ORIGINAL ARTICLE

In vitro effects of nicotine on the non-small-cell lung cancer line A549

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Abstract

Objective: To investigate in vitro effects of nicotine on the non-small-cell lung cancer line A549.

Methods: The case-control study was conducted at the First Affiliated Hospital of Nanchang University from 1st January to 30th June, 2014 and comprised A549 cells which were treated with a series of concentrations of nicotine (0.01 μM, 0.1 μM, 1 μM and 10 μM) for 24 hours. Control cells were incubated under the same conditions without the addition of nicotine. Cell growth was detected by monotetrazolium salt [3-(4,5-dimethyl-2-thiazolyl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay. Cell apoptosis was detected by Haematoxylin and Eosin staining, immunofluorescence analysis of Filamentous actin and electron microscope observation.

Results: Nicotine had no significant effect on A549 cell growth at the dose of 0.01μM ($p>0.05$), but had significant growth inhibitory effects at the doses of 0.1μM, 1μM and 10μM ($p<0.05$ each). A significant decrease in cell numbers was observed on staining ($p<0.05$). Significant changes in the size and shape of cells and concomitant changes in cytoskeletons and organelles were observed by immunofluorescence and electron microscope observation ($p<0.05$).

Conclusion: The growth inhibitory effects of nicotine on A549 cells were found to be dose-dependent.

Keywords: Nicotine, A549 cell line, Apoptosis, F-actin. (JPMA 66: 368; 2016)

Introduction

Lung cancer is one of the most frequent malignant tumours responsible for more than 1.3 million deaths worldwide annually.[1] Among all causes, tobacco use is believed to be the most common one, causing 80-90% of lung cancers.[2,3] Cigarette smoke is the predominant form of tobacco consumption, consisting of approximately 95% of tobacco use.[4] There are an estimated 7,000 compounds in cigarette smoke, including at least 60 known carcinogens,[5] among which nicotine is the primary psychoactive chemical component associated with addiction.[6] It was found that an average cigarette yields about 1mg absorbed nicotine.[7] Importantly, nicotine can bind to and activate nicotinic acetylcholine receptors (nAChRs) that are expressed on both normal and cancerous tissues in the body, thereby exerting many biological effects.[8,9]

Nicotine has been found to induce proliferation and angiogenesis in different cellular models although it is not carcinogenic itself.[10,11] Several studies showed that nicotine can be rapidly absorbed and distributed throughout the body after entering the body at concentrations of 10-8-10-7 M in the blood stream of smokers.[8,11] In addition, nicotine was found to act as a stimulant in mammals in small doses (several mg) while high amounts (50-100mg) can be harmful.[12]

The non-small-cell lung cancer (NSCLC) line A549, which is known to have diverse functions, was first developed in 1972.[13] These alveolar epithelial cells can be cultured in vitro easily and are widely used as an in vitro model for drug metabolism and function assessment.[14] Although there are many studies supporting the significant role of nicotine in lung cancer, but there are few that report on the effects of different concentrations of nicotine on A549 (NSCLC) directly.

According to the statistical data on patient survival rates and prognoses, lung cancer treatments are still unsatisfactory so far.[1] In fact, there is no single ideal therapy yet that could be considered curative for lung cancer. In this sense, fighting against addiction to cigarette smoking is of great significance in decreasing the mortality of lung cancer. Studying the growth-modulatory effects of nicotine on A549 cell line may provide a better understanding of the mechanisms related to lung cancer and also allow us to develop new drugs that can inhibit and eliminate lung tumour cells in the near future.

The present study was planned to investigate in vitro effects of nicotine on NSCLC line A549 and to observe the concomitant morphological changes of A549 under the microscope.

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Materials and Methods

The case-control study was conducted at the First Affiliated Hospital of Nanchang University from 1st January to 30th June, 2014, and comprised A549 cells which were treated with a series of concentrations of nicotine (0.01 µM, 0.1 µM, 1 µM and 10 µM) for 24 hours. Control cells were incubated under the same conditions without the addition of nicotine.

NSCLC A549 cell lines (American Type Culture Collection [ATCC]) were cultured in DMEM medium (Gibco) supplemented with 10% (m/v) foetal bovine serum (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin on culture plates at 37°C in a 5% carbon dioxide (CO_2) atmosphere with stable humidity. The density of cells was 1×105 cells/ml before starting the culture.

Monotetrazolium salt (MTS) growth inhibition assay. (3-[4, 5-dimethyl-2-thiazolyl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) was used to assess effects of nicotine on cell growth according to the instructions provided by the manufacturer (Promega). Briefly, the same number of cells were seeded into each well of a 96-well plate on day 1 (1×10^5 cells/ml). On day 2, the cells were either treated with different concentrations of nicotine (0.01 µM, 0.1 µM, 1 µM and 10 µM) for 24 h or were left as untreated controls. Control cells were grown under the same conditions but without the addition of nicotine to the medium. At the end of incubation time, fresh complete medium containing 10 µL of MTS solution was added and cells further incubated for 2 hours. Optical density of each culture was then recorded at 490nm using a microplate reader (Bio-Rad, model 550). Each experiment was performed six times at different concentrations level. Results are calculated as percentage growth inhibition with respect to the untreated cells.

Haematoxylin and Eosin (H&E) staining was performed as described in literature.[15] Cultured cells were fixed with 4% paraformaldehyde in Hanks-buffered salt solution (HBSS) for 15 min and afterwards rinsed with phosphate-buffered saline (PBS) for 5 min at room temperature (RT). After fixation, cells were embedded in 0.1 M glycine solution for 5 min at RT. Following a triple rinsing in PBS, the cells were stained with H&E) for 5 min at RT. After washing the cells for 25 min under running water, cells were dehydrated in a series of alcohols and xylenes. Finally, the fixed cells were examined using an Eclipse E800 microscope (Nikon, Tokyo, Japan) with computer imaging system analysis.

For fluorescence observation, cells were fixed with 4% paraformaldehyde in HBSS for 15 min at RT and afterwards rinsed with PBS (3×5 min, RT). Then the cells were incubated with 0.1 M glycine for 5 min at RT and rinsed in PBS (3×5 min, RT). Phalloidin conjugated with a derivative of rodamin was used to stain the filamentous actin (F-actin). The cells were incubated in CO_2 incubator with 10 µM of stock phalloidin/tetramethylyrhodamine-5-isothiocyanate (TRITC) solution diluted 1:5 in 20% methanol for 20 min. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) at a dilution of 1:25,000 for 20 min at RT. In the end, the material was embedded in Gelvatol. F-actin was examined in an Olympus IX83 fluorescence microscope (Olympus, Tokyo, Japan). Computer analysis of fluorescent imaging was done with the software Image-Pro Plus 6.0 (Media Cybernetics, American).

For electron microscopy, A549 cells were fixed for 1 hour in 3.6% glutaralehyde and then moved to 0.1 M cacodylate buffer (pH 7.4). Following post-fixing for 1.5 h with 2% osmium tetrooxide in 0.1 M cacodylate buffer, the cells were dehydrated in a graded series of alcohols and embedded in Epon 212. A Reichert OmU3 ultramicrotome was used to make semi-thin sections. Parts of the material selected from the semi-thin sections were cut into ultrathin sections and then stained with uranyl acetate and lead citrate. The prepared material was analysed with a JEM 100 CX electron microscope (JEOL, Tokyo, Japan).

All materials, unless otherwise stated, were obtained from Sigma-Aldrich (Shanghai, China).

Statistical analyses were performed with OriginPro 8.0 (OriginLab, America). Data was expressed as means±standard deviation (SD). Statistical significance was carried out using one-way analysis of variance (ANOVA) and Dunnetts post-hoc test. Differences were considered significant at $p<0.05$.

Results

Growth inhibitory effects of nicotine on A549 cells were observed. To test the growth-modulatory effects of nicotine on A549 cells, a wide concentration range of nicotine (0.01 µM, 0.1 µM, 1 µM and 10 µM) was used. The original raw data of optical density recorded at 490nm by MTS method is shown in Table. In control group, the mean OD490nm measured was 1.303, while in nicotine treated group, the mean OD490nm measured at concentrations of 0.01 µM, 0.1 µM, 1 µM and 10 µM was 1.298, 0.7622 0.5550 and 0.3350, respectively. The results showed that nicotine had significant growth inhibitory effects on A549 cells at concentrations of 0.1 µM, 1 µM and 10 µM, with an average growth inhibition rate of 40.79%, 56.58% and 72.93% respectively compared with control cells. However, low concentration (0.01 µM) of nicotine had no significant growth modulatory effect compared with

Table: Raw data of optical density recorded at 490nm by MTS method.

Nicotine concentration	OD_{490nm}						Mean	SD
0	1.28	1.32	1.29	1.33	1.31	1.29	1.303	0.0196
0.01 μM	1.27	1.28	1.3	1.31	1.32	1.31	1.298	0.0194
0.1 μM	0.78	0.76	0.75	0.76	0.76	0.77	0.7633	0.0103
1 μM	0.58	0.54	0.53	0.56	0.55	0.57	0.5550	0.0187
10 μM	0.35	0.34	0.32	0.33	0.33	0.34	0.3350	0.0105

SD: Standard Deviation.
MTS: Monotetrazolium salt

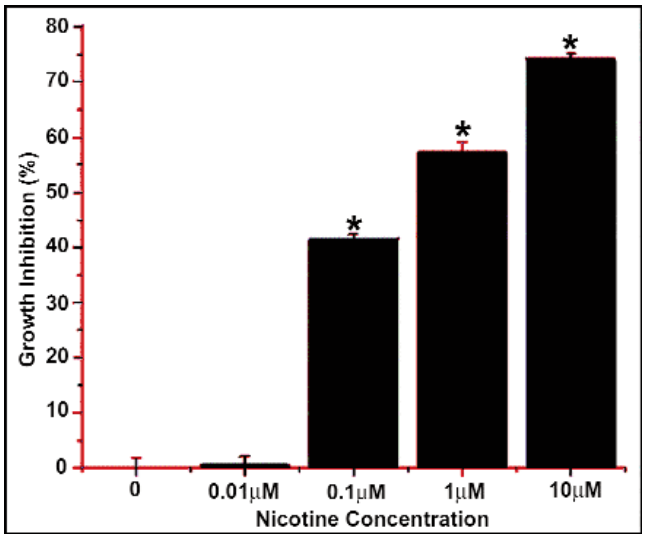

Figure-1: Growth inhibitory effect of nicotine (0.01 μM, 0.1 μM, 1 μM and 10 μM) on A549 cells. Data expressed as means ± SD, n=6. *p<0.05 represents significant differences when compared with control cells.

control. The data indicate that the growth inhibitory effect of nicotine on A549 cells was concentration dependent (Figure-1).

In terms of H&E staining, significant changes in number, shape and size of nicotine-treated cells were observed compared to the control cells (Figure-2A). HE staining revealed decreased total numbers of cells, increased intracellular spaces, and cell shrinkage occurring after 1 μM and 10 μM nicotine treatment for 24 h (Figures-2B, 2C). Cells treated with 10 μM nicotine showed a tendency to form clusters and multiplied nucleoli were observed in comparison with the control cells (Figure-2C).

Fluorescence microscopic studies showed the effect of nicotine (1 μM and 10 μM) on F-actin distribution in the A549 cells. In the control cells, F-actin was evenly distributed; the nuclei were distinct and had regular shapes (Figure-3A). After treatment of nicotine for 24 h, cells were shrunken and highly developed stress fibres were observed in large cells (Figures-3B, 3C). Lobe-shaped nuclei and micronuclei also appeared and F-actin accumulation was observed in the central part in the large cells under 1 μM nicotine treatment (Figure-3B). However, after 10 μM nicotine treatment, the cells became more shrunken. A549 cells with condensed chromatin and apoptotic blebs were observed (Figure-3C).

Electron microscopy was used to analyse the ultrastructural changes in the cell area after 1 μM and 10 μM nicotine treatment. In the control group, round and clear nuclei were observed (Figure-4A). However, after

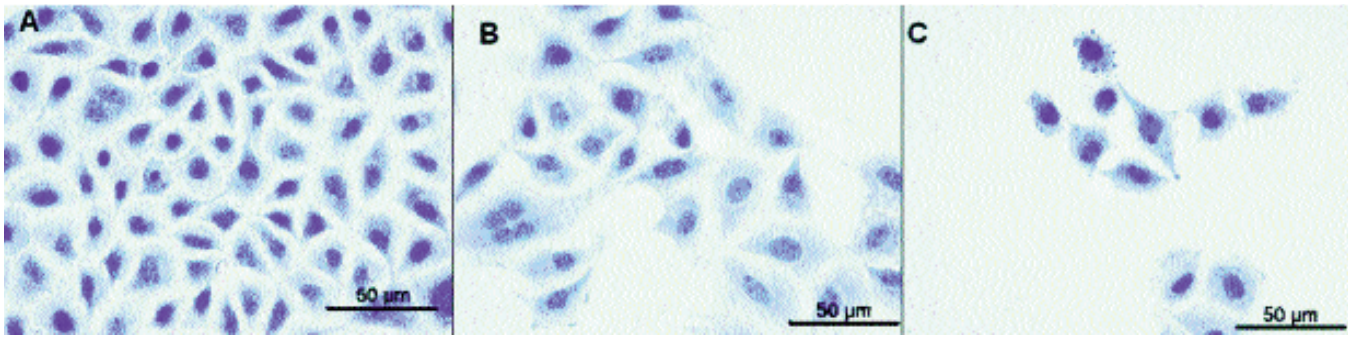

Figure-2: Haematoxylin and Eosin staining. (A) Control: A549 cells incubated without nicotine. (B) A549 cells incubated with 1 μM nicotine for 24 h. Intracellular spaces and the size of cells became larger, nucleus multiplied and the number of cells decreased after 1 μM nicotine treatment. (C) A549 cells incubated with 10 μM nicotine for 24 h. Cells with condensed chromatin and apoptotic blebs were observed. The number of cells decreased significantly after 10 μM nicotine treatment.

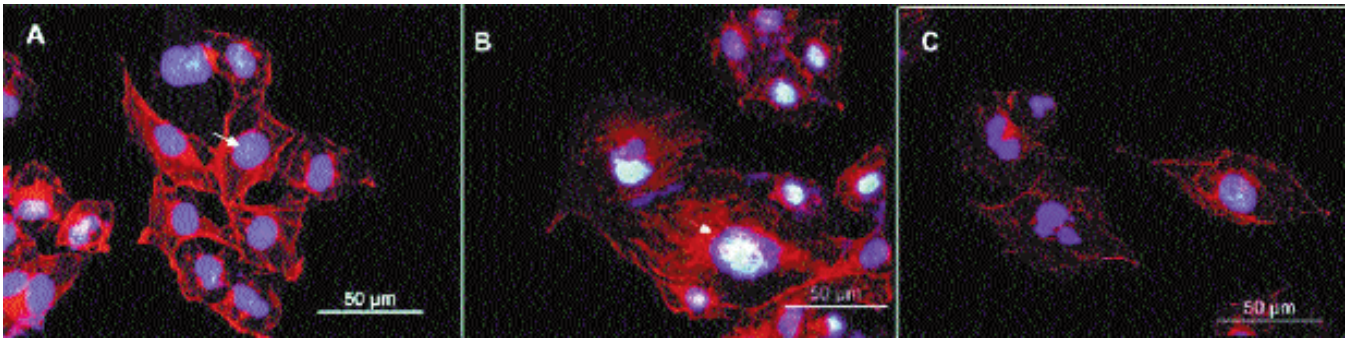

Figure-3: Analysis of Filamentous actin organisation in fluorescence microscopy (Phalloidin/TRITC - F-actin, DAPI - nuclei). (A) Control group: A549 cells incubated without nicotine. Distinct nuclei with regular shapes and evenly distributed F-actin were observed. (B) Cells treated with 1 µM nicotine. Cells were shrunken and F-actin accumulation was observed. (C) Cells treated with 10 µM nicotine. Cells were shrunken and chromatin became condensed.

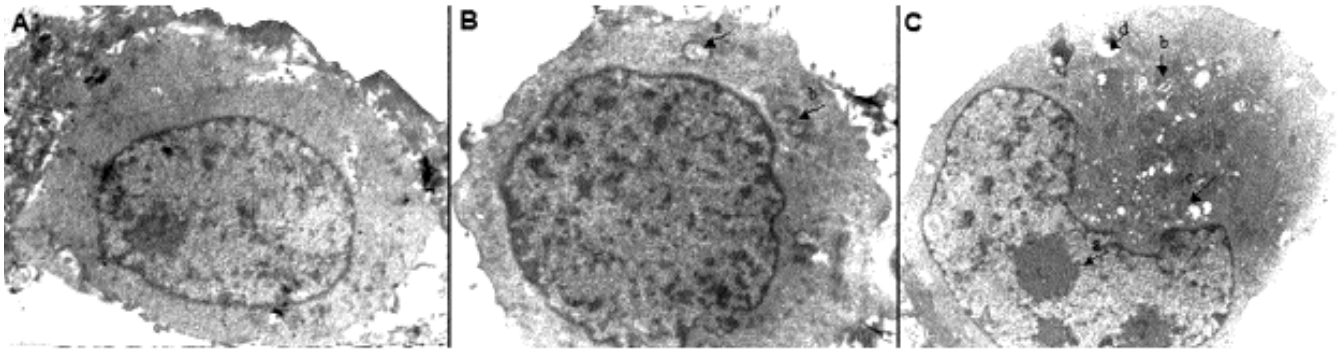

Figure-4: Ultrastructure analysis of A549 cells by electron microscopy; Magnification×12,000. (A) Control group: A549 cells incubated without nicotine. Round nucleus and regularly shaped organelles were observed. (B) Cells treated with 1 µM nicotine. Swollen nucleus, augmented mitochondria (arrow a) and lysosomes (arrow b) were observed. (C) Cells treated with 10 µM nicotine. Shrunken nucleus, nucleoli in the nucleus (arrow a), dilated endoplasmic reticulum (arrow b), augmented mitochondria (arrow c) and lysosomes (arrow d) were observed.

nicotine treatment, significant changes were observed under electron microscopy scanning. The shape of nuclei changed dose-dependently after nicotine treatment, with swollen and shrunken nucleus observed in cells treated with nicotine at concentrations of 1 µM and 10 µM, respectively (Figures-4B, 4C). At the higher concentration (10 µM) of nicotine, markedly increased numbers of lysosomes and nucleoli were observed compared to the control cells. Swollen mitochondria and morphological alterations of the endoplasmic reticulum in cytoplasmic area were also observed (Figure-4C).

Discussion

The study was conducted to investigate the effect of nicotine on A549 cells, an NSCLC line. Due to its good properties in culture, A549 cell line is an attractive research model for studying the metabolism and mechanism of different drugs.[14,16] Tobacco use is believed to be one of the most common causes of lung cancer nowadays.[2,3] Nicotine is one of the over 7,000 compounds in tobacco and is the principal chemical associated with addiction.[5,17] Several studies have demonstrated that tobacco can decrease overall survival and quality of life, and reduce survival in patients with tobacco-related cancers such as lung cancer.[18-20] There is little data assessing the effects of nicotine on outcome in lung cancer patients, but data shows that nicotine has deteriorating effects.[21] Therefore, smoking cessation is particularly important for lung cancer and other cancer patients, as well as for healthy smokers. Nicotine is one of the most commonly used and well-supported drugs in tobacco cessation. In vitro, similar results related to the increased number of apoptotic cells after nicotine treatment have been reported.[22,23]

We investigated the influence of nicotine, the main compound and principal chemical associated with addiction in tobacco on A549 cells. The concentrations of nicotine used in the present study were chosen to encompass a wide range of environmentally realistic

exposures. We found that nicotine has no significant growth inhibitory effects at the dose of 0.01 μM but has significant growth inhibitory effects at the doses of 0.1 μM, 1 μM and 10 μM on A549 cells. After nicotine treatment, the morphology changed significantly, which may suggest a cellular response to nicotine stress. Apoptosis observed by characteristic changes in cellular cytoskeleton and organelles suggest that nicotine may promote apoptosis at high concentrations. However, further studies are required to confirm the results and to investigate the underlying mechanism.

Conclusions

Nicotine had no significant growth modulatory effects at low concentration (0.01 μM), but had significant growth inhibitory effects at high doses (0.1 μM, 1 μM and 10 μM) on A549 cells. A significant decrease in cell number was observed by H&E staining after nicotine treatment. Changes in the size and shape of cytoskeletons and organelles were significantly observed by immunofluorescence and electron microscope observation. Specifically, shrunken cells and F-actin accumulation were observed after nicotine treatment. Furthermore, the shape of nuclei and mitochondria changed and the number of lysosomes and nucleoli increased significantly when stressed with nicotine.

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