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ORIGINAL ARTICLE

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

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Abstract *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.
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′-bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein-labeled U937 cells to EAhy926 endothelial cells that were pretreated with various concentrations of arecoline was further studied.
Results: The addition of arecoline at concentrations >0.4 mM significantly decreased cellular viability. EAhy endothelial cells showed marked morphological changes, and cellular migration

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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.

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 sulfoxide (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, trypan 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% CO_2 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% CO_2 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