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Cytokines secreted by arecoline activate fibroblasts that affect the balance of TH17 and Treg

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Abstract

Background: Oral submucous fibrosis (OSF) is a chronic progressive oral disease with cancerous tendency. Arecoline plays an important role in the pathogenesis of OSF. Fibroblasts (FBs) are the primary cells involved in the pathogenesis of OSF. There is a change in $CD4^+IL\text{-}17^+$ helper T cells (Th17) and $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg) in OSF patients, but the molecular mechanisms of are not clearly understood. In this work, we studied the molecular mechanisms.

Methods: Enzyme digestion was used to extract primary FBs, and immunofluorescence was used to identify FBs. Cytotoxic experiment was then performed to determine the effect of arecoline on FB activity. Enzyme-linked immunosorbent assay (ELISA) was used to detect changes in the amount of cytokines. In addition, we treated human peripheral blood mononuclear cells (PBMC) with the above cytokines and detected their changes. Flow cytometry was used to detect the changes of Th17 and Treg, and quantitative-polymerase chain reaction (Q-PCR) was used to detect the changes of RORγt and Foxp3.

Results: We have found that the stimulation of arecoline on FBs increased interleukin-2, interleukin-6, and interleukin-21 (IL-2, IL-6, and IL-21) while decreased transforming growth cytokine-β (TGF-β). After the cytokine-containing supernatant was co-cultured with PBMC, the cytometry results showed that Th17 was increased, while Treg was significantly decreased and Q-PCR results showed that RORγt expression was increased and Foxp3 expression was decreased.

Conclusion: The arecoline can affect inflammatory cytokines produced by FBs, which then act on immune cells Th17 and Treg, and make them change.

KEYWORDS

arecoline, fibroblast, oral submucosal fibrosis, Th17, Treg

1 | INTRODUCTION

Oral submucous fibrosis (OSF) is a chronic progressive disease in the oral mucosa. It is a fibrotic disease characterized by atrophic oral epithelium, collagen accumulation in the submucosal lamina propria, gradual occlusion, and even reduction of blood vessels.[1] Elucidating the related mechanism of OSF can provide an effective target for the prevention and remission of OSF. Changes in the immune microenvironment are closely related to the occurrence and development of OSF, but the related mechanism is still not fully understood.

Currently, most scholars believe that OSF is caused by several components of the areca nut, mainly arecoline and areca nut's thick

fibers, which repeatedly stimulate oral mucosa, resulting in excessive collagen synthesis and reduced degradation in local mucosa, both of which are out of balance.[2] It has been reported that arecoline induces apoptosis of vascular endothelial cells and oral epithelium,[3] but it is rarely reported whether it regulates the changes of oral mucosa fibroblasts (FBs).

Some studies have found that the changes of FBs in OSF tissue are strictly related to the collagen accumulation in the submucosal lamina propria.[4] Studies have shown that chewing areca nut act directly on the oral submucosal tissues and produce cytotoxic effects on subepithelial FBs in contact with it.[5] Inflammatory cytokines secreted by FBs can act on immune cells, such as T lymphocytes and B lymphocytes, changing their original balance mechanism.[6] However, its molecular mechanism is not clear.

Inflammatory cytokines secreted by FBs not only act on immune cells and regulate the immune environment of the body, but they also lead to a persistent chronic inflammatory reaction. Growth factors and cytokines secreted by FBs, and endothelial cells have been suggested as contributing to initiation and progression of connective tissue fibrosis.[7] It has been found that many cytokines and inflammatory mediators and their related receptors are differentially expressed at different stages of OSF.[8]

The important role of immune regulation in tumorigenesis is gaining more and more attention. Initially, $CD4^+$ T lymphocytes can differentiate into different subtypes of T lymphocytes under different conditions, including Th1, Th2, Th17, and Treg.[9,10] It is generally believed that TGF-β and IL-6 are the most critical cytokines to induce the differentiation of initial $CD4^+$ T cells into Th17 cells. In the presence of both, the activated $CD4^+$ T cells differentiated into Th17 cells via the STAT3 pathway by activating the orphan nuclear receptor (RORγt).[11] IL-21, another highly expressed cytokine in Th17 cells, plays a key regulatory role in the differentiation, development, and function of T cells through the autocrine pathway.[12] Cytokines secreted by Th17 cells such as IL-17 have a powerful pro-inflammatory effect and can induce a variety of cells to release inflammatory cytokines and produce inflammatory responses.[13] Some studies have shown that IL-2 is essential for the function of Treg cells. IL-2 and TGF-β induce the transformation of initial $CD4^+$ T cells into $CD4^+CD25^+$ T cells and express the forkhead transcription cytokine 3 (Foxp3).[14] Th17 cells are a pro-inflammatory subpopulation that promotes autoimmune and tissue damage, while Treg cells control the balance between immune activation and tolerance.[15] Treg has the function of inhibiting the inflammatory response caused by Th17.[16] It has been found that Th17 and Treg play a role in the fibrosis of lung, liver, and kidney tissues, and induce tissue fibrosis by activating FBs, liver stellate cells, inflammatory response, and overactivated injury repair.[17-19]

In this study, FBs was used as a cell model to explore the changes and regulatory mechanism of Th17 and Treg in the body immune environment under the action of arecoline and to clarify the involvement of cytokines produced by FBs in this change.

2 | MATERIALS AND METHODS

2.1 | Extraction and culture of primary FBs cells

The experimental specimen was obtained from healthy mucosal tissue resected during oral and maxillofacial surgery. Patients had no systemic disease or recent medication history, their buccal mucosa was normal, without keratosis, and no previous history of chewing areca nut. All the patients signed the informed consent form provided by the Xiangya Stomatological Hospital of Central South University. The study was approved by the Medical Ethics Committee of Xiangya Stomatological Hospital of Central South University, Hunan Province (20190036). Extraction, culture, and passage of primary FBs were performed following the enzymatic digestion method.[20] FBs were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) with 10% fetal bovine serum (FBS; Invitrogen), and cells from the third to the seventh generation were used in the study.

2.2 | Cell immunofluorescence experiments

The sixth-generation FBs were inoculated into a 6-well plate for immunocytochemical staining. The antibody was a mouse anti-human vimentin monoclonal antibody (ABCAM) and will not contain antibody was used as a negative control. The experiment was performed using an immunohistochemical staining kit, and cells were observed under the inverted fluorescence microscope (NIKON).

2.3 | Cytotoxicity analysis

Fibroblasts transmitted to the fifth generation were inoculated into 96-well plates, and the number of cells in each hole was adjusted to 1×10^3. The 96-well plates were divided into five groups and treated with arecoline at different concentrations (0, 10, 20, 40, and 80 μg/mL), and cells with arecoline concentration of 0 μg/mL were set as the control group. A medicated base of 100 μL was added to each hole, and 6 repeated holes were set for each group. After 4 days culture, 20 μL CCK-8 cell counting kit was added into each well. Then, the absorbance value was measured at 450 nm wavelength with the microplate reader (Rayto RT-6100), calculating cell viability, and IC50 value according to Equation (1), Equation (2), and Equation (3). The arecoline concentration that did not affect cell proliferation in the previous experiment was selected to treat the cells and was cultured for 0 day, 1 day, 2 days, 3 days, 4 days, and 5 days, respectively. The cells cultured on 0 day were set as the control group. The absorbance was measured according to the same procedure above. Then, cell viability and IC50 value were calculated using the following formula.

$$\text{Cell viability} = \frac{\text{OD value (Experimental group)}}{\text{OD value (Control group)}} \quad (1)$$

$$\text{Cell inhibition rate} = 1 - \text{Cell viability} \quad (2)$$

$$\lg IC50 = X_m - \frac{I[P-(3-P_m-P_n)]}{4} \quad (3)$$

where $X_m = \lg \text{Maximum dose}$; I = lg (maximum dose/adjacent dose); P is the sum of cell inhibition rates; P_m is the maximum cell inhibition rate; and P_n is the minimum inhibition rate.

2.4 | ELISA analysis

The supernatant of the cultured sixth-generation FBs was taken out and placed in EP tube in the cell chamber. The EP tube with supernatant was placed in a cryogenic centrifuge (Eppendorf company) and centrifuged at 200 *g* for 20 minutes. ELISA kit (Shanghai zcibio technology) was taken out of the refrigerator at 4°C. Diluent was prepared with 2.5 mL original solution, and 50 mL ultra-clean water and samples were added according to the procedure provided by the manufacturer. Then, the absorbance value was measured at 450 nm wavelength with the microplate reader.

2.5 | Flow cytometry analysis

Peripheral blood mononuclear cell (PBMC) was extracted from human whole blood and cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (containing 10% FBS). In the experimental group, 3 mL of FB supernatant was added after the withdrawal of arecoline. In the control group, 3 mL of the supernatant of FBs was added without the intervention of arecoline, which was cultured for 4 days for flow cytometry experiments. Th17 was stained with FITC anti-human CD4, and PE anti-human IL-17a (BioLegend company), and Treg was stained with FITC anti-human CD4, PE anti-human CD25, and AF647 anti-human Foxp3 (Biolegend company) according to the manufacturer's instructions. Later, these cells were detected with flow cytometry (Cyto-Flex Beckman).

2.6 | Q-PCR analysis

Total RNA was extracted from cells using Trizol reagent (Invitrogen GT1402). The cDNA was synthesized using reverse transcription following the manufacturer's procedure. The standard product was purified, and the fluorescence quantitative PCR reaction system was prepared on ice. The primers of RORγt and Foxp3 were synthesized by Wuhan Sevier biotechnology company after searching the gene pool, and the synthesized primers were as follows: hFoxp3-FGGAG-GATGGACGAACAGG; hFoxp3-R: GCAGGCAAGACAGTGGAA; hRORγt-F:ACCTCACCGAGGCCATTCCAG; and hRORγt-R: TAGGCCCGGCACATCCTAAC. The repeat holes were set in each sample tube, and the relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7 | Data analysis and statistics

All statistical analyses were performed with SPSS 25.0 and Origin 8.0 software, and measurement data were expressed as $\overline{X} \pm S$. One-way ANOVA was used for statistical test among multiple experimental groups, and LSD multiple comparisons or independent sample *t* test was used for testing differences between the two groups. $P \leq .05$ was considered statistically significant.

3 | RESULTS

3.1 | Extraction, culture, and identification of primary FBs

As shown in Figure 1A, under the optical microscope (NIKON Eclipse E100 Japan), the cells were star-shaped or long fusiform, with round or elliptical nucleus. There were 2 to 4 cytoplasmic processes, and the nucleolus was visible. As shown in Figure 1B, the staining result of anti-vimentin antibody was positive, which demonstrated that FBs culture was successful.

3.2 | Cytotoxicity analysis

To investigate the effect of arecoline on FB proliferation, FBs was cultured for 4 days at different arecoline concentrations (0, 10, 20, 40, and 80 μg/mL) and compared with the control group. As shown in Figure 2A, the cell viability of FBs were 100%, 97.20%, 96.34%, 91.12%, and 89.36% when arecoline concentrations were 0, 10, 20, 40, and 80 μg/mL, respectively. Statistical test results showed that

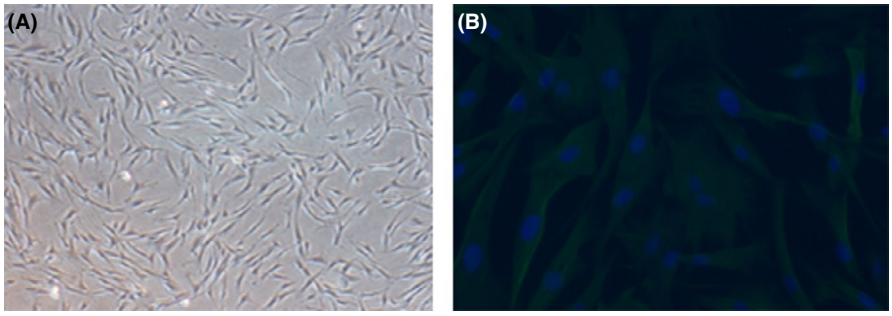

FIGURE 1 (A). Optical microscope (40×): The cells had stellate or long fusiform shape, with rounded or elliptic nuclei, and 2 ~ 4 cytoplasmic processes, which were typical fibroblasts (B). Inverted fluorescence microscope (200×): The vimentin is expressed in the FBs as the corresponding fluorescein-labeled green, and the nuclei are marked blue by the DAPI dye

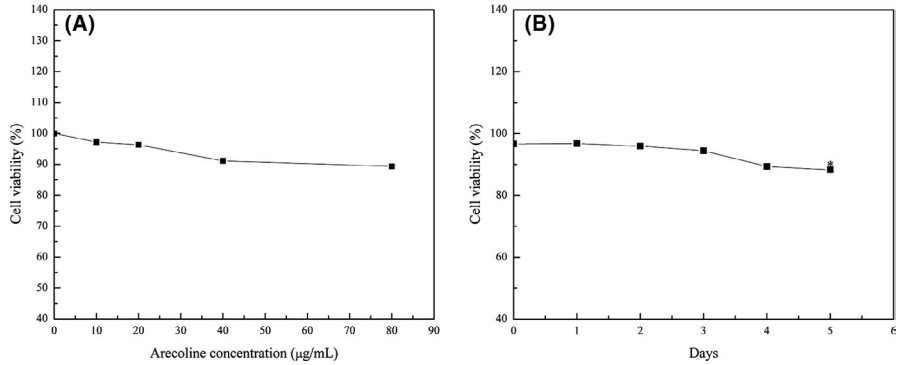

FIGURE 2 (A). FBs were cultured for 4 d with arecoline concentrations of 0, 10, 20, 40, and 80 μg/mL. Cell viability was measured with the CCK-8 method. Arecoline can affect the cell viability of FBs, and FBs are dose-dependent on arecoline (compared to the control group, $P < .05$). (B). FBs were cultured at different times (0, 1, 2, 3, 4, and 5 d) with arecoline concentration of 80 μg/mL. Cell viability was measured with the CCK-8 method. When cultured for 5 d, cell viability was significantly different from the control group (compared to the control group, $^*P < .05$)

there was no significant difference ($P > .05$) in cell viability between the experimental groups and the control group. It can be concluded that arecoline at a concentration below 80 μg/mL has no obvious effect on FB proliferation, and the IC50 of arecoline was about 90.00 μg/mL after calculation. Therefore, 0-80 μg/mL was selected as the arecoline concentration in subsequent experiments.

Fibroblasts was cultured with arecoline concentration of 80 μg/mL at 0, 1, 2, 3, 4, and 5 days, respectively, and compared with the control group. Experimental results as shown in Figure 2B, the cell viability was 100% for 0 d, 96.77% for 1 d, 95.94% for 2d, 94.46% for 3d, 89.64% for 4d, and 88.24% for 5d, respectively. There was a significant difference ($P < .05$) in cell viability between the control group and the culture at day 5, while there was no significant difference ($P > .05$) in cell viability between the culture of day 4 and the control group, which proves that FB proliferation is significantly inhibited by arecoline on the 5th day of cell culture. Therefore, 0-4 days was selected as the culture time in subsequent experiments.

3.3 | ELISA analysis

To investigate the effect of arecoline on the production of IL-2, IL-6, IL-21, and TGF-β in FBs, the content changes of IL-2, IL-6, IL-21, and TGF-β in FBs were measured in arecoline concentrations ranging from 0 to 80 μg/mL, and culture time ranging from 0 to 4 days.

Figure 3 shows the results for the measurement of IL-2, IL-6, IL-21, and TGF-β in FBs. As can be seen from Figure 3A-C, within the range of experimental conditions, the production of IL-2, IL-6, and IL-21 in FBs during 1-4 days increased with the increase of arecoline concentration, and as shown in Figure 3E-G, the content of IL-2, IL-6, and IL-21 in the experimental groups was significantly different ($P < .05$) from the control group. As can be seen from Figure 3D, within the range of experimental conditions, the production of TGF-β in FBs during 1-4 days decreased with the increase of arecoline concentration, and as shown in Figure 3H, the content of TGF-β in the experimental groups was significantly different ($P < .05$) from the control group.

By investigating the changing trend of arecoline on the production of IL-2, IL-6, IL-21, and TGF-β in FBs, it can be seen that arecoline had the most significant effect on the production of four types of inflammatory cytokines when arecoline concentration was 80 μg/mL and culture time was 4 days. To investigate if arecoline could continuously affect the production of IL-2, IL-6, IL-21, and TGF-β in FBs, in the subsequent experiments, the experimental group with arecoline concentration of 80 μg/mL and cultured for 4 days was selected and continued to culture for 3 days after removing arecoline. Simultaneously, the control group cultured for 4 days in the above experiment was also continued to culture for 3 days. Experimental results are shown in Figure 4, and after the removal of arecoline, the contents of IL-2, IL-6, IL-21, and TGF-β in FBs were still significantly different ($P < .05$) from those of the control group, indicating that the effect of arecoline on IL-2, IL-6, IL-21, and TGF-β was still present when continued to culture for 3 days after removing arecoline.

3.4 | Flow cytometry analysis

To investigate whether inflammatory cytokines produced by FBs under the action of arecoline would induce changes in Th17 and Treg, the supernatant without the above-mentioned inflammatory cytokines was co-cultured with PBMC as the control group, and the supernatant containing the above-mentioned inflammatory cytokines was co-cultured with PBMC as the experimental group. Experimental results are shown in Figure 5. Figure 5A shows that the proportion of Th17 cells in the control group was 1.67%, and Figure 5B shows that the proportion of Th17 cells in the experimental group was 3.03%. Figure 5C shows that the proportion of Treg cells in the control group was 3.13%, and Figure 5D shows that the proportion of Treg cells in the experimental group was 1.91%. As shown in Figure 5E,F, there were significant differences ($P < .05$) between the experimental group and the control group. Therefore,

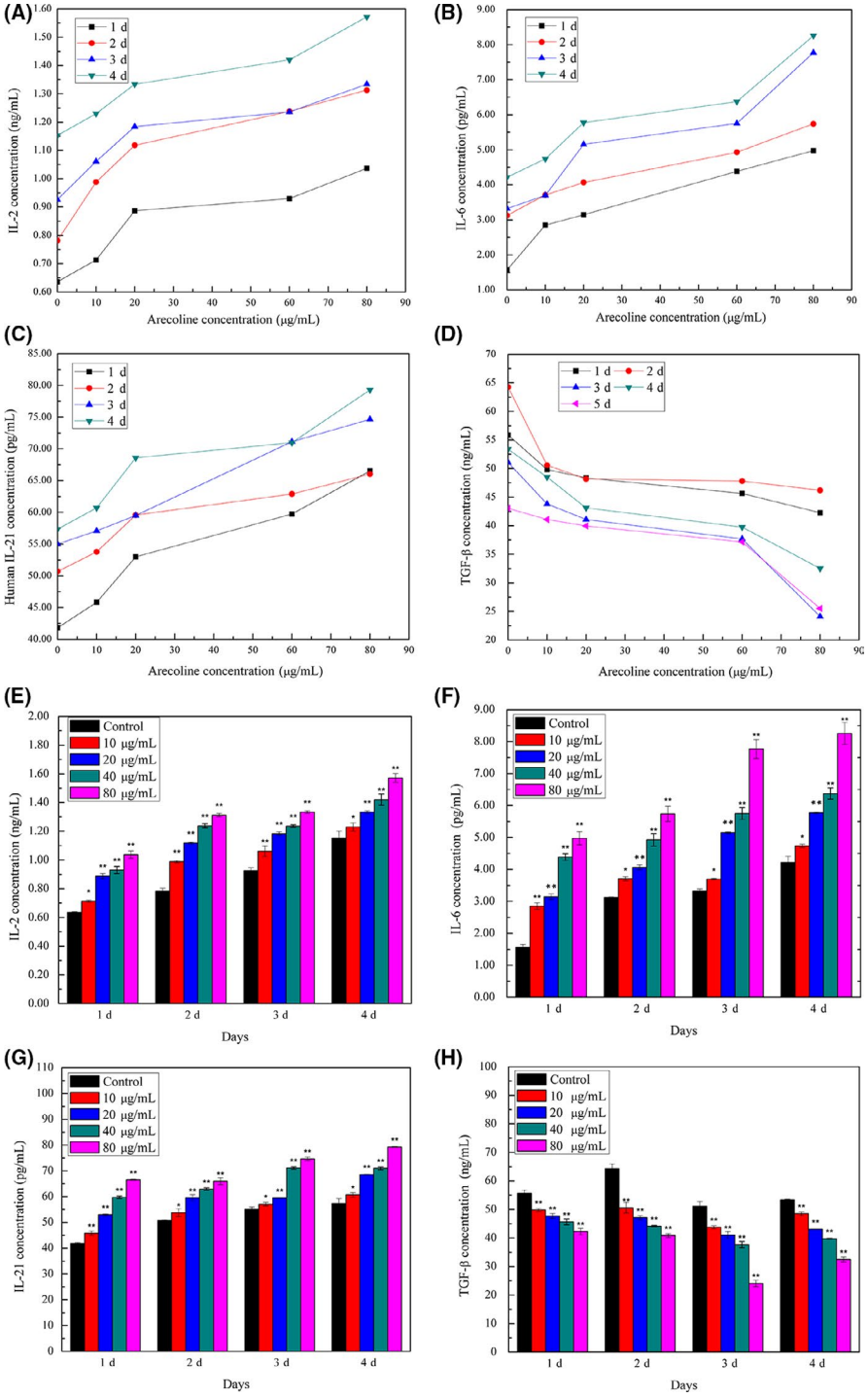

FIGURE 3 The change of IL-2, IL-6, IL-21, and TGF-β produced by FBs under the influence of different arecoline concentrations (0, 10, 20, 40, and 80 μg/mL), and different culture time (0, 1, 2, 3, and 4 d) was detected with ELISA (compared to the control group, $^{*}P < .05$, $^{**}P < .01$; IL-2: A and E, IL-6 : B and F,IL-21: C and), TGF-β: D and H)

it can be concluded that IL-2, IL-6, IL-21, and TGF-β produced by FBs, under the action of arecoline, could affect the changes of Th17 and Treg.

3.5 | Q-PCR experiment

To verify the mechanism of Th17 and Treg changes, the supernatant of FBs without arecoline effect was co-cultured with PBMC for 4 days as the control group, and the supernatant of FBS with arecoline effect was co-cultured with PBMC for 4 days as the experimental group to compare the changes of expression levels between the two. The experimental results are shown in Figure 6. As can be seen from Figure 6A, the RORγt's relative expression level of the experimental group was higher than those in the control group, and as can be seen from Figure 6B, the Foxp3's relative expression level of the experimental group was lower than those in the control group. Statistical test results show that there was a significant difference ($P < .05$) between the experimental group and the control group.

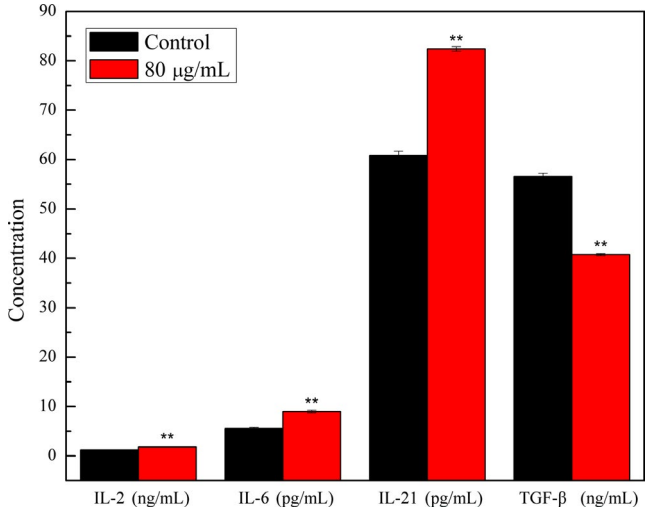

FIGURE 4 The change of IL-2, IL-6, IL-21, and TGF-β produced by FBs after removing arecoline was detected with ELISA (compared to the control group, *P < .05, **P < .01)

4 | DISCUSSION

The pathogenesis of OSF is unclear, and the effect of arecoline on the disease is suspected of playing an important role. Some scholars, after many epidemiological investigations, have found that the incidence of OSF is closely related to chewing betel nut.[21] Chewing betel nut for a long time can cause oral mucosal tissue lesions and change the levels of cytokines and pro-inflammatory factors, such as IL-1, IL-6, IL-8, TNF-α, and TGF-β1.

Fibroblast is the leading cell type present in connective tissue, leading cell for the synthesis of collagen, and is also involved in the degradation and phagocytosis of collagen. Some studies have shown that fibroblasts are associated with fibrosis. Therefore, FB could be the main participating cell in the pathogenesis of OSF. In this study, we investigated the changes of cytokines IL-2, IL-6, IL-21, and TGF-β produced by fibroblasts after arecoline treatment on fibroblasts.

The relationship between FBs, arecoline, Th17, and Treg deserves our attention. In the present study, we first studied the FB cell

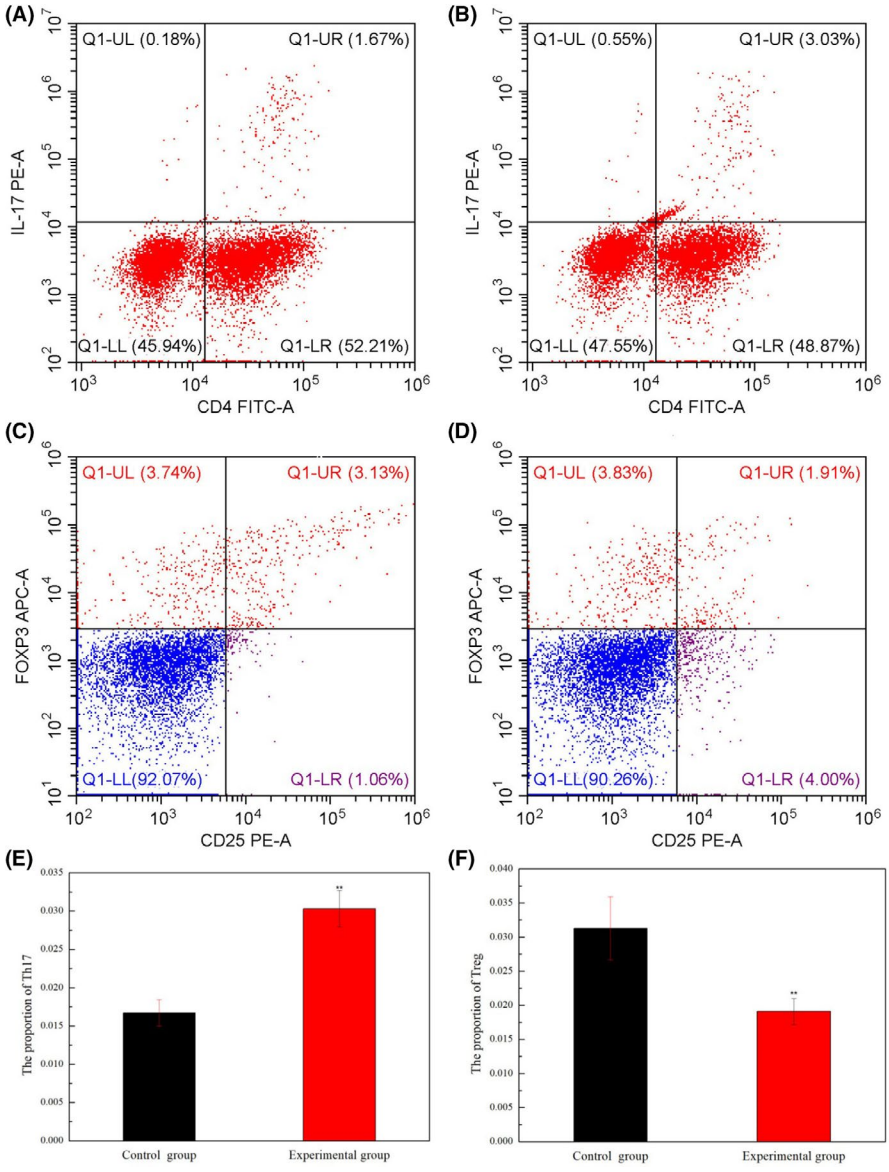

FIGURE 5 The proportion of Th17 and Treg in the control group and the experimental group were analyzed with flow cytometry. A and B shows the control group and experimental group of Th17, respectively. The x-coordinate was CD4+ T cells, the y-coordinate was IL-17+ T cells, and the upper right value was Th17 proportion. C and D shows the control group and experimental group of Treg, respectively. The x-coordinate was CD25+ T cells, the y-coordinate was Foxp3+ cells, and the upper right value was Treg proportion. E shows the comparison in Th17 proportion between the control group and experimental group, and F shows the comparison in Treg proportion between the control group and experimental group (compared to the control group, P < .05)

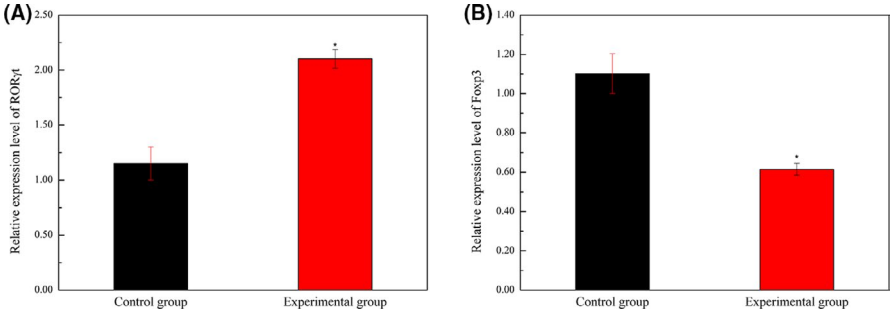

FIGURE 6 After the removal of arecoline to intervene FBs, the supernatant containing cytokine was co-cultured with PBMC for 4 d, and the expression of RORγt and Foxp3 was analyzed with Q-PCR. A shows the changes of RORγt expression in the control group and the experimental group. B shows the changes of Foxp3 expression in the control group and the experimental group (compared to the control group, $^*P < .05$)

viability after the intervention of arecoline at different concentrations in different culture time. Altered cultured fibroblast behavior is reported above 0.1 μg/mL (0.64 μmol/L), and cell death commences at doses of 10-30 μg/mL (64-193 μmol/L).[22-25] The concentration of arecoline in this experiment starts from 10 μg/mL. The results showed that arecoline has time- and dose-dependent inhibition of FB cell viability. FBs produced the highest cytokine concentration of arecoline 80 μg/mL, and the intervention time of 4 days was selected as the intervention condition for subsequent experiments. To avoid the change of Th17 and Treg caused by the direct action of arecoline, we chose to continue the culture of 3 days FBs after the withdrawal of arecoline for the study of the subsequent experiments.

Th17 and Treg play a role in tissue fibrosis, and the role of Th17 and Treg cells in OSF is also possible. In this study, we concluded that arecoline could cause changes in inflammatory cytokines produced by FBs, and when supernatant of cytokines containing the most significant changes was applied to peripheral blood, Th17 and Treg changed, compared with the control group. Th17 increased while Treg decreased. The increase of Th17 was related to the transcription factor (RORγt), and the decrease of Treg was related to the transcription factor (Foxp3). It was speculated that the up-regulated expression of RORγt resulted in the up-regulated expression of Th17 cells, and the down-regulated expression of Foxp3 resulted in the down-regulated expression of Treg cells. This guess is consistent with previous research.[26-29]

In conclusion, this study shows that arecoline can affect inflammatory cytokines produced by FBs, which then act on immune cells Th17 and Treg, and make them change. This could be part of the OSF pathogenesis.

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CONFLICT OF INTEREST

None.

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REFERENCE

1. Warnakulasuriya S, Tilakaratne WM, Kerr A. *Oral submucous fibrosis*, (p. 102); 2017.
2. Varghese K, Anuram R, Kumar TM. A sectional splint for maintaining surgically enhanced vertical height in an oral submucous fibrosis patient: a case report. *J Ind Prosthodon Soc*. 2014;14:287-292.
3. Kamath VV, Krishnamurthy S, Satelur KP, Rajkumar K. Transforming growth cytokine-β1 and TGF-β2 act synergistically in the fibrotic pathway in oral submucous fibrosis: an immunohistochemical observation. *Ind J Med Paediatr Oncol*. 2015;36:111-116.
4. Krithika C, Ramanathan S, Koteeswaran D, Sridhar C. Ultrasonographic evaluation of oral submucous fibrosis in habitual areca nut chewers. *Dentomaxillofac Rad*. 2013;42:20120319.
5. Patel U, Patel V. Oral submucous fibrosis: recent modalities of treatment. *J Int Med Res*. 2014;2:79-80.
6. Lindebo HT, Ditte T, Henrik S, Kvist PH. Evaluating IL-21 as a potential therapeutic target in Crohn's disease. *Gastroent Res Pact*. 2018;2018:1-22.
7. Jimenez SA, Hitraya E, Varga J. Pathogenesis of scleroderma: collagen[J]. *Rheum Dis Clin North Am*. 1996;22:647-674.
8. Haque MF, Meghji S, Khitab U, Harris M. Oral submucous fibrosis patients have altered levels of cytokine production. *J Oral Pathol Med*. 2010;29:123-128.
9. Schroder WA, Gardner J, Le TT, et al. SerpinB2 deficiency modulates Th1/Th2 responses after schistosome infection. *Parasite Immunol*. 2010;32:764-768.
10. Singh TP, Schön MP, Wallbrecht K, Gruber-Wackernagel A, Wang XJ, Wolf P. Involvement of IL-9 in Th17-associated inflammation and angiogenesis of psoriasis. *PLoS ONE*. 2013;8:1-11.
11. Brockmann L, Giannou AD, Gagliani N, Huber S. Regulation of Th17 cells and associated cytokines in wound healing, tissue regeneration, and carcinogenesis. *Int J Exp Pathol*. 2017;18:1033.
12. Raveney BJE, Shinji O, Takashi Y. Nuclear receptor NR4A2 orchestrates Th17 cell-mediated autoimmune inflammation via IL-21 signalling. *PLoS ONE*. 2013;8:1-10.
13. Tosa M, Ghazizadeh M, Murakami M. Involvement of Th17/IL-17 in the pathogenesis of keloid. *J Dermatol Sci*. 2013;69:e12.
14. Wang K, Zhu T, Wang H, et al. Adoptive transfers of $CD4^+$ $CD25^+$ Tregs raise Foxp3 expression and alleviate mouse enteritis. *Biomed Res Int*. 2018;2018:9064073.
15. Arce-Sillas A, Álvarez-Luquín DD, Tamaya-Domínguez B, et al. Regulatory T cells: molecular actions on effector cells in immune regulation. *J Immunol Res*. 2016;2016:1-12.

16. Ma L, Xue H, Gao T, Gao M, Zhang Y. Notch1 signaling regulates the Th17/Treg immune imbalance in patients with psoriasis Vulgaris. *Mediat Inflamm*. 2018;2018:3069521.
17. Richards CD. Innate immune cytokines, fibroblast phenotypes, and regulation of extracellular matrix in the lung. *J Interf Cytok Res*. 2017;37:52-61.
18. Li K, Liu H, Guo T. Th17/Treg imbalance is an indicator of liver cirrhosis process and a risk factor for HCC occurrence in HBV patients. *Clin Res Hepatol Gas*. 2017;41:399-407.
19. Mehrotra P, Collett JA, McKinney SD, Stevens J, Ivancic CM, Basile DP, Stevens J. IL-17 mediates neutrophil infiltration and renal fibrosis following recovery from ischemia-reperfusion: compensatory role of natural killer cells in athymic rats. *Am J Physiol-renal*. 2017;312:F385-F397.
20. Paw BH, Zon LI. Primary fibroblast cell culture. *Method Cell Biol*. 1999;59:39.
21. Reichart PA, Nguyen XH. Betel quid chewing, oral cancer and other oral mucosal diseases in Vietnam: a review. *J Oral Pathol Med*. 2010;37:511-514.
22. Harvey W, Scutt A, Meghji S, Canniff JP. Stimulation of human buccal mucosa fibroblasts in vitro by betel-nut alkaloids. *Arch Oral Biol*. 1986;31:45-49.
23. Tsai CL, Kuo MY, Hahn LJ, Kuo YS, Yang PJ, Jeng JH. Cytotoxic and cytostatic effects of arecoline on oral mucosal fibroblasts. *Proc Natl Sci Counc Repub China B*. 1997;21:161-167.
24. Sundqvist K, Liu Y, Erhardt P, Nair J, Bartsch H, Grafstrom RC. Areca-nut toxicity in cultured human buccal epithelial cells. *IARC Sci Publ*. 1991;105:281-285.
25. Chang YC, Tai KW, Cheng MH, Chou LSS, Chou MY. Cytotoxic and non-genotoxic effects of arecoline on human buccal fibroblasts in vitro. *J Oral Pathol Med*. 1998;27:68-71.
26. Spits H, Di Santo JP. The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. *Nat Immunol*. 2011;12:21-27.
27. Lochner M, Peduto L, Cherrier M, et al. In vivo equilibrium of proinflammatory IL-17$^+$ and regulatory IL-10$^+$ Foxp3$^+$ RORgamma t$^+$ T cells. *J Exp Med*. 2008;205:1381-1393.
28. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for scurfin in CD4$^+$CD25$^+$T regulatory cells. *Nat Immunol*. 2003;4:337-342.
29. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4$^+$CD25$^+$ regulatory T cells. *Nat Immunol*. 2003;4:330-336.

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