Cytotoxic Effect of Nicotine and Cotinine on Primary Mouse Embryonic Fibroblasts *in Vitro*

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Abstract

The aim of this study was to investigate the effect of nicotine and its major metabolized form, cotinine, on primary mouse embryonic fibroblasts (PMEF cells). The cytotoxic effects were evaluated by cell viability and apoptosis assay correlating to morphological observation. Reactive oxygen species (ROS) production and apoptosis-related genes expression were evaluated to gain some insight into the underlying mechanisms of nicotine and cotinine effecting on PMEF cells. Results showed that nicotine and cotinine significantly decreased cell viability and promoted cell apoptosis in a dose-dependent manner at 24 h. PMEF cells were more sensitive to nicotine than cotinine. Interestingly, after the combined treatment of cotinine to 5 mM nicotine for 6 h, cytotoxicity level was increased conducting with a progression of morphological apoptotic appearance compared to a single nicotine treatment. The 5 mM nicotine and cotinine significantly induced overproduction of ROS. Their combination effects promoted ROS overproduction and significantly increased the apoptosis-related genes expression such as Bax, Caspase-3 and P53 compared to a single nicotine treatment.

In conclusion, data showed adverse effect of nicotine and cotinine on PMEF cells. Cotinine could enhance the cytotoxic effect of nicotine to induce apoptosis.

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Introduction

Smoking is one of environmental risk factors that has been widely investigated on its adverse effects on fetal development.[1-3] For example, active maternal cigarette smoking has affected pregnancy in every trimester ranging from increased miscarriages in the first trimester [4] to increased premature delivery and decreased fetal growth in the third trimester.[5] Studies in mouse experiments reported that components in tobaccos directly affected the craniofacial development and obviously interrupted morphological process formation.[6, 7] Nicotine is the principal alkaloid in tobacco and it comprises approximately 95% of the total alkaloid.[8] Nicotine is considered the main teratogenic substance that alters and delays embryonic development.[9] Several authors stated an evidence supporting the nicotine accumulation in fetal serum and amnionic fluid since slightly higher concentrations of nicotine were found in fetal serum and amnionic fluid than that found in maternal serum.[10, 11] Nicotine is extensively metabolized in liver and approximately 70%-80% of nicotine is converted to cotinine. The cotinine levels are of particular interest as qualitative markers of tobacco use and quantitative indicators of nicotine intake.[12] Like nicotine, cotinine can rapidly pass the placenta from mother to child through transplacental transfer occurring throughout pregnancy.[13-15] The research of epidemiology found the teratogenic effects of nicotine on maternal smoking, specifically showing statistically significant association of the 1.5-fold increased risk of orofacial cleft from maternal smoking compared to non-smoking mothers in early pregnancy.[16] However, the underlying mechanisms have not been clarified.

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To gain some insight into the cytotoxic effects of nicotine and cotinine on mesenchymal cells developing in palate formation, PMEF cells representing the mesenchymal cells comparable to critical stage of palate formation (E13) were chosen. Our study aims were to investigate the effect of nicotine, cotinine and their combinations on cell viability, cell apoptosis, ROS generation and apoptosis-related genes expression of PMEF cells *in vitro*.

Materials and methods

Chemicals

Nicotine ((-)-Nicotine 36733, Sigma®, USA) was dissolved in Special Dulbecco's modified Eagle medium (DMEM, Merck®, USA) and cotinine ((-)-Cotinine 74003, Sigma®, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma®, USA) for 100 mM stock solution followed by dilution in DMEM to a desired concentration prior to use.

PMEF cells and nicotine, cotinine treatments

PMEF cells isolated from CF-1 mouse embryos at day 13 (E13, Merck®, USA) were cultured in Special DMEM supplemented with 10% embryonic stem (ES) cell qualified FBS, 1% penicillin-streptomycin and 1% L-glutamine (Merck®, USA) and maintained at 37°C in a humidifier incubator (5% CO_2 /95% air). The cells at the third-forth passages were used for the experiments. PMEF cells were treated with 3, 4, 5 mM nicotine, 0.1, 0.5, 1, 5 mM cotinine and 5mM nicotine combined with 0.1, 0.5, 1, 5 mM cotinine. The 0.001, 0.005, 0.01, and 0.05 % DMSO were performed as vehicle controls for cotinine treatments, respectively. In this study, the results indicated that DMSO yielded no toxicity to cells after 24 h incubation, which was in agreement with previous studies suggesting that a concentration of DMSO used as vehicle control in embryotoxic parameters study *in vitro* should not increase 0.1%.[17, 18]

Cell viability assay

Optimal concentrations of nicotine and cotinine on cell survival were determined using the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay. The 5 x 10^4 PMEF cells/well were seeded in to 48-well cell culture plate. PMEF cells were treated with the indicated concentrations of nicotine and cotinine. After cultured for 24 h, the cells were incubated with 0.5 mg/mL MTT solution (USB Corporation, USA) for 30 min at 37°C and the formation of formazan crystal was evaluated by dissolving in dimethylsulfoxide (DMSO, Sigma®, USA). The optical density was evaluated at 570 nm on a SpectraMax® M3 microplate reader (Molecular Devices, USA).

Observation of cell morphology under microscopy

PMEF cells were observed under an Inverted microscope (IX70, Olympus®, Japan) at 6 and 24 h after nicotine and cotinine treatments. Images were taken using fluorescence microscope (BX60, Olympus®, Japan).

Cell apoptosis assay

Cells were seeded at 2.5 x 10^4 cells/well in to 96-well cell culture plate. At 24 h of nicotine and cotinine treatments, apoptotic analysis was performed using a HT Titer TACS™ Apoptosis Detection Kit (R&D®, USA). Positive control was treated with TAC nuclease. The cells were quenched with 3% hydrogen peroxide. Cells were incubated with labelling reaction mix (deoxynucleotidyl transferase) at 37°C for 60 min in humidity chamber and incubated with Strep-HRP solution for 10 min at room temperature. After incubation, TACS-Sapphire substrate was added and incubated for 30 min in the dark at room temperature. The reaction was ceased with 0.2 M HCl. The absorbance was read at 450 nm on a SpectraMax® M3 microplate reader (Molecular Devices, USA).

Reactive Oxygen Species (ROS) detection

ROS-Glo H_2O_2 Assay (Promega®, USA) were used to assess the effects of nicotine and cotinine on ROS generation in PMEF cells. Cells were treated with the indicated concentrations of nicotine and cotinine and H_2O_2 substrate solution. At 6 h, ROS-Glo H_2O_2 assay was performed according to the manufacturer's protocol. H_2O_2, were used as positive control. The produce generating a luminescent signal that is proportional to H_2O_2 concentration and relative luminescence units (RLU) was read at 450 nm on a SpectraMax® M3 microplate reader (Molecular Devices, USA).

Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis

Cells were evaluated for the expression of apoptosis-related genes including Bax, Bcl-2, Caspase-3, P53 after treatment of indicated

nicotine and cotinine concentrations for 6 h. Briefly, total RNA was extracted using the NucleoSpin® RNA Plus (Macherey-Nagel, Germany) in accordance with the manufacturer's protocols. The total RNA was reverse transcribed into the first strand cDNA via iScript™ reverse transcription Supermix for RT-qPCR (Bio-Rad®, USA) according to the manufacturer's protocol. Reverse transcription reactions were performed using Thermocycler (Veriti™ Thermal Cycler, USA). The relative mRNA expression levels were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and evaluated with a LightCycler® 480 SYBR Green I Master (Roche®, Germany) in accordance with the manufacturer's protocols. A real time PCR was detected with Roche LightCycler 480 real time PCR system machine (Roche®, Germany). The results were analysed using the $2^{-\Delta\Delta CT}$ method.[19] The forward and reverse primers for the target genes and GAPDH were listed in Table 1.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product length (bp)
Bax	CTCAAGGCCCTGTGCACTAA	GAGGCCTTCCCAGCCAC	120
Bcl-2	CTCGTCGCTACCGTCGTGACTTCG	ACCCCATCCCTGAAGAGTTCC	112
Caspase-3	TCTGACTGGAAAGCCGAAACTC	TCCCACTGTCTGTCTCAATGCCAC	83
P53	GTACCTTATGAGCCACCCGA	AGAAGGTTCCCACTGGAGTC	143
GAPDH	AGAACATCATCCCTGCATCCAC	GTCAGATCCACGACGGACAC	127

Table 1. Primer sequences used for quantitative real-time PCR[37].

Data Analysis

Data were presented as the means and standard deviation of three identical experiments made in triplicate. Statistical significance was analyzed using one-way ANOVA followed by Tukey's test for multiple comparison for cell viability, apoptosis and ROS production. Apoptosis-related gene expressions were analysed using Kruskal-Wallis followed by Mann-Whitney U test for comparison between groups. The level of statistically significant difference was set at $p < 0.05$.

Results

Effect of nicotine and cotinine on cell viability and apoptosis

We found that all nicotine tested doses were significantly toxic to cells and 5 mM nicotine yielded the highest effects on PMEF cells resulting in decreased cell viability and increased cell apoptosis. All cotinine treated cells have shown a significance of decreased cell viability and increased apoptosis. To prove their combination effects on PMEF cells, highest cytotoxic effect dose of nicotine (5 mM) combined with 0.1, 0.5, 1, and 5 mM cotinine was chosen. We found all cotinine concentrations significantly enhanced 5 mM nicotine in decreasing cell viability and promoting cell apoptosis (Figure 1).

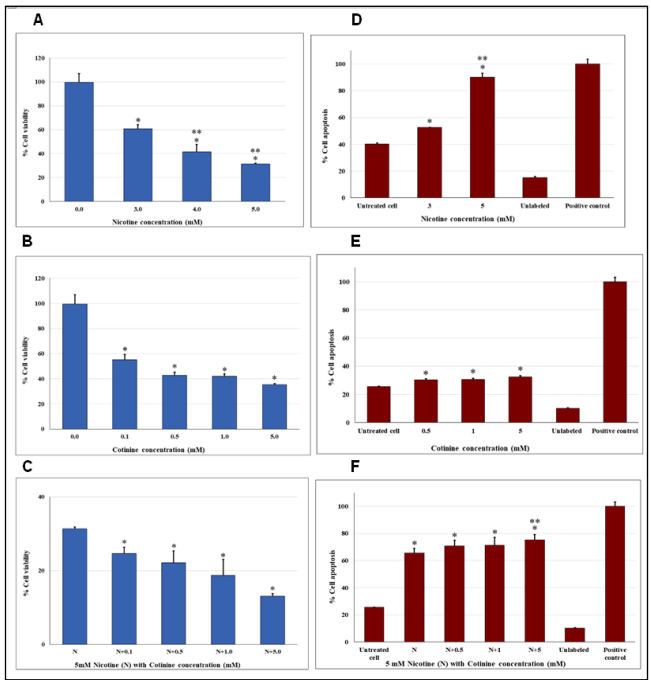

Figure 1. Cell viability and apoptosis of PMEF cells cultured in nicotine (A, D), cotinine (B, E), and 5 mM nicotine combined with cotinine (C, F) at 24 hours determined using MTT assay and HT Titer TACS™, respectively. Note: *p < 0.05 vs. control; *p < 0.05 vs. 5 mM nicotine in C; **p < 0.05 vs. 3 mM nicotine in A, D; **p < 0.05 vs. 5 mM nicotine in F.

Observation of cell morphology under microscopy

At 24 h, morphological observation of nicotine-treated cells was recorded. Cells treated with 3 mM nicotine showed shrinkage and condensation indicating the first stage of apoptosis while 4 and 5 mM nicotine-treated cells appeared to be in late stage apoptosis showing many blebbings and apoptotic bodies. Most of treated cells with cotinine exhibited shrinking with an increased degree of condensation. Some apoptotic bodies have been found in a dose-dependent manner (Figure 2A). We found an interesting action of nicotine-cotinine combination

treatments at 6 h (Figure 2B). Cells displayed an irregular form, increased the degree of condensation and shrinkage, indicating a sign of apoptosis, while the 5 mM nicotine-treated cells just exhibited swelling and polymorphic vacuole formation in cytoplasm, reflexing the damaged cell responded to cytotoxic stimuli. If the cytotoxic stimuli was persistent, the starting of cell apoptosis could subsequently occur. At 24 h, a number of apoptotic bodies were found in all treated groups, characterizing as a late apoptosis (Figure 2B).

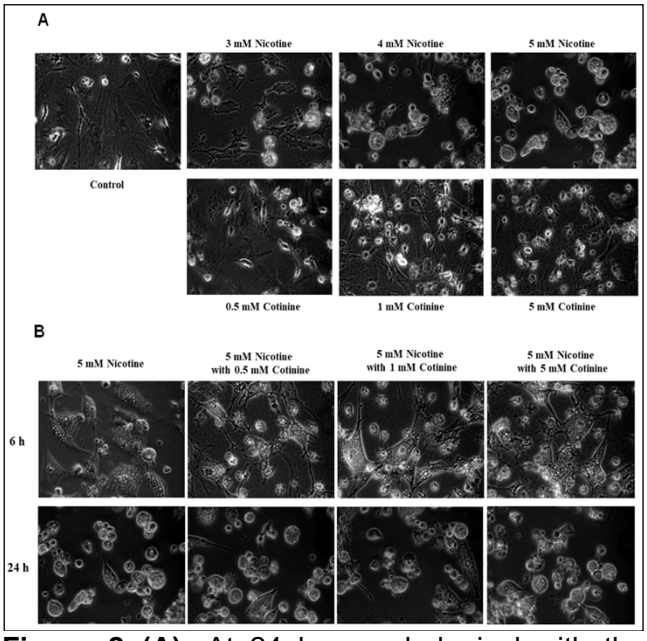

Figure 2 (A). At 24 h, morphological with the treatment of nicotine at 3 mM, cell exhibited irregular form, shrinking and increased cell condensation indicating the first stage apoptosis. Late stage apoptosis was found at 4 and 5 mM nicotine treatments showing plasma membrane blebbing and numerous apoptotic bodies. All cotinine treatments showed changed in cell shape through shrinking and an increased degree of cell condensation with some apoptotic bodies found in a dose-dependent manner. **(B)** At 6 h, the 5 mM nicotine treated-cells exhibited swelling and polymorphic vacuole formation, suggesting an earlier sign of damage cell prior to apoptosis while as the first stage of apoptosis was shown at 5 mM nicotine combined with 0.5, 1 and 5 mM cotinine. At 24 h, all of treated cells showed the characterised morphologically apoptosis, indicating by plasma membrane blebbing and numerous apoptotic bodies. (×400 magnification, Fluorescence microscope BX60, Olympus®, Tokyo, Japan).

Effect of nicotine and cotinine on the generation of ROS

Nicotine had a potential to induce ROS production in a dose-dependent manner and the 5 mM nicotine treated group showed the highest significant ROS generation (Figure 3A). All cotinine treated group showed a significantly increased ROS production compared to the control (Figure 3B). All combined treatment group showed a significantly elevated ROS production compared to the control. Cotinine could promote and sustain ROS production when combined with 5 mM nicotine, showing a higher mean with contracted SD of ROS production than those observed in a single treatment of 5 mM nicotine. (Figure 3C).

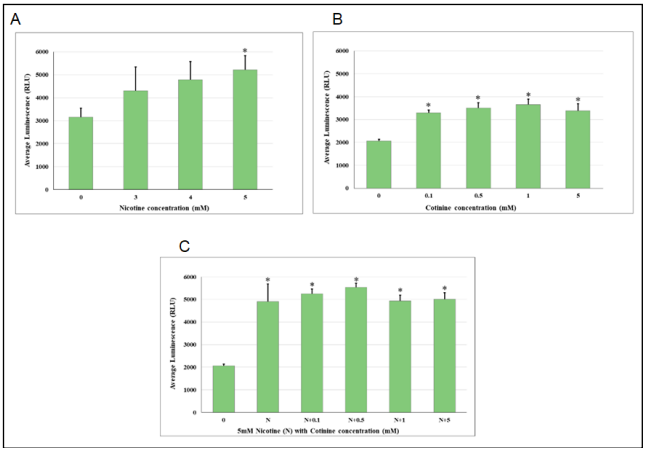

Figure 3. ROS generation of PMEF cells cultured in nicotine (A), cotinine (B), and 5 mM nicotine combined with cotinine (C) at 6 hours determined using ROS-Glo H_2O_2 assay. Note: *p < 0.05 vs. control.

Expression of apoptosis-related genes

Treatment of 0.5 mM cotinine, 5 mM nicotine and 5 mM nicotine combined with 0.5 and 1 mM cotinine significantly increased the expression of Bax, compared to the control. In addition, 5 mM nicotine combined with 0.5 and 1 mM of cotinine significantly increased Bax expression compared to the 5 mM nicotine concentration (Figure 4A). The mRNA expression level of Bcl-2 was increased significantly in comparison to that of the control with the treatment of 0.5, 1 mM cotinine, 5 mM nicotine and 5 mM nicotine combined with 0.5 and 1 mM cotinine. In comparison to the 5 mM nicotine treatment, 5 mM nicotine combined with 1 mM of

cotinine significantly increased Bcl-2 expression (Figure 4B). The mRNA expression level of Caspase-3 and P53 was increased significantly in comparison to that of the control with the treatment of 5 mM nicotine and 5mM nicotine combined with 0.5 and 1 mM cotinine. Furthermore, treatment with 5 mM nicotine combined with 1 mM cotinine was significantly induced Caspase 3 and P53 gene expression compared to the 5 mM nicotine concentration (Figure 4C, D).

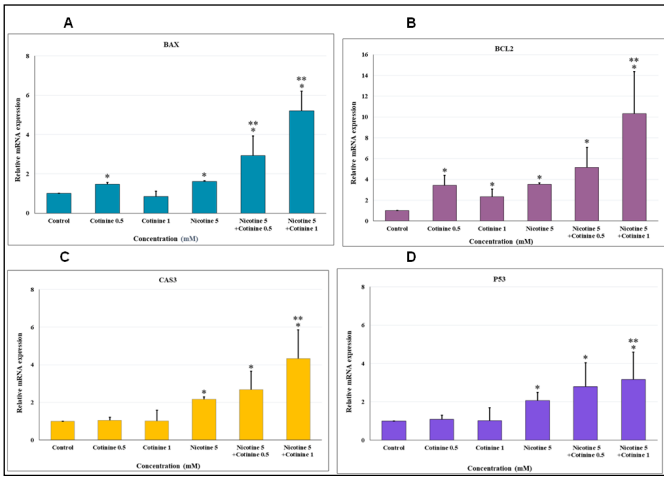

Figure 4. The relative mRNA expression level of Bax (A), Bcl-2 (B), Caspase-3 (CAS3) (C), and P53 (D) of PMEF cells cultured in cotinine (0.5 and 1 mM), nicotine (5 mM) and nicotine (5 mM) combined with cotinine (0.5 and 1 mM) at 6 hours determined using RT-qPCR. Note: *p < 0.05 vs. control; **p < 0.05 vs. 5 mM nicotine.

Discussion

Nicotine has cytotoxic effect which increases cell death in a dose dependent manner of various cells especially on mesenchymal derived cells such as human mesenchymal stem cells (MSCs) derived from the human periodontal tissues,[20] and human periodontal ligament cells (hPDLCs) in a low dose concentration.[21] The study in pulmonary fibroblast cell line (MRC-5) showed the similar results as high nicotine concentration (2 mM) induced approximately 50% cell death after 24 h of treatment.[22] The survival and proliferation of human alveolar bone marrow-derived mesenchymal stem cells decreased when the cells were exposed to 5 mM of nicotine.[23] Our studies also showed significantly decreased cell viability and increased apoptosis of PMEF cells in a high dose of nicotine treatments (3-5 mM). 5 mM nicotine obviously marked 68.59% of decreased cell viability and 90.03% of cell apoptosis, showing an inhibition of cell proliferation and a trigger of cell apoptosis events. All cotinine treatments caused cell damage showing more than 40% reduction of cell viability similarly to the previous studies. Toxic effect of cotinine was found in vascular smooth muscle cells *in vitro*,[24] and a high concentration of cotinine may induce malformation at the cranial part of the thoracic neural tube in a chick embryo model.[25]

Effect of cotinine on PMEF cells in our study showed a significant cell apoptosis compared to control group. Nevertheless, the quantitative apoptosis cell responses (about 25%-30%) in cotinine-treated groups were lower than those found in nicotine-treated PMEF cells (about 50%-90%), indicating that cotinine showed lower toxicity than nicotine regarding the enhancement of PMEF cell apoptosis. In accordance with a previous studies, cotinine showed lower toxicity than nicotine on the MRC-5 cells and the early stage of embryogenesis. However, the harmful effect of low dose of cotinine during embryogenesis i.e. inhibition of embryos blastulation was observed in a laboratory study by adding 0.8 mM cotinine to media containing the 0.5 mM nicotine. This study showed an enhanced effects of cotinine combined with low dose nicotine on cell damage. Low level of nicotine without cotinine could not exert this harmful capability.[26] This finding is similarly found in our studies when PMEF cells exposed to 5 mM nicotine combined with 0.1 mM - 5.0 mM cotinine leading to more sensibility in reduced cell viability and induced apoptosis. Morphological findings in our study displayed that nicotine and cotinine had teratogenic effect in inducing cell apoptosis in a dose-dependent manner and nicotine exhibited severely adverse effects than that of cotinine. At 6 h of their combination treatments, we found interesting cell response compared to 5 mM nicotine-treated cells.

The 5 mM nicotine-treated cells exhibited swelling, polymorphic vacuole formation, showing a sign of damaged cell from teratogens prior to the stage of program cell death or apoptosis.[27] This was an earlier event than those observed in a combination treated-groups that already showed changed in cell shape through shrinking and an increased degree of cell condensation

indicating the first stage of apoptosis. Our finding at 6 h of treatments revealed an enhanced effect of cotinine with nicotine to promote the program cell death progression.

The ROS can be produced in cells following exposure to exogenous and endogenous stimuli. The exogenous agents include chemicals found in cigarette smoke, environmental toxins, medications, UV, and radiation.[28, 29] The cytotoxic effects of cigarette smoke are associated with its action to augment the intracellular ROS level.[30] Our results is consistent with previous studies, showing that nicotine treatments can induce an ROS production in a dose dependent manner and a significant ROS overproduction was found in 5 mM nicotine. All cotinine treated group significantly induced ROS formation compared to the untreated control. Notably, all cotinine treatments can induce a low level ROS production, showing a significant ROS product than that of ROS production of treated cell with 3 or 4 mM nicotine (Figure 3A-B). The results revealed that cotinine had a potential to consistently induce ROS overproduction on treated cells. Moreover, combined treatment of 5 mM nicotine with cotinine clearly showed a high ROS generation, suggesting that cotinine can enhance nicotine effect in promoting ROS production.

The RT-qPCR in our study showed a significant altered apoptosis-related genes (Bax, Bcl-2, Caspase-3, P53) induced by 5 mM nicotine treatment. Interestingly, the combined 5 mM nicotine with 1 mM cotinine treatments was significantly increased all apoptosis-related genes compared to the treatment of nicotine solely, suggesting that cotinine can enhance nicotine effect in inducing cell apoptosis. It has also been reported that ROS can induce changes in pro-apoptotic Bcl-2 family proteins (Bax, Bak). BAX is directly responsible for breaching the mitochondrial outer membrane, interacting with the mitochondria to induce a permeability transition and releasing cytochrome C, which sequentially activates a downstream caspase program.[31-34] Our study hinted this cascade supported by a significantly increased Caspase-3 expression in 5 mM nicotine and a combined 5mM nicotine with cotinine treatments. The results indicated that PMEF treated cells have been damaged and circled into late apoptosis stage. BCL-2 serves an anti-apoptotic role, inhibiting apoptosis by regulation of mitochondrial outer membrane permeabilization (*MOMP*).[35, 36] In our study, treatments of 5 mM nicotine and a combined 5 mM nicotine with cotinine increased expression of Bax. This may increase Bcl-2 expression by restraining the MOMP. The results showed an association of increased expression between Bax and Bcl-2. Previous studies provided an insight into the molecular mechanisms of nicotine pro-apoptotic effects on the liver and kidney.[37] Results showed a significant decreased in Bax/Bcl-2 ratio with DNA fragmentation indicating cell apoptosis in the nicotine treated mice. However, contrast results were reported in human oral mucous fibroblasts study in which smokeless tobacco was found to induce ROS and cell apoptosis with increased in Bax/Bcl-2 ratio.[38]

Therefore, nicotine have pro-apoptotic effect depending on the concentration of the substance used, species-related variations in the metabolism of nicotine and the target cells. However, anti-apoptotic Bcl-2 proteins also contain other genes besides Bcl-2 such as Bcl-X large, Bcl-2-like protein 2, and Bcl-2-like protein 10.[39] These genes may reduce expression and result in increased Bax expression, which is an interesting point for further study. Closely related to P53 mRNA expression in this study, the mRNA expression level of PMEF treated cells at 5 mM nicotine and combined 5mM nicotine with cotinine was significantly increased. P53 is a key protein mediating cell response to stresses and DNA damage, associating with genes in cell cycle arrest and apoptosis.[40] Previous studies investigated the effects of nicotine on cell apoptosis via ROS production reported that ROS could trigger cytoplasmic membrane damage and DNA fragmentation.[41] Elevated ROS can directly oxidizes DNA and triggers genotoxicity to induce apoptosis.[42, 43] In consistent with our study, apoptotic stimuli (ROS) can causes DNA damage through induction the expression of P53. P53 mediated apoptotic signalling which will further affect the mitochondria membrane transition pore complex resulting in increased inner mitochondria membrane permeability allowing free passage of molecules (i.e. protons) into the mitochondrial matrix. This cause osmotic swelling of the mitochondrial matrix. Then, increased MOMP accelerates transportation of cytochrome C, apoptosis inducing factor (AIF) and endonuclease G (endo) into cytosol to induce

apoptosis.[44-46]

Adverse effect of ROS production in 5 mM nicotine and 5 mM nicotine combined with cotinine treatments in PMEF cells in our study was interesting. The mechanism supported these effects by severe mitochondrial dysfunction, accelerating MOMP through the opening of the mitochondria permeability transition pores, since we found the associated ROS overproduction and increased levels of Bax, Caspase-3 and P53 mRNA expression.

Conclusions

Nicotine and cotinine had an adverse effect on PMEF cells through cell apoptosis induction in a dose-dependent manner. Cotinine could enhance the cytotoxic effect of nicotine treatment by decreasing cell viability, increasing ROS production and Bax, Caspase-3 and P53 expression to promote cell apoptosis.

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Declaration of Interest

The authors report no conflict of interest.

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