Original Article

Deleterious Effect of Toluene on Rat Cerebrum Cortex and the Exert Effect of Resveratrol: (Histological, Immunohistochemical and Ultrastructural Study)

Rania Said Moawad, Eman Ramadan Abd El Fattah and Amira Ebrahim Alsemeh

Department of Human Anatomy and Embryology, Faculty of Medicine, Zagazig University, Egypt

ABSTRACT

Background: Toluene is a chemical hydrocarbon that affects mainly the central nervous system. Resveratrol has a large spectrum of beneficial health effect.

Aim: The study aimed to assess the protective effect of Resveratrol (RES) on cerebrum injury in rat caused by toluene (TN).

Materials and Methods: Thirty adult male Wistar albino rats were used in this study, each weighting 200-250 g, aging 4-6 months. They were separated into five equal groups, Control group: The animals had only regular diet and tap water, Corn oil group: was given 0.1 ml/10 g/day corn oil, intraperitoneally, TN-treated group: The animals received Toluene intraperitoneally with a dosage of 500 mg/kg/day (n ¼ 7) (0.1 g/ml, in corn oil) (0.5ml/kg/day) for 14 days, RES group: The animals received resveratrol 10 mg/kg/day intraperitoneally for 14 days, and TN+RES treated group: The animals received toluene alongside supplemented with resveratrol (as previous doses) for 14 days. All rats were anesthetized, scarified and the brain was dissected out. Parts of brain tissues were processed for tissue homogenates for measuring MDA, SOD, Gpx. A nd other parts of the brain tissues were subjected to histopathological examination (light and electron microscopic examination). Immunohistochemical staining for BCL2, BAX, GFAP and synaptophysin were performed with morphometric and statistical analysis.

Results: Toluene revealed increment in MDA together with a decrement of SOD and Gpx activity. Moreover, Toluene exhibited obvious histopathological deterioration with over expression of Bax protein and glial fibrillary acidic protein (GFAP) and down expression of Bcl2and synaptophysin. RES restored the biochemical profile of oxidative damage and improved the histopathological and immunohistochemical picture.

Conclusion: In conclusion, our results speckled a bright on the probable effective role of RES on cerebrum injury induced by toluene and provide available suggestion on the potential clinical effectiveness of RES supplementation in case of cerebrum injury.

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Key Words: Cerebrum, morphology, rat, resveratrol, toluene.

Corresponding Author: Rania Said Moawad, MD, Department of Human Anatomy and Embryology, Faculty of Medicine, Zagazig University, Egypt, **Tel.**: +2055 3651701, **E-mail:** karimwaleed62@gmail.com

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INTRODUCTION

In nature, in crude oil and tolu trees, toluene is available and is delivered through the discharge of gasoline and other fuels from crude oil and burning of coke[1]. Gasoline (main source of toluene exposure) is delivered to the air during creation, transportation, and consuming. There for, toluene is mainly present in refineries, around gas stations and areas with heavy traffic. In addition, when a person smoked out a cigarette, he exposed to 80–100 µg of toluene[2]. Paints, varnish, paint thinners, rust preventers, shellac, solvent based cleaners, glues, and some cleaning products are the main products containing toluene. It is utilized additionally as a dissolvable in beautifying products and normally in I benzyl chloride, benzoic acid, vinyl toluene, phenol cresol, toluene diisocyanate construction, and trinitrotoluene (TNT)[3].

TN is a chemical hydrocarbon which is quickly absorbed through the respiratory system and gastrointestinal tract and, to a lesser degree, through the skin. The main target of TN is the central Nervous System (CNS). Symptoms that may occur are fatigue, easy drowsiness, headaches, and nausea[4].

Toluene has a lipophilic nature, so it changes the structure of the cell wall lipid. In acute doses, toluene elevates Na/K-ATPase activity, so it increases membrane fluidity[5].

Toluene crosses the blood brain barrier with subsequent effects on behavior, cognition, and neuronal integrity[6]. Cheerfulness followed by relaxation and lethargy are the acute effects of toluene exposure. Motor impairment in the form of ataxia and confusion can also develop, which might proceed to coma and death[7]. Long term abuse of toluene results in serious and long-lasting effects on the central nervous system, including white matter abnormalities, brain atrophy, leukoencephalopathy, and dementia[8].

Oxidative stress has been concerned with toluene neurotoxicity. Studies shown that a toluene dose 0.5–1.5 g/kg via intraperitoneal injection increase the generation

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of reactive oxygen species (ROS) in crude mitochondrial fractions from rat cerebellum, striatum, and hippocampus[9]. Lipid peroxidation products increased in the hippocampus, cortex, and cerebellum[10] and protein carbonyls increased in the frontal cortex and cerebellum of rats following weeks of toluene inhalation[11] Oxidative DNA damage has also been detected in the liver and kidney of rats after toluene inhalation for 7 days[12].

Resveratrol (RES) is a polyphenolic phytoalexin which richly found in plants such as plums, berries grapes, and peanuts and found in many commercial products such as grape juice and red wine with relatively high concentration[13].

Resveratrol has a wide health property including cardioprotective[14] antiproliferative[15] an neuroprotective properties[16] as it has antioxidant and anti-inflammatory effects, and also it has a regulatory effect of apoptosis and autophagy[17-20].

In the current work we inspected the toluene exposure effects on cerebrum of adult male rats and assess the possible protecting effect of resveratrol.

MATERIAL AND METHODS

Chemicals

1. Toluene, was gotten from Sigma Chemical (St. Louis, MO, USA). Toluene was freshly prepared in corn oil to obtain the required doses.
2. Corn oil: was brought from commercial sources.
3. Resveratrol: was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Experimental animals: Thirty healthy adult male Wistar albino rats were used in this study, gotten from the animal house" Faculty of Medicine, Zagazig University, Egypt" weighing 200-250 g, aging 4-6 months. Animals were group-kept under temperature and light-regulated environments with typical laboratory rodent chow and water provided ad libitum. All exploratory strategies were acted as per the rules of the Institutional Animal Care and the norms of Ethical Committee of Faculty of Medicine; Zagazig University (IACUC approval: ZU-IACUC/3/F/135/2020). The rats were divided into five groups (n = 6 per group) as follows:

Control group: The animals had only regular diet and tap water to determine the basic parameters.

Corn oil group: was given 0.1 ml/10 g/day corn oil, intraperitoneally (TN vehicle).

TN-treated group: The animals of this group received Toluene intraperitoneally with a dosage of 500 mg/kg/day (n ¼ 7) (0.1 g/ml, in corn oil) (0.5ml/kg/day) for 14 days[21].

TN+RES treated group: The animals of this group received toluene 0.5ml/kg/day intraperitonially[21] alongside supplemented with resveratrol 10 mg/kg/day intraperitoneally[22] for 14 days.

RES group: The animals of this group received resveratrol 10 mg/kg/day intraperitoneally[22] for 14 days.

After 14 days of the experiment, the rats in all groups were anesthetized and all animals were sacrificed. The cerebrum samples were then quickly dissected and existed carefully. The brain divided mid sagittal, the right half of the cerebrum from all groups were immediately immersed in 10% formol saline for light microscopic examination. Left half was divided into two parts, one washed with ice-cold phosphate-buffered saline (pH 7.4) and stored at −80 °C for biochemical analysis of oxidative stress (MDA, SOD, and Gpx) and the other part cut into small pieces and immediately fixed in 3% glutaraldehyde-formaldehyde at 4 °C for electron microscopic examination.

Biochemical analysis

The portions from the left half of cerebrum from all groups that were saved for biochemical analysis of oxidative stress, were homogenized by a tissue homogenizer. The homogenates were centrifuged at 10,000g for 20 min at 4°C, and the supernatants were gotten; malondialdehyde (MDA), superoxide dismutase (SOD) activity and glutathione peroxidase (Gpx) levels were resolved as recently depicted[23].

Tissue preparation for histopathological and immunohistochemical analyses

Light Microscope Techniques

Right half of the cerebrum from all groups was immediately immersed in 10% formol saline for 12 hours to be processed and embedded in paraffin according to paraffin technique. Thereafter, 5-µm-thick sections were cut and stained with H&E[24]. Light microscopy (Leica ICC50W) in the Image analysis unit of the Department of Anatomy and Embryology, "Faculty of Medicine, Zagazig University" was used to examine all stained sections.

Electron Microscope Techniques

The other portion of the left half of cerebrum from all groups were immediately fixed in 3% glutaraldehyde-formaldehyde at 4 °C for 18–24 h, rinsed in phosphate buffer, then post fixed in 1% osmium tetroxide. The specimens were then dehydrated in a series of alcohols, cleared in propylene oxide, and finally embedded in Epon epoxy resin. After that, the blocks were trimmed and sectioned with glass knives by an ultra-microtome. Semithin sections (1 mm) were stained with toluidine blue and examined on light microscope to select the suitable area for the ultrathin sections. Ultrathin sections (70–90 nm) were cut on the same ultramicrotome and stained with uranyl acetate and lead citrate[25]. The ultrathin sections were studied and photographed using a JEOL 1010 electron microscope at the Mycology and Regional Biotechnology Center "Al Azhar University, Cairo, Egypt".

Immunohistochemical analysis

Immuno-staining was done on paraffin blocks sections that have 4 µm thickness to identify Bcl-2, Bax, SYN and

GFAP immunoreactivity. After that, the sections were then submerged in 10 mM citrate buffer (pH 6.0) and heated in a water bath at 98 °C for 30 min. The sections washed under running tap water after allowed them to cool in 20 min. Then the sections were preserved with 3% hydrogen peroxide in methanol for 15 min to block endogenous peroxidase activity. The horse serum was used to block at room temperature for 10 min, then, the primary monoclonal antibody (Mouse anti-EMA, ICN, Costa Mesa, CA, USA and Sigma Chem. Comp.) of anti-Bcl-2, anti-Bax[26], anti-GFAP[27], or anti-SYN[28] was incubated at 4 °C in a humidified chamber for 24 h. Using 3,3-diaminobenzidine (DAB) solution to develop the color. 0.1% hematoxylin was used for 5 min to stain the sections.

Morphometric analyses

For morphometrical study, ImageJ software analyzer computer system (Wayne Rasband, NIH, Bethesda, Maryland, USA) was used to analysis the 5 perceptive non over lapping fields from Bcl-2, Bax, SYN and GFAP immunostaining sections from 6 rats /group at 400× magnification. The area percentage of both GFAP and SYN immunoreactions were analyzed and the numbers of Bcl2 and Bax immuno-positive cortical neurons were calculated according to the method described by Varghese *et al*.[29].

Statistical analysis

Continuous variables were represented by the mean ± SE as the data showed normal distributions (parametric). The Kolmogorov-Smirnov test was used for checking the normality. Testing the significant differences between groups, One-way ANOVA was used. Post hoc Tukey's test was used for multiple comparisons between groups. At $P < 0.05$, the differences were considered significant. All statistical comparisons were two tailed. All statistical assessment was done using Graph pad Prism software, version 5.0 (Graph Pad Software, San Diego, CA, USA)

RESULT

I- Biochemical analysis

Regarding to antioxidant biomarkers levels (SOD, Gpx), there were non-significant differences among control group, corn oil and RES groups (P>.05). In TN treated group, SOD and Gpx levels were significantly decreased compared to control values (P<0.05). But SOD and Gpx levels were significantly increased in TN + RES treated group compared to TN treated group value (P<0.05). Regarding brain lipid peroxidation biomarker level (MDA), there were no statistically significant differences among control group, corn oil and RES groups (P>.05). MDA level was significantly increased in TN treated group compared to control values (P<0.05). In TN+RES group, MDA level was significantly decreased compared to TN treated group (P<0.05). These resulted as illustrated in (Table 1)

II- Histopathological analysis

Histological results using Hematoxylin and eosin staining:

H&E-stained parasagittal sections of the control and corn oil cerebrum showed nearly similar normal neuronal configuration and cortical morphology with intact pia matter attachment. Cerebral cortex was divided into 6 layers, 3 outer and 3 inner cortical layers. The outer cortex showed small cortical neuronal cells. The inner cortex showed large cortical neurons that had vesicular nuclei with rim of basophilic cytoplasm. These cortical neurons were embedded in normal homogenous acidophilic neuropil with intact blood vessels that had narrow perivascular spaces. Neuroglial cells had well demarcated lightly or darkly stained nuclei (Figures 1A-C).

TN treated group revealed a clear disturbance in the layers of the cerebrum cortex with dilated and congested blood vessels and pia matter detachment (Figure 2A). The outer &inner cerebrum cortex demonstrated dark shrunken cortical neurons that had deeply stained pyknotic nuclei with pericellular vacuolations. Vacuolization within neuropils with dilated and congested blood vessels and many glial cells' nuclei were noticed. Few normal cortical neurons were seen (Figures 2A-C).

Alongside treatment of RES with TN in rats revealed nearly similar normal histological morphology of the frontal cortex and better influence on the cortical neurons which exhibited normal appearance with vesicular nuclei and basophilic cytoplasm, but few cortical neurons were still degenerated with pyknotic and darkly stained nuclei. Congested dilated blood vessels and few normal ones can be observed. Glial cells appeared normal that had well demarcated lightly or darkly stained nuclei with pericellular space embedded in acidophilic neuropil (Figures 3A-C).

Histological results using Toluidine blue staining

Semithin sections of toluidine blue of control groups reveal intact cortical neurons with open-faced nuclei encircled by pale cytoplasm that having basophilic Nissl's granules with intact long process. Glial cell with sharply rounded demarcated nuclei of glial cells and normal blood vessel can be observed within the intact neuropil (Figure 4A). In contrary, TN treated group showed deeply stained cortical neurons and few normal neurons were also noticed. Vacuolated neuropils with dilated blood vessels that encircled by a wide perivascular space could be seen. Glial cells and perineural glial cells beside the degenerated neurons could be observed (Figure 4B). However, alongside treatment of RES with TN showed that the most cortical neuronal cells were normal and scarce of them with intensely stained nuclei. Blood vessels with slightly wide perivascular space embedded in intact neuropil could be noticed. Glial cells and perineural glial cells beside the degenerated neurons and the intact neurons could be observed (Figure 4C).

III. Immunohistochemical analysis

Bax and Bcl2 immunoreactivity were evaluated to assess the apoptotic and antiapoptotic status of the cerebral cortical neurons, respectively. Negative Bax immunoreactivity

and positive brown Bcl2 immunoreactivity were noticed in the cortical neurons' cytoplasm of control group (Figures 5A,6 A). While in TN treated group, the cytoplasm of apoptotic cortical neurons showed positive brown immunoreactivity for Bax and negative immunoreactivity for Bcl2 (Figures 5B,6B) However, in TN+RES group, most cortical neurons were exhibited negative immunoreactivity for Bax and positive immunoreactivity for Bcl2 (Figures 6C,7C). These results were confirmed by morphometrical and statistical analysis by counting the immuno-positive neurons of both Bax and Bcl2 as shown in (Figures 5D,6D) respectively.

GFAP immunostaining of astrocytes were performed to evaluate the compensatory mechanism of the astrocyte to the cerebral injury. In control group, the astrocytes looked small and scarce in number with short thin processes (Figure 7A). In contrast, the TN treated group revealed higher immuno-expression of GFAP in the astrocytes that increased in size and number with elongated numerous processes (Figure 7B). However, in TN+RES treated group the GFAP immuno-expression in the astrocytes decrease where most of them appeared small with few short thin processes and few others still large with thick multiple processes (Figure 7C). These results were confirmed morphometrical and statistical where the area % of GFAP immuno-expression in the astrocyte increased significantly compared to the control group. Otherwise, in TN+RES treated group the area % decrease significantly compared to TN treated group as presented in (Figure 7D).

Synaptophysin immunostaining was performed to evaluate the integrity of the cortical neurons synapse, the immuno-reactivity was observed at the outer surface of the neuron in the form of dense coarsely fine beaded appearance in control group. The scattering of the reactive granules was also noticed in the neuropil between the neurons (Figure 8A). In TN treated group the synaptophysin reactivity was decreased at the margin of the affected neurons and within the neuropil (Figure 8B). However, Alongside treatment of RES with TN increased synaptophysin immuno-expression nearly as control group (Figure 8C). These results were ascertained morphometrically and statistically where the area % of synaptophysin reactivity was decreased in TN treated group compared to control group however, treatment with RES alongside the TN increased the area % of synaptophysin reactivity that still revealed a significant difference from the control group as shown in (Figure 8D).

IV. Electron microscopic results

In control group, all normal ultrastructural cytoplasmic organelles were appeared. Cortical neurons had well demarcated euchromatic nuclei with normal nuclear demarcation. Normal mitochondria were observed. Rough endoplasmic reticulum were present as parallel tubules near the nucleus (Figures 9a,b,c). The axon`s cores were covered with thick myelin sheathes (Figure 9d). Astrocytes formed from rounded euchromatic well demarcated nucleus that surrounded by translucent cytoplasm (Figure 9e). However, in TN treated group, there were signs of neural degeneration. The nucleus was small with nuclear membrane breakdown and irregular shaped clumps of condensed chromatin. There were shrunken cytoplasm, swollen mitochondria, dilated cisternae of endoplasmic reticulum, lysosomes, and vacuolization in the cytoplasm (Figure 10a). There was thinning irregular myelin sheath and some nerve fibers showed loss of myelin sheath (Figure 10 b). Astrocyte had small shrunken heterochromatic nucleus with irregular nuclear membrane (Figure 10c). In TN+RES group, the cortical neurons preserved most of the normal appearance with rounded euchromatic nucleus. Some cytoplasmic organelles showed signs of degeneration as moderately swollen mitochondria and slightly dilated cisterns of endoplasmic reticulum (Figures 11a,b). Neuronal axon showed normal mitochondria and most myelinated nerve fibers were surrounded by thick regular myelin sheath and few myelinated nerve fibers were surrounded by thin myelin sheath (Figure 11c). Astrocyte with normal euchromatic nucleus (N) surrounded by electron lucent cytoplasm was observed (Figure 11d).

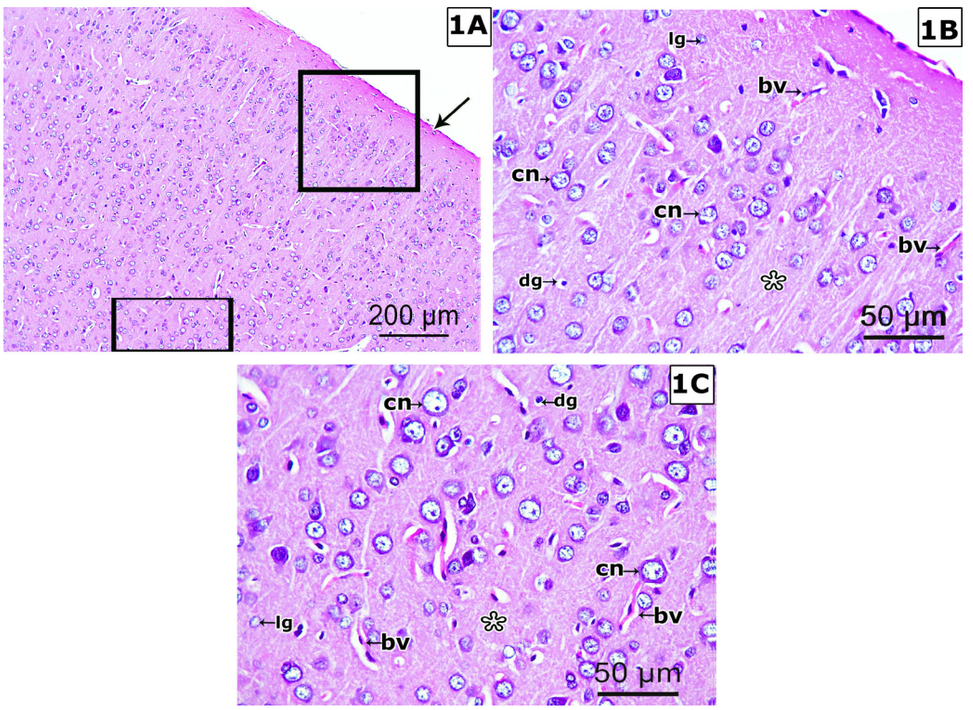

Fig. 1: Photomicrographs of sections in rat's cerebral cortex of the control group showing: A) well organized frontal cortex layers and normally attached pia matter (arrow). B) Higher magnification of the upper square in outer layers of cerebral cortex showing small cortical neuronal cells (cn) with vesicular nuclei and basophilic cytoplasm. Blood vessels with narrow perivascular spaces (bv) and glial cells with either lightly (lg) or dark (dg) stained nuclei are seen within the acidophilic neuropil (*). C) Higher magnification of the lower square showing large neuronal cortical cells with vesicular nuclei and basophilic cytoplasm (cn). The acidophilic neuropil (*) contains blood vessels with narrow perivascular spaces (bv) and glial cells with either lightly (lg) or dark (dg) stained nuclei. H&E Scale bar A, = 200 µm x100, B and C, 50 µm x400

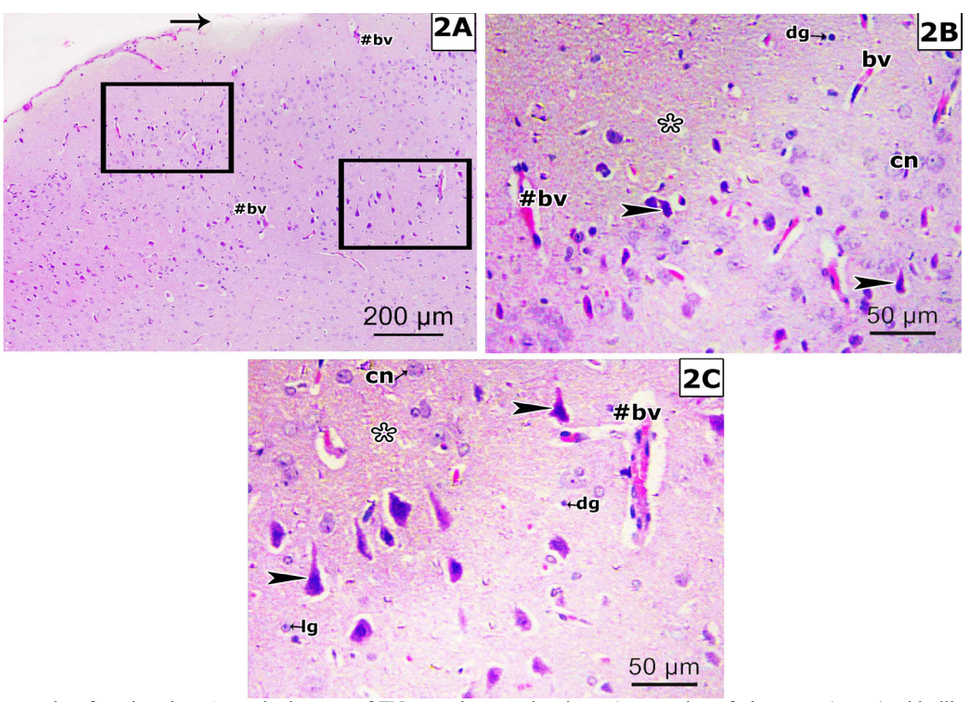

Fig. 2: Photomicrographs of sections in rat's cerebral cortex of TN treated group showing: A) separation of pia matter (arrow) with dilated and congested blood vessel (#bv). B) Higher magnification of the upper square in the outer cortex shows pyknotic cortical neurons (arrowhead) with congested and dilated blood vessel (#bv). Vacuolated neuropils (*) with many glial cells that have dark stained nuclei (dg) are noticed. Few small cortical neuronal cells (cn) and blood vessel appear (bv) normal. C) Higher magnification of the lower square in the inner cortex shows degenerated cortical neurons with darkly stained nuclei and surrounded by vacuolations (arrowhead). Few small cortical neuronal cells (cn) are still normal. Congested and dilated blood vessels (#bv) in vacuolated neuropils (*) with many glial cells that have dark (dg), or light (lg) stained nuclei are noticed. H&E Scale bar A, = 200 µm x100, B- and C, 50 µm x400

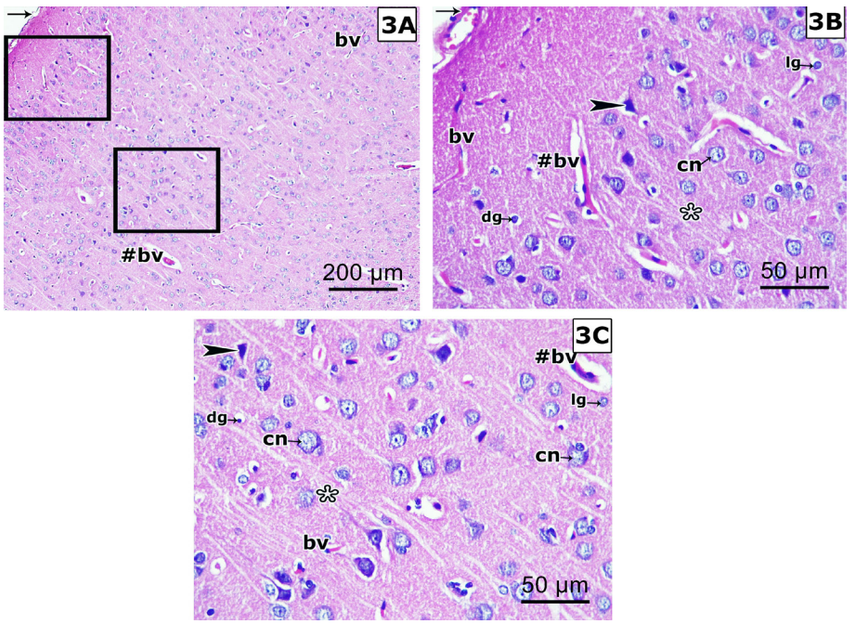

Fig. 3: Photomicrographs of sections in rat's cerebral cortex of TN+RES treated group showing: A) Nearly similar normal arrangement of cortical layers blood vessels either with narrow (bv) or wide perivascular space(#bv) B) Higher magnification of the upper square in outer layers of cerebral cortex showing most cortical neurons (cn) is normal. Few cortical neurons still exhibit dark stained nuclei (arrowhead). Some blood vessels still congested with wide perivascular spaces (#bv) and others with narrow perivascular space (bv). Glial cells with either lightly(lg) or dark (dg) stained nuclei are seen within the acidophilic neuropil (*). C) Higher magnification of the lower square showing most cortical neurons with vesicular nuclei and basophilic cytoplasm (cn). The acidophilic neuropil (*) contains blood vessels with narrow perivascular spaces (bv) and others with wide perivascular space (#bv). Glial cells with either lightly(lg) or dark (dg) stained nuclei can be noticed. H&E Scale bar A, = 200 µm x100, B- and C, 50 µm x400

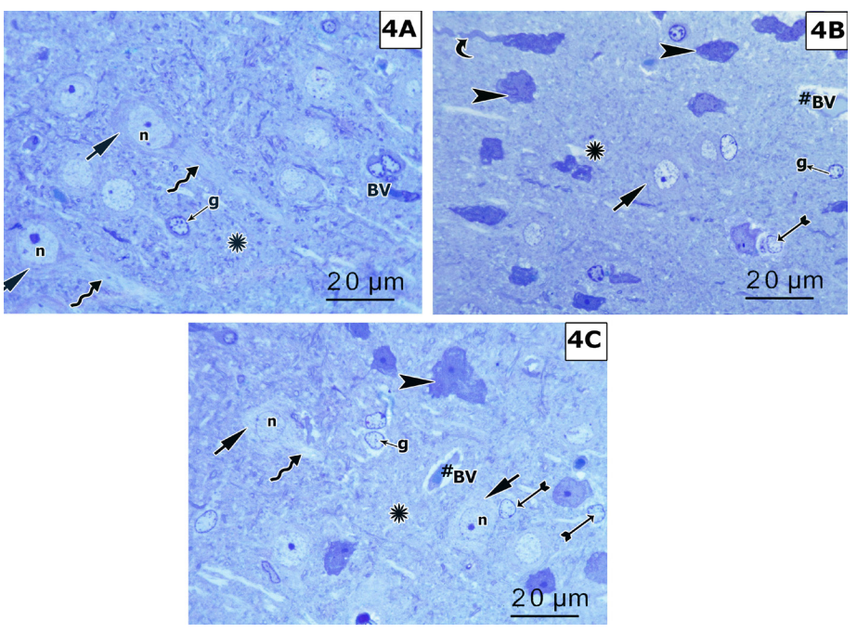

Fig. 4. photomicrographs of a semithin section in the cerebral cortex of the different groups showing: A) Control groups show intact cortical neurons with open-faced nuclei (n) encircled by pale cytoplasm (arrow) that having basophilic Nissl's granules with intact long process (wavy arrow). Glial cell with sharply rounded demarcated nuclei of glial cells (g) and normal blood vessel (BV) can be observed within the intact neuropil (*). B) TN treated group showed deeply stained cortical neurons (arrowhead) with irregular process (curved arrow) and few normal neurons (arrow) were also noticed. Vacuolated neuropils (*) with dilated blood vessels (#BV) that encircled by a wide perivascular space can be seen. Glial cells (g) and perineural glial cells (bifid arrow) beside the degenerated neurons can be observed. C) TN+ RES treated group shows that the most cortical neurons were normal with open-faced nuclei (n) encircled by pale cytoplasm (arrow) that having basophilic Nissl's granules with intact long process (wavy arrow). Few neurons with deeply stained nuclei (arrowhead) can be detected. Blood vessels with slightly wide perivascular space (#BV) embedded in intact neuropil (*) can be noticed. Glial cells(g) and perineural glial cells (bifid arrow) beside the degenerated neurons and the intact neurons can be observed. Toluidine blue Scale bar= 20 µm x1000

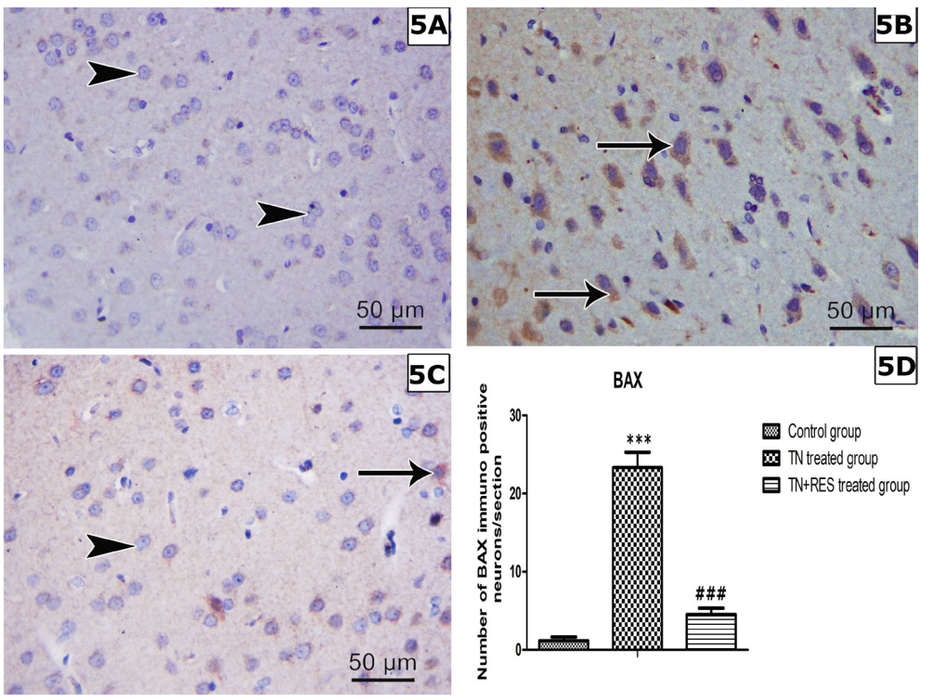

Fig. 5: Representative photomicrographs identify the immunoreactivity of the apoptotic neuronal cells in different groups via BAX immunostaining of cortical neurons (A) Control group; (B)TN treated group; (C) TN+RES treated group. Arrow refer to dark brown staining of the cytoplasm of immuno-positive affected cortical neurons and arrowhead refers to the negative immunostaining of the normal cortical neurons. Scale bar; 50 µm x400. (D) Bar graph showing differences in the number of BAX positive affected neurons in all studied groups. The immuno-positive neurons were counted from 6 animals/group in perceptive fields at ×400 magnification. One-way ANOVA was used for statistical analysis *Significant difference from the control group, $p<0.05$.# Significant difference from the TN treated group, $p<0.05$.

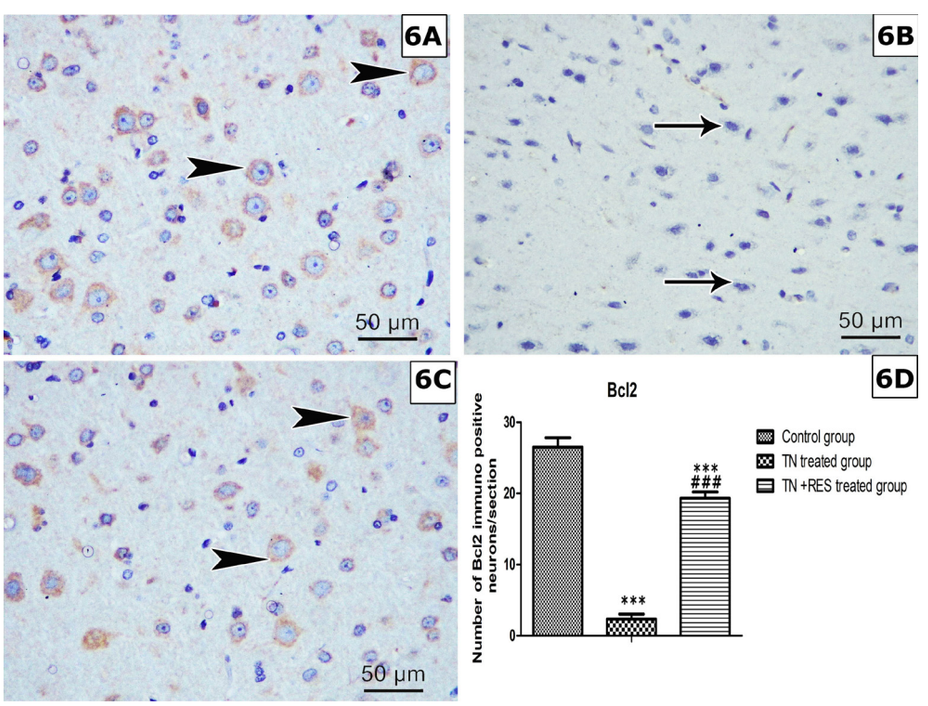

Fig. 6: Representative photomicrographs identify the immunoreactivity of the apoptotic neuronal cells in different studied groups through Bcl2 immunostaining of cortical neurons (A) Control group; (B)TN treated group; (C) TN+RES treated group. Arrowhead indicating dark brown staining of the cytoplasm of immuno-positive normal cortical neurons and arrow indicating the negative immunostaining of the affected cortical neurons. Scale bar; 50 µm x400. (D) Bar graph presenting the changes in the number of Bcl2 positive normal neurons in all different studied groups. The immuno-positive neurons were counted from 6 animals/group in perceptive fields at ×400 magnification. One-way ANOVA was used for statistical analysis.*Significant difference from the control group, $p<0.05$.# Significant difference from the TN treated group, $p<0.05$

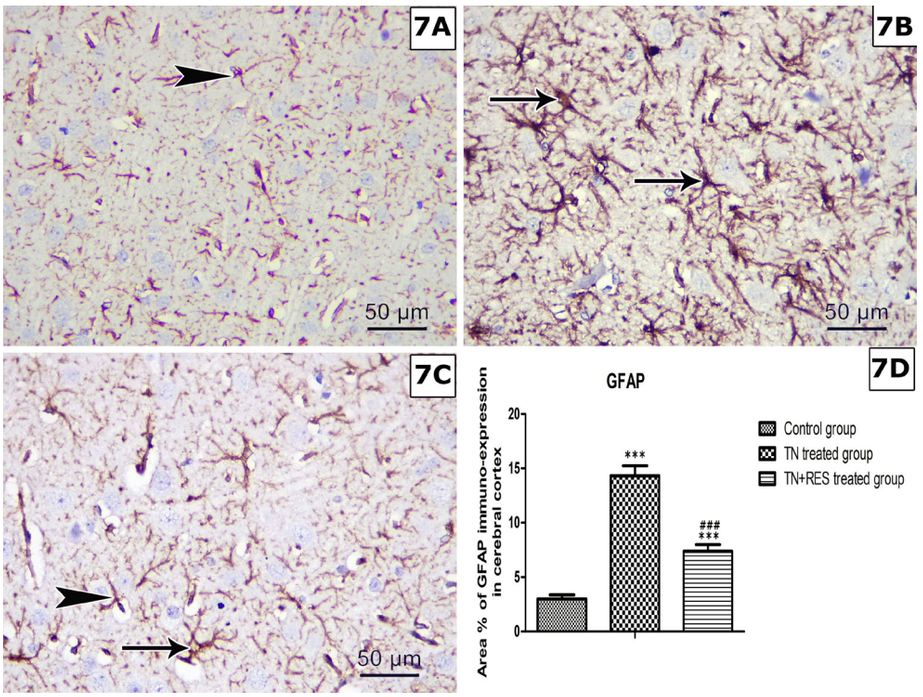

Fig. 7: Representative photomicrographs identify the immunoreactivity of astrocyte for GFAP in different groups (A) Control group; (B)TN treated group; (C) TN+RES treated group. Arrowhead is indicating thin small astrocytes and arrow is indicating the thick and large astrocytes. Scale bar; 50 µm x400. (D) Bar graph showing the area % of GFAP immuno-expression in the astrocyte from 6 animals/group in- perceptive field sat×400 magnification. One-way ANOVA was used for statistical analysis. *Significant difference from the control group, p<0.05.# Significant difference from the TN treated group, p.<0.05

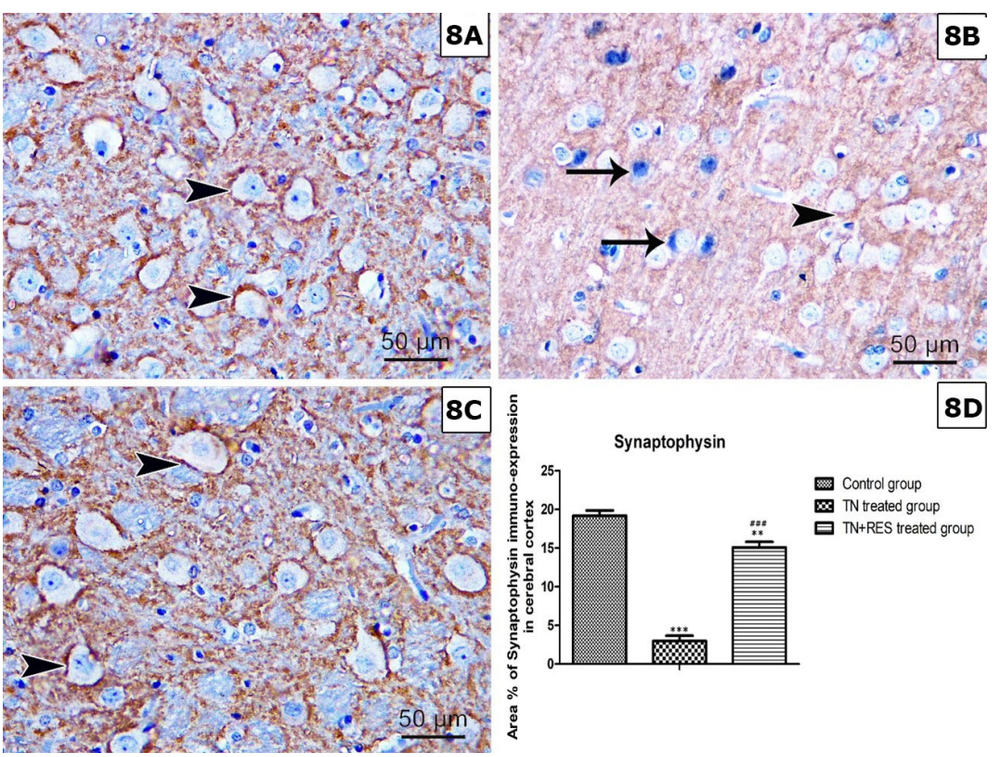

Fig. 8: Representative photomicrographs identify the immunoreactivity of cortical neurons to the synaptophysin in different groups (A) control group; (B) TN treated group; (C) TN+RES treated group. Arrowheads indicate the dense coarsely fine beaded appearance on the surface of the normal cortical neurons and arrows indicate the faint immunoreactivity of the affected neurons. The scattering of the reactive granules is also observed in the neuropil between the neurons Scale bar; 50 µm x400. (D) Bar graph showing the area % 0f synaptophysin immuno-expression from 6 animals/group in perceptive fields at ×400 magnification. One-way ANOVA was used for statistical analysis.*Significant difference from the control group, p<0.05.# Significant difference from the TN treated group, p<0.05.

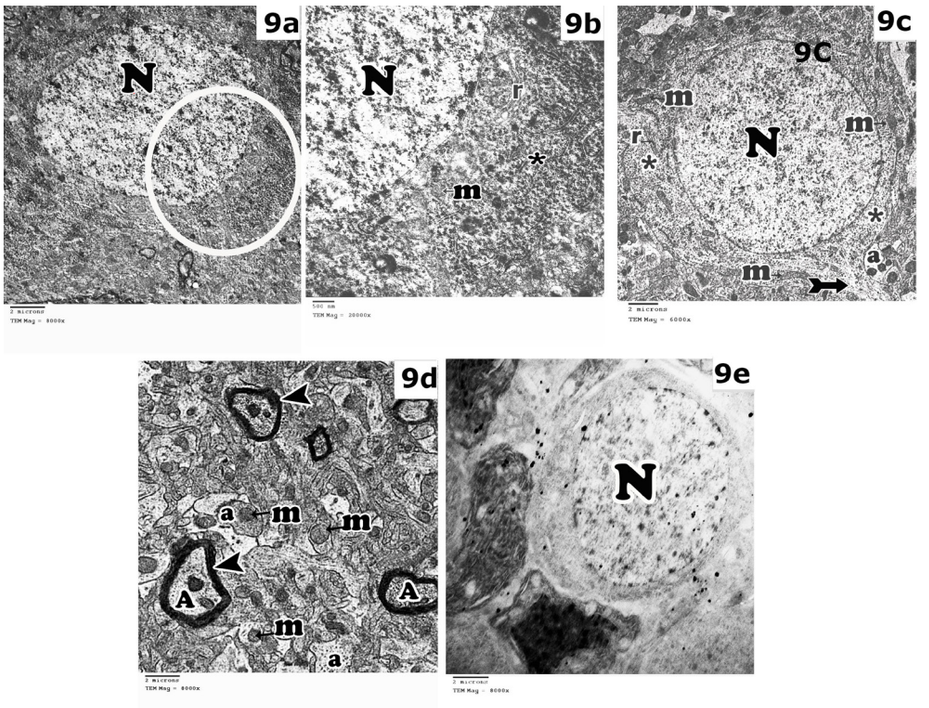

Fig. 9: Transmission electron micro-photographs showing the ultra-structure of the cerebral cortex of control group a) Cortical neuron showing normal appearance of the nucleus (N) and all cytoplasmic organelles. b) Higher magnification of the circled area showing round euchromatic nucleus (N), normal mitochondria (m), rough endoplasmic reticulum (r) and Nissl's granules (*) can be noticed. c) Other cortical neurons with well demarcated rounded euchromatic nucleus (N), normal mitochondria (m), rough endoplasmic reticulum (r) and Nissl's granules (*) are Noticed. Long axon of the cortical neuron (bifid arrow) and non-myelinated nerve fibers (a) can be observed. d) Neuronal axon section showing normal mitochondria (m), myelinated nerve fibers (A) surrounded by thick myelin sheath (arrowhead), non-myelinated nerve fibers (a) can be noticed. e) Astrocyte is seen with normal euchromatic nucleus (N) surrounded by electron lucent cytoplasm with few organelles. TEM Scale bar = a, d, and e- 2 microns x8000, b- 500nm x20000 and, C- 2 microns x6000.

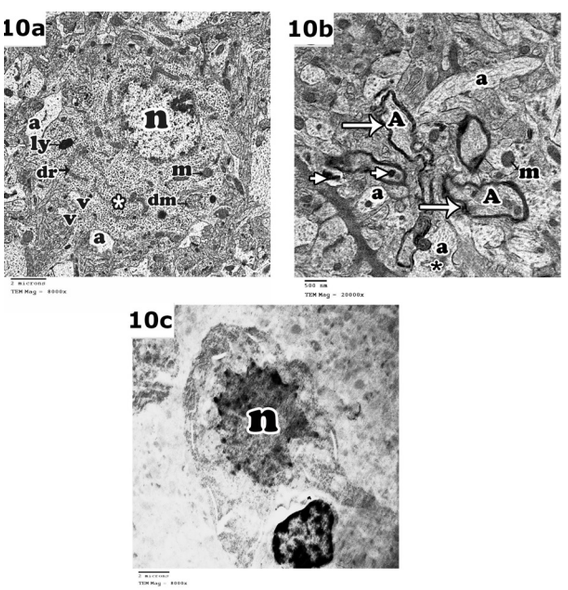

Fig. 10: Transmission electron micro-photographs showing the ultra-structure of the cerebral cortex of TN treated albino rat a) Cortical neuron showing small shrunken nucleus (n) with irregular membrane and condensed chromatin fragments, swollen mitochondria (dm), dilated cisternae of endoplasmic reticulum (dr), lysosomes (Ly), vacuoles (V). Nissl's granules (*), mitochondria (m) and nonmyelinated nerve fibers (a) can be seen. b) Neuronal axon section showing swollen mitochondria (m), irregular mitochondria (short arrows). Thin myelinated nerve fibers (A) with irregularity (long arrows) and nonmyelinated nerve fibers (a). c) Astrocyte is showing shrunken heterochromatic nucleus (n) surrounded by electron lucent cytoplasm. TEM Scale bar = a and c- 2 microns x8000 and, b- 500 nm x20000.

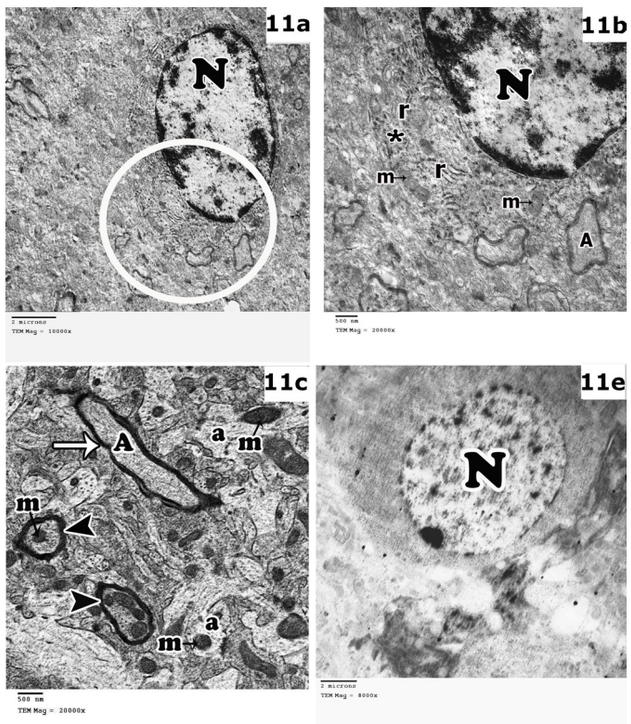

Fig. 11: Transmission electron micro-photographs showing the ultra-structure of the cerebral cortex of TN+RES group a) Cortical neuron showing nearly normal appearance of the nucleus and all cytoplasmic organelles. b) Higher magnification of the circled area showing round heterochromatic nucleus (N), normal mitochondria (m), rough endoplasmic reticulum (r), Nissl's granules (*) and myelinated nerve fibers (A) can be noticed. c) Neuronal axon section showing normal mitochondria (m), most myelinated nerve fibers surrounded by thick regular myelin sheath arrowheads few myelinated nerve fibers (A) surrounded by thin myelin sheath (arrow), non-myelinated nerve fibers (a) can be noticed. d) Astrocyte with normal euchromatic nucleus (N) surrounded by electron lucent cytoplasm. TEM Scale bar = a, 2 microns x10000, b and c- 500 nm x20000, d-2 microns x8000.

Table 1: levels of MDA, SOD& Gpx in cerebral tissue in different studied group (Mean± SE)

Variable \ Group		Control (n=6)	Corn oil (n=6)	RES	TN treated (n=6)	TN&RES treated(n=6)
SOD (ng/ml)	Mean± SE	0.2554±0.03274	0.2464±0.005023	0.2649±0.009738	0.1018±0.01041**	0.2031±0.007181##
Gpx (ng/ml)	Mean± SE	7.659±0.2464	7.579±0.3442	7.682±0.3178	4.799±0.2223***	6.073±0.3047**##
MDA (nmol/mg)	Mean± SE	2.006 ± 0.1343	2.006±0.07334	1.908±0.1058	3.750±0.1012***	1.872±0.2640###

Rats number in each group (n=6). Statistical analysis was performed using One-way ANOVA followed by Tukey's post hoc test. Values are signified as Mean± SE. Statistical significance was set at *p value* <0.05.
* significant *P value* compared to control group. # significant *P value* compared to TN group.

DISCUSSION

Reactive oxygen species (ROS) are generated by organic solvents which interfere with the body's normal defense mechanisms through numerous processes, mainly in the brain and liver[30-32].

Toluene (TN) is one of the most abused organic solvents worldwide. Occurrence of disorders in pyramidal cells, peripheral neuropathy, atrophy in optic nerve, neural audio loss, cognitive disturbance has been reported due to chronic abuse of toluene[33; 21].

Some young people abused volatile substances containing TN which may cause a neurological damage. Glue inhaling is used frequently between teenagers and young adults as it is cheap and simply found[34; 35; 21].

TN and its metabolites may produce injury in many tissues mainly the brain also, affect the defense system in the body by means of oxidative stress[30; 36-38].

In the view of the previous studies, oxidative stress is resulted from an imbalance between intracellular production of free radicals and cellular defense mechanisms. An increase in free radicals or reducing anti-oxidative substances impaired this balance between oxidant and antioxidant agents. Oxidative stress can trigger several potentially harmful biochemical reactions. So, exposure to TN lead to changes in the levels of the antioxidant enzymes.

In this study, we found that animals exposed to TN with a dosage 500 mg/kg bw/day for 14 days intraperitoneally showed a significant increase in MDA levels and a

significant reduction in mean enzymatic antioxidant levels of SOD and Gpx activities compared to the animals in the control and TN+RES groups.

Our data were in consistent with Abdel-Salam *et al*.[39] who proved that administration of TN in a dosage 900 mg/kg bw intraperitoneally every day for 6 days, produced markedly increased oxidative stress as revealed by rising lipid peroxidation (MDA) in the brain and with Coskun *et al*.[40] who examined the lethal effects of high concentration of TN inhalation (3000 ppm, 8 hours per day for 6 days a week for 16 weeks) on rats' sciatic nerves and institute that TN reduced tissue SOD, GSH-Px and increased the blood and tissue MDA values.

Coskun *et al*.[40] that tissue CAT did not decrease as tissue decreased tissue SOD and GSH-Px in TN treated group in comparison with control group.

Our finding was supported by ÇÖMELEKOĞLU *et al*.[41] who added that reduction in SOD, GSH-Px and CAT with the increased concentrations of ROS, suggesting that oxidative stress might be the earliest mechanism for apoptotic changes in cortical neurons following their exposure.

Also, Meydan *et al*.[21] proved that there was rise in the degree of MDA with decrease in the levels of both SOD and GSH-Px in groups treated with TN compared to control group.

These data strongly suggested that free radicles directly or indirectly generated by TN through reduction of antioxidant defense mechanisms depending on the dose that caused lowering the activity of antioxidant systems.

Co-administration of RES with TN showed decrease of MDA level compared to it in TN treated group. The current results were supported by Kasdallah-Grissa[42] who found that RES intake after chronic ethanol administration in rats significantly reduced MDA levels in the liver, brain, heart, and testis, and the strongest suppressive effect of RES on MDA levels was achieved in the brain.

Singh *et al*[17] added that RES intake reduced free radical formation and increased GSH-Px and SOD levels in rodent stroke models. It also lowered the oxidative stress as revealed by reducing the level of MDA because of its nature as an antioxidant and its free-radical scavenging action.

However, animal treated with RES showed significant improvement in the activity of SOD and CAT as antioxidant biomarkers compared to TN treated group. The current results were supported by Mokni *et al*.[43] who found that the intake of RES in a dose 12.5 mg/kg bw/day for 7 days intraperitoneally is significantly and dose dependently in decreasing brain MDA level and increasing brain superoxide dismutase, catalase, and peroxidase activities.

Apoptosis is a controlled (programmed) cell death without producing inflammation and it is distinguished by shrinkage of cytoplasm, membrane alterations, and destruction of DNA, apoptosis may be caused by different pathways[44;2].

Releasing of apoptogenic factors like, cytochrome c, by mitochondria-mediated apoptosis is the most common path by which apoptosis is started[45;46].

Our histopathological observations concerning the TN treated group, might be related to reactive oxygen species (ROS) formation. We observed pia detachment, congested dilated blood vessels with perivascular spaces, vacuolated neuropil and degenerated cortical neuronal cells with darkly stained nuclei and neuroglial cells which had darkly stained shrunken nuclei

Our results agreed with the earlier findings of Kanter[35] who noticed that there were severe deteriorating changes, shrunken cytoplasm and extensively dark pyknotic nuclei in neurons of the frontal cortex and brain stem tissues of rats exposed to TN in a dose of 3,000 ppm toluene, in an 8 h/day and 6 day/week order for 12 weeks by inhalation.

Demir *et al*.[2] were reported, in rabbits' cerebral cortexes intake toluene, an area of focal vacuolar degeneration, gliosis, and numerous apoptotic cells and necrosis.

Our finding was in consistent with Abdel-Salam *et al*.[47] who proved that with short-term exposure to high dose of TN neuronal loss could be occurred. They stated that a single injection of TN (a dose 500 mg/kg, intraperitoneally) caused cerebral neuronal necrosis. The previous authors demonstrated Shrinkage of neurons and vacuolation in neuropil in prefrontal cortex While a report by Abdel-Salam *et al*.[48] found that, intraperitoneal injection of TN at a dose of 900 mg/kg for two consecutive days, revealed death and apoptotic neurons, perineuronal vacuolation and glial cells in the cerebral cortex.

Atef *et al*.[49] and Abdel-Salam *et al*.[39] found that intra-peritoneal injection of TN at a dose 900 mg/kg for 6-9 consecutive days led to DNA fragmentation of DNA, a darkly stained neurons, cerebral in brain of the rats treated by TN.

In the current work, co administration of RES with TN improved the great pathological changes caused by the later. RES led to decreasing the number of pyknotic cells indicating to reduction of the rate of apoptosis. Some of cortical neurons and glial cells returned to its normal appearance.

Neuro-protective effects of RES on brain were before proved in some studies. The study of Tabrizian, *et al*.[22] showed the toxicity on male rat caused by Carbon Monoxide (CO) which improved with RES treatment in a dose of (1, 5 and 10 mg/kg bw) intraperitoneal. It decreased the apoptosis of cerebral cells caused by CO, cellular damage, and oxidative stress.

The previous author added that, BAX/BCL2 ratio is one way of assessment of apoptosis in the brain. If the ratio increases, apoptosis state increases and vice versa. So, it was proved that RES reduced this ratio.

Mokni *et al*.[43] who stated that RES protected against neurotoxicity produced by kainic acid and functioned as a multifunctional cytoprotective mediator. RES stimulated several antioxidant enzymes. The strongest rise focused on CAT followed by SOD.

In the current study, immunohistochemical staining was to determine the apoptotic status of rat brain by detecting proapoptotic protein as Bax using Bax immune reactivity. There was increasing in Bax immunoreactivity in the cell cytoplasm in TN treated group compared to control group. Bax immunoreactivity decreased in TN+RES group denoting that RES was antiapoptotic agent through its antioxidant role.

Demjr *et al*[2] also reported that, the apoptosis is triggered by toluene even in about 3 hours as it increases the levels of the pro-apoptotic proteins, caspase 3 and Bax, in the brain cortex, hippocampal area, entorhinal cortex, substantia nigra of rabbits' brain that were administrated toluene.

Tas *et al*[31] concluded that TN long-lasting exposure accumulated in certain organs especially in the brain and liver. It was able to increase the apoptotic activity in the liver tissue through increasing Bax immunoreactivity

Singh *et al*[17] emphasize the significance to use RES as antiapoptotic to attenuate apoptosis by administration of 30 mg/kg of RES to a rat model subjected to transient middle cerebral artery occlusion. This reduced the ischemia and led to up-regulating Bcl-2 and down-regulating Bax in the hippocampus.

Authors revealed several different mechanisms of action of TN. Calderón-Guzmán *et al*[5] stated that TN increases the membrane fluidity by modifying the cell membrane lipid structure and affects the Na / K-ATPase activity. Balster[50] and Bowen *et al*.[51] have stated that TN may affect GABAergic, glutamatergic, serotonergic, dopaminergic pathways. Additionally, Lee *et al*.[52] and Tas *et al*.[31] revealed that toluene increases the pro-apoptotic proteins (Bax) and may cause mitochondrial damage in the cytoplasm and thus increases apoptotic activity.

RES has a neuroprotective effect by its strong antioxidant properties and by its capability in decreasing stimulation of glial cells and ischemia-stimulated astrocytes, reducing infarct size and lessening in mitochondrial cytochrome C oxidase[53-57].

Bcl-2 expression, which is a regulatory protein of cell death, has also been indicated to estimate the pathway of neuronal cell death[58]. During normal brain development, Bcl-2 inhibits neuronal apoptosis as well as that persuaded by cytotoxic drugs or growth factor deficiency[59]. Offen *et al*[59] also described that Bcl-2 upregulation in neuronal cells gives safety against neurotoxins, while neuronal cells lacking Bcl-2 are further vulnerable to neurotoxins apoptosis[60]. Moreover, Hossain *et al*[61] added that decrease in Bcl-2 protein preceded the apoptosis and could be believed as an significant step in pro-apoptotic reaction to neurotoxicity.

This study showed that toluene decreased the expression of Bcl-2 and increased the expression of pro-apoptotic Bax genes in rat frontal cortex.

Tian *et al*[62] reported that resveratrol reverses the alteration of Bcl-2, Bax and Caspase-3 levels in diabetic rat. This indicates that resveratrol ameliorates the cognitive decline in STZ-induced diabetic rats, at least partly through inhibiting hippocampal apoptosis via Bcl-2, Bax and Caspase-3 signaling pathways.

Zhang *et al*[63] also reported that the doses of resveratrol between 2.5 and 10 μmol/L prevent apoptosis, increase Bcl-2 protein expression, decrease Bax and Caspase 3 protein expression, which showed that the regulation of RES on neuron apoptosis is mainly by increasing the ratio of Bcl-2 / Bax and inhibiting the activation of Caspase pathway. Moreover, RES suppressed oxidative stress, due to its antioxidant effect, it reduced the content of ROS that formed by corticosterone induced PC12 cells. Therefore, they also found that RES could inhibit mitochondrial apoptotic pathways by reducing Bax and caspase-3 expression and increasing Bcl-2 expression.

In the present study, the apoptotic neurons were significantly increased in numbers in TN treated group compared to control and TN+RES group. RES causes structural enhancement on neuro degeneration in the cerebral cortex after exposure of TN in rats. This indicates its useful effect as an effective treatment on neuro degeneration after exposure to TN in rats.

In this experimental work, a significant rise in the ratio of Bax immune reactivity in rat brain exposed to toluene were observed. We reached the conclusion that toluene could increase the apoptotic activity in brain tissue.

Astrocytes play a vital role in persistence of neurons through regulating the ionic environments which are needed to neurons` function. Cellular response occurred early following any CNS damage[35;64].

According to the current study, there was immunoreactivity to GFAP illustrating the shape of astrocytes as cell body and branching processes in control group, but the immunoreactivity increased as intensity and thickness of processes in TN treated group.

These results were in consistent with Yu *et al*.[65] who noticed the increase in GFAP immunoreactivity as a sensitive marker of reactive astrocytosis. Reactive astrocytosis is astrocytes reaction to neuronal injury in response to hypoxia-ischemia, cerebral hypoperfusion and exposure to neurotoxicants.

Our results agreed with the earlier findings of Gotohda *et al*.[66] and Filley *et al*.,[8] who noticed alterations in astrocytes than other neuronal cells during their research on human. Toluene disrupted differentiation of astrocyte precursor cells. They also added that activation of astrocytes with enhancement of GFAP immunoreactivity occurred after toluene inhalation in rats' brain.

The study of Means *et al*.[64] showed that in an Alzheimer's disease rat model GFAP expression was increased and when treated with Curcumin, an agent used to treat tumorigenesis, oxidation, and apoptosis, GFAP expression was significantly reduced. But this result was not in agreement of Abdel-Salam *et al*.[39] who mentioned that TN induced marked decrease in immuno-histochemical staining for GFAP. They suggested inhibition of GFAP in glial cells by toluene.

Fukui *et al*[66] stated that, in TN treated rats, the numbers of GFAP-immunoreactive (IR) astrocytes were not changed, whereas the area and intensity of GFAP-IR processes were increased markedly in the dentate gyrus. In addition, dense, thick and highly stained GFAP-IR processes appeared entering into the granular cell layer, compared to controls.

Coadministration of RES to TN decreased the intensity of GFAP if compared to TN treated group. This result was supported by Means *et al*.[64] who emphasized the ability of RES to reduce the increased levels of GFAP during oxidative stress.

Synaptophysin is a chief membrane glycoprotein of synaptic vesicles that is expressed in all neurons[67]. Moreover, it delivers a beneficial tissue marker of synaptogenesis during normal situations and may deliver signs to cerebral pathogenesis[68]. Most researches have evaluated SYN as a measure of synaptic integrity[69].

In the current study, synaptophysin was used to measure synapse loss following toluene administration. In the present study there were scattering of the reactive granules in the neuropil between the neurons in the control group. However, in TN treated group the synaptophysin reactivity was decreased at the periphery of the affected neurons and within the neuropil. Moreover, alongside treatment with RES increased synaptophysin immuno-expression nearly as control group.

Lai *et al*.,[28] demonstrated that fetal acrylamide exposure during pregnancy was associated with reduced expression of GAP43 and SYP in the hippocampus of offspring 21 postnatal days, which may be correlated with inhibition of neuronal proliferation and differentiation and synaptic function.

TN toxicity was noticed to cause synapse loss or injury. The current study revealed that RSE can increase the levels of synaptic proteins in the cerebral cortex. Furthermore, we observed that following TN toxicity, RES administration was able to down regulate the neuronal autophagy in the rat cerebral cortex. Lin *et al*[70] reported that RES can increment cell existence by inhibition the glycogen synthase kinase-3-intermediated autophagy and apoptosis by using models of traumatic brain injury in *vivo* and in *vitro*.

In the current study, exposure to 500 mg/kg/day of TN for 14 days intraperitoneally leads to multiple neural degenerative changes, markedly swollen mitochondria, dilated cisternae of rough endoplasmic reticulum, breakdown of nuclear membrane. These findings agreed with the results of other investigators on TN induced neuro-toxicity in different animals[35].

Moreover, in TN treated group, there were nuclear membrane breakdown and irregular shaped clumps of condensed chromatin, shrunken cytoplasm, markedly swollen mitochondria, dilated cisternae of endoplasmic reticulum, lysosomes and vacuoles in the cytoplasm. There was thinning irregular myelin sheath and loss of myelin to some axons. Astrocyte had small shrunken heterochromatic nucleus with irregular nuclear membrane.

Our results were in agreements of Filley *et al*.[8] who proved that on TN exposure destruction of myelin axons, and endoneuria structures. Distortion in some nerve fibers and vacuolization in the cytoplasm of nerve cells would be occurred

Filley *et al*.[8] and Gotohda *et al*[71] added that, there were thinning of the cerebellar white matter and myelin axonal loss in toluene-exposed rats. Myelin loss in patients was highly varying, probably due to the level of exposure, age of beginning of toluene abuse, abuse of other substances with TN.

Moreover, our ultrastructural finding was supported by the results of Coskun *et al*.[40] who added that on chronic toluene inhalation in high concentration there were myelin destructions with onion-bulb and bubble forming myelin sheath protrusion and some nerve fibers showed swollen and shrunken axons in the rat sciatic nerve.

In addition, our result is in parallelism with the observation of Kanter[35] who worked on biopsies of the rat sural nerve illustrated distension of the axons with very slim lamella of the myelin sheath. Also added that Nigella sativa as antioxidant and neuroprotective was effective in preventing mitochondrial degeneration and irregularly shaped nuclei. Demjr *et al*.[2] noticed perivascular demyelination in rabbits' brains that were administrated toluene.

CONCLUSION

In conclusion our work proved that TN mediates apoptotic and degenerative effect on the cells of cortical neurons most probably due to the release of ROS. The combination of antioxidant like RES to TN causes some protection to the cortical neurons. So, the results suggest that RES has protective role against TN induced apoptosis and neural toxicity via an antioxidant mechanism.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

التأثير الضار للتولوين على قشرة دماغ الجرذ وتأثير ممارسة الريسفيراترول: (دراسة نسيجية ومناعية كيميائية وبنية تحتية)

رانيا سعيد معوض، ايمان رمضان عبد الفتاح، اميرة ابراهيم السمح

قسم التشريح وعلم الاجنة، كلية الطب البشرى، جامعة الزقازيق، مصر.

خلفية: البحث: التولوين هو هيدروكربون كيميائي يؤثر بشكل رئيسي على الجهاز العصبي المركزي. الريسفيراترول يحتوي على مجموعة كبيرة من التأثيرات الصحية المفيدة.

الهدف من العمل: هدفت الدراسة إلى تقييم التأثير الوقائي للريسفيراترول (RES) على إصابة الدماغ في الجرذ بسبب التولوين (TN).

طرق و مواد البحث: استخدم في هذه الدراسة ثلاثون جرذا من الذكور ، وزن كل منها 250-200 جم، تتراوح أعمارهم بين 6-4 أشهر. تم فصلهم إلى خمس مجموعات متساوية، المجموعة الضابطة: كان للحيوانات نظام غذائي منتظم وماء من الصنبور، مجموعة زيت الذرة: تم إعطاؤها 0.1 مل / 10 جم / يوم من زيت الذرة، بالحقن البريتونى، المجموعة المعالجة بـ TN: تلقت الحيوانات التولوين بالحقن البريتونى مع جرعة 500 مجم / كجم / يوم (ن ¼ 7) (0.1 جم / مل، في زيت الذرة) (0.5 مل / كجم / يوم) لمدة 14 يومًا، مجموعة الريسفيراترول: تلقت الحيوانات ريسفيراترول 10 مجم / كجم / يوم بالحقن البريتونى لمدة 14 يومًا، والمجموعة المعالجة بـالتولوين مع الريسفيراترول: تلقت الحيوانات التولوين جنبًا إلى جنب مع الريسفيراترول (كجرعات سابقة) لمدة 14 يومًا. تم تخدير جميع الجرذان وذبحها وتشريح المخ. تمت معالجة أجزاء من أنسجة المخ لمجانسات الأنسجة لقياس Gpx، SOD، MDA. وأجزاء أخرى من أنسجة المخ تم إخضاعها لفحص الأنسجة (الفحص المجهري بالضوء والإلكترون). تم إجراء تلطيخ كيميائي مناعي لـ BCL2 وBAX وGFAP وsynaptophysin مع التحليل المورفومتري والإحصائي.

النتائج: كشفت النتائج فى مجموعة التولوين عن زيادة في MDA مع انخفاض نشاط SOD و Gpx. علاوة على ذلك، أظهر التولوين تدهورًا نسيجيًا واضحًا مع الإفراط في التعبير عن بروتين Bax والبروتين الحمضي الليفي الدبقي (GFAP) والتعبير المنخفض عن Bcl2 و synaptophysin. أعاد الريسفيراترول المظهر الكيميائي الحيوي للضرر التأكسدي وحسن الصورة النسيجية المرضية والكيميائية المناعية.

الخلاصة: أظهرت نتائجنا ضوءًا ساطعًا حول الدور الفعال المحتمل للريسفيراترول في إصابة الدماغ التي يسببها التولوين وقدمت اقتراحًا متاحًا حول الفعالية السريرية المحتملة لمكملات الريسفيراترول في حالة إصابة الدماغ.