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Research article

Comparative cytotoxicity study of nicotine and cotinine on MRC-5 cell line

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Abstract

Nicotine has several health hazards regarding carcinogenic potential. It also imparts increased risk for respiratory, cardiovascular, and gastrointestinal disorders. Several mechanisms have been proposed for the carcinogenic potential, including effects on cell proliferation, inducing oxidative stress, DNA mutation, or inhibition of apoptosis. The cotinine metabolite is generally thought to have effects similar to nicotine in some experimental systems.

The purpose of this study was to assess the nicotine and cotinine cytotoxicity on MRC-5 lung fibroblasts. The pulmonary fibroblasts were treated with various concentrations of nicotine or cotinine (in the range 1 μM – 2 mM) for 24 or 48 h and analyzed for cell viability by MTT test.

The results indicated that high nicotine concentrations (2 mM) induced marked cell death (about 50%) in MRC-5 cell line. Cotinine showed lower toxicity than nicotine on the MRC-5 cells. In contrast to nicotine treatment, cells treated with cotinine continued to proliferate after the 48h incubation period.

Keywords : nicotine, cotinine, cytotoxicity, MRC-5, MTT-assay

Highlights

- ✓ At concentrations similar to the levels observed in the plasma of smokers, nicotine and cotinine were not cytotoxic on MRC-5 cell line
- ✓ Higher concentrations (2mM) of both nicotine and cotinine decreased the viability of MRC-5 cells.
- ✓ Nicotine proved to be more cytotoxic than cotinine. Unlike nicotine, the proliferative capacity of MRC-5 cells is maintained after 48 hours of cotinine treatment.

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Introduction

Smoking is the only cause of premature death that can be totally prevented. Morbidity induced by tobacco smoking refers in particular to diseases of the respiratory system and the cardiovascular system. In addition, increased prevalence of cancer at different levels in the body, urinary tract damage, gastrointestinal disorders, ocular damage, endocrine and metabolic alterations, and adverse effects on pregnancy have been reported. An alarming aspect is that smoking also affects the health of non-smokers who are passively exposed to tobacco smoke (1, 2).

Nicotine alkaloid is the major addictive component of tobacco. Nicotine is a highly toxic compound that exerts its effects on almost every organ and system in the body. It is absorbed through the entire respiratory tract, through the oral and nasal mucosa, into the gastrointestinal tract, and even through the skin. In vivo, nicotine is extensively metabolized by cytochrome P450 in the liver, to the major metabolite cotinine (Figure 1). Cotinine metabolite has a relatively long half-life (~17 h) compared to nicotine (1–2 h) and it is used as a biomarker for smoking, including passive smoking (3,4).

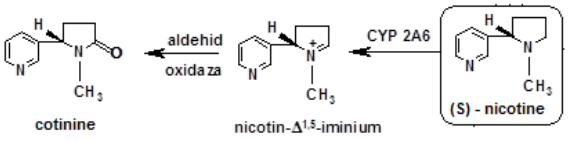

Figure 1. Oxidative biotransformation of nicotine to cotinine

The carcinogenic potential of nicotine is supported by current studies. Many types of cancers, including small-cell and non-small-cell lung carcinomas, head and neck, gastric, pancreatic, colon, breast, cervical, urinary, bladder, and kidney cancers have been associated with nicotine (5).

Several studies indicate that nicotine is toxic and addictive, but it is not carcinogenic (6).

Current in vitro and in vivo studies demonstrate that nicotine and its main metabolite cotinine have tumor-promoting activities. In addition, tobacco smoke contains a variety of proved carcinogens including polycyclic aromatic hydrocarbons (i.e. 3,4-benzo(a)pyrenes), and tobacco-specific nitrosamines, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN) (7).

The current research is focused on the molecular mechanisms by which nicotine and tobacco smoke can initiate tumor formation, influence cell cycle progression and proliferation, and promote tumor progression in multiple cancer types (7). The potential of nicotine in inducing DNA mutations as a step towards cancer initiation is still controversially discussed (8).

Nicotine was shown to have an important role in regulating cellular processes such as proliferation, differentiation, and migration. In normal cells, nicotine can stimulate mechanisms consistent with cell transformation and the early stages of cancer development (9).

On in vitro systems, it has been demonstrated that nicotine increased cell proliferation, inhibited apoptosis, stimulated cancer cell growth, and enhanced or inhibited angiogenesis. Nicotine was also reported to promote tumor growth and metastasis in cancer xenograft models (10).

Nicotine exerts its effects by activating the nicotinic acetylcholine receptors (nAChRs) that are expressed in neurons and neuromuscular junctions and in a wide variety of non-neuronal cells, including those of epithelial and endothelial origin.

Nicotine has been demonstrated to have tumor-promoting properties by activating the nAChRs; this may result in activation of several signaling pathways that can have tumorigenic effects. It has been shown that nicotine (at concentrations similar to those in the bloodstream of smokers, ranged from 10 nmol/L to 10 μmol/L) or tobacco-specific nitrosamines such as NNK could induce proliferation in various *in vitro* cell culture models, and this effect could be canceled by nAChR antagonists (7).

Recent studies demonstrated that nicotine induces multiple cancer cell proliferation via α7-nAChR subunit. Thus, nicotine was shown to induce a dose-dependent increase in proliferation of lung cancer cells, breast cancer cells, and pancreatic cancer cells via α7-nAChRs-mediated signal transduction pathways (11). In addition, nicotine induced epithelial-to-mesenchymal transition (EMT) in breast and lung cancer (11).

It has been demonstrated that nicotine induces cell proliferation via α7-nAChR subunit in mouse lung cancer and enhances the growth of tumors induced by tobacco carcinogens. In addition, nicotine promotes re-growth and metastasis of tumors in mice (12).

Nicotine was demonstrated to promote proliferation, invasion, and angiogenesis in tumor cells while inhibiting apoptosis (13).

The cotinine metabolite was also shown to induce tumor promotion in human lung adenocarcinoma cells by inhibiting apoptosis and increasing cellular proliferation (14).

There are conflicting reports regarding the beneficial effects of nicotine and its main metabolite cotinine on the human body. Cotinine has been reported to have some pharmacologic activity that decreases vascular resistance, decreases blood pressure in animals, and affects cognitive performance (15).

Nicotine (10-7 – 10-5 M) and cotinine (10-6 – 10-8 M) exhibited neuroprotective properties in SH-SY5Y neuroblastoma cell line. Cotinine also protected against 6-hydroxydopamine (6-OHDA)-induced cell damage in neuroblastoma cells (16).

Studies have revealed the citotoxic effects of nicotine in different experimental systems while there are limited data regarding the citotoxic effects of cotinine. Nicotine exerts dose- and time-dependent cytotoxic effects on the mesenchymal stem cells derived from human periodontium (17). Nicotine and cigarette extract (CE) were demonstrated to induce cytotoxicity in human embryonic lung fibroblast, MRC-9 (18).

The present study investigated the in vitro effects of nicotine and cotinine on proliferation of MRC-5 cell line, a diploid human cell culture line composed of fibroblasts derived from normal lung tissue of a 14-week-old male fetus.

Materials and method

This study was performed on the continuous cell line of pulmonary fibroblasts MRC-5 (ATCC – American Type Culture Collection). According to the manufacturer's datasheet, these cells exhibit typical fibroblast morphology.

To spread the culture, the cells were cultivated in Eagle's Minimum Essential Medium (EMEM) supplemented with 1 % antibiotic/antimichotic (ABAM) and 10% fetal bovine serum (SFB), put in sterile culture vials. The cells were incubated at 37°C, in humid atmosphere of 5% CO2 during the entire experimental period. In order to multiply, the cells adherent to the culture substrate were detached by means of enzimatic treatment with trypsin at 80 % confluency. Effectively, for the multiplication, the cells were detached from the culture surface with a solution of trypsin –EDTA. The cell sediment following the centrifugation was re-suspended in fresh culture medium, and the cell suspension was distributed in new culture vials.

(-)-Nicotine (≥99%, Sigma-Aldrich) and (-)-Cotinine (≥98%, Sigma-Aldrich) were used to prepare stock solutions in DMSO. Both nicotine and cotinine have been tested in the following dilutions: D1 = 2 mM, D2 = 1 mM; D3 = 750 μM; D4 = 500 μM; D5 = 100 μM; D6 = 10 μM and D7 = 1 μM.

For determining the optimal working dose and also for evaluating the proliferation of the cells under treatment with nicotine and cotinine, viability of the pulmonary fibroblasts MRC-5 was assessed. Cytotoxicity was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. The quantitative colorimetric MTT is based on the reduction of a yellow tetrazolium salt MTT to a blue-violet formazan in the culture medium. The reduction reaction is made by the mytochondrial enzymes (especially hydrogenase succinate) and is an index of the cell/mitocondriale integrity. The formazan may be solubilised with izopropanol or DMSO, and the optic density (D.O.) of the solution obtained this way may be spectrophotometrically evaluated at 550nm.

Briefly, the MRC-5 cells were seeded at a density of 103 cells/well into 96-well microtiter plates in three replicates and left for 24 hours to adhere. The next day, the culture medium was replaced with a fresh one, which contained different concentrations of nicotine or cotinine followed by incubation for 24 or 48 hours. After 24 or 48 hours, the treatment was removed and the cell mono-layers were incubated for 4 h with a 1 mg/ml MTT solution in serum-free medium. The formazan crystals formed, in the meantime, by the active metabolic cells, have been solubilised in DMSO, and the D.O. of the resulting solution was determined at 550 nm.

Statistics

Quantitative data were processed and represented graphically by means of the soft GraphPad Prism 3.03.

Data are expressed as mean ± SE (SEM). Grouped data were compared with one-way ANOVA with Bonferroni adjustment. P-values < 0.05 were considered statistically significant.

Results

Dose screening

In order to examine if nicotine or cotinine is cytotoxic to MRC-5 cells, the cell line was treated with various concentrations of nicotine or cotinine in the range 1 μM – 2 mM for 24 h and analyzed for cell viability by MTT-assay.

Dose screening for nicotine has shown that the use of a 2 mM dose lowers the viability of the cells to 50% compared to the non-treated control, and the treatment with concentrations which are lower or equal to 750 μM does not induce modifications of the cell viability (Figure 2). Lung fibroblast cell line MRC-5 was largely

unaffected by the nicotine treatment in the range of concentration 1 µM – 750 µM. Therefore, following the dose screening, the concentration of 1mM of nicotine was selected for the further study of the cell proliferation.

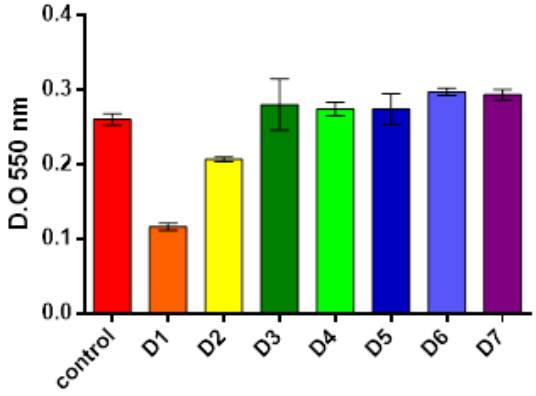

Figure 2. The viability of the MRC-5 cells treated for 24h with nicotine D1 = 2 mM, D2 = 1 mM, D3 = 750 µM, D4 = 500 µM, D5 = 100 µM, D6 = 10 µM and D7 = 1 µM in culture medium

The viability of the MRC-5 cells treated with cotinine was unaffected in the range of concentration 1 µM – 1 mM (Figure 3). Cotinine showed lower toxicity than nicotine on the MRC-5 cells. The dose screening for cotinine shows that the use of a dose smaller or equal to 1mM does not induce modifications of the cell viability. Thus, following the dose screening, the concentration of 2mM of cotinine was selected for the further study of the cell proliferation.

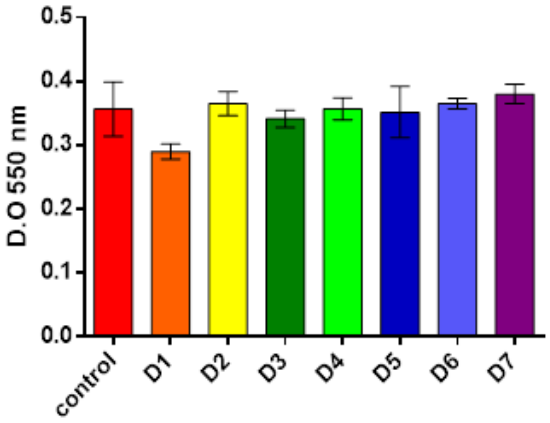

Figure 3. The viability of the MRC-5 cells treated for 24 h with D1 = 2 mM, D2 = 1 mM, D3 = 750 µM, D4 = 500 µM, D5 = 100 µM, D6 = 10 µM si D7 = 1 µM cotinine in culture medium

Evaluation of proliferative potential

The evaluation of proliferative potential has shown that non-treated control MRC-5 cells proliferate significantly ($p<0.0001$) in culture for 48h (Figure 4). Once exposed to the treatment with 1mM nicotine, the proliferative potential of the MRC-5 cells is inhibited, but we did not detect statistically significant changes between the viability of the cells treated with 1 mM nicotine for 24h and for 48h.

In terms of treatment with 2 mM cotinine, the proliferative potential of the MRC-5 cells is inhibited compared to the non-treated control, with statistically significant differences ($p<0.05$) between the viability of the cells treated with cotinine for 24h and 48h (Figure 4).

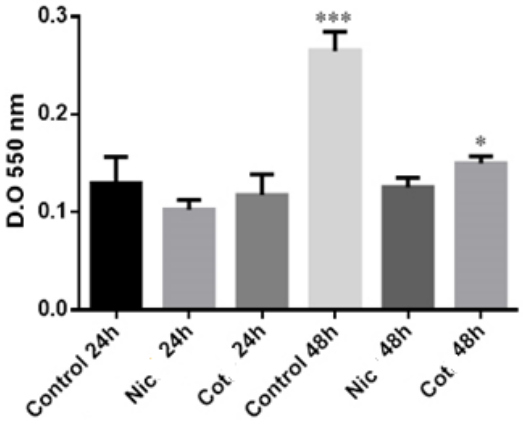

Figure 4. The proliferative potential of MR-5 cells treated for 24h and 48h with 1 mM ncotine or 2 mM cotinine (Nic = Nicotine; Cot = Cotinine)

*** $p<0.0001$ control 48h versus control 24h;
* $p<0.05$ cotinine 48h versus cotinine 24h)

The results indicated that the MRC-5 cells treated with 1mM nicotine do not proliferate after 48h of treatment, in contrast to those treated with 2 mM cotinine that retain some weak proliferative potential if treated for 48h.

Discussions

Evidence confirms harmful effects of tobacco use and nicotine. Smoking is demonstrated as a risk factor for various types of cancer, including lung, oral cavity, or breast cancer. Nicotine has been shown to have tumor-promoting activity. In addition, growing evidence suggests that the use of tobacco can reduce the efficacy of cancer medicines and may increase the risk of recurrence, while migration to other geographical areas is able to influence the cancer incidence (19).

In recent years, research has provided findings regarding the mechanism of nicotine toxicity. Furthermore, evaluation of the toxicological profile of cotinine, the main metabolite of nicotine, is under current investigation. Controversy over the toxicity of cotinine continues. Cotinine is less lipophilic than nicotine and has been found to be less cytotoxic than nicotine on fibroblast (HFF), melanoma (SK-Mel/27), and hepatoma (HepG2) cell lines (20). Nicotine and cotinine were not cytotoxic to Chinese hamster ovary (CHO) cells (21).

The present study evaluated the toxicity of nicotine and its metabolite cotinine on normal human lung cell line MRC-5. Limited data are published with respect to the cytotoxic effects of nicotine and cotinine on normal cells. Our study investigated the cytotoxic potential of nicotine compared to cotinine on MRC-5 human lung fibroblast cell line by using the MTT assay.

Our overall results are in line with studies investigating the cytotoxicity of nicotine in various in vitro systems. Nicotine, at a concentration of 1μM, was shown to exert a minor effect on the viability of MRC-5 normal fibroblasts, while the proliferation of both human lung cancer cell lines A549 and SKMES-1 (13% for A549 and 14% for SKMES-1) was slightly enhanced (22).

The treatment of MRC-5 cells with nicotine-free tobacco extract (1 mg/mL, but no 0.1 mg/mL) reduced ($p < 0.05$) the viability of MRC-5 cells by 42%, indicating that components other than nicotine in tobacco leaves exhibited cytotoxic effect (23).

Current reports indicate that the toxic effect exerted in vitro by nicotine depends on the type of the cell tested. At a concentration higher than 2.5 mM, nicotine inhibited cell proliferation in human periodontal ligament fibroblast (PDLFs) (24), suggesting that nicotine may contribute to the progression of periodontal disease.

It has been demonstrated that nicotine is not cytotoxic on MCF-7 human breast cancer cells and KB-3.1 human nasopharyngeal carcinoma cells. Furthermore, pretreatment with nicotine (10 μM) determined significant protection to MCF-7 cells and KB-3.1 cells treated with doxorubicin (25).

The results of our study indicate that high nicotine concentrations (2 mM) induced marked cell death (about 50%) in MRC-5 cell line. Similar results have been reported in experiments on alveolar cell line (A549). Nicotine was shown to act in a concentration-dependent manner on A549 lung cells and concentrations beginning with 4mM showed a significant toxic effect on the cell viability (26).

Cotinine was less cytotoxic than nicotine on MRC-5 cell line. The results indicate that the MRC-5 cells treated with 1mM nicotine do not proliferate after 48h of treatment, in contrast to those treated with 2 mM cotinine that retain weak proliferative potential if treated for 48h.

The present study demonstrated that, at physiologically relevant concentrations, both nicotine and cotinine would have no significant toxic effect on MRC-5 pulmonary fibroblasts. High concentrations decreased the cell viability, and cotinine was proved to be less toxic than nicotine in MRC-5 cells. In contrast to nicotine treatment, cells treated with cotinine continue to proliferate after 48h incubation period.

Conclusions

Our data indicate that, at concentrations similar to those reported in the bloodstream of smokers, nicotine and cotinine have not been shown to be cytotoxic on the tested MRC-5 normal human lung cell line.

The viability of the MRC-5 cells treated with higher concentration of nicotine or cotinine was decreased, and nicotine was shown to be more toxic than cotinine.

The results of the study provide additional information on the toxicity of cotinine and nicotine on normal cells.

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