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ORIGINAL ARTICLE

Arecoline induced cell cycle arrest, apoptosis, and cytotoxicity to human endothelial cells

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Abstract Betel quid (BQ) chewing is a common oral habit in South Asia and Taiwan. BQ consumption may increase the risk of oral squamous cell carcinoma (OSCC), oral submucous fibrosis (OSF), and periodontitis as well as systemic diseases (atherosclerosis, hypertension, etc.). However, little is known about the toxic effect of BQ components on endothelial cells that play important roles for angiogenesis, carcinogenesis, tissue fibrosis, and cardiovascular diseases. EAhy 926 (EAHY) endothelial cells were exposed to arecoline, a major BQ alkaloid, for various time periods. Cytotoxicity was estimated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay. The cell cycle distribution of EAHY cells residing in sub-G0/G1, G0/G1, S-, and G2/M phases was analyzed by propidium iodide staining of cellular DNA content and flow cytometry. Some EAHY cells retracted, became round-shaped in appearance, and even detached from the culture plate after exposure to higher concentrations of arecoline (> 0.4 mM). At concentrations of 0.4 and 0.8 mM, arecoline induced significant cytotoxicity to EAHY cells. At similar concentrations, arecoline induced G2/M cell cycle arrest and increased sub-G0/G1 population, a hallmark of apoptosis. Interestingly, prolonged exposure to arecoline (0.1 mM) for 12 and 21 days significantly suppressed the proliferation of EAHY cells, whereas EAHY cells showed adaptation and survived when exposed to 0.05 mM arecoline. These results suggest that BQ components may contribute to the pathogenesis of OSF and BQ chewing-related cardiovascular diseases via toxicity to oral or systemic endothelial cells, leading to impairment of vascular function. During BQ chewing, endothelial damage may be induced by areca nut components and associate with the pathogenesis of OSF, periodontitis, and cardiovascular diseases.

Keywords Arecoline · Cardiovascular diseases · Cell cycle · Cytotoxicity · Endothelial cells · Oral submucous fibrosis

Mei-Chi Chang and Ming-Lun Hsu made an equal contribution to the first author.

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Introduction

Betel quid (BQ) chewing is the fourth most common oral habit in the world [1]. BQ chewing habit is very popular in many countries of Southeast Asia including India, Sri Lanka, Philippines, Mainland China, and Taiwan [2–4].

There are about 200–600 million BQ chewers in the world [1, 4, 5] and the prevalence rate of BQ chewing is about 10% in Taiwan [6]. BQ chewing habit is associated with the elevated risk of localized oral mucosal diseases such as oral squamous cell carcinoma, oral submucous fibrosis (OSF), and oral leukoplakia [1, 4, 6, 7]. Recently, BQ chewing habit has been linked to the enhanced risk of systemic diseases such as atherosclerosis, hypertension, and all-cause mortality [8–10].

Histologically, an increase of vascular density in the early stage of OSF is observed, whereas the vascular density decreased markedly in the middle and late stages of OSF [11]. Similarly, a decline or complete loss of endothelial cells in the juxtaepithelial region of OSF tissue is also noted [12]. The vascular effects by BQ components are suggested to contribute to the localized nutritional deprivation and mucosal atrophy in OSF, a precancerous condition. However, the precise mechanisms responsible for the BQ chewing-related oral mucosal diseases and vascular changes in localized oral mucosa or systemic circulation are not fully clear. BQ ingredients released during chewing are proposed to be the major contributing factors for blood vessel dysfunction [1, 4].

In Taiwan, BQ contains mainly areca nut (AN), inflorescence *Piper betle* and lime with/without wrapping by a *P. betle* leaf. AN contains several alkaloids such as arecoline, arecaidine, guvacoline, and guvacine. Arecoline is the most abundant areca alkaloid [13]. During BQ chewing, the salivary concentration of arecoline may reach 140 μg/ml [14]. At clinically relevant concentrations, arecoline may induce cytotoxicity to oral mucosal epithelial cells and fibroblasts [15–18]. Whether BQ components are toxic to vascular endothelial cells should be further addressed.

The mechanisms responsible for arecoline cytotoxicity are not fully clarified. AN ingredient may induce prostaglandin E_2 (PGE_2) production, cell cycle arrest, and cytotoxicity to primary oral keratinocytes and KB cancer cells [17]. The prolonged exposure of human KB epithelial cells to arecoline results in growth inhibition and cellular apoptosis [19]. Recently, AN extract has been shown to stimulate platelet aggregation and thromboxane B_2 production [20], which may potentially elevate the risk of cardiovascular diseases. Since the metabolism of chemicals shows some differences among cells, we propose that arecoline may induce cytotoxicity to endothelial cells to mediate the decline of localized vascularity in OSF and elevate the risk of systemic cardiovascular diseases. In this study, we therefore used EAhy926 (EAHY) endothelial cells [21], which are derived from a fusion of human umbilical vein endothelial cells with the lung adenocarcinoma hybrid cell line. EAHY cells showed endothelial cell characteristics such as the expression of endothelin-1, Weibel–Palade bodies, prostacyclin, factor VIII-related antigen, and endothelial adhesion molecules ICAM-1 and VCAM-1 [22–24]. The effects of arecoline on morphologic change, cytotoxicity, apoptosis, and changes in cell cycle distribution in EAHY cells were investigated. Moreover, the toxic effect of long-term exposure of EAHY cells to low-dose arecoline was also evaluated.

Materials and methods

Chemicals

Arecoline hydrobromide and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma (Sigma Chemical Company, St. Louis, MO, USA). Trypan blue, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin/ streptomycin and other cell culture biologicals were purchased from Gibco (Life Technology, NY, USA). Reagents for flow cytometric analysis were from Becton Dickinson (San Jose, CA, USA).

Cell culture

EAHY cells were kindly given by Dr. Cora-Jean S. Edgell (North Carolina University, NC, USA) [21, 22]. They were cultured in DMEM containing 10% fetal bovine serum (FBS), 5% CO_2, at 37°C.

Arecoline cytotoxicity to EAHY endothelial cells

For short-term exposure to higher concentrations of arecoline, 5×10^5 EAHY cells were seeded onto 6-well culture plates. After 24 h, the culture medium was changed and then various amounts of arecoline (final 0, 0.1, 0.2, 0.4, and 0.8 mM) were added. Cells were further incubated for 24 or 72 h. MTT was added into each well and cells were further cultured for 3 h. The culture medium was decanted. The insoluble formazan generated by viable cells was dissolved in DMSO and read against reagent blank (DMSO) at a wavelength of 540 nm by a microplate reader for estimation of cell viability. Results were expressed as mean±SEM (% of control).

Long-term exposure to arecoline on the proliferation of EAHY cells

For long-term exposure to lower concentrations of arecoline, 5×10^5 EAHY cells were plated onto 10-cm Petri-dishes and treated with various concentrations of arecoline (final 0, 0.01, 0.025, 0.05, and 0.1 mM). They were subcultured every 3 days at the same cell density, followed by the addition of new arecoline. This was continued for seven passages (21 days). After exposure to arecoline for 3,

12, and 21 days, both floating and attached cells were collected and viable cell numbers were counted by trypan blue dye exclusion technique as described before [25].

Flow cytometric analysis of cell cycle distribution of EAHY cells

Flow cytometric analysis was performed as described previously [26]. Briefly, EAHY cells (5 x 10^5 cells) were exposed to various concentrations of arecoline for 24 h. Then, both floating and attached cells were collected and centrifuged in tubes. Cells were re-suspended and fixed in 70% ice-cold ethanol for 30 min, washed with phosphate-buffered saline (PBS), and then stained with propidium iodide (PI) (50 μg/ml) in PBS containing 2 mg/ml ribonuclease A for 30 min. Cellular DNA content was measured by using a FACS Calibur flow cytometer (Beckman Coulter, Fullerton, CA, USA) to evaluate cell cycle distribution. A total of 10,000 events were determined for each sample. The percentages of cells residing in G0/G1, S, or G2/M and sub-G0/G1 (a hallmark of cellular apoptosis) phases were automatically calculated from histograms by CellQuest software (BD Sciences, San Jose, CA, USA) and ModFit LT version 2.0 software (Verity Software House, ME, USA).

Statistical analysis

Four or more independent experiments were performed and used for statistical evaluation. The results were expressed as median of maxima and 25% and 75% percentile values. Statistical analysis was conducted by one-way ANOVA and post hoc Tukey test to check the difference between groups. A p value<0.05 was considered to indicate a statistically significant difference.

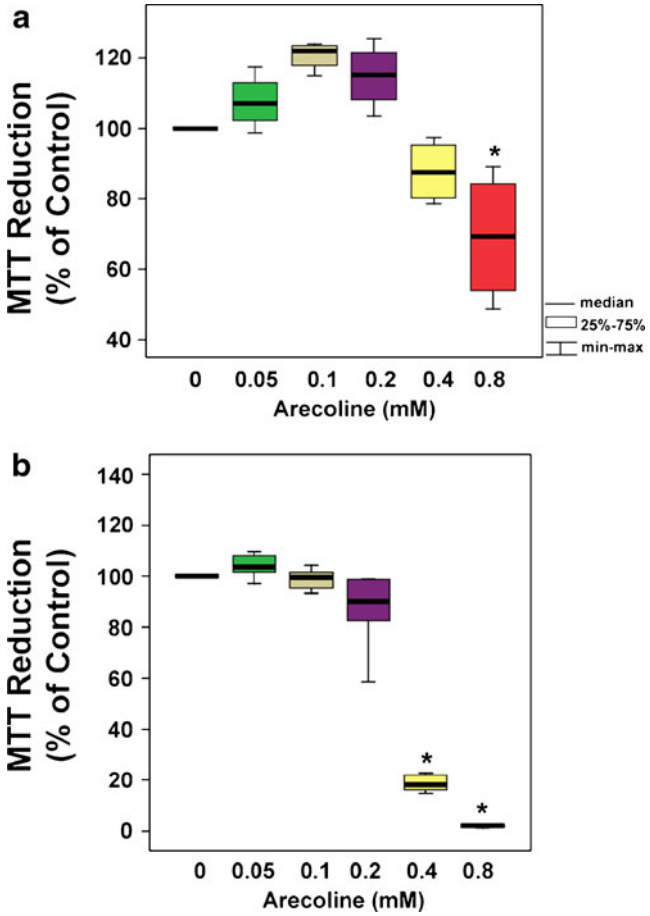

Fig. 1 Cytotoxicity of arecoline to EAHY endothelial cells as estimated by MTT assay. **a** Viable cell numbers (% of control) after exposure of EAHY cells to arecoline for 24 h (n=4); **b** viable cell numbers (% of control) after exposure of EAHY cells to arecoline for 72 h (n=6). Results were shown as mean±SEM (% of control). *Asterisk* denotes a significant difference when compared with the untreated group ($p<0.05$)

Results

Cytotoxicity of arecoline on EAHY endothelial cells

Arecoline was cytotoxic to EAHY cells and decreased the number of viable cells by 31% at a concentration of 0.8 mM after 24 h of exposure (Fig. 1a). After 72 h of exposure, arecoline further decreased the viable cells to 18% and 2% of control at concentrations of 0.4 and 0.8 mM, respectively (Fig. 1b).

Morphological changes of EAHY cells following exposure to arecoline

Morphologically, EAHY endothelial cells were generally cuboidal or polygonal in appearance (Fig. 2a). Most of the EAHY cells became retracted with poorly developed pseudopodia and loss of extended cellular processes (arrows) after exposure to 0.4 mM arecoline for 24 h. Some cells became round (arrowheads) and even detached from the culture plate (Fig. 2b). Concomitant with cell retraction and rounding, the density of EAHY cells decreased and many cells detached from the culture plates by exposure to 0.8 mM arecoline (data not shown).

Arecoline induced the cell cycle alteration of EAHY cells

Cytotoxicity of arecoline was associated with the induction of cell cycle alteration. As shown in Fig. 3, control (no arecoline) cells showed the percentage of cells residing in G0/G1, G2/M, and S phases to be 68.9%, 10.2%, and 21.1%, respectively. Exposure to 0.8 mM arecoline altered the percentage of G0/G1, G2/M, and S phase population to 54.8%, 29.5%, and 12.7%, respectively, indicating the induction of G2/M cell cycle arrest.

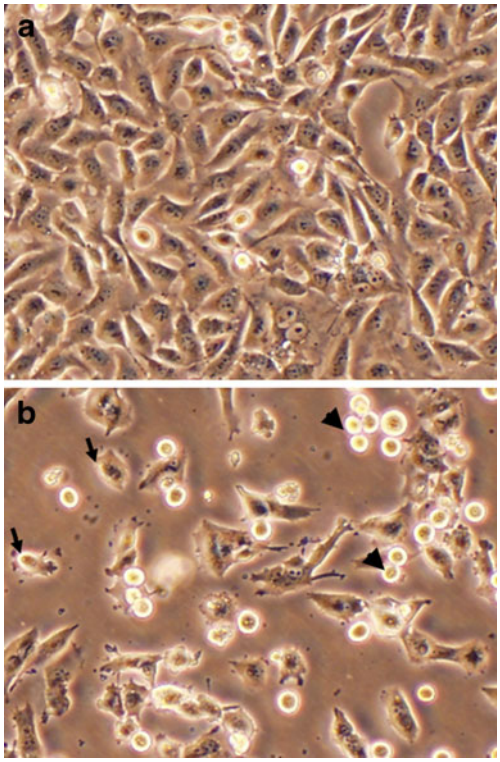

Fig. 2 **a** Morphology of untreated EAHY cells; **b** morphology of EAHY cells after exposure to 0.4 mM arecoline for 24 h. More cells became retracted (*arrows*) or round-shaped (*arrowheads*) with an increase of intercellular space (×100, original magnification)

Arecoline induced the apoptosis of EAHY cells

At higher concentrations, arecoline induced the apoptosis of EAHY cells as indicated by an increase in sub-G0/G1 population, the hallmark of cellular apoptosis. Quantitatively, arecoline increased the sub-G0/G1 population of EAHY endothelial cells in a dose-dependent manner (Fig. 4). When EAHY cells were treated with 0.4 and 0.8 mM arecoline for 24 h, the percentage of sub-G0/G1 population elevated to 9.9% and 37.2%, respectively.

Effect of lower concentrations of arecoline on the long-term viability of EAHY cells

After exposure to low-dose arecoline (0–0.1 mM) for 3 days (Fig. 5a), 12 days (Fig. 5b), and 21 days (Fig. 5c), cytotoxicity to EAHY cells was determined by counting the number of viable cells. There was no significant difference of viable cell counts after exposure to a low dose of arecoline (0–0.1 mM) for 3 days. Exposure of EAHY cells to 0.05 and 0.1 mM arecoline for 12 days decreased the viable cells from 3.75 (control) to 3.12 and 1.6×10^6 cells, respectively (Fig. 5b). After exposure of EAHY cells to 0.05 and 0.1 mM arecoline for 21 days, the viable cell counts were 5.02 and 1.2×10^6 cells, respectively, relative to 5.03×10^6 cells (control). A statistically significant inhibition on cell growth was noted when EAHY cells were exposed to 0.1 mM of arecoline for 12 and 21 days (Fig. 5b, c).

Discussion

BQ chewing has been a public health issue in many countries not only due to its elevation of the risk of oral and pharyngeal cancers but also due to the risk of systemic diseases, including cardiovascular diseases. Endothelial cells are important in the control of vascular tone and

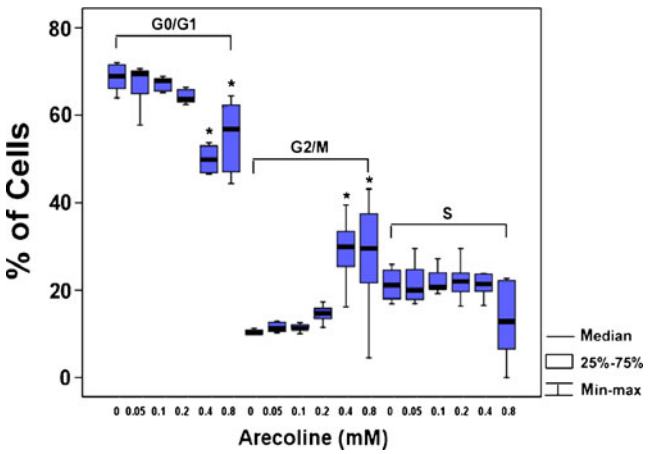

Fig. 3 Quantitative analysis of cell cycle distribution of EAHY cells after exposure to various concentrations of arecoline for 24 h (n=7). Results were expressed as the percentage of cells residing in the G0/G1, G2/M, and S phase of the cell cycle. *Asterisk* denotes a significant difference (p<0.05) when compared with the untreated control

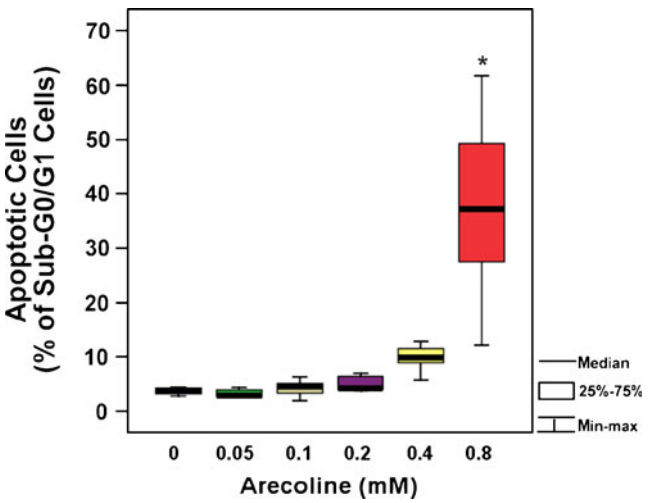

Fig. 4 Quantitative analysis of the percentage of apoptotic EAHY cells (sub-G0/G1 population) after exposure to various concentrations of arecoline for 24 h (n=7). *Asterisk* denotes a significant difference (p<0.05) when compared with the untreated control

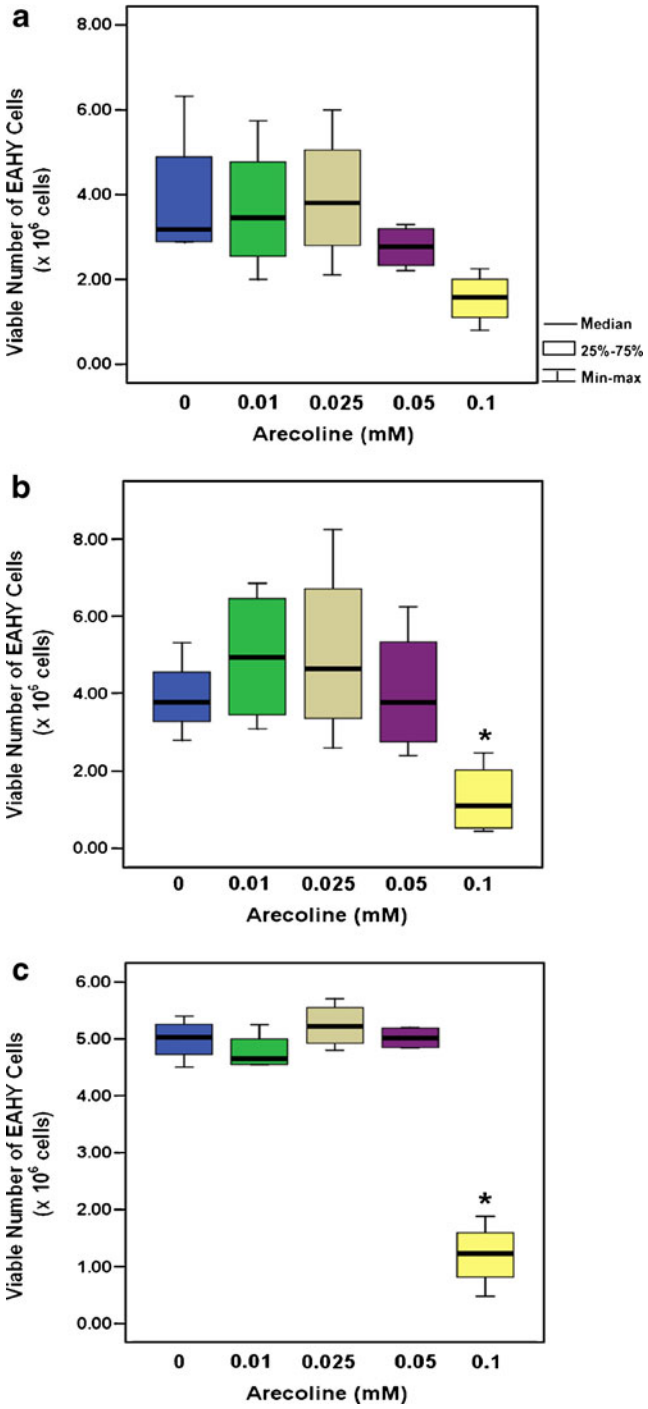

Fig. 5 Cytotoxicity to EAHY endothelial cells after a long-term exposure to a low dose of arecoline. EAHY cells (5×10^5) were seeded onto 10-cm culture dishes and treated with various concentrations of arecoline (0.01–0.1 mM) for **a** 3, **b** 12, and **c** 21 days, with a change of fresh medium and arecoline every 3 days. The number of viable cells was measured by the trypan blue dye exclusion technique. The results were expressed as median of maxima, 25% and 75% percentile (n=4). *Asterisk* denotes a statistically significant difference when compared to the untreated control (p<0.05)

blood flow in vessels [27, 28]. The impairment of endothelial cell functions has been associated with the diseased processes of atherosclerosis, hypertension, diabetes, coronary artery disease, and tissue fibrosis [27–30]. Nair et al. have examined the salivary arecoline content to be 0–89.8 μg/ml among BQ chewers, with an average of 29.69 μg/ml [14]. Cox et al. also found that the highest salivary arecoline concentration could reach 5.66 to 97.39 μg/ml [31]. In this study, we demonstrated that arecoline was cytotoxic to endothelial cells at feasible salivary concentrations. Cytotoxicity of arecoline was concomitant with marked morphological changes of EAHY cells, e.g., retraction and rounding, indicating the presence of endothelial cell damage such as loss of substrate adhesion or cell–cell adhesion, increases vascular permeability, and tissue edema [32–34]. The untreated endothelial cells showed an intact monolayer to serve as a barrier to selective across of vascular wall by fluid or inflammatory cells [32]. The damage and dysfunction of endothelium may expose subendothelial connective tissue and induce platelet activation and procoagulant, prothrombotic, and pro-inflammatory events that contribute to clinical cardiovascular changes [28, 29, 33, 34]. Persistent vascular endothelial injury may also result in chronic tissue ischemia leading to a decrease in new vessel formation, vascular fibrosis, and platelet activation as observed in scleroderma [30]. These results suggest that toxic injury to endothelium by BQ components may potentially lead to a decline of oral mucosa vascularity, impairment of the nutritional supply, and eventually mucosa atrophy as noted in OSF [11, 12].

Why BQ chewing may increase the risk of atherosclerosis, hypertension, and all-cause mortality is currently not clear [8–10]. BQ components may induce oral mucosa inflammatory responses and oral inflammation has been linked to the incidence of atherosclerosis [16, 35, 36]. AN components and arecoline may stimulate catecholamine and glucagon secretion, contributing further to the pathogenesis of diabetes and cardiovascular diseases [37]. The mean arterial blood pressure and heart rate increased significantly after exposure to arecaidine and arecoline, possibly due to the stimulation of muscarinic receptor in the sympathetic ganglia [38]. Arecoline can diffuse through the buccal mucosa or be absorbed from the gastrointestinal tract into the bloodstream [39]. Arecoline can soon cross the blood–brain barrier and be metabolized in the liver, kidney, and buccal mucosal fibroblasts by carboxylesterase to produce arecaidine [4, 40]. The metabolism of arecoline in mice is mediated by glutathione conjugation, esterase, flavin-containing monooxygenases, etc. to generate 11 major metabolites including arecaidine, arecoline-1-oxide, *N*-methylnipecotic acid, *N*-methylnipecotylglycine, arecaidinylglycine, arecaidinylglycerol, arecaidine mercapturic acid, arecoline mercapturic acid, and arecoline *N*-oxide mercapturic acid [41, 42]. The concentration of arecoline and arecaidine in the blood of BQ chewers has been

determined to be 7 and 143 ng/ml, respectively [43]. The analysis of urine in BQ chewers further confirms the presence of arecoline, arecaidine, and *N*-methylnipecotic acid [44]. The exposure of cardiovascular cells to the BQ components is generally low relative to oral mucosal endothelial cells. However, the possibility of accumulated effects on vascular cells in systemic circulation by exposure to low-dose BQ components cannot be overlooked. To the best of our knowledge, this is the first report to explore the long-term effects of low doses of arecoline on vascular endothelial cells. Incubation of EAHY cells to a low concentration of arecoline (0.1 mM) for 12 days caused cytotoxicity and marked suppression of cell proliferation. These events may further contribute to pathologic vascular changes in localized oral mucosa and systemic circulation, leading to clinical diseases. Interestingly, a 0.05-mM arecoline showed mild cytotoxicity to EAHY cells at 3 days of exposure, whereas this event became less evident after exposure for 21 days. This reveals the adaptive response of endothelial cells to exogenous toxicant and 0.05–0.1 mM arecoline is the critical concentration for the survival or death of EAHY cells. This may explain why the quantity, duration, and accumulated exposure to BQ chewing markedly elevated the risk of cardiovascular diseases. Moreover, in addition to current BQ chewers, former BQ chewers may still have a higher risk of cardiovascular disease [9, 10].

The toxicity of arecoline to EAHY cells may be due to deregulation of cell cycle progression. Cell cycle checkpoints are regulatory pathways to ensure the progression and completion of critical events such as DNA replication and chromosome segregation [45]. Checkpoint loss may result in genomic instability and apoptosis to prevent the accumulation of genetic defect in normal tissues. However, the over-induction of cellular apoptosis may cause tissue damage [46]. Previous studies have found that exposure of oral mucosa fibroblasts, human KB cancer epithelial cells, and Chinese hamster ovary-K1 cells to arecoline can elicit G2/M cell cycle arrest and even apoptosis [19, 47, 48]. In this study, exposure to>0.2 mM arecoline decreases the proportion of EAHY cells residing in S phase but increases the cells arresting in G2/M phase. This suggests that the anti-proliferative and cytotoxic effects of arecoline are possibly associated with the alteration of specific cell cycle regulatory proteins such as ATM, checkpoint kinases, p53, and cdc25C for G2/M checkpoint [16, 49]. However, arecoline induced DNA damage, G0/G1 arrest, and p21 and p53 expression in cultured rat hepatocytes [50]. This indicates that cell cycle response to arecoline shows some differences between cells possibly due to diverse cellular metabolic enzymes present in differential tissues. The prolonged deregulation of cell cycle by arecoline may further lead to genome instability, chromosome aberration, and aneuploidy [51, 52].

In conclusion, arecoline showed cytotoxic and growth inhibitory effects to vascular endothelial cells. This is due to the induction of cell cycle arrest and apoptosis to endothelial cells by arecoline. Long-term exposure to lower concentrations of arecoline is also toxic to vascular endothelial cells. These results indicate that BQ components may damage local and systemic vascular endothelial cells, contributing to the pathogenesis of BQ chewing-related cardiovascular diseases, OSF, and periodontal diseases.

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Conflicts of interest The authors declare that there are no conflicts of interest for this submission.

References

1. Jeng JH, Chang MC, Hahn LJ (2001) Role of areca nut in betel quid-associated chemical carcinogenesis: current awareness and future perspectives. Oral Oncol 37:477–492
2. Gupta PC, Ray CS (2004) Epidemiology of betel quid usage. Ann Acad Med Singapore 33(4 Suppl):31–36
3. Zhang X, Reichart PA (2007) A review of betel quid chewing, oral cancer and precancer in Mainland China. Oral Oncol 43:424–430
4. IARC (2004) Betel-quid and areca-nut chewing and some areca-nut-derived nitrosamines. In: IARC monographs on the evaluation of carcinogenic risks to humans, vol 85. IARC, World Health Organization, Lyon, France
5. Nelson BS, Heischober B (1999) Betel nut: a common drug used by naturalized citizens from India, Far East Asia, and the South Pacific Islands. Ann Emerg Med 34:238–243
6. Ko YC, Chiang TA, Chang SJ, Hsieh SF (1992) Prevalence of betel quid chewing habit in Taiwan and related sociodemographic factors. J Oral Pathol Med 21:261–264
7. Trivedy CR, Craig G, Warnakulasuriya S (2002) The oral health consequences of chewing areca nut. Addict Biol 7:115–125
8. Jeng JH, Chang MC (2007) Betel quid chewing and cardiovascular diseases: a traditional oral habit with potential new health issues. Atherosclerosis Commentary, September
9. Lin WY, Chiu TY, Lee LT, Lin CC, Huang CY, Huang KC (2008) Betel nut chewing is associated with increased risk of cardiovascular disease and all-cause mortality in Taiwanese men. Am J Clin Nutr 87:1204–1211
10. Yen AM, Chen LS, Chiu YH, Boucher BJ, Chen TH (2008) A prospective community-population-registry based cohort study of the association between betel-quid chewing and cardiovascular disease in men in Taiwan. Am J Clin Nutr 87:70–78
11. Fang CY, Han WN, Fong DY (2000) A morphometric study on the microvessel in oral submucous fibrosis. Hunan Yi Ke Da Xue Bao 25:55–57
12. Desai RS, Mamatha GS, Khatri MJ, Shetty SJ (2010) Immunohistochemical expression of CD34 for characterization and quantification of mucosal vasculature and its probable role in malignant transformation of atrophic epithelium in oral submucous fibrosis. Oral Oncol 46:553–558
13. Arjungi KN (1976) Areca nut: a review. Arzneimittelforschung 26:951–956

14. Nair J, Ohshima H, Friesen M, Croisy A, Bhide SV, Bartsch H (1985) 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 6:295–303
15. Jeng JH, Kuo ML, Hahn LJ, Kuo MY (1994) Genotoxic and non-genotoxic effects of betel quid ingredients on oral mucosal fibroblasts in vitro. J Dent Res 73:1043–1049
16. Jeng JH, Ho YS, Chan CP et al (2000) Areca nut extract up-regulates prostaglandin production, cyclooxygenase-2 mRNA and protein expression of human oral keratinocytes. Carcinogenesis 21:1365–1370
17. Chang MC, Wu HL, Lee JJ et al (2004) The induction of prostaglandin E2 production, interleukin-6 production, cell cycle arrest, and cytotoxicity in primary oral keratinocytes and KB cancer cells by areca nut ingredients is differentially regulated by MEK/ERK activation. J Biol Chem 279:50676–50683
18. Van Wyk CW, Olivier A, Miranda CM, Bijl P, Grobler-Rabie AF (1994) Observations on the effect of areca nut extracts on oral fibroblast proliferation. J Oral Pathol Med 23:145–148
19. Lee PH, Chang MC, Chang WH et al (2006) Prolonged exposure to arecoline arrested human KB epithelial cell growth: regulatory mechanisms of cell cycle and apoptosis. Toxicology 220:81–89
20. Jeng JH, Chen SY, Liao CH et al (2002) Modulation of platelet aggregation by areca nut and betel leaf ingredients: roles of reactive oxygen species and cyclooxygenase. Free Radic Biol & Med 32:860–871
21. Edgell CJ, Mcdonald CC, Graham JB (1983) Permanent cell line expressing human factor VIII-related antigen established by hybridization. Proc Natl Acad Sci USA 80:3734–3737
22. Edgell CJ, Haizlip JE, Bagnell CR, Packenham JP, Harrison P, Wilbourn B, Madden VJ (1990) Endothelium specific Weibel–Palade bodies in a continuous human cell line, EA.hy926. In Vitro Cell Dev Biol 26:1167–1172
23. Staton CA, Brown NJ, Lewis CE (2003) The role of fibrinogen and related fragments in tumour angiogenesis and metastasis. Expert Opin Biol Ther 3:1105–1120
24. Aranda E, Owen GI (2009) A semi-quantitative assay to screen for angiogenic compounds and compounds with angiogenic potential using the EA.hy926 endothelial cell line. Biol Res 42:377–389
25. Jeng JH, Lu FJ, Hahn LJ, Wang YJ, Kuo MYP (1994) Eugenol triggers different pathobiological effects on oral mucosal fibroblasts in vitro. J Dent Res 73:1050–1055
26. Chang MC, Chen LI, Chan CP et al (2010) The role of reactive oxygen species and hemeoxygenase-1 expression in the cytotoxicity, cell cycle alteration and apoptosis of dental pulp cells induced by BisGMA. Biomaterials 31:8164–8171
27. Iwakiri Y, Groszmann RJ (2007) Vascular endothelial dysfunction in cirrhosis. J Hepatol 46:927–934
28. Balakumar P, Chakkarwar VA, Krishan P, Singh M (2009) Vascular endothelial dysfunction: a tug of war in diabetic nephropathy? Biomed Pharmacother 63:171–179
29. Sitla S, Tomasoni L, Atzeni F et al (2010) From endothelial dysfunction to atherosclerosis. Autoimmun Rev 9:830–834
30. Kahaleh B (2008) The microvascular endothelium in scleroderma. Rheumatology 47(Suppl 5):v14–v15
31. Cox S, Vickers ER, Ghu S, Zoellner H (2010) Salivary arecoline levels during areca nut chewing in human volunteers. J Oral Pathol Med 39:465–469
32. Zeng Q, Lagunoff D, Masaracchia R, Goeckeler Z, Cote G, Wysolmerski R (2000) Endothelial cell retraction is induced by PAK2 monophosphorylation of myosin II. J Cell Sci 113:471–482
33. Garcia JG, Verin AD, Schaphorst KL (1996) Regulation of thrombin-mediated endothelial cell contraction and permeability. Semin Thromb Hemost 22:309–315
34. Onoda JM, Kartak SS, Diglio CA (1999) Radiation induced endothelial cell retraction in vitro correlation with acute pulmonary edema. Pathol Oncol Res 5:49–55
35. Jeng JH, Wang YJ, Chiang BL et al (2003) Roles of keratinocyte inflammation in oral cancer: regulating the prostaglandin E2, interleukin-6 and TNF-alpha production of oral epithelial cells by areca nut extract and arecoline. Carcinogenesis 24:1301–1315
36. Offenbacher S, Beck JD (2005) A perspective on the potential cardioprotective benefits of periodontal therapy. Am Heart J 149:950–954
37. Boucher BJ, Mannan N (2002) Metabolic effects of the consumption of *Areca catechu*. Addict Biol 7:103–110
38. Wess J, Lambrecht G, Moser U, Mutschler E (1987) Stimulation of ganglionic muscarinic M1 receptors by a series of tertiary arecaidine and isoarecaidine esters in the pithed rat. Eur J Pharmacol 134:61–67
39. Vander B, Van Eyk AD, Van Wyk CW, Stander IA (2001) Diffusion of reduced arecoline and arecaidine through human vaginal and buccal mucosa. J Oral Pathol Med 30:200–205
40. Harvey W, Scutt A, Meghji S, Canniff JP (1986) Stimulation of human buccal fibroblasts in vitro by betel-nut alkaloids. Arch Oral Biol 31:45–49
41. Giri S, Idle JR, Chen C, Zabriskie TM, Krausz KW, Gonzalez FJ (2006) A metabolomic approach to the metabolism of the areca nut alkaloids arecoline and arecaidine in the mouse. Chem Res Toxicol 19:818–827
42. Giri S, Krausz KW, Idle JR, Gonzalez FJ (2007) The metabolomics of (+/−)-arecoline 1-oxide in the mouse and its formation by human flavin-containing monooxygenases. Biochem Pharmacol 73:561–573
43. Wu IC, Chen PH, Wang CJ et al (2010) Quantification of blood betel quid alkaloids and urinary 8-hydroxydeoxyguanosine in humans and their association with betel chewing habits. J Anal Toxicol 34:325–331
44. Hu CW, Chang YZ, Wang HW, Chao MR (2010) High-throughput simultaneous analysis of five urinary metabolites of areca nut and tobacco alkaloids by isotope-dilution liquid chromatography–tandem mass spectrometry with on-line solid-phase extraction. Cancer Epidemiol Biomarkers Prev 19:2570–2581
45. Elledge S (1996) Cell cycle checkpoints: preventing an identity crisis. Science 274:1664–1672
46. Thangjam GS, Kondaiah P (2009) Regulation of oxidative-stress responsive genes by arecoline in human keratinocytes. J Periodont Res 44:673–682
47. Lin CC, Chang MC, Chang HH et al (2009) Areca nut-induced micronuclei and cytokinesis failure in Chinese hamster ovary cells is related to reactive oxygen species production and actin filament deregulation. Environ Mol Mutagen 50:367–374
48. Chang MC, Ho YS, Lee PH et al (2001) Areca nut extract and arecoline induced the cell cycle arrest but not apoptosis of cultured oral KB epithelial cells: association of glutathione, reactive oxygen species and mitochondrial membrane potential. Carcinogenesis 22:1527–1535
49. Tsai YS, Lee KW, Huang JL et al (2008) Arecoline, a major alkaloid of areca nut, inhibits p53, represses DNA repair, and triggers DNA damage response in human epithelial cells. Toxicology 249:230–237
50. Chou WW, Guh JY, Tsai JF et al (2008) Arecoline-induced growth arrest and p21WAF1 expression are dependent on p53 in rat hepatocytes. Toxicology 243:1–10
51. Wang YC, Tsai YS, Huang JL et al (2010) Arecoline arrests cells at prometaphase by deregulating mitotic spindle assembly and spindle assembly checkpoint; implication for carcinogenesis. Oral Oncol 46:255–262
52. Nojima H (2006) Protein kinases that regulate chromosome stability and their downstream targets. Genome Dyn 1:131–148