Development of a mouse model of arecoline-induced oral mucosal fibrosis

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Objective: To develop a BALB/c mouse model of oral submucous fibrosis (OSF) induced by arecoline and to exhibit an accumulation of collagen and angiogenesis changes.

Methods: BALB/c mice were randomly assigned to either the control (distilled water) or experimental group (arecoline) (n = 40). Eight mice from each group were sacrificed every 4 weeks since 8 weeks post treatment. Changes in histopathologic features, levels of collagen type I and collagen type III, and angiogenesis were measured.

Results: In the 8th week, epithelium atrophy, collagen cumulation and micrangium pathologic changes in the lamina propria were observed in the oral mucosa. In the 20th week, hyaline degeneration of the connective tissues was observed on the tongue and palate mucosa. The angiogenesis and collagen type I changed significantly as the diseases advanced (P < 0.05); however, collagen type III was not statistically different.

Conclusions: An OSF model involving mice can be rapidly induced by drinking a high-dose of arecoline. OSF angiogenic changes in mice primarily decrease and collagen accumulation is mainly collagen type I.

1. Introduction

Oral mucosal fibrosis (OSF) is a potentially malignant mucosal disorder, which is chronic, insidious and progressive in nature. The main clinical symptoms of OSF include a pale and stiff mucosa, limitation of mouth opening and the inability to eat spicy food. The most prominent pathological feature of OSF is the over-accumulation of collagen in the oral mucosa lamina propria [1]. Therefore, OSF is considered to be a topical collagen metabolism disorder. The pathogenesis of OSF remains unclear and most of the drug therapies or other systemic medical methods that have been attempted over the past 30 years are associated with poor efficacy. Therefore, the lack of an ideal animal model limits the pathogenesis and clinical treatment of OSF.

Although some animal models have been developed for OSF in the past, a simple and reliable animal model that can be used for therapeutic development is unsatisfactory. In 1960, Sirsat et al. used betel nut and pan masala solutions injected into the buccal mucosa of Sprague–Dawley rats over a period of 48...
weeks [5]. However, this method was time-consuming, expensive and technically difficult to cause its application to be limited. In 2016, Zhang et al. injected bleomycin into the buccal mucosa of Sprague–Dawley rats for 8 weeks, which successfully induced OSF [6]; however, the active ingredients of bleomycin and areca nut were not the same and it was difficult to simulate the clinical process of OSF. Moreover, all established animal models of OSF were lesions induced at a single site.

Areca nut has been identified in four main types of alkaloids: arecoline, arecaidine, guvacine and guvacoline, of which arecoline plays the most important role in the induction of OSF [7]. Therefore, we attempted to feed BALB/c mice with high concentrations of arecoline to develop a murine model with features characteristic of OSF. Moreover, detection indicators of angiogenesis and collagen were determined through immunohistochemical staining and quantitative PCR. Based on the controversies associated with collagen and vascular changes, we discussed their place in the occurrence and development of OSF.

2. Materials and methods

2.1. Ethical permission

Ethical permission for undertaking this study was granted by the Committee of the Guangxi Medical University Laboratory Animal Centre (No. 201511031).

2.2. Animals and reagents

Eight male BALB/c mice (6 weeks old) were obtained from the Experimental Animal Centre of Guangxi Medical University. Mice were treated according to the guidelines for the use of animal protection in Guangxi Medical University. The animals were housed under controlled conditions, including a 24-h alternate light and a room with a relative humidity level of 30%–50%. The mice were fed with standard mouse fodder. Arecoline (Sigma, USA) was diluted to a concentration of 1000 mg/L in the drinking water for the experimental group (we separately used 250, 500, 1000 and 2000 mg/L arecoline in the preliminary experiments) and distilled water for the control group. The mice were allowed to drink water, which was replaced once a week and the chow diet ad libitum at all times.

2.3. Experimental protocol

A total of 80 mice were randomly selected and divided into control group and experimental group, each consisting of 40 animals. The experimental group was treated for 20 weeks and the control group was treated with distilled water over the same period. According to the duration of treatment, these groups were then further divided into subgroups of 10 animals per group, namely, 8, 12, 16, or 20 weeks and eight mice of each subgroup were sacrificed.

2.4. Experimental observation

Mice vitality was observed daily during the course of experiment and the water intake, food consumption and body weight changes were recorded weekly. Mouth opening was measured every 2 weeks. Before the mice were sacrificed by administering sodium pentobarbital anesthesia of intraperitoneal injection, changes of the oral mucosa were detected.

2.5. Pathological examination

Oral mucosal lesion specimens (containing partial deep muscle tissue) were dissected. A portion of the tissue sample was fixed in 10% formalin, embedded in paraffin, sectioned into 4-μm sections and stained with haematoxylin and eosin (HE). Van Gieson was used to stain the collagen fibres and Masson's trichrome was used to stain for newly synthesized collagen. The classification and pathological diagnosis of OSF was made according to the HE, Van-Gieson and Masson staining [4,6]. The digestive tract, heart, lung, liver and other organs were also collected and stained with HE. The remaining tissue was stored in RNA later for gene expression studies.

2.6. Immunohistochemistry and scoring

Paraffin sections were subjected to immunohistochemical staining. Peroxidase activity was quenched by treatment with 0.2% H2O2 for 3 h. The sections were incubated with monoclonal CD34 (0.004 mg/mL, ZSGB-Bio, China), overnight at 4 °C. In addition, 0.01 mol/L PBS and normal mouse oral mucosal mucous tissue sections were used as negative and positive controls, respectively. The immunostaining was visualised with an SP kit (ZSGB-Bio, China) using a peroxidase and dianinobenzidine substrate. The sections were counterstained (excluding CD34) with haematoxylin.

The calculation method of angiogenesis was based on immunohistochemically stained by CD34 [8]. To assess CD34 expression in OSF, all morphological structures with cavities surrounded by CD34-positive endothelial cells were considered microvessels. Vascular endothelial cells were stained brown (cytoplasmic expression), alone or in clusters that were distinctly separate from adjacent microvessels. Vessels of the muscular layer were not counted. The highest density of staining was measured by an preliminary scan at 100× magnification in the OSF tissue and three areas were randomly selected under high power (400×). The number of blood vessels positive for CD34 expression was evaluated by two independent observers. To evaluate the immunohistochemical expression of CD34, the mean of the three fields was taken as the final score.

2.7. Quantitative real time PCR

The mouse tissues were weighed and pulverized into powder in liquid nitrogen and then transferred to a homogenization tube. The homogenized tissue was processed for RNA isolation. Reverse transcription of 2 μg of total RNA was performed using a high-capacity cDNA synthesis kit (Takara, Japan). PCR reactions were performed in conjunction with a gene-specific primer pair. The normalizing gene was glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The product size and primer sequences are as follows: collagen type I (Col I, 148 bp) 5'-CAC TGC CCT CCT GAC GCA TGG-3' (forward) and 5'-CAC GATC ATC GCA CAC AGC CG-3' (reverse); collagen type III (Col III, 173 bp) 5'-CAG GCC AGT GGC AAT GTA AAG A-3' (forward) and 5'-CTC ATT GCC TGT CTT TGA TA-3' (reverse); G3PDH (233 bp) 5'-ACC ACA GTC CAT GCC ATC AC-3' (forward) and 5'-TCC ACC ACC CTG TTG CTG TA-3' (reverse).
The reaction was performed in a 7500 Real-Time PCR System (Life Technologies) using SYBR Premix Ex Taq (Takara, Japan) according to the manufacturer’s protocol. All calculated concentrations of the target genes were divided by the amount of the endogenous reference G3PDH to obtain the normalized \( \text{Col I} \) and \( \text{Col III} \) expression values. The reactions were performed in triplicate for each sample. All of the primers used in the study provided a single peak in the dissociation curve, suggesting that there is a single amplicon.

2.8. Scoring and statistical analysis

SPSS version 17.0 was used for the statistical analyses. The data are presented as the mean ± SEM. The evaluation of statistical significance was determined by a one-way analysis of variance (ANOVA) followed by multiple comparison tests. For all tests, a \( P \)-value of less than 0.05 was considered to be significant.

3. Results

3.1. General observations

The experimental group exhibited weight loss at the beginning of the experiment. Compared with the control group, the experimental group exhibited a dim coat colour, activity decline and lack of appetite during the late stage of the experiment. Three mice died in the experimental group in the 5th, 11th and 17th weeks, respectively. No mice were found dead in the control group during the experiment.

3.2. Macroscopic observations

The oral mucosa of the experimental group gradually became pale and rigid from 8 to 20 weeks. Although all mice demonstrated the above changes to varying degrees, a fibrous band was not discovered. No obvious change was found in the mice of the control group. The mouth opening of the experimental group gradually decreased over time compared to that of the control group. The mean mouth opening of the experimental group was significantly lower than the control group from 16 weeks onward (\( P < 0.05 \); Table 1).

3.3. Pathological examination

3.3.1. HE staining

The tongue tissue of the experimental group began to exhibit the early stages of OSF lesions in the 8th week and the buccal and palatal tissue showed early OSF lesions in the 12th weeks. In the 8th week, the tongue mucosa of the experimental group began to show orthokeratosis and atrophy of keratosis layer in the epithelial layer. Lymphocytic infiltration and thickening of the lamina propria were observed. A significant increase in the number of vessels, vascular congestion and diameter changes were observed in lamina propria (Figure 1A and E). With prolonged treatment, the atrophic epithelium lost its rete pegs and the lamina propria became thicker. The number of inflammatory cells was reduced in the lamina propria. The blood vessels revealed a gradual progression from hyperaemia to atrophy from 12 to 16 weeks (Figure 1B, C, F and G). In the 20th week, age-related changes in the control group were observed, the number of inflammatory cells increased and the lamina propria became thicker; however, the blood vessels did not change significantly. The collagen was compact and disorderly in the lamina propria, the number of cells was significantly reduced and local hyalinization was apparent in the experimental group. Blood vessels were obliterated in the lamina propria and muscular atrophy was visible in most cases (Figure 1D and H). Buccal and palate mucosa displayed pathological changes similar to the tongue mucosa.

Liver and lung damage occurred during the later period. Liver sinusoidal, central vein and interlobular pathological congestion was identified. Congestion of the blood vessels contained a large number of red blood cells. Liver cell shrinkage and steatosis of the surrounding hepatic lobules was observed (Figure 2A and C). The lungs showed signs of alveolar wall telangiectasia congestion, small number of alveolar red blood cells, and partial alveolar expansion indicated a state of hyperventilation (Figure 2B and D). The remaining organs (i.e., heart, oesophagus, large intestine, small intestine) did not show any distinct abnormalities.

3.3.2. Van Gieson and Masson staining

Masson staining revealed that the distribution of collagen was stained in blue and purple. The tongue mucosa of the control mice displayed a small amount of blue-violet stained collagen fibres in the lamina propria. The collagen fibres were loosely and regularly arranged and many elongated fibres extended to the papillary epithelial (Figure 3A and F). The terminal branch of the elongated fibres was woven into the elastic web beneath the basal epithelial cells. The tongue mucosa of the control mice displayed thick and dense collagen fibres that were stained blue, indicating thickening of the lamina propria. The collagen fibres that were close to the subepithelial layer exhibited a dense and bright blue collagen fibrous area as time increased (12, 16 and 20 weeks). In comparison to the control group, collagen fibres increased significantly and the staining also became deeper (Figure 3B–E). The Van Gieson’s stain showed densely-deposited collagen in bright red, which corresponded to the blue areas of the Masson stain. The photomicrographs showed some areas stained in red comparison with the associated control (Figure 3G–J). The buccal and palate mucosa exhibited pathological changes similar to the tongue mucosa.

3.3.3. Quantitative results of angiogenesis

CD34 is specifically expressed in vascular endothelial cells and is well-established for testing angiogenesis changes in the

<table>
<thead>
<tr>
<th>Observation period</th>
<th>Mouth opening (mm)</th>
<th>( P ) Values</th>
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</thead>
<tbody>
<tr>
<td>Control group</td>
<td>Experimental group</td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>10.6 ± 0.4</td>
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<tr>
<td>2 weeks</td>
<td>10.8 ± 0.9</td>
<td>11.2 ± 1.0</td>
</tr>
<tr>
<td>4 weeks</td>
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<td>6 weeks</td>
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<td>11.7 ± 1.1</td>
</tr>
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<td>8 weeks</td>
<td>12.3 ± 0.7</td>
<td>12.4 ± 1.2</td>
</tr>
<tr>
<td>10 weeks</td>
<td>12.8 ± 1.1</td>
<td>11.9 ± 0.8</td>
</tr>
<tr>
<td>12 weeks</td>
<td>13.2 ± 0.7</td>
<td>11.4 ± 0.5</td>
</tr>
<tr>
<td>14 weeks</td>
<td>13.7 ± 1.1</td>
<td>10.7 ± 0.6</td>
</tr>
<tr>
<td>16 weeks</td>
<td>14.1 ± 0.3</td>
<td>9.4 ± 1.1</td>
</tr>
<tr>
<td>18 weeks</td>
<td>13.9 ± 1.0</td>
<td>9.8 ± 0.8</td>
</tr>
<tr>
<td>20 weeks</td>
<td>14.6 ± 0.5</td>
<td>8.2 ± 1.2</td>
</tr>
</tbody>
</table>

\( P < 0.05. \)
Figure 1. Histopathological changes in mice of the tongue mucosa in different observation periods (HE × 200).
In the 8th week, compared with the control group (A), the experimental group (E) showed atrophic and thin epithelium, orthokeratosis, shallowed rete pegs, changed vascular caliber (black arrow), congestion, lymphocytic infiltration, lamina propria light degree of thickening (red arrow), mild changes in collagen and dispersion extending downward to muscle layer, and unobvious abnormalities root of tongue glands (yellow arrow). In the 12th week, compared with the control group (B), the experimental group (F) showed orthokeratosis are aggravated in epithelial, and lamina propria greatly thickened, and collagen edema, blood vessels surrounded by fibre. In the 16th week, compared with the control group (C), the experimental group (G) showed decreased number of vessels, shrinking diameter, mild hyaline change (red arrow), disordered collagen and reducing cellularity. In the 20th week, the control group (D) showed the age change, thickened lamina propria and increased fibroblasts (red arrow), but the blood vessels did not change remarkably (black arrow). The experimental group (H) showed vascular occlusion disappearance, dense collagen depositioned in connective tissue, and clear hyaline change (red arrow), and accumulation of collagen in the layers begins to invade the muscle.

Figure 2. Histopathological changes of the liver and lung in mice (HE × 200).
The liver of experimental group in the 16th week (A) showed that hepatic congestion, liver cell mild shrinkage and no significant hepatic steatosis. The liver in the 20th week (C) showed sinusoids, dilated central veins and interlobular veins, congestion, a large number of red blood cells, liver cell shrinkage, and a small amount of liver cell steatosis surrounding lobular. The lungs of experimental group in the 16th week (B) showed alveolar wall telangiectasia congestion and alveolar cavity filled with red blood cells. The lungs in the 20th week (D) showed part of alveolar cavity expansion and alveolar expansion, which are the state of hyperventilation.
Compared with mice of the control group, the tongue mucosa of the mice in the experimental group showed an increased number of blood vessels since 8 weeks and the vessel number was significantly different between the two groups ($P < 0.05$). However, with prolonged administration, the lesions gradually increased and the number of blood vessels in the experimental group began to decline in the 16th week. Compared with the control group, the blood vessels in the experimental group showed a significant reduction in the 20th week ($P < 0.05$; Figure 4; Table 2).

3.3.4. Expression of Col I and Col III

The mRNA expression of Col I and Col III in the lamina propria at different times was detected by quantitative PCR. The mRNA expression of Col I in the tongue mucosa was significantly higher than that in the control group by the 8th week and the mRNA expression increased with the severity of the lesion ($P < 0.05$). Although the immunohistochemical staining

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<table>
<thead>
<tr>
<th>Observation period</th>
<th>Position</th>
<th>Control group</th>
<th>Experimental group</th>
<th>$P$ Values</th>
</tr>
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<tbody>
<tr>
<td>8 weeks</td>
<td>Tongue</td>
<td>8.2 ± 1.2</td>
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<td></td>
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<td>7.3 ± 0.6</td>
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<tr>
<td></td>
<td>Palate</td>
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<td>6.8 ± 0.5</td>
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<tr>
<td>12 weeks</td>
<td>Tongue</td>
<td>9.3 ± 0.7</td>
<td>13.5 ± 0.6</td>
<td>0.034*</td>
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<tr>
<td></td>
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<td>7.3 ± 0.6</td>
<td>0.894</td>
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<tr>
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<td>7.4 ± 0.6</td>
<td>8.9 ± 0.9</td>
<td>0.003*</td>
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<tr>
<td>16 weeks</td>
<td>Tongue</td>
<td>8.5 ± 0.2</td>
<td>11.6 ± 0.2</td>
<td>0.099</td>
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<tr>
<td></td>
<td>Buccal</td>
<td>7.7 ± 0.8</td>
<td>9.6 ± 0.4</td>
<td>0.036*</td>
</tr>
<tr>
<td></td>
<td>Palate</td>
<td>6.4 ± 0.4</td>
<td>5.4 ± 1.4</td>
<td>0.078</td>
</tr>
<tr>
<td>20 weeks</td>
<td>Tongue</td>
<td>8.9 ± 0.4</td>
<td>5.4 ± 0.6</td>
<td>0.013*</td>
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<tr>
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<td>Buccal</td>
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<td>8.8 ± 0.2</td>
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<tr>
<td></td>
<td>Palate</td>
<td>6.3 ± 0.4</td>
<td>3.6 ± 0.8</td>
<td>0.000*</td>
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</tbody>
</table>

$*P < 0.05.$
revealed a slight increase of Col III in the early lesions, the mRNA expression of Col III was not significantly correlated with the degree of the lesion. Moreover, there was no significant change compared with the control group. The mRNA expression of Col I and Col III in the buccal and palate mucosa was similar to that of the tongue mucosa (Figure 5).

4. Discussion

In this study, we observed the early appearance of OSF-like changes in the tongue mucosa in the 8th week through the induction of a high concentration of arecoline in the drinking water. Such OSF-like changes manifested as the gradual flattening and eventual disappearance of the mucosal epithelium rete pegs, epithelial layer atrophy and inflammatory cell infiltration in the lamina propria and submucosa. Over time, there was a large amount of collagen deposition and reduced number of blood vessels. These pathological changes are consistent with the clinical characteristics of human OSF and pathological stage is close to that exhibited by human OSF lesions. In addition, the buccal and palate mucosa also began to display OSF-like changes in the 12th week. After 20 weeks post induction, hyaline changes were observed in the tongue and palate mucosa, indicating moderately advanced OSF. Thus, this model of OSF shortened the time required to induce OSF symptoms and the disturbance caused by age-related changes was excluded. The model also has the advantage of a simple method of induction, the administration of a single dose and control, as well as drug action on the entire mouth mucosa. Traditionally, the physical and chemical stimuli (e.g., betel coarse fibre stabbed, stuffed with lime) in the process of betel nut chewing are factors involved in the induction of OSF occurrence and development [9]. However, the administration of arecoline in the drinking water completely avoided physical stimulation. This experimental model proved the premise that OSF-like changes can be induced in mice by avoiding physical stimulation and a mixture of lime, using the administration of pure drinking water containing arecoline. Therefore, we believe that physical stimulation, quicklime and other factors are not necessary for inducing OSF and are merely cofactors. The chemical stimuli of alkaloids are the primary factors which cause OSF to occur and develop.

We first validated this conclusion in our in-vivo mouse experiments. Some studies used areca nut extract to induce an animal model of OSF; however, this method is extremely long. We considered the fact that areca nut contains between 5% and 40% polyphenols and alkaloids, whereas arecoline constitutes only 1% of the net weight of dry areca nut, and the active ingredient in areca nut could easily be dissolved in water [10]. Thus, due to the excessive number of ingredients in areca nut extracts, the effective concentration of OSF is too low, thereby affecting the time taken to develop the symptoms of OSF in an animal model. This may also explain why the use of areca nut extracts to induce OSF samples takes such a long period.

In this study, we also found that the mouse tongues (root of the tongue) first began to exhibit OSF-like changes (8 weeks), followed by the buccal region and finally, the palate (soft palate). Different results appeared for each part of the oral mucosa, which may be due to the drinking habits of the mice.
and the duration of drug exposure in the oral mucosa. In the existing clinical studies, the predilection site of OSF remains controversial and the buccal mucosa was found to be the most commonly involved site in patients, followed by the palate [11]; however, Reichart et al. found that the tongue is the most commonly affected [12]. Each mouse exhibited OSF-like changes at multiple oral locations after 12 weeks of the experiment, whereas humans typically develop OSF at a single location. In one study, the simultaneous occurrence of OSF at the buccal and palatal mucosa was found to be only 7.9% [13]. The site of human OSF may also be considered as multi-
position; however, since the clinical diagnosis were pathology-based, with no obvious OSF location identified without a biopsy, human OSF-like changes can not be considered to involve only a single location. This may explain why different clinical studies have presented different results.

Vascular occlusion and reduction are considered to be one of major pathological changes of OSF, as well as contributing factors leading to subsequent disease [7]. However, recent studies have challenged this prevailing concept, stating that the development of disease does not lead to a decrease in vascularity [14]. CD34 is considered to be an important indicator of tissue angiogenesis and vascular endothelial cell markers can be used to calculate the change of angiogenesis [15]. Thus, we assessed angiogenesis changes using CD34 staining in the establishment of an animal model of OSF. We found that while the tongue exhibited the earliest OSF-like lesions, the palatal tissue lesions grew rapidly.

We speculate that the speed of lesion growth and angiogenesis changes are consistent with the differences in vasculopathy sensitivity. In addition, we found that with the progression of OSF lesions, the number of presenting vessels first increased and then decreased. This may be attributed to the early stimulation of inflammatory factors by OSF, which promotes the increase in angiogenic factors, accelerates the regeneration of capillaries and relieves the symptoms of ischemia. During pathological deterioration, angiogenesis factor production is inhibited, which promotes platelet adhesion and thrombus formation, leading to a reduction in the number of vessels. However, clinical studies have found increased angiogenesis during the advanced stages of OSF [8], which may have resulted in malignancies. Such malignancies are thought to be due to the impact of carcinogens on the induction of angiogenesis factors [16], which lead to the formation of blood vessels, thereby increasing the number of vessels. If the assumption is true, perhaps the malignant trend of OSF could be monitored by detecting vascular changes. In order to observe whether the blood vessel is involved in the development or malignant trend of OSF, future experiments should extend the exposure time or append carcinogenic substances.

The increased collagen exhibited in OSF is due to the imbalance in collagen of diseased tissue, which is mainly type I and III [17]. Thus, we further examined the expression levels of Col I and Col III in the mouse oral mucosa. Our results revealed that Col I within the tissue lesions gradually increased the deposition. By 20 weeks, Col I appeared to be the sole and major constituent in the lamina propria. Col III showed some increase in early treatment, but noticeably decreased with the extension of the overall treatment time. Arecoline has been shown to enhance the biosynthesis of Col I and the proliferation of fibroblasts [18] when exposed to arecoline, demonstrating the ability of arecoline to disrupt the balance between the synthesis and degradation of collagen. Our research shows a decline in Col III, which indicates that it does not play an important role in fibrosis and Col I is essential in OSF progression. Moreover, the experiments of Utsunomiya et al. [19] on human OSF also demonstrated similar results. However, there has been no reasonable explanation for this phenomenon and a more detailed investigation is required to determine the elastin-specific metabolic pathway during the later stages of fibrosis.

In this study, the development of a mouse model of OSF induced by arecoline based on light microscopy findings was examined in detail. This animal model of OSF exhibited consistency and stability throughout the treatment period. While physical stimulation factors were found to be unnecessary for the induction of OSF, the presence of an alkaloid substance, particularly arecoline derived from areca nut, was a key factor. Experimental data also revealed angiogenic changes in the lamina propria, which promote the relationship between OSF lesions, as well as the collagen type involved in OSF lesions. Therefore, an OSF mouse model was successfully constructed for the future treatment of OSF and provides the foundation for future study.

Conflict of interest statement

We declare that we have no conflict of interest.

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