Arecoline inhibits endothelial cell growth and migration and the attachment to mononuclear cells

Shuei-Kuen Tseng\textsuperscript{a,b,}\textsuperscript{1}, Mei-Chi Chang\textsuperscript{c,}\textsuperscript{2}, Ming-Lun Hsu\textsuperscript{a,}\textsuperscript{1}, Cheng-Yao Su\textsuperscript{a,}, Lin-Yang Chi\textsuperscript{a,}, Wen-Chien Lan\textsuperscript{d,}, Jiing-Huei Jeng\textsuperscript{b,}\textsuperscript{e,}\textsuperscript{*}

\textsuperscript{a} School of Dentistry, National Yang-Ming University, Taipei, Taiwan
\textsuperscript{b} Department of Dentistry, National Taiwan University Hospital, Taipei, Taiwan
\textsuperscript{c} Biomedical Science Team, Chang Gung University of Science and Technology, Taoyuan, Taiwan
\textsuperscript{d} School of Dentistry, Taipei Medical University, Taipei, Taiwan
\textsuperscript{e} Graduate Institute of Clinical Dentistry, National Taiwan University Medical College, Taipei, Taiwan

Received 22 May 2012; Final revision received 14 December 2012
Available online 10 June 2013

**KEYWORDS**
arecoline; betel quid; cardiovascular diseases; endothelial cells; oral cancer; oral submucous fibrosis

**Abstract**

\textbf{Background/purpose:} Betel quid (BQ) chewing is a popular habit in South-Asian and Southeast Asian countries, and Taiwan. BQ chewing can cause oral cancer and oral submucous fibrosis, and increases the risk of cardiovascular diseases. However, how BQ chewing affects endothelial cells and is involved in cardiovascular diseases and vascular changes is not fully understood. The effects of arecoline, a component of BQ, on the growth and migration of endothelial cells (EAhy 926), and their adherence by U937 cells were investigated.

\textbf{Materials and methods:} EAhy926 endothelial cells were cultured and exposed to various concentrations of arecoline for 24 hours. Morphological changes were observed using phase-contrast microscopy. Cytotoxic effects were analyzed using a 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay. A wound closure assay was used to evaluate the cellular migration of EAhy926 cells. The attachment of \( 2',7'-\text{bis}(2\text{-carboxyethyl})-5\text{-}(6\text{-carboxyfluorescein}) \) labeled U937 cells to EAhy926 endothelial cells that were pretreated with various concentrations of arecoline was further studied.

\textbf{Results:} The addition of arecoline at concentrations >0.4 mM significantly decreased cellular viability. EAhy endothelial cells showed marked morphological changes, and cellular migration
Introduction

Betel quid (BQ) chewing is a popular oral habit in India, Sri Lanka, Southeast Asian countries, and Taiwan.1–3 The prevalence rate of the BQ chewing habit in Taiwan is estimated to be about 10%; i.e. 2.0–2.8 million people have experience of this habit.4 BQ is usually comprised of an areca nut (AN, Areca catechu), slaked lime (calcium hydroxide) inside the betel leaf (Piper betle leaf), and catechu, with or without tobacco.5 BQ chewing has become the fourth most common oral habit in the world.3 In 2003, the International Agency for Research on Cancer announced that BQ and AN were confirmed to be carcinogens.6 This is because the BQ chewing habit shows an intimate relationship with the occurrence of oral cancer, oral leukoplakia, and oral submucous fibrosis (OSF).2,3,7,8

BQ chewing increases the risk of cardiac arrhythmias, sinus tachycardia,9 and cardiac dysrhythmias.10 BQ chewing was shown to cause myocardial infarction, and arecoline is considered to be a possible contributing factor to coronary artery spasms due to its parasympathomimetic effects on the vascular endothelium.11 The concentration of arecoline in the ripe AN extract was 9.1 mg/g and up to about 140 mg/L in the saliva during BQ chewing.12 Arecoline, as a major component of ANs, is a cholinergic alkaloid with the ability to stimulate the central nervous system.13 Interestingly, while the vascular density increased in the early stage of OSF, the number of blood vessels obviously decreased in the middle and advanced stages of OSF.14 Accordingly, the distribution of vascular endothelial cells in the juxtaepithelial region of OSF tissues decreased as observed histologically.15

The recruitment, migration, and adhesion of monocytes to endothelial cells are early steps in many inflammatory disorders including atherosclerosis.16 The adhesion of monocytes to the vascular endothelium and their subsequent migration into the vessel wall are early events in this disease process. In this study, we used EAhy 926 endothelial (EAHY) cells,17 derived from the fusion of human umbilical vein endothelial cells with a lung adenocarcinoma hybrid cell line. EAHY cells express the characteristics of human vascular endothelial cells.18

We recently demonstrated that arecoline inhibited the proliferation of EAHY cells. Long-term exposure to arecoline may potentially damage the vascular endothelium. These results may lead to vascular changes and cytotoxic effects and potentially contribute to BQ chewing-related cardiovascular diseases (CVDs).19 However, the precise factors and reasons responsible for the vascular changes in OSF and the increased risk of CVD mortality with the BQ chewing habit are still not fully clear. In this study, we propose that arecoline may contribute to CVDs and oral mucosal alterations by causing endothelial cell damage and dysfunction. We therefore evaluated the effects of arecoline in inhibiting the growth and migration of human endothelial cells and their attachment to U937 mononuclear cells.

Materials and methods

Chemicals

Arecoline hydrobromide, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), and dimethyl sulf oxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS) penicillin/streptomycin, trypsin/EDTA, phosphate-buffered saline (PBS), glutamine, trypsin blue, and RPMI 1640 culture medium were obtained from Gibco (Life Technologies, Grand Island, NY, USA). Molecular Probes (Eugene, OR, USA) supplied 2’,7’-Bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein acetoxy methyl ester (BCECF-AM).

Culture of EAHY and U937 cells

EAHY cells were derived from a human endothelial hybrid cell line. They were kindly provided by Dr Cora-Jean S. Edgell (University of North Carolina, Chapel Hill, NC, USA)17 and cultured in DMEM containing 10% FBS and 5% CO2 at 37 °C. Human monocytic U937 cells were a kind gift from Professor Shan-Ling Hung (Dental School, National Yang Ming University, Taipei, Taiwan). They were maintained in RPMI 1640 medium containing 10% FBS and 5% CO2 at 37 °C in an incubator. U937 cells were cultured in suspension in plastic culture flasks. Cells were split with fresh media 1: 5 every 3–4 days. The viability of U937 cells was examined by a trypan blue dye exclusion method and found to exceed 95%.

Effects of arecoline on the growth of EAHY cells

Viable cell numbers were measured by a modified MTT assay.20 Briefly, EAHY cells were seeded at an initial density of 5 × 10^5 cells/well in a 6-well plate for 24 hours. Cells were then exposed to fresh medium containing various concentrations of arecoline (0 mM, 0.1 mM, 0.2 mM, 0.4 mM, and 0.8 mM) for 1 day or 5 days. MTT (at a final concentration of 0.5 g/L) was then added to the wells and decreased after 24 hours and 48 hours of exposure to arecoline. The number of U937 cells attached to EAhy 926 cells increased when endothelial cells were pretreated by arecoline.

Conclusion: Arecoline impaired vascular endothelial cells by inhibiting their growth and migration and their adhesion to U937 mononuclear cells. These results reveal that arecoline may contribute to the pathogenesis of oral submucous fibrosis and cardiovascular diseases by affecting endothelial cell function in BQ chewers.
cultured for a further 3 hours. The culture medium was decanted. The insoluble formazan produced by viable cells was dissolved in DMSO and read against a blank (DMSO) at OD540 using a plate reader (Dynatech Microwell, Dynatech, Alexandria, VA, USA) Results are expressed as MTT reduction (mean ± standard error of mean as a percent of the control).

Effects of arecoline on the migration of EAHY cells

EAHY cells were cultured on six-well plates at a concentration of 5 × 10⁵ cells/well and cultured for 48 hours. The cell layer on the plates was scratched with a 100-μL pipette tip. The wounded areas were marked at the start time and photographed with a microscope (Olympus TH4-100 and Olympus IX71; Olympus America Inc., Center Valley, PA, USA). EAHY cells were then exposed to medium with/without different concentrations of arecoline (0 mM, 0.1 mM, 0.2 mM, and 0.4 mM) for 16 hours and 24 hours. Cells were photographed at the same sites for comparison.

Effects of arecoline treatment of EAHY cells on the adhesion to U937 cells

An adhesion study of U937 cells to endothelial cells was performed using an established model. Briefly, U937 cells were labeled with the membrane-permeable dye, BCECF-AM (3 μM), in RPMI-1640 for 30 minutes in a 37 °C incubator. Cells were then centrifuged and resuspended in serum-free medium before use. Confluent EAHY cells grown in six-well tissue culture plates were treated with various concentrations of arecoline (0 mM, 0.01 mM, 0.05 mM, 0.1 mM, 0.2 mM, and 0.4 mM) for 24 hours. Control wells received only vehicle and were used as the negative control. After arecoline stimulation, EAHY cells were washed with PBS, and then 4 × 10⁵ BCECF-labeled U937 cells/well were added and allowed to attach at 37 °C for 30 minutes. Thereafter, the medium was decanted, and cells were washed with RPMI-1640 medium. Unbound U937 cells were removed by washing with serum-free medium, and attached U937 cells (green fluorescence) were observed and photographed under a fluorescent microscope (Olympus TH4-100 and Olympus IX71; Olympus America).

For quantification of the fluorescence of attached BCECF-labeled U937 cells, the emitted BCECF fluorescence of cultured wells was measured with a Gemini XPS Microplate Spectrofluorometer (Molecular Devices, North America, Sunnyvale, CA, USA). The fluorescence of BCECF-labeled U937 cells attached to untreated EAHY cells was used as the control.

Statistical analysis

At least three or more separate experiments were performed for all tests. Results were analyzed by one-way analysis of variance (ANOVA) and post hoc Tukey’s test to check differences between groups. A P value of <0.05 was considered to indicate a statistically significant difference.

Figure 1  (A) Morphology of untreated EAHY cells after 24 hours of culture. Cell growth had reached confluence. EAHY cells were cuboid or spindle-shaped in appearance. (B) Morphology of EAHY cells after exposure to 0.4 mM arecoline for 24 hours. Some EAHY cells had become retracted and rounded after exposure to 0.4 mM arecoline for 24 hours. (C) Morphology of EAHY cells after 5 days of culture. (D) Morphology of EAHY cells after exposure to 0.2 mM arecoline for 5 days. The cell density had decreased, and some floating cells were noted (100×, original magnification).
Results

Morphological changes to EAHY cells

Cultured EAHY endothelial cells were generally spindle or cuboid in appearance. (Fig. 1A) Some of the EAHY cells became retracted and lost the extended cellular processes. Some cells were rounded and had even detached from the culture plate after exposure to 0.4 mM arecoline for 24 hours (Fig. 1B). EAHY cells were confluent after 72 hours in culture (Fig. 1C). After 72 hours in culture, even 0.2 mM arecoline induced morphologic changes in EAHY cells with a decrease in the cell density and some floating cells being noted (Fig. 1D).

Cytotoxicity of arecoline to EAHY endothelial cells

Quantitatively, 24 hours of exposure to arecoline was cytotoxic and decreased the number of viable EAHY cells by 32% at a concentration of 0.8 mM as analyzed by the MTT assay (Fig. 2A). After incubation for 5 days, arecoline at concentrations of 0.2–0.8 mM caused a further decline in viable cells to <20% of the control (Fig. 2B).

Arecoline inhibits the migration of EAHY cells

A wound closure assay was used to evaluate EAHY cell migration. The migration of EAHY cells toward the wounded area was noted in the control culture after 16 hours. The wound had almost closed after 24 hours of incubation (Fig. 3A). Similarly, EAHY cells migrated to the wound area after exposure to 0.1 mM arecoline for 16 hours and 24 hours, but the extent of wound closure was less than that of the control (Fig. 3B). At a concentration of 0.2 mM, arecoline further attenuated the wound closure activity by EAHY cells (Fig. 3C). Marked cytotoxicity was noted when cells were exposed to 0.4 mM arecoline (data not shown).

Figure 2  Cytotoxicity of arecoline to EAHY endothelial cells as estimated by an MTT assay. (A) Viable cell numbers (% of control) after exposure of EAHY cells to arecoline for 24 hours (n = 5). (B) Viable cell numbers (% of control) after exposure of EAHY cells to arecoline for 5 days (n = 3). * Denotes a significant difference compared to the untreated group (P < 0.05).

Figure 3  Closure of a wound by EAHY cells photographed initially (0 hours) and after 16 hours and 24 hours of incubation with or without arecoline. (A) Control cells, (B) 0.1 mM arecoline. Compared to the control, fewer EAHY cells had migrated into the wounded area after exposure to 0.1 mM arecoline for 16 hours and 24 hours. (C) Arecoline at 0.2 mM. The migration of EAHY cells into the wounded area was markedly inhibited. Dotted lines (- - -) indicate the original wounded area. (40×, original magnification).
Arecoline enhances the adhesion of U937 cells to EAHY cells

Treatment of EAHY cells with arecoline enhanced their attachment to BCECF-labeled U937 mononuclear cells. As observed under fluorescence microscopy, BCECF-labeled U937 cells showed green fluorescence. Some U937 cells may have attached to untreated EAHY cells (Fig. 4A). Exposure of EAHY cells to 0.1 mM and 0.4 mM arecoline provided a cell layer for the attachment of U937 cells, as indicated by an increase in the number of attached U937 cells (green fluorescence) under fluorescence microscopy (Fig. 4B,C).

Quantitatively, exposure of EAHY cells to arecoline promoted the adhesion of U937 mononuclear cells with a maximal stimulation at a concentration of 0.1 mM (P < 0.05). The fluorescence of culture wells increased from 287 relative fluorescent units (in the control) to 477 relative fluorescent units (in the 0.1 mM arecoline-treated EAHY cell group; Fig. 5). No further increase was noted when the concentrations of arecoline were elevated to 0.2 mM and 0.4 mM, possibly due to cytotoxicity.

Discussion

In the present study, we clearly demonstrated that arecoline is cytotoxic to EAHY endothelial cells in a dose-dependent manner. EAHY cells showed morphological changes and detached from the culture dish after exposure to 0.4–0.8 mM arecoline. Viabilities of EAHY cells were 88% and 68% of the control, respectively. Arecoline inhibited the migration of EAHY cells, but increased the adhesion of U937 cells to EAHY cells. Effects of arecoline on the cytotoxicity, migration, and mononuclear cell adhesion to endothelial cells may contribute to the pathogenesis of periodontal disease, OSF, and BQ chewing-related CVDs via toxicity to oral or systemic endothelial cells, leading to the impairment of vascular function. Arecoline inhibited the growth, attachment, spread, and collagen synthesis of human gingival fibroblasts (GFs). Chang et al also demonstrated that arecoline inhibited the proliferation and collagen synthesis of human GFs. Arecoline significantly inhibited human periodontal ligament fibroblast proliferation, decreased protein synthesis, and significantly depleted intracellular thiols. Moreover, arecoline induced cell cycle deregulation and apoptosis in GFs. Arecoline may suppress mitochondrial activation, deplete intracellular thiols, and influence the cell function of GFs. Accordingly, arecoline was cytotoxic to endothelial cells in this study.
Endothelial cell damage was shown to promote thrombosis and increase vascular permeability that is crucial for tissue inflammation. This result suggests that, during BQ chewing, endothelial cell damage may be induced by BQ components, leading to oral mucosal inflammatory responses, and inflammation of the vasculature may increase incidences of atherosclerosis, thrombosis, hypertension, and other CVDs. 28–31

Nair et al examined the salivary arecoline content and found it to be 0–89.8 mg/L among BQ chewers, with an average of 29.69 mg/L. 12 AN components and arecoline may stimulate glucagon and catecholamine secretions which contribute to diabetes and CVDs. 32 Arecoline directed leukocyte migration and exhibited an inhibitory effect on the functions of neutrophils at high concentrations.33 Endothelial migration is important for endothelial repair after arterial injury,34 and this event is impaired during aging and diseased conditions such as oxidative stress, atherosclerosis, diabetes mellitus, prehypertension, etc. 35–37 In this study, we found that arecoline at 0.1–0.2 mM may have adversely affected endothelial migration. This might partly explain why BQ chewing elevates oxidative stress and increases the risk of atherosclerosis, diabetes mellitus, hypertension, and heart disease in epidemiological studies.38,39

Atherosclerosis is a chronic low-grade inflammatory disorder.40 Activated endothelial cells secrete interleukin-8, an inflammatory cytokine that can induce adhesion molecules.41 The adhesion of monocytes to the vascular endothelium and their subsequent migration into the vessel wall may change the integrity and are pivotal early events in tissue inflammation and CVDs.42 These migrating inflammatory cells include monocytes, macrophages, neutrophils, and lymphocytes.43 These inflammatory cells may further generate various mediators and growth factors involved in the pathogenesis of vascular diseases. Interestingly, we found that arecoline treatment of endothelial cells promotes their attachment to U937 mononuclear cells as observed under fluorescence microscopy and quantification of attached fluorescent-labeled U937 mononuclear cells. Chang et al pointed out that abnormal growth of attached fluorescent-labeled U937 mononuclear cells promotes their attachment to U937 mononuclear cells.42 These results suggest that arecoline may be involved in the disease processes of atherosclerosis and oral mucosal diseases by inducing vascular changes and tissue inflammation.

In conclusion, arecoline may inhibit the growth and migration of human endothelial cells. Exposure to arecoline potentially damages the vascular endothelium and induces tissue inflammation via increasing the attachment to U937 monocytes. These effects of arecoline on endothelial cells may be associated with BQ chewing-related CVDs, OSF, and periodontal diseases.

Acknowledgments

This study was supported by a grant from the National Science Council, Taiwan (NSC97-2320-B-255-001-MY3).

References


