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

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ABSTRACT

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 2% capsaicin to coat the rat palatal mucosa surface and claimed to establish a model of OSF; however, the typical pathological and clinical manifestations were lacking [2]. In 1987, MacDonald used arecoline to hamster cheek pouches to induce OSF, which failed [3]. Until 2007, Perera *et al*. applied an aqueous areca nut extract to the buccal mucosa of BALB/c mice for 300–600 d, which successfully induced pathological changes similar to that of human OSF [4]. Recently, Maria *et al*. used betel nut and pan masala solutions injected into the buccal mucosa of Sprague–Dawley rats over a period of 48

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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 1 000 mg/L in the drinking water for the experimental group (we separately used 250, 500, 1 000 and 2 000 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 mucousal 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 RNAlater 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% H_2O_2 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 diaminobenzidine 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 GTC 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 TTG CGT GTT 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).