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Research Article

# Resveratrol Attenuates the Nicotine-Induced Neurotoxicity in Rats

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## Abstract

Neurotoxicity is one of the side effects of nicotine (NIC). We investigated the protective role of resveratrol (RES) in the brain tissue of NIC-induced rats. Male albino rats were divided into four groups (n=10): control, NIC, RES and NIC + RES. All the treatments were applied intraperitoneally. The effects of NIC on some brain metabolic enzyme activities such as glucose-6-phosphate dehydrogenase (G6PD) and antioxidant enzymes as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), myeloperoxidase (MPO) and xanthine oxidase (XO) were evaluated. Lipid peroxidation (LPO) and nitric oxide (NO) levels were determined as well as the quantitative histopathological analysis was performed. All the biochemical parameters determined in the brain supernatant at the end of 4 weeks of exposure.NIC caused a significant decreased in SOD, CAT, GPx and G6PD activities and increased in LPO levels as well as MPO and XO activities compared to control group. Antioxidant enzymes activities were significantly increased with the depression of LPO level and the activities of MPO and XO in NIC + RES group compared to NIC group. The results indicate that treatment with RES ameliorate the brain injury and reduce oxidative stress and lesion induced by NIC.

**Keywords:** Nicotine; Resveratrol; Brain; Xanthine oxidase; Nitric oxide; Myeloperoxidase

## Introduction

Nicotine (NIC), 3-(1-methyl-2-pyrrolidinyl) pyridine disperses extensively to different organs, including the liver, kidney, spleen, lung, and brain [1]. The time required for NIC accumulation in different body organs is highly dependent on route and rate of dosing. NIC reaches the brain in 10 - 20 seconds [2].NIC in tobacco smoke inhaled into the lung is rapidly absorbed because of the large surface area of the alveoli and small airways as well as the dissolution of NIC in the fluid coating the epithelial layer of lungs. In our previous study, we found that resveratrol exerts its protective effect by improving the enzymatic/non-enzymatic antioxidant defense system as well as decrease the pathological changes in animals against the lung damage caused by NIC [3]. Hritcu., *et al.* [4] found that NIC-induced memory impairment is due to an increase in the brain oxidative stress in rats by reducing antioxidant enzymes activity, and increasing production of lipid peroxidation (LPO) and reactive oxygen species (ROS). Increasing the LPO plays an important role in causing of numerous human diseases. The initiation of LPO is caused by increasing the free radicals as superoxide, hydroxyl radicals and other ROS like $H_2O_2$ causing cellular injury. LPO levels increased in tissues (liver, lung, and heart) after intraperitoneal administered of NIC (0.6 mg/kg body weight) to rats [5]. NIC is oxidized to cotinine in the liver and causes the formation of ROS in tissues. The formation of these ROS parallel with depletion in glutathione (GSH) content in tissues causes oxidative damage [6]. It has been shown that NIC treatment at a dose of 2 mg/kg induced oxidative damage in both liver and kidney which were attenuated by the GSH supplementation [7]. Oxidative stress is a major mechanism for cellular damage associated with a wide variety of neurotoxicants [8]. Another study demonstrated deleterious effects of NIC in old rats as augmented by DNA damage, ROS concentration and lipid peroxides levels [9].

The brain is very sensitive to oxidative stress because it has abundant of polyunsaturated fatty acid and lack of antioxidant defense. Moreover, it is utilized a high content of $O_2$, because of a high metabolic rate and a high level of transition metals, that could lead to the formation of hydroxyl radical by Fenton reaction [10].

Resveratrol (RES) is a phenolic phytoalexin [11] that present in various foods, as grapes, plums, cranberries, and peanuts. RES has antioxidant properties [12] by regulating the endogenous cellular antioxidant systems. Grapeseed extracts containing RES were found to protect glial cells against oxidative stress [13] and exhibit neuroprotective properties [14]. However, its mode of action needs more investigation [15]. Robb., *et al.* [16] found that RES has the ability to increase the action of superoxide dismutase (SOD)

by a fourteen-fold. It has been reported in our laboratory that RES reduced lung injury induced by NIC through oxidative/antioxidant defence system and decrease the pathological changes [4].

However, brain tissue of rats after NIC administration. Therefore, we investigated the histopathological and biochemical effects of RES on NIC-induced the brain injury in rats by measuring stable metabolites of nitric oxide and major enzymes involved in the antioxidant defence of rat brain.

## Materials and Methods

### Chemicals

NIC hydrogen tartrate was obtained from Sigma Chemical Company, St. Louis, Missouri, USA. Trans-Resveratrol (> 98% purity) was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). All other chemicals and reagents used were of analytical grade and were purchased from Sigma Chemical Company, St. Louis, MO, USA.

### Sample Size

Male Wistar albino rats weighing 150-180g were purchased from the animal house of Faculty of Pharmacy, Zagazig University, Zagazig, Egypt and housed in the animal laboratory. The animals were fed a standard pellet diet (Valley Group Co., Egypt) and water *ad libitum* as well as kept on a physiological day-night rhythm. We have followed the European Community Directive (86/609/EEC) and national rules on animal care that was carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals 8th edition. The animals were acclimatized for 14 days prior to their use in experiments.

Animals were randomized into four groups of 10 rats each and were treated intraperitoneally (i.p) as below for a month: control group was received physiological saline (NaCl 9%; 1 mL/kg b.w.), NIC group was received NIC (2.5 mg/kg/ b.w.) in physiological saline [17], RES group was treated with 20 mg/Kg/ b.w. [18], finally, NIC + RES group was received the same dose of NIC and followed by RES at the same doses.

### Preparation of Homogenate Brain

Forebrain tissues were obtained according to the anatomical structure of the brain in Figure 1 for biochemical analysis; the specimens were washed out from contaminating blood with ice-cold buffered saline. They were weighed and cut very thinly with a clean scalpel blade and then homogenized in 0.15M ice-cold KCl for 3 min at 16,000 rpm with a homogenizer (Ultra Turrax Type T-25-B; Labortechnic, Staufen, Germany). The homogenates were then centrifuged for 1h at 4°C at 5000 xg. All the oxidative/antioxidant parameters were done at the supernatant.

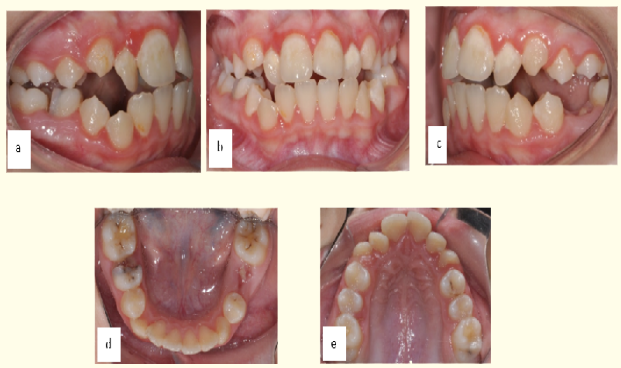

**Figure 1:** Diagram Showing Different Parts of the Vertebrate Brain and the Obtained Part is Forebrain.

### Evaluation of Oxidative Stress

MPO is a peroxidase enzyme that was determined using fluorometric assay kit [19]. The MPO presented as nmol/min/mL.

The LPO was estimated as the concentration of thiobarbituric acid reactive product malondialdehyde (MDA) by using the method of Ohkawa., *et al.* [20]. The amount of MDA was calculated as nmol/g.

The levels of nitrite, a stable end-product of nitric oxide (NO) production, were measured spectrophotometrically based on the Griess reaction [18] and expressed as µmol/g.

Total oxidative status (TOS) was measured in the brain using a commercially available kit from Rel Assay Diagnostics [21]. This assay was calibrated with hydrogen peroxide ($H_2O_2$), and the results were expressed as µmol $H_2O_2$ eq./L.

### Evolution of Enzymatic Antioxidant

Xanthine oxidase (XO) activity was assayed spectrophotometrically by the reaction of the enzyme with xanthine, as a substrate, and the absorbance was measured at 650 nm, according to the method described in Litwack., *et al.* [22]. 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.

SOD activity was measured according to Marklund and Marklund [23] and calculated as the amount of protein that caused 50 % pyrogallol auto-oxidation inhibition. SOD activity is expressed as nmol/g tissue. CAT activity was determined according to Aebi [24] and expressed as U/g tissue. GPx activity was determined as described by Hafeman., *et al.* [25] and expressed in mol/g GSH consumed min/g/wet weight tissue. Tissue glucose-6-phosphate dehydrogenase (G6PD) activity was measured in the brain homogenates using the methods of Aebi [26]. The results were expressed as U/g tissue.

### Evaluation of Non-Enzymatic Antioxidant

Total antioxidant status (TAS) was measured in the brain using a commercially available kit from Rel Assay Diagnostics (Gaziantep, Turkey) [27]. The method was calibrated using the vitamin E analog Trolox, and data were expressed as mmol Trolox eq./L.

Total thiols level was determined according to Hu [28] and results are expressed in mM/g. Protein concentration in the homogenate of the brain was determined by the method of Bradford [29] using bovine serum albumin as standard.

### Histological Evaluation

For histological examination, a portion of the brain was fixed in 10% neutral buffered formalin embedded in paraffin, sectioned and stained with hematoxylin and eosin as described by Gabe [30]. Then Semi-thin sections (0.5-1 microns) were prepared by using LKB ultramicrotome. The sections were stained with toluidine blue, examined with a light microscope and photographed. Semi-quantitative histopathologic assessment of slides evaluated while blinded to exposure status, followed by nonparametric statistical analysis, is an established standard technique for evaluating morphologic changes in tissue sections from toxicology studies [31].

### Statistical Analysis

Data were presented as means ± standard error. The one-way ANOVA analyses of variance followed by Tukey-Kramer multiple comparisons test were performed on the data of biochemical variables to examine differences among groups. The difference was considered statistically significant if $p < 0.05$.

## Results

Effect of NIC or/and RES on enzymatic antioxidant (SOD, CAT, GPx, and G6PD) activity of the brain rats are shown in Figure 2. There were significant falls ($p < 0.05$) in SOD, CAT, GPx and G6PD activity of the brain after nicotine administration. A significant increase was observed in the NIC + RES group compared to the NIC-treated group ($P < 0.05$) for all the antioxidant enzymes activities.

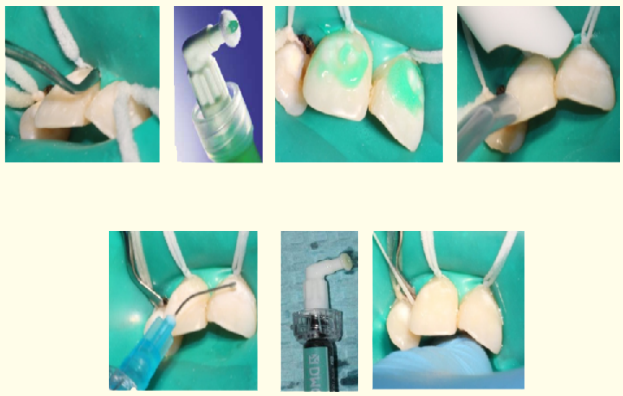

**Figure 2:** Effect of resveratrol on SOD, CAT, GPx and G6PD activity in the brain after nicotine administration to rats. The data are expressed as mean ± SE (n = 10). aP < 0.05, compared with control group. bP < 0.05 compared with nicotine-treated group.

As the inspection of Figure 3 indicates tissue MPO and XO activities were significantly higher in the NIC group in relation to the control group. Tissue MPO and XO activities decreased significantly in the NIC + RES group compared to the NIC-treated animals ($P < 0.05$).

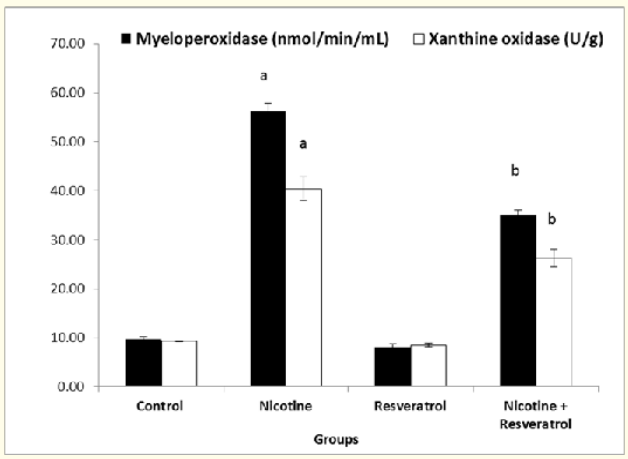


**Figure 3:** Effect of Resveratrol on the Activity of MPO and XO in the Brain after Nicotine Administration to Rats. The Data are Expressed as Mean ± SE (N = 10). Ap < 0.05, Compared with Control Group. Bp < 0.05 Compared with Nicotine-Treated Group.

No significant difference was determined in the brain tissue NO, MDA, TOS, thiol and TAS levels between RES and control animals (P > 0.05). MDA is an important marker of LPO. Tissue MDA as well as NO, and TOS levels were increased significantly in NIC group compared to control rats, while Thiol and TAS levels were decreased significantly (Table 1). Levels of NO, MDA and TOS were decreased significantly in the NIC + RES group compared to the NIC-treated animals (P < 0.05), while thiol and TAS levels rose significantly.

**Table 1:** Effect of resveratrol on oxidant- antioxidant levels in brain tissue of rats treated with nicotine.

| Parameters | Groups | | | |
|---|---|---|---|---|
| | Control | Nicotine | Resveratrol | Nicotine + Resveratrol |
| Nitrite (µmol/g) | 1.29 ± 0.14 | 3.10 ± 0.22 [a] | 0.99 ± 0.17 | 1.37 ± 0.31 [b] |
| MDA (nmol/g) | 4.62 ± 0.33 | 41.53 ± 5.05 [a] | 3.66 ± 0.39 | 17.09 ± 1.06 [b] |
| TOS (µmol $H_2O_2$ eq./L) | 0.85 ± 0.12 | 2.85 ± 0.43 [a] | 0.80 ± 0.08 | 1.81 ± 0.09 [b] |
| Thiol (mmol/mL) | 0.48 ± 0.09 | 0.25 ± 0.05 [a] | 0.49 ± 0.04 | 0.43 ± 0.04 [b] |
| TAS (mmol Troloxeq./L | 0.69 ± 0.07 | 0.27 ± 0.05 [a] | 0.74 ± 0.04 | 0.49 ± 0.04 [b] |

The data are expressed as mean ± SE (n = 10). aP < 0.05, compared with the control group. bP < 0.05 compared with nicotine-treated group.

## Histopathological Observation

Effects of RES on the brain histology of rats that treated with NIC are presented (Figure 4). Control brain tissue formed of round and pyramidal-shaped neurons surrounded by eosinophilic glial fibres (Figure 4A, 4B). Histological sections of control rats showed normal architecture with distinct cortical layers: outer molecular, inner granular cell layer, between which is the single layer of large neurons called Purkinje cells; the central medullary region was also observed. Resveratrol group showed neuron bodies with rounding to oval nuclei, a glial cell in fibrillary background and sulci of brain tissues (Figure 4C,4D).

Congestion with a mild area of haemorrhage between the compartments of the brain tissues has been observed in NIC-treated animals with disorganization of normal architecture (Figure 4E,4F). Few classic degenerating Purkinje neurons with a condensed nucleus and eosinophilic cytoplasm were seen. After 30 days of NIC treatment, many neutrophils and monocytes were observed in the cerebral cortex. Treatment the rats with NIC and RES improved the brain tissues as compared to NIC-group, there are fragments separated by very mild congested area (Figure 4G,4H).

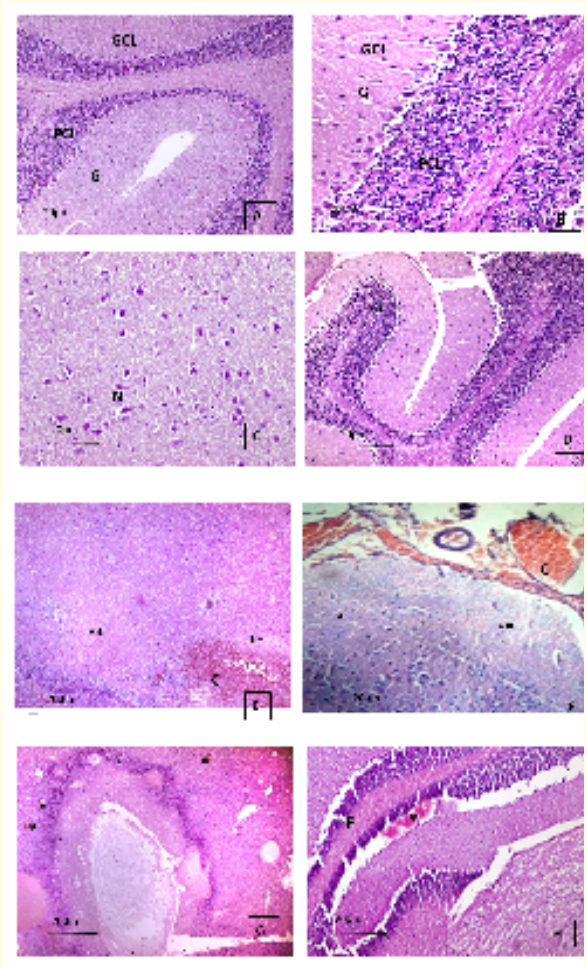


**Figure 4:** Photomicrographs of cerebral cortex stained with hematoxylin and eosin after 30 days of resveratrol or/and nicotine administration to rats. Figs. 4A and 4B: Control brain tissue formed of round and pyramidal-shaped neurons surrounded by eosinophilic glial fibers (G); light area presents the granular cell layer (GCL) and the dark area present pyramidal cell layer (PCL) Figs. 4C and 4D: Resveratrol group showed neuron bodies (N) with rounding to oval nuclei, a glial cell in fibrillary background and sulci of the cerebral cortex in brain tissues. Figs. 4E and 4F: Nicotine group showed necrotic area (**) in the brain tissues with pyknotic nuclei and congestion (C) with a mild area of hemorrhage between the compartments of brain tissues with the appearance of neutrophils as a marker of inflammation. Figs. 4G and 4H: Nicotine + resveratrol group showed restoration of brain tissues to a normal level and fragment (F) separated by very mild congested area (*). All the photomicrographs are with scale bar 20 µm. (Figs. 4A, 4C, 4E, 4G are X100) and (4B, 4D, 4F, 4H are X400).

**Table 2:** Histopathological Findings in Brain Tissue of Rats Treated with Nicotine or/and Resveratrol.

| Findings Control | Groups | | | |
|---|---|---|---|---|
| | Control | Nicotine | Resveratrol | Nicotine + Resveratrol |
| **Round and pyramidal shaped neurons** | ++++ | ------ | ++++ | +++- |
| **Eosinophilic glial fibers** | ++++ | ++– | ++++ | +++- |
| **Congestion with mild area of hemorrhage** | ------ | ++++ | ------ | —+ |
| **Neuron bodies with rounded to oval nuclei** | ++++ | ------ | ++++ | +++- |
| **Normal brain tissues fragment** | ++++ | ------ | ++++ | +++- |
| **Congested brain tissues** | ------ | ++++ | ------ | —+ |

| Symbol | meaning |
|---|---|
| ------ | The absence of the change in the animals of the studied group. |
| ++++ | A change which was often found in all the studied animals. |
| +++- | A change which was observed in almost all the studied animals. |
| ++– | A change not so often observed in all animals of a group. |
| —+ | A change which was rare within a group |

## Discussion

Nicotine-induced oxidative stress in the brain that was prevented by RES in this study. Although it is known that RES prevents oxidative stress in different tissues [32-34] to our knowledge there was no study reporting the counteracting effects of RES against NIC that induced oxidative stress in rat brain.

Our data confirmed previous work about NIC-induced toxicity in the brain [35]. In the present study, a significant increase in MDA levels was observed in the NIC group, indicating increased LPO. This result is in agreement with the previous study that i.p. nicotine administration increased TBARS levels of mitochondrial and microsomal fractions of the brain tissue in (2)-nicotine hydrogen tartrate administered (1.6 mg/kg) rats for 10 days [36]. Elevation of LPO could be due to excessive production of ROS. Previous studies have reported that oxidative stress is the mechanism responsible for toxicity developing in the brain tissue due to NIC [7, 35].

There are various antioxidant mechanisms in the cell to reduce the levels of excess ROS induced by NIC as antioxidant enzymes. In the present study, decreasing the SOD, CAT and GPx activity in NIC-treated rats suggested membrane damage may be due to free radical generation. SOD and CAT react against radicals such as superoxide and hydrogen peroxide, respectively. However, GPx has a scavenging effect against alkyl, alkoxyl and peroxyl radicals that can form from oxidized membrane compounds, and uses GSH as a substrate [35]. The current study confirms the previous work of Jain and Flora [37] who found that increasing the concentration of LPO in nicotine treated rats is associated with decreasing the activities of SOD and CAT. Parallel to our findings, those recorded by Helen., *et al.* [5] who found that SOD and CAT decreased in different tissue in treated rats with NIC.

Tian., *et al.* [38] found that G6PD is very important in mammalian cell growth and it is the only source of NADPH for erythrocytes to control the oxidative stress [39]. The decreasing of NADPH production is direct affects the production of both GSH and CAT, thus inhibiting both pathways that discard $H_2O_2$. In the present study, NIC administration at a dose of 2.5 mg/kg body weight resulted in a significant decrease in G6PD activity in the rat brain compared to control, that could be due to depletion of GSH.

The study showed a significant increase in NO production in the brain tissue as the effect of NIC. This data is paralleled with Das., *et al.* [35] who found that NIC produces NO, which extinguished the mitochondrial oxidative stress scavenger system in different brain parts. The formation of NO in the brain mitochondria may have an important consequence because this compound binds to the haem group in cytochrome oxidase and inhibits respiration [40]. This may stimulate O2”- formation, which may react with more NO,

forming peroxynitrite, an oxidant capable of inhibiting important enzymes and affecting mitochondrial integrity.

MPO measurement act as a marker for quantification of infiltrated inflammatory cells in the brain [41].

The correlation between MPO activity (neutrophil infiltration) and brain injury after cerebral ischemia had been studied before where Chang., *et al.* [42] reported that over-activation or deficiency of MPO caused pathological symptoms in the brain. The present study was designed to evaluate the MPO activity after NIC or NIC with RES treatment. This is the first time to study that correlation between RES ameliorative effect and NIC toxicity so it is very difficult to compare our result with other studies.

Increase the brain MPO activity was correlated with neutrophil infiltration in histological observation of NIC-treated rats and depletion of neutrophils by treatment RES significantly observed. These results strongly suggest that the invasion of neutrophils into the ischemic areas is implicated in the development of brain injury.

Under the pathological condition, the XO is formed by oxidation or proteolysis [43]. XO is able to catalyze the reduction of oxygen causing the formation of superoxide and $H_2O_2$ that play a crucial role in the mechanism of oxidative injury [43]. It is also broken down the xanthine and formed $H_2O_2$ and hydroxyl radicals. XO has several mechanisms to induced neuronal damage [44]. Therefore, increasing the XO in NIC-treated rats in the present study caused oxidative injury in the brain.

Resveratrol has protective properties on neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease due to its antioxidant effect. In our study, brain tissue MDA levels were significantly lower in the NIC + RES group, while GPx and other antioxidant enzymes activity increased. Previous studies have reported that RES lowers MDA levels and reduces oxidative stress by increasing GSH levels in ethanolic induced-rats [45] and RES may also function as the antioxidant enzymes [46]. Moreover, the NO levels of NIC +RES group were significantly lower than those of the NIC animals.

Previous studies have also shown the antioxidant effects of RES via elevation of the antioxidant status such as GSH and SOD [46,47]. Venturini., *et al.* [48] observed that decreasing LPO and increasing the antioxidant activity in hippocampus and frontal cortex as the result of treating the diabetic rats with RES.

It also improved rat dorsal neuronal function after spinal cord injury by improving the energy metabolism [49]. This effect could be due to the RES acts as the SOD1 and GPx1 [46]. Yulug., *et al.* [50] reported that the protective effect of RES could be due to its preservation of cellular membranes against oxidative damage. These effects are related to its properties as anti-oxidation, anti-inflammation, and anti-apoptosis [49].

Moreover, RES protects against rotenone-induced neuronal injury through MPO and NO [42]. Thus, it could be its mechanism to control the toxicity of NIC in the brain of rats in the current study where RES + NIC reduced XO and NO level. However, RES + NIC increased significantly G6PD activity compared to NIC group. This effect of RES seems to be essential in response to oxidative stress.

Reducing the XO activity in NIC + RES group decreased the free radical production. Our results are in parallel with Kanemitsu., *et al*. [51] who found that inhibiting XO activity prevents generation of uric acid in the rat brain after cerebral ischemia.

Microscopic examinations of the brain tissue showed significant changes as the effect of NIC. Apoptosis which appear in NIC-treated rats could be due to the interaction of NIC receptors and NIC results in cell death in the brain tissue as previously described by Denissenko., *et al.* [52]. We found that NIC causes histopathological lesions in cerebral cortex including neuronal degeneration as cytoplasmic vacuolization hemorrhage, ghost cell, and gliosis. Our histopathological findings are correlated to those of Tewari., *et al.* [53] found that the adult cerebellum is damaged by decreasing the white core of cerebellum as the effect of NIC treatment. The treatment of rats with RES previous to NIC reduced most of the observation in NIC group as the effect of NIC on hippocampus MPO activity have been previously documented [54]. Yin., *et al.* [55] found that resveratrol has protective effect on the nervous system by regulating the potassium channels that effect on various physiological functions.

## Conclusion

Impairment the antioxidant system could be the mechanism for cellular damage associated with the neurotoxic effect of NIC. RES showed the neuroprotective effect against NIC toxicity by reducing oxidative stress and lesion, therefore, it could be used as therapeutic against nicotine toxicity to the brain.

## Conflicts of Interest

The authors have no conflicts of interest relevant to this article.

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