# Original Article Effect of astragaloside IV on diabetic gastric mucosa in vivo and in vitro

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Abstract: This study evaluated the effects of AS-IV on high glucose induced-gastric mucosal lesions compared to the effects of the Bu-zhong-yi-qi pill and the Xiaoke pill, which are already in clinical use. STZ-induced diabetic SD rats were treated with drugs for 12 weeks. Diabetes serum (DS) was used to mimic high glucose conditions in GES-1 cells in vitro at different concentrations and time points. The drugs were set at three concentration gradients for 24, 48 and 72 h before being added to DS-induced GES-1 cells. The proliferation activity and inhibition ratio of cells were measured by the CCK-8 assay. Gastric tissues were examined by H&E staining. Cell morphology was observed by inverted phase contrast microscopy. Apoptosis of cells was detected by annexin V-FITC/PI. In addition, expression levels of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and mucin1 (MUC1) were measured by Western blots. We found that the drugs significantly alleviated the pathological damage in STZ-induced gastric mucosal lesions. DS inhibited the viability of the cells in a dose and time-dependent manner, while pretreatment with drugs reversed these effects. Pretreatment with drugs also ameliorated the changes in cell morphology and inhibited cell apoptosis. Furthermore, the drugs decreased the expression levels of iNOS, COX-2 and MUC1. AS-IV showed the most beneficial effect compared with buzhong and xiaoke. These results suggest that AS-IV had a significant effect on high glucose-induce gastric mucosal lesions compares with buzhong and xiaoke. We speculate that this is in part through restoring the balance of iNOS, COX-2 and MUC1 expressions. Thus, AS-IV may be a potential antioxidant drug for treating diabetic gastropathy.

Keywords: Diabetic rat, astragaloside IV, bu-zhong-yi-qi pill, xiaoke pill

#### Introduction

In 1958, the "diabetes gastrointestinal disease" concept was first proposed by Kassander. The main manifestations of this disease include dyspepsia, low gastrointestinal motility, watery diarrhea and constipation. A clinical study found that about 75% of diabetic patients exhibited severe gastrointestinal symptoms [1]. One complication of diabetes mellitus (DM) is gastric ulcers. Gastric mucosal lesions in patients with DM can further affect gastrointestinal function and the absorption of hypoglycemic drugs, thus affecting the quality of life, leading to unstable blood sugar levels, and increasing the risk of DM complications and severity.

Diabetes gastrointestinal disease is a common complication of diabetes and encompasses

diabetic autonomic neuropathy, endocrine dysfunction, gastrointestinal microvascular disease, susceptibility to Helicobacter pylori and other factors related to metabolic disorders of diabetes [2]. The gastrointestinal barrier is primarily dependent on the gastrointestinal mucosa for its first line of defense. The core units of the gastrointestinal mucosa is mucin (MUC) which is secreted by the epithelial cells of the gastrointestinal (including mucous neck cells, goblet cells, etc.) tract [3]. These form a dynamic and interactive mucosal defense system that acts on the gastrointestinal mucosal surface [4]. Studies have shown that the balance of nitric oxide (NO) and prostaglandins (PGs) that are produced by constitutive and inducible NOS and COX [5, 6], comprise the gastrointestinal mucosal defense system. NOS and COX play key roles in maintaining the basic gastric muco-



Figure 1. The structure of astragaloside IV.

sal blood flow, in developing a congestive response to harmful stimuli and in repairing the gastric mucosa [7, 8]. In recent years, a large number of studies have found that DM can lead to apoptosis of different cells including myocardial cells, endothelial cells, ganglion cells, retinal endothelial neurons, and gastric smooth muscle cells [9-11]. Studies have found that the expression of cytochrome C in DM rat gastric mucosal cells is enhanced, suggesting that apoptosis of gastric mucosal cells plays a key role in gastric mucosal injury in diabetic rats [12].

The primary cause of disability and premature death among DM patients is caused by complications associated with poor blood glucose control. However, traditional Chinese medicine (TCM) has been shown to have beneficial effects in the treatment of diabetes gastrointestinal disease [13, 14]. The Chinese Medicine Research Institute used 591 modern diabetes prescriptions for treatments and 53.19 percent of these drugs contain astragalus as the primary ingredient. Astragalus has significant pharmacological effects on the cardiovascular, renal and nervous systems and the gastrointestinal tract [15-18]. A recent study showed that Astragalosides promoted gastrointestinal absorption effects [19], but the specific mechanism remains unknown. Two herbal drugs, the Bu-zhong-yi-qi pill and the Xiao Ke pill contain Astragalus as the principal ingredient. The Bu-zhong-yi-qi pill is composed of eight herbs, such as Radix Astragali (Astragalus membranaceus (Fisch) Bunge), Radix Codonopsis (Codonopsis pilosula (Franch.) Nannf.), Rhizoma

Atractylodis Macrocephalae (Atractylodes macrocephala Koidz.), Radix Angelicae Sinensis (Angelicae Sinensis (Oliv.) Diels), Rhizoma Cimicifugae (Cimicifuga heracleifolia Kom.), Radix Bupleuri (Bupleurum chinense DC.), Pericar*pium Citri Reticulatae* (Citrus reticulata Blanco) and Radix Glycyrrhizae (Glycyrrhiza uralensis Fisch.), and is traditionally used to treat deficiency of spleen and stomach Qi or middle Qi collapse syndrome [20]. A modern study found that Bu-zhong-yi-qi pill can also be used to treat diabetes [21]. The Xiaoke pill, which was approved by the SFDA for commercial use several years ago, is a Sino-western medicine (including glibenclamide 0.1%) used for the widespread clinical treatment of Yin and Oi Deficiency in diabetes [22]. It consists of the Chinese herbal drugs, Radix Astragali (Astragalus membranaceus (Fisch) Bunge), Radix Rehmanniae (Rehmannia glutinosa (Gaerhn) DC.), Radix Puerardiae (Pueraria lobata (Willd) Ohwi), Rhizoma Dioscoreae (Dioscorea opposita Thunb.), Stylus et Stigma ZeaeMaydis (Zea mays L.), Fructus Schisandrae Chinensis (Schisandras phenanthera Reher & E.H.Wilson), Radix Trichosanthis (Anguinakirilowii (Maxim.) Kuntze) and glibenclamide (a chemical drug). Astragaloside IV (AS-IV) is an active ingredient of the herb Astragalus, which can inhibit other diabetes complications, like apoptosis [23]. However, no research has been conducted on apoptosis of diabetic gastric mucosal cells. The aim of this study was to explore the preventive effects of AS-IV on high glucose-induced gastric mucosal lesions compared to the effects with buzhong and xiaoke in vivo and in vitro, and to provide a novel drug therapy for the prevention of diabetic gastropathy.

# Materials and methods

# Drug preparation

Astragaloside IV (AS-IV) was purchased from Chendu King-tiger Pharm-Chem. Tech. Co., Ltd (Chengdu, China), with a purity of above 98%. The chemical structure of AS-IV has been depicted in **Figure 1**. ( $C_{41}H_{68}O_{14}$ , molecular weight = 784, CAS: 84687-43-4). The Bu-Zhong-Yi-Qi pills, Xiao Ke pills were purchased from JiuZhiTang Co., Ltd (Guangzhou, China).

Preparation of drug-containing serums: male Sprague-Dawley (SD) rats weighing about 200 g were randomly assigned to Bu-Zhong-Yi-Qicontaining serum, Xiao Ke-containing serum and blank serum (n = 15), and were given Buzhongyiqi pill with 9.25 g·kg<sup>1</sup>·d<sup>1</sup>, Xiao Ke pill with 3.85 g·kg<sup>1</sup>·d<sup>1</sup> and normal saline at 10 mL/ kg respectively according to quintuple optimal dosing for adults. All rats were given intragastric administration of the drugs twice daily for 5 d. One hour after the last gavage, abdominal aortic blood of rats was taken and sterile separation of serum was performed. The serum was stored at -80°C for subsequent use.

### In vivo studies: animal studies and drugs treatment

All work with rats was approved by the Animal Ethics Committee of Guangzhou University of Chinese Medicine, Guangzhou, China. Healthy male SD rats weighing between 220 to 260 g, were purchased from the Experimental Animal Center, Guangzhou University of Chinese Medicine, Guangzhou, China. Animal Production license number: SCXK (Guangdong, China) 2013-0020. Rats were housed in an air-conditioned room maintained at 21±2°C with a humidity of 58%±5%, and on a 12-h light/dark cycle. Animals were acclimatized for 7 days in the research facility before initiation of the experiments. Intraperitoneal injections of STZ (Sigma-Aldrich, St. Louis, MO, USA) were administered to fasted rats after 12 h, at 55 mg/kg, while, age-matched control rats received an equal volume of vehicle (0.1 M citrate buffer, PH 4.2). Seventy-two hours after STZ injection, the blood glucose levels were measured from blood obtained from the tail vein. Rats with three times the blood glucose levels, over 16.8 mmol/L, and that exhibited polydipsia, polyuria, and polyphagia symptoms were selected as diabetic rats. After four weeks, the rats were randomly divided into normal control (control). untreated diabetic rats (non-treat), xiaoke pills (xiaoke), bu-zhong-yi-qi pills (buzhong) and AS-IV treated groups (n = 8/each group). The drug regimen lasted 12 weeks, the rats in the control group and untreated groups were given oral gavage consisting of equal volume of saline (20 mL/kg per day), the rats in the xiaoke group were given gavage with Xiao Ke pills (1.54 g/kg per day), the rats in the buzhong group were given Bu-Zhong-Yi-Qi pills fed (3.75 g/kg per day), and the rats in the AS-IV group were given Astragaloside IV gavage (40 mg/kg per day). All rats had free access to food and drinking water.

The body weights and blood glucose levels were monitored, and the appetite, water intake and urine output for 24 h were recorded monthly by metabolic cages.

#### In vitro studies: cell culture and CCK-8 assay

Gastric epithelial cells (GES-1) (Pericells) were thawed and cultured in RPMI-1640 medium (Hyclone) and supplemented with 5% fetal bovine serum (FBS, Gibco) at 37°C under a humidified atmosphere of 5% CO2. Cells were washed 2 times with PBS followed by digestion with 0.25% trypsin. The cells were then seeded at a concentration of 10<sup>4</sup> cells/mL in 96-well plates. GES-1 cells were stimulated with diabetic serum (DS) at different concentrations (10%, 20% and 30%) and at different timepoints (24 h, 48 h and 72 h). The control cells were treated with 10% normal same aged-rats serum, and all cells were cultured in FBS-free RPMI-1640 medium. Finally, 100 ul of cell counting kit-8 (CCK-8) was added to each well and the cells were further incubated at 37°C for 4 h. The absorbance of CCK-8 was detected at 450 nm by a microplate reader, and the viability of cells was determined by the CCK-8 assay (Dojindo). The cells were divided into the following groups: (1) normal control group (control), (2) untreated group (non-treat), (3) Buzhongyiqi pill-containing serum (buzhong) group, (4) xiaoke pill-containing serum group (xiaoke), (5) astragaloside IV (AS-IV) group. Cells were pretreated with blank serum, Buzhongyiqi pill-containing serum and xiaoke pill-containing serum at different concentrations (10%, 20% and 30%), and with AS-IV (25, 50 and 100 µg/mL) for 24, 48 and 72 h before 20% diabetic serum was added for 24 h. The control group was pretreated with 10% blank serum before 20% normal same aged-rat serum was added for 24 h. Furthermore, the proliferation activity of cells was detected by the CCK-8 assay and the best concentration and timepoint for treatments were selected for the subsequent study. All experimental groups were cultured in triplicate.

# Pathological examination

At the end of the *in vivo* study, stomach tissue of rats was removed and cut along the greater curvature and flipped open with 4°C saline rinse. The tissue was then dried with filter paper, paraffin-embedded after 4% paraformal-



Figure 2. Effect of AS-IV on body weight (A) and blood glucose (B) after treatment with drugs at different times. Results are expressed as the mean  $\pm$  SD. \*\*indicates P<0.01 vs Control.

dehyde fixation and sections 4 um in thickness were prepared for hematoxylin and eosin (H&E) staining. Pathological changes were observed via microscopic photographs. In addition, 1/3 of the stomach tissue was cut and cryopreserved at -80°C for later use in Western blots.

# Morphological observations and apoptosis assay

The 20% drug-containing serums and AS-IV with 25 µg/mL were selected and pretreated for 24 h before being incubated with diabetic serum-induced GES-1 cells. Cells were washed twice by PBS suspended and cell morphology was observed using inverted phase contrast microscopy. Moreover, after 24 h of incubation with the different drugs, cells were washed with PBS, trypsinized, and centrifuged at 1,000 rpm for 5 min. Cells were washed twice with PBS and resuspended in binding buffer. Subsequently, cells were stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) (UNOCI biological). The stained cells were then analyzed for apoptosis using flow cytometry. Cells positive for annexin V-FITC and negative for PI were considered apoptotic.

# Western blotting

Stomach tissue or cultured GES-1 cells under the different experimental conditions were lysed in lysis buffer with a sonicator. Crude lysates were centrifuged at 14,000 rpm for 10 minat 4°C. Total protein concentration from the supernatant was determined by the bicinchoninic acid (BCA) protein assay (Pierce, MT, USA). Protein samples were separated by 10% SDS-PAGE and transferred to a PVDF membrane (Millipore) by electroblotting. The membranes were blocked with 5% fat-free milk at room temperature for 1 h and then incubated with primary antibodies (1:400 for anti-COX2 antibody (Boster Company, Hunan, China), constant flow membrane 69 min; 1:300 for anti-iNOS antibody (Boorson company, Beijing, China), constant flow membrane for 130 min; 1:1000 for anti-MUC1 antibody (Boorson company, Beijing, China), constant flow membrane 230 min) at room temperature for 1 h, followed by three washes in TBST. The membranes were incubated with anti-rabbit IgG (Boster) at room temperature for 40 min. After washing, the signals were visualized with enhanced Chemiluminescence and X-ray film, and gel image processing system (Image-Pro Plus 6.0) for analysis. GAPDH served as an internal control, and protein expression was quantified as the ratio of the specific band to GAPDH.

#### Statistical analysis

Statistical analysis was conducted by using LSD of the ANOVA test. Data is expressed as mean  $\pm$  SD with P<0.05 considered statistically significant. Application of the SPSS 17.0 statistical software was used for data processing.

# Results

Effect of AS-IV on body weight and blood glucose in vivo

We established a rat model of diabetes by intraperitoneally injecting STZ. Blood glucose levels

# Effect of astragaloside IV on diabetic gastric mucosa in vivo and in vitro



**Figure 3.** Effect of AS-IV on gastric histopathology (A) and cell morphological (B). (A) Using HE staining (200×) of gastric tissue from normal or diabetic rats with drug treatment for 12 weeks. (B) Using inverted phase contrast microscope to observe cell morphology and recording the photographs for each group of drug treatment for 24 h.

were significantly increased in STZ-induced diabetic rats at different stages (*P*<0.01 vs. normal control rats). However, no differences in blood glucose levels were observed between drug- treated and untreated STZ-induced diabetic rats (**Figure 2A**). In diabetic rats, a decrease in body weight was observed compared with normal rats. In addition, no change was observed compared with rats that were pretreated with AS-IV, buzhong and xiaoke (**Figure 2B**). Moreover, drugs did not reduce the levels of water intake, appetite and urination for 24 h in diabetic rats. AS-IV significantly ameliorated the gastric histopathology in STZ-induced diabetic rats and the cell morphology in DS-induced GES-1 cells

At 16 weeks after STZ injection, gastric tissues from diabetic rats were stained with H&E. We found that the gastric epithelial cell mucus was thinner, deletion, the cell gap was larger, mild necrosis was present, cytoplasmic vacuoles were deformed, capillaries were expanded and inflammatory cell infiltration was present. In contrast, the normal control rats exhibited continuity in gastric epithelial cell, neatly arrayed

Table 1. Cell viability of DS-induced GE	S-1
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Control	Model		
	10%	20%	30%
0.67±0.002	0.518±0.007	0.373±0.029	0.271±0.005
1.067±0.041	0.648±0.019	0.418±0.035	0.335±0.005
1.505±0.019	0.791±0.028	0.340±0.040	0.181±0.010
	Control 0.67±0.002 1.067±0.041 1.505±0.019	Control 10%   0.67±0.002 0.518±0.007   1.067±0.041 0.648±0.019   1.505±0.019 0.791±0.028	Model   10% 20%   0.67±0.002 0.518±0.007 0.373±0.029   1.067±0.041 0.648±0.019 0.418±0.035   1.505±0.019 0.791±0.028 0.340±0.040

Results are expressed as the mean  $\pm$  SD.

glands, clear hierarchy of mucosa and muscularis mucosa and submucosa with no telangiectasia congestion. Treatment with AS-IV, buzhong and xiaoke ameliorated the damage observed in the pathological lesions compared with the untreated STZ-induced diabetic rats with AS-IV showing the most beneficial effect (Figure 3A). Moreover, the cell morphology in DS-induced GES-1 cells comprised of cells with adherent growth that were flat polygon or nearly fusiform. Some pseudopodia showing island-like growth, and some round and broken off cells were also observed. Pretreatment with AS-IV and drugcontaining serums significantly alleviated these features of cell morphology (Figure 3B), and AS-IV showed the most improved effect similar to the observations in vivo.

Effect of AS-IV on the viability of DS-induced GES-1 by CCK-8 assay

GES-1 cells were induced at different concentrations of diabetic serums (10%, 20% and 30%) and at different timepoints (24 h, 48 h and 72 h). We found that the proliferative activity of cells was 30% in diabetic serum. The inhibition rate of cells was both dose and timedependent (Table 1; Figure 4A). We selected 20% as the optimal concentration of diabetic serum for treating GES-1 cells for 24 h. Furthermore, GES-1 cells were pretreated with AS-IV and drug-containing serums before being exposed to diabetic serum. We found that the effect of drugs on the viability of DS-induced GES-1 cells was concentration and time-dependent, in contrast to untreated DS-induced GES-1 cells, which inhibited the proliferative activity of cells, regardless of concentration (Table 2: Figure 4B-E). However, AS-IV more effectively enhanced the viability of DS-induced GES-1 cells compared to buzhong and xiaoke, which affect the viability of DS-induced GES-1 cells in a similar manner (Table 2; Figure 4F). These findings indicated that AS-IV significantly prevented diabetic serum from inhibiting the proliferative activity of GES-1 cells.

Antiapoptotic effect of AS-IV on DSinduced GES-1

We selected 20% of xiaoke and buzhong containing serums and AS-IV at 25 ug/mL to examine apoptosis of DS-induced GES-1. The results of annexin V-FITC/PI staining are shown in **Figure 6**. GES-1 cells exposed to diabetic serum for 24 h resulted in significant apoptosis compared with control cells (P<0.01, **Figure 5**). However, AS-IV significantly inhibited DS-induced GES-1 apoptosis (early apoptosis, late apoptosis and total apoptosis) compared with buzhong and xiaoke. These findings indicate that AS-IV protects against DS-induced GES-1 apoptosis.

# AS-IV regulated the expressions of iNOS, COX-2 and MUC1 by Western blot in vivo and in vitro

At 16 weeks after diabetic rats were induced with STZ, we observed an increase in the expression levels of iNOS and COX-2 and a dramatic reduction in the expression level of MUC1. These changes were not observed in the drug treated animals and AS-IV showed the most significant efficacy (Figure 6A). Consistent with these effects on protein expressions in STZ-induced diabetic rats, DS-induced GES-1 cells also showed elevated expressions of iNOS and COX-2 and decreased MUC-1 expression compared with normal controls. In addition, AS-IV pretreatment lowered the increased levels of iNOS, COX-2 and elevated the decreased level of MUC1 compared with buzhong and xiaoke. Moreover, buzhong and xiaoke have similar efficacy and no significant differences were observed in their regulation of the expressions of iNOS, COX-2 and MUC1 (Figure 6B). These findings suggest that AS-IV restores the balance of iNOS, COX-2 and MUC1 protein expressions in gastric mucosa to repair gastric mucosal lesions in STZ-induced diabetic rats.

# Statistical analysis

All data in this research were expressed as means  $\pm$  SD. SPSS 19.0 version was used for data analysis using Dunnett t test or Two-way ANOVA methods. Images were done using



**Figure 4.** Effect of AS-IV on the cell growth inhibition rate by the CCK-8 assay. (A) Shows the cell growth inhibition rate of diabetic serum-induced GES-1 at different concentrations and timepoints, (B-E) Shows blank serum, buzhongyiqi pill-containing serum, xiaoke pill-containing serum and AS-IV at different concentrations pretreated for 24, 48 and 72 h before DS-induced GES-1, respectively. (F) Shows 20% blank serum, buzhong and xiaoke-containing serums and AS-IV at 25 ug/mL that were selected for the CCK-8 assay (F). Growth inhibition ratio =  $(OD_{control} OD_{value})/OD_{control} \times 100\%$ .

Group	Concentration	24 h	48 h	72 h
Non-treat	20%	0.492±0.007	0.432±0.013	0.304±0.003
	10%	0.484±0.008	0.411±0.007	0.292±0.008
	5%	0.490±0.004	0.400±0.015	0.294±0.009
Buzhong	20%	0.596±0.003**	0.659±0.003**	0.69±0.012**
	10%	0.559±0.009**	0.592±0.023**,&	0.651±0.006**
	5%	0.526±0.009**	0.553±0.009**	0.620±0.008**
Xiaoke	20%	0.594±0.014**	0.66±0.005**	0.680±0.009**
	10%	0.562±0.004**	0.624±0.006**	0.664±0.004**
	5%	0.529±0.012**	0.565±0.009**	0.606±0.016**
AS-IV (ug/mL)	100	0.695±0.007**,##	0.768±0.007**,##	1.044±0.032**,##
	50	0.678±0.009**,##	0.735±0.009**,##	0.846±0.022**,##
	25	0.662±0.004**,##	0.692±0.011**,##	0.780±0.011**,##
Control		0.916±0.005**	1.026±0.019**	1.497±0.033**

Table 2. AS-IV enhanced cell viability of DS-induced GES-1 by CCK-8 assay

Results are expressed as the mean  $\pm$  SD. \*P<0.05, \*\*P<0.01 represent cell viability of DS-induced GES-1 in other groups compared to the untreated group; #P<0.05; ##P<0.01 represents cell viability of DS-induced GES-1 in AS-IV compared to buzhong and xiaoke; &P<0.05 represent the cell viability of DS-induced GES-1 in buzhong compared to xiaoke.

GraphPad Prism 6. P<0.05 was regards to be statistically significant.

#### Discussion

In this study, we demonstrated for the first time that astragaloside IV acts directly on gastric

mucosal cells. Addition of AS-IV resulted in an increase in the cell proliferation rate in a dose and time-dependent manner, inhibition of cell apoptosis and reduction in pathological lesions in diabetic gastropathy. We speculate that these effects occurred in part, through the decrease in expression levels of iNOS and



**Figure 5.** Effect of AS-IV on cell apoptosis DS-induced GES-1 cells and this was measured using annexin V-FITC/PI. A and B: Cell apoptosis was measured using flow cytometry with annexin V-FITC/PI staining, and cell apoptosis was inhibited after treated with AS-IV for. \*indicates P<0.05, \*\*indicates P<0.01 vs untreated group; #indicates P<0.05, ##indicates P<0.01 vs untreated group; #indicates P<0.05, ##indicates P<0.01 represent the cell viability of DS-induced GES-1 in buzhong compared to xiaoke.

COX-2 and an increase in the expression of MUC1. Both *in vivo* and *in vitro* studies suggest

that these results have potentially important implications in a clinical setting.



**Figure 6.** AS-IV downregulated iNOS and COX-2 expressions and upregulated MUC1 expression *in vitro* (A) and *in vivo* (B). Western blot detected the protein expression levels of iNOS, COX-2 and MUC1 in diabetic rats at 16 weeks after STZ injection and in DS-induced GES-1 after treating with drugs for 24 h. Results are expressed as the mean  $\pm$  SD. \*indicates P<0.05, \*\*indicates P<0.01 vs. the Control group; #P<0.05, ##P<0.01 vs. the untreated group.

Diabetes is closely associated with a range of gastrointestinal symptoms. A large number of studies show that STZ-induced DM rats have oxidative stress and injury, which is widespread in the stomach and small intestinal mucosa [24, 25]. Whereas a weakened and an increased intestinal mucosal permeability barrier increases the risk of type 1 DM [26], bleeding ulcers or gastrointestinal disorders have a negative effect on diabetes stability [27]. Our previous study found that diabetes metabolic disorders result in an increase in NO production, a decrease in PGE2, and an increase in 6-keto-PGF1 $\alpha$  levels. The interaction of NO and PGs, result in increased STZ-induced gastric muco-

sal lesions in diabetic rats. In addition, serum  $\alpha$ -mannosidase and serum amylase activity in DM rats was significantly decreased compared with normal rats. The resulting gastric lectin binding disorders, lead to cell proliferation, transitional defects and cell death that affect tissue repair.

In this study, we found that STZ-induced diabetic rats had reduced body weight and increased blood glucose levels (**Figure 2**). However, AS-IV had no effect on blood glucose levels, thus we concluded that the preventive effects of AS-IV on gastric mucosal lesions were independent of blood glucose levels.

Moreover, the pathological changes in STZinduced gastric mucosa as observed by H&E staining included gastric mucosal thinning, necrosis and inflammatory cell infiltration compared with normal controls (Figure 3A). AS-IV treated rats exhibited ameliorated pathological damage of the gastric mucosa. Furthermore, the proliferative activity of GES-1 cells that were cultured in diabetic serum was inhibited in a dose and time-dependent manner (Table 1; Figure 4A). Pretreatment with AS-IV for 24 h in the CCK-8 assay significantly enhanced the viability of DS-induced GES-1 cells compared with untreated DS-induced GES-1 cells (Table 2: Figure 4). The antiapoptotic effect of AS-IV is shown in Figure 5, where AS-IV dramatically inhibited diabetic serum (DS)-induced GES-1 apoptosis in vitro. Pretreatment with AS-IV also significantly ameliorated DS-induced GES-1 changes in cell morphology and prevented apoptosis compared with the untreated group (Figure 3B). These findings were associated with the downregulation of the expressions of iNOS and COX-2 proteins and the upregulation of the expression of MUC1. The balance between these molecules was partially restored by AS-IV in both in vivo and in vitro studies (Figure 6).

Proteins rarely work alone and protein-protein interactions play a very important role in cell activity and life processes [28]. Any interference in the bio-molecular interaction network, often leads to disease [29, 30]. Studies found that iNOS defects are associated with MUC defects [31], resulting in thinning of the mucosa and the cumulative reduction in mucin. MUC and COX act together, therefore an increase in COX-2 activity was accompanied by an increase in MUC secretion. PGs in COX source also promotes MUC glycosylation [32, 33]. On the other hand, MUC can induce iNOS and COX-2 activity [34, 35]. The interaction between COX and NOS has been confirmed, iNOS is an upstream signal of COX-2, iNOS via S-nitro activates COX-2 and iNOS and COX-2 coexist [36]. It was found that COX-2 enhances iNOS-induced phospholipase A2 S-nitration, and the largest PGs are synthesized by the interaction of iNOS, phospholipase A2 and COX-2 [37]. In the diabetic gastrointestinal mucosa, MUC, NOS, COX expressions undergo significant changes. The current study in STZ-induced diabetic rats and DS-induced GES-1 cells has demonstrated an increase in expression of iNOS and COX-2 and a decrease in expression of MUC1. The beneficial effect of AS-IV on high glucose-induced gastric mucosal cells is most likely because the balance of iNOS, COX-2 and MUC1 levels is restored.

Even though a similar protective effect, like AS-IV is exerted by Bu-Zhong-Yi-Qi pills and Xiao Ke pills on gastric mucosal lesions, we did not observe any significant differences in vivo and in vitro studies, in terms of alleviating high glucose-induced gastric mucosal lesions. Numerous studies found that AS-IV strongly stimulates the immune system, has anti-inflammatory and anti-oxidative properties, and inhibits apoptosis [23], aldose reductase inhibitors, and free radical scavengers [38]. Moreover, AS-IV inhibited oxidative stress in renal proximal tubular cells by reducing glycated albumin induced epithelial cells into mesenchymal cell transformation, and prevented in vivo and in vitro glucose-induced generation of podocytes and improved diabetic nephropathy [39, 40]. AS-IV also improved STZ-induced diabetic kidney lesions by inhibiting the expression of the inflammatory transcription factor NF-Kb [41], and inhibiting diabetic peripheral neuropathy [38]. These findings demonstrate that AS-IV has a wide range of pharmacological effects on various diabetic complications. In both in vivo and in vitro studies, we show evidence of AS-IV significantly preventing high glucose-stimulated gastric mucosal cell apoptosis by partly restoring the balance of iNOS, COX-2 and MUC1 proteins.

In summary, our results demonstrate for the first time that astragaloside IV acts directly on gastric mucosal cells. AS-IV promotes cell proliferation and inhibits cell apoptosis and ameliorates pathological lesions in diabetic gastropathy in part by decreasing the levels of iNOS and COX-2 expressions while increasing the level of MUC1 expression. These findings strengthen the rationale for using AS-IV in the treatment of diabetic gastropathy and also provides new insights into the development of a gastric-protective drug.

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#### Disclosure of conflict of interest

None.

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#### References

- [1] Tsimmermanla S, Zinnatullin MR. Diabetes mellitus and functional and structural changes in the gastrointestinal tract: a clinical and epidemiological study (a review and the authors' data). Ter Arkh 2011; 83: 71-75.
- [2] Quan C, Talley NJ, Jones MP, Spies J, Horowitz M. Gain and loss of gastrointestinal symptoms in diabetes mellitus: associations with psychiatric disease, glycemic control, and autonomic neuropathy over 2 years of follow-up. Am J Gastroenterol 2008; 103: 2023-2030.
- [3] Perez-Vilar J, Mabolo R. Gel-forming mucins. Notions from in vitro studies. Histol Histopathol 2007; 22: 455-464.
- [4] Wesseling J, van der Valk SW, Hilkens J. A mechanism for inhibition of E-cadherin-mediated cell-cell adhesion by the membrane-associated mucinepisialin/MUC1. Mol Biol Cell 1996; 7: 565-577.
- [5] Burdan F, Szumilo J, Gajjar B, Dudka J, Korobowicz A, Patel S, Nat A, Nat AS, Dworzanski W, Kwasniewski W. Immunoexpression of constitutive and inducible cyclo-oxygenase isoforms in the rat foetal and maternal digestive tract. Folia Morphol 2008; 67: 24-31.
- [6] Vador N, Jagtap AG, Damle A. Vulnerability of gastric mucosa in diabetic rats, its pathogenesis and amelioration by cuminumcyminum. Indian J Pharm Sci 2012; 74: 387-396.
- [7] Ehrlich K, Sicking C, Respondek M, Peskar BM. Interaction of cyclooxygenase isoenzymes, nitric oxide, and afferent neurons in gastric mucosal defense in rats. J Pharmacol Exp Ther 2004; 308: 277-283.
- [8] Samonina GE, Zhuikova SE. [Homeostasis in the gastric mucosa and blood circulation. Part 1. Mechanisms of the adequate blood flow maintenance in the gastric mucosa]. Usp Fiziol Nauk 2001; 32: 60-72.
- [9] Cai L, Wang Y, Zhou G, Chen T, Song Y, Li X, Kang YJ. Attenuation by metallothionein of early cardiac cell death via suppression of mitochondrial oxidative stress results in a pre-

vention of diabetic cardiomyopathy. J Am Coll Cardiol 2006; 48: 1688-1697.

- [10] Eriksson L, Erdogdu O, Nystrom T, Zhang Q, Sjoholm A. Effects of some anti-diabetic and cardioprotective agents on proliferation and apoptosis of human coronary artery endothelial cells. Cardiovasc Diabetol 2012; 11: 27-35.
- [11] Kim J, Kim CS, Sohn E, Lee YM, Jo K, Shin SD, Kim JS. Aminoguanidine protects against apoptosis of retinal ganglion cells in Zucker diabetic fatty rats. Eur Rev Med Pharmacol Sci 2014; 18: 1573-1578.
- [12] Chen X, Fu XS, Li CP, Zhao HX. ER stress and ER stress-induced apoptosis are activated in gastric SMCs in diabetic rats. World J Gastroenterol 2014; 20: 8260-8267.
- [13] Ji L, Tang XQ, Peng JY. Research progress of effect of anti-diabetic traditional Chinese medicines based on regulation of glucose metabolic enzym. Zhongguo Zhong Yao Za Zhi 2012; 37: 3519-3525.
- [14] Li YJ, Xu HX. Research progress on anti-diabetic Chinese medicines. Zhongguo Zhong Yao Za Zhi 2006; 29: 621-624.
- [15] Agyemang K, Han L, Liu E, Zhang Y, Wang T, Gao X. Recent advances in astragalusmembranaceus anti-diabetic research: pharmacological effects of its phytochemical constituents. Evid Based Complement Alternat Med 2013; 2013: 654-643.
- [16] Chen W, Li YM, Yu MH. Astragalus polysaccharides inhibited diabetic cardiomyopathy in hamsters depending on suppression of heart chymase activation. J Diabetes Complications 2010; 24: 199-208.
- [17] Li M, Wang W, Xue J, Gu Y, Lin S. Meta-analysis of the clinical value of astragalusmembranaceus in diabetic nephropathy. J Ethnopharmacol 2011; 133: 412-419.
- [18] Nie Y, Li S, Yi Y, Su W, Chai X, Jia D, Wang Q. Effects of astragalus injection on the TGFbeta/ Smad pathway in the kidney in type 2 diabetic mice. BMC Complement Altern Med 2014; 14: 148.
- [19] Jing Q, Wu GT, Du LD, Liu WZ, Li YD, Ren Y. Therapeutic effects of ethanol extracts of angelica sinensis and astragalus mongholicus in diabetic gastroparesis rats. Zhong Yao Cai 2014; 37: 1415-1420.
- [20] Zheng XF, Tian JS, Liu P, Xing J, Qin XM. Analysis of the restorative effect of Bu-zhong-yi-qitang in the spleen-qi deficiency rat model using (1)H-NMR-based metabonomics. J Ethnopharmacol 2014; 151: 912-920.
- [21] Liu XQ, Wu L, Guo XJ. Effect of Bu-Zhong-Yi-Qi-Tang on deficiency of N-glycan/nitric oxide and islet damage induced by streptozotocin in diabetic rats. World J Gastroenterol 2009; 15: 1730-1737.

- [22] Ji L, Tong X, Wang H, Tian H, Zhou H, Zhang L, Li Q, Wang Y, Li H, Liu M, Yang H, Gao Y, Li Y, Li Q, Guo X, Yang G, Zhang Z, Zhou Z, Ning G, Chen Y, Paul S; Evidence-Based Medical Research of Xiaoke Pill Study Group. Efficacy and safety of traditional Chinese medicine for diabetes: a double-blind, randomised, controlled trial. PLoS One 2013; 8: e56703.
- [23] Gui D, Guo Y, Wang F, Liu W, Chen J, Chen Y, Huang J, Wang N. Astragaloside IV, a novel antioxidant, prevents glucose-induced podocyte apoptosis in vitro and in vivo. PLoS One 2012; 7: e39824.
- [24] Hep A, Dolina J, Prasek J, Dite P. [Dyspeptic complaints in diabetic patients]. Vnitr Lek 2000; 46: 782-784.
- [25] Bhor VM, Raghuram N, Sivakami S. Oxidative damage and altered antioxidant enzyme activities in the small intestine of streptozotocin-induced diabetic rats. Int J Biochem Cell Biol 2004; 36: 89-97.
- [26] Koch CA, Uwaifo GI. Are gastrointestinal symptoms related to diabetes mellitus and glycemic control? Eur J Gastroenterol Hepatol 2008; 20: 822-825.
- [27] Hung CR. Effect of lysozyme chloride on betel quid chewing aggravated gastric oxidative stress and hemorrhagic ulcer in diabetic rats. World J Gastroenterol 2005; 11: 5853-5858.
- [28] Brinda KV, Vishveshwara S. Oligomeric protein structure networks: insights into protein-protein interactions. BMC Bioinformatics 2005; 6: 296-310.
- [29] Berg T. Small-molecule inhibitors of proteinprotein interactions. Curr Opin Drug Discov Devel 2008; 11: 666-674.
- [30] Charbonnier S, Gallego O, Gavin AC. The social network of a cell: recent advances in interactome mapping. Biotechnol Annu Rev 2008; 14: 1-28.
- [31] Phillipson M, Johansson ME, Henriksnas J, Petersson J, Gendler SJ, Sandler S, Persson AE, Hansson GC, Holm L. The gastric mucus layers: constituents and regulation of accumulation. Am J Physiol Gastrointest Liver Physiol 2008; 295: G806-812.
- [32] Kim HJ, Kim JS, Kim KO, Park KH, Yim HJ, Kim JY, Yeon JE, Park JJ, Shim JJ, Byun KS, Bak YT, Lee CH. [Expression of MUC3, MUC5AC, MUC6 and epidermal growth factor receptor in gallbladder epithelium according to gallstone composition]. Korean J Gastroenterol 2003; 42: 330-336.

- [33] Wagner S, Enss ML, Cornberg M, Mix H, Schumann S, Kirchner G, Jahne J, Manns MP, Beil W. Morphological and molecular characterization of human gastric mucous cells in long-term primary culture. Pflugers Arch 1998; 436: 871-881.
- [34] Inaba T, Sano H, Kawahito Y, Hla T, Akita K, Toda M, Yamashina I, Inoue M, Nakada H. Induction of cyclooxygenase-2 in monocyte/ macrophage by mucins secreted from colon cancer cells. Proc Natl Acad Sci U S A 2003; 100: 2736-2741.
- [35] Tan XD, Liu QP, Hsueh W, Chen YH, Chang H, Gonzalez-Crussi F. Intestinal trefoil factor binds to intestinal epithelial cells and induces nitric oxide production: priming and enhancing effects of mucin. Biochem J 1999; 338: 745-751.
- [36] Atar S, Ye Y, Lin Y, Freeberg SY, Nishi SP, Rosanio S, Huang MH, Uretsky BF, Perez-Polo JR, Birnbaum Y. Atorvastatin-induced cardioprotection is mediated by increasing inducible nitric oxide synthase and consequent S-nitrosylation of cyclooxygenase-2. Am J Physiol Heart Circ Physiol 2006; 290: H1960-1968.
- [37] Li X, Xu L, Gao H, Li B, Cheng M. Effects of grape seed proanthocyanidins extracts on AGEs and expression of bone morphogenetic protein-7 in diabetic rats. J Nephrol 2008; 21: 722-733.
- [38] Yu J, Zhang Y, Sun S, Shen J, Qiu J, Yin X, Yin H, Jiang S. Inhibitory effects of astragaloside IV on diabetic peripheral neuropathy in rats. Can J Physiol Pharmacol 2006; 84: 579-587.
- [39] Chen J, Chen Y, Luo Y, Gui D, Huang J, He D. Astragaloside IV ameliorates diabetic nephropathy involving protection of podocytes in streptozotocin induced diabetic rats. Eur J Pharmacol 2014; 736: 86-94.
- [40] Lu WS, Li S, Guo WW, Chen LL, Li YS. Effects of Astragaloside IV on diabetic nephropathy in rats. Genet Mol Res 2015; 14: 5427-5434.
- [41] Gui D, Huang J, Guo Y, Chen J, Chen Y, Xiao W, Liu X, Wang N. Astragaloside IV ameliorates renal injury in streptozotocin-induced diabetic rats through inhibiting NF-kappaB-mediated inflammatory genes expression. Cytokine 2013; 61: 970-977.