Abstract

AIM: To investigate the effect of Tangweian Jianji (TWAJJ) on the biomechanical and morphometrical remodeling of the upper gastrointestinal tract in diabetic rats.

METHODS: Diabetes was induced in 27 rats by injecting streptozotocin (40 mg/kg body weight), the animals were then divided into three groups (n = 9 in each group), i.e., diabetic control (DM); high dose (10 g/kg, T1) and low dose (5 g/kg, T2). Another 10 rats acted as normal controls (Control). TWAJJ was administered by gavage once daily. Blood glucose and serum insulin levels were measured. Circumferential length, wall thickness and opening angle were measured from esophageal, duodenal, jejunal and ileal ring segments. The residual strain was calculated from the morphometric data. Step-wise distension was carried out on esophageal and jejunal segments. The obtained data on the length, diameter and pressure changes were then used to calculate the circumferential and longitudinal stresses and strains. Real-time reverse transcription polymerase chain reaction was used to detect the receptor of advanced glycation end-products (RAGE) mRNA level in jejunal tissues.

RESULTS: At the end of the experiment, the blood glucose level was significantly higher and the serum insulin level was significantly lower in DM, T1 and T2 groups than in the control group (Glucose: 30.23 ± 0.41 μmol/L, 27.48 ± 0.27 μmol/L and 27.84 ± 0.29 μmol/L, respectively; Insulin: 1.47 ± 0.32 μg/L, 2.66 ± 0.44 μg/L, 2.03 ± 0.29 μg/L and 4.17 ± 0.54 μg/L, respectively). However, these levels did not differ among the DM, T1 and T2 groups. The wet weight per unit length, wall thickness and opening angle of esophageal and jejunal segments in the DM group were significantly higher than those in the control group (from P = 0.009 to P = 0.004). These parameters in the T1 group were significantly lower than those in the DM group (wet weight, duodenum: 0.147 ± 0.003 g/cm vs 0.151 ± 0.002 g/cm, P = 0.017; ileum, 0.127 ± 0.004 g/cm vs 0.139 ± 0.003 g/cm, P = 0.046; wall thickness, esophagus: 0.94 ± 0.03 mm vs 0.94 ± 0.02 mm, P = 0.014; duodenum: 1.27 ± 0.06 mm vs 1.39 ± 0.05 mm, P = 0.031; jejenum: 1.19 ± 0.07
CONCLUSION: TWAJJ (high dose) treatment partly restored the morphometric and biomechanical remodeling of the upper gastrointestinal tract in diabetic rats.

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Key words: Biomechanics and morphometric remodeling; Diabetes rats; Gastrointestinal tract; Mechanism; Tangwejian Jianji

INTRODUCTION
Diabetic gastrointestinal disorder (DGID) is a common complication of diabetes. Up to 76% of diabetic patients express gastrointestinal symptoms including dysphagia, early satiety, reflux, constipation, abdominal pain, nausea, vomiting and diarrhea[^1,2]. Duration of the disease and poor glycemic control seem to be associated with the severity of gastrointestinal (GI) problems. In general, the pathogenesis of DGID includes high blood glucose level, smooth muscle degeneration, abnormal autonomic neuropathy, gastrointestinal hormone secretion disorders and oxidative stress[^3,4].

The GI tract is functionally subjected to dimensional changes. Hence, biomechanical properties such as the stress-strain relationships are of particular importance[^5]. These properties are remodeled in response to growth[^6], fasting[^7] and disease[^8]. The biomechanical properties are crucial for GI motor function because peristaltic motion which propels the food through the GI tract is a result of an interaction between the passive and active tissue forces and the hydrodynamic forces in the food bolus. Remodeling of the mechanical properties reflects the changes in the tissue structure that determine a specific motor dysfunction. During the past few years, several studies have demonstrated that experimental diabetes induces GI morphological and biomechanical remodeling[^9-13]. Following the development of diabetes, the GI wall becomes thicker and stiffer in a time-dependent manner. Therefore, diabetic gastrointestinal morphological and biomechanical remodeling play an important role in DGID and has become the new perspective of diabetic gastrointestinal pathogenesis[^10].

Some studies on diabetic arteries have demonstrated that non-enzymatic glycation of arterial wall tissues is associated with remodeling of the wall[^14,15]. We believe that the same applies to the diabetic GI wall. Advanced glycation end-products (AGEs) may contribute to diabetic GI morphological and biomechanical remodeling by two major mechanisms. The first is receptor-independent alteration of the extracellular matrix architecture by non-enzymatic glycation and the formation of protein cross-links. The second mechanism is receptor-dependent and consists of modulation of cellular functions through ligation of specific cell surface receptors[^16].

Western medical treatment of DGID is mainly focused on symptomatic control with improvement of gastric motility, using promoting agents and supportive measures based on blood glucose control[^17]. Although these therapies can partially improve the clinical symptoms, they do not fundamentally reverse the diabetes-induced changes and result in a very high relapse rate. In clinics it was shown that Tangwejian Jianji (TWAJJ) significantly improved DGID with a low relapse rate, however, the mechanism involved in this improvement is not fully understood. Therefore, the aim of the present study was to investigate whether TWAJJ treatment can improve the morphometric and biomechanical remodeling of the GI tract in streptozotocin (STZ)-induced diabetic rats. Furthermore, the receptor of advanced glycation end-products (RAGE) mRNA level in the jejunal tissues was also investigated to explore the possible mechanism of TWAJJ in the treatment of DGID.

MATERIALS AND METHODS
Animal model and groups
Forty male SD rats weighing 220-250 g were included in this study. Diabetes was induced in 30 rats by a single tail vein injection of 40 mg/kg body weight of strepto-
zotocin (STZ, Sigma-Aldrich, China). This dose of STZ resulted in a random blood glucose level ≥ 16.7 mmol/L in 90% of rats 7 d after the injection. The remaining 10% of rats were excluded from this study. Twenty-seven STZ-induced diabetic rats were subdivided into three groups (n=9 in each group), i.e., diabetic control group (DM); high dose of TWAJJ group (T1) and low dose of TWAJJ group (T2). Another 10 rats of similar age and body weight from the same vendor were used as a non-diabetic control group (Control).

Drugs and administration methods
TWAJJ is composed of Citrus aurantium, Codonopsis, fried Atractylodes and wine Rhubarb and was provided by Guang’anmen Hospital, China Academy of Chinese Medical Sciences. The medicine was administered by gavage which passed though the esophagus and reached the stomach lumen. The drug was perfused directly into the stomach once daily from the beginning of the experiment. The dosage was 10 g/kg for T1 and 5 g/kg for T2, respectively. The rats in the DM and control groups were perfused with physiological saline.

Chemical analysis of TWAJJ
The formulation of TWAJJ was prepared according to the corresponding monograph in the 2005 Edition of Chinese Pharmacopoeia. There were quantitative control limits for the raw herbs (for example, the content of Naringin in Citrus aurantium was not less than 4.0% and Neohesperidin was not less than 3.0%) and for the final drug product. The chemical composition of TWAJJ was determined by using an ultra-fast high performance liquid chromatography-electrospray-ionization-quadrupole time-of-flight mass spectrometry method. An example chromatographic fingerprint is shown in Figure 1. The major compounds in the final dosage form were identified as Naringin (peak 12), Neohesperidin (peak 15), Lobetylolin (peak 16), Atractylenolide (peak 26) and Emodin (peak 23). WC: Wave chromatogram.

Experimental procedures
Weight and blood glucose levels were measured at 2-wk intervals after initiating the experiment. For blood glucose measurement, one drop of blood was obtained from rat tail vein and blood glucose was measured by a John-
son and Johnson One Touch Ultra Blood Glucose Meter. For insulin measurement, blood was obtained from the abdominal aorta and 0.2 mL serum was separated. The serum insulin was measured by radioimmunoassay (rat insulin radioimmunoassay kit, Linco Company, United States) at the end of the experiment.

The experimental period was 60 d. At the end of the experiment, the rats were fasted overnight and anesthetized with 4% Chloral hydrate (10 mL/kg, ip). Following laparotomy, the whole esophagus and ten centimeters of duodenum, jejunum and ileum were harvested. The duodenum was taken from the descending section, 1 cm down from the pylorus; the jejunum from 5 cm distal to the ligament of Treitz, and the ileum from 5 cm proximal to the ileocecal valve. After gently cleaning the lumen of the segments with saline, the length and wet weight were measured.

The esophageal and jejunal segments were divided into three sections, the proximal 1 cm segment was immediately stored at -70 °C for protein and RAGE mRNA detection. The adjacent 1 cm long segment was used for the zero-stress state experiment. The remaining section was used for the distension test. The duodenal and ileal segments were divided into two and used for protein and mRNA detection and the zero-stress state experiment. In this experiment, RAGE mRNA detection was performed only on the jejunal segments.

**Zero-stress state experiment**

To obtain data on the zero-stress state, three 1-2 mm wide esophageal and intestinal rings were cut and placed in Krebs solution at room temperature. The composition of Krebs solution (mmol/L) was: NaCl, 118; KCl, 4.7; NaHCO₃, 25; NaH₂PO₄, 1.0; MgCl₂, 1.2; and ascorbic acid, 0.11. A photograph was taken of the cross-section of the rings using a Canon camera (Canon, Japan) and was presented as the no-load state. Each ring-shaped segment was then cut radially from the opposite mesentery site and the photographs were taken about 60 min after the radial cutting to allow viscoelastic creep to take place. This is presented as the zero-stress state.

**Distension test**

The distal end of the remaining esophageal and jejunal segments was tied with a suture and the proximal end was cannulated with a tube for the distension experiment. After preconditioning of the segments, they were inflated with Krebs solution using a step-wise distension protocol to allow viscoelastic creep to take place. This is presented as the no-load state. Each ring-shaped segment was then divided into three sections, the proximal 1 cm segment was immediately stored at -70 °C for protein and RAGE mRNA detection. In this experiment, RAGE mRNA detection was performed only on the jejunal segments.

**Mechanical data analysis**

The morphometric data were obtained from digitized images of the segments in the zero-stress, no-load and pressurised states. Measurements were undertaken using image analysis software (Sigmascan ver. 4.0, Sigma Corp., San Rafael, CA, United States). The following data were measured from each specimen: the circumferential length (Lₒ), the wall thickness (h), the area (A), and the opening angle at zero-stress state (αₒ). The subscripts i, o, n, z and p refer to the inner (mucosal) surface, outer (serosal) surface, no-load state, zero-stress state and pressurised condition.

The stress and strain of the esophageal and intestinal segments in the pressurised state were determined under the assumptions that the wall was homogenous and the organ shape was cylindrical. Calculations were performed knowing the no-load state dimensions, the outer diameters and lengths of the specimens at varying pressures, and assuming incompressibility of the wall. The longitudinal stretch ratio, \( \lambda_{L} = \frac{L}{L_{n}} \); the luminal radius, \( r_{i-p} = \sqrt{r_{i}^{2} - \frac{A_{i}}{\pi \lambda_{o}}} \); the wall thickness, \( h_{p} = r_{o-p} - r_{i-p} \); the mucosal circumferential length, \( C_{p} = 2 \pi \times r_{o-p} \); the serosal circumferential length, \( C_{o-p} = 2 \pi \times r_{o} \); the mid-wall circumferential length, \( C_{m-p} = C_{p} + C_{o-p} \); the circumferential stretch ratio, \( \lambda_{C} = \frac{C_{m-p}}{C_{o-p}} \); (where the middle-wall circumferential length at zero-stress state, \( C_{m-o} = \frac{C_{o}}{C_{o-p}} \) ) were calculated.

Then the Kirchhoff’s stress and Green’s strain in a wall at a given pressure were calculated according to the following equations:

**Circumferential Kirchhoff’s stress:** \( S_{C} = \frac{\Delta P r_{o-p}}{h_{p} \lambda_{o}} \) (3)

**Longitudinal Kirchhoff’s stress:** \( S_{L} = \frac{\Delta P r_{o-p}^{2}}{h_{p} \lambda_{o}(r_{o-p} + r_{i-p})} \) (4)
RESULTS

Blood glucose and serum insulin levels

Blood glucose and serum insulin levels at the end of the experiment are shown in Figure 2. The blood glucose level was 4-fold higher in the DM group compared with the control group (Figure 2A, *P* < 0.01). The serum insulin level was significantly lower in the DM group compared with the control group (Figure 2B, *P* < 0.01). Compared with the DM group, the blood glucose and insulin levels did not significantly change in the T1 and T2 groups.

The weight/cm, wall thickness and wall area

The wet weight per unit length (Figure 3A), no-load wall thickness (Figure 3B) and cross-sectional wall area (Figure 3C) of the esophageal and intestinal segments increased in the DM group compared with the control group (*P* < 0.01, *P* < 0.05). After treatment with T1, the wall thickness decreased in all segments (Figure 3B, *P* < 0.05), whereas the wet weight and wall area decreased in the duodenal, jejunal and ileal segments (Figure 3A, D, *P* < 0.01, *P* < 0.05), but not in the esophageal segment (*P* = 0.12). All the above parameters did not change significantly in the T2 group.

Opening angle and residual strain

At the end of the experiment, the opening angle of all three intestinal segments was significantly increased in...
the DM group compared with the control group (Figure 4A, \( P < 0.05 \)). However, the opening angle in the esophageal segment did not change. Treatment with high dose TWAJJ decreased the opening angle in all segments studied (Figure 4A, \( P < 0.05 \)). Interestingly, the opening angle of the esophageal segment was also decreased in the T2 group (\( P < 0.05 \)).

A similar pattern to that found in the opening angle was found in the inner residual strain for all segments and groups, with the exception of the ileal segment (Figure 4B), where no difference was found between the different groups (\( P > 0.05 \)). The outer residual strain did not differ between the groups and segments (Figure 4C).

**Stress-strain distribution**

At the end of the experiment, the stress-strain analysis showed that the circumferential stress-strain curves of esophageal and jejunal segments (Figure 5A, C), and the longitudinal stress-strain curve of the esophageal segment (Figure 5B) in the DM group shifted to the left
compared with those in the control group, indicating that the diabetic esophageal and intestinal wall became stiffer. Calculation of the mechanical constants demonstrated a difference between the DM group and the control group (P < 0.05, Table 1). However, the longitudinal stress-strain distribution of the jejunal segment did not differ between the DM and control groups (P > 0.05, Figure 5D). High dose TWAJJ (T1) decreased the stiffness of the esophageal wall in the longitudinal direction (Figure 5B and Table 1, P < 0.05) and the intestinal wall in the circumferential direction (Figure 5C and Table 1, P < 0.05). Low dose TWAJJ did not improve the stiffening of the esophageal and jejunal wall caused by diabetes (P > 0.05, Figure 5 and Table 1).

Table 1 Comparison of constant a among the different groups

|                        | Circumferential direction | Longitudinal direction |
|------------------------|---------------------------|------------------------|
|                        | Esophagus | Jejunum | Esophagus | Jejunum |
| Control (n = 10)       | 3.56 ± 0.35 | 2.65 ± 0.37 | 18.33 ± 2.36 | 13.51 ± 3.66 |
| DM (n = 9)             | 4.59 ± 0.79 | 3.81 ± 0.31a | 27.62 ± 3.93* | 14.79 ± 2.26 |
| T1 (n = 9)             | 3.77 ± 0.39 | 2.55 ± 0.51c | 19.43 ± 4.13 | 14.67 ± 1.56 |
| T2 (n = 9)             | 3.84 ± 0.44 | 4.02 ± 0.71a | 27.82 ± 1.74c | 15.41 ± 4.36 |

*P < 0.05 vs control group; †P < 0.05 vs DM group. DM: Diabetic control; T1: High dose; T2: Low dose.

Compared with those in the control group, indicating that the diabetic esophageal and intestinal wall became stiffer. Calculation of the mechanical constants demonstrated a difference between the DM group and the control group (P < 0.05, Table 1). However, the longitudinal stress-strain distribution of the jejunal segment did not differ between the DM and control groups (P > 0.05, Figure 5D). High dose TWAJJ (T1) decreased the stiffness of the esophageal wall in the longitudinal direction (Figure 5B and Table 1, P < 0.05) and the intestinal wall in the circumferential direction (Figure 5C and Table 1, P < 0.05). Low dose TWAJJ did not improve the stiffening of the esophageal and jejunal wall caused by diabetes (P > 0.05, Figure 5 and Table 1).
movement; therefore the biomechanical properties of the wall are important for this function. The biomechanical properties of the esophagus and small intestine depend on their structure and can be evaluated by the opening angle, residual stress and strain, and stress-strain relationship. Previous studies have demonstrated that the esophageal wall and intestinal wall were remodeled during the development of diabetes. Therefore, it is important to determine whether TWAJJ can improve DGID through a pathway involved in changing the biomechanical properties of the GI tract.

The present study confirmed previous findings that morphometric and biomechanical remodeling of the esophageal and intestinal wall occur in STZ-induced diabetic rats. Although the treatment with TWAJJ did not significantly change the blood glucose and serum insulin levels, high dose TWAJJ, to a large extent, improved the morphometric and biomechanical remodeling caused by diabetes. The improvement in morphometric remodeling was expressed as reduced wall thickness and area. Improvements in biomechanical remodeling were expressed as reduced opening angle, reduced absolute value of residual strain and decreased wall stiffness. The present study indicated that the effect of TWAJJ on improvements in DGID in the clinic may be partially associated with improvements in biomechanical remodeling of the GI wall. Due to the complex geometry of the stomach, we did not focus on the biomechanical parameters of the gastric wall in the present study. Future studies may focus on the effect of TWAJJ on the stomach due to the importance of stomach function in diabetic patients.

The alterations in residual strain and wall stiffness in diabetes will change the tension and stress distribution in the location of mechanosensitive afferents in the GI tract. Therefore, biomechanical remodeling of the diabetic GI wall indirectly affects GI motor function. High dose TWAJJ partially restored the changes in biomechanical properties caused by diabetes. This may be associated with improved motor and sensory function of the diabetic GI tract.

AGEs is a heterogeneous group of macromolecules formed by the non-enzymatic glycation of proteins, lipids and nucleic acids. Endogenous AGEs are generated at higher rates in diabetes due to abnormal glucose metabolism. Chronic high blood glucose can cause excessive glycoprotein accumulation in the body including the GI wall causing GI wall stiffening. There is growing evidence to show that AGEs and RAGE are implicated in various disorders. AGE levels in serum and tissues are associated with chronic complications of DM including DGID. Furthermore, RAGE is involved in signal transduction of AGEs in a variety of cells. Our previous studies showed that AGE and RAGE were over-expressed in diabetic GI tissues. In the present study, we found that the RAGE mRNA level in the jejunal segment increased in diabetic rats compared with normal rats. Following high dose TWAJJ administration, this alternation was reversed to the normal state. Therefore, the observed
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improvement in the mechanical factors due to TWAJJ treatment in DGID may be through the inhibition of AGEs accumulation. However, in future studies we need to clarify this mechanism by immunohistochemistry of RAGE to demonstrate whether the change in mRNA expression actually translates to protein expression. Furthermore, it is also important to study whether specific knockdown of RAGE in the GI mucosa can improve biomechanical remodeling of the GI tract in diabetes and reverse DGID.

In conclusion, high dose TWAJJ treatment improves biomechanical and morphometric remodeling of the diabetic esophageal and intestinal wall. The mechanism may, at least partly, be explained by decreased RAGE mRNA levels in STZ-induced diabetic rats. Therefore, it seems feasible to develop Chinese herbs, such as TWAJJ, to improve the morphometric and biomechanical remodeling caused by diabetes. This may have an impact on GI dysfunction in diabetes and be used in clinical practice.

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