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Research Article

Effect of Ketogenic Diet on Pentylenetetrazole (PTZ) Kindled Rats with Medial Prefrontal Cortex Damage: Behavioral, Morphological and *In Silico* Analysis

***Danladi J.[1,5], Suleiman Y. A.[2,5], Jahun A. S.[3], Dzekashu B. B.[1], Umar N.[4], and Lawal A. A.[6]**

[1]Department of Human Anatomy, Faculty of Basic Medical Sciences, College of Basic Medical Sciences, Ahmadu Bello University, Zaria, Nigeria
[2]Department of Human Anatomy, Abubakar Tafawa Balewa University, Bauchi, Nigeria
[3]Department of Human Anatomy, Faculty of Basic Medical Sciences, College of Medical and Allied Sciences, Federal University, Dutse, Jigawa, Nigeria
[4]Department of Human Physiology, Faculty of Basic Medical Sciences, College of Basic Medical Sciences, Ahmadu Bello University, Zaria, Nigeria
[5]Molecular Genetics and Infectious Disease Laboratory, Abubakar Tafawa Balewa University, Bauchi, Nigeria
[6]Department of Human Anatomy, Federal University, Lafia, Nasarawa, Nigeria
****Corresponding Author's email*: jibrin.danladi00@gmail.com**

ABSTRACT
Ketogenic diet (KD), has gained attention as an adjunct therapy for refractory epilepsy due to its anticonvulsant and neuroprotective effects. This study investigated the neuroprotective potential of different KD formulations in a pentylenetetrazol kindling (PTZK (35 mg/kg) every other day for 20 days. To promote kindling, PTZ was increased by 5 mg/kg in subsequent intraperitoneal administrations after the fourth injection. Rats were fed various diets 10 days before Kindling and during the Kindling. Thirty (30) young adults female Wistar rats were divided into six groups (n=5 per group). Group 1, Control (normal feed); Group 2, PTZK (35 mg/kg); Group 3, PTZK + levetiracetam (10 mg/kg); Group 4, PTZK + palm-kernel oil-KD; Group 5, PTZK + castor oil-KD; and Group 6, PTZK + olive-KD. Seizure severity was assessed using Racine's scoring, cognitive function was evaluated using the novel object-recognition test. Number of neurons in the supralimbic medial prefrontal cortex (smPFC) layer 2/3 was estimated using unbiased stereological methods, including histology and physical dissector technique. DNA-methylation was assessed using Feulgen reaction and molecular docking analysis evaluated the binding-affinities of ketone-bodies and levetiracetam. Results demonstrates that Ketone-bodies' strong bond to HCR2 and NMDA receptor may protect against neurodegeneration as KD-treated groups showed reduced seizure severity, delayed seizure onset, and improved behavioral outcomes compared to the PTZK group. Number of neurons and DNA integrity were preserved in KD groups almost similar to control group compared to marked neurodegeneration in smPFC layer 2/3 of PTZK group. KDs, provide neuroprotection against PTZK-neurodegeneration in smPFC layer 2/3.

Keywords: DNA methylation; Epilepsy; Ketogenic diets; mPFC; PTZ-kindling; Stereology

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INTRODUCTION

Epilepsy is a neurological disorder orchestrated by recurrent, unprovoked seizures as a result of abnormal hyperexcitation and hypersynchronicity of neuronal activity within the brain. The mechanisms of seizure are multifactorial, ranging from imbalances between excitatory and inhibitory neurotransmission, altered ion channel function, and structural and functional changes in neural networks (Fisher *et al.,* 2014; Löscher, 2011). Understanding the pathways associated with epilepsy is key for developing successful therapeutic options and improving patient outcomes. One of the fundamental mechanisms in epilepsy is the interference in the balance of excitatory and inhibitory neurotransmission mediated by excessive glutamatergic activity and reduced GABAergic inhibition, which facilitate seizure generation and propagation (Treiman, 2001; Stafstrom, 2007). Excessive glutamate and reduced GABA in epilepsy may be associated with altered receptor expression, blockade of neurotransmitter release and synaptic dysfunction. Ion channel dysfunction also plays a critical role in epileptogenesis. Voltage-gated sodium (Na^+), potassium (K^+), and calcium (Ca^{2+}) channels regulate neuronal excitability and action potential generation. Mutations or functional modifications in any of the ion channels might result in aberrant neuronal firing, hence facilitating seizure activity. More activity in sodium channels can make neurons depolarize more, while less activity in potassium channels can make repolarization harder; both of these effects make neurons more likely to stay excited (Catterall, 2014; Noebels, 2015). The ion channel pathologies have been reported in both genetic and acquired forms of epilepsy. In addition to the imbalance between excitatory and inhibitory neurotransmission, ion channels and alterations in the brain's structure and synapses significantly contribute to neuronal loss, gliosis, synaptic reorganizations, and abnormal axon sprouting, which can result in hyperexcitable neural circuits (Pitkänen and Engel, 2014). Neuroinflammation and oxidative stress are acknowledged as significant factors in epileptogenesis. The activation of microglia and astrocytes triggers the release of pro-inflammatory cytokines, which can modify neuronal excitability and compromise blood–brain barrier integrity (Vezzani *et al.,* 2011). Simultaneously, the excessive generation of reactive oxygen species during seizures promotes oxidative damage to lipids, proteins, and DNA, furthering neuronal injury and dysfunction that perpetuates and amplifies seizure activity leading to neurodegeneration (Patel, 2004). Understanding these pathways is key for identifying novel therapeutic targets and the management of epilepsy using animal models.

Among the models of epilepsy, chemical method involving the use of the PTZK mimics progressive development of seizures and long-term neurobiological changes similar to human epilepsy (Samokhina and Samokhin, 2018; Singh *et al.,* 2021). It involved a repeated administration of subconvulsive doses of PTZ, leading to a gradual escalation in seizure severity, a phenomenon known as 'kindling', which reflects enduring alterations in neuronal circuitry and cortical excitability (Löscher, 2011; Samokhina and Samokhin, 2018). This model is particularly valuable for investigating structural and functional impairments in specific brain regions, including smPFC, a region critically involved in executive function, decision-making, and emotional regulation. Damage to the mPFC in PTZ-kindled animals has been associated with cognitive deficits and behavioural abnormalities, further emphasizing its relevance in epilepsy research (Barkmeier and Loeb, 2009).

Recent research work has focused on interventions that are solely based on non-pharmacological interventions as alternative strategies for epilepsy management. One such approach is the use of the ketogenic diet (KD), which comprises a high-fat, low-carbohydrate, and adequate-protein formulation and has gained substantial attention for its anticonvulsant and neuroprotective effects. KD was initially developed for the treatment of drug-resistant epilepsy and has demonstrated a significant reduction in seizure frequency and severity in both clinical and preclinical contexts; however, its exact mechanisms of action remain inadequately elucidated (Neal *et al.,* 2008; Rho & Stafstrom, 2012; He *et al.,* 2024; Zhang *et al.,* 2024). In PTZ-induced kindled rat models, KD has demonstrated robust antiepileptic potential, which is characterized by increases in both electrographic and behavioural seizure thresholds, delays the onset of seizures, and reduces their duration and severity (Simeone *et al.,* 2017). These results indicate the ability of KD to influence neuronal excitability and synaptic function, thereby enhancing its protective effects against the onset and progression of seizures. Beyond seizure suppression, emerging evidence suggests that the KD exerts significant beneficial effects on cognitive and behavioural outcomes associated with epilepsy-induced brain damage, including improvements in

memory, learning and overall cognitive function in affected individuals. At the morphological level, the KD plays a significant role in preserving neuronal integrity and enhancing synaptic plasticity in epileptic conditions, suggesting that KD may modulate key neurotransmitter systems and ion transport mechanisms, exploring the regulation of cation–chloride co-transporters such as potassium–chloride co-transporter 2 (KCC2) and sodium–potassium–chloride co-transporter 1 (NKCC1). The upregulation of KCC2 alongside the downregulation or inhibition of NKCC1 promotes a shift toward enhanced inhibitory γ-aminobutyric acid (GABA)-mediated neurotransmission, thereby reducing neuronal hyperexcitability and seizure susceptibility (Kim *et al.,* 2015; Kahle *et al.,* 2016). Furthermore, KD has been shown to promote the expression of synaptic proteins and intracellular signaling pathways critical for neuronal communication and plasticity. Notably, alterations in glutamate receptor subunits, such as GluR1 (AMPA receptor) and NR2B (NMDA receptor), as well as modulations of mitogen-activated protein kinase (MAPK) signaling pathways, have been reported in epilepsy models (Simeone *et al.,* 2017). These molecular adaptations contribute to improved synaptic stability, reduced excitotoxicity, and enhanced neuroprotection. Collectively, highlighting the capacity of KD to mitigate structural and functional neuronal damage, particularly within vulnerable brain regions such as the medial prefrontal cortex, in PTZ-kindled models.

From an *in-silico* perspective, computational modelling and molecular pathway analyses further illuminate the mechanistic basis of the KD in epilepsy. These approaches have identified several key pathways through which KD exerts its neuroprotective effects, including metabolic reprogramming, enhancement of mitochondrial function, reduction of oxidative stress, and modulation of neurotransmitter systems. Specifically, bioinformatics and network-based analyses reveal that KD promotes a shift from glucose-dependent metabolism to ketone body utilization, thereby improving cellular energy efficiency and mitochondrial bioenergetics (Bough and Rho, 2007; Simeone *et al.,* 2018). Additionally, *in silico* studies have highlighted the role of KD in regulating oxidative stress pathways by upregulating antioxidant defence mechanisms and reducing reactive oxygen species (ROS) production, which are critical factors in seizure-induced neuronal damage (Masino and Rho, 2019). Computational analyses also suggest that KD influences key neurotransmitter networks, particularly by enhancing GABAergic activity and reducing glutamatergic excitotoxicity, thereby stabilizing neuronal excitability (Ruskin *et al.,* 2017). Importantly, the integration of *in vivo* behavioural and morphological findings with *in silico* modelling offers a more comprehensive understanding of KD's therapeutic potential. Such integrative approaches demonstrate how KD mitigates PTZ-induced neurotoxicity and cortical damage, particularly within the medial prefrontal cortex, by targeting multiple interconnected molecular and cellular pathways involved in epileptogenesis.

In the context of smPFC injury, the KD demonstrates multifaceted therapeutic effects, ranging from seizure suppression to cognitive enhancement and molecular regulation. Experimental epilepsy models demonstrate that the KD not only mitigates neuronal hyperexcitability but also enhances behavioural outcomes and preserves cortical architecture, particularly in areas susceptible to seizure-related damage, such as the smPFC (Löscher, 2011; Rho and Stafstrom, 2012). Furthermore, KD-mediated modulation of neurotransmitter balance, mitochondrial function, and oxidative stress pathways contributes to its neuroprotective profile (Masino and Rho, 2019; Simeone *et al.,* 2017). Therefore, investigating the effectiveness of KD in PTZ-kindled rat models of smPFC damage using an integrative approach that combines behavioral, morphological, and *in silico* analyses is of significant scientific importance. This comprehensive framework not only elucidates the mechanisms underlying KD's antiepileptic and neuroprotective effects but also offers significant insights into the formulation of innovative therapeutic strategies for epilepsy and related cognitive impairments (Kahle *et al.,* 2016; Ruskin *et al.,* 2017).

MATERIALS AND METHODS

Ketogenic diets formulation

The three oils; Palm kernel oil (PKO), Castor oil (CTO), Olive oil (OLO), were purchased from Samaru market in Zaria, Kaduna State were formulated by percentage composition of oils, proteins and carbohydrate (**Table 1**) with a custom-formulated powdered diet mixture containing nutrients, fiber, minerals and vitamins per one kilogram of standard and ketogenic diets were formulated (**Table 2**). The premixes for the KDs and ready-to-use control diet was obtained from vital feed, Zaria, Nigeria. Analysis of the three ketone bodies (acetoacetate, acetone and β-Hydroxybutyrate) and standard anti-seizure

medication (Levetiracetam) were carried out using molecular docking.

Table 1: The comparison of carbohydrate, fat, and protein contents of standard and ketogenic diets

Diet	Carbohydrate (%)	Fat (%)	Protein (%)
Standard	52.20	7.00	15.25
Ketogenic	5.66	86.19 (PKO, CTO, OLO)	8.15

(Arsyad *et al.,* 2020)

PKO: Palm kernel oil, CTO: Castor oil, OLO: Olive oil.

Table 2: Standard diets and ketogenic diets formulation for 1kg.

Standard diets formulation		ketogenic diets formulation	
Ingredients	Quantity/g	**Ingredients**	Quantity/g
Maize	522	**Maize**	50
Groundnut	35	**Groundnut**	250
Soybeans	35	**Soybeans**	250
Soybeans meal	100	**Soybeans meal**	80
Groundnut cake	52.5	**Palm kernel**	100
Lysine	50	**Lysine**	1
Methionine	50	**Methionine**	1
Wheat hoofers	50	**Wheat hoofers**	1
Limestone	20	**Limestone**	1
Salt	20	**Salt**	1
Bone Ash	30	**Bone Ash**	1
Premix(grower)	20	**Premix(grower)**	13
Enzymes	5.5	**Enzymes**	1
CTO, PKO and OLO	10ml	**CTO, PKO and OLO**	250 ml

PKO: Palm kernel oil, CTO: Castor oil, OLO: Olive oil

Molecular docking of ketone bodies and standard drug ligands

The 3D structures of Acetoacetate (PubChem CID: 6971017), Acetone (PubChem CID: 180), β-Hydroxybutyrate (PubChem CID: 3541112), and Levetiracetam (PubChem CID: 5284583) were retrieved from the PubChem database and were saved in SDF format. Preparation of ligands was carried out using PyRx v0.8; ligands were imported into PyRx and energy minimized using the MMFF94 force field with steepest descent followed by conjugate gradient algorithms. Hydrogen atoms were added automatically at physiological pH, and Gasteiger charges were assigned before the conversion of the SDF to PDBQT format for docking.

The crystal structures of HCR2 (PDB ID: 81JA), and NMDA (PDB ID: 7SAD) were retrieved from the RCSB PDB database in PDB format. Protein preparation was performed in PyMOL v3.1.6.1 by isolating chain A, removing water molecules, ions, and other non-protein residues, and deleting co-crystallized ligands. Polar hydrogens were added, and the structures were subjected to gentle energy minimization using Open Babel (PyRx backend) to relieve steric clashes while maintaining crystallographic geometry.

Molecular docking was carried out using AutoDock Vina (PyRx v0.8). Grid boxes were defined around the binding pockets identified from co-crystallized ligands, with an additional 5–8 Å margin. Docking parameters were set to an exhaustiveness of 16, an energy range of 4 kcal/mol, and 8 output poses per ligand. Each ligand (acetoacetate, acetone, β-hydroxybutyrate, and levetiracetam) was docked separately against each protein target (HCR2 and NMDA).

Animal Welfare and Ethic: Thirty young adult female Wistar rats (*Rattus norvegicus*), weighing between 90 to 100 g, were purchased from the Faculty of Pharmaceutical Sciences, Ahmadu Bello University (ABU), Zaria. The rats were maintained at the Department of Human Anatomy animal facility, Ahmadu Bello University Zaria. The rats were housed in a standard rat plastic cage containing sawdust bedding, wooden sticks, and nests at room temperature and were subjected to natural light-dark cycle. Rats were allowed regular free access to water and food during the period of acclimatization for 2 weeks before the commencement of the experiment. Animals were treated in accordance with the guidelines of the Ahmadu Bello University, Zaria

committee on Animal Use and Care (Approval No: ABUCUAC/2025/105). With all efforts, animal suffering was minimized to reduce the number of animals used for this experiment.

PTZ Kindling: PTZ (Sigma Aldrich, St. Louis, USA) was used to induce kindling. PTZ was injected intraperitoneally (ip) at a dose 35 mg/kg (subconvulsive dose). PTZ dose was increased (5 mg/kg) in subsequent injection to encourage the progression of kindling (Shimada and Yamagata, 2018).

Experimental animals: Adult male Wistar rats weighing 90 to 100 grams were raised at room temperature and subjected to a natural light-dark cycle at the animal house of the Department of Human Anatomy, Faculty of Basic Medical Sciences, Ahmadu Bello University, Zaria, Nigeria. Rats received water and food ad libitum. Rats were divided into six (6) groups, each containing five (5) Wistar rats per group. To promote kindling, PTZ was increased by 5 mg/kg in subsequent intraperitoneal administrations after the fourth (Table 3). The Wistar rats were weighed before kindling seizure induction and at an interval of 5 days during the experiment. Behavioral testing was conducted within the animal facility, during the light-phase (9:00 AM to 5:00 PM).

Table 3. **Experimental design**

S/N	Group	Treatment
1	Control	Normal feed, No PTZ
2	Experimental control	Normal feed + PTZ (35mg/kg)
3	Standard control	Normal feed + Levetiracetam + PTZ (35mg/kg)
4	KD (Palm kernel oil)	KD + PTZ (35mg/kg)
5	KD (Castor oil)	KD + PTZ (35mg/kg)
6	KD (Olive oil)	KD + PTZ (35mg/kg)

(Bough and Rho, 2007).

Post-injection, rats were observed in clear chambers for 30 minutes, with seizure severity scored using a modified Racine's scale (Racine, 1972)

i. Stage 0: No seizure activity.
ii. Stage 1: Immobility, prone posture.
iii. Stage 2: Head nodding, myoclonic twitches.
iv. Stage 3: Unilateral forelimb clonic seizures.
v. Stage 4: Rearing, generalized clonic seizures, tonic posture.
vi. Stage 5: Tonic-clonic seizures with loss of balance and falling

Novel Object Recognition Test (NORT)

Novel object recognition test (NORT) is a test for assessing recognition memory, the NORT was conducted in a box measuring 80 cm × 80 cm × 50 cm, according to the method described (Sadegzadeh *et al.,* 2020). NORT consist of the training and test phase, during the training phase two similar objects are introduced by placing them on two opposite angles of the central arena forming an imaginary hypotenuse, the test phase involves using an identical object from the training phase and a novel object, still placing them as mentioned above. At the beginning of both the training and the test, each rat was placed in one of the corners of the box and monitored by a camera placed above the box for a duration 5 minutes; while taking note of the times the animal sniffs a particular object and the time it spent doing so. Following each trial, both the inner and outside arena, as well as the objects being used were cleaned with 75% alcohol to ensure cleanliness. The center of the open field apparatus, which covered approximately 25% of the total area, was marked out as a square measuring roughly 40 cm × 40 cm. The animals were habituated in the platform using the open field test, after habituation.

Figure 1: Experimental rats exploring novel object, in the NORT apparatus

Animal Euthanasia and Brain Fixation: At the end of the experiment, within twenty-four hours (24 h), animals were sacrificed by decapitation. The animal skulls were dissected, brain were collected and immersed in 10% buffered formalin at 4^{o}C for 48 hours and further processed for morphological assessment. The brain tissues were grossed, dehydrated in a graded percentage of alcohol (70%, 80%, 100%), cleared in xylene, infiltrated in molten wax, and then embedded to form a block.

Morphological Analysis of mPFC

Brain tissue processing and Thionine staining for cell number estimation: The embedded brain tissue was sectioned to produce 10 microns sections using a rotary microtome, and every 1 of 20 sections was collected and stained with 2.5% thionine. The sections were dewaxed using xylene, followed by rehydration in a descending grade of alcohol, and then the section was dipped in water and stained with thionine. The stained section was dehydrated with ascending graded alcohol, cleared with xylene, and mounted with DPX mountant and then left to dry at room temperature for further stereological analysis.

Estimation of number of neurons in the mPFC: Using an unbiased design-based stereological method (Schmit & Hof, 2005; West & Gundersen, 1990), with physical dissector, the number of thionine (2.5%) stained neurons in the SNr was estimated from every 1/20 sections. The region of interest was viewed and captured under a light microscope. A counting frame was superimposed on the captured region of interest, and the number of neurons in the SNr was counted by following the counting rule (only neurons within the counting frame and neurons touching the dash lines were counted).

Data Analysis

The data were presented as mean ± standard error of mean (SEM). One-way and two-way ANOVAs were used appropriately, followed by the Tukey post hoc test and multiple comparisons between groups. Results were considered significant when the p-value was less than 0.05 ($p < 0.05$). The data obtained was analyzed using GraphPad Prism version 8.4.

RESULTS

Molecular Docking of Ketones Bodies and Standard Drugs Ligand: The docking analysis of HCR2 receptor, levetiracetam exhibited one of its strongest interactions with a binding energy of −5.9 kcal/mol, suggesting high receptor–ligand stability. Among the test ligands, acetoacetate (−4.8 kcal/mol) showed the highest affinity, followed by β-hydroxybutyrate (−4.5 kcal/mol), whereas acetone (−3.1 kcal/mol) was the weakest. The higher affinities of acetoacetate and β-hydroxybutyrate suggest favorable orientation and possible engagement of hydrophilic residues via their carboxyl and hydroxyl groups, though still weaker than levetiracetam's robust interaction.

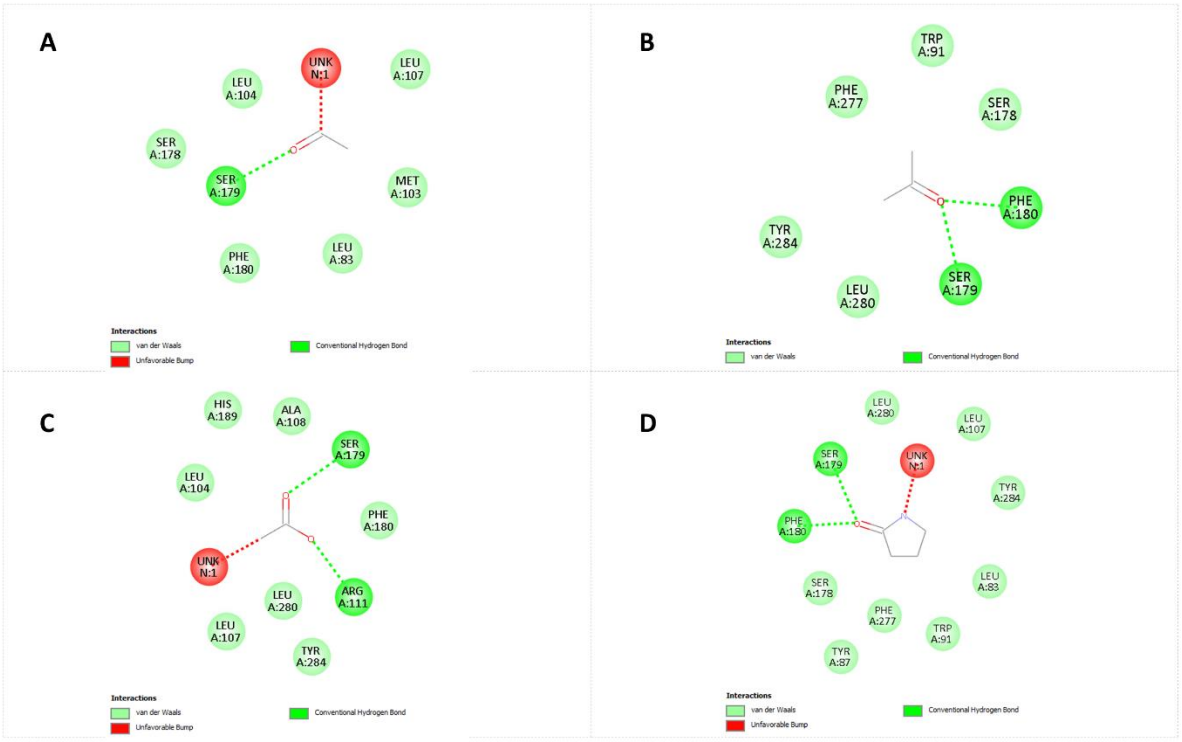

Figure 2. **Molecular docking binding affinities ketone bodies (A) Acetoacetate (B) Acetone, (C) β-Hydroxybutyrate and (D) Levetiracetam against HCR2**

For the NMDA receptor, levetiracetam demonstrated a strong binding affinity of −5.2 kcal/mol, consistent with its known neuromodulatory effects on glutamatergic signaling. Among the ketone bodies, β-Hydroxybutyrate (−3.9 kcal/mol) and Acetoacetate (−3.7 kcal/mol) showed comparable moderate interactions, while Acetone (−3.2 kcal/mol) was weaker. These results suggest that the ketone bodies may occupy peripheral or allosteric regions within the NMDA receptor, whereas levetiracetam likely interacts within the main modulatory domain, enhancing binding stability.

Across all receptor systems, levetiracetam consistently demonstrated the strongest binding affinities (−4.4 to −6.2 kcal/mol). Among the physiological ligands, acetoacetate and β-hydroxybutyrate showed moderately stable and reproducible interactions, whereas acetone had the weakest affinities in all simulations. These findings suggest that while ketone bodies may act as mild receptor modulators, levetiracetam exhibits significantly stronger binding and receptor stabilization, supporting its potential role as a broad neuromodulatory and anti-inflammatory agent. However, synergistic action of the three ketone bodies may provide a robust neuromodulatory, anti-oxidant and anti-inflammatory activity.

Ketogenic diets do not alter somatic and brain weight in female Wistar rats: On Day 1, there was noticeable changes in body weight across all groups ($p = 0.999$). By Day 10, however, the row factor analysis showed that the FSC group had a significant increase in body weight compared to the FN group ($p = 0.0211$). The FC group also showed an increase relative to FN and [$F(5,54) = 11.34$], a significant increase when compared to the FEC group ($p = 0.0459$).

By Day 20, the FSC group continued to show a significant increase in body weight compared to the FN group ($p = 0.0024$). Overall, the column factor analysis revealed a highly significant difference across all groups [$F(5,54) = 11.34$, $p = 0.0001$] (Figure 4A).

Further analysis across time points showed that from Day 1 to Day 10, there was a significant increase in body weight. Specifically, the FSC ($p = 0.0082$) and FC ($p = 0.0066$) groups showed significant increases compared to FN. This trend was also evident in additional comparisons, where FSC ($p = 0.0001$), FP (p

= 0.0019), and FC (p = 0.0313; p = 0.0142) all showed significant increases relative to FN (Figure 4A).

Between Day 5 and Day 10, body weight increased significantly across several groups. The FSC (p = 0.0002), FP (p = 0.0066), and FO (p = 0.00418) groups all showed significant increases compared to FN. Additionally, FSC (p = 0.0004) and FP (p = 0.0155) were significantly higher than FEC. However, FP (p = 0.0418) and FO (p = 0.0380) also showed significant increases when compared to FSC (Figure 4A).

Regarding brain weight, only the standard control group showed a significant increase compared to the normal group [F (5,18) =3, p=0.0463], as indicated (Figure 4B). In contrast, the mean brain-to-body weight ratio did not show any significant differences across the groups (Figure 4C).

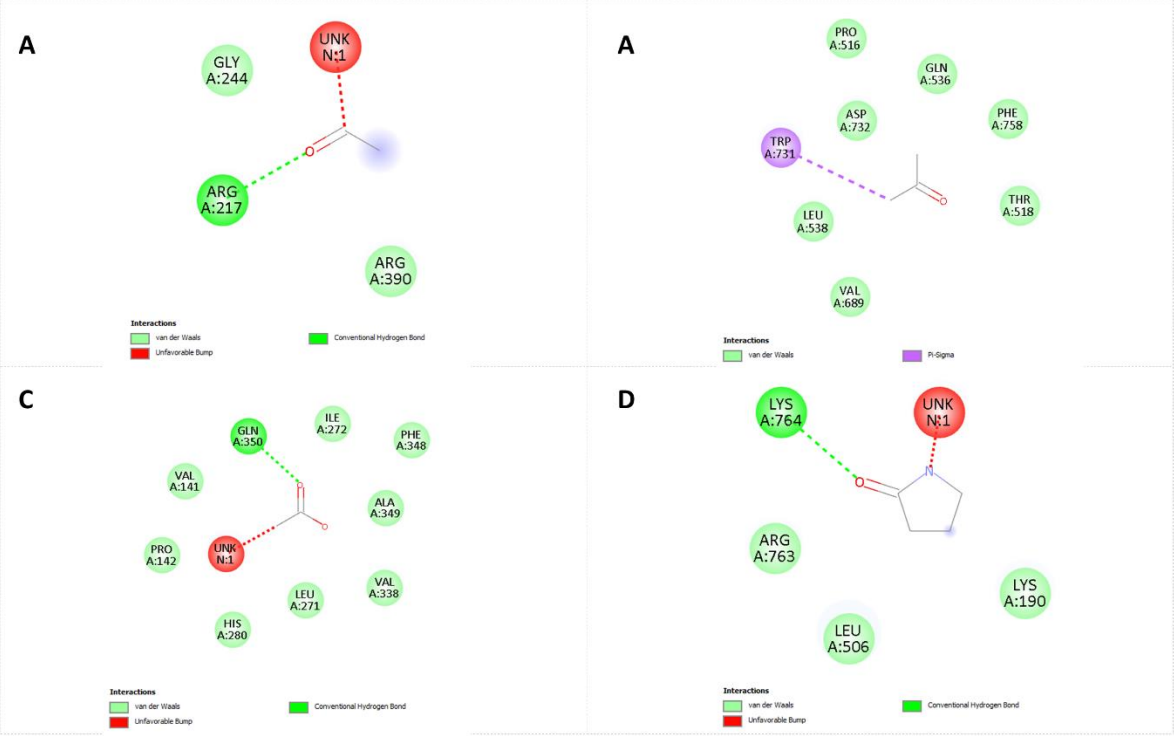

Figure 3. Molecular docking binding affinities ketone bodies (A) Acetoacetate (B) Acetone, (C) β-Hydroxybutyrate and (D) Levetiracetam against NMDA receptor.

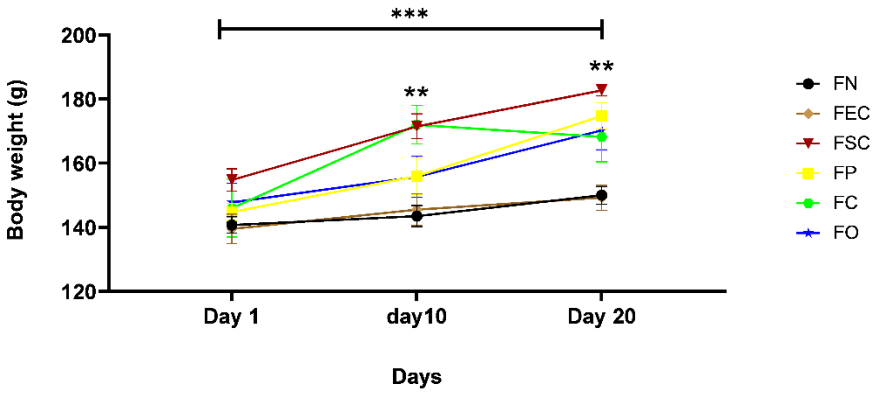

Figure 4A. Effect of different ketogenic diets on PTZ-kindling body weight. FN = Normal; FEC = PTZ-kindling; FSC = Standard diet + PTZ-kindling; FP = Palm Kernel Oil ketogenic diet + PTZ-kindling; FC = Castor Oil ketogenic diet + PTZ-kindling; FO = Olive Oil ketogenic diet + PTZ-kindling. Data expressed as Mean ± SEM; n = 5; two-way ANOVA

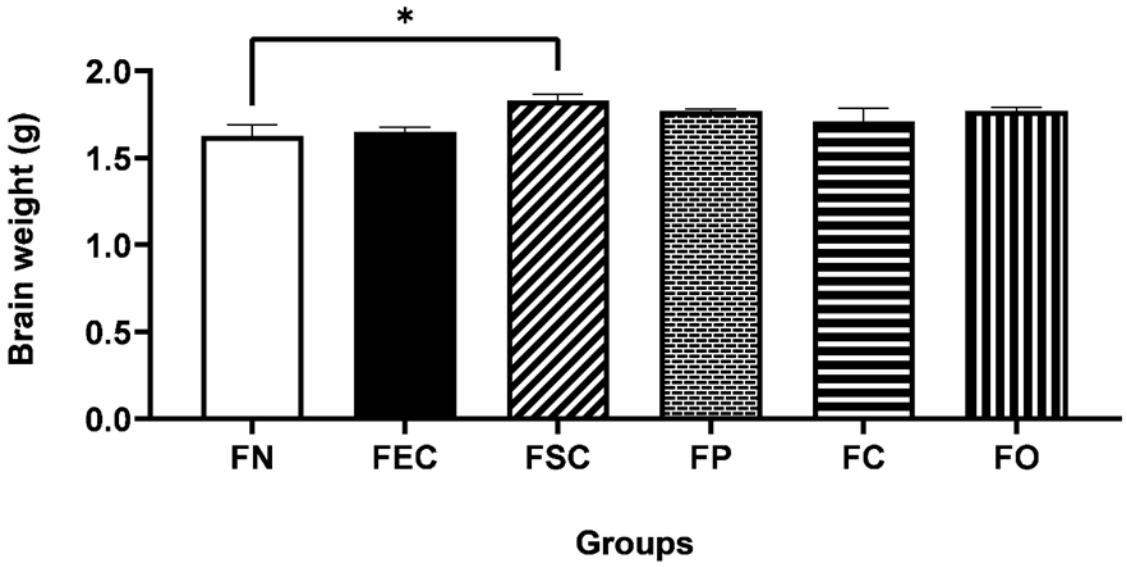

Figure 4B: Effect of treatment on the Brain weight of animals. FN=Normal; FEC=Experimental control; FSC=standard control; FP=palm kernel oil; FC=Castor oil; FO=olive oil. One-way ANOVA, *P<0.05

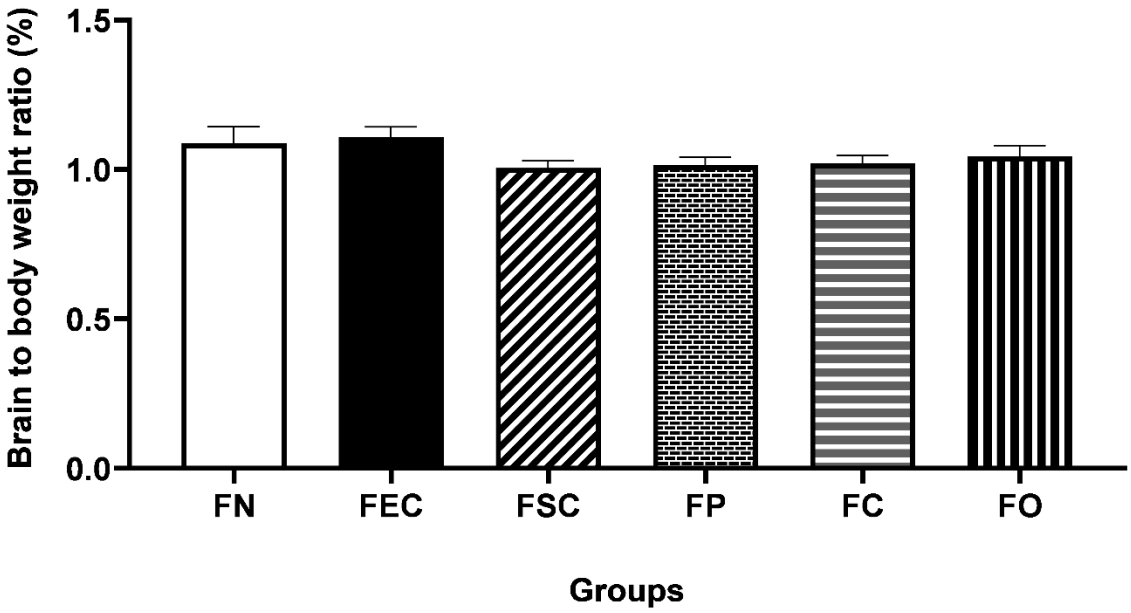

Figure 1C: Brain-to-Body Weight ratio. FN=Normal; FEC=Experimental control; FSC=standard control; FP=palm kernel oil; FC=Castor oil; FO=olive oil. *p= 0.2739

Neurobehavioral Observation

Day 1 object recognition

The analysis of the Novel Object Recognition test in day 1 revealed no significant interaction between the variables [F (5,31) = 1.847, *P* =0.1327]. Additionally, there was no significant effect of exploratory time [F (5,31) = 0.9842, *P* =0.1327] or group differences [F (5,31) = 1.512, *P* =0.2148] across the experimental groups (Figure 5A).

Day 1 Number of NOR sniffs

The analysis of the Novel Object Recognition of **number of NOR sniffs between OA1-SD and OA2-SD** showed no interaction between the variables [F (5, 30) =1.035, (p=0.4154)]. The effect of exploration time was not statistically significant [F (1, 30) =0.2818, (p=0.5994)]. However, FP showed a significant increase in number of new sniffs of OA2SD compared with FN, FEC, FSC and FO groups [F (5,30) = 6.757, (P= 0.0003)] (Figure 5B).

Novel object recognition analysis

Day 2 object recognition

The analysis of the Novel Object Recognition test on the day 2 showed no significant interaction between the variables [F (5, 31) =0.8766, (p=0.5081)]. The effect of exploration time was not statistically significant [F (1, 31) =0.008819, (p=0.9258)]. However, there was a significant difference between FC and FSC groups [F (5,31) = 5.097, (P= 0.0016)] (Figure 6A).

Day 2 Number of NOR sniffs

The novel object recognition test revealed no significant interaction between the variables [F (5, 30) =1.291, (p=0.2940)]. However, there was a significant main effect of the row factor on exploration time, indicating clear differences across the groups [F (1, 30) =7.080, (p=0.0124)]. In addition, the column factor also showed a significant decrease in number of NOR sniffs times in FEC and FO compared to FN group [F (5,30) = 4.174, (P= 0.0084)] (Figure 6B).

Morphological Analysis

Thionine Stereology (neuronal cell density)

Thionine sections for stereology revealed a significant decrease in number of neurons in mPFC supra-limbic layer 2/3 of FEC [F (5, 18) = 8.532, P < 0.0001), FSC (P = 0.0232), FCD (P = 0.0069), and FOD (P = 0.0442) compared with FNC group. Additionally, FPD showed a significant increase compared to FEC (P = 0.0079), all based on the same ANOVA result [F (5, 18) = 8.532)] (Figure 7)

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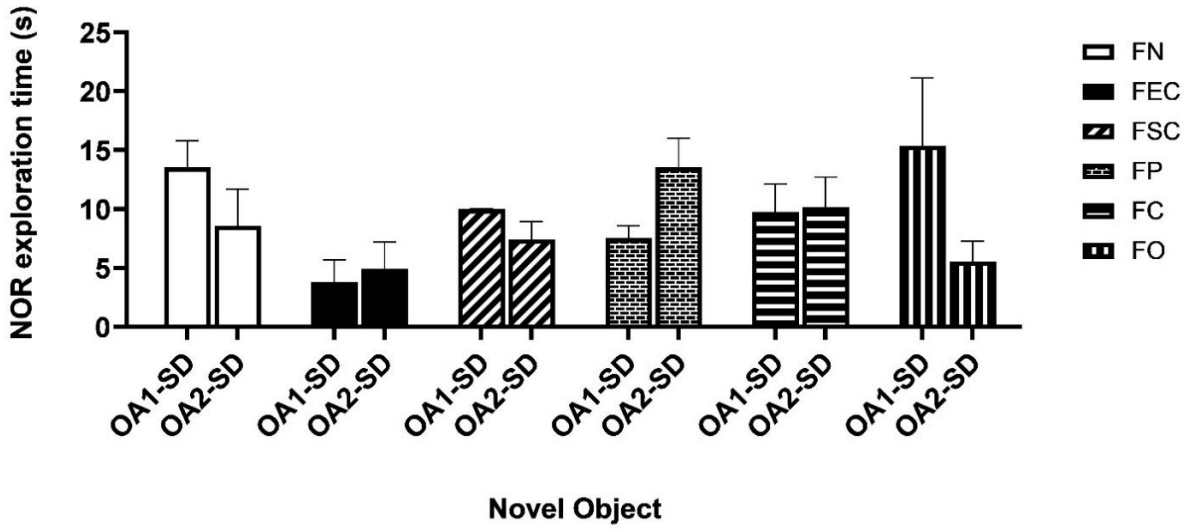

Figure 5A: The novel object recognition analysis of day one and two across groups; OA1-SD=object exploration day 1, OA2-SD= object exploration day 2

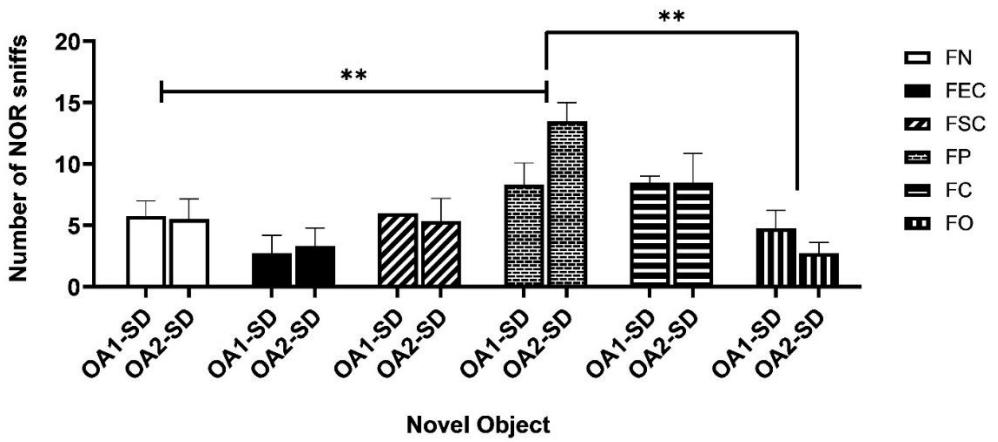

Figure 5B: Number of novel object recognition sniff; NO-SD=number of sniffs. Two-way Anova **p=0.0053, **p=0.0016

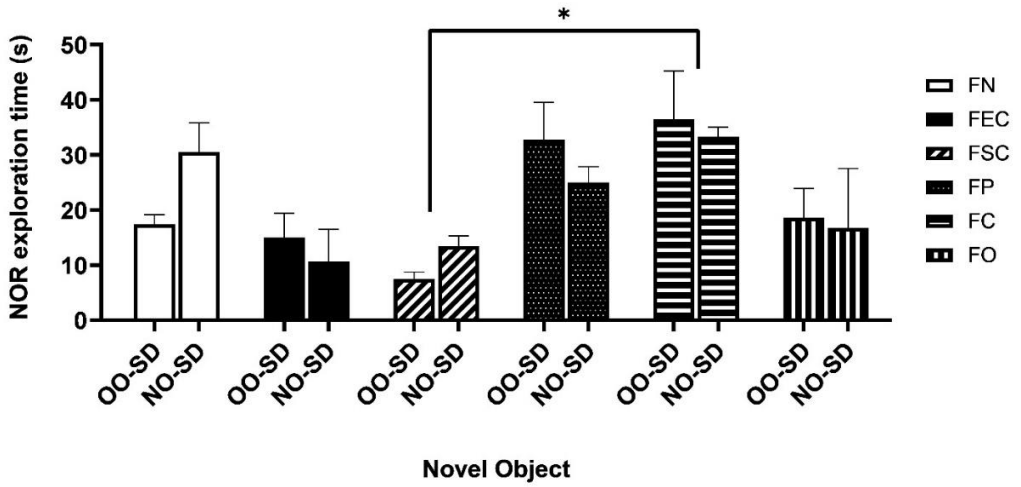

Figure 6A. Novel object exploration test; OO-SD=old object, NO-SD= new object. Two-way Anova; * p= 0.0210

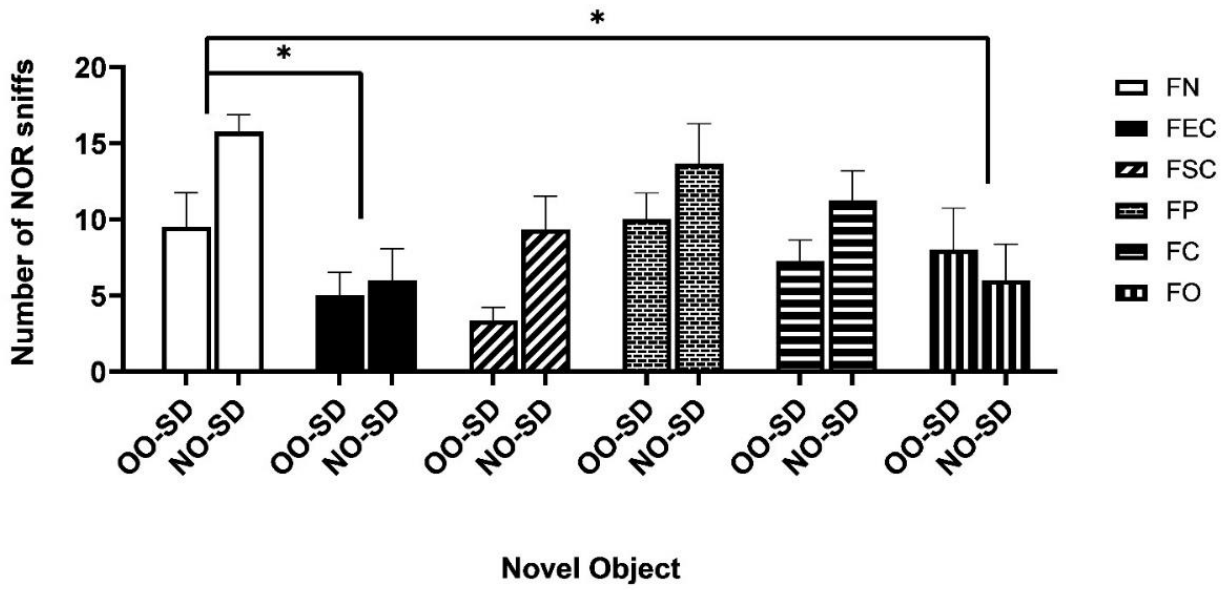

Figure 6B. number of NOR sniffing; OO-SD = old object sniffing, NO-SD = New object sniffing. Two -way Anova *p=0.0237, *p= 0.0122

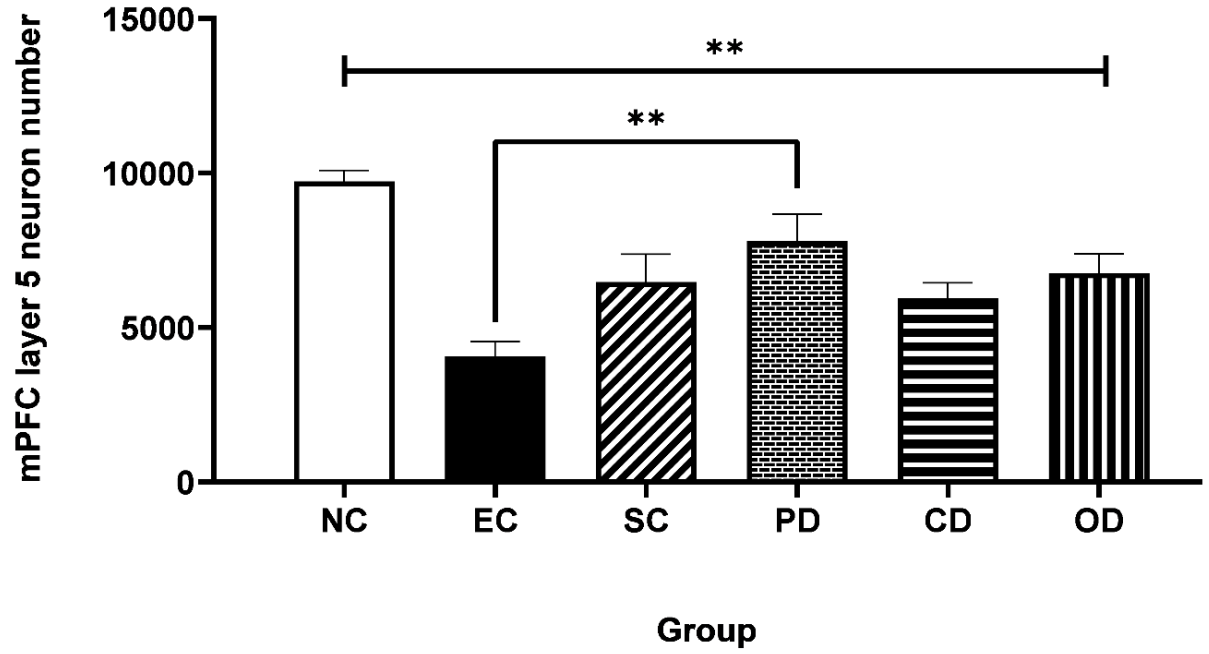

Figure 7: the effect of pentylenetetrazol (PTZ), levetiracetam, ketogenic diet on neuronal density number of the medial Prefrontal Cortex (mPFC) Layer 5. NC= Normal control; EC=Experimental Control; SC=Standard Control; PD=Palm kernel Oil Diet; CD=Castor Oil diet; OD=Olive Oil Diet. One-way ANOVA, **P<0.005

DISCUSSION

The present docking results provide important insight into how ketone bodies may interact with the **HCRTR2 (orexin-2) receptor** in comparison to a known anticonvulsant drug, levetiracetam. Among all ligands evaluated, levetiracetam demonstrated the strongest binding affinity (−5.9 kcal/mol), indicating the formation of a relatively stable receptor–ligand complex. Although levetiracetam is primarily known to act via synaptic vesicle protein SV2A, evidence suggests that it can influence broader neuromodulatory systems, including pathways

involved in arousal and excitability, which may indirectly involve orexin signaling (Lynch et al., 2004; Löscher et al., 2016). The strong binding observed in this study may therefore reflect favorable interactions within the receptor binding pocket, contributing to its overall anticonvulsant efficacy. Among the endogenous ketone bodies, acetoacetate (−4.8 kcal/mol) exhibited the highest binding affinity, followed closely by β-hydroxybutyrate (−4.5 kcal/mol). These relatively stronger interactions, compared to acetone, are likely attributable to their chemical structures, particularly the presence of functional groups such as carboxyl and hydroxyl moieties. These groups facilitate hydrogen bonding and electrostatic interactions with amino acid residues within the receptor binding site, thereby enhancing ligand stability and proper orientation (Paoli *et al.,* 2014). The ability of these metabolites to engage in such interactions suggests a plausible mechanism through which ketogenic states may influence receptor activity at a molecular level. In contrast, acetone exhibited the weakest binding affinity (−3.1 kcal/mol), likely due to its simpler molecular structure and lack of functional groups capable of forming strong intermolecular interactions. This reduced binding capacity suggests that acetone may play a limited role in directly modulating HCRTR2 receptor activity, despite its presence during ketosis (Likhodii *et al.,* 2003). Although the ketone bodies did not achieve binding affinities comparable to levetiracetam, their moderate interactions with the HCRTR2 receptor are noteworthy. The orexin (hypocretin) system, particularly through HCRTR2, is known to regulate arousal, wakefulness, and neuronal excitability, and has been implicated in seizure susceptibility and epileptogenesis (Sakurai, 2007; Zheng *et al.,* 2025). Therefore, even modest modulation of this receptor by ketone bodies may contribute to stabilizing neuronal networks and reducing hyperexcitability associated with PTZ-induced seizures. Furthermore, the combined presence of β-hydroxybutyrate and acetoacetate during ketogenic states may produce synergistic neuromodulatory effects, enhancing their overall impact despite individually moderate binding affinities. Such synergistic interactions, coupled with their established roles in improving mitochondrial function, reducing oxidative stress, and modulating neurotransmitter balance, may contribute significantly to the anticonvulsant and neuroprotective effects of the ketogenic diet (Masino & Rho, 2012; Hartman et al., 2007). Overall, these findings highlight the importance of molecular structure and functional groups in determining ligand–receptor interactions. While levetiracetam exhibits strong and direct receptor engagement, ketone bodies appear to exert more subtle modulatory effects on HCRTR2. In the context of PTZ-induced kindling, where dysregulation of excitatory and arousal pathways contributes to seizure generation, such modulation may play a supportive role in reducing neuronal hyperexcitability and protecting medial prefrontal cortex integrity.

The molecular docking results for the NMDA receptor provide important insight into the differential interactions between levetiracetam and key ketone bodies. Levetiracetam demonstrated a relatively strong binding affinity (−5.2 kcal/mol), which is consistent with its established role in modulating excitatory neurotransmission and synaptic activity. Although levetiracetam is primarily known to act via synaptic vesicle protein SV2A, emerging evidence suggests that it can indirectly influence glutamatergic signaling, including NMDA receptor-mediated pathways (Lynch *et al.,* 2004; Rogawski & Löscher, 2004). The relatively strong binding observed in this study may therefore reflect a stabilizing interaction within modulatory regions of the receptor, potentially contributing to its anticonvulsant and neuroprotective effects. Among the ketone bodies, β-hydroxybutyrate (−3.9 kcal/mol) and acetoacetate (−3.7 kcal/mol) exhibited moderate and comparable binding affinities. Although weaker than levetiracetam, these interactions are notable and support the growing body of evidence that ketone bodies can influence neuronal excitability and synaptic function. Rather than acting as direct antagonists at the NMDA receptor, ketone bodies are more likely to exert modulatory effects through allosteric interactions or indirect metabolic pathways (Masino & Rho, 2012; Paoli *et al.,* 2014). Their binding profiles suggest a preference for peripheral or regulatory sites, which may allow them to subtly alter receptor conformation and reduce excitotoxic signaling without interfering with normal neurotransmission. In contrast, acetone showed the weakest interaction (−3.2 kcal/mol), likely due to its simpler molecular structure and limited capacity for stable receptor binding. This observation is consistent with previous reports indicating that acetone plays a less prominent role in receptor-level modulation compared to other ketone bodies, despite its presence during ketosis (Likhodii *et al.,* 2003). When considered collectively, levetiracetam consistently exhibited the strongest binding affinities across receptor systems evaluated in this study (−4.4 to −6.2

kcal/mol), reinforcing its robust pharmacological profile as an anticonvulsant agent. In contrast, β-hydroxybutyrate and acetoacetate demonstrated moderate but reproducible interactions, suggesting that they may function as mild neuromodulators. Importantly, while individual ketone bodies display relatively weak binding, their combined elevation during ketogenic states may produce synergistic effects. Such synergy has been proposed to enhance mitochondrial function, reduce oxidative stress, and attenuate glutamate-induced excitotoxicity, all of which contribute to neuroprotection in epilepsy models (Masino & Rho, 2012; Hartman *et al.,* 2007). In the context of PTZ-induced kindling, where excessive glutamatergic activity and NMDA receptor overactivation play a central role in seizure generation and neuronal damage, these findings are particularly relevant (Pitkänen & Lukasiuk, 2011). The ability of both levetiracetam and ketone bodies to modulate NMDA receptor activity—either directly or indirectly—may underlie their observed protective effects on neuronal integrity in the medial prefrontal cortex. Overall, these results highlight the importance of both binding affinity and cooperative interactions in determining the functional impact of endogenous and exogenous compounds on receptor systems. While levetiracetam demonstrates strong and direct receptor engagement, ketone bodies appear to exert subtler, yet potentially synergistic modulatory effects. This complementary interaction may contribute significantly to the therapeutic efficacy observed in ketogenic diet interventions for epilepsy and associated neurodegenerative changes.

The changes in body weight observed in this study provide important insight into the physiological and metabolic effects of the different treatments over time. At baseline (Day 1), there were no significant differences in body weight across all groups, indicating that the animals were comparable prior to the interventions. This initial uniformity strengthens the internal validity of the study, as subsequent changes can be more reliably attributed to PTZ-induced kindling and treatment effects rather than pre-existing differences (Löscher, 2011). By Day 10, clear variations in body weight began to emerge. The FSC group showed a significant increase compared to the normal (FN) group, suggesting that the treatment may have influenced metabolic activity, nutrient utilization, or energy balance. Similarly, the FC group demonstrated increased body weight relative to FN, and this increase was also significant when compared to the FEC group. These findings may reflect alterations in metabolic homeostasis, possibly due to the interaction between seizure activity and dietary or pharmacological interventions. It is well established that seizure models, including PTZ kindling, can disrupt metabolic processes, while certain treatments—particularly dietary interventions—may counteract these effects (Kandratavicius *et al.,* 2014; Hartman et al., 2007). By Day 20, this trend became more pronounced, with the FSC group continuing to exhibit a significant increase in body weight compared to FN. The overall column factor analysis further confirmed a highly significant difference across groups, indicating that the treatments exerted sustained and cumulative effects on body weight. Temporal analysis revealed that the most notable increases occurred between Day 1 and Day 10, particularly in the FSC and FC groups, suggesting a rapid physiological adaptation to the interventions. Such early changes are consistent with reports that metabolic shifts—especially those induced by dietary modifications like the ketogenic diet—can occur within days of initiation (Paoli *et al.,* 2014). Additional comparisons between Day 5 and Day 10 further supported this trend, with groups such as FSC, FP, and FO showing significant increases relative to FN. Interestingly, some groups (FP and FO) exhibited higher body weights compared to FSC, indicating possible differences in the magnitude or mechanisms of action of the treatments. In the context of ketogenic diet intervention, changes in body weight may be associated with shifts in energy substrate utilization, including increased reliance on ketone bodies and altered lipid metabolism (Masino & Rho, 2012). However, it is important to note that ketogenic diets can produce variable effects on body weight depending on composition, duration, and experimental conditions. In contrast to body weight, changes in brain weight were minimal. Only the standard control group showed a modest but significant increase compared to the normal group. This suggests that while systemic growth and body mass were influenced by the treatments, brain weight remained relatively stable and tightly regulated. Furthermore, the brain-to-body weight ratio did not differ significantly across groups, indicating that brain development remained proportionate to overall body growth. This is a critical observation, as it suggests that neither PTZ-induced seizures nor the administered treatments caused disproportionate brain atrophy or hypertrophy (Pitkänen & Lukasiuk, 2011). Overall, these findings indicate that the treatments—particularly those involving dietary modulation—primarily influenced systemic metabolic processes rather than organ-specific

development. The consistent increase in body weight observed in certain groups may reflect improved metabolic efficiency or adaptive energy utilization. Importantly, the stability of the brain-to-body weight ratio suggests that these interventions do not adversely affect relative brain development, supporting their physiological safety profile. In the context of epilepsy research, such outcomes are valuable, as they indicate that therapeutic strategies like the ketogenic diet may confer metabolic and neuroprotective benefits without compromising brain integrity (Masino & Rho, 2012; Hartman *et al.,* 2007).

The findings from the Novel Object Recognition (NOR) test on Day 1 suggest that there were no major differences in cognitive performance across the experimental groups at the early stage of assessment. Specifically, the absence of significant interaction effects and group differences indicates that baseline recognition memory and exploratory behavior were comparable among all groups. This is an important observation, as it confirms that subsequent behavioral differences are unlikely to be due to pre-existing cognitive disparities but rather to the effects of PTZ-induced kindling or therapeutic interventions (Löscher, 2011; Bevins & Besheer, 2006). Further analysis of sniffing behavior between familiar (OA1-SD) and novel (OA2-SD) objects also revealed no significant interaction or overall exploration effect, suggesting limited discrimination between objects at this early time point. This may reflect the short interval between exposure and testing, during which memory consolidation processes are still developing (Antunes & Biala, 2012). However, a notable exception was observed in the FP group, which demonstrated a significant increase in exploration of the novel object compared to the FN, FEC, FSC, and FO groups. This preference for novelty is widely interpreted as an indicator of intact or enhanced recognition memory, as rodents naturally exhibit a bias toward unfamiliar stimuli (Ennaceur & Delacour, 1988). The improved performance in the FP group may therefore reflect enhanced cognitive processing, potentially linked to the neuroprotective effects of the ketogenic diet. Ketogenic diets have been shown to improve synaptic function, enhance mitochondrial efficiency, and reduce oxidative stress, all of which can contribute to improved memory encoding and retrieval (Masino & Rho, 2012; Neal *et al.,* 2008). Thus, the early enhancement observed in this group may represent a protective effect against PTZ-induced cognitive disruption. On Day 2, the NOR test provided further insight into the progression of cognitive changes. Similar to Day 1, no significant interaction between variables was observed, and overall exploration time remained comparable across groups, suggesting stable general exploratory behavior. However, a significant difference between the FC and FSC groups emerged, indicating that treatment conditions may differentially influence recognition memory over time. Such differences are consistent with previous reports that seizure activity and its modulation can produce variable cognitive outcomes depending on intervention strategies (Holmes, 2015). Analysis of sniffing behavior on Day 2 revealed no significant interaction effects but demonstrated a significant main effect of exploration time, indicating that temporal dynamics such as habituation or sustained interest influenced object exploration across groups (Antunes & Biala, 2012). More importantly, a significant reduction in novel object exploration was observed in the FEC and FO groups compared to the FN group. This decrease suggests impaired recognition memory or reduced novelty preference, which is commonly associated with hippocampal and prefrontal cortical dysfunction following recurrent seizures (Kandratavicius *et al.,* 2014; Pitkänen & Lukasiuk, 2011). In contrast, the relatively preserved or enhanced performance observed in treatment groups such as FP further supports the potential cognitive benefits of the ketogenic diet. The ability of ketogenic interventions to mitigate seizure-induced cognitive deficits has been attributed to their role in stabilizing neuronal networks, reducing excitotoxicity, and enhancing neuroplasticity (Hartman et al., 2007; Masino & Rho, 2012). Overall, these findings demonstrate that while baseline cognitive performance was similar across groups, PTZ-induced kindling led to progressive impairments in recognition memory in specific groups, particularly FEC and FO. In contrast, the improved or preserved performance observed in the FP group highlights a potential neuroprotective and cognitive-enhancing effect of the ketogenic diet. These results underscore the sensitivity of the NOR test in detecting subtle, time-dependent changes in memory and exploratory behavior and emphasize the importance of longitudinal behavioral assessment in evaluating therapeutic interventions in epilepsy models.

The stereological analysis revealed marked alterations in neuronal density within the medial prefrontal cortex (mPFC), particularly in the supralimbic layers 2/3. The significant reduction in neuronal number observed in the FEC, FSC, FCD, and

FOD groups relative to the normal control (FNC) group suggests that exposure to PTZ-induced kindling and associated experimental conditions may have resulted in neuronal loss or compromised neuronal integrity within this critical region (Löscher, 2011; Kandratavicius *et al.,* 2014). PTZ kindling is well known to induce repeated seizure activity, leading to excitotoxicity, oxidative stress, and progressive neuronal damage in vulnerable brain regions, including the prefrontal cortex. The mPFC, particularly layers 2/3, plays a crucial role in higher-order cognitive processes such as decision-making, working memory, and executive function (Miller & Cohen, 2001; Goldman-Rakic, 1996; Lewis & Anderson, 1995). Therefore, the observed reduction in neuronal density may have important functional implications, potentially contributing to seizure-associated cognitive and behavioral deficits (Holmes, 2015). The consistency of neuronal loss across multiple experimental groups further supports the presence of a robust neurotoxic and neurodegenerative effect associated with PTZ-induced epileptogenesis (Pitkänen & Lukasiuk, 2011). Interestingly, the FPD group demonstrated a significant increase in neuronal number compared to the FEC group, suggesting a potential neuroprotective or restorative effect of the intervention, likely attributable to the ketogenic diet. The ketogenic diet has been widely reported to exert anticonvulsant and neuroprotective effects through multiple mechanisms, including enhancement of mitochondrial function, reduction of oxidative stress, modulation of neurotransmitter balance, and inhibition of excitotoxic pathways (Neal et al., 2008; Masino & Rho, 2012). The observed increase in neuronal density in the FPD group may therefore reflect improved neuronal survival and attenuation of PTZ-induced neurodegeneration. Additionally, emerging evidence suggests that ketogenic interventions may support synaptic plasticity and, under certain conditions, promote neurogenesis, although this remains to be fully elucidated (Paoli et al., 2014). Overall, these findings demonstrate a clear pattern of neuronal vulnerability in PTZ-treated groups, alongside evidence of partial neuroprotection in the ketogenic diet-treated group (FPD). This contrast underscores the therapeutic potential of the ketogenic diet in mitigating neuronal loss within the mPFC. Such neuroprotective effects are particularly relevant in epilepsy and related neurological disorders, where preservation of prefrontal cortex integrity is essential for maintaining cognitive function and behavioral regulation (Hartman et al., 2007; Masino & Rho, 2012).

CONCLUSION

The present study demonstrates that KDs protects mPFC against PTZK model induced risk like taking behavior, neurodegeneration and DNA fragmentation, in particular ketogenic diet formulation from palm kernel oil.

Conflict of interest

All the authors declare no conflicting interests.

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