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Prolonged exposure to arecoline arrested human KB epithelial cell growth: Regulatory mechanisms of cell cycle and apoptosis

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Abstract

Arecoline, the main areca alkaloid in betel quid (BQ), is reported to have cytotoxic, genotoxic, and mutagenic effects in various cells. It shows strong correlation to the incidence of oral submucous fibrosis, leukoplakia, and oral cancer. To clarify the role of arecoline in BQ-induced carcinogenesis, primary human gingival keratinocyes (GK) and human KB epithelial cells were used for studying the molecular mechanisms of arecoline-mediated cell cycle deregulation for comparison. After 24 h of exposure, arecoline (0.2–0.8 mM) inhibited KB cell growth in a dose- and time-dependent manner with a reduction in cell number by 27–37 and 37–58%, respectively, as determined by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and sulforhodamine B (SRB) assays. Incubation of KB cells with arecoline (0.1–0.4 mM) caused late-S and G2/M phases' cell cycle arrest. Western blot analysis revealed that arecoline induced cyclin Bl, Wee 1, and phosphorylated cdc2 protein levels whereas it declined p21 protein expression in KB cancer cells. Nevertheless, arecoline induced p21, but decreased cdc2 and cyclin B1 protein levels in GK. We demonstrated that higher concentrations of arecoline (0.2–1.2 mM) induced both cell necrosis and apoptosis as detected by DNA fragmentation and Annexin V–PI staining after long-term (48 h) treatment. Our results suggest that differential regulation of S and/or G2/M cell cycle-related proteins in the GK and KB cells play a crucial role in different stages of BQ-mediated carcinogenesis.

Keywords: Areca nut; Apoptosis; Arecoline; Betel quid; Cell cycle; Epithelial cells; Carcinogenesis

1. Introduction

Betel quid (BQ) chewing is a popular oral habit in Taiwan, India, and many Southeast Asian countries. This habit is closely associated with the high incidence of oral leukoplakia, oral submucous fibrosis (OSF), and oral cancer (Trivedy et al., 2002; Reichart, 1995; IARC, 2004; Jeng et al., 2001). However, the precise mechanisms of BQ chewing-mediated oral cancer are not fully clear. In Taiwan, the BQ consists of fresh areca nut (AN)

Abbreviations: AN, areca nut; BQ, betel quid; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GK, gingival keratinocytes; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; OMF, oral mucosal fibroblasts; OSF, oral submucous fibrosis

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with a piece of *inflorescence Piper betle* and lime mixture. AN extract was found to be cytotoxic and genotoxic to oral mucosal fibroblasts, oral keratinocytes and inhibit the growth, attachment, and matrix protein synthesis of cultured human gingival fibroblasts (Jeng et al., 2001; IARC, 2004). These AN-induced effects could be partially explained by its content of arecoline (Jeng et al., 2001; IARC, 2004).

Arecoline, the most abundant areca alkaloid, has been suggested as a possible carcinogen (IARC, 2004; Jeng et al., 2001; Shirname et al., 1983). Arecoline is mutagenic in the Ames test (Shirname et al., 1983), and can induce sister chromatid exchanges, chromosomal aberrations, and micronuclei in several types of cells (IARC, 2004; Jeng et al., 2001). However, it is not able to induce DNA breaks and unscheduled DNA synthesis in oral epithelial cells (Sundqvist et al., 1989; Jeng et al., 1999). Therefore, it is important to study the mechanism responsible for arecoline-induced cytotoxicity.

Short-term exposure of human KB cancer epithelial cells and oral mucosal fibroblasts (OMF) to arecoline has been shown to elicit cell cycle deregulation (Chang et al., 2001a). In eukaryotic cells, cell cycle checkpoints help to ensure the orderly progression and completion of critical events, such as DNA replication and chromosome segregation (Elledge, 1996). Upon DNA damage, eukaryotic cells can arrest in G1, S, or G2 phase of the cell cycle, allowing the cells to repair damaged DNA (Smith and Fornace, 1996). Defects in the checkpoint can result in enhanced sensitivity to DNA damaging agents, increase genomic instability (Alby et al., 2001), apoptosis (Smith and Fornace, 1996), gene mutation, cell death, and even cancer (Shackelford et al., 1999).

The cell cycle is controlled by periodic regulation of the highly conserved cyclin-dependent kinases (CDKs). In eukaryocytes, the G2/M checkpoint is controlled by the Cdc2/cyclin B complex, whose activity is required for entry into mitosis. Conditions such as oxidative stress and radiation have been shown to initiate a G2 checkpoint response that causes a delay in the activation Cdc2/cyclin B kinase activity at the G2/M border (Shackelford et al., 2000). Since arecoline has been found to elicit mutagenicity, genotoxicity, cytotoxicity, and chromosomal aberrations in different assay system (IARC, 2004; Jeng et al., 2001), the molecular mechanisms of arecoline-mediated events awaits delineation, in this study, we therefore evaluate the toxic mechanisms of arecoline on cell growth, cell cycle kinetics, and apoptosis by using human KB epithelial cells and primary GK.

2. Materials and methods

2.1. Materials

Propidium iodide, sulforhodamine B (SRB), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), and arecoline hydrobromide were obtained from Sigma (Sigma Chemical Company, St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Gibco (Life Technology, USA). Reagents for flow cytometric analysis were purchased from Becton Dickinson (CA, USA).

2.2. Cell cultures

KB epithelial cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in DMEM with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO, Life Technologies Inc., Taipei, Taiwan) at 37 °C in a 5% CO_2 atmosphere. Primary human gingival keratinocytes (GK) were as described previously (Jeng et al., 1999, 2000, 2003). Briefly, human gingival tissues were obtained during clinical crown lengthening procedures with proper informed consent of the patients. They were cultured by explant technique in collagen/fibronectin coated culture dishes in KGM-SFM medium containing epidermal growth factor and pituitary gland extract.

2.3. Cell growth assay

The viable cell numbers were first measured by a modified 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Mosmann, 1983). Briefly, KB cells were seeded at an initial density of 1×10^5 cells/well in a 6-well plate for 24 h. Cells were then exposed to fresh medium containing various concentrations of arecoline for 12, 24, 36, 48, and 72 h. After various time period of incubation, a final concentration of 0.5 mg/ml MTT was added into each well, and production of insoluble formazan by viable cells were finally dissolved in dimethylsulfoxide and read against blank using a microplate reader (Dynatech, UK) at 540 nm.

On the other hand, cell growth was also estimated by measuring the cellular protein contents, as assessed after staining with sulforhodamine B (SRB) (Skehan et al., 1990). Briefly, 5000 cells in 96-well plates were treated with various concentrations of arecoline for 12–72 h. Cells were fixed in 50% trichloroacetic acid at 4 °C for 1 h, rinsed three times with tap water and then stained with 0.4% SRB/1% acetic acid. After 30 min, unbound SRB dye was removed by three washes with 1% acetic acid. Finally, the protein-bounded dye was dissolved in 100 μl of 10 mM Tris buffer (pH 10.5). Absorbance at wavelength of 540 nm was read using a microplate reader.

2.4. Cell cycle analysis

For measurement of cellular DNA content, flow cytometric analysis was used (Chang et al., 2001a, 2002). Briefly, 5×10^5 KB cells were exposed to various concentration of arecoline in DMEM with 10% FBS for 12, 24, 36, 48, and 72 h. The cell cycle stages in drug-treated groups were measured by flow cytometric analysis. Both floating and attached cells were harvested and stained with propidium iodide (50 μg/ml) (Sigma Chemical Co., St. Louis, MO), and the DNA content was measured using a FACScan laser flow cytometer analysis system (Becton-Dickinson, San Jose, CA); and 15,000 events were analyzed for each sample. The percentage of cells residing in different phases of cell cycle was determined using Mofit *LT* version 2.0 software (Verity Software House Inc., USA).

2.5. Annexin V–PI staining assay

For apoptotic assay, 5×10^5 KB cells were exposed to various concentrations of arecoline for 24, 48 or 72 h. After incubation, floating and attached cells were harvested. Cells were washed with PBS, suspended in 400 μl HEPES (10 mM HEPES–NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) solution, and the Annexin V–FITC (Becton Dickson)/PI (50 μg/ml) staining solution was added in dark for 30 min. The Annexin V–FITC and PI fluorescence of cultured cells were analyzed by FACS Calibur Flow Cytometry (Becton Dickinson) immediately. In each analysis, 15,000 events were recorded.

2.6. Analysis of DNA fragmentation

KB cells (5×10^6 cells) were exposed to various concentrations of arecoline for 24, 48, and 72 h. After incubation, both floating and attached cells were harvested, washed with PBS, and dissolved in 100–200 μl of lysis buffer (50 mM Tris–HCI buffer (pH 8.0) containing 10 mM EDTA, 0.1% SDS, and 0.5 mg/ml proteinase K) at 55 °C for overnight. Ten microliters of 2 mg/ml RNase A was added and incubated for additional 3 h. The residual materials were extracted with 200 μl of phenol/chloroform/isoamyl alcohol (25:24:1), and electrophoresis in 1.8% agarose gels. The DNA was visualized by ethidium bromide (0.5 μg/ml) and detected using Alphalmager 3300 (Alpha Innotech Corp., San Leandro, CA).

2.7. Western blotting

After exposure to arecoline, both floating and attached cells were harvested and Western blotting analysis was performed. Briefly, cell lysates were prepared, electrotransferred, immunoblotted with antibodies against human cyclin B1 (1:1000, GNS1), human cdc2 (1:2000, clone 17), human Wee 1, human p-cdc2, human G3PDH (Santa Cruz Biotechnology Biotech, Santa Cruz, CA, USA) or human p21 (1:250, Ab-11) (NeoMarkers, Fremont, CA, USA). The primary antibodies were detected with HRP-conjugated anti-mouse, anti-rabbit, or anti-goat secondary antibody (1:7000, Santa Cruz). Results were visualized on X-ray film using enhanced chemiluminescence reagent (NEN™ Life Science) as described previously (Jeng et al., 2003; Chang et al., 2004).

2.8. Statistical analysis

Each experiment was repeated for at least three times and results were expressed as mean ± S.E.M. in some quantitative experiments. Student's *t*-test was employed and *P*-values were determined to evaluate the statistical significance ($P < 0.05$) where necessary.

3. Results

3.1. Morphological changes of KB cells following exposure to arecoline

KB cells were cuboid or polygonal in appearance. Marked intercellular clear space could be observed under phase contrast microscope (Chang et al., 2001a). Some rounded KB cells were noticed after exposure to 0.1 mM arecoline for 24 h. More evident cell rounding and decrease in density of KB cells were found following incubation in 0.2 mM arecoline (data not shown). Concomitant with cell rounding, some KB cells seem increased in cell size by exposure 0.4–0.8 mM arecoline. Further elevation of arecoline concentration to 1.2 mM led to retraction and rounding of KB cells (data not shown).

3.2. Effect of arecoline on the growth of KB cells

Human KB epithelial cells were treated with medium containing arecoline (0.1–1.2 mM) for 12, 24, 36, 48, or 72 h. The growth inhibitory effect of arecoline was evaluated by either MTT assay for cell viability or by SRB assay for cellular protein content. As shown in Fig. 1A, arecoline treatment of KB cells resulted in a decrease of cell viability in a dose- and time-dependent manner. Compared with the untreated control, 0.2, 0.4, and 0.8 mM arecoline suppressed the cell viability by 27, 33, and 37% after 24 h, respectively. As shown in Fig. 1A, a significant inhibitory effect was noted when KB cells were exposed to different doses (0.2–0.8 mM) of arecoline for as long as 36–72 h. In addition, arecoline suppressed the cellular protein content of KB cells in a time- and dose-dependent manner as determined by SRB assay. At concentrations ranging from 0.2 to 0.8 mM, long-term exposure (24–72 h) of arecoline decreased the cellular protein content in KB cells by 39–83, 47–88, and 58–90%, respectively (Fig. 1B).

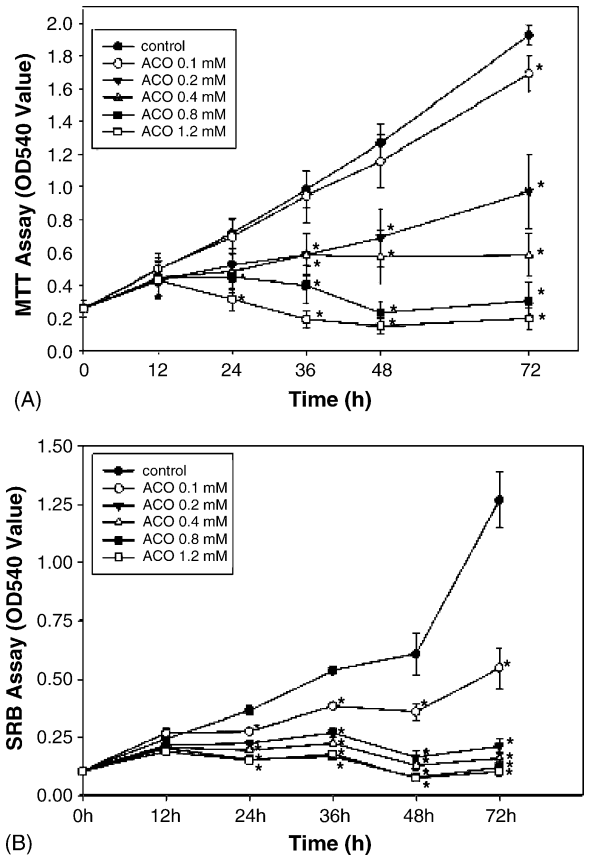

Fig. 1. Effect of arecoline (ACO) on the growth of KB cells. In (A), KB cells were exposed to arecoline (0.1–1.2 mM) for 12–72 h. Cell number was measured with MTT assay. In (B), as the same as (A), KB cells were exposed to various concentrations of arecoline for 12–72 h, fixed with 50% TCA and stained with SRB for analysis of cellular protein content. Data was represented as means ± S.E. for three independent determinations. * Denotes marked difference ($P < 0.05$) between arecoline-treated and untreated cells.

3.3. Effect of arecoline on cell cycle

The cell growth and inhibition are both tightly mediated through cell cycle control (Sanchez and Dynlacht, 2005). Based on the data shown in Fig. 1, we examined the effect of arecoline on cell-cycle progression by using flow cytometric analysis of cellular DNA content. As shown in Fig. 2, the percentage of cells in G2/M phase was increased from 14% in untreated control cells to 19–52% in cells treated with arecoline (0.1–0.4 mM) after 24 h of exposure. We also observed an increase in the proportion of cells arresting in S phase (Fig. 2). The percentage of KB cells in G2/M decreased time-dependently (36–72 h) when exposed to 0.4 mM arecoline. Interestingly, the increase of cells in G2/M phase by 0.4 mM arecoline was observed simultaneously with

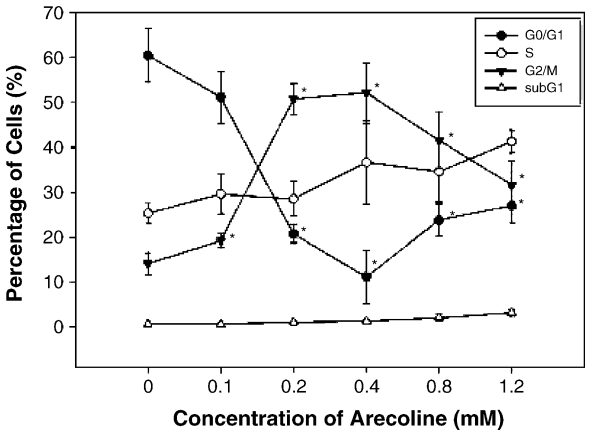

Fig. 2. Effects of arecoline on the cell cycle progression of KB cells. Human KB cells were exposed to arecoline (0.1–1.2 mM) for 24 h. Different phases of cell cycle were measured by flow cytometry. Data were represented as means ± S.E. for three independent determinations. * Denotes marked difference ($P < 0.05$) between arecoline-treated and untreated cells.

an increase of the cells in S phase, and a decrease in G1 phase. In addition, cells showed apparent apoptosis as indicated by the presence of a sub-G1 peak after 48 h of arecoline treatment (data not shown).

3.4. Detection of necrosis and apoptotic features by Annexin V–PI dual staining

The deregulation of cell cycle has been reported to be correlated with the induction of apoptosis (Eastman and Rigas, 1999). Based on the apparent sub-G1 peak shown in flow cytometric analysis after 48 and 72 h treatment (data not shown), we further demonstrated the occurrence of arecoline-induced apoptosis by DNA fragmentation and Annexin V–PI staining methods. Flow cytometric analysis with Annexin V–PI staining showed the proportion of necrotic cells (upper right) was increased at 48 h after exposure to arecoline (0.8–1.2 mM) (Fig. 3). Moreover, apoptotic cells (lower right) significantly elevated in arecoline (0.4–1.2 mM)-treated cells from 3.7% in untreated cells to 24–49% after treatment with 0.2–0.8 mM arecoline for 72 h (Fig. 3). These results suggested that both necrosis and apoptosis contributed to the arecoline-induced death of KB cancer cells with different exposure periods. Such hypotheses were further confirmed by DNA fragmentation analysis as illustrated in Fig. 4. Obvious DNA fragmentation was only observed in KB cells when treated with arecoline (0.4–1.2 mM) for a long-term period of more than 72 h. However, short-term (24–48 h) exposure of KB cells to arecoline showed little DNA laddering formation.

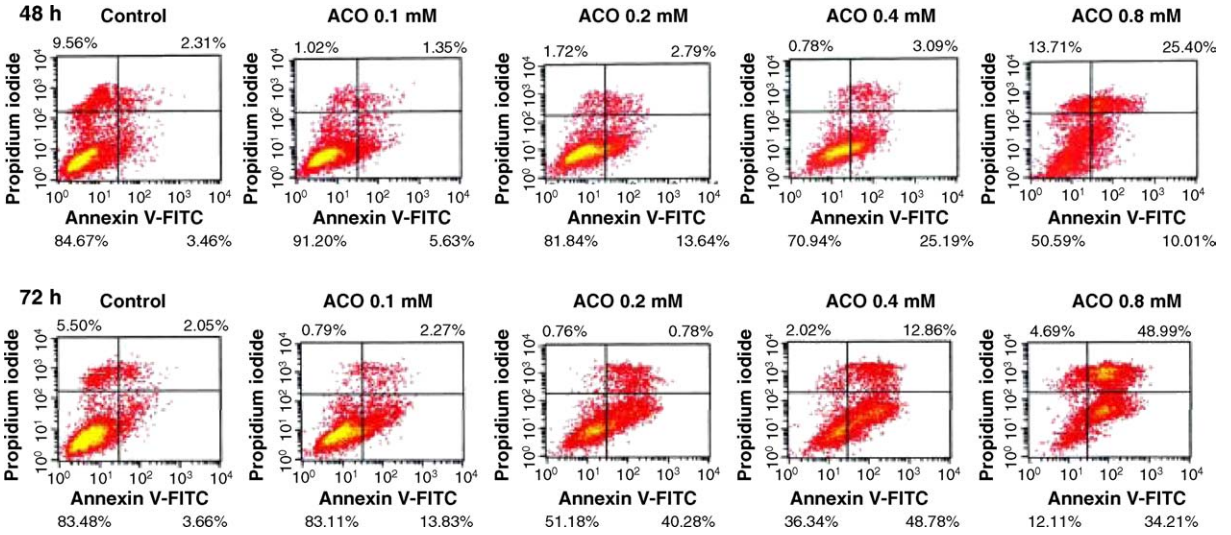

Fig. 3. Detection of apoptosis feature by Annexin V–PI staining. KB cells were exposed to arecoline (0.1–0.8 mM) for 48 and 72 h. Cells were harvested at the indicated time points, and stained with Annexin V/PI for FACS analysis. As shown, the cell populations shown in the lower left represent living cells, lower right represents apoptosis cells, upper right represents necrotic cells and upper left represents pre-necrotic cells. Three samples were analyzed in each group, and values represent the mean ± S.E.

3.5. Differential regulation of cell cycle-related proteins by arecoline on human GK and KB cells

To investigate the mechanisms of arecoline-induced S- and G2/M phase arrest in KB epithelial cells, we examined the protein levels of Cdc2, cyclin B1, phospho-cdc2, Wee1, and $P21^{waf1/cip1}$ by immunoblotting. The Cdc2 activity was inhibited through site-specific phosphorylation by two major regulators including Wee1, which phosphorylates Cdc2 at Tyr-15, and Myt1, which phosphorylates Cdc2 at Thr-14 and, to a lesser extent, Tyr-15 (Li and Brooks, 1999). As shown in Fig. 5, arecoline treatment for 24 h at doses of (14 mM or higher significantly increased the phosphorylation of $Cdc2^{Tyr15}$, with no changes in total Cdc2 protein level. Consistent with the effect on phosphorylation of $Cdc2^{Tyr15}$, arecoline (0.4 mM) treatment resulted in a marked induction of Wee1. The level of cyclin B1 was also increased after arecoline treatment, presumably reflecting an increase in the proportion of cells arrested in G2/M phase. Unexpectedly the $p21^{waf1/cip1}$ a specific inhibitor of CDKs in the G1 and G2/M phase of cell cycle (Harper et al., 1995), was significantly down-regulated after exposure to arecoline (Fig. 5). In addition, we further tested whether primary-cultured human GK cells showed differential responses of cell cycle-related proteins to arecoline in comparison with KB cancer epithelial cells. Interestingly, the cellular responses of the arecoline-induced cell cycle arrest were different in these two types of human epithelial cells. We found that the $p21^{waf1/cip1}$ level was significantly induced by arecoline (>0.1 mM) in GK, whereas the levels of cyclin B1 and Cdc2 was inhibited (>0.2 mM) (Fig. 6).

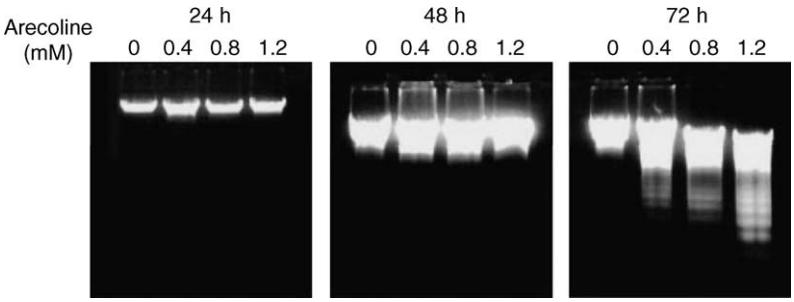

Fig. 4. Detection of apoptosis feature by agarose gel analysis of DNA fragmentation. KB were exposed to arecoline (0.4–1.2 mM) for 24, 48, and 72 h. The DNA was extracted at the indicated time points, subjected to 1.8% of agarose gel electrophoresis and then photographed under UV light.

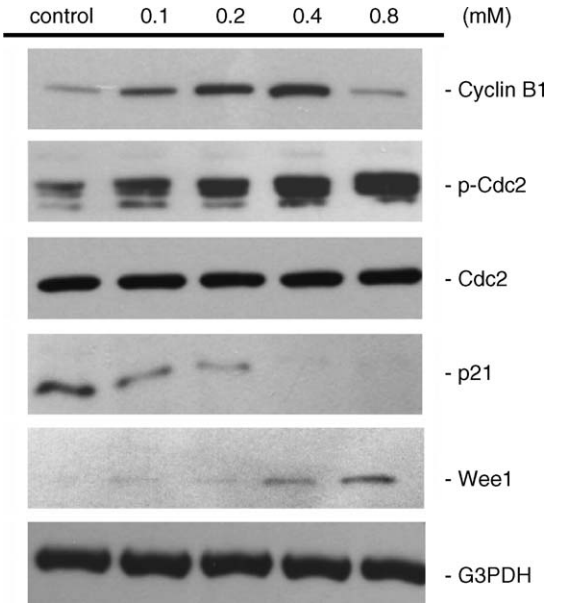

Fig. 5. Effect of arecoline on cell cycle-related proteins expression in KB epithelial cells. Cells were treated with arecoline (0.1–0.8 mM) for 24 h. The treated cells were collected, lysed, and the level of phosphorylated pCdc2 (Tyr-15), as well as the level of Cdc2, cyclin B1, Wee1 and $p21^{waf1}$ in each sample was determined by immunoblotting analysis with specific antibodies. The expression level of the G3PDH protein was selected as a loading control. Immunoreactive bands were finally developed with ECL system. One representative Western blotting data were shown with more than three similar results.

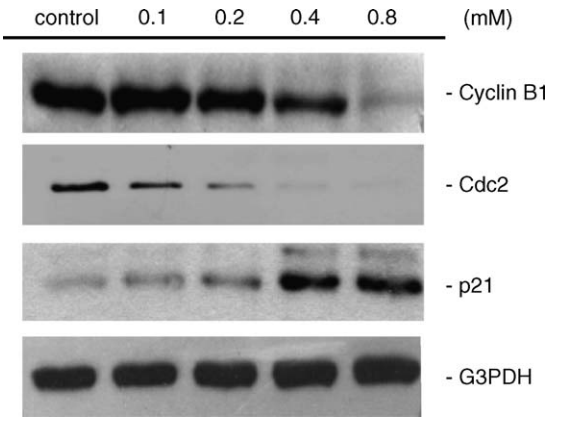

Fig. 6. Effect of arecoline on expression of cell cycle-related protein expression in primary GK. Cells were treated with arecoline (0.1–0.8 mM) for 24 h. The treated cells were collected, lysed, and the level of Cdc2, cyclin B1, and $p21^{waf1}$ in each sample was determined by immunoblotting analysis with specific antibodies. The expression level of the G3PDH protein was selected as a loading control. Immunoreactive bands were finally developed with ECL system. One representative Western blotting data were shown with more than three similar results.

4. Discussion

Arecoline is shown to induce various kinds of genetic damages, such as chromosomal aberrations, sister chromatid exchange, DNA breaks and DNA–protein cross-links on different target cells (IARC, 2004; Jeng et al., 2001). Our recent studies demonstrated that arecoline and AN extract also exhibit cytotoxicity and inhibit the growth of oral fibroblasts and keratinocytes (IARC, 2004; Jeng et al., 1999; Chang et al., 1998). Salivary concentration of arecoline during BQ chewing has been detected to be up to about 140 μg/ml (0.9 mM) (Nair et al., 1985). Besides, Sundqvist et al. (1989) have found that the areca alkaloids, such as arecoline, in the millimolar concentration range is important for the long-term effects of BQ chewing on buccal mucosa. We therefore tested the effects of arecoline on human KB epithelial cells and GK at concentrations ranging from 0.1 to 1.2 mM.

In this study, arecoline suppressed the proliferation of KB cells as analyzed by MTT and SRB toxicity assays. Arecoline and arecaidine have been shown to stimulate the proliferation of OMF at concentrations of 10 μg/ml or higher (Harvey et al., 1986). However, different results were found in our study and indicated that 15.5 μg/ml arecoline (0.1 mM) inhibited the growth of KB cells after 3 days of exposure. This result strongly implies that cytotoxic and cytostatic effect of arecoline and AN components toward oral mucosa cells (IARC, 2004; Jeng et al., 1999) may contribute to the pathogenesis of epithelial atrophy, and a decrease in epithelial thickness as noted histologically in mucosa of many BQ chewers with OSF (Reichart, 1995; IARC, 2004; Jeng et al., 1999).

Previous studies have found the induction of cell cycle deregulation on human epithelial cells and mice bone marrow cells by AN extract, arecoline as well as hydroxychavicol (HC), a betel leaf component (Chang et al., 2001a, 2002; Mukherjee and Giri, 1991). AN extract and arecoline induced the S and G2/M phases cycle arrest of KB cells time-dependently within 24 h of exposure (Chang et al., 2001a,b). In addition, exposure of JB6 cells to lime-piper-betel quid extract elicits transformed foci with the concomitant decrease in G1 phase and increase in S-phase populations (Lin et al., 2003). In our study, arecoline induced S-phase arrest at concentration of 0.2–0.4 mM, whereas G2/M arrest was induced by higher contraction of arecoline (0.8–1.2 mM) after 24 h of incubation. All these results implied that cellular responses to toxic BQ components were different in a dose-dependent manner. Deregulation of cell cycle control by arecoline may contribute to chemical carcinogenesis in BQ chewers.

Annexin V has been proven as a useful marker for cellular apoptosis, and the binding of cells by FITC-labeled annexin V reveals the early changes of apoptosis. In this study, exposure to arecoline (0.4–1.2 mM) within 48 h elicited mainly cell necrosis, whereas further prolonged the incubation time to 72 h induced both necrotic and apoptotic changes as indicated by annexin V staining, suggesting both necrosis and apoptosis that occurred in different time points contributed to arecoline-mediated cytotoxicity. These findings were further confirmed by the presence of DNA laddering formation in KB cells after 72 h of arecoline (0.4–1.2 mM) exposure. Such results implied that the presence of late induced signal transduction cascades (such as poly-ADP ribosylation of histones) was involved in arecoline-induced apoptosis. Accordingly, chronic exposure of Swiss albino mice to arecoline (10 μg/ml) in vivo has been shown to down-regulate the poly-ADP ribosylation of cellular histones and to inhibit the poly-ADP-ribosyl-polymerase leading to chromatin relaxation observed in spleen and bone marrow cells (Saikia et al., 1999).

Recently, deregulation of genes that involved in cell growth and apoptosis has been observed in BQ-chewing related oral cancer specimens (Chiang et al., 2002). BQ also contains other chemicals (such as hydroxychavicol), which may induce apoptosis of epithelial cells (Chang et al., 2002). Apoptosis that occurred in normal tissue can prevent accumulation of genetic defects that lead to cancer or other diseases. However, excessive induction of cellular apoptosis may cause tissue destruction (Nagata et al., 2003). More clinical studies are therefore needed to evaluate the apoptotic changes of oral mucosa epithelium in BQ chewers exposed to BQ within 6 months–1 year.

In KB cancer epithelial cells, we found that the $p21^{waf1}$ protein expression was not induced following exposure to arecoline, which was possibly resulted from either transcriptional inactivation or by shortening the half-life of $p21^{waf1}$ mRNA similar to that induced by UV-irradiation on fibroblasts (McKay et al., 1998). This insufficient induction of $p21^{waf1}$ by arecoline may also explain why KB cells would not efficiently arrest in G0/G1 phase. Similar results shown in the MCF-7 breast cancers indicated that S-phase arrest with stabilization of p53 but not induction of p21 was observed by treatment with *anti* benzo[*g*]chrysene 11,12-dihydrodiol-13,14-epoxide, a potent mammary carcinogen (Khan et al., 1997). Delayed-induction of KB cancer epithelial cell apoptosis by arecoline may also be explained by reduction in anti-apoptotic function of p21.

Chemical carcinogens (such as chromium) and radiation have been shown to initiate a G2 checkpoint response that causes a delay activation of cyclin B/cdc2 kinase activity at the G2/M border (O'Brien et al., 2003; Shackelford et al., 2000). Since the cdc2/cyclin B1 complex is known as the primary regulator of G2/M progression, inhibition the supply of cyclin B1 seems not a crucial factor in arecoline-induced G2/M arrest KB cancer cells in our study. To clarify the mechanisms responsible for arresting KB cells in G2/M by arecoline, Western blot analysis was performed in KB cells treated with arecoline. We found that the protein levels of cyclin B1 was not induced by arecoline in a significant level. Furthermore, we also found that cdc2 protein level showed no evident changes. Interestingly, an increased level of cdc2 (Tyr 15) phosphorylation was found in arecoline-treated KB cells. Such results were consistent with previous studies demonstrated that the kinase activity of cdc2/cyclin B1 complex is negatively regulated by phosphrylation at Try 15 through different kinases including Wee 1 kinase, Chk1 or Cd1 proteins activated by DNA damaging agent and unreplicative DNA (Yarden et al., 2002). The results described above suggest that arecoline-induced S and G2/M phase arrest in KB epithelial cells was associated mainly with phosphorylation of cdc2 (Tyr 15) by Wee 1 kinase resulting in a decrease of cdc2 activity without change of cyclinB1/cdc2 protein levels.

On the other hand, human primary GK were selected as another research cell model to investigate the arecoline-mediated cytotoxicity in oral epithelial cells. We found that arecoline induced a significant elevation of the $p21^{waf1}$ and a decline of cdc2 and cyclin B1 in GK in our study. $p21^{waf1}$, a CDK inhibitor, may form complex with the cdc2 and cyclin B and thus reduce the kinase activity, which implied that arecoline-induced cell cycle arrest can be mediated by cdc2/cyclin B1 complex inhibition through induction of p21 protein expression in normal (GK) epithelial cells. Differential cell cycle regulatory proteins responses between primary oral epithelial (GK) cells and malignant epithelial (KB) cells by arecoline may play some important roles in early and late stages of multi-step chemical carcinogenesis. For example, the $p21^{waf1}$ protein level is usually undetectable in normal oral mucosa epithelium, whereas the $p21^{waf1}$ expression is markedly elevated in pre-malignant lesions of BQ chewers in India (Agarwal et al., 1998). Increased level of p21 protein expression is also found associated with most of well-differentiated SCC, whereas poorly differentiated SCCs do not (Agarwal et al., 1998). These results indicated that BQ contained ingredients which may involve in oral carcinogenesis through induction of $p21^{waf1}$ expression in oral epithelium.

In conclusion, our results suggest that arecoline inhibits the growth of KB cancer epithelial cells by

arresting cells in S and G2/M phase, and prolonged exposure to higher concentrations (>0.4 mM) led to apoptosis. Differential alterations of cell cycle-related proteins can be crucial in different stages of BQ-mediated carcinogenesis. Further studies must be necessary to clarify the BQ ingredients on the sequential changes of cell cycle-related proteins in different stages of BQ-mediated carcinogenesis.

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