# Therapeutic effect of low molecular weight chitosan containing sepia ink on ethanol-induced gastric ulcer in rats[1]

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**ABSTRACT**

**PURPOSE:** To evaluate the role of low molecular chitosan containing sepia ink (LMCS) in ethanol-induced (5 ml/kg) gastric ulcer in rats.

**METHODS:** Animals were divided into four groups (n = 12): normal group (Normal), negative control group (Con), experiment group (LMCS) and positive control Omeprazole group (OMZ). Gastric empty rate was detected in the first 7 days. Rats were sacrificed at 7, 14 and 21 day for histology and ELISA detections.

**RESULTS:** Gastric empty was no significant differences among the groups ($P > 0.05$). Histological observation showed gastric mucosal LMCS treated had better healing effect. Hydroxyproline (Hyp) was significantly increased from 7 day ($P < 0.05$). LMCS significantly inhibited malondialdehyde (MDA) generation for lipid peroxidation from 7 day ($P < 0.05$). LMCS significantly promoted the activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) at the earlier stage ($P < 0.05$). OMZ had the similar effects above. As for myeloperoxidase (MPO), LMCS significantly decreased and restored it to normal levels from 7 day ($P < 0.05$), it is earlier than OMZ which is from 14 day.

**CONCLUSION:** LMCS can improve gastric mucosa tissue repair, exert significant influences on oxidative and antioxidant enzyme activities and neutrophil infiltration.

**Key words:** Chitosan. Sepia. Stomach Ulcer. Hydroxyproline. Malondialdehyde. Rats.

## Introduction

Gastric ulcer is a common gastrointestinal disease with multiple etiologies and it is prevalent worldwide[1]. The pathogenesis of gastric ulcers includes stress, smoking, alcohol consumption, infections caused by *Helicobacter pylori*, and frequent and indiscriminate use of non-steroidalanti-inflammatory drugs (NSAIDs)[2].

The gastric mucosa is important tissue because of its structure and function, which can reflect the pathological processes by the inflammation changes of gastric ulcer[3]. The mucosal defense has been best characterized in the gastric, which exhibits remarkable resistance to the damaging effects of acid and pepsin[4].

Currently, many different anti-ulcer drugs including proton pump inhibitors and $H_2$ receptor antagonists are available for the treatment of peptic ulcer disease, however, clinical evaluation has shown various side effects and incidence of relapse of these drugs[5]. The ever-increasing problem of gastric ulcer necessitated the development and screening novel gastroprotective agents from natural polymers, which might be less toxic and cost-effective[6].

Natural polymers are widely used in medical fields[7]. Especially, chitosan is biodegradable, biocompatibile and bioadhesive, and it increases the solubility of hydrophobic drugs[8]. It's biological application depends on its molecular weight and its deacetylation degree[9]. Chitosan with high molecular weight displays high viscosity and low water solubility, which limits its use[10]. Low molecular weight chitosan (LMC) has overcome these limits, and it is more readily to be absorbed by organism. Moreover, it has been proved that LMC possesses many outstanding health benefits such as anti-inflammatory, immunity regulation, antibacterial, antioxidant, and antilipid peroxidation[11].

Also, sepia ink is a traditional medicine used in the treatment of hemostasis for centuries in China[12], and some researchers have founded that sepia ink has anti-radiation activity, antioxidant activity, antitumor activity, immunomodulatory activity, procoagulant function, etc[13]. It can improve the functional status of the whole body[14].

In this experiment, a novel biocompatible material, low molecular weight chitosan containing sepia ink (LMCS) was prepared for treating ethanol-induced gastric ulcer, which mainly based on two considerations. LMC can form adhesive colloidal solution under acidic conditions in gastric to protect the gastric mucosa from persistent injury, and promote the tissue repair; sepia ink can be used as a hemostatic and antioxidant agent in the ulcer site. The main purpose of this study was to detect whether LMCS has a positive therapeutic effect in gastric ulcer of rats and the potential underlying mechanism.

## Methods

This project was reviewed and approved by the committee of experimental animals of Lingnan Normal University and conformed to National Institutes of Health guidelines.

Wistar rats (200 ± 20 g) were purchased from the Medical Experimental Animal Center of Guangdong Province. Animals were provided with standard pellet diet and water *ad libitum* during the experiment period. Animals were maintained in 12 h light dark cycle and room temperature of 24 ± 3°C.

### *Surgical procedures and groups formation*

Forty eight rats were deprived of food but had *ad libitum* access to tap water for 48 h before ulcer induction. Gastric ulcer model was induced in conscious rats by gavages of absolute ethanol (5 ml/kg)[15]. The rats were randomized into four groups (n = 12): Normal group (Normal), negative control group (Con), experiment group (LMCS) and positive control Omeprazole group (OMZ). An hour later, treatment of each group carried out simultaneously. The gavage dosage of LMCS in experiment group is 7.6 mg/kg which was counted from the mount of human requirement[12]; and OMZ in positive control group is 5.5 mg/kg which was calculated from the medication instructions; negative control group was without treatment process, but with a standard dose of saline gavage; normal group did not been treated for gastric ulcer, which was only taken samples for detection at each time point. Finally, all rats were gavage once a day and then free access to food and water.

In parallel, each group of rats was sacrificed on 7, 14 and 21 day. The rat gastric was rapidly removed and opened along the greater curvature and rinsed with normal saline. Thereafter, each gastric was dichotomized, with one moiety of gastric immersed in neutral formalin for histological evaluation and gastric mucosa from the other moiety stored at -80°C for biochemical determinations.

### *Determination of gastric emptying rate*

As an indication of the restoration of gastric function, gastric emptying rate was detected on the seventh day after ulcer induction.After fasting for 48 h, thenrats were given 30 g standard pellets and water ad libitum. 3 three hours later, the spilled pellets were removed and food intake was recorded. Water was prohibited for next 5 h, then rats were decapitated and the gastric was removed and weighed. Then opened along the greater curvature and rinsed thoroughly with normal saline, gastric was blotted dry

and weighed again. Gastric emptying rate was calculated through the formula:

Gastric emptying (%) = (1 − gastric content/food intake) × 100

*Pathology assessment*

For histological evaluations, gastric was opened by an incision along the greater curvature, fastens and expanded on a plexiglass. Then gastric tissues were then fixed in 10 % neutral formalin and embedded in paraffin wax. Sections were made and stained by routine Hematoxylin and Eosin (H&E) staining for histological evaluation and Masson's Trichrome staining for collagen evaluation.

*Hydroxyproline content*

Hydroxyproline (Hyp) content was determined through alkaline hydrolysis method by ELISA (Shanghai, China). 50 mg gastric tissue sample and 1.0 ml hydrolyzate were mixed in a test tube, and put into boiling water bath for 20 min. According to the instruction, blank tube, standard tube and measuring tube were respectively added 1.0 ml distilled water, 1.0 ml standard solution and 1.0 ml test sample. Followed by, 0.5 ml regent I was added and standing for 10 min after mixing; 0.5 ml regent II was added and standing for 5 min after mixing; 0.5 ml regent III was added and kept in water bath at 60°C for 15 min after mixing.Finally, the tubes were cooled in water and centrifuged at 3500 rpm for 10 min to obtain supernatants, then absorbance values was measured at 550 nm and Hyp content was calculated according to the ELISA kit.

*Oxidative damage and antioxidant radical scavenging enzymes determination*

Gastric tissues washed with PBS, homogenized on ice for 10 min. Supernatant fraction collected over from 15 min of centrifugation at 12,000 rpm was accordingly extracted in lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mMv NaCl, 1% Triton X-100, and protease inhibitors). The extracts were separated by centrifugation at 12.000 rpm for 15 min. The viabilities of different antioxidant enzymes were measured by conducting the homogenization of rat gastric tissue. Malondialdehyde (MDA), Superoxide dismutase (SOD) and Catalase (CAT) activity were detected using the commercial assay kits (Shanghai, China). Glutathione peroxidase (GPx) activity in tissue homogenate was determined according to literature[16].

*Myeloperoxidase assay*

Myeloperoxidase (MPO) is used as a quantitative index of inflammation. 0.3g gastric tissue samples were homogenized in 10 volumes of ice-cold potassium phosphate buffer (20 mM $K_2HPO_4$, pH 7.4). The homogenate was centrifuged at 10,000 rpm for 20 min at 4°C. Then pellet was homogenized with an equivalent volume of 50 mM acetic acid (pH 6.0) containing 0.5% (w/v) hexadecyltrimethyammonium bromide (HETAB). MPO activity was assessed by measuring $H_2O_2$-dependent oxidation of 3,3',5,5-tetramethylbenzidine. One unit of MPO activity was defined as the MPO present that caused a change inabsorbance of 1.0 min at 655 nm and 37°C.

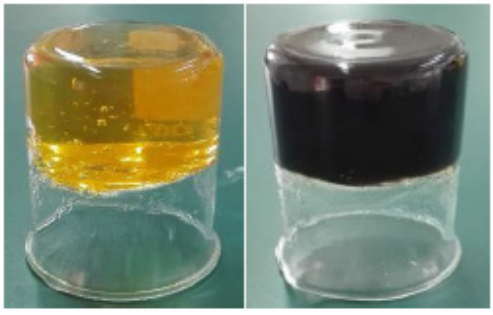
*Statistical analysis*

All results were expressed as means ± standard deviation (S.D.) and SPSS 17.0 software was used to analyze the data of the test. The means of the different groups were using one-way Kruskal-Wallis test. Values of $P < 0.05$ were regarded as significant.

## Results

*Properties of low molecular weight chitosan containing sepia ink*

The dry low molecular weight chitosan containing sepia ink (LMCS) used in this experiment is black powder substance. The viscosity of 3% LMCS under the same acidic condition in gastric of rats (pH 3.2) is 25.2 mPa.s (Figure 1). It exhibits good adhesion, which would protect the inner surface ulcers of the gastric.



A B

**FIGURE 1 - A.** Cross-linked low molecular weight chitosan. **B.** Cross-linked low molecular weight chitosan containing purified sepia ink.

*Gastric emptying rate assessment*

From the experimental results, gastric emptying rate in Normal group, Control group and OMZ group was respectively 81.0 ± 2.9 %, 72.4 ± 4.3% and 80.5 ± 3.5%. Comparing to LMCS group 79.8 ± 3.8 %, there was no significant difference (P > 0.05), although an increasing tendencywas obvious.

*Pathology evaluation of gastric ulcers*

Histological observation showed absolute ethanol caused severe lesion and extensive damage to the gastric mucosa (Figure 2b). On 7 day in Con group, the symptom of edema, severe hemorrhage, leukocytes infiltration and destruction of the mucosa surface were relatively obvious (Figure 2A7). Until 21 day in Con group, the gastric mucosa epithelium still had obvious edema and partial defect, and accompanied by mild mucosal hemorrhage (Figure 2A21). In LMCS group on 7day, it was seen that edema symptom was mild and gastric mucosa was relatively intact, mucosal columnar epithelial cell arranged neatly (Figure 2B7). When came to 21 day in LMCS group, the gastric mucosa was nearly no major differences compared with normal group (Figure 2B21, a). OMZ group and LMCS group had similar healing effect, but the differences also existed. LMCS had a certain inflammatory response in early time (Figure 2B7), which nearly disappeared from 14 to 21day (Figure 2B14, B21). As for OMZ group, the inflammatory response was observed obviously from 14 to 21 day (Figure 2C14, C21), but the early time was slight (Figure 2C7).

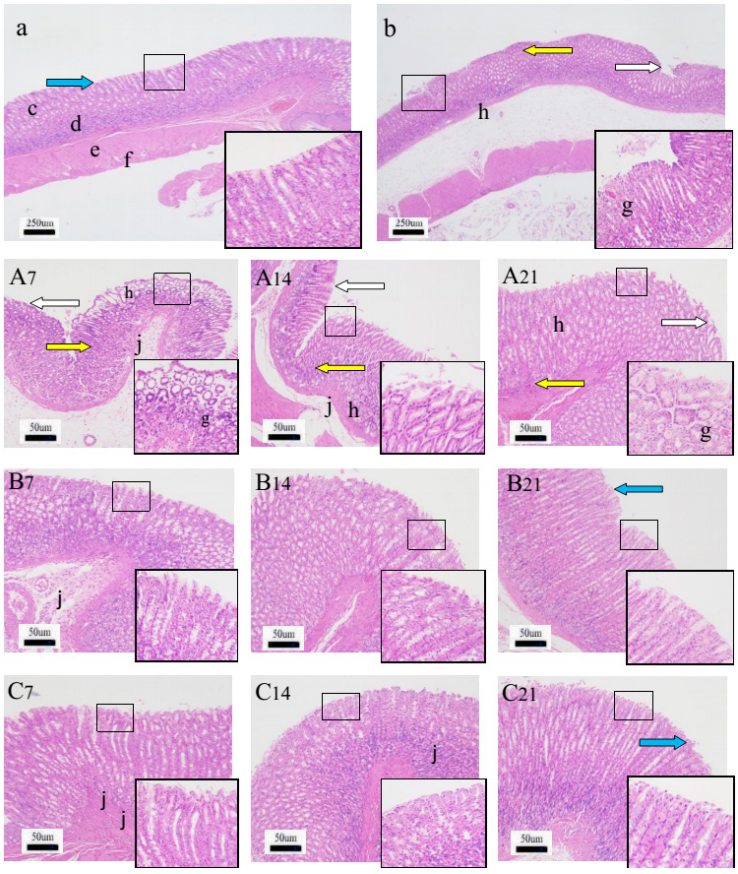



**FIGURE 2** - Histological detection of gastric mucosa in the ethanol-induced ulcerated rats (H&E staining). (**a**) Gastric mucosa of normal rats, (**b**) Gastric ulcers model of ethanol-induced rats, (**A7-A21**) Gastric mucosa of negative control group on 7, 14 and 21day, (B7-B21) Gastric mucosa of LMCS group on 7, 14, 21 day, (**C7-C21**) Gastric mucosa of OMZ group on 7, 14 and 21 day. Yellow arrow indicates severe hemorrhageand disruption to the deep mucosa layer. White arrow indicates disruption to the surface epithelium. Blue arrow shows intact appearance of histological structureof the epithelium and mucosa layer; e-mucosa; d-submucosa; e-muscularis; f-serosa; h-edema; j-leukocyte infiltration;g-hemorrhage.

Collagen can be dyed blue through Masson staining, by which we can roughly evaluate the healing effect at the macro level. Figure 3 indicated the synthesis of new collagen at different time points. On 7 day, both LMCS and OMZ groups obviously contained more collagen than Con group (Figure 3D7, E7, F7), and the similar results also appeared on the 14 day (Figure D14, E14, F14). By the 21 day, there was an obvious increase of the content of collagen in Con group than earlier time (Figure 3D21). Meanwhile, the content of collagen in LMCS and OMZ groups decreased (Figure E21, F21).

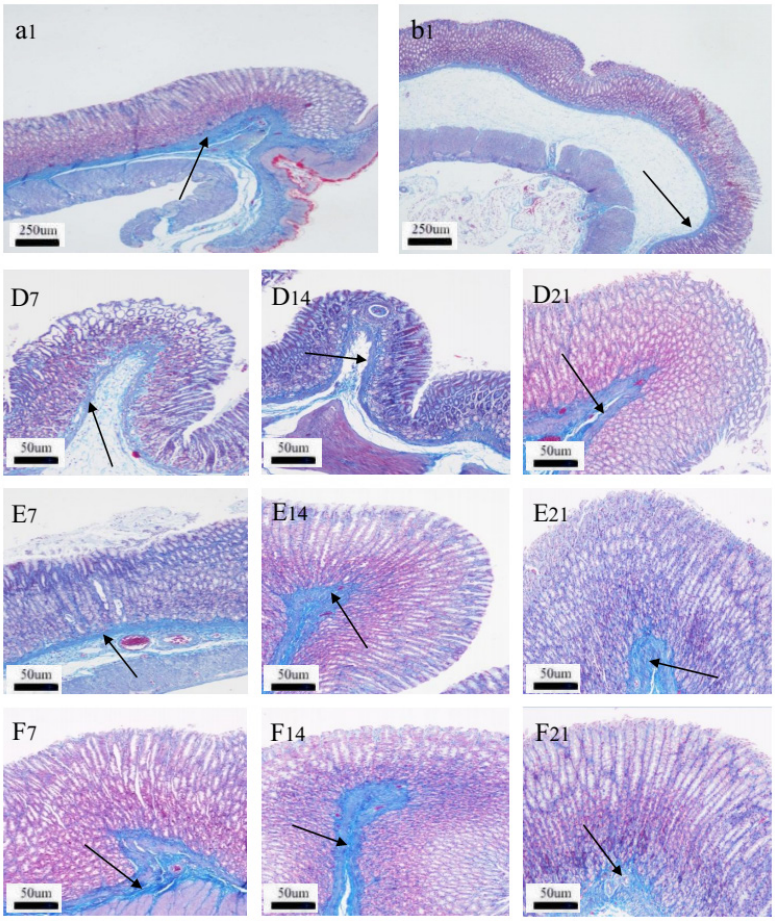



**FIGURE 3** - Histological evaluation of gastric mucosa in the ethanol-induced ulcerated rats (Masson staining). (**a**) Gastric mucosa of normal rats, (**b**) Gastric mucosa of absolute ethanol-induced rats, (**D7-D21**) Gastric mucosa of negative control group on 7, 14 and 21 day, (**E7-E21**) Gastric mucosa of LMCS group on 7, 14, 21 day, (**F7-F21**) Gastric mucosa of OMZ group on 7, 14 and 21 day. The black arrow is the new collagen fiber in the healing process of gastric mucosa, which was dyed blue.

*Content of hydroxyproline assays*

As shown in Figure 4, Hyp content in LMCS group was significantly stimulated comparing to Con group from 7 to 14 day ($P<0.05$). There was a similar variation trend in OMZ group. But both LMCS and OMZ group dropped to normal level on 21 day.

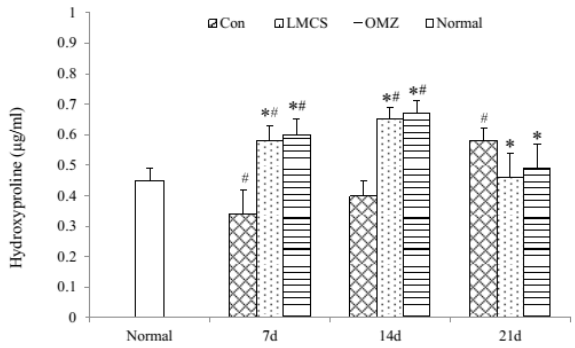



**FIGURE 4** - Effects of LMCS, OMA on the Hyp content in stomach tissue of ethanol-induced gastric ulcer in rats. Results are expressed as mean ± SD (n=3). (*p<0.05 *vs*. Con group and #p<0.05 *vs*. Normal group).

*Oxidative damage and antioxidant enzymes determination*

Figure 5 indicated that ethanol caused a significant increase of MDA from 7 to 21 day in Con group ($P < 0.05$). Compared to Con group, there was an significant inhibitory effect in LMCS and OMZ group on the content of MDA ($P < 0.05$), which also earlier decreased to normal level on 21 day.

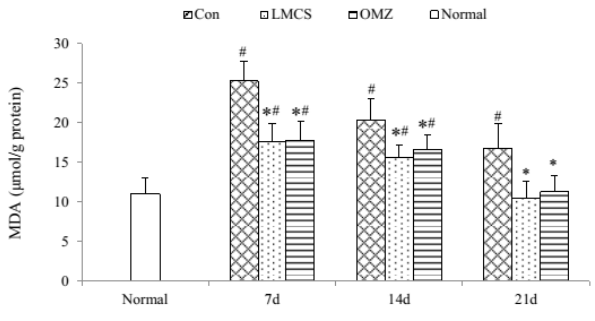



**FIGURE 5** - Effects of LMCS, OMZ on the MDA content in stomach tissue of ethanol-induced gastric ulcer in rats. Results are expressed as mean ± SD (n=3). (*p<0.05 *vs*. Con group and #p<0.05 *vs*. Normal group).

The results of Figure 6 suggested that LMCS and OMZ played a similar regulatory role on the activities of CAT, SOD and GPx enzymes, which were all significantly enhanced at earlier time on 7day ($P<0.05$), and came to normal level on 14 day, except GPx on 21 day.

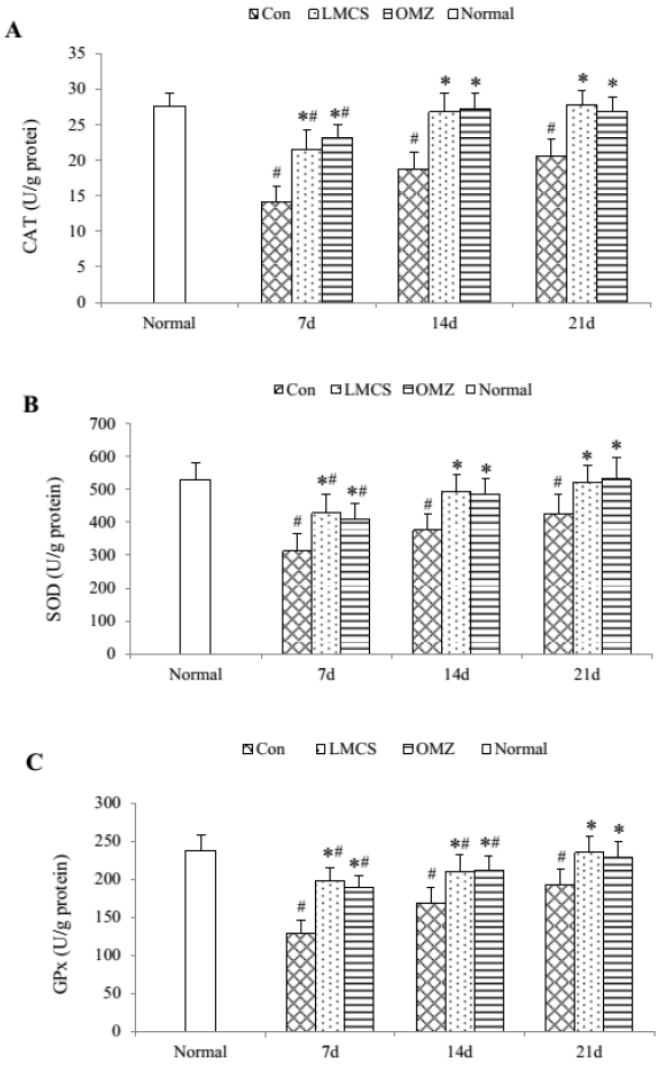



**FIGURE 6** - Effects of LMCS, OMA on activities of free radical scavenging enzyme in the gastric tissue of ethanol-induced gastric ulcer in rats. (**A**) Catalase activity. (**B**) Superoxide dismutase activity. (**C**) Glutathione peroxidase activity. Results are expressed as mean ± SD (n=3). (*p<0.05 *vs*. Con group and #p<0.05 *vs*. Normal group).

*Myeloperoxidase assay*

Figure 7 depicted that the activity of MPO comparing to normal group were all significantly inhibited on 7 day ($P < 0.05$), and this inhibition degree in LMCS group was more significant comparing to Con group ($P < 0.05$). When came to the 21 day, the content of MPO in Con group was still significantly higher than Normal group ($P < 0.05$).

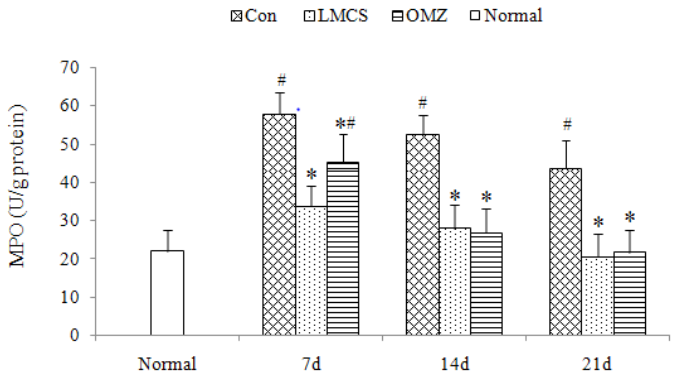



**FIGURE 7** - Effects of LMCS, OMZ on the MPO content in stomach tissue of ethanol-induced gastric ulcer in rats. Results are expressed as mean ± SD (n=3). (*p<0.05 *vs*. Con group and #p < 0.05 **vs**. Normal group).

## Discussion

Ethanol-induced gastric ulceration is a classic model that is widely used to investigate the gastroprotective activity of drugs[17]. The mechanisms of ethanol-induced gastric ulcer mainly includes breaking the mucosal barrier, back diffusion of acid, increased gastric mucosal permeability and depletion of certain oxygen free radical scavengers[18]. Also, metabolism of ethanol also releases free radicals[19]. In the present study, we evaluated the therapeutic effect of LMCS on ethanol-induced gastric ulcer in rats, and results manifested that oral administration of LMCS (7.6 mg/kg) has obviously promoting healing effect. Our previous work had illuminated that chitosan and sepia ink has good biocompatibility and regulation effect for gastrointestinal diseases[12]. But the underlying therapeutic mechanism of compound LMCS in gastric ulcer is still unclear. Hence, we carried out a preliminary analysis in this experiment.

Results showed that gastric emptying rate in LMCS-treated group at the first week reached to normal levels, which meant that LMCS had good effect on the regulation of gastric functions. Microscopical assessment revealed that at tissue level, LMCS had a healing promoting effect and the healing level of gastric ulcer was close to normal tissue on 21 day (Figure 2B21). Also, it exhibited less edema and bleeding point in the early stage (Figure 2B7, B14). Collagen expression is often used to evaluate the healing rate of wounds. Collagen is mainly secreted by fibroblasts, which is critical for ulcer healing[20]. Hydroxyproline (Hyp), as a characteristic amino acid of collagen, are often used as an indicator for detecting the collagen content[21]. Present study in Figure 3 showed that the fibroblasts in mucosa lamina of LMCS group secreted more collagen fibers compared to Con group at the testing time. Collagen fibers contained in OMZ

group exhibited also increased (Figure 3F7), but it is more orderly distribution in LMCS group (Figure 3E7). What's more, through the determination of Hyp showed that LMCS and OMZ indeed promoted collagen fiber growth in the ulcer (Figure 4).

Experimental studies had indicated that reactive oxygen species (ROS) play an important role in ethanol-induced gastric ulcers[22]. The gastric mucosal injury is closely related to an antioxidant action, increased lipid peroxidation and generation of free-radicals[23]. Organisms itself have enzymatic and non-enzymatic defenses, including CAT, SOD and GPx, which could reduced or prevent the injury of gastric tissue caused by ROS[24]. Therefore, we appraised kinds of oxidant-antioxidant parameters in rats gastric tissues to explore the role of oxidant stress in our experiment.

An effective indicator of oxidative stress and mucosal injuries is MDA, which is a major metabolite of lipid peroxidation. Hence, determination of MDA levels can be used to access lipid peroxidation[25]. Present study showed that MDA was sharply increased by ethanol, but LMCS significantly decreased the MDA content in gastric tissue. As for endogenous antioxidants CAT, SOD and GPx, the content varieties of which indicated the ability of the organism to scavenge the ROS[26]. An obvious evidence observed is that ethanol inhibited the antioxidant enzymes activities of CAT, SOD and GPx in gastric tissues, which is consistent with the previous studies of alcoholic gastric ulcer symptoms[27]. Also, it was clear that treatment with LMCS significantly promoted the expressions of CAT, SOD and GPx at the early stage in the healing process of gastric ulcer, and reached the normal level more quickly. Researchers found that induction of antioxidant enzyme systems is important in modulation of intracellular stress, which can eliminate the ROS before they damaged the critical cellular macromolecules[2]. Seen in this light, LMCS prepared in this study regulated the antioxidants enzymes of CAT, SOD and GPx and their activities were increased in advanced in the process of gastric mucosal tissue repair.

There is increasing evidence that major source of ROS in gastric mucosal injury is the activated neutrophlis[28], which play an vital role in the development of gastric damage by their aggregation and release of tissue-disrupting substance, such as oxygen free radicals and proteases[29]. The neutrophil infiltration into the gastric mucosal tissues is assessed by MPO[30]. Our data found that LMCS significantly inhibited MPO activity and restored it to normal levels from 7 day, which was earlier than OMZ form 14 day. The reason for this discrepancy could be related to the good antiinflammatory of chitosan and sepia ink[18].

Present study showed that LMCS may be used as a potential therapeutic drug for gastric ulcer in clinical, but further investigations should be studied.

## Conclusions

LMCS had significantly improved therapeutic effects on ethanol-induced gastric ulceration in rats. The underlying mechanisms of its promoted therapeutic effects might involve the good ability of gastric tissue repair, reduction of oxidative damage, high radical scavenging activity and its inhibitory effects on neutrophil infiltration.

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