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Original Article Research Journal of Health Sciences

Memory, neurogenic protein and oxidative deficits of frontal cortex following chlorpyrifos/dichlorvos exposure in rats

***Kadir R.E.[1], Gegele T.A.[1], Kola-Taiwo I.O.[1], Oyewole A.L.[2], Ibrahim A.[1,4], Imam A.[1.3], Chengetanai S.[5], Ajao M.S.[1].**

Abstract

Objective: The use of xenobiotics to boost agricultural productivity has led to toxic chemicals exposure including organophosphates, causing adverse health outcomes including behavioral and neuronal impairments. This study aimed to evaluate the memory indices, possible oxidative and cholinesterase outturns on the frontal cortices of rats exposed to organophosphates.

Methodology: Thirty-two Wistar rats were grouped into four. They received 1ml/kg of Normal, 8.8 mg/kg dichlorvos, 14.9 mg/kg chlopyrifos, and 8.8 mg/kg dichlorvos plus 14.9mg/kg chlorpyrifos respectively. They had training trials in the Y Maze paradigm then spatial working memory assessment. They were euthanized 24hours following exposure and tissues excised for analysis.

Results: A marked reduction in metabolic markers, Acetylcholine Esterase (AChE) activity, spatial memory indices and proliferative neuron marker (Ki67) were observed. Also, increase in oxidative stress markers in the frontal cortices of the organophosphates exposed rats.

Conclusion: The findings demonstrated neurotoxic effects of organophosphates in rats.

Keywords: Organophosphates, Oxidative stress, Acetylcholinesterase, Spatial memory

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Article original

Research Journal of Health Sciences

Mémoire, proteines neurogènes et déficits oxydatifs du cortex frontal suite a une exposition au chlorpyrifos/dichlorvos chez le rat

***Kadir R.E.[1], Gegele T.A.[1], Kola-Taiwo I.O.[1], Oyewole A.L.[2], Ibrahim A.[1,4], Imam A.[1.3], Chengetanai S.[5], Ajao M.S.[1].**

Résumé

Introduction : L'utilisation de xénobiotiques pour stimuler la productivité agricole a entraîné une exposition à des produits chimiques toxiques, notamment des organophosphates, entraînant des effets néfastes sur la santé, notamment des troubles comportementaux et neuronaux. Cette étude visait à évaluer les indices de mémoire, les éventuels résultats oxydatifs et cholinestérases sur le cortex frontal de rats exposés à des organophosphorés.

Méthode de l'étude : Trente-deux (32) rats Wistar ont été regroupés en quatre. Ils ont reçu 1 ml/kg de Normal, 8,8 mg/kg de dichlorvos, 14,9 mg/kg de chlopyrifos et 8,8 mg/kg de dichlorvos plus 14,9 mg/kg de chlorpyrifos respectivement. Ils ont eu des essais de formation dans le paradigme Y Maze puis une évaluation spatiale de la mémoire de travail. Ils ont été euthanasiés 24 heures après l'exposition et les tissus ont été excisés pour analyse.

Résultats de l'étude : Une réduction marquée des marqueurs métaboliques, de l'activité de l'acétylcholine estérase (AChE), des indices de mémoire spatiale et du marqueur neuronal prolifératif (Ki67) a été observée. Aussi, augmentation des marqueurs de stress oxydatif dans le cortex frontal des rats exposés aux organophosphorés.

Conclusion : Les résultats ont démontré des effets neurotoxiques des organophosphorés chez le rat

Mots-clés : Organophosphorés, stress oxydatif, acétylcholinestérase, mémoire spatiale

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INTRODUCTION

Pesticides are chemicals used to control a variety of pests that damage crops, livestock and reduce farm productivity. Organophosphates compounds are a group of pesticides that includes some of the most toxic chemicals used in agriculture. Organophosphorus chemicals (OPs) were developed more than a century ago and are used as insecticides, medications and nerve agents (1). OPs are especially efficacious in disabling insects via inhibition of Acetylcholinesterase (AChE), leading to the upsurge of acetylcholine (ACh) in the body (2). AChE hydrolyzes the neurotransmitter ACh into acetic acid and choline after the completion of neurochemical transmission. OP compounds bind covalently to the active sites of AChE, transforming them into enzymatically inert proteins. Inhibition of AChE leads to the continual buildup of ACh at the nerve and neuromuscular synapses. This overabundance of ACh produces acute cholinergic symptoms, including salivation, lacrimation, blurred vision, and tremors that, if left untreated, may evolve into seizures (3). Being potent AChE inhibitors, OPs are also shown to inhibit mitochondrial enzymes and their exposure even in non-target animals can induce perturbation of physiological processes which may lead to death.

The two commonly used organophosphate chemicals in Nigeria are chlorpyrifos (CPF) and dichlorvos (DDVP). CyaPF and DDVP ubiquity stems from their availability, affordability and most importantly, their efficacy in the control of household insects, agricultural and urban public health pests (4). Neurotoxicity and oxidative damage are some of the documented characteristics common to both chemicals following acute exposure (5). Occupational exposure of OP is widespread, particularly amongst farmers, where the combination of absence of personal protection equipment during handling and a high frequency of usage may lead to acute and long-term poisoning (6,7). Worldwide, around 350,000 cases of self-poisoning with pesticides are recorded per year, and it is estimated that nearly 30% of global suicides are due to pesticide self-poisoning, with most cases recorded in low- and middle-income countries including Nigeria (8).

In the present study using the rat model, we therefore hypothesized that combined exposure to DDVP and CPF will have more pronounced neurotoxic effects on the rat's brain than those administered with either of the chemicals separately.

MATERIALS AND METHODS

Ethical Approval: The University of Ilorin Ethical Review Committee (UERC) (UERC/ASN/2017/856) approved this research on July 27, 2017 in compliance with the Institutional Animal Care and Use Committee (IACUC).

Chemicals and Drugs: Dichlorvos (PubChem Substance ID 329756736) and Chlorpyrifos (PubChem Substance ID 329756699) PESTANAL®, were procured at Sigma Aldrich.

Animal Preparation: Thirty-two (32) rats with average weight of 200 $\pm$ 20g were used for this study. All rats were housed in the animal building of College of Health Sciences, University of Ilorin, where they were allowed to acclimatize for 7 days prior to the experiment. Animals had unlimited access to clean water and standard pelletized rat feed during and after the acclimatization period. Rats were then randomly divided into four (4) groups of eight (n = 8) rats and labeled according to the precise treatment of the group. Briefly, the first group was given DDVP at a dose of 8.8 mg/kg, the second group was treated with CPF at a dose of 14.9 mg/kg, the third group was administered with combined DDVP and CPF at doses of 8.8 mg/kg and 14.9 mg/kg respectively while the fourth group was treated with normal saline at a dose of 1ml/kg. All treatments were carried out orally, once daily and repeated for 14 days. On the 15th day, relevant behaviors were assessed, this was followed by collection of samples (plasma and brains) from sacrificed animals. The plasma and brain homogenate were used for biochemical study while part of the brains were used for histological study.

Behavioral Assessment

To evaluate the effect of lone and co-administration of DDVP and CPF on the spatial working memory of the rats, the Y-maze behavioral paradigm was used.

Procedure for Y Maze: Three training trials on Day 11, and a single trial on day 12 was used to determine the spatial working memory. The spatial memory of the animals following treatments were assessed using the Y-maze apparatus. All the events were observed personally, and with the aid of a stop watch, the behaviours were scored and documented. Spontaneous alternation in rats was also carried out using the Y-maze apparatus, that has a floor width of 5cm and having saw shaves for its

levelling. To determine this, a rat is stationed at a time in one of the arms, and given time (5 minutes) to move freely in the apparatus. An alternation is determined by the order or consecutive entrance into the apparatus arms. The spontaneous alternation was then calculated by subtracting two from the total number of times it entered the arms. The percentage alternation was determined by dividing the actual alternations by the maximum alternations, and multiplying the result by 100%. The correct and wrong alternations are determined from the recorded data of the total arm entries (ABCBCABACBCA is 12 entries) (12).

Measurement of NO and ROSs Levels

Using 50 mM sodium phosphate-buffered saline (100mM Na_2HPO_4/NaH_2PO_4 at pH 7.4), the frontal cortices were washed in an ice-containing medium, with 0.1 mM EDTA, with the aim of removing any blood clots and cells. Ice cold buffer solution was used to homogenize the tissues, at a ratio of a gram of tissue to 10ml fluid, then centrifuged at 5000 revolutions per minute. The supernatant was stored in Eppendorf tube and refridgerated at -80°C. This was later thawed and used for the spectrophotometric estimation of NO and ROS in the tissues. Determining the ROS level was via monitoring the increasing fluorescence of DCFH-DA as described by Ahadpour et al, aided by flow cytometry (Partec, Deutschland) that has an argon ion laser of 488mm via a Flomax software. A 530-nm band pass filter (FL-1 channel) was used to obtain the signals. The mean intensity of the fluorescence was 10,000 counts that was used for each determination (13). In order to obtain the measurement of the nitrate production (nitric oxide metabolites), the remaining tissue homogenate was added to the Griess reagents, with sulfanilamide and naphthyl ethylene diamine solutionse nitrate/nitrite production (nitric oxide metabolites). A microplate reader was used to determine the absorbance levels, then the nitric oxide metabolites were read based on a standard curve (14).

Measurement of AChE Activity

The dissected frontal cortices were homogenized in ice cold Tris-buffered saline (pH 7.4), then diluted in a 1 to 25 ratio of 0.1% saponin. A duration of 10minutes was used for the incubation on ice followed by freezing -20℃. The protein concentration was determined from the brain homogenate using a bicinchoninic acid assay, procured from Sigma Aldrich. Bovine serum albumin, which are standard samples were incubated with bicinchoninic acid reagents, at a ratio of 1:20 at 37℃ for 30 minutes, then read at 562nm absorbance. The activity of acethycholinesterase was quantified in the brain homogenate via a modification of Ellmans colorimetric assay. 10ml of the further diluted homogenate (dilution with 0.1% saponin at a ratio of 1:5) was added to the well with of 110ml phosphate buffered saline at 0.1 M and pH 7.4). Further addition of 99ml 5,5-Dithio-bis-2 nitro-benzoate (DTNB) at 0.25 mM, serving as chromatogen (Sigma-Aldrich), and 11ml acetylthiocholine iodide at 155 mM, serving as substrate (Greyhound Chroma- tography, UK) were made. The asorbance of DTNB was interpreted at 412 nm for 30 min at 25℃. Thus, this AChE activity from the brain homogenate was expressed in nmol min_1 mg_1 protein.

Histology and Immunohistochemistry

Twenty mg/kg of intraperitoneal ketamine was used to anaesthesize the rats after 24 hours of behavioural test. The brains were then carefully excised and fixed in 4% paraformaldehyde solution, embedded in paraffin, allowed to cool then coronal sections of the frontal cortices made at 8 μm each with the aid of a rotary microtome (MK 1110). Cresyl fast violet (CFV) stain was employed for the overall neural architecture then Nissl granules after observing standard laboratory procedures and anti-Ki-67 to reveal the expression of Ki-67 proteins. Sections of the paraffin embedded frontal tissues now incubated in citrate buffered solution for epitope retrieval at pH 6.0 of 90 ℃ temperature for forty minutes. This was then transferred to an endogenous blocking reagent (peroxidase) 0.6% hydrogen peroxide in TBS`-Triton (0.05% Triton X-100 in TBS, pH 7.4) for thirty minutes. The sections were now incubated in 2% normal goat serum, 0.1% bovine serum albumin (BSA) and 0.25% Triton in TBS, for sixty minutes at room temperature. A preincubation solution served as a solvent for incubation of polyclonal rabbit-anti-lyophilized-Ki-67p antibody (Novocastra, Newcastle, 1:5000), which was carried out through the night at 4℃. Further incubation for 2 hours in biotinylated goat anti-rabbit IgG of 1:1000 (2% goat serum and 0.1% BSA in TBS Vector lab, CA, USA;1:250) follows the primary antibody incubation. These sections were then transferred into a streptavidin-biotin complex (Vectastain Elite ABC kit) solution, then stained with 3,3'-

diaminobenzidine. TBS Triton was used as rinses before incubating in the primary antibody, while TBS only was used afterward. PhotoAnaltysis and Imaging of the cytoarchitecture and the distribution of the Ki-67 immunoreactive proteins in the sections were captured using AmScope MD500 camera attached to Olympus light microscope.

Statistical Analysis

Data was reported as mean ± standard error of mean. Data was analysed using Analysis of Variance (ANOVA), followed by a Bonfferoni posthoc test. Statistical significance was considered at P value of ≤ 0.05. The software package Graph Pad Prism was used to study and for graphical representation of the data.

RESULTS

Animal exposure to the organophosphates employed in this study led to observable effects in indirect metabolic markers (body, brain and brain-body weights ratios). AChE, oxidative markers, histoarchitecture and distribution of proliferation of nuclei proteins in the frontal cortex and spatial memory and alternation in exposed rats. (The animals exposed to the organophosphates used in this research have resulted in noticeable effects on the indirect metabolic markers).

Effects of DDVP+CPF exposure on indirect metabolic markers

The outcome of this research presented an extreme loss in body and brain weights in all rats that were exposed to the organophosphates, with more noticeable decrease in DDVP treated rats (Figure 1a and 1b). However, the control group showed negligible changes (Figure 1c).

Effects of DDVP+CPF exposures on Oxidative Markers (NO and ROS) in the Frontal cortices

DDVP with joint DDVP+CPF exposure in the study amounted to a substantial rise in the overall levels of the oxidative markers in the frontal cortices of the exposed rats, with no notable changes when the CPF treated rats were compared to the control group (Figures 2a-b).

Effects of DDVP and CPF exposures on AChE activities in the Frontal cortices

AChE activity in the frontal cortices was drastically reduced in rats treated with DDVP, CPF and also both when they were compared to the control group. Exposure to both organophosphates however resulted in decreased cholinesterase activity on comparison with the DDVP, CPF and NS treated rats (Figure 3).

Effects of DDVP and CPF exposures on Memory Indices

Treatment with both DDVP and CPF separately or combined significantly impaired the two indices of activity and spatial memory. A noticeable ($*p<0.05$) reduction in percentage alteration and natural alteration in the treated group as in the treated groups when varied with the control (Figure 4b)

Effects of DDVP and CPF exposure on the Histoarchitecture of the Frontal cortices and distribution of Neurogenic protein (Ki-67)

Treatment with both chloropyrifos and dichlorvos interrupts the components of the frontal cortices in the exposed rats, with noticeable canalizations and degeneration (Figure 3). The treatment groups DDVP, CPF and DDVP+CPF shows scanty granule cells and numerous degenerating pyramidal cells. The relative Ki- 67 density was characterized by a marked reduction with no visible immunoreactive cells in any of the treatment groups in comparison with the control (Figure 5b).

DISCUSSION

Organophosphates (OP) generally are commonly used as pesticides and insecticides to enable increased agricultural output. However, in some developing countries, OP poisoning causes more death than infectious diseases (15). In this research, we aimed at comparing the neurotoxicity in rats that have been treated with DDVP and CPF separately, and CPF+DDVP. The sub-chronic oral exposure to OPs resulted in marked depletion of AChE levels, induce oxidative stress, affect the neurohistoachitecture of the frontal cortices of exposed animals, alter spatial memory by influencing the percentage spontaneous alternation and deplete body weight. After treatment, the exposure group when compared to control animals, showed a marked inhibition in frontal cortices' AChE activity in all the DDVP and/or CPF exposed rats. Several independent studies reported similar findings (11,16), though their findings were on CPF or DDVP separately and not on both chemicals at different exposure periods and dose. However, rats co-administered with both CPF+DDVP show most deleterious effects on AChE activity in this study,after being varied with those that were treated with CPF only. This implies that exposure

to various types of organophosphates is very likely to reduce AChE activities in the frontal cortices which may expose the brain to more significant damages. AChE activity in the brain is necessary for control of behavioral processes (17), the reduction in AChE activity is evident in its deleterious out-turn on the memory indices that were being estimated.

Increased production of ROS in the frontal cortices following organophosphate exposure was recorded. Generally, oxidative stress is an important pathophysiological mechanism in OP toxicity (18), and oxidative damage by OP is usually sequel to ROS upsurge and this has been shown to play a pivotal role in initiating secondary brain damage (19). Hence, the elevated ROS level we observed agrees with previous studies (5,20). Our administered doses and duration of exposure might not be enough to produce observable significant increases in ROS levels OP exposed rats. The relative outburst in NO levels in DDVP only and those co-administered with CPF is in correspondence with the increase ROS production in this study. NO being a ubiquitous gaseous cellular messenger, plays significant roles in a variety of neurobiological processes (21). This include relaxation of blood vessels and increase blood supply to the brain (22). Therefore, NO may have a role in exaggerating the impaired cholinergic activities as result inducing neuronal activity (23). Oxidative stress is said to be a result of increased free radicals occuring in addition to reduced antioxidant enzymes. thereby resulting in varied macromolecules oxidation of DNA, protein and lipids (24). Oxidative stress thus lead to an imbalance between ROS and antioxidants, resulting in impairment in cellular functions and leading to potential tissue damage (25). It has also been reported that the oxidative stress can occur as a result of microglia stimulation which initiates neuronal damage (14). Impairment of synaptic plasticity as well as functions of cognition have been linked with oxidative stress, which has been demonstrated in some ageing process and neurodegenerative disorders (26).

In consistence with the oxidative and cholinesterase damage reported above, exposure to both DDVP and CPF resulted in a degenerative effect of frontal tissues of the rats exposed, coupled with an impaired distribution of neurogenic marker (Ki67). The reduced number of Ki-67 immunopositive cells suggests a lower distribution of integrated dividing progenitor cells in the frontal cortices of the exposed rats. Our result suggests a possible drastic effect on propagation of neural cells and removal of regenerative activities in the frontal cortices of exposed rats. This position can be further strengthened according to findings of previous works where toxic substances were reported to have showed marked loss of neurogenic cells or alteration of neurogenesis in laboratory rats (9,27).

A healthy frontal cortex with preserved pyramidal neurons and para- and non-paravalbumin-immunoreactive interneurons has many functions including maintaining attention, planning complex movements, speech, temporal perception, working memory, executive function and impulsivity (28). This prompted us to investigate its effect of exposure on motor, spontaneity and spatial memory functions. We observed significant reductions in both percentage, and spontaneous alternation of the maze learning in all treatment groups compared to control animals. Our observation is not unconnected to oxidative damage, weight loss, distorted neuroarchitecture and neuronal proliferation. OPs toxicity have been implicated in learning and memory processes in rats through association between cholinergic neurons and NO (24). This cognitive dysfunction has been reported to be associated with impairment in central cholinergic system which plays an important role in learning and memory (29). This finding agrees with a study by Kanu et al., that reported a relative neuro-cognitive deficit that followed exposure to OP compounds (11).

Our findings showed an expected reduction in body weight and brain weight at the end of the research. It presented a notable decrease in body weight supported by a corresponding significant brain weight decrease in the DDVP, CPF and DDVP+CPF treatment groups. However, a more significant drop was recorded in the DDVP group as against the expectation of a more deleterious effect be recorded in the DDVP+CPF group. The mechanism underpinning this observation was not investigated and this is open to more research. The drop in body weight is suggested to be the induced AChE imbalance and oxidative stress by the exposure which have been reported to yield a metabolic dysfunction as implicated in other metabolic related diseases (30).

CONCLUSION

Our results show a trend that is consistent across our main objective measures, the neurotoxicity markers and behavioural markers. Our findings confirm the established effects of OPs toxicity in the rat brain with disruptions of

AChE activity, NO and ROS molecules physiology, and neurodegeneration. In addition, our study shows that exposure to more than one type of OP chemicals could have more deleterious effects on the brain as evidenced by weight loss, marked AChE inhibition, higher NO and ROS, reduced Ki67 immunoreactive cells and poorer spatial memory outcomes in the rats that have been synchronously exposed to CPF and DDVP.

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Conflict of interest: We do not have any conflicting interest in the pursuit of this research work and article.

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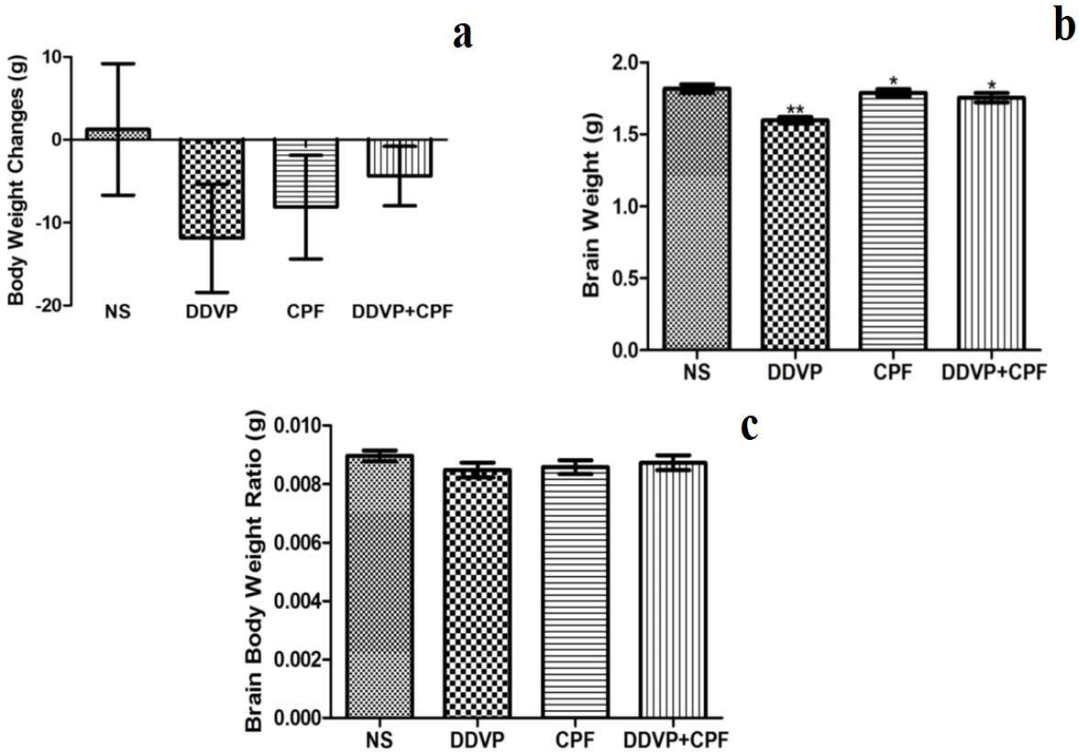


Figure 1: Exposure to dichlorvos (DDVP) and chlorpyrifos (CPF) results in loss of body weight and brain weight. (a) Body weight of control (NS) and treatment groups (b) Brain weights of control and treatment groups (c) Brain-Body weight ratio of control and treatment groups.
** indicates a significant (*p<0.05) reduction as compared to NS (control group), while
* indicates significant increase (*p<0.05) as compared to the NS control group.

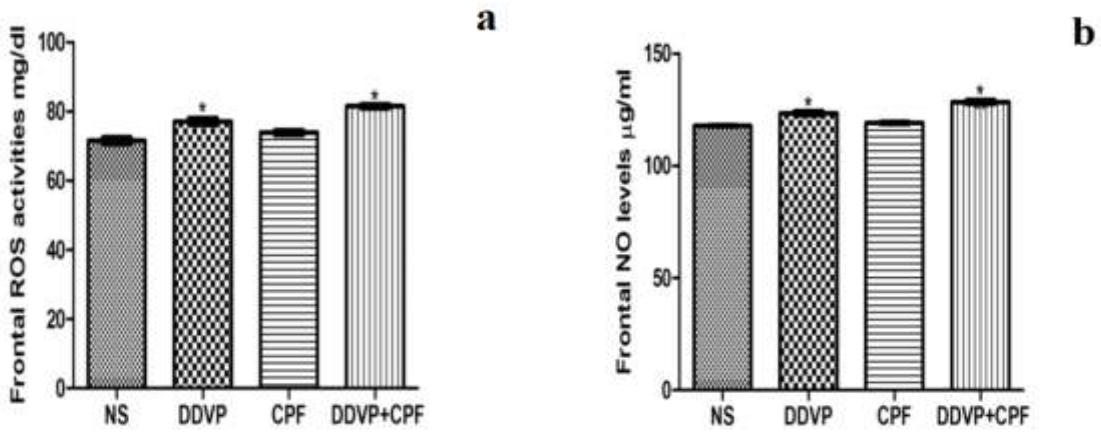


Figure 2: The results of administration (oral) of normal saline (NS), dichlorvos (DDVP) with/without chlorpyrifos (CPF) on frontal reactive oxygen species (ROS) levels (Figure 2a); and nitric oxide (NO) levels (Figure 2b) in the treated rats. Single asterisk (*) indicates a significant (*p<0.05) increase as compared to the Normal Saline exposed rats.

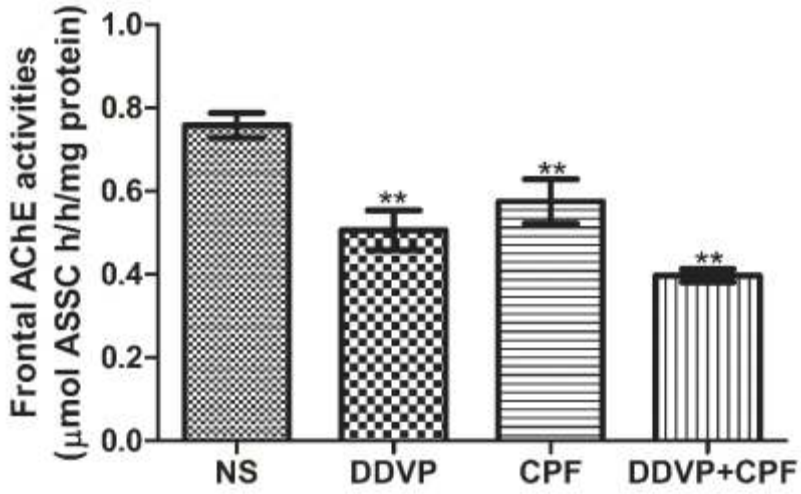


Figure 3: The results of administration (oral) of normal saline (NS), dichlorvos (DDVP) with/without chlorpyrifos (CPF) on levels of acetylcholinesterase in the frontal cortices of exposed rats. ** indicates a significant (*p<0.05) reduction in comparison with NS rats.

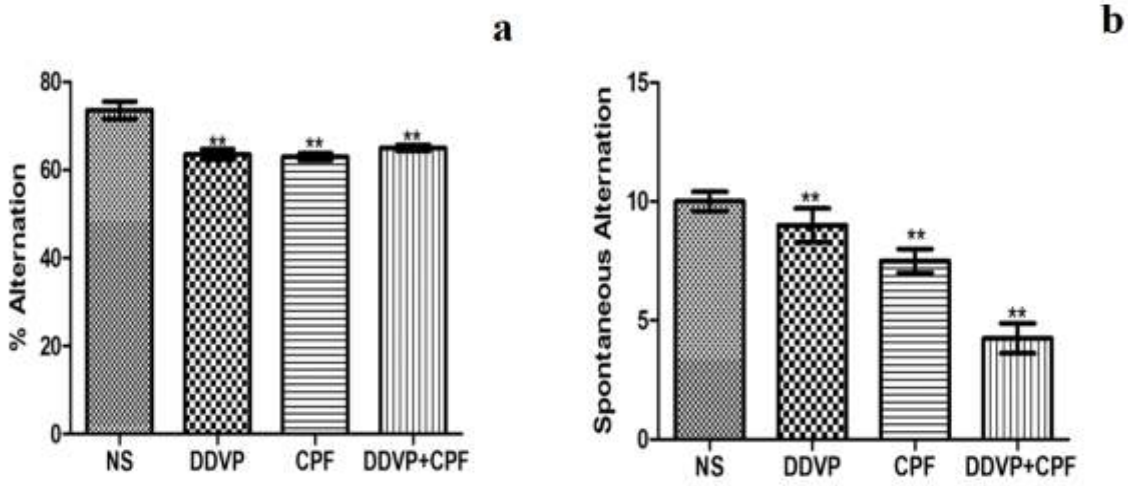


Figure 4: Exposure to DDVP and CPF impaired percentage alternation and spontaneous alternation. The results of administration (oral) of normal saline (NS), dichlorvos (DDVP) with/without chlorpyrifos (CPF) on percentage alternation (Figure 4A); and spontaneous alternation (Figure 4b) in a YMAZE paradigm in the exposed rats. ** indicates a significant (*p<0.05) decrease in comparison to NS (control) rats.

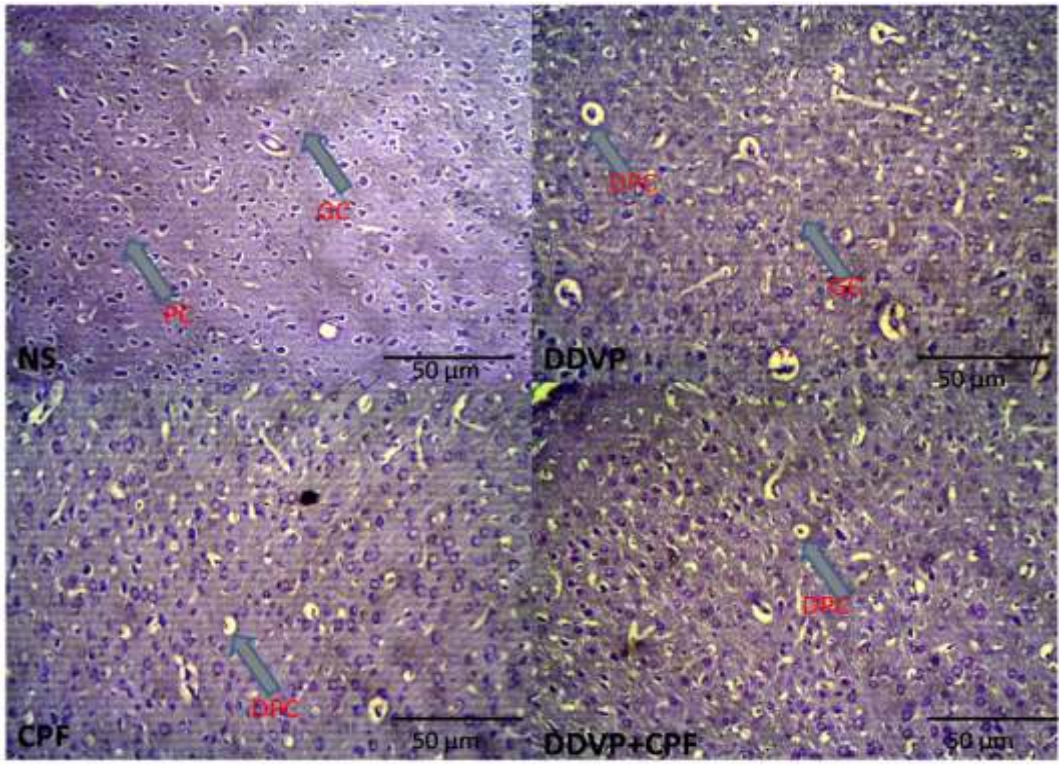


Figure 5A: Effect of oral exposure of NS, DDVP, CPF and DDVP+CPF on histoarchitecture of the frontal cortices of exposed animals. The organophosphate groups show degenerating granule and pyramidal cells. Red arrows show degenerating pyramidal cells (DPC) and granular cells (GC). Scale Bar 50µm.

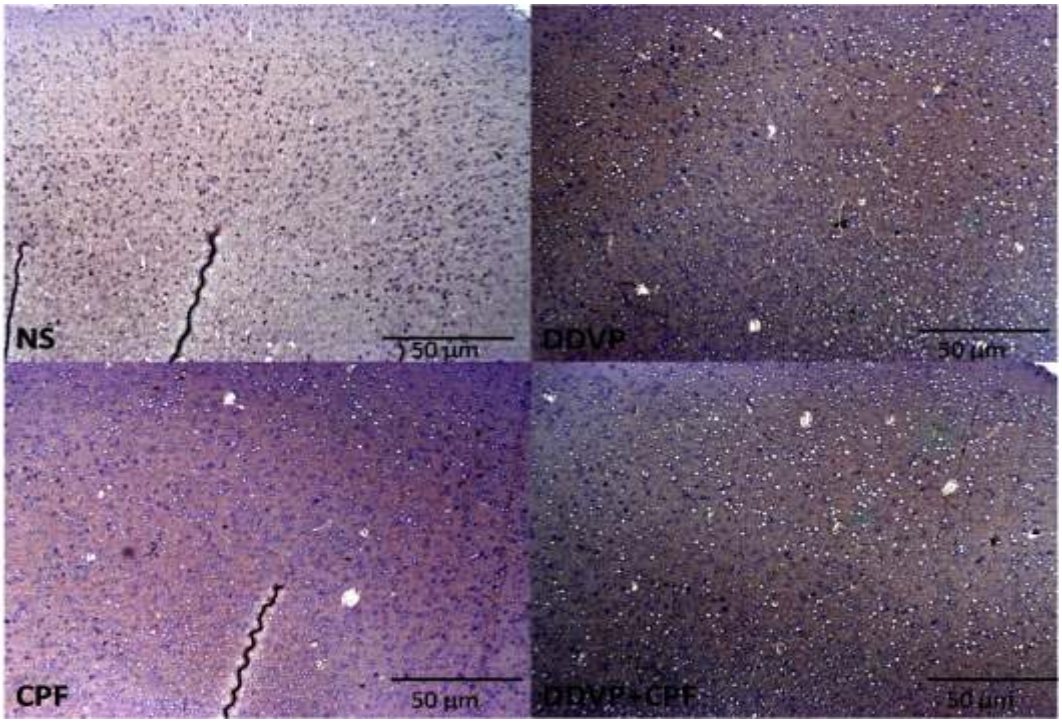


Figure 5B: Effect of exposure of NS, DDVP, CPF, and DDVP+CPF on the distribution of neurogenic protein Ki-67 on the frontal cortices of treated rats. Scale Bar 50µm.