

Acidified Pepsin Promotes Laryngeal Precancerosis by Upregulating H⁺/K⁺-ATPase and Activating Mitophagy in Laryngeal Epithelial Cells

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Research

Keywords: Laryngopharyngeal reflux, gastroesophageal reflux, pepsin, autophagy, mitophagy, H⁺/K⁺-ATPase

Posted Date: December 4th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-117704/v1>

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Abstract

Background: Although laryngopharyngeal reflux (LPR) has been implicated in various upper aerodigestive tract and laryngeal diseases, the underlying mechanisms remain elusive. In this study, we investigated the role of gastric acidified pepsin in laryngeal precancerosis.

Methods: The *in vitro* and *in vivo* effects of acidified pepsin on H^+/K^+ -ATPase expression and autophagy/mitophagy induction in mouse laryngeal epithelial cells were assessed by hematoxylin and eosin staining, immunohistochemistry, CCK-8 assay, flow cytometry, Western blotting, and quantitative real-time PCR. Additionally, the levels of pepsin and H^+/K^+ -ATPase α and β subunits in 31 human laryngeal mucosal specimens were assessed by immunohistochemical staining.

Results: Acidified pepsin (pH=3) enhanced the growth and survival of mouse laryngeal epithelial cells *in vitro* and promoted laryngeal mucosal thickening and laryngeal epithelial cell growth *in vivo*. Furthermore, acidified pepsin promoted autophagy/mitophagy induction, accompanied by a significant decrease in mitochondrial membrane potential (MMP). Inhibition of autophagy by chloroquine abolished the ability of acidified pepsin to promote mitophagy and cell growth in laryngeal epithelial cells. Additionally, chloroquine promoted cell apoptosis and further reduced MMP in laryngeal epithelial cells treated with acidified pepsin. The expression levels of pepsin and H^+/K^+ -ATPase α and β subunits in 31 human laryngeal mucosa specimens were 51.6%, 48.4%, and 48.4%, respectively. Importantly, the pepsin level was correlated with the H^+/K^+ -ATPase β subunit level. H^+/K^+ -ATPase upregulation in laryngeal epithelial cells in response to acidified pepsin was essential for the mitophagy-promoting effect of acidified pepsin. H^+/K^+ -ATPase knockout or inhibition further reduced MMP in the presence of acidified pepsin.

Conclusions: Our findings suggest that in an acidic environment, pepsin promotes laryngeal epithelial cell growth and survival by upregulating H^+/K^+ -ATPase and activating mitophagy, potentially leading to laryngeal precancerosis.

Background

Although numerous studies have shown that tobacco and alcohol are risk factors for laryngeal cancer, the molecular mechanisms underlying laryngeal carcinogenesis remain unclear. Laryngopharyngeal reflux (LPR) is a common gastroesophageal reflux disease (GERD) resulting from gastrointestinal motility dysfunction [1]. GERD may progress to esophageal adenocarcinoma, significantly impacting quality of life [2]. Mounting evidence indicates that distal esophageal reflux increases the risk of extra-esophageal manifestations of GERD, such as precancerous lesions or laryngopharyngeal squamous cell carcinoma [3]. Although patients with typical GERD symptoms are empirically treated with proton pump inhibitors (PPIs) [4], the effects of PPIs on LPR remain unclear.

LPR entails the flow of gastric acid into the laryngopharynx [5, 6]. Gastric acid reflux promotes histological changes in the pharynx, larynx, and lower airways [7]. Additionally, gastric acid has been

shown to induce changes in the laryngopharyngeal mucosa [5, 8, 9]. Pepsin is a critical digestive enzyme found in gastric acid [10]. Secreted acid (HCl 0.1 mmol/L) and pepsin are predominant defense mechanisms protecting the gastrointestinal epithelium from potentially harmful microbes and toxins [11]. However, reflux and pulmonary aspiration of pepsin-containing gastric acid can lead to a wide range of diseases [12], including pulmonary fibrosis [13]. The combination of acid and pepsin's enzymatic activity can cause severe damage to the esophageal mucosa [14]. Notably, pharyngoesophageal reflux of HCl and pepsin has been shown to promote cancer development and progression in rat models [15], suggesting that acidified pepsin may play a role in fibrosis and carcinogenesis. Additionally, salivary pepsin has emerged as a novel biomarker of LPR [16], impairing the function of the upper respiratory tract and initiating inflammatory changes within the larynx [17]. A recent study confirmed that LPR is common among patients with neoplastic lesions and is a significant risk factor for squamous cell carcinoma of the pharynx or larynx [18]. Given the pro-inflammatory and pro-tumorigenic effects of acidified pepsin, we hypothesized that pepsin is a key determinant of the LPR-mediated alternations in the laryngeal mucosa.

In the present study, we assessed the expression pattern and role of acidified pepsin in laryngeal epithelial cell apoptosis and proliferation. We also investigated the effect of acidified pepsin on mitophagy in laryngeal epithelial cells. Furthermore, we explored the relevance of H^+/K^+ -ATPase in pepsin-mediated alterations in the proliferation and mitophagy of laryngeal epithelial cells. The findings of this study provide further insight into the roles of acidified pepsin and H^+/K^+ -ATPase in laryngeal cancer.

Methods

This study was approved by the Institutional Review Board of The First Affiliated Hospital, College of Medicine, Zhejiang University, China. All authors had access to the study data and reviewed and approved the final manuscript. The informed consent was obtained for experimentation with human subjects. The animals' care and use involving experiments were in accordance with institution guidelines for the care and use of laboratory animals.

Patients and patient samples

Specimens (n=31) were acquired from normal laryngeal mucosa far from the negative margin during open partial or total laryngectomy for tumor excision or microsurgery for benign laryngeal lesions. Operations were performed in the Department of Otolaryngology of The First Affiliated Hospital, College of Medicine, Zhejiang University between October 2018 and May 2019. Specimens were frozen immediately in liquid nitrogen following surgical removal and stored until further analysis. Among the 31 patients, 28 were males, and 3 were females, with a mean age of 58.1 (range, 32–81) years. Exclusion criteria were as follows: 1) preoperative radiotherapy, chemotherapy, or immunotherapy, 2) treatment with PPIs, and 3) underlying metabolic or autoimmune diseases.

All patients voluntarily received 24 h combined multichannel intraluminal impedance and pH monitoring (MMS Ohmega Ambulatory Impedance and pH Recorder, the Netherlands preoperatively (after 4 h of fasting) and completed the self-administered reflux symptom index (RSI; Chinese version, 2015), which contains nine items. The maximum RSI was 45, with an RSI >13 considered suggestive of LPR. All patients underwent laryngoscopic examination and evaluation for the reflux finding score (RFS). The RFS is based on eight components (maximum score, 26), and an RFS >7 was considered suggestive of LPR. Abnormal gastroesophageal reflux was defined as a DeMeester score of ≥ 14.7 and syndrome association probability of $\geq 95\%$ (gastroesophageal acid reflux) or syndrome association probability $\geq 95\%$ (abnormal non-acid gastroesophageal reflux).

Animal models

Seven-week-old male C57BL/6 mice (20–25 g) were purchased from Hubei Provincial Center for Disease Control and Prevention. Mice were housed in a specific-pathogen-free room with a controlled temperature ($20 \pm 2^\circ\text{C}$). The LPR animal model was established as described previously [19,20]. All mice were maintained under a 12 h light/dark cycle with free access to standard rodent chow and water. After 1 week of acclimation, the mice were randomly divided into three groups: control (treated with PBS), HCl (pH=3) treatment, and acidified pepsin (pH=3) treatment. HCl and acidified pepsin were administered by esophageal acid perfusion. Briefly, mice were anesthetized by intraperitoneal injection of pentobarbital sodium (Sigma, P3761). Subsequently, 0.15 mL HCl (1 M, pH=3) with or without 2.5 mg/g pepsin (SINOPHARM, 7647-01-0) was perfused (8 drops/min) into the mid- and lower esophagus using a feeding tube. Drug perfusion was performed twice daily for 45 consecutive days. Control mice were perfused with PBS instead of HCl. After treatment, throat specimens were obtained and washed with 0.9% saline solution. The specimens were stored at -80°C until further analysis.

Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC)

Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. After sectioning (5 μM thick), the tissues were deparaffinized and rehydrated. Antigen retrieval was performed by incubating the sections at high pressure. After blocking with 3% hydrogen peroxide, all sections were incubated with normal non-immune serum for 10 min at room temperature. Subsequently, slides were incubated overnight with antibodies against gastric H^+/K^+ -ATPase α subunit (D031-3, Clone 1H9; MBL International Corp., Woburn, MA, USA; 1:285 dilution), gastric H^+/K^+ -ATPase β subunit (D032-3, Clone 1B6; MBL International Corp.; 1:285 dilution), polyclonal pepsin (PAA632Hu01, Cloud-Clone Corp.; 1:100 dilution), and Ki67 (Abcam, ab15580, 1:200 dilution), followed by incubation with the appropriate biotin-labeled secondary antibody. After adding the chromogen diaminobenzidine, sections were stained with H&E and dehydrated. After mounting in neutral balsam, the slides were observed under a microscope. Regions of interest were analyzed using Image-Pro Plus 6.0 software. The sections were evaluated by two experienced

pathologists (YHT and YWW), who were blinded to the study treatments. Cell staining was scored as negative (0–10%), weakly positive (10–25%), moderately positive (26–50%), or strongly positive (50–100%).

For H&E staining, 5- μ M-thick sections were deparaffinized, rehydrated, and stained with hematoxylin (Sigma, H9627) for 5 min. After plating in HCl alcohol for 10 s, the sections were stained with 0.5% eosin for 1 min, incubated with ammonium hydroxide for 10 s, washed under running water, and mounted in neutral balsam. Experienced pathologists evaluated the histology.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Apoptosis in the throat tissues of mice was assessed using the *In Situ* Cell Death Detection Kit-POD (Roche, Shanghai, China). Briefly, 5- μ M-thick sections were deparaffinized, rehydrated, and incubated with proteinase for 25 min. After a 20-min incubation in cell-penetrating solution, sections were incubated in TUNEL reaction mixture at 37°C for 2 h in the dark. Subsequently, the sections were stained with 4',6-diamidino-2-phenylindole (DAPI), mounted in mounting medium, and observed under a fluorescence microscope.

Isolation of laryngeal epithelial cells

Primary laryngeal epithelial cells were isolated from C57BL/6 mice. Briefly, mice were anesthetized by intraperitoneal injection of pentobarbital sodium (Sigma, P3761). Laryngeal mucosal specimens were obtained under a dissecting microscope and washed with Hanks' solution

on ice. After cutting into 1 mm³ blocks, the specimens were treated with 10 mg/mL dispase II (Sigma-Aldrich, St. Louis, MO, USA) for 48 h at 4°C. The cell pellet was enzymatically dissociated in 0.05% Trypsin/EDTA at 37°C for 2–3 min and resuspended in 2 mL RPMI-1640 (SH30809.01; HyClone, USA) medium. The medium was replaced every 3 days, and the cells were expanded upon reaching 90% confluence.

Generation of H⁺/K⁺-ATPase- α -knockout (KO) cells and treatment

To establish an H⁺/K⁺-ATPase- α -subunit-KO cell line using CRISPR, we cloned a guide RNA (gRNA) targeting the H⁺/K⁺-ATPase α subunit (designed and synthesized by RIO Biotech) into the pUC57-T7-gRNA plasmid. The gRNA was amplified (forward, CACCGTATCAGACCAGCGCCACCA; reverse, AAAGTGGTGGCGCTGGTCTGATAC), and the target and control gRNAs were cloned into the BsaI sites of the pUC57-T7-gRNA plasmid. Plasmids were transfected into laryngeal epithelial cells using

Lipofectamine 2000 reagent (Invitrogen). After selecting for the transfected (puromycin-resistant) cells and confirming the knockout efficiency by western blotting, cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (HyClone, Waltham, MA, USA) at 37°C in a 5% CO₂ humidified atmosphere. KO cells were treated with acidic medium (pH=3 or 5) or normal medium (pH=7) with or without pepsin for 1 h, followed by a 24 h incubation in fresh RPMI-1640. Pepsin was inactivated using 10 M NaOH (pH=8) at 37°C for 30 min, and the pH of the medium was adjusted to 7 using 1 M HCl. To induce autophagy and inhibit proton pumps, we also treated cells with 20 µM chloroquine (CQ) and 10 µg/mL pantoprazole, respectively.

CCK-8 assay

Cells were seeded in 96-well plates (5×10^5 /well) and treated with acidic medium containing pepsin and CQ/pantoprazole for 24 h. Subsequently, 20 µL cell counting solution (Beyotime Biological Technology Co. Ltd, C0037) was added, and the cells were incubated in the dark for an additional 4 h. The optical absorbance at 450 nm was measured using the Spectra Plus microplate reader (Multiskan MK3, Thermo).

Flow cytometry

For cell cycle analysis, we fixed cells in 700 µL ice-cold 80% ethanol at 4°C for at least 4 h. After incubating with 10 µL RNase (1 mg/mL) and 10 µL propidium iodide (APOAF, Sigma) in the dark, the cells were analyzed by flow cytometry. For apoptosis measurement, we incubated the cells in 500 µL Annexin V Binding Buffer. After adding 5 µL Annexin V–FITC and 5 µL propidium iodide, the cells were incubated for 10 min at room temperature in the dark. The percentage of apoptotic cells was determined by flow cytometry. To measure mitochondrial membrane potential (MMP), we incubated the cells in 1000 µL JC-1 solution (Beyotime Biological Technology) for 20 min at 37°C. After centrifugation, the cells were resuspended in 200 µL 10× incubation buffer and analyzed by flow cytometry. All flow cytometry data were analyzed using ModFit LT software (Becton Dickinson, Mountain View, CA, USA).

Western blotting

Total protein was extracted using RIPA lysis buffer. Equal amounts of protein (30 µg) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes (IPVH00010; Millipore Co., Boston, MA, USA). Subsequently, the membranes were incubated with primary antibodies against caspase-3 (ab214430, Abcam; 1:1000), LC3 (ab48394, Abcam; 1:1000), p62 (ab109012, Abcam; 1:1000), H⁺/K⁺-ATPase α subunit (D031-3, MBL International Corp.; 1:1000), and H⁺/K⁺-ATPase β subunit (D032-3, MBL International Corp.; 1:1000) at 4°C overnight. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; ab181602, Abcam) served as a loading control. After incubating with the appropriate secondary antibodies for 1 h, the signal was developed using an

enhanced chemiluminescence assay kit (Beyotime Biological Technology) and visualized using the ChemiDoc XRS+ System (Bio-Rad Laboratories, Hercules, CA, USA).

Immunofluorescence (IF) assay

LC3 and H⁺/K⁺-ATPase (α subunit) expression was assessed by IF. Briefly, cells were grown on glass slides for 24 h, fixed in 3% paraformaldehyde for 30 min at 4°C, and neutralized with 50 mM NH₄Cl. After permeabilizing with 0.1% Triton X-100 for 15 min and washing with PBS, cells were incubated with antibodies against H⁺/K⁺-ATPase (α subunit) and LC3 at room temperature for 1 h, followed by incubation with the appropriate secondary antibody for 1 h and then with DAPI for 5 min. Stained cells were visualized under the LSM5 EXCITER laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

GFP-RFP-LC3 expression was also assessed by IF. Cells were treated with pepsin, CQ, and pantoprazole, followed by a 24 h incubation with adenoviruses carrying GFP-RFP-LC3. After fixing with 4% paraformaldehyde, LC3 expression was assessed by confocal laser scanning microscopy (LSM 800; Carl Zeiss). For analysis of mitophagy, cells were seeded on glass slides and, after the appropriate treatments, incubated with 100 nmol/L mitophagy dye for 30 min at 37°C. Fluorescence was measured by confocal laser scanning microscopy.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using the TRIzol reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. After reverse transcription using the first-strand cDNA synthesis kit (Transgene, China), qRT-PCR was performed using SYBR green (Roche). The primer sequences used for PCR were as follows: mouse H⁺/K⁺-ATPase α subunit, 5'-GGAGATGGAGATTAACGACCACC-3' (forward) and 5'-ACGGGCAAACCTTCACATACTC-3' (reverse); H⁺/K⁺-ATPase β subunit, 5'-TTCCGGCACTACTGTTGGAAC-3' (forward) and 5'-TGACCACATAGAAACCTGCGTA-3' (reverse); GAPDH, 5'-AGGTCGGTGTGAACGGATTTG-3' (forward) and 5'-GGGGTCGTTGATGGCAACA-3' (reverse). Target gene expression levels were normalized to GAPDH mRNA levels, and relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method.

Transmission electron microscopy

Cells grown on glass slides were fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, and gradually dehydrated in ethanol and acetone. Subsequently, the cells were embedded in epoxy resin and stained with uranyl acetate and lead citrate. Autophagy was assessed by transmission electron microscopy (HITACHI, HT7700-SS, Japan).

Statistical analysis

All experiments were independently performed at least three times. Data are expressed as means \pm standard error. Statistical analyses were performed using SPSS 25.0 software (IBM Corp., Armonk, NY, USA) or GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). IHC and IF images were analyzed using Image-Pro Plus 6.0 software. Statistical significance was determined using one-way analysis of variance (ANOVA) or Student's *t*-test. *P*-values <0.05 were considered statistically significant.

Results

Acidified pepsin enhances laryngeal epithelial cell growth in vitro and in vivo

Non-acidic pepsin (pH = 7) has been shown to increase the proliferation of laryngeal carcinoma cells [21]. We previously showed that the pepsin level was elevated in vocal cord polyps and leukoplakia and was correlated with the grade of dysplasia [22]. Here, we assessed the effects of acidic pepsin on the growth and viability of laryngeal epithelial cells and found that at low (pH = 3) and moderate (pH = 5) pH, increasing pepsin levels significantly increased cell viability (Fig. 1A). However, neutral pepsin (pH = 7) and inactivated pepsin (pH = 7) did not affect laryngeal epithelial cell proliferation or viability. These findings suggest that only activated pepsin can promote laryngeal epithelial cell proliferation. Thus, in subsequent experiments, we used acidified pepsin (pH = 3, 0.05–0.5 mg/mL).

Next, we performed flow cytometry to investigate the effect of acidified pepsin on cell cycle in laryngeal epithelial cells. Although the population of cells in S phase was decreased under acidic conditions (pH = 3), treatment with high-dose acidified pepsin (pH = 3; 0.1–0.5 mg/mL) significantly increased the proportion of cells in S phase ($P < 0.01$; Fig. 1B). Cell proliferation was also analyzed by Ki67 staining. In line with the flow cytometry findings, the number of Ki67-positive cells was lower under acidic than neutral conditions; medium and high doses of pepsin significantly increased the number of Ki67-positive cells (Fig. 1C). Low (0.05 mg/mL) and medium (0.1 mg/mL) concentrations of acidified pepsin had no effect on laryngeal epithelial cell apoptosis (Fig. 1D, E). However, a high dose (0.5 mg/mL) of acidified pepsin significantly increased the active caspase-3 level and promoted apoptosis in laryngeal epithelial cells (Fig. 1D, E). Given that the medium concentration (0.1 mg/mL) of pepsin promoted cell proliferation without significantly affecting apoptosis, we selected this dose to further explore the effect of pepsin on the growth of laryngeal epithelial cells.

To confirm the effects of pepsin on the laryngeal mucosa *in vivo*, we established an acid perfusion model using acidified pepsin and HCl (pH = 3), given that acidic medium (pH = 3) did not affect cell viability in laryngeal epithelial cells *in vitro*. Acidified pepsin significantly increased the thickness of the laryngeal mucosa and the number of Ki67-positive cells in the larynx (Fig. 2A, B), in contrast to perfusion with HCl alone (Fig. 2A, B). In accordance with our *in vitro* findings, HCl and acidified pepsin did not affect the

proportion of apoptotic cells in the laryngeal mucosa (Fig. 2C). These findings suggest that acidified pepsin induces laryngeal epithelial cell proliferation without affecting cell survival.

2Acidified pepsin induces mitophagy and mitochondrial injury in laryngeal epithelial cells

It has become increasingly evident that autophagy has a dual role in cell growth [23]. Mitophagy is a form of selective autophagy responsible for removing dysfunctional mitochondria via the autophagosome–lysosome system [24]. To assess the effect of acidified pepsin on autophagy in laryngeal epithelial cells, we evaluated autophagic flux by measuring microtubule-associated protein light chain 3 (LC3) levels. Stronger LC3 signals were observed in acidified pepsin-treated cells than in those treated with acidic medium alone (Fig. 3A), suggesting that acidified pepsin promotes the formation of autolysosomes. Consistently, Western blotting revealed that treatment with acidified pepsin increased the LC3II/LC3I ratio and decreased p62 levels in laryngeal epithelial cells (Fig. 3B). Additionally, acidified pepsin significantly increased the fluorescence intensity of a mitophagy dye probe and the number of autophagosomes in mitochondria (Fig. 3C, D). These findings indicate that acidified pepsin induces autophagy and mitophagy in laryngeal epithelial cells. Furthermore, we found that treatment with acidified pepsin significantly decreased MMP (Fig. 3E), which is essential for energy storage during oxidative phosphorylation and for the elimination of dysfunctional mitochondria [25]. Overall, these results suggest that acidified pepsin induces mitophagy and the accumulation of dysfunctional mitochondria accompanied by the reduction in MMP in laryngeal epithelial cells.

Autophagy inhibition abolishes acidified pepsin-induced cell growth in laryngeal epithelial cells

Considering the role of mitophagy/autophagy in cell growth and differentiation [26], we investigated the relevance of autophagy in the growth-promoting effects of acidified pepsin in laryngeal epithelial cells. The autophagy inhibitor CQ increases the pH of lysosomes and prevents the fusion of lysosomes and autophagosomes. CQ treatment (at pH = 3) of laryngeal epithelial cells significantly decreased autophagic flux, as shown by the increased LC3II and p62 levels (Fig. 4A). Importantly, autophagy inhibition by CQ significantly abolished the effects of acidified pepsin on the LC3II/LC3I ratio and p62 levels (Fig. 4A). Consistently, CQ treatment significantly suppressed the increase in mitophagy in response to acidified pepsin (Fig. 4B). Although CQ treatment alone had little effect on the proportion of J-monomer-positive cells, cells treated with CQ combined with pepsin had a significantly higher number of J-monomer-positive cells than that of cells treated with acidified pepsin alone (Fig. 4C), suggesting that autophagy inhibition by CQ augments the effect of pepsin on MMP. Interestingly, CQ treatment significantly abolished the ability of acidified pepsin to promote the growth of laryngeal epithelial cells (Fig. 4D). Although acidified pepsin alone had no effect on laryngeal cell apoptosis, the combination of CQ with acidified pepsin profoundly enhanced cell apoptosis, as reflected by the increase in the cleaved

caspase-3 level (Fig. 4E, F). These findings suggest that mitophagy plays a critical role in acidified pepsin-induced laryngeal cell growth.

H⁺/K⁺-ATPase is essential for the acidified pepsin-mediated induction of autophagy/mitophagy

The H⁺/K⁺-ATPase proton pump expressed in laryngeal cells has been implicated in the abnormal mucus secretion frequently seen in patients with chronic laryngitis, since laryngeal epithelial cells are more sensitive to alterations in pH than are esophageal epithelial cells [27, 28]. To assess the relationship between H⁺/K⁺-ATPase and pepsin in normal laryngeal mucosa, we analyzed normal laryngeal mucosal specimens obtained from 31 patients who underwent 24 h combined multichannel intraluminal impedance and pH monitoring preoperatively. pH monitoring indicated GERD in seven patients. Six patients exhibited an RSI > 13, and two patients an RSF > 7. However, there were no significant correlations among pH monitoring findings, RSI, and RSF (*P* > 0.05; Table 1). IHC revealed that pepsin and H⁺/K⁺-ATPase α and β subunits were expressed in 51.6%, 41.9%, and 48.4% of laryngeal mucosal cells, respectively (Fig. 5). There was a significant correlation between the levels of the H⁺/K⁺-ATPase β and α subunits, as well as between the levels

Table 1
The correlations among pH monitoring findings, RSI, and RSF

		RSI>13	RSF>7	GERD
RSI>13	Pearson Correlation	1	-0.129	0.271
	<i>p</i> value		0.49	0.141
	Ratio of Positive		6/31	
RSF>7	Pearson Correlation	-0.129	1	-0.155
	<i>p</i> value	0.49		0.405
	Ratio of Positive		2/31	
GERD	Pearson Correlation	0.271	-0.155	1
	<i>p</i> value	0.141	0.405	
	Ratio of Positive		8/31	

of H⁺/K⁺-ATPase β and pepsin (*P* < 0.01, Table 2). IHC in tissues from an LPR mouse model confirmed the elevated expression of H⁺/K⁺-ATPase α and β subunits in the laryngeal mucosa in response to acidified pepsin (Fig. S1A, B). Consistently, mouse laryngeal epithelial cells treated with acidified pepsin exhibited a higher H⁺/K⁺-ATPase fluorescence intensity (for both α and β subunits) than that in cells treated with acidic medium (pH = 3) alone (Fig. S1C, D). qRT-PCR and Western blotting confirmed the acidified pepsin-mediated upregulation of H⁺/K⁺-ATPase α and β subunits at the mRNA and protein levels, respectively (Fig. S1E, F).

Table 2
The correlations among pepsin, H⁺/K⁺-ATPase α and β subunits

		Pepsin	H ⁺ /K ⁺ -ATPase α subunit	H ⁺ /K ⁺ -ATPase β subunit
Pepsin	Pearson Correlation	1	0.169	0.550**
	<i>p</i> value		0.364	0.001
	Ratio of Positive		16/31	
H ⁺ /K ⁺ -ATPase α subunit	Pearson Correlation	0.169	1	0.485**
	<i>p</i> value	0.364		0.006
	Ratio of Positive		13/31	
H ⁺ /K ⁺ -ATPase β subunit	Pearson Correlation	0.550**	0.485**	1
	<i>p</i> value	0.001	0.006	
	Ratio of Positive		15/31	

The β subunit of H⁺/K⁺-ATPase has a regulatory role, whereas the α subunit is the catalytic core of the pump. To investigate the relevance of H⁺/K⁺-ATPase in acidified pepsin-induced laryngeal epithelial cell growth, we established an H⁺/K⁺-ATPase-α-subunit-KO laryngeal cell line using CRISPR/Cas9 (Fig. S2A). Expectedly, acidified pepsin did not upregulate the H⁺/K⁺-ATPase α subunit in the H⁺/K⁺-ATPase-KO laryngeal epithelial cells (Fig. S2A). In contrast to wild-type laryngeal epithelial cells, acidified pepsin exposure in H⁺/K⁺-ATPase-α-subunit-KO laryngeal epithelial cells did not promote cell growth but significantly enhanced cell apoptosis, as indicated by the increased level of cleaved caspase-3 (Fig. S2B–D).

Given the similar effects of H⁺/K⁺-ATPase deletion and autophagy inhibition on pepsin-mediated cell growth, we investigated the effect of H⁺/K⁺-ATPase α subunit depletion on acidified pepsin-induced autophagy. Acidified pepsin treatment in wild-type laryngeal cells significantly increased H⁺/K⁺-ATPase expression and LC3 accumulation; in these cells, H⁺/K⁺-ATPase largely co-localized with LC3 (Fig. 6A). However, H⁺/K⁺-ATPase depletion abrogated the acidified pepsin-induced formation of autophagosomes and accumulation of LC3 (Fig. 6B). Additionally, acidified pepsin treatment in H⁺/K⁺-ATPase-KO laryngeal epithelial cells failed to induce mitophagy and reduce the p62 level (Fig. 6C, D). Consistent with the effects of CQ treatment, H⁺/K⁺-ATPase depletion reduced MMP in laryngeal epithelial cells, possibly due to the decrease in mitophagic activity (Fig. 6E). These findings suggest that mitophagy induction in laryngeal epithelial cells in response to acidified pepsin is dependent on H⁺/K⁺-ATPase.

Proton pump inhibition by pantoprazole suppresses mitophagy induction and laryngeal epithelial cell growth in

response to acidified pepsin

PPIs targeting H^+/K^+ -ATPases are widely used to treat acid-related diseases, including LPR. To assess the effect of proton pump inhibition on acidified pepsin-mediated laryngeal epithelial cell growth, we treated laryngeal epithelial cells with different PPIs. Treatment with omeprazole (10 and 50 $\mu\text{g/mL}$), lansoprazole, rabeprazole, esomeprazole, and SCH-28080, but not pantoprazole, significantly reduced laryngeal epithelial cell growth ($P < 0.05$; Fig. S3). The combination of acidified pepsin with each of the PPIs significantly suppressed the pepsin-mediated increase in laryngeal epithelial cell growth ($P < 0.01$), with pantoprazole (50 $\mu\text{g/mL}$), rabeprazole, and esomeprazole providing the most potent growth inhibitory effects (Fig. S3). These results indicate that pantoprazole abrogates acidified pepsin-induced cell growth in laryngeal epithelial cells without significantly affecting the growth of normal laryngeal epithelial cells. Consistent with our findings in H^+/K^+ -ATPase-KO cells, pantoprazole treatment suppressed autophagy/mitophagy induction in response to acidified pepsin, reflected by the increased LC3II/LC3I ratio and p62 level (Fig. S4A, B). However, no changes in these indicators were observed in cells treated with pantoprazole alone.

Similarly, although treatment with pantoprazole alone did not affect MMP in laryngeal epithelial cells (at $\text{pH} = 3$), pantoprazole hindered the ability of acidified pepsin to decrease MMP in laryngeal epithelial cells (Fig. 7A). In addition, pantoprazole significantly abrogated the effect of acidified pepsin on cell viability in laryngeal epithelial cells (Fig. 7B). Consistently, cells treated with pantoprazole combined with acidified pepsin exhibited enhanced apoptosis and a higher level of cleaved caspase-3 compared with cells treated with pepsin alone (Fig. 7C, D). These findings suggest that proton pump inhibition by pantoprazole suppresses mitophagy induction and laryngeal epithelial cell growth in response to acidified pepsin.

Pantoprazole suppresses acidified pepsin-induced cell proliferation in the laryngeal mucosa *in vivo*

Next, we assessed the effects of pantoprazole combined with acidified pepsin on laryngeal mucosal cell proliferation *in vivo*. Acidified pepsin alone resulted in profound laryngeal mucosa thickening and increased Ki67 expression in the laryngeal mucosa (Fig. 8A, B) but did not induce cell apoptosis in the laryngeal mucosa. The combination of pantoprazole with acidified pepsin significantly suppressed the thickening of the laryngeal mucosa and increased Ki67 expression (Fig. 8A–C). Additionally, pantoprazole markedly enhanced cell apoptosis in the laryngeal mucosa when used in combination with acidified pepsin (Fig. 8C). These results indicate that proton pump inhibition by pantoprazole suppresses acidified pepsin-induced laryngeal mucosa alterations by inducing apoptosis and inhibiting proliferation of laryngeal epithelial cells.

Discussion

In this study, we found that pepsin levels were elevated in normal laryngeal mucosal specimens obtained from laryngeal carcinoma patients and that acidified pepsin promoted laryngeal cell survival and proliferation via mitophagy induction. We also identified H⁺/K⁺-ATPase as a link between pepsin and mitophagy induction and showed that proton pump inhibition impaired laryngeal epithelial cell growth in the presence of acidified pepsin. PPIs are commonly used to treat acid-related diseases, including GERD and LPR [29]. However, the efficacy of PPIs in LPR remains poorly demonstrated due to the influence of non-acid or mixed reflux, such as pepsin or bile salt reflux. The findings presented here provide strong evidence supporting the ability of acidified pepsin to promote cell proliferation, which possibly hinders the therapeutic effect of PPIs on LPR. Our findings also suggest that the combination of PPIs with pepsin-targeting agents may improve the treatment of LPR and prevent acidified pepsin-mediated inflammation and damage in laryngeal epithelial cells.

Gastric pepsin internalized by airway epithelial cells promotes cell damage, oxidative stress, and inflammation, which could lead to cancer development [30, 31]. Acid and pepsin have also been shown to induce gastric epithelial cell damage in rat models, and administration of epidermal growth factor or sucralfate prevented acid/pepsin-induced damage in rat gastric cells [32]. Furthermore, pepsin has been demonstrated to increase the risk of laryngeal carcinoma development by promoting IL-8-induced epithelial–mesenchymal transition [33]. Non-acidic pepsin (pH = 7) has also been shown to increase the proliferation of laryngeal epithelial cells and human hypopharyngeal squamous cell carcinoma cells [21]. In contrast to these reports, we found that non-acidic pepsin had no effect on the viability of laryngeal epithelial cells. On the other hand, we found that a high dose of acidic pepsin promoted laryngeal epithelial cell proliferation and survival. Although low doses of acidic pepsin enhanced the proliferation of laryngeal epithelial cells, they failed to promote cell survival *in vitro* or *in vivo*. These findings suggest that the effects of acidified pepsin on laryngeal epithelial cell viability are concentration dependent.

Autophagy exerted cytoprotective effects in laryngeal squamous cell carcinoma cells treated with recombinant human arginase, which induced cytotoxicity [34]. Similarly, mitophagy induction after exposure to hydrogen peroxide has been demonstrated to drive cell survival in laryngeal cancer cells, promoting laryngeal cancer progression [35]. By activating autophagy and inhibiting the release of gliadin peptides, gastrointestinal and pancreatic enzymes alleviate celiac disease [36]. Here, we show that treatment with acidified pepsin promoted autophagy and mitophagy in laryngeal epithelial cells. Therefore, the ability of pepsin to promote laryngeal epithelial cell survival might be linked to the induction of mitophagy.

Alterations in MMP strongly indicate cell dysfunction [37]. Notably, MMP disruption has been shown to induce mitochondrial damage and mitophagy in human gastric epithelial cells [38]. In this study, we found that, in addition to inducing mitophagy, pepsin treatment significantly reduced MMP in laryngeal epithelial cells. Mitochondrial dysfunction due to alterations in MMP has been shown to promote mitochondrial biogenesis and induce mitophagy [39, 40], further supporting that the reduction in MMP by acidified pepsin might be linked to mitophagy induction. Mitophagy is critical for removing dysfunctional mitochondria, promoting cell survival, and protecting cells from oxidative stress [41–43]. Typically, MMP

alterations activate the mitochondria-mediated apoptosis pathway [44]. Additionally, mitophagy inhibition has been shown to induce apoptosis in epithelial cells [45]. In this study, we found that mitophagy inhibition using CQ promoted the accumulation of dysfunctional mitochondria, further reducing the MMP and ultimately causing apoptosis. Thus, pharmacological inhibition of autophagy may represent a promising strategy to treat laryngeal cancer [46]. Additionally, mitophagy inhibition may suppress acidified pepsin-induced cell proliferation, preventing the development of precancerous lesions in the larynx.

The H^+/K^+ -ATPase proton pump comprises α and β subunits [47]. Gastric H^+/K^+ -ATPase plays a key role in regulating gastric acid secretion [48]. H^+/K^+ -ATPase is strongly expressed in the laryngeal mucosa [27, 49] and is elevated in LPR and laryngeal cancer patients [28]. In this study, we confirmed high H^+/K^+ -ATPase expression levels in the laryngeal mucosa. Interestingly, a diet high in phytate has been shown to reduce pepsin activity, thereby downregulating H^+/K^+ -ATPase expression [50]. Consistent with these findings, we found that pepsin treatment increased H^+/K^+ -ATPase levels. Similar to H^+/K^+ -ATPase, ATP13A2 is a lysosomal P-type ATPase. Notably, ATP13A2 deficiency has been shown to decrease autophagic flux accompanied by accumulation of mitochondrial mass, implying that proton pump inhibition induces mitochondrial injury [51]. Here, we show that H^+/K^+ -ATPase depletion markedly reduced autolysosome formation and autophagic flux in pepsin-treated cells, as well as promoted mitochondrial damage and MMP disruption, which led to apoptosis. These findings strongly suggest that acidified pepsin-induced mitophagy is dependent on H^+/K^+ -ATPase.

Phagophores play an essential role in autophagy [52]. After nucleation, the membrane of phagophores expands to generate autophagosomes, which mature into autolysosomes that function to degrade different cellular components [53]. During autophagy, cytosolic LC3I is conjugated to phosphatidylethanolamine to form LC3II, which is recruited to the autophagosomal membrane [54]. After fusing with lysosomes, LC3II in the autolysosomal lumen is degraded along with other intra-autophagosomal components [55]. p62, commonly used as a marker of autophagy, is also degraded during autophagy [56]. In this study, we found that acidified pepsin increased LC3II and decreased p62 levels. Moreover, we found that LC3 co-localized with H^+/K^+ -ATPase in cells undergoing autophagy, suggesting the involvement of H^+/K^+ -ATPase in the process of autophagosome/autolysosome formation in cells treated with acidified pepsin. Inhibition of autolysosome formation by CQ results in the accumulation of p62 and LC3II [57]. Here, we show that H^+/K^+ -ATPase depletion abrogated mitophagy induction induced by acidified pepsin, as indicated by the increased levels of LC3II and p62. It is possible that H^+/K^+ -ATPase depletion inhibits mitophagy by impairing autolysosome formation. However, future studies are required to elucidate the role of H^+/K^+ -ATPase in autolysosome formation.

Additionally, proton pump inhibition by pantoprazole inhibited mitophagy, enhanced mitochondrial damage, and promoted mitochondrial-mediated apoptosis in laryngeal epithelial cells treated with acidified pepsin. Consistent with these findings, pantoprazole has been previously shown to augment the anti-tumor effect of docetaxel by inhibiting autophagy [58]. Hence, PPIs may prevent the development of

LPR-associated laryngeal precancerous lesions by activating mitochondrial-mediated apoptosis. However, the ability of PPIs to prevent laryngeal cancer development and progression requires further investigation. Furthermore, given the importance of H^+/K^+ -ATPase in gastric acid release, the role of H^+/K^+ -ATPase in establishing an acidic environment in the larynx and the relevance of this effect in the growth-promoting effects of pepsin merit further investigation.

Conclusions

In this study, we found that pepsin levels were elevated in the laryngeal mucosa, promoting laryngeal epithelial cell growth and survival by activating autophagy and mitophagy under acidic conditions. We also found that acidified pepsin-mediated mitophagy flux was dependent on the proton pump H^+/K^+ -ATPase. These findings strongly support that inhibition of mitophagy and proton pumps may represent a promising approach to treat patients with LPR and prevent the development of laryngeal cancer.

Abbreviations

LPR laryngopharyngeal reflux

GERD gastroesophageal reflux disease

PPIs proton pump inhibitors

RSI reflux symptom index

RFS reflux finding score

H&E hematoxylin and eosin

IHC immunohistochemistry

TUNEL terminal deoxynucleotidyl transferase dUTP nick end labeling

gRNA guide RNA

CQ chloroquine

MMP mitochondrial membrane potential

qRT-PCR quantitative real-time PCR

LC3 microtubule-associated protein light chain 3

Declarations

Ethics approval and consent to participate:

This study was approved by the Institutional Review Board of The First Affiliated Hospital, College of Medicine, Zhejiang University, China. The informed consent was obtained for experimentation with human subjects. The animals' care involving experiments was in accordance with institution guidelines.

Consent for publication:

The informed consent was obtained for experimentation with human subjects.

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests:

The authors declare that they have no competing interests

Funding:

This work was supported by Health Commission of Zhejiang Province, China (No. 2020KY996)

Authors' contributions:

Ke-Jia Cheng: Conceptualization, Methodology, Writing - Original Draft,

Qiong Xu: Validation, Formal analysis,

Zhi-Mei Li: Resources,

Shui-Hong Zhou: Conceptualization, Methodology, Writing- Reviewing and Editing,

Yang-Yang Bao: Data Curation,

Zhe Chen: Supervision,

Jun Fan: Methodology, Investigation

Acknowledgements:

Not applicable

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Figures

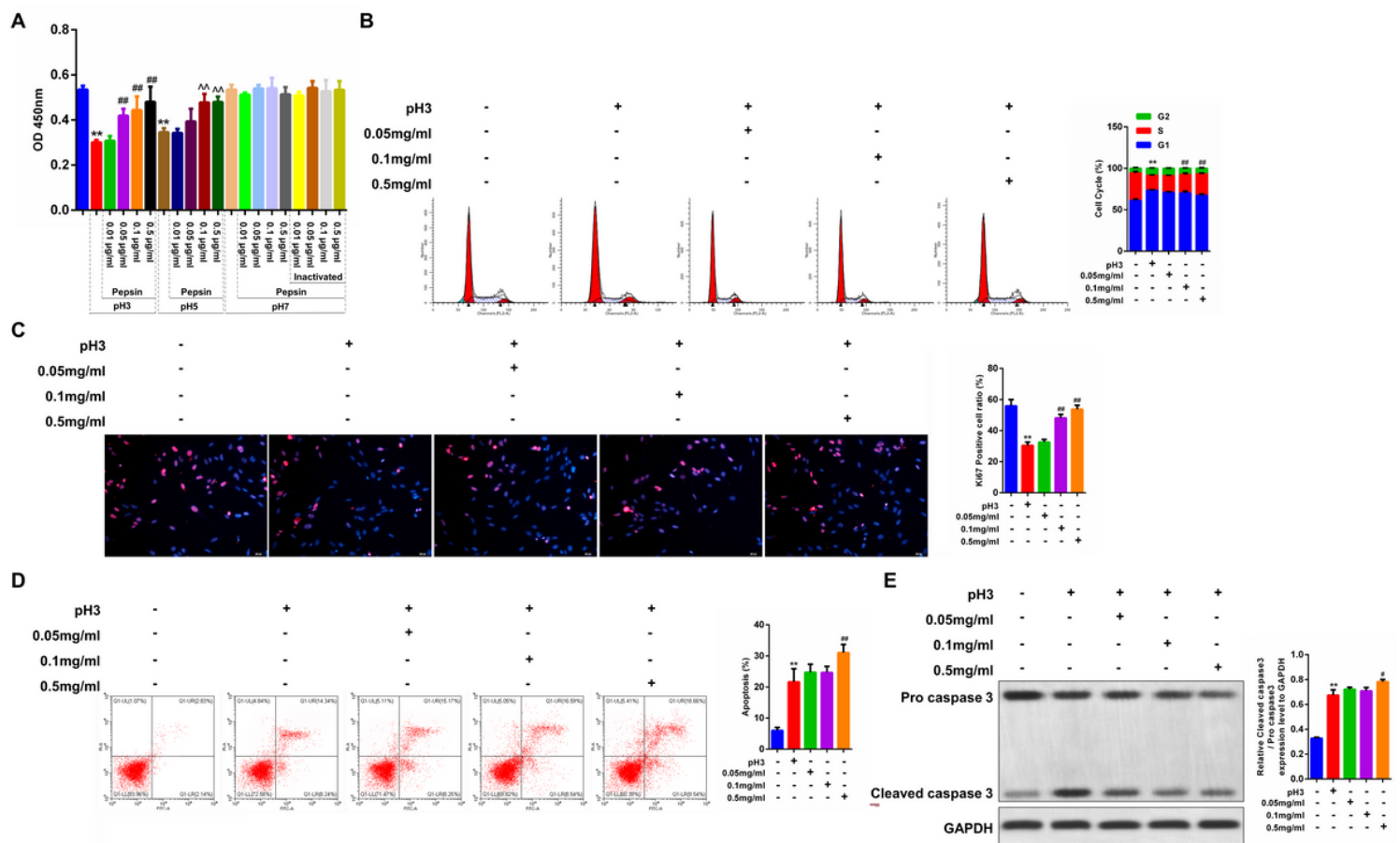


Figure 1

Effect of acidified pepsin on laryngeal epithelial cell viability. (A) Laryngeal epithelial cells were cultured in normal medium or acidic medium. Cells were treated with 0.01, 0.05, 0.1, or 0.5 mg/mL pepsin at pH 3, 5, or 7 or with inactivated pepsin at pH7. Cell proliferation was determined by CCK-8 assay. * indicates acidic versus control medium. # and ^ indicate pepsin versus acidic medium treatment at pH 3 and 5, respectively. (B) Cells grown in normal or acidic medium were treated with 0.05, 0.1, or 0.5 mg/mL pepsin at pH 3. The proportion of cells in different cell cycle phases was determined by flow cytometry. (C) Ki67-positive cells were detected by IHC. Ki67-positive cells are shown in red. The right panel shows the relative number of Ki67-positive cells. (D) Cell apoptosis was assessed by flow cytometry. The right panel shows the relative number of apoptotic cells. (E) Procaspace-3 and cleaved caspace-3 levels were determined by Western blotting. The right panel shows the ratio of cleaved caspace-3 to procaspase-3. **, ##, and ^^ indicate significance at $P < 0.01$.

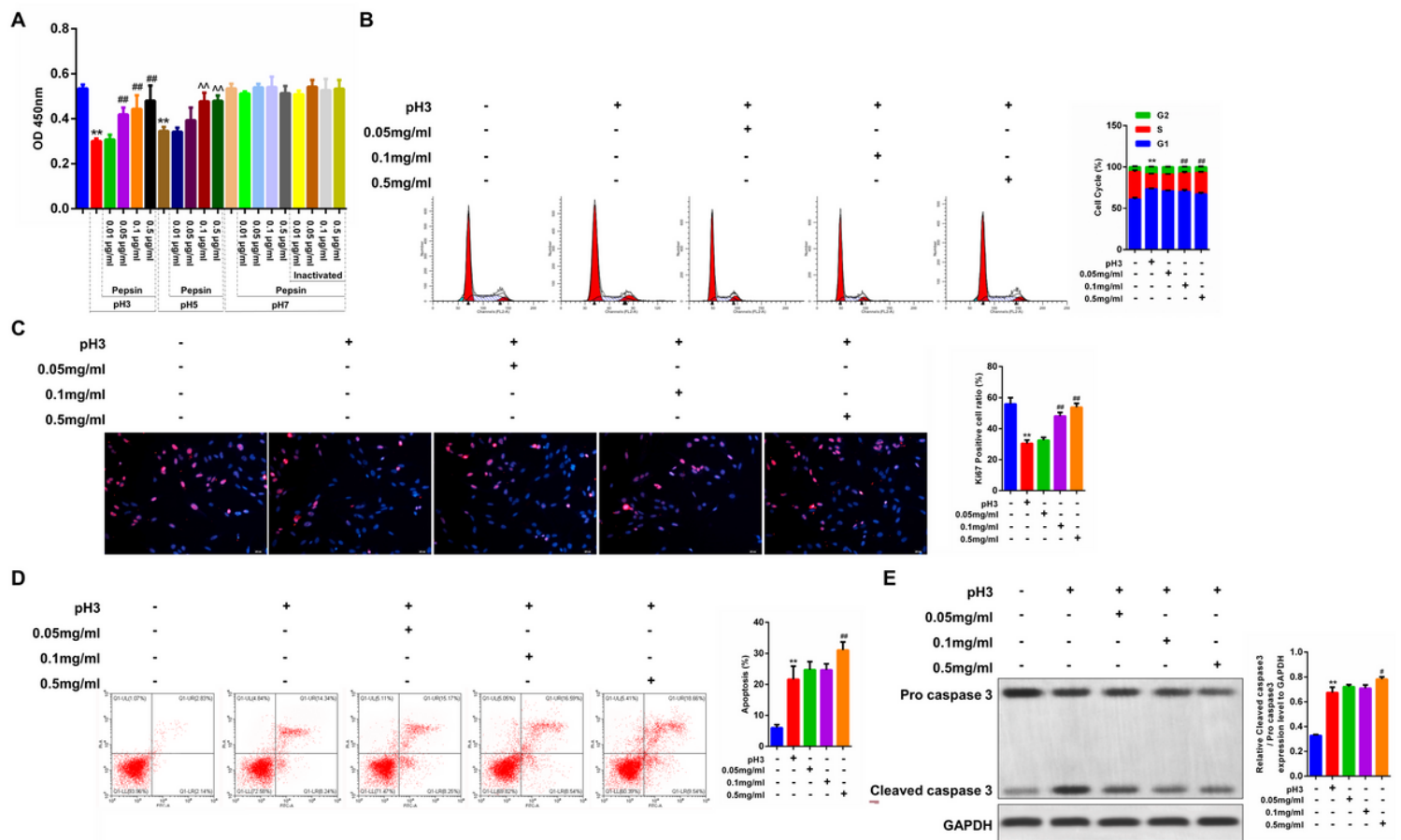


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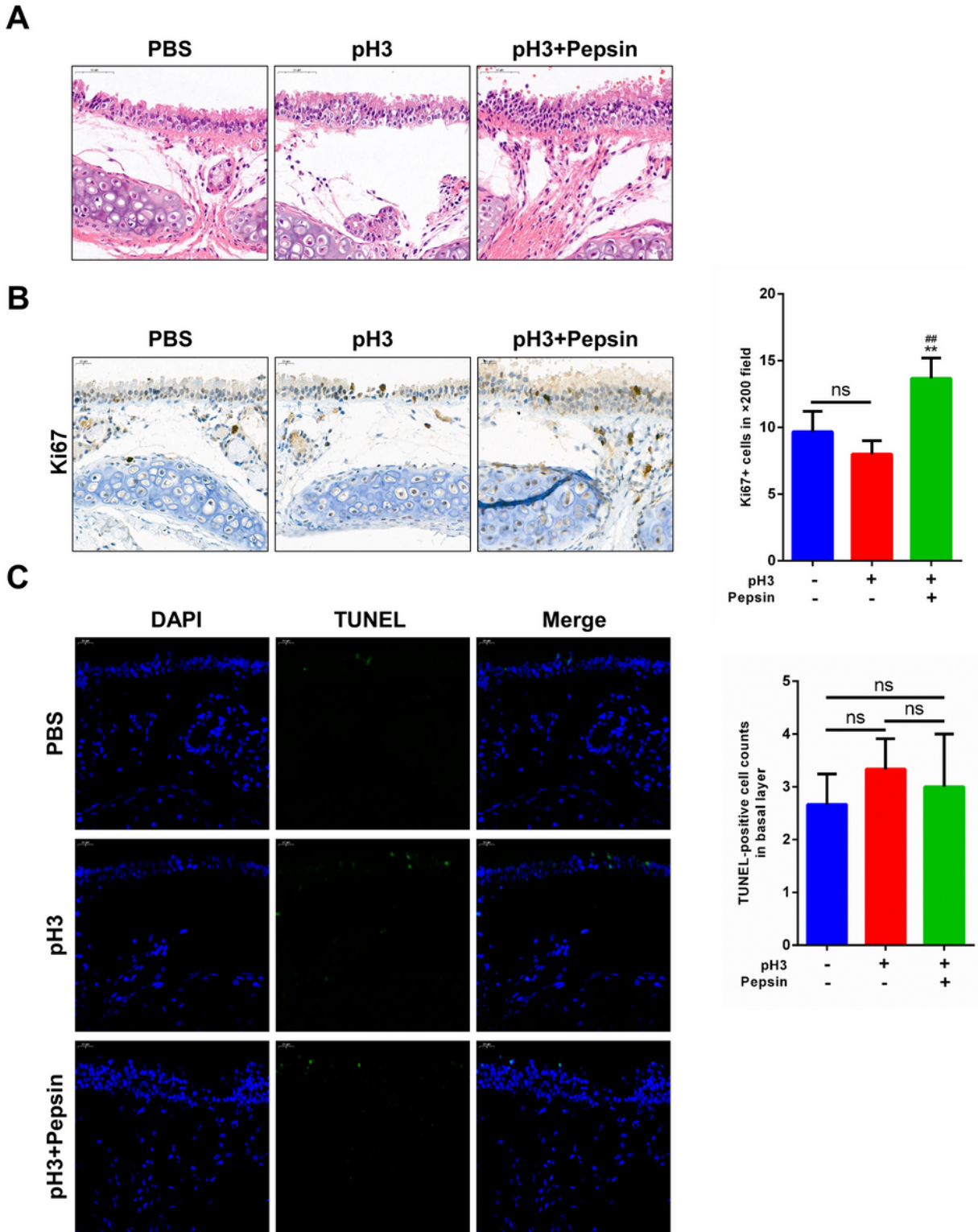
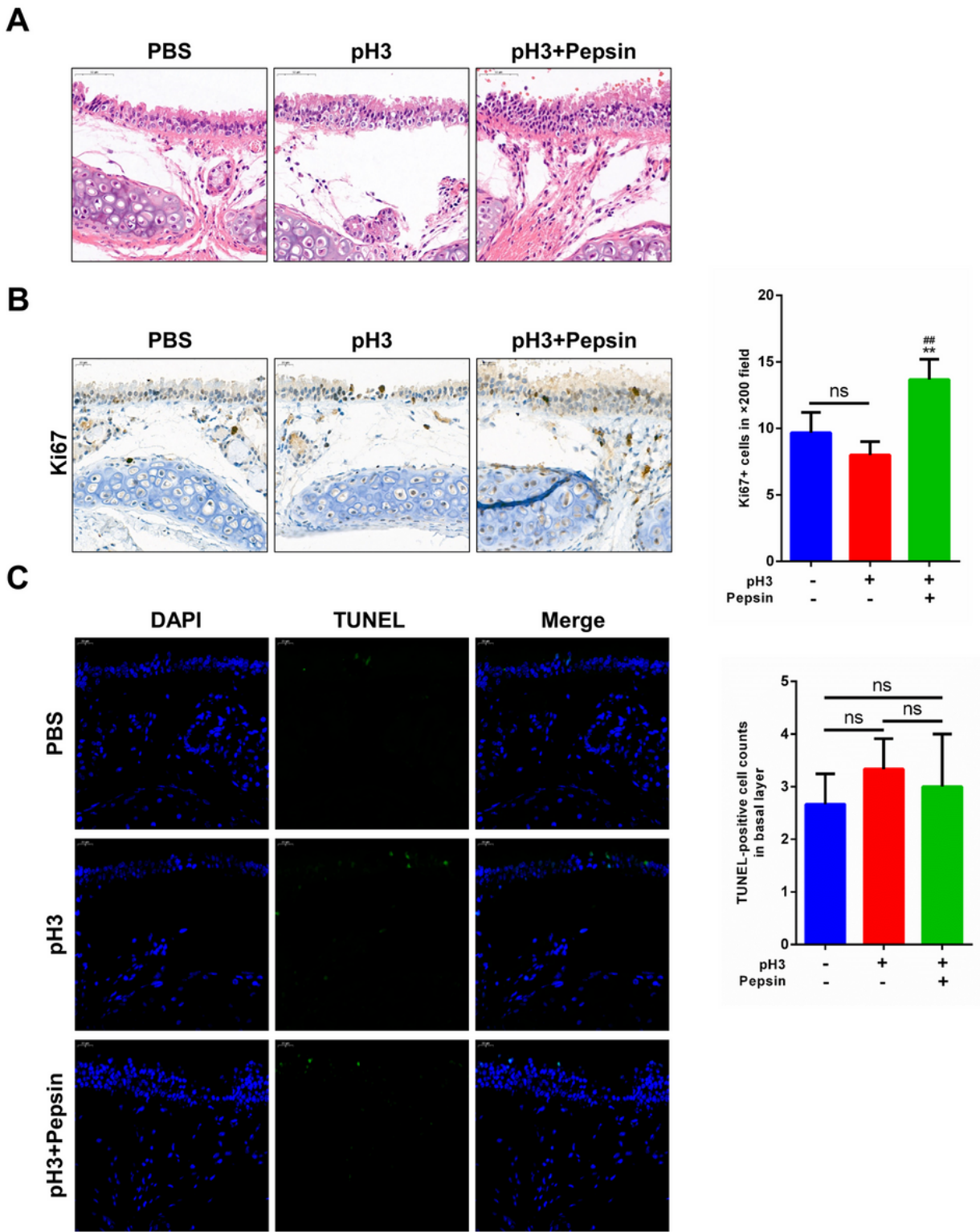


Figure 2

Effect of pepsin on the viability of laryngeal mucosal epithelial cells. (A) An esophageal acid perfusion mouse model was established by perfusing 0.15 mL HCl (1 M, pH=3) with or without 2.5 mg/g pepsin. Mice were divided into three groups: control (treated with PBS), HCl (pH=3) treatment, and acidified pepsin (pH=3) treatment (n=8 mice per group). The histological structure of laryngeal mucosal tissues was determined by H&E staining. (B) Ki67-positive cells in laryngeal mucosal tissues were detected by

IHC. The right panel shows the number of Ki67-positive cells in 200 representative fields in IHC images. (C) Apoptotic cells in laryngeal mucosal tissues were detected by TUNEL. The right panel shows the relative numbers of apoptotic cells. * indicates pepsin-treated versus control mice. # indicates pepsin-treated versus acid-treated mice. ** and ## indicate significance at $P < 0.01$; ns, not significant.



DAPI

TUNEL

Merge

PBS

pH3

pH3+Pepsin

Group	TUNEL-positive cell counts in basal layer
PBS	~2.7
pH3	~3.4
pH3+Pepsin	~3.0

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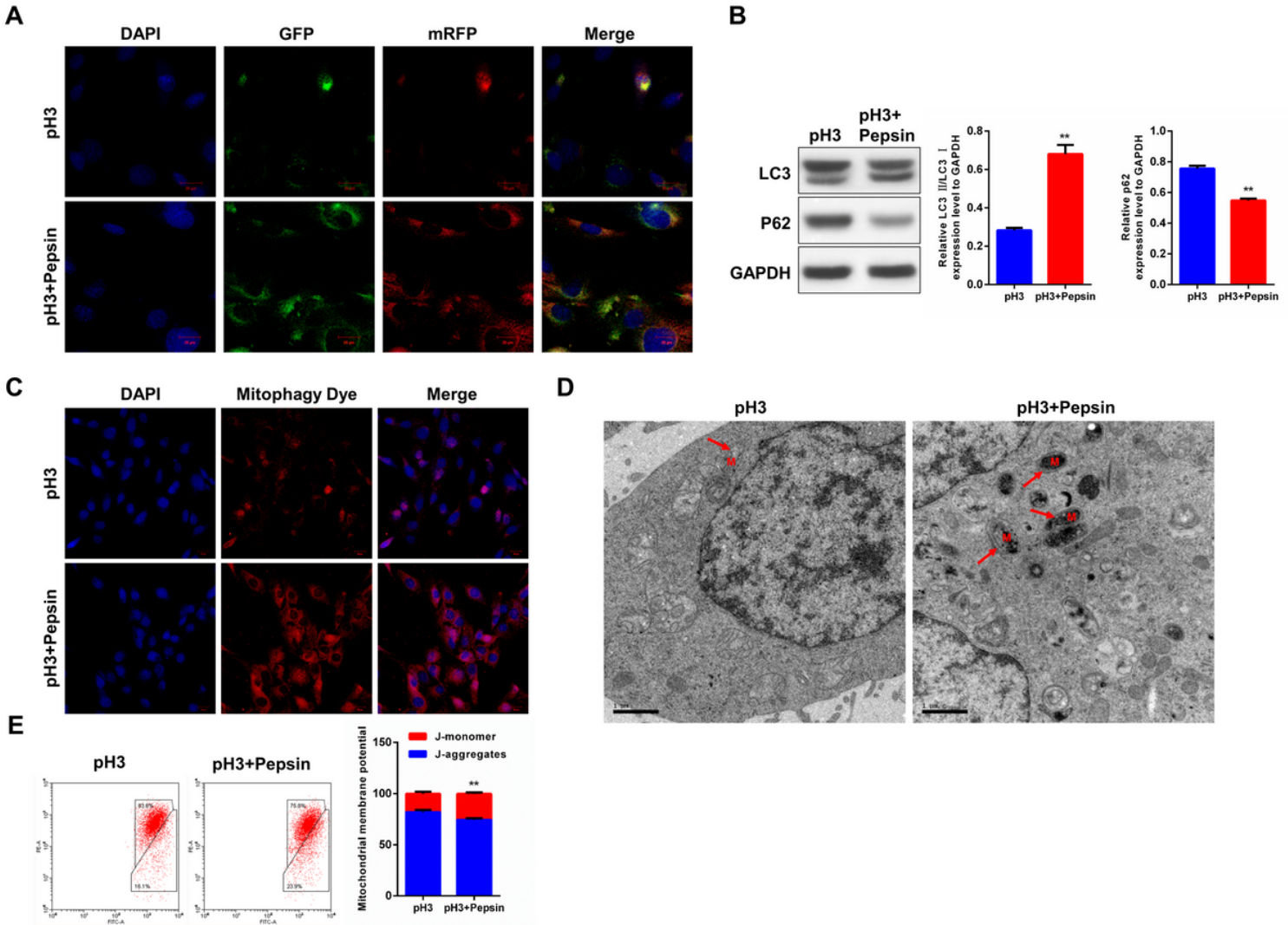


Figure 3

Effects of pepsin on autophagy and mitophagy in laryngeal epithelial cells. (A) Cells were transfected with GFP-mRFP-LC3 and then treated with acidic medium or pepsin. The fluorescence intensities of GFP and mRFP were measured by IF. (B) LC3 and p62 protein levels in cells treated with acidic medium or acidified pepsin were determined by Western blotting. The LC3II/LC3I ratio and relative p62 level were determined. (C) Mitophagy induction was assessed by IF using a fluorescent mitophagy dye. (D) Induction of mitochondrial autophagy in cells treated with acidic medium or acidified pepsin was

determined by transmission electron microscopy. (E) After treatment with acidic medium or acidified pepsin, cells were stained for JC-I. The percentage of JC-I-positive cells was determined by flow cytometry.

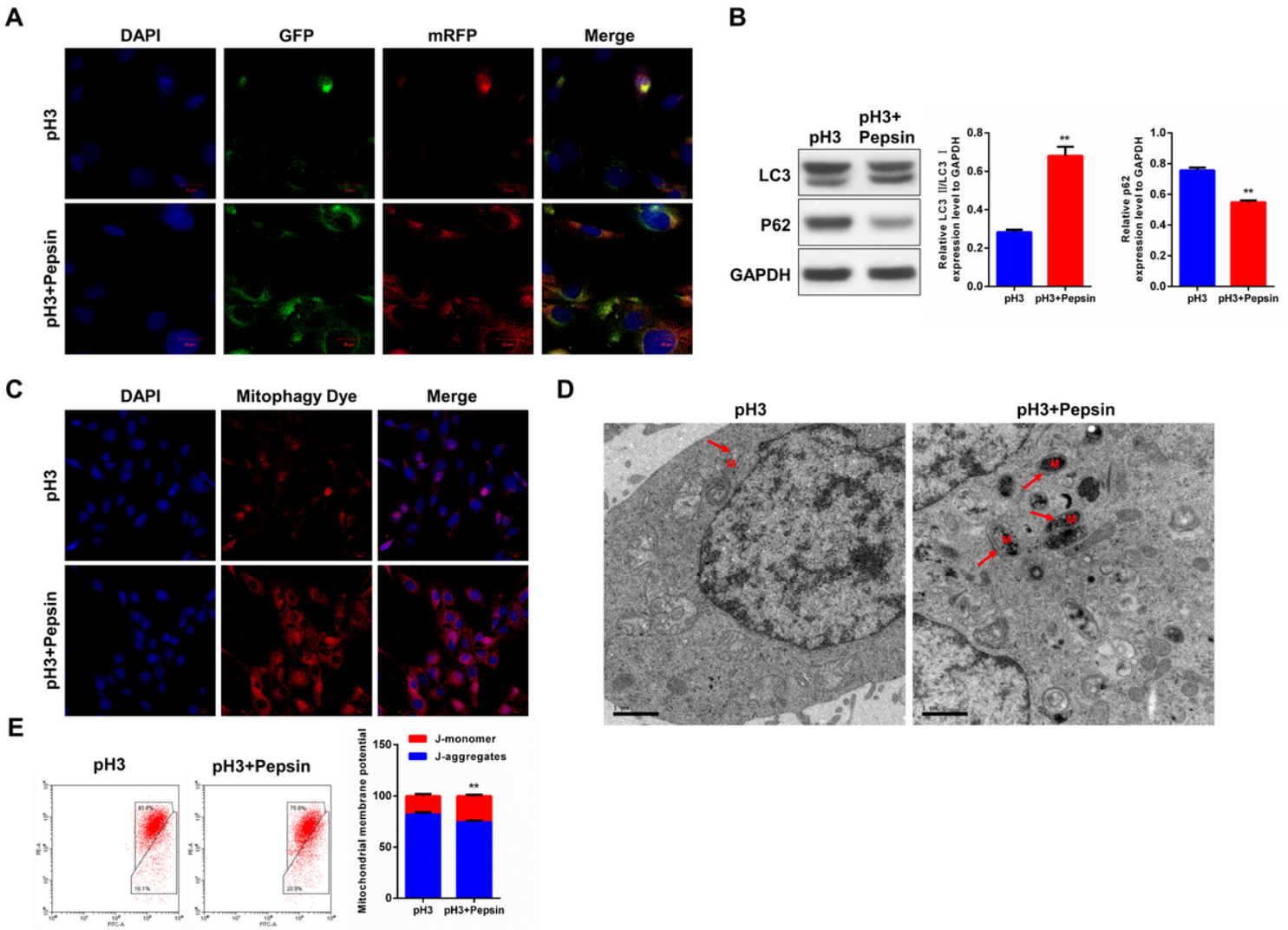


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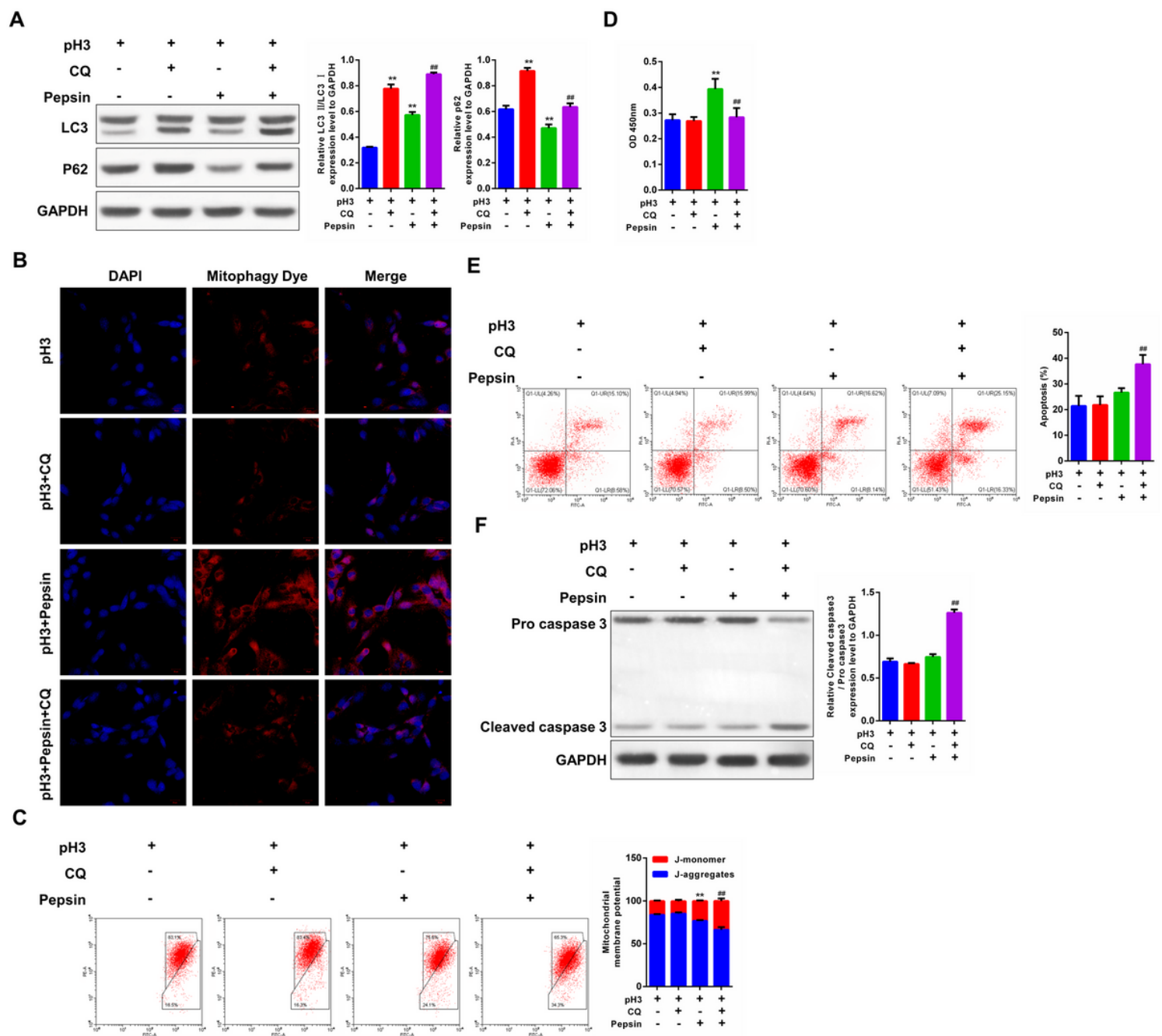


Figure 4

Effect of autophagy inhibition by CQ on acidified pepsin-induced autophagy in laryngeal epithelial cells. (A) LC3 and p62 protein levels in cells treated with acidic medium or acidified pepsin in the presence/absence of CQ were determined by Western blotting. The right panel shows the LC3II/LC3I ratio and relative p62 levels. (B) Mitophagy induction was assessed by IF using a fluorescent mitophagy dye. (C) The proportion of cells with J-monomers and J-aggregates was determined by flow cytometry. (D) Cell viability in the different groups was assessed by CCK-8 assay. (E) Cell apoptosis after treatment with pepsin or CQ was measured by flow cytometry. The right panel shows the relative number of apoptotic cells. (F) The levels of procaspase-3 and activated caspase-3 were determined by Western blotting. The

right panel shows the ratio of cleaved caspase-3 to procaspase-3 levels. ** and ## indicate significance at P<0.01.

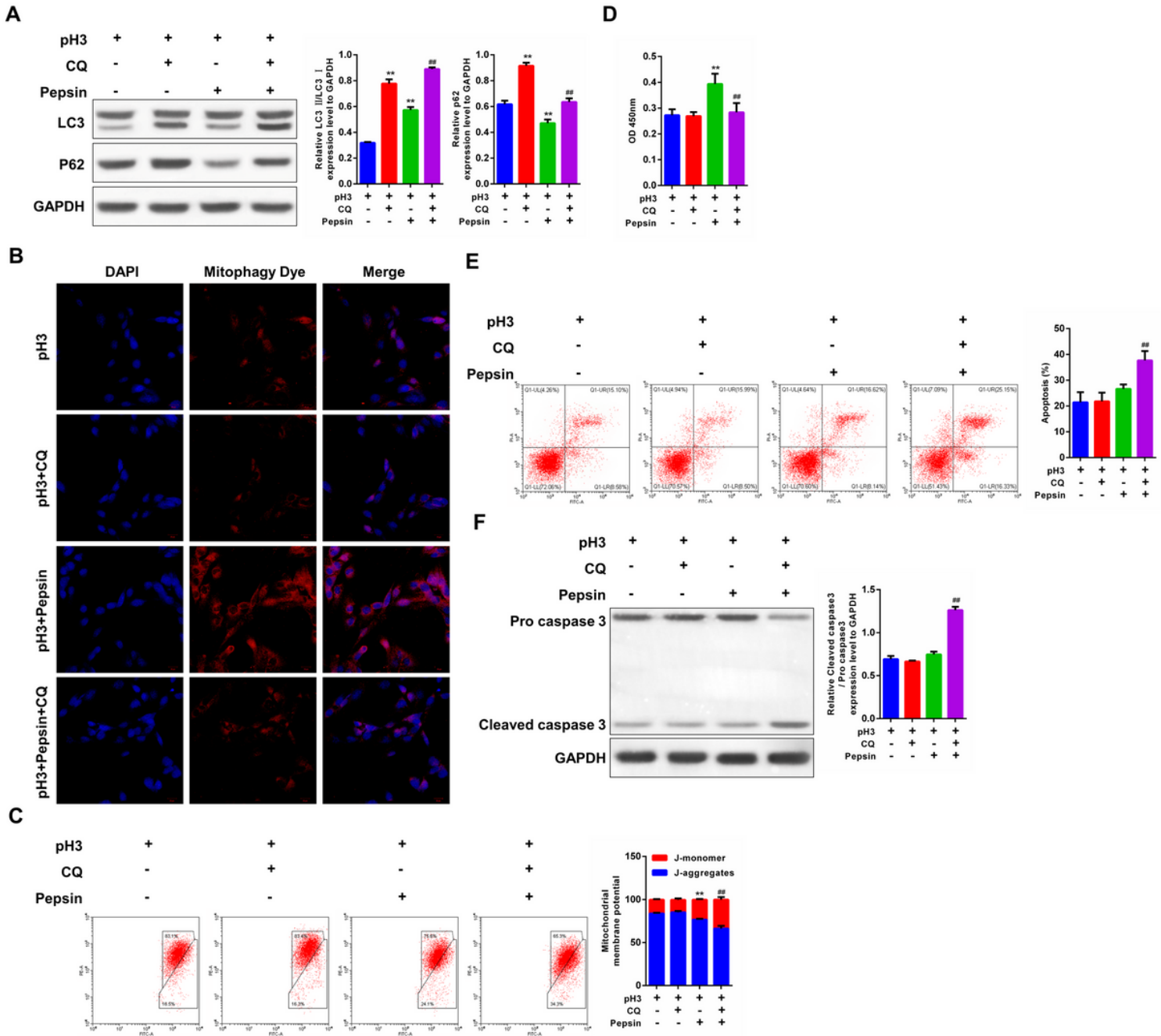


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Effect of autophagy inhibition by CQ on acidified pepsin-induced autophagy in laryngeal epithelial cells. (A) LC3 and p62 protein levels in cells treated with acidic medium or acidified pepsin in the presence/absence of CQ were determined by Western blotting. The right panel shows the LC3II/LC3I ratio and relative p62 levels. (B) Mitophagy induction was assessed by IF using a fluorescent mitophagy dye. (C) The proportion of cells with J-monomers and J-aggregates was determined by flow cytometry. (D) Cell viability in the different groups was assessed by CCK-8 assay. (E) Cell apoptosis after treatment with pepsin or CQ was measured by flow cytometry. The right panel shows the relative number of apoptotic

cells. (F) The levels of procaspase-3 and activated caspase-3 were determined by Western blotting. The right panel shows the ratio of cleaved caspase-3 to procaspase-3 levels. ** and ## indicate significance at $P<0.01$.

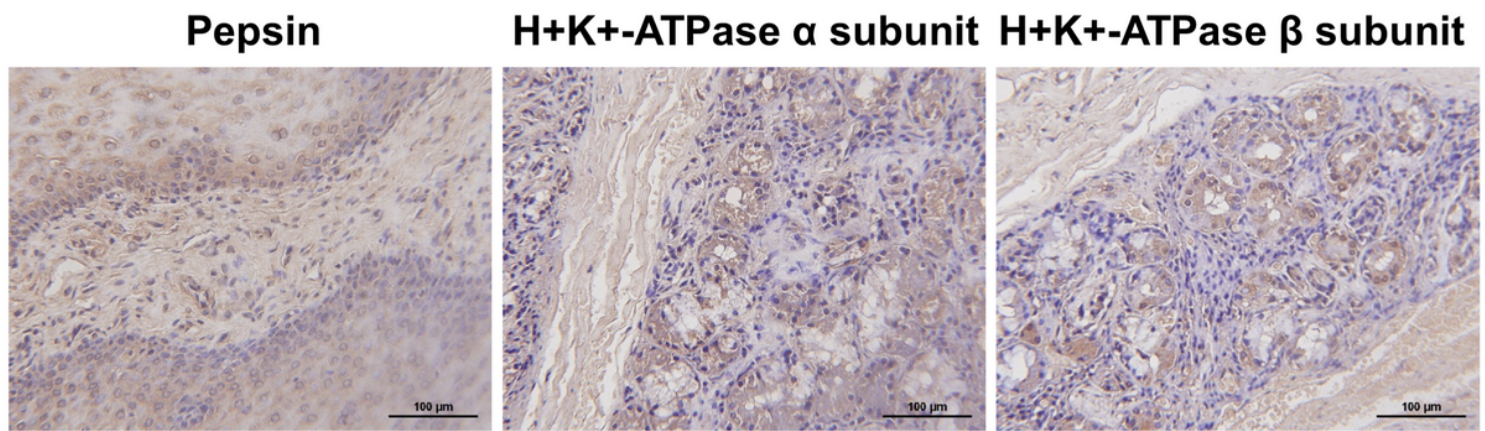


Figure 5

Expression of pepsin and H+/K+-ATPase α and β subunits in human laryngeal mucosa. The levels of pepsin (left panel), H+/K+-ATPase α subunit (middle panel), and H+/K+-ATPase β subunit (right panel) in human laryngeal mucosa tissues were determined by IHC.

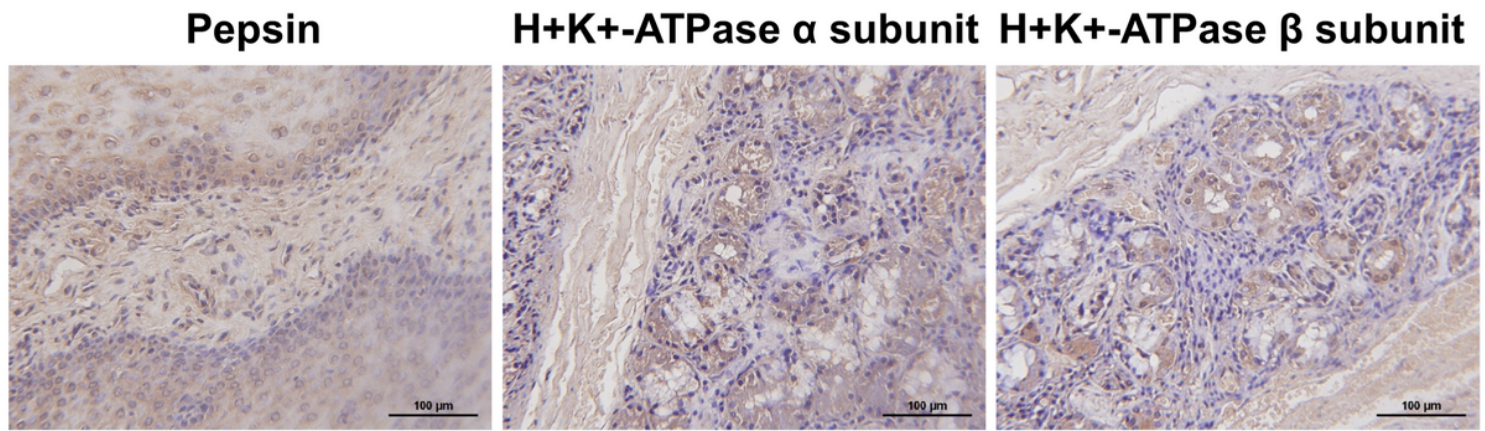


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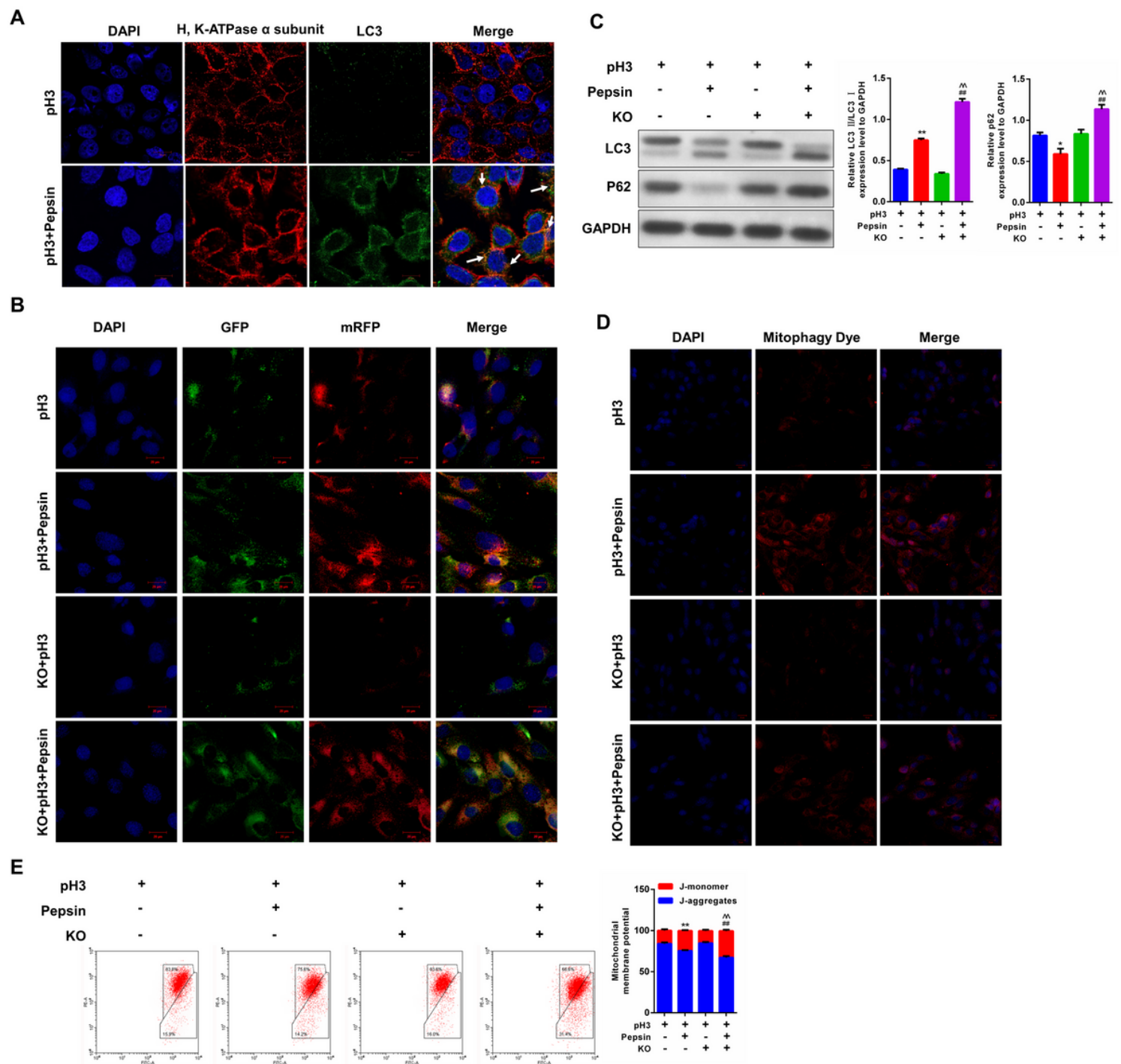


Figure 6

Effect of the H⁺/K⁺-ATPase α subunit on the acidified pepsin-mediated induction of mitophagy. (A) The levels of LC3 and H⁺/K⁺-ATPase α subunit in laryngeal cells treated with pepsin were determined by IF staining. Green, LC3-expressing cells; red, H⁺/K⁺-ATPase α subunit-expressing cells. (B) The H⁺/K⁺-ATPase α subunit was depleted in laryngeal epithelial cells using CRISPR/Cas9. Cells were transfected with GFP-mRFP-LC3 and then treated with acidic medium or acidified pepsin. The fluorescence intensities of GFP and mRFP were determined by IF. (C) LC3 and p62 protein levels in cells treated with acidic medium or acidified pepsin in the presence/absence of the H⁺/K⁺-ATPase α subunit were determined by

Western blotting. The LC3II/LC3I ratio and relative p62 levels are also shown. (D) Mitophagy induction was assessed by IF using a fluorescent mitophagy dye. (E) After treatment of wild-type or H+/K+-ATPase-KO cells with acidic medium or acidified pepsin, cells were stained for JC-I. The percentage of JC-I-positive cells was determined by flow cytometry. **, ##, and ^^ indicate significance at P<0.01.

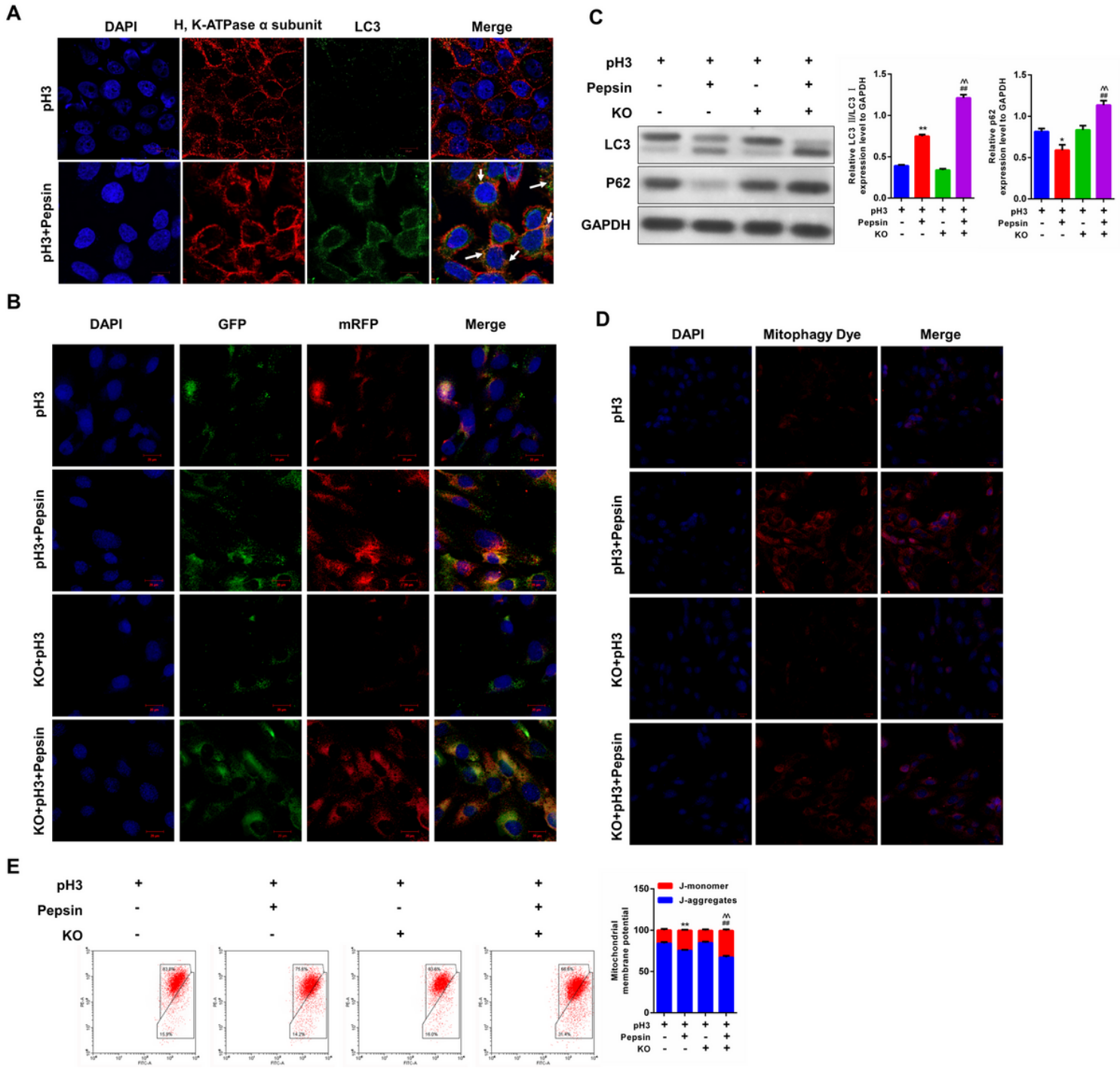


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Effect of the H+/K+-ATPase α subunit on the acidified pepsin-mediated induction of mitophagy. (A) The levels of LC3 and H+/K+-ATPase α subunit in laryngeal cells treated with pepsin were determined by IF staining. Green, LC3-expressing cells; red, H+/K+-ATPase α subunit-expressing cells. (B) The H+/K+-

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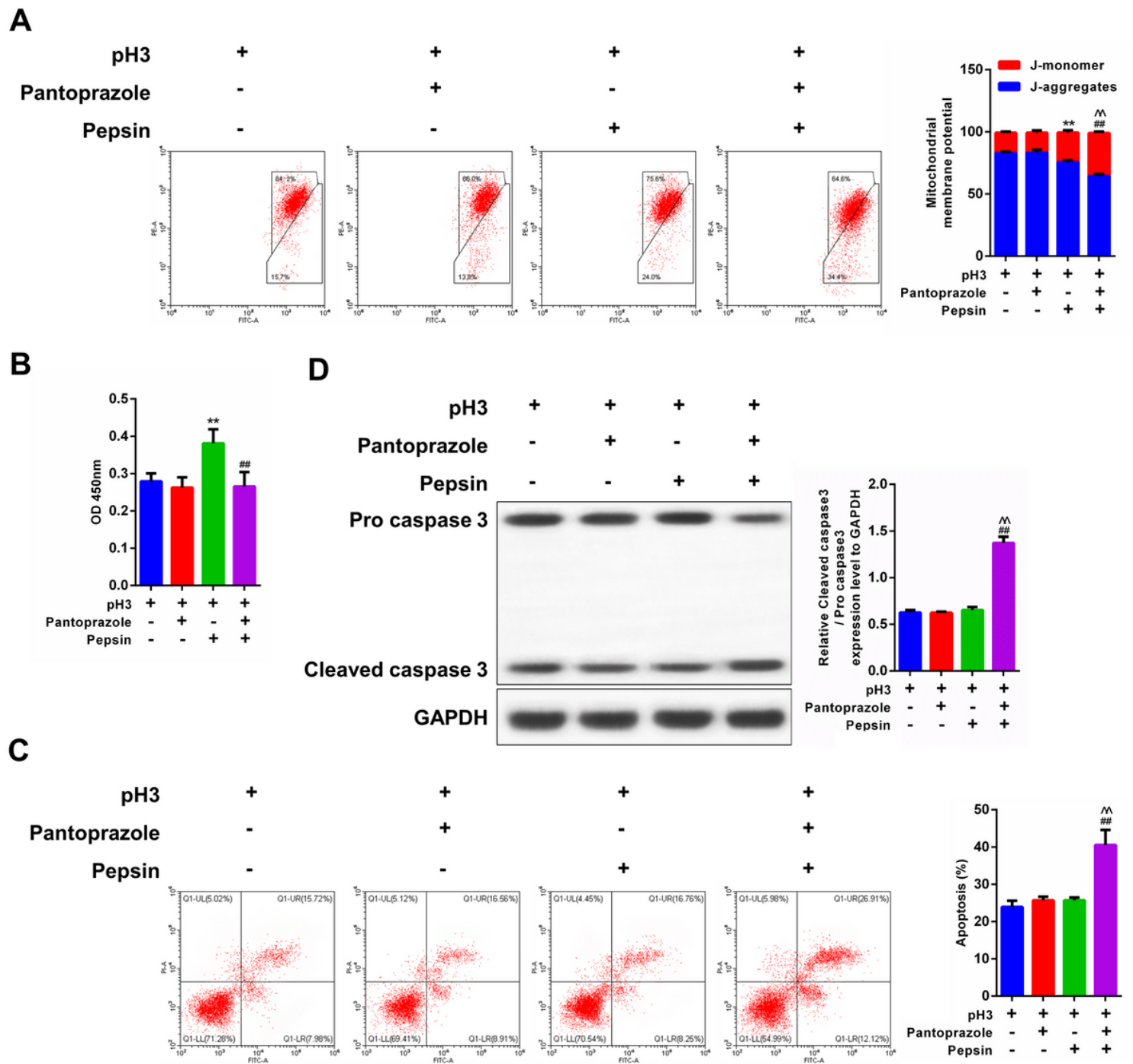


Figure 7

Effects of proton pump inhibition by pantoprazole on mitophagy induction and laryngeal epithelial cell growth in response to acidified pepsin. (A) Laryngeal epithelial cells were treated with acidic medium or acidified pepsin in the presence or absence of pantoprazole. The MMP level was determined by measuring the proportion of JC-1-positive cells using flow cytometry. (B) Cell proliferation was determined by CCK-8 assay. (C) Cell apoptosis was determined by flow cytometry. The right panel shows the relative numbers of apoptotic cells. (D) The level of cleaved caspase-3 was determined by Western blotting. The cleaved caspase-3 to procaspase-3 ratio is also shown. **, ##, and ^^ indicate significance at P<0.01.

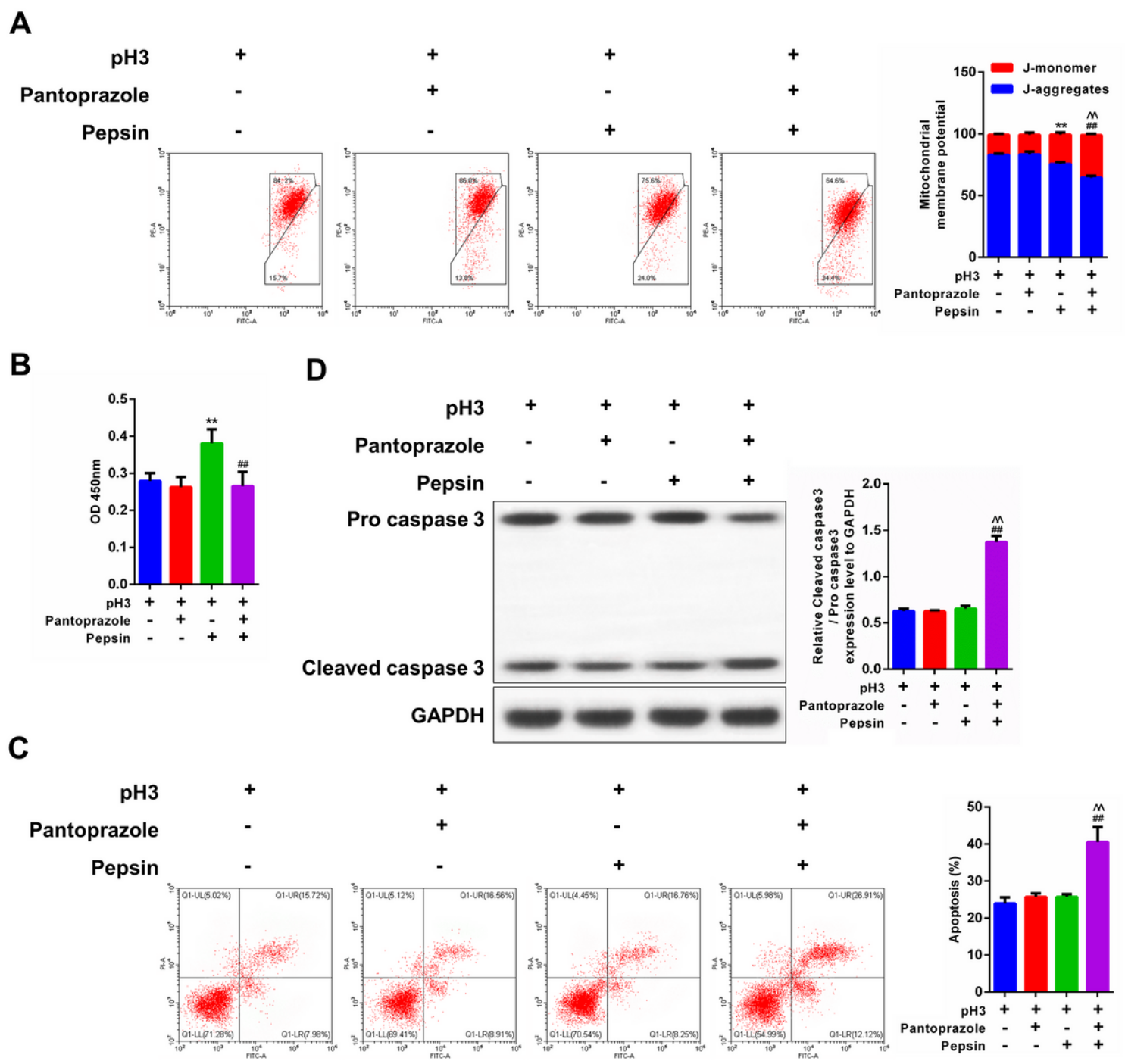


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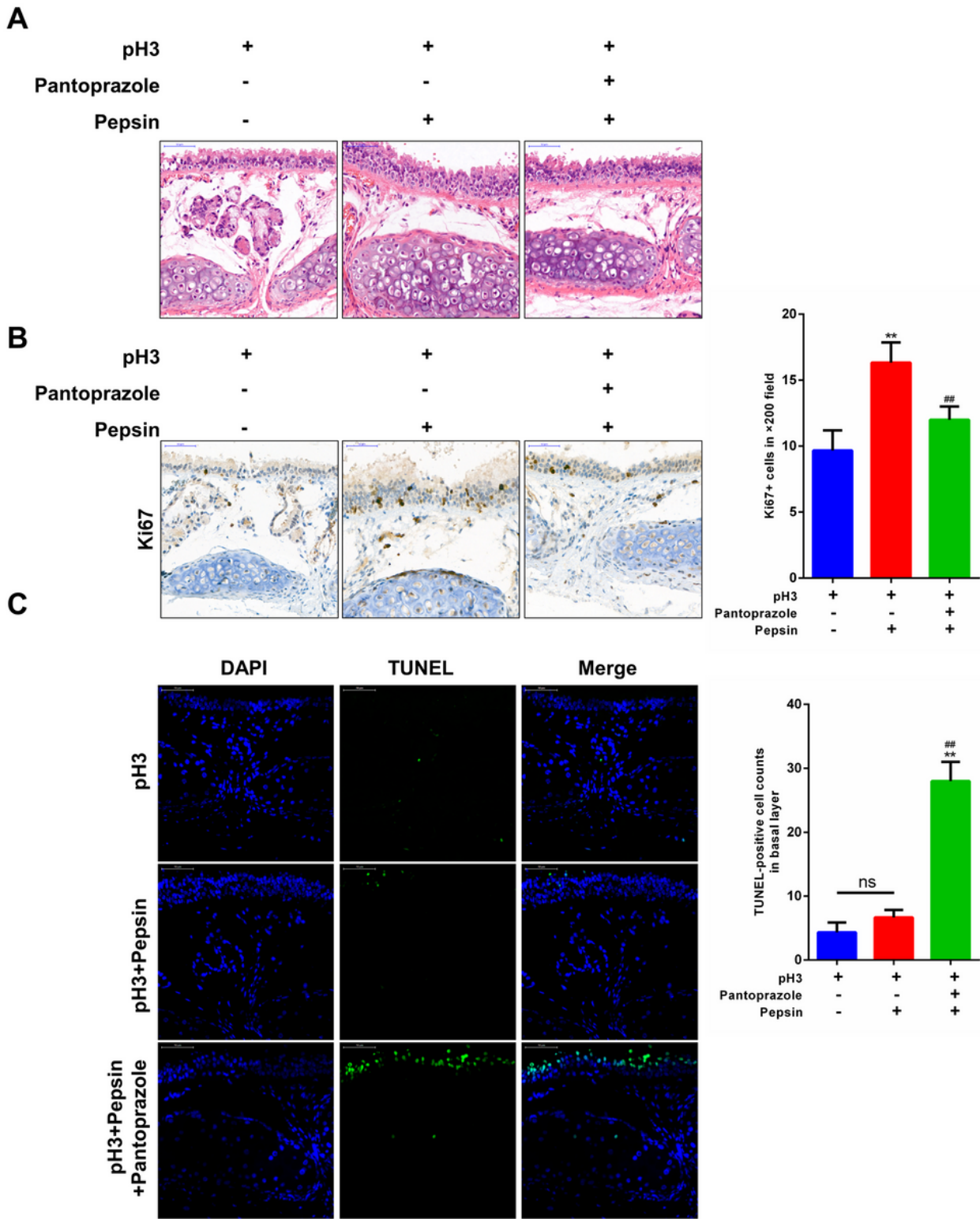


Figure 8

Effect of pantoprazole on acidified pepsin-induced cell proliferation in laryngeal mucosa in vivo. (A) An esophagus acid perfusion mouse model was established by perfusing 0.15 mL HCl (1 M, pH=3) with or without 2.5 mg/g pepsin and pantoprazole. Mice were divided into three groups: HCl (pH=3) treatment, acidified pepsin (pH=3) treatment, and acidified pepsin plus pantoprazole treatment; n=8 mice per group. The histological structure of laryngeal mucosal tissues was determined by H&E staining. (B) Ki67-positive cells were detected by IHC. The right panel shows the numbers of Ki67-positive cells in 200 representative fields of IHC images. (C) Apoptotic cells in laryngeal mucosal tissues were detected by TUNEL. The right panel shows the relative number of apoptotic cells. * indicates pepsin-treated versus control mice. # indicates the pepsin-treated versus the HCl-treated group. ** and ## indicate significance at $P < 0.01$; ns, not significant.

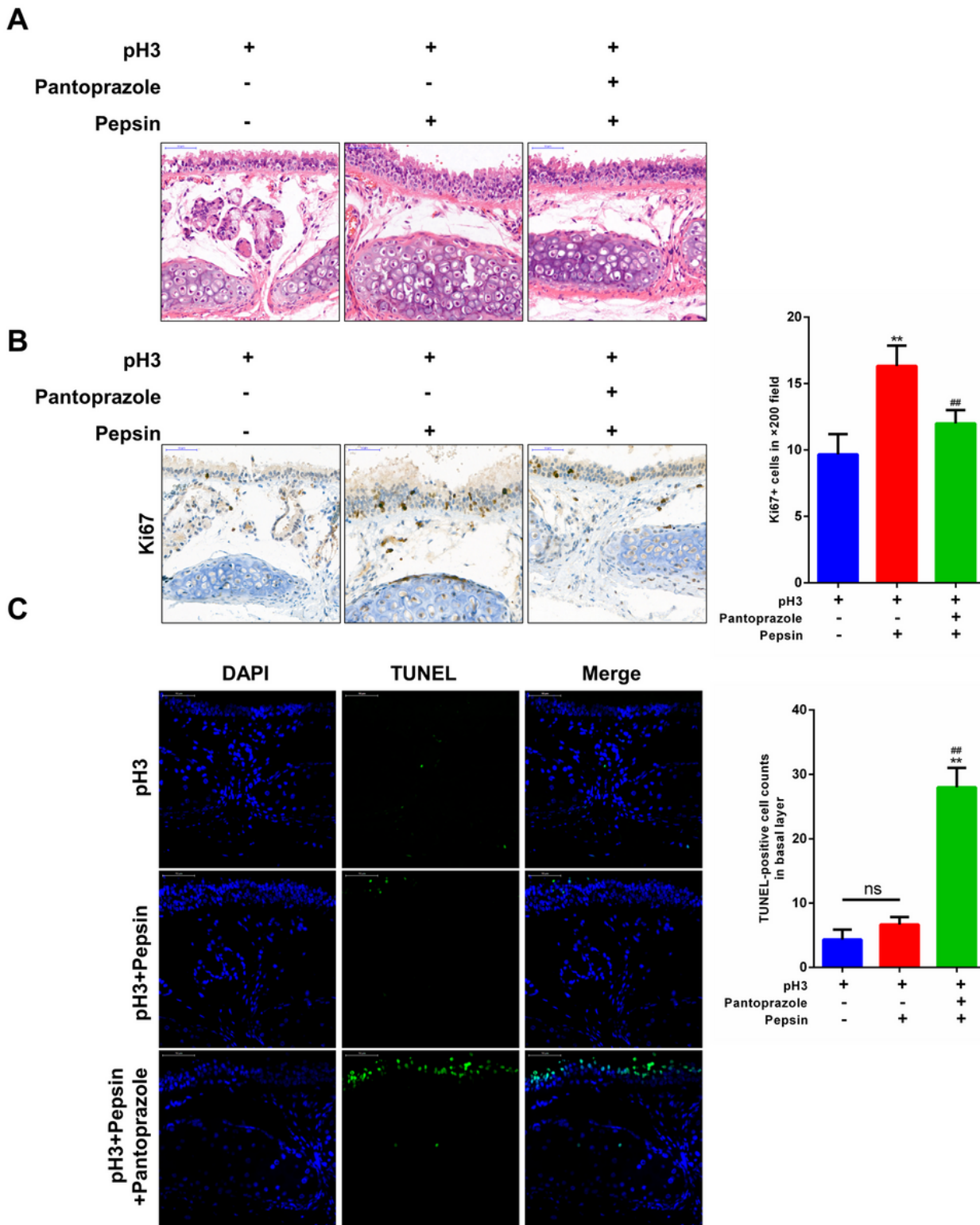


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