J Oral Pathol Med (2004) 33: 581–7

www.blackwellmunksgaard.com/jopm

Effects of arecoline, safrole, and nicotine on collagen phagocytosis by human buccal mucosal fibroblasts as a possible mechanism for oral submucous fibrosis in Taiwan

Dean-Hwa Shieh[1], Lien-Chai Chiang[2], Chien-Hung Lee[3], Yi-Hsin Yang[4], Tien-Yu Shieh[5]

[1]Graduate Institute of Dental Sciences, [2]Department of Microbiology, [3]School of Public Health, [4]Graduate Institute of Oral Health Sciences and [5]Oral Health Research Center, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan

BACKGROUND: Oral submucous fibrosis (OSF) is associated with the betel quid chewing habit, and 86% of betel quid chewers in Taiwan are also smokers. Arecoline and safrole are major principles in the composition of betel quid, and nicotine is the main toxic ingredient of cigarettes.

METHODS: To explore the pathogenesis of OSF, flow cytometry was used to compare collagen phagocytosis by fibroblasts from the normal and the OSF region of the same 15 OSF patients.

RESULTS: The results indicated that heterogeneity of fibroblasts existed because collagen phagocytosis by fibroblasts from the normal region was higher than from the OSF region in the same patient. The percentage of phagocytic cells was significantly inhibited by 10, 25 and 50 μg/ml arecoline, safrole and nicotine in normal fibroblast cultures, respectively, and the percentage of phagocytic cells was significantly reduced by 25, 25 and 50 μg/ml arecoline, safrole and nicotine in OSF fibroblast cultures, respectively. Collagen phagocytosis by fibroblasts exhibited prominent dose-dependent inhibition as the concentration of arecoline, safrole, and nicotine increased. Besides, nicotine had a synergistic effect on arecoline- or safrole-inhibited collagen phagocytosis.

CONCLUSIONS: The present study concludes that even in the same person, the collagen phagocytosis by fibroblasts is different between normal and OSF region. The deficiency in collagen phagocytosis by fibroblasts of the lesion might participate in the pathogenesis of OSF. Arecoline, safrole and nicotine, which are released in saliva during BQ chewing plus cigarette smoking, inhibit collagen phagocytosis by fibroblasts in a dose-dependent manner and may induce OSF formation in Taiwan's patients.

J Oral Pathol Med (2004) **33**: 581–7

Correspondence: Yi-Hsin Yang, Graduate Institute of Oral Health Sciences, Kaohsiung Medical University, No. 100, Shih-Chuan 1st Road, Kaohsiung, Taiwan. Tel: +886-7-312-1101 (ext) 2271, Fax: +886-7-322-3870. E-mail: tiyush@kmu.edu.tw
Accepted for publication January 26, 2004

Keywords: arecoline; fibroblast; nicotine; oral submucous fibrosis; phagocytosis; safrole

Introduction

Oral submucous fibrosis (OSF) is an insidious disease and a chronic inflammatory lesion characterized by deposition of fibrous contents in the submucous layer of oral connective tissues that leads to restriction of mouth opening and increasing difficulty in phonation, mastication and swallowing (1). It results from an increased collagen synthesis (2), and a decreased collagenase activity (3). Collagen is degraded via the intracellular phagocytosis route (4) and by the extracellular collagenase-dependent route (5). Phagocytosis of collagen is considered to be a major pathway of collagen digestion (6). OSF is an oral premalignant condition (7), which is associated with the betel quid chewing habit (8). Betel quid is a mixture of natural substances whose contents vary in different areas. In Taiwan, there are more than 2 million people who are in the habit of chewing betel quid, and most of them are smokers and/or drinkers (9). They consume betel quid by putting the inflorescence of *Piper betle* Linn. between two halves of unripe areca fruit with a lime dressing or wrapping a raw areca fruit and a lime paste with a piece of betel leaf (9). Epidemiologic studies revealed that the incidence rate of oral mucosa lesions varied with different compositions of betel quid, which varied with geography (10).

Many studies have been concerned about the effects of the components of betel quid on the oral mucosa. Arecoline is a main alkaloid of areca nut, which may inhibit fibroblast proliferation and collagen synthesis (11). Fibroblasts metabolize arecoline to arecaidine, which may enhance fibroblast proliferation (12). Safrole is the major ingredient of inflorescence of *P. betle* Linn., which is known as a rodent hepatocarcinogen (13). It forms stable safrole-DNA adducts in human oral tissue and may be associated with oral carcinogenesis (14). Besides, cigarette smoking is a risk factor in

carcinogenesis, and nicotine is a major constitute in cigarettes. Nicotine may inhibit the proliferation, attachment and migration of human periodontal ligament fibroblasts (15), and may be synergistic to the effects of arecoline-induced cytotoxicity (16).

Our previous study demonstrated that the ability of collagen phagocytosis by fibroblasts was different between healthy and OSF patients (17). The present study focused on the collagen phagocytosis by fibroblasts of normal region and OSF lesion from the same patient. We used paired samples from the same patients to avoid interferences such as age, gender, race, betel quid chewing, alcohol drinking, and cigarette smoking. Furthermore, the previous study discussed the effects of arecoline and arecaidine, contents of betel nut, on the collagen phagocytosis by fibroblasts (17). This study elucidated the effects of arecoline, safrole and nicotine on the phagocytic activity of cultured human oral fibroblasts that were more coincident with the betel quid chewing habits plus cigarette smoking in Taiwan.

Materials and methods

Fibroblast cultures

Fifteen OSF patients aged between 41 and 63 years old were selected from the Department of Oral and Maxillofacial Surgery of Chung-Ho Memorial Hospital, Kaoshiung Medical University and consented to participate in this study. All participants were male and had chewed betel quid and smoked cigarettes for more than 10 years. One OSF lesion and one normal mucosa (NM) were taken from each patient, and oral pathologists confirmed all tissues. The tissue specimens were minced and washed several times with Dulbecco's phosphate-buffer saline (PBS, Gibco, BRL Life Technologies, Grand Island, NY, USA). Minced tissues were seeded into 35 mm Petri dishes and cultured in Dulbecco's modification of Eagle's minimum essential medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS, Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone (Gibco). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The outgrown cells were detached with trypsin-ethylenediaminetetraacetic acid (Trypsin–EDTA, Gibco) and were subcultured at T-25 flask (Falcon; Becton Dickinson, Lincoln Park, NY, USA). The early passage fibroblasts were certified with the monoclonal antibodies of antifactor VIII (Dakopatts, Glostrup, Denmark), anticytokeratin (ZYMED Laboratory, San Francisco, CA, USA) and antivimentin (Sigma, St Louis, MO, USA) as described previously (17). The human skin epithelial cells (BCC-1/KMC) and human endothelial cells (HUV-EC-C) were used as negative controls, whereas the human gingival fibroblasts (HGF-1) were used as positive controls. The cells were cultured for 24 h before harvesting. The slides were fixed for 5 min at room temperature in methanol-acetic acid (3:1), washed three times in PBS, and subsequently permeabilized by treatment with acetone at 4°C. After brief drying, cells were layered with 10 μl of diluted antiserum and incubated in a humidified box for 20 min at 37°C. After washing three times in PBS, slides were mounted on coverslips with buffered glycerol and screened for fluorescence using a Nikon Fluophot (Nikon, Tokyo, Japan) with epi-illumination (18). Fibroblasts of sixth to eighth passages were used in the phagocytic assay.

Bead preparation

Fluorescent latex beads (2.0 μm, Polysciences, Warrington, PA, USA) were used in this study for the measurement of phagocytic activity. The method of collagen-coated beads preparation was described by Knowels (19). Briefly, 5.7×10^6 beads was put in 1 ml of soluble calf skin collagen (3 mg/ml collagen S, collagen type I; Boehringer Mannheim, Mannheim, Germany) for 30 min at room temperature and then neutralized with 0.1 M NaOH in PBS for 10 min at 37°C. For analysis of non-specific phagocytosis by starved fibroblasts, bovine serum albumin (BSA) coated beads were prepared by incubating beads with 1 ml of 1% (w/v) BSA (Sigma) in PBS for 30 min at room temperature. Following these procedures, the beads were centrifuged at 5000 *g*, the supernatant was removed, and the beads were then resuspended in PBS and sonicated for 15 s to disperse the bead aggregates.

Analysis of phagocytosis

Fibroblasts from 15 paired samples were starved for 48 h by the replacement of normal growth medium with serum-free DMEM medium after being washed twice with PBS washing. The procedure of serum deprivation increased the proportion of phagocytic cells (20). Cells were incubated with collagen-coated fluorescent beads in T-25 flask at a bead/cell ratio of 4:1 for 3 h at 37°C. Fibroblasts were detached with 0.01% trypsin (Gibco) for 10 min at 37°C to eliminate surface-bound but not internalized beads (20), and neutralized with growth medium. The cell suspension was transferred to a polystyrene tube and centrifuged at 200 *g* for 5 min. The supernatant was removed and the pellet was fixed with 80% ethanol for 30 min at 4°C. The suspension was centrifuged at 60 *g* for 5 min and resuspended with 1 ml PBS. The phagocytic activity of fibroblasts were analyzed by a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems) using 488 nm laser excitation and measuring emission with 530 nm band pass filter to detect the beads' fluorescence. The analysis was processed by assessing 10 000 cells for each sample. Data presented with the percentage of phagocytosis by the cell numbers with internalized beads ÷ total cell numbers.

Viability assay

The NM and the OSF fibroblasts from six OSF patients were included in this assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) cell proliferation kit (Boehringer Mannheim) was used as the manufacturer described to access the fibroblasts viability. Arecoline (Sigma) and nicotine (Sigma) were diluted in DMEM medium at final concentrations of 5, 10, 25, 50, 75, 100, and 200 μg/ml. Safrole (Sigma) was

dissolved with dimethyl sulfoxide (DMSO) and then diluted with DMEM medium at final concentrations of 5, 10, 25, 50, 75, 100, and 200 μg/ml with 1% (v/v) DMSO in all culture media. Co-treatment of different concentrations (5–200 μg/ml) of arecoline or safrole with 25 μg/ml nicotine were diluted with medium or 1% (v/v) DMSO in medium, respectively. Control groups for arecoline, nicotine and arecoline/nicotine contained only medium, whereas the control groups for safrole and safrole/nicotine were medium with 1% (v/v) DMSO. Fibroblasts were plated at 10^4 cells in 96-well plates and left overnight to attach. The culture medium was changed to medium containing treated compounds and supplemented with 2% FCS, and fibroblasts were cultured for 24 h. MTT labeling reagent (final concentration 0.5 mg/ml) was added to individual seeded wells for 4 h, then solubilization solution [10% sodium dodecyl sulfate (SDS) in 0.01 M HCl] was added to each well for 24 h. MTT was selectively taken up by living cells and converted to purple formazan crystals, which were measured spectrophotometrically in a microplate reader (SLT 400ATC EIA reader, SLT Labinstruments, Salzburg, Austria) with a test wavelength of 550 nm and a reference wavelength of 690 nm.

Effect of arecoline, safrole or nicotine on collagen phagocytosis

The effects of arecoline, safrole, nicotine, arecoline/nicotine, and safrole/nicotine on collagen phagocytosis by fibroblasts were evaluated by flow cytometry analysis. There were six pairs of NM and OSF fibroblasts included in this assay. Fibroblasts were cultured in DMEM medium supplemented with 2% FCS and 0, 5, 10, 25, and 50 μg/ml arecoline, safrole and nicotine, and these concentrations of arecoline or safrole were co-treated with 25 μg/ml nicotine for 24 h. Fibroblasts were starved for 48 h before manipulation for phagocytosis analysis.

Statistical analysis

All experiments were performed three times. Mean values and SD were calculated for phagocytic percentage of each sample. Wilcoxon's signed rank test was used to compare significant differences between all paired samples.

Results

After incubation with collagen-coated beads, phagocytic fibroblasts derived from normal (NM) regions and OSF regions exhibited significant differences as shown in Fig. 1. Fibroblasts from NM regions contained more cells with better ability to internalize beads than those from OSF regions. Figure 2 revealed that fibroblasts from NM presented higher phagocytic activity (72.8 ± 5.1%) than fibroblasts from OSF sites (40.0 ± 4.3%). When cells were incubated with BSA-coated beads to assess non-specific phagocytosis, fibroblasts derived from NM and OSF regions showed only 9.8% and 9.6% of cells with internalized beads, respectively (Fig. 2).

Table 1 depicted that there were no significant differences in proliferation rate between OSF and NM

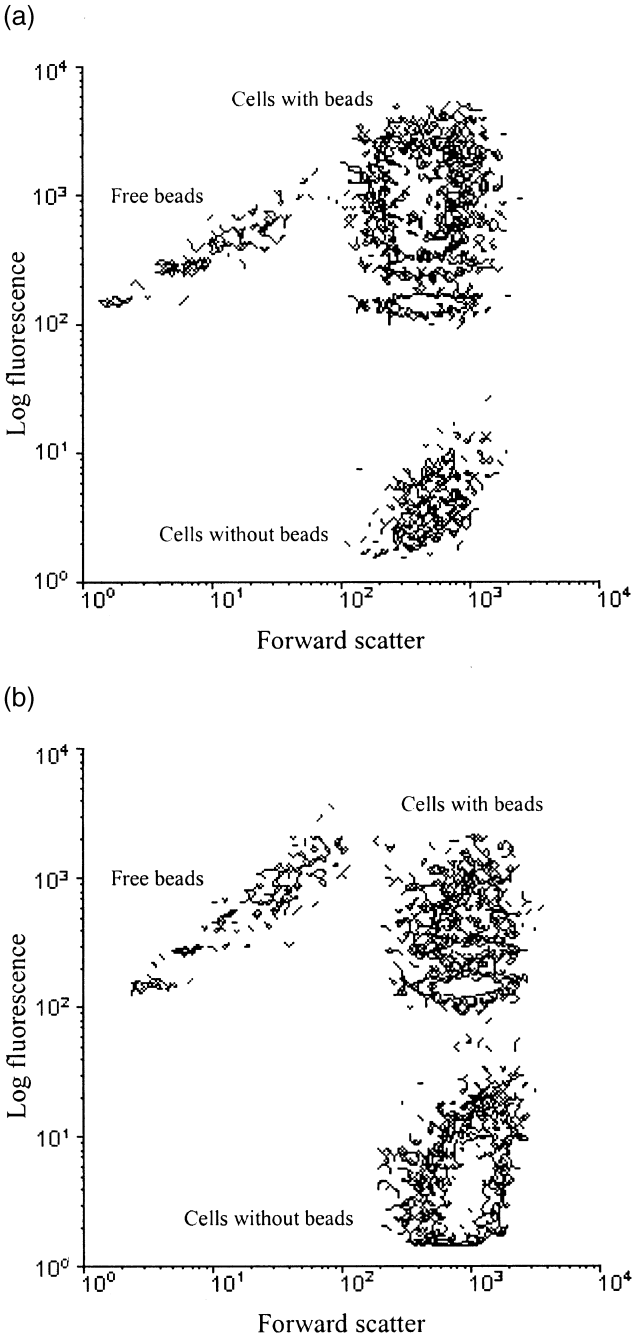

Figure 1 Flow cytograph of collagen phagocytosis by fibroblasts from normal and oral submucous fibrosis (OSF) regions of the same 15 patients. (a) Pattern of collagen phagocytosis in normal regions. (b) Pattern of collagen phagocytosis in OSF regions. The percentage of phagocytic cells in normal regions was higher than in OSF regions, and fibroblasts from normal regions contained more cells with higher ability to internalize beads than those from OSF regions.

fibroblasts at the same concentration of arecoline, safrole, and nicotine. At concentrations above 50 μg/ml, arecoline significantly inhibited the proliferation of fibroblasts from both sites ($P < 0.05$). Safrole at concentrations of 50–200 μg/ml inhibited NM and OSF fibroblast proliferation in a dose-dependent manner ($P < 0.05$). Nicotine at concentrations more than 100 μg/ml inhibited NM and OSF fibroblast growth by

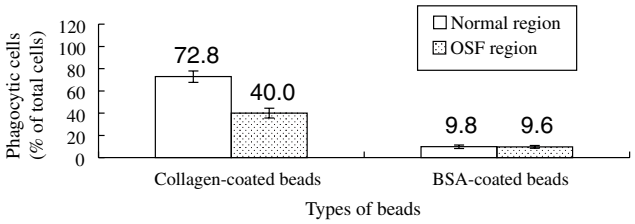

Figure 2 Comparison of collagen-coated and bovine serum albumin (BSA)-coated beads phagocytosis by fibroblasts from normal and oral submucous fibrosis (OSF) regions of the same 15 patients. The values represented mean ± SD of 15 samples. The percentage of collagen-coated beads phagocytic cells in normal regions was higher than in OSF regions, and the percentage of BSA-coated beads phagocytic cells was not significantly different between normal and OSF regions.

Table 1 Cell viability of NM and OSF fibroblasts from the same patients by arecoline, safrole and nicotine treatments

	Normal fibroblasts (%)	*OSF fibroblasts (%)*
Arecoline		
5 μg/ml	100.9 ± 2.0	100.6 ± 1.4
10 μg/ml	102.7 ± 3.8	105.1 ± 5.6
25 μg/ml	94.6 ± 4.2	95.5 ± 4.0
50 μg/ml	80.6 ± 4.8*	78.1 ± 3.1*
75 μg/ml	66.3 ± 3.4*	65.8 ± 3.0*
100 μg/ml	53.7 ± 3.6*	51.3 ± 1.4*
200 μg/ml	32.1 ± 1.2*	31.6 ± 1.5*
Safrole		
5 μg/ml	99.9 ± 0.2	100.0 ± 0.3
10 μg/ml	98.1 ± 2.5	98.6 ± 2.3
25 μg/ml	95.3 ± 4.1	95.0 ± 4.3
50 μg/ml	82.0 ± 3.4*	78.4 ± 1.5*
75 μg/ml	69.1 ± 5.4*	66.9 ± 3.9*
100 μg/ml	58.0 ± 2.4*	55.4 ± 5.1*
200 μg/ml	32.3 ± 1.6*	30.6 ± 3.2*
Nicotine		
5 μg/ml	99.3 ± 1.0	99.4 ± 0.7
10 μg/ml	99.0 ± 1.1	98.2 ± 1.9
25 μg/ml	98.9 ± 1.4	99.7 ± 0.7
50 μg/ml	97.7 ± 1.8	97.8 ± 3.1
75 μg/ml	94.9 ± 3.9	94.7 ± 4.2
100 μg/ml	86.6 ± 1.4*	86.3 ± 3.3*
200 μg/ml	75.9 ± 3.7*	76.1 ± 4.6*

Values represented percentage of treated group/control group (mean ± SD of six samples). Control groups for arecoline and nicotine contained only medium, whereas the control groups for safrole was medium with 1% (v/v) Dimethyl sulfoxide (DMSO). There was no significant difference between Normal mucosa (NM) and Oral submucous fibrosis (OSF) fibroblasts at the same dosage of reagent treatment in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay by using Wilcoxon's signed rank test.
*$P < 0.05$ represented significant difference between control and treated fibroblasts by using Wilcoxon's signed rank test.

approximately 13.4–24.1% and 13.7–23.9% ($P < 0.05$), respectively. Table 2 showed that fibroblast viability was not obviously changed when co-treated with 5–200 μg/ml arecoline plus 25 μg/ml nicotine or 5–200 μg/ml safrole plus 25 μg/ml nicotine.

A preliminary MTT assay revealed that more than 50 μg/ml arecoline or safrole and 100 μg/ml nicotine significantly decreased fibroblasts viability. This study used arecoline, safrole, and nicotine at various concentrations of 0–50 μg/ml as candidates to explore the collagen phagocytosis by fibroblasts. Figure 3 showed the effects of various dosages of arecoline, safrole, and nicotine on collagen phagocytosis by fibroblasts in normal or OSF regions. There was a significant difference in phagocytic activity between NM and OSF regions ($P < 0.05$). The percentages of fibroblasts from normal regions with collagen phagocytic activity were also higher than in OSF regions at any concentration of arecoline-, safrole-, and nicotine-treated ($P < 0.05$). Figure 3a revealed that the percentages of phagocytic cells were significantly reduced in fibroblasts from the NM and OSF regions treated with more than 10 and 25 μg/ml arecoline, respectively. Analogously, the percentages of both NM and OSF fibroblasts with phagocytic activity were significantly decreased when treated with 25 μg/ml safrole and 50 μg/ml nicotine, respectively (Fig. 3b,c). As arecoline or safrole with 25 μg/ml nicotine co-treatment did not obviously affect fibroblast viability, this nicotine concentration was used to evaluate the synergistic effect on collagen phagocytosis. Figure 4 revealed that 5–50 μg/ml arecoline plus 25 μg/ml nicotine significantly inhibited the amounts of phagocytic cells in both NM and OSF sites, whereas 10–50μg/ml safrole plus 25 μg/ml nicotine had similar effects.

Table 2 Cell viability of NM and OSF fibroblasts from the same patients by arecoline plus nicotine and safrole plus nicotine treatments

	Normal fibroblasts (%)	*OSF fibroblasts (%)*
Arecoline + nicotine		
5 μg/ml + 25 μg/ml	99.9 ± 0.3	99.9 ± 0.1
10 μg/ml + 25 μg/ml	99.5 ± 0.8	99.6 ± 0.6
25 μg/ml + 25 μg/ml	93.8 ± 4.7	93.9 ± 4.6
50 μg/ml + 25 μg/ml	77.5 ± 3.6*	76.6 ± 2.9*
75 μg/ml + 25 μg/ml	63.5 ± 1.6*	62.6 ± 1.8*
100 μg/ml + 25 μg/ml	49.9 ± 0.8*	48.7 ± 3.0*
200 μg/ml + 25 μg/ml	27.3 ± 3.2*	26.9 ± 3.1*
Safrole + nicotine		
5 μg/ml + 25 μg/ml	98.9 ± 1.6	97.8 ± 2.5
10 μg/ml + 25 μg/ml	95.9 ± 3.4	97.3 ± 3.1
25 μg/ml + 25 μg/ml	94.7 ± 4.1	94.6 ± 4.1
50 μg/ml + 25 μg/ml	78.5 ± 4.7*	77.2 ± 3.2*
75 μg/ml + 25 μg/ml	66.8 ± 2.1*	65.4 ± 3.7*
100 μg/ml + 25 μg/ml	54.2 ± 1.9*	52.3 ± 1.4*
200 μg/ml + 25 μg/ml	28.9 ± 1.3*	28.4 ± 3.8*

Values represented percentage of treated group/control group (mean ± SD of six samples). Control group for arecoline plus nicotine contained only medium, whereas the control group for safrole plus nicotine was medium with 1% (v/v) Dimethyl sulfoxide (DMSO). There was no significant difference between Normal mucosa (NM) and Oral submucous fibrosis (OSF) fibroblasts at the same dosage of reagent treatment in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay by using Wilcoxon's signed rank test.
*$P < 0.05$ represented significant difference between control and treated fibroblasts by using Wilcoxon's signed rank test.

Discussion

The etiology of OSF has been thought to be associated with betel quid chewing habit and a genetic predisposition (21). The etiology of OSF might also be affected

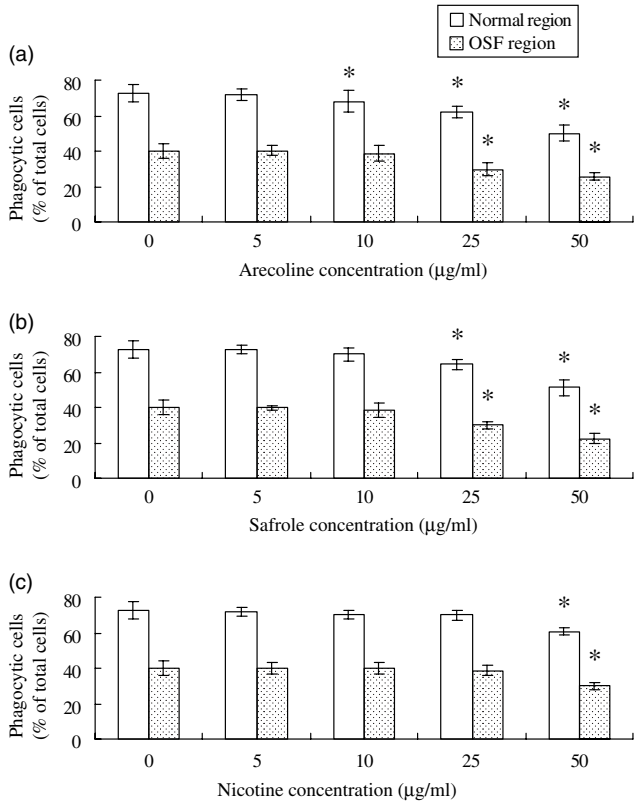

Figure 3 Phagocytic cells (percentage of total cells) in normal mucosa (NM) and oral submucous fibrosis (OSF) fibroblasts from the same patients. Values represented mean ± SD of six samples. All of the phagocytic cell numbers were significantly different between NM and OSF fibroblasts after treatment with the same concentration of reagent. $*P < 0.05$ represented significant difference between control and treated fibroblasts by using Wilcoxon's signed rank test.

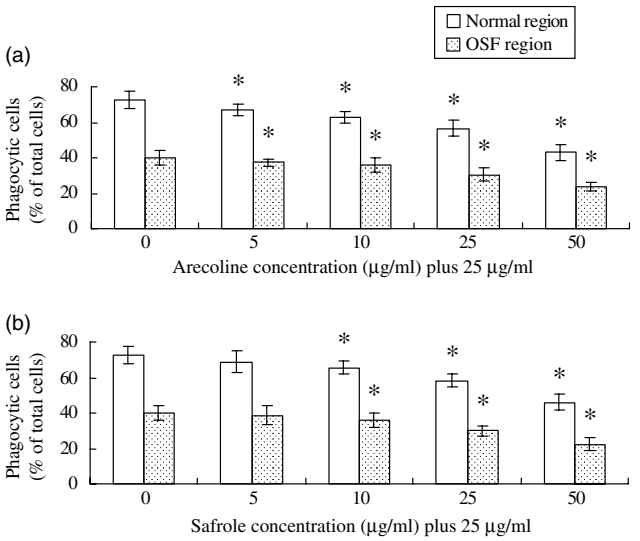

Figure 4 Phagocytic cells (percentage of total cells) in normal mucosa (NM) and oral submucous (OSF) fibroblasts from the same patients. Values represented mean ± SD of six samples. All of the phagocytic cell numbers were significantly different between NM and OSF fibroblasts after treatment with the same concentration of reagent. $*P < 0.05$ represented significant difference between control and treated fibroblasts by using Wilcoxon's signed rank test.

by other factors including age, gender and race of the donors, dose and duration of betel quid chewing, contents of betel quid, additional habits of drinking or smoking. In addition, it has been reported that fibroblasts heterogeneity exists (22), and that the susceptibility of fibroblasts to areca quid varies between different individuals (23). To avoid the above factors to influence the results, we used the NM and OSF fibroblasts from the same persons.

Betel quid chewing is a popular habit in Taiwan, and 86% of betel quid chewers are also smokers (9). Epidemiologic evidence demonstrated that betel quid chewing plus cigarette smoking promoted the relative risk for oral cancer by way of the formation of *N*-nitroso compounds were the possible causes (24). The ingredients of BQ may contribute to the development of OSF, while the effect of cigarette smoking in the pathogenesis of OSF was still unknown. Arecoline and safrole were thought to be major toxic components in the composition of betel quid, whereas nicotine was the main constitute in cigarettes. Previous reports have demonstrated that arecoline, safrole, and nicotine were cytotoxic to oral mucosal cells, and the effects might result from the cellular depletion of gluthinone (GSH), which would render the cells more vulnerable to other stimulation from betel quid or cigarette contents (25–27). The concentration of arecoline is as high as 89.89 μg/ml (mean 29.69 μg/ml) in saliva during betel quid chewing (24). Arecoline may increase tissue inhibitor of metalloproteinase-1 secretion and decrease gelatinase A activity in buccal mucosal fibroblasts (28), which may lead to accumulation of extracellular matrix in BQ-associated OSF formation. The salivary safrole concentration is about 70 μg/ml (420 μM) when chewing the type of betel quid that was sandwiched with the inflorescence of *P. betle* Linn. between a raw areca fruit (29). Safrole might form stable safrole-DNA adducts in OSF tissues (14). Nicotine may inhibit collagen production as well as increase collagenase activity, which was associated with gingival destruction. Nicotine may uptake by gingival fibroblasts with non-specific binding and intracellular nicotine levels in fibroblasts are maintained over 4 h (30). The salivary concentration of nicotine that leached out from cigarette after smoking one cigarette was reported at levels ranging from 0 to 4.42 μg/ml (mean 0.45 μg/ml) in saliva (24). In Taiwan, the BQ chewers smoke the cigarette instead of chewing the tobacco. Taken together, the salivary nicotine concentration did not excess 90 μg/ml when one pack of cigarette (20 cigarettes) was consumed per day. Although the concentrations of these reagents in saliva were established, the permeability of them in oral mucosa is not clearly known and need to be further studied. The MTT assay showed that more than 50 μg/ml arecoline, 50 μg/ml safrole, and 100 μg/ml nicotine were significantly reduced cell viability. Therefore, we used 50 μg/ml each of arecoline, safrole, and nicotine to evaluate the phagocytosis activity in fibroblasts were within the salivary concentration range and suited for exploration

of OSF pathogenesis in Taiwan. As previous evidence revealed that arecoline decreased the phagocytic activity by fibroblasts (17), the present data further proved that safrole and nicotine also decreased collagen phagocytosis by fibroblasts. The results indicated that the amounts of phagocytic cells were inhibited by 10, 25 and 50 μg/ml arecoline, safrole and nicotine in NM fibroblasts culture, respectively, and were reduced by 25, 25 and 50 μg/ml arecoline, safrole, and nicotine in OSF fibroblast culture, respectively. The decrement of collagen phagocytosis by fibroblasts was inversely dose-dependent by the increment of arecoline, safrole, and nicotine. It is similar to gingival hyperplasia induced by cyclosporin A (31), dilatin, and nifedipine (32), which decreased collagen phagocytosis by fibroblasts is in a dose-dependent manner. When arecoline or safrole plus 25 μg/ml nicotine was used as candidate to treat fibroblasts, the phagocytic activity decreased more apparently than those only treated with arecoline or safrole. This means that nicotine had a synergistic effect on arecoline- or safrole-inhibited collagen phagocytosis. As the deficiency in collagen phagocytosis by fibroblasts was the possible mechanism in OSF formation, these reagents might play important roles and be involved in the pathogenesis of OSF.

Several reports have indicated that the etiology of OSF resulted from an increase of collagen deposition and a decrease of collagen degradation. Our previous study had demonstrated a decrease of phagocytic activity of fibroblasts in OSF patients when compared with normal persons (17). The present study revealed the number of phagocytic cells and phagocytic activity in cultured human oral fibroblasts from OSF region was lower than the fibroblasts from normal region of the same person. This result demonstrated that the heterogeneity of fibroblasts existed in different areas of oral mucosa from the same patients as the previous reports (22). The decrease in collagen phagocytosis of OSF lesions may represent that the OSF fibroblasts with selective subpopulation contribute to mass collagen deposition. This coincided with other similar lesions such as scleoderma that are also caused by the collagen over-deposition (33). This finding also represented that OSF was just the solitary lesion in chronic irritation region of oral mucosa instead of a general condition existing in all oral mucosa.

The mechanism of OSF formation was unknown clearly by the association of complex factors, but our results indicated that betel quid indeed decreased the percentage of phagocytic cells, which might result in collagen deposition and contribute to the development of OSF. This study also confirmed the existence of the heterogeneity of fibroblasts. Fibroblasts of normal regions exhibited a greater proportion of cells with high phagocytic activity than that of OSF regions even in the same persons. The present study concluded that arecoline, safrole, and nicotine, which are released in saliva during BQ chewing plus cigarette smoking inhibit collagen phagocytosis by fibroblasts in a dose-dependent manner and may induce OSF formation in Taiwan's patients.

References

1. Sirsat SM, Pindborg JJ. Subepithelial changes in oral submucous fibrosis. *Acta Pathol Microbiol Scand* 1967; **70**: 161–73.
2. Meghji S, Scutt A, Harvey W, Canniff JP. An in-vitro comparison of human fibroblasts from normal and oral submucous fibrosis tissue. *Arch Oral Biol* 1987; **32**: 213–5.
3. Shieh TY, Yang JF. Collagenase activity in oral submucous fibrosis. *Proc Natl Sci Counc Repub China* 1992; **16**: 106–10.
4. Everts V, Beertsen W, Tigchelaar-Gutter W. The digestion of phagocytosed collagen is inhibited by the proteinase inhibitors leupeptin and E-64. *Collagen Relat Res* 1985; **5**: 315–36.
5. Murphy G, Reynolds JJ. Current views of collagen degradation. Progress towards understanding the resorption of connective tissues. *BioEssays* 1984; **2**: 55–60.
6. Yajima T. Acid phosphatase activity and intracellular collagen degradation by fibroblasts in vitro. *Cell Tissue Res* 1986; **245**: 253–60.
7. Pindborg JJ, Murti PR, Bhonsle RB, Gupta PC, Daftary DK, Mehta FS. Oral submucous fibrosis as a precancerous condition. *Scand J Dent Res* 1984; **92**: 224–9.
8. Sinor PN, Gupta PC, Murti PR, et al. A case-control study of oral submucous fibrosis with special reference to the etiologic role of areca nut. *J Oral Pathol Med* 1990; **19**: 94–8.
9. Ko YC, Chiang TA, Chang SJ, Hsieh SF. Prevalence of betel quid chewing habit in Taiwan and related sociodemographic factors. *J Oral Pathol Med* 1992; **21**: 261–4.
10. Ko YC, Huang YL, Lee CH, Chen MJ, Lin LM, Tsai CC. Betel quid chewing, cigarette smoking and alcohol consumption related to oral cancer in Taiwan. *J Oral Pathol Med* 1995; **24**: 450–3.
11. Chang YC, Tai KW, Lii CK, Chou LS, Chou MY. Cytopathologic effects of arecoline on human gingival fibroblasts in vitro. *Clin Oral Invest* 1999; **3**: 25–9.
12. 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**: 45–9.
13. Daimon H, Sawada S, Asakura S, Sagami F. In vivo genotoxicity and DNA adduct levels in the liver of rats treated with safrole. *Carcinogenesis* 1998; **19**: 141–6.
14. Chen CL, Chi CW, Chang KW, Liu TY. Safrole-like DNA adducts in oral tissue from oral cancer patients with a betel quid chewing history. *Carcinogenesis* 1999; **20**: 2331–4.
15. Giannopoulou C, Geinoz A, Cimasoni G. Effects of nicotine on periodontal ligament fibroblasts in vitro. *J Clin Periodontol* 1999; **26**: 49–55.
16. Chang YC, Hu CC, Tseng TH, Tai KW, Lii CK, Chou MY. Synergistic effects of nicotine on arecoline-induced cytotoxicity in human buccal mucosal fibroblasts. *J Oral Pathol Med* 2001; **30**: 458–64.
17. Tsai CC, Ma RH, Shieh TY. Deficiency in collagen and fibronectin phagocytosis by human buccal mucosa fibroblasts in vitro as a possible mechanism for oral submucous fibrosis. *J Oral Pathol Med* 1999; **28**: 59–63.
18. Chiang LC, Chiang W, Chang SF, Chen HY, Yu HS. Characterization of an immortalized human cell line derived from neonatal foreskin diploid fibroblasts. *J Dermatol* 1992; **19**: 1–11.
19. Knowles GC, McKeown M, Sodek J, McCulloch CAG. Mechanism of collagen phagocytosis by human gingival fibroblasts: importance of collagen structure in cell recognition and internalization. *J Cell Sci* 1991; **98**(Pt 4): 551–8.

20. McKeown M, Knowles GC, McCulloch CAG. Role of the cellular attachment domain of fibronectin in the phagocytosis of beads by human gingival fibroblasts in vitro. *Cell Tissue Res* 1990; **262**: 523–30.
21. Canniff JP, Harvey W, Harris M. Oral submucous fibrosis: its pathogenesis and management. *Br Dent J* 1986; **160**: 429–34.
22. Hassell TM, Stanek EJ III. Evidence that healthy human gingiva contains functionally heterogeneous fibroblast subpopulations. *Arch Oral Biol* 1983; **28**: 617–25.
23. van Wyk CW, de Miranda CM, Olivier A, van der Bijl P. The effect of baked areca nut extract on the growth of buccal mucosa fibroblasts from healthy non-areca nut chewers. *J Dent Assoc S Afr* 1996; **51**: 29–31.
24. Nair J, Ohshima H, Friesen M, Croisy A, Bhide SV, Bartsch H. Tobacco-specific and betel nut-specific N-nitroso compounds: occurrence in saliva and urine of betel quid chewers and formation in vitro by nitrosation of betel quid. *Carcinogenesis* 1985; **6**: 295–303.
25. Jeng JH, Hahn LJ, Lin BR, Hsieh CC, Chan CP, Chang MC. Effects of areca nut, inflorescence *Piper betle* extracts and arecoline on cytotoxicity, total and unscheduled DNA synthesis in cultured gingival keratinocytes. *J Oral Pathol Med* 1999; **28**: 64–71.
26. Jeng JH. *Genotoxic and non-genotoxic effects of betel quid constituents in Taiwan*. PhD thesis, National Taiwan University, 1994.
27. Chang YC, Huang FM, Tai KW, Yang LC, Chou MY. Mechanisms of cytotoxicity of nicotine in human periodontal ligament fibroblast cultures in vitro. *J Periodontol Res* 2002; **37**: 279–85.
28. Chang YC, Yang SF, Tai KW, Chou MY, Hsieh YS. Increased tissue inhibitor of metalloproteinase-1 expression and inhibition of gelatinase A activity in buccal mucosal fibroblasts by arecoline as possible mechanisms for oral submucous fibrosis. *Oral Oncol* 2002; **38**: 195–200.
29. Wang CK, Hwang LS. Phenolic compounds of betel quid chewing juice. *Food Sci* 1993; **20**: 458–71.
30. Hanes PJ, Schuster GS, Lubas S. Binding, uptake, and release of nicotine by human gingival fibroblasts. *J Periodontol* 1991; **62**: 147–52.
31. Kataoka M, Shimizu Y, Kunikiyo K, et al. Cyclosporin A decreases the degradation of type I collagen in rat gingival overgrowth. *J Cell Physiol* 2000; **182**: 351–8.
32. McCulloch CAG, Knowles GC. Deficiencies in collagen phagocytosis by human fibroblasts in vitro: a mechanism for fibrosis? *J Cell Physiol* 1993; **155**: 461–71.
33. Botstein GR, Sherer GK, Leroy EC. Fibroblast selection in scleroderma. An alternative model of fibrosis. *Arthritis Rheum* 1982; **25**: 189–95.

Acknowledgment

This study was supported by a grant from National Science Council, ROC (grant no. NSC 86-2314-B-037-059).