

## Original Article

# Effect of water immersion restraint stress on gastric mucosa in rats with removed salivary glands

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**Abstract:** Objective: To explore the effect of salivary gland removal on water immersion restraint stress (WIRS)-induced gastric mucosal injury (GMI) in rats. Methods: Eighty male WISTAR rats were allocated into sham operation (sham) group, sham + WIRS group, salivary gland removal group, and salivary gland removal + WIRS group, with 20 rats in each group. In the sham group, skin and subcutaneous tissues were cut to expose glands. The rats in other three groups were subjected to a total salivary gland resection, and all their submandibular glands and sublingual glands and part of the parotid glands were removed, and the parotid duct was ligated. Afterwards, rats were exposed to WIRS for one and a half months and sacrificed. The severity of GMI was scored, and any histologic and ultrastructural changes were monitored. Results: There were differences in the Guth index (GMI evaluation) scores among the four groups ( $P=0.000$ ), which were higher in salivary gland removal + WIRS group than those in the other three groups (all  $P<0.001$ ), and were higher in the sham + WIRS group and salivary gland removal group than those in the sham group (all  $P=0.000$ ). Compared with other groups, the gastric mucosa was severely injured in the salivary gland removal + WIRS group, with obvious congestion, edema, inflammatory cell infiltrate, mucosal shedding, bleeding ulcers, enlarged intercellular spaces, and damaged organelles. Conclusion: Salivary gland removal aggravates WIRS-induced GMI to a certain extent.

**Keywords:** Water immersion restraint stress, salivary gland removal, gastric mucosal injury, rat model

## Introduction

Psychological factors and drugs such as anti-platelet drugs and acid suppressants are inducers of gastric mucosal injury (GMI), and unhealthy lifestyle habits are responsible for its increasing prevalence [1, 2]. Modern medicine believes that GMI is the result of the loss of balance between invasive factors and mucosal defense. In the case of enhanced damaging factors and/or weakened protective factors, injuries or even ulcers may occur in the gastric mucosa, eventually resulting in irreversible damage to the body [3, 4]. Saliva, an important protective factor composed of water and a few ions, enzymes, amino acids and bioactive regulators, is a mixed liquid secreted by the parotid gland, submandibular gland, sublingual gland, and minor salivary gland. It has the functions of moistening the mouth, dissolving and digesting food, generating taste, resisting bacteria, cleaning and pro-

tecting oral cavity, and excreting [5, 6]. By binding to their own receptors, epidermal growth factors (EGFs) in saliva promote cell proliferation, differentiation, and migration, inhibit gastric acid secretion, induce angiogenesis, as well as accelerate the self-repair and reconstruction of gastric mucosa and enhance the capability of resisting injury. These indicate that salivary glands play a vital role in maintaining epithelial integrity and preventing mucosal injury [7-9]. However, animal experiments mostly histologically demonstrate the protective effects of saliva on GMI, and rarely determine the changes in the level of organelle injury [10]. Based on this, the present study established rat models of GMI with water immersion restraint stress (WIRS), and comprehensively explored the protective effect of salivary glands on gastric mucosa from the level of tissues to organelles, to enlighten a new target for clinical research.

## Materials and methods

### Materials

**Laboratory animals:** Eighty healthy male WISTAR rats, weighing  $350 \pm 10$  g (clean grade, LuKang Pharmaceutical Co., Ltd., Shandong, China, SCXK (Shandong) 2008002), were raised in Central Laboratory of Qingdao University (clean grade) at room temperature of 20–25°C. All rats were maintained on a 12-h/12-h light/dark cycle and reared in separate cages (5 per cage), with free access to a nutritionally complete, pelleted diet. They were fasted for 10 hours and water withheld for 1 hour without restriction of activities before the experiment. The experimenter touched them once a day for one week, 2 min/time, for their better adaptation. This study was approved by the Ethics Committee of The Affiliated Hospital of Qingdao University.

### Methods

**Grouping and surgical treatment:** Eighty male rats were divided into four groups (sham operation (sham) group, sham + WIRS group, salivary gland removal group, and salivary gland removal + WIRS group) by a random number table, with 20 rats in each group. All rats were anesthetized with intraperitoneally administered 10% chloral hydrate (3 mL/kg, Beijing Baiaolaibo Technology Co., Ltd., China). The rats in surgery groups were subjected to a total salivary gland resection, and all their submandibular glands and sublingual glands and part of the parotid glands were removed, and the parotid duct was ligated. In the sham group, skin and subcutaneous tissue were cut to expose glands. 30 minutes before and 24 hours after the surgery, intramuscular injection of penicillin (30,000 units/kg, Harbin Pharmaceutical Group Pharmaceutical General Pharm. Factory, China) and hypodermoclysis of glucose-saline solution (2 mL, Baxter, USA) were performed for infection prevention and rehydration. After the surgery, the rats rested for 10 days for healing.

**WIRS and specimen acquisition:** The rats in the sham + WIRS group and salivary gland removal + WIRS group were tied up on a stress plate (20 × 30 cm), and were immersed in 20°C water (surface flush with the xiphoid process) for about half an hour, once every other day, for one and a half months [10]. After fast-

ing for 10 hours, the rats were anesthetized with intraperitoneally administered 10% chloral hydrate (3 mL/kg), and the whole stomach was taken out from the incision of the upper abdomen. Finally, rats were sacrificed by spinal dislocation.

**Histological sectioning and HE staining:** The gastric specimens of each group were taken along the lesser and greater curvatures, fixed with 10% formaldehyde solution for 48 hours, dehydrated and paraffin embedded, sectioned (4  $\mu$ m in thickness) and hematoxylin-eosin stained. Afterwards, the histologic changes were monitored under an optical microscope.

**Observation of ultrastructure:** Gastric samples of each group were taken (1 mm × 1 mm × 1 mm in size), fixed with 10% glutaraldehyde at 4°C for 2 h, dehydrated with gradient ethanol series, infiltrated in epoxy resin, embedded and semithin-sectioned. Following re-thinning and uranyl acetate-lead citrate staining, the ultrastructure changes were observed under a transmission electron microscopy (TEM).

**Outcome measures:** Main outcome measures: GMI evaluation by Guth index: The whole stomach was cut along the greater curvature and washed with normal saline. The gastric mucosa was monitored with a magnifying glass (10 × magnification), and the degree of mucosal injury was scored. Guth index: ulcer length less than 1 mm was scored 1, 1–2 mm (including 1 mm) scored 2, 2–3 mm (including 2 mm) scored 3, 3–4 mm (including 3 mm) scored 4, more than 4 mm (including 4 mm) scored 5. The score was multiplied by 2 for width greater than 1 mm [11]. The cumulative score was obtained from observations of the whole stomach.

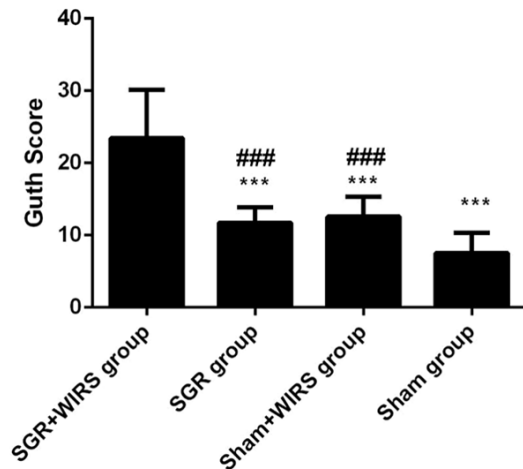
Secondary outcome measures: (1) Histologic evaluation (hematoxylin-eosin staining): Gastric specimens were taken along the lesser and greater curvatures, stained with hematoxylin-eosin (Xinfan Biotechnology Co., Ltd., Shanghai, China). Histologic changes were monitored under an optical microscope; (1) Observation of ultrastructure under an electron microscope: The stomach specimens (1 mm × 1 mm × 1 mm) were stained with uranyl acetate-lead citrate (HEDE Biotechnology Co., Ltd., Beijing, China), and the ultrastructural changes were observed under a TEM.

## WIRS effect on gastric mucosa with removed salivary glands

**Table 1.** Injury scores of rats in each group ( $\bar{x} \pm sd$ )

Group	Injury score	F-value	P-value
Salivary gland removal + WIRS group	23.45±6.65		
Salivary gland removal group	11.76±2.11 <sup>***,###</sup>	67.342	0.000
Sham + WIRS group	12.59±2.72 <sup>***,###</sup>		
Sham group	7.65±2.76 <sup>***</sup>		

Note: Compared with salivary gland removal + WIRS group, <sup>\*\*\*</sup>P<0.001; compared with sham group, <sup>###</sup>P<0.001. WIRS: water immersion restraint stress.



**Figure 1.** Guth index of gastric mucosa ( $\bar{x} \pm sd$ ). Compared with SGR + WIRS group, <sup>\*\*\*</sup>P<0.001; compared with sham group, <sup>###</sup>P<0.001. WIRS: water immersion restraint stress. SGR: salivary gland removal. SGR+WIRS group: salivary gland removal combined with WIRS group; SGR group: salivary gland removal group; Sham + WIRS group: sham group combined with WIRS group.

**Statistical methods:** SPSS22.0 was used for statistical analysis. The measurement data were expressed by mean  $\pm$  standard deviation ( $\bar{x} \pm sd$ ), and were statistically processed by one-way analysis of variance. Pairwise comparisons were processed by LSD-t. For all analyses, statistical significance was tested using an alpha value of 0.05, and P<0.05 was considered significant.

### Results

*Comparison of general conditions of rats - mental status and motor activity in sham group were better than those in the other three groups*

Approximately one week after surgery, the motor activity, mental status and food intake of rats in sham group had no significant change,

while surgically treated rats showed listlessness, decreased activity, decreased food intake, yellowish and dull coat color.

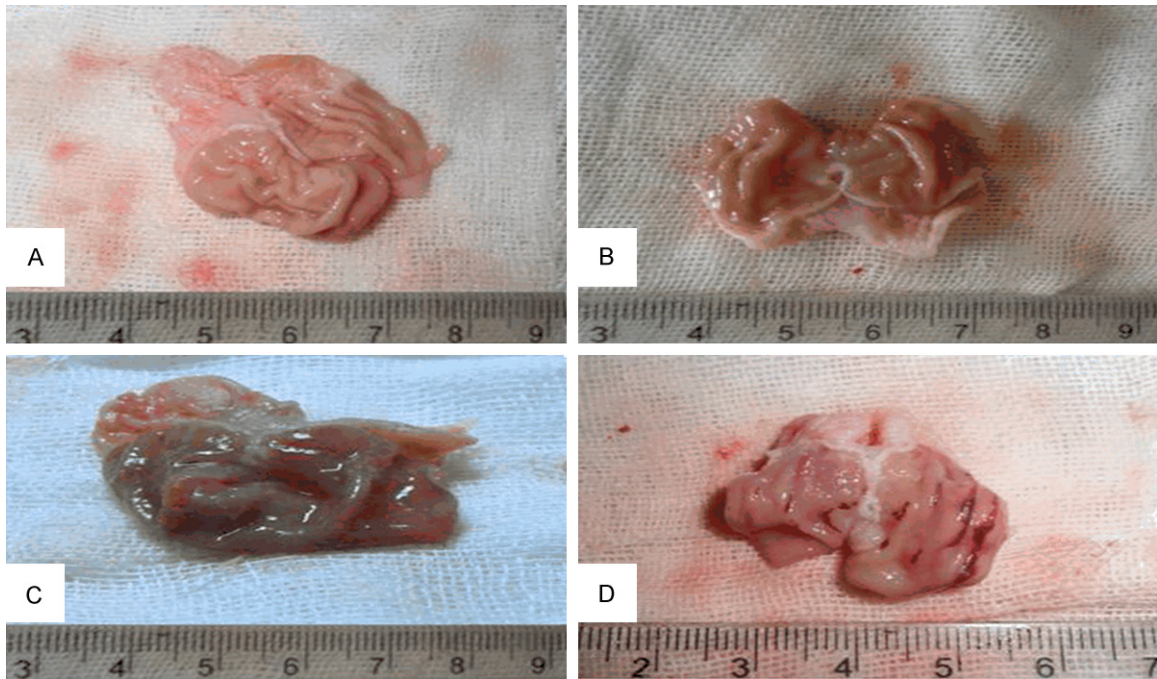
*Guth index and the condition of the gastric mucosa - the gastric mucosa was more severely injured in salivary gland removal + WIRS group than that in other three groups*

There were differences in Guth index scores among the four groups (F=67.342, P=0.000), as shown in **Table 1**. It can be seen from **Table 1** and **Figure 1** that Guth index scores in the salivary gland removal + WIRS group were higher than those in the other three groups (all P=0.000). There was no statistical difference between salivary gland removal group and sham + WIRS group (P=0.672), but they were higher than the sham group (all P=0.000). The gastric mucosa of rats in sham group, salivary gland removal group, and sham + WIRS group (**Figure 2A-C**) was generally normal, with occasional congestion and edema, without bleeding spots, ulcers, or notable exudation. Histologic analyses demonstrated that the gastric mucosa in salivary gland removal + WIRS group showed congestion, edema, redness, punctate hemorrhage and erosion, diffuse lesions, and occasional punctate ulcers. Also, it was covered with gray-yellow and gray-white mucinous exudate, accompanied by mucosal shedding (**Figure 2D**).

*Pathological evaluation of the gastric mucosa - the most severe pathologic injury was observed in salivary gland removal + WIRS group*

The relatively normal gastric mucosa epithelium presented crisscross folds and a large number of irregular pits, which were surrounded by regularly arranged near-circular cells of roughly equal size. In the salivary gland removal + WIRS group, the lesions were mainly located in the superficial mucosa, namely the upper third of the mucosa, showing diffuse distribution, with partial necrosis and shedding of the superficial epithelium. There was infiltration and accumulation of lymphocytes and plasma cells in the lamina propria, thinning of mucosal glands, reduction of the number of glands, shallowing of gastric pits, occasional cystic dila-





**Figure 2.** Comparison of gastric mucosa of four groups. A: Sham group; B: Salivary gland removal group; C: Sham + WIRS group: the gastric mucosal surface of rats is generally in good condition, with mild edema, no ulcers and with or without bleeding spots; D: Salivary gland removal + WIRS group: rat gastric mucosa is obviously red and swollen, edematous, accompanied by bleeding spots and erosions, partial mucosal denudation, and obvious damage. WIRS: water immersion restraint stress.

tation, and no intestinal metaplasia (**Figure 3A** and **3B**). Mild edema, hyperemia and inflammatory cell infiltration were not found in the gastric mucosa of rats in the sham group (**Figure 3C** and **3D**), while edema and congestion were occasionally seen in the salivary gland removal group and sham + WIRS group (**Figure 3E** and **3F**), with or without notable inflammatory cell infiltration, and with no superficial epithelial necrosis and shedding.

*Ultrastructure of the gastric mucosa - the mucosa was more severely damaged in salivary gland removal + WIRS group*

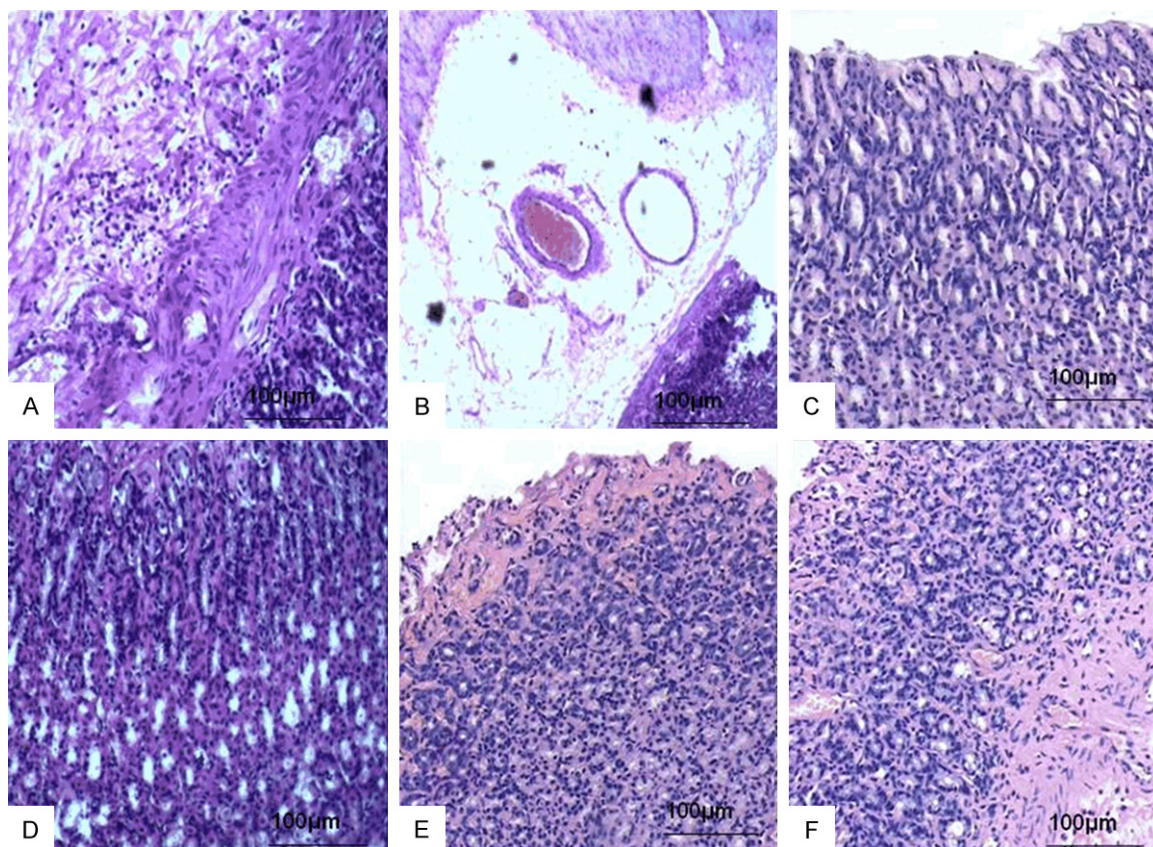
As revealed by the electron microscope, intercellular spaces between mucosal epithelial cells in the sham + WIRS group and salivary gland removal group were slightly wider than those in the sham group, with intact organelles (**Figure 4A** and **4B**). In the sham group, gastric mucosal epithelial cells were tightly connected, with intact mitochondria, endoplasmic reticulum, and Golgi apparatus (**Figure 4C**). However, in the salivary gland removal + WIRS group, the intercellular spaces were widened, resulting in poor connection between cells.

Other manifestations were as follows: Nucleus/cytoplasm ratio greater than 1 and increased lobulated nuclei; enlarged nucleoli and the appearance of nucleolar margination; Swelling, constriction, partial hyaline or even vacuolar degeneration of mitochondria, abnormal mitochondrial morphology; disordered and ruptured mitochondrial cristae, decreased mitochondrial cristae and mitochondria, and expanded rough endoplasmic reticulum in a circular arrangement; atrophic and structure-altered Golgi apparatus; evidently decreased cytoplasmic secretory granules (**Figure 4D-F**).

## Discussion

GMI is one of the common digestive tract diseases induced by various acute and chronic stress factors, which may lead to gastric dysfunction, bleeding, and ulcers caused by mucosal tears, and even death in severe cases [12, 13]. Therefore, it is of great significance to explore the pathogenesis of GMI for improving its treatment.

In previous studies, WIRS has been used as a stimulus to establish the model of GMI, indicat-



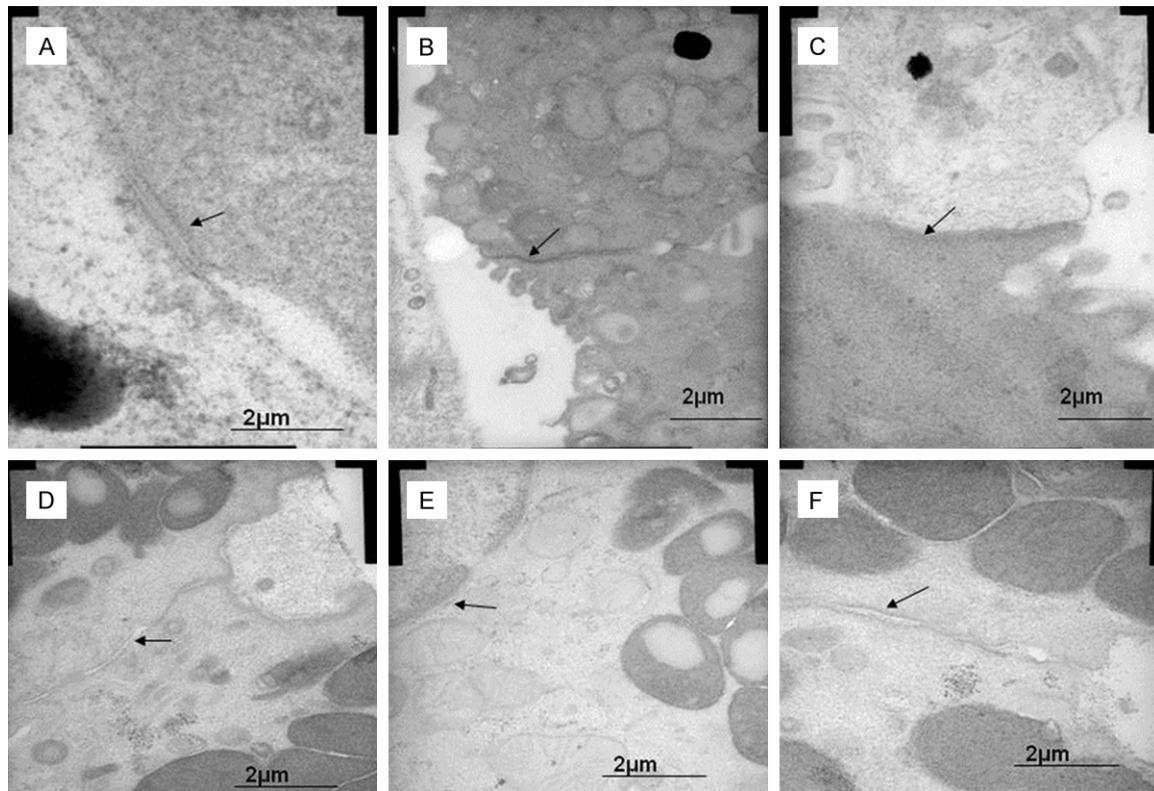
**Figure 3.** Histology of gastric mucosa of rats in each group ( $\times 100$ ). A: H&E staining demonstrates lymphocyte and plasma cell infiltration in the salivary gland removal + WIRS group; B: Thinning of mucosal glands, superficial epithelial necrosis and shedding, and tissue edema and congestion in the salivary gland removal + WIRS group; C, D: The gastric mucosa of rats in the sham group shows no mild edema, hyperemia, and inflammatory cell infiltration; E, F: The gastric mucosa of rats shows mild edema, congestion, and inflammatory cell infiltration in the salivary gland removal group and sham + WIRS group. WIRS: water immersion restraint stress.

ing the feasibility of WIRS in inducing GMI [14]. The present study proposes that rats suffer from more severe GMI after WIRS treatment. The mechanism may be that the excitement of sympathetic-adrenal medullary system caused by stress redistributes the blood and constricts the blood vessels of digestive tract mucosa more strongly, thereby reducing blood perfusion and inducing mucosal ischemia, further resulting in energy metabolism disorder of mucosal epithelium, as well as the disturbance of tight junctions between mucosal cells and the destruction of the “mucus-bicarbonate” barrier covering the mucosal membrane [15].

Previous studies have confirmed the protective effect of salivary glands on GMI [16]. This may be due to the fact that saliva contains epidermal growth factors (EGFs), which are

mainly synthesized by human submandibular gland, duodenal Brunner’s gland, kidney tissue, prostate, and brain. After synthesis, they are released in body fluids and platelets, and cause a variety of substrate phosphorylation reactions through their receptors (EGFRs), leading to signaling cascade and increased synthesis and secretion of mucus glycoprotein [16]. In addition, by promoting the mitosis of DNA, RNA, and related proteins and cells, EGFRs enhance the proliferation activity of epithelial cells, stimulate the migration, proliferation, and differentiation of gastrointestinal epithelial cells, and promote angiogenesis. They also play an important role in accelerating the self-repair and reconstruction of mucosal tissues, maintaining the integrity of epithelial mucosal barriers, and enhancing the capability of resisting injury [17]. We employed the Guth index to evaluate the degree of GMI after





**Figure 4.** Electron microscopy findings in the gastric mucosa (TEM  $\times 200K$  (L), TEM  $\times 20K$  (R), 200  $\times$  magnifications). A: Sham + WIRS group; B: Salivary gland removal group; C: Sham group; D-F: Salivary gland removal + WIRS group; TEM showed that the intercellular space was widened, the ratio of nucleus to cytoplasm  $>1$ , and the number of lobulated nuclei increased; the nucleoli were increased and nucleolar margination appeared. Mitochondrial swelling, Golgi loss of typical structures, and other changes were identified. WIRS: water immersion restraint stress. TEM: transmission electron microscopy.

salivary gland removal; it turned out that the Guth index score in the salivary gland removal + WIRS group was significantly higher than that in other groups, indicating a protective role of salivary glands against GMI. The positive and effective role of saliva in self-repair of oral mucosa and maintaining the homeostasis of oral environment has been reported previously, and available components have been extracted and applied to clinical treatment. The mucosal injury caused by head and neck radiotherapy can be alleviated by exogenous supplement of those components [18]. Both oral cavity and gastrointestinal tract are derived from the extraembryonic mesoderm, so their cells have the same or similar origin, indicating that saliva can also protect and repair histologically homologous gastrointestinal epithelium, consistent with previous conclusions [19].

In the present study, it was found by electron microscopy, an important tool for ultrastructural damage assessment, that due to the lack of

protective effect of saliva in salivary gland removal + WIRS group, the gastric mucosa lost the function of self-repair under cold stress, resulting in the severe injury of gastric mucosal cells. This led to the appearance of GMI manifestations, that is, diffuse congestion, edema, mucosal necrosis, and inflammatory cell infiltration. Furthermore, as shown by the electron microscope, organelle damage occurred in this group, which further confirms the protective effect of salivary glands on gastric mucosa [20, 21].

To sum up, WIRS is a damaging factor inducing significant damage to gastric mucosa of rats with removed salivary glands. Saliva may directly facilitate the endogenous repair of injured mucosa, but related pathway studies are required to confirm the protective effect of salivary glands on GMI.

#### Disclosure of conflict of interest

None.

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