Chlorpyrifos- and dichofos-induced oxidative and neurogenic damage elicits neuro-cognitive deficits and increases anxiety-like behaviors in wild-type rats

Aminu Imam¹,⁴*, Nafeesah Abdulkareem Sulaiman¹, Aboyeji Lukuman Oyewole², Samson Chenganai³,⁴, Victoria Williams⁴, Musa Iyiola Ajibola⁵, Royhaan Olamide Folarin⁵, Asma'u Shehu Muhammad⁵, Sheu-Tijani Toyin Shittu⁸ and Salihu Moyosore Ajao¹

¹ Neuroscience Unit, Department of Anatomy, College of Health Sciences, University of Ilorin, P.M.B 1515, Ilorin 240003, Nigeria; imam.a@unilorin.edu.ng; abdulkareem99mona@gmail.com; moyoajao@yahoo.com
² Neurophysiology Unit, Department of Physiology, College of Health Sciences, University of Ilorin, P.M.B 1515, Ilorin 240003, Nigeria; oyewole.al@unilorin.edu.ng
³ Division of Basic Medical Sciences, National University of Science and Technology, Zimbabwe; schengetani@gmail.com
⁴ Comparative Neurobiology Unit, School of Anatomical sciences, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown 2193, Johannesburg, Republic of South Africa; victoriamarywilliams@gmail.com;
⁵ Institute of Neuroscience, National Yang-Ming University, Shih-Pai, Taipei 11221, Taiwan; musaiyiola@gmail.com
⁶ Department of Anatomy, University of Ilorin, Ilorin, Kwara State, Nigeria; royhaan.folarin@oouagoiwoye.edu.ng
⁷ Department of Human Anatomy, Faculty of Basic Medical Sciences, Federal University of Dutse, Jigawa state, Nigeria. Asmau.shehu@fud.edu.ng
⁸ Endocrinology and metabolism Research Unit, Department of Physiology, College of Medicine, University of Ibadan, Ibadan, Nigeria; toyinsts@yahoo.com

* Correspondence: imam.a@unilorin.edu.ng; Tel.: +234-81-6566-3947

Abstract: The mechanization of agricultural activities has led to indiscriminate deposition of toxic xenobiotics, including organophosphates in the biomes, and this has led to intoxication characterized with deleterious oxidative and neuronal changes. This study investigated the consequences of oxidative and neurogenic disruptions that follow exposure to two organophosphates, chlorpyrifos (CPF) and dichlorvos (DDVP) on neuro-cognitive performance and anxiety-like behaviors in rats. Thirty-two adult male Wistar rats (150 – 170g) were randomly divided into 4 groups, orally exposed to normal saline (NS), DDVP (8.8mg/kg), CPF (14.9mg/kg) and DDVP+CPF for 14 consecutive days. On day 10 of exposures, anxiety-like behaviors and amygdala dependent fear learning were assessed using Open Field and Elevated Plus Maze paradigms respectively, while spatial working memory was assessed on day 14 in the Morris water maze paradigm, following 3 training trials each on days 11, 12 and 13. On day 15, the rats were euthanized, and their brains excised, hippocampus and amygdala removed, 5 of which were homogenized and centrifuged to analyze nitric oxide (NO) metabolites, total reactive oxygen species (ROS), and acetylcholinesterase (AChE) activity, and the other three processed for histology (cresyl violet stain) and proliferative marker (Ki67 immunohistochemistry). Marked (p<0.05) loss in body weight, AChE depletion, and overproduction of both NO and ROS were observed after repeated exposure to individual and combined doses of CPF and DDVP. Insults from DDVP exposure appeared more severe owing to the observed greater losses in the body weights of exposed rats. There was also a significant (p<0.05) effect on the cognitive behaviors recorded from the exposed rats, and these deficits were related to the oxidative damage and neurogenic cell loss in the hippocampus and the amygdala of the exposed rats. Taken together, these results provided an
insight that oxidative and neurogenic damages are central to the severity of neuro-cognitive
dysfunction and increased anxiety-like behaviors that follow organophosphate poisoning.

Keywords: oxidative damage; organophosphates; neurotoxicity; spatial working memory;
anxiety-related behaviors

1. Introduction

Indiscriminate deposition of xenobiotics into the environment has been associated with the increase
in accidental poisoning and non-specific multi-organ toxicity. Oxygen stress, a product of the
imbalance between the antioxidant systems of the body and the generation of free radicals, has been
implicated in the pathophysiology of the subsequent toxicity from exposure to many xenobiotics
and also in the development of many diseases [1-4]. Organophosphate pesticides are one major
example of xenobiotics that are intentionally released into the environment to control pests and
insects in households and agriculture, and their use has been accompanied by burdens of diseases
that result from accidental poisoning due to deposition in food substances, water and through
inhalation for the occupational workers [3]. Although the primary mechanisms of OPs poisoning is
through their irreversible inhibition of acetylcholinesterase (AChE), leading to cholinergic
dys-homeostasis [5], most of the destructive activities of these substances have been largely linked to
the oxidative damages, one of the widely implicated factors that complicate OPs induced toxicity
[6-12].

In addition OPs have been implicated in the induction of deleterious oxidative changes in various
organs in the body, their activities on antioxidant free-radical balance are of vital importance, since
free radicals are important mediators in the pathophysiology of most neurodegenerative diseases.
[13]. The neurologic effects of OPs toxicity is manifested as chronic organophosphate-induced
neuropsychiatric disorder (COPIND), which is characterized by cognitive deficits, depression,
anxiety and some personality problems [14,15]. All of these are associated with excessive generation
of reactive oxygen and nitrogen species (ROS and RNS), and or nitric oxides in the brain, as well as
reduction in anticholinesterase activities [8,9,16].

Evidently, increased oxidative damages have been implicated in adversely affecting psychological
and cognitive related functions through disruptions of normal neurogenesis in the hippocampus
and other potential hotspots within the brain [8,9,17-19]. Chronic and subchronic exposures to both
CPF and DDVP have resulted in wide range toxicity, including cardiotoxicity, neurotoxicity,
hepatotoxicity, renal toxicity, haematological toxicity, and immune system toxicity among others
[8,9,20-23]. Besides cholinesterase inhibition, these substances caused marked disruptions in normal
oxidative functions [8,9,20,21,24]. Thus, in this study, we investigated the neuro-cognitive
consequences of uptake of two commonly used OPs, chlorpyrifos (CPF) and dichlorvos (DDVP) in
rats, with possible effects on oxidative stress and proliferative functions in the hippocampus and the
amygdala.

2. Materials and Methods

2.1. Chemicals and drugs

DDVP (PubChem Substance ID 329756736) and CPF (PubChem Substance ID 329756699)
Pestanal®, analytical standard were purchased from Sigma (Sigma-Aldrich)(St. Louis, MO, USA),
while the normal saline solution was prepared in our laboratory.
2.2. Animals and experimental design

Thirty-two adult male Wistar rats weighing between 150g and 170g were obtained from the University of Ilorin biological garden, Ilorin. They were housed in cages and fed with standard laboratory diet and water ad libitum, in the animal holding unit of the Faculty of Basic Medical Sciences, College of Health Sciences, University of Ilorin, Ilorin. The rats were exposed to a 12 hours’ light/dark cycle at room temperature for 7 days before the commencement of the experiments. All rats were handled in accordance with the standard guide for the care and use of laboratory animals.

2.3. Treatment Schedule

The rats were randomly divided into four groups (n=8) as follows:

Group 1 (control)- were given normal saline (1 ml/kg orally) daily for 14 days

Group 2- were given DDVP (8.8 mg/kg orally) daily for 14 days [8,20,21]

Group 3- were given CPF (14.9 mg/kg orally) daily for 14 days [9]

Group 4- were given DDVP (8.8 mg/kg orally) plus CPF (14 mg/kg orally) daily for 14 days

All procedures were scheduled and carried out during the early phase of the day between 07:00 and 08:30 hours, and treatments were given for fourteen consecutive days.

2.4. Ethical approval

This research work was approved by the University of Ilorin ethical review committee (UERC) (UERC/ASN/2017/856), following the recommendation of the College of health sciences ethical review committee, in compliance with the Institutional Animal Care and Use Committee (IACUC).

2.5. Body and brain weight evaluation

The body weights of all the rats were recorded after acclimatization at the first day of the exposures as initial weight and at the last day of exposure as the final weight. Thus, the differences between the two weights were calculated and recorded as the weight changes. The brain weights of all rats were recorded after the sacrifice, and a ratio of the brain to final body weight was calculated and recorded.

2.6. Behavioral evaluations

The rats were subjected to behavioral evaluations on the 14th day of the treatment to assess, short term memory, long term memory and reference memory in the Morris water maze paradigm.

2.6.1. Morris water maze procedure

The Morris water maze (MWM) apparatus is the most commonly used model to test spatial learning and memory. To evaluate spatial memory, rats were tested in a circle shaped black pool filled with 23–24°C water (pool dimensions: 60cm deep × 136cm diameter). The pool was divided to four quadrants with boundaries labelled north (N), east (E), south (S) and west (W) and a circular platform (10cm diameter, 28cm high) was submerged about 2cm below water surface in the central area of the southwest quadrant of the pool. Animals were allowed to swim until they found, mount and remained on the platform for15s. If they were not able to find the platform after 60s of swimming, they were guided to the platform by examiner and were allowed to stay on it for 15s. The rats were then removed from the pool, dried and placed in their holding bin for 5 min. Trials were recorded by a video system. Animals received a training session consisting of three trials per session.
once from each starting point) for 3 days (days 11, 12 and 13), with each trial having a maximum duration of 60s and a trial interval of approximately 30s. Twenty-four hours after the acquisition phase, the time taken to locate the hidden platform (escape latency) was recorded as long term memory (LTM), an average of the escape latency of the two subsequent trials was recorded as the short term memory (STM). A probe test was conducted by removing the platform, and allowing the rats to swim freely in the pool for 60s; the time spent in the target quadrant which had previously contained the hidden platform was recorded as the reference memory (14th day). The time spent in the target quadrant indicated the degree of relative memory consolidation which had taken place after learning [25].

2.6.2. Anxiety-like behaviors and fear learning

The rats were subjected to behavioral evaluations on the 13th day of the exposures to evaluate, anxiety related behaviors and fear related learning in the open field test (OFT) and the elevated plus maze (EPM) paradigms.

OFT Procedure:
The animals were exposed to a trial in the OFT to evaluate anxiety related behaviors in rats following DDVP and/or CPF exposures. The rats were individually placed in the centre of the apparatus and time spent in the centre and immobility period were recorded in a 5 minute session and all animals were monitored in a balanced design during the procedures. For analysis, trial was performed in a well illuminated wooden box, divided into 4 × 4 squares. It has been reported that preference or avoidance of central squares may provide an evaluation of the anxiety level in the rats [26,27].

EPM Procedure:
To evaluate amygdala dependent or fear related learning, the rats were exposed to two trials in the EPM paradigm. The consisted of 2 open arms, surrounded by a short edge to prevent falls, and two enclosed arms erected in such a way that the 2 open arms were opposite each other. The maze was raised about 35cm above the ground with a stable stand and the arms of the maze were connected by a central platform. At each of the two trials, each rat was gently placed on an open arm, positioned to face away from the central platform and the closed arms. The time it takes the rats to recognise the treat and move to the closed arms was recorded as the transfer latency, while the first trial was for acquisition; the second was used as a measure of fear learning. The principle of this experiment is primarily based on the aversion of rats to heights and open spaces [9].

2.7. Biochemical evaluation
At the end of the treatment period, the animals were euthanised with an overdose of ketamine (10 mg/kg ip) and the brains were quickly dissected out and weighed. Blocks of hippocampal and amygdala tissues(from Bregma –2.5 mm to –4.5 mm) were removed from the brains of five rats from each group, dipped in 30% sucrose solution, homogenized and portions centrifuged at 2500 revolutions per minute for 10 minutes and the supernatant collected into tubes containing the reagents for the NO and ROS analysis.
ROS was measured by monitoring the increasing fluorescence of DCFH-DA following a previously described procedure using flow cytometry (Partec, Deutschland) equipped with a 488 nm argon ion laser and supplied with the Flomax software and the signals were obtained using a 530 nm band pass filter (FL-1 channel). Each determination was based on the mean fluorescence intensity of 10,000 counts [28]. The remaining tissue homogenate was added to the Griess reagents, sulfanilamide and naphthyl ethylene diamine solutions to measure nitrate/nitrite production (NO metabolites). Absorbance was measured with the aid of a microplate reader and the levels of NO metabolites were calculated from standard curve [29]. The remaining portions of the homogenized hippocampal tissues were placed in phosphate buffer with 1% Triton-X 100 and centrifuged at 5000rpm for 10 minutes. The following reagents were used; 35μL of 5mM dithio-bisnitrobenzoic acid, also known as Ellman’s reagent (DTNB), 10μL of 75 mM acetylthiocholine (ATCh) and 50mM phosphate buffer (pH 8.0). Protein concentration in brain homogenates was quantified using a Bradford assay and AChE activity was calculated in micromoles of ATCh hydrolysed per hour per milligram of protein and was expressed as percentage of control activity and measured values in micromole per hour per milligram of protein.

2.8. Tissue processing and Histopathology

After euthanasia and extraction the brains of three rats from each groups, the brains were fixed in 10% formalin for 24 hours, hippocampal and amygdala blocks (from Bregma –2.5 mm to –4.5 mm) were removed, dehydrated through ascending grades of alcohol, cleared in xylene and embedded in paraffin blocks. Every second and third hippocampal and amygdala tissue sections (5μm in thickness) were stained with Nissl stain and/or immunostained to reveal Ki67 proliferative nuclei protein, analyzed under an AmScope 40X-2500X LED Lab Compound microscope, and photographed using the AmScope 5.0 MP USB Still Photo & Live Video Microscope Imager Digital Camera 5MP, manufactured by iscope corp., USA.

2.8.1. Immunohistochemistry for Ki-67

The Ki-67 is a chromosome-associated protein present during division (G1, S, G2, and M phases but absent from cells at rest, G0). Sections from paraffin embedded hippocampal blocks were incubated for epitope retrieval in citrate buffer, pH 6.0, at 90˚C for 40 minutes, followed by incubation in endogenous peroxidase blocking reagent, 0.6% H2O2 in Tris-buffered saline (TBS)-Triton (0.05% Triton X-100 in TBS, pH 7.4) for 30 minutes at room temperature. Thereafter, sections were pre-incubated in 2% serum (normal goat serum) + 0.1% bovine serum albumin (BSA) + 0.25% Triton in TBS for 60 minutes at room temperature. Afterward, sections were incubated with polyclonal rabbit-anti-lyophilized-Ki-67p antibody (Novocastra, Newcastle, UK; 1:5,000 in preincubation solution) overnight at 4˚C. Incubation with biotinylated goat anti-rabbit IgG (1:1,000 + 2% normal goat serum + 0.1% BSA in TBS; Vector lab, CA, USA;1:250) was performed for 2 hours at room temperature followed by incubation with streptavidin-biotin complex (Vectastain Elite ABC kit) and stained with 3,3’-diaminobenzidine (DAB) as chromogen. Until incubation with primary antibody, all rinses in between incubations were made with TBS-Triton, afterwards with TBS alone.
2.8. Statistical Analysis

Data from the morphometry, behavior and biochemical assays were analyzed using one-way analysis of variance (ANOVA) and subjected to post hoc Bonferroni’s multiple comparison test. The results are expressed as mean±SEM. Statistical analyses were performed using Graphpad Prism software (version 5.0, La Jolla, CA). Values of p≤0.05 were considered statistically significant.

3. Results

The exposures to both DDVP and CPF in the present study resulted in differential effects on indirect metabolic markers (body weight, brain weight and brain-body weight ratio), AChE activities, ROS levels, NO levels, histoarchitecture, an distributions of proliferative nuclei proteins in the hippocampus and the amygdala, and the anxiety-related behaviors, fear learning and spatial working memory in the exposed rats.

3.1. Morphometric changes following exposure to DDVP and CPF

Subchronic exposures to 1/10 ratios of the oral highest tolerable dosages of both CPF and DDVP, separately and in combination markedly caused loss of body weight over a period of 14 consecutive exposures (Figure 1A). But, the observed body weight loss was more in the DDVP only exposed rats, and what may be a conflicting effect with less weight loss in the combined exposed rats (Figure 1A). There was also a significant (p≤0.05) loss in brain weight of the exposed rats, with relatively more loss observed in the DDVP only exposed rats’ brains (Figure 1B).

![Figure 1: Exposures to DDVP and CPF results in loss of body and brain weight](image)

3.2. Effects of DDVP and CPF exposures on spatial working memory

Exposures to DDVP and/or CPF significantly (p≤0.05) delayed the latency to the submerged platform (escape latency) in the exposed rats in both tests for LTM (Figure 2A), STM (Figure 2B), and MWM paradigm. Although, this effect is relative to the three exposure modalities in the LTM, the combined exposures to DDVP and CPF caused more (p≤0.05) delay in the latency to the hidden platform, followed by the DDVP only exposure, when compared with the control (Figure 2A and B). The separate exposures to DDVP or CPF consequently resulted in avoidance (p≤0.05) of the platform.
quadrant, during the probe test for reference memory (R, while their combination surprisingly did have no effect on RF (Figure 2C).

![Figure 2](image)

Figure 2: Exposure to DDVP and CPF impaired LTM, STM and reference memory (A) Long-term memory (escape latency); (B) Short-term memory (escape latency); and (C) Reference memory (% time in the platform-quadrant) tests in the MWM paradigm. Double asterisks (**) indicates significant (p≤0.05) reduction when compared with NS and DDVP+CPF rats (Fig. 2C), while single asterisk (*) indicates significant (p≤0.05) increase when compared with NS (Fig. 2A) and or CPF (Fig. 2B).

3.3. DDVP and CPF exposures increased anxiety-like behaviors

The latency to the closed arm, an indirect measure of fear learning, in the EPM paradigm, was significantly (p≤0.05) delayed by exposures to both DDVP and CPF, seperately and in combination (Figure 3A) in the exposed rats. Both DDVP and CPF also caused marked increase in freezing periods, an indication of fear, in the exposed rats. This observation was corroborated by the significant (p≤0.05) reduction in time spent at the centre squares by the rats, indicating anxiety-related responses (Figures 3B and C).
Figure 3: The effects of oral exposures to normal saline (NS), dichlorvos (DDVP) or/and chlorpyrifos (CPF) on: A) fear learning (transfer latency) in the elevated plus maze paradigm; B and C) anxiety related behaviors (freezing period and time in center squares) in the open field test paradigm. Double asterisks (**) indicates significant (p≤0.05) increase (Fig. 3A and B) or decrease (Fig. 3C) when compared with NS, other groups and/or CPF rats only; while single asterisk (*) indicates significant (p≤0.05) increase when compared with NS (Fig. 3A and B). Using one-way analysis of variance (ANOVA) and subjected to post hoc Bonferroni’s multiple comparison test.

3.4. DDVP and CPF exposures inhibit Anticholinesterase in the Amygdala and hippocampus

Exposures to the two OPs used in this study, DDVP and CPF, either seperately or combined resulted in a significant depletion in both amygdaloid (Figure 4A) and hippocampal (Figure 4B) AChE levels in the exposed rats when compared with the control’s. Although the inhibition of AChE activities in both brain regions are in relative patterns, the basal (control) AChE activities was more in the hippocampal region, thus the inhibiting effects of the OPs on the hippocampal may be more.

Figure 4: The effects of oral exposures to normal saline (NS), dichlorvos (DDVP) or/and chlorpyrifos (CPF) on: A) amygdaloid AChE activities; and B) hippocampal AChE activities in the exposed rats.
Double asterisks (**) indicates significant (p≤0.05) decrease when compared with the NS rats. Using one-way analysis of variance (ANOVA) and subjected to post hoc Bonferroni’s multiple comparison test.

3.5. Effects of DDVP and CPF exposures on oxidative stress markers (ROS and NO) in the Amygdala and hippocampus

Consecutive oral DDVP and/or CPF exposure in rats, caused a relative (p≤0.05) increase in both nitric oxide (NO) and total reactive oxygen species (ROS) levels in the amygdala and the hippocampus of the exposed rats (Figures 5A-D). Although, no marked differences were observed in the pattern of the effects on both NO and ROS levels, CPF exposure did not result in a significant change in the hippocampal ROS level (Figure 5D).

Figure 5: The effects of oral exposures to normal saline (NS), dichlorvos (DDVP) or/and chlorpyrifos (CPF) on: NO levels (A: amygdala and B: hippocampus); and ROS levels (C: amygdala and D: hippocampus) in the exposed rats. Single asterisk (*) indicates significant (p≤0.05) increase when compared with the NS rats. Using one-way analysis of variance (ANOVA) and subjected to post hoc Bonferroni’s multiple comparison test.

3.6. Effects of DDVP and CPF exposures on the distributions of proliferative nuclei (Ki67) in the hippocampus and the histoarchitecture

Histological Nissl granulation stain revealed no marked effects on either the connus ammonis regions (CA1 and 3) and the dentate gyrus following exposures to DDVP, CPF or combined when compared with the control (NS). However, there is qualitatively more glia-like small sized intensely stained cells in the DDVP and/or CPF exposed CA regions and the dentate gyrus (glia activation)
(Figure 6), with also some vacuolations mostly in the DG of the exposed rats. Furthermore there was a reduced presence of proliferative cells marker, Ki67 immunoreactive nuclei proteins in the CA1 and 3, and DG of the DDVP and/or CPF exposed rats, most especially in the subgranular zone of the dentate gyrus (Figure 7).

Figure 6: The effects of oral exposures to normal saline (NS), dichlorvos (DDVP) or chlorpyrifos (CPF) on: the hippocampal connus ammonis 1 and 3 (CA1 and 3), and the dentate gyrus (DG) in the exposed rats. There was no marked changes following either DDVP and/or CPF when compared with the control (NS). Scale bar 50μm.
Figure 7: The effects of oral exposures to normal saline (NS), dichlorvos (DDVP) or/and chlorpyrifos (CPF) on: the distributions of Ki67 nuclei proteins in the hippocampal cornus ammonis regions (CA1 and 3), and the dentate gyrus (DG) in the exposed rats. White arrows indicate the Ki67 immunoreactive proteins in the respective regions, with reduced nuclei in the DDVP and/or CPF exposed rats compared to the control. Scale bar 50μm.
4. Discussion

Organophosphates poisoning account for a high percentage of reported toxicities from chemical exposure around the world, posing growing threats to public health, and with more concerns as they are continuously deposited in water bodies and the biomes [30,31]. Toxicities from these substances are primarily linked to the irreversible inhibition effects on acetylcholinesterase (AChE) in the blood and the nervous systems, thus having the ability to affect general body functions and personality related functions [8,9,30-32]. In the present study sub chronic oral exposures to two most commonly used broad spectrum OPs worldwide, separately, and in combination was sufficient to markedly deplete the levels of AChE in the hippocampus and the amygdala, in a pattern relative to what we recently found with CPF only exposure on the amygdala, and with the dichlorvos in discrete brain regions, including the cerebellum, hippocampus, frontal cortex, medulla, spinal cord and occipital cortex [9,32]. This is no surprise, as it further confirms the earlier established mechanism of OPs activities in the brain. In separate studies in the literature, DDVP and CPF have been reported to cause significant inhibition of AChE in the brains of rats [8,9,33,34], of which most of its induced toxicities have been attributed.

However, there is growing evidence suggesting that, although AChE inhibition contributes greatly to the toxicities and remains the primary mechanism of action of OPs, their effects on redox processes, antioxidant functions and on lipid peroxidation, are greatly implicated in the chronic outcomes following poisoning [8-12,32]. Exposing rats to 1/10th of the oral tolerable dosages of DDVP, CPF and their combination in the present study, significantly caused an increase in total reactive oxygen species (ROS) and nitric oxides (NO) levels in the hippocampus and amygdala of the exposed rats. Further corroborating previous findings on the activities of OPs on anti-oxidant defense and on general oxidative functions, and more than the AChEI, these are very much implicated in the neurotoxic effects of OPs poisoning, including the neuro-cognitive impairments and cell death [9,32,35-37]. The oxidative damages following exposures to OPs may further contribute to its detrimental effects on health, as it has been linked to loss of biological functions in cells, and contributing to the pathophysiological factors for various life threatening diseases, like respiratory, cardiovascular and renal diseases, carcinogenesis and neurodegenerative disorders [3,4].

It is expected, that the induced AChE dys-homeostasis and most importantly, the oxidative dysfunctions may affect metabolic functions, since it has been implicated in different metabolic related diseases [3,4,11]. Thus, we recorded the changes in body weight at the initiation and termination of the experiment, and this revealed a significant loss in body weight, supported by a subsequent low brain weight following exposures to DDVP, CPF and their combination, with more effects observed with DDVP exposure. These findings are affirmed by previous findings, where a loss in both body and brain weights were recorded following exposures to different OPs, including CPF and DDVP [9,32,38-40].

An observation into the possible effects of these substances on neural functions and survival related proteins, and structures, revealed a consequent qualitative depletion of the proliferative nuclei marker (Ki67 proteins) in the hippocampal CA regions and the dentate gyrus of the DDVP, CPF and combined DDVP+CPF exposed rats. This was complemented by the observed increase in intensely stained nuclei-like glia, most especially in the dentate gyrus. These suggest possible damaging effects on potential neurogenesis and a build up of a possible shut down of regenerative activities in the brains of the exposed rats. This can be strengthened with findings from previous studies, where exposures to neurotoxic compounds have reported to result in mark loss neurogenic cells in laboratory rodents [41,42]. Our previous examination of effects of CPF exposure on amygdala AChE activities, oxidative markers and expression of Ki67 proteins, further support these findings [9].
A healthy hippocampus, with preserved neurogenesis is linked to enhancing psycho-cognitive functions, while any damage that affects this, have been claimed to affect cognitive activities [43]. Thus, we investigated possible effects on anxiety-like behaviors and spatial working memory in the exposed rats. In congruence with the above, sub chronic exposures to either of DDVP and/or CPF significantly increased anxiety-like behaviors and impaired spatial working memory behaviors respectively. These dysfunctions in psychosocial related and cognitive functions following exposure to the two OPs used in this study cannot be unrelated to the combined effects of the observed oxidative damages, weight loss, diminished proliferative nuclei in the hippocampus and the amygdala. And these can be strongly supported by the relative neuro-cognitive deficits that follows exposures to different insecticidal compounds, including OPs [9,17,37,44,45].

5. Conclusions

In conclusion, sub chronic oral exposures to DDVP and CPF, separately or in combination imposed hippocampal and amygdala oxidative damages and subsequent depletion of neurogenic nuclei in the hippocampus and dentate gyrus. These might have contributed to the psycho-cognitive deficits and increased anxiety-like behaviors that were observed following AChE inhibitions in the studied brain regions.

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