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Cypermethrin Exacerbates Pentylenetetrazole-induced GABAergic Interneuron Loss and Neuroinflammation in the Rat Amygdala: Neuroprotection by Valproate and Vitamin E Co-therapy

Alli-Oluwafuyi Abdulmusawwir*[1], Abikoye Jesutofunmi Grace[2], Owolabi David Olamilekan[2], Olanrewaju Ridwan[2], Omole Precious[2], Oloyede Priscilla[2], Oladimeji Mufutau Oladele[2], Oyeniyi Joseph Oludare[2], Moyosore S. Ajao[2,3], Imam Aminu[2]

[1]Department of Pharmacology and Therapeutics, [2]Neuroscience Unit, Department of Anatomy, College of Health Sciences, University of Ilorin, Ilorin; [3]Department of Anatomy, College of Health Sciences, Kwara State, Malete, Ilorin 240003, Nigeria.

ORCIDs: AA-O - 0000-0002-1769-1747; MSA - 0000-0002-9074-1405; IA - 0000-0003-2371-3065

***Corresponding Author:** Email: aoluwafuyi.am@unilorin.edu.ng; Tel: +2348060459854

ABSTRACT

The comorbidity of epilepsy with exposure to cypermethrin may exacerbate neuropathology and compromise treatment outcomes. This study investigated the synergistic neurotoxic effects of pentylenetetrazole and cypermethrin on the amygdala and evaluated the neuroprotective potential of valproic acid (VPA) and vitamin E (Vit E). In the first phase, male Wistar rats were divided into four groups (n=9): control (corn oil), cypermethrin (4.4 mg/kg, oral), pentylenetetrazole (35 mg/kg, i.p.), and cypermethrin + pentylenetetrazole. In the second phase, four groups (n=9) received cypermethrin + pentylenetetrazole and were co-treated with VPA (100 mg/kg), Vit E (100 mg/kg), or VPA+Vit E. Amygdala tissues were analyzed for cyclooxygenase-2 (COX-2) levels, neuronal morphology (H&E), GABAergic interneuron density (parvalbumin, PV), microglial activation (Iba-1), and astrogliosis (GFAP) using immunohistochemistry and biochemical assays. Combined cypermethrin + pentylenetetrazole exposure increased brain COX-2 levels and significantly reduced the density of PV+ GABAergic interneurons and healthy neurons (H&E) in the amygdala, accompanied by a trend towards increased gliosis (GFAP, Iba-1). In the intervention phase, VPA treatment alone significantly reduced body weight. While Vit E alone modestly reduced COX-2, the combination of VPA and Vit E most effectively restored PV+ and H&E-stained cell counts and attenuated GFAP expression, suggesting enhanced neuroprotection and reduced astrogliosis. The VPA+Vit E combination also mitigated the body weight loss associated with VPA monotherapy. Cypermethrin exposure exacerbates pentylenetetrazole-induced GABAergic interneuron loss, microgliosis, and astroglyosis in the amygdala. Combined antiepileptic drug and antioxidant supplement therapy offers superior neuroprotection compared to monotherapy by ameliorating neuroinflammation, restoring inhibitory neuronal populations, and suppressing gliosis.

Keywords: epilepsy, neuroinflammation, amygdala, valproic acid, Vitamin E

INTRODUCTION

Epilepsy is a chronic neurological disorder affecting over 50 million people worldwide, characterized by recurrent, unprovoked seizures[1]. While its etiology is multifactorial, encompassing genetic, structural, and metabolic causes, emerging evidence highlights the significant role of environmental factors in modulating disease onset, progression, and treatment resistance[2,3]. Among these, exposure to neurotoxic pesticides is a growing concern, particularly in agricultural communities where the burden of both epilepsy and pesticide exposure can be high[4].

Cypermethrin, a widely used type II pyrethroid insecticide, is a known neurotoxicant. Its primary mechanism involves prolonging sodium channel opening, leading to neuronal membrane depolarization, hyperexcitability, and repetitive neuronal firing[5]. Beyond this, cypermethrin induces oxidative stress and neuroinflammation, contributing to neuronal damage[6,7]. The amygdala, a key limbic structure involved in emotional regulation and seizure genesis and propagation, particularly in temporal lobe epilepsy, is highly susceptible to such insults[8]. Concurrent exposure to a proconvulsant agent like pentylenetetrazole, a GABA-A receptor antagonist commonly used to model epilepsy, and a

neurotoxicant like cypermethrin may create a "double-hit" phenomenon, where neuroinflammation and excitotoxicity are synergistically amplified, leading to exacerbated pathology[9]. This comorbidity model is clinically relevant, as individuals with epilepsy may have ongoing or past exposure to such environmental pollutants, potentially influencing their disease course and response to therapy.

The neuropathological hallmarks of epilepsy and excitotoxic damage include neuroinflammation, gliosis, and the loss of specific neuronal subpopulations[10]. Cyclooxygenase-2 (COX-2), a key enzyme in the inflammatory cascade, is rapidly upregulated in the brain following seizures and contributes to the production of pro-inflammatory prostaglandins, thereby perpetuating neuronal injury[11]. Furthermore, the loss of GABAergic interneurons, particularly the fast-spiking parvalbumin (PV)-positive subtype, is a critical event that disrupts the inhibitory-excitatory balance within limbic circuits, lowering the seizure threshold and promoting hyperexcitability[12]. This neuronal injury is often accompanied by reactive gliosis, marked by the activation of microglia (Iba-1-positive) and astrocytes (GFAP-positive), which, while initially neuroprotective, can become chronically detrimental and contribute to pharmacoresistance[13].

Valproic acid (VPA) is a first-generation, broad-spectrum antiepileptic drug with a complex mechanism of action, including GABA potentiation, sodium channel blockade, and histone deacetylase (HDAC) inhibition[14]. Vitamin E (α-tocopherol) is a potent lipophilic antioxidant that protects cell membranes from lipid peroxidation and has demonstrated anti-inflammatory properties[15]. Given the role of oxidative stress and inflammation in seizure-induced pathology, adjunctive antioxidant therapy has been proposed as a neuroprotective strategy. While VPA is effective, its use is associated with side effects, and its neuroprotective efficacy in the context of combined seizure and toxicant exposure is not fully understood. The potential synergistic effect of combining VPA with an antioxidant like vitamin E to enhance neuroprotection and mitigate toxicity remains an important area of investigation.

We hypothesized that exposure to cypermethrin would exacerbate pentylenetetrazole-induced neuroinflammation and neurodegeneration in the rat amygdala. Furthermore, we hypothesized that co-treatment with VPA and vitamin E would provide superior neuroprotection against this combined insult compared to either agent alone, by synergistically targeting both excitotoxic/inflammatory and oxidative stress pathways. This study was designed to first establish the pathological impact of combined exposure to cypermethrin and pentylenetetrazole on the amygdala, and second to evaluate the therapeutic efficacy of VPA and vitamin E, both individually and in combination, in ameliorating these deficits.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were conducted at the Animal House, College of Health Sciences, University of Ilorin, in strict accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC). The study protocol was approved by the University of Ilorin Ethical Review Committee (UERC) (approval number: UIL/UERC/21/68LD001, September 2024).

Animals

Seventy-two adult male Wistar rats (weighing 100-120 g at the start) were obtained from the University of Ilorin Central Research Laboratory. Animals were housed in wire-gauzed plastic cages with sawdust bedding under standard laboratory conditions (natural light/dark cycle). They were provided with standard laboratory chow and water *ad libitum* and were acclimatized for one week prior to the commencement of experiments.

Chemicals and drugs

Pentylenetetrazole (pentylenetetrazole), cypermethrin (cypermethrin), Valproic acid (VPA), and Vitamin E (Vit E; α-tocopherol) were used. Pentylenetetrazole was dissolved in normal saline for intraperitoneal (i.p.) injection. Cypermethrin, VPA, and Vit E were dissolved in corn oil for oral administration via gavage. The doses were selected based on previous studies: pentylenetetrazole (35 mg/kg)[16], cypermethrin (4.4 mg/kg, 1/10th of the LD50)[17], VPA (100 mg/kg), and Vit E (100 mg/kg)[9].

Experimental design

The study was conducted in two phases.

Phase 1: Characterization of the cypermethrin + pentylenetetrazole Comorbidity Model

Thirty-six rats were randomly assigned to four groups (n=9): Group 1 (Control): Received 1 ml/kg/day of corn oil (oral); Group 2 (cypermethrin): Received 4.4 mg/kg/day of cypermethrin (oral); Group 3 (pentylenetetrazole): Received 35 mg/kg of pentylenetetrazole (i.p.) every 48 hours; Group 4 (cypermethrin + pentylenetetrazole): Received cypermethrin (4.4 mg/kg/day, oral) and pentylenetetrazole (35 mg/kg, i.p. every 48 hours).

Phase 2: Evaluation of Neuroprotective Interventions

Based on the results from Phase 1, the cypermethrin + pentylenetetrazole co-exposure model was used to test the interventions. Thirty-six rats were randomly assigned to four groups (n=9): Group 1 (Control/cypermethrin + pentylenetetrazole): Received cypermethrin + pentylenetetrazole as in Phase 1; Group 2 (VPA + cypermethrin + pentylenetetrazole): Received VPA (100 mg/kg/day, oral) 30 minutes before cypermethrin + pentylenetetrazole administration; Group 3 (VitE + cypermethrin + pentylenetetrazole): Received Vit E (100 mg/kg/day, oral) 30 minutes before cypermethrin + pentylenetetrazole administration; Group 4 (VPA + VitE + cypermethrin + pentylenetetrazole): Received

both VPA and Vit E (100 mg/kg/day each, oral) 30 minutes before cypermethrin + pentylenetetrazole administration.

In both phases, oral treatments (corn oil, cypermethrin, VPA, Vit E) were administered once daily for 10 consecutive days. Pentylenetetrazole injections were given on days 1, 3, 5, 7, and 9. Body weight, food, and water intake were monitored throughout the study.

Animal sacrifice and tissue collection

Twenty-four hours after the last treatment (day 11), all rats were euthanized with an intramuscular injection of ketamine (20 mg/kg). For each group, three rats were transcardially perfused with normal saline followed by 10% buffered formalin. Their brains were excised and post-fixed in 10% buffered formalin for histological and immunohistochemical analyses. The brains from the remaining six rats were rapidly removed, and the amygdala was dissected on ice, weighed, and stored in cold sucrose solution for biochemical analyses.

Tissue processing and histology

Formalin-fixed brains were processed through graded alcohols (50%-100%) for dehydration, cleared in xylene, and embedded in paraffin. Mid-coronal sections (5.8 µm thick) containing the amygdala were cut using a rotary microtome. Sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E) to assess general neuronal morphology and cellular density.

Immunohistochemistry

For IHC, 8-µm-thick paraffin-embedded amygdala sections were used. After deparaffinization, rehydration, and heat-mediated antigen retrieval (citrate buffer, pH 6.0), endogenous peroxidase activity was blocked with 0.3% H_2O_2. Sections were then incubated with 2.5% normal horse serum to block non-specific protein binding. Primary antibody incubation was performed for 2 hours at room temperature using the following antibodies: goat polyclonal anti-Iba-1 (1:250, Abcam, USA, ab5076) for microglia, mouse monoclonal anti-Parvalbumin (1:150, Santa Cruz, USA, sc-33673) for GABAergic interneurons, and rabbit polyclonal anti-GFAP (1:1000, Novus Biologicals, USA, NB120-11427) for astrocytes. Following PBS washes, sections were incubated with the appropriate ImmPRESS™ HRP Polymer Reagents (Vector Labs, USA) for 30 minutes. Immunoreactivity was visualized using 3,3-diaminobenzidine (DAB) Peroxidase Substrate Kit (Vector Labs, USA). Sections were counterstained with hematoxylin, dehydrated, cleared in xylene, and mounted with Permount.

COX-2 analysis

Brain tissue from the amygdala region was homogenized, and COX-2 levels were quantified using a standard enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's protocol. Results were expressed as concentration per gram of protein.

Microscopy, image acquisition, and quantification

Stained sections were examined under an AmScope compound microscope. For each stain and each brain, 9 sections (3 sections per brain, 3 brains per group) were analyzed. Approximately 27 images per group per stain were captured at 400x magnification. Photomicrographs were taken using an AmScope 5.0 MP digital camera. Cell counting and densitometric analysis of staining intensity were performed manually by a blinded observer using ImageJ software (NIH, USA). A grid was overlaid on each image, and positively stained cells were counted within a defined area.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism (Version 8.4.2). Comparisons among multiple groups were made using a one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant.

RESULTS

No significant differences were observed in body weight gain, daily food intake, or daily water intake among the groups ($p > 0.05$; Fig. 1A-C). While not statistically significant, a clear trend was observed in brain COX-2 levels (Fig. 1D). The cypermethrin + pentylenetetrazole co-exposure group showed an increase in COX-2 concentration compared with the control, pentylenetetrazole-alone, and cypermethrin-alone groups, suggesting a trend toward an enhanced inflammatory response in the comorbidity model.

GABAergic interneuron density and neuronal morphology in the amygdala of cypermethrin &/or pentylenetetrazole-exposed Rats

Quantification of PV-positive interneurons in the amygdala revealed a significant main effect of treatment ($p < 0.05$). Post-hoc analysis confirmed a significant reduction in PV-positive cell density in the cypermethrin + pentylenetetrazole group compared to the control group ($p < 0.05$), indicating a loss of inhibitory neurons (Fig. 2). A similar pattern was observed with H&E staining, where the density of healthy-appearing neurons was significantly reduced in the cypermethrin group ($p < 0.05$ vs. control) and showed a non-significant decreasing trend in the pentylenetetrazole and cypermethrin + pentylenetetrazole groups (Fig. 2).

Glial activation in the amygdala following exposure to cypermethrin and pentylenetetrazole

Exposure to pentylenetetrazole, cypermethrin, and their combination led to noticeable trends in glial activation (Fig. 3). The cypermethrin + pentylenetetrazole group exhibited the highest numbers of GFAP-positive astrocytes and Iba-1-positive microglia, suggesting reactive astrogliosis

and microgliosis. However, these changes did not reach statistical significance ($p > 0.05$).

Metabolic markers and COX-2 expression in the brains of VPA &/or Vit. E treated co-morbid rats.

In the intervention phase, treatment with VPA alone (VPA + cypermethrin + pentylenetetrazole) resulted in a significant loss of body weight compared to the cypermethrin + pentylenetetrazole control group ($p = 0.0309$, Fig. 4A). This weight loss was not observed in the Vit E or combination therapy groups. No significant differences in food or water intake were observed across the treatment groups ($p > 0.05$, Fig. 4B-C).

Analysis of brain COX-2 levels (Fig. 4D) showed that treatment with VPA alone non-significantly increased COX-2. In contrast, Vit E treatment reduced COX-2 levels. Notably, the VPA+Vit E combination group had a significantly lower COX-2 level compared to the VPA monotherapy group ($p = 0.0278$), indicating that Vit E effectively counteracted the pro-inflammatory COX-2 trend associated with VPA.

Integrity of GABAergic interneurons and neuronal morphology in the amygdala of VPA &/or Vit. E treated co-morbid rats.

Quantification of PV-positive interneurons (Fig. 5) revealed that treatment with Vit E alone resulted in a non-significant increase in cell density compared to the cypermethrin + pentylenetetrazole control group. The combination therapy (VPA+VitE+cypermethrin + pentylenetetrazole) showed the most pronounced increase in PV-positive cell counts, trending towards restoration of this inhibitory neuronal population, although this did not reach statistical significance. A similar pattern was observed for H&E-positive cell counts, with the combination therapy group exhibiting the highest density of healthy neurons, suggesting improved neuronal preservation compared with controls and monotherapy groups (Fig. 5).

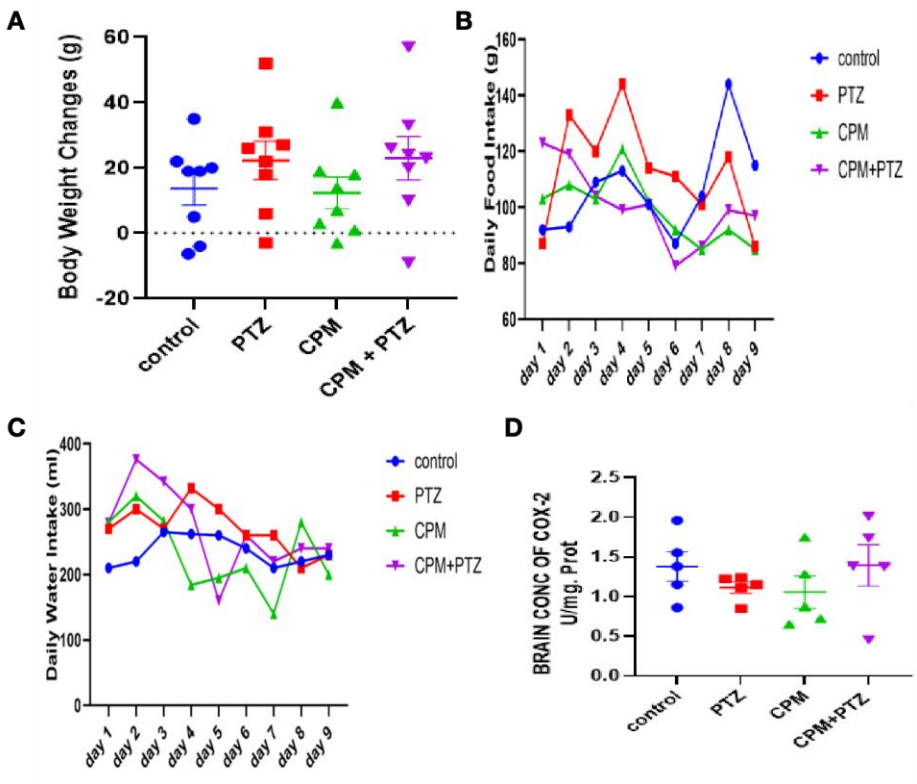

Figure 1: Graphical representation of Body weight (A), Food intake (B), Water intake (C), and COX 2 concentrations (D) in the brain of rats exposed to pentylenetetrazole, cypermethrin, and pentylenetetrazole + cypermethrin—one-way ANOVA. No significant differences were observed in body weight gain, daily food intake, or daily water intake among the groups ($p > 0.05$; Fig. 1A-C).

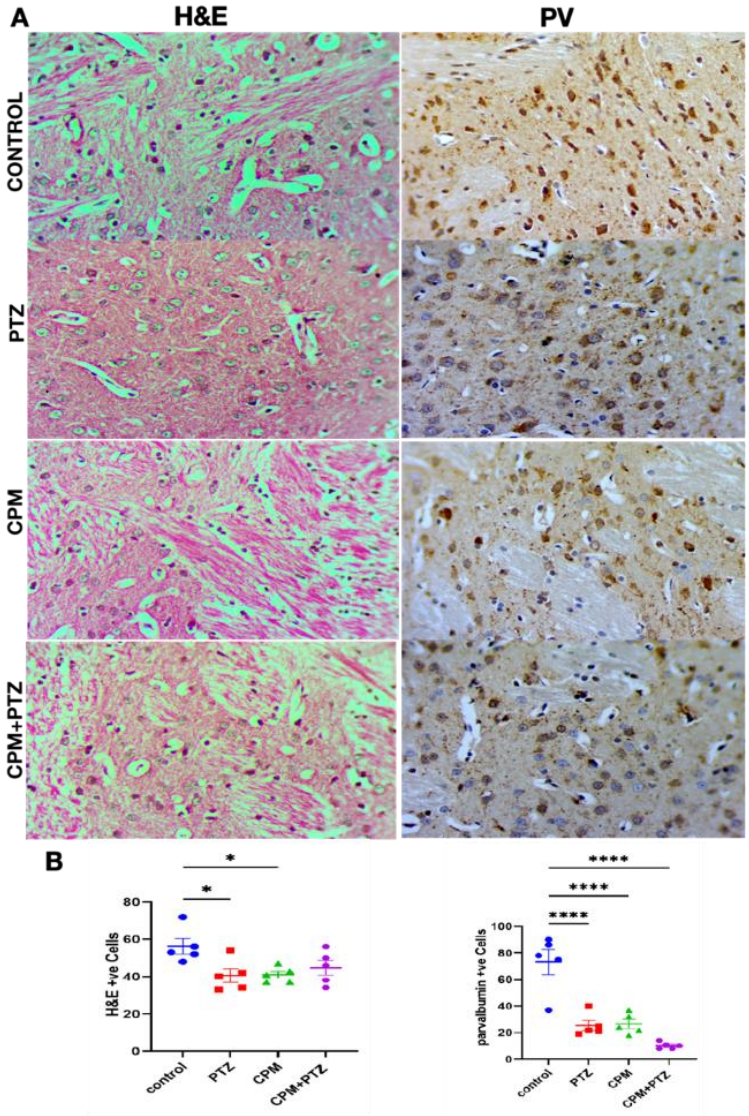

Figure 2: Representative Photomicrographs of H&E and PV staining (A), and the corresponding quantifications (B), in the amygdala of rats exposed to pentylenetetrazole, cypermethrin, and pentylenetetrazole+cypermethrin. one-way ANOVA. Asterisk (*) indicates a significant difference. H&E, anti-PV immunohistochemistry, x400. Based on H&E PV analysis, PTZ and CPM treatment significantly reduced the number of intact cells compared to controls.

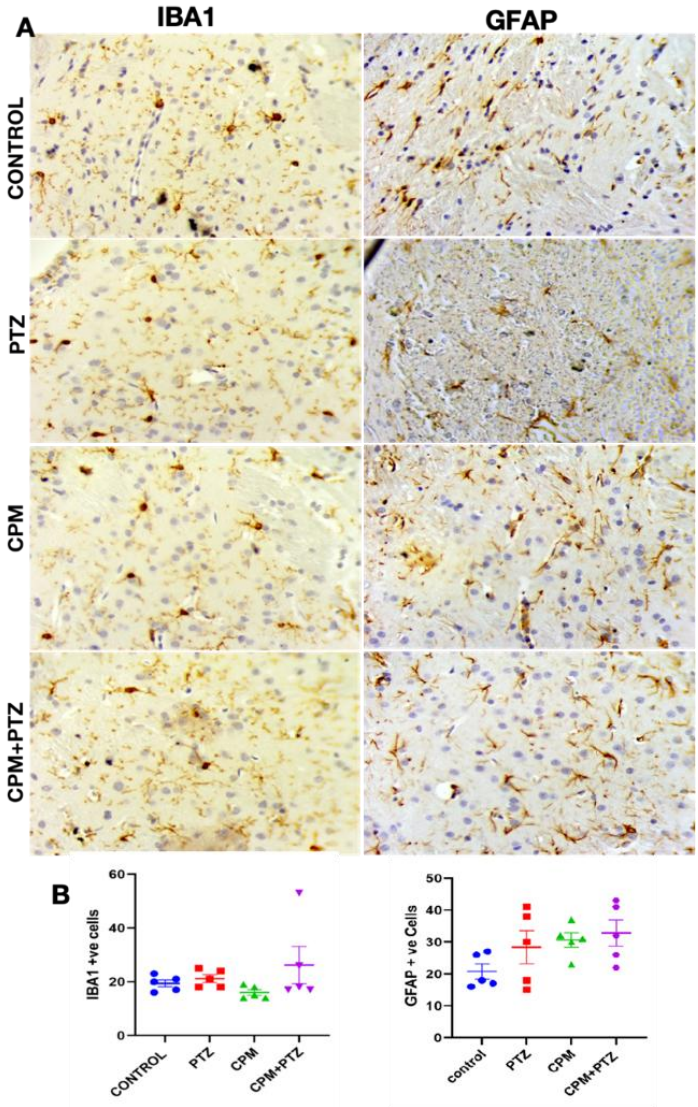

Figure 3: Representative Photomicrographs of GFAP and IBA1 staining (A), and the corresponding quantifications (B), in the amygdala of rats exposed to pentylenetetrazole, cypermethrin, and pentylenetetrazole+cypermethrin. one-way ANOVA. Asterisk (*) indicates a significant difference. Anti-GFAP, anti-IBA1 immunohistochemistry, x400. There was a trend showing increasing glial activation based on GFAP analysis.

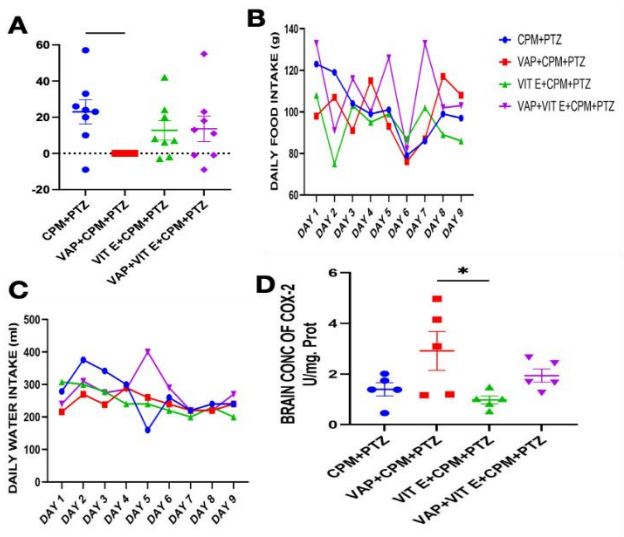

Figure 4: Graphical representation of Body weight (A), Food intake (B), Water intake (C), and COX 2 concentrations (D) in the brain of rats treated with Valproic acid and Vitamin E and further exposed to cypermethrin + pentylenetetrazole using one-way ANOVA. Asterisk (*) indicates a significant difference. VPA induced a significant loss in body weight in cypermethrin + pentylenetetrazole-treated animals, which was not associated with food or water intake.

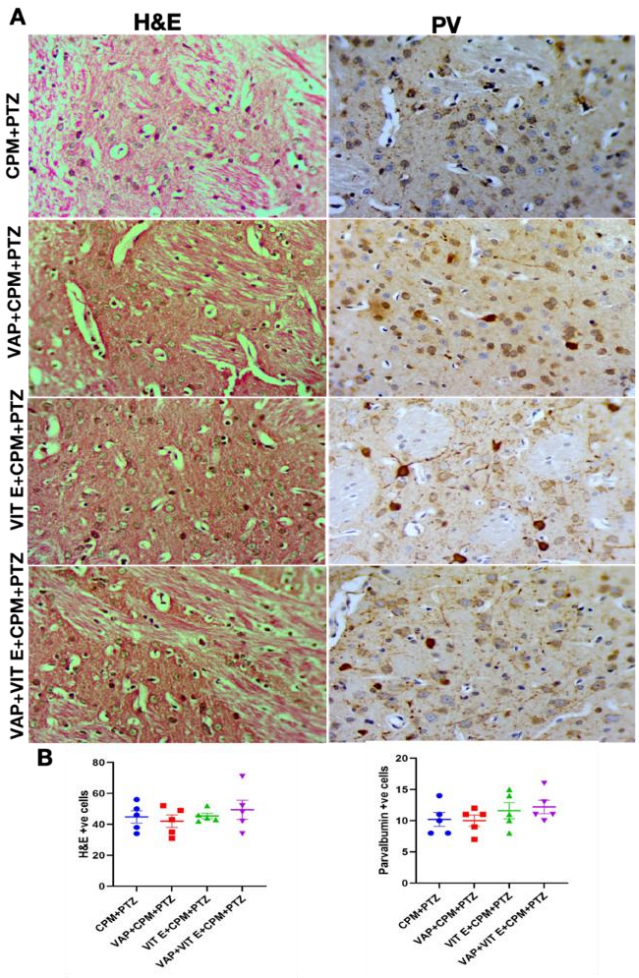

Figure 5. Representative Photomicrographs of H&E and PV staining (A), and the corresponding quantifications (B), in the amygdala of rats treated with Valproic acid and Vitamin E and further exposed to cypermethrin + pentylenetetrazole. one-way ANOVA. Asterisk (*) indicates a significant difference. H&E, anti-PV immunohistochemistry, x400. No significant effect of either VPA or Vit E treatment on cell density in cypermethrin + pentylenetetrazole rats.

Glia integrity in the amygdala of VPA &/or Vit. E treated co-morbid rats.

Analysis of glial markers (Fig. 6) showed that VPA alone treatment appeared to reduce GFAP-positive astrocyte density compared with the cypermethrin + pentylenetetrazole control. The most striking effect was observed in the combination therapy group (VPA+VitE+cypermethrin + pentylenetetrazole), which showed a marked, though non-significant, decrease in GFAP-positive astrocytes compared to the control and Vit E monotherapy groups, suggesting a synergistic suppression of astrogliosis. No clear pattern was observed for Iba-1-positive microglia across the treatment groups in this phase.

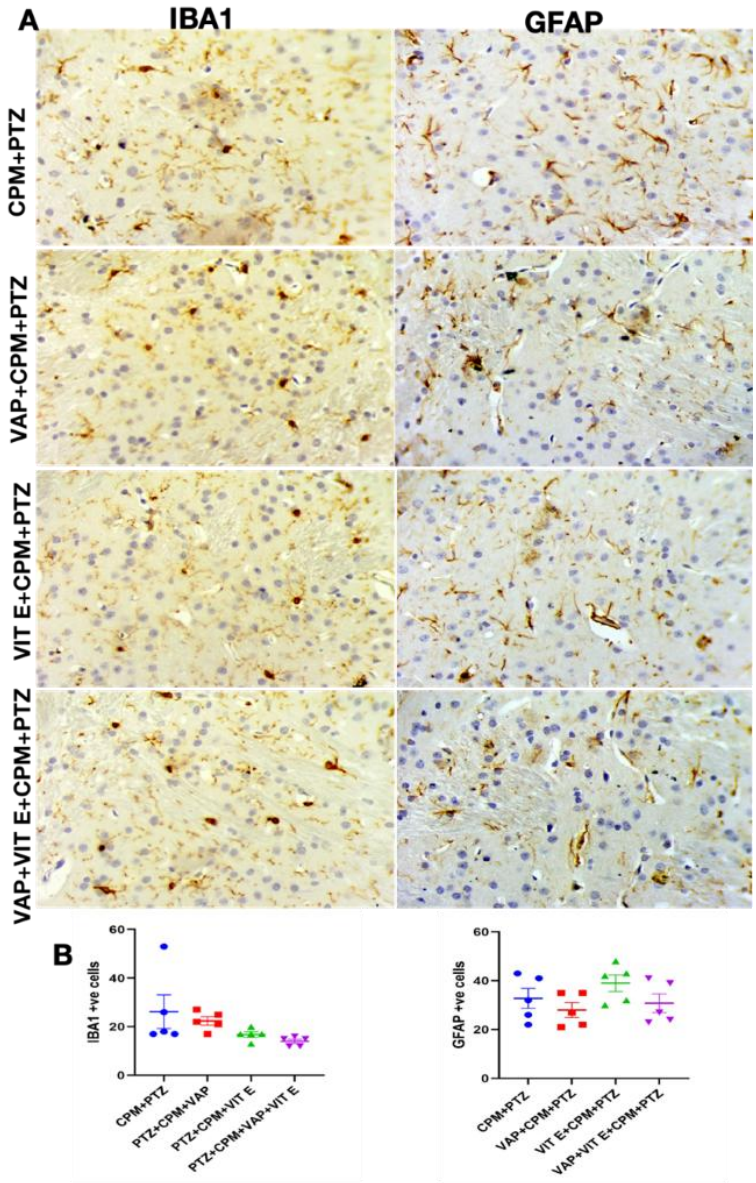

Figure 6. Representative Photomicrographs of H&E and PV staining (A), and the corresponding quantifications (B), in the amygdala of rats treated with Valproic acid and Vitamin E and further exposed to cypermethrin + pentylenetetrazole. one-way ANOVA. Asterisk (*) indicates a significant difference. H&E, anti-PV immunohistochemistry, x400. No significant effect of either VPA or Vit E treatment on glial markers in cypermethrin + pentylenetetrazole rats.

DISCUSSION

This study provides critical insights into the neuropathological consequences of combined seizure activity and pesticide exposure, and it highlights the potential of a combinatorial neuroprotective strategy. The principal findings are threefold. First, co-exposure to pentylenetetrazole and the pyrethroid insecticide cypermethrin exacerbates neurotoxicity in the rat amygdala, characterized by a trend towards increased inflammation (COX-2), a significant loss of PV-positive GABAergic interneurons, and a trend towards increased gliosis. Second, while valproate (VPA) monotherapy was associated with significant body weight loss, it showed limited efficacy in reversing the core neuropathologies. Third, the combination of VPA with vitamin E (Vit E) demonstrated superior neuroprotective effects, including a significant reduction in COX-2 compared to VPA alone, a trend towards restoring GABAergic interneurons and neuronal health, and a synergistic suppression of astrogliosis, all while mitigating the body weight loss associated with VPA.

The initial phase of our study successfully established a model of exacerbated neuropathology. The non-significant increase in COX-2 in the cypermethrin + pentylenetetrazole group suggests that the combination may initiate a more pronounced inflammatory cascade than either insult alone. COX-2 is a key mediator of neuroinflammation, and its upregulation is a well-documented consequence of prolonged seizures, contributing to neuronal injury and blood-brain barrier dysfunction[18,19]. The significant loss of PV-positive interneurons in the amygdala of cypermethrin + pentylenetetrazole-exposed rats is a pivotal finding. PV interneurons are the primary source of fast perisomatic inhibition in cortical and limbic circuits, and their loss is a hallmark of epileptogenesis, leading to network hyperexcitability[12,20]. The vulnerability of these neurons to excitotoxicity and oxidative stress is well-established[7], and our data suggest that cypermethrin exposure potentiates this damage. The concurrent trend towards gliosis (increased GFAP and Iba-1) indicates a state of reactive gliosis, which, while initially an attempt at repair, can chronically release pro-inflammatory factors and further destabilize neuronal networks[21,22]. This "double-hit" of excitotoxicity (from pentylenetetrazole) and oxidative/inflammatory stress (from cypermethrin) likely overwhelmed endogenous protective mechanisms, leading to the observed pathology.

The second phase of the study evaluated clinically relevant interventions. The significant body weight loss observed with VPA monotherapy is a recognized side effect in both clinical and preclinical settings, potentially related to metabolic and endocrine effects.[9,23]. Importantly, this effect was absent in the Vit E and combination groups, suggesting Vit E may have a protective effect against this adverse reaction.

The therapeutic mechanisms of the interventions are distinct but complementary. VPA's primary antiepileptic action involves enhancing GABAergic transmission and blocking voltage-gated sodium channels[14]. Its HDAC-inhibitory activity may also promote neuroprotective gene expression.[14] However, VPA can also induce oxidative stress in some contexts[24]. In our study, VPA alone failed to restore PV interneurons and did not significantly increase COX-2, suggesting that its anti-inflammatory effects may be insufficient to counter the severe oxidative and inflammatory insult in this comorbidity model. In contrast, vitamin E is a chain-breaking antioxidant that protects lipid membranes from peroxidation, a key mechanism of cell death in excitotoxicity[9,25,26]. It also has direct anti-inflammatory properties, which may explain its modest ability to lower COX-2 levels and its trend towards increasing PV cell counts when used alone[9].

The most compelling finding is the enhanced neuroprotection conferred by the VPA+Vit E combination. The significant reduction in COX-2 levels in the combination group compared to VPA alone indicates a synergistic anti-inflammatory effect. By neutralizing oxidative stress that can drive COX-2 expression and its downstream effects, Vit E may have unmasked or complemented the neuroprotective potential of VPA[27]. This combined anti-inflammatory and antioxidant action likely created a more favorable environment for neuronal survival, explaining the trend towards restoration of PV and H&E-positive cells. Furthermore, the pronounced suppression of GFAP-positive astrocytes in the combination group is clinically significant. Reactive astrogliosis contributes to the formation of glial scars, which can impede neuronal repair and have been linked to pharmacoresistance in epilepsy[28]. Attenuating this response could improve the long-term prognosis and drug responsiveness.

The clinical importance of these findings is substantial. They suggest that individuals with epilepsy who have significant exposure to neurotoxicants like pyrethroids may present with a more severe neuropathology that is less responsive to standard antiepileptic monotherapy. In such cases, an adjunctive therapy with a safe antioxidant, such as vitamin E, could offer multiple benefits: enhancing seizure control by protecting inhibitory neurons, reducing neuroinflammation that may contribute to disease progression, and potentially mitigating some of the adverse effects of first-line drugs, such as valproate.

CONCLUSION

Cypermethrin co-exposure worsens pentylenetetrazole-induced amygdala neuropathology, depleting GABAergic interneurons and promoting inflammation/gliosis. Valproate + vitamin E combination therapy outperforms monotherapy, offering superior neuroprotection and potential as an adjunct in epilepsy, pending further mechanistic studies.

Acknowledgement

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Ethical Approval
This research was approved by the University of Ilorin ethical review committee (UERC) (UIL/UERC/21/68LD001) in September 2024, following the recommendation of the College of Health Sciences ethical review committee, in compliance with the Institutional Animal Care and Use Committee (IACUC).

Data availability declaration
The authors declare that the data supporting the findings of this study are available within the paper. Should raw data files be needed in another format, they are available from the corresponding author.

Competing interests
The authors declare that they have no competing interests.

Funding declaration
Not applicable

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