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Silent Neurotoxicity: Neurobehavioral Effects of Repeated Low-Dose Chlorpyrifos Exposure and Zinc-Mediated Protection

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Abstract

Chronic exposure to chlorpyrifos (CPF), a widely used organophosphorus insecticide, poses significant neurobehavioral risks, potentially mediated by oxidative stress and neuroinflammation. This study investigates the impact of repeated exposure to low dose of CPF exposure on neurobehavioral functions and hippocampal astrocyte activation and assesses the protective role of zinc supplementation. Adult male albino rats were assigned to control, CPF-treated, and CPF+zinc-treated groups. CPF was administered orally (18 mg/kg/day) for three weeks, with and without zinc sulfate (15 mg/kg/day). Neurobehavioral assessments included the Passive Avoidance and Open Field tests. Immunohistochemistry for Glial Fibrillary Acidic Protein (GFAP) was performed to evaluate astrocyte activation, with morphometric analysis quantifying GFAP immunoreactivity and the percentage of GFAP-positive astrocytes. The results show that CPF-treated rats exhibited significant memory and exploration deficits and increased anxiety-like behaviors compared to controls. These changes coincided with elevated GFAP immunoreactivity and an increased number of GFAP-positive astrocytes. Zinc co-administration ameliorated neurobehavioral impairments, reduced GFAP expression, and decreased astrocyte activation compared to CPF-only treatment. In conclusion, Repeated exposure to CPF was associated with neurobehavioral deficits and hippocampal astrocyte activation. Zinc supplementation showed a potential neuroprotective role, as evidenced by reduced astrocyte reactivity and partial improvement in behavioral performance.

Keywords: Chlorpyrifos, zinc, neurobehavioral deficits, astrocytes, GFAP, oxidative stress

1. Introduction

Organophosphorus compounds (OPs) as widely used insecticides are a major cause of global poisoning and mortality, with approximately 3 million exposures and over 2.2 thousand deaths annually, according to WHO. In Egypt, OPs are the leading cause of poisoning-related deaths, accounting for 23.8% of all fatalities. (Shah et al.2016; Abdelhamid 2021)

Chlorpyrifos (CPF) is one of the most hazardous organophosphorus compounds, with possible adverse health effects. (Rathod et al.2017)

Chlorpyrifos (CPF), commonly used in agriculture, disperses into the environment, contaminating air, soil, water, and food. Its lipophilic nature allows it to penetrate the skin, accumulating in tissues, organs, milk, and body fluids. (Lu C et al.2020) Farm workers often underestimate CPF's hazards and use limited protective measures due to cultural and financial factors. (Salvi et al.2003) Studies indicate that prolonged CPF exposure poses significant health risks, including potential neurobehavioral and cognitive impairments. (Cañadas et al.2005) (Rauh 2018).

Acetylcholinesterase inhibition plays a crucial part in the long-term toxicity of OPs. However, over twenty years of experimental research support the idea that this mechanism cannot alone account for the large spectrum of symptoms that have been described. (Burke et al. 2017) (Mense et al. 2006).

Zinc (Zn), an essential trace element, plays a vital role in numerous metabolic, structural, and functional processes in the body. It is a key component of various enzymes, including hydrolases, transferases, oxidoreductases, ligases, isomerases, and lyases. (Rusch et al.2021) (Maywald et al. 2022) Additionally, zinc is critical for both physiological and pathological

processes in the central nervous system, with its homeostasis being essential for normal brain function. (Li et al.2022)

The telencephalon is the brain's principal zinc storage site, notably in regions such as the cortex, hippocampus, and amygdala. (Choi et al.2020)

Therefore, the present study aimed to study the neurobehavioral changes of exposure to repeated low doses of chlorpyrifos and investigate the possible ameliorative effect of zinc on such changes. Furthermore, we aimed to explore the potential reversibility of these changes after chlorpyrifos withdrawal, offering new insights into protective strategies and recovery pathways

2. Materials And Methods

2.1. Animals and Housing

Forty male Wister albino rats, aged approximately 6 weeks and weighing 150–200 g, were obtained from the Animal House Center in faculty of medicine- Alexandria university. All rats were confirmed to be in good health at the start of the experiment, showing no clinical signs of disease as assessed by veterinary examination. The rats were kept under controlled conditions, including a closed environment with a temperature of 24°C, 52% humidity, a 12-hour light/dark cycle, and unrestricted access to food and water.

2.2. Experimental Design

The rats were randomly assigned to three groups:

Group I (Control): Ten rats received corn oil (5 mL/kg BW) orally via an oro-gastric tube daily for three weeks.

Group II (Chlorpyrifos-Treated): This group included 20 rats, divided into two subgroups of 10 rats each:

- **Subgroup IIa**: Rats received daily oral doses of chlorpyrifos (97% purity, purchased from Egypt-Chem International for Agrochemicals, Egypt.) in a dose of 18 mg/kg BW for three weeks and were subsequently sacrificed after a three-week period. (Voorhees et al. 2019) (Middlemore-Risher et al.2010)
- **Subgroup IIb**: Rats received daily oral doses of chlorpyrifos (18 mg/kg BW) for three weeks, followed by a two-week withdrawal period without exposure. During this phase, the rats were kept in a low-stress environment with ad libitum access to food and water. (Middlemore-Risher et al.2010)

Group III (Chlorpyrifos + Zinc):

- Ten rats received daily oral doses of chlorpyrifos (18 mg/kg BW) and zinc sulfate (purchased from International Pharma Company, Egypt) in a dose of 15 mg/kg BW concurrently via an oro-gastric tube for three weeks.(Cavalcanti et al.2020)(Joshi et al.2012) (Goel et al.2000).

2.3.Assessment

At the end of the experimental period, rats in all groups were assessed using a combination of biochemical, behavioral, and histopathological methods tailored to the specific exposure durations and treatment protocols for each group.

Neurobehavioral tests:

Passive Avoidance Task (Ishiyama et al.2007)**:** The Passive Avoidance Task evaluates learning and memory using a fear-based model. Rats explore a two-compartment apparatus during training and receive a mild foot shock upon entering the dark compartment. In the testing phase, 24 hours later, the latency to re-enter the dark compartment is measured. Increased latency compared to training indicates memory retention and successful learning. The task stops if the rat does not enter within 300 seconds.

Open field test (Yang et al.2017)**:** The open field test evaluates exploration and anxiety in rats when exposed to a novel environment. The test is conducted in arena that is consisted of an open wooden box, whose dimensions were 75 (length) x 75 (width) x 35 (height) cm, and its floor was divided into 16 squares. Each rat is placed at the center of the arena and observed for 5 minutes. Behavioral parameters include Latency times: Time taken to move from the center to the first square and to rear up on hind legs for the first time, Number of squares crossed and Number of rears.

Reduced movement, fewer rears, and longer latencies suggest higher anxiety and hesitancy, while increased exploration indicates lower anxiety and curiosity.

Biochemical assay: After behavioral tests, the rats were sacrificed using 4% isoflurane inhalation followed by cervical dislocation to ensure humane euthanasia in accordance with ethical research guidelines, and then blood samples were collected for biochemical analysis.

Serum Zinc levels were measured using a colorimetric assay kit following the manufacturer's instructions. The method is based on the formation of a red chelate complex between zinc and 2-(5-Bromo-2-pyridylazo)-5-(N-propyl-N-sulfopropylamine)-phenol (5-Br-PAPS), with absorbance measured at 546 nm.(Johnsen et al.1987)

Butyryl cholinesterase activity was measured using a colorimetric assay kit following the manufacturer's instructions. The method is based on the hydrolysis of butyryl thiocholine by butyryl cholinesterase, producing thiocholine, which reacts with DTNB to form a yellow compound measured at 405 nm. (Jońca et al.2015)

The assays were validated for linearity (zinc up to 200 µg/dL, cholinesterase up to 0.4 ΔA/min), precision (consistent absorbance readings), and accuracy (calculated concentrations matching expected values). No significant interference from hemolysis was observed. Samples remained stable at 2–8°C for 24 hours without degradation

Histological examination:

1) **Light Microscopic Study:** The left half of the cerebral hemisphere from each animal was fixed in 10% formol saline and processed for light microscopic study. Paraffin sections (5 µm thick) were stained with Haematoxylin and eosin stain (Bancroft et al.2013) and Immunohistochemical stain for Glial Fibrillary Acidic Protein (GFAP) of the astrocytes.(Jackson et al.2013)

2) **Immunohistochemical stain:** Paraffin sections on poly-L-lysine-coated slides were deparaffinized, rehydrated, and treated with 3% H2O2 to inhibit endogenous peroxidase. Sections were incubated with a primary anti-GFAP antibody (1/500 dilution) to detect astrocyte activity, followed by a biotinylated secondary antibody and DAB chromogen for visualization. Slides were counterstained with hematoxylin, with dark brown cytoplasmic staining indicating positive immunoreactivity. Saline phosphate buffer replaced the primary antibody for negative controls, while brain white matter served as a positive

control. A blinded observer examined the slides using an Olympus digital microscope. (Goyal et al. 2015)

3) **Morphometric and statistical Analysis**: With the use of Image J software, version K 1.45, the color intensity of GFAP immune-positive areas and the number of immune-positive cells were measured. Ten nonoverlapping fields (x400) were examined for all rats/ each experimental group using Leica Qwin 500 LTD image analysis. The mean area percent of GFAP-positive immune reaction and the number of GFAP-positive cells were counted per 5 high power fields (x400) in slides from each animal in each group using (CX21, Japan. Light microscope). (Marques et al.2023)

1. **Statistical analysis :** Data were analyzed using the IBM SPSS software package version 23.0. Statistical significance was determined at the 5% level. The Kruskal-Wallis test was used for non-normally distributed quantitative variables to compare more than two groups, with Bonferroni adjustments for pairwise comparisons. For normally distributed quantitative variables, the F-test (ANOVA) was applied, followed by the Tukey Post Hoc test for pairwise comparisons. (Kotz et al.2006)

3. Results

3.1. Neuro-behavioral study

1. Passive avoidance test:(Table 1)

Chlorpyrifos exposure significantly impaired memory, evidenced by reduced latency to enter the dark compartment in Subgroup IIa compared to controls ($p < 0.001$). Two weeks of recovery (Subgroup IIb) showed memory improvement, but this was not statistically significant compared

to Subgroup IIa (p = 0.819). Zinc co-administration (Group III) significantly improved memory compared to Subgroup IIa (p = 0.001) and Subgroup IIb (p = 0.026), highlighting its protective effect.

2. Open field test: (Table 2)

Chlorpyrifos exposure (Subgroup IIa) induced anxiety-like behavior, shown by increased latency times to cross and to first rear compared to controls ($p < 0.001$). Recovery from chlorpyrifos (Subgroup IIb) did not result in significant changes in these latencies compared to Subgroup IIa ($p = 0.579$ and 0.728, respectively).

Zinc co-administration (Group III) significantly reduced latency to cross ($p = 0.018$) compared to Subgroup IIa but did not significantly affect latency to first rear ($p = 0.105$). No significant differences were observed between Group III and Subgroup IIb in latencies ($p = 0.071$ and 0.202).

Chlorpyrifos exposure also significantly reduced the number of crosses and rears compared to controls ($p < 0.001$). Recovery for two weeks resulted in non-significant increases in these parameters compared to Subgroup IIa ($p = 0.286$ and 0.249). Zinc co-administration significantly increased both the number of crosses and rears compared to Subgroup IIa ($p < 0.001$) and Subgroup IIb ($p = 0.022$ and 0.018, respectively).

3.2. Biochemical tests

1- Serum butyrylcholinesterase activity (U/L): (Table 3)

A significant reduction in serum butyrylcholinesterase activity was observed in the chlorpyrifos-treated group (Subgroup IIa; 257.6 ± 24.0 U/L) compared to the control group (587.8 ± 54.0 U/L; $p < 0.001$). Recovery from chlorpyrifos exposure for two weeks (Subgroup

IIb) significantly improved enzyme activity (555.8 ± 51.9 U/L) compared to Subgroup IIa ($p < 0.001$). Zinc co-administration (Group III) did not result in a significant increase in enzyme activity (310.8 ± 24.4 U/L) compared to Subgroup IIa ($p = 0.070$). However, a significant difference was noted between Subgroup IIb and Group III ($p < 0.001$

2- Serum zinc level (µg/dl): (Table 4)

Serum zinc levels significantly decreased in the chlorpyrifos-treated group (Subgroup IIa; 83.0 ± 9.3 µg/dL) compared to controls (94.6 ± 4.5 µg/dL; p = 0.002). Recovery from chlorpyrifos exposure for two weeks (Subgroup IIb) resulted in a non-significant change in zinc levels (85.2 ± 5.8 µg/dL) compared to Subgroup IIa (p = 0.17). In the group receiving both chlorpyrifos and zinc (Group III), a significant increase in zinc levels was observed (93.1 ± 3.1 µg/dL) compared to Subgroup IIa (p = 0.011). Additionally, a significant difference in zinc levels was noted between Subgroup IIb and Group III (p < 0.04).

3.3. Histological results

Group I (Control group)

Examination of hippocampus of the control group showed normal structure of hippocampus proper (cornu-ammonis) and its subregions CA1, CA2, CA3 & CA4 areas and a dark C shaped dentate gyrus. CA1 & CA3 areas are formed of; stratum alveolus (SA), stratum oriens (SO), stratum pyramidale (SP), and stratum radiatum (SR). The stratum pyramidale contains rows of small pyramidal neurons having large vesicular nuclei with prominent nucleoli and pale basophilic cytoplasm. The pyramidal neurons of SP were seen in 4-6 rows in CA1, on the other hand, they were large arranged in 2-5 rows in CA3. (**Fig. 1**).

Regarding Immunohistochemical results, CA1 and CA3 histological sections, group I showed mild immunoreaction in the star shaped astrocytes cytoplasm that revealed as scattered brownish small cells and their thin short branching processes in SO, SP and SR layers of hippocampus that reached the near blood capillaries. (**Fig. 2**).

Group II (Chlorpyrifos-treated group)

Sections of subgroup IIa (Group treated with chlorpyrifos) in CA1 and CA3 were examined, and the results showed areas of neuronal loss along with disarray in the pyramidal cell layers of CA1 and CA3. Disintegrated chromatin is seen in many pyramidal neurons, and some of them have pyknotic nuclei with shrunken cell bodies. In the neuropil, there were also some vacuoles visible between the pyramidal neurons (**Fig. 3**).

Examination of hippocampus of rats of subgroup IIb (the Recovery group) revealed similar histological changes as subgroup IIa. Most pyramidal nerve cells revealed shrunken cell bodies and deeply stained nuclei. Only a few pyramidal neurons appeared with lightly stained nuclei (**Fig. 5**). Regarding Immunohistochemical results, subgroups IIa and IIb revealed strong moderate positive immunoreaction in the star-shaped astrocytes' cell body and in their thick cell processes **(Fig. 4&6).**

Group III (chlorpyrifos + zinc group):

Examination of hippocampal sections of rats of subgroup III (given zinc as a co-administration with chlorpyrifos) revealed almost restored architecture of hippocampus proper. Most pyramidal neurons in CA1 and CA3 appeared with vesicular nuclei and prominent nucleoli. Focal regions of neuronal loss were observed in a few numbers of pyramidal neurons with deeply stained nuclei and shrunken cell bodies (**Fig. 7**). Regarding Immunohistochemical results, the

GFAP immunoreaction markedly declined in group III that revealed less cytoplasmic reaction in the cell bodies of astrocytes in addition to their thin processes (**Fig 8**).

3.4.Histo-morphometric results

Area percent of GFAP (Fig.9)

Subgroup IIa (chlorpyrifos exposure) showed a significant increase in GFAP levels compared to the control, with mean values of 94.61 ± 3.08 (CA1) and 94.50 ± 2.98 (CA3), both $p < 0.001$.

Subgroup IIb (withdrawal group) exhibited similar GFAP levels to subgroup IIa, with no significant differences ($p = 0.998$ in CA1, $p = 0.817$ in CA3).

Group III (chlorpyrifos with zinc co-administration) showed a significant reduction in GFAP immunoreactivity compared to subgroup IIa, with mean values of 87.42 ± 1.72 (CA1, $p = 0.001$) and 88.24 ± 1.68 (CA3, $p = 0.021$). When compared to subgroup IIb, group III also showed a significant decrease in GFAP levels ($p < 0.001$ in CA1, $p = 0.002$ in CA3).

The number of GFAP positive astrocytes (Fig.10)

Subgroup IIa (chlorpyrifos-treated) showed a significant increase in astrocytes with GFAP immunoreaction compared to control, with mean values of 27.93 ± 0.80 in CA1 and 28.40 ± 0.51 in CA3 ($p < 0.001$).

After 2 weeks of withdrawal (subgroup IIb), no significant reduction was noted compared to subgroup IIa, with mean values of 27.27 ± 1.39 (CA1) and 27.40 ± 1.12 (CA3), and p values of 0.952 and 0.979, respectively.

Group III (zinc co-administration) demonstrated a significant reduction in GFAP-positive astrocytes compared to subgroups IIa and IIb, with mean values of 19.53 ± 1.92 in CA1 and 21.47 ± 1.30 in CA3 ($p < 0.001$ vs. IIa, $p = 0.003$ vs. IIb).

4. DISCUSSION

Studying repeated OP toxicity in humans is challenging due to variable exposures, genetic differences, and environmental factors. Experimental animal studies offer a controlled environment to better understand OP toxicity and its long-term effects. The current study was conducted on albino rats due to their neuroanatomical and neurophysiological similarities to humans.(Costa et al.2006) (Huff et al.2008)

A low dose of CPF at 18 mg/kg body weight, approximately 1/13 of the acute oral LD50 for rats, was selected to avoid acute toxicity and severe harm. Administered orally over three weeks, this dose aligns with prior research and models repeated, low-level exposure similar to that experienced by humans in agricultural settings or through pesticide-contaminated products.(Mohamed et al.2018) (Terry et al.2012)

Over the 3-week treatment period with CPF in the current study, all rats survived without showing significant signs of cholinergic overstimulation despite about 40% decrease in cholinesterase enzyme activity. This can be explained by the difference in the body's response to acute versus small doses in repeated exposure to organophosphorus compounds. Repeated- small dose exposure leads to a gradual decrease in cholinesterase activity, allowing the body to adapt through different mechanisms. Additionally, the slower progression of exposure provides time for the body to metabolize and eliminate the compounds, preventing the severe symptoms of typical acute poisoning. (Naughton et al.2018)

In the present study, the passive avoidance test was used to evaluate memory function after repeated-CPF-exposure. In CPF group, the short latency to enter the dark room indicates significant memory impairment in the rats. They are unable to recall the learned avoidance behavior. This result highlights potential issues with memory function, cognitive processing, or learning capability, which can be attributed to CPF.

These results align with prior studies demonstrating CPF-induced memory deficits, increased impulsivity, and cognitive impairments in rodents at various doses and durations.(Cavalcanti et al.2020) (Terry et al.2012) (Nikbin et al.2022) Similarly, human studies have reported poorer memory and attention in children living near agricultural areas with high organophosphate use, emphasizing the potential cognitive risks associated with CPF exposure.(Rohlman et al.2005)

To assess behavioral changes and anxiety-related behaviors following CPF exposure, the present study utilized the open field test which is a widely used experimental paradigm. (Kraeuter et al. 2019) The observed reductions in locomotor activity and exploratory behavior in the CPF-treated rats suggest heightened anxiety levels, which can be attributed to the neurotoxic effects of CPF.

These findings align with Aminu et al. 2018 , who observed anxiety-like behaviors in rats exposed to CPF (14.9 mg/kg) over two weeks, evidenced by decreased locomotor activity and reduced rearing in the open field test conducted on days 10 and 14. Similarly, Silva et al. 2017 reported that CPF exposure during gestational days 14–20 at doses of 0.1, 1, and 10 mg/kg/day caused anxiogenic-like behavior in offspring at 21 days of life.

In contrast, Savy et al. 2015 found no significant effects on locomotor activity or anxiety-like behavior in rats given a lower CPF dose (1 mg/kg via intraperitoneal injection for five days). This discrepancy may be attributed to the lower dose used in their study compared to the current research.

Moreover, Behavioral testing conducted after a two-week withdrawal period revealed no significant improvement in anxiety-like behavior or memory function in rats previously exposed to CPF, despite a marked recovery in cholinesterase (ChE) activity. These findings are consistent with Ribeiro et al. 2022 , who reported that after a 12-week washout period following 21 days of CPF exposure, rats showed full recovery of ChE activity but continued to exhibit anxiety-related behaviors (open-field and elevated plus-maze) and despair (forced swim test). This suggests that behavioral impairments from CPF exposure may persist beyond biochemical recovery.

The mechanisms underlying neurobehavioral deficits caused by chronic exposure to CPF remain uncertain and controversial. While these deficits are often linked to acetylcholinesterase (AChE) inhibition, resulting in overstimulation of cholinergic receptors in the hippocampus and amygdala, some studies suggest non-cholinergic pathways may also contribute. Researchers have proposed mechanisms such as oxidative stress-induced neuronal damage, reduced mitochondrial function, altered N-methyl-D-aspartate (NMDA) receptor activity, disrupted glucocorticoid levels, and impaired microtubule motility, indicating that CPF's neurotoxicity extends beyond AChE inhibition emphasizing the complex nature of CPF neurotoxicity. (Slotkin et al.2012) (Lee et al. 2012) The study reveals a significant reduction in zinc levels in the group exposed to CPF compared to controls. Zinc supplementation restored serum zinc concentrations to normal levels but did not significantly improve serum butyrylcholinesterase activity compared to the CPF-only group.

Increased antioxidant defences in response to CPF toxicity could explain this zinc deficiency in CPF group. The levels of most zinc-containing proteins, metallothioneins, and antioxidant enzymes are significantly elevated to counter the toxicity. It may result in a net consumption of zinc in this process.(Jarosz et al.2017)

Moreover, CPF-induced hepato-renal dysfunction alters zinc metabolism and excretion; liver injury reduces the synthesis of proteins such as albumin and metallothioneins, which are essential for zinc transport and buffering, while kidney damage may contribute to increased urinary zinc loss, further depleting systemic zinc levels.(Verma et al.2007)

In agricultural countries, the widespread use of organophosphorus compounds for pest control makes avoiding exposure challenging, raising concerns about their neurobehavioral effects. Consequently, current research emphasizes identifying protective agents to mitigate these adverse outcomes.

Zinc supplements have emerged as a potential candidate for this purpose as it is distributed throughout the body, with especially high concentrations in the limbic system, including the hippocampus, hypothalamus, and amygdala. The crucial role of zinc in neurogenesis, neuronal migration, synaptogenesis, and neurotransmission makes it closely related to learning and memory function.(Gower-Winter et al.2012)

Consistent with the present study, Goel et al. 2000 demonstrated significant reductions in serum and hepatic zinc concentrations in animals treated with chlorpyrifos (13.5 mg/kg body weight) for eight weeks. Zinc supplementation normalized these levels in both liver and serum. Similarly, Abass et al. 2016 linked low serum zinc levels to organophosphorus-induced delayed

polyneuropathy in farmers, highlighting zinc's role in the activity of antioxidant and esterase enzymes.

Increased antioxidant defences in response to CPF toxicity could explain this zinc deficiency in CPF group. The levels of most zinc-containing proteins, metallothioneins, and antioxidant enzymes are significantly elevated to counter the toxicity. It may result in a net consumption of zinc in this process.(Abass et al.2016)

In the current study, a zinc supplement dose of 15 mg/kg was chosen to balance efficacy and safety, offering potential protective benefits without toxicity. This suggests that AChE inhibition may not be the sole mechanism behind neurobehavioral deficits. Consistent with these findings, Franco et al. 2009 reported that zinc did not affect AChE activity or reverse malathion-induced AChE inhibition. Similarly, Tahmasebi et al. 2022 observed that antioxidants failed to restore plasma cholinesterase activity reduced by organophosphates. However, Singla and Dhawan 2017 demonstrated that zinc co-administration in aluminum-treated rats restored AChE activity after 8 weeks, possibly by displacing aluminum from AChE active sites or preventing its binding, enhancing zinc's role in biochemical processes.

Regarding behavioral testing, the co-administration of zinc to the CPF-administered group showed an improvement in retention latency time to enter the dark room in the passive avoidance test, compared to the group administered CPF alone. This suggests that zinc may protect memory function from the deteriorating effects of CPF. Furthermore, the zinc-supplemented group exhibited improvements in latency time to cross, as well as in the number of crossings and rears, which can be attributed to the anxiolytic effects of zinc.

This study aligns with findings by Abass et al. 2016, who highlighted zinc's neuroprotective potential against pesticide-induced neurodegenerative and behavioral disorders. Rivers-Auty et al. 2021 demonstrated that zinc deficiency leads to cognitive deficits, while adequate zinc intake reduced cognitive decline in Alzheimer's patients. Akintoye et al. 2023 further noted zinc's role in improving memory deficits, its anxiolytic, antioxidant, and anti-inflammatory properties, and its ability to alleviate anxiety by increasing gamma-aminobutyric acid (GABA) concentrations.

Zinc also enhances antioxidant generation capacity by inhibiting NMDA receptors, which play a pivotal role in inhibiting the transcription of pro-inflammatory mediators such as TNF and IL-6. (Krall et al. 2020)

Banks and Lein 2012 reported that CPF exposure increased neuroinflammatory markers in the hippocampus and cortex, regions responsible for cognitive function. CPF can trigger neuroinflammation by activating microglia and astrocytes, causing the release of pro-inflammatory cytokines like TNF-α and IL-1β. Chronic neuroinflammation impairs neurogenesis and synaptic plasticity and contributes to anxiety-like behaviors as mentioned by Réus et al. 2015.

The current results were confirmed by **histological analysis** to determine whether the observed neurobehavioral deficits have a structural basis. By examining brain tissue at the microscopic level, we aimed to identify any potential changes in neuronal architecture, cell density, or other structural alterations that may underlie the cognitive and behavioral impairments detected in CPF-treated subjects.

This approach helps to determine whether the neurobehavioral deficits are linked to visible structural damage or primarily to functional disruptions at the molecular or cellular levels. The susceptibility of various hippocampal subfields to damage remains a topic of debate. While some

studies suggest that CA1 neurons are the most vulnerable, others indicate that CA3 and DG subfields are more prone to damage. (Huang et al.2013)

This study revealed that repeated exposure to CPF caused significant structural damage in the hippocampus, including neuronal loss, apoptotic changes, and distorted pyramidal cells, likely due to oxidative stress and free radical production. CPF penetrates the blood-brain barrier, disrupting brain integrity, decreasing antioxidant enzyme activity, and triggering apoptosis. (Elmorsy et al.2022) (Barrera 2012) (Nakagami et al.2009)

Zinc administration in the study nearly restored normal hippocampal architecture, showing sporadic apoptotic pyramidal cells and focal neuronal loss due to zinc's antioxidant and anti-inflammatory properties. Zinc acts as a cofactor for superoxide dismutase (SOD) and stimulates metallothionein, scavenging hydroxyl radicals and reversing oxidative stress. Szewczyk et al. 2013 highlighted Zinc’s neuroprotective role in conditions like depression, anxiety, learning impairments, and memory loss. Similarly, Hafez et al. 2023 reported modest histological improvements in the hippocampus with zinc supplementation in obese male rats.

Astrocytes, essential glial cells in the CNS, support neuronal survival by providing structural and metabolic support, regulating the synaptic microenvironment, maintaining the blood-brain barrier, vascular reactivity, energy metabolism, and protecting against oxidative stress and reactive oxygen species. In response to brain damage, astrocytes activate and undergo morphofunctional remodelling, exhibiting resilience to toxins and enhanced proliferation. They protect neurons and fill spaces left by neuronal death through glial scar formation. Astrogliosis, a reaction to CNS injury, involves increased expression of intermediate filaments and GFAP, hypertrophy, and functional adaptations.(Fernandez et al.2010) (Vincent et al. 2010)

GFAP, an intermediate cytoskeleton protein in astrocytes and ependymal cells, regulates astrocyte motility and shape by stabilizing structural processes and serves as a marker for astrocyte maturity in the CNS. During astrogliosis, GFAP synthesis increases and can be detected through immunostaining. In the current study, chlorpyrifos exposure activated astrocytes, marked by elevated GFAP expression, is likely as a compensatory response to neuronal damage. Zinc co-administration with chlorpyrifos reduced GFAP activity, indicating its reparative effects on neurons. Histopathological findings further support Zinc's role in mitigating gliosis and inflammation, consistent with previous studies showing its regulatory influence on GFAP activity.(Vazi et al.2021)

5. Conclusion

The study demonstrates that repeated low-dose exposure to chlorpyrifos impairs neurobehavioral functions in rats, accompanied by hippocampal histopathological alterations and potential involvement of non-cholinergic mechanisms, including reduced zinc levels. A two-week withdrawal period was insufficient for spontaneous recovery. Concurrent zinc supplementation during CPF exposure showed a protective effect, improving behavioral outcomes and reducing histopathological changes.

While these findings support zinc's potential neuroprotective role, further studies are needed to confirm long-term benefits and to explore optimal dosing strategies.

6. Recommendations

To mitigate the risks associated with CPF exposure, regular health screenings to detect early signs of neurotoxicity are recommended. Zinc supplementation is recommended as a cost-effective protective measure for those frequently exposed. Further research is essential to understand CPF

toxicity mechanisms beyond cholinergic pathways, evaluate long-term effects and recovery, and determine zinc's optimal dosage and efficacy through larger studies and clinical trials.

References

Abass, M.A., Arafa, M.H., Elfakharany, Y.M. and Atteia, H.H. (2016) 'Role of zinc deficiency in the susceptibility to delayed polyneuropathy in organophosphorous intoxicated patients in Zagazig university hospitals, Zagazig, Egypt', *Egyptian Journal of Forensic Sciences and Applied Toxicology*, 16(2), pp. 17–33.

Abdelhamid, W. (2021) 'Evaluation of severity of poisoning exposures among patients presented to Poison Control Center, Ain Shams University Hospitals, Egypt during 2019', *AJFM*, 36(1), pp. 106–112.

Akintoye, O.O., Ajibare, A.J., Folawiyo, M.A., Jimoh-Abdulghaffaar, H.O., Asuku, A., Owolabi, G.A. and Olajide, O.J. (2023) 'Zinc supplement reverses short-term memory deficit in sodium benzoate-induced neurotoxicity in male Wistar rats by enhancing anti-oxidative capacity via Nrf2 up-regulation', *Behavioural Brain Research*, 437, p. 114163.

Aminu, A.I., Sulaiman, N.A., Oyewole, A.L., Chengetanai, S., Williams, V., Ajibola, M.I., Folarin, R.O., Muhammad, A.S., Shittu, S.T. and Ajao, M.S. (2018) 'Chlorpyrifos- and dichlorvos-induced oxidative and neurogenic damage elicits neuro-cognitive deficits and increases anxiety-like behavior in wild-type rats', *Toxics*, 6(4), p. 71.

Bancroft, J.D. and Layton, C. (2013) 'The hematoxylins and eosin', in Suvarna, S.K., Layton, C. and Bancroft, J.D. (eds.) *Bancroft's Theory and Practice of Histological Techniques*. 7th edn. Oxford: Churchill Livingstone, pp. 173–186.

Banks, C.N. and Lein, P.J. (2012) 'A review of experimental evidence linking neurotoxic organophosphorus compounds and inflammation', *NeuroToxicology*, 33(3), pp. 575–584.

Barrera, G. (2012) 'Oxidative stress and lipid peroxidation products in cancer progression and therapy', *ISRN Oncology*, 2012, p. 137289.

Burke, R.D., Todd, S.W., Lumsden, E., Mullins, R.J., Mamczarz, J., Fawcett, W.P., Kelly, M.E., McCullough, P.M. and Tjalkens, R.B. (2017) 'Developmental neurotoxicity of the organophosphorus insecticide chlorpyrifos: From clinical findings to preclinical models and potential mechanisms', *Journal of Neurochemistry*, 142, pp. 162–177.

Cañadas, F., Cardona, D., Dávila, E. and Sánchez-Santed, F. (2005) 'Long-term neurotoxicity of chlorpyrifos: Spatial learning impairment on repeated acquisition in a water maze', *Toxicological Sciences*, 85(2), pp. 944–951.

Cavalcanti, C.L., Gonçalves, M.C.R., Alves, A.F., de Araújo, E.V., Carvalho, J.L.P., Lins, P.P., Alves, R.C., Soares, N.L., Pordeus, L.C.M. and Aquino, J.S. (2020) 'Antidepressant, anxiolytic and neuroprotective activities of two zinc compounds in diabetic rats', *Frontiers in Neuroscience*, 13, p. 1411.

Choi, S., Hong, D.K., Choi, B.Y. and Suh, S.W. (2020) 'Zinc in the brain: Friend or foe?', *International Journal of Molecular Sciences*, 21(23), p. 8941.

Costa, L.G. (2006) 'Current issues in organophosphate toxicology', *Clinica Chimica Acta*, 366(1), pp. 1–13.

Elmorsy, E., Al-Ghafari, A., Al Doghaither, H., Salama, M. and Carter, W.G. (2022) 'An investigation of the neurotoxic effects of malathion, chlorpyrifos, and paraquat to different brain regions', *Brain Sciences*, 12(8), p. 975.

Fernandez, L.L., Carmona, M., Portero-Otin, M., Naudi, A., Pamplona, R., Schröder, N., Moreira, J.C.F., Perry, G., Zhu, X. and Smith, M.A. (2010) 'Effects of increased iron intake during the neonatal period on the brain of adult AβPP/PS1 transgenic mice', *Journal of Alzheimer's Disease*, 19(3), pp. 1069–1080.

Franco, J.L., Posser, T., Mattos, J.J., Trevisan, R., Brocardo, P.S., Rodrigues, A.L.S., Leal, R.B., Farina, M. and Dafre, A.L. (2009) 'Zinc reverses malathion-induced impairment in antioxidant defenses', *Toxicology Letters*, 187(3), pp. 137–143.

Goel, A., Chauhan, D.P. and Dhawan, D.K. (2000) 'Protective effects of zinc in chlorpyrifos induced hepatotoxicity', *Biological Trace Element Research*, 74(2), pp. 171–183.

Goyal, R., Mathur, S.K., Gupta, S., Goyal, R., Kumar, S., Batra, A., Kumar, V., Singh, P., Sharma, S. and Jain, M. (2015) 'Immunohistochemical expression of glial fibrillary acidic protein and CAM5.2 in glial tumors and their role in differentiating glial tumors from metastatic tumors of central nervous system', *Journal of Neurosciences in Rural Practice*, 6(4), pp. 499–503.

Gower-Winter, S.D. and Levenson, C.W. (2012) 'Zinc in the central nervous system: From molecules to behavior', *BioFactors*, 38(3), pp. 186–193.

Hafez, L.M., Aboudeya, H.M., Matar, N.A., El-Sebeay, A.S., Nomair, A.M., El-hamshary, S.A., El-Sayed, R.M. and Mansour, D.F. (2023) 'Ameliorative effects of zinc supplementation on

cognitive function and hippocampal leptin signaling pathway in obese male and female rats', *Scientific Reports*, 13(1), p. 5072.

Huang, Y., Coupland, N.J., Lebel, R.M., Carter, R., Seres, P., Wilman, A.H. and Malykhin, N.V. (2013) 'Structural changes in hippocampal subfields in major depressive disorder: A high-field magnetic resonance imaging study', *Biological Psychiatry*, 74(1), pp. 62–68.

Huff, J., Jacobson, M.F. and Davis, D.L. (2008) 'The limits of two-year bioassay exposure regimens for identifying chemical carcinogens', *Environmental Health Perspectives*, 116(11), pp. 1439–1442.

Ishiyama, T., Tokuda, K., Ishibashi, T., Ito, A., Toma, S. and Ohno, Y. (2007) 'Lurasidone (SM-13496), a novel atypical antipsychotic drug, reverses MK-801-induced impairment of learning and memory in the rat passive-avoidance test', *European Journal of Pharmacology*, 572(2), pp. 160–170.

Jackson, P. and Blythe, D. (2013) 'Immunohistochemical techniques', in Suvarna, S.K., Layton, C. and Bancroft, J.D. (eds.) *Bancroft's Theory and Practice of Histological Techniques*. 7th edn. Oxford: Churchill Livingstone, pp. 381–426.

Jarosz, M., Olbert, M., Wyszogrodzka, G., Młyniec, K. and Librowski, T. (2017) 'Antioxidant and anti-inflammatory effects of zinc: Zinc-dependent NF-κB signaling', *Inflammopharmacology*, 25(1), pp. 11–24.

Johnsen, O. and Eliasson, R. (1987) 'Evaluation of a commercially available kit for the colorimetric determination of zinc in human seminal plasma', *International Journal of Andrology*, 10(2), pp. 435–440.

Jońca, J., Żuk, M., Wasąg, B., Janaszak-Jasiecka, A., Lewandowski, K., Wielgomas, B., Kołodziejczyk, A., Dąbrowska, A., Nowak, M., Kowalska, K. and Wójcik, A. (2015) 'New insights into butyrylcholinesterase activity assay: Serum dilution factor as a crucial parameter', *PLoS ONE*, 10(10), p. e0139480.

Joshi, M., Akhtar, M., Najmi, A., Khuroo, A. and Goswami, D. (2012) 'Effect of zinc in animal models of anxiety, depression and psychosis', *Human and Experimental Toxicology*, 31(12), pp. 1237–1243.

Kotz, S., Balakrishnan, N., Read, C.B. and Vidakovic, B. (2006) *Encyclopedia of Statistical Sciences*. 2nd edn. Hoboken, NJ: Wiley-Interscience.

Kraeuter, A.K., Guest, P.C. and Sarnyai, Z. (2019) 'The open field test for measuring locomotor activity and anxiety-like behavior', *Methods in Molecular Biology*, 1916, pp. 99–103.

Krall, R.F., Moutal, A., Phillips, M.B., Asraf, H., Johnson, J.W., Khanna, R. and Tzounopoulos, T. (2020) 'Synaptic zinc inhibition of NMDA receptors depends on the association of GluN2A with the zinc transporter ZnT1', *Science Advances*, 6(27).

Lee, J.E., Park, J.H., Shin, I.C. and Koh, H.C. (2012) 'Reactive oxygen species regulated mitochondria-mediated apoptosis in PC12 cells exposed to chlorpyrifos', *Toxicology and Applied Pharmacology*, 263(2), pp. 148–162.

Li, Z., Liu, Y., Wei, R., Yong, V.W. and Xue, M. (2022) 'The important role of zinc in neurological diseases', *Biomolecules*, 13(1), p. 28.

Lu, C., Yang, Z., Liu, J., Liao, Q., Ling, W., Waigi, M.G., Wang, Y., Zhang, Y., Chen, X., Li, H. and Zhao, J. (2020) 'Chlorpyrifos inhibits nitrogen fixation in rice-vegetated soil containing *Pseudomonas stutzeri* A1501', *Chemosphere*, 256, p. 127098.

Marques, S.I., Carmo, H., Carvalho, F., Sá, S.I. and Silva, J.P. (2023) 'A semi-automatic method for the quantification of astrocyte number and branching in bulk immunohistochemistry images', *International Journal of Molecular Sciences*, 24(5), p. 4508.

Maywald, M. and Rink, L. (2022) 'Zinc in human health and infectious diseases', *Biomolecules*, 12(12).

Mense, S.M., Sengupta, A., Lan, C., Zhou, M., Bentsman, G., Volsky, D.J., Whyatt, R.M., Perera, F.P. and Zhang, L. (2006) 'The common insecticides cyfluthrin and chlorpyrifos alter the expression of a subset of genes with diverse functions in primary human astrocytes', *Toxicological Sciences*, 93(1), pp. 125–135.

Middlemore-Risher, M.L., Buccafusco, J.J. and Terry, A.V. Jr. (2010) 'Repeated exposures to low-level chlorpyrifos results in impairments in sustained attention and increased impulsivity in rats', *Neurotoxicology and Teratology*, 32(4), pp. 415–424.

Mohamed, W.R., Mehany, A.B.M. and Hussein, R.M. (2018) 'Alpha lipoic acid protects against chlorpyrifos-induced toxicity in Wistar rats via modulating the apoptotic pathway', *Environmental Toxicology and Pharmacology*, 59, pp. 17–23.

Nakagami, A., Negishi, T., Kawasaki, K., Imai, N., Nishida, Y., Ihara, T., Kuroda, Y. and Yoshikawa, Y. (2009) 'Alterations in male infant behaviors towards its mother by prenatal

exposure to bisphenol A in cynomolgus monkeys (*Macaca fascicularis*) during early suckling period', *Psychoneuroendocrinology*, 34(8), pp. 1189–1197.

Naughton, S.X. and Terry, A.V. Jr. (2018) 'Neurotoxicity in acute and repeated organophosphate exposure', *Toxicology*, 408, pp. 101–112.

Nikbin, S., Jameie, S.B., Azarbayjani, M.A. and Hosseini, N. (2022) 'The effects of chlorpyrifos on hippocampal caspase-3 levels and passive avoidance memory in rats', *Thrita Journal of Neurology*, 11(1), p. e123356.

Rathod, A.L. and Garg, R.K. (2017) 'Chlorpyrifos poisoning and its implications in human fatal cases: A forensic perspective with reference to Indian scenario', *Journal of Forensic and Legal Medicine*, 47, pp. 29–34.

Rauh, V.A. (2018) 'Polluting developing brains — EPA failure on chlorpyrifos', *New England Journal of Medicine*, 378(13), pp. 1171–1174.

Réus, G.Z., Fries, G.R., Stertz, L., Badawy, M., Passos, I.C., Barichello, T., Kapczinski, F. and Quevedo, J. (2015) 'The role of inflammation and microglial activation in the pathophysiology of psychiatric disorders', *Neuroscience*, 300, pp. 141–154.

Ribeiro, A.C.R., Hawkins, E., Jahr, F.M., McClay, J.L. and Deshpande, L.S. (2022) 'Repeated exposure to chlorpyrifos is associated with a dose-dependent chronic neurobehavioral deficit in adult rats', *Neurotoxicology*, 90, pp. 172–183.

Rivers-Auty, J., Tapia, V.S., White, C.S., Daniels, M.J.D., Drinkall, S., Kennedy, P.T., Spencer, N.G., McAlpine, C.S., Amrita, Malik, A., Lavelle, E.C., McColl, B.W., Freeman, S., Kirov, A., Taylor, P.R., Allan, S.M. and Brough, D. (2021) 'Zinc status alters Alzheimer's disease

progression through NLRP3-dependent inflammation', *Journal of Neuroscience*, 41(13), pp. 3025–3038.

Rohlman, D.S., Arcury, T.A., Quandt, S.A., Lasarev, M., Rothlein, J., Travers, R., Tamulinas, A., Scherer, J., Early, J., Marín, A., Phillips, J. and McCauley, L. (2005) 'Neurobehavioral performance in preschool children from agricultural and non-agricultural communities in Oregon and North Carolina', *Neurotoxicology*, 26(4), pp. 589–598.

Rusch, P., Hirner, A.V., Schmitz, O., Kimmig, R., Hoffmann, O. and Diel, M. (2021) 'Zinc distribution within breast cancer tissue of different intrinsic subtypes', *Archives of Gynecology and Obstetrics*, 303(1), pp. 195–205.

Salvi, R.M., Lara, D.R., Ghisolfi, E.S., Portela, L.V., Dias, R.D. and Souza, D.O. (2003) 'Neuropsychiatric evaluation in subjects chronically exposed to organophosphate pesticides', *Toxicological Sciences*, 72(2), pp. 267–271.

Savy, C.Y., Fitchett, A.E., McQuade, R., Gartside, S.E., Morris, C.M., Blain, P.G. and Judge, S.J. (2015) 'Low-level repeated exposure to diazinon and chlorpyrifos decrease anxiety-like behaviour in male rats as assessed by marble burying behaviour', *Neurotoxicology*, 50, pp. 149–156.

Shah, N.M. and Mundura, S.H. (2016) 'Clinical profile of organ phosphorous poisoning in a tertiary care centre', *International Journal of Medical Science and Public Health*, 5(8), pp. 1621–1625.

Silva, J.G., Boareto, A.C., Schreiber, A.K., Redivo, D.D.B., Gambeta, E., Vergara, F., Morais, H., Zanoveli, J.M. and Dalsenter, P.R. (2017) 'Chlorpyrifos induces anxiety-like behavior in offspring rats exposed during pregnancy', *Neuroscience Letters*, 641, pp. 94–100.

Singla, N. and Dhawan, D.K. (2017) 'Zinc improves cognitive and neuronal dysfunction during aluminium-induced neurodegeneration', *Molecular Neurobiology*, 54(1), pp. 406–422.

Slotkin, T.A. and Seidler, F.J. (2012) 'Developmental neurotoxicity of organophosphates targets cell cycle and apoptosis, revealed by transcriptional profiles in vivo and in vitro', *Neurotoxicology and Teratology*, 34(2), pp. 232–241.

Szczewczyk, B. (2013) 'Zinc homeostasis and neurodegenerative disorders', *Frontiers in Aging Neuroscience*, 5, p. 33.

Tahmasebi, K., Jafari, M., Heydari, J., Asgari, A., Salehi, M., Khazaie, S. and Ghorbani, M. (2022) 'Tissues toxicity attenuation by vitamin E on oxidative damage induced by diazinon', *Environmental Analysis, Health and Toxicology*, 37(4), p. e2022036.

Terry, A.V. Jr., Beck, W.D., Warner, S., Vandenhuerk, L. and Callahan, P.M. (2012) 'Chronic impairments in spatial learning and memory in rats previously exposed to chlorpyrifos or diisopropylfluorophosphate', *Neurotoxicology and Teratology*, 34(1), pp. 1–8.

Vazi, E., Holanda, F., Santos, N., Cardoso, C., Martins, M. and Bondan, E. (2021) 'Short-term systemic methotrexate administration in rats induces astrogliosis and microgliosis', *Research in Veterinary Science*, 138, pp. 39–48.

Verma, R.S., Mehta, A. and Srivastava, N. (2007) 'In vivo chlorpyrifos induced oxidative stress: Attenuation by antioxidant vitamins', *Pesticide Biochemistry and Physiology*, 88(2), pp. 191–196.

Vincent, A.J., Gasperini, R., Foa, L. and Small, D.H. (2010) 'Astrocytes in Alzheimer's disease: Emerging roles in calcium dysregulation and synaptic plasticity', *Journal of Alzheimer's Disease*, 22(3), pp. 699–714.

Voorhees, J.R., Remy, M.T., Erickson, C.M., Dutca, L.M., Brat, D.J. and Pieper, A.A. (2019) 'Occupational-like organophosphate exposure disrupts microglia and accelerates deficits in a rat model of Alzheimer's disease', *NPJ Aging and Mechanisms of Disease*, 5, p. 3.

Yang, Y., Hu, Z., Du, X., Davies, H., Huo, X. and Fang, M. (2017) 'miR-16 and Fluoxetine both reverse autophagic and apoptotic change in chronic unpredictable mild stress model rats', *Frontiers in Neuroscience*, 11, p. 428.

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Author Contributions

Abeer Sheta: Conceptualization, Methodology, Supervision, Writing – review & editing. Eman Allam: Methodology, Investigation, Data analysis, Writing – original draft. Yassmin H. Khalifa: Histopathological examination, Immunohistochemistry, Morphometric analysis, Writing – review & editing. Rania Mohamed: Investigation, Behavioral testing, Data collection and writing the draft. Maram Atef: Conceptualization, Data analysis, Supervision, Writing – review & editing,

Corresponding author. All authors contributed to the interpretation of the data, revised the manuscript critically, and approved the final version for publication.

Ethical approval

All experimental procedures were conducted in accordance with the guide for care and use of laboratory animals. The study proposal was approved by the research ethics committee of faculty of medicine, (Serial NO: 0107403, IRB NO: 00012098, FWA NO:00018699); membership in international Council of laboratory animal science organization, ICLAS).

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Consent to Participate

Not applicable

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Competing interests

The authors declare no conflict of interest. The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

Table 1: Comparison between the different studied groups in passive avoidance test

	Control (Group I) n = 10	Chlorpyrifos (Subgroup IIa) n = 10	Recovery (Subgroup IIb) n = 10	Chlorpyrifos & Zinc (Group III) n = 10
Retention latency time in seconds				
Min. – Max.	280.0-300.0	90.0-240.0	180.0-280.0	200.0-300.0
Mean ± SD	292.0±8.56	183.0±49.23	201.0±32.47	254.8±37.8
F (p)		17.433*(<0.001*)		
P1		<0.001*	<0.001*	0.156
Sig. bet. groups		**P_2**=0.819, **P3** = 0.001*, **P_4** = 0.026*		

Min.: Minimum Max.: Maximum SD: Standard Deviation

F: F for ANOVA test, pairwise comparison between each 2 groups was done using Post Hoc Test (Tukey)

p_1: p value for comparing between control and each other group.

P_2: p value for comparing between CPF and Recovery group.

P_3: p value for comparing between CPF and CPF & Zinc group.

P_4: p value for comparing between CPF & Zinc and Recovery group.

*: Statistically significant at $p \leq 0.05$

Table 2: Comparison between the different studied groups in open field test.

	Control (Group I) n = 10	Chlorpyrifos (Subgroup IIa) n = 10	Recovery (Subgroup IIb) n = 10	Chlorpyrifos & Zinc (Group III) n = 10
Latency time to cross (seconds)				
Min. – Max.	2.0-4.0	3.0-15.0	2.0-9.0	3.0-5.0
Median (IQR)	3.0 (2.0-3.3)	7.0 (4.8-12.8)	6.0 (5.0-7.5)	4.0 (3.0-4.0)
H (p)	29.828*(<0.001*)			
P1		<0.001*	0.001*	0.116
Sig. bet. Groups	**P_2**=0.579, **P3** =0.018*, **P_4** = 0.071			
Latency time to rear (seconds)				
Min. – Max.	3.0-5.0	12.0-90.0	5.0-26.0	5.0-20.0
Median (IQR)	5.0 (3.0-4.0)	18.5 (15.8-64.8)	19.0 (15.0-25.0)	14.0 (8.8-18.5)
H (p)	36.413*(<0.001*)			
P1		<0.001*	<0.001*	0.010*
Sig. bet. Groups	**P_2**=0.728, **P3** =0.105 , **P_4** = 0.202			
Number of crosses				
Min. – Max.	45.0-58.0	15.0-30.0	20.0-50.0	38.0-54.0
Median (IQR)	52.0 (52.5-57.0)	24.0(19.5-26.0)	29.0 (23.8-33.5)	44.5(29.3-36.5)
H (p)	35.552*(<0.001*)			
P1		<0.001*	<0.001*	0.234
Sig. bet. Groups	**P_2**=0.286, **P3** =0.001*, **P_4** = 0.022*			
Number of rears				
Min. – Max.	25.0-30.0	6.0-16.0	11.0-17.0	20.0-29.0
Median (IQR)	27.0 (27.0-28.5)	9.0 (6.8-12.0)	14.5 (12.8-17.0)	24.5(14.0-28.3)
H (p)	39.487*(<0.001*)			
P1		<0.001*	0.001*	0.296
Sig. bet. Groups	**P_2**=0.249, **P3** <0.001* , **P_4**=0.018*			

Min.: Minimum **Max.: Maximum** **IQR: Interquartile range**

H: Kruskal Wallis test for comparing between the different groups and parwise comparison between group using Adjustment Bonferroni

p_1: p value for comparing between control and each other group.

P_2: p value for comparing between CPF and recovery group.

P_3: p value for comparing between CPF and CPF & Zinc group.

P_4: p value for comparing between CPF & Zinc and recovery group.

*: Statistically significant at $p \leq 0.05$

Table 3: Comparison between the different studied groups in serum butyrylcholinesterase activity (U/L)

	Control (Group I) n = 10	Chlorpyrifos (Subgroup IIa) n = 10	Recovery (Subgroup IIb) n = 10	Chlorpyrifos & Zinc (Group III) n = 10
Min. – Max.	466.0–615.0	237.0-298.0	480.0-606.0	280.0-356.0
Mean ± SD	578.8±54.0	257.6±24.0	555.8±51.9	310.8±24.4
F (p)		128.942*(<0.001*)		
P1		<0.001*	0.770	<0.001*
Sig. bet. groups		**P_2**<0.001*, **P3** = 0.070, **P_4** <0.001*		

Min.: Minimum Max.: Maximum SD: Standard Deviation
F: F for ANOVA test, Pairwise comparison between each 2 groups was done using Post Hoc Test (Tukey)
p_1: p value for comparing between control and each other group.
P_2: p value for comparing between CPF and Recovery group.
P_3: p value for comparing between CPF and CPF & Zinc group.
P_4: p value for comparing between CPF & Zinc and recovery group.
*: Statistically significant at $p \leq 0.05$

Table 4: Comparison between the different studied groups in serum Zinc level(µg/dl)

	Control (Group I) (n = 10)	Chlorpyrifos (Group IIa) (n = 10)	Recovery (Group IIb) (n = 10)	Chlorpyrifos & Zinc (Group III) (n = 10)
Zinc level(µg/dl)				
Min. – Max.	90.0- 102.0	69.0-100.0	82.0-101.0	89.0-98.0
Mean ± SD	94.6±4.5	83.0±9.3	85.2±5.8	93.1±3.1
F (p)	6.104* (<0.001*)			
p_1		0.002*	0.204	0.908
Sig. bet. groups	**P_2** = 0.175, **P_3** =0.011*, **p_4** = 0.04*			

Min.: Minimum Max.: Maximum SD: Standard Deviation
F: F for ANOVA test, Pairwise comparison between each 2 groups was done using Post Hoc Test (Tukey)
p_1: p value for comparing between control and each other group.
P_2: p value for comparing between CPF and Recovery group.
P_3: p value for comparing between CPF and CPF & Zinc group.
P_4: p value for comparing between CPF & Zinc and recovery group.
*: Statistically significant at $p \leq 0.05$

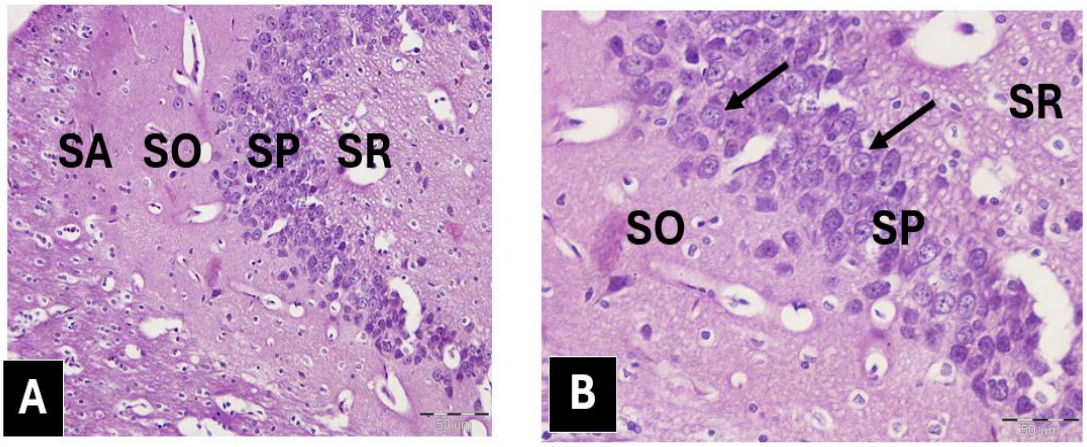


Figure 1: Light micrographs of the hippocampus of **control** sections of **CA3** area showing **A**; stratum alveolus (SA), stratum oriens (SO), stratum pyramidale (SP) and stratum radiatum (SR**). B;** SP shows larger pyramidal neurons with vesicular nuclei, prominent nucleoli and pale basophilic cytoplasm in rows (arrow). H&E stain, scale bar A; 100 µm, B; 50 µm.

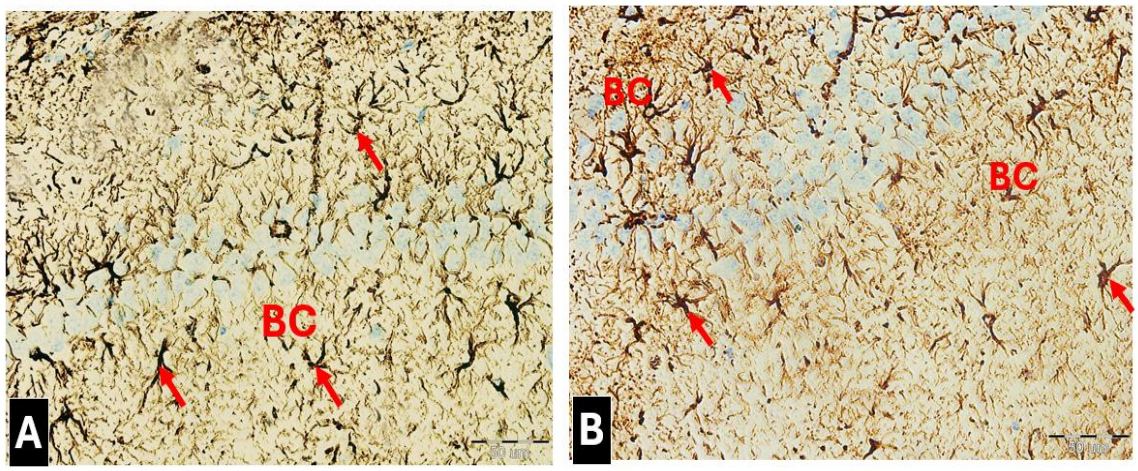


Figure 2: Photomicrographs of GFAP immune-stained sections of rat hippocampus of **control group**. **A (CA1) and B (CA3**): showing mild positive reaction in the astrocytes appearing as scattered brownish star shaped cells. They exhibited small cell body with thin branching processes

(arrow) in SO, SP and SR layers of hippocampus and extending near blood capillaries (BC). scale bar 50 µm.

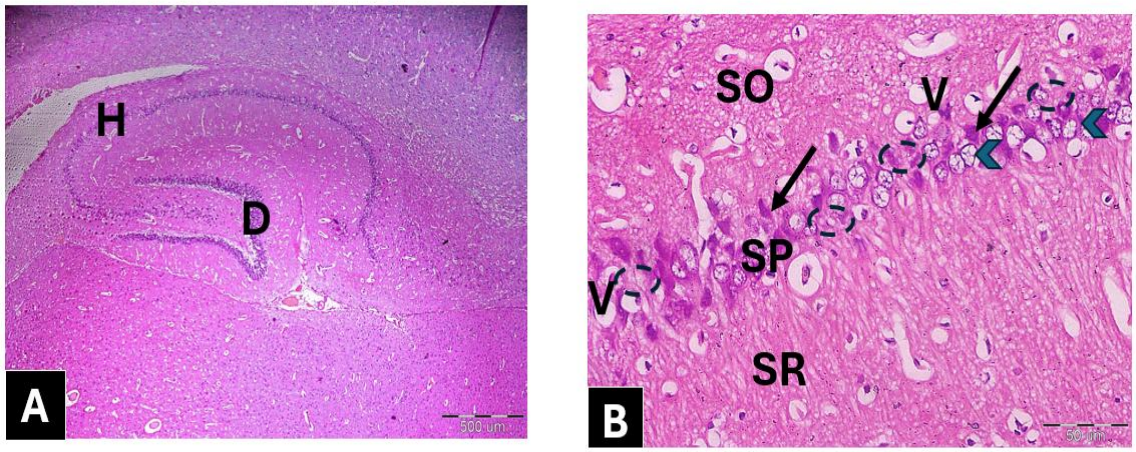


Figure 3: Light micrographs of the hippocampus of **subgroup IIa.** A; hippocampus proper (H). D; dentate gyrus. **B (CA1);** stratum oriens (SO), stratum pyramidale (SP) and stratum radiatum (SR) of hippocampus proper, showing some vacuoles (V) in between the pyramidal neurons in SP. Evident loss of pyramidal neurons (dotted circle). Some pyramidal neurons were seen with shrunken cell bodies and pyknotic nuclei (arrows) and most pyramidal neurons show nuclei with disintegrated chromatin (chevron arrow). H&E stain, scale bar A; 500 µm, B; 50 µm.

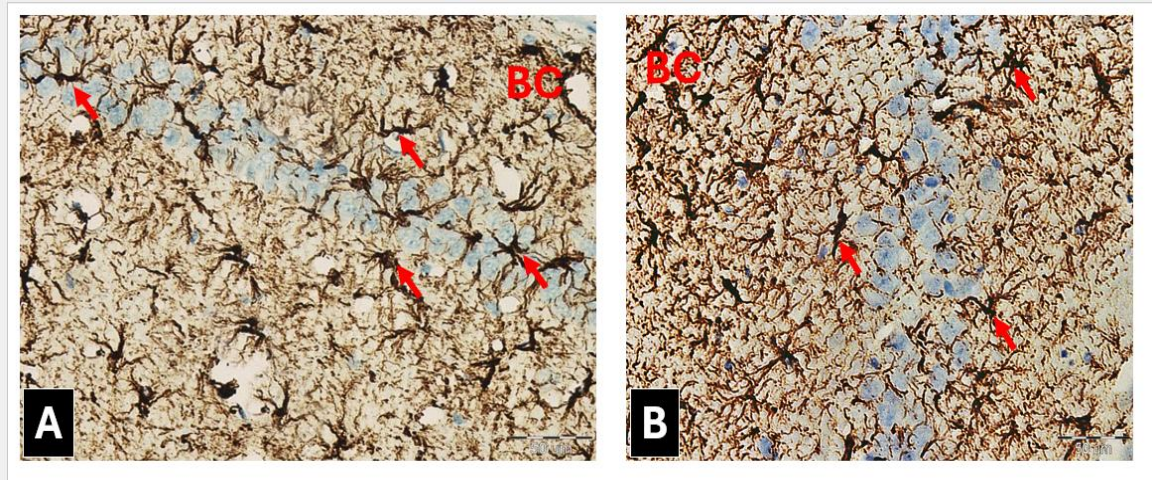


Figure 4: Photomicrographs of GFAP immune-stained sections of rat hippocampus of **subgroup IIa. A (CA1) and B (CA3)**: showing severe immune-positive reaction in star-shaped astrocytes having numerous branching and thick cell processes (arrow). BC; Blood capillary. scale bar 50 µm

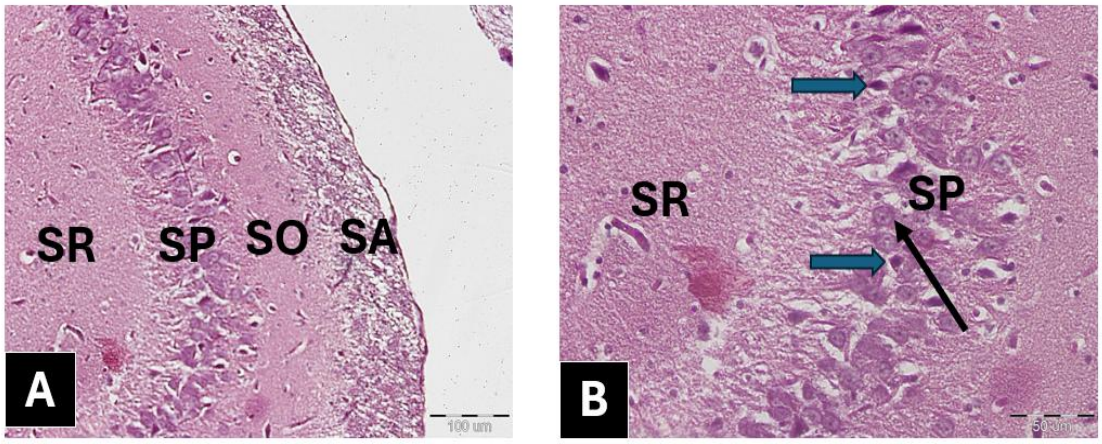


Figure 5: Light micrographs of the **CA3** of **subgroup IIb**. **A;** showing stratum alveolus (SA), stratum oriens (SO), stratum pyramidale (SP) and stratum radiatum (SR) of the hippocampus proper. **B;** SP appears thin with most pyramidal neurons having shrunken cell bodies and deeply stained nuclei (blue arrows). Few pyramidal neurons appear with lightly stained nuclei (black arrow).H&E stain, scale bar A; 100 µm, B; 50 µm.

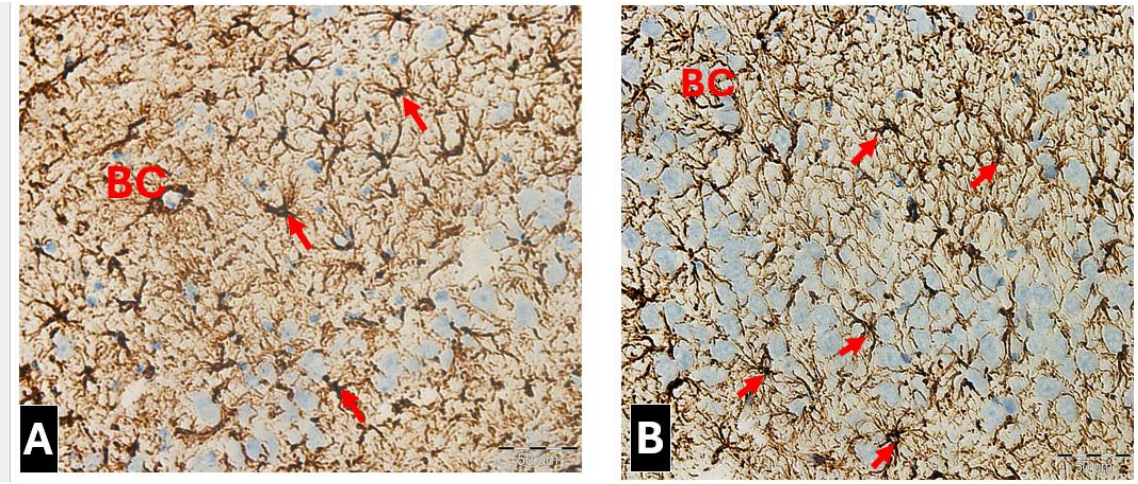


Figure 6: Photomicrographs of GFAP immune-stained sections of rat hippocampus of **subgroup IIb** received chlorpyrifos 18 mg kg for 3 weeks then left for 2 weeks of withdrawal period). **A (CA1) and B (CA3):** showing moderate positive brown color reaction in cytoplasm and the branching thick processes (arrow) of astrocytes. BC; Blood capillary. scale bar 50 µm

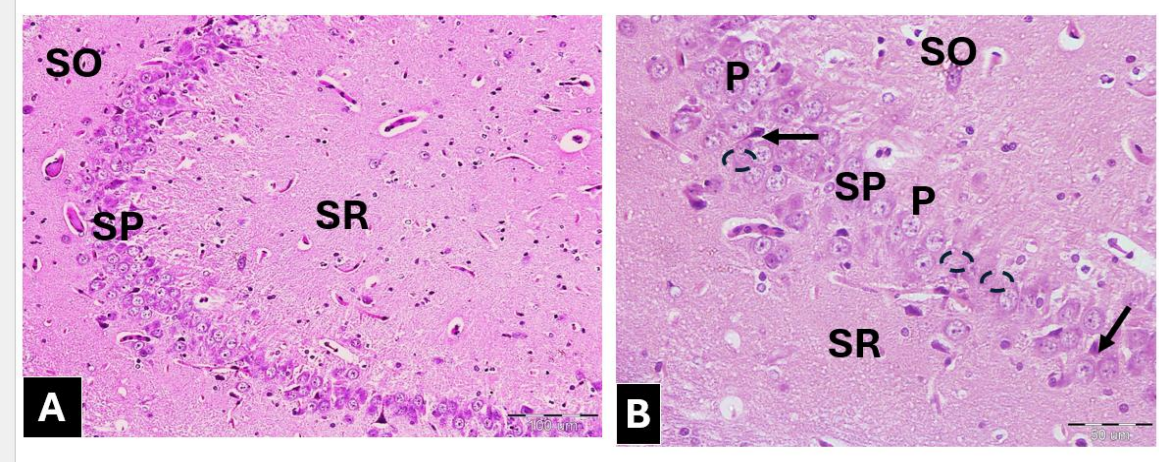


Figure 7: Light micrographs of the **CA3** of the hippocampus of **group III** showing A, B; stratum oriens (SO), stratum pyramidale (SP), and stratum radiatum (SR) of hippocampus proper. SP reveals many pyramidal neurons (P) with vesicular nuclei, prominent nucleoli, and pale basophilic cytoplasm, few with deeply stained nuclei and shrunken cell bodies (arrows). Focal areas of neuronal loss (dotted circle) are noted. H&E stain, scale bar A; 100 µm, B; 50 µm.

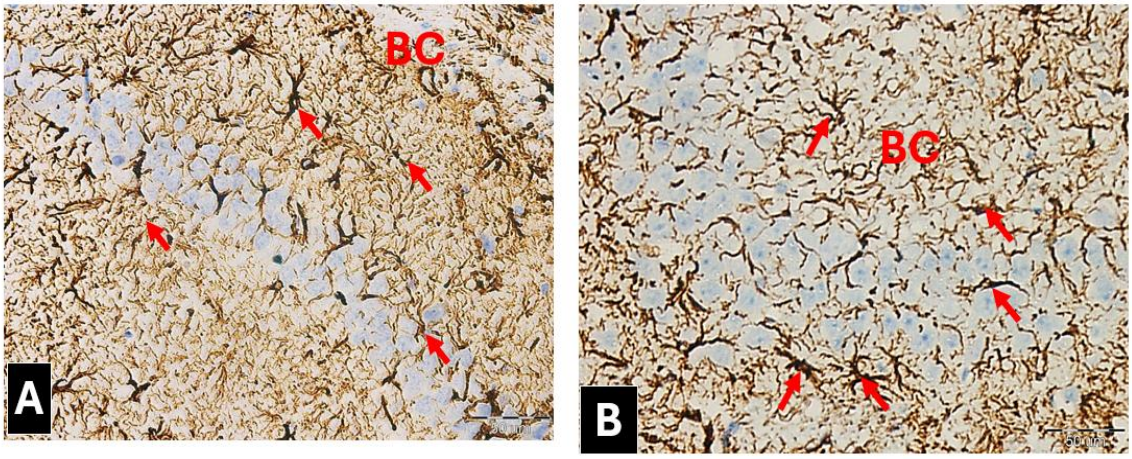
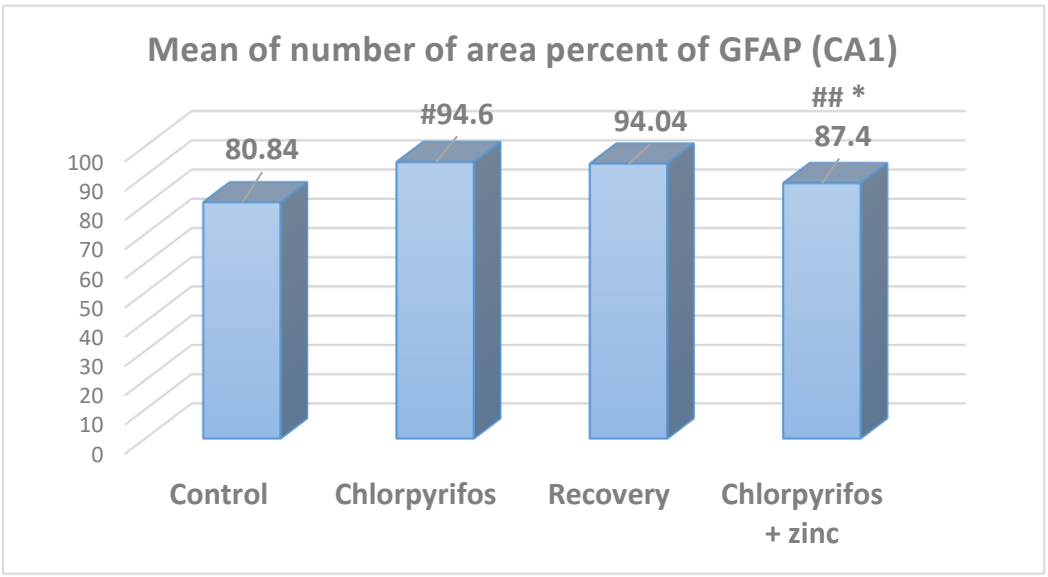


Figure 8: Photomicrographs of GFAP immune-stained sections of rat hippocampus of **group III**. A (CA1) and B (CA3): showing an apparent decrease in the GFAP immuno-reactive astrocytes (arrow). BC; Blood capillary. scale bar 50 µm.



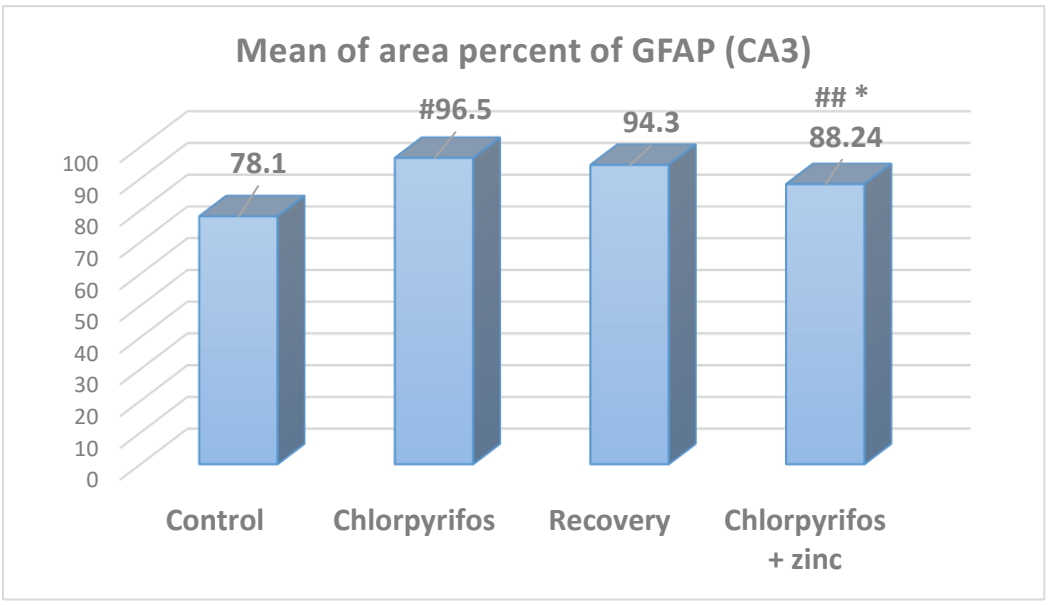
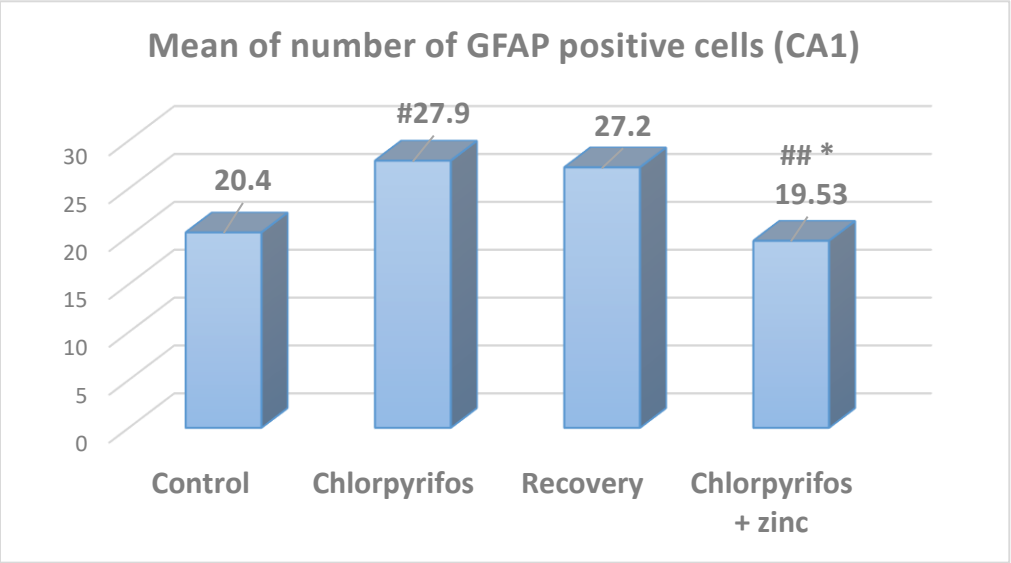


Figure 9: A bar chart demonstrating a comparative morphometric statistical analysis of the groups studied according to the area percentage of GFAP reaction in CA1 & CA3.

Significant when compared to control group

Significant when compared to Chlorpyrifos group.

*Significant when compared to Recovery group IIb



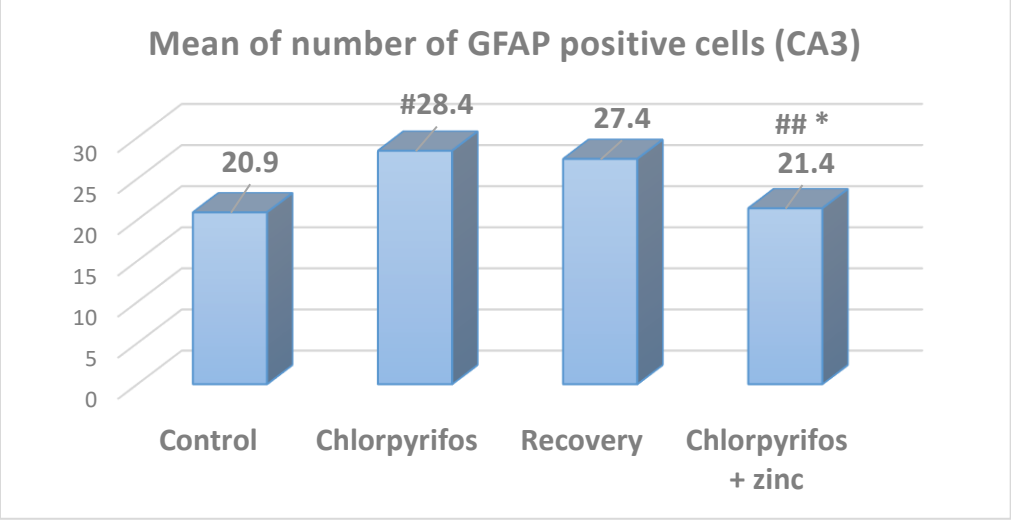


Figure 10: A bar chart demonstrating a comparative morphometric statistical analysis of the groups studied according to the number of GFAP positive cells in CA1 & CA3.

Significant when compared to control group

Significant when compared to Chlorpyrifos group.

*Significant when compared to Recovery group IIb