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Adverse effects of arecoline and nicotine on human periodontal ligament fibroblasts in vitro


Abstract
Background, aims: The habit of betel nut chewing impinges on the daily lives of approximately 200 million people. Betel quid chewers have a higher prevalence of periodontal diseases than non-chewers. This study examined the pathobiological effects of arecoline, a major component of the betel nut alkaloids, on human periodontal ligament fibroblasts (PDLF) in vitro.

Method: Cell viability, proliferation, protein synthesis, and cellular thiol levels were used to investigate the effects of human PDLF exposed to arecoline levels of 0 to 200 µg/ml. In addition, nicotine was added to test how it modulated the effects of arecoline.

Results: Arecoline significantly inhibited cell proliferation in a dose-dependent manner. At concentrations of 10 and 30 µg/ml, arecoline suppressed the growth of PDLF by 20% and 50% (p<0.05), respectively. Arecoline also decreased protein synthesis in a dose-dependent manner during a 24-h culture period. A 100 µg/ml concentration level of arecoline significantly inhibited protein synthesis to only 50% of that in the untreated control (p<0.05). Moreover, arecoline significantly depleted intracellular thiols in a dose-dependent manner. At concentrations of 25 µg/ml and 100 µg/ml, arecoline depleted about 18% and 56% of thiols (p<0.05), respectively. This suggests that arecoline itself might augment the destruction of periodontium associated with betel nut use. Furthermore, the addition of nicotine acted with a synergistic effect on the arecoline-induced cytotoxicity. At a concentration of 60 µg/ml, arecoline suppressed the growth of PDLF by about 33%, and 5 mM nicotine enhanced the arecoline-induced cytotoxic response to cause about 66% cell death.

Conclusion: During thiol depletion, arecoline may render human PDLF more vulnerable to reactive agents within cigarettes. Taken together, people who combine habits of betel nut chewing with cigarette smoking could be more susceptible to periodontium damage than betel nut chewing alone.

Key words: betel nut; arecoline/adverse effects; nicotine; fibroblasts; periodontium

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Betel nut chewing, like cigarette smoking, is a popular oral habit in Taiwan, India and Southwest Asian countries (IARC 1986). It is estimated that at least 200 million people practice betel nut chewing worldwide (Barton-Bradley 1979). Epidemiological studies have clearly associated betel nut chewing with oral mucosal lesions (IARC 1986). However, the role of betel nut chewing in the etiology of periodontal diseases is not clear. Early studies have shown a higher prevalence of gingivitis and periodontal diseases among betel nut chewers than non-chewers (Mehta et al. 1955). In addition, Waerhaug (1967) found increasing amounts of plaque and debris in betel nut chewers. While much evidence suggests a detrimental relationship between betel nut use and periodontal health; the actual nature of the relationship in periodontal therapy still remains unclear.

Betel nut contains a complex mixture of substances including alkaloids as well as polyphenols (Mujumder et al. 1979, IARC 1986). Cellular responses to these substances vary widely and may relate to specific components of the betel nut. Many of the undesirable ef-
fects of betel nut have been attributed to arecoline, a major component of betel nut alkaloid (Jeng et al. 1994, Chang M C et al. 1998). Arecoline has been detected in saliva obtained during betel nut chewing in concentrations of up to 140 μg/ml (Nair et al. 1985). In vitro, arecoline is a cytotoxic agent to human oral epithelial cells (Sundqvist et al. 1989, Jeng et al. 1999) and fibroblasts (Jeng et al. 1994, Jeng et al. 1996, Chang Y C et al. 1998, 1999).

In recent decades, several studies have shown that the cell type most likely to provide a major contribution to periodontal regeneration is the human periodontal ligament fibroblast (PDLF) (Boyko et al. 1981, Egelberg 1987). It was suggested that any factor able to inhibit the functions of such cells, would also impair tissue repair and regeneration. The potential toxicological implications of arecoline on PDLF remain to be elucidated. The objective of the study was undertaken to address the cytotoxic effects and possible mechanisms of arecoline on human PDLF. In Taiwan, there are two million people who have the betel nut chewing habit and about 86% of the chewers are also smokers (Ko et al. 1992). Furthermore, nicotine, a reaction product from cigarette smoking, was added to test how it modulated the cytotoxicity of arecoline on human PDLF.

Material and Methods

Cell Culture

Fibroblasts were obtained from the periodontal ligament of premolar teeth from three patients undergoing extraction for orthodontic reasons. After extraction, the teeth were placed in Petri dishes containing Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO; Grand Island, NY, USA) and 100 units of penicillin and 100 μg of streptomycin/ml. To avoid contamination from gingiva, the periodontal ligament was carefully removed from the middle third of the root by scalpel. The fragments were grown in DMEM supplemented with 10% fetal calf serum (FCS) and antibiotics. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Confluent cultures of periodontal ligament cells were obtained at 14 to 21 days. Confluent cell layers were treated with 0.25% trypsin and 0.05% EDTA for 5 min; aliquots of separated cells were subcultured and then used between the 3rd to 8th passages.

Cell proliferation assay

PDLF were seeded into 6-well culture plates (GIBCO; Grand Island, NY, USA) with 0–200 μg/ml arecoline in DMEM. Arecoline (Sigma; St. Louis, MO, USA), in medium was prepared from a 1 mg/ml stock solution of pure arecoline in distilled water, which was prepared fresh prior to each experiment. [3H]-thymidine incorporation into cellular DNA was used as a measure of cell proliferation. The cells were plated at an initial density of 5×10^4 cells/well into 6-well culture plates. After overnight attachment, [methyl-3H]-thymidine (Amersham, Buckinghamshire, UK) (0.5 μCi/ml) was added, and cells were exposed to various concentrations of arecoline for 4 days. Finally, the radioactive medium was discarded and cells were washed three times with 5% trichloroacetic acid at 4°C. Cells were solubilized with 1 ml of 0.1 N NaOH for 15 min at room temperature. Aliquots of the cell lysates were counted in a liquid scintillation counter (Packard model 2100TR; USA).

Protein synthesis

Protein synthesis was measured in 24-well culture plates (GIBCO; Grand Island, NY, USA). Each well was incubated with 5×10^4 cells in 1 ml of DMEM with 10% FCS and incubated overnight before replacement with 1 ml fresh medium containing 2% FCS and various concentrations of arecoline. The rate of protein synthesis was estimated by the incorporation of [3H]-leucine (Amersham, Buckinghamshire, UK). After labeling with triated leucine for 24 h, the radioactive medium was decanted. Monolayer cells were removed by trypsinization, isolated by centrifugation and cellular proteins were precipitated with 5 ml of cold 5% trichloroacetic acid. The final precipitates were each dissolved in 0.5 ml 2 M NH4OH and transferred to scintillation vial inserts containing 3 ml of a scintillation cocktail. Radioactivity was measured in a liquid scintillation counter (Packard model 2100TR; USA) and results were expressed as a percent inhibition of leucine incorporation.

Assay for cellular thiols

For measurement of cellular thiol levels, fibroblasts were incubated at a concentration of 1×10^6 cells/ml with different concentrations of arecoline for 24 h. Intracellular thiols were determined by adding 1 ml 5% perchloric acid containing 2.5 mM phenanthroline (Sigma; St. Louis, MO, USA) to each petri dish. The plates were then scraped and homogenates were centrifuged at 10,000 g for 10 min. The acid-soluble thiols were in the supernatant fraction and the pellets were used for protein assay. The acid-soluble thiols were measured by the high liquid performance chromatography (HPLC) method as described by Reed et al. (1980). The amounts of thiols were expressed as nmol/mg protein.

Effects of nicotine on arecoline-induced cytotoxicity

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay was developed to monitor mammalian cell survival and proliferation. The MTT assay was measured by dehydrogenase activity as described by Mosmann (1983), with minor modification (Alley et al. 1988). The MTT assay was used to evaluate the cytotoxicity of nicotine on human PDLF as described previously during a 2 h, 6 h or 24 h incubation period. Subsequently, nicotine was added to wells to test its synergistic effect. Briefly, cells were incubated with arecoline for 24 h then with added nicotine (Sigma; St. Louis, MO, USA) for 4 h. The MTT (Sigma; St. Louis, MO, USA) solution was prepared in 5 mg/ml of phosphate-buffered saline just before use and filtered through a 0.22 μm filter. Cells were seeded 5×10^4 cells/well into a 24-well culture plates and incubated to attach for 24 h. After a 24-h incubation in various concentrations of arecoline, 10 μl of MTT solution was added to each well. Plates were incubated in a CO2 incubator for 4 h, then the medium was discarded by inverting and tapping the plates, and 100 μl of dimethyl sulfoxide was added to each well. The spectrophotometric absorbance at 540 nm was then measured by an ELISA reader (Hitachi, U2000, Tokyo, Japan). The % of dehydrogenase activity at each concentration, compared with that of the control, was calculated from the absorbance values.

Statistical analysis

TriPLICATE or more separate experiments were performed throughout this study.
The significance of the results obtained from control and treated groups was statistically analyzed by the paired Student t-test. A p-value of <0.05 was considered to be statistically significant.

Results

Fig. 1 shows the effects of arecoline on incorporation of [3H]-thymidine for cell proliferation. Arecoline inhibited the DNA synthesis of PDLF in a dose-dependent manner (p<0.05) and was cytotoxic at concentration levels >10 μg/ml. Arecoline at the concentration level of 30 μg/ml inhibited DNA synthesis to only 50% of that in the untreated control. Elevating the arecoline concentration to 60 μg/ml almost completely inhibited DNA synthesis.

Effects of arecoline on protein synthesis of human PDLF are shown in Fig. 2. Arecoline inhibited protein synthesis at 25 μg/ml and higher concentrations in a dose-dependent manner, as determined by [3H]-leucine incorporation. A 100 μg/ml concentration level of arecoline significantly inhibited the protein synthesis to only 50% of that in the untreated control.

Fig. 3. Depletion of intracellular thiols in human PDLF after exposure to various concentrations of arecoline for 24 h. The intracellular thiols were expressed as nmol/mg protein. All values are shown as mean ±SD. *, ** Significant differences from control values with p<0.05 and p<0.001, respectively.

The status of thiols in PDLF was determined by the HPLC method (Fig. 3). In this study, arecoline significantly depleted intracellular thiols in a dose-dependent manner. At the concentrations of 25 μg/ml and 100 μg/ml, arecoline depleted about 18% and 56% of thiols, respectively. Fibroblasts in the control group maintained their original thiol levels during the incubation period.

Cytotoxic effects of nicotine on human PDLF by MTT assay is shown in Fig. 4. At a concentration higher than 5 mM, nicotine was cytotoxic to human PDLF in a concentration- and time-dependent manner. Nicotine at a concentration of 5 mM caused about 15% of cell death over the 4 h incubation period (Fig. 5). Human PDLF incubation with various concentrations of arecoline for 24 h showed synergistic cytotoxic effects upon addition of 5 mM nicotine for 4 h (Fig. 5). Arecoline at a concentration of 60 μg/ml caused about 33% of cell death over the 24 h incubation period. However, nicotine enhanced the cytotoxic response to cause about 66% of cell death on arecoline-induced cytotoxicity.

Discussion

Betel nut and some of its components such as arecoline, therefore, may contribute to the development of periodontal diseases by its direct effects on a variety of host maintenance and defense systems. Normal fibroblast func-
tion is crucial for maintenance of periodontal health. Previous studies have shown that betel quid and arecoline could inhibit human gingival fibroblast growth, proliferation and collagen synthesis in vitro (Jeng et al. 1996, Chang Y C et al. 1998, 1999). As with other tissue responses, normal fibroblast function is critical for the maintenance of periodontal tissues and for optimal wound healing responses. Although arecoline has been found to have adverse effects on many types of cells, including gingival fibroblasts, differences between PDLF and gingival fibroblasts do exist. Mariotti & Cochran (1990) have reported that PDLF appear to be more differentiated and are responsible for normal maintenance of the periodontal ligament, remodeling of adjacent bone and cementum, and repair and the actual regeneration of the periodontal ligament. Both of the cell types exhibit variations in growth rate, but their doubling times are similar and they also synthesize collagen type I and III (Hou & Yeager 1993). Furthermore, a previous study demonstrated that gingival fibroblasts lack the ability to stimulate reattachment to the root surface (Nyman et al. 1980). Differences in response of both cell types may be an important factor in periodontal wound healing, and should be considered by clinicians. In addition, earlier in vivo studies have shown that the cell type most likely to provide a major contribution to periodontal regeneration is the PDLF (Boyko et al. 1981, Egelberg 1987).

In the present study, we have focused on the effects of arecoline and the role it could play in periodontal breakdown via its direct effects on human PDLF. We found that arecoline inhibits cell growth, proliferation and protein synthesis on human PDLF. The ultimate goal in periodontal therapy is the attachment and regeneration of the attachment apparatus (Diem et al. 1974, Pitaru et al. 1984). Studies have suggested that cell growth, proliferation and matrix syntheses of fibroblasts are necessary for regeneration of a connective tissue attachment (Boyko et al. 1980, 1981, MacNeil & Somerman 1993). Therefore a direct toxic effect on host cells can retard periodontal wound healing during periodontal therapy. This will impair the reparative and regenerative potential of periodontal tissues of those who chew betel quid.

Thiols have been known to participate in numerous and diverse cellular processes, such as, in detoxification, in drug metabolism, and in nucleic acid and protein synthesis. In this study, arecoline in a dose-dependent manner significantly decreased the intracellular thiol levels. Since arecoline is reported to react with free thiols using chromatography in vitro (Boylan & Nery 1969, Sundqvist et al. 1989) and in vivo (Boylan & Nery 1969), formation of conjugates is a possible mechanism for thiol depletion in arecoline-treated human PDLF. Thiols are involved in a variety of intercellular functions and also play an important role in the cellular defense against many reactive foreign compounds (Kosower & Kosower 1978, Shaw & Chou 1986). Thiol depletion might render the cells more vulnerable to other reactive agents present in dental plaque from betel nut chewing.

Cigarette smoking is a risk factor for periodontitis. Many of the undesirable effects of smoking have been attributed to nicotine. Earlier studies have shown that nicotine was a cytotoxic agent on fibroblasts derived from periodontium (Hanes et al. 1991, Peacock et al. 1993, Tipton et al. 1995, Alpar et al. 1998, Giannopoulou et al. 1999, James et al. 1999, Checchi et al. 1999). In this study, nicotine was also found to exert cytotoxicity on human PDLF in a concentration- and time-dependent manner. The cytotoxic nature of nicotine on cultured cells has clearly been shown.

Betel chewing is a popular, islandwide habit in Taiwan and the number of users was estimated to be two million; among these chewers, 86% were also smokers (Ko et al. 1992). Little is known about the association between betel chewing and cigarette smoking. In this present study, nicotine had a synergistic effect on arecoline-induced cytotoxicity on human PDLF. During thiol depletion, arecoline may render the cells more vulnerable to reactive agents within cigarettes. This result might partly explain why patients who combined the habits of betel quid chewing with cigarette smoking took a great risk of contracting periodontitis in Taiwan (Wu et al. 1998).

The health of the periodontium depends, among other things, on normal functions of the immune system and gingival epithelial and connective tissue cells. A previous study has suggested that cell growth, proliferation and matrix synthesis play an important role in periodontal wound healing as well as regeneration (MacNeil & Somerman 1993). Impairment of any factors might alter the normal metabolism of periodontal tissues as well as periodontal therapy. In this study, it was found that the concentration level of arecoline in the oral cavity during betel nut chewing could easily reach the effective cytotoxic level. This indicated that betel nut chewing might be another risk factor in the pathogenesis of periodontal diseases. In addition, nicotine had a synergistic effect on arecoline-induced cytotoxicity. Thus, those who combine habits of betel nut chewing with cigarette smoking could be more susceptible to periodontal damage than betel nut chewing alone. However, the pathological mechanism of periodontal diseases triggered by various betel nut constituents as well as tobacco products might be one important issue for advanced studies.

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Zusammenfassung
Ungünstige Auswirkung von Arekolin und Nikotin auf menschliche Desmodontalfibroblasten in vitro.


Ergebnisse: Arekolin hemmt die Zellproliferation signifikant in dosisabhängiger Weise. Bei Konzentrationen von 10 und 30 μg/ml unterdrückt Arekolin das Wachstum der PDLF um 20% bzw. 50% (p<0.05). Arekolin unterdrückt ebenfalls dosisabhängig die Proteinsynthese während der 24-stündigen Kultivierungsperiode. Ein Arekolinspiegel von 100 μg/ml reduzierte die Proteinsynthese auf 50% im Vergleich zur unbehandelten Kontrollkulturn (p<0.05). Auch die intrazellulären Thiolspiegel wurden dosisabhängig reduziert. Bei
Konzentrationen von 25 und 100 µg/ml wurden die Thioyangie um 18% bzw. 56% reduziert (p<0.05). Bei einer Konzentration von 60 µg/ml unterdrückte das Arecolin das PDLF-Wachstum um 33%. Die Zugabe von 5 mM Nikotin verstärkte die durch Arecolin induzierte zytotoxische Wirkung, so daß es zum Zelltod von 66% kam.

Schlußfolgerungen: Es scheint, daß Arecolin selbst zu der Schädigung des Parodonts beiträgt, die der Betelnuß zugeschrieben wird. Außerdem deuten die Ergebnisse darauf hin, daß Personen, die Betelnüßkauen mit Nikotinkonsum kombinieren, empfindlicher für Schädigungen des Parodonts sind als solche, die nur Betelnüsse kauen. Während der Inkubation des Thiois könnte das Arecolin PDLF verletzlicher für andere reaktive Substanzen wie Nikotin machen.

Résumé
Effets adverses de l’arécoline et de la nicotine sur les fibroblastes du ligament parodontal humain in vitro

L’habitude de mastiquer de la noix de betel affecte la vie quotidienne de près de 200 millions de personnes. Les mâchoires de bérets présentent une prévalence plus élevée de maladies parodontales. Cette étude examine les effets pathologiques de l’arécoline, un composant majeur des alcaloïdes de la noix de betel, sur des fibroblastes du ligament parodontal humain (PDLF) in vitro. La viabilité cellulaire, la prolifération, la synthèse protéique et les niveaux cellulaires de thiol ont été utilisés pour observer les effets de l’exposition à des doses d’arécoline de 0 à 200 µg/ml. De plus, de nicotine fut ajouté pour tester la façon dont cela modulait les effets de l’arécoline. L’arécoline inhibait significativement la prolifération cellulaire de façon dose dépendante. À des concentrations de 10 à 30 µg/ml, l’arécoline supprimait la croissance des fibroblastes par 20 et 50% (p<0.05), respectivement. L’arécoline diminuait également la synthèse des protéines de façon dose dépendante pendant une période de culture de 24 h. Une concentration de 100 µg/ml d’arécoline inhibait la synthèse protéique à seulement 50% de celle du groupe contrôle non traité (p<0.05). De plus, l’arécoline réduit les thiol intracellulaires de façon dose dépendante. À des concentrations de 25 µg/ml et 100 µg/ml, l’arécoline réduit environ 18 à 56% des thiol, respectivement (p<0.05). Cela suggère que l’arécoline, elle même, peut augmenter la destruction du parodonte en association avec l’utilisation de noix de betel. De plus, l’addition de nicotine entraînaient un effet synergique sur la cytotoxicité induite par l’arécoline. A une concentration de 60 µg/ml, l’arécoline supprimait la croissance des PDLF d’environ 33% et 5 mM de nicotine augmentait cette réponse cytotoxicité induite par l’arécoline, jusqu’à entrainer 66% de morts cellulaires. Lors de la réduction des thiol, l’arécoline pourrait rendre les PDLF humains plus vulnérables à des agents réactifs entrant dans la composition des cigarettes. Pris ensemble, les gens qui combinent des habitudes de mastication de noix de betel et de tabagisme, pourrait être plus susceptibles à des dommages parodontaux, que les gens qui utiliserait uniquement la noix de betel, mais sans fumer.

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