogen peroxide (H_2O_2), nitric oxide (NO), malondialdehyde (MDA) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Preparation of DG

The dried *Salvia miltiorrhiza Bunge* and *Radix Puerariae* were shattered to powder and 500 g powder (1:1 w/w; 250 g each) was dunked into 6000 mL water at room temperature for 30 min and then extracted under 100 °C for 45 min. The extraction procedure was repeated. The extracts was freeze-dried, producing a powder and stored at 4 °C.

2.3. Analysis of DG constitutes

The assay was performed using an ultimate 3000 hyperbaric LC system coupled to an LTQ Orbitrap mass spectrometer via an ESI interface. The chromatography system consisted of an autosampler, a diode-array detector, a column compartment and two pumps. Xcalibur, Metworks and Mass Frontier 7.0 software packages were used for data collection and data analysis. Liquid chromatographic separations of the analysts were performed using a Thermo Hypersil BDS C18 column (150 mm × 4.6 mm, 3.5 μm). The mobile phase consisted of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). The gradient elution was as follows: 0–2 min, held at 5% B; 2–4 min, linear from 5% to 10% B; 4–5 min, linear from 10% to 20% B; 5–12 min, linear from 20% to 25% B; 12–17 min, linear from 25% to 50% B; 17–30 min, linear from 50% to 80% B; 30–35 min, linear from 80% to 95% B, 35–40 min, 95% B. The flow rate was 0.5 mL/min. The injection volume was 5 μL. The temperature controlled column oven was set at 30 °C and the sampler was set at 4 °C. The ESI source parameters were as follows:

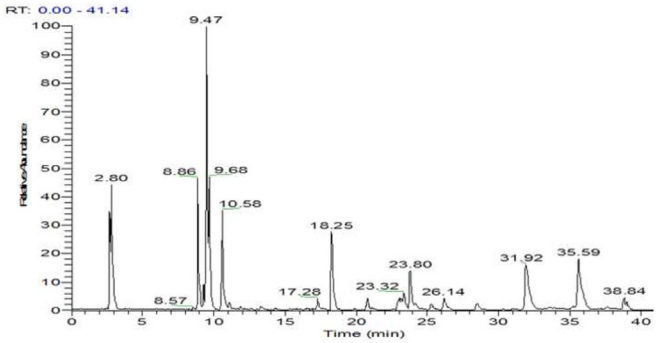

Fig. 1. The chemical components of DG. DG, aqueous extract of *Salvia miltiorrhiza* Bunge-*Radix Puerariae* herb pair.

capillary temperature, 350 °C; source voltage and is pray voltage, 5 kV; sheath gas (N2) flow, 35 psi; and aux gas flow, 10 psi. The ESI source was operated in the positive ionization mode. In the Fourier transform (FT) cell, full MS scans were acquired in the range of m/z 50–2000 for LMs. The MS/MS experiments were set as data-dependent scans.

2.4. Animals

Male SD rats (250–280 g, 8 weeks old) purchased from the Experimental Animal Center of Daping Hospital (Chongqing, China) were housed under standard environmental conditions (22 ± 2 °C, 55–60% relative humidity, and 12 h light/12 h dark cycle) and allowed free access to tap water and food. Great care was taken to minimize their suffering and this study was approved by the Animal Ethics Committee of Zunyi Medical University.

2.5. Experimental design

Diabetic rat model was established according to our previous report (Li et al., 2016) with minor revisions. Thirty-six SD rats were administrated of freshly prepared STZ in citrate buffer (dissolved in 0.1 mM citrate buffer, pH 4.2–4.5) at a dosage of 50 mg/kg/day for 2 consecutive days by intraperitoneal injection. Nine rats received an equal volume of citric buffer as control. Rats with blood glucose level ≥11.1 mM after 72 h administration of STZ were considered as diabetic rats, which were randomly divided into 3 groups and were orally administered with or without DG (50 and 200 mg/kg/day, dissolved in saline water) for 7 weeks. The aorta and serum were collected for the following experiments.

2.6. Measurement of body weight and FBG

The body weights and FBG were determined once a week using an electronic balance and an ONETOUCH Ultra Glucometer (Johnson & Johnson, USA) in accordance with the manufacturer's instructions, respectively.

2.7. Determination of GHb, INS, MDA, SOD, H_2O_2, NO, s-ICAM-1 and s-VCAM-1

The serum levels of GHb, INS, s-ICAM-1, s-VCAM-1 were determined with commercial ELISA kits in accordance with the manufacturer's instructions. Serum levels of NO, SOD, H_2O_2 CAT, and MDA were determined by commercial Kits in accordance with the manufacturer's instructions.

2.8. Measurement of aorta relaxation

The measurement of aorta relaxation was as a previous report (Ishida et al., 2014), Briefly, after the rats were sacrificed, the aorta was

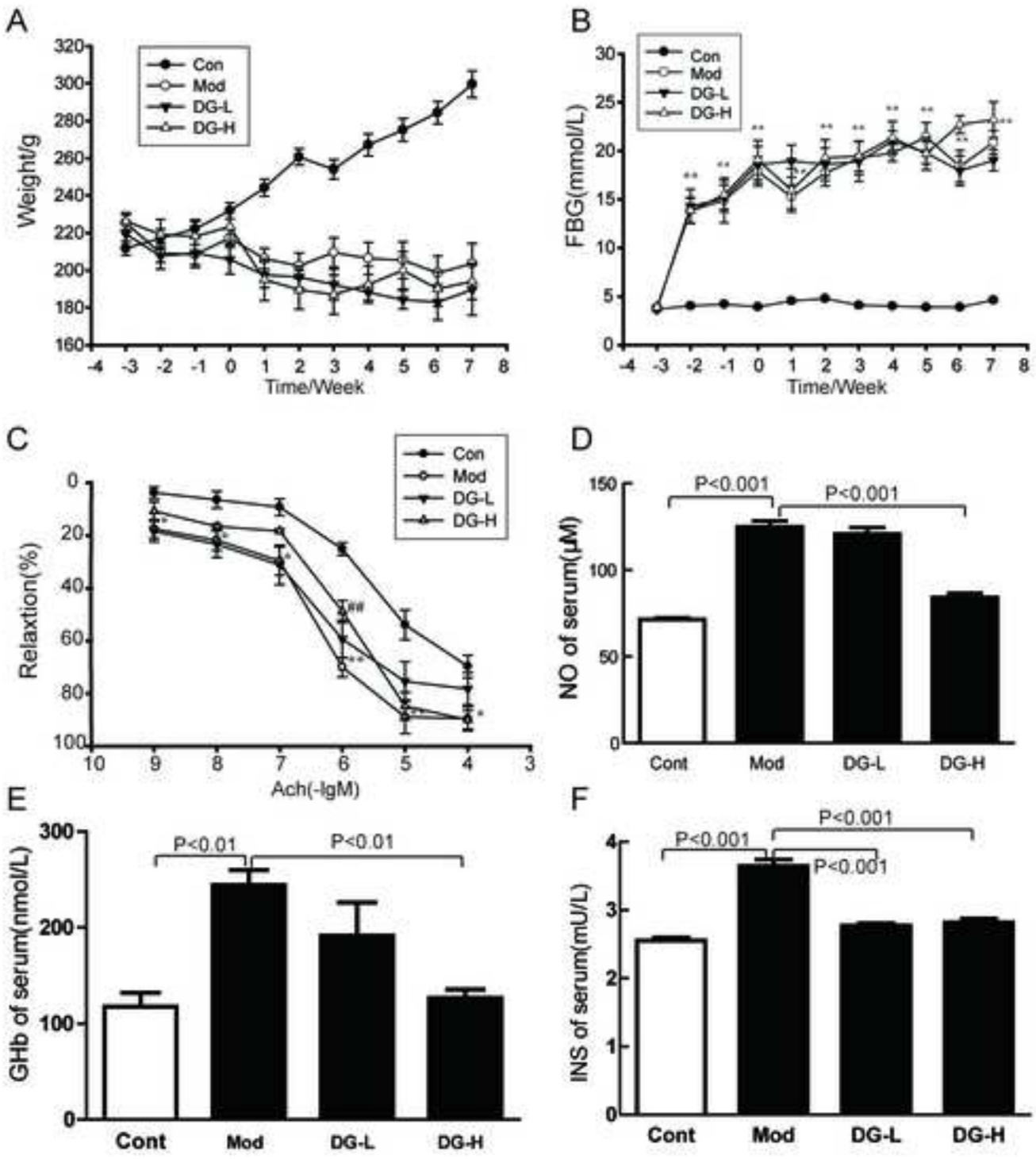

Fig. 2. Effect of DG on body weight, FBG, aorta relaxation, and serum levels of NO, GHb, and INS. Diabetic rats were treated with DG for 7 weeks and the body weight (A) and FBG (B) were measured every week. The response of aorta to Ach was measured at the end of experiment (C). Serum levels of NO (D), GHb (E), and INS (F) were determined by commercial kits. $^{*}p < 0.05$, $^{**}p < 0.01$ versus control group; $\#\#p < 0.01$ versus model group. DG, aqueous extract of *Salvia miltiorrhiza* Bunge-*Radix Puerariae* herb pair; FBG, fasting blood glucose; Ach, acetylcholine; NO, nitric oxide; GHb, glycohemoglobin; INS, insulin; Cont, control; Mod, Model; DG-L, DG (50 mg/kg); DG-H, DG (200 mg/kg).

carefully isolated, trimmed free of surrounding fats and connective tissues, cut into circular segments (2–3 mm long) and immediately placed in Krebs-Henseleit Solution (KHS) containing (mM): NaCl: 118.0; KCl: 4.7; $NaHCO_3$: 25.0; $CaCl_2$: 1.8; NaH_2PO_4: 1.2; $MgSO_4$: 1.2; glucose: 11.0. After 1h equilibration, cumulative dose-response curves were performed using noradrenaline bitartrate (10^{-6} M). When noradrenaline bitartrate induced contraction reached a plateau level, relaxation response curves for ACh (10^{-9}-10^{-4} M) were determined.

2.9. H&E staining

Aorta tissues were fixed in 4% neutral formaldehyde solution and

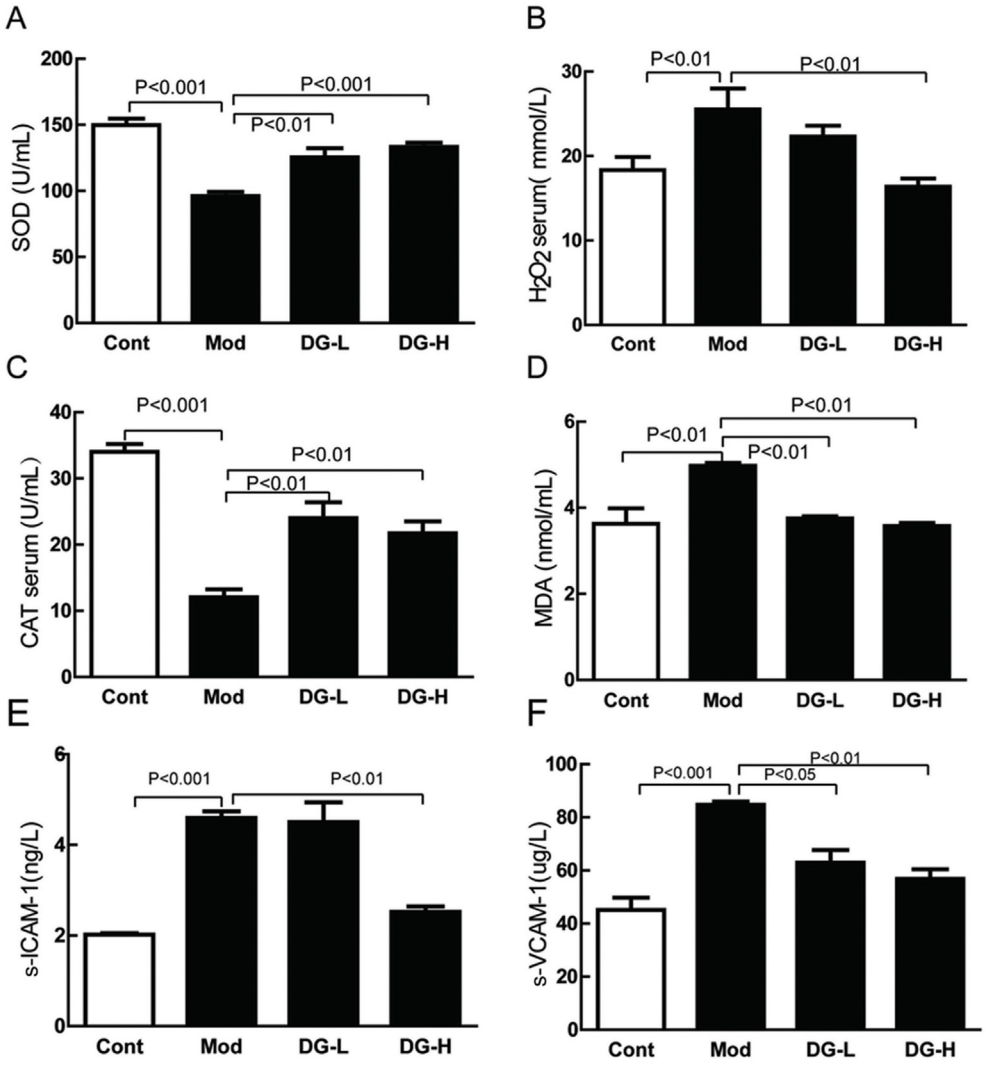

Fig. 3. Effect of DG on serum levels of SOD, H_2O_2,CAT, MDA, s-ICAM-1 and s-VCAM-1. Diabetic rats were treated with DG for 7 weeks and the levels of SOD (A), H_2O_2 (B), CAT (C), MDA (D), s-ICAM-1 (E) and s-VCAM-1 (F) in serum were determined by commercial kits. DG, aqueous extract of *Salvia miltiorrhiza* Bunge-*Radix Puerariae* herb pair; SOD, superoxide dismutase; H_2O_2, hydrogen peroxide; CAT, catalase; MDA, malondialdehyde; s-ICAM-1, soluble intercellular cell adhesion molecule-1; s-VCAM-1, soluble vascular cell adhesion molecule-1; Cont, control; Mod, Model; DG-L, DG (50 mg/kg); DG-H, DG (200 mg/kg).

the H&E staining was performed as our previous reports (Li et al., 2016; Zhao et al., 2014).

2.10. Immunohistochemistry

The immunohistochemistry was performed as our previous reports (Li et al., 2016; Zhao et al., 2014). Briefly, after deparaffinization, deactivation of endogenous peroxidase with H_2O_2 (3%), and antigen block with SA-PBS (5%), the sections were incubated with ICAM-1, VCAM-1, and NOX4 antibodies (1:50 dilution) at 37 °C for 2 h. Then, the sections were incubated with secondary antibody (Gene Tech, Shanghai, China) for 30 min at 37 °C after rinsing with PBS. The sections were then stained with DAB chromogen kit.

2.11. Cell culture

Human umbilical vein endothelial cells (HUVECs) purchased from ATCC (Rockville, MD, USA) were cultured in Vascular Cell Basal Medium with Endothelial Cell Growth Kit-BBE at 37 °C in a humidified atmosphere of 5% CO_2. THP-1 cells obtained from ATCC were cultured in DMEM medium containing 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin under a humidified atmosphere containing 5% CO_2 and 95% air.

2.12. MTT assay

HUVECs monolayers in 96-well microplates were exposed to DG (solved in serum-free medium, 0–50 μg/mL) for 24 h and the cytotoxic effect of DG was determined with MTT assay.

2.13. Measurement of intracellular ROS production

The intracellular ROS production was determined as our previous reports (Zhao et al., 2014, 2016). Briefly, cells were seeded in 12-well plates (3.0×10^5/well) for overnight and then treated with high glucose (HG) (30 mM) for 12 h followed by CM-$DCFH_2$-DA incubation (final concentration 10 μM) in the dark at 37 °C for 30 min. After washed twice with PBS, the cellular fluorescence was observed under a fluorescent microscopy and quantitatively determined by a flow cytometry. To explore the effect of DG and NAC on HG-induced ROS formation, cells were pretreated with DG (25, 50 μg/mL), NAC (1 mM), respectively.

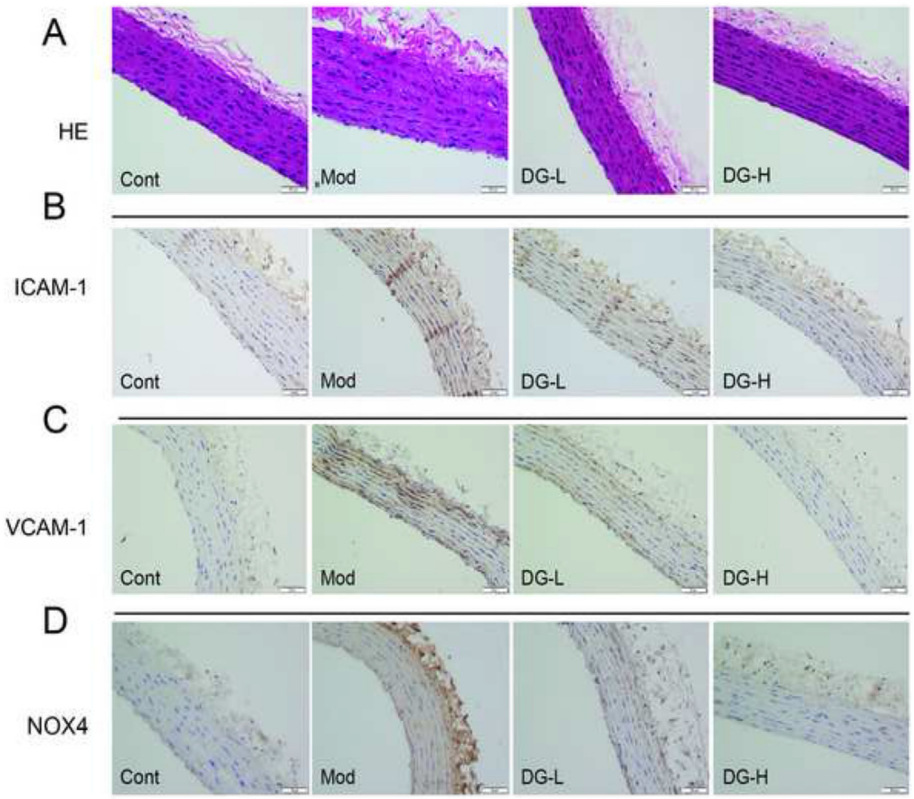

Fig. 4. DG alleviated pathological alterations and inhibited expression of ICAM-1, VCAM-1 and NOX4 in diabetic aorta. Diabetic rats were treated with DG for 7 weeks and H&E staining (A) and immunohistochemical staining of ICAM-1 (B), VCAM-1 (C) and NOX4 (D) were performed. DG, aqueous extract of *Salvia miltiorrhiza* Bunge-*Radix Puerariae* herb pair; ICAM-1, intercellular cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; Cont, control; Mod, Model; DG-L, DG (50 mg/kg); DG-H, DG (200 mg/kg).

2.14. Cell adhesion assay

The endothelial-monocyte adhesion was determined as our previous report (Zhao et al., 2014). Briefly, the THP-1 cells were labeled with DAPI in serum-free medium for 30 min. After extensive washing with PBS to remove the free DAPI, the labeled THP-1 cells were incubated with HG-treated endothelial cells for 1 h at 37 °C. The non-adherent cells were removed by gentle washing with PBS. The adhesion cells was measured with a fluorescent microscopy. To explore the role of NOX4 and NF-κB in HG-induced cell adhesion, NOX4 inhibitor GKT (10 nM) and NF-κB inhibitor PDTC (10 μM) were pretreated for 1 h before THP-1 cells were co-incubated with endothelial cells.

2.15. Western blotting

For animal study, the aorta was chopped into small pieces and homogenized with cold lysis buffer (150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40 dissolved in 50 mM Tris pH 8.0) for 30 min at 4 °C. Then the homogenized tissues were centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was collected and the protein content were determined with BCA protein assay kit. For cultured cells, after different treatment cells were collected and then co-incubated with cold cell lysis buffer for 30 min at 4 °C. After centrifugation at 12,000 rpm for 15 min at 4 °C, The supernatant was collected and the protein content were determined with BCA protein assay kit. Equal proteins were subjected to 8–10% SDS-PAGE electrophoresis and transferred onto PVDF membrane and incubated with primary antibodies (1:500–1:1000) and secondary antibodies (1:5000). The protein-antibody complexes were detected by ECL Advanced Western Blot detection Kit.

2.16. Statistical analysis

Data were expressed as the means ± SD from at least three separate experiments. The differences between groups were analyzed using SPSS 17.0, and differences between groups were analyzed by one-way ANOVA (LSD test). $p < 0.05$ was considered statistically significant.

3. Results

3.1. The chemical content of DG

As shown in Fig. 1 and Supplementary Table 1, a total of 14 compounds were identified from DG using ultra-high-performance liquid chromatography coupled with high-resolution LTQ-Orbitrapmass spectrometry. They were saccharose, rosmarinic acid, 3′-hydroxypuerarin, puerarinxyloside, puerarin, 3′-methoxydaidzin, daidzin, ononin, daidzein, dihydrotanshinone I, cryptotanshinone, tanshinone I, dihydrotanshinone I and tanshinoneIIA. Among which, 9 components were from pueraria and 6 from salvia.

3.2. Effect of DG on body weight, aorta relaxation, FBG, NO, GHb, and INS

Compared with the control rats, the diabetic rats showed significant decrease of body weights (Fig. 2A), approximate 3 folds increase of FBG (Fig. 2B), and increased serum levels of NO, GHb, and INS (Fig. 2D–F). DG showed no effect on either body weight or FBG (Fig. 2A and B) but significantly suppressed NO, GHb, and INS (Fig. 2D–F). Interestingly, diabetic rats showed increased relaxation of aorta to Ach, which was partially inhibited by DG treatment (Fig. 2C).

3.3. DG inhibited oxidative stress and adhesion molecules

The serum levels of SOD and CAT were decreased in diabetic rats, which was partially restored by DG (Fig. 3A and C). Furthermore, the levels of H_2O_2 and MDA increased in diabetic rats was significantly inhibited by DG (Fig. 3B and D). In addition, the increased levels of s-ICAM-1 and s-VCAM-1, two important biomarkers for endothelial dysfunction, were significantly inhibited by DG as well (Fig. 3E and F).

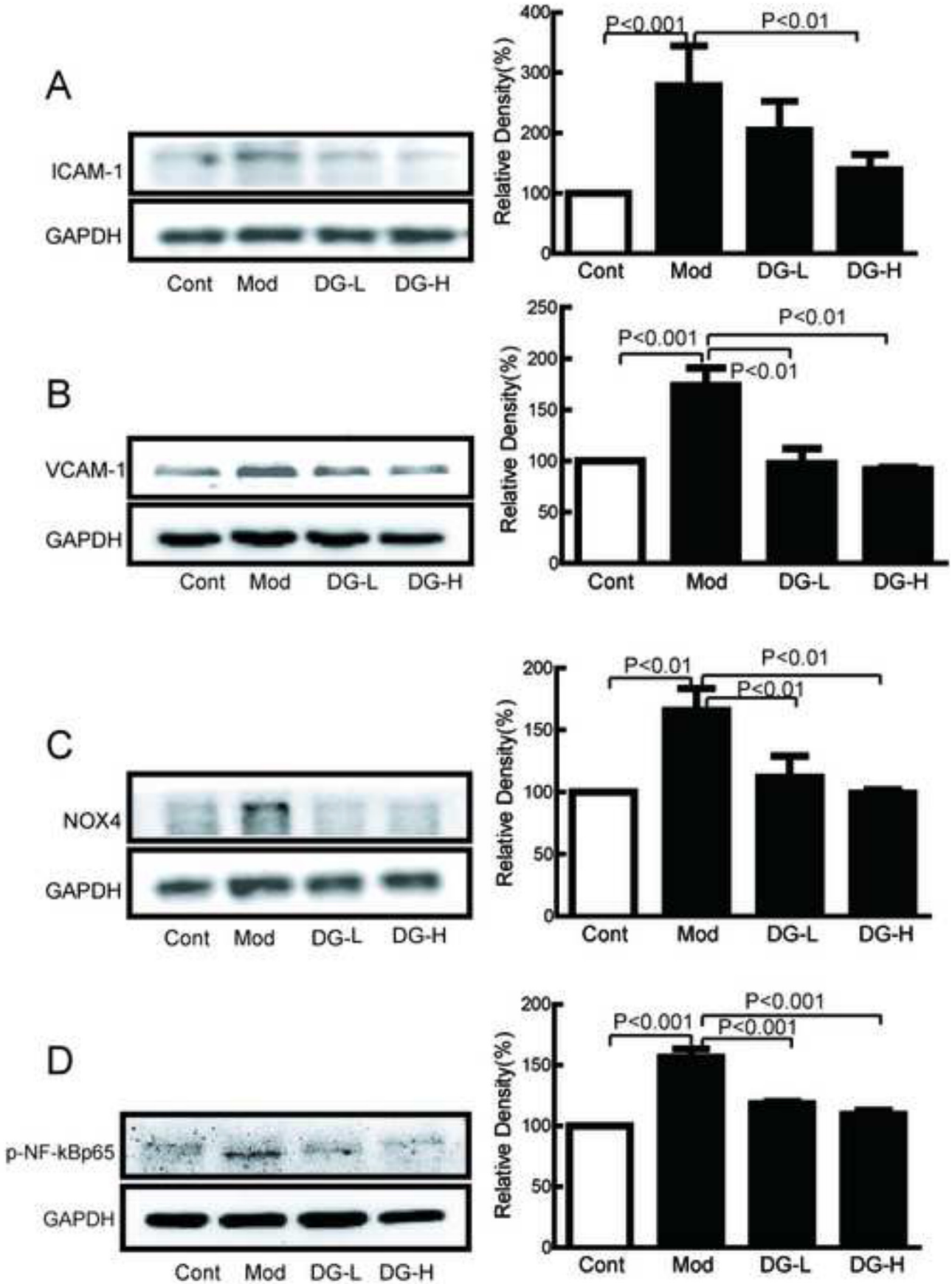

Fig. 5. DG inhibited aorta expression of ICAM-1, VCAM-1, NOX4 and p-p65 in diabetic aorta. Diabetic rats were treated with DG for 7 weeks and the protein expression of ICAM-1 (A), VCAM-1 (B), NOX4 (C), and p-p65 (D) were determined by Western blotting. DG, aqueous extract of *Salvia miltiorrhiza* Bunge-*Radix Puerariae* herb pair; ICAM-1, intercellular cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; p-p65, phosphorylated NF-κB p65; Cont, control; Mod, Model; DG-L, DG (50 mg/kg); DG-H, DG (200 mg/kg).

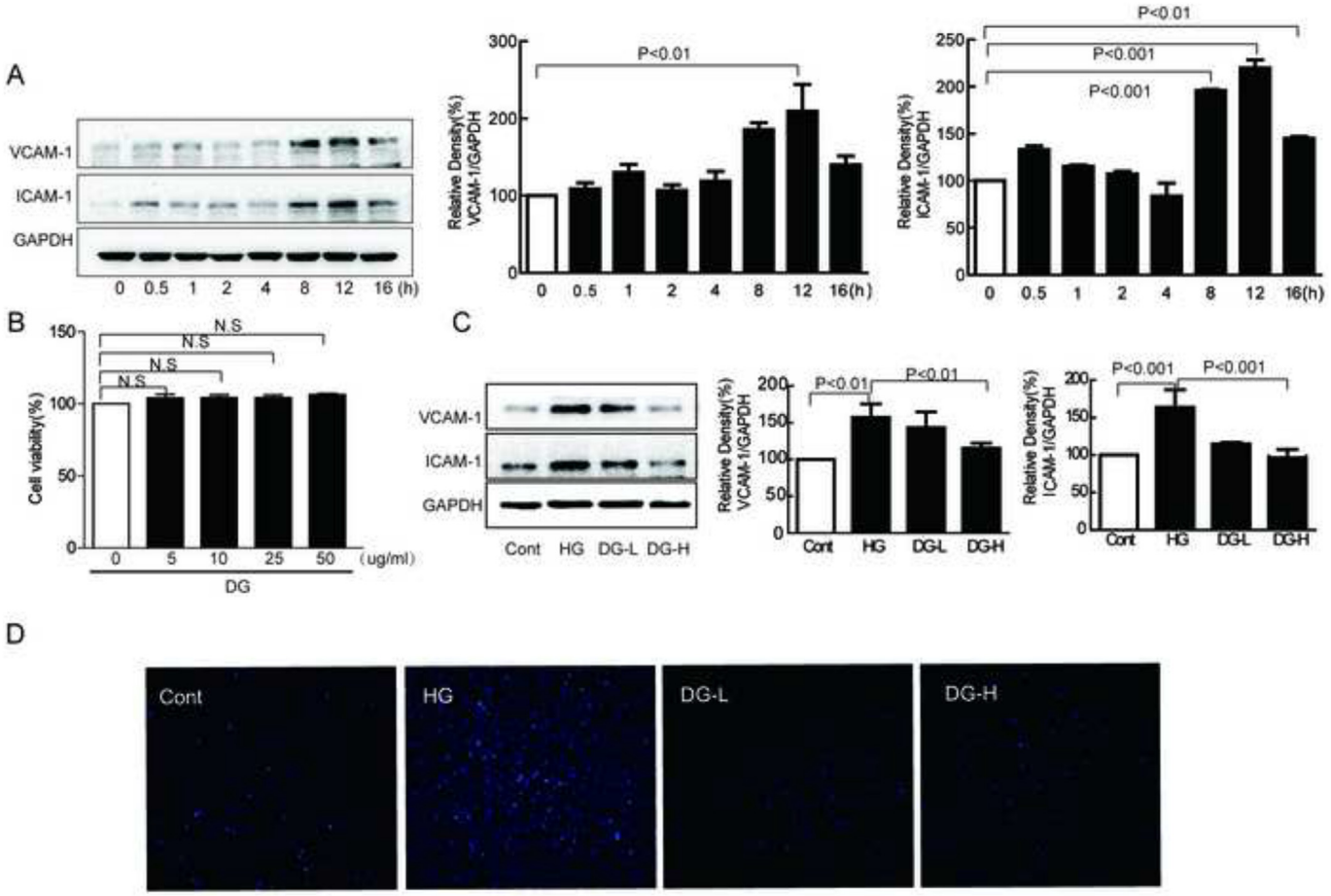

Fig. 6. DG inhibited HG-induced expression of ICAM-1, VCAM-1 and cell adhesion. Endothelial cells were treated with HG and the protein expression of ICAM-1 and VCAM-1 was determined by Western blotting (A). Endothelial cells were treated with DG for 24 h and the cell viability was determined by MTT assay (B). Endothelial cells were treated with HG for 12 h with or without DG (25, 50 μg/mL) pretreatment for 1 h, the protein expression of ICAM-1 and VCAM-1 and the adhesion of THP-1 cell were determined by Western blotting (C) and a fluorescent probe (D). DG, aqueous extract of *Salvia miltiorrhiza* Bunge-*Radix Puerariae* herb pair; ICAM-1, intercellular cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; HG, high glucose (30 mM); Cont, control; DG-L, DG (25 μg/mL); DG-H, DG(50 μg/mL).

3.4. DG alleviated aorta damage and inhibited ICAM-1, VCAM-1, and NOX4 expression

H&E staining showed that the aorta was normal in the control rats while the aortic wall was thickened in the diabetic rats. Furthermore, ruptured smooth muscles and increased number of nucleus were observed. These pathological alterations were alleviated by DG (Fig. 4A). Immunohistochemistry results showed that positive brown-yellow signals were increased for ICAM-1, VCAM-1, and NOX4 in diabetic aorta compared with control aorta suggesting the increased expression of these proteins. DG administration could dramatically inhibited the expression of these proteins (Fig.4B–D).

3.5. DG decreased aorta expression of ICAM-1, VCAM-1, NOX4, and NF-κB

Diabetic aorta showed increased protein expression of ICAM-1, VCAM-1, and NOX4, which was inhibited by DG in a dose-dependent manner (Fig. 5A–C). Complete inhibition was observed in high dose of DG on VCAM-1 and NOX4. In addition, increased phosphorylation of NF-κB p65 in diabetic aorta was inhibited by DG (Fig. 5D).

3.6. DG inhibited HG-induced adhesion molecule expression and cell-cell adhesion

HG treated endothelial cells showed increased expression of ICAM-1 and VCAM-1 in a time-dependent manner, which reached the highest levels at 12 h (Fig. 6A). DG pretreatment dramatically inhibited HG-induced cell adhesion molecules expression in a concentration-dependent manner (Fig. 6C). Furthermore, HG-induced adhesion of THP-1 cells to endothelial cells was suppressed by DG (Fig. 6D). No cytotoxicity was observed after DG (0–50 μg/mL) treatment in endothelial cells (Fig. 6B).

3.7. DG decreased ROS and inhibited NOXs expression

HG induced ROS formation in endothelial cells as indicated by the green fluorescence in microscope images and the right shift of histograms in flow cytometry. The ROS generation was suppressed by DG pretreatment (Fig. 7A and B). HG treatment increased protein expression of NOX1, NOX2, and NOX4. DG pretreatment concentration-dependently inhibited NOX4 expression without affecting NOX1. Weak inhibitory effect of DG on NOX2 was observed (Fig. 7C).

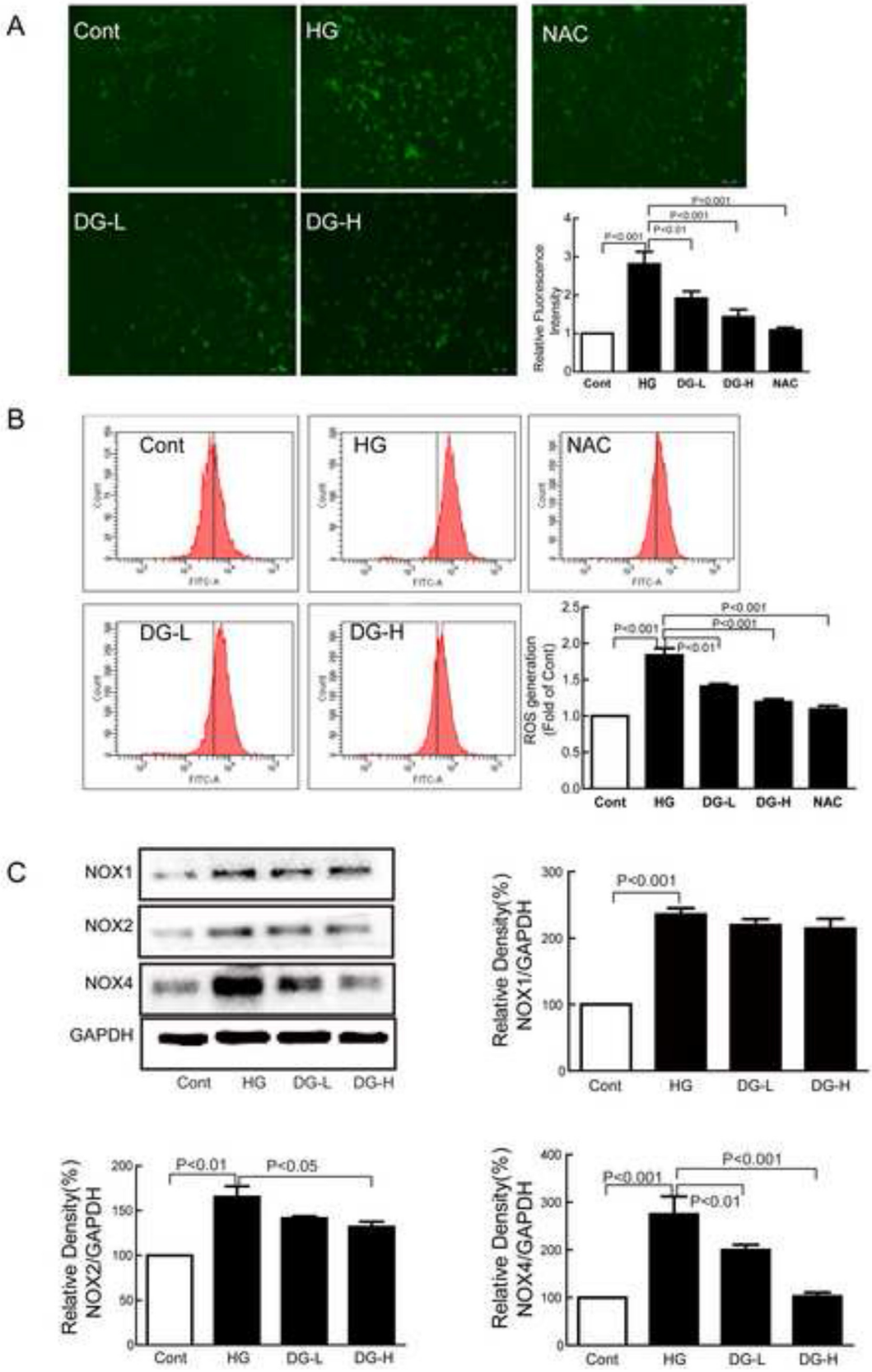

Fig. 7. DG inhibited HG-induced ROS generation and NOXs expression in cultured endothelial cells. Endothelial cells were treated with HG with or without DG (25, 50 μg/mL), NAC (1 mM), the ROS generation was indicated with fluorescence probe detected with a microscope (A) and a flow cytometry (B). The expression of NOXs was determined by Western blotting (C). DG, aqueous extract of *Salvia miltiorrhiza* Bunge-*Radix Puerariae* herb pair; HG, high glucose (30 mM); ROS, reactive oxygen species; NAC, N-Acetyl-L-cysteine (NAC); Cont, control; DG-L, DG (25 μg/mL); DG-H, DG (50 μg/mL).

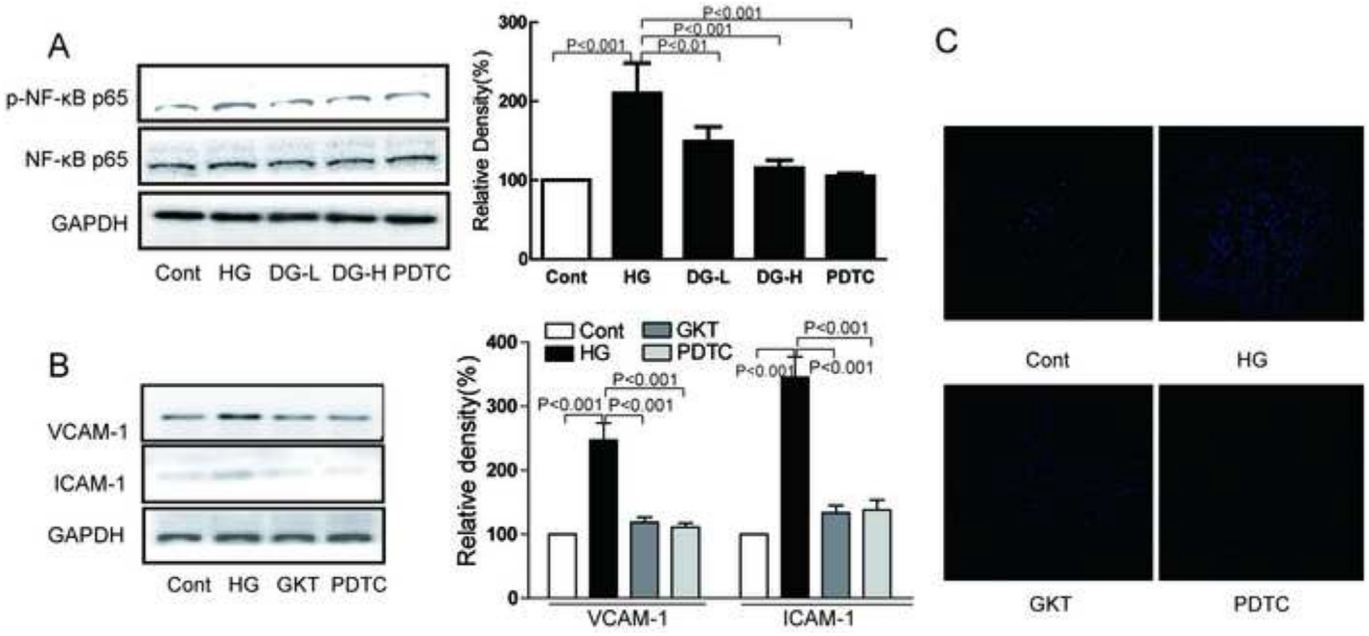

Fig. 8. DG inhibited HG-induced expression of ICAM-1, VCAM-1 and cell adhesion mediated by NOX4 and NF-κB. Endothelial cells were treated with HG for 12 h with or without DG (25, 50 μg/mL), or PDTC (10 μM) pretreatment for 1 h and the phosphorylation of p65 was determined by Western blotting (A). Endothelial cells were treated with HG for 12 h with or without GKT (10 nM), or PDTC (10 μM) pretreatment for 1 h and the expression of ICAM-1 and VCAM-1 was determined by Western blotting (B). Endothelial cells were treated with HG for 12 h with or without GKT (10 nM), or PDTC (10 μM) pretreatment for 1 h, the adhesion of THP-1 cells were determined by a fluorescent probe (C). DG, aqueous extract of *Salvia miltiorrhiza* Bunge-*Radix Puerariae* herb pair; ICAM-1, intercellular cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; HG, high glucose (30 mM); Cont, control; DG-L, DG (25 μg/mL); DG-H, DG(50 μg/mL); GKT, GKT137831; PDTC, pyrrolidine dithiocarbamate.

3.8. DG inhibited HG-induced adhesion molecule expression and cell-cell adhesion mediated by NF-κB and NOX4

HG-induced phosphorylation of NF-κB p65 was inhibited by both DG and PDTC, a NF-κB inhibitor (Fig. 8A). Furthermore, HG-induced expression of VCAM-1 and ICAM-1 was inhibited by PDTC and GKT, a NOX4 inhibitor (Fig. 8B). In addition, two inhibitors suppressed HG-induced adhesion of THP-1 cells to endothelial cells (Fig. 8C).

4. Discussion

Salvia miltiorrhiza Bunge and *Radix Puerariae* have been used for the treatment of diabetes in TCM for centuries. Here, we reported the protective effects and mechanisms of aqueous extract of this herb pair on diabetic vascular injury.

To follow the clinical prescriptions, DG was prepared as the aqueous extract of *Salvia miltiorrhiza* Bunge and *Radix Puerariae* with the ratio 1:1 (w/w), which was quite different from previous report (Chiu et al., 2012). STZ administration induced sustained loss of body weights and increase of FBG suggesting the establishment of diabetic model. DG treatment showed of no effect on either weight or FBG, suggesting that it has no direct anti-diabetic effect. Though decreased relaxation of diabetic aorta to Ach was reported (Durante et al., 1988; Endo et al., 1995; Wang et al., 2014), unaffected or increased response of diabetic aorta to Ach was observed (Joshi and Woodman, 2012; Pieper and Lai, 1999; Shen et al., 2003; Ye et al., 2005). Here, we observed increased aorta relaxation in response to Ach in STZ treated mice, which might be caused by the compensatory increase of endothelial function in the early stage of diabetes (Ye et al., 2005). Beside, increased NO might contribute to this. Improvement of aorta relaxation to Ach by DG, especially at the high dosage, suggesting that DG mitigated this vascular reaction. Similar to our previous report (Li et al., 2016), STZ induced aorta damage as indicated by morphological alterations, which was improved by DG treatment. The significant decreased of serum GHb and INS by DG might be partially due to puerarin as our previous report (Li et al., 2016).

Oxidative stress, increased ROS levels due to excessive ROS production and/or decrease in endogenous antioxidant defenses, has been implicated in diabetic complications (Sedeek et al., 2012; Tiwari et al., 2013). Consistent with previous reports (Bacanli et al., 2017; Li et al., 2016; Zhang et al., 2014), serum levels of SOD and CAT, two antioxidant enzymes, were significantly decreased while the levels of H_2O_2 and MDA, two oxidative biomarkers, were dramatically increase. The inhibitory effects of DG on these parameters suggested that DG might improve vascular injury by decreasing oxidative stress. This was confirmed by in vitro experiments. DG dramatically inhibited intracellular ROS. Especially, this inhibitory effect was comparable with that of NAC, a potent antioxidant. The NADPH oxidase (NOXs) enzymes are main sources for vascular ROS and NOX1, NOX2, NOX4, and NOX5 are expressed in the vascular, including endothelium, vascular smooth muscle cells, fibroblasts, etc (Drummond and Sobey, 2014; Konior et al., 2014). Consistent with previous reports (Gray et al., 2013; Taye et al., 2010; Williams et al., 2012), we found that HG-induced protein expression of NOX1, NOX2, and NOX4 in endothelial cells. However, only the increase of NOX4, but not NOX1 or NOX2, was suppressed by DG. Collectively, this suggested that DG improve vascular injury possibly mediated by inhibition of NOXs derived ROS, especially NOX4.

Endothelial dysfunction, characterized by decreased endothelial generation of NO, enhanced increased expression of cell adhesion molecules and binding of circulating leukocytes to these cells, is an early indicator of diabetic vascular disease (Hadi et al., 2005; Hamilton and Watts, 2013; Shi and Vanhoutte, 2017). Soluble cell-surface adhesion molecules (soluble E-selectin, s-ICAM-1, and s-VCAM-1) are important biomarkers for endothelial dysfunction (Page and Liles, 2013). Here, we reported the increased s-ICAM-1 and s-VCAM-1 in STZ-induced diabetic rats. Furthermore, increased expression of ICAM-1 and VCAM-1 was observed both in diabetic aorta and HG treated endothelial cells. The inhibitory effect of DG on them suggested that DG could improve diabetic endothelial dysfunction. As these adhesion molecules are key mediators of monocyte-endothelial cell interactions (Mestas and Ley,

2008), the inhibitory effect of DG on THP-1-endothelial cells interaction possibly mediated by regulating them. Furthermore, both GKT, an inhibitor for NOX4, and PDTC, an inhibitor for NF-κB, could inhibited HG-induced expression of ICAM-1 and VCAM-1 and the adhesion of THP-1-to endothelial cells. In view of the transcriptional regulation of NF-κB on ICAM-1 and VCAM-1 (Chen and Manning, 1995) and the effect of NOX4 on NF-κB activation in endothelial cells (Maloney et al., 2009; Zhao et al., 2014), it is possibly that the inhibitory effect of DG on THP-1-endothelial cells adhesion was mediated by decreasing NOX4 derived ROS generation and subsequent NF-κB activation.

In summary, our data demonstrated that DG, an aqueous extract of *Salvia miltiorrhiza* Bunge-*Radix Puerariae* herb pair significantly improved vascular injury in diabetic mouse model possibly through decreasing oxidative stress derived from NOX4. These results provide scientific evidence for the clinical application of this herb pair for the treatment of diabetic vascular complications.

Conflict of interest

We declared that there was no any conflict of interest.

Author Contributions

X.P.C. and Y.T.H. conceived the study, designed experiments, supervised all research and revised the manuscript. Y.Y. and W.W.Z. carried out the animal and cell experiments respectively, analyzed the data and contributed equally to the study. H.Y.Z. performed the chemical analysis of DG.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (no. 81774200) and the Research Fund of University of Macau (MYRG2016-00043-ICMS-QRCM).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.04.018.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.04.018.

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