Original Article
Toxicology International, *Jan-April 2016 / Vol-23 / Issue-1, 79-88*
ISSN (Print) : 0971-6580
ISSN (Online) : 0976-5131
DOI: 10.22506/ti/2016/v23/i1/146674

Protective effect of Resveratrol against spleen toxicity of nicotine in male rat: Antioxidant and Histopathological approaches

Mohammad S. Al-Harbi[1], Reham Z. Hamza[1,2*]

[1]Taif University, Faculty of Science, Biology Department, Taif 888, Saudi Arabia.
[2]Zagazig University, Faculty of Science, Zoology Department, Zagazig, Egypt.
*dr_reham_z@yahoo.com

Abstract

Nicotine is a tertiary amine, nicotine can freely cross the biological membranes of body organs. During smoking, nicotine is rapidly absorbed into the circulatory system, and blood concentration of nicotine rise quickly during and peak at the completion of cigarette smoking. Resveratrol is a compound is a member of a group of plant compounds called polyphenol which possess a free radical scavenger that increases the activity of several antioxidant enzymes. The study aimed to evaluate the influence of Resveratrol (Res) in rat with Nicotine (Nic) induced spleen toxicity, by evaluating the biochemical and histopathological changes. The experiment was carried out on four groups of male rats as follows; group 1 served as a control group, group 2 was treated with Nic (2.5 mg kg^{-1}) in physiological saline, group 3 was received Res (20 mg Kg^{-1}), group 4 was treated with combination of Nic (2.5 mg Kg^{-1}) and Res (20 mg Kg^{-1}). The results indicated that Nic increased lipid peroxidation, Myloperoxidase, xanthine oxidase, nitrite level, TOS level and decrease the antioxidant enzymes as well as decrease the thiol and G6PD levels . A serious congestion and stasis, correlated with congested red pulp and numerous secondary lymphoid follicles were observed. All the lymphatic nodules appeared smaller with multiple variable sized reactive germinal centers with peripherally located central arteriole in the animals treated with Nic. Nic/Res treatments improve all the oxidative damage and the histopathological changes. In the light of the present results, it is evident that Res was able to reduce the toxic effect of Nic in rat. Conclusion: Resveratrol had found to effect on antioxidant parameters of the spleen tissue. The present study indicates that a decrease in the antioxidant status is one of the main factors contributing to Nicotine toxicity to the spleen. Res treatment found to remove the continuously generated free radicals, to prevent the endogenous antioxidant enzymes decrease and act to prevent oxidative cell damage induced by Nic.

Keywords: Nicotine, Resveratrol, Spleen, Oxidative stress, Histopathology.

Received: 22-01-2016 **Revised:** 11-03-2016 **Accepted:** 01-04-2016

1. Introduction

Cigarette smoking is the major preventable cause of lung cancer in developed and developing countries alike. Epidemiological studies have shown a strong relationship between smoking and increased risks of lung cancer and pulmonary diseases [1-2]. Moreover, it has been proven that cigarette smoking strongly induces apoptosis in alveolar epithelium[3]and accelerates the progression of pulmonary fibrosis[3].

Nicotine is the most abundant alkaloid of tobacco and its major pharmacologically active component [4] responsible for harmful effects in smokers. The addictive propriety of nicotine is the cause of the compulsive use of tobacco products, which in turn results an incessant exposure to a mixture of bioactive and carcinogens compounds in tobacco including the tobacco-specific nitrosamine NNK and polycyclic aromatic hydrocarbons. Besides tobacco dependence, nicotine alters the equilibrium in the various organ systems such as cardiovascular, endocrine and the male reproductive system [5].

Nicotine exerts pharmacological effects that could

* *Author for correspondence*

contribute to acute cardiovascular events and accelerated atherogenesis experienced by cigarette smokers and thus there are few reports about the effect of nicotine toxicity on spleen. Studies of nicotine medications and smokeless tobacco indicate that the risks of nicotine without tobacco combustion products (cigarette smoke) are low compared to cigarette smoking[6].

The detrimental effects of nicotine have been extensively investigated in human, animal and in a variety of cell systems. The predominant effects of nicotine in the whole intact animal or human consist of an increase in heart rate (10 to 20 beats/min), blood pressure (5 to 10 mmHg), release of catecholamines and free fatty acids and mobilization of blood sugar [7-8].

Numerous experimental and clinical evidences have supported the key role of nicotine exposure in the pathogenesis of organ disorders by promoting an oxidative stress [9–12]. In fact, it has been shown that nicotine exposure significantly increased oxidative stress by generating reactive oxygen species [13,14], and lipid peroxidation [15,17].

Numerous investigations have revealed that nicotine treatment might augment toxicity and oxidative damage in rat tissues [18]. In reality, it has been demonstrated that nicotine as individual source for ROS production cause oxidative damage to important components of the cellular machinery. In this respect and for the increasing rate of co-use and co-abuse of smoking cigarette [19-20].

Resveratrol is a compound obtained from the roots of the Polygonum cuspidatum plant used in traditional Eastern medicine for the treatment of fungal diseases, skin inflammation, and cardiovascular and liver diseases[21]. Resveratrol (trans- 3,5,40 trihydroxystilbene) is a phenolic phytoalexin[22] that occurs naturally in various foods, including grapes, plums, cranberries, and peanuts.

Its antioxidant effects have been demonstrated in the brain[23], the liver[24], and the kidneys[25]. Resveratrol is a free radical scavenger that increases the activity of several antioxidant enzymes [26].

Resveratrol is a free radical scavenger that increases the activity of several antioxidant enzymes.Resveratrol (3,5,40-trihydroxystibene) possesses antioxidant activity, due to its polyphenolic structure. It exists as trans-and cis-isomers. The trans-isomer is the naturally occurring form produced by plants and it is converted to the cis-isomer under exposure to light [27].

It was imperative to investigate the effects of nicotine administration and these interactions with Resveratrol supplementation.

2. Materials and methods

2.1 Chemicals

All reagents were of the highest purity available. Nicotine hydrogen bitartrate was obtained from Sigma Chemical Company, St.- Louis, Missouri, USA. All other chemicals and reagents used were of analytical grade. Other chemicals for measurements were purchased from Sigma Chemical Company, St. Louis, MO, USA , trans-Resveratrol (>98% purity) was obtained from Cayman Chemical Company (Ann Arbor, MI, USA).

2.2 Animals

Male albino Wistar rats, of 8 weeks of age and weighing about 150–180 g, were obtained from the king Fahad center for scientific purposes - King abdulaziz university, Jeddah, Saudi Arabia. Animals were housed into a polypropylene cage and provided with food and water ad libitum. Before beginning the experiment, all animals were acclimated for 2 weeks under well-controlled conditions of temperature (22- 25°C) and a 12/12 h light-dark cycle. Animals were fed standard pellet diet.

2.3 Routes of administration and Experimental design

The dosages of the two chemicals were determined according to **Kalpana and Menon**[28] and **Oktem *et al.***[29]. Rats were randomly distributed into four groups (n=10). And received their respective intraperitoneally (i.p) treatment for 4 weeks for sub-chronic study as follows: group 1 served as non treated control group which received physiological saline (NaCl 9%) (1 ml kg^{-1}), group 2 treated with Nic (2.5 mg kg -1) dissolved in physiological saline, group 3 treated with Res in a dose of (20 mg Kg-1).Finally , group 4 was treated with Nic plus Res at the same doses.

2.4 Blood collection

Blood samples of the fasted rats were collected from the medial retro-orbital venous plexus immediately with capillary tubes (Micro Hematocrit Capillaries, Mucaps, Jiangsu, China (Mainland) under ether anesthesia (Boussarie [30]). Then, the blood was centrifuged at 1000 xg for 15 min and serum was collected for different biochemical analyses.

2.5 Tissue preparation

Spleen tissue (about 0.25 g) was used for the analysis of oxidative stress parameters.

Prior to dissection, tissue was perfused with a 50 mM (sodium phosphate buffer saline (100 mM Na_2HPO_4/ NaH_2PO_4) (pH 7.4) and 0.1 m ethylenediamine tetraacetic acid (EDTA) to remove any red blood cells and clots. Then, tissues were homogenized in 5 mL cold buffer per gram tissue and centrifuged at 2700 xg for 1hr. The resulting supernatant was transferred into Eppendorf tubes and preserved in a deep freeze until used for various oxidative assays.

2.6 Total antioxidant status assay (TAS)

Total antioxidant status (TAS) was measured in spleen using a commercially available kit from Rel Assay Diagnostics (Gaziantep, Turkey) ***Erel,***[31]. The method was based on the reduction of colored 2,20-azino-bis(3-ethylbenzotiazoline-6-sulfonic acid) (ABTS) radical to a colorless reduced form by antioxidants present in the sample. Absorbance was measured spectrophotometrically at a wavelength of 660 nm. The method was calibrated using the vitamin E analog Trolox, and data were expressed as mmol Trolox equivalent (eq.) per liter (mmol Trolox eq./L).

2.7 Total oxidant status assay (TOS)

Total antioxidant status (TOS) was measured in spleen using a commercially available kit from Rel Assay Diagnostics ***Erel,***[32]. The method was based on the principle that the oxidants in the sample oxidized ferrous ions, previously bounded to a chelator, toferric ions. In the acidic medium of the assay, these ferric ions formed a colored complex with a chromogen. The color intensity was measured spectrophotometrically at a wavelength of 530 nm. This assay was calibrated with hydrogen peroxide (H_2O_2), and the results were expressed as l mol H_2O_2 eq./L.

2.8 Determination of oxidative and antioxidant parameter

Lipid peroxidation was determined by the procedure of **Esterbauer and Cheeseman**[33]. Tissue supernatant thiobarbituric acid-reactive substances (TBARS) were measured at 532 nm by using 2-thiobarbituric acid (2,6-dihydroxypyrimidine-2-thiol; TBA)MDA concentrations were determined using 1,1,3,3-tetraethoxypropane as standard and expressed as µmol/g tissue.

Superoxide dismutase (SOD) activity was measured according to the method described by **Marklund and Marklund**[34] by assaying the auto oxidation of pyrogallol at 440 nm for 3 min. One unit of SOD activity was calculated as the amount of protein that caused 50% pyrogallol autooxidation inhibition. A blank without homogenate was used as a control for non-enzymatic oxidation of pyrogallol in Tris–EDTA buffer (50 Mm Tris, 10 mM EDTA, pH 8.2). The SOD activity is expressed as U/mg protein.

Catalase (CAT) activity was measured, determined according to the method described by **Aebi** [35] by assaying the hydrolysis of H_2O_2 and the resulting decrease in absorbance at 240 nm over a 3 min period at 25˚C. Before determination of the CAT activity, samples were diluted 1:9 with 1% (v/v) Triton X-100. CAT activity is expressed as mmol/mg protein.

Glutathione peroxidase (GPx) activity was determined by the assay described by **Hafeman *et al.***[36]. The peroxide substrate (ROOH), GRx and NADPH are included in the reaction mixture. The formation of the oxidized form of glutathione (GSSG) that is catalyzed by GPx is coupled to the recycling of GSSG back to the reduced form of glutathione (GSH) using GRx. NADPH is oxidized to $NADP^+$. The change in A 340 due to NADPH oxidation is monitored and is indicative of GPx activity. The activity of GPx was expressed in terms of mmol GSH consumed/ min/g wet weight tissue

Total antioxidant capacity was determined using ferric reducing antioxidant power assay according to the method of **Prieto *et al.***[37]. FRAP reagent (300 mM acetate buffer, pH 3.6, 10 mM 2,4,6-Tri (Z-pyridy)-S (riazine, 99 %) (TPTZ) in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in the ratio of 10:1:1 was prepared. 1.5 mL FRAP reagent was added to 50 µL of homogenated liver, brain, testis and kidney tissues and incubated at 37 ºC for exactly 5 min. The change in absorbance was measured at 593 nm due to the formation of a blue colored FeII-tripyridyltriazine complex from the colorless oxidized Fe^{III} form by the action of electron donating antioxidants. The absorbance of the sample was read against reagent blank (1.5 mL FRAP reagent and 50µL distilled water) at 593 nm.

Myeloperoxidase (MPO) is a peroxidase enzyme (EC 1.11.1.7) most abundantly present in neutrophil granulocytes. It is a green hemoprotein found in

neutrophils and monocytes that catalyzes the reaction of hydrogen peroxide and halide ions to form cytotoxic acids and other intermediates that play a role in the oxygen-dependent killing of tumor cells and microorganisms. Myeloperoxidase (MPO) Activity Assay Kit (Fluorometric) (ab111749) provides a rapid, simple and reliable method to study MPO activity. **Suzuki *et al.*** [38]. Briefly, 10 ml sample was combined with 80 ml 0.75 mM H_2O_2 (Sigma) and 110 ml TMB solution (2.9 mM TMB in 14.5% DMSO [Sigma] and 150 mM sodium phosphate buffer at pH 5.4), and the plate was incubated at 37˚C for 5 min. The reaction was stopped by adding 50 ml2MH_2SO_4 (Sigma), and absorption was measured at 450 nm to estimate MPO activity.

Xanthine oxidase (XO) activity was assayed spectrophotometrically by the reaction of enzyme with xanthine, as a substrate, and the absorbance was measured at 650 nm, according to the method described in **Kamel and Mohamed,** [39]. In two test tubes, 15 µl phosphate buffer (0.1 M, 7.4 pH) and 30 µl water (control) or substrate (test; xanthine, 38 mM, dissolved in distilled water by adding alkali and gentle heating) was added to 0.5 ml homogenates (spleen) and incubated for 40 min at 37 °C. Then, 0.1 ml of this solution was mixed with 0.1 ml Na-tungstate 40%, 0.5 ml water and 0.1 ml 2N H_2SO4 complete to 1 ml and incubated for 1 h at 37°C then centrifuged at 3000 rpm for 10 min. 0.15 ml of supernatant was mixed with 0.75 ml water, 0.3 ml Foline reagent and 1.5 ml saturated Na-carbonate solution. The absorbance was measured at 650 nm after 10 min. XO activity was determined using the following equations: Concentration of xanthine in control or test = A (sample) /A (standard) × concentration of standard × 48. Xanthine oxidase activity (U/g tissue) = concentration of test concentration of control/0.284.

The levels of nitrite, a stable end-product of nitric oxide (NO) production, were measured spectrophotometrically based on the Griess reaction **Oktem *et al.*** [40]**; Sosroseno *et al.*** [41], in which equal volume of deproteinized tissue homogenates (spleen) were mixed with acidic Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid). The absorbance was measured at 546 nm.

2.9 Determination of glucose-6 phosphate dehydrogenase (G6PD):

Tissue glucose-6-phosphate dehydrogenase (G6PD) activities were measured in liver, kidney, lung, spleen and brain homogenates using the methods described by Deutsch ***(Aebi,*** [35]***)***. The results were expressed as U/g protein.

2.10 Determination of non enzymatic antioxidant (Thiol level)

Total thiols level was determined according to **Hu** [42]. An aliquot of Liver , brain , spleen , kidney and lung homogenate (50 mL) was mixed with 1 mL of Tris base (0.25 M) – EDTA (20 mM) buffer, pH 8.2, and the absorbance at 412 nm was determined. To this was added 20 mL of 10 mM DTNB. After 15 min at ambient temperature, the absorbance was measured again with DTNB blank. Results are expressed in [mM].

2.11 Histological evaluation

For histological examination, a portion of the spleen was fixed in 10% neutral buffered formalin embedded in paraffin, sectioned and stained with Haematoxylin and Eosin as described by **Gabe,**[43]. Then semi-thin sections (0.5-1 microns) were prepared by using LKB ultra microtome. The sections were stained with toluidine blue, examined with a light microscope and photographed.

3. Results

All the parameters of antioxidant enzymes (SOD, CAT, and GPx) were decreased except the XO was increased when the rat was exposed to the dose of Nic (Table 1). Administration of Res to the Nic-treated group with dose (2.5 mg Kg-1) had restored SOD, GPx, and XO significantly ($p < 0.05$) as compared to Nic treated group alone. Treating the rat with the Nic decreased the activity of SOD that has been increased again when Nic group has been treated with Res for 4 weeks. The activity of CAT had depressed significantly ($p < 0.01$) in the spleen tissues of Nic treated group as compared to control by 75.91 %.

The same observation has been noticed in the GPx activity that decreased by Nic treated group and increased by the treatment of the rat with Res after Nic.

To investigate the oxidative stress in spleen tissues, we measured the levels of LPO and NO in all treated groups. Table 1 represents the levels of MDA (index of lipid peroxidation) in normal and experimental rat. It was found that MDA levels in Nic treated rat were increased in spleen tissues by approximately 26.99

Table 1. Effect of Nicotine and/ or Resveratrol on some antioxidant /oxidative stress parameters in spleen of male rat

Parameters	SOD (U g^{-1})	CAT (U g^{-1})	GPx (U g^{-1})	MDA (U g^{-1})	TCA %	XO (U g^{-1})	Myloperoxidase (U g^{-1})	NO (µmol g-1)
Control group	30.41±2.12^{a}	18.65±1.54^{a}	15.36±1.25^{b}	10.26±2.36^{cd}	97.26±3.25^{a}	4.25±0.25^{c}	10.45±1.25^{c}	1.20±0.05^{c}
Nic group	16.25±1.23^{c}	4.56±0.58^{c}	4.39±2.25^{d}	37.25±3.62^{a}	71.29±2.52^{c}	11.35±1.05^{a}	35.25±3.25^{a}	3.74±0.78^{a}
Res group	31.65±2.65^{a}	18.78±1.69^{a}	15.97±1.87^{ab}	8.59±1.36^{d}	97.66±3.65^{a}	4.21±0.68^{c}	9.57±2.25^{c}	1.35±0.06^{c}
Nic +Res group	27.35±2.65^{b}	12.58±2.54^{b}	10.38±1.24^{c}	15.38±2.25^{b}	85.34±2.89^{b}	8.65±1.25^{b}	15.35±2.56^{b}	1.78±0.69^{b}

Nic: Nicotine; Res: Resveratrol; SOD: superoxide dismutase; CAT: catalase; MDA: malondialdehyde; XO: xanthine oxidase; No: nitric oxide; TAC: total antioxidant capacity.
Values are expressed as means SE; n = 10 in each treatment group.
Significant difference are arranged alphabetically from higher to lower.

-fold as compared to control after 4 weeks of treatment with Nic. The increments of NO levels were noticed by 2.54-folds as compared to control for Nic treated group (Table 1). Treatment of the rat with Nic /Nic decreased the NO levels by approximately same degree (48.33 %) as compared to Nic treated group. The values of TAC were 97.26%, 71.29%, 97.66% and 85.34% for control, Nic, Res and Nic and Res respectively.

Table 2. Effect of Nicotine and/ or Resveratrol on TAS and TOS levels in spleen of male rat

Parameters	TAS (mmol Trolox eq./L)	TOS (µmol H_2O_2 eq./L)
Control group	2.01±0.98^{a}	0.78±0.05^{c}
Nic group	1.11±0.78^{c}	3.56±0.52^{a}
Res group	2.00±0.78^{a}	0.71±0.02^{c}
Nic +Res group	1.78±0.88^{b}	1.89±0.38^{b}

TAS : Total antioxidant status
TOS:Total oxidant status
Values are expressed as means SE; n = 10 in each treatment group.
Significant difference are arranged alphabetically from higher to lower.

Data presented in (Table.2) showed that treatment of the rat with Nic caused a significant increase in the level of TAS. It increased by 0.9 fold as compared to control group. However, the concurrent administration of Res to the rat treated with Nic significantly ($p < 0.001$) increased the level of TOS by 2.78 - fold as compared to its related group.but the levels were restored to near to normal values in group treated with Nic and Res respectively in both parameters (TAS and TOS) levels.

Thiol and G6PD levels were decreased by 0.67 and 0.98 -folds in Nic treated rat (Table. 3). Co-administration of Res with Nic practically prevented the changes in Thiol and G6PD levels.

Table 3. Effect of Nicotine and/ or Resveratrol on Thiol (SH) (mmole/ml) and G6PD (U/g) levels in spleen of male rat

Parameters	Thiol (mmole/ml)	G6PD (U/g)
Control group	0.89±0.05^{a}	1.54±0.14^{a}
Nic group	0.22±0.09^{c}	0.56±0.01^{c}
Res group	0.87±0.04^{a}	1.55±0.23^{a}
Nic +Res group	0.69±0.03^{b}	1.01±0.18^{b}

Thiol: Thiol level
G6PD: Glucose -6-Phosphate dehydrogenase
Significant difference are arranged alphabetically from higher to lower.

3.1 Histological finding

The histopathological changes in spleen after 4 weeks of Nic or/and Nic treatment are presented in Fig. 1 (A-D). All control rat and Res group had a normal splenic tissue with numerous small primary follicles with intervening red bulb and normal multiple variable sized reactive germinal (Fig 1A-1C). The splenic tissues in Nic group had multiple variable sized reactive germinal centers with peripherally located central arteriole with congested red pulp numerous secondary lymphoid follicles with pale germinal centers surrounded by dark mantle zone (Fig. 1B). Evident histological alterations already appeared 4 weeks after the treatment, a serious congestion and stasis, correlated with a decrease in lymphocyte count in the germinal centers in the white pulp, so that all the lymphatic nodules appeared smaller (Fig. 1D). These modifications were more evident to the oxidative stress that has been taken place in splenic tissues of Nic treated group. Moreover, There were primary rounded to oblong germinal centers with mild congested red pulp in Nic/ Res group (Fig. 1E). Restoration of spleen tissues to the normal structure with the central reactive rounded

pale germinal center, which is large to some extent, was observed in Nic/Res than observed in Nic treated group (Fig. 1B).

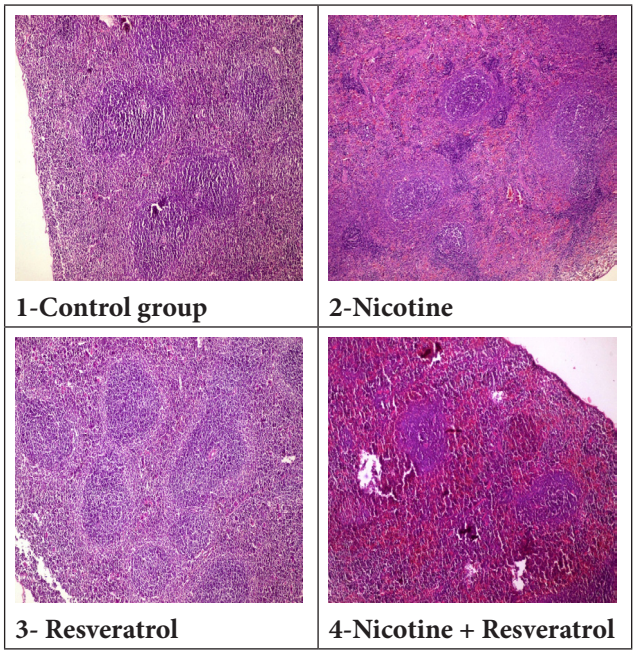


Figure 1. Effect of Resveratrol and Nicotine on spleen tissue. (A) **Control group**: histological section from control with normal splenic tissue with numerous small primary follicles with intervening red bulb (Rb) (H&E x 400);(B) **Nicotine group**:showing multiple variable sized reactive germinal centers with peripherally located central arteriole with congested red pulp numerous secondary lymphoid follicles with pale germinal centers surrounded by dark mantle zone (H&E x 400) .(C) **Resveratrol group**: Showed Splenic tissues appear to be normal with multiple variable sized reactive germinal (H&E x 400). (D) **Nicotine + Resveratrol group:**Showed primary rounded to oblong germinal centers with mild congested red pulp (H&E x 400).

Table (4) showed the recording of different histopathological findings in different slides obtained from the four different groups and the variation from absence of change ----+ , change that was observed in almost groups +++-, change that is often observed in all animals ++- till the change that is often found ++++ .

4. Discussion

The spleen is the largest secondary immune organ in the body and is responsible for initiating immune reactions to blood-borne antigens and for filtering the blood of foreign material and old or damaged red blood cells. These functions are carried out by the 2 main compartments of the spleen, the white pulp (including the marginal zone) and the red pulp, which are vastly different in their architecture, vascular organization, and cellular composition (Cesta, [44]).

The doses of Nic that was used, induced significant oxidation status and histological modifications in the spleen.The increased LPO and TOS concentration , while decreasing TAS level observed in this study resulted from oxidative damage to the spleen tissue after Nic-treatment. It is evident that Nic caused prooxidative activity.

Nicotine, once absorbed, is mainly metabolized by the liver to a number of major and minor metabolites [45,46]. It has been considered a highly susceptible organs to free radical generation and toxic effect of nicotine. In fact, numerous experimental and clinical evidences have been shown that nicotine through smoking induces an inflammatory response in the lung [47], promote pathogenesis of obstructive pulmonary diseases [48] and accelerate progression of pulmonary fibrosis [20] and thus the new concept in the present study is the effect of nicotine on splenic tissues from the oxidation aspect and histopathological approach through measuring also thiol and G6PD levels which are new concepts in the present finding and confirming the occurance of cell damage in Nic treated group which is diminished by administration of Res and thus proving its antioxidant activity.

In accordance with the present study findings , numerous investigations have revealed that nicotine treatment might augment toxicity and oxidative damage in rat tissues [12]. In reality, it has been demonstrated that nicotine as individual source for ROS production [13,15] cause oxidative damage to important components of the cellular machinery [12]. In this respect and for the increasing rate of co-use and co-abuse of smoking cigarette [16,17].

Resveratrol is a compound obtained from the roots of the Polygonum cuspidatum plant used in traditional Eastern medicine for the treatment of fungal diseases, skin inflammation, and cardiovascular and liver diseases[21]. Resveratrol (trans- 3,5,40 trihydroxystilbene) is a phenolic phytoalexin [22]that occurs naturally in various foods, including grapes, plums, cranberries, and peanuts.

Its antioxidant effects have been demonstrated in the brain [23], the liver [24], and the kidneys[25]. Res is a free radical scavenger that increases the activity of several antioxidant enzymes [26]and the present finding revealed its antioxidant activity against spleen toxicity induced by nicotine in male rats and this appear greatly and consequently through increasing the level of antioxidant enzymes and

Table 4. Histopathological findings in spleen of rats treated with Nicotine and Resveratrol

Findings	Groups			
	Control	Nicotine	Resveratrol	Nicotine + Resveratrol
Normal splenic tissue	++++	------	++++	+++-
small primary follicles	++++	+++-	++++	++++
multiple variable sized reactive germinal centers	------	+++-	------	++–
central arteriole with congested red pulp	------	++++	------	++–
numerous secondary lymphoid follicles with pale germinal centers	------	++++	------	++–
congested red pulp	------	++++	------	—+
Symbol	**meaning**			
------	Absence of the change in the animals of the studied group.			
++++	A change which was often found in all the studied animals of a group.			
+++-	A change which was observed in almost all the studied animals of a group.			
++–	A change not so often observed in all animals of a group.			
—+	A change which was rare within a group			

decreasing the final product of lipid peroxidation which reflect the antioxidant ability of Res against toxicity of Nic.

The present findings concides with obtained by (***Leonard* et al.**,[26]) as Res is a free radical scavenger that increases the activity of several antioxidant enzymes. Resveratrol (3,5,40-trihydroxystibene) possesses antioxidant activity, due to its polyphenolic structure. It exists as trans-and cis-isomers. The trans-isomer is the naturally occurring form produced by plants and it is converted to the cis-isomer under exposure to light .

It was imperative to investigate the effects of nicotine administration and these interactions with resveratrol supplementation.

The obtained results cam in accordance with that obtained by **Mosbah *et al.***,[56] who reported that Nicotine is an active substance present in tobacco that causes oxidative stress and tissues damages leading to several diseases. Natural antioxidants that prevent or slow the progression and severity of nicotine toxicity may have a significant health impact. They used green tea extract against nicotine toxicity and they proved that green tea extract played a protective role against nicotine-induced oxidative stress as well as the reproductive effects by improving the oxidative status, semen quality and the testicular histological damage.

The present work also showed that the changes in LPO were accompanied by a concomitant decrease in the activities of antioxidant enzymes, namely SOD, CAT and GPx after 4 weeks of Nic treatment. SOD, CAT, and GPx constitute a mutually supportive team of enzymes which provide a defense against the intermediates of dioxygen.

SOD constitutes an important link in the biological defense mechanism through

dismutation of endogenous cytotoxic superoxide radicals to H_2O_2 and O_2.–, which are deleterious to polyunsaturated fatty acids and proteins [49].

Nic administration to male rats induces free radical generation and hence the first line defense comes to the rescue as shown by the significant decrease in SOD in Nic-animals. Moreover, the reduced in CAT or GPx activity to degrade H_2O_2, more H_2O_2 could be converted to toxic hydroxyl radicals that may contribute to oxidative stress due to Nic as shown before in methanol intoxication with antioxidant enzymes [50].

A decline in the activities of these enzymes could be due to their inactivation by excess ROS production. The antioxidant enzymes CAT and GPx protect SOD against inactivation by H_2O_2. Reciprocally, SOD protects CAT and GPx against superoxide anion. The role of H_2O_2 in ROS generation has been observed in other studies [51].

In the present study, the activity of GPx decreased as the effect of Nic. A reduction in GPx activity results in increased H_2O_2 levels and hence severe cellular damage is observed in splenic tissue. This result is in line with the

observation of Cheeseman and Slater [52]. Thus, the balance of this enzyme system is essential to dispose of superoxide anion and peroxides generated in the spleen. The reduction in the activities of these enzymes and increase in LPO could reflect the adverse effects of Nic on this finely balanced antioxidant system in the spleen tissues, a result that could lead to impaired immune functions.

Free radicals initially increase due to induction of Nic as the levels of NO and LPO increased as well as the activity of XO. LPO is associated with a wide variety of toxic effects, including decreased membrane fluidity and function, impaired functions of the mitochondria and Golgi apparatus and inhibition of enzymes.

In agreement with the obtained results, **Ahmed*et al*.**[57] showed that treatment with Vitamin C or Selenium plays a significant role in elevation of SOD and CAT activities after decreasing by Nicotine and so, Vitamin C supplementation to nicotine treated rats was more effective than selenium in attenuation of nicotine-induced oxidative stress, in rat spleen tissues and this proves the role of natural compound in attenuating nicotine toxicity.

Confirming the obtained results and the role of natural compounds , **Neogy *et al.*,**[58] revealed the ameliorative properties of bioactive compound andrographolide (ANDRO), aqueous extract of Andrographis paniculata (AE-AP) and vitamin E (vit.E) against nicotine induced liver, kidney, heart, lung and spleen toxicity. The significantly increased levels of lipid peroxidation, protein oxidation and the decreased antioxidant enzyme status were noted in nicotine treated group as compared to vehicle treated group. ANDRO, AE-AP and vit.E significantly reduced the lipid peroxidation, protein oxidation and increased the antioxidant enzyme status.

The results of the present study go parallel with the results obtained by **Salahshoor *et al*.**[59]who indicated the effect of nicotine on vital organs and decreasing its antioxidant activity and the role of natural compound like curcumin against nicotine toxicity and they proved that nicotine administration significantly decreased liver weight and increased the mean diameter of hepatocyte, central hepatic vein, liver enzymes level, and blood serum nitric oxide level compared with the saline group. However, curcumin and curcumin plus nicotine administration substantially increased liver weight and decreased the mean diameter of hepatocyte, central hepatic vein, liver enzymes, and nitric oxide levels in all groups compared with the nicotine group.

The normal structure of splenic tissue characterizes with the red pulp that is composed of the three-dimensional meshwork of splenic cords and venous sinuses. The splenic cords are composed of reticular fibers, reticular cells, and associated macrophages [53]. The reticular cells are considered to be myofibroblasts and may play a role in splenic contraction [53].

The red pulp macrophages are actively phagocytic and remove old and damaged erythrocytes and blood-borne particulate matter [54].

Under the treatment of the splenic tissue with Nic, the follicles are variable in size and contain occasional plasma cells, however, follicular dendritic cells are absent since B and T cells are required for the development of these cells as it has been described before for abnormal splenic tissues[55]. The marginal zone is markedly decreased in size and is poorly demarcated from the follicles and these architecture changes have been greatly improved after Res treatment after Nic administration.

5. Conclusion

Resveratrol had found to effect on antioxidant parameters of the spleen tissue. The present study indicates that a decrease in the antioxidant status is one of the main factors contributing to Nicotine toxicity to the spleen. The observed significant increase in the LPO and nitrite levels, as well as XO and Myloperoxidase activities in the immune tissues of Nic-exposed animals, suggests that the tissues are subjected to increased oxidative stress. Reversible oxidative/antioxidant and histological modifications were observed when Res used after Nic treatment. Res treatment found to remove the continuously generated free radicals, to prevent the endogenous antioxidant enzymes decrease and act to prevent oxidative cell damage induced by Nic.

6. Conflict of Interest

None Declared

7. References

1. Boyle, P., 1997.Cancer, cigarette smoking and premature death in Europe: a review including the Recommendations of European Cancer Experts Consensus Meeting, Helsinki, October 1996. Lung Cancer.,17:1–60.
2. Fagerstrom, K.,2002. The epidemiology of smoking: health consequences and bene-fits of cessation. Drugs.,62:1–9.
3. Piipari, R., K.Savela, T.Nurminen, J.Hukkanen, H.Raunio,

J.Hakkola, 2000. Expression of CYP1A1, CYP1B1 and CYP3A and polycyclic aromatic hydrocar-bon-DNA adduct formation in bronchoalveolar macrophages of smokers and non-smokers. Int J Cancer.,86:610–6.
4. Jung, B.H., B.C. Chung, S.Chung, C.Shim, 2001. Different pharmacokinetics of nicotine following intravenous administration of nicotine base and nicotine hydrogen tartarate in rats. J Control Release .,77:183–90.
5. Kovacic, P., A.Cooksy,2005. Iminium metabolism for nicotine toxicity and addiction: oxidative stress and electron transfer. Med Hypotheses .,64:104–11.
6. Neal,L., Benowitz, D.Andrea, 2016. Burbank, Cardiovascular toxicity of nicotine: Implications for electronic cigarette use. Trends in Cardiovascular Medicine,.Accepted manuscript in press.
7. Van der ,V.H., D.S. Postma, W.Timens, 2004. Acute effects of cigarette smoke on inflammation and oxidative stress: a review. Thorax .,59:713–21.
8. Ben Ahmed, H., S.Khlifi, K.Rtibi, S.El Fazaa, N.Gharbi, 2010. Indicators of oxidative stress in weanling and pubertal rats following exposure to nicotine via milk. Hum Exp Toxicol.,29(6):489–96.
9. Kalpana,C., V.P.Menon,2004. Protective effect of curcumin on circulatory lipid peroxidation and antioxidant status during nicotine-induced toxicity. Toxicol Mech Methods .,14:339–43.
10. Husain, K., R.B. Scott, K.S.Reddy, S.M.Somani,2001. Chronic ethanol and nicotine interaction on rat tissue antioxidant defense system. Alcohol .,25(2): 89–97.
11. Cahill A, Cunningham C, Adachi M. 2002. Effects of alcohol and oxidative stress on liver pathology: the role of the mitochondrion. Alcoholism Clin Exp Res .,26:907–15.
12. Anthony,J.C., F.Echeagaray-Wagner.2000. Epidemiologic analysis of alcohol and tobacco use. Alcohol Res Health ., 24:201–8.
13. Falk DE, Yi HY, Hiller-Sturmho¨ fel S ,2006. An epidemiologic analysis of co-occurring alcohol and tobacco use and disorders: findings from the National Epidemiologic Survey on alcohol and related conditions. Alcohol Res Health .,29: 162–71.
14. Bradford, M.,1976. A rapid and sensitive for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem .,72:248–51.
15. Mamdouh Y. Kamel, R.Ragaa. Hamed,1980. A colorimetric method for the determination of guanine and xanthine and its application to the assay of xanthine oxidase, Microchemical Journal, 25(3):323-329.
16. Sekhon,H., B.J.Proskocil, J.A.Clark, 2004.Spindel ER. Prenatal nicotine exposure increases connective tissue expression in fetal monkey pulmonary vessels. Eur Respir J.,23:906–15.
17. Schievelbein, H., D.J.K. Balfour,1984. Nicotine and the tobacco smoking habit. Oxford: Pergamon Press;1–15.
18. Favaro WJ, Cagnon VH. Morphometric and morphological features of the ventral prostate in rats submitted to chronic nicotine and alcohol treatment. Tissue Cell 2006;38:311–23.
19. Benowitz NL. Nicotine and coronary heart disease. Trends Cardiovasc Med 1991;1:315–21.
20. Benowitz NL. Pharmacological aspect of cigarette smoking and nicotine ad-diction. N Engl J Med 1988;319:1318–30.
21. Arichi, H., Kimura, Y., Okuda, H., Baba, K., Kozawa, M., Arichi, S., 1982. Effects of stilbene components of the roots of Polygonum cuspidatum Sieb et Zucc on lipid metabolism. Chem. Pharm. Bull. 30, pp. 1776–1770.
22. Wang, Y., Catana, F., Yang, R., Roderick, R., Van Breemen, R.B., 2002. Analysis of resveratrol in grape products, cranberry juice and wine using liquid chromatography-mass spectrometry. J. Agric. Food Chem. 50, 431–435.
23. Ates, O., Cayli, S., Altinoz, E., Gurses, I., Yucel, N., Sener, M., Kocak, A., Yologlu, S.,2007. Neuroprotection by resveratrol against traumatic brain injury in rats. Mol.Cell. Biochem. 294, 137–144.
24. Dalaklioglu, S., Genc, G.E., Aksoy, N.H., Akcit, F., Gumuslu, S., 2013. Resveratrol ameliorates methotrexate-induced hepatotoxicity in rats via inhibition of lipid peroxidation. Hum. Exp. Toxicol. 32, 662–667.
25. Silan, C., Uzun, O., Comunog˘lu, N.U., Golcen, S., Bedirhan, S., Cengiz, M., 2007. Gentamicin-induced nephrotoxicity in rats ameliorated and healing effects of resveratrol. Biol. Pharm. Bull. 30, 79–83.
26. Leonard, S., Xia, C., Jiang, B.H., Stinefelt, B., Klandorf, H., Harris, G.K., Shi, X., 2003. Resveratrol scavenges reactive oxygen species and effects radical-induced cellular responses. Biochem. Biophys. Res. Commun. 309, 1017–1026.
27. Wetscher GJ, Bagchi M, Bagchi G, Perdikis G, Hinder PR, Glaser K, et al. Free radical production in nicotine treated pancreatic tissue. Free Radic Biol Med 1995;18:877–82.
28. Kalpana, C., V.P.Menon, 2004. Protective effect of curcumin on circulatory lipid peroxidation and antioxidant status during nicotine-induced toxicity. Toxicol Mech Methods.,14:339–43.
29. Oktem G, Uysal A, Oral O, Sezer ED, Olukman M, Erol A. Resveratrol attenuatesdoxorubicin-induced cellular damage by modulating nitric oxide and apoptosis.Exp Toxicol Pathol 2012;64:471–9.
30. Boussarie D., 1999.Hématologie des rongeurs et lagomorphes de compagnie- Bull. Acad. Vét. De France., 72: 209-216.
31. Erel, O., 2004. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin. Biochem., 37: 277–285.
32. Erel, O., 2005. A new automated colorimetric method for measuring total oxidant status. Clin. Biochem., 38:1103–1111.
33. Esterbauer, H., Cheeseman, K.H., 1990. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Methods Enzymol., 186: 407–421.
34. Marklund S, Marklund G., 1974. "Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase". Europ. J. Biochem.,47: 469–474.

35. Aebi, D.J.,1986. Glucose-6-phosphate dehydrogenase. In: Bergmeyer HU, editor. Methodsof enzymatic analysis. 2nd ed. Weinheim: VCH Verlagsgesellschaft mbH., 190–6.
36. Hafeman D. G., Sunde R. A. and Hoekstra W. G. 1974."Effect of dietary selenium on erythrocyte and liver glutathione peroxidase in the rat". J. Nulr., 104, 580-587.
37. Prieto, P., M.Pineda, M.Aguilar, 1999. "Spectrophotometric quantitation of antioxidant capacity through the formation of a Phosphomolybdenum complex specific application to the determination of vitamin E". Anal. Biochem. ,269:337–341.
38. Suzuki, K., H.Ota, S.Sasagawa, T.Sakatani, T.Fujikura, 1983. Assay method for myeloperoxidase in human polymorphonuclear leukocytes. Anal Biochem., 132: 345–352.
39. Kamel MY, Hamed RR, A.,1980. colorimetric method for the determination of guanine and xanthine and its application to the assay of xanthine oxidase, Microchemical Journal, 25(3): 323-329.
40. Oktem, C., Uysal, A., Oral, O., Sezer, D.E., Olukman, M., Erol, A., 2012. Resveratrol attenuates doxorubicin-induced cellular damage by modulating nitric oxide and apoptosis. Exp. Toxicol. Pathol.,64:471–479.
41. Sosroseno, N., E.Sugiatno, A.R. Samsudin, M.F.Ibrahim, 2008. The role of nitric oxide on the proliferation of a human osteoblast cell line stimulated with hydroxyapatite. J. Oral Implantol., 34 (4): 106–202.
42. Hu, M.L.,1994. Measurement of protein thiol groups and glutathione in plasma. Methods Enzymol ., 233:380–5.
43. Gabe, M., 1968. Techniques histologiques [Histological Techniques]. Paris: Masson Publisher.
44. Cesta, M.F., 2006. "Normal structure, function, and histology of the spleen." Toxicologic Pathology .,34 (5): 455-465.
45. Yildiz,D.,2004. Nicotine, its metabolism and an overview of its biological effects. Toxicon ., 43:619–32.
46. Snyder, M.J., E.Hsu, R.Feyereisen,1993. Induction of cytochrome P-450 activities by nicotine in the tobacco hornworm, manduca sexta. J Chem Ecol .,19: 2903–10.
47. Carpagnano, G.E., S.A. Kharitonov, M.P. Foschino-Barbaro, O.Resta, E. Gramiccioni, P.J.Barnes,2003. Increased inflammatory markers in the exhaled breath condensate of cigarette smokers. Eur Respir J.,21(4):589–93.
48. Hackett NR, A.Heguy, B.G.Harvey, R.Kalpan,2003. Variability of antioxidant related gene expression in the airway epithelium of cigarette smokers. Am J Respir Cell Mol Bio.,29(3):733–44.
49. El-Shenawy, N.S., M.S. AL-Harbi, and R.Z. Hamza. 2014. Effect of vitamin E and selenium separately and in combination on biochemical, immunological and histological changes induced by sodium azide in male mice. Experimental and Toxicologic Pathology.,67: 65–76.
50. Paula, M.E., D.C. Mathangi and A. Namasivayam. 2003. "Free radical changes in methanol toxicity."Indian Journal of Physiology and Pharmacology.,47:207-211.
51. Hamza, R.Z., H.A.A. Ismail, H.E. Khaled, and N.S. El-Shenawy. 2015. "Antioxidant effect of Lcysteine on sodium-valproate-induced oxidative stress in rat liver: biochemical and ultrastructural approaches." Toxicological and Environmental Chemistry 97(8): 1041-1056.
52. Cheeseman, K.H. and T.F. Slater. 1993. "An introduction to free radical biochemistry". British Medical Bulletin., 49: 481-493.
53. Saito, H., Y. Yokoi, S. Watanabe, J. Tajima, H. Kuroda, and T. Namihisa. 1988. "Reticular mesh-work of the spleen in rats studied by electron microscopy." American Journal Anatomy .,181: 235–252.
54. Mebius, R.E. and G. Kraal. 2005. "Structure and function of the spleen." Nature Reviews Immunology., 5: 606–616.
55. Seymour, R., J.P. Sundberg, E.H. Hogen, 2006. Abnormal lymphoid organ development in immunodeficient mutant mice. Veterinary Pathology 43(4): 401-423.
56. Mosbah, R., M.I.Yousef, A.Mantovani,2015.Nicotine-induced reproductive toxicity, oxidative damage, histological changes and haematotoxicity in male rats: The protective effects of green tea extract. Experimental and Toxicologic Pathology., 67(3): 253-259.
57. Ahmed, M.A., K.H. Hassan, M.A. Khaled, H.W. Hassanein, 2014.Role of vitamin C and selenium in attenuation of nicotine induced oxidative stress, P53 and Bcl2 expression in adult rat. Pathophysiology., 21(3): 211-217.
58. Neogy,S., D.Subhasis, K.M. Santanu, M.Nirjal, R.Somenath, 2008.Amelioratory effect of Andrographis paniculata Nees on liver, kidney, heart, lung and spleen during nicotine induced oxidative stress. Environmental Toxicology and Pharmacology, 25(3):321-328.
59. Salahshoor, M., S.Mohamadian, S.Kakabaraei, S.Roshankhah, C.Jalili,2016. Curcumin improves liver damage in male mice exposed to nicotine. Journal of Traditional and Complementary Medicine., 6(2): 176-183.