Abstract: Oral submucous fibrosis (OSF) is a serious, potentially malignant oral disorder. It is histopathologically characterized by chronic inflammation and atrophic epithelium accompanied by the accumulation of collagen fibers in the lamina propria. The molecular mechanisms leading to atrophic epithelium remain poorly understood. Therefore, the present study investigated the role of autophagy and apoptosis in atrophic epithelium in OSF. The expression of Caspase-3 and autophagy-related proteins (LC3 and P62) in OSF epithelial tissues was quantified by immunohistochemistry. The analysis demonstrated that, compared with normal oral mucosal tissues, autophagy and apoptosis increased with the progression of OSF. Flow cytometry and Western blotting showed that arecoline induces apoptosis in human oral keratinocytes (HOKs) in a time-dependent manner in vitro. Arecoline-induced autophagy was confirmed by transmission electron microscopy and Western blotting. When chloroquine was used as an inhibitor of autophagy, the apoptosis rate and Caspase-3 expression decreased compared with the use of arecoline alone. Thus, autophagy and apoptosis may be involved in atrophic epithelium in OSF, and arecoline-induced autophagy promotes apoptosis in HOKs.

Keywords: apoptosis, arecoline, autophagy, oral submucous fibrosis

Introduction

Oral submucous fibrosis (OSF) is a serious, potentially malignant oral disorder [1]. The malignant conversion rate of OSF in India was 7.6% in a 17-year longitudinal study [2]. The common signs and symptoms of OSF include a burning sensation, dry mouth, and progressive inability to open the mouth. OSF is histopathologically characterized by chronic inflammation and atrophic epithelium with the accumulation of collagen fibers in the lamina propria [3]. Although OSF is typically a disease of fibroblasts, epithelial changes could be important in these processes.

Epidemiological studies provide overwhelming evidence that areca nut chewing is a major etiological factor for OSF and carcinogenesis [4]. An areca extract-induced oral mucosa fibrosis rat model showed progressive changes in epithelial thickness (ET); this leads to atrophy and cytoskeletal infiltration into the subepithelial layer to promote fibroblast proliferation and collagen formation [5,6]. Recent studies have shown that arecoline, the main areca alkaloid, is cytotoxic to epithelial cells and fibroblasts. Arecoline-induced epithelial cell apoptosis could contribute to atrophic epithelium in OSF [7,8]. However, the exact mechanisms underlying arecoline-induced epithelial cell apoptosis remain poorly understood.

Autophagy is a process of self-digestion in which abnormal cytoplasmic macromolecules and organelles are encapsulated in autophagosomes of double-membrane structures [9]. Then, autophagosomes fuse with lysosomestom form autophagosomes, which degrade and recover their contents. Therefore, autophagy is essential for maintaining cellular metabolism and homeostasis in eukaryotic cells. Autophagy and apoptosis are two forms of programmed cell death, and many studies have shown a complex relationship between them [10]. Autophagy activation has been proposed to function as a cell survival mechanism under metabolic stress [11]. Conversely, excessive autophagy can promote apoptosis by actively or non-selectively degrading essential cellular components [12]. Oxidative stress has been implicated as a major contributor to the pathogenesis of arecoline-induced human keratinocyte apoptosis [13]. Multiple lines of evidence indicate that elevated reactive oxygen species or oxidative stress can trigger autophagy [14]. Thus, autophagy may be involved in the progression of arecoline-induced epithelial cell apoptosis.

To further study the possible mechanism underlying atrophic epithelium in OSF, the present study investigated the expression of autophagy and apoptosis marker proteins in OSF epithelial and normal oral mucosal tissues using immunohistochemistry. In addition, the influence of apoptosis and autophagy on human oral keratinocytes (HOKs) treated with arecoline and role of autophagy in arecoline-induced apoptosis were investigated.

Materials and Methods

Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of Xiangya Hospital, Central South University (IRB No.201609053), and informed consent obtained from participants.

Materials and reagents

Chloroquine (CQ) and methyl thiazolyl tetrazolium (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-P62, mouse anti-β-actin, and horseradish peroxidase (HRP) goat anti-rabbit IgG were purchased from Proteintech (Chicago, IL, USA). Fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco (New York, NY, USA). Further, arecoline was purchased from Abcam (Cambridge, MA, USA). Rabbit anti-LC3 and rabbit anti-cysteine-aspartic acid protease (Caspase)-3 were purchased from Cell Signal Technology (Danvers, MA, USA).

Tissues

This study included a total of 80 total samples: 20 normal oral mucosal tissue samples and 60 OSF epithelial tissue samples (including samples from 20 patients with early-, 20 with moderate-, and 20 with advanced-stage OSF). All 60 patients were clinically and histopathologically diagnosed with OSF and showed no comorbidity with other oral mucosal diseases. OSF samples were graded using the Fndborg and Sirsat histopathological classification system as well as by measuring the interincisal mouth opening (stage I, >3 cm; stage II, 2-3 cm; and stage III, <2 cm) according to the report by Haider et al. [15,16]. The normal oral mucosal tissue samples were obtained during surgical removal of third molars. All samples for this study were obtained from the Centre of Stomatology, Xiangya Hospital, Central South University (Changsha, P. R. China). The clinical characteristics of all samples are listed in Table 1.

Assessment of epithelial changes by hematoxylin and eosin (H&E) staining

Epithelial changes in H&E-stained sections were assessed based on ET following the method reported by Rajiv et al. [17]. Epithelial layers were counted in the thinnest and thickest areas of each section and averaged.

Immunohistochemistry

The collected tissue samples were fixed in 10% formalin, embedded in paraffin, and sectioned at 4-μm thickness. The sections were dewaxed with
xylene, rehydrated using gradient alcohol, and incubated in 3% H₂O₂ for 10 min. Subsequently, the sections were incubated with LC3B (1:50, ab51520, CST), P62 (1:50, 18420-1-AP, Proteintech), and Caspase-3 (1:50, ab4051, CST) primary antibodies overnight at 4°C, followed by incubation with goat anti-rabbit immunoglobulin secondary antibody for 30 min at 37°C. Then, the samples were stained with 3,3-diaminobenzidine, counterstained with Harris hematoxylin, mounted with Permount TM Mounting Medium, and observed under a microscope. PBS was used instead of the primary antibody as a negative control, and normal oral mucosal tissues were used as a positive control. The staining results were quantitatively evaluated using the Image-Pro-Plus 6.0 Image analysis software (Media Cybernetics, Rockville, MD, USA) to measure the mean optical density (MOD) of OSF epithelial tissues.

### Cell culture
HOKs (ATCC) were grown on Dulbecco’s modified Eagle’s medium (HyClone, Logan, UT, USA) containing 10% heat-inactivated certified FBS, 1,000 IU/mL penicillin, and 1,000 IU/mL streptomycin. Cells were cultured in a humidified incubator containing 5% CO₂ at 37°C.

### MTT assay
Cells were seeded in 96-well culture plates at a density of 1×10³ cells per well and exposed to various concentrations of arecoline for 24 h. Thereafter, 0.5 mg/mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well and incubated for 4 h. Then, 200 μL DMSO was added to each well to dissolve formazan crystals, and the absorbance was measured at 490 nm using a microplate reader (Bio-tek, Winooski, VT, USA).

### Transmission electron microscopy (TEM)
HOKs were treated with arecoline (15 μg/mL) for 0, 3, 6, 12, and 24 h. Cells were harvested by centrifugation and prefixed with 2.5% glutaraldehyde. After post fixing with 1% osmium tetroxide for 1 h and washing with PBS, cells were dehydrated in a graded series of ethanol and embedded in Epon 812. Ultrathin sections (60-70 nm) were cut with an ultramicrotome and post-stained with 1% uranyl acetate and lead citrate. Finally, the sections were observed and photographed using TEM.

### Western blotting
The harvested cells were washed twice with PBS, lysed on ice for 10 min with RIPA lysis buffer, and centrifuged at 12,000 rpm for 15 min. The supernatant was collected and protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific, MA, USA). Equal amounts of protein were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk for 1.5 h at room temperature and then incubated with primary antibodies against LC3B (1:1,000, CST), Caspase-3 (1:1,000, CST), P62 (1:1,000, Proteintech), and β-actin (1:5,000, Proteintech) overnight at 4°C. Then, the membranes were washed thrice with TBST and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Finally, protein bands were visualized using an enhanced chemiluminescence kit (Thermo Fisher Scientific). The relative quantity of proteins was analyzed using the Quantity One software, and β-actin was used as the loading control.

### Annexin V-FITC staining
HOKs were cultured in a medium containing 15 μg/mL arecoline for 0, 3, 6, 12, and 24 h or 10 μmol/L CQ for 2 h before treatment with 15 μg/mL arecoline for 24 h. The harvested cells were washed twice with ice-cold PBS and then stained with 5 μL annexin V-FITC (KeyGEN Biotech, Jiangsu, P. R. China) and 5 μL propidium iodide (KeyGEN Biotech). After incubation for 15 min in the dark at room temperature, the samples were analyzed by BD FACSCalibur flow cytometry.

### Statistical analysis
All data represent at least three independent experiments and are presented as mean ± standard error of the mean. Statistical differences were determined using one-way analysis of variance. Data analysis was performed using the SPSS 22.0 software (IBM, Armonk, NY, USA) and GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). P < 0.05 was considered statistically significant.

### Results
The ET of normal oral mucosal and OSF epithelial tissues was analyzed to assess changes in epithelial tissues (Table 2). The ET of OSF epithelial tissues significantly reduced compared with that of normal oral mucosal tissues. Across the three stages of OSF, the mean ET gradually decreased and reached the lowest level at the advanced stage. Epithelial dysplasia was not observed in OSF.

### Expression of LC3, P62, and Caspase-3 in normal oral mucosal and OSF epithelial tissues
H&E and immunohistochemical staining were performed on normal oral mucosal and OSF epithelial tissues. H&E staining showed that OSF epithelial tissues were characterized by collagen hyalinization, obliterated or narrowed blood vessels, and atrophic epithelium unlike normal oral mucosal tissues (Fig. 1A). Immunohistochemical staining showed that the positive expression of LC3, P62, and Caspase-3 was localized in the cytoplasm and nucleus in OSF epithelial tissues. In normal oral mucosal tissues, LC3 and Caspase-3-positive cells were mainly present in the spinous and granular layers and were minimally expressed in the basal layer. However, LC3 and Caspase-3 expression levels were elevated, and they were expressed in the basal layer in OSF epithelial tissues (Fig. 1B, D). P62-positive cells were found in the entire epithelial layer of the normal

### Table 1 Clinical characteristics of the samples included in the study
<table>
<thead>
<tr>
<th></th>
<th>NOM</th>
<th>OSF1</th>
<th>OSF2</th>
<th>OSF3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Areca nut chewing</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Intercinal mouth opening</td>
<td>&gt;4 cm</td>
<td>&gt;3 cm</td>
<td>2-3 cm</td>
<td>&lt;2 cm</td>
</tr>
<tr>
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<td>18</td>
<td>20</td>
</tr>
<tr>
<td></td>
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<td>6</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Age (years)</td>
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<td>20-60</td>
<td>20-60</td>
<td>20-60</td>
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<tr>
<td>Mean ± SD</td>
<td>37.1 ± 11.8</td>
<td>39.5 ± 15.1</td>
<td>45.3 ± 12.7</td>
<td>40.3 ± 11.7</td>
</tr>
</tbody>
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NOM, normal oral mucosa; OSF1, early stages of OSF; OSF2, moderate stages of OSF; OSF3, advanced stages of OSF.

### Table 2 Epithelial thickness in normal oral mucosal and OSF epithelial tissues
<table>
<thead>
<tr>
<th></th>
<th>NOM</th>
<th>OSF1</th>
<th>OSF2</th>
<th>OSF3</th>
</tr>
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<td>Number of samples</td>
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<td>20</td>
<td>20</td>
<td>20</td>
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<tr>
<td>Mean SD</td>
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<td>24.6</td>
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<td>P values</td>
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</table>

NOM, normal oral mucosa; OSF1, early stages of OSF; OSF2, moderate stages of OSF; OSF3, advanced stages of OSF; a Compared with normal oral mucosa.
oral mucosal tissues, whereas P62 expression was restricted to the basal layer in advanced-stage OSF epithelial tissues (Fig. 1C). Quantitative analysis of LC3, P62, and Caspase-3 expression shown in Fig. 1E-G. Compared with normal oral mucosal tissues, OSF epithelial tissues had increased LC3 expression and decreased P62 expression. Moreover, there was a significant difference between the average expression levels of LC3 and P62 at all OSF stages in OSF epithelial tissues (Fig. 1E, F). All results indicate that autophagy is activated in OSF, and greater autophagy activity occurs in advanced-stage OSF. LC3 expression levels were significantly higher in OSF epithelial tissues than in normal oral mucosal tissues, and they further increased with the progression of OSF (Fig. 1G). LC3, P62, and Caspase-3 expression levels were not significantly different between normal oral mucosal and OSF epithelial tissues in the early stages of OSF.

Relationship among the expression of LC3, P62, and Caspase-3 in normal oral mucosal and OSF epithelial tissues

The relationship among the expression of LC3, P62, and Caspase-3 was determined using two-tailed Pearson’s correlation between normal oral mucosal and OSF epithelial tissues. Linear regression lines were fit to analyze the predictive relationships of the immunohistochemical staining cores. Caspase-3 expression was positively correlated with LC3 expression \( (P < 0.001, r = 0.6036, n = 80) \) and negatively correlated with P62 expression \( (P < 0.001, r = -0.4601, n = 80) \) (Fig. 1H). Moreover, LC3 expression was negatively correlated with P62 expression \( (P < 0.001, r = -0.4395, n = 80) \) (Fig. 1I). These results indicate that autophagy and apoptosis play important roles in OSF epithelial tissues.

Effects of arecoline on the cell viability of HOKs

The ability of arecoline to reduce the cell viability of HOKs was detected using MTT assay. Cells were treated with different concentrations of arecoline (0, 7.5, 15, 30, 60, and 120 μg/mL) for 24 h. As shown in Fig. 2A, arecoline decreased the cell viability of HOKs in a concentration-dependent manner. Because cells treated with 15 μg/mL arecoline showed approximately 50% inhibition, this concentration was used for further experiments.

Arecoline induced apoptosis in a time-dependent manner

To investigate the influence of arecoline on apoptosis, cell apoptosis was assessed using annexin V-FITC/PI staining assay after treatment with 15 μg/mL arecoline for 0, 3, 6, 12, and 24 h. Flow cytometry showed that arecoline induced apoptosis in HOKs in a time-dependent manner (Fig. 2B). Next, the protein expression of the apoptosis executioner Caspase-3 was analyzed using Western blotting. Consistent with annexin V-FITC staining results, arecoline increased Caspase-3 expression and cleaved it in a time-dependent manner (Fig. 2C).

Arecoline induced autophagy in a time-dependent manner

The present results demonstrate autophagy in OSF epithelial tissues. To investigate whether arecoline can induce autophagy in HOKs, TEM was used to observe the presence of autophagic vacuoles (Fig. 3A). HOKs treated with arecoline for 6, 12, or 24 h demonstrated a greater number of autophagic vacuoles than HOKs treated with arecoline for 0 or 3 h. LC3 is the most widely used autophagy marker in current research. Western blotting showed that the LC3-II/LC3-I ratio increased significantly in a time-dependent manner (Fig. 3B). Similarly, P62 (an autophagy marker) expression increased in a time-dependent manner (Fig. 3C). These results suggest that arecoline induces autophagy in HOKs and P62 was not completely degraded and accumulates moderately in cells.

Arecoline-induced autophagy promotes the action of apoptosis

To evaluate whether autophagy affects arecoline-induced apoptosis, HOKs were pretreated with CQ before treatment with arecoline. Western blotting showed that CQ inhibited the LC3-I to LC3-II conversion and increased P62 expression (Fig. 4A). These results indicate that CQ can inhibit arecoline-induced autophagy. Moreover, the expression of Caspase-3 and cleaved Caspase-3 decreased following co-treatment with CQ and arecoline compared with that following treatment with arecoline alone.
Therefore, the study of the response of HOKs to arecoline could provide insights into the mechanisms of epithelial changes before damaged keratinocytes release a variety of cytokines that result in fibroblast proliferation and accumulation of collagen fibers in the lamina propria in OSF. Connective tissue fibrosis and increased vascularity lead to epithelial ischemia and hypoxia, further promoting the action of apoptosis.

**Discussion**

The outermost layer of the oral mucosa is a protective barrier composed of epithelial tissues. Long-term areca nut chewing stimulation first causes epithelial changes before damaged keratinocytes release a variety of cytokines that result in fibroblast proliferation and accumulation of collagen fibers in the lamina propria in OSF. Connective tissue fibrosis and decreased vascularity lead to epithelial ischemia and hypoxia, further aggravating atrophic epithelium and thereby forming a vicious cycle [18-20]. The present study supports the findings that OSF epithelial tissues are atrophic, which is more pronounced in the advanced stages of OSF. Therefore, the study of the response of HOKs to arecoline could provide a deeper understanding of OSF pathogenesis. The present study used in vitro methods to demonstrate the effects of arecoline and confirmed the mechanism of pathogenesis in OSF epithelial tissues.

The Caspase family plays a crucial role in apoptosis, and Caspase-3 acts as the key executioner [21]. In the present study, Caspase-3 expression in OSF epithelial tissues was significantly higher than that in normal oral mucosal tissues, and its upregulation was greater in the presence of advanced-stage OSF pathology. Arecoline-induced apoptosis was also investigated in HOKs. Flow cytometry and Caspase-3 expression measured by Western blotting showed that arecoline induced apoptosis in HOKs in a time-dependent manner. Cheng et al. also demonstrated that arecoline induces apoptosis by Caspase-3 activation and decreased the expression of anti-apoptotic proteins such as Bcl-2 and Bcl-XL [22]. Besides inducing apoptosis, arecoline also disrupts the cell cycle. Previous research has shown that arecoline inhibits the proliferation of epithelial cells by arresting cells in S and G2/M phases, thereby damaging the repair process [23]. These data suggest that increased epithelial apoptosis and inhibition of cell proliferation is a potential pathogenesis of atrophic epithelium and that this process is associated with arecoline cytotoxicity.

Autophagy is a lysosome-dependent degradation process that widely exists in eukaryotic cells and causes the digestion of damaged proteins and organelles and recycling [24]. Recent studies have demonstrated that autophagy plays an important role in OSF, and the inhibition of autophagy promotes apoptosis of fibroblasts while reducing collagen fiber synthesis [25]. However, the role of autophagy in OSF epithelial tissues has not been well understood. LC3 is an autophagy marker protein that is a mammalian autophagosomal homologue of Atg8 in yeast [26]. After autophagy activation, ubiquitinated LC3-I is covalently linked to phosphatidylethanolamine to form LC3-II on the autophagosome membrane [27]. P62 is also an autophagy marker protein. During autophagy, P62 is associated with different ubiquitin-tagged cargos and is degraded in autophagosome/lysosome fusion [28]. The present results confirmed that LC3 expression increased and P62 expression decreased in OSF epithelial tissues compared with their expressions in normal oral mucosal tissues. These changes were significantly correlated with the pathological stage of OSF.

To further examine whether arecoline induced autophagy in HOKs, TEM was used to investigate autophagic vacuole structures. LC3 and P62 expression was quantified to further confirm arecoline-induced autophagy. The results showed that the LC3-II/LC3-I ratio increased significantly in a time-dependent manner, suggesting that arecoline induced autophagy in HOKs. However, P62 expression was upregulated after treatment with arecoline for 24 h. P62 is not completely degraded and accumulates moderately in cells, potentially impairing proteasome activity [29].

Autophagy and apoptosis are important for maintaining cell metabolism and homeostasis, but there is a complex relationship between them [30]. Du et al. indicated that TGF-β inhibits an autophagic response in renal tubular epithelial cells (RTECs), which activates the TGF-β/Smad4 signaling pathway. Enhanced autophagy activation contributes to the proliferation, migration, and anti-apoptosis of RTECs in renal fibrosis [31]. Interestingly, another study revealed that cigarette smoke extract transiently induces autophagy activation in bronchial epithelial cells followed by the accumulation of P62 and ubiquitinated proteins, resulting in increased cell death [32]. Because autophagy acts as a double-edged sword in apoptosis, the relationship between autophagy and apoptosis was explored in HOKs treated with arecoline. CQ was used as an inhibitor of arecoline-induced autophagy to investigate the correlation between autophagy and apoptosis. CQ effectively inhibited arecoline-induced autophagy in HOKs. Moreover, autophagy inhibition reduced arecoline-stimulated apoptosis and decreased the expression of cleaved Caspase-3. These results suggest that autophagy has a pro-apoptosis effect on HOKs treated with arecoline. The anti-apoptotic protein Bcl-2 can inhibit autophagy by binding to Beclin-1, which is a crucial regulator of autophagy [33]. It was speculated that Bcl-2 expression is reduced in OSF epithelial tissues and autophagy is induced by the release of Beclin-1 from Bcl-2, resulting in increased apoptosis. The specific mechanism underlying apoptotic induction will be elucidated in further studies. The present results also demonstrated that autophagy activity in OSF epithelial tissues was significantly higher than that in normal...
oral mucosal tissues. Further, LC3 was mainly expressed in the epithelial spinous and granular layers at the early stage of OSF, which may be related to the cells of the outer layer being more susceptible to areca nut chewing stimulation. In addition, increased autophagy promotes the apoptosis of outer epithelial cells, which allows areca nut to penetrate the atrophic epithelium enter basal cells more easily. The basal layer may be mutated under the long-term stimulation of arecoline to form atypical hyperplasia and carcinogenesis.

In conclusion, the present study revealed that arecoline induces apoptosis and autophagy in HOKs in vivo and in vitro. Furthermore, these results suggest that arecoline-induced autophagy promotes apoptosis in HOKs, but the exact mechanism remains to be elucidated.

Acknowledgments
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Conflict of interest
There is no conflict of interest.

References