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Continuous exposure of nicotine and cotinine retards human primary pterygium cell proliferation and migration

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Abstract

Pterygium is a triangular-shaped hyperplastic growth, characterized by conjunctivalization, inflammation and connective tissue remodeling. Our previous meta-analysis found that cigarette smoking is associated with a reduced risk of pterygium. Yet, the biological effect of cigarette smoke components on pterygium has not been studied. Here we reported the proliferation and migration properties of human primary pterygium cells with continuous exposure to nicotine and cotinine. Human primary pterygium cells predominantly expressed the $\alpha 5$, $\beta 1$ and γ subunits of nicotinic acetylcholine receptor. Continuous exposure to the mixture of 0.15 μM nicotine and 2 μM cotinine retarded pterygium cell proliferation by 16.04% ($p = 0.009$) and hindered their migration by 11.93% ($p = 0.039$), without affecting cell apoptosis. SNAIL and α -SMA protein expression was significantly downregulated in pterygium cells treated with 0.15 μM nicotine-2 μM cotinine mixture by 1.33 ($p = 0.036$) and 1.31 folds ($p = 0.001$) respectively. Besides, the 0.15 μM nicotine-2 μM cotinine mixture also reduced matrix metalloproteinase (MMP)-1 and MMP-9 expressions in pterygium cells by 1.56 ($p = 0.043$) and 1.27 folds ($p = 0.012$) respectively. In summary, this study revealed that continuous exposure of nicotine and cotinine inhibited human primary pterygium cell proliferation and migration *in vitro* by reducing epithelial-to-mesenchymal transition and MMP protein expression, partially explaining the lower incidence of pterygium in cigarette smokers.

Graphical Abstract

Continuous exposure of nicotine and cotinine retards human primary pterygium cell proliferation and migration with reduction in EMT marker and MMP expressions. There could be an inverse relationship between cigarette smoking and pterygium incidence.

Keywords: primary pterygium; nicotine; cotinine; proliferation; migration

1. Introduction

Pterygium is a triangular-shaped hyperplastic growth, occurring medially or laterally in the palpebral aperture. It is characterized by conjunctivalization, inflammation and connective tissue remodeling (Coroneo et al., 1999). As the pterygium encroaches the cornea, it induces astigmatism and eventually blocks the visual axis (Chen et al., 2014). The prevalence of pterygium in over 50 year-old Chinese adults is about 11%, ranging from 2.9% to 39.0% (Ma et al., 2007; Zhong et al., 2012).

The pathogenesis of pterygium remains elusive. Multiple signaling pathways have been suggested to be involved in pterygium development, including nuclear factor kappa B (NF- κ B) pathway (Siak et al., 2011), p38 MAPK pathway (Nuwormegbe et al., 2017) and mTOR pathway (Liu et al., 2017). We have previously reported that the involvement of p53/MDM2 pathway (Cao et al., 2018) and microRNA-145 (Teng et al., 2018) in the pathogenesis of pterygium, and the head region of pterygium tissues has higher expression of matrix metalloproteinase-2 (MMP-2) and MMP-9 than the neck and body regions (Bai et al., 2010). Coincidentally, aberrant accumulation of extracellular matrix molecules and elastotic degeneration in the stroma of pterygium tissues has been reported (Wang et al., 2000). There are myofibroblastic differentiation and high expression of α -smooth muscle actin (α -SMA), vimentin and matrix metalloproteinases

(MMPs; Touhami et al., 2005; Tsai et al., 2010), indicating the fibrotic changes in pterygium. Besides, the occurrence of pterygium could also be associated with age, gender, ultraviolet light exposure and cigarette smoking (Saw and Tan, 1999; Gazzard et al., 2002).

Our previous meta-analysis suggests that cigarette smoking is associated with a reduced risk of pterygium (Rong et al., 2014). However, the biological effect of cigarette smoke components on pterygium has not been studied. Nicotine, one of the major components in the cigarette smoke, accelerates corneal angiogenesis and fibrosis by increasing the number of α -SMA positive cells in corneal stroma and upregulating the expression of transforming growth factor- β (TGF- β), vascular endothelial growth factor (VEGF) and MMP-9 in an alkali burn model (Kim et al., 2017). Nicotine concentration in the plasma of daily cigarette smokers ranges from 0.08 – 0.15 μ M with a half-life of 2 hours. It is continuously catabolized in the liver into cotinine, which has a concentration range 1.02 – 1.73 μ M and a half-life of 19 hours (Nakajima et al., 1996). Our previous studies showed that cigarette smoking contributes to the development of age-related macular degeneration (Tam et al., 2008; Liang et al., 2014). We also found cotinine and nicotine-cotinine mixture induce human retinal pigment epithelial (RPE) cell dysfunction (Zhang et al., 2017). In this study, based on the results of our meta-analysis, we hypothesize that nicotine and cotinine could retard

pterygium development and progression. The proliferation and migration properties of human primary pterygium cells was evaluated under continuous exposure of nicotine and cotinine. Their effects on the epithelial-to-mesenchymal transition (EMT) marker expression and MMP levels were also studied.

2. Materials and Methods

2.1 *Human primary pterygium cell culture*

The study protocol was approved by the Ethics Committee for Human Research of the Chinese University of Hong Kong, which is in accordance with the tenets of the Declaration of Helsinki. Informed consent was obtained from all study subjects before inclusion into the study. Human primary pterygium cell lines ($n = 6$) have been established and characterized as previously described (Cao et al., 2018). Briefly, full-length primary pterygium, freshly collected from surgical removal, was first digested with 50 µg/ml dispase (Gibco®, Rockville, MD) and 100mM D-sorbitol (Sigma-Aldrich) in Dulbecco's Modified Eagle Medium (DMEM; Gibco®) at 37°C for one hour, and further dissociated into single cells by 0.05% trypsin at 37°C for 5 min with the cell strainer (40 µm, Nunc™). The isolated cells were cultured in advanced DMEM medium supplemented with 5% fetal bovine serum (FBS; Gibco®) and 1x penicillin-streptomycin (Gibco®) in 5% CO₂ incubator at 37°C. The medium was changed in every 2 – 3 days, and the

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pterygium cells with passage 3 – 5 were used in this study. Each experiment was repeated in all 6 pterygium cells.

2.2 Nicotine and cotinine treatments

(-)-Nicotine (catalog number: N3876; Sigma-Aldrich, St. Louis, MO) and (-)-cotinine (catalog number: C5923; Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). The pterygium cells were treated continuously for 7 days with 0.08 or 0.15 μM nicotine, 1 or 2 μM cotinine, 0.08 μM nicotine-1 μM cotinine (1 molar equivalent of nicotine and 12.5 molar equivalents of cotinine) or 0.15 μM nicotine-2 μM cotinine (1 molar equivalent of nicotine and 13.3 molar equivalents of cotinine) mixture in serum-supplemented advanced DMEM medium (final concentration of DMSO was 0.1%). The control group was the cells treated with 0.1% DMSO in advanced DMEM medium. The treatment medium was changed daily to mimic the continual exposure situation in smokers.

2.3 Nicotinic acetylcholine receptor expression

The gene expressions of nicotinic acetylcholine receptor subunits were analyzed. Briefly, 2×10^5 cells per dish were seeded on the 60-mm dishes one day before the start of the treatments and treated with nicotine and/or cotinine for 7 days. After the 7-day treatment, total RNA was extracted and purified with the TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad,

CA). One μg of total RNA was reverse-transcribed by SuperScript® III reverse transcriptase (Invitrogen). The expression of nicotinic acetylcholine receptor subunit genes was evaluated by Sybr green PCR with specific primers (**Supplementary Table 1**). Housekeeping gene (*GAPDH*) was used for normalization. The relative expression levels (ΔC_t) of the treatment groups were compared to that of the control group.

2.4 Cell proliferation analysis

The proliferation property of pterygium cells was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Invitrogen, Carlsbad, CA). Briefly, 10,000 cells per well (3 wells for each treatment group) were seeded on the 24-well plates (Corning Life Sciences, Lowell, MA) one day before the start of the treatments and treated with nicotine and/or cotinine for 7 days. MTT analysis was performed on Day 0, 1, 3, 5 and 7. The MTT signal was measured at the wavelength of 570 nm with reference to that of 650 nm by a plate reader (Powerwave XS, Bio-Tek Instruments). The percentage of cell viability was calculated by the equation of $\text{OD}_{570} \text{ sample} / \text{OD}_{570} \text{ control} \times 100\%$.

2.5 Cell cycle analysis

The cell cycle distribution of pterygium cells was measured by the propidium iodide (PI) staining with flow cytometry analysis. Briefly, 100,000 cells per dish were seeded on the 60-mm dishes (Corning Life Sciences) one day before the start

of the treatments and treated with nicotine and/or cotinine for 7 days. After the 7-day treatment, pterygium cells were trypsinized and immediately fixed with 70% ethanol at 4 °C for at least 24 hours. The fixed cells were treated with RNase A (Pure link™, Invitrogen) and PI (Sigma-Aldrich) in PBS for 20 mins at room temperature in dark. The cell cycle was analyzed by the flow cytometry machine (Cytomics FC500; Beckman Coulter, Indianapolis, IN), and the percentage of cells in the 4 phases of cell cycle (sub-G1, G1, S and G2/M phases) was calculated.

2.6 Cell migration analysis

The migration property of pterygium cells was evaluated by the scratch wound assay. Briefly, 10,000 cells per well were seeded on the 12-well plates (Corning Life Sciences) one day before the start of the treatments and treated with nicotine and/or cotinine for 7 days. After the 7-day treatment, scratch wounds were created with 200- μ l pipette tips on the nicotine/cotinine-treated cells, and the cells were incubated for 24 hours. Photomicrographs were taken at time 0 (immediately following the scratch wound), 6, 12 and 24 hours. The wound gaps were measured by ImageJ (version 1.47; NIH, Bethesda, MD). The percentage migration was calculated by the average area reduction at 6, 12 or 24-hour as compared to time 0. Every well has 6 scratch wounds.

2.7 Epithelial-to-mesenchymal transition status

The EMT marker expression was determined by the immunoblotting analysis. Briefly, 2×10^5 cells per dish were seed on the 60-mm dishes one day before treatment and treated with nicotine and/or cotinine for 7 days. After the 7-day treatment, the pterygium cells were lysed with the RIPA buffer (Sigma-Aldrich) supplemented with protease and phosphatase inhibitors (Roche). Total protein concentrations of the cell lysates were measured by Protein assay (BioRad). After denaturation, equal amount of total proteins (20 μ g) was resolved in 12.5% SDS-polyacrylamide gel and electro-transferred to nitrocellulose membranes for sequential probing with the primary antibodies for the EMT markers (α -SMA, SNAIL and VIMENTIN; **Supplementary Table 2**) and respective secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology). The signals were detected by enhanced chemiluminescence (Amersham Pharmacia, Cleveland, OH) with the ChemiDocTM XRS⁺ system (BioRad). β -ACTIN was used as housekeeping protein for normalization.

2.8 Matrix metalloproteinase expression

MMP expression was quantified by the multiplex ELISA array (Quansys Biosciences, Logan, UT), which measured 6 human MMPs (MMP-1, MMP-2, MMP-3, MMP-7, MMP-9 and MMP-13). Briefly, 10,000 cells per well (6 repeats for each treatment group) were seeded on 24-well plates one day before treatment

and treated with nicotine and/or cotinine for 7 days. Afterwards, the culture medium was collected, and the MMP expression in the culture medium was measured by the multiplex ELISA array according to the manufacturer's protocol. Multiplex ELISA signals were imaged by the ChemiDocTM XRS⁺ system (BioRad, Hercules, CA), and the spot intensities were analyzed by the Q-View software (Quansys Biosciences).

2.9 Statistical analysis

One-way analysis of variance (ANOVA) with post-hoc Tukey's test (for multiple testing correction) was used to compare the means among different treatment groups. All statistical analyses were performed by the commercially available software (IBM SPSS Statistics 22; SPSS Inc., Chicago, IL). Significance was defined as $p < 0.05$.

3. Results

3.1 The expression of nicotinic acetylcholine receptor subunits in human primary pterygium cells

As nicotine binds to the nicotinic acetylcholine receptor (Brunzell et al., 2015), we examined the expression of nicotinic acetylcholine receptor subunit genes in order to validate the responsiveness of human primary pterygium cells to nicotine and cotinine. Sybr green PCR showed the pterygium cells highly expressed the $\alpha 5$ (*CHRNA5*), $\beta 1$ (*CHRNBI*) and γ (*CHRNG*) subunits, moderately

expressed $\alpha 1$ (*CHRNA1*), $\alpha 6$ (*CHRNA6*), $\alpha 9$ (*CHRNA9*) and $\beta 2$ (*CHRNA2*) subunits, and slightly expressed $\alpha 10$ (*CHRNA10*) and $\beta 3$ (*CHRNA3*) subunits (**Figure 1**). There were very low to no expressions of $\alpha 2$ (*CHRNA2*), $\alpha 3$ (*CHRNA3*), $\alpha 4$ (*CHRNA4*), $\alpha 7$ (*CHRNA7*), $\beta 4$ (*CHRNA4*), δ (*CHRNA4*) and ϵ (*CHRNA4*) subunits. The $\beta 1$ subunit was upregulated in 0.15 μ M nicotine, 1 μ M cotinine, 2 μ M cotinine and 0.15 μ M nicotine-2 μ M cotinine mixture by 4.76 folds ($p < 0.001$), 3.37 folds ($p = 0.022$), 2.04 folds ($p = 0.037$) and 1.60 folds ($p = 0.044$), respectively. In addition, 1 μ M cotinine increased $\alpha 10$ subunit expression by 3.52 folds ($p = 0.016$). These results confirmed biological responses of human primary pterygium cells to nicotine and cotinine.

3.2 The effect of nicotine and cotinine on human primary pterygium cell proliferation

The neoplastic-like properties of pterygium are determined by multiple factors. One factor is related to the abundance of pterygium cells along the hyperplastic process, which can be determined by the proliferation of the pterygium cells and analyzed by MTT assay. Upon nicotine and cotinine treatments, human primary pterygium cells showed similar proliferation rates as the vehicle-treated control (0.1% DMSO), with no significant morphology change (**Figure 2**). Yet, pterygium cells treated with 0.15 μ M nicotine-2 μ M cotinine mixture exhibited a significant reduction in MTT signal by 16.04% at Day 7 ($p =$

0.009), compared to the control group (**Figure 3**). Therefore, exposure to higher concentrations of nicotine and cotinine retarded the proliferation rate of human primary pterygium cells.

3.3 The effect of nicotine and cotinine on human primary pterygium cell apoptosis

To delineate the reduced proliferation in pterygium cells, the cell cycle of nicotine and cotinine-treated pterygium cells were evaluated by PI staining and flow cytometry analyses (**Figure 4**). The apoptotic indicator in the cell cycle analysis (sub-G1 population) showed no significant difference among the nicotine and cotinine treatment groups ($0.92 \pm 0.41\%$ – $1.58 \pm 0.90\%$), compared to the vehicle control ($1.19 \pm 0.66\%$; **Table 1**). Similarly, there was also no significant difference in G1, S and G2/M populations of the nicotine and cotinine-treated pterygium cells (G1: $84.67 \pm 3.16\%$ – $86.30 \pm 2.97\%$; S: $3.18 \pm 1.56\%$ – $3.64 \pm 1.82\%$; G2/M: $9.42 \pm 2.15\%$ – $10.38 \pm 2.50\%$), compared to the vehicle-treated control (G1: $85.69 \pm 3.07\%$; S: $3.34 \pm 1.74\%$; G2/M: $9.85 \pm 2.03\%$). Our results suggested that the reduced pterygium cell proliferation under $0.15 \mu\text{M}$ nicotine- $2 \mu\text{M}$ cotinine mixture treatment was not due to cell apoptosis.

3.4 The effect of nicotine and cotinine on human primary pterygium cell migration

Another determining factor is related to the movement of pterygium cells and their capacity to migrate towards the central cornea. This characteristic can be examined by cell migration analysis, which can be evaluated by the scratch wound assay (**Figure 5**). Scratch wound was induced after 7-day nicotine and cotinine treatment. The migration of pterygium cells was not affected by the 0.08 μ M nicotine, 1 μ M cotinine and 2 μ M cotinine treatments. However, pterygium cell migration was significantly inhibited by 0.15 μ M nicotine, 0.08 μ M nicotine-1 μ M cotinine mixture, and 0.15 μ M nicotine-2 μ M cotinine mixture by 8.12% ($p = 0.042$), 7.14% ($p = 0.014$) and 11.93% ($p = 0.039$), respectively, at 24 hours, when compared to the vehicle control. These results showed that nicotine and cotinine hindered human primary pterygium cell migration.

3.5 The effect of nicotine and cotinine on epithelial-to-mesenchymal transition status in human primary pterygium cells

To delineate the mechanism of the nicotine and cotinine effects on human primary pterygium cells, we evaluated the EMT status in nicotine and cotinine-treated pterygium cells as the expression of EMT markers (α -SMA, SNAIL and VIMENTIN) correlates with the cell proliferation and migration properties (Zhang et al., 2016; Zhang et al., 2017). Immunoblotting analyses

showed that SNAIL expression was significantly reduced in pterygium cells treated with 0.15 μ M nicotine-2 μ M cotinine mixture by 1.33 ($p = 0.036$), compared to the vehicle control (**Figure 6**). Similarly, α -SMA expression was also significantly decreased in 0.15 μ M nicotine-2 μ M cotinine mixture group by 1.31 folds ($p = 0.001$). In contrast, the expression of vimentin was not significantly altered in any treatment groups. Our results suggested that the retarded pterygium cell proliferation and migration by nicotine and cotinine could be associated with the reduced expression of the EMT markers.

3.6 The effect of nicotine and cotinine on matrix metalloproteinase expression in human primary pterygium cells

To further validate the retarded cell migration property after 7-day nicotine and cotinine treatment, we determined the MMP production by the pterygium cells. Multiplex ELISA assay was adopted to quantify the expression of 6 MMPs (MMP-1, MMP-2, MMP-3, MMP-7, MMP-9 and MMP-13; **Figure 7**). Human primary pterygium cells highly expressed MMP-1, MMP-2 and MMP-3, and slightly expressed MMP-9. Significant reduction in MMP-1 expression was detected in 2 μ M cotinine (13074.42 ± 3433.62 μ g/ml; $p = 0.023$), 0.08 μ M nicotine-1 μ M cotinine mixture (10947.1 ± 2291.84 μ g/ml; $p = 0.001$) and 0.15 μ M nicotine-2 μ M cotinine mixture groups (11555.08 ± 6393.68 μ g/ml; $p = 0.043$), compared to the control (18062.04 ± 3699.17 μ g/ml). Moreover, MMP-9

expression was significantly decreased in 1 μ M cotinine (334.34 ± 52.73 μ g/ml; $p = 0.043$), 0.08 μ M nicotine-1 μ M cotinine mixture (262.29 ± 109.72 μ g/ml; $p = 0.013$) and 0.15 μ M nicotine-2 μ M cotinine mixture groups (308.04 ± 61.66 μ g/ml; $p = 0.012$), compared to the control (392.40 ± 43.06 μ g/ml). Our results suggested that the reduction in MMP-1 and MMP-9 expression by nicotine and cotinine exposure could be correlated with the retarded migration property of human primary pterygium cells.

4. Discussion

Epidemiological studies showed inconsistent contributions of cigarette smoking to the incidence of pterygium. The Southern Harbin Eye Study reported a significant association between cigarette smoking and pterygium in rural adult northern Chinese population with odds ratio (OR) of 1.90 (Li and Cui, 2013). Similarly, cigarette smoking was significantly associated with higher incidence of pterygium (OR = 5.46) in a dry and high-altitude province of Iran (Malekifar et al., 2017). The Singapore Malay Eye Study found that cigarette smoking is not associated with the incidence of pterygium in adult Malay population but increased the odds of bilateral pterygium (OR = 1.50; Cajucom-Uy et al., 2010). In contrast, a population-based survey (Proyecto VER) of adult Latinos in Arizona reported that current smokers were less likely to develop pterygium (OR = 0.75), compared to the pterygium patients who never smoke (West and Munoz, 2009).

Moreover, the Korean National Health and Nutrition Examination Survey showed that the lifetime smokers have a reduced risk to develop pterygium (OR = 0.70; Rim et al., 2013), and the Handan Eye Study in northern China found that current smoking is a protective factor for pterygium (OR = 0.50; Sun et al., 2013). However, no mechanism between cigarette smoking and pterygium has been suggested (Asokan et al., 2012; Zhong et al., 2012; Tano et al., 2013). Our previous systemic review and meta-analysis demonstrated that cigarette smoking was associated with a reduced risk of pterygium in current smokers (OR = 0.68), but not in ex-smokers (OR = 1.05), and the association is independent of ultra-violet light exposure and gender (Rong et al., 2014). The biological effects of cigarette smoking components, including nicotine, have still not been evaluated in human primary pterygium cells before the current study.

Cigarette smoke contains over 4,000 chemicals. This study focused on nicotine, which is the determinant factor for addiction (Prochaska and Benowitz, 2016) and the major component in cigarette replacements, including electronic cigarette and nicotine patches. Nicotine binds to and activates the pentameric nicotinic acetylcholine receptors, composed of α , β , γ , δ and ϵ subunits (Lukas et al., 1999). Nicotine can accelerate corneal angiogenesis and fibrosis by upregulating the expression of α -SMA, TGF- β , VEGF and MMP-9 in the alkali burn model (Kim et al., 2017). Under normal physiological conditions, nicotine

absorbed into the bloodstream would be metabolized and detoxified in the liver, where hepatic cytochrome P450 enzyme CYP2A6 catabolizes nicotine into cotinine (Nakajima et al., 1996). Plasma levels of nicotine and cotinine in daily cigarette smokers range from 0.08 – 0.15 μM and 1.02 – 1.73 μM , respectively (Nakajima et al., 1996), indicating that cotinine has higher concentration and stays longer in the tissue cells than nicotine. Low concentration of cotinine (313 $\mu\text{g/ml}$ or below) stimulates secretory epithelial cell viability, whereas high concentrations of cotinine (1250 $\mu\text{g/ml}$ or above) significantly decreases the cell numbers, metabolic activity and the secretory component (Gregory and Gfell, 1996). Moreover, 10 nM cotinine is a mitogen for human vascular smooth muscle cells, but becomes toxic at higher concentrations (Carty et al., 1997). Our previous study also demonstrated that cotinine as well as nicotine-cotinine mixture, but not nicotine alone, induce human RPE cell dysfunction (Zhang et al., 2017). In this study, human primary pterygium cells predominantly expressed the $\alpha 5$, $\beta 1$ and γ subunits of the nicotinic acetylcholine receptor (**Figure 1**), confirming that human pterygium cells respond to nicotine stimulation. Neither nicotine nor cotinine alone showed influence on pterygium cell proliferation and migration, but higher concentration of nicotine-cotinine mixture inhibited proliferation (**Figure 3**) and retarded migration (**Figure 5**). The effects of nicotine and cotinine on pterygium cells could be independent but additive. The additive effect of nicotine and

cotinine could be driven by the effect of cotinine because of the higher molar equivalents of cotinine in the nicotine-cotinine mixture. Moreover, a dose-dependent response could also be observed in individual treatment group (nicotine, cotinine or nicotine-cotinine mixture), confirming the effect of nicotine and cotinine on human primary pterygium cells. Yet, we found that our tested nicotine and cotinine concentrations did not induce pterygium cell apoptosis (**Figure 2 and 4**). Cell apoptosis is therefore not responsible for the reduction in pterygium cell proliferation.

The pathological mechanisms of pterygium growth and development remain elusive. mTORC1 activation has been reported to inhibit pterygium apoptosis by regulating the Beclin 1-dependent autophagy and targeting Bcl-2, and to stimulate pterygium cell proliferation by negatively regulating fibroblast growth factor receptor 3 and inhibiting p73 (Liu et al., 2017). The p53 dysregulation has been suggested to be involved in the uncontrolled cell proliferation in pterygium (Chowers et al., 2001; Cao et al., 2018). Moreover, the excessive proliferation of pterygium tissues can be caused by aberrant fibrotic proliferation beneath the pterygium epithelium, which correlates with the expression of EMT markers, α -SMA, SNAIL and VIMENTIN (Kato et al., 2007). In this study, we confirmed the high expression of EMT markers in human primary pterygium cells (**Figure 6**). A significant downregulation of α -SMA and SNAIL was observed after higher

concentration of nicotine-cotinine mixture treatment. This implied that reduced expression of EMT markers could be correlated with retarded cell proliferation and migration of the pterygium cells. EMT is therefore a critical mechanism for the effect of nicotine and cotinine on human primary pterygium.

Extracellular matrix (ECM) remodeling is another mechanism involved in the progression of pterygium (Zada et al., 2018). ECM proteins, regulated by metalloproteinases, can provide the structure and biochemical support for cells adhesion and migration (Barker, 2011). Expressions of MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-10 and MMP-13 were detected in human pterygium. Their expressions are associated with the invasion and migration abilities of pterygium cell as well as the disease progression (Di Girolamo et al., 2001; Li et al., 2001; Yang et al., 2009; Tsai et al., 2010). Inhibition of pterygium cell migration in response to cyclosporine A has been demonstrated to be correlated with the reduced expression of MMP-3 and MMP-13 (Kim et al., 2015). Our previous study also found that MMP-2 and MMP-9 were expressed in pterygium cells with higher proliferative and migration properties (Bai et al., 2010). In this study, based on the multiplex ELISA, we detected high expression of MMP-1, MMP-2 and MMP-3, as well as low expression of MMP-9 in our cultured human primary pterygium cells (**Figure 7**), but not MMP-7 and MMP-13. Significant reduction in MMP-1 and MMP-9 expression was found in pterygium cells after

nicotine and cotinine treatments, suggesting that the retarded cell proliferation and migration properties could be explained by reduced expressions of MMP-1 and MMP-9 proteins in pterygium cells.

From other perspective, pterygium has been linked to the wound healing process (Di Girolamo et al., 2004). Our results, in this study, demonstrated the retarded proliferation and migration properties of human primary pterygium cells by nicotine and cotinine. This indicates that the wound healing process by pterygium cells could be reduced upon nicotine and cotinine exposure.

Coherently, our previous studies found that cigarette smoking, nicotine and cotinine hinders the wound healing potential of human periodontal ligament-derived stem cells (Ng et al., 2013; Ng et al., 2015) and RPE cells (Zhang et al., 2017). Therefore, cigarette smoking as well as nicotine and cotinine exposure could have a generalized effect on wound healing delay. Further investigations are needed to delineate the contribution and mechanism of wound healing process in pterygium development.

In summary, this study revealed that nicotine and cotinine mixture retards human primary pterygium cell proliferation and migration properties *in vitro* through reduction in EMT and MMP expressions. Our results affirm the inverse association of cigarette smoking and pterygium incidence.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Q.Y. and T.K.N. mainly performed the experiments. S.Q.T., K.P.C., D.C. and W.K.C. helped with the experiments. V.J. recruited study subjects. V.J., M.Z., C.P.P. and T.K.N. supervised the project. Q.Y., V.J., C.P.P. and T.K.N. interpreted the results and wrote the manuscript.

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Figures

Figure 1: **Nicotine acetylcholine receptor subunit gene expression in human primary pterygium cells.** The expression of α (*CHRNA1-10*), β (*CHRNB1-4*), δ (*CHRND*), ϵ (*CHRNE*) and γ (*CHRNG*) subunit genes in human primary pterygium cells was determined by Sybr green PCR. *CHRNA5*, Human primary pterygium cells highly expressed *CHRNB1* and *CHRNG*, moderately expressed *CHRNA1*, *CHRNA6*, *CHRNA9* and *CHRNB2*, and slightly expressed *CHRNA10* and *CHRNB3*. Very low/no expression of *CHRNA2*, *CHRNA3*, *CHRNA4*, *CHRNA7*, *CHRNB4*, *CHRND* and *CHRNE* was found in human primary pterygium cells. The relative gene expression was presented as the mean of 6 pterygium cells $\pm$ standard deviation. * $p < 0.05$, *** $p < 0.001$.

Figure 2: **Human primary pterygium cell culture along the nicotine and cotinine treatments.** The morphology of human primary pterygium cells under the nicotine and cotinine stimulation along the 7-day treatment. The pterygium cells showed no significant morphology change in different treatment groups. Scale bar: 200 μ m.

Figure 3: **The effect of nicotine and cotinine on human primary pterygium cell proliferation.** Pterygium cell proliferation was assessed by the MTT assay on Day 0, 1, 3, 5 and 7. Pterygium cells treated with 0.15 μ M nicotine-2 μ M cotinine mixture showed reduced cell proliferation rate, compared to the vehicle control group (0.1% DMSO). The data was presented as the mean of 6 pterygium cells $\pm$ standard deviation. $**p < 0.01$

Figure 4: **The effect of nicotine and cotinine on human primary pterygium cell apoptosis.** Cell cycle analysis was evaluated by PI staining and flow cytometry analysis. The apoptotic indicator in the cell cycle analysis (sub-G1 population) showed no significant difference among the nicotine and cotinine treatment groups, compared to the vehicle control group (0.1% DMSO). No significant difference was observed in G1, S and G2/M populations of the nicotine and cotinine-treated pterygium cells.

Figure 5: **The effect of nicotine and cotinine on human primary pterygium cell**

migration. Pterygium cell migration after the 7-day nicotine and cotinine treatments was evaluated by scratch wound assay. Pterygium cell migration was significantly inhibited in 0.15 μM nicotine, 0.08 μM nicotine-1 μM cotinine mixture as well as 0.15 μM nicotine-2 μM cotinine mixture groups, compared to the vehicle control group (0.1% DMSO). The data was presented as the mean of 6 pterygium cells $\pm$ standard deviation. $*p < 0.05$. Scale bar: 200 μm .

Figure 6: The effect of nicotine and cotinine on epithelial-to-mesenchymal transition status in human primary pterygium cells. The expression of epithelial-to-mesenchymal transition marker protein (α -SMA, SNAIL and VIMENTIN) in human primary pterygium cells after the 7-day nicotine and cotinine treatments was evaluated by the immunoblotting analysis. The expression of α -SMA and SNAIL was significantly reduced in the 0.15 μ M nicotine-2 μ M cotinine mixture groups, compared to the vehicle control group (0.1% DMSO). β -actin was used as housekeeping protein for normalization. The data was presented as the mean of 6 pterygium cells $\pm$ standard deviation. * $p < 0.05$; ** $p < 0.01$.

Figure 7: The effect of nicotine and cotinine on matrix metalloproteinase expression in human primary pterygium cells. The expression of matrix metalloproteinase (MMP-1, 2, 3, 7, 9 and 13) in human primary pterygium cells after the 7-day nicotine and cotinine treatments was determined by Multiplex ELISA assay. Human primary pterygium cells expressed MMP-1, MMP-2, MMP-3 and MMP-9. Significant reduction in MMP-1 and 9 expressions were detected in 1 μ M cotinine, 2 μ M cotinine, 0.08 μ M nicotine-1 μ M cotinine and 0.15 μ M nicotine-2 μ M cotinine mixture groups, compared to the vehicle control group (0.1% DMSO). The data was presented as the mean of 6 pterygium cells $\pm$ standard deviation. * $p < 0.05$; ** $p < 0.01$.

Table 1: Cell cycle analysis of primary human pterygium cells treated with nicotine and cotinine

Treatments	sub-G1 phase	G1 phase	S phase	G2/M phase
0.1% DMSO	1.19 ± 0.66%	85.69 ± 3.07%	3.34 ± 1.74%	9.85 ± 2.03
0.08 µM nicotine	1.58 ± 0.90%	85.05 ± 2.98%	3.32 ± 1.64%	9.96 ± 2.50%
0.15 µM nicotine	1.28 ± 0.69%	85.39 ± 3.00%	3.31 ± 1.45%	10.08 ± 2.44%
1 µM cotinine	1.30 ± 0.65%	84.67 ± 3.16%	3.64 ± 1.82%	10.38 ± 2.50%
2 µM cotinine	1.20 ± 0.63%	86.30 ± 2.97%	3.18 ± 1.56%	9.42 ± 2.15%
0.08 µM nicotine-1 µM cotinine	1.33 ± 0.81%	85.22 ± 2.99%	3.47 ± 1.72%	10.01 ± 2.28%
0.15 µM nicotine-2 µM cotinine	0.92 ± 0.41%	85.32 ± 3.35%	3.41 ± 1.65%	10.38 ± 2.44%

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