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Neuroprotective Effects of Ketogenic Diets Against Neuronal Death in the Substantia Nigra in Pentylenetetrazol-Kindling-induced Seizure in Female Wistar Rats

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ABSTRACT

Little is known about the ketogenic diet (KD)'s seizure-fighting properties. The substantia nigra pars reticulata (SNr) is a "gate" site for seizures. The neuroprotective effect of KDs on PTZ-induced epilepsy (35 mg/kg) every other day for 20 days was examined. The thirty mature female Wistar rats were separated into six five-rat per group. Group 1 control (normal feed); Group 2 PTZK; Group 3 PTZK and levetiracetam (10 mg/kg); Group 4 PTZK and palm-kernel oil-KD; Group 5 PTZK and castor oil-KD; and Group 6 PTZK and olive-KD. To promote kindling, PTZ was increased by 5 mg/kg in subsequent intraperitoneal administrations after the fourth. Rats were fed various diets. 10 days before and during Kindling. The Racine scale gave seizure severity scores. Ketone bodies (KB) (Acetoacetate, Acetone, β-Hydroxybutyrate) and levetiracetam's GABA binding-affinity were determined by molecular docking. Elevated plus-maze assessed anxiety/risk-taking. The number of neurons (unbiased design-based stereology) and DNA fragmentation (Feulgen reaction) were estimated. *In silico* analysis of levetiracetam showed high binding affinity for FFR2 and FFR3, but weaker bonding than KB. KD groups (4, 5, and 6) had seizure scores of 3, while the PTZK group (2) had 4. Contrary to groups 2 and 3, the KD's neuroprotective benefits may improve cognition, lower impulsivity, and reduce risk-taking. Compared to the KD groups (4, 5, and 6), the PTZK group (2) had decreased neuronal number and an increase in SNr DNA-fragmentation. KBs' connection to FRR2 and FFR3 may protect against neurodegeneration, risk-taking, and seizures.

Keywords: PTZ-Kindling seizure, Ketogenic diets, substantia nigra pars reticulata, DNA fragmentation, Stereology

INTRODUCTION

It is well-known that ketogenic diets (KD) can effectively treat epilepsy. The ketogenic diet's mechanism of action is still not well understood, despite the many theories surrounding it [1-4]. However, exposure to KD increases levels of its essential components, the ketone bodies, and its potential anti-seizure effect [5]. To mimic KD's effects, synthetic ketones have been the subject of prior research [6, 7]. However, it has also been shown that cutting calories or engaging in intermittent fasting can help prevent seizures [8-11]. Finding the anatomical structure or neuronal population within the central nervous system that is impacted by KD in a way that results in an anti-seizure effect is of immense interest, in addition to identifying the anti-seizure molecules present in KD. Studies have shown that ketone bodies derived from KD can influence the spontaneous excitation rate of neurons in the SNr in vitro [12-14], which serves as a focal point for seizures [6, 15-19]. The pathophysiology of epilepsy interplay between excitatory and inhibitory neurotransmission imbalance is key in epilepsy [20–22]. This imbalance results from dysregulated ion channels, impaired GABAergic inhibition, and glutamate excitotoxicity [23–25]. Excitotoxicity activates glial cells, and the generation of pro-inflammatory mediators and oxidative stress significantly contributes to neuronal hyperexcitability and progressive damage in the epileptic brain [26–28]. Understanding the neurochemical and neuroinflammatory pathologies of epilepsy, neuroprotective remedies for those with epilepsy can be targeted. This necessitates the use of valid

experimental models to accomplish neuroprotection. Over the years, numerous experimental models, both *in vitro* and *in vivo* models, have been used to study epilepsy.

To mimic a human-like model of epilepsy, the use of Pentylenetetrazol (PTZ) kindling model in rodents provides a robust experimental platform for investigating therapeutic and preventive interventions [29]. PTZ, a GABA-A receptor antagonist, induces progressive kindling, leading to a chronic epileptic state characterized by established seizures, behavioral deficits, and neuropathological changes in structures like the SNr. PTZ acts on the $GABA_A$ receptor complex, as a result, blocks the benzodiazepine site, the picrotoxin site, and the GABA site. Blocking these stops the GABAA receptor from being activated, which stops chloride ions from getting into neurons. This stops GABAergic inhibition [28, 30]. Because of the overactivation of glutamatergic signaling, the removal of GABAergic inhibition will cause progressive hyperexcitation, which will eventually lead to epilepsy and a slow progression of epileptogenesis [31, 32]. This model allows for the assessment of interventions administered before and after the disease is established, thereby evaluating their capacity to therapeutically reverse or ameliorate existing neurological dysfunction, a critical and underexplored area in epilepsy research [33].

Given that SNr is a hub to GABAergic neurons, making it vulnerable to seizure-mediated dysfunction and its contribution to midbrain pathology, exploring neuroprotective strategies in this region holds translational significance. Therefore, this study aimed to evaluate whether different ketogenic diet interventions can protect the SNr against structural and functional damage in adult female Wistar rats subjected to PTZ-induced epilepsy, providing insights into both the anticonvulsant and neuroprotective capacity of KD at a regional brain level.

MATERIALS AND METHODS

Ketogenic diets formulation

The three ketogenic oils: Palm kernel oil (PKO), Castor oil (CTO), Olive oil (OLO), were purchased from Samaru market in Zaria, Kaduna State. The ketogenic diets were formulated by percentage composition of fat/oils, proteins, and carbohydrates (Table 1), with a specially prepared powdered mixture of vitamins, minerals, fiber, and nutrients per one kilogram of standard and ketogenic diets were formulated (Table 2). The premixes for the KDs and the ready-to-use control diet were obtained from Vital Feed, Zaria, Nigeria. Analysis of the three ketone bodies (acetoacetate, acetone, and β-Hydroxybutyrate) and standard anti-seizure medication (Levetiracetam) was carried out using molecular docking.

Molecular docking of ketone bodies and standard drug ligands

By utilizing the PubChem database, 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 GABA (PDB ID:6D6T) 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 (GABA).

Animal welfare and ethics

Thirty-five young adult albino female Wistar rats (Rattus norvegicus), weighing between 90 and 100 grams, 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 a 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.

Table 1: The comparison of the carbohydrate, lipid, 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 oil)	8.15

[34]

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

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

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
CT, PK, and OL	10ml	CT, PK, and OL	250 ml

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

Pentylenetetrazol Kindling

Pentylenetetrazol (PTZ) (Sigma Aldrich, St. Louis, USA) was used to induce kindling. PTZ was injected intraperitoneally (i.p.) at a dose of 35 mg/kg (subconvulsive dose). PTZ dose was increased incrementally (5 mg/kg) in subsequent injections to encourage the progression of kindling [35].

Animal grouping

Rats were divided into six (6) groups, each containing five (5) Wistar rats per group. Control for Group 1 (normal feed); Group 2 received PTZK (35 mg/kg) every other day; Group 3 received PTZK and levetiracetam (10 mg/kg); Group 4 received PTZK and palm kernel oil-KD; Group 5 received PTZK and castor oil-KD; and Group 6 received PTZK and olive-KD. To promote kindling, PTZ was increased by 5 mg/kg in subsequent intraperitoneal administrations after the fourth [36].

Post-injection, rats were observed in clear chambers for 30 minutes, with seizure severity scored using a modified Racine's scale [37]:

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.

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).

Behavioral evaluations

Behavioral tests were performed at day 21 of the experimental period (i.e., after the 20-day PTZK) during the light phase (09:00 AM to 03:00 PM) in a controlled, quiet environment. Wistar rats were acclimatized to the testing room for 30 minutes before the commencement of the behavioral test.

Elevated plus maze test

Two open arms (16 x 5 cm) and two enclosed arms (16 x 5 x 12 cm) with an open ceiling make up the elevated plus maze. Both arms are attached to a central platform (5 x 5 cm), and the entire device is raised to a height of 25 cm. Each animal is positioned with its head facing an open arm in the middle of the elevated plus maze. Black paint was applied to the apparatus's floor and walls. The equipment was stored in a soundproof room. A 200-lux lamp was used to light the space [38]. For five minutes, the amount of time spent in both open and closed arms was noted. In order to remove olfactory signals, 70% ethanol was used to clean the equipment in between trials. Manual records of the alternation activity were made. Each animal's proportion of time spent in the open arms [100 × open / (open + enclosed)] and the percentage of open arm entries (100 × open/total entries) were then computed. After every trial, the equipment was carefully cleaned. When all four paws have crossed the boundary between an arm and the center area, it is referred to as arm entrance [39].

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, the brains 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 substantia nigra pars reticulata

Brain tissue processing and thionine staining for cell number estimation: The embedded brain tissue was sectioned at 10 microns using a rotary microtome, and every 1 in 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 sections were 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 substantia nigra pars reticulata: Using an unbiased design-based stereological method [40-42], a thionine-stained section, and a physical dissector, the number of neurons in the SNr was estimated from every 1/20 thionine (2.5%) stained 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 dashed lines were counted).

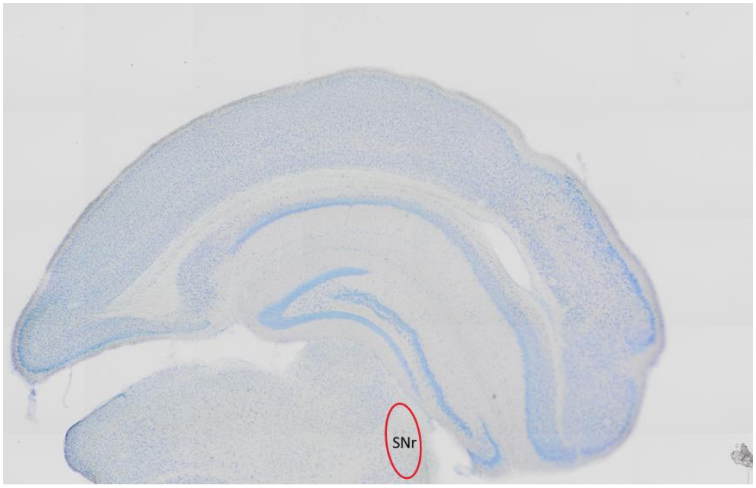

Figure 1: Photomicrographs of thionine-stained sections showing the anatomical location of Substantia Nigra Pars Reticulata.

Feulgen Reaction for DNA fragmentation in the subiculum pyramidal cell layer: Every 20th 8-micron-thick section was dewaxed in xylene, rehydrated in descending graded alcohol, and dipped ten times in distilled water before washing in a cold-prepared 1N HCL, followed by incubation at 60 degrees centigrade. Then the sections were later washed in cold-prepared 1N HCl before being directly placed in Schiff's reagent solution, followed by washing in tap water, potassium metasulfite, counterstaining with light green, washing in distilled water, and then dehydrating and clearing in xylene. Then it was mounted with DPX. Sections were allowed to dry at room temperature and viewed and captured under the microscope using an OMAX camera at X400. For DNA fragmentation analysis using ImageJ optical density, one section at a time was uploaded into ImageJ to determine the optical density, which involved color deconvolution, light Feulgen stain with the appreciated cell of interest with stained nucleus and washed cytoplasm, then the mean gray value MGV. The mean values were calculated using the formula log10(255/MGV) and the MOD in Excel.

Data analysis

The data obtained were analyzed using GraphPad Prism version 8.4.3; the data were presented as mean ± SEM. One-way and two-way ANOVA were used appropriately, followed by the *Tukey post-hoc* test for numerous group comparisons. When $p<0.05$, the results were deemed significant.

RESULT

Levetiracetam demonstrated a strong binding affinity with the $GABA_A$ receptor

For the $GABA_A$ receptor, Levetiracetam demonstrated a strong binding affinity of −5.2 kcal/mol, consistent with its known neuromodulatory effects on glutamatergic signaling (Figure 2-D). Among the ketone bodies, β-Hydroxybutyrate (−3.9 kcal/mol) (Figure 2-C) and Acetoacetate (−3.7 kcal/mol) (Figure 2-A) showed comparable moderate interactions, while Acetone (−3.2 kcal/mol) (Figure 2-B). 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.

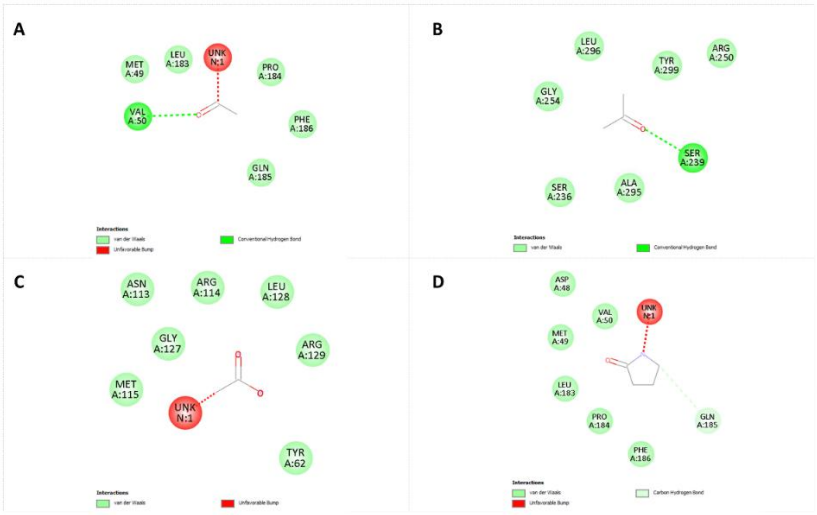

Figure 2. Molecular docking binding affinities of ketone bodies (A) Acetoacetate, (B) Acetone, (C) β-Hydroxybutyrate, and (D) Levetiracetam against the GABA receptor.

Effects of the ketogenic diet on seizure resistance and onset

Rats fed the KDs showed a noticeable resistance to seizure progression [$F(45, 180) = 28.26$, $P < 0.0001$] and onset time [$F(5, 180) = 5567$, $P < 0.0001$] following i.p. injection of PTZ every other day for 20 days (Figure 3A). Although the ketogenic diet significantly [$F(9, 180) = 421.9$, $P < 0.0001$] delayed

the onset of PTZ-induced seizures compared with the PTZ group, the ketogenic diet also showed a significant delay in the onset of PTZ-induced seizures compared with the standard drug group. That is, ketogenic diets delayed seizure onset and prevented it from becoming progressively more severe (Figure 3A).

Increased intraperitoneal injection of PTZ every other day showed a significant [F (45, 180) = 2.054, P < 0.0005] interaction with increased neurological score to stage 4 in the PTZK group compared with other groups. The ketogenic diet groups significantly maintained neurological scores at stage 3 compared to stage 4 of the PTZK group [F (45, 180) = 2.054, P < 0.0398]. However, at the end of the treatment, no noticeable change was observed between the standard drug group and the PTZK group (P<0.2327) (Figure 3B).

Ketogenic diets do not change short-term and special memory in Y-maze analysis

Time spent in EPM open arm showed no significant change across groups (Figure 4A). There was a significant increase in time spent in the EPM closed arm in the PTZK group compared with the control group (Figure 4B). These findings indicate that PTZK does induce anxiety.

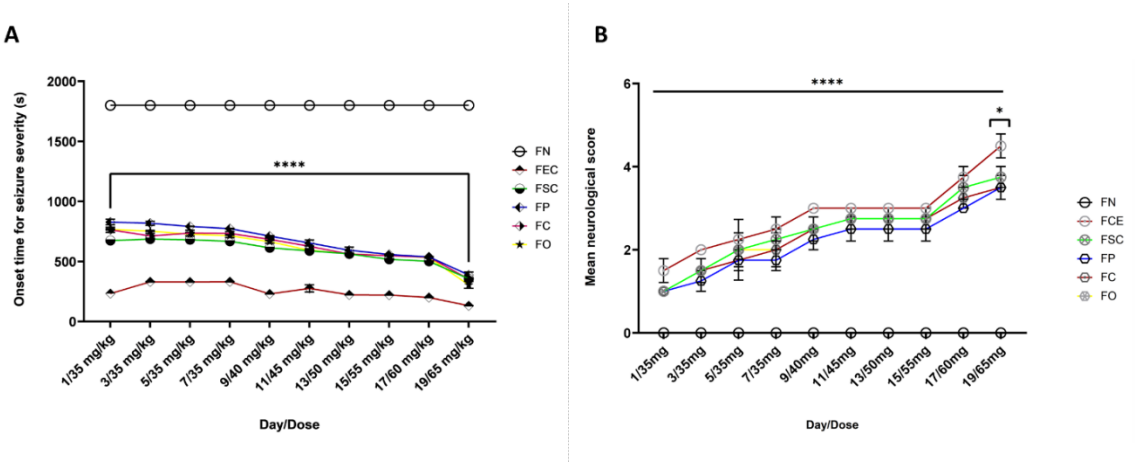

Figure 3. Effect of different ketogenic diets on PTZK seizure onset (3A) and seizure score (3B). FN = Normal; FEC = PTZK; FSC = Standard diet + PTZK; FP = Palm Kernel Oil ketogenic diet + PTZK; FC = Castor Oil ketogenic diet + PTZK; FO = Olive Oil ketogenic diet + PTZK. Data are presented as Mean ± SEM; n = 5; * p < 0.05 **** p < 0.0001, two-way ANOVA.

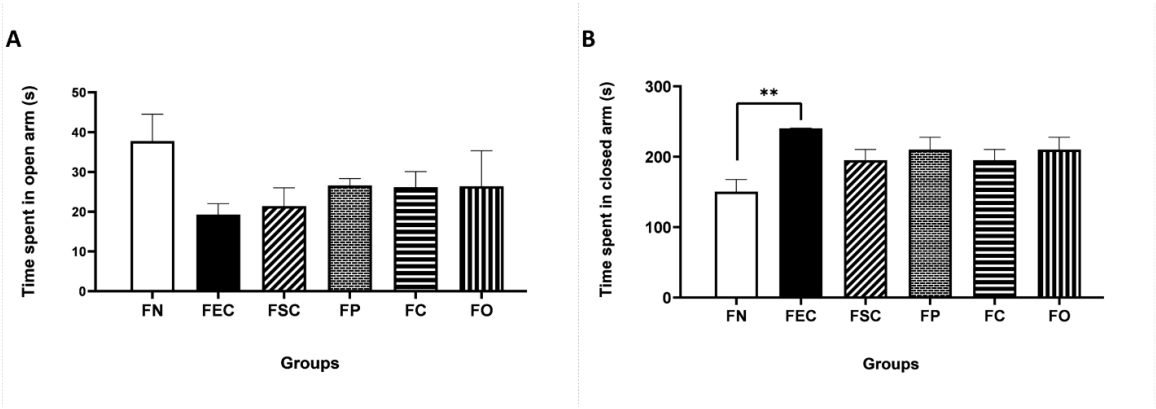

Figure 4. Effect of different ketogenic diets on PTZK-induced anxiolytic phenotype on EPM time spent in open arm (4A) and closed arm (4B). FN = Normal; FEC = PTZK; FSC = Standard diet + PTZK; FP = Palm Kernel Oil ketogenic diet + PTZK; C = Castor Oil ketogenic diet + PTZK; FO = Olive Oil ketogenic diet + PTZK. Data are presented as Mean ± SEM; n = 5; ** p < 0.001, one-way ANOVA.

Ketogenic diets do not alter somatic and brain weight in female Wistar rats

The mean body weight analysis on day 1 showed no noticeable change (p=0.999) across the groups. The row factor for day 10 showed the FSC had a significant increase compared to FN (p = 0.0211). The FC showed an increase compared to FN. FC also showed an increase compared to FEC (p=0.0459). The row factor for day 20 showed FSC significantly

increased compared to FN (p=0.0024). The column factor showed a highly significant difference across the groups, F (5, 54) =11.34, p=0.0001 (Figure 5A).

The column factor between day 1 and day 10 showed a significant increase. FSC showed a significant increase compared to FN (p=0.0082). FC showed a significant increase compared with FN (p=0.0066). The column factor between day 1 and day 10 showed a significant increase. The FSC showed a significant increase compared to FN (p=0.0001), FP showed a significant increase compared to FN (p=0.0019), FC showed a significant increase compared to FN (p=0.0313), and also a noticeable increase compared to FN (p= 0.0142) (Figure 5A).

The column factor between day 5 and day 10 showed a significant increase. The FSC showed a marked increase compared to FN (p= 0.0002), FP showed a significant increase compared to FN (p= 0.0066), FO showed a pronounced increase compared with FN (p=0.00418), FSC showed a significant increase compared to FEC (p=0.0004), FP showed a significant increase compared to FEC (p=0.0155). However, there is a marked increase in FP compared to FSC (p=0.0418), and also FO had a noticeable increase compared to FSC (p=0.0380) (Figure 5A). The mean brain-to-body ratio showed no significant change across the groups (Figure 5B).

Ketogenic diets protect against PTZ-kindling induced neurodegeneration

Neuroprotective effect of ketogenic diets were investigated on every 1/20 thionine stained subiculum sections and unbiased designed-based stereology method, showed significant decreased in PTZK (FEC) Wistar rats [F (5, 18) = 4.634, p=0.0082], standard anti-seizure medication (FSC) Wistar rats [F (5, 18) = 4.634, p= 0.0299] and castor oil ketogenic diet formulation (FC) Wistar rats [F (5, 18) = 4.634, p=0.0261] (Figure 6A). Suggesting that not the standard anti-seizure medication but ketogenic diet formulations from palm kernel oil (MP) and olive oil (OL) that protect against PTZK-induced neurodegeneration. To further support this finding, DNA fragmentation using the Feulgen reaction stain—a primary biomarker for apoptosis showed that PTZK (FEC) had a significant increase in mean optical density of fragmented DNA compared to the normal control (FN) group [F (8, 16) =3.866, p=0.0081] (Figure 6B).

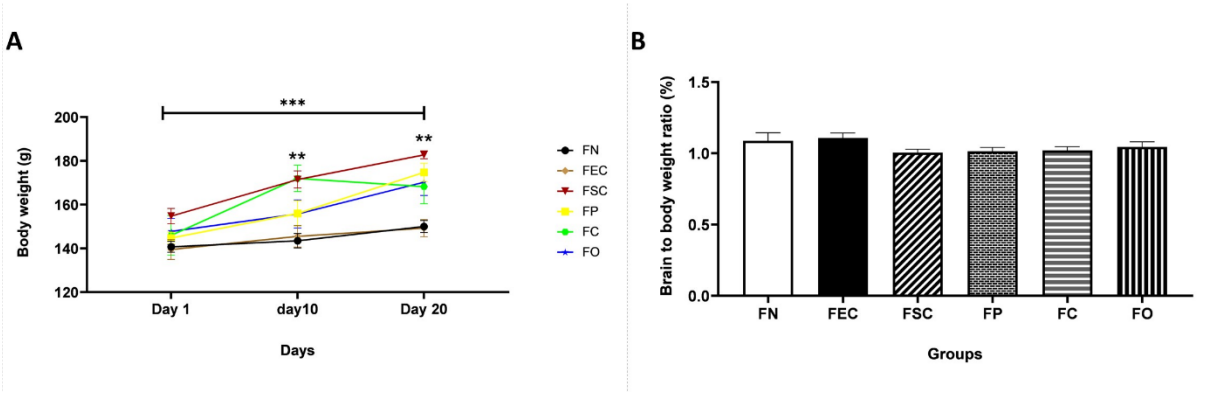

Figure 5. Effect of different ketogenic diets and PTZK-induced body weight changes (5A) and brain-to-body ratio (5B). FN = Normal; FEC = PTZK; FSC = Standard diet + PTZK; FP = Palm Kernel Oil ketogenic diet + PTZK; FC = Castor Oil ketogenic diet + PTZK; FO = Olive Oil ketogenic diet + PTZK. Data are presented as Mean ± SEM; n = 5; two-way ANOVA

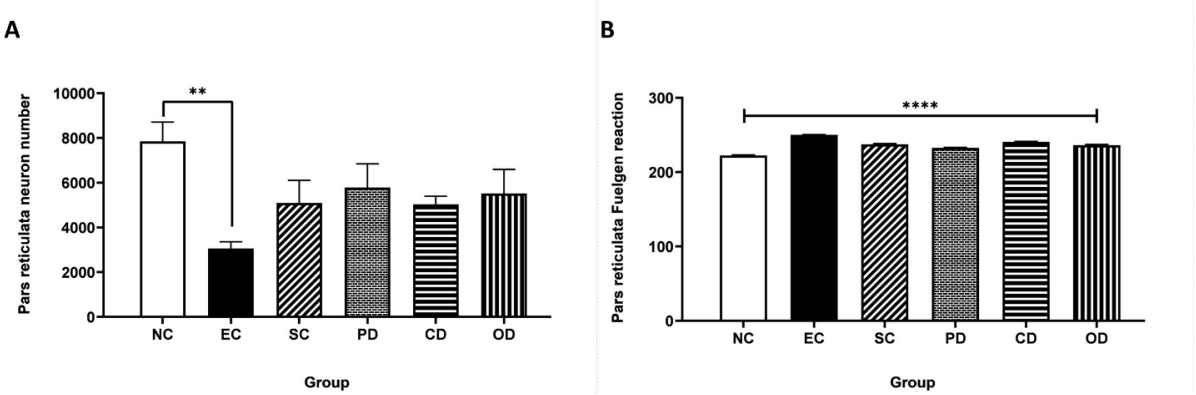

Figure 6. Effect of different ketogenic diets against PTZK-induced neurodegeneration (6A) and DNA fragmentation in the SNr (6B). NC = Normal control; EC = PTZK; SC = Standard diet + PTZK; PD = Palm Kernel Oil ketogenic diet + PTZK; CD = Castor Oil ketogenic diet + PTZK; OD = Olive Oil ketogenic diet + PTZK. Data are presented as Mean ± SEM; n = 5; ** $p < 0.01$, **** $p < 0.00$, one-way ANOVA.

DISCUSSION

Ketogenic diets have been widely characterized because of their neuroprotective properties across several models of brain injury, hypoxia, and oxidative stress, where their antioxidant and metabolic capacity is associated with enhanced neuronal survival and reduced damage. However, the physiological potentials of ketogenic diets formulated from palm kernel oil and castor oil remain largely unexplored. To provide novel insights into the biological functions of ketogenic diets against PTZK-induced neurodegeneration, we investigated *in silico* binding affinity of ketone bodies and standard anti-seizure medication vs. well-established receptors involved in seizure, neurological score/behavioral, biochemical, and morphological alterations in the PTZK model of Wistar rats.

The *in-silico* comparison of binding affinities between ketone bodies and standard anti-seizure medications helps uncover the molecular mechanisms by which the ketogenic diet (KD) works, especially for patients with drug-resistant epilepsy [43-45]. While Chronic recurrent epilepsy can be effectively treated with the ketogenic diet, its exact mechanisms have remained a long-standing question. Therefore, comparing the binding profiles of ketone bodies (beta-hydroxybutyrate, acetoacetate, and acetone) to known drug targets helps identify potential direct interactions with seizure-related receptors and ion channels, such as GABA-A receptors, enhancing inhibitory neurotransmission of AMPA and NMDA glutamate receptors, thus attenuating excitatory neurotransmission and ion channels that open and close based on voltage, like sodium and potassium channels [46, 47]. The molecular docking analysis for standard anti-seizure medication and ketone bodies against GABA-A receptors revealed that the standard drug (Levetiracetam) exhibited the strongest binding affinity for GABA-A receptor, however, this binding affinity is of weak van der Waals force, that is the strong binding affinity observed between the standard drug (Levetiracetam) and GABA-A receptor can be displaced, therefore questioning the standard drug ability to completely treat epilepsy. The moderate and consistent affinities demonstrated by acetoacetate and β-hydroxybutyrate are fortified with a strong hydrogen bond. This suggests that ketone bodies (acetoacetate and β-hydroxybutyrate) may act as mild modulators of neuronal and inflammatory pathways, possibly contributing to seizure suppression and neuroprotection observed *in vivo* [48, 49]. Also, the synergistic effect of the three ketone bodies might provide a more robust anti-seizure suppressor than the standard drugs, as observed in this study.

When the animals exhibit Stage 4 or Stage 5 traits for at least two consecutive PTZ administrations, the kindling model is deemed successful [50-52]. The PTZ model greatly cut down on the number of injections needed to reach the fully ignited condition when compared with the ketogenic diets and standard anti-seizure medication. The PTZ model thereby shortens the time it takes to establish chronic epilepsy, which in turn shortens the length of investigations. It's equally vital to remember that the ketogenic diets and standard anti-seizure medication had a higher seizure onset time compared to PTZ, despite the same number of PTZ injections across the groups.

The delay in achieving the fully kindled state was observed in both the ketogenic diet group and the standard anti-seizure medication group. The confirmed anti-seizure activity of ketogenic diets and standard anti-seizure medication in protecting against PTZ strongly sensitized the SNr neurons, which may result in increased threshold of excitability, especially in the SNr, which is known for having generally high limitations on excitability [53]. If the given dose had been subconvulsive, this increase in the threshold of excitability would not have been substantial enough to overcome the natural compensating process. Therefore, raising this excitability threshold might delay the onset of kindling in these animals by attenuating an upsurge of the epileptogenesis process.

The average number of animals that reached stages 4 and 5, and the time it took, were used to gauge seizure severity. The use of Racine's scale clearly shows that phases 4 or 5 are the ones that show successful kindling [50, 51, 54]. This study shows that the PTZK group had reached stage 4 compared to the ketogenic diets and standard anti-seizure medication groups. Furthermore, the PTZK group had decreased onset time across the stages 1-4 compared to the ketogenic diets and standard anti-seizure medication groups. That is the anti-seizure activity of ketogenic diet (KD) and standard anti-seizure medication may be associated with the sensitization of GABAergic neurons or glutamatergic synaptogenesis, which stems from enhanced the activation time of GABAergic neurotransmission and attenuated glutamatergic activity, leading to a more stable, less excitable brain state, hence an occurrence of less abundant and late or absence of seizures [43, 50, 51, 55-57]. These results may be associated with PTZK, which amplifies glutamatergic signaling, as demonstrated by Samokhina *et al*.[50].

Anxiolytic-like phenotype is characterized by a decreased time taking in the open arms of the Elevated Plus Maze (EPM), and consequently a longer period of time spent in closed arms. The increased time in the closed arms is an anxiogenic (anxiety-producing) impact of PTZ, stemming from complex neurobiological changes in the brain that affect the balance of neurotransmission between excitatory and inhibitory. The EPM test relies on the rodents' innate

dislike for open spaces, exposed spaces, and their preference for enclosed areas. Increased time in the closed arms in this study suggests higher anxiety levels. This PTZK anxiety phenotype can occur via several mechanisms, including GABAergic dysfunction [50, 58, 59]. PTZ acts as an adversary that doesn't compete at the $GABA_A$ receptor complex, inhibiting the brain's primary inhibitory neurotransmitter system [57, 59, 60]. The kindling process leads to a persistent imbalance, with a reduction in GAD65 expression (which is key for activity-dependent GABA synthesis) and lower overall GABA levels in certain brain regions, such as the hippocampus [61-63]. An imbalance between GABAergic and glutamatergic concentrations, such as a decrease in GABAergic inhibition, results in hyperexcitability and the overactivation of glutamatergic signaling, the primary excitatory system that enhances the excitability of neurons and the onset of anxiety-related behaviors [64, 65]. This imbalance contributes to increased neuronal excitability and the development of anxiety-related behaviors [66, 67]. The repeated seizures in the kindling model led to long-term functional and structural alterations in neural circuits, particularly in the limbic system (e.g., amygdala and hippocampus), which are involved in emotional processing and anxiety [68, 69]. PTZK can also alter levels and turnover rates of monoamine neurotransmitters in the prefrontal cortex and hippocampus, which are linked to emotional behavior and anxiety [50, 70].

Moreover, these significant anti-seizure effects of the ketogenic diet groups were observed in the SNr, following a lower number of pyramidal neurons in the SNr and a significant increase in DNA fragmented optical density in the SNr. That is, PTZK may cause increased DNA fragmentation primarily due to the generation of oxidative assault and subsequent triggers of apoptosis (cell death) pathways in the SNr. Oxidative stress is associated with seizures, especially those induced by PTZK, which lead to an imbalance in the brain's antioxidant defense and the generation of reactive oxygen species (ROS)[71, 72]. This excessive ROS can directly damage cellular components, including DNA bases and strands, before significant breaks even appear [73]. In this context, PTZ glutamatergic excitotoxicity works by antagonizing the $GABA_A$ receptor, which lifts GABAergic inhibition and results in progressive neuronal hyperexcitation [50, 73]. This overactivation of glutamatergic signaling contributes to excitotoxicity, a process known to cause neuronal damage and cell death. Apoptosis activation is associated with significantly low neuronal number and DNA damage observed in the SNr. Studies have indicated heightened expression of pro-apoptotic genes such as Bax and caspase-3 in PTZ-treated animals, which leads to the characteristic "laddering pattern" of DNA fragments [74, 75]. It is worth knowing that SNr is considered among the most vulnerable brain structures during epileptogenesis.

CONCLUSION

The present study demonstrates that KDs have protected against PTZK model-induced risk, such as taking behavior, neurodegeneration, and DNA fragmentation, particularly the ketogenic diet formulation from palm kernel oil.

Conflict of interest

All authors declare they have no conflicting interests.

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