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Original Research

The effect of Heweijiangni-decoction on esophageal morphology in a rat model of OVA-induced visceral hypersensitivity followed by acid exposure

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Abstract: Heweijiangni decoction (HWJND) is an effective traditional Chinese medicine prescription in clinical treatment of nonerosive reflux disease (NERD). Esophageal hypersensitivity and acid contribute to the disease. However, the exact underlying mechanism of action remains unclear. In this study, we observed the effect of HWJND on esophageal morphology in a rat model of ovalbumin (OVA)-induced visceral hypersensitivity followed by acid exposure. Esophageal morphology was assessed by measuring the extent of dilated intercellular spaces (DIS), desmosome disruption, and mitochondrial fragmentation. HWJND in low, moderate, and high doses relieved DIS and desmosome disruption in esophageal epithelium compared with model group ($P<0.05$ for all doses). In addition, HWJND in high dose protected mitochondria from fragmentation ($P<0.05$). Other findings suggest that DIS and mitochondrial fragmentation are independent events, and that omeprazole protects mitochondria. Overall, HWJND significantly resists esophageal morphology changes in OVA-induced and acid exposure rat model.

Key words: Heweijiangni decoction (HWJND); Gastroesophageal reflux disease; OVA.

Introduction

Gastroesophageal reflux disease (GERD) is defined as the reflux of stomach contents causes troublesome symptoms and/or complications. It is common in adults, with about 40% of United States population suffering from GERD symptoms. 14% of them complain of GERD symptoms daily (1). The most common symptoms are heartburn and regurgitation (2). GERD can be divided into several subgroups: erosive esophagitis (EE), Barrett's esophagus, and nonerosive reflux disease (NERD) (3, 4).

Currently, the definition of NERD includes patients with negative endoscopy but with abnormal esophageal acid exposure time (5). However, the pathophysiology of NERD is considered complex and still unclear. The diagnosis and treatment are far from satisfactory. Acid was considered as an important factor in NERD (6) but many patients do not respond very well to proton pump inhibitors (PPI), which is responsible for reducing stomach acid production. For example, only 43–46% of NERD patients report to be free of symptoms after the treatment with PPI (2).

Current NERD pathophysiology involves mucosal changes, peripheral factors (nonacid reflux, gas reflux, and proximal distension of the esophagus), and visceral hypersensitivity (VH) (2).

Mucosal morphology changes can be observed in many aspects, using light and electron microscopy, such as intercellular junctions and organelles. Intercellular junctions include desmosomes, intercellular space, adherence, and tight junctions (7). Desmosomes are considered to be an important component to the epithelial defense of the esophageal mucosa (8). Moreover, the most reported histological change observed in the mucosa of NERD patients is the presence of dilated intercellular spaces (DIS). Recent research suggested that DIS may be the most sensitive and objective marker of NERD (9-13). DIS can be considered as one of the earliest morphological features of cell damage (12). Acid was previously thought to be contributing to DIS (14). However, it is now known that regardless of pathological acid exposure, DIS are present in NERD patients, both adults and children. This indicates an "intrinsic" vulnerability (9, 15), which could be defined as having a hypersensitive esophagus (9). This suggests that other impair factors, such as bile reflux and VH, may play a role in inducing DIS in NERD patients. One of the important mechanisms of VH may be contributed by transient receptor potential vanilloid subfamily member-1 receptors (TRPV1) in the esophageal mucosa (6), as heartburn and esophageal sensitization can be induced by TRPV1 activation (16). Guarino et al. (17) also found that NERD patients presented an increased TRPV1 receptor mRNA and protein levels in esophagus.

Chinese herbal medicine uses several herbs (herbal cocktail) to ameliorate a series of symptoms associated with a particular disease and has been used in China for thousands of years (18, 19). Heweijiangni Decoction (HWJND) is a new and effective traditional Chinese medicine prescription formulated by Li Jun-Xiang, a professor at the Beijing University of Chinese Medi-

cine. Our previous studies have shown significant clinical efficacy of HWJND in patients with GERD (20). In animal research, HWJND could reduce 5-hydroxytrypamine (5-HT) levels in OVA-combined acid exposure rats (21). However, the exact mechanism is yet unclear. Therefore, the present study aims to evaluate the esophageal morphology changes in OVA-induced visceral hypersensitivity combined acid exposure in rat model and following dose-dependent application of HWJND.

Materials and Methods

Preparation of HWJD

HWJND granules were purchased from the Pharmacy Department of Dongfang Hospital, Beijing University of Chinese Medicine (Beijing, China). The granules consisted of the following ingredients of the HWJND formula: Astragalus 9g, Coptis Chinensis 6g, Ginger 9g, Pinellia Sinensis 9g, Fritillaria japonica 9g, Dandelion 9g, Gentiana 9g, Gorgonian 9g, Whole Gualou 9g, Zhigancao 3g

Animals preparation

48 male Sprague-Dawley rats (7 weeks old; weight, 200±20 g) were supplied by SPF Biological Technology Co., Ltd., Beijing, China. All animal experimental procedures were approved by the Animal Ethics Committee of Beijing University of Chinese Medicine under the guidelines issued by Regulations of Beijing Laboratory Animal Management. Rats were randomly divided into eight groups: control group, model group, sham-operated group, HWJND low dose group (HWJNDL), HWJND moderate dose group (HWJNDM), HWJND high dose group (HWJNDH), omeprazole group (OME), and SB705498 group (a selective and orally bioavailable TRPV1 antagonist (22)). Rats were housed in a specific pathogen-free animal room with the temperature maintained at 20–24°C, 50–60% humidity, and a light-controlled environment (12/12 h light/dark cycle), with free access to food and sterile tap water. All animals were allowed to adapt to the environment for 5 days before the experiments were started.

Model establishment

The rat model was established by intraperitoneal injection (i.p.) of OVA and acid exposure (23). Briefly, on day 0, animals received an intraperitoneal injection of 100 mg OVA plus aluminum hydroxide (200 mg/mL in 0.9% NaCl, Sigma) 1.5ml. Rats in sham-operated group received 0.9% NaCl instead of OVA plus aluminum hydroxide. Each drug group was administered by intragastric administration (i.g.) twice a day for 13 days (HWJNDH 14.54g/kg·d, HWJNDM 9.72 g/ kg·d, HWJNL4.86 g/kg·d, OME 4.17mg/kg·d, SB705498 5.12mg/kg·d). On day 14, 0.1N hydrochloric acid was used for esophageal acid infusion. Specific methods: anesthetized animal fixed supine position, head elevation of 20-30 °. abdominal wall and stomach wall were cut and placed in a drainage tube at the fontanelle to collect fluid from the esophagus. A single lumen perfusion tube was placed in the esophagus orally, and the catheter opening was located in the esophagus and the stomach junction at 2 to 3 cm. The fixed catheter was connected to the continuous infusion pump on the other end and used 0.1mol/L hydrochloric acid infusion, with the drip temperature maintaining 37 °C, speed at 10mL / h, for a total of 30min.

Tissue specimens and routine histology

Tissue specimens were collected from 2 to 3 cm at the esophagus and stomach junction and were processed by standard formalin fixation and paraffin embedding. They were cut into 5 mm tissue sections and stained with hematoxylin-eosin. Each group was collected 3 specimens from different rats. Esophagus tissue was observed using electron microscopy (Hitachi H7650) (13).

Electron microscopy and quantitative analysis

From each specimen, intercellular spaces were evaluated in five photomicrographs. At least 40 randomly selected perpendicular trans-sections to adjacent membranes were drawn and measured in each image (2000 magnification) (24). Desmosomes were evaluated in five photomicrographs per specimen, counting the amount of desmosome in each photomicrograph (8000 magnification). Mitochondria were evaluated in six photomicrographs per specimen, in 25μm2 randomly selected area (1500 magnification), counting the amount of all mitochondria and fragmented mitochondria separately and calculating the percentage of structurally altered mitochondria (25).

Statistical analysis

All data are expressed as median (interquartile range, IQR) values due to the non-normal distribution of the data. SPSS v23.0 (IBM Corp., Armonk, NY, USA) was used for statistical analyses. The data were compared between groups using rank sum test. $P < 0.05$ was considered statistically significant.

Results

Intercellular spaces

The esophageal mucosae of all the groups were examined by gross inspection and no evident inflammation or lesions were found in all rats. Using transmission electron microscopy (TEM), dilated intercellular spaces were noticed in model group. The intercellular diameters in model group were significantly larger than those in control group (model group median: 0.207 μm [IQR 0.126-0.401 μm] vs. Control group median: 0.064 μm [IQR 0.050-0.084 μm]; P<0.05). Compared with model group, the sham-operated group showed relatively integrated mucosa sham-operated (median, 0.076 μm [IQR 0.059-0.092 μm]), HWJNDH (median, 0.053 μm [IQR 0.044-0.064 μm]), HWJNDM (median, 0.053 μm [IQR 0.043-0.066 μm]), HWJNDL (median, 0.049 μm [IQR 0.040-0.059 μm]), SB705798 (median, 0.054 μm [IQR 0.043-0.067 μm]) (P<0.05). Moreover, there was no significant difference between OME group and model group (median, 0.169 μm [IQR 0.127-0.230 μm]; P>0.05). In these two groups, the distribution of DIS was non-normal (Figure 1, Figure 2).

Desmosomal disruption and quantity reduction

Desmosomal disruption could be found in the model group and OME group — disruption of desmosomes as

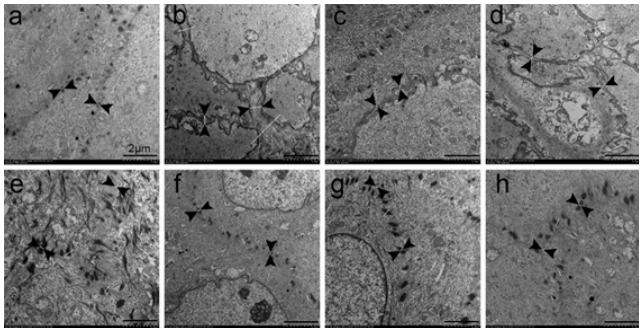

Figure 1. Transmission electron microscopy images of specimens showing the method used for the measurement of dilated intercellular spaces. (2000 magnification). A, control group; b, model group; c, sham-operated group; d, omeprazole group; e, low dose of HWJND group; f, moderate dose of HWJND group; g, high dose of HWJND group; h, SB705798 group. Transects perpendicular to opposing cell membranes are randomly drawn across the intercellular spaces. Intercellular space diameters (ISD) in model group (b) and OME group (d) are largely dilated and uneven. ISD in other groups are similar. (n=5 per group).

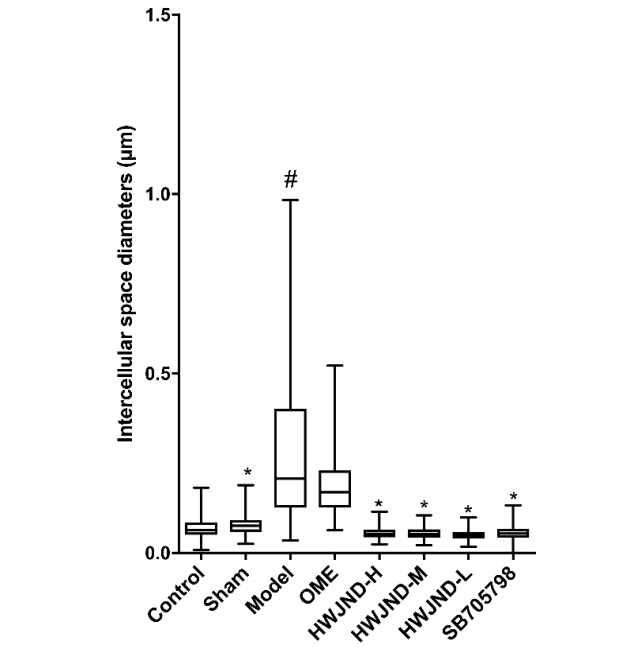

Figure 2. Mucosal intercellular space diameter in biopsies from different groups. #P<0.05 vs. control; *P<0.05 vs. model. OME, omeprazole; HWJND-H, Heweijiangni decoction high dose; HWJND-M, Heweijiangni decoction moderate dose; HWJND-L, Heweijiangni decoction low dose. (n=5 per group).

well as DIS were markedly visible at higher magnification. The desmosomal architecture remains intact after treatment with low dose of HWJND, moderate dose of HWJND, high dose of HWJND and SB705798, similar to the control (Figure 3). Desmosomes were reduced in model and OME group. Compared with control group, the quantity of desmosome in model group was significantly lower (median, 1 [IQR 0.5-3] vs. median, 6 [IQR 5-6]; P<0.05). However, the sham-operated group (median, 6 [IQR 5-6]), HWJNDH group (median, 5 [IQR 4-6.5]), HWJNDM group (median, 4 [IQR 0.043-0.066]), HWJNDL group (median, 5 [IQR 5-6]), SB705798 group (median, 5μm [IQR 4-5]) showed significant difference compared to the model group respectively (P<0.05) (Figure 4).

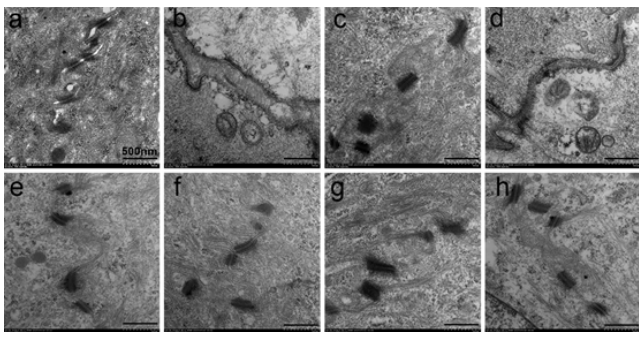

Figure 3. Desmosome morphological changes observed by transmission electron microscopy (8000 magnification). A, control group; b, model group; c, sham-operated group; d, omeprazole group; e, low dose of HWJND group; f, moderate dose of HWJND group; g, high dose of HWJND group; h, SB705798 group. Desmosomal disruption were found in the model group (b) and OME group (d) — disruption of desmosomes as well as DIS is markedly visible at higher magnification. The desmosomal architecture remains intact after treatment with low dose of HWJND (e), moderate dose of HWJND (f), high dose of HWJND (g) and SB705798 (h), similar to the control (a). (n=5 per group).

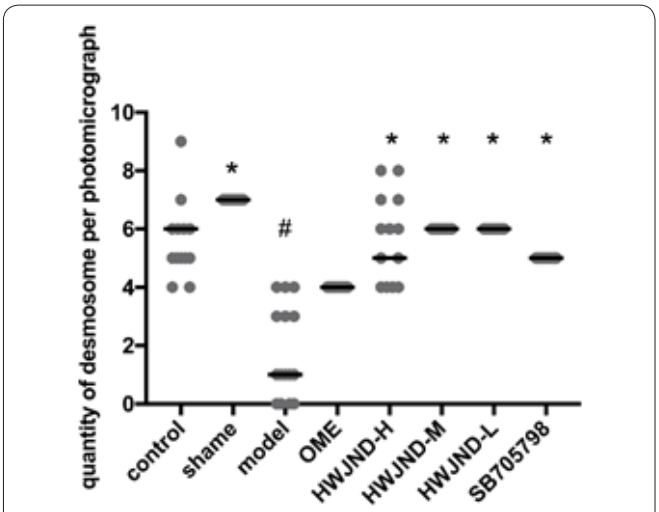

Figure 4. Desmosomes are not only disruption in morphology but also reduced in quantity while the intracellular spaces are enlarged. #P<0.05 vs. control; *P<0.05 vs. model. OME, omeprazole; HWJND-H, Heweijiangni decoction high dose; HWJND-M, Heweijiangni decoction moderate dose; HWJND-L, Heweijiangni decoction low dose. (n=5 per group).

Structure of mitochondria

In our research, it was observed that the formation of mitochondria in the model group is highly fragmented. In low dose HWJND group, moderate dose of HWJND group, and SB705798 group, mitochondria were observed to be swollen with a visible disappearance of cristae. Mitochondria in the control group, sham-operated, OME group, and high dose of HWJND group had similar morphology, with regular shape and clear cristae. Interestingly, the severity of fragmented mitochondria was not accompanied by DIS (Figure 5). In addition, the percentage of structurally altered mitochondria in the model group was significantly higher than control group (model group median, 81% [IQR 66%-100%] vs. control group median, 21% [IQR 5%-34%]; P<0.05). Mitochondria in sham-operated group (median, 33% [IQR 20%-50%]), OME group (median, 50% [IQR 40%-75%]), and HWJNDH group (median,

Table 1. Structurally altered mitochondria (%) in different groups.

control	sham-operated	model	OME	HWJND-H	HWJND-M	HWJND-L	SB705798
21(5, 34)	33(20, 5^0)*	81(66, 100)#	50(40, 75)*	27(14, 42)*	68(57, 77)	75(56, 100)	73(57, 84)

Model group showed the highest percentage change. OME group and HWJNDH group showed a degree of protection against structural alteration. #P<0.05 vs. control; *P<0.05 vs. model. OME, omeprazole; HWJND-H, Heweijiangni decoction high dose; HWJND-M, Heweijiangni decoction moderate dose; HWJND-L, Heweijiangni decoction low dose. (n=5 per group).

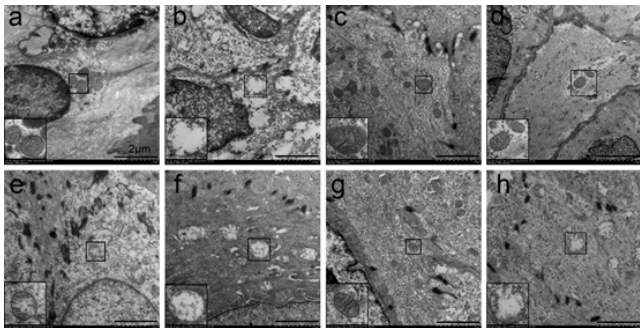

Figure 5. Mitochondria morphological changes observed by transmission electron microscopy (1500 magnification). A, control group; b, model group; c, sham-operated group; d, omeprazole group; e, low dose of HWJND group; f, moderate dose of HWJND group; g, high dose of HWJND group; h, SB705798 group. In model group (b) the formation of mitochondria is highly fragmented. In low dose of HWJND group (e), moderate dose of HWJND group (f) and SB705798 group (h), mitochondria are swollen with a visible disappearance of cristae. Mitochondria in control group (a), sham-operated group(c), omeprazole group (d), high dose of HWJND group (g) are similar, with regular shape and clear cristae. The severity of fragmented mitochondria was not present along with DIS. (n=5 per group).

75% [IQR 56%-100%]) were protected compared with model group. (P<0.05) (Table 1).

Discussion

Although NERD pathophysiology is complicated, involving mucosal changes, peripheral factors, esophageal visceral hypersensitivity has been proposed to be a pathogenesis in nonerosive reflux disease (NERD), but its exact mechanisms are unclear (26, 27). In the present study, SD rats were sensitized by intraperitoneal injection of OVA. In order to simulate acid reflux, acid exposure was applied. Our study investigated the effects of visceral hypersensitivity combined with acid exposure on the esophagus and the effect of HWJND on morphology changes. We found that intercellular spaces in the model group are significantly larger than the control group, demonstrating that visceral hypersensitivity combined with acid exposure could dilate esophageal intercellular space. As DIS provide an appropriate parameter for damage (12), we accessed the DIS values in different treatment groups. DIS in all the HWJND treated groups were relieved, both in high and low dose, suggesting that HWJND pretreatment before acid exposure could improve DIS, through VH mechanism. According to the results of the sham-operated group, mechanical damage to the upper esophagus did not significantly affect the lower esophagus compare to the model group. This finding suggests that VH and acid exposure are the main reasons of DIS. Zhang, D. H. et al. (13) pointed out that pretreatment with esomeprazole had no effect on DIS of rat esophageal epithelium. Our study also showed that there was no difference in the intercellular space diameter between Omeprazole and model group. However, the DIS of esophageal epithelium in NERD and EE (erosive esophagitis) patients could be improved after the treatment with omeprazole (28). This contradiction may be explained by the fact that laboratory rats and mice lack vomiting reflex response (29). Proton pump inhibitors (PPIs), such as omeprazole, offer rapid symptomatic relief in GERD patients due to their inhibition of gastric acid secretion with suppression of esophageal acid exposure (13). This mechanism is invalid in rats as DIS relief was not observed. Therefore, it can be supposed that omeprazole does not improve VH. This might partly explain why some NERD patients do not see any improvement through PPI treatment. SB805798 is a selective and orally bioavailable TRPV1 antagonist (22). DIS disappeared in SB805798 group, indicating VH is an independent factor in DIS, and DIS is related to TRPV1 mechanism.

In our results, DIS showed non-normal distribution, or so-called “Radial distribution”, consistent with the findings of Vieth et al. (11). This means that DIS can vary significantly even within a small area. Tobey et al. found that DIS could be observed within normal-appearing mucosa of patients with both EE and NERD (30). This finding indicates DIS might be a link between NERD and EE. We suspect that those with serious DIS may develop into erosive epithelial mucosa.

Desmosomes are important intercellular junction which not only provide mechanical integrity but also limit the movement of molecules across the monolayer (7). In our results, along with the observation of DIS, disruption and decreased number of desmosomes were found. Although it cannot be made certain as to which one is the initial trigger, this finding suggests that desmosome disruption plays a role in affecting DIS mechanism. Desmosomes in both HWJND groups and SB805798 group were protected from disintegration. Further studies using SB805798 as a selective TRPV1 antagonist would help investigate the effect of HWJND on TRPV1 protein.

GERD can develop into Barrett’s esophagus, which is a premalignant condition (31). PPIs decrease the risk of oesophageal adenocarcinoma in patients with Barrett's esophagus (32). But the mechanism is still unclear. O'Farrell et al. (33) demonstrated that increased mitochondrial instability is an early event in the Barrett's disease sequence. In our results, omeprazole protected against mitochondrial fragmentation in esophageal epithelium cells, indicating that omeprazole decreases oesophageal adenocarcinoma risk. Another interesting finding is that DIS are not accompanied by the mitochondria fragmentation, suggesting that these two phenomena have different mechanisms. Furthermore, SB805798 cannot prevent mitochondria from fragment-

ing. This may be due to TRPV1 not being a factor in protecting mitochondria. Strikingly, HWJND in high dose could not only ameliorate DIS but also protect mitochondria. This finding suggests that HWJND has multiple biological targets and is dose dependent. In other studies (34), it is shown that TCM formulations which could smooth the liver can relieve NERD. In our decoction, herb Whole Gualou has the function of smooth the liver too. More than this, Coptis Chinensis can ameliorate the disharmony between the liver and stomach (zang-fu organs in TCM). These may partly explain why HWJND could relieve NERD.

Thus, our data shows that VH and acid exposure could cause DIS, which might be related with TRPV1 protein. DIS may be a link between NERD and EE. Desmosome disruption plays a role in DIS mechanism. DIS are not accompanied by the mitochondria fragmentation, and omeprazole could protect mitochondria in esophageal epithelium. DIS, desmosome disruption and mitochondria fragmented could get relief when applying HWJND in high dose. Based on the data of the present study, HWJND could ameliorate esophageal morphology changes in OVA-induced and acid exposure rat model.

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The data used to support the findings of this study are available from the corresponding author upon request.

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Conflict of interest

There are no conflict of interest in this study.

Author's contribution

All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Jun-xiang Li; Bo-yi Jia, Chun-e Xie, Jun-xiang Li collected and analysed the data; Bo-yi Jia wrote the text and all authors have read and approved the text prior to publication.

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