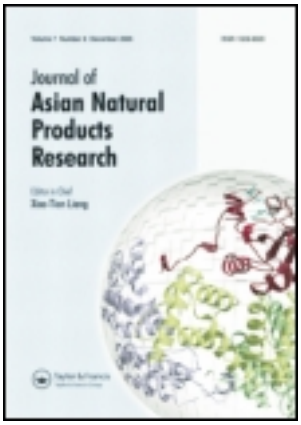

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Salvianolic acid A protects against vascular endothelial dysfunction in high-fat diet fed and streptozotocin-induced diabetic rats

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[a] Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, 100050, China

[b] Aerospace Center Hospital, Beijing, 100049, China

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[a]*Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China;* [b]*Aerospace Center Hospital, Beijing 100049, China*

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Salvianolic acid A (SalA) is one of the main active ingredients of *Salvia miltiorrhizae*. The objective of this study was to evaluate the effect of SalA on the diabetic vascular endothelial dysfunction (VED). The rats were given a high-fat and high-sucrose diet for 1 month followed by intraperitoneal injection of streptozotocin (30 mg/kg). The diabetic rats were treated with SalA (1 mg/kg, 90% purity) orally for 10 weeks after modeling, and were given a high-fat diet. Contractile and relaxant responses of aorta rings as well as the serum indications were measured. Our results indicated that SalA treatment decreased the level of serum Von Willebrand factor and ameliorated acetylcholine-induced relaxation and KCl-induced contraction in aorta rings of the diabetic rats. SalA treatment also reduced the serum malondialdehyde, the content of aortic advanced glycation end products (AGEs), and the nitric oxide synthase (NOS) activity as well as the expression of endothelial NOS protein in the rat aorta. Exposure of EA.hy926 cells to AGEs decreased the cell viability and changed the cell morphology, whereas SalA had protective effect on AGEs-induced cellular vitality. Our data suggested that SalA could protect against vascular VED in diabetes, which might attribute to its suppressive effect on oxidative stress and AGEs-induced endothelial dysfunction.

Keywords: salvianolic acid A; diabetes; vascular endothelial dysfunction; oxidative stress; advanced glycation end products

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia followed by micro- and macrovascular complications, which are the leading causes of morbidity and mortality in patients with DM [1]. Vascular endothelium participates in the control of the vascular tone, coagulation, thrombosis, inflammation, and vascular smooth muscle cell growth as well as migration [2,3]. Vascular endothelial dysfunction (VED) is present at the onset of diabetes and is associated with the long-term adverse outcomes. Increased oxidative stress, via augmentation of reactive oxygen species (ROS), has been implicated as one of the major pathological mechanisms underlying VED in DM [4].

The effect of hyperglycemia on intracellular advanced glycation end products (AGEs) formation also reflects the increase of ROS by mitochondrial overproduction [5]. In endothelial cells exposed to high glucose, intracellular AGEs formation occurs within a week.

*Corresponding author. Email: dugh@imm.ac.cn

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AGEs can decrease the elasticity of large vessels of diabetic rats, even after vascular tone is abolished, also increase the fluid filtration across the carotid artery [6]. Furthermore, the increased serum AGEs in DM patients is associated with VED [7].

Nitric oxide (NO) pathway is suggested to be related to augmenting oxidative stress [8]. NO is synthesized from L-arginine by the action of nitric oxide synthase (NOS), an enzyme existing in three isoforms, termed endothelial NOS (eNOS), neuronal NOS, and inducible NOS (iNOS). NO is very reactive and readily sequestered by superoxide anions to form peroxynitrite which can change the function of protein by modifying the tyrosine residues. Numerous evidence indicate that eNOS and iNOS play important roles in the pathogenesis of diabetic cardiovascular complications [9]. Studies demonstrated that diabetes resulted in the increased eNOS and iNOS levels in the heart, whereas the amount of NO remained unaltered [10].

Salvianolic acid A (SalA) ((2*R*)-3-(3,4-dihydroxyphenyl)-2-[(*E*)-3-[2-[(*E*)-2-(3,4-dihydroxyphenyl) ethenyl]-3,4-dihydroxyphenyl]prop-2-enoyl]oxypropanoic acid, see Figure 1, is one of the main active, water-soluble components in *Salvia miltiorrhizae*, which has been widely used as a traditional Chinese medicine for more than 2000 years [11]. Previous studies have indicated the beneficial effects of SalA on preventing oxidative stress, platelet aggregation, ischemia, and hepatocirrhosis [12,13]. To date, however, fewer reports are available with respect to its pharmacological properties on diabetes, in which endothelial dysfunction plays a key role and is thought to be a major cause of the associated vascular complications. Our previous study had demonstrated that SalA had beneficial effect on vascular reactivity in streptozotocin (STZ)-diabetic rats [14]. In this study, we investigated the protective effect of SalA on VED in high-fat diet fed and STZ-induced diabetic rats and the possible mechanisms. The results suggested that SalA exerted beneficial effects on endothelial dysfunction in diabetes.

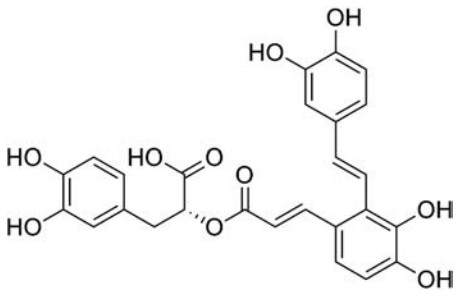

Figure 1. Chemical structure of SalA.

2. Results

2.1 Effects of SalA on survival rate, body weight, blood glucose, and blood lipid levels

Initially, 40 rats were employed in this study to establish the diabetic rats; fasting blood glucose level was higher than 10 mmol/l in 36 rats. Throughout the whole experimental period, hyperglycemia and hyperlipidemia were predominantly present in diabetic rats. Compared with the normal control (NC) group, DM rats had a lower survival rate. A relatively higher proportion of DM + SalA rats survived compared with DM rats, but there was no significant difference between the two groups. The levels of blood glucose, total cholesterol, triglyceride, and low-density lipoprotein (LDL) were lower in DM + SalA group than in DM group (decreased by 19.3, 33.5, 42.9, and 37.1%, respectively), whereas there was no difference in body weight and high-density lipoprotein (HDL) between DM and DM + SalA groups (Table 1).

2.2 SalA reduced the level of serum Von Willebrand factor in diabetic rats

The level of serum Von Willebrand factor (vWF) was significantly higher in all

Table 1. Effects of SalA on survival rate, body weight, blood glucose, and blood lipids.

	NC	DM	DM + SalA
Survival rate (%)	100*	62	81
Body weight (g)	559 ± 18**	322 ± 14	329 ± 15
Blood glucose (mmol/l)	7.21 ± 0.64**	26.6 ± 0.99	22.8 ± 0.35**
Triglyceride (mmol/l)	1.61 ± 0.16**	7.48 ± 0.89	4.27 ± 0.88*
Total cholesterol (mmol/l)	1.75 ± 0.15**	27.8 ± 2.18	18.5 ± 2.65*
HDL (mmol/l)	0.54 ± 0.07	0.39 ± 0.07	0.41 ± 0.08
LDL (mmol/l)	1.3 ± 0.12**	21.3 ± 1.66	13.4 ± 2.30*

Note: Values are means ± SEM for 10–13 animals per group. *$P < 0.05$, **$P < 0.01$ compared with DM group.

diabetic rats than in NC rats, whereas vWF level decreased in DM + SalA group compared with DM group ($P < 0.05$; Figure 2). This suggested that the diabetic rats had vascular endothelial damage that was improved by SalA.

2.3 SalA ameliorated the vascular function in diabetic rats

As shown in Figure 3, compared with NC rats, in DM rats the contractile responses of aorta ring to noradrenaline (NA) or KCl decreased with significant difference ($P < 0.01$). Compared with vehicle treatment, SalA treatment ameliorated the abnormal contractile response to KCl ($P < 0.05$) in DM rats and had a trend to ameliorate the contractile response to NA, but with no significant difference ($P > 0.05$). Thus, SalA treatment, to some extent, ameliorated the contractile reactivity of aortic ring in diabetic rats.

Acetylcholine-induced relaxations of aorta ring in DM rats significantly decreased compared with that in NC rats ($P < 0.01$). The impaired relaxant response to acetylcholine in aortic rings of DM + SalA rats was ameliorated toward the level of NC group ($P < 0.05$).

2.4 Histological analysis of rat aorta

Compared with NC rats, in DM rats the aorta intima showed hyperplasia and the smooth muscle cells were indiscriminate. Distinct edema was observed in endothelium and subendothelium, as well as in injured and desquamated endothelium cells, accumulated foam cells in the aorta in DM group. In DM rats, SalA alleviated these pathological changes (Figure 4).

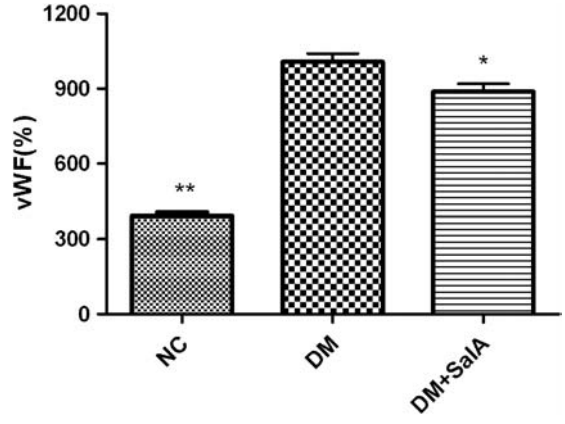

Figure 2. Effect of SalA on the level of serum vWF in diabetic rats. Values are means ± SEM for 10–13 animals per group. *$P < 0.05$, **$P < 0.01$ compared with DM group.

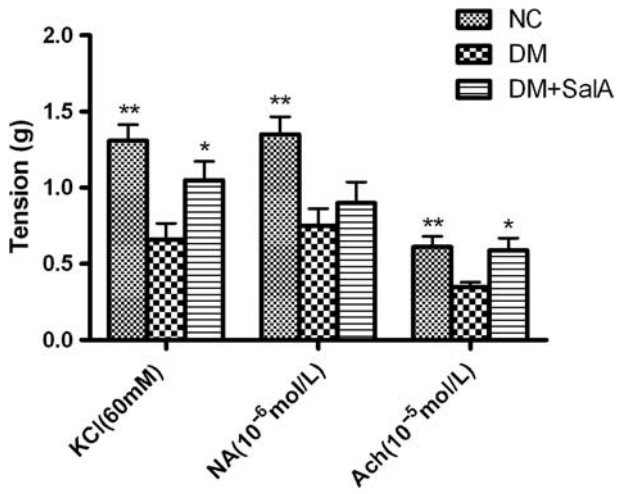

Figure 3. Effects of SalA on the reactivity of contraction and relaxation of aorta ring in diabetic rats. After the rats were sacrificed, the thoracic aortas were isolated immediately. The reactivity of contraction and relaxation of aorta ring was evaluated using the aortic ring assay. The contractile responses to NA (10^{-6} mol/l) and KCl (60 mmol/l) and relaxant response to Ach (10^{-5} mol/l) were measured, respectively. Values are means ± SEM for 10–13 animals per group. $*P < 0.05$, $**P < 0.01$ compared with DM group.

2.5 SalA reduces oxidative stress in diabetic rats

2.5.1 Effect of SalA on the level of serum malondialdehyde

The level of serum malondialdehyde (MDA) was significantly higher in diabetic rats than in NC rats, whereas MDA level fell in DM + SalA group compared with that in DM group ($P < 0.05$; Figure 5).

2.5.2 Effect of SalA on the activity of NOS in aortas

Compared with NC rats, in all diabetic rats the activities of total NOS, eNOS, and

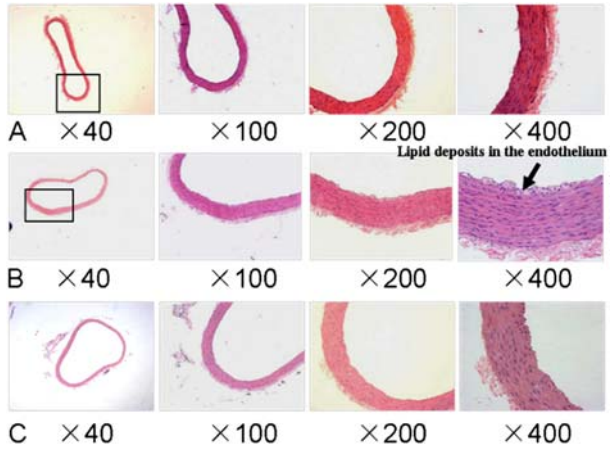

Figure 4. Pathological changes of rat aorta by H&E staining. A, NC group; B, DM group; C, DM + SalA group.

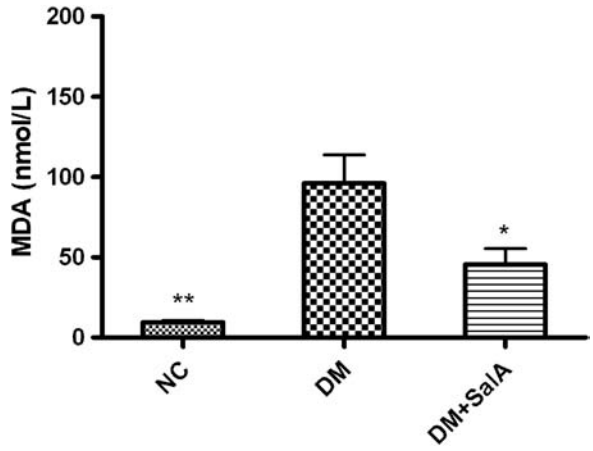

Figure 5. Effect of SalA on the level of serum MDA. Values are means ± SEM for 10–13 animals per group. $*P < 0.05$, $**P < 0.01$ compared with DM group.

iNOS in aorta significantly increased, whereas in DM + SalA group total NOS and iNOS activities were lower than in DM group ($P < 0.05$), and eNOS activity had no significant change (Figure 6).

2.5.3 Effect of SalA on the expression of eNOS protein in aortas

Compared with NC rats, in diabetic rats the expression of eNOS protein in aortas increased by 218%. SalA treatment decreased the expression of aortic eNOS protein by 34.4% compared with no treatment (Figure 7). Thus, SalA could have an inhibitory effect on the expression of eNOS protein in aorta.

2.6 SalA reduced AGEs-induced injury

2.6.1 Effect of SalA on the level of AGEs in aorta of diabetic rats

The level of AGEs in aorta of diabetic rats was significantly higher than NC rats, whereas SalA decreased the level of AGEs by 31.2% ($P < 0.01$; Figure 8(A)).

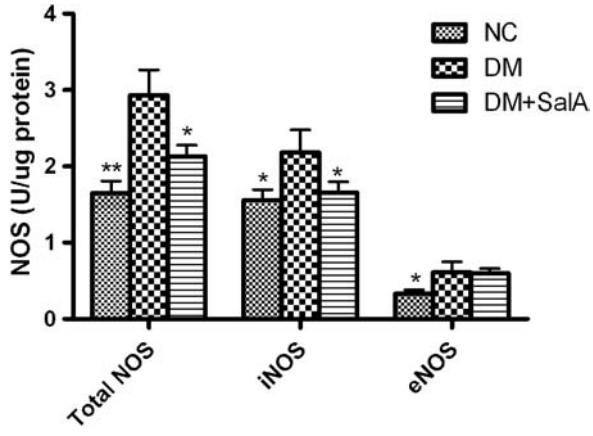

Figure 6. Effect of SalA on NOS activity in aorta. Values are means ± SEM for 10–13 animals per group. $*P < 0.05$, $**P < 0.01$ compared with DM group.

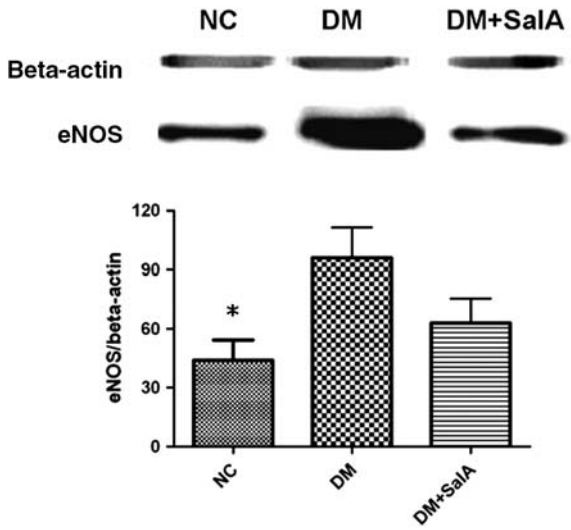

Figure 7. Effect of SalA on the expression of eNOS protein in aorta. (A) Protein expression was detected by Western blot; (B) blots were quantified using Gel-Pro analyzer 4.0 system. Values are mean ± SEM ($n = 10–13$) expressed as a ratio of eNOS expression to beta-actin in each sample. Values are means ± SEM for 10–13 animals per group. $*P < 0.05$ compared with DM group.

2.6.2 Effect of SalA on AGEs-induced injury to EA.hy926 cells

After EA.hy926 cells were incubated with AGEs, the production of ATP was significantly reduced. Compared with AGEs group, in SalA-treated groups the production of ATP was significantly increased, especially at 10^{-5} and 10^{-6} mol/l concentrations ($P < 0.01$; Figure 8(B)). The morphology of EA.hy926 cells changed after incubation with AGEs. And the cells exhibited severe changes: the bulk of cells significantly reduced, plasma became condensed, membrane was crimpled, cell cavity was increased, and vacuole was formed. SalA alleviated the AGEs-induced injury, especially at 10^{-5} and 10^{-6} mol/l concentrations (Figure 8(C)).

3. Discussion

In this study, we observed the effect of SalA on VED in high-fat diet fed and STZ-induced diabetic model. SalA treatment attenuated diabetes-induced VED, concomitantly with markedly decreased oxidative stress and AGEs injury. SalA also decreased the blood glucose and lipid levels in diabetic animals.

Changes in vascular responsiveness to several vasoconstrictors and vasodilators are mainly responsible for the development of vascular complications of diabetics [15]. Compared with the NC rats, the diabetic rats exhibited the abnormal contractile and relaxant responses of aorta ring, as well as morphological damage in aorta vascular endothelium. SalA treatment ameliorated Ach-induced relaxation and KCl-induced contraction of aorta ring, consistent with some previous studies [16]. Although increased contraction to NA in STZ diabetic rats has been observed in most studies [17], we found that SalA did not significantly affect rat aortas NA-induced contractile responses. The mechanism was not completely understood.

vWF is known as an important adhesion protein in the endothelium. On endothelial cell stimulation, vWF is released into the blood, in which it acts as a marker of endothelial damage and activation of blood coagulation [18]. Our

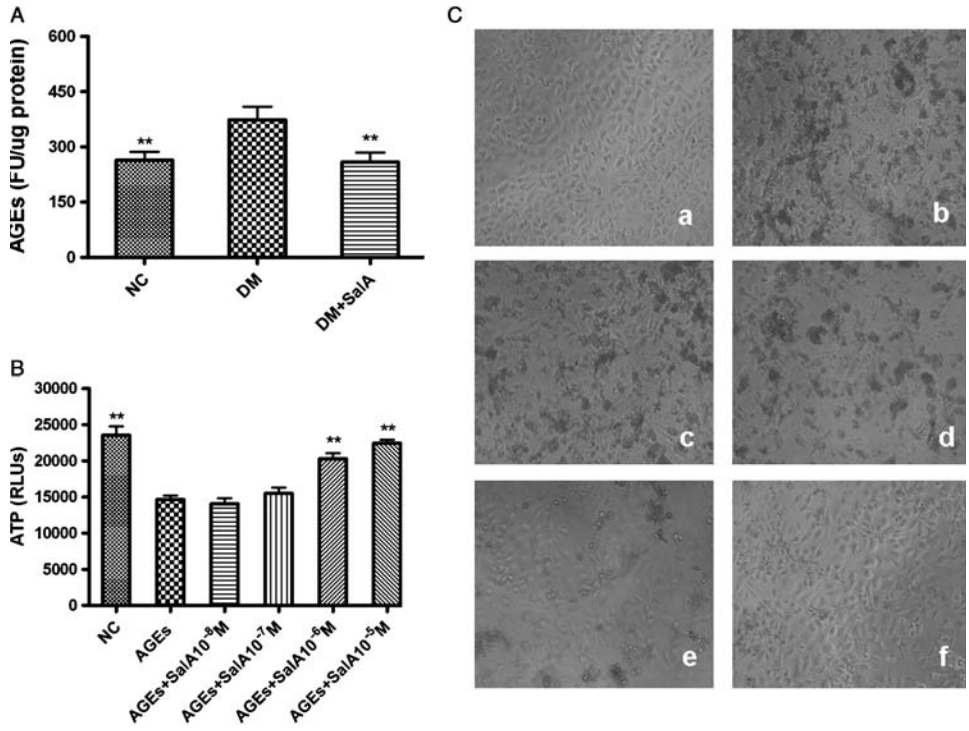

Figure 8. Protective effect of SalA against AGEs injury. The content of AGEs in aorta (A) was measured by detecting the fluorescence intensity in aortic homogenate and corrected by protein concentration. Data are means ± SEM of 12–16 animals per group. $*P < 0.05$, $**P < 0.01$ compared with DM group. (B) EA.Hy926 cells were incubated with different concentrations of SalA in 1% FBS RPMI-1640 medium for 2 h and then treated with AGEs for 30 h. NC and all AGEs-treated cells were analyzed for cell viability using CellTiter-Glo Luminescent Cell Viability Assay. Data are mean ± SEM of three separate experiments. $*P < 0.05$, $**P < 0.01$ compared with AGEs group. (C) The morphological changes of EA.hy926 cells treated with AGEs and different concentrations of SalA were examined a microscope at 250 × magnification. a, NC; b, AGEs; c, AGEs + SalA 10^{-8} mol/l; d, AGEs + SalA 10^{-7} mol/l; e, AGEs + SalA 10^{-6} mol/l; f, AGEs + SalA 10^{-5} mol/l.

results showed that the level of serum vWF increased in diabetic rats, which also exhibited the abnormally contractile and relaxant responses of aorta ring, as well as morphological aortavascular endothelium damage. SalA treatment reduced the level of serum vWF, ameliorated the vascular reactivity of aorta ring, and alleviated the pathological changes in diabetic rats, indicating the beneficial effect of SalA on vascular endothelial function.

Substantial evidence indicates that oxidative stress is augmented in diabetic complications, including VED [4]. Our observation was consistent with earlier findings that a balance between NOS and ROS was crucial for the maintenance of vascular function. We found that diabetes leads to the decreased vasorelaxant and vasocontractile response, accompanied by augmented oxidative stress as indicated by increased activity of MDA and NOS. Treatment of diabetic animals with SalA decreased the diabetes-induced MDA and NOS upregulation, suggesting an antioxidative stress activity of SalA.

eNOS plays a key role in the endothelial dysfunction. STZ-induced diabetic rats exhibited an increase in the expression of eNOS in endothelial

cells [19]. However, importantly, eNOS expression does not always correlate with eNOS activity. The generation of NO from L-arginine by eNOS requires the presence of cofactors that can be inactivated by oxidants, and when eNOS is 'uncoupled', superoxide opposed to NO will be generated. Brodsky *et al.* [20] found that high glucose induced uncoupling of eNOS, which caused a reduction in NO bioavailability and a concurrent increase in superoxide production. As eNOS activation also requires its translocation into the plasma membrane in its coupled form, it is likely that the increased level of eNOS in diabetic rats might be in the inactivated form of this enzyme. Thus, in our study, we found that SalA treatment had a trend to decrease the expression of eNOS protein, but had no effect on the activity of eNOS.

AGEs can produce ROS and increase oxidative stress by activation of nicotinamide adenine dinucleotide phosphate oxidase through AGEs receptors [21]. It was also shown that antioxidants not only reduced the formation of AGEs but also suppressed the AGE-mediated intracellular effects [22]. In clinical patients with diverse cardiovascular risk factors, AGEs inhibitors were shown to ameliorate the endothelial dysfunction [23]. Our results showed that the elevated level of AGEs was present in the aorta of diabetic rats. SalA could reduce the aortic AGEs levels in diabetic rats and alleviate the AGEs-induced injury to Hy926 cells. Moreover, the increase of ATP level in the SalA-treated cells indicated that SalA may ameliorate the mitochondrial function, subsequently promote the cell viability and finally alleviate the AGEs-induced injury to Hy926 cells. The suppressive effect of SalA on AGEs-induced injury and oxidative stress at least partially explained the protective role of SalA against the endothelial dysfunction in diabetic rats.

In addition, SalA treatment ameliorated the abnormal blood glucose and lipid levels in diabetic rats, which may contribute to the reduced production of AGEs and ROS. The mechanism underlying the improved metabolic disorder by SalA may be related to the amelioration of mitochondrial function, which needs to be further studied.

In conclusion, the beneficial effects of SalA in the experimental model of diabetes-induced VED suggest that this agent may have important implication for the treatment of diabetes. Nevertheless, more experimental and clinical evidence will be required to completely understand the role of SalA in the pathogenesis of diabetic vasculopathy.

4. Materials and methods

4.1 Animals and treatments

Male Wistar rats (body weight, 180–200 g) were obtained from Vitalriver Company (Beijing, China). This study was approved by the Ethics Committee of the Institute of Material Medica, Chinese Academy of Medical Sciences. The rats were randomly divided into NC group and diabetic group. The rats in the NC group were fed with a standard diet (41.47% carbohydrate, 14.42% fat, and 21.06% protein), and the rats in diabetic group were given a high-fat and high-sucrose diet (standard rat diet supplemented with 10% sucrose, 10% lard, 2% cholesterol, and 0.2% cholic acid) for 4 weeks, and then the rats in the diabetic group were treated with a single intraperitoneal injection of STZ (30 mg/kg, Sigma, St. Louis, MO, USA) [24,25]. The NC rats (NC group) were injected with equal volume of vehicle. After 7 days following STZ injection, the animals with fasting blood glucose higher than 10 mmol/l were considered as diabetic rats.

The diabetic rats were equally assigned into two groups according to blood glucose levels and body weights: untreated

diabetic group (DM group, $n = 16$) and SalA-treated diabetic group (DM + SalA group, $n = 16$). Rats in both DM and DM + SalA groups were given a high-fat diet (standard rat diet supplemented with 10% lard, 2% cholesterol, and 0.2% cholic acid), whereas rats in the NC group ($n = 12$) were given a standard diet. All rats had free access to water and food.

Rats in DM + SalA group orally received SalA (above 90% purity, Beijing Collab Co., Beijing, China) daily with a dose of 1 mg/kg throughout the experimental period (10 weeks), and rats in NC and DM groups received equal amounts of water intragastrically.

4.2 Blood collection and tissue preparation

At the end of the 10-week treatment, all rats were anesthetized with 25% urethane (0.5 ml/100 g body weight, i.p.) after overnight fasting. Blood samples were collected from femoral artery. The thoracic aortas from aortic arch to diaphragm were isolated immediately after blood collection for the preparation of paraffin slices, the for the determination of contractile and relaxant response, and the assays of AGEs and NOS.

4.3 Measurement of blood lipid and glucose

Blood lipids, including total cholesterol, triglyceride, LDL, and HDL, were measured according to the protocol of the kits (Beijing BHKT clinical reagent company, Beijing, China). Blood glucose was measured according to Hexokinase method (Beijing Biosino Co., Beijing, China).

4.4 Measurement of vWF in serum

Serum vWF level in all rats was measured according to the protocol of the enzyme-linked immunosolvent assay kit (Shanghai Sun Biotech Co. Ltd, Shanghai, China).

4.5 Aortic ring preparations and recording of contraction–relaxation

The thoracic aortas were cut into approximately 3-mm wide rings. Great care was taken to avoid damaging the luminal surface of endothelium. The aortic rings were mounted on a stainless steel hook in bath chambers (10 ml) containing modified Krebs–Henseleit solution (in mmol/l, NaCl 120, KCl 4.8, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, $CaCl_2$ 2.5, $MgCl_2$ 1.4, and EDTA 0.01), which was maintained at 37°C and bubbled with 95% O_2 and 5% CO_2. The tension was recorded using the MP100 system (BIOPAC Systems, Goleta, CA, USA).

The aortic rings were given a tension of 1.2 g during the 60-min equilibration period and were exposed to two successive stimulations with high K^+ (60 mmol/l) solution. At the end of equilibration, the contractile responses to NA (10^{-6} mol/l) and KCl (60 mmol/l) were measured, respectively. To assess the endothelium-dependent relaxation, we pretreated the rings with NA (10^{-6} mol/l) followed by the addition of acetylcholine (10^{-5} mol/l) into the chambers. At the end of the experiment, the aortic rings were dried at 55°C for 5 min and weighed.

4.6 Histopathological examination

The upper portion of thoracic aorta was harvested, immediately fixed in 10% buffered formalin, and embedded in paraffin blocks. Slices were stained with hematoxylin and eosin stain (H&E). Stained sections were examined with light microscopy and the images were captured.

4.7 Measurement of MDA in serum

Serum MDA level was measured as an index of lipid peroxidation using the colorimetric methods (Nanjing Jiancheng Co., Nanjing, China). MDA and thiobarbituric acid were condensed to red

products, which have the absorption maximum at 532 nm.

4.8 Examination of total NOS, iNOS, and eNOS activities in rat aorta

Total NOS, iNOS, and eNOS activities were measured from aortic homogenates using an NOS assay kit (Nanjing Jiancheng Co.). Briefly, NOS converts L-arginine to L-citrulline, leading to the generation of free radical NO. NOS activity was determined by the production of NO absorbed at 530 nm.

4.9 Expression of eNOS protein in rat aorta by Western blot

The expression of eNOS protein in rat aortas was examined by Western blot. Total protein in aorta was extracted in 1 × RIPA buffer (50 mmol/l Tris HCl [pH 8], 150 mmol/l NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with complete protease inhibitor cocktail (Biochem, California, Germany), then centrifuged at 12,000*g* for 10 min. Equal amounts of protein were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotted with antibodies specific for eNOS (1:400, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and beta-actin (1:2000, Santa Cruz Biotechnology). The blots were exposed to ECL (Fujifilm, LAS 3000, Tokyo, Japan) and were quantified using Gel-Pro analyzer 4.0 system and expressed as a ratio of eNOS expression to beta-actin.

4.10 Assay of AGEs in rat aorta

AGEs emit fluorescence at Ex 370 nm/Em 440 nm, and the fluorescence intensity represents the level of AGEs. We detected the fluorescence intensity in aortic homogenate directly by Fluorescence Microplate Reader and corrected by protein concentration to represent the content of AGEs.

4.11 Effect of SalA on EA. Hy926 cells subjected to AGEs stimulation

AGEs were prepared by incubating 5 g/l BSA (Amresco, Inc., Cleveland, OH, USA) with 0.5 mol/l glucose (Sigma) at 37°C for 6 months.

EA.hy926 cell line was cultured in RPMI-1640 supplemented with 15% FBS at 37°C and 5% CO_2. The cells were planted at a density of 4×10^3 cells/well in a 96-well culture plate. When the cells reached the subconfluent state (70–80%), the medium was changed to RPMI-1640 with 1% FBS, which was supplemented with SalA (10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} mol/l). After 2 h, AGEs were added into the medium with a final concentration of 10% and incubated for another 30 h. AGEs-stimulated and untreated groups were regarded as AGEs group and NC group, respectively. The cells were observed under a microscope and then harvested to measure the ATP content according to the protocol of CellTiter-Glo Luminescent cell viability assay (Promega, Madison, WI, USA).

4.12 Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM) and analyzed using the statistical package SPSS 13.0. One-way ANOVA followed by Dunnett's multiple comparison test was used to determine the statistically significant differences among the three groups.

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Note

1. Xiuying Yang and Guifen Qiang contributed equally to this work.

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