ORIGINAL ARTICLE

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Protective effect of Petroselinum crispum (parsley) extract on alveolar stage of rat lung development after perinatal nicotine exposure

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SUMMARY

Nicotine exposure during pregnancy is linked to multiple obstetrical, fetal, and developmental complications. Parsley (Petroselinum crispum) is an aromatic herb, which has well-known potent anti-inflammatory and anti-oxidative effects. The purpose of this study was to investigate, for the first time, the protective effect of parsley extract on alveolar stage of lung development in rats exposed to perinatal nicotine. Thirty Sprague-Dawley adult female rats were randomly divided into five main groups after being pregnant: control, sham control, parsley-treated (5mg /kg/day), nicotine-treated group (1mg /kg/day), and protected (nicotine + parsley extract) groups. Nicotine was injected subcutaneously, while parsley extract was given orally by gastric tube from the 7th day of pregnancy until the 21st day postnatally. At the end of the experiment, lungs of 21-day-old male offspring were subjected to biochemical, histological, and immunohistochemical analyses. Our results revealed toxic effects of nicotine on alveolar stage of rat lung development. These were indicated by histopathological alterations, including poorly developed primary and secondary septa; interstitial tissue infiltration with inflammatory cells, atypical features appeared in some cells of bronchioles and blood vessels. In addition, a reduction in elastic fibers contents and in alpha smooth muscle expression, an increase in surfactant protein B expression, and changes of oxidative stress indices and tumor necrosis factor alpha level in lung tissue were detected. Co-administration of parsley extract ameliorated nicotine-induced toxic alterations on the development of the lung. Therefore, parsley can be a promising candidate for the prevention of nicotine-induced toxicity in the developing lung.

Key words: Antioxidant – Secondary septation – Alveolar myofibroblast – Lung – Nicotine

INTRODUCTION

Nicotine exposure during pregnancy continues to be a major global public health concern, and is an important risk factor for poor maternal and infant health outcomes. It may cause miscarriage, ectopic pregnancy, antepartum hemorrhage, increased risk of perinatal mortality, preterm delivery, and fetal growth restriction (Huihuang et al., 2017) with

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increased incidence of lifelong respiratory infections and asthma (Mcevoy and Spindel, 2017).

Fetal lung development is greatly disturbed by nicotine exposure, leading to retardation of its maturation (Banderali et al.,2015). These adverse effects are owed to its ability to create an environment of oxidant-antioxidant imbalance by increasing the reactive oxygen species [ROS] and reducing the antioxidant capacity (Martiz, 2013). In addition, nicotine induces inflammatory effects on the fetal lung and significantly increased circulating serum pro-inflammatory cytokines, as tumor necrosis factor alpha (TNF-α) in rodents after prenatal exposure (Wong et al., 2015).

Petroselinum crispum (parsley) is a bright green herb, which belongs to the family Apiaceae that originates from the central Mediterranean regions, especially southern Italy, Algeria, Tunisia and Egypt (Awe and Banjoko, 2013). It has a wide range of pharmacological activities including antidiabetic, analgesic, laxative, estrogenic, diuretic, hypotensive, and antibacterial activities (AbdRabou and Eid, 2017). It contains many important active ingredients, such as cumarins, vitamin C and flavonoids, mainly luteolin, apigenin and its glycosides. These flavonoids have anti-inflammatory and antioxidant activities that combat the inflammatory and oxidative imbalance caused by nicotine (Papay et al., 2012).

Despite the fact that the anti-inflammatory and antioxidant effects of parsley are well-established, its ability to ameliorate nicotine toxicity have not been evaluated before. Therefore, the main aim of this study was to evaluate, for the first time, the possible protective effects of parsley extract on nicotine-induced lung toxicity.

MATERIALS AND METHODS

Animals and experiment design

This study was carried out on thirty Sprague-Dawley adult female rats aged 3 months old. They were obtained from the Theodore Bilharz Research Institute, Egypt. The animals were maintained in the animal house of the Faculty of Medicine, Menoufia University, Egypt. The rats were subjected to a 12:12-h daylight/darkness and allowed unlimited access to chow and water. The procedure was approved by the ethics committee for animal experimentation of the Faculty of Medicine, Menoufia University, Egypt, in accordance with the international regulation on care and use of laboratory animals.

The female rats were kept overnight with adult male rats. Vaginal smears were taken every day; the day in which the smear was sperm positive, it was designated as day 0 of gestation (Mohsenzadeh et al., 2014).They were allowed to acclimatize until the 6th day of the gestational period. On the 7th day, the pregnant rats were classified into five groups (n=6) as follows: control, sham control, parsley-treated, nicotine-treated, and parsley + nicotine-treated groups. Control group was kept without any treatment all through the experiment, while sham control was injected with 1ml distilled water subcutaneously daily. Parsley group received parsley extract orally by a gastric tube at a dose of 5 mg/kg/day (Rezdad and Farokhi, 2014), and nicotine group was injected subcutaneously with nicotine at a dose of 1mg/Kg/day (Adeyemi, 2017).Treatment continued from 7th day of pregnancy until the 21st day postnatally. Male offspring (n=5) from each group were sacrificed at 21st day postnatally.

Chemicals

Parsley seeds were brought from local market in Shebein El-Kom, Menoufia. They were identified in the Faculty of Agriculture, Menoufia University. About one hundred grams of powdered seeds of parsley were mixed with 750 ml of 70% ethanol for 72 hours at room temperature in an air tight container. Then, it was filtered through filter paper. The filtrate was evaporated using a rotary evaporator and the resulted material was kept in the refrigerator for usage (Jassim, 2013). Nicotine (a vial of 1ml) was purchased from Sigma Aldrich Chemical Co. Mouse monoclonal anti-alpha smooth muscle actin (α-SMA) (Dako Cytomation, glostrup, Denmark, Cat # M0851) and rabbit polyclonal antibody surfactant protein B (SP-B) (biorbyt Cat # orb 11406) were purchased for immunohistochemical studies. Rat glutathione peroxidase (GPx) ELISA kit and rat malondialdehyde (MDA) ELISA kit (MyBiosource, SanDeigo , CA , USA, Catalog # MBS 727547 & MBS 046356 respectively), and tumor necrosis factor- alpha (TNF-α) ELISA kit (Sigma Aldrich, Catalog # RAB0480) were purchased to assess oxidative stress and inflammatory status.

Histological and immunohistochemical studies

At the end of the experiment, 21 day old male offspring (n=5) from each group were anaesthetized using diethyl ether inhalation, then sacrificed, and thorax was opened. The two lungs were extracted, one of them was preserved by injection of 6 ml of 10% neutral buffered formalin through the trachea and then it was stored in 10% neutral formalin solution; this one was processed to paraffin blocks for histological and immunohistochemical studies, sections were dehydrated using ethanol, stained with hematoxylin & eosin and orcein stain . For immunohistological staining, paraffin sections (5 µm thick) were deparaffinized in xylene, subjected to immunohistochemical studies α-SMA and SP-B.

The other lung was cut into two pieces: one piece was kept in glutaraldehyde solution, processed to polythene beam capsule that was polymerized into blocks, these blocks were trimmed and cut into semithin sections by using ultramicrotome,

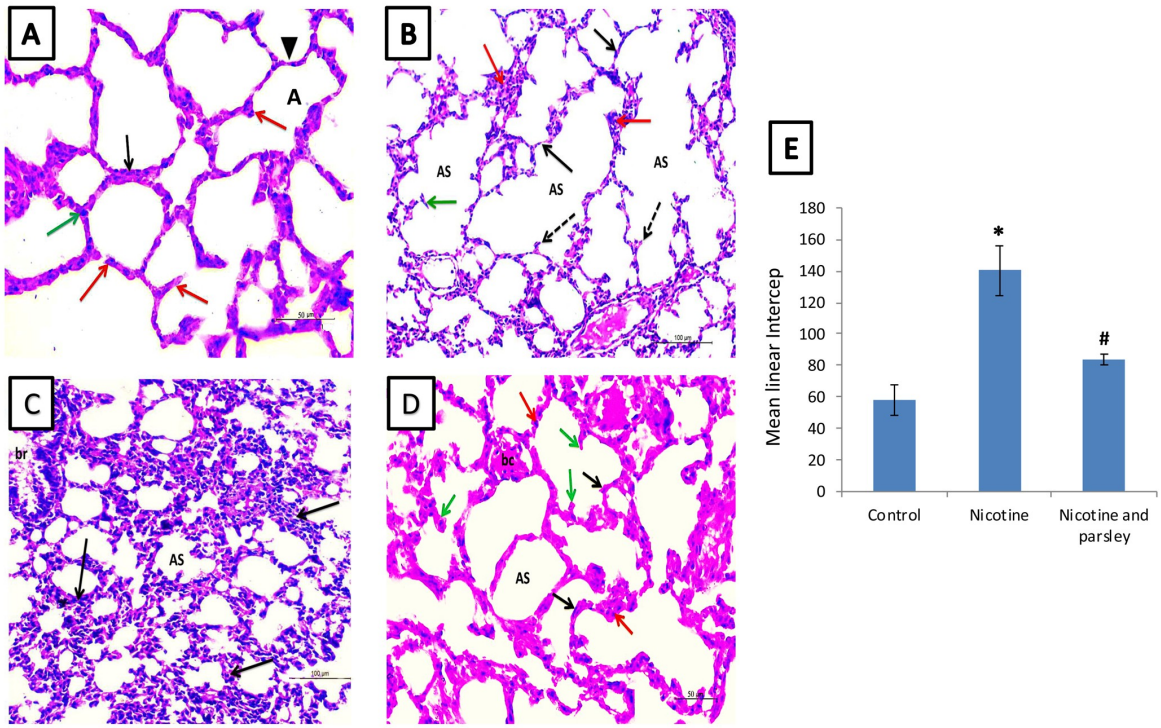

Fig 1. Representative hematoxylin and eosin (H&E) staining (**A-D**) and mean linear intercept (**E**) of lungs from the control (**A**), nicotine-treated group (**B & C**), and protected group (**D**). *P<0.05, compared with the control; and #P< 0.05, compared with the nicotine group. Data are expressed as means ± SEM. Scale bar = 50 µm (**A, C**) and 100 µm (**B, D**).

stained with toluidine blue then examined under the light microscope; the other was put in a foil paper and freezed at -20°C for biochemical study.

Quantitative assessments

For histological and immunohistochemical quantitative assessment, five non-overlapping fields (400×) per section were randomly captured by a Leica Microscope DML B2/11888111 equipped with a Leica camera DFC450. The mean linear intercept, surface area percentage of elastic fibers, and the percentage of immune-positive cells in the fields taken from at least three sections/animal were measured using image J software (Maryland, USA) and averaged per field for each animal. The numbers obtained from at least five animals/ experimental group were considered for comparison and statistical analyses.

Statistical analysis

Results were expressed as mean ± SEM. One way-ANOVA followed by a post hoc Bonferroni test was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA. The level of significance of P ≤ 0.05 was considered to be statistically significant.

RESULTS

Parsley ameliorated histopathological changes in nicotine-induced lung toxicity

H&E-stained lung sections from offspring of control and parsley groups showed the same picture of normal architecture of lung parenchyma with multiple air spaces separated by thin well-developed primary septa (arrow head, Fig. 1A). Multiple developing secondary septa projected into these air spaces, dividing them into smaller alveolar spaces (red arrows, Fig. 1A). Type I pneumocyte with a flat nucleus (black arrow, Fig. 1A) and type II pneumocyte with rounded nucleus (green arrow, Fig. 1A) were found. Bronchioles were free of secretions, having folded mucosa lined with simple cuboidal epithelium (double head black arrow, Fig. 2A), musculosa with regularly arranged muscle fibers having characteristic spindle-shaped nuclei (double head red arrow, Fig. 2A) and adventitia. Blood vessels showed normal endothelial lining, tunica media and adventitia (black, red, and green arrows, respectively; Fig. 2A).

H&E-stained lung sections from offspring of nicotine-treated group showed disturbance in the architecture of lung parenchyma with multiple irregular air spaces(AS Fig. 1B), separated by thin poorly-developed primary septa (black arrows, Fig. 1B)

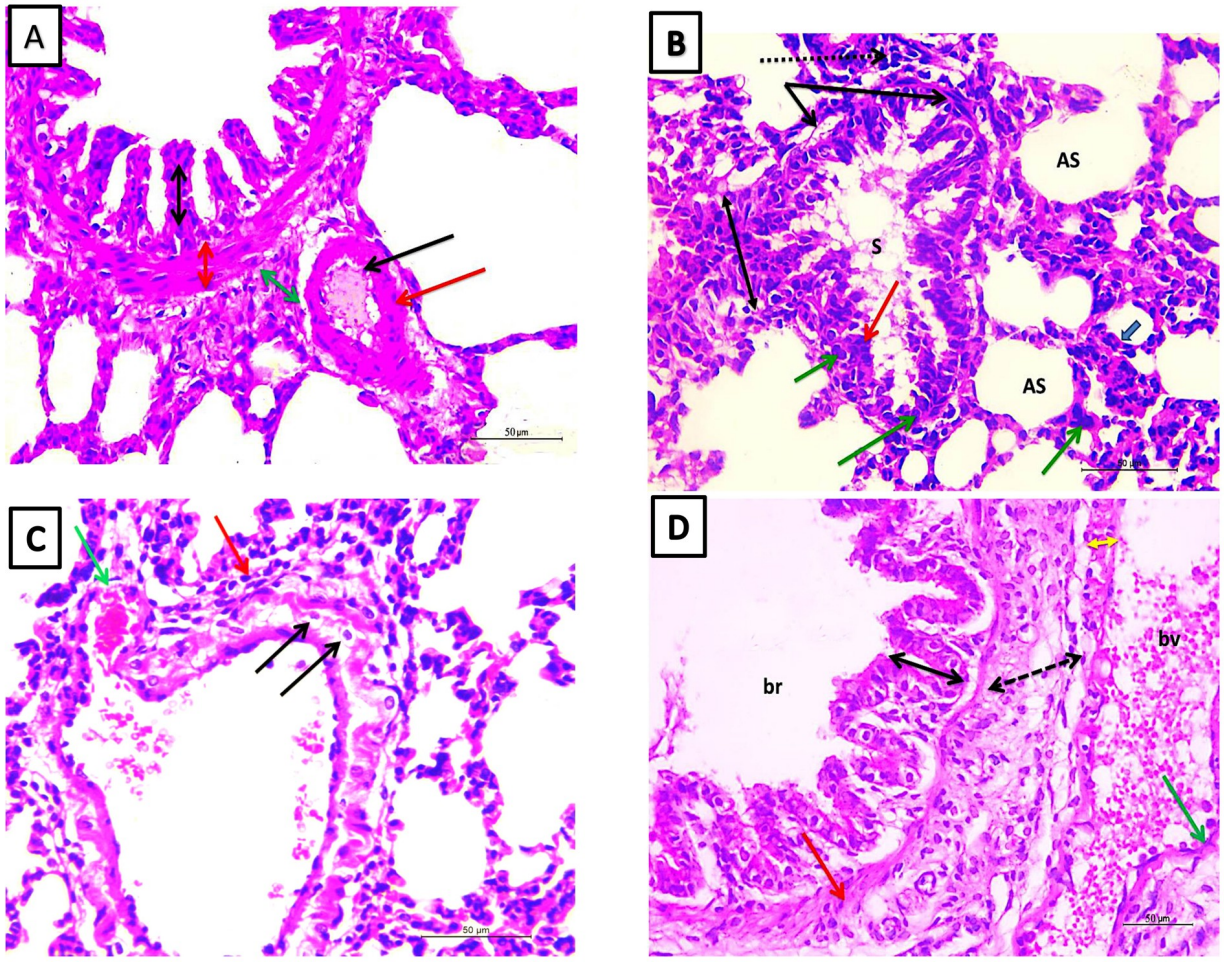

Fig 2. Representative hematoxylin and eosin (H&E) staining of lungs from the control (**A**), nicotine-treated group (**B and C**), and protected-group (**D**). Scale bar = 50 µm.

and crested weak irregular poorly-developed secondary septa (green and dashed arrows, Fig. 1B). The interstitial tissue was infiltrated with multiple inflammatory cells (red arrows, Fig. 1B and black arrows, Fig. 1C). The bronchiole was full of secretions, and had a disorganized lining epithelium (red arrow, Fig. 2B); some of these lining cells showed mild atypia with increased nucleo-cytoplasmic ratio and hyperchromatism (green arrows, Fig. 2B). In addition, muscle layer was poorly-developed with peribronchiolar lymphocytic aggregation (dashed arrow, Fig. 2B). The blood vessel wall also showed complete distortion with intimal injury (green arrow, Fig. 2C) and a peri-vascular infiltration with inflammatory cells (red arrow, Fig. 2C). The media of the blood vessel also showed disarrangement with pyknotic nuclei (black arrow, Fig. 2C). On the other hand, parsley extract co-treatment showed a considerable improvement in architecture of lung parenchyma with significant decrease in mean linear intercept (the average distance between the walls of an alveolus that is considered as an index of alveolar wall size) (Fig.1E). Almost normal well-developed secondary septa compared to nicotine-treated group were observed (green arrow, Fig. 1D), with nearly normal type I pneumocyte with a flat nucleus (black arrow, Fig. 1D) and type II pneumocyte with a rounded nucleus (red arrow, Fig. 1D). pneumocyte with a rounded nucleus.

In control group, orcein-stained sections showed considerable amount of elastic fibers deposited in primary septa (black arrows, Fig. 3A) and at the tips of the developing secondary septa (red arrows, Fig. 3A), while there was a significant decrease in amount of these fibers in nicotine-treated group (black arrows, Fig. 3B). Parsley co-treatment exerted a significant increase in their amount compared to nicotine-treated group (black and red arrows; Fig. 3C).

Parsley attenuated the increase in type II pneumocytes and inflammatory infiltrate in nicotine-induced lung toxicity

In control group, alveolar spaces were lined by type I pneumocyte with a flat nucleus (black arrows, Fig. 4A), type II pneumocyte with rounded nucleus and vacuolated cytoplasm (red arrows, Fig. 4A), with some secondary septa projecting into alveolar lumen. Nicotine-treated group showed increase in number of type II pneumocyte (black arrow, Fig. 4B) with some detached cells inside alveolar lumen (red arrow, Fig. 4B). Interstitial tis-

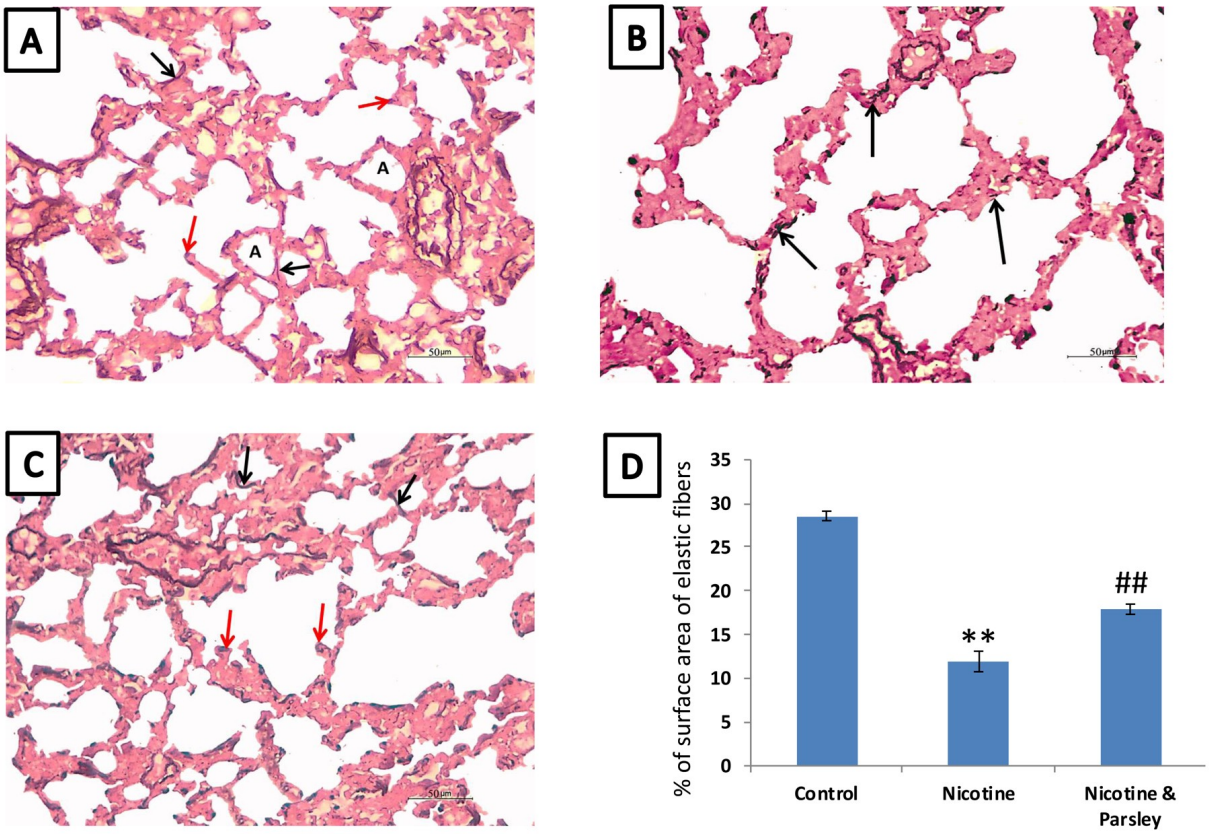

Fig 3. Representative orcein staining (**A-C**) and surface area percentage of elastic fibers (**D**) of lungs from the control (**A**), nicotine treated (**B**), and protected-group (**C**). **P<0.001, compared with the control; and ##P< 0.001, compared with the nicotine group. Data are expressed as means ± SEM. Scale bar = 50 µm.

sue showed multiple inflammatory cellular infiltrations. Interalveolar septa was overcrowded by inflammatory cells with variable shaped nuclei (black arrow, Fig. 4C). Parsley co-treatment showed significant decrease in number of type II cells and inflammatory cells compared to nicotine-treated group (red and black, Fig. 4D).

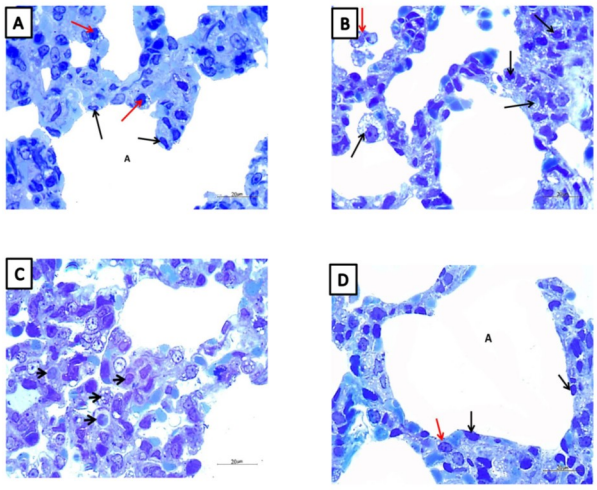

Fig 4. Representative toluidine blue-staining from lungs of the control group (A), nicotine-treated group (B-C), and protected-group (D). Scale bar = 20 µm.

Parsley up-regulatedα-SMA and down-regulated SP-B expression in nicotine-induced lung toxicity

There was a significant decrease in α-SMA expression in nicotine-treated group compared to control group (Figs. 5A, B, and D; P < 0.01). Parsley extract co-treatment significantly upregulated nicotine-induced decrease in α-SMA expression (Figs. 5B-D). Nicotine-treated group revealed a significant up-regulation in SP-B expression compared to control group (Fig. 6A, B-D; P < 0.01). This upregulation was significantly downregulated in parsley + nicotine-treated group (Fig. 6A, B-D; P < 0.05).

Parsley reduced oxidative stress and TNF-α level in nicotine-induced lung toxicity

To evaluate whether oxidative stress played a role in the pathogenesis of nicotine-induced toxic effects, we measured levels of MDA and GPx in lung tissue and we found that parsley group showed no significant difference in their levels when compared to the control group, while nicotine -treated group showed significant increase in lung MDA level (Fig . 7; P<0.01) and significant decrease in lung GPx level when compared to the control group (Fig. 7; P<0.01). Their levels returned nearly to normal values after co-administration of parsley (Fig. 7; P<0.01). In addition, level of TNF-α

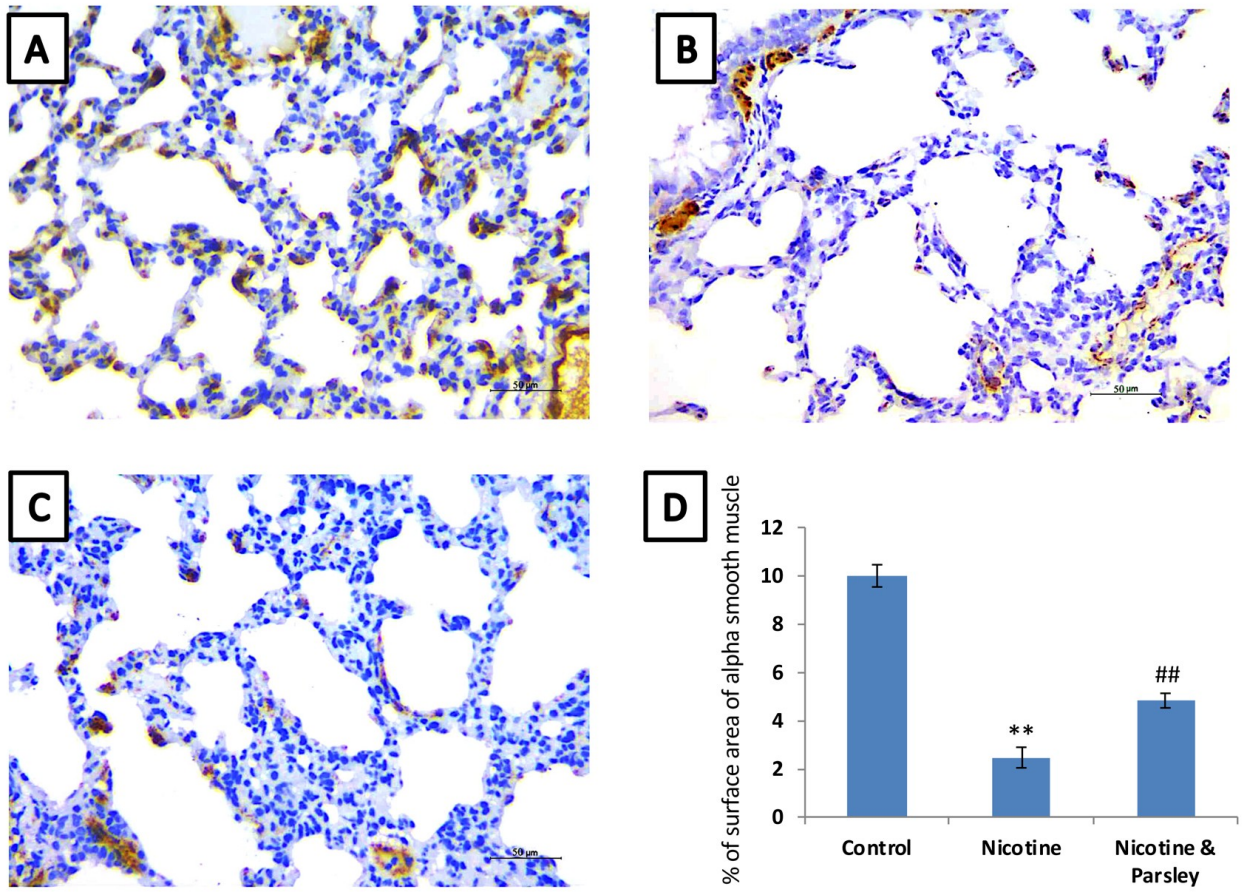

Fig 5. Expression of α-SMA in the lungs of the control (**A**), nicotine-treated group (**B**), and protected group (**C**). **P<0.001, compared with the control group; and ##P<0.001, compared with the nicotine group. Data are expressed as means ± SEM. Scale bar = 50 µm.

showed no significant difference in parsley group when compared to control group, while nicotine-treated group showed significant increase when compared to control group (Fig. 8; P< 0.01). Its level returned nearly to normal values after co-administration of parsley extract (Fig. 8; P<0.01).

DISCUSSION

Multiple studies have revealed that tobacco is the most commonly used drug during pregnancy and, despite of its harmful effects on various body systems, this habit still remains a public health concern worldwide (Adeyemi, 2017). There was considerable evidence that increased oxidative stress might be a potential mechanism underlying nicotine toxic effects (Conceicao et al., 2015). This directs the attention to the role of antioxidants, such as flavonoids, as a potential candidate in reducing the health risk of tobacco consumption (Mohsenzadeh et al., 2014).

The selected nicotine dose in the current study was based on previous ones (Sakurai et al., 2016): this dose corresponds to the range of nicotine intake by habitual smokers. In our study, nicotine greatly affected alveolar stage of rat lung development indicated by H&E-stained lung sections of offspring of nicotine-treated group. The lungs of this group displayed irregular air spaces (alveoli), weak irregular poorly developed and destructed secondary septa. These findings were in agreement with Ibrahim and Selim (2012) who reported widening in alveoli and thinning of interalveolar septa in the offspring of 21 -day-old rat exposed to perinatal nicotine. Nicotine-induced inhibition of secondary septation could be explained by inhibition of elastic fiber formation (Martiz, 2008). Our results obtained from orcein staining showed a dramatic decrease in elastic fiber content of lungs of nicotine group when compared to the control group. Therefore, it could be postulated that the observed failure of normal process of secondary septation and alveolar development was due to a defect in the elastic fiber formation.

The lung elastic fibers were formed by alveolar myofibroblasts, which express α-SMA protein. Our results revealed a significant reduction in the number of α-SMA positive cells in the lungs of nicotine-treated group. The dramatic decrease in the number of these cells could be explained by the toxic effect of nicotine, which may be direct or secondary to nicotine-induced oxidative stress in lung tissue. Therefore, inhibition of alveolar myofibroblast by toxic effect of nicotine led to decrease in

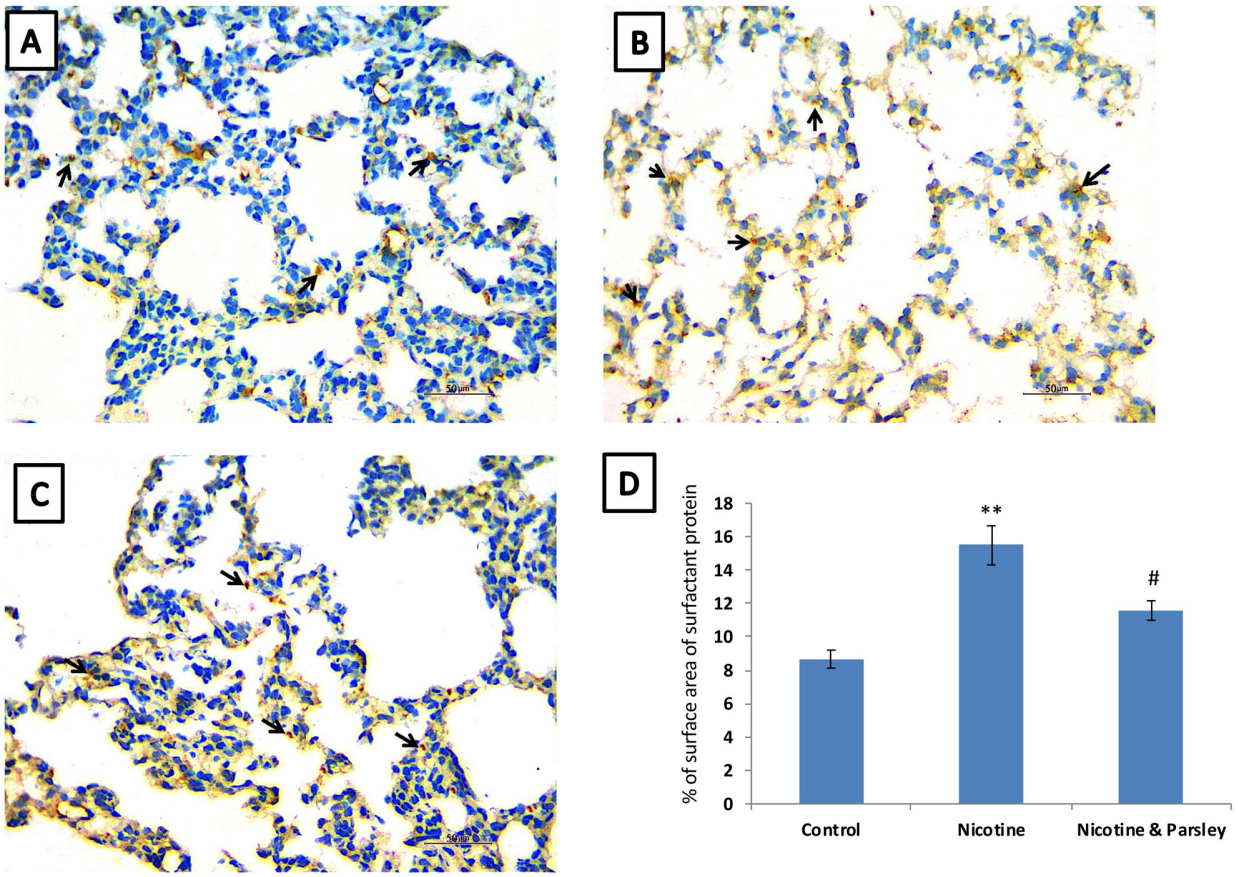

Fig 6. Expression of SP-B in the lungs of the control (**A**), nicotine-treated group (**B**), protected group (**C**). **P<0.001 compared with the control; #P<0.05 compared with the nicotine group. Data are expressed as means ± SEM. Scale bar = 50 µm.

elastic fibers formation and subsequent inhibition of the process of secondary septation.

In nicotine-treated lungs, histological investigations showed multiple inflammatory cells infiltrating the interstitial tissue. This could be explained by nicotinic toxic and destructive effects on lung tissue, which induce inflammatory reaction in lung with recruitment of multiple inflammatory cells. This finding was in agreement with Wong et al. (2015) who demonstrated that fetal exposure to nicotine was associated with increased inflammation in the offspring. This histological finding was further supported by a marked increase in TNF-α level in the lung tissue of nicotine-treated group when compared to the control group.

Our results also revealed hyperchromatism and increased nucleo-cytoplasmic ratio in some cells of nicotine-treated group which indicated a toxic effect of nicotine on the normal cell growth. Similar findings of nicotine on cell growth were reported in breast epithelium by Phaniendra et al. (2015) who found that damage to the breast epithelium by reactive oxygen species (ROS) led to hyperplasia of the epithelium, cellular atypia and breast cancer. Moreover, blood vessel walls showed complete distortion and a perivascular infiltration with inflammatory cellsPrevious studies showed that nicotine toxicity was associated with loss of lung endothelial barrier function and acute lung inflammatory reaction (Schweitzer et al. 2015).

Furthermore, the nicotine-treated group showed a significant increase in type II pneumocytes compared to type I pneumocytes. This proliferation might be a response to type I cell injury, as one of the functions of type II alveolar epithelial cells is to proliferate and differentiate to replace damaged type I cells. Previous studies showed similar findings (El-Aasar et al., 2007; Ibrahim and Selim, 2012). Moreover, this proliferation was associated with significant increase in SP-B expression as indicated by immunohistochemical examination. This finding was in agreement with Rehan et al. (2007) who demonstrated that in utero nicotine exposure significantly increased SP-B protein level in the cultured alveolar type II cells.

We also found a marked increase in MDA level and marked decrease in GPx level in lung tissue of nicotine-treated group. Therefore, we could conclude that oxidative distress was one of the underlying mechanisms underpinning the observed histopathological alterations in nicotine-treated group. Banderali et al. (2015) reported that in utero nicotine exposure increased oxidative stress in

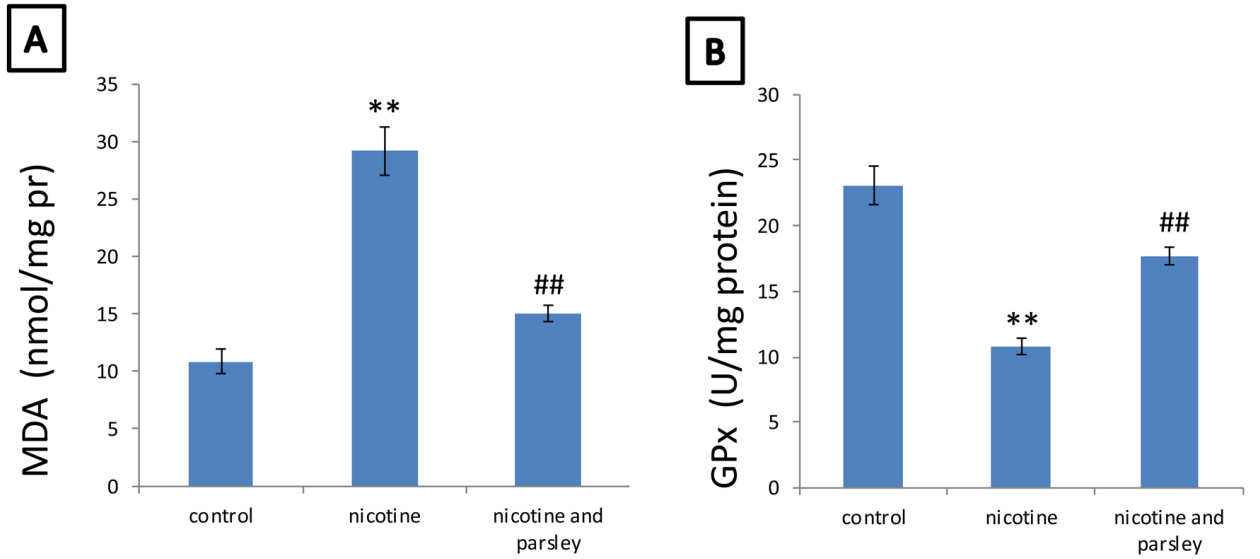

Fig 7. Status of malondialdehyde (MDA; **A**) and glutathione peroxidase (GPx; **B**) in the lungs of control, nicotine-treated group, and protected group. **P<0.001, compared with the control; and ##P< 0.001, compared with the nicotine group. Data are expressed as means ± SEM.

lungs with a consequent reduced alveolarization and impaired lung development.

Administration of Petroselinum crispum (parsley) extract along with nicotine remarkably ameliorated the histopathological alterations induced by nicotine, as indicated by hematoxyline and eosin results that revealed almost nearly normal appearance of lung tissue with development of secondary septa. In addition, there was an improvement in the amount of elastic fibers in protected group when compared to nicotine-treated group. Moreover, a significant improvement in other examined parameters, such as α -SMA and SP-B expression when compared to nicotine-treated group. These results indicated that parsley exerted a protective effect against nicotine-induced toxicity. This might be explained by high contents of antioxidant and anti-inflammatory substances in petroselinum crispum that antagonized the toxic effects of nicotine. Our results revealed that MDA, GPx , and TNF -α levels returned to almost normal range in parsley-protected group, indicating that parsley induced an anti-oxidant and anti-inflammatory effects on fetal lungs exposed to nicotine. Petroselinum crispum was effective in reducing stress -induced gastric oxidative damage (Akinci et al., 2017). Its antioxidant properties protected against DNA damage and inhibited proliferation and migration of cancer cells (Tang et al., 2015). The anti-inflammatory effects of parsley could be explained by either inhibiting the synthesis or release of inflammatory mediators (Al-khazraji, 2015).

Lung development is characterized by rapid cell proliferation and sensitivity of the cellular DNA to toxic oxidant effects. Therefore, nicotine gestational exposure can lead to lung pathology in the offspring on long-term. Our results showed that parsley exerted a protective effect on the lungs of

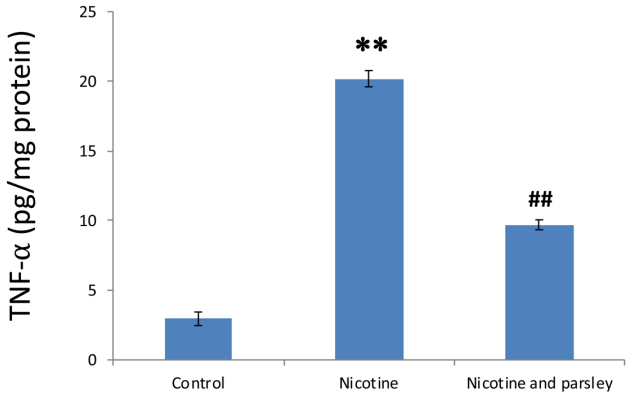

Fig 8. Tumor necrosis factor- alpha (TNF-α) level in the lungs of control, nicotine-treated, and protected group. **P<0.001, compared with the control; and ##P< 0.001, compared with the nicotine group. Data are expressed as means ± SEM.

nicotine-treated group via its antioxidants and anti-inflammatory actions, suggesting that diet supplementation containing parsley during gestation can prevent the adverse effects of nicotine on lung of the offspring. However, it is important to note that while parsley supplementation can improve respiratory outcome, further studies are needed to evaluate whether it has beneficial effects on prematurity and fetal growth restriction associated with nicotine consumption during pregnancy.

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