Synergistic effects of nicotine on arecoline-induced cytotoxicity in human buccal mucosal fibroblasts

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Abstract
Areca quid chewing has been linked to oral submucous fibrosis and oral cancer. Arecoline, a major areca nut alkaloid, is considered to be the most important etiologic factor in the areca nut. In order to elucidate the pathobiological effects of arecoline, cytotoxicity assays, cellular glutathione S-transferase (GST) activity and lipid peroxidation assay were employed to investigate cultured human buccal mucosal fibroblasts. To date, there is a large proportion of areca quid chewers who are also smokers. Furthermore, nicotine, the major product of cigarette smoking, was added to test how it modulated the cytotoxicity of arecoline. At a concentration higher than 50 μg/ml, arecoline was shown to be cytotoxic to human buccal fibroblasts in a dose-dependent manner by the alamar blue dye colorimetric assay (P<0.05). In addition, arecoline significantly decreased GST activity in a dose-dependent manner (P<0.05). At concentrations of 100 μg/ml and 400 μg/ml, arecoline reduced GST activity about 21% and 46%, respectively, during a 24 h incubation period. However, arecoline at any test dose did not increase lipid peroxidation in the present human buccal fibroblast test system. The addition of extracellular nicotine acted synergistically on the arecoline-induced cytotoxicity. Arecoline at a concentration of 50 μg/ml caused about 30% of cell death over the 24 h incubation period. However, 2.5 mM nicotine enhanced the cytotoxic response and caused about 50% of cell death on 50 μg/ml arecoline-induced cytotoxicity. Taken together, arecoline may render human buccal mucosal fibroblasts more vulnerable to other reactive agents in cigarettes via GST reduction. The compounds of tobacco products may act synergistically in the pathogenesis of oral mucosal lesions in areca quid chewers. The data presented here may partly explain why patients who combined the habits of areca quid chewing and cigarette smoking are at greater risk of contracting oral cancer.

Key words: arecoline; buccal mucosal fibroblasts; cytotoxicity; nicotine; synergistic effect

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It was estimated that at least 200 million people in the world chewed areca quid (1). In Taiwan, the number of areca quid chewers was esti-
mated to be two million among the twenty million inhabitants (2). Unfortunately, epidemiological studies have shown that areca quid chewing increases the risk and incidence of oral cancer and oral submucous fibrosis (3, 4); about 80 percent of all oral cancer deaths in Taiwan are associated with this habit (5). The majority of oral mucosal lesions arise in the buccal mucosa in contact with the areca quid. However, how areca quid chewing could induce alterations of oral mucosa is still not fully understood. The toxic effects of areca quid components are considered to be the main causative factors.

The composition of the areca quid shows regional variation. In Taiwan, people chew areca quid with two halves of a fresh areca nut that was sandwiched with a piece of inflorescence of *Piper betle* and lime paste containing some secret additives. Areca nut is the endosperm of the fruit of the *Areca catechu* tree. The pharmacological effects of areca nut include addiction, euphoria, excessive salivation and tremor, attributable to the parasympathomimetic action of arecoline, the major alkaloid of the areca nut (6).

Many of the undesirable effects of areca nut have been attributed to arecoline. It is mutagenic in both bacteria and mammalian cells (7, 8). It also increases the frequency of chromosome aberrations, sister chromatid exchanges and micronuclei in Chinese hamster ovary cells (9, 10). Moreover, arecoline is a cytotoxic agent to primary human buccal mucosal fibroblast cultures (11–13).

During cellular stress, many adverse effects are triggered, including lipid peroxidation, protein oxidation and interference with cellular homeostasis. Protein thiol is one of the targets that can easily be attacked under oxidative stress. To date, the potential toxicological implications of arecoline on buccal mucosal fibroblasts remain to be elucidated. The aim of the present study was to address the cytotoxic effects and possible mechanisms of arecoline on human buccal mucosal fibroblasts. In this study, cytotoxicity assays, glutathione S-transferase (GST) activity and lipid peroxidation assay were used to investigate the pathobiological effects of arecoline on cultured human buccal mucosal fibroblasts.

Convincing evidence has shown the interrelationship between cigarette smoking and various forms of cancer, including oral cancer (14). Areca quid chewing is a popular, islandwide habit in Taiwan and about 86% of chewers are also smokers (2). Tobacco smoke contains several classes of carcinogens, such as polycyclic aromatic hydrocarbons, volatile and tobacco-specific nitroamines, that affect humans (3). Little is known about the association between areca quid chewing and cigarette smoking on human oral mucosa, and the combined effects of areca quid and tobacco compounds on human buccal mucosal fibroblasts are worthy of further investigation.

Nicotine, the major product of cigarette smoking, serves as a precursor for highly carcinogenic nitrosamines (15). Furthermore, nicotine was added to test how it modulated the cytotoxicity of arecoline.

### Material and methods

#### Chemicals

Arecoline, nicotine, Hoechst 33258 (H33258), phosphotungstic acid, and thioarbitratric acid were purchased from Sigma (St. Louis, MO, USA). Alamar blue dye was obtained from Alamar Biosciences Inc. (Sacramento, CA, USA). All culture materials were obtained from Gibco (Grand Island, NY, USA). Arecoline was dissolved in sterile distilled water and adjusted to pH 7.4. The final concentrations were 0–400 μg/ml.

#### Cell culture

Six healthy patients (3 men and 3 women, 18–32 years old, average 24 years) were selected from the Department of Oral and Maxillofacial Surgery, Chung Shan Medical & Dental College Hospital, Taichung, Taiwan for this study. All patients were duly informed of the nature and extent of the study, and their informed consent was obtained according to the Helsinki Declaration. Biopsy specimens were derived from histologically normal areas of surgical third molar extraction specimens from patients without areca quid chewing habits. The tissues were minced using sterile techniques and washed twice in phosphate buffered saline (PBS) supplemented with antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml of fungizone). Explants were placed into 60 mm petri dishes and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics, as described above. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Once the fibroblasts were established, the fungizone was omitted from the medium. Confluent cells were detached by treatment with 0.25% trypsin and 0.05% EDTA for 5 min, and aliquots of separated cells were subcultured with the same medium. Cell cultures between the third and eighth passages were used in this study.

#### Cytotoxicity assay

Cytotoxicity was evaluated by the alamar blue dye assay. Alamar blue is an oxidation-reduction indicator for eukaryotic cells. It is a recently developed extension of the cytotoxicity assay based on the reduction of tetrazolium salts by the mitochondrial cytochromoxidase system (16). Briefly, fibroblasts were seeded 5×10⁴ cells per well into 24-well culture plates and incubated for 24 h so that they would become attached. The culture medium was replaced with fresh DMEM plus 0.5% FCS and various concentrations of arecoline. After trypsinization, 25 μl of alamar blue dye was added to each
well for 2 h at 37°C. The colorimetric determination was done at 570 nm and 600 nm on a plate reader (CytoFluor 4500, Millipore, Bedford, MA, USA). The percent of inhibition of mitochondria activity in response to a test agent as compared with untreated cells was calculated by the formula:

\[ \text{percent of inhibition} = \left(1 - \frac{\text{OD}_{570} \text{ of test agent dilution}}{\text{OD}_{570} \text{ of untreated control}} \right) \times 100 \]

where OD is the optical density.

**Intracellular activity of glutathione S-transferase**

Cells were lysed and homogenized in Tris-sucrose buffer (pH 7.5) at 4°C and centrifuged at 10,000×g. The resulting supernatants were used to determine the activity of GST (1-chloro-2,4-dinitrobenzene as substrate), according to Habig et al. (17). The protein concentration was determined with a commercial kit (Bio-Rad Lab. Ltd., Watford, Herts., UK) and bovine serum albumin as standard. The data are expressed as a fraction of individual control (nmol/min/mg protein).

**Lipid peroxidation assay**

Production of triobarbituric acid-reactive substances (TBARS) was used as an indicator of lipid peroxidation and was determined as described by Fraga et al. (18). Briefly, 1 ml (10⁶ cells) was pelleted and resuspended in ice-cold PBS. The TBARS reaction was performed by adding reaction buffer (0.5 ml 3% SDS, 2 ml 0.1 N HCl, 0.3 ml 10% phosphotungstic acid and 1 ml 0.7% thiobarbituric acid) to Eppendorf tubes and then heating the tubes in a boiling water bath for 30 min. After cooling, 5 ml 1-butanol was added to the mixture to extract TBARS, followed by centrifugation at 1100×g for 15 min. The fluorescence of the supernatant was detected at an excitation wavelength of 515 nm and an emission wavelength of 555 nm in a fluorescence spectrophotometer (F4500 Hitachi, Tokyo, Japan). 1,1,3,3-tetramethoxypropane was used as a malondialdehyde precursor and a TBARS standard. An aliquot of cell homogenate was used to measure the concentration of protein, as described above. Values were expressed as nmol of TBARS per mg of protein.

**Effects of nicotine on arecoline-induced cytotoxicity**

A H33258 fluorescence assay was used to evaluate the cytotoxicity of nicotine on human buccal mucosal fibroblasts, according to a method of Skehan et al. (19). H33258 is an UV-excited blue bisbenzimidazole dye that selectively intercalates into A-T rich regions of DNA, undergoing a fluorescence enhancement in the process. It provides a fluorescence signal that is linearly proportional to cellular DNA content over a wide range of DNA values (19). Briefly, cells were plated at an initial density of 5×10⁴ cells/well into 24-well culture plates and allowed to attach for 24 h. Then cells were exposed to various concentrations of nicotine for 6 or 24 h. Finally, the medium was removed, the plates frozen at −80°C and stored frozen until ready for further processing. After thawing plates, each well received 100 µl of distilled water and was incubated for 1 h at room temperature. They were refrozen at −80°C for 90 min and thawed under room temperature conditions. Then 100 µl of TNE buffer (10 mM Tris, 1 mM EDTA, 2 M NaCl, pH 7.4) containing 20 µg/ml of H33258 dye was added. After a 90-min incubation period in the dark, the DNA content was measured using a fluorescence plate reader (CytoFluor2300, Millipore, Bedford, MA, USA) at an excitation wavelength of 350 nm and an emission wavelength of 460 nm.

Subsequently, nicotine was added to wells to test its synergistic effect. Briefly, cells were incubated with arecoline for 24 h and then nicotine was added for 6 h. The H33258 fluorescence assay was used, as described previously.

**Statistical analysis**

Triplicate (or more) experiments were performed throughout this study. All assays were repeated three times to ensure reproducibility. Statistical analysis was performed by a one-way analysis of variance. Tests of differences of the treatments were analyzed by Duncan’s test and were considered to be significant at \( P < 0.05 \).

**Results**

Metabolic activity of cells, as assessed by alamar blue absorbance, was inhibited when compared with the control (Fig. 1). Arecoline was cytotoxic to human buccal fibroblasts at a concentration of 50 µg/ml (\( P < 0.05 \)). The effect of inhibition was dose dependent. The amount of alamar blue absorbance was about 62% at a concentration of 100 µg/ml compared with control.

One route for GSH depletion is via the GST reaction. As shown in Fig. 2, arecoline reduced the intracellular activity of GST on human buccal mucosal fibroblasts in a concentration-dependent manner (\( P < 0.05 \)). At concentrations of 100 µg/ml and 400 µg/ml, arecoline reduced GST activity about 21% and 46%, respectively, during the 24 h incubation period.

The level of TBARS production was used as an indicator of
Effects of nicotine on arecoline-induced cytotoxicity

Fig. 1. Effect of arecoline on human buccal mucosal fibroblasts in alamar blue assay. The percentage of absorbance at each concentration, compared with that of the control, was calculated. Each point and bar represent a mean ± SD value. * indicates significant differences from control values with \( P < 0.05 \).

Table 1. Effects of arecoline on cellular lipid peroxidation in human buccal mucosal fibroblasts

<table>
<thead>
<tr>
<th>Arecoline (µg/ml)</th>
<th>TBARS (nmol/mg protein)</th>
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<tbody>
<tr>
<td>0</td>
<td>0.34 ± 0.025</td>
</tr>
<tr>
<td>25</td>
<td>0.36 ± 0.031</td>
</tr>
<tr>
<td>50</td>
<td>0.39 ± 0.009</td>
</tr>
<tr>
<td>100</td>
<td>0.34 ± 0.012</td>
</tr>
<tr>
<td>200</td>
<td>0.38 ± 0.029</td>
</tr>
</tbody>
</table>

Lipid peroxidation was evaluated by TBARS production. The pink-colored TBARS were measured and expressed as nmol/mg protein. All values represent the mean value and SD. No difference in the TBARS production was noted between the control and arecoline-treated cells \((P > 0.05)\).

lipid peroxidation. Unexpectedly, arecoline at any test dose did not increase lipid peroxidation in the present human buccal fibroblast test system (Table 1). No difference in the TBARS production was noted between the control and arecoline-treated cells \((P > 0.05)\).

The effect of nicotine on human buccal mucosal fibroblasts, as measured by H33258 fluorescence assay for 6 h and 24 h incubation periods, is shown in Fig. 3. Nicotine was cytotoxic to human buccal mucosal fibroblasts in a concentration- and time-dependent manner. Nicotine at a concentration of 2.5 mM caused about 32% of cell death over the 6 h incubation period (Fig. 3). Human buccal mucosal fibroblast incubation with various concentrations of arecoline for 24 h showed a synergistic cytotoxic effect with 2.5 mM nicotine added.

Fig. 3. Effect of nicotine on human buccal mucosal fibroblasts by H33258 fluorescence assay for 6-h and 24-h incubation period. The percentage of fluorescence at each concentration, compared with that of controls was calculated. Each point and bar represent a mean ± SD value. * denotes significant differences from control values with \( P < 0.05 \).
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Fig. 4. Synergistic effects of nicotine on arecoline-induced cytotoxicity to human buccal mucosal fibroblasts by H33258 fluorescence assay. Cells were incubated with arecoline for 24 h, then nicotine was added for 6 h. The percentage of fluorescence compared with that of controls was calculated. Each point and bar represent a mean ± SD value. * denotes significant differences from control values with *P*, 0.05.

for 6 h (Fig. 4). Arecoline at a concentration of 50 μg/ml caused about 30% of cell death over the 24 h incubation period. However, the nicotine-enhanced cytotoxic response caused about 50% of cell death on 50 μg/ml arecoline-induced cytotoxicity.

Discussion

In this study, at a concentration higher than 50 μg/ml, arecoline was cytotoxic to buccal mucosal fibroblasts and decreased GST activity in a dose-dependent manner (*P*<0.05). Furthermore, the addition of extracellular nicotine acted synergistically on the arecoline-induced cytotoxicity.

Previous studies have reported that arecoline could react with glutathione (GSH) both in vitro (20, 21) and in vivo (20) to produce cysteine β-alkylation adducts. Thus, formation of conjugates is a likely mechanism for GSH depletion in arecoline-treated human oral mucosal fibroblasts. This route for GSH depletion is via the GST reaction (22). In this study, arecoline inhibited cellular GST activity, corresponding to GSH depletion, as shown by previous studies (20, 21). It is reported that the reaction of glutathione conjugation with a large number of foreign compounds with electrophilic centers is catalyzed by GST (23–25). The cytotoxic and thiol-depleting properties of arecoline appear to be chemically related to the presence of a methylester group (21). The lower polarity and lower degradation of arecoline due to ionization in aqueous solution might be contributory to its passage over cell membranes. Moreover, in addition to detoxification of foreign compounds, GST also participated in metabolism of many endogenous compounds (24, 26). Taken together, the formation of the GS-arecoline conjugate may be through GST catalysis in detoxification reaction. Therefore, GST would provide an important detoxification pathway in arecoline-induced cytotoxicity.

In this study, decreased GST activity was found to be one of the inhibitory mechanisms of arecoline on human buccal mucosal fibroblasts, since one route for GSH depletion is via the GST reaction (22). A previous study has reported that local production of GSH is deficient in patients suffering from pulmonary fibrosis (27). Consistently, Wong et al. (28) have found that the GSH level is decreased in areca quid chewers with oral submucous fibrosis, a precancer condition, compared with normal buccal mucosa of healthy individuals. Taken together, thiol depletion might play an important role in the pathogenesis of fibrosis. In addition, arecoline decreased intracellular thiol activity in human buccal mucosal fibroblasts might render the cells more vulnerable to other reactive agents within the areca quid. Moreover, intermediates produced during the detoxification of xenobiotics via the GST reaction have also been implicated in carcinogenesis (22). Thus, this might be the reason why areca quid chewers suffer from OSF at a high risk ratio for oral cancer.

A previous study has shown that thiol depletion by exogenous chemicals could increase the intracellular concentration of reactive oxygen species and cause subsequent lipid peroxidation on cell membranes (27). Unexpectedly, arecoline at any test dose did not increase lipid peroxidation in the present human buccal fibroblast test system. The possible explanation of this result may be that the metabolism of arecoline is not a free-radical-generation system in human buccal fibroblasts. These results are in general agreement with those reported by Sundqvist et al. (21) and our recent study (29) that arecoline can delete free low-molecular-weight thiols in human oral cells and that thiol depletion was not coupled with oxidative stress in these cells.

Cigarette smoking is known to be a risk factor in cancer development. Many of the undesirable effects of smoking have been attributed to nicotine. It has been reported that nicotine could cause epithelial alterations when applied topically to the oral mucosa (30). Earlier studies have shown that nicotine was a cytotoxic agent on oral fibroblasts (31–35). In the present study, nicotine was also found to exert cytotoxicity on human buccal mucosal fibroblasts in
a concentration- and time-dependent manner. The cytotoxic nature of nicotine on cultured oral fibroblasts has clearly been shown.

Epidemiological studies have clearly associated areca quid chewing plus cigarette smoking with an increased risk for oral cancer, and the formation of N-nitroso compounds is thought to be a direct cause of such cancer (36). However, the mechanism of the combined effect still remains to be established. In the present study, nicotine acts synergistically with arecoline to induce cytotoxicity on human buccal mucosal fibroblasts. During GST reduction, arecoline may render the cells more vulnerable to other reactive agents within cigarettes. This result may partly explain why patients who combined the habits of areca quid chewing with cigarette smoking were at greater risk of contracting oral cancer in Taiwan (37). Earlier reports have demonstrated consistently that a synergistic increase in risk of oral cancer has been shown among people who chew areca quid and smoke cigarettes (38–40).

The results of the present study indicate that arecoline participates in the pathogenesis of areca quid chewing-related oral mucosal lesions, possibly through cytotoxic mechanisms. Arecoline has been detected in saliva obtained during chewing in concentrations up to about 140 μg/ml (36). From the present study, it was cytotoxic at the concentration of 50 μg/ml, comparable to the saliva levels of arecoline achieved during areca quid chewing. In addition, 2.5 mM nicotine, which is lower than the concentration of nicotine detected in the saliva (as high as 9.62 mM) during smoking (41), acts synergistically on arecoline-induced cytotoxicity. Thus, daily consumption of milligrams of arecoline or nicotine, culminating in a cumulative dose, should be considered for its adverse effects. We suggest that people who combine the habits of areca quid chewing and cigarette smoking are more susceptible to oral mucosal damage than people who chew areca quid alone. Further studies will help address the contribution of various areca nut constituents, as well as tobacco products, in areca quid chewing-related oral mucosal lesions.

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