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Arecoline, a main alkaloid in *Areca catechu*, induces biological changes in human papillomavirus 16–positive cervical cancer cells *via* upregulation of viral oncogenes and cellular transcriptional factors

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ABSTRACT

Objective: To investigate the effects of arecoline on HPV-positive cervical cells and unveil its underlying mechanism in cervical carcinogenesis.

Methods: The cytotoxicity of arecoline was determined and the effect of subtoxic concentrations of arecoline on the expression of viral oncoproteins and transcriptional factors was examined in CaSki and SiHa cells. HPV16 promoter activity was evaluated in a plasmid containing HPV16 long control region (pGL3-HPV16LCR)-transfected cells. Cell proliferation, cell migration, and number of colonies were assessed by MTT, wound healing assay, and colony-forming assay, respectively.

Results: Arecoline at 0.01 µg/mL significantly upregulated HPV16 E6 and E7 oncoproteins in both CaSki and SiHa cells. It also upregulated the expression level of *c-Fos* and *c-Jun* mRNAs, and c-Myc protein in CaSki and SiHa cells. In addition, arecoline at subtoxic concentrations (0.0025 and 0.01 µg/mL) significantly induced HPV16 promoter activity in pGL3-16LCR-transfected cells. It also promoted SiHa and CaSki cell proliferation, migration, and colony formation.

Conclusions: Arecoline at subtoxic concentrations promotes the proliferation, migration, and colony formation of CaSki and SiHa cells *via* upregulation of c-Fos, c-Jun, c-Myc, and HPV16 E6 and E7 expressions.

KEYWORDS: Arecoline; Human papillomavirus; Oncogenes; Cervical cancer; c-Fos

1. Introduction

Cervical cancer is the fourth most common cancer in women

worldwide with 661 021 cases and 348 189 death in 2022. The age-standardized incidence and mortality rates of cervical cancer were 14.1% and 7.1%, respectively[1]. It is well known that more than 90% of oncogenic human papillomavirus (HPV) infection is a necessary cause of cervical cancer[2]. Furthermore, smoking, long-term oral contraceptives, unvaccinated people, unsafe sex, multiple sexual partners, and multiple childbirths are risk factors for cervical cancer[3]. Notably, betel quid chewing has been linked to uterine cervical dysplasia and cervical cancer[4].

The areca nut (*Areca catechu*) is the main ingredient in betel quid

Summary

Question: How can arecoline promote HPV-associated cervical cancer development?

Findings: Arecoline at subtoxic concentrations upregulated the cellular transcriptional factors c-Fos, c-Jun, c-Myc, and HPV16 E6/E7 expression, and increased viral LCR activity in cervical cancer cell lines. Low-dose of arecoline increased cell proliferation, cell migration, and colony formation in HPV16-positive cervical cancer cells.

Meaning: Arecoline at subtoxic concentrations promotes cellular and viral oncogenes expression and cervical carcinogenesis.

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chewing and is wrapped in the leaf of the vine *Piper betle* which is chewed by over 600 million people in Asia[5]. Arecoline is the main alkaloid in the areca nut and induces various cellular pathways, especially involving carcinogenesis including oral cancer, hepatoma, and lung cancer[4]. Betel quid chewing has shown a significant, dose-dependent association with invasive cervical cancer[4]. In addition, the invasive cervical cancer risk seemed to be attributable to a higher prevalence of cervical HPV infection in women who engaged in betel quid chewing[4]. However, the exact mechanism of betel quid chewing has not been elucidated. Here, we investigated the effect of arecoline on cervical cancer cell lines with HPV infection.

The long control region (LCR), also known as the upstream regulatory region of HPV, is a non-coding region that regulates viral DNA replication and transcription[6]. LCR contains the HPV early promoter that controls the transcription of all early genes including the *E6* and *E7* oncogenes[6]. Activator protein-1 (AP-1), which is a transcription factor composed of members of the Jun and Fos families, is known to control the early promoter through their binding to specific sites in the LCR[7,8]. c-Jun positively regulated HPV oncogene *E6/E7* expression in cervical cancer cell lines[9]. Myc is also well known as a transcriptional factor promoting oncogenesis in many cancers and in particular, directly suppressing *p53* mRNA expression[10]. A previous study demonstrated the effect of c-Myc on the regulation of HPV LCR activity[11]. In contrast, some other studies demonstrated that HPV *E6/E7* promoted c-Myc expression and directly interacted with c-Myc promoting oncogenesis[12]. Moreover, HPV DNA integration promoted c-Myc expression in the nucleus[13]. Similar to c-Myc, c-Fos and c-Jun proteins also directly bind p97 enhancer/promoter region of HPV16 to activate viral oncogene expression[13]. In addition, arecoline directly induced c-Myc, c-Fos, and c-Jun expression in oral cancer, epithelial, and oral mucosal fibroblast cells at a low concentration, respectively[14–16].

Many compounds, including cigarette smoke condensate and andrographolide (androg), can modulate HPV LCR activity, contributing to the alteration of HPV replication and transcription in various cancer cell lines, including lung and cervical cancer cells[17,18]. However, the effect of arecoline on altering HPV *E6/E7*, c-Fos, c-Jun, and c-Myc expression has not been examined in HPV-infected cervical cancer cells.

In the present study, we aimed to study the effect of various concentrations of arecoline on HPV16-positive cervical cancer cells and to explore the biological changes promoting cervical cancer development.

2. Materials and methods

2.1. Cell culture and reagents

The cervical cancer cell lines: HPV-positive cell lines (CaSki and SiHa) and HPV-negative cell line (C33A) were kindly provided by

Prof. Tohru Kiyono (National Cancer Center Research Institute, Japan) and were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco; Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (HiMedia Laboratories; Mumbai, Maharashtra, India) and antibiotics (2.5 µg/mL amphotericin B, 40 µg/mL gentamicin, 100 unit/mL penicillin G and 100 µg/mL streptomycin) at 37 °C in a humidified incubator.

2.2. Compound

Arecoline was purchased from Sigma-Aldrich (purity > 98%, St. Louis, MO, USA). A stock of arecoline solution was prepared and dissolved in sterile water at a concentration of 5 mg/mL and stored at –20 °C until used.

2.3. MTT assay for cytotoxicity

C33A, CaSki, and SiHa cells were seeded into 96-well plates at a density of 10 000 cells/well, incubated overnight, and maintained in DMEM with 10% FBS. Various concentrations of arecoline were treated in each cell line for 48 h. Then, 10 µL of 5 mg/mL of the MTT solution was added to each culture well, followed by incubation at 37 °C for 4 h. Purple formazan was dissolved in DMSO (Sigma-Aldrich; St. Louis, MO, USA). The absorbance of the color was measured at 540 nm using a microplate spectrophotometer (Multiskan GO; Thermo Fisher Scientific; Vantaa, Finland). The assay was performed in triplicate. Relative viability was calculated by subtracting the blank control (no cells) and normalizing to untreated cells. The subtoxic concentration (SC) under 50% cytotoxic concentration (CC_{50}) was used for further experiments.

2.4. The biological effect of arecoline-treated cells

Low FBS was used to determine the biological changes including cell proliferation, cell migration, and colony formation compared with complete medium and control to minimize its effect on biological activity[19,20].

2.4.1. Cell proliferation assay

A low density of cervical cancer cell lines was cultured in DMEM with 2.5% and 10% FBS and treated with arecoline at SCs to determine the percentage of a cell population using MTT in a cell proliferation assay. Briefly, CaSki and SiHa cells were seeded into 96-well plates at a density of 5 000 cells per well, incubated overnight, and maintained in DMEM with 2.5% or 10% FBS. Each cell line was treated with arecoline at SCs for 24 and 48 h. Then 10 µL of 5 mg/mL of the MTT solution was added to each culture well, followed by incubation at 37 °C for 4 h. Purple formazan was dissolved in DMSO (Sigma-Aldrich). The absorbance of the color was measured at 540 nm using a spectrophotometer (Multiskan GO; Thermo Fisher Scientific). The assay was performed in triplicate. The percentage of cell population was calculated by subtracting the

blank control (no cells) and normalizing it to untreated cells.

2.4.2. Cell migration by wound healing assay

CaSki and SiHa cells were seeded into a 24-well plate at a density of 150 000 cells per well and maintained in the media containing 2.5% or 10% FBS for 24 h. Arecoline at SCs was subjected to the cells and continuously incubated for 48 h. The wound was made using a 200 μ L sterile tip on day 0 after the arecoline treatment. The photo of wound healing was captured on days 0, 1, and 2 after arecoline treatment under an inverted microscope (Nikon Eclipse TS100, Tokyo, Japan). Wound distance was measured using the ImageJ software (1.51j8; Wayne Rasband; National Institutes of Health, Bethesda, MD, USA).

2.4.3. Colony forming assay

CaSki and SiHa cells were seeded into 12-well plates at a density of 300 cells per well, incubated overnight, and maintained in DMEM with 2.5% or 10% FBS. Arecoline (0–25 μ g/mL) was treated in each cell line for 96 h and then replaced with a complete medium for 5 d. Colonies were fixed and stained with acetic acid/methanol (3:1, *v/v*), and crystal violet, respectively. Colonies were counted to calculate the relative number of colonies.

2.5. Cell morphology after arecoline treatment using acridine orange/ethidium bromide (AO/EB) staining assay

Each CaSki and SiHa was seeded in a 24-well plate at a density of 100 000 cells in a complete medium and incubated for 24 h. Cells were treated with 0–25 μ g/mL arecoline for 48 h, and then were harvested using trypsinization. Cell suspension was dually stained with AO/EB and immediately observed under a fluorescent microscope (Olympus BX51, Olympus, Tokyo, Japan) to distinguish viable, dying, and dead cells.

2.6. Total RNA and protein isolation from arecoline-treated/untreated cell lines

Each CaSki and SiHa cell line was seeded into a 24-well plate at a density of 150 000 cells and maintained in a complete medium for 24 h. Various SCs of arecoline were added to the cell culture and incubated for 48 h. The cell pellet was lysed using Trizol (Invitrogen; Carlsbad, CA, USA), according to the manufacturer's protocol. Briefly, the cell pellet was added to 500 μ L of Trizol reagent, followed by 100 μ L of chloroform, and then mixed vigorously. After incubation on ice for 30 min, the layer in the solution was separated by centrifugation at 12 000 rpm for 15 min. The upper and lower phase layers were collected to extract RNA and protein, respectively.

2.7. Detection of HPV16 E6/E7, *c-Fos* and *c-Jun* expression using RT-PCR

Total RNA samples from the treated/untreated cell lines were used

as a template to synthesize cDNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific; Waltham, MA, USA) with oligo (dT) primer, according to the manufacturer's protocol. Diluted 1:10 cDNA was subjected to LightCycler[®] 480 SYBR Green I Master (Roche Diagnostics; Mannheim, Germany) to investigate HPV16 E6/E7, *c-Fos*, *c-Jun*, and *GAPDH* (an internal control) expression with specific primers (Supplementary Table 1). The reactions were carried out in the CFX96 Touch[™] Real-Time PCR Detection System (Bio-Rad; Hercules, CA, USA). The relative quantification was determined using $2^{-\Delta\Delta CT}$.

2.8. Luciferase activity of HPV16 LCR in arecoline-treated pGL3-16LCR-transfected C33A cells

The HPV16 LCR containing the early gene promoter was cloned into the pGL3 luciferase reporter vector (Promega; San Luis Obispo, CA, USA). The pGL3-Basic and pGL3-16LCR vectors were transformed into competent *Escherichia coli* TOP10 and extracted using an ENZA[™] Plasmid Miniprep Kit (Omega Bio-tek Inc; Doraville, GA, USA). C33A cell line was seeded into a 96-well plate and maintained in a complete medium for 24 h. Then, 200 ng of each pGL3-Basic vector and pGL3-16LCR vector were subjected to the reaction mixture of transfection using Lipofectamine 2000 (Invitrogen; San Diego, CA, USA) according to the manufacturer's protocols and added into each well. After 6 hours of transfection, the SCs of arecoline were added into transfected cells and incubated for 48 h. Cell supernatant was provide using the Bright-Glo[™] Luciferase Assay kit (Promega, Madison, WI) and subjected to GloMax 20/20 luminometer (Promega, Madison, WI, USA) to measure luciferase activity. Luciferase activity of pGL3-16LCR-transfected cells was measured relative to normalized luciferase activity of pGL3-Basic-transfected cells.

2.9. Western blot

Proteins from the arecoline-treated/untreated cell lines were subjected to electrophoresis, followed by Western blot analysis. The HPV16 E6, c-Myc and β -actin proteins were detected using the monoclonal antibody from the C1P5, 9E10, and C4 clones from Santa Cruz Biotechnology Inc. (CAT No. sc-460, sc-40, and sc-47778 respectively, Santa Cruz, Cal, USA) in dilutions of 1:400, 1:500, and 1:1 000, respectively. The IgG κ binding protein-HRP (m-IgG κ BP-HRP) (CAT No. sc-516102; Santa Cruz Biotechnology Inc.; Santa Cruz, CA, USA) was used as the secondary antibody. The signal was produced using ECL Prime Western Blotting Reagent (GE Healthcare UK Ltd; Chalfont St. Giles, UK). The protein bands on the nitrocellulose membrane were analyzed using an ImageQuant LAS 4000 (GE Healthcare, Fairfield, CT, USA). The intensity of protein bands was measured using the Image J 1.49v software (National Institutes of Health, Bethesda, MD, USA).

2.10. Statistical analysis

Data were expressed as mean $\pm$ standard error of the mean (SEM) and analyzed using one-way ANOVA analysis. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using the Prism5 software (GraphPad, San Diego, CA, USA).

3. Results

3.1. Cytotoxicity of arecoline against CaSki, SiHa, and C33A

To investigate the cytotoxicity of arecoline against cervical cancer cell lines, CaSki, SiHa, and C33A cell lines were treated with various concentrations of arecoline and analyzed for cell viability using an MTT reagent. Based on the results, the SCs of the arecoline-treated CaSki and SiHa cell lines were $\leq 25 \mu\text{g/mL}$ and $\leq 50 \mu\text{g/mL}$,

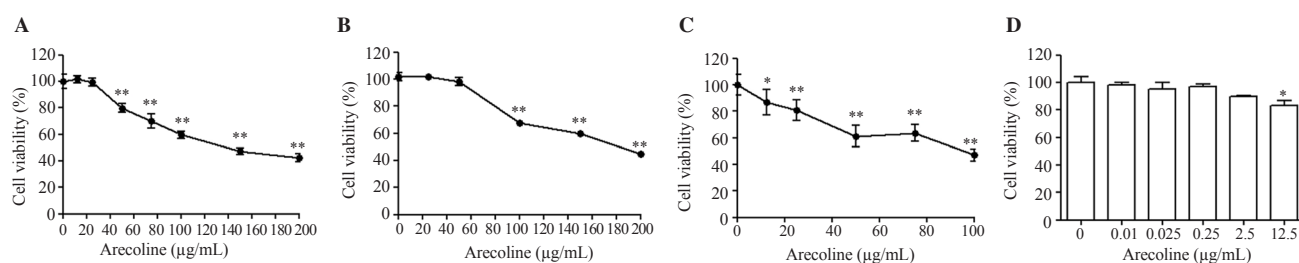


Figure 1. Cytotoxicity of arecoline against CaSki, SiHa, and C33A cells. CaSki (A), SiHa (B), and C33A (C-D) were treated with arecoline at various concentrations for 48 h and then cell viability was measured using an MTT assay. Absorbance of color was detected at 540 nm. Data are expressed as mean $\pm$ SEM and analyzed by one-way ANOVA. * and ** indicate significant differences at $P < 0.05$ and 0.001 , respectively.

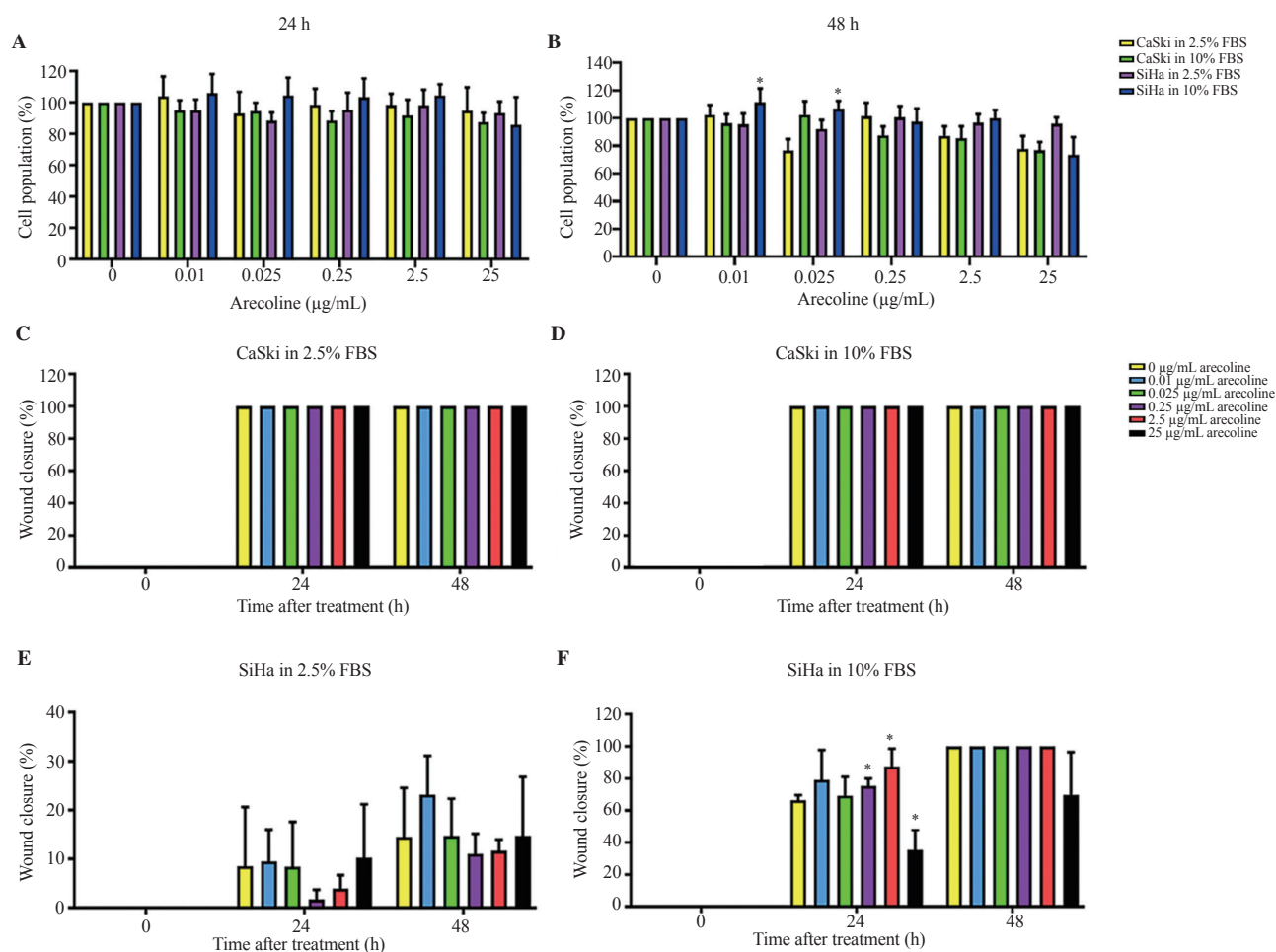


Figure 2. Effect of arecoline on cervical cancer cell proliferation and migration. The percentage of cell population was analyzed using an MTT assay in arecoline-treated CaSki and SiHa at 24 (A) and 48 h (B). CaSki (C-D) and SiHa (E-F) cell migration were determined by wound healing assay. Data are expressed as mean $\pm$ SEM and analyzed by two-way ANOVA. * indicates a significant difference at $P < 0.05$.

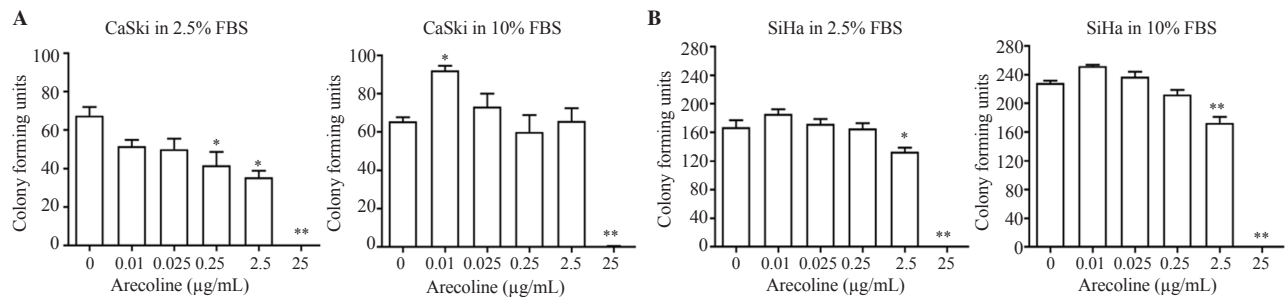


Figure 3. Effect of arecoline on colony formation in CaSki and SiHa cells. CaSki and SiHa cells were treated with arecoline for 96 h. Visible colonies in CaSki (A) and SiHa (B) cells were counted after staining with crystal violet. Data are expressed as mean $\pm$ SEM and analyzed by one-way ANOVA. * and ** indicate significant differences at $P < 0.05$ and 0.001 , respectively.

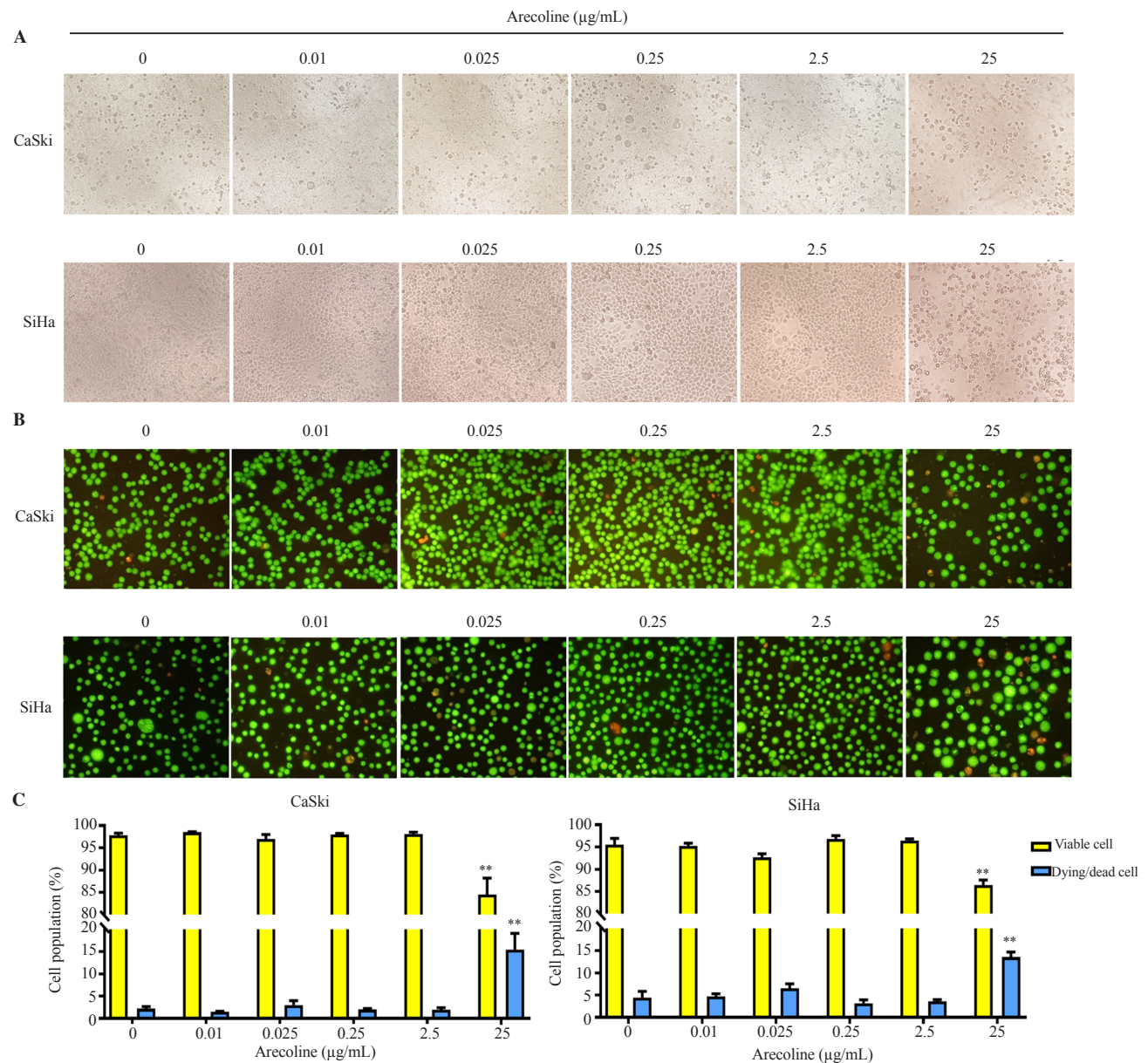


Figure 4. Effect of arecoline on CaSki and SiHa cell morphology. CaSki and SiHa cells were treated with 0–25 µg/mL arecoline for 48 h and then observed under a light microscope (A). Viable (rounded shape, normal nucleus with green fluorescence), dying (irregular shape with green fluorescence), and dead (orange or red nucleus with/without green, yellow, orange or red cytoplasm) cells were distinguished by acridine orange/ethidium bromide staining and observed under a fluorescent microscope (B). The number of viable and dying/dead cells was presented in percentage of cell population (C). Data are expressed as mean $\pm$ SEM and analyzed by one-way ANOVA. ** indicates a significant difference at $P < 0.001$.

respectively (Figure 1A-B). Because the percentage of cell viability in C33A treated with 12.5 $\mu\text{g/mL}$ arecoline was less than 90% (Figure 1C), the viability of C33A cells was additionally determined after treatment with 0–12.5 $\mu\text{g/mL}$ arecoline. The SC of the arecoline treatment in C33A cells was ≤ 2.5 $\mu\text{g/mL}$ (Figure 1D). From this information, arecoline at concentrations of 0.01–25 $\mu\text{g/mL}$ was used for further experiments.

3.2. Biological activity of SCs of arecoline in HPV16–positive cervical cancer cell lines

3.2.1. Effect of arecoline on cell population and cell migration

Arecoline at concentrations of 0.01–2.5 $\mu\text{g/mL}$ tended to increase

the percentage of cell population in SiHa cells at 24 h without significant differences compared with untreated control cells (Figure 2A), whereas, at 48 h, 0.01 and 0.025 $\mu\text{g/mL}$ of arecoline significantly enhanced SiHa cell population (Figure 2B). In contrast, arecoline at 0.01–2.5 $\mu\text{g/mL}$ did not increase cell population in CaSki whereas 25 $\mu\text{g/mL}$ tended to decrease cell population at 48 h (Figure 2B). Simultaneously, it increased cell migration in SiHa cells, whereas these results were not observed in CaSki cells because the wound had completely closed within 24 h, as shown in Figure 2C–F, Supplementary Figure 1, and Supplementary Table 2. This study demonstrated that arecoline treatment for 48 h significantly increased cell population (at 0.01 and 0.025 $\mu\text{g/mL}$) and cell migration (at 0.25 and 2.5 $\mu\text{g/mL}$) in SiHa cells.

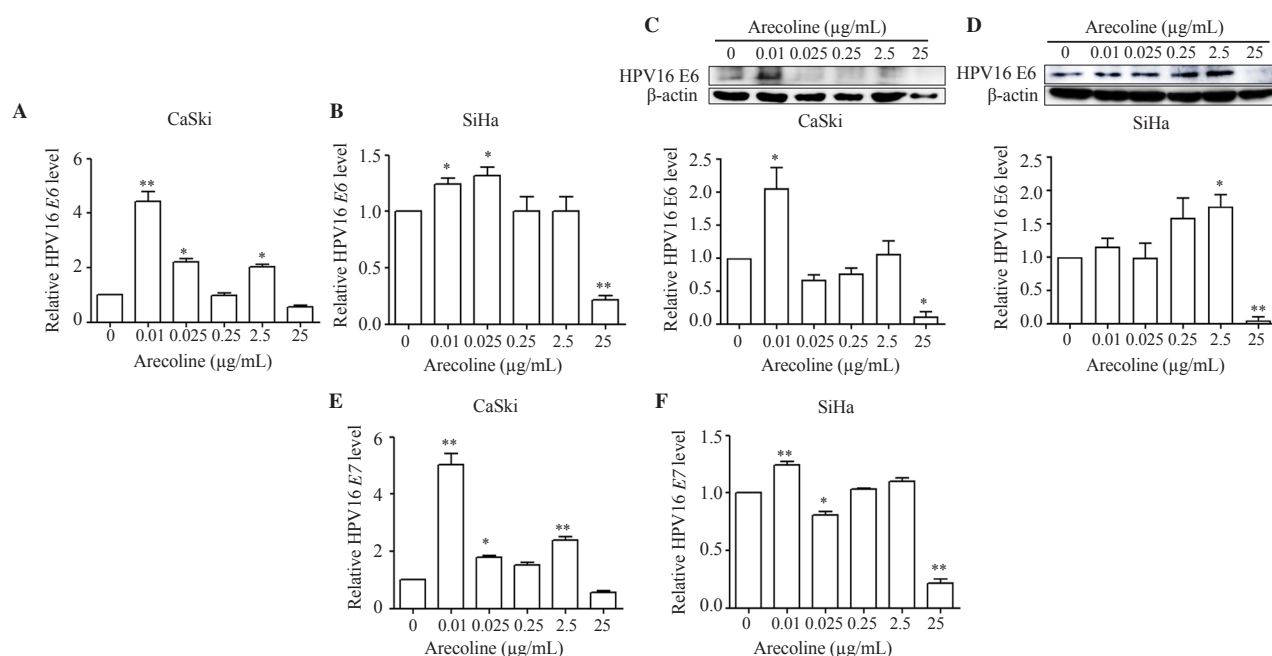


Figure 5. HPV16 E6 and E7 expression in CaSki and SiHa cells after arecoline treatment. CaSki and SiHa cells were treated with various subtoxic concentrations (0.01–25 $\mu\text{g/mL}$) of arecoline for 48 h. The mRNA and protein expressions of HPV16 E6 were determined using RT-PCR (A and B) and Western blot analysis (C–D). HPV16 E7 mRNA expression was also examined in CaSki (E) and SiHa (F) cells treated with various subtoxic concentrations of arecoline for 48 h. *GAPDH* was used as an internal control. Data are expressed as mean $\pm$ SEM and analyzed by one-way ANOVA. * and ** indicate significant differences at $P < 0.05$ and 0.001, respectively.

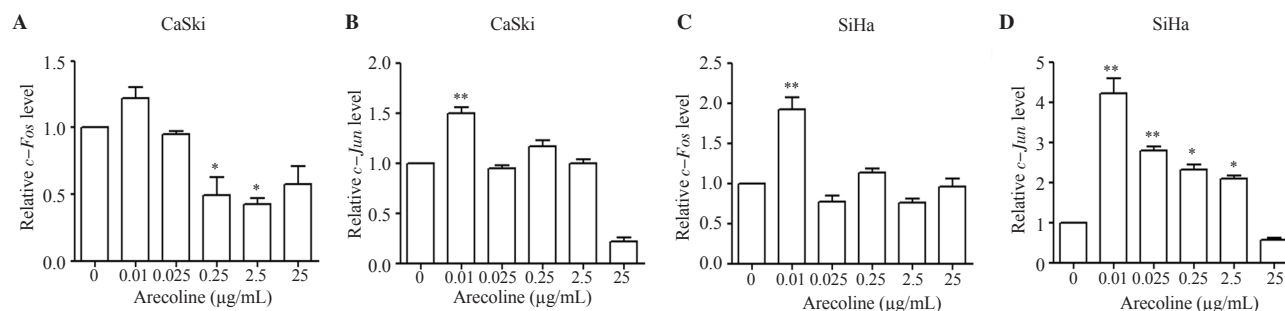


Figure 6. mRNA level of *c-Fos* and *c-Jun* in CaSki (A–B) and SiHa (C–D) cells treated with various subtoxic concentrations of arecoline for 48 h. *GAPDH* was used as an internal control. Data are expressed as mean $\pm$ SEM and analyzed by one-way ANOVA. * and ** indicate significant differences at $P < 0.05$, and 0.001, respectively.

3.2.2. Effect of arecoline on colony number

We found that 0.01 $\mu\text{g/mL}$ of arecoline significantly increased colony number in CaSki cells maintained in 10% FBS (Figure 3A and Supplementary Figure 2A). Moreover, low doses of arecoline increased the number of colonies in SiHa cells that were maintained in 2.5% and 10% FBS without significant difference compared with the untreated control (Figure 3B and Supplementary Figure 2B).

3.3. Effect of arecoline on CaSki and SiHa cell death

To confirm SCs used in this study that did not cause cell death in arecoline-treated CaSki and SiHa, the cells were stained with AO/EB to determine viable and dying/dead cells. Figure 4A shows the morphology of 0.01–2.5 $\mu\text{g/mL}$ arecoline-treated cells did not differ from untreated cells whereas 25 $\mu\text{g/mL}$ caused higher number of detached and rounded cells than untreated control. In addition, 0.01–2.5 $\mu\text{g/mL}$ arecoline did not induce any significant effect on CaSki and SiHa cell death, but a high dose (25 $\mu\text{g/mL}$) of arecoline significantly reduced cell viability (Figure 4B–C). These results demonstrated that 0.01–2.5 $\mu\text{g/mL}$ arecoline did not cause cytotoxic effect against CaSki and SiHa.

3.4. Effect of arecoline on HPV16 E6 expression

HPV16 E6 expression was examined using RT-PCR and Western blot analysis. HPV16 E6 mRNA expression in arecoline (0.01 and 0.025 $\mu\text{g/mL}$)-treated CaSki (Figure 5A) and SiHa cells (Figure 5B) was significantly increased. The level of HPV16 E6 protein was increased in 0.01 $\mu\text{g/mL}$ arecoline-treated CaSki (Figure 5C), whereas in SiHa cells, the level of HPV16 E6 protein gradually increased with increasing arecoline concentration from 0.01 to 2.5 $\mu\text{g/mL}$ (Figure 5D). Conversely, the level of HPV16 E6 expression

was markedly downregulated after treatment with 25 $\mu\text{g/mL}$ of arecoline in CaSki and SiHa cells. These results demonstrated that arecoline at a low concentration of 0.01 $\mu\text{g/mL}$ could upregulate HPV16 E6 mRNA expression. In addition, an upregulation of HPV16 E6 in arecoline-treated CaSki corresponded to an increase in colony formation maintained in DMEM with 10% FBS whereas E6 upregulated in arecoline-treated SiHa was associated with an increase in cell population (0.01 and 0.025 $\mu\text{g/mL}$ arecoline maintained in DMEM with 10% FBS), cell migration (0.01–2.5 $\mu\text{g/mL}$ arecoline maintained in DMEM with 10% FBS), and colony formation (0.01 $\mu\text{g/mL}$ arecoline maintained in DMEM with 2.5 and 10% FBS) (Figures 2–3). LCR or upstream regulatory region (URR) directly regulates HPV16 E6 and E7. HPV16 E7 mRNA expression was therefore examined using RT-PCR to confirm arecoline's effect on viral oncogenes. The highest level of HPV16 E7 was found in CaSki (Figure 5E) and SiHa cells (Figure 5F) treated with 0.01 $\mu\text{g/mL}$ of arecoline. These results suggested that low concentrations of arecoline treatment promoted HPV16 E7 expression in HPV16-positive cervical cancer cell lines.

3.5. Effect of arecoline on *c-Fos* and *c-Jun* expression in HPV16-positive cervical cancer cell lines

We found that 0.01 $\mu\text{g/mL}$ of arecoline increased *c-Fos* and *c-Jun* expression in CaSki cells compared with untreated cells (Figure 6A–B). Simultaneously, arecoline treatment significantly upregulated *c-Fos* (at 0.01 $\mu\text{g/mL}$) and *c-Jun* (at 0.01–2.5 $\mu\text{g/mL}$) expression in SiHa cells (Figure 6C–D). These findings demonstrated that arecoline at low concentrations increased *c-Fos* and *c-Jun* expression in HPV16-positive cervical cancer cell lines. The activity of the p97 promoter in HPV16 on LCR was evaluated in pGL3-16LCR-transfected HPV-negative C33A cells, with pGL3-Basic-transfected

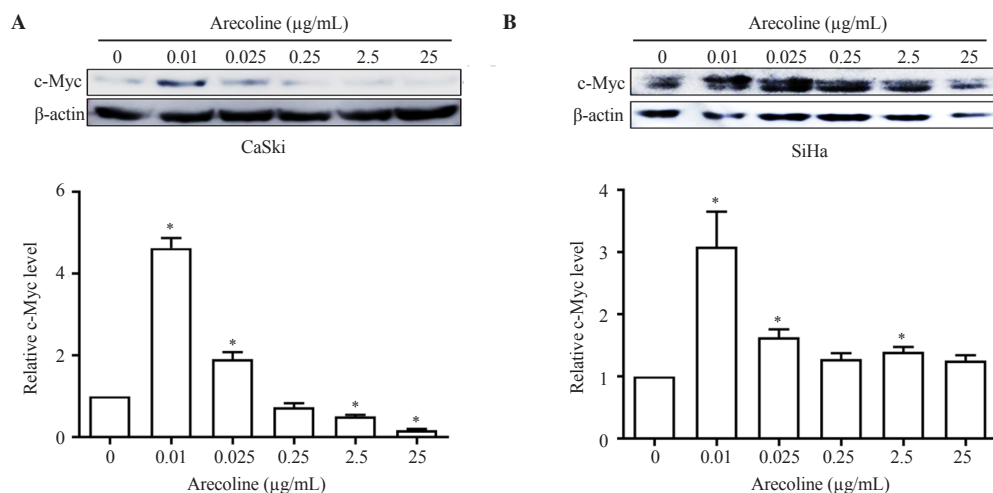


Figure 7. Expression of c-Myc protein in arecoline-treated CaSki and SiHa cells. CaSki (A) and SiHa (B) cells were treated with various concentrations of arecoline for 48 h. Data are expressed as mean $\pm$ SEM and analyzed by one-way ANOVA. * indicates a significant difference at $P < 0.05$.

cells used as a control following arecoline treatment. We observed that 0.0025, and 0.01 $\mu\text{g/mL}$ of arecoline could increase luciferase activity in C33A cells (Supplementary Figure 3).

3.6. Effect of arecoline on *c-Myc* expression in cervical cancer cell lines

Expectedly, arecoline at 0.01 and 0.025 $\mu\text{g/mL}$ markedly upregulated *c-Myc* expression in CaSki and SiHa cells (Figure 7). These data suggested that arecoline at SCs could promote *c-Myc* expression.

4. Discussion

A previous study has already reported that low concentrations of arecoline could promote oral carcinogenesis[14]. Conversely, there have been reported effects of arecoline-induced apoptosis in various cell types[21,22]. The different results from using this compound may have been caused by the balance between pharmacological and toxic effects. A high dose of arecoline could induce cytotoxic effects and cell death through apoptotic pathways, whereas a low dose that did not affect cytotoxicity could promote cancer development through oncogene upregulation[23]. Therefore, we considered evaluating the effect of arecoline at SCs on HPV oncogene expression as well as cellular transcriptional factors on cervical cancer cell lines.

Arecoline is an alkaloid produced during areca nut chewing. This compound is circulated through the blood in the chewer's body, including the nervous, cardiovascular, digestive, and endocrine systems[24]. The level of blood arecoline may depend on the frequency of betel quid chewing. The mean level of blood arecoline was reported as (7.0 ± 10.7) ng/mL after areca nut chewing[25]. Therefore, this level of blood arecoline may induce the cell signaling cascades on cervical cells that affect its biological pathway.

There have been few studies on arecoline-treated cervical cell lines. The previous study suggested that the half maximal inhibitory concentration (IC_{50}) of arecoline on HeLa cells was 462 μM (109.08 $\mu\text{g/mL}$) and that this compound also induced cell death at 1–1000 μM (0.23–236.11 $\mu\text{g/mL}$)[26]. However, we found that the IC_{50} of arecoline on HeLa cells was (28.95 ± 2.10) $\mu\text{g/mL}$ (Supplementary Figure 4A). This different IC_{50} may have resulted from the different conditions of cell cultivation including 1) the percentage of FBS-containing medium; 2) the cell density; and 3) the time for incubation. However, the effects of arecoline on SiHa, CaSki, and C33A cell lines have not been reported.

The current study used 0–25 $\mu\text{g/mL}$ of arecoline for treatment. In the arecoline-treated cells, the expression of transcriptional factors (*c-Fos* and *c-Jun* genes, and *c-Myc* protein) was induced in cervical cancer cell lines, corresponding to the previous study in

skin keratinocyte (HaCaT)[27], oral mucosal fibroblast[16], and oral cancer (ORL-48T)[14]. As expected, low doses of arecoline treatment increased HPV16 p97 promoter activity and HPV16 *E6/E7* oncogene expression which might be related to arecoline-promoted *c-Fos*, *c-Jun* and *c-Myc* upregulation[11,28]. Other researchers have reported that cigarette smoke condensate could upregulate p97 promoter in a dose-dependent manner[17]. The upregulation of HPV16 *E6* not only inhibited apoptosis, but also promoted cell migration through *E-cadherin* and *p-cadherin* regulation at 24 and 48 h[29], which was consistent with our findings. Our study demonstrated that arecoline upregulated HPV16 *E6* protein, and increased cell migration and the number of colonies that were correlated with upregulation of *c-Fos* and *c-Jun* mRNAs, and *c-Myc* protein in HPV16-positive cells, corresponding to the previous studies that demonstrated the upregulation of *c-Fos*, *c-Jun*, and *c-Myc* proteins was associated with the growth of cancers[30–32].

Nevertheless, there are some limitations in this study including the cell types and *in vitro* experiments alone. The expression levels of *E6* and *E7* mRNAs in CaSki (200–400 copies of HPV16 per cell) were approximately 5.7 and 10-fold higher than in SiHa (1–2 copies of HPV16 per cell), respectively[33,34]. Previous study demonstrated that high levels of HPV16 *E6* and *E7* increased cell proliferation, migration, and invasion[35]. The high levels of HPV16 oncogenes in CaSki promoted its ability to grow faster than in SiHa, which aligns with our findings that may reflect the different results between both arecoline-treated cell lines. In addition, *in vitro* study alone cannot entirely predict the overall outcomes when compared with animal models and human participants after arecoline treatment.

The effect of arecoline has been studied elsewhere and has been suggested to be an anti-cancer drug; however, these studies have investigated a higher arecoline concentration (≥ 109.08 $\mu\text{g/mL}$ in HeLa and ≥ 30 $\mu\text{g/mL}$ HA22T/VGH cells) than our experiments (≤ 25 $\mu\text{g/mL}$ at SCs)[22,26]. Simultaneously, from our findings, its pharmacology at below cytotoxic level should be considered due to the induction of oncogenesis on cervical cells and did not increase cell death in 0–2.5 $\mu\text{g/mL}$ arecoline-treated CaSki, SiHa, and HeLa (Figure 1, 4 and Supplementary Figure 4B–D).

In conclusion, this study provides insight into the effect of arecoline at SCs on cervical cancer development and its oncogenes. The pharmaceutical role of arecoline might depend on its concentration in the body and synergistic combination of other compounds. To elucidate the effect of arecoline on its pharmaceutical role, low and high concentrations of this compound need to be further investigated in *in vivo* studies.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Funding

The study received no extramural finding.

Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

JC, CL, TE, and CP designed and performed the experiments. JC and CP wrote the manuscript. JC and CL analyzed the data and revised the manuscript. JC, CL, TE, and CP revised the manuscript. CP supervised the manuscript. All authors read and approved the final manuscript.

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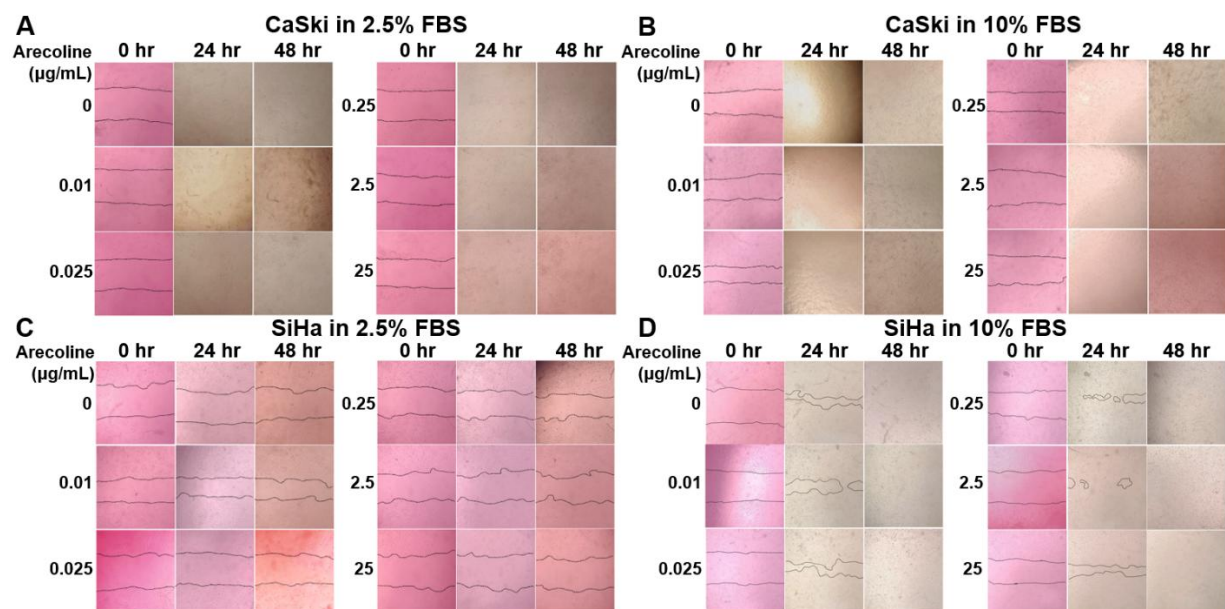
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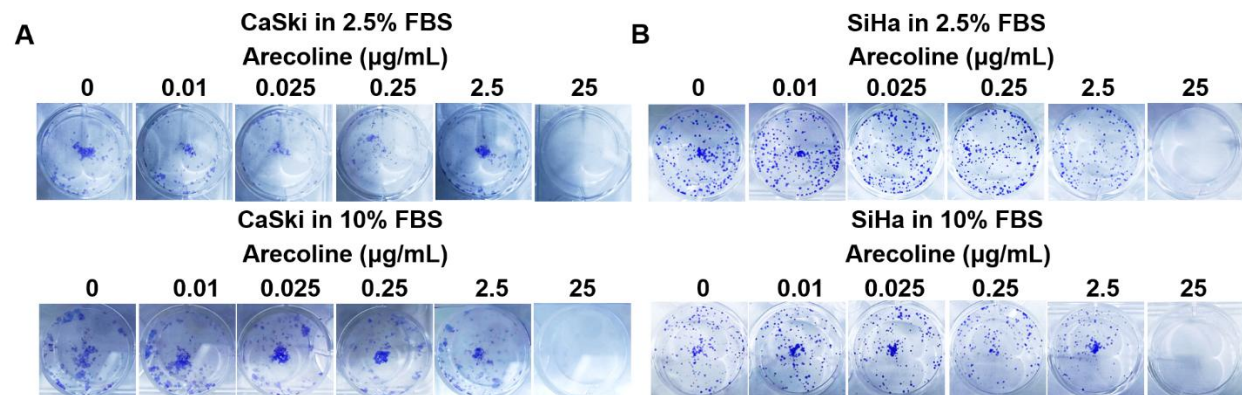
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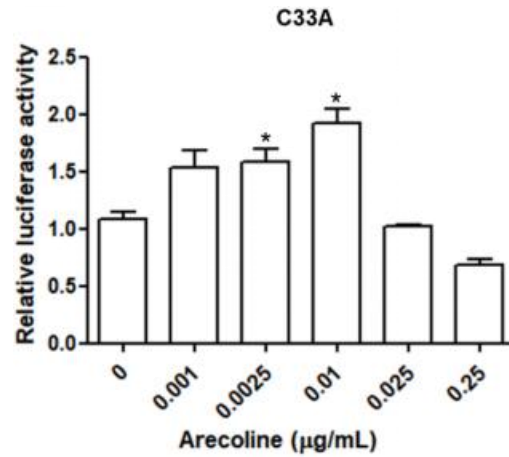


Supplementary Figure 1. Cell migration in arecoline-treated CaSki and SiHa based on wound healing assay.

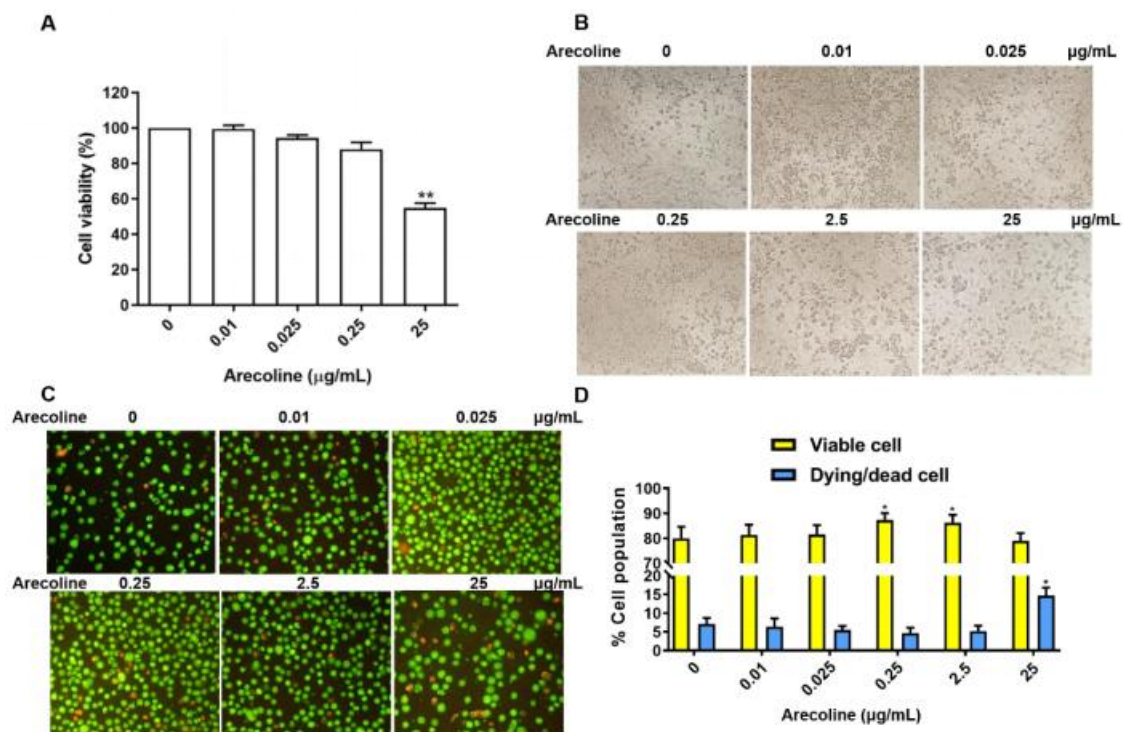
CaSki (A-B) and SiHa (C-D) cells were treated with 0-25 µg/mL arecoline maintained in medium supplemented in 2.5% (A and C) and 10% (B and D) FBS for 24 and 48 h.



Supplementary Figure 2. Colony numbers in arecoline-treated CaSki and SiHa cells. CaSki and SiHa cells were treated with arecoline for 96 h. Visible colonies in CaSki (A) and SiHa (B) cells were counted after staining with crystal violet.



Supplementary Figure 3. Luciferase activity of HPV16LCR in pGL3-16HPVLCR-transfected C33A cell line following arecoline treatment. Significant differences in luciferase activity were analyzed using one-way ANOVA, where * indicates a significant difference at $P < 0.05$.



Supplementary Figure 4. Cytotoxicity and cell morphology in HeLa after arecoline treatment. HeLa cell line was treated with 0-25 µg/mL arecoline for 48 h to determine cell viability and cell morphology using MTT (A)

and acridine orange/ethidium bromide staining (B-D) assays. Data are expressed as mean $\pm$ SEM and analyzed by one-way ANOVA. * and ** indicate significant differences at $P < 0.05$ and $P < 0.001$, respectively.

Supplementary Table 1. Primer sequences.

Primer	Oligosequence	Size
<i>HPV16 E6</i>	Sense: 5'-TCAAAAGCCACTGTGTCCTG-3'	139 bp
	Antisense: 5'-CAGCTGGGTTTCTCTACGTGT-3'	
<i>HPV16 E7</i>	Sense: 5'-CCGGACAGAGCCCATTACAA-3'	75 bp
	Antisense: 5'-GCTTTGTACGCACAACCGAA-3'	
<i>c-Fos</i>	Sense: 5'-AGAATCCGAAGGGAAAGGAA-3'	150 bp
	Antisense: 5'-CTTCTCCTTCAGCAGGTTGG-3'	
<i>c-Jun</i>	Sense: 5'-CAGGTGGCACAGCTTAAACA-3'	80 bp
	Antisense: 5'-GTTTGCAACTGCTGCGTTAG-3'	
<i>GAPDH</i>	Sense: 5'-TCATCAGCAATGCCTCCTGCA-3'	117 bp
	Antisense: 5'-TGGGTAGCAGTGATGGCA-3'	

Supplementary Table 2. Percentage of wound healing in arecoline-treated CaSki, and SiHa cells.

Day	Cell	Percentage of wound healing distance					
		0 $\mu\text{g/mL}$	0.01 $\mu\text{g/mL}$	0.025 $\mu\text{g/mL}$	0.25 $\mu\text{g/mL}$	2.5 $\mu\text{g/mL}$	25 $\mu\text{g/mL}$
24 h	CaSki in 2.5%FBS	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00
48 h	CaSki in 2.5%FBS	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00
24 h	CaSki in 10% FBS	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00
48 h	CaSki in 10% FBS	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00
24 h	SiHa in 2.5% FBS	8.47 $\pm$ 6.06	9.51 $\pm$ 3.23	8.37 $\pm$ 4.59	1.68 $\pm$ 0.98	3.94 $\pm$ 1.36	10.28 $\pm$ 5.44
48 h	SiHa in 2.5% FBS	14.47 $\pm$ 5.03	23.11 $\pm$ 3.99	14.70 $\pm$ 3.81	11.05 $\pm$ 2.05	11.65 $\pm$ 1.15	14.74 $\pm$ 6.00
24 h	SiHa in 10% FBS	66.33 $\pm$ 1.60	78.95 $\pm$ 9.37	69.24 $\pm$ 5.82	75.23 $\pm$ 2.31 ^a	87.58 $\pm$ 6.29 ^a	35.45 $\pm$ 6.25 ^a
48 h	SiHa in 10% FBS	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	69.79 $\pm$ 13.30

^a indicates a significant difference compared with untreated control.