Original Research

Autophagy protects gastric mucosal epithelial cells from ethanol-induced oxidative damage via mTOR signaling pathway

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Impact statement

The effect and mechanism of autophagy on ethanol-induced cell damage remain controversial. In this manuscript, we report the results of our study demonstrating that autophagy can protect gastric mucosal epithelial cells against ethanol toxicity *in vitro* and *in vivo*. We have shown that ethanol can activate autophagy via downregulation of the mTOR signaling pathway, serving as an adaptive mechanism to ameliorate ethanol-induced oxidative damage in gastric mucosal epithelial cells. This study brings new and important insights into the mechanism of alcoholic gastric mucosal injury and may provide an avenue for future therapeutic strategies.

Abstract

Alcohol abuse is an important cause of gastric mucosal epithelial cell injury and gastric ulcers. A number of studies have demonstrated that autophagy, an evolutionarily conserved cellular mechanism, has a protective effect on cell survival. However, it is not known whether autophagy can protect gastric mucosal epithelial cells against the toxic effects of ethanol. In the present study, gastric mucosal epithelial cells (GES-1 cells) and Wistar rats were treated with ethanol to detect the adaptive response of autophagy. Our results demonstrated that ethanol exposure induced gastric mucosal epithelial cell damage, which was accompanied by the downregulation of mTOR signaling pathway and activation of autophagy. Suppression of autophagy with pharmacological agents resulted in a significant increase of GES-1 cell apoptosis and gastric mucosa injury, suggesting that autophagy could protect cells from ethanol toxicity. Furthermore, we evaluated the cellular oxidative stress response following ethanol treatment and found that autophagy induced by ethanol inhibited generation of reactive oxygen species and degradation of antioxidant and lipid peroxidation. In conclusion, these findings provide evidence that ethanol can activate autophagy via downregulation of the mTOR signaling pathway, serving as an adaptive mechanism to ameliorate oxidative damage induced by ethanol in gastric mucosal epithelial cells. Therefore, modifying autophagy may provide a therapeutic strategy against alcoholic gastric mucosa injury.

Keywords: Autophagy, ethanol, gastric mucosal epithelial cells, mTOR signaling pathway, oxidative stress

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Introduction

Alcohol is consumed all over the world every day. Though a moderate intake is thought to be beneficial to human health,[1] excessive alcohol consumption, both chronic and binge-drinking, is detrimental to our physical constitution and causes serious health problems. Alcohol abuse not only damages our nervous system, cardiovascular system and immune system, but also injures the digestive system.[2,3] Specifically, binge drinking can cause acute fulminant pancreatitis and chronic drinking can lead to the formation of chronic liver fibrosis. Both acute and chronic excessive alcohol consumption can cause severe gastric mucosal injury and even hemorrhagic ulcers.[4,5]

The gastric mucosa has evolved numerous defensive mechanisms to counter the harsh environment in the stomach, including the mucus-bicarbonate-phospholipid barrier, slime layer, mucosal microcirculation, epithelial cell renewing, endogenous prostaglandins, and epidermal growth factor. Exogenous factors such as ethanol, *Helicobacter pylori* (*H. pylori*) and non-steroidal anti-inflammatory drugs (NSAIDs) can cause an imbalance in this equilibrium, leading to disorders of the gastric mucosa.[6] The effect of ethanol on the gastric mucosa is multifactorial and complicated. Specifically, ethanol promotes the secretion of gastric acid and pepsin, impairs the mucus bicarbonate barrier, disturbs mucosal microcirculation and induces the

infiltration of inflammatory cells in the gastric mucosa.[7,8] Recent research shows that ethanol-induced damage to the gastric mucosa likely occurs as a result of oxidative stress due to excessive production of reactive oxygen species (ROS) during ethanol metabolism.[9,10] To prevent the generation of ROS, mammalian cells have developed many antioxidant defense molecules, including glutathione, superoxide dismutase, catalase, peroxidase, and thioredoxin protein. However, if ROS cannot be degraded effectively by this antioxidant system, the result is oxidative damage to indispensable biomolecules, including proteins,[11] lipids,[12] and nucleic acids.[13,14] The resulting oxidized proteins and lipid peroxidation products cross-link each other, leading to their dysfunction and the formation of toxic aggregates, as seen with lipofuscin.[15] Moreover, this process leads to dysregulation of lysosomal activity, resulting in cell death.[16] An excess of ROS can also cause mitochondrial damage, including mitochondrial DNA (mtDNA) mutation, lipid peroxidation and altered mitochondrial membrane channel activity. This in turn decreases the quantity of ATP and increases the release of calcium from mitochondria, followed by mitochondrial swelling and cytochrome C release, ultimately leading to the induction of apoptosis and cell death.[14,17]

While ethanol exposure can lead to cell death, cells can activate complex protective systems to minimize the damage. There is increasing evidence that moderate levels of autophagy can be an important cellular protective mechanism contributing to cell survival.[18,19] Autophagy, or cellular self-digestion, is an evolutionarily conserved mechanism for the regulation of cellular stress. It controls cellular homeostasis and provides energy by degrading proteins, protein aggregates, and defective organelles.[20,21] This dynamic process is regulated by several cell signaling pathways, including the mammalian target of rapamycin (mTOR) pathway. Constitutive inhibition of mTOR signaling can lead to the initiation of autophagy.[22,23] Once activated, autophagy is coordinated by more than 30 autophagy-related (Atg) genes, which were originally identified in yeast and most have mammalian homologues.[24] The high degree of conservation among eukaryotes points to the importance of this system for cellular development and stability.[25] Furthermore, a low-level of basal autophagy has an important cytoprotective effect on maintaining cell function.[26] Autophagy can be activated in response to a number of insults, including excessive ROS as a consequence of oxidative stress to eliminate toxic polymers and damaged organelles.[27,28] Therefore, autophagy has emerged as a focus of research and as a potential therapeutic target to protect cells from oxidative damage.

Although autophagy is an important survival mechanism for cells, it is not known whether it plays a role in the response to ethanol-induced damage in gastric mucosal epithelial cells. Here, we have used cell and animal models to explore the role of autophagy in ethanol-induced injury to gastric mucosal epithelial cells and the relationship between autophagy and oxidative damage following ethanol exposure. Our findings present new insights into the mechanism of gastric mucosal epithelial cell injury caused by alcohol toxicity and point to autophagy modification as a potential therapeutic strategy for patients with alcoholic gastric mucosa injury.

Materials and methods

Cell culture and ethanol treatment

Human gastric mucosal epithelial cell (GES-1 cell), one of the most commonly used cell-lines for the study of gastric mucosal injury,[29,30] was cultured in Roswell Park Memorial Institute 1640 (RPMI1640) complete media, supplemented with 100 units/mL penicillin, 100 g/L streptomycin, and 10% fetal bovine serum (Hyclone). Cells were maintained in a humidified incubator at 37°C and 5% CO_2, and media were changed every 48 h. To determine the appropriate experimental conditions for our autophagy research, GES-1 cells were treated with increasing concentrations of ethanol for 6 h at 37°C. For subsequent experiments, 200 mmol/L of ethanol was selected as the experimental condition to mimic ethanol exposure *in vitro*. Where indicated, rapamycin (RAPA, 10 μmol/L, Selleck Chemicals, Houston, TX), 3-methyladenine (3-MA, 10 mmol/L, Sigma-Aldrich, Saint Louis, MO, USA), chloroquine (CQ, 10 μmol/L, Sangon Biotech, Shanghai, China), E64d (10 μg/ml, Selleck Chemicals) and pepstatin A (10 μg/ml, Selleck Chemicals) were applied to media, respectively.

MTT assay

The MTT assay was used to estimate cell viability in the presence of ethanol.[31] GES-1 cells were plated in 96-well plates incubated at 37°C prior to use. After 24 h, they were incubated in the presence and absence of ethanol and 3-MA in the concentrations indicated for 6 h. The culture media were then carefully removed and replaced with culture media containing 0.5% MTT in the dark for 4 h at 37°C to allow formazan crystal formation. The formazan crystals were dissolved completely in dimethyl sulfoxide (DMSO), and the optical density in each well was measured using a plate reader at 570 nm.

Annexin V-FITC/PI staining assay

An Annexin V-FITC/PI Apoptosis Detection Kit (KeyGEN BioTECH, Nanjing, China) was used to evaluate the apoptosis rate of GES-1 cells under different treatment conditions as per our previous study.[32] Briefly, cells were washed in phosphate buffered saline (PBS), re-suspended in annexin V binding buffer and then incubated with annexin-V FITC and propidium iodide (PI) for 20 min at room temperature. Finally, the samples were analyzed using a BD FACSCanto II Flow Cytometer.

Western blot analysis

Western immunoblotting was used to detect the expression of LC3, Beclin-1, Atg12-Atg5, Phospho-mTOR, mTOR, Phospho-p70S6 kinase, p70S6 kinase, Bcl-2 and Bax (Cell Signaling Technology, Danvers, MA) in GES-1 cells. GES-1 cells were lysed in radio immunoprecipitation assay (RIPA) lysis buffer and the protein extract concentration was estimated using a BCA protein assay kit

(Beyotime, Shanghai, China). Total protein (50 μg) was separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE). The separated protein was electro-transferred to polyvinylidene difluoride (PVDF, Millipore, Bedford, MA, USA) membranes. Next, membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST), incubated with the primary antibody overnight at 4°C, and then incubated with horseradish peroxidase-conjugated secondary antibody (Boster, Wuhan, China) after washing with TBST. Membranes were visualized using an enhanced chemiluminescence (ECL) detection system. The density of each band was estimated using the Image Lab software. All target proteins were normalized against the loading control, β-actin.

Immunofluorescence staining

Expression of LC3 in GES-1 cells was analyzed by immunofluorescence to detect LC3-positive intracellular puncta, which correspond to autophagic structures. After fixation with cold methanol at −20°C for 20 min, the treated cells were blocked with goat serum (3 mL/L) in PBS for 30 min at 37°C. Cells were subsequently incubated with anti-LC3 (Cell Signaling Technology) antibody overnight at 4°C, washed with PBS, and then incubated for 1 h at room temperature with Cy3-conjugated secondary antibody (Boster, Wuhan, China). Afterwards, all of the cells were stained with PBS containing 4′,6-diamidino-2-phenylindole (DAPI, Roche, Branford, CT) for 10 min to enable visualization of the nuclei. Autophagic vesicles were immediately visualized, and fluorescent images were taken using a fluorescence microscope (Olympus, Japan).

Animals and ethanol feeding

Male Wistar wild-type rats (Hubei Center for Disease Control and Prevention, Wuhan, China) (200–230 g) were housed in an environmentally controlled facility (temperature and humidity controlled, 12 h light-dark cycle, water and rodent diet *ad libitum*) and allowed to acclimatize for a week prior to starting the experiment. Animal experiments were conducted according to the guidelines of the National Institutes of Health and approved by the Ethic Committee of Tongji Medical College, Huazhong University of Science and Technology (Permit Number: S351). After 24 h of fasting and 6 h of water deprivation, the rats were randomly divided into four groups ($n = 6$ per group). Experimental rats were gavaged with 1 ml 70% ethanol (vol/vol) and control rats received the same volume of saline. Where indicated, chloroquine (CQ, Sangon Biotech, Shanghai, China; 60 mg/kg) was administered by gavage twice within 10 h prior to the administration of ethanol. Control animals received the same volume of saline. All of the rats were sacrificed 3 h after ethanol administration and the stomachs were immediately excised to assess the lesions.

Histopathological examination and immunohistochemical staining

Rat gastric mucosa was excised and fixed in a formalin solution for 24 h before being embedded in paraffin. Tissue sections (4 μm) were then prepared for hematoxylin-eosin (H&E) staining and immunohistochemical staining with LC3 antibody to evaluate the gastric mucosa damage and to label the autophagosomes. H&E-stained and LC3-stained tissue sections were imaged using a light microscope (Nikon, Japan).

Determination of gross gastric mucosal lesions

Following ethanol treatment, gross gastric mucosal injury appeared as elongated bands of hemorrhagic lesions. The damage was therefore classified according to the Guth standard[33]: 0 points for intact gastric mucosa without any lesions; 1 point for petechial hemorrhage; 2 points for a lesion length shorter than 1 mm; 3 points for a lesion length from 1 mm to 2 mm; 4 points for a lesion length from 2 mm to 4 mm; and 5 points for a lesion length longer than 4 mm. The partial points were then summed to obtain the lesion index, and the mean values of each group reflected the results of the various treatments.

Measurement of ROS expression

Intracellular ROS expression was measured using an oxidation sensitive non-fluorescent probe 2′, 7′-dichlorofluorescin diacetate (DCFH-DA, Sigma-Aldrich), which turns to highly fluorescent 2′, 7′-dichlorofluorescin (DCF) upon oxidation. GES-1 cells were incubated with 10 μmol/L of DCFH-DA in the dark for 30 min at 37°C and then washed carefully using serum-free media. ROS generation was detected using a spectrophotometer at a 488 nm excitation wavelength and a 525 nm emission wavelength.

Detection of MDA contents and SOD activity

The tissue homogenate of gastric mucosa was collected to measure the expression of malondialdehyde (MDA) and superoxidase dismutase (SOD) activity. The lipid peroxidation MDA assay kit and total superoxide dismutase assay kit with NBT (Beyotime) were used according to the manufacturer's instructions.

Statistical analysis

Each experiment was performed at least three times, and all results were presented as mean ± SEM. Statistical analysis was examined using a Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Ethanol exposure-induced GES-1 cell damage

To determine whether ethanol could inhibit gastric epithelial cell viability and induce apoptosis, GES-1 cells were incubated with ethanol at concentrations of 0, 50, 100, 200, 400, and 800 mmol/L for 6 h. The results demonstrated that ethanol treatment significantly decreased cell viability in a dose-dependent manner (Figure 1(a)). Although there was no statistical difference at 50 mmol/L ethanol, cell viability was only 42.86 ± 5.73% ($P < 0.05$) at concentration of 800 mmol/L. The cellular apoptosis rate, detected by flow cytometry, gradually increased from 4.13 ± 0.22% to

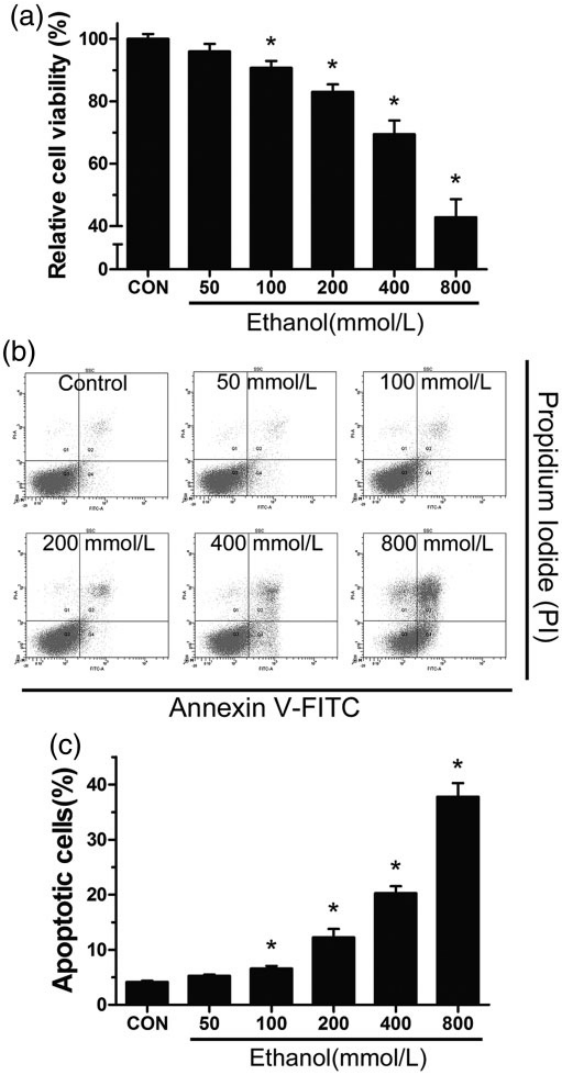

Figure 1 Ethanol decreases GES-1 cell viability and induces apoptosis in a dose-dependent manner. GES-1 cells were incubated with the indicated concentrations of ethanol in growth media for 6 h. (a) Cell viability was measured using the MTT assay. (b, c) Apoptotic cells were analyzed by flow cytometry using annexin V-FITC/PI staining assay, and the percentage of apoptotic cells is presented as a histogram. Data are shown as means ± SEM of three independent experiments; *$P < 0.05$ as compared with control group

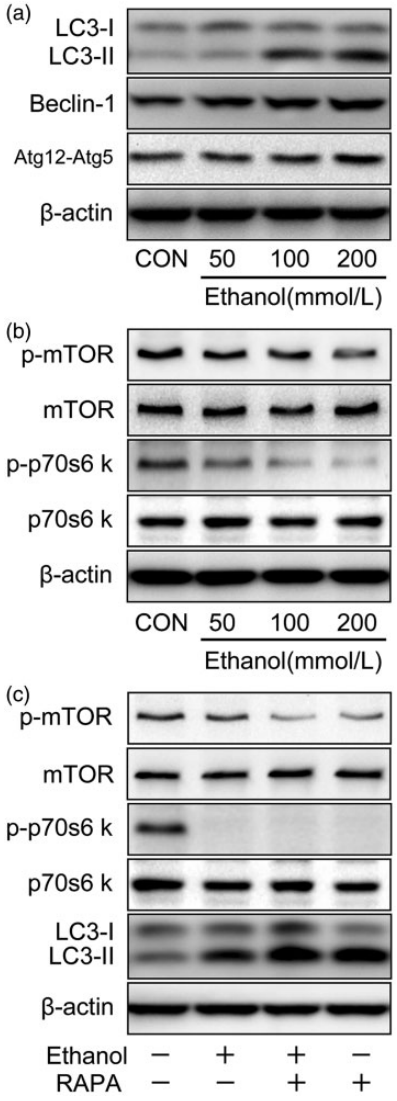

Figure 2 Ethanol exposure increased the relative expression of marker proteins for autophagy and reduced the relative levels of mTOR signaling proteins. GES-1 cells were incubated in the presence or absence of indicated concentrations of ethanol and/or RAPA (10 μmol/L). (a to c) Total cell lysates were prepared for Western blot analysis with antibodies against LC3, Beclin-1, Atg12-Atg5, p-mTOR, mTOR, p-p70S6 kinase, p70S6 kinase and β-actin

38.03 ± 2.62% with incremental doses of ethanol (Figure 1(b) and (c)). These results indicate that ethanol can reduce cell viability and induce apoptosis in a dose-dependent manner in GES-1 cells.

Ethanol-induced autophagy via downregulation of the mTOR signaling pathway in GES-1 cells

Ethanol treatment significantly affected GES-1 cell viability and death. As an important regulatory mechanism of stress, autophagy is a cellular reaction against outside intervention.[34] In order to determine whether ethanol could induce autophagy, GES-1 cells were treated with different concentrations of ethanol for prior to Western blot analysis using antibodies against a number of autophagic markers (Figure 2(a)). After 6 h of ethanol exposure, 200 mmol/L ethanol treatment was found to significantly increase the levels of Beclin-1, Atg12-Atg5, and LC3-II, which is the membrane-bound form of LC3 associated with autophagic activity. This reflected a remarkable dose-dependent activation of autophagy induced by ethanol in the range 0–200 mmol/L, and ethanol-induced cellular autophagy was most activated at the concentration of 200 mmol/L.

The mTOR signaling pathway is a key regulator of autophagy induction, with phosphorylation and activation of the signaling pathway being associated with the inhibition of autophagy. Western blots of GES-1 cell lysates with antibodies against mTOR and phosphor-mTOR showed that ethanol treatment significantly inhibited the phosphorylation of mTOR and that maximal inhibition occurred when cells were incubated with 200 mmol/L ethanol (Figure 2(b)). Ethanol also significantly inhibited the phosphorylation of p70S6 kinase, which is a substrate of mTOR (Figure 2(b)).

To further prove this point, we performed *in vitro* experiments to examine the inhibit effect of ethanol. Rapamycin (RAPA), an mTOR inhibitor, was used in GES-1 cells. Cells were incubated in the presence of ethanol and RAPA simultaneously for 6 h before processing for Western blotting. Compared with the ethanol treatment group, phosphor-mTOR and phosphor-p70S6 kinase were inhibited simultaneously after RAPA co-treatment, and the level of LC3-II was increased as expected (Figure 2(c)).

Our findings suggest that ethanol-induced autophagy is associated with inhibition of the mTOR signaling pathway in GES-1 cells.

Inhibition of autophagy enhanced ethanol-induced gastric epithelial cell injury

In order to determine whether the upregulation of autophagy in GES-1 cells was protective against ethanol-induced cell damage, 3-MA, a commonly used inhibitor in the regulation of intracellular autophagy, was used to suppress autophagy in GES-1 cells. Cells were incubated in the presence of ethanol and 3-MA simultaneously for 6 h before processing for Western blotting. LC3-II level was significantly decreased in cells treated with ethanol and 3-MA versus ethanol alone, indicating that 3-MA treatment inhibited autophagy (Figure 3(a)). Cell viability and levels of apoptosis were determined using the MTT assay, annexin V-FITC/PI staining assay, and Western blot analysis (Figure 3(b) to (f)). Cell viability significantly decreased after incubating with ethanol and 3-MA compared with ethanol alone (Figure 3(b)), while the rate of apoptosis significantly increased (Figure 3(c) and (d)). More importantly, the expression levels of the apoptosis-related proteins Bcl-2 and Bax were altered following treatment with ethanol and 3-MA (Figure 3(e) and (f)). Ethanol treatment resulted in decreased levels of Bcl-2/Bax ratio, while the ratio is lower following treatment with ethanol and 3-MA. Immunocytochemistry using anti-LC3 antibodies was used to confirm the effects of ethanol and 3-MA on autophagy in GES-1 cells. LC3 positive puncta (white arrow), corresponding to autophagic structures, were abundant in the cytoplasm of cells in the presence of ethanol compared to control cells (Figure 4). In contrast, LC3 immunoreactivity was rarely detected in GES-1 cells co-treated with 3-MA and ethanol. To further evaluate of LC3-II processing from LC3-I after treatment with ethanol, GES-1 cells

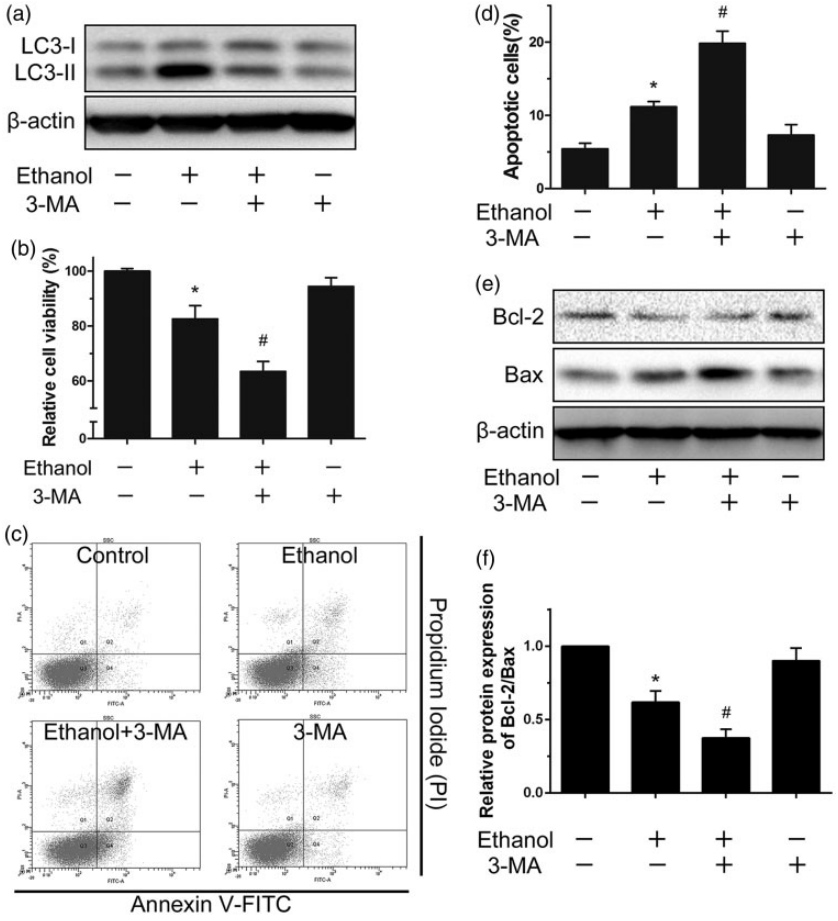

Figure 3 Autophagy plays an essential role in ethanol-induced GES-1 cell injury. GES-1 cells were incubated in the presence or absence of ethanol (200 mmol/L) and/or 3-MA (10 mmol/L). (a) Total cell lysates were prepared for Western blot analysis with antibodies against LC3. (b) Cell viability was determined by MTT assay. (c, d) Apoptosis was measured by flow cytometry with annexin V-FITC/PI staining, and the percentage of apoptotic cells is presented as a histogram. Western blot (e) and semiquantitative analysis (f) of Bcl-2 and Bax in GES-1 cell lysates. Error bars are represented as means ± SEM for the three independent experiments. $^{*}P < 0.05$ as compared with the control group. $^{\#}P < 0.05$ as compared with ethanol alone

were incubated with lysosomal inhibitors, E64d/pepstatin A and CQ, for analysis of autophagic flux. LC3-II significantly accumulated in the presence of lysosomal inhibitors (Supplementary Figure). Taken together, our data indicate a role for autophagy in protecting GES-1 cells from ethanol-induced apoptosis.

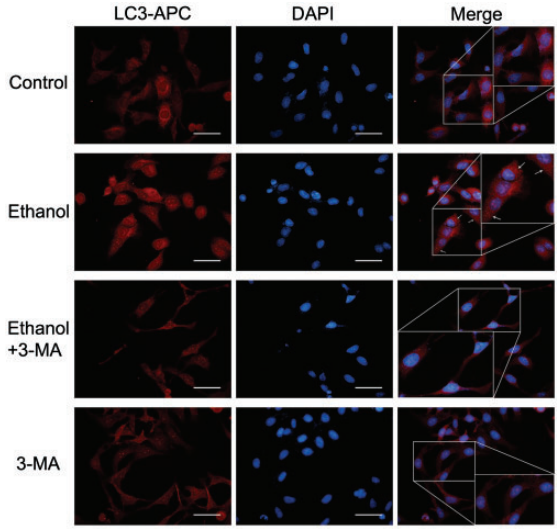

Figure 4 Ethanol regulates autophagosome formation. Autophagosomes were localized in the cytoplasm of GES-1 cells. GES-1 cells were treated with 200 mmol/L ethanol in the presence or absence of 10 mmol/L 3-MA for 6 h, and the distribution of endogenous LC3 (red channel) was visualized by immunofluorescence followed by nuclear DAPI staining (blue channel). White arrows indicate LC3 positive puncta corresponding to autophagosomes. LC3 puncta at a higher magnification are shown in the insets. The scale bar represents 40 μm. (A color version of this figure is available in the online journal.)

In order to determine whether autophagy serves the same role *in vivo*, rats were exposed to ethanol by intragastric administration. Chloroquine (CQ), an inhibitor of autophagosome-lysosome fusion and autophagic protein degradation, was also commonly used to inhibit autophagy.[35] As shown in Figure 5(a) and (b), ethanol treatment induced hemorrhagic lesions of the gastric mucosa, and CQ pretreatment was associated with an increased lesion index. Moreover, histological sections of gastric mucosa clearly revealed denudation of the surface epithelium, mucosal hemorrhage, and disruption of gastric pits following ethanol treatment (Figure 5(c)). This was exacerbated with prior administration of CQ. Treatment with CQ alone did not cause lesions and the gastric surface epithelium and pits remained intact (Figure 5(a) to (c)). Afterwards, immunohistochemistry was performed for LC3 on gastric mucosal tissue (Figure 5(d)). The sections showed that the number of LC3-positive cells significantly increased after ethanol treatment. High LC3 levels could represent excessive induction or reduced completion of autophagy.[36] Taken together, the evidence indicates an important role for autophagy in limiting ethanol-induced damage of the gastric mucosa *in vivo*.

Autophagy limits damage due to ethanol-induced oxidative stress

It is known that ethanol can stimulate ROS generation in gastric epithelial cells.[37,38] To investigate whether

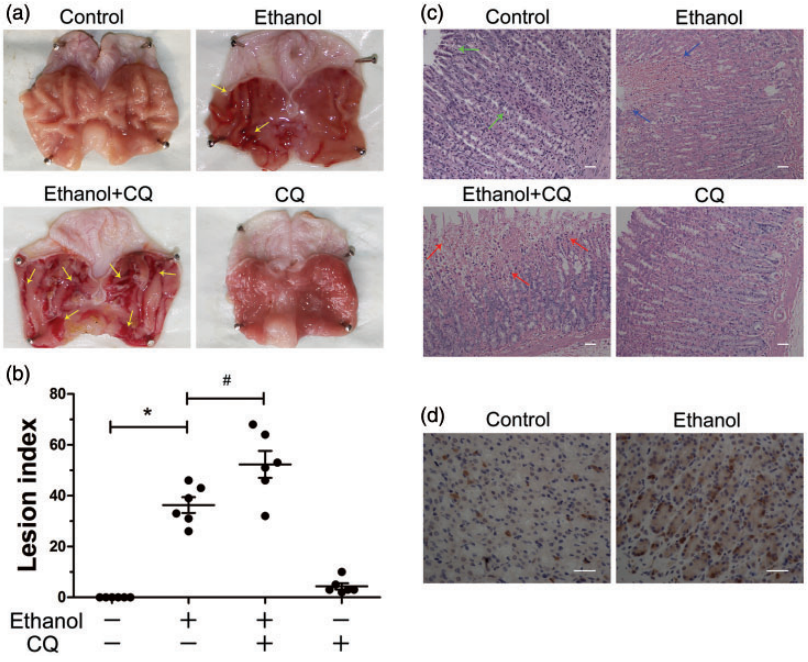

Figure 5 Inhibition of autophagy enhances ethanol-induced damage of rat gastric mucosa. Rats received intragastric administration of 70% ethanol or saline as control, with or without pre-treatment with 60 mg/kg chloroquine (CQ). (a) Gross appearance of rat gastric mucosa. Yellow arrows point to the obvious hemorrhagic lesions of the gastric mucosa. (b) The lesion index was calculated according to the Guth standard based on the gastric mucosal gross appearance. (c) H&E staining of gastric mucosal tissue sections. Green arrows point to healthy epithelium cells and gastric pits in the control group. Blue arrows show the denudation of surface epithelium, mucosal hemorrhage, and disruption of gastric pits by ethanol treatment alone, while red arrows indicate surface epithelium and gastric pits with serious erosions in the CQ pre-treated group. (d) Immunohistochemical staining in stomach sections was performed using anti-LC3 antibodies. Results are presented as means ± SEM ($n = 6$). $^{*}P < 0.05$ as compared with the control group. $^{\#}P < 0.05$ as compared with ethanol alone. Scale bar represents 250 μm. (A color version of this figure is available in the online journal.)

ethanol-induced autophagy can alter ROS production, GES-1 cells were treated with 200 mmol/L ethanol in the presence or absence of the autophagy inhibitor 3-MA. In addition, ROS production was determined using DCFH-DA, which converts to the fluorescent DCF in the presence of ROS. The intensity of DCF fluorescence increased by 1.70 ± 0.06 fold in the presence of ethanol compared to the control group (Figure 6(a)). Treatment with ethanol and 3-MA led to a 2.17 ± 0.10 fold increase compared to control, which was significantly higher than ethanol alone. Thus, autophagy limits ethanol-induced ROS production which could relate to cell apoptosis.

In order to investigate the antioxidant effect of autophagy *in vivo*, extracts of gastric mucosal tissues were collected following ethanol administration with or without CQ pre-treatment to determine the activity of SOD and the concentrations of MDA. SOD, an important intracellular antioxidant, was significantly decreased after ethanol treatment, and inhibiting autophagy further reduced its level (Figure 6(b)). MDA, a product of lipid oxidation due to excessive generation of ROS, increased from 11.51 ± 1.30 to 18.92 ± 2.64 μmol/mg protein following ethanol treatment. Levels were significantly increased in animals exposed to ethanol and CQ pre-treatment (Figure 6(c)). These data suggest that autophagy is involved in reducing ethanol-induced oxidative stress, thereby limiting damage to gastric mucosal epithelial cells *in vitro* and *in vivo*.

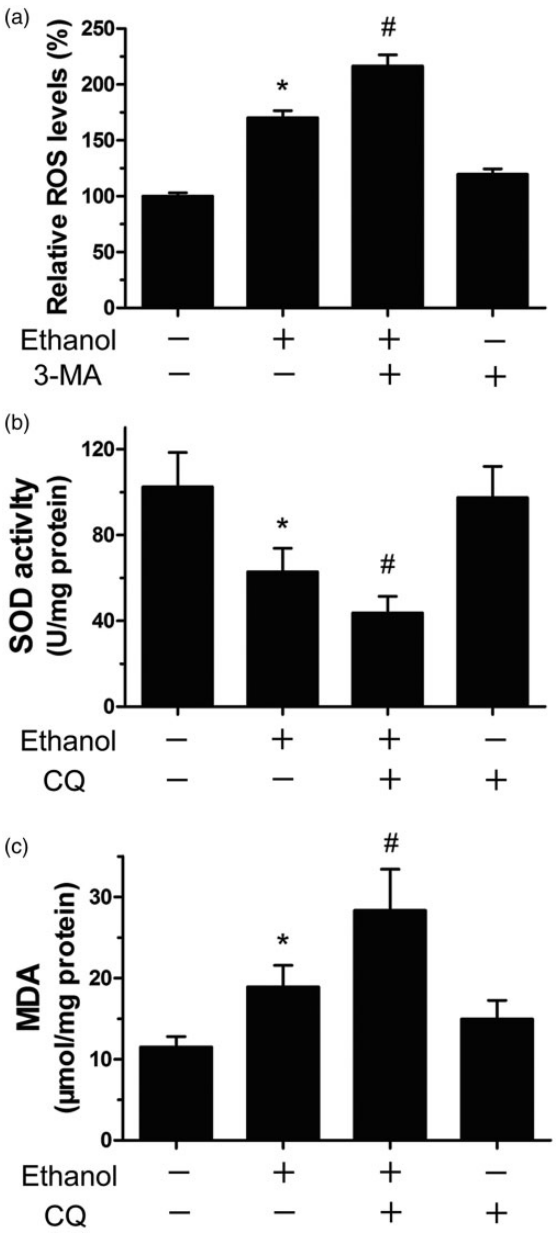

Figure 6 Autophagy inhibition increases ethanol-induced oxidative stress *in vitro* and *in vivo*. GES-1 cells were treated with 200 mmol/L ethanol and/or 10 mmol/L 3-MA. (a) ROS production was detected by staining with DCFH-DA, and fluorescence intensity of DCF was measured using a spectrophotometer. Components of the antioxidant response were assessed biochemically in rat gastric mucosa following intragastric administration of 70% ethanol, with or without CQ pretreatment. The histograms show SOD activity (b) and the concentrations of MDA (c) in gastric mucosa homogenate. Data are presented as means ± SEM for the three independent experiments. $^{*}P < 0.05$ as compared with the 0control group. $^{\#}P < 0.05$ as compared with ethanol alone

Discussion

In this study, we show that cellular autophagy is induced in gastric mucosal epithelial cells upon exposure to ethanol both *in vitro* and *in vivo* and that ethanol-induced damage is exacerbated when autophagy is inhibited. These results indicate a role for autophagy as a defense mechanism against ethanol toxicity. In GES-1 cells, autophagy activities increased gradually with the escalating concentrations of ethanol, combined with inhibition of the mTOR signaling pathway apparently, suggesting that this protective cell response is promoted by the downregulation of mTOR signaling pathway after ethanol exposure. Suppressing autophagy using the chemical inhibitors 3-MA *in vitro* and CQ *in vivo* exacerbates ethanol-induced gastric mucosal epithelial cell damage, causing increased apoptosis of GES-1 cells and increased gastric mucosal injury. Moreover, ethanol-induced ROS production, and consequently oxidative damage, are significantly increased when autophagy is inhibited, suggesting that autophagy acts to inhibit ethanol-induced ROS generation, degradation of antioxidant enzymes, and lipid peroxidation which could lead to oxidative stress.

Alcohol is one of the major exogenous factors that can lead to the damage of gastric mucosal epithelial cells and even gastric ulcers.[39] In our study, we successfully established a model for ethanol-induced gastric mucosal epithelial cell injury *in vitro* and *in vivo*, which are in agreement with the findings in human alcohol-induced gastric damages.

Autophagy is a dynamic, evolutionarily conserved process that is promoted under adverse conditions to maintain the balance between protein anabolism and catabolism, as well as cell homeostasis. The escalating levels of LC3-II, Beclin-1, and Atg12-Atg5 proteins reflect the activation state of autophagy,[40–43] and the mTOR intracellular signaling pathway is recognized as a major negative regulator of autophagy, which is the main degradative process in cells.[44,45] Therefore, targeting the mTOR pathway may have a critical impact on regulating autophagy activation

and alteration. In this study, we demonstrated that ethanol could induce autophagy via downregulation of mTOR pathway and increase of autophagic markers in gastric mucosal epithelial cells.

Nevertheless, whether autophagy plays a protective or a harmful role is not clearly established for most diseases.[46] The emerging role of autophagy in response to stress is a double-edged sword; on one hand, autophagy helps to maintain intracellular recycling and metabolic regulation, and on the other hand, excessive autophagy may also contribute to cell apoptosis or cell death.[47] Our study has demonstrated that ethanol could induce autophagy against gastric mucosal epithelial cell damage. Suppression of autophagy with inhibitor led to further cellular damage, including reduced cell viability, induction of apoptosis, and increased gastric mucosal disruption and hemorrhage. Therefore, the activated autophagy plays protective role in ethanol-induced gastric mucosal epithelial cells damage. Hernández et al.[48] also found that aspirin treatment downregulated basal autophagy activity in the gastric mucosa, and inhibition of autophagy exacerbated rat gastric damage and aspirin-induced gastric epithelial cell apoptosis; therefore, it is concluded that autophagy is a protective mechanism against aspirin-induced gastric epithelium injury, which is consistent with our study.

There are various mechanisms involved in ethanol-induced damage, including ROS generation, extracellular matrix protein degradation, and mitochondria damage.[38,49] In particular, ROS plays a dominating role in tissue damage during the pathogenesis of various disorders in the gastrointestinal tract.[50,51] Although oxidative stress may be a trigger to induce autophagy,[52] the relationship between ROS and autophagy in gastric mucosal epithelial cells is still poorly defined. Here, we showed that suppressing autophagy in GES-1 cells resulted in a further increase of ROS induced by ethanol treatment. In addition, the lipid peroxidation reaction mediated by ROS can be a major cause of cell destruction and damage.[53,54] Chen et al.[26] also reported that ethanol can induce ROS generation in SH-SY5Y cells, application of antioxidants block ethanol-induced LC3 lipidation suggested that autophagy is activated in response to ethanol-induced oxidative stress. Our data showed that MDA and SOD activity were altered after suppressing autophagy with ethanol treatment. Thus, we propose that inhibition of ROS generation, antioxidant enzyme degradation, and lipid peroxidation reaction by autophagy may be an essential part of the defensive mechanism against ethanol toxicity on gastric mucosa.

In conclusion, it is the first study to demonstrate a role for autophagy in protecting gastric mucosal epithelial cells against ethanol-induced apoptosis and gastric mucosal injury. This protective mechanism could be mediated via inhibiting ethanol-induced ROS generation, degradation of antioxidant enzymes, and lipid peroxidation. Our findings revealed that a proper autophagy capability may be crucial against ethanol toxicity and that regulation of autophagy could be a novel therapeutic strategy to mitigate ethanol-induced damage on gastric mucosa.

Authors' contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. WLC, JB, SBT, MYM, RD, and JYC conducted the experiments, and WLC, PZ and KXT wrote the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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