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Ameliorative effect of boric acid against nicotine-induced cytotoxicity on cultured human primary alveolar epithelial cells

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ABSTRACT

Nearly six million people die from smoking due to addiction to nicotine. Because of high toxicity by nicotine, main alkaloid in tobacco, the action modes of nicotine have been analyzed comprehensively by scientists in different organisms and cell cultures. One of the main cytotoxicity mechanisms of nicotine is that activating lipid peroxidation by inducing production of reactive oxygen species (ROS). Recent investigations support that boric acid exhibits cyto-protective properties on different cell types via its antioxidant nature. Thus, in this study boric acid (BA) was assessed as a potential cyto-protective agent against nicotine-induced cytotoxicity. Therefore, toxic concentrations of nicotine were evaluated on human primary alveolar epithelial cells (HPAEpiC) and BA was applied against toxic dose of nicotine to analyze whether cytotoxicity could be attenuated or not. Wide spectrum nicotine hydrogen tartrate concentrations (0.312 mM to 20 mM) were used to investigate the cytotoxicity. 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) release assays were used to analyze cytotoxicity after exposure to nicotine and boric acid, and their combinations. In addition, Hoechst 33258 (bis-Benzimide) dye was used to analyze genome integrity under fluorescent microscope. According to the results of MTT cell viability assay, IC50 concentration of nicotine was determined as 1.71 mM. Different concentration of BA (0.625 µg/ml to 20 µg/ml) were applied into cultured HPAEpiC cells with nicotine to prevent cellular damages by nicotine. MTT and LDH assays clearly showed that 5 µg/ml of BA supplementation increased cell viability against nicotine exposure. Again, Hoechst 33258 test revealed that chromosome structure was preserved significantly after BA exposure. As a conclusion, our results revealed for the first time that BA could be used as a protective agent against nicotine-induced toxicity on human lung alveolar cells.

1. Introduction

Today, cigarette smoking and health risks associations are well known. Bronchogenic carcinoma development is one of the most important side effects of smoking. Also, pulmonary diseases as centrilobular and panacinar emphysema, pulmonary Langerhans cell histiocytosis and chronic bronchitis are correspondent to cigarette smoking [1]. Furthermore, there are various important functions of alveolar epithelial cells such as regulating inflammation via releasing growth factors and cytokines, alveolar structure constitution with proteinases and matrix proteins, and prevent external toxic molecules to damage lung components. Alveolar cell damages can cause several consequences such as alveolar structure demolition resulted from growth factors and cytokines insufficiency and, alveoli destruction induced by increased permeability of epithelial [2–5]consisting of 90% lipid and 10% protein, and of calf lung surfactant extract (CLSE. Cigarette smoke can damage epithelial cell in different ways; (I) prevent collagen production and surfactant synthesis, (II) suppression of cell proliferation and attachments and (III) increase single strand breaks in DNA which are lead lung diseases in wide spectrum aspect [6,7].

Tobacco products contain 2000 different potential toxic molecules and one of the most cytotoxic molecules is nicotine which was found very high concentration in saliva and teeth root surface of tobacco addicts [8,9]. Moreover, analysis showed that nicotine can affect many cellular pathways. According to researches fibroblasts could collect nicotine molecules in high levels and the cells keep most of them inside of different compartments [10]. Most important effects of nicotine on the fibroblast were cell attachment and growth disfi-

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guration which are crucial events for cell functions and metabolisms to survive [11]. Besides, thiol depletion was analyzed to have an important role in nicotine-induced cytotoxicity. Researches indicated that inhibition of thiol activity could cause susceptibility in cells against reactive agents which resulted in prevention of protein synthesis, growth and proliferation of fibroblasts [12].

Boron compounds are abundant in earth surface and have an important role in plant growth. Also, deficiency of boron can affect human health adversely including brain function, plasma lipid level, arthritis and osteoporosis. Boron is generally found as boric acid (BA) form in environment and BA can be used in variety of industrial product such as cosmetics, textile, fiberglass, jewelry, glass, electroplating, furnace linings and ceramics [13–15]. Likewise, analysis on human blood cells exhibits that BA has non-genotoxic properties. Thus BA is reported that it can be used in pharmacological and medical applications due to its antioxidant feature [16,17].

In the present study, the protective effect of BA was tested against nicotine induced cytotoxicity in the human primary epithelial cells. Firstly, toxic concentrations of nicotine compound were determined via using MTT assay. After toxic environment (IC50) being created for the cells, wide range of BA was applied to find out protective potential. MTT and LDH assays were used to analyze cell viability for BA exposure against nicotine-induced toxicity. In addition, nucleus integrity of HPAEpiC was investigated by using Hoechst 33258 (bis-Benzimide) fluorescent dye.

2. Material and methods

Cell culture

Human Pulmonary Alveolar Epithelial Cells (HPAEpiC) was obtained from American Type Culture Collection (ATCC, USA). The cells were grown and maintained in AEpiCM consists of 500 ml of basal medium, 10 ml of fetal bovine serum, 5 ml of epithelial cell growth supplement and 5 ml of penicillin-streptomycin at 37°C in a 90% humidified incubator with 5% CO_2.

Determination of toxic nicotine concentration

$2x10^4$ cells/well were seeded in 96-well plate and incubated at 37 °C with 5% CO_2 for 24 h to allow cell attachment. Then, the cells were incubated with 20, 10, 5, 2.5, 1.25, 0.612, 0.362 mM of nicotine hydrogen tartrate (Sigma®, USA). After 48 h of incubation, IC_{50} values of nicotine were determined by MTT assay. Briefly, 10 µl of MTT (5 mg/ml MTT in PBS) was added to each well and incubated for 4 h. Then, 100 µl of DMSO was added to each well to dissolve any formazan crystals formed and the optical density was measured at 570 nm by a microplate reader.

MTT assay

$2x10^4$ cells/well were seeded in 96-well plate and incubated at 37 °C with 5% CO_2 for 24 h to allow cell attachment. Then, the cells were incubated with 1.71 mM (IC_{50} value) of nicotine and six different concentrations of boric acid (20; 10; 5; 2.5; 1.25 and 0.625 mg/ml). After 48 h of incubation, cell viability was determined by MTT assay that is mentioned previously.

LDH cytotoxicity assay

$2x10^4$ cells/well were seeded in 96-well plate and incubated at 37 °C with 5% CO_2 for 24 h to allow cell attachment. Then, the cells were incubated with 1.71 mM of nicotine hydrogen tartrate and six different concentrations of boric acid (20; 10; 5; 2.5; 1.25 and 0.625 mg/ml). After 48 h of incubation, LDH assay was performed using CytoSelect™ LDH Cytotoxicity Assay kit according to the manufacturer's instructions. 90 µl of the supernatant from each well was transferred to a new plate. Afterwards, 10 µl of LDH Cytotoxicity Assay reagent was added into each well and incubated at 37 °C for 30 minutes. The absorbance was determined at 450 nm by a microplate reader.

Hoechst 33258 assay

After protective concentration of BA was determined against Nicotine induced cytotoxicity, positive control, negative control and BA/nicotine treated cells were fixed with 4% paraformaldehyde in phosphate buffered saline at 4 °C for 30 min. After washing cells with PBS, the nuclear DNAs were incubate with 1 mM Hoechst 33258 fluorescent dye for 5 min at room temperature (excitation/emission wavelength=365/420 nm) and observed under the fluorescent microscope (Leica® DM IL LED).

Statistical analysis

Statistical analysis was performed using Excel (Microsoft©, USA, 2010) software. $P<0.05$ was considered to represent statistically significant result.

3. Results and discussions

Recently, it is investigated that nicotine can induce oxidative stress in U937 monocytic cells via activating cytochrome P450 (CYP) 2A6-mediated pathway [18]. In addition to oxidative stress induction which interfere with redox homeostasis, cigarette smoke can cause carcinogenicity, upregulation of pro-inflammatory gene expressions and downregulation of antioxidant gene expressions [19–22]. In this research, we show that nicotine molecules can cause high toxicity on HPAEpiC cells and over 10 mM concentration of nicotine can induce nearly % 100 cytotoxicity by using MTT assay (Figure 1). According to MTT cell viability assay IC_{50} value for nicotine on lung epithelial cells calculated from regression analysis as 1.71 mM which is shown in Figure 2.

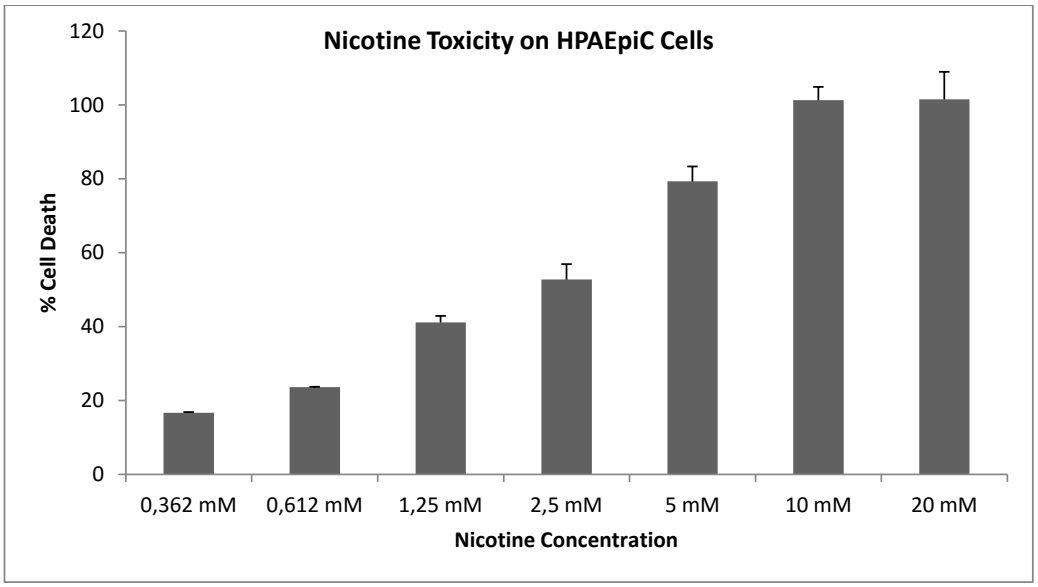

Figure 1. The cytotoxic effects of nicotine application on HPAEpiC cultures via using MTT cell viability assay (IC_{50}= 1.71 mM)

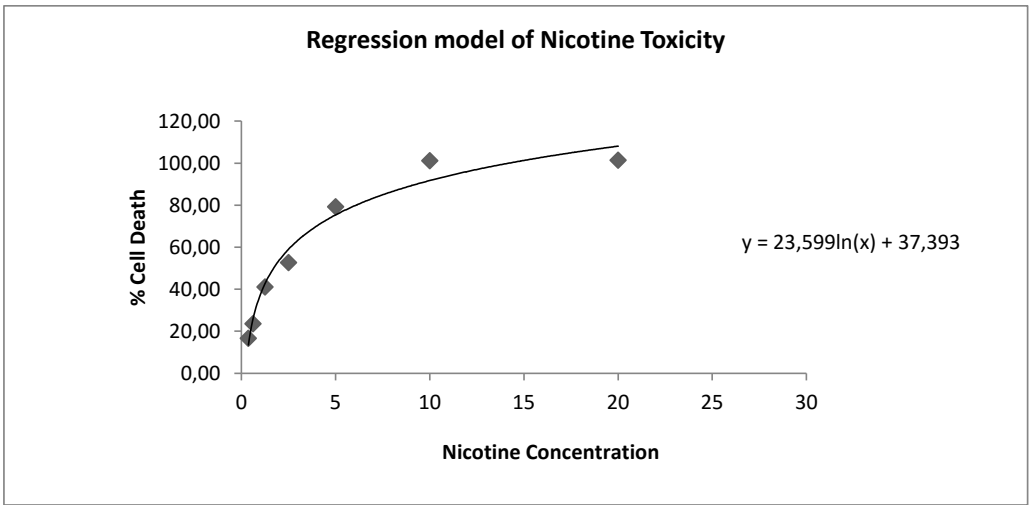

Figure 2. Regression model for nicotine induced cytotoxicity (Graphic equation is y=23,599ln(x)+37,393 and IC_{50}=1,71 mM)

After IC_{50} value of nicotine molecule was found out, different concentrations of BA were applied against 1.71 mM nicotine. In Figure 3, higher and lower concentrations (≥5 µg/ml and ≤2.5 µg/ml) of BA didn't show any ameliorative effect against nicotine-induced cytotoxicity. On the other hand, 5 µg/ml and 2.5 µg/ml concentrations of BA significantly decrease toxic effect of nicotine. These results correlated via using both MTT and LDH viability assays, which indicated complementary results in Figure 3. Since, nicotine application could lead to production of reactive oxygen species and oxidative stress [23], one of the possible mechanism underlying the protective effect might be antioxidant activity of BA molecules [16,17]sister-chromatid exchanges (SCEs. Furthermore, researches demonstrated that DNA double strand breaks reduced and also, wound healing process improved when BA applied to cell culture [24].

In Figure 4a, Hoechst 33258 (bis-Benzimide) fluorescent staining analysis exhibited that genome integrity of the HPAEpiC cells were disturbed and cell density was decreased after nicotine exposure. According to studies, nicotine could activate GRP78 and NF-κB through oxidative stress induction. Thus, production of reactive molecules could increase apoptosis and genotoxic sensitivity in cancer cells [25–27]. Moreover, micronuclei frequency and DNA single strand breaks was shown to increase significantly peripheral blood of rats after nicotine treatment [28,29]. On the contrary, application of BA (5 µg/ml concentration) against nicotine exposure shown to protect genome constituent and decrease cytotoxicity significantly (Figure 4b). Supporting to these, scientists suggested that BA treatment could prevent micronuclei and DNA strand breaks inductions in V79 cell cultures [29].

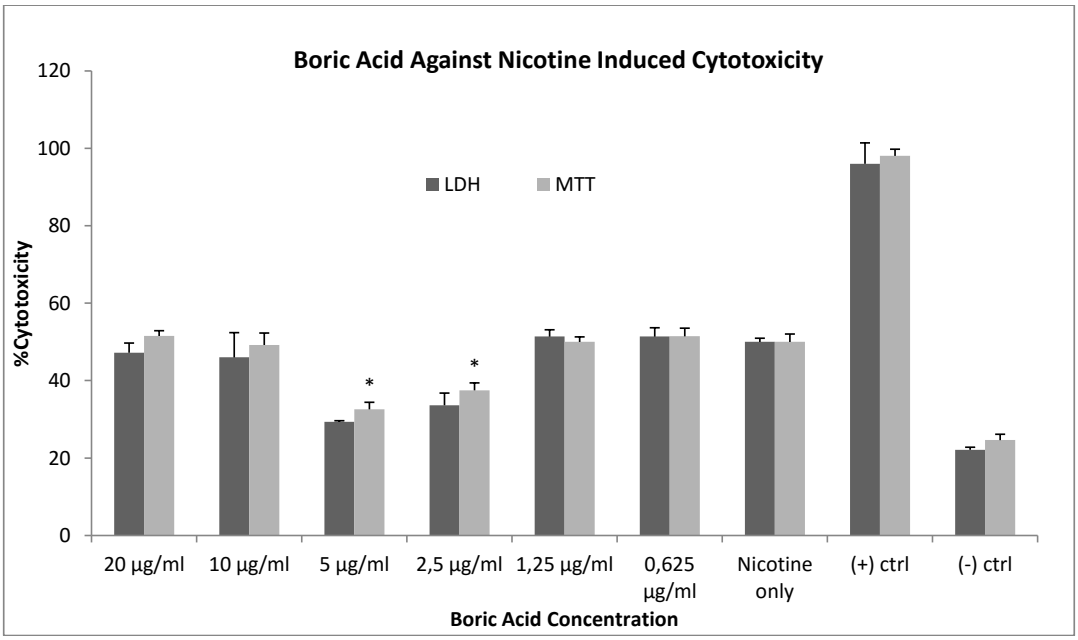

Figure 3. MTT and LDH viability test results after boric acid application to nicotine induced cytotoxic cells. Symbol (*) indicates significantly decrease of % cytotoxicity against nicotine only samples

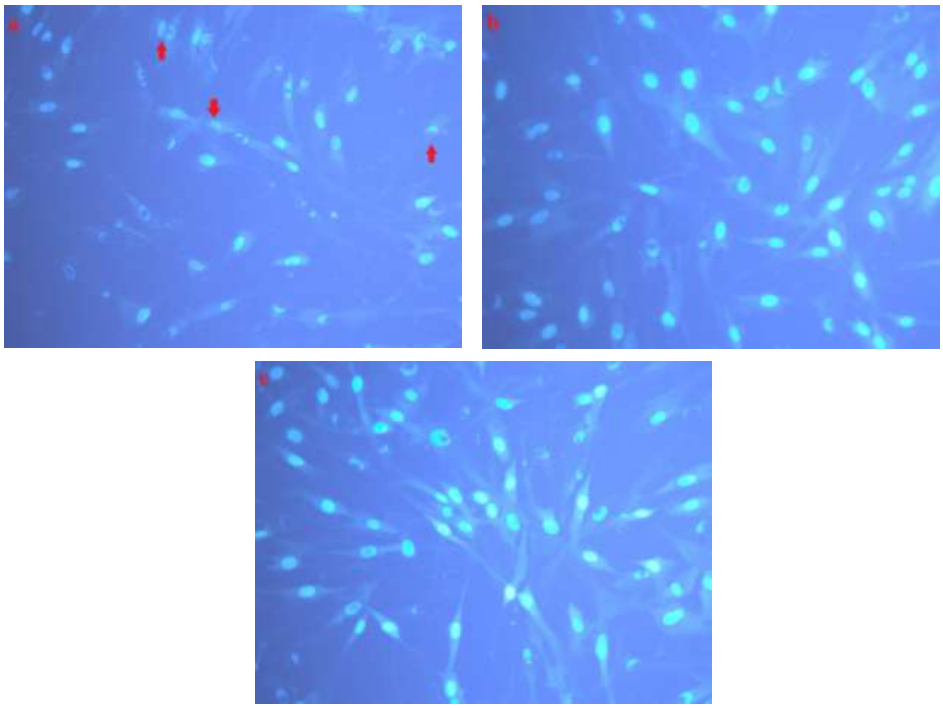

Figure 4. Fluorescent microscope image of Hoechst 33258 stained HPAEpiC cells. a- Nicotine (1.71 mM) only treated cells (Red arrow indicates some of the degenerated cells), b- Nicotine (1.71 mM) + BA (5 µg/ml) treated cells, and c- Negative (-) control

4. Concluding remarks

In the present study, high toxicity of nicotine on HPA-EpiC cells were investigated via MTT and Hoechst 33258 assays. It could be interpreted that nicotine induced cytotoxicity and genome destruction were reduced significantly through BA treatment (Figure 3 and 4). According to MTT and LDH cell viability tests, 5 µg/ml concentration of BA was effective quantity for reducing toxicity by nicotine. In Hoechst 33258 assay, BA treated cells with nicotine (Figure 4b) and negative (-) control group cells were seemed similar in aspect of genome integrity and cell density which correlated to cell viability tests. As a conclusion it might be said that antioxidant properties of BA prevent cytotoxicity and DNA damage resulted from nicotine treatment [30]. In a conclusion, our results supported for the first time that BA has protective effects against nicotine induced cytotoxicity on the human primary alveolar epithelial cells.

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