Supplementation with Pearl Millet Enhances Hippocampal GABA-ergic and Suppresses Glutamatergic Systems in Pentylenetetrazole-Kindled Wistar Rats

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Abstract
Background: Pearl millets (PMS) has been reported to exert various health benefits, including anti-diabetic, anti-microbial and anti-convulsant effects.
Objectives: To evaluate the effect of PMS supplementation in Hippocampal GABA-ergic and Glutamatergic Systems in Pentylenetetrazole (PTZ) kindled Wistar rats
Methodology: Induction of PTZ-kindled seizures in Wistar rats involves the grouping of 40 rats into 5 groups (normal saline, 200 mg/kg sodium valproate, 25% PMS, 50 % PMS and 100 % PMS) with each group receiving PTZ (35 mg/kg) on every alternate day for 30 days. Thirty minutes after each PTZ injection, the rats were observed for seizure behaviour using the Racine scale. The hippocampal tissues were isolated, homogenised and used to determine GABA levels, GABA-A receptor, GABA-3-transporter, glutamate levels, NMDA receptor, and glutamate-2-transporter.
Results: PMS Supplementation significantly increased the mean amount of hippocampal GABA \[F(4,20)=10.39, p<0.01\] compared to 100% PGM + NS + PTZ. PMS also significantly \[F(4,20)=3.22, p=0.03\] up-regulated mean GABA-A receptor but the decreased mean GAT-3 compared to rats treated with 100 % PGM + NS + PTZ was not statistically significant.
Low levels of mean hippocampal glutamate were observed in PTZ-kindled Wistar rats fed with all doses of PMS compared to 100 % PGM + NS + PTZ-treated rats. However, the difference was not statistically significant. At 25 % PMS \[F(4,20)=9.65, p<0.01\] significantly down-regulated mean hippocampal N2-NMDA receptor. All PMS-treated groups significantly \[F(4,20)=14.57, p<0.01\] increased the mean quantity of hippocampal glutamate-2-transporter as compared to 100 % PGM + NS + PTZ.
Conclusion: PMS supplementation increased hippocampal GABA-ergic transmission while suppressing
glutamatergic signalling in PTZ-kindled Wistar rats.

**Keywords:** GABA-ergic, Glutamatergic, Hippocampal, Pearl millet, Pentylenetetrazole, Wistar rat

**Abbreviation**

ELISA: Enzyme-linked immunoassay

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**Introduction**

Epilepsy is a chronic brain dysfunction characterised by multiple unprovoked seizures induced by abnormal neuronal discharge\(^1\). The symptoms of epilepsy include altered levels of consciousness, disturbances of movements, abnormal sensory phenomena, autonomic changes, transient disturbances of behaviour (like mood) and physical problems. A core factor for epilepsy development and its progression is the imbalance between the brain inhibitory and excitatory systems resulting from a deficient GABAergic and/or enhanced glutamatergic signalling involving altered neurotransmitter levels, expressions and/or activities of receptors and transporters, among others. The number of global epileptics has been projected to be one billion by 2030\(^2\). The current status is about 70 million\(^3\) with 60 million residing in low- and middle-income countries\(^3,4\).

Pearl millet is the most widely grown millet used for human consumption\(^5\) and it represents up to 40% of the global millet production\(^6\). PMS, being gluten-free with high antioxidant activity and polyunsaturated fatty acids\(^7,8\) has been reported to exert health benefits, including the recent anti-convulsant in PTZ models\(^9\). Our previous study found that PMS suppressed seizure severity in PTZ-kindled Wistar rats, among other seizure models used in that study\(^9\). Since agents with the potential to prevent or delay seizure onset (anti-epileptogenesis) in susceptible individuals or reverse seizure progression and/or improve its associated co-morbidities (disease-modifying) are of utmost clinical need\(^10\) and PMS has proven positive by retarding seizure from the most severe stage\(^9\). We hypothesised that the efficacy of PMS may be associated with its role in inhibiting modulating GABA and blockade of glutamatergic neurotransmission mediated by NMDA receptor; this present study was designed to validate these claims.

**Materials and Methods**

**Experimental Animals**

Forty (40) male Wistar rats weighing (140-180g) were chosen based on high frequency and severity of seizures than females. They were obtained from the Animal House of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria. The rats were kept in a well-ventilated room, maintained at room temperature 25.0 ± 2.0°C, fed on standard pellets of grower’s mash (vital feeds, Jos, Plateau state) and given access to water *ad libitum*.

**Ethical Approval**

This study was carried out under the approval of the Ahmadu Bello University Ethical Committee on Animal Use and Care, ABUCAUC/2021/008.

**Instruments**

Rayto-RT-2100C microplate reader (absorbance of 450nm), DHP-9035A
heating incubator, ELISA microplates (rat GABA-ER 1707, rat IL-1β-ER 1094, rat NMDA receptor associated protein 1-ER 0669, rat GABAA-CK bio 14669, rat glutamate-CK bio 20422, SLC6A11-CK bio20530) Wuhan Fine Biotech Co., Ltd, China

**Drugs and treatment**
Pentylenetetrazole (Sigma-Aldrich, St. Louis, USA, CAS No: 54-95-5), Sodium valproate (Epilim) (Sanofi aventis Riells, Spain, CAS No: 1069-66-5)

**Plant material**
Millet was purchased from Samaru Market, Sabon Gari Local Government Area, Kaduna State, Nigeria, in June 2020. Identified and authenticated as the pearl millet (PM) variety by a taxonomist at the Herbarium unit of the Department of Biological Sciences, ABU, Zaria, Nigeria, where it was given the voucher number (1824).

**Preparation of supplement**
The identified Pearl Millet was soaked in water for 12 hours, paste grinded, sieved with a muslin cloth, residue discarded, and filtrate allowed to settle in a container. It was then decanted, drained with muslin, shade dried into powder, measured and added to pellets grower mash at 25 %, 50 % and 100 %.

Pentylenetetrazole-induced kindling seizures in Wistar rats.
Forty male rats were divided into 5 groups of 8 each, with groups 1 and 2 fed on pellet grower mash (PGM) only while 3-5 with 25 %, 50 % and 100 % PMS, respectively. On every alternate day, groups 1 and 2 were orally administered normal saline and sodium valproate an hour before all 5 groups were injected with freshly prepared PTZ (35mg/kg) and observed for 30 minutes for the presence/absence seizure and its severity using seizure evaluating scale as described by Racine RJ for 30 days.

**Group 1**-100 % PGM + normal saline (1mg/kg, Oral) + PTZ (35mg/kg, I.p)
**Group 2**-100 % PGM + sodium valproate (200mg/kg, Oral) + PTZ (35mg/kg, I.p)
**Group 3**-75% PGM+ 25 % PMS + PTZ (35mg/kg, I.p)
**Group 4**- 50 % PGM+ 50 % PMS + PTZ (35mg/kg, I.p)
**Group 5**- 100 % PMS + PTZ (35mg/kg, I.p)

Rats were considered fully kindled when they showed stages 4 and 5 on two consecutive trials on the 15th day of administration of PTZ (the 30th day of the experiment).

**Sample collection and Preparation**
All rats were cervically dislocated (not anesthesized with a mixture of ketamine and diazepam since ketamine acts on NMDAR and diazepam is an anticonvulsant drug; thus the mixture could alter the result), decapitated, brains dissected, cortices exposed, and hippocampi isolated while placed over ice. Five hippocampal tissues from each group were washed with phosphate buffer, homogenized, collected into labelled vials, frozen and used within two weeks for the determination of GABA and glutamate concentrations, GABA and NMDA receptors, GABA-3- and glutamate-2-transporters using their specific rat enzyme-linked immune-sorbent assay (ELISA) kit according to the manufacturer’s instruction using sandwich principle.

**Assay principle (Sandwich)**
As described by the manufacturer’s manual, Antibody specific for GluN2-NMDAR has been pre-coated onto a microplate. Biotin-conjugated detection antibody specific for GluN2 were added to the wells and washed with wash buffer. HRP-Streptavidin was subsequently added
while free conjugates were washed away with wash buffer. TMB catalysed by HRP produces a blue-coloured product which changes to yellow on addition of stop solution were added to the wells. Finally, optical density was read at 450nm absorbance, and the concentration of GluN2-NMDAR calculated. (ELISA kit user manual).

**Statistical Analysis**

Data collected were analyzed using one way analysis of variance, ANOVA, expressed as mean ± SEM followed by Tukey’s post-hoc test using SPSS version 23.0. Values of $p \leq 0.05$ were considered significant.

**Results**

**Effect of Pearl Millet Supplement (PMS) on Mean Hippocampal GABAergic system in Pentylentetrazole-kindled Wistar Rats**

The results showed a significant $[F(4, 20)=10.389; \ p<0.001]$ increase in the amount of hippocampal GABA in rats treated with sodium valproate ($205.92 \pm 5.52$pg/ml) when compared to that of NS-treated rats ($157.46 \pm 12.32$ pg/ml). PMS supplementation at all doses showed a significant increase in hippocampal concentrations of GABA 25 % ($215.20 \pm 7.26$pg/ml), 50 % ($235.46 \pm 9.41$ pg/ml) and 100% ($215.58 \pm 9.25$ pg/ml) with respect to NS treated rats ($157.46 \pm 12.32$ pg/ml). However, no significant difference was observed within, and between PMS groups 25 % ($215.20 \pm 7.26$pg/ml), 50 %($205.92 \pm 5.52$pg/ml), and 100 % ($215.58 \pm 9.25$ pg/ml) when compared to sodium valproate treated group ($205.92 \pm 5.52$pg/ml) though the highest increase in GABA level was seen with hippocampal tissues of 50 % PMS ($235.46 \pm 9.41$ pg/ml) rats while sodium valproate treated showed the least GABA levels ($205.92 \pm 5.52$pg/ml).

The number of hippocampal GABA$_{A}$ sensitive receptors was significantly $[F(4,20)=3.224, \ p=0.034]$ up-regulated in the valproate-treated rats ($232.68 \pm 4.16$ pg/ml) in relation to that of NS-treated rats ($187.58 \pm 19.15$ pg/ml). All PMS-treated groups, including 25 % ($207.66 \pm 8.54$pg/ml), 50% ($207.46 \pm 5.98$pg/ml) and 100% ($227.32 \pm 3.12$ pg/ml), significantly showed an up-regulation of GABA$_{A}$ receptor in the hippocampi of PTZ-kindled Wistar rats compared to NS treated group ($187.58 \pm 19.15$ pg/ml). The hippocampi of sodium valproate-treated rats ($232.68 \pm 4.16$ pg/ml) showed the highest number of GABA$_{A}$ receptors, followed by 100% PMS ($227.32 \pm 3.12$ pg/ml), while 50% PMS had the least quantity ($207.46 \pm 5.98$pg/ml).

Though, no significant difference was observed within PMS-treated groups 25 % ($207.66 \pm 8.54$pg/ ml),50% ($207.46 \pm 5.98$pg/ml),100% ($227.32 \pm 3.12$ pg/ml) and with valproate treated group ($232.68 \pm 4.16$ pg/ml).

The result showed a decrease in the mean amount of hippocampal GAT-3 recorded in the rats administered sodium valproate ($235.07 \pm 16.40$ pg/ml) relative to those that received NS ($242.40 \pm 6.61$pg/ml). A decrease was observed in the mean quantity of hippocampal GAT-3in all three doses of PMS supplementation 25% ($225.54 \pm 13.18$pg/ml), 50% ($207.58 \pm 6.62$pg/ml) and 100% ($200.06 \pm 11.16$pg/ml) when compared to those of rats administered NS ($242.40 \pm 6.61$pg/ml) and the difference was not statistically significant $[F(4,20)=2.460, \ p=0.79]$. Compared to sodium valproate ($235.07 \pm 16.40$ pg/ml), PMS supplementation at the tested doses showed a dose-dependent decrease in the amount of hippocampal GAT-3 with the highest decrease in 100 % ($200.06 \pm$...
11.16 pg/ml) and lowest in 25% (225.54 ± 13.18 pg/ml) (Table 1).

Table 1: Effect of Pearl Millet Supplement (PMS) on Mean Hippocampal GABAergic system in Pentylenetetrazole-kindled Wistar Rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>GABA conc. (pg/ml)</th>
<th>GABAAR (pg/ml)</th>
<th>GABA-3-Tp (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 % PGM +NS + PTZ</td>
<td>157.46 ± 12.32</td>
<td>187.58 ± 19.15</td>
<td>207.58 ± 06.62</td>
</tr>
<tr>
<td>100 % PGM +NaVal + PTZ</td>
<td>205.92 ± 5.52a</td>
<td>232.68 ± 4.16b</td>
<td>235.07 ± 16.40</td>
</tr>
<tr>
<td>75 % PGM+25% PMS +PTZ</td>
<td>215.20 ± 7.26a</td>
<td>207.66 ± 8.54b</td>
<td>225.54 ± 13.18</td>
</tr>
<tr>
<td>50 % PGM+25 % PMS +PTZ</td>
<td>235.46 ± 9.41a</td>
<td>207.46 ± 5.98b</td>
<td>242.40 ± 06.61</td>
</tr>
<tr>
<td>100% PMS + PTZ</td>
<td>215.58 ± 9.25a</td>
<td>227.32 ± 3.12b</td>
<td>200.06 ± 11.16</td>
</tr>
</tbody>
</table>

Superscripts a, and b indicate a significant difference p≤ 0.05

Effect of Pearl Millet Supplement (PMS) on Mean Hippocampal Glutamatergic system in Pentylenetetrazole-kindled Wistar Rats

PTZ-kindled Wistar rats treated with sodium valproate showed a decrease in the amount of hippocampal glutamate (9.76 ± 0.98 pg/ml) relative to those that received NS (13.20 ± 1.42 pg/ml). Hippocampal glutamate level was higher in 25% PMS supplemented rats (10.16 ± 0.87 pg/ml) compared to sodium valproate treated rats (9.76 ± 0.98 pg/ml), whereas in rats fed with 50% PMS (9.74 ± 0.94 pg/ml) and 100% PMS (8.76 ± 0.98 pg/ml) lower levels of hippocampal glutamate were recorded compared to sodium valproate treated rats (9.76 ± 0.98 pg/ml). The amount of hippocampal glutamate was highest with 25% PMS-fed rats and lowest with 100% PMS (8.76 ± 0.98 pg/ml), even though the difference was not statistically significant. A significant down-regulation of hippocampal N2-NMDA receptor was observed in sodium valproate-treated group (255.00 ± 10.91 pg/ml) compared to NS-treated rats (342.68 ± 13.53 pg/ml).
50% (442.20 ± 33.82 pg/ml) and 100% (427.68 ± 4.47 pg/ml) demonstrated significant [F(4,20)=14.565, p<0.001] increase in the quantity of hippocampal glutamate-2-transporter as compared to NS treated group (190.00 ± 22.23 pg/ml). Only 25% PMS supplemented rats had a slightly higher amount of hippocampal glutamate-2-transporter as compared to that of sodium valproate treated rats (457.70 ± 34.14 pg/ml), while lower levels of hippocampal glutamate-2-transporter were recorded in rats fed with 50% (442.20 ± 33.82 pg/ml) and 100% PMS (427.68 ± 4.47 pg/ml) respectively in relation to sodium valproate treated rats (457.70 ± 34.14 pg/ml) (Table 2).

Table 2: Effect of Pearl Millet Supplement (PMS) on Mean Hippocampal Glutamatergic system in Pentylentetrazole-kindled Wistar Rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Glut conc.(pg/ml)</th>
<th>NMDAR (pg/ml)</th>
<th>Glut-2-Tp(pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 %PGM +NS + PTZ</td>
<td>13.20 ± 1.42</td>
<td>342.68 ± 13.53</td>
<td>190.00 ± 22.23</td>
</tr>
<tr>
<td>100 %PGM +NaVal + PTZ</td>
<td>09.76 ± 0.98</td>
<td>255.00 ± 10.91</td>
<td>457.70 ± 34.14</td>
</tr>
<tr>
<td>75 %PGM +25% PMS +PTZ</td>
<td>10.16 ± 0.87</td>
<td>297.46 ± 07.87</td>
<td>458.10 ± 11.24</td>
</tr>
<tr>
<td>50 %PGM +50 % PMS + PTZ</td>
<td>09.74 ± 0.94</td>
<td>254.76 ± 11.55</td>
<td>442.20 ± 33.82</td>
</tr>
<tr>
<td>100 % PMS + PTZ</td>
<td>08.74 ± 0.94</td>
<td>301.20 ± 14.30</td>
<td>427.68 ± 04.47</td>
</tr>
</tbody>
</table>

Superscripts a, and b indicate significant differences (p≤ 0.05).

Discussion

GABA is the main endogenous inhibitory neurotransmitter that produces inhibitory post-synaptic potentials and can act as a natural anticonvulsant\(^1\)\(^2\) thus, inhibition of GABA-ergic neurotransmission or activity has been shown to promote and facilitate seizures, while enhancement of GABA-ergic neurotransmission is known to inhibit or attenuate seizures\(^1\)\(^3\). Hence, the ability of PMS to increase the amount of GABA in the hippocampus of PTZ-kindled rats proves its GABA-enhancing effect.

The activation of GABA receptors ultimately decreases neuronal excitation. The up-regulation of GABA\(_A\) receptors observed in the hippocampus of PTZ-kindled rats treated with various doses of PMS clearly points out the ability of PMS to enhance hippocampal GABA-ergic neurotransmission by increasing the number of sensitive receptors, which mediates the inhibitory transmission to endogenous GABA.

GATs are known to terminate GABA-ergic neurotransmission through the removal of synaptic GABA after its phasic release\(^1\)\(^4\) back into the neuron/glia\(^1\)\(^5\). A decrease in the expression of GATs by PMS ensures continuous inhibitory transmission.

Glutamate is the key excitatory neurotransmitter in the brain that results in fast/prolonged neuronal excitation\(^1\)\(^6\), with elevated glutamate levels reported from the sera of human epileptics\(^1\)\(^7\). The decrease in hippocampal glutamate in the PTZ-kindled rats treated with PMS suggests its possible role in weakening hippocampal glutamatergic transmissions, thereby promoting anti-convulsant effects. Studies have shown that activations of N-methyl-D-aspartate (NMDA) receptors are
involved in the initiation and generalisation of PTZ-induced seizures. Therefore, the down-regulation of N2-NMDA receptor in the hippocampus of PTZ-kindled rats demonstrated by PMS implies some shortened/fewer stimulations of the NMDA receptor that mediates hippocampal excitatory transmission. The increase in the quantity of glutamate-2-transporter (EAAT-2) seen with the hippocampi of the PMS-treated PTZ-kindled rats suggests its possible role in modulating glutamatergic neurotransmission since EAATs are known to be responsible for the active reuptake of synaptic glutamate into the neuronal cell (neurons and/or glia), keeping low levels of extracellular glutamate. Our result is in line with that of previous workers, who demonstrated that loss of EAAT-2 is seizuregenic while over-expression is protective against seizures and epilepsy.

Conclusion
Our results support the neuro-protective potential of PMS, pointing to a number of contributing mechanisms involving the enhancement of hippocampal GABA concentration, upregulation of GABA receptor and glutamate-2-transporter, downregulation of NMDA receptor.

Conflict of interest: The authors declare no conflict of interest

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References


