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ARAŞTIRMA

Protective effects of Nigella sativa on formaldehyde-induced neuronal injury in frontal cortex

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Abstract

Purpose: **The aim of this study was designed to evaluate the possible protective effects of Nigella sativa (NS) on the neuronal injury in the frontal cortex after formaldehyde (FA) toxicity in rats.**

Methods: **The rats were randomly allotted into one of three experimental groups: control, FA treated and FA treated with NS; each group contain 10 animals. FA treated and FA treated with NS groups received intraperitoneal injection of 10 mg/kg FA for 10 days. The rats in NS treated group was given NS (in a dose of 400 mg/kg body weight) once a day orally for 10 days starting just after FA injection. Control and FA untreated rats were injected with the same volume of isotonic NaCl as the FA treated animals that received NS. Tissue samples were obtained for histopathological investigation. To date, no histopathological changes of neurodegeneration in the frontal cortex after FA exposure in rats by NS treatment have been reported.**

Results: **In the FA treated group, the neurons of frontal cortex tissue became extensively dark and degenerated with picnotic nuclei. The morphology of neurons in FA treated with NS group was protected well, but not as neurons of the control group. The number of apoptotic neurons in frontal cortex tissue of FA treated group was significantly more than both control and FA treated with NS groups. The caspase 3 immunopositivity was increased in degenerating neurons of the frontal cortex tissue following FA treatment. Treatment of NS markedly reduced the immunoreactivity of degenerating neurons after FA treatment. The distorted nerve cells were mainly absent in the NS treated rats.**

Conclusion: **We conclude that NS therapy causes morphologic improvement on neurodegeneration in frontal cortex after FA treatment in rats.**

Key Words: ***Formaldehyde, Nigella sativa, morphology, neuronal injury, rat***

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Formaldehitin neden olduğu frontal korteksteki nöronal hasara karşı nigella sativa'nın koruyucu etkileri

Özet

Amaç: **Bu çalışma, formaldehite (FA) maruz bırakılan sıçanların frontal kortekslerindeki nöronal hasara karşı Nigella sativa'nın (NS) muhtemel koruyucu etkilerini araştırmak amacıyla planlandı.**

Gereç ve Yöntem: **Sıçanlar, herbir grup 10 hayvan içerecek şekilde biri kontrol ikisi deney olmak üzere rastgele 3 gruba ayrıldı; Kontrol grubu, FA uygulanan grup ve FA+NS uygulanan grup. FA, 10 gün boyunca günde 10 mg/kg intraperitoneal uygulandı. NS uygulanan gruptaki ratlara, 400 mg/kg NS 10 gün boyunca ağızdan günde bir kez, FA uygulanmasından hemen sonra verilmeye başlandı. Kontrol ve tedavi uygulanmayan FA gruplarındaki sıçanlara, NS tedavisi alan gruptaki sıçanlarla aynı dozda izotonik NaCl uygulandı. Histopatolojik incelemeler için doku örnekleri toplandı. Bugüne kadar FA'e maruz bırakılan sıçanların frontal kortekslerindeki nöronal hasara**

karşı NS'nin koruyucu etkileri ile ilgili herhangi bir bilgiye rastlanmamıştır.

Bulgular: **FA uygulanan gruba ait frontal korteksteki piknotik ve dejenere olmuş nukleuslara sahip nöronların oldukça koyu görünümlü olduğu görüldü. FA ile birlikte NS tedavisi uygulanan gruptaki nöronların morfolojilerinin kontrol grubundaki nöronlar kadar olmasa da iyi korundukları tespit edildi. FA uygulanan grubun frontal korteksindeki apoptotik nöron sayısı kontrol ve FA ile birlikte NS tedavisi uygulanan gruptakilere oranla daha fazlaydı. FA uygulanmasından sonra frontal korteksteki dejenere olmuş nöronlarda kaspaz 3 immunreaktivitesinin arttığı görüldü. NS ile tedavinin FA uygulanmasından sonra oluşan dejenere nöronlardaki immunreaktivitenin belirgin bir şekilde azaldığı gözlendi.**

Sonuç: **Biz NS tedavisinin FA uygulanan sıçanların frontal kortekslerinde oluşan nörodejenerasyonu morfolojik olarak iyileştirdiği kanaatindeyiz.**

Anahtar Kelimler: ***Formaldehit, Nigella sativa, morfoloji,nöronal hasar, sıçan.***

Formaldehyde (FA), a member of the aldehyde family and one of the simplest organic molecules, is a pungent, irritant and colourless gas. It is found in nature in domestic air, cigarette smoke, and the polluted atmosphere of cities due to the incomplete combustion of organics, photochemical smog and by release from FA-containing products (1, 2, 3). Thus, everyone living in society may be exposed to it. FA is also widely used in industrial and medical settings and employees in these areas may be highly exposed to it. In particular, anatomists and medical students having dissection lectures are the most common subjects that can be exposed to FA gas (4, 5). FA is accepted as toxic over certain doses and the chances of harmful effects are increased at room temperatures because of its volativity (1, 2, 6, 7, 8, 9). It is known that inhaled FA gas has negative effects on the central nervous system, and these effects may appear acutely in the form of headaches, malaise, insomnia, anorexia and dizziness (10). Long-term exposure to FA such as 14–30 years, may cause irreversible neurotoxicity16 and is related to brain cancer (astrocytoma) (11). In addition, inhaled FA has been shown to cause behavioural and memory disorders in rats and has been classified as 'probably neurotoxic' (12, 13). However, its morphological effects in the frontal cortex and brain stem have not yet been clarified (14).

Apoptosis, a type of programmed cell death, is a major event in normal development of the nervous system, playing an important role in the establishment of neuronal connections (15, 16, 17). Apoptotic cell death is executed via molecular pathways that are mediated by the activation of caspases, a family of cysteine proteases (18). Caspase-3, a main effector caspase, is strongly implicated in neuronal apoptosis (19), which occurs due to competition for, or limited supply of, neurotrophins that suppress the endogenous genetic death program. Evidence suggests that experimental axotomy or target elimination and withdrawal of neurotrophins result in an increase in apoptosis during development and in the adult (20, 21).

The black seed, *Nigella sativa* (NS), family Ranunculaceae has been shown to contain > 30% of fixed oil and 0.4-0.45% wt/wt of volatile oil. The volatile oil has been shown to contain 18.4-24% thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone) and 46% monoterpenes such as *p*-cymene and α-piene (22). Recently, clinical and animal studies have shown that the extracts of the black seeds have many therapeutic effects such as bronchodilatation, immunomodilative (23), antibacterial (24), hypotensive (25), antidiabetic (26, 27), hepatoprotective (26, 28), gastroprotective (29), antihistaminic and antioxidative (30) and neuroprotective (31). In the present study, it was aimed to investigate the possible protective effects of NS on the neuronal injury in frontal cortex after FA treatment in rats.

Material and methods

Experimental procedure

Plant material and extraction procedure: The NS seeds were purchased from a local herb store in Zonguldak, Turkey. Sample specimens have been kept at the Department of Histology and Embryology, Zonguldak Karaelmas University Medical Faculty, Zonguldak, Turkey for future reference. The seeds of NS were powdered in a mixer. 20 g of the powdered seeds were added to 400 ml of distilled water and extraction was carried out by steam distillation. The distillation process was continued until about 200 ml of distillate was collected. The distillate was extracted three times with chloroform. Moisture was removed by anhydrous sodium sulphate and the resultant extract was evaporated using a 40°C water bath leading to the appearance of the volatile oil. 500 mg of the volatile oil were dissolved in 1 ml of dimethyl sulphoxide (DMSO) then 9 ml of normal saline was added to yield a concentration of 50 mg volatile oil per 1 ml solution (32).

Animals: Thirty healthy male Wistar albino rats, weighing 250-300 g and averaging 12 weeks old were utilized in this study. The animals were

purchased from Zonguldak Karaelmas University Medical Faculty Experimental Research Center and housed in individual cages (360 x 200 x 190 mm) 1 month before the start of the experiments. Food and tap water were available ad libitum. In the windowless animal quarter automatic temperature (22±2ºC) and lighting controls (light on at 07 AM and off at 09 PM: 14 h light/10 h dark cycle) was performed. Humidity ranged from 50% to 55%. All animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health.

Experimental design: The rats were randomly allotted into one of three experimental groups: control, FA treated and FA treated with NS; each group contains 10 animals. FA treated and FA treated with NS groups received intraperitoneal injection of 10 mg/kg FA for 10 days at the same time in the morning. The rats in NS treated group was given NS (in a dose of 400 mg/kg body weight) once a day orally for 10 days starting just after FA injection. Control and FA untreated rats were injected with the same volume of isotonic NaCl as the FA treated animals that received NS. After the treatment, the animals were sacrificed and frontal cortex tissue were removed for histological investigation.

Histological examinations: Frontal cortex tissue were harvested from the sacrificed animals, and the tissues were fixed in 10% neutral buffered formaline, embedded in paraffin, sectioned at 5 mm thickness and then, stained with heamatoxyline and eosine. Histological specimens were examined in light microscopy (Nikon Optiphot II, Japan).

Immunohistochemical procedures: Harvested frontal cortex tissue were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5 µm thickness. Immunocytochemical reactions were performed according to the ABC technique described by Hsu et al. (33). The procedure involved the following steps: 1) endogenous peroxidase activity was inhibited by 3% H_2O_2 in distilled water for 30 minutes; 2) the sections were washed in distilled water for 10 minutes; 3) nonspecific binding of antibodies was blocked by incubation with normal goat serum (DAKO X 0907, Carpinteria, CA) with PBS, diluted 1:4; 4) the sections were incubated with specific a rabbit polyclonal anti-caspase-3 antibody (Cat. # RB-1197-P, Neomarkers, USA), diluted 1: 50 for 1 hour, and then kept at room temperature; 5) the sections were washed in PBS for 3x3 minutes; 6) the sections were incubated with biotinylated anti-mouse IgG (Dako LSAB 2 Kit, prepared according to kit instructions) for 20 min; 7) the sections were washed in PBS for 3x3 minutes; 8) sections were incubated with ABC (Dako LSAB 2 Kit prepared according to kit instructions) for 20 min; 9) the sections were washed in PBS for 3x3 minutes; 10) peroxidase was detected with diaminobenzidine as substrate for 10 min; 11) the sections were washed in in PBS; 12) the sections were counterstained with hematoxylin for 1 min, dehydrated through a graded ethanol series; and 13) the sections were mounted in DAKO paramount. As a negative control, primary antibody was replaced with PBS.

Microscopic examination: Histological specimens of the frontal cortex were examined under light microscopy, with the examination carried out at a magnification of 400 and the counts of neurons determined per square millimeter with the use of a standardized ocular grid. Apoptotic neurons (strong caspase 3 immunopositive) were counted. The distribution of neurons was examined in the sections from the specimens were subjected to immunohistochemical staining by using anti-caspase-3 antibody. Tissue sections were examined under light microscopy (×400) and the number of the neurons counted within random high-power fields using a Nikon Optiphot 2 light microscope incorporating a square graticule in the eyepiece (eyepiece ×10, objective ×40, a total side length of 0.25 μm^2). Neuron density was assessed by counting the number of cells in 400 high power fields amongst the frontal cortex tissue preparations of each group. The neuron density in each site was calculated and recorded as the number of neurons /μm^2. The tissue compartments were used to record neuron distribution in the frontal cortex tissue.

Statistical analysis: All statistical analyses were carried out using SPSS statistical software (SPSS for windows, version 11.0). All data were presented in mean (±) standard deviations (S.D.). Differences in measured parameters among the three groups were analyzed with a nonparametric test (Kruskal-Wallis). Dual comparisons between groups exhibiting significant values were evaluated with a Mann–Whitney *U*-test. These differences were considered significant when probability was less than 0.05.

Results

Histopathological findings: In the control group, the morphology of neurons in the frontal cortex tissues was normal (Figure 1a). In the FA treated group, the most consistent findings

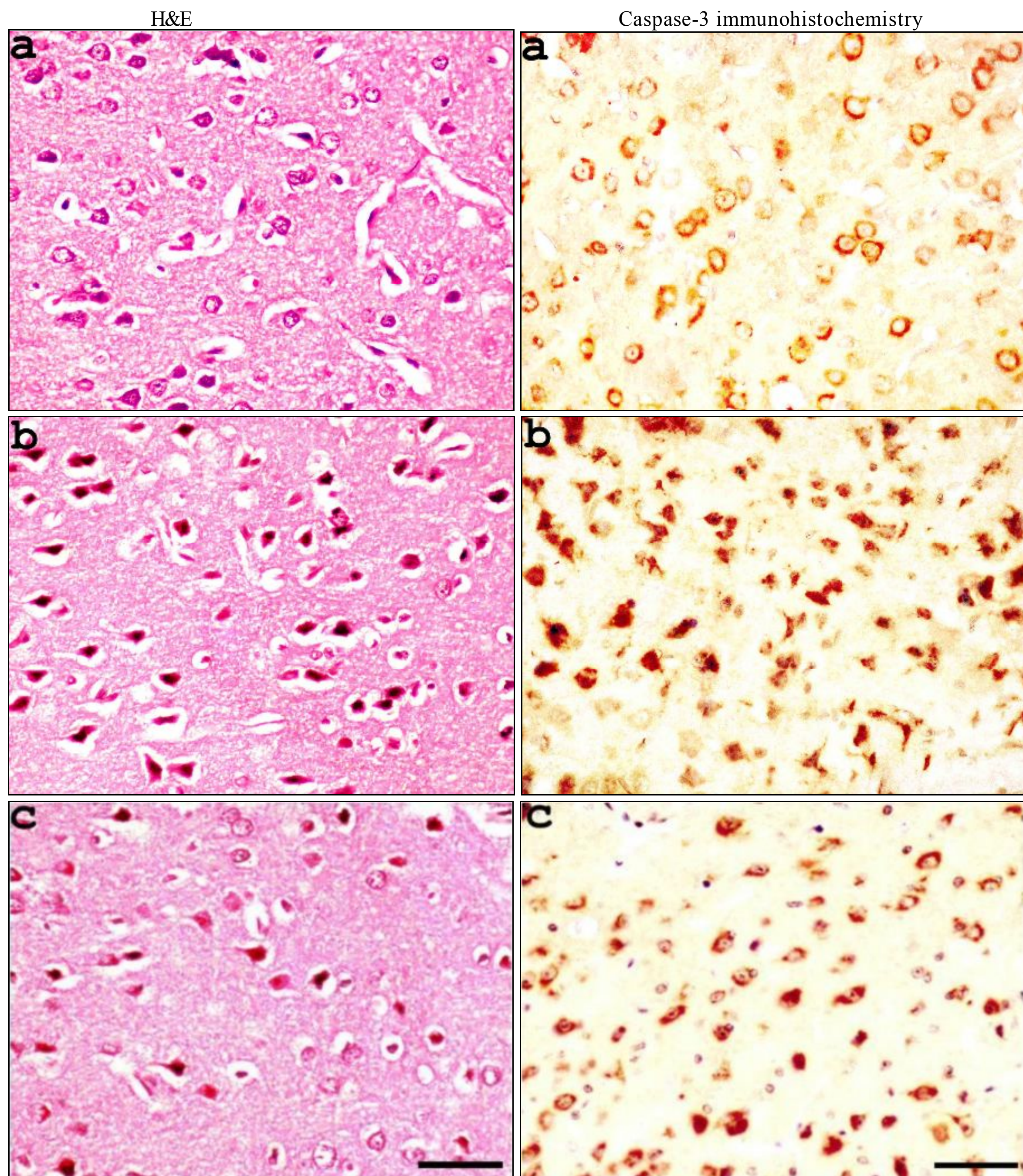

Figure 1. Representative light microphotographs showing the morphology and apoptosis (a–c) of the frontal cortex tissue after FA exposure by hematoxylin-eosin and caspase-3 immunohistochemistry. (a) Control rats: Normal frontal cortex tissue histology is seen. (b) FA treated rats: Severe degenerative changes, shrinkage cytoplasma and extensively dark picnotic nuclei in neurons of the frontal cortex tissue are seen. (c) FA treated rats with NS: Effective preventing of degenerative changes and shrunken in cytoplasma and nuclei are prominent in neuronal cells. (H&E, scale bar, 50 μm). (a) Control rats: Neuronal cells are mildly stain with the anti-caspase-3 antibody. (b) FA treated rats: The caspase-3 immunopositivity was strongly increased in degenerating neurons of the frontal cortex tissue after FA exposure. (c) FA treated rats with NS: Treatment of NS markedly reduced the immunoreactivity of degenerating neurons after FA exposure. (Immunoperoxidase, haematoxylin counterstain, scale bar, 50 μm)

occurring in the histological tissue sections stained with hematoxylin-eosin were those indicating severe degenerative changes, shrunken cytoplasma and extensively dark picnotic nuclei in neurons of the frontal cortex tissues (Figure 1b). In the NS-treated rats' frontal cortex tissues, the intensity of neuronal changes was less than in the only FA treated group. The dark stained

nucleus and the distorted nerve cells were mainly absent in the NS-treated rats. In these groups, the severity of degenerative changes in the cytoplasma and especially in the nuclei of cells was less than that in the only FA treated group (Figure 1c). On the other hand, the number of apoptotic neurons were increased significantly in FA treated rats compared to control ($p < 0.0001$) and FA treated with NS ($p < 0.01$) rats' frontal cortex tissues (Table 1).

Immunohistochemical findings: Mild caspase-3 immunoreactivity was observed in the cytoplasm of neurons in control rats (Figure 1a). The micrographs showed apoptotic neurons by caspase-3 immunohistochemistry after FA exposure in rats. The caspase 3 immunopositivity was increased in degenerating neurons of the frontal cortex tissues following FA exposure (Figure 1b). Treatment of NS markedly reduced the immunoreactivity of degenerating neurons after FA exposure (Figure 1c).

Table 1. The numbers (number/μm^2) of apoptotic neurons (strong caspase 3 immunopositive) in the frontal cortex tissues of A (control), B (FA treated) and C (FA treated with NS) groups.

Groups	Frontal cortex tissue
A	11.7±1.2
B	87.3 ± 9.3[a]
C	49.8±4.5[b]

Kruskal-Wallis test was used for statistical analysis. Values are expressed as means ± SD, n = 10 for each group; [a]$p < 0.0001$ compared to A group, [b]$p < 0.001$ compared to A group, [b]$p < 0.01$ compared to B group

Discussion

FA has many detrimental effects on various tissues of the body including skin, eye, gonads, gastrointestinal system and respiratory tract (33). Additionally, the central nervous system is affected by the toxicity of FA. Histology, pathology, cadaver embalming technicians, dissection students and nurses working at dialysis units are occupationally subject to FA exposure (34, 35). The neurotoxic effects of FA have been shown in experimental studies. It was reported that acute FA administration at low concentrations increases the stimulation rate of the central nervous system, while applied in higher concentrations, it acts as a depressant on the central nervous system (3). The studies on rats showed that exposure to FA slows down motor activity (3, 36). In the study of Pitten et al. (13) inhalation by rats of FA inhibited learning capacity. Furthermore, other studies on rats have also reported that exposure to FA causes behavioural sensitivity and hesitation (12, 37). Similarly in our study, the rats that were systemically exposed to FA had several symptoms of disorder such as marked decline in food and water consumption, lethargy, loss of appetite, and slow motor activity (38).

FA, after administration, fastly diffuses to many tissues, including brain. In a postmortem study FA and its metabolites, methanol and formic acid, were found at the similar concentration in the brain (39). When ingested, FA is rapidly taken up and metabolized as shown by the blood increase of formic acid. There are at least seven enzymes that catalyze the oxidation of FA in animal tissues, namely aldehyde dehydrogenase, xanthine oxidase, catalase, peroxidase, aldehyde oxidase, glyceraldehyde-3-phosphate dehydrogenase, and a specific NAD-dependent FA dehydrogenase (40).

This experiment was carried out to evaluate the histopathological changes in frontal cortex tissues due to oxidative damage by FA and protective effect of NS. FA has a neurotoxic and a slight carcinogenic effect. Although the neuron cytotoxic action of FA is not fully understood, it is thought to be mediated by the activation of free radical producing enzymes, and also, by the inhibition of free radical scavenger systems, thereby enhancing the production of the ROS. It has also been shown that FA and methanol are substrates for cytochrome P-450 monooxygenase system II E1 isozyme (CYP 2E1), which is important in the brain (41) and can thus be oxidised by the endoplasmic reticulum by peroxidase, aldehyde oxidase and xanthine oxidase (40). Activity of these enzymes have previously been shown to exhibit an unusually high rate of oxidase activity with the subsequent formation of ROS. The result of ROS formation is damage to an array of biomolecules found in tissues, including membrane lipids, proteins and nucleic acids. The brain and nervous system may be especially prone to radical damage, as brain has a high content of easily peroxidizable unsaturated fatty acids and as brain requires very high amounts of oxygen per unit weight. Membrane-associated polyunsaturated fatty-acids are readily attached by ROS in a process that results in the peroxidation of lipids. Peroxidation of membrane lipids can disrupt membrane fluidity and cell compartmentation, which can result in cell lysis. Thus oxygen radical-initiated lipid peroxidation and protein oxidation may contribute to the impaired cellular function and necrosis associated with toxicity of FA or its derivates (42). Therefore, FA administration caused

neurodegenera-tion, which is associated with the generation of ROS, resulting in oxidative damage (43).

In this study, FA administration caused severe degenerative changes, shrunken cytoplasma and extensively dark picnotic nuclei in neurons of the frontal cortex tissues. This observation is in agreement with previous studies, which suggest that FA treatment caused severe degenerative changes in neurons of frontal cortex and hippocampal tissues and vit E treatment prevented this neuronal damage (43).

In a previous immunohistochemical and biochemical study, Zararsiz et al. (38) have shown that melatonin treatment prevents FA-induced neuronal damage in the prefrontal cortex of rats. It has been reported that the tissue sections of the FA-administered group contained apoptotic cells with broken nuclei and irregular membranes. There were also pycnotic cells and apoptotic body formations (14).

Likewise, in previous studies, FA has been reported to cause DNA damage, thereby leading to apoptosis (44, 45, 46). Mitochondria play an important role in apoptotic process. Death signals cause an increase in the permeability of the outer mitochondrial membrane, which in turn causes apoptosis. Some proteins regulate the permeability of the outer membrane of mitochondria. The most important of them are the proteins of the Bcl-2 family. Some of the proteins in this family are pro-apoptotic, whereas others are anti-apoptotic. Bax is a pro-apoptotic protein and causes cytochrome c release into cytoplasm

from the mitochondrial membrane. Cytchrome c then initiates the apoptotic process by activating caspase in the cytoplasm. However, Bcl-2 is an anti-apoptotic protein and it inhibits cytochrome c release by preventing Bax insertion into the mitochondrial membrane. To sum up, signs of immunohistochemical Bax staining in the cytoplasm of the cells indicates that apoptosis has occurred (47, 48). In the present study, we showed that FA caused apotosis in the prefrontal cortex.

In the present study, the caspase 3 immunopositivity was increased in degenerating neurons of the frontal cortex tissues following FA exposure. Treatment of NS markedly reduced the immunoreactivity of degenerating neurons after FA exposure.Our results are in agreement with reports of other workers, which suggest that FA caused apotosis in the prefrontal cortex.

In conclusion, the findings obtained in the present study indicate that FA causes neuronal damage in the frontal cortex tissues of rats which can be prevented by NS.

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