Cerebral cortex alterations induced via sub-acute exposure of albino rat to fipronil insecticide (Histopathological and immunohistochemical study)

Mohamed A. Awad[1], Zainab Sabry Othman Ahmed[1*], Gehad Abd El-Fattah Hassan Elbargeesy[1], Moukhtar H. G. Moussa[1]

1. Department of Cytology and Histology, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt.

* Correspondence author; Zainab Sabry Othman Ahmed, Email: zainab.sabry@cu.edu.eg, tel. +201093084062

1. Abstract

Fipronil (FIP) is a globally utilized insecticide that leads to environmental pollution. Exposure of non-target organisms to FIP causes occupational and non-occupational neurological hazard. Therefore, this study aimed to assess the histopathological and immunohistochemical changes in the cerebral cortex of adult albino rats exposed to FIP. Twenty adult male albino rats were divided into 2 groups: control and experimental groups. Each rat of the experimental group received a daily oral dose of FIP at 10 mg /kg body weight. After 45 days of FIP daily administration, samples from the cerebral cortex were collected from both groups and were processed for histopathological and immunohistochemical examination. The cerebral cortex of treated rats showed severe neuronal degeneration, reactive gliosis, neuropil vacuolation and elevated immunoreactivity to GFAP, iNOS and Caspase 3 (active form). In conclusion, sub-acute FIP exposure has neurotoxic effect that appeared in our study in the form of apoptosis and tissue reactivity of the cerebral cortex.

Key words: Fipronil, neurons, neurotoxicity, cerebral cortex, GFAP, iNOS, active caspase-3.

2. Introduction

Fipronil,(±)-5-amino-1-(2,6-dichloro-α,α,α-trifluorop-tolyl)-4 trifluoromethylsulfinylpyrazole-3-carbonitrile [1] , (CAS number 120068-37-3) [2], is a synthetic [3] second-generation insecticide of phenylpyrazole derivatives class (4). It was originally discovered and produced between 1985 and 1987 by the French company Rhône-Poulenc Agro (now Bayer Crop Science) [4].Then, it reached the market in 1993 [5] and was formally registered by the U.S. Environmental Protection Agency in the United States three years later [2]. FIP is a broad-spectrum insecticide which has been applied in Veterinary Medicine; it has an excellent therapeutic action and persistent activity against fleas and ticks of domestic dogs and cats [6]. In agriculture, it is accustomed to fight insects in sugarcane, rice, cotton, potato, maize and soybean crops [3]. In public health, it is used to control the main anthropozoonosis vectors [7]. The broad range of application and usage of FIP leads to possible exposure of mammals and non-target organisms to its toxic effect. In this context, FIP exposure may occur through its application on animals as insecticide [8,9] and consumption of different food products containing FIP residues [10, 11], indicating the risk to human health [12]. Several studies have reported that FIP potentially induces specific neurotoxicity in mice [13], rat [14] and human [15]. The cerebral cortex is the largest portion of vertebrate brain that plays crucial role in neural transactions which enhance memory, plasticity, cognition, speech and mental activity. Moreover, it was considered in several studies as a site to explore insecticides induced neurotoxicity. Many cellular and tissue alterations may occur in cerebral cortex via sub-acute exposure to

FIP. For this purpose, histopathological examination was performed in this study to evaluate the histological alterations of the cerebral cortex. Then, immunohistochemical analysis for iNOS, GFAP and caspase-3 (active form) was conducted. Inducible nitric oxide synthase (iNOS) is not expressed in normal brain, while astroglia and microglia express it following ischemic, traumatic, neurotoxic, or inflammatory conditions [16]. For immunohistochemical detection of reactive gliosis (astrogliosis) as a prevalent reaction to various sorts of brain injury, GFAP antibodies were used [17- 19]. Eventually, immunoreactivity to the active form of caspase-3 was screened and quantified to check apoptosis in paraffin sections [20-23] of cerebral cortex.

3. Materials and Methods:

3.1. Contrast Agent:

The chemical formula of technical grade FIP insecticide is: C12H4Cl2F6N4OS, purity: 92% WG, a product of Crop Life Science LTD. Pesticides Company, India.

3.2. Lab Animals:

20 Wistar strain (*Rattus norvegicus*) healthy adult (age of 7-8 weeks) male rats of 160–180 g body weight were purchased from Vaccera EGY VAC (Helwan, Egypt). The animals were acclimatized for 2 weeks in the Animal House of Pharmacology Department, Faculty of Veterinary Medicine, Cairo University. The rats were housed in plastic cages (dimensions 43cm × 40cm × 29cm), five animals were housed in each cage, with stainless steel top grills (netted cover) and soft wood shavings employed as bedding. They were maintained under good hygienic conditions and standard laboratory conditions (controlled atmosphere; standardized temperature, humidity, and light conditions) at a temperature of 22± 2°C, a dark/light cycle of 12 and 50–70% humidity. Standard food and water were provided ad libitum.

3.3. Study Setup:

The twenty rats were randomly allocated to two groups (10 rats for each group); control (CTR) and treated (FIP) groups. The control group received corn oil (0.5 ml), while the treated group received FIP (technical grade) at 10 mg/kg body weight [24] daily for 45 days [25]. Both corn oil and FIP were administrated by oral gavage. Then, the animals were euthanized by Ketamine (5mg/kg body weight) and Xylazine (90mg/kg body weight) taken 1:1 ratio and injected at 0.1ml/100 gram body weight. Each rat was rapidly exsanguinated by incision of the jugular blood vessels. Brains were immediately excised, weighed, and washed. Then, they were immediately immersed in 10% neutral buffered formalin (10%NBF) for histopathological and immunohistochemical examination.

3.4. Histopathological Analysis:

After routine dehydration with various ascending grades of alcohol, brain tissues were cleared by immersion in xylene and finally embedded in paraffin (melting point 56°C) to prepare paraffin blocks. Then, serial coronal sections (4 µm) were obtained by using microtome (Leica, Germany) for hematoxylin and eosin (H&E) [26] and toluidine blue [27] staining. The stained slides were examined via using the light microscope (LEICA DM500), then images were captured with the camera (LEICA ICC50 HD) attached to the microscope and finally examined by image analysis software (Leica microsystems, LAS version 3.8.0 [build:878] Leica Ltd image analyzer computer system) at Cytology and Histology Department, Faculty of Veterinary Medicine, Cairo University.

3.5. Immunohistochemical Analysis:

5 µm thick deparaffinized sections were prepared for the immunohistochemical analysis, according to the manufacturer's protocol. Deparaffinized retrieved tissue sections were treated with 0.3% H_2O_2 in PBS for 20 minutes to block endogenous peroxidase activity, followed by incubation with anti-iNOS (PA1-036) (1:20) Thermofisher Inc., anti-GFAP – Glial Fibrillary Acidic Protein - by using Mouse monoclonal antibody, Cat. No. MS-280-P1 from thermo scientific Co. (1:100) and with Mouse Anti-Active Caspase 3 antibody (bsm33199M- Sun Long Biotech Co., LTD) 1:100 for 1hr. Tissue sections were washed out by PBS followed by incubation with secondary antibody HRP Envision kit (DAKO) for 20 mins, washed out and incubated with diaminobenzidine (DAB) for 15 mins. Then, they were washed by PBS and counter stained with hematoxylin, dehydrated, cleared in xylene and covered with cover slip for microscopic examination [28]. Different fields (five fields) from cerebral cortex of immunostained sections were scanned for determination of the area percentage of the expression levels of iNOS, Caspase 3 (active form) and GFAP by using Leica Queen 500 software for morphological analysis (Leica Microsystems GmbH, Germany).

3.6. Statistical analysis:

Comparisons between control and fipronil treated groups in cerebral cortex were performed using independent t test. Shapiro–Wilk and Levene's tests were used to check normal distribution and equality of variance assumptions, respectively. The analyses and graphs were performed in R-studio [29, 30]. *P*-value of ≤ 0.01 was accepted as statistical significance for the mentioned tests.

3.7. Ethical statement:

The study was conducted in the Faculty of Veterinary Medicine, Cairo University, Giza, Egypt. The maintenance of experimental rats and all implemented experimental procedures were approved by Institutional Animal Care and Use committee (IACUC). Reference No. is Vet CU20022020135**.**

4. Results:

4.1. Histopathological examination:

4.1.1. Hematoxylin and eosin staining:

Histological study of the control group exhibited normal histological appearance of the cerebral cortex (Fig.1.a), while fipronil treated group showed significant histological alterations which appeared in the form of histological disorganization, neurons in different degrees of degeneration, highly neuroglial infiltration (diffuse gliosis), minute neuropil vacuolation and hemorrhages in the cortex parenchyma (Fig.1.b).

4.1.2. Toluidine blue staining:

Light microscopic examination of the control group revealed normal histoarchitectural structure of the cerebral cortex (Fig.2.a), while fipronil treated group showed significant histoarchitectural alterations appeared as histoarchitectural disorganization, neurons (granular neurons) in different stages of degeneration appeared darkly stained with ill-defined nuclei, neuropil and perineural vacuolation and glia cell infiltrations (Fig.2.b).

4.2. Immunohistochemical findings:

4.2.1. Caspase 3 (active form):

The control group revealed low positive immunostaining reaction to caspase 3 (active form) observed in neurons of the cerebral cortex (Fig.3.a), while fipronil treated group exhibited positive immunostaining (Fig.3.b). Moreover, the mean area percentage of caspase 3 (active form) was significantly increased ($p \leq 0.05$) in fipronil treated group when compared to the control group (Fig.3.c).

4.2.2. inducible nitric oxide synthase (iNOS):

Low positive iNOS immunoreactivity was observed in the cerebral cortex of the control group (Fig.4.a), while fipronil treated group showed high positive immunostaining in neurons and glia cells (Fig.4.b). Moreover, the mean area percentage of iNOS was significantly increased ($p \leq 0.05$) in fipronil treated group when compared to the control group (Fig.4.c).

4.2.3. Glial fibrillary acidic protein (GFAP):

The control group showed mild positive immunoreaction to GFAP which represented the normal morphological pattern in astrocytes and glia fibers in cerebral cortex (Fig.5.a), while fipronil treated group exhibited strong positive immunoreaction to GFAP (Fig.5.b) as astrocytes appeared hypertrophic with extensive overlapping and interdigitations of their branching processes. Moreover, the mean area percentage of GFAP was significantly increased ($p \leq 0.05$) in fipronil treated group when compared to the control group (Fig.5.c).

5. Discussion:

FIP is a widely used insecticide in agriculture, veterinary and household applications. This extensive usage may lead to high rates of contamination and exposure. Therefore, public health questions for its adverse effects on non-target organisms have been raised [1]. The response to its exposure varies between experimental studies and case reports; it depends mainly on the duration and the level of exposure as well as the route of exposure. However, neurological response is prominent. In the current study, we focused on investigating the possible effect on the cerebral cortex following sub-acute exposure of albino rats to 10 mg/kg body weight of fipronil. Cerebral cortex has vital functions in the fine motor control, motion fractionation and sensorimotor incorporation [31,32]. Our data showed that rats treated for 45 days with fipronil at 10 mg/kg body weight developed several histopathological alterations in the cerebral cortex in the form of histoarchitectural disorganization, different degrees of neuronal degeneration, highly neuroglial infiltration (diffuse gliosis), minute neuropil vacuolation and hemorrhages in cortical parenchyma. These results agree with previous studies that showed nuclear pyknosis and degeneration in the cerebral cortex neurons [24,25]. In this study, three different antibodies [caspase 3 (active form), iNOS and GFAP] have been used to test the effect of fipronil on the cerebral cortex. The current study revealed that iNOS expression in fipronil treated group had statistically significant positive cytoplasmic immunoreaction in cerebral cortex compared to the control group. These findings are in line with a study that revealed increased levels of iNOS in the substantia nigra (SN) and in the striatum of FIP treated rats after infusion of fipronil in SN [33]. iNOS expression in neurons has possible impact on inflammation and neurodegeneration of human brain [34]. Moreover, other analysts have stated that the expression of iNOS may be one of the coordinate impacts of inflammatory process [35]. In addition, another study revealed elevated nitric oxide NO in brain tissue upon fipronil exposure [36]. Nitrosylation reactions which results from the action of excessive production of reactive nitrogen species could alter the protein structure and thereby impede their normal function [37]. Moreover, many investigators explained the presence of immunoreaction to the role of iNOS in inducing protein nitration, which may be associated with changes in cell morphology [38, 39]. Therefore, fipronil may induce generation of NO mediated by overexpression of inducible form of nitric oxide synthase (iNOS) resulting in morphological alterations in cerebral cortex. The second antibody is anti-glial fibrillary acidic acid protein (GFAP) which is a specific marker protein for astrogliosis and reactive astrocytosis [40]. Our findings revealed immunohistochemically

overexpression of GFAP in the cerebral cortex of fipronil exposed group when compared to the control one. These results are consistent with another study that exhibited via western blot analysis the upregulation of GFAP in SN and striatum after fipronil injection in SN of rats [41]. Therefore, fipronil exposure may lead to astrocytic hyperplasia and hypertrophy in cerebral cortex. Caspase activation is an integral process of apoptosis during neural tissue damage [42]. For this reason, the last studied antibody in the current work was the activated caspase 3 which is crucial for the execution of apoptosis [43]. Moreover, both internal and external apoptotic pathways meet at the level of caspase 3 [44]. The present study exhibited elevated immunoreactivity of caspase 3 (active form) in cerebral cortex of rats exposed to fipronil. This result supports investigations which suggested apoptosis upon fipronil exposure mediated by elevation of Caspase 3 expression [25, 33, 45]. Therefore, we suggest the ability of fipronil to induce apoptosis in cerebral cortex mediated by caspase 3 activation.

6. Conclusion:

The present study documented the ability of fipronil to induce tissue reaction and apoptosis in the cerebral cortex of adult male albino rats upon their subacute exposure to fipronil at 10 mg/ kg body weight. Tissue reaction appeared histopathologically as neuron degeneration and neuroglia infiltration and immunohistochemically through upregulation of GFAP and iNOS. Moreover, apoptosis appeared though elevated caspase 3 (active form) expression.

7. References

1. K. A. Jennings, T. Canerdy, R. Keller, B. Atieh, R. Doss, and R. Gupta (2002): Human exposure to fipronil from dogs treated with Frontline. *Vet. Hum. Toxicol.*44, 301.
2. E. N. P. F. Sheet (1996): EPA-737-F-96-005.*Washington, DC: United States Environmental Protection Agency*.
3. J. Z. Magalhães, T. M. Sandini, M. S. B. Udo, A. R. Fukushima, and H. de Souza Spinosa (2018): Fipronil: usos, características farmacológicas e toxicológicas.
4. C. C. Tingle, J. A. Rother, C. F. Dewhurst, S. Lauer, and W. J. King (2003):Fipronil: environmental fate, ecotoxicology, and human health concerns. *Rev Environ Contam Toxicol*, ed: Springer,1-66.
5. C. Tomlin (2000):The pesticide manual 12th Edition. *British Crop Protection Council.* 9, 647-648.
6. P. R. de Oliveira, G. H. Bechara, and M. I. Camargo-Mathias (2008): Evaluation of cytotoxic effects of fipronil on ovaries of semi-engorged Rhipicephalus sanguineus (Latreille, 1806)(Acari: Ixodidae) tick female. *Food Chem. Toxicol.* 46,2459-2465.
7. J. W. Pridgeon, R. M. Pereira, J. J. Becnel, S. A. Allan, G. G. Clark, and K. J. Linthicum(2008): Susceptibility of Aedes aegypti, Culex quinquefasciatus Say, and Anopheles quadrimaculatus Say to 19 pesticides with different modes of action. J. Med. Entomol.45, 82-87.
8. J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald, and W. J. Rutter(1979):Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.*18,5294-5299.
9. J. Sambrook, E. F. Fritsch, and T. Maniatis(1989):*Molecular cloning: a laboratory manual*: Cold spring harbor laboratory press.

10. T. Yoshioka, K. Kawada, T. Shimada, and M. Mori(1979):Lipid peroxidation in maternal and cord blood and protective mechanism against activated-oxygen toxicity in the blood. *Am. J. Obstet. Gynecol*.135,372-376.
11. D. Jollow, J. Mitchell, N. a. Zampaglione, and J. Gillette(1974):Bromobenzene-induced liver necrosis. Protective role of glutathione and evidence for 3, 4-bromobenzene oxide as the hepatotoxic metabolite. *Pharmacology*.11,151-169.
12. R. Altenburger, W. Boedeker, M. Faust, and L. Grimme(1996): Regulations for combined effects of pollutants: Consequences from risk assessment in aquatic toxicology. *Food Chem. Toxicol.* 34,1155-1157.
13. S. Mondot and M. Dange, "Acute oral LD50 in the mouse(1995): *Unpublished report No. R&D/CRSA/TO-PHA3 from Rhone-Poulenc Agrochimie Toxicology (Research Triangle Park, NC, USA, Submitted to WHO by Rhone-Poulenc, Inc.)*.
14. D. Ray (1997): Report of the toxicity of fipronil.*Unpublished report from Medical Research Council (Leicester, United Kingdom, Submitted to WHO by MRC Toxicology)*.
15. F. Mohamed, L. Senarathna, A. Percy, M. Abeyewardene, G. Eaglesham, R. Cheng, *et al.*(2004):Acute human self-poisoning with the n-phenylpyrazole insecticide fipronil—A GABAA-gated chloride channel blocker," *J. Toxicol. Clin. Toxicol.*42, 955-963.
16. S. R. Jaffrey and S. H. Snyder (1995): Nitric oxide: a neural messenger. *Annu. Rev. Cell Dev. Biol.*11,417-440.
17. J. P. O'Callaghan and K. Sriram (2005):Glial fibrillary acidic protein and related glial proteins as biomarkers of neurotoxicity. *Expert Opin Drug Saf.* 4, 433-442.
18. K. Sriram and J. P. O'Callaghan (2005): Signaling mechanisms underlying toxicant-induced gliosis. *The role of glia in neurotoxicity*. 2, 141-171.
19. W. T. Norton, D. Aquino, I. Hozumi, F.-C. Chiu, and C. Brosnan (1992): Quantitative aspects of reactive gliosis: a review. *Neurochem. Res.*17,877-885.
20. A. M. Gown and M. C. Willingham (2002): Improved detection of apoptotic cells in archival paraffin sections: immunohistochemistry using antibodies to cleaved caspase 3. *J. Histochem. Cytochem*.50,449-454.
21. W. R. Duan, D. S. Garner, S. D. Williams, C. L. Funckes-Shippy, I. S. Spath, and E. A. Blomme (2003): Comparison of immunohistochemistry for activated caspase-3 and cleaved cytokeratin 18 with the TUNEL method for quantification of apoptosis in histological sections of PC-3 subcutaneous xenografts. *The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland*.199,221-228.
22. A. Resendes, N. Majo, J. Segales, J. Espadamala, E. Mateu, F. Chianini, *et al.*(2004): Apoptosis in normal lymphoid organs from healthy normal, conventional pigs at different ages detected by TUNEL and cleaved caspase-3 immunohistochemistry in paraffin-embedded tissues. *Vet. Immunol. Immunopathol.*99,203-213.
23. S. Jakob, N. Corazza, E. Diamantis, A. Kappeler, and T. Brunner (2008): Detection of apoptosis in vivo using antibodies against

caspase-induced neo-epitopes. *Methods*.44,255-261.
24. P. C. Badgujar, N. N. Pawar, G. A. Chandratre, A. Telang, and A. Sharma(2015): Fipronil induced oxidative stress in kidney and brain of mice: protective effect of vitamin E and vitamin C. *Pestic Biochem Physiol.*118,10-18.
25. A. Khalaf, M. K. Galal, M. A. Ibrahim, A. Abd Allah, M. M. Afify, and R. Refaat (2019):The Terminalia laxiflora modulates the neurotoxicity induced by fipronil in male albino rats. *Biosci. Rep.*39.
26. J. D. Bancroft and M. Gamble (2008): *Theory and practice of histological techniques*: Elsevier health sciences.
27. J. A. Kiernan (1999): Histological and histochemical methods: theory and practice. *Shock.*12,479.
28. K. S. Suvarna, C. Layton, and J. D. Bancroft (2018): *Bancroft's Theory and Practice of Histological Techniques E-Book*: Elsevier Health Sciences.
29. R. Core Team (2013): R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. *Supplementary Figure S.*2.
30. R. Team (2015): RStudio: integrated development for R.*RStudio, Inc., Boston, MA URL http://www. rstudio. Com.*42,14.
31. E. V. Evarts, C. Fromm, J. Kroller, and V. Jennings (1983): Motor cortex control of finely graded forces. *Journal of Neurophysiology*.49,1199-1215.
32. J. N. Sanes and J. P. Donoghue (2000): Plasticity and primary motor cortex. *Annu. Rev. Neurosci.*23,393-415.
33. Y.-W. Ki, J. E. Lee, J. H. Park, I. C. Shin, and H. C. Koh (2012): Reactive oxygen species and mitogen-activated protein kinase induce apoptotic death of SH-SY5Y cells in response to fipronil. *Toxicol. Lett.* 211,18-28.
34. M. T. Heneka and D. L. Feinstein (2001): Expression and function of inducible nitric oxide synthase in neurons. *J. Neuroimmunol.*114,8-18.
35. C. V. Suschek, O. Schnorr, and V. Kolb-Bachofen (2004): The role of iNOS in chronic inflammatory processes in vivo: is it damage-promoting, protective, or active at all. *Curr. Mol. Med.*4, 763-775.
36. M. M. Abdel-Daim, H. M. Shaheen, A. I. Abushouk, E. A. Toraih, M. S. Fawzy, W. S. Alansari, *et al.* (2018): Thymoquinone and diallyl sulfide protect against fipronil-induced oxidative injury in rats. *Environ Sci Pollut Res Int.* 25, 23909-23916.
37. M. Valko, D. Leibfritz, J. Moncol, M. T. Cronin, M. Mazur, and J. Telser (2007): Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.*39,44-84.
38. S. Blanco, F. J. Molina, L. Castro, M. L. Del Moral, R. Hernandez, A. Jimenez, *et al.* (2010): Study of the nitric oxide system in the rat cerebellum during aging. *BMC Neurosci.*11,1-14.
39. J. Rodrigo, D. Alonso, A. P. Fernández, J. Serrano, A. Richart, J. C. López, *et al.* (2001):Neuronal and inducible nitric oxide synthase expression and protein nitration in rat cerebellum after oxygen and glucose deprivation. *Brain research.*909, 20-45.
40. L. F. Eng and R. S. Ghirnikar, "GFAP and astrogliosis (1994): *Brain Pathol.*4,229-237.
41. J. H. Park, Y. S. Park, and H. C. Koh (2016): Progressive loss of nigrostriatal dopaminergic neurons induced by inflammatory responses to fipronil. *Toxicol. Lett.*258,6-45.

42. A. G. Yakovlev and A. I. Faden (2001): Caspase-dependent apoptotic pathways in CNS injury. *Mol. Neurobiol.*24,131-144.
43. A. G. Porter and R. U. Jänicke (1999): Emerging roles of caspase-3 in apoptosis. *Cell Death Differ.*6,99-104.
44. M. Guo and B. A. Hay (1999): Cell proliferation and apoptosis. *Current opinion in cell biology.*11,745-752.
45. S. Khan, M. Jan, D. Kumar, and A. Telang (2015): Firpronil induced spermotoxicity is associated with oxidative stress, DNA damage and apoptosis in male rats. *Pestic Biochem Physiol.*124,8-14.

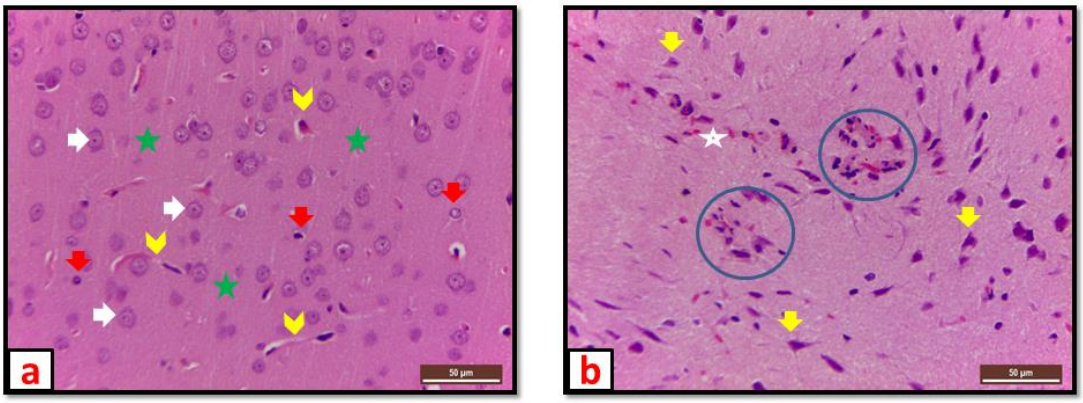

Fig .1. Photomicrographs of cerebral cortex section of adult male albino rat stained by H& E stain showing: (a) Control group with normal intact histological structure of cerebral cortex; it shows its normal structural components including granular neurons with round open face vesicular nuclei and prominent nucleoli surrounded by pale basophilic cytoplasm (white arrow). Neurons are disposed within abundant neuroglia with dense small nuclei without properly seen cytoplasm (red arrow) and acidophilic neuropil (green star). Blood vessels are also present (yellow arrow head).(b) Fipronil treated group: cerebral cortex shows different degrees of neuronal degeneration appear as deeply stained shrunken neurons with contracted dense hyperchromatic pyknotic nuclei (yellow arrow), highly neuroglial infiltration (diffuse gliosis) (blue circle) and hemorrhage in cortex parenchyma (white star). (H&E, 400X).

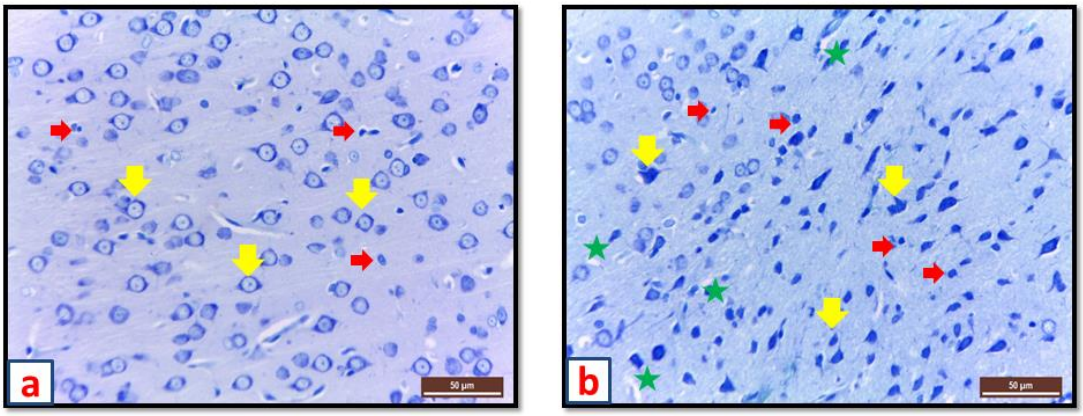

Fig .2. Photomicrographs of cerebral cortex section of adult male albino rat stained by Toluidine blue stain showing: (a) Control group: normal intact histoarchitectural structure of cerebral cortex that shows the main cellular components including granular neurons with round vesicular nuclei and prominent nucleoli surrounded by rim of darkly stained scanty cytoplasm (yellow arrow). Neurons are disposed within abundant neuroglia with dense small nuclei without properly seen cytoplasm (red arrow). (b) Fipronil treated group: cerebral cortex shows area of neuronal degeneration appear as deeply stained shrunken neurons with ill- defined darkly stained nuclei (yellow arrow), extensive neuropil, perineural vacuolation (green star) and neuroglial infiltration (red arrow). (toluidine blue, 400X).

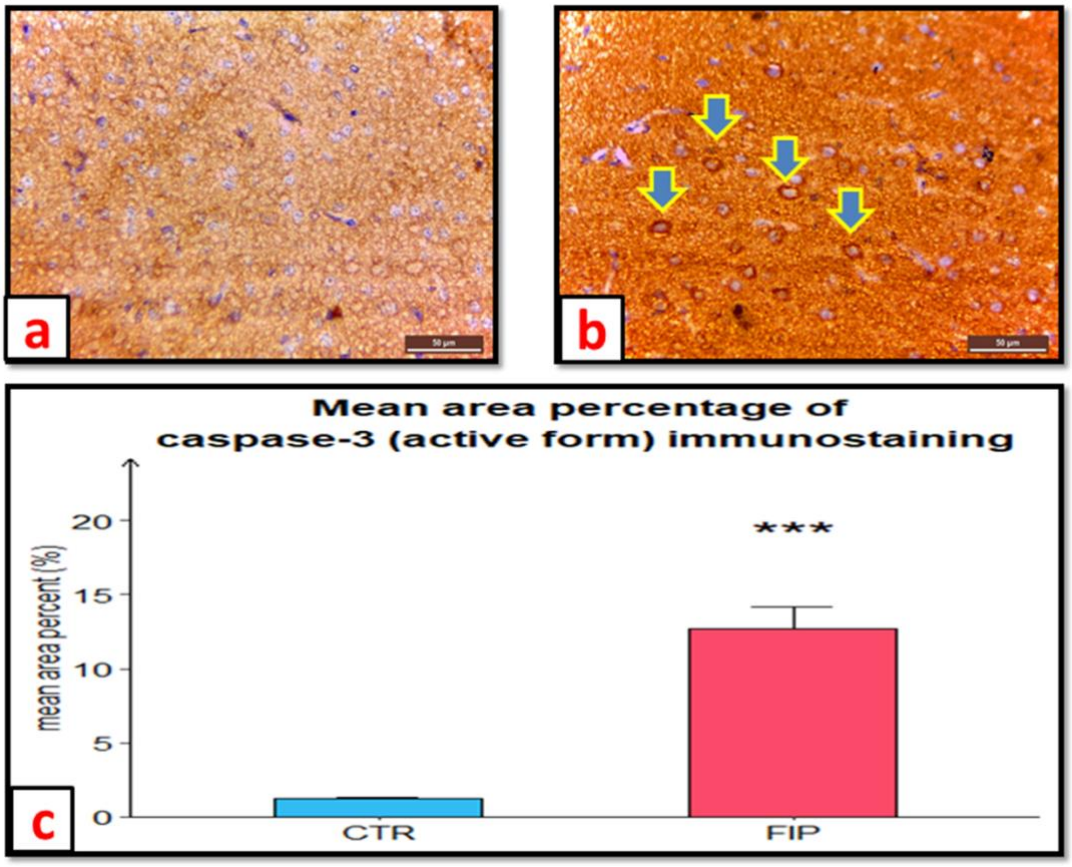

Fig .3. Representative micrographs of immunostained specimens of cerebral cortex from the control (a) and the experimental (b) groups of rats (n = 5 animals/group). Specimens were probed with antibody specific for Caspase 3 (active form). Magnification, 400 X; scale bar, 50 µm. Immunostaining was quantified by mean area percentage per slide in each section in cerebral cortex for active caspase 3 (c). Values are expressed as means, n = 5. Significance at $P \leq 0.01$.

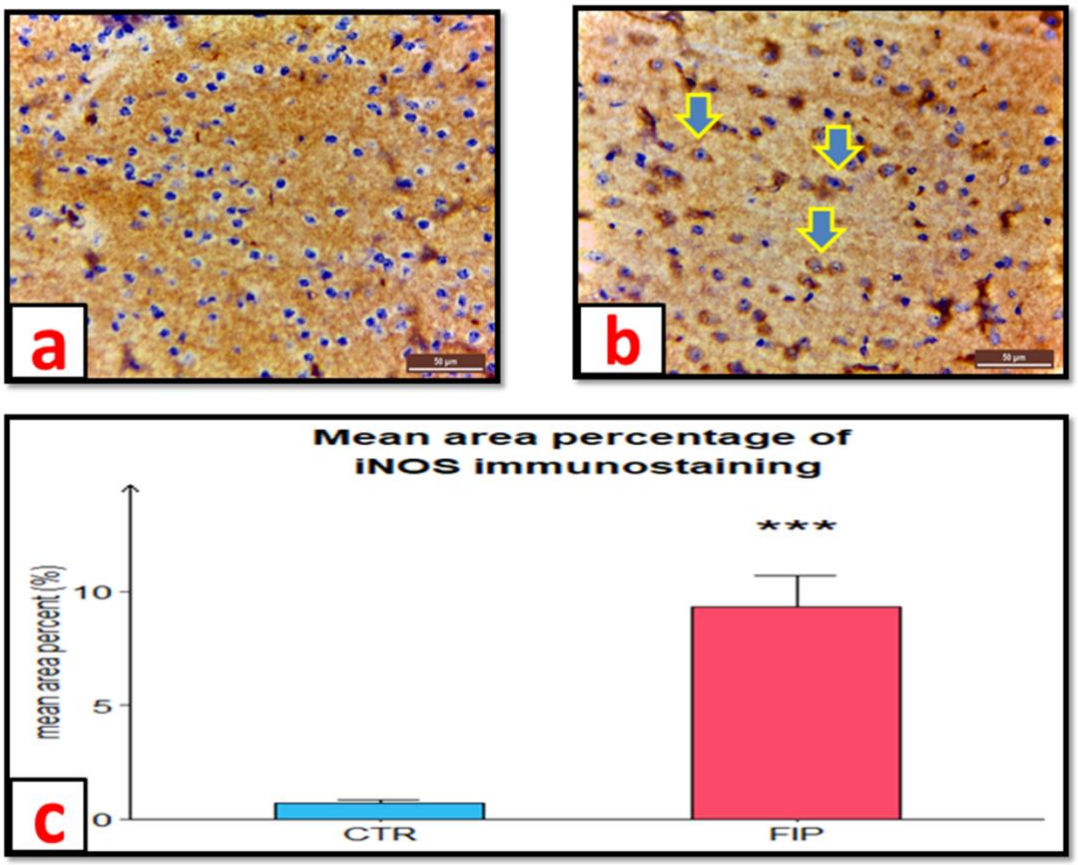

Fig .4. Representative micrographs of immunostained specimens of cerebral cortex from the control (a) and the experimental (b) groups of rats (n = 5 animals/group). Specimens were probed with antibody specific for iNOS. Magnification, 400 X; scale bar, 50 µm. Immunostaining was quantified by mean area percentage per slide in each section in the cerebral cortex for iNOS (c). Values are expressed as means, n = 5. Significance at $P \leq 0.01$.

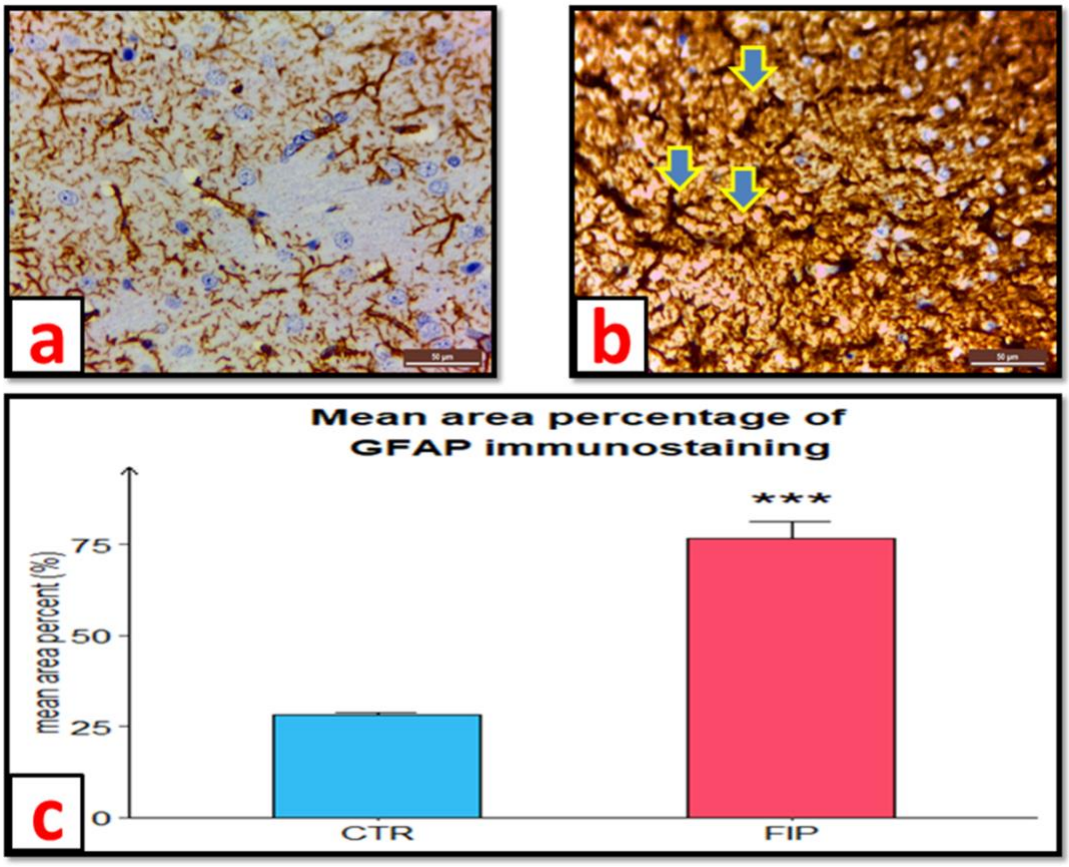

Fig .5. Representative micrographs of immunostained specimens of cerebral cortex from the control (a) and the experimental (b) groups of rats (n = 5 animals/group). Specimens were probed with antibody specific for GFAP. Magnification, 400 X; scale bar, 50 µm. Immunostaining was quantified by mean area percentage per slide in each section in the cerebral cortex for GFAP (c). Values are expressed as means, n = 5. Significance at $P \leq 0.01$.