

Original Article

Ameliorating effect of vitamin C and selenium against nicotine induced oxidative stress and changes of p53 expression in pregnant albino rats

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ABSTRACT

Objective: This study was aimed to evaluate the effects of daily intake of vitamin C or selenium against deleterious effects of nicotine toxicity on pregnant albino rats.

Materials and methods: Forty albino pregnant rats were equally distributed into four groups. Group A was considered as control. Group B was administered with nicotine dosed at 1 mg/kg body weight (bwt) daily for 7 weeks (wks) from 1st day of gestation until the postnatal 4 wks. Group C was treated with nicotine and vitamin C dosed at 1 mg/kg bwt orally for 7 wks, group D was treated with nicotine and sodium selenite dosed at 1 ug/100 g bwt concurrently for 7 wks. The levels of catalase (CAT), superoxide dismutase (SOD), protein carbonyl (PC) and thiobarbituric acid reactive substances (TBARS), were estimated in homogenates of the lung, kidney and liver. Histopathological studies using hematoxylin and eosin as well as immunohistochemical studies using p53 antibody were also done.

Results: Nicotine significantly elevated the levels of TBARS and PC as compared to control rats. Groups C and D showed decrease in these levels significantly. CAT and SOD activities of group B were decreased significantly. Significant elevation of CAT and SOD activities was detected in both groups C and D. Vitamin C elevated the antioxidant enzymes activities to normal levels, however selenium administration improved these levels but still lower than those of group A. Expression of p53 was decreased in group B as compared to group A. Vitamin C completely reversed the expression of p53 as group A. However, group D did not showed any significant changes in expressions as compared to group B.

Conclusion: It is concluded that vitamin C intake was useful than selenium in prevention against nicotine-induced oxidative stress including p53 expression in the lung, kidney and liver of pregnant rats.

KEYWORDS

Histopathology, Nicotine, Pregnant rats, p53, Selenium, Vitamin C

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INTRODUCTION

The deleterious effects of Tobacco exposure is of health concern for newborns and adults ([Mathers et al., 2006](#); [Rogers, 2008](#)). Cigarette smoke constitutes 5000 organic and metallic chemical compounds ([Cay et al., 2009](#)). Nicotine is the most toxic compound of cigarette ([Helen et al., 2000](#)). Cigarette smoking is the major sources of human exposure to nicotine tobacco ([Muthukumaran et al., 2008](#)). In pregnant smoker women, nicotine is absorbed from the respiratory tract of smokers. Nicotine crosses the placenta and enters the amniotic fluid and absorbed through the skin of the fetus. During gestation and early postnatal life, nicotine exposure through maternal milk may interfere with growth and development, where mother's milk contains 2-3 times of nicotine elevated than mother's plasma ([Maritz and Harding, 2011](#)).

Nicotine causes oxidative stress results in oxidant/antioxidant imbalance in blood cells and tissues. Whereby, nicotine increased the production of hydrogen peroxide and superoxide anions which leads to impairment of the mitochondrial respiratory chain ([Suleyman et al., 2002](#)). [Kurt et al. \(2016\)](#) reported that active or passive smoker mothers and neonates have decreased antioxidant concentrations. Oxidative stress has impressive role in regulation of apoptosis and affects the apoptotic signaling by agonistic and antagonistic ([Kalpana and Menon, 2004](#)).

p53 is a transcriptional activator can induce gene expression that regulate to cell cycle, apoptosis and DNA repair in response to many cellular stimuli as hypoxia and DNA damage (synergetic induction). Lack of functional p53 in the cells leading to its resistant to chemotherapy. However, restoration of p53 to the normal level decreases the tumor incidence ([Bykov et al., 2002](#); [Aubrey et al., 2016](#)).

Ascorbic acid (vitamin C) is an important component of the body's antioxidant defense mechanisms; it can decrease the oxidative effects of toxic substances ([Gunes et al., 2008](#)). Smoking causes lowering the levels of serum ascorbic acid ([Raitakari et al., 2000](#)). Vitamin C ameliorates nicotine induced oxidative stress and p53 expression in rat spleen ([Ahmed et al., 2014](#)). Selenium (Se) is essential for the immune system in both animals and humans. It is known an antioxidant in the cytosol and the extracellular space ([Arthur et al., 2003](#)). It can prevent damage of sub-cellular membranes and unsaturated fatty acid caused by free radicals. [Charab et al. \(2016\)](#) reported that administration of 1.78 µg sodium selenite/kg bwt had protective effects against oxidative stress induced by exposure to waterpipe smoke in mice.

Often, the best way to ensure the health of the fetus is to ensure the health of the pregnant mother. The current study aimed to evaluate the effects of daily intake of natural antioxidants (vitamin C or Se) against deleterious effects of nicotine toxicity on some organs of pregnant rats.

MATERIALS AND METHODS

Ethical approval: All experimental procedures were approved by the Institutional Animal Research Committee of the Faculty of Medicine, Assiut University, Egypt, and followed the published guidelines and regulations.

Animals: Forty adult time-dated pregnant Wistar albino rats weighing 200-220 gm were obtained from the Animal House, Faculty of Medicine, Assiut University, Egypt. Female rats were mated with adult male rats over 2 days for copulation in the proportion of two females for every male rat. The presence of a vaginal plug of semen was checked in the breeding cage the following morning ([Inaloz et al., 2000](#)). The pregnant rats were housed individually and allowed food and water *ad libitum*. They were distributed into 4 groups of 10 animals each.

Group A: Female pregnant rats served as control and administered physiological saline S/C daily for 7wks.

Group B: The pregnant female rats were injected S/C with nicotine (Sigma Aldrich, UK), dissolved in physiological saline, (at 1 mg/kg bwt/day) for 7 wks from 1st day of gestation and for 4 wks postnatally ([Maritz and Dennis, 1998](#)).

Group C: The rats received daily oral dose of vitamin C (Sigma Chemical Company, USA), (at 1 mg/kg bwt) prior administrated with the same dose and the same periods of nicotine ([Maritz and Van Wyk, 1997](#)).

Group D: Daily oral dose of Se (Gibco, UK), (at 1 µg sodium selenite/100g bwt) was received to rats prior administrated with the same dose and the same periods of nicotine ([Sreekala and Indira, 2009](#)).

Sampling: At the end of the experiment, animals were scarified under anesthesia with sodium pentobarbital after an overnight fast. Liver, kidney and lung tissues were collected and stored at -20°C for biochemical studies. For the histological and immunohistochemical investigations, pieces of the lung, liver and kidney were fixed in 10% buffered formalin.

Biochemical determinations: TBARS were measured in tissues homogenates as per the method of Ohkawa et al. (1979). The method of [Kakkar et al. \(1984\)](#) was used to measure SOD. CAT was assayed by the method of [Aebi \(1984\)](#). The protein concentration in all samples was determined by the method of [Lowry et al. \(1951\)](#). Protein carbonyl (PC) was estimated by spectrophotometric

detection of the reaction of 2,4-dinitrophenylhydrazine with PC to form protein hydrazones according to method of [Reznick and Packer \(1994\)](#).

Histopathology: All mother rats were scarified under anaesthesia with sodium pentobarbital. The specimens was obtained from the lung, liver and kidney were fixed in 10% buffered formalin, processed, embedded in paraffin and sections of 4-5 μm thickness were done. Tissues were stained with hematoxylin and eosin (HE) for histopathological examination by light microscopy ([Bancroft et al., 1996](#)).

Immunohistochemistry: Paraffin sections of the lung, liver and kidney were cleared in xylene, rehydrated in graded ethanol, immersed in water for 5-10 min and incubated in 0.3% H_2O_2 in 70% methanol for 20 mins to inhibit endogenous peroxidase activity. The specimens were then rinsed three times for 5 min in phosphate-buffered saline solution (PBS), and epitopes were unmasked by boiling in citrate buffer (pH 6.0) for 10-15 min, when necessary. After rinsing in PBS, the sections were blocked for 30-60 min in 3% bovine serum albumin (BSA) in PBS or in 5% goat or rabbit serum in PBS, and were incubated with primary antibody against p53 in 0.1% BSA in PBS overnight at 4°C in a humidified chamber. The samples were then rinsed in PBS and incubated with 7.5 g/mL of biotinylated secondary antibody in 0.1% BSA in PBS for 1 h at room temperature, followed by avidin-biotin amplification (ABC Elite) for 30 min, and were developed with 3,3-diaminobenzidine peroxidase substrate. Sections were counterstained with Mayer hematoxylin for 3 min and mounted. Negative controls were obtained by substituting the primary antibody with PBS ([Kazi et al., 2003](#)).

Statistical Analysis: Results were represented as mean \pm SE. Data were analyzed by One way ANOVA test for multiple comparisons followed by the least significant difference (LSD) was performed ([Knapp and Miller, 1992](#)).

RESULTS

Biochemical results

Lipid peroxidation (LPO): The levels of LPO was significantly increased in the lung, kidney and liver tissues of rats of group B than group A ($P<0.001$). LPO levels significantly decreased in rats treated with vitamin C to normal levels in both the lung and kidney but in the liver tissues decreased but higher than group A ($P<0.05$). However, Se administration with nicotine decreased these levels significantly in tissues homogenates but significantly higher than group A. (**Table 1-3**).

Superoxide dismutase: SOD was lowered significantly in the lung, kidney and liver tissues of group B compared to the other groups ($P<0.001$). Groups C and D showed a significant raise of SOD levels but significantly lower than group A. SOD levels in rats of group C were significantly higher than group D (**Table 1-3**).

Catalase: **Table 1, 2 and 3** showed that CAT activities were declined significantly in tissue homogenates after nicotine administration compared to group A ($P<0.001$). Treatment with vitamin C caused elevation of CAT activity to normal levels. While the treatment with Se increased the level of CAT activity but lower than group A.

Protein Carbonyl: PC levels were significantly elevated ($P<0.001$) in group B in the lung, liver and kidney compared to group A. However, addition of vitamin C lowered these levels significantly to the control group levels. Intake of Se decreased these levels significantly than those of group B but still significantly higher than the group A.

Histopathological results

Lung: The lung in control group showed normal appearance (**Figure 1A**). Examination of the lung tissues in the nicotine treated group showed marked tissue damage in the form of vascular and parenchymal changes. The vascular changes consisted of congestion, hemorrhage and associated with lymphocytic infiltration. Compensatory emphysema was also noticed (**Figure 1B,C**). Hyperplasia of the peribronchial lymphoid aggregation (PBLA) was seen (**Figure 1D**). The parenchymal changes were consisted of degeneration and desquamation of the bronchiolar epithelium. Moreover, thickening of the interalveolar septa was seen infiltrated with eosinophils and lymphocytes.

Liver: The liver in control negative group showed normal appearance (**Figure 2A**). Histological examination of the liver tissues in the nicotine treated group showed congestion of the central vein and dilatation of the blood sinusoids (**Figure 2B**). Proliferation of the Kupffer cells and degenerative changes of the hepatocytes were seen (**Figure 2C**). The portal area revealed congestion of the blood vessels and mononuclear cell infiltration (**Figure 2D**).

Kidney: The kidney in control negative group showed normal structure (**Figure 3A**). Histological examination of the kidney tissues in the nicotine treated group showed vascular and parenchymal changes. The vascular changes were seen in the form of congestion and thrombosis of

Table 1. Lipid peroxidation (LPO) levels, superoxide dismutase (SOD), catalase (CAT) and protein carbonyl (PC) activities in the lung tissues homogenates of the studied groups.

Group	LPO (nmol/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	PC (µmol/mg protein)
A	0.92± 0.01	0.53± 0.01	3.44± 0.01	1.14 ±0.023
B	2.32± 0.06 ^{#####}	0.25± 0.01 ^{#####}	2.75± 0.05 ^{*** #####}	2.17±0.167 ^{#####} +++
C	0.96± 0.02 ^{NS +}	0.48± 0.01 ^{* +}	3.46± 0.01 ^{NS ++}	1.15±0.024 ^{NS ++}
D	1.16± 0.06 ^{**}	0.43± 0.01 ^{**}	3.34± 0.03 ^{**}	1.17±0.03 [*]

Table 2. Lipid peroxidation (LPO) levels, superoxide dismutase (SOD), catalase (CAT) and protein carbonyl (PC) activities in the hepatic tissues homogenates of the studied groups.

Group	LPO (nmol/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	PC (µmol/mg protein)
A	0.91 ± 0.01	1.05 ± 0.01	1.65 ± 0.01	1.66 ±0.082
B	1.15 ±0.04 ^{#####} +++	0.64 ± 0.02 ^{#####} +++	0.81 ± 0.02 ^{#####} +++	2.69 ±0.092 ^{#####} +++
C	0.92± 0.01 ^{NS +}	1.04 ± 0.02 ^{NS +}	1.66± 0.01 ^{NS ++}	1.69 ±0.11 ^{NS ++}
D	0.99± 0.03 [*]	0.83± 0.05 [*]	1.6± 0.01 ^{**}	1.8 ±0.11 [*]

Table 3. Lipid peroxidation (LPO) levels, superoxide dismutase (SOD), catalase (CAT) and protein carbonyl (PC) activities in the renal tissues homogenates of the studied groups.

Group	LPO (nmol/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	PC (µmol/mg protein)
A	1.33±0.01	2.14± 0.05	1.07± 0.03	1.07±0.08
B	2.45± 0.04 ^{*** #####} +++	1.42± 0.06 ^{#####} ++	0.66± 0.02 ^{#####} +++	2.45±0.14 ^{#####} +++
C	1.45± 0.08 ^{NS ++}	2.17± 0.05 ^{NS, ++}	1.15± 0.03 ^{NS, +}	1.1±0.09 ^{NS ++}
D	2.29± 0.09 ^{**}	1.81± 0.07 ^{**}	0.94± 0.03 [*]	1.17±0.08 [*]

Legend to Tables (1-3):

Data are expressed as mean±SE.

*=significance compared to A group, *=*P*<0.05, **=*P*<0.01 and ***=*P*<0.001

#=significance compared to C groups, ###=*P*<0.001

+ =significance compared to D groups, +=*P*<0.05 and +++=*P*<0.001

NS=non significant compared to group A

Table 4. Incidence of histopathological lesions in the lung, liver and kidney of the studied groups.

Lesions	Group A	Group B	Group C	Group D
Lung				
Congestion and hemorrhage	-	+++	-	-
Emphysema	-	+++	+	+
Degeneration of bronchiolar epithelium	-	++	-	-
Hyperplasia of PBLA	-	+++	-	+
Inflammatory cell infiltration	-	+++	+	+
Liver				
Congestion	-	+++	-	+
Degeneration of hepatocytes	-	+++	-	-
Proliferation of Kupffer cells	-	+++	+	+
Inflammatory cell infiltration	-	+++	-	+
Kidney				
Congestion	-	+++	+	+
Thrombosis	-	+++	-	-
Glomerular swelling	-	++	-	-
Degeneration of tubular epithelium	-	+++	-	+
Inflammatory cell infiltration	-	+++	+	+

- No lesions

+ Lesions found in 1-2 rats

++ Lesions found in 3-6 rats

+++ Lesions found in 7-10 rats

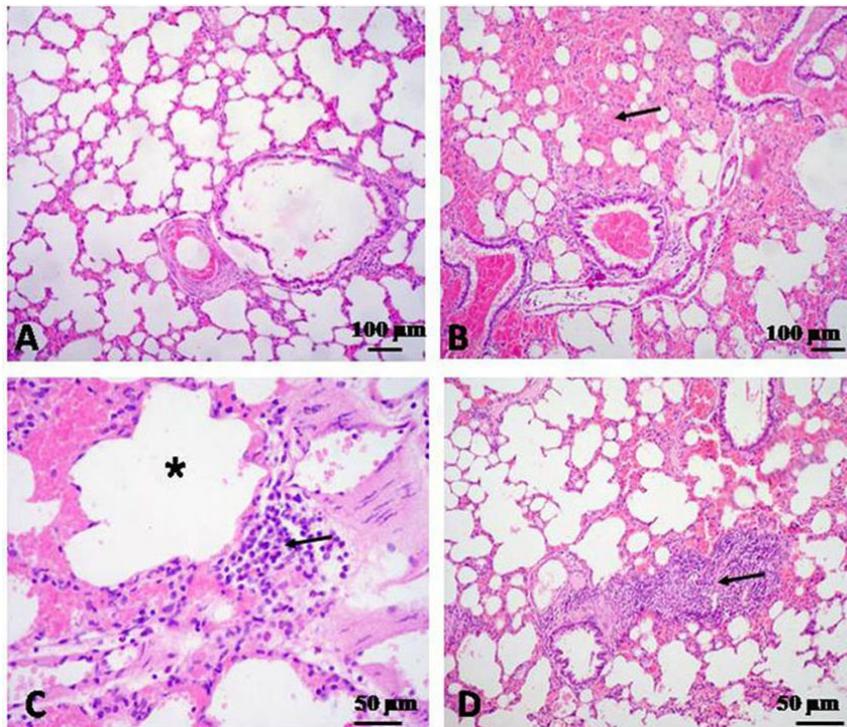


Figure 1. Photomicrographs of the rat lung. (A) Control group. (B) Nicotine-treated group showing hemorrhage (arrow) and congestion. (C) Emphysema (asterisk) and lymphocytic infiltration. (D) Hyperplasia of the peribronchial lymphoid aggregation. HE.

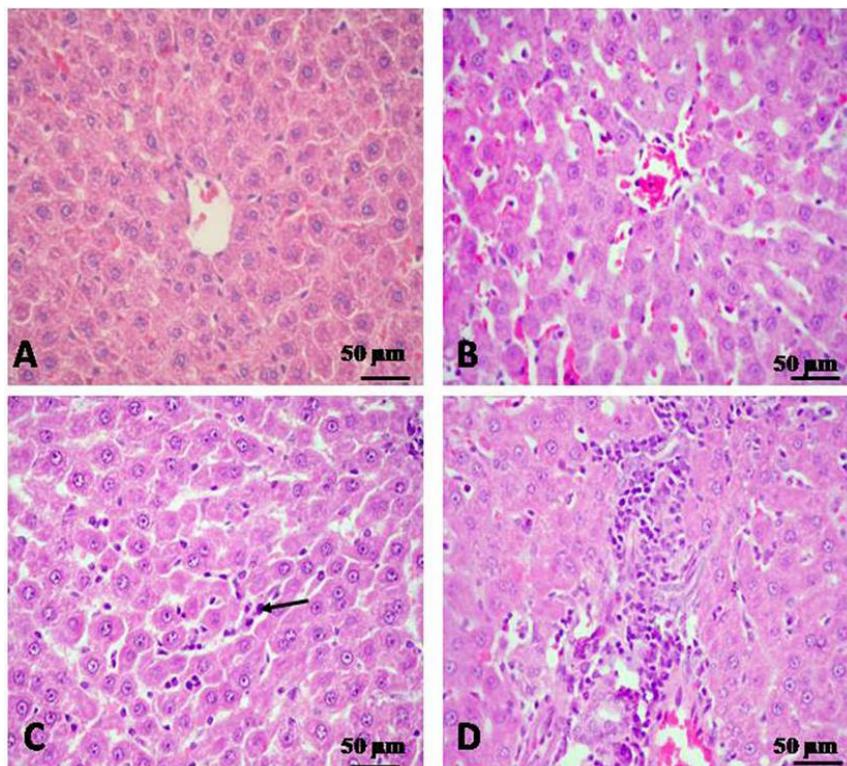


Figure 2. Photomicrographs of the rat liver. (A) Control group. (B) Nicotine-treated group showing congestion. (C) Hyperplasia of Kupffer cells (arrow). (D) Mononuclear cell infiltration in the portal area. HE.

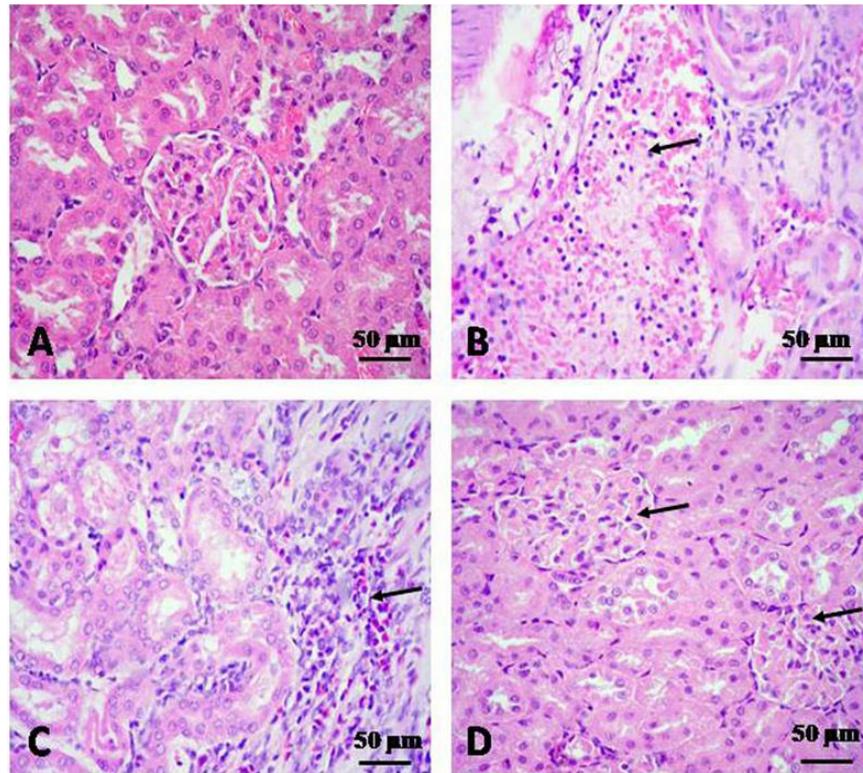


Figure 3. Photomicrographs of the rat kidney. (A) Control group. (B) Nicotine-treated group showing congestion and thrombosis (arrow). (C) Infiltration of eosinophils (arrow). (D) Glomerular swelling (arrows). HE.

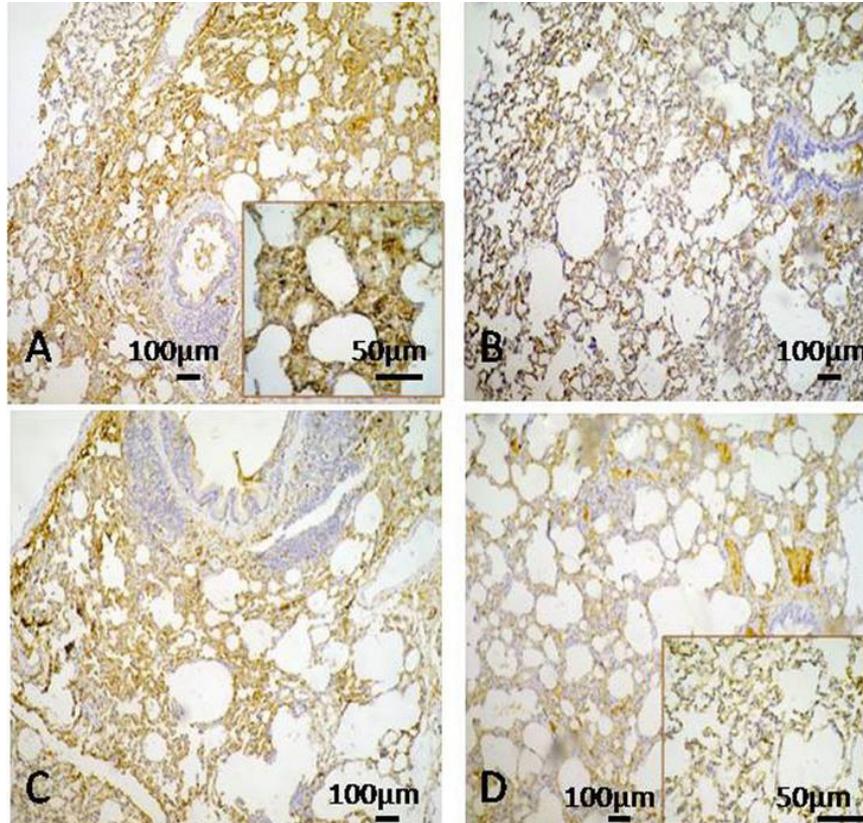


Figure 4. Immunohistochemical technique for demonstration of P53 protein levels in the lung tissues. control group (A), Nicotine-treated group (B), Nicotine + vitamin C group (C) and Nicotine + Se group (D) respectively showing; (A, C) Strong reaction. (B, D) Weak reaction. Bar=50μm and in the window, Bar=100 μm.

the blood vessels (**Figure 3B**). The parenchymal changes consisted of degeneration of the renal tubules and the interstitial tissues infiltrated with eosinophils and lymphocytes (**Figure 3C**). The glomerulus revealed swelling due to congestion and edema (**Figure 3D**).

Histopathological examination of HE-stained section from the examined organs (lung, liver and kidney) in both C and D groups revealed minor changes of the tissues. The incidence of the most common lesions in different groups was summarized in **Table 4**.

Immunohistochemical results: Immunohistochemical method for detection of p53 in the lung, liver and kidney tissues revealed that the expression of p53, in the control group, and nicotine+vitamin C was strong positive reaction to p53 antibody appeared as multiple small dark brown granules. In contrast, only nicotine treated group and the group treated with nicotine and Se revealed weak reactions (**Figure 4-6**).

DISCUSSION

This study was aimed to evaluate the effect of vitamin C and Se against nicotine-induced oxidative stress and expression of p53. Lipid peroxidation disturbs the cellular membrane integrity resulted in escape of cytoplasmic enzymes ([Husain et al., 2001](#)). The data have shown that rat treated with nicotine have significantly elevated level of LPO in all tissue homogenates. High level of LPO products were demonstrated in smokers by many authors ([Petruzzelli et al., 2000](#); [Suleyman et al., 2002](#); [Kavin et al., 2016](#)). These results were explained by that administration of nicotine leads to develop the Reactive Oxygen Species (ROS) by various mechanisms enhances the oxidative stress ([Yildiz et al., 1998](#)). These elevations of TBARS in tissue homogenates of nicotine group might be explained by increased levels of free radicals by nicotine ([Muthukumaran et al., 2008](#)). Vitamin C and Se intake caused to decrease the levels of LPO; however,

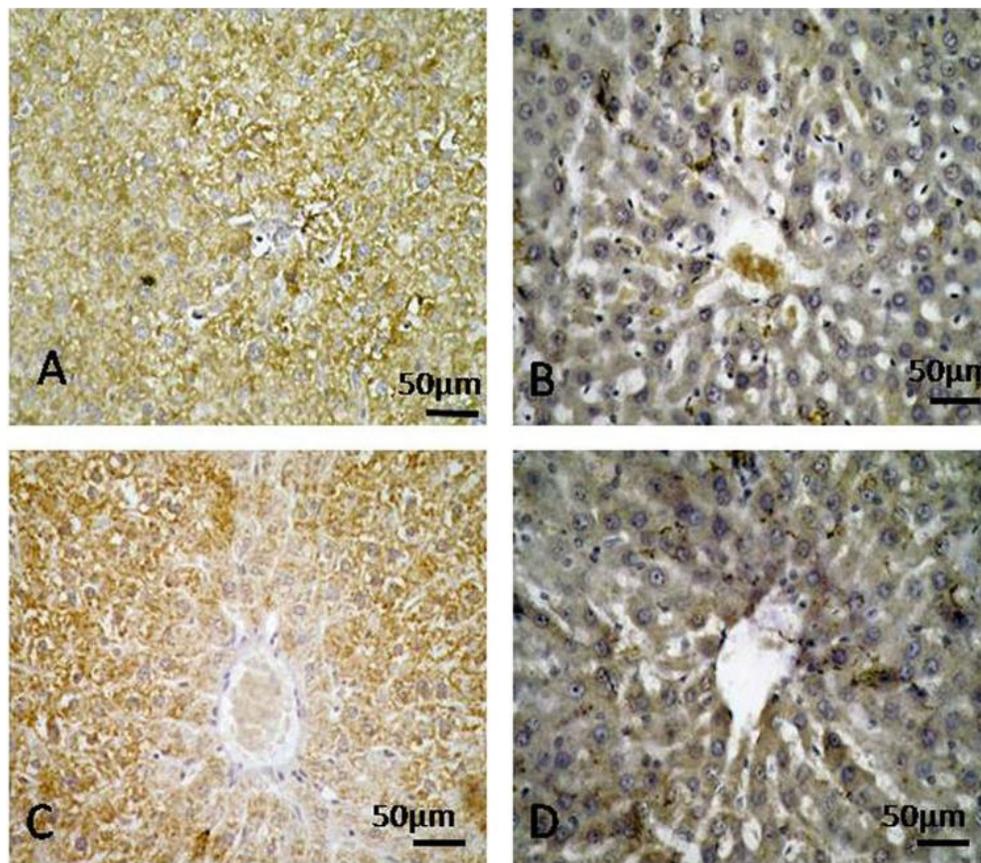


Figure 5. Immunohistochemical technique for demonstration of P53 protein levels in liver tissues of control group (**A**), Nicotine-treated group (**B**), Nicotine + vitamin C group (**C**) and Nicotine + Se group (**D**) respectively showing; **A, C** Strong reaction. **B, D** Weak reaction. Bar = 50µm.

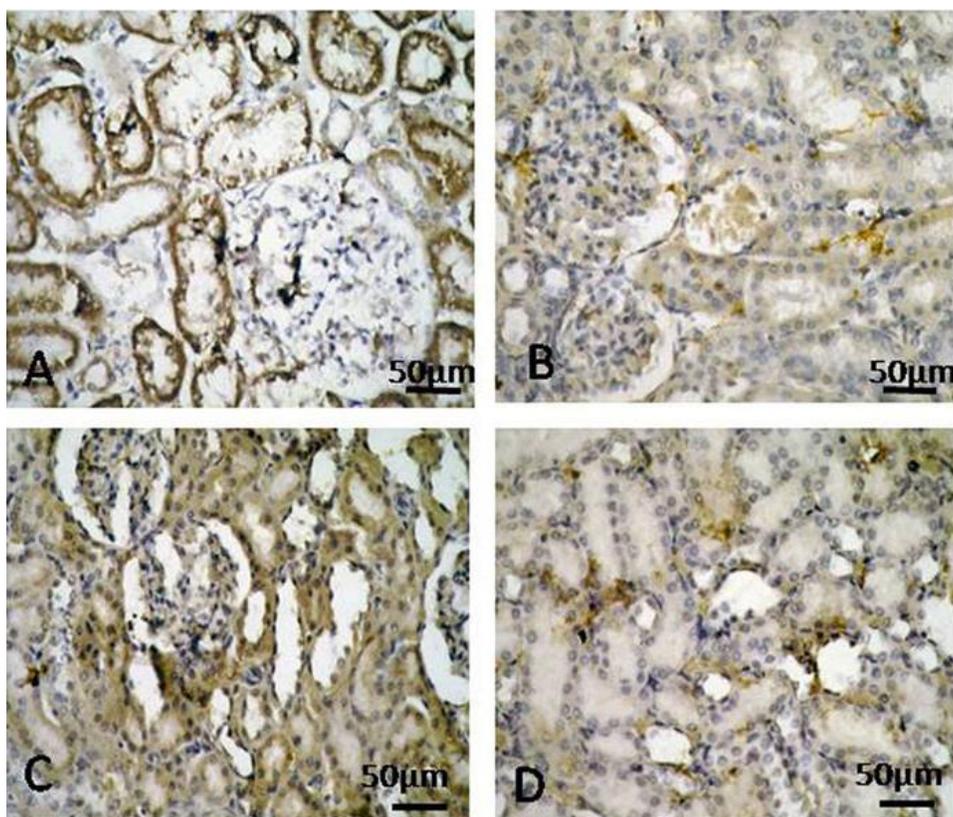


Figure 6. Immunohistochemical technique for demonstration of p53 protein levels in kidney tissues of control group (A), Nicotine-treated group (B), Nicotine + vitamin C group (C) and Nicotine + Se group (D) respectively showing; A, C Strong reaction. B, D Weak reaction. Bar=50 µm.

this effect was more prominent in group C. Lowered levels of TBARS was due to vitamin C supplementation to smokers for 4 wks, as reported by [Harats et al. \(1990\)](#).

In the current study, the levels of SOD, CAT and PC were decreased significantly in the group B compared to group A. These results may be due to decreased activities of the scavenging enzymes ([Ozokutan et al., 2005](#); [Erat et al., 2007](#)).

Histopathological examination showed that nicotine caused tissue damage in the lung, liver and kidney. The lesions were found in the form of vascular changes, parenchymatous changes with infiltration of inflammatory cells. Administration of vitamin C and Se revealed minor changes of the tissues. The changes in the pulmonary tissues were previously reported ([Valença et al., 2004](#); [Demiralay et al., 2006](#); [Gawish et al., 2012](#)). [Sheng et al. \(2001\)](#) reported that rat treated with nicotine developed severe liver damage. [Arany et al. \(2011\)](#) reported that smoking had a negative effect on renal function progression of acute and chronic renal injury. [Akkoyun and Karadeniz \(2016\)](#) mentioned that the antioxidant activity of ellagic acid had a protective effect against nicotine-induced nephrotoxicity and oxidative stress in rats.

In the present study, Se supplementation lowered the levels of LPO and raised the levels of the antioxidant enzymes as compared to group B. Contrary, administration of vitamin C was more effective than Se in elevating the CAT and SOD levels towards the normal values.

Formation of PC is a prior marker of protein oxidation ([Reznick and Packer, 1994](#)). Oxidative changes of proteins may result in functional inactivation and structural alteration of several enzyme proteins ([Davies, 1988](#)). In this study, PC levels in the tissue homogenates was increased significantly in group B as compared to group A. This finding was observed by [Neogy et al. \(2008\)](#) who suggested that nicotine might induce oxidation of the modified proteins caused by excessive oxidation of proteins.

P53 is a very important key in apoptosis ([Agarwal et al., 1998](#)). Extrinsic factors, such as genotoxic agents can cause gathering of p53 in the nucleus resulted in apoptosis and arrest of growth ([Asiri, 2010](#); [Kiraz et al., 2016](#)). The findings obtained from immunohistochemical examination of the lung, liver and kidney in the studied groups showed that nicotine administration significantly lowered the level of p53. [Asiri \(2010\)](#) suggested that the

decrease of p53 expression might be due to lowered antioxidant enzyme levels following nicotine administration which results in altered gene expression. Interestingly, in the present study, vitamin C treatment elevated the p53 expression to the normal values. However, in Se group, the expression of p53 in tissues of the rats did not differ from those of nicotine group. Similarly, [Chen et al. \(2008\)](#) reported that pharmacological ascorbic acid concentration resulted in effective apoptosis and inhibition of tumor development. Besides, it has been reported that high dose intravenous vitamin C improved quality of life in cancer patients ([Takahashi et al., 2012](#)).

CONCLUSION

Our study identifies a detrimental role of nicotine administration on the lung, kidney and liver via oxidative stress, histopathology and immunohistochemistry. Also, the study reveals a protective role of vitamin C against nicotine-induced oxidative stress, which was more impressive than Se. Addition of ascorbic acid for smokers, seems to be much warranted.

CONFLICT OF INTEREST

There is no conflict of interest.

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