Tetramethylpyrazine contributes to the neuroprotection in a rodent epileptic model of pentylenetetrazole-induced kindling

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Running title: Neuroprotection against PTZ induced kindling

Abstract:

Objectives: In the present study, TMP was evaluated for its therapeutic potential as an alternative therapy for epileptogenesis and its associated comorbidities in rats.

Methods: The sub-convulsant dose of Pentylenetetrazole (PTZ) (35 mg/kg, i.p) was injected on alternative days to produce kindling for 32 days and observed for seizure score percent of kindled animals in each group. After kindling, the animals were evaluated in models of anxiety, memory, and predictive of depression. The neuroprotective effect of TMP was assessed by estimating the biochemical parameters in the cortex and hippocampus of the brain. Histopathological alterations were also observed in the cortex and hippocampus (CA1, CA3, and DG).

Key findings: The administration of TMP reduced the seizure score and percentage of kindled animals dose-dependently. Furthermore, TMP significantly improved the behavioural parameters measured in the predictive models of depression but not in the anxiety and cognitive performances of the animals. The oxidative-nitrosative stress, excitotoxicity, neuroinflammation, and histological alterations in the brain induced by PTZ were significantly mitigated by administering the TMP high dose of 60 mg/kg.

Conclusion: In conclusion, the TMP attenuated the depression behaviour in the PTZ induced kindled rats, and reduced the oxidative-nitrosative stress, excitotoxicity, neuroinflammation, and histological alterations of the brain.

Introduction

Epilepsy is a chronic neurological disorder characterized by synchronizing abnormal electrical discharge of a group of excitable neurons, manifested as seizures.[1] Epilepsy is known for its behavioural abnormalities, which are estimated to affect 70 million population worldwide.[2] The ultimate goal in treating epilepsy is to offer a good quality of life without seizures and associated comorbidities. Epilepsy and its associated neuropsychiatric manifestations like depression, anxiety, and cognitive impairments have been recognized for decades. Despite having several new antiepileptic agents, the management of epilepsy was still inadequate due to the side effects and high rate of refractoriness to the existing drugs.[3,4] So, the present study was intended to investigate alternative medicine, which can provide a good quality of life without seizures.

Kindling is a widely used chronic animal model for investigating epileptogenesis and helps in evaluating the novel antiepileptic agents in the drug discovery process. The repeated administration of the sub-convulsive Pentylenetetrazole (PTZ) dose as a $GABA_A$ receptor antagonist is commonly used for kindling the animals in experimental studies.[5] Furthermore, the PTZ induced kindling exhibits behavioural alterations, revealing that this model also mimics the comorbidities of epilepsy in animal models.[6] Researchers proposed various pathophysiological mechanisms like oxidative stress, mitochondrial dysfunction,[7] neuroinflammation,[8] and imbalance in the excitatory and inhibitory neurotransmitters[9] as the underlying cause of the seizures and its comorbidities.[4] Therefore, targeting the neurochemical balance, mitigating reactive oxygen species (ROS) and reactive nitrogen species (RNS), and attenuation of neuroinflammation may be a useful preventive or treatment approach in managing epilepsy and related comorbidities.

Tetramethylpyrazine (TMP) is one of the principal active compounds isolated from *Ligusticum wallichii* (Chuan Xiong), a Chinese herbal medicine.[10] TMP has been demonstrated in the treatment of several neurovascular and cardiovascular diseases and is popularly known to exhibit anti-oxidant, anti-apoptotic and anti-inflammatory properties. Previous experimental studies have reported that TMP exerts significant neuroprotection in models of global and focal cerebral ischemia,[11,12] Parkinson's disease (PD),[13] Alzheimer's disease,[14] and traumatic brain injury (TBI).[15] In addition, TMP has been proven for its neuroprotective activity against various neurotoxic agents like 3-nitropropionic acid,[16] and also kainate-induced excitotoxicity models.[17] Literature supports that TMP's protective potential may be due to its high antioxidant potential and downregulation of pro-inflammatory cytokine production.[18,19] TMP is reported to have an ameliorating effect on mitochondrial dysfunction by promoting the biogenesis of mitochondria.[20] Furthermore, TMP showed an anti-depressant effect[21] and attenuates memory impairment in animal models. Since these reports of central nervous system effects are strongly supported that the TMP may effectively penetrate the blood brain barrier. The continuous monitoring of TMP concentrations in the blood and brain samples of rats with the help of micro-dialysis indicates that the unbound TMP best fit to a two compartment model and the elimination half-life were found to be 82.1 and 184.6 min in rat's blood and brain, respectively.[22] So the pharmacokinetic data, brain/blood concentration ratios of TMP suggested that the effective penetration of TMP through the blood brain barrier. However, whether TMP could prevent the PTZ induced kindling associated neurodegeneration and behavioural alterations is not yet known. To our knowledge, no study has explained the effect of TMP on PTZ induced kindling. Hence, keeping the above literature in mind, the present study was designed to evaluate TMP's effect on PTZ-kindling induced seizures by evaluating the behavioural, bio-chemical, pro-

inflammatory cytokines, and histopathological studies in rats. The results might implicate a new therapeutic agent with lower side effects.

Materials and methods

Animals: Adult male Wistar rats weighing 150-200 g were procured from Mahaveer Enterprises, Hyderabad, India. All the animals were maintained at standard temperature (25°C ± 5°C) and humidity (55 ± 10%), with a 12:12 h light-dark cycle. The rats were allowed to have free access to food and water ad libitum. The experimental protocol was approved by the Institutional Animal Ethical Committee (1725/GO/Re/S/13/CPCSEA) of Acharya Nagarjuna University College of Pharmaceutical Sciences, dated 29/01/2019 with an approval number ANUCPS/IAEC/AH/P/9/2019. All the experiments were performed following the committee for the purpose of control and supervision of experiments on animals (CPCSEA), India.

Drugs: Tetramethylpyrazine (Sigma-Aldrich, USA), PTZ (Alfa aesar, India), Valproic acid (Sun Pharmaceutical Industries Ltd, India), TBA (Otto Chemical, India). 5,5' dithiobis-(2-Nitrobenzoic acid) DTMB (Loba Chemie, India) and all other chemicals were purchased from Hi-Media Laboratories Pvt, Ltd., Mumbai.

Dose selection: The dose of PTZ[23] and TMP[16] was selected from the previous literature.

Experimental design: Animals were randomly divided into five groups, and each group consisted of 15 animals. The treatment schedule was summarized as follows.

Group I: normal saline (i.p)

Group II: PTZ (35 mg/kg, i.p)

Group III: Valproic acid (150 mg/kg, i.p) + PTZ (35 mg/kg, i.p)

Group IV: TMP (30 mg/kg, i.p) + PTZ (35 mg/kg, i.p)

Group V: TMP (60 mg/kg, i.p) + PTZ (35 mg/kg, i.p)

The first group of animals served as a normal control group and was administered with normal saline intraperitoneally (i.p). The second group of animals was administered with PTZ (35 mg/kg, i.p) and served as a seizure control group. The third group was administered with a standard drug, valproic acid (150 mg/kg, i.p), along with PTZ (i.p), and served as standard treatment. The fourth and fifth groups were administered with TMP (30 mg/kg and 60 mg/kg, i.p) suspended in normal saline, respectively, along with PTZ (35 mg/kg, i.p). In all the experimental groups, the treatments were given 30 min before the administration of PTZ. The respective treatments were given for 32 days, with 16 alternative injections of PTZ.[24] After 24 hours of the last PTZ injection, behavioural performances were assessed in all the animals, but the forced swim test was performed 48 hours after kindling. After 48 hours, the animals were sacrificed to estimate biochemical and histological alterations in the brain.

Kindling procedure: The sub-convulsant dose of PTZ (35 mg/kg, i.p) was dissolved in saline and injected on alternative days to all the animals except the normal control group. The animals were observed for 30 min for seizure scores by placing the animals in individual boxes after the PTZ injection. The intensity of the seizure was recorded according to Racine's seizure score[25] as follows:

Score 0- No response

Score 1- Hyperactivity, restlessness, and vibrissae twitching

Score 2- Head nodding, head clonus, and

Score 3- Myoclonic jerks

Score 4- Forelimb clonic seizure with rearing

Score 5- Generalized tonic-clonic seizures with falling.

Animals were considered kindled when the seizure control group presented a seizure score of 4 or 5 in three consecutive PTZ injections.

Behavioural parameters: Each group of animals was divided into two subgroups with an equal probability of having kindled and non-kindled animals randomly to assess the behavioural parameters. The first subgroup of animals was used to determine the open field test,[26] novel object recognition test (NOR),[27] and novel place recognition test (NPR)[27], elevated plus maze test for anxiety.[24] The second subgroup of animals from each group was assessed for the Y maze test,[16] elevated plus maze test for memory,[16] and forced swim test.[28] The tests were performed in the same order as described in the previous literature.

Biochemical estimations: All the subgroups of animals were euthanized with ketamine 60 mg/kg/i.p and decapitated for harvesting the brain samples. Six brain samples from the first subgroup were used to estimate oxidative stress parameters, nitrite levels, and acetylcholinesterase activity (AChE) in the half of the cerebral hemisphere. The remaining cerebral hemisphere was used for the estimations of GABA and glutamate. The biochemical oxidative stress parameters like malondialdehyde (MDA),[29] nitrite,[30] reduced glutathione (GSH),[31] catalase (CAT)[32] and superoxide dismutase (SOD),[32] along with AChE[33] were estimated in the brain homogenate of the hippocampus and cortex, as described in the previous literature. The amino acid neurotransmitters like GABA and glutamate were evaluated in all the hippocampus and cortex of the brain by using paper chromatography, as described in the previous literature.[34]

Estimation of pro-inflammatory cytokines: The remaining six animals from the second subgroup were euthanized with ketamine 60 mg/kg/i.p and decapitated for harvesting the brain samples. The brain samples were sectioned into the hippocampus and cortex from each cerebral

hemisphere, and used to estimate pro-inflammatory cytokines. The remaining half of the cerebral hemisphere from each animal was used for the histological studies.

The dissected hippocampus and cortex were rinsed with 0.9% cold saline and homogenized (10% w/v) individually in ice-cold phosphate buffer (0.1 M, pH 7.4), centrifuged at 10,000 RPM to collect the supernatant for the estimations of IL-β and TNF-α in the hippocampus and cortex. The analysis of IL-β and TNF-α was done using ELISA kits and the protocol of the (ELAB-sciences, China).[35]

Histopathological study: The remaining half of each group's cerebral hemispheres were stored in the formalin (10 % v/v) solution. After 24 hours, the brain samples were embedded in the paraffin wax, dehydrated with a series of alcohol with different concentrations, and cleaned the samples with xylene. Three coronal sections from each brain sample were dissected into 5 µm thickness and stained with hematoxylin and eosin (H & E) to observe the viable neuronal cell count in the hippocampus and cortex.[34]

Statistical analysis

All the values were expressed as Mean ± standard error mean (SEM). The seizure score was analyzed using two-way ANOVA, followed by Bonferroni's post hock test for multiple comparisons. The behavioural parameters were analyzed by Kruskal-Wallis test followed by Dunn's Multiple Comparison test. All the other parameters were analyzed by one-way ANOVA followed by Tukey's test by using Graph pad prism. The significance was set at $P \leq 0.05$.

Results

Effect of TMP on seizure score: The sub-convulsive dose of PTZ for 32 days gradually increased the seizure score in 1st, 2nd, 3rd and 4th week significantly ($P<0.01$, $P<0.001$, $P<0.001$

and P<0.001 respectively) in the PTZ group of animals compared to the control group of animals [F (4, 55) = 46.7]. TMP 60 mg/kg showed a significant reduction in seizure score and % kindling in the 3rd (P<0.001) and 4th (P<0.001) week of the study period compared to the PTZ treatment group. However, the low dose of TMP 30 mg/kg decreased the seizure score and % kindling, but the results were not significant compared to the PTZ treatment group at the end of the treatment schedule. The valproate treatment group showed efficient protection against PTZ induced kindling. It also showed a significant difference in seizure scores at 3rd (P<0.001) and 4th (P<0.001) weeks than the PTZ treatment group of animals (**Table 1 and Figure 1**).

Effect of TMP on open field test: The number of crossings indicates the exploratory behaviour of the animals in the open field test. In this test, we observed a significant (P<0.05) decrease in the number of crossings in the PTZ control group of animals compared to the normal control group of animals. The treatment groups valproate and TMP did not present any significant increase in the number of crossings compared to the PTZ control group of animals (**Figure 2A**).

The anxiety-like behaviour of animals was assessed by the % of time spent in the central square of the open field. The % time spent in the central square represents the anxiolytic behaviour of the animals. In the present study, the PTZ control group of animals reduced the % time spent in the central square than the control group of animals, but not significantly. The VPA and TMP treatment groups were also not significantly different from the PTZ control group of animals in % of the time spent in the central square of the open field test. (**Figure 2B**).

Effect of TMP on the elevated plus-maze test: The elevated plus-maze test was performed to evaluate the anxiety-like behaviour of the animals. In our study, the PTZ control group of

animals showed a significant ($P<0.05$) decrease in the % of time spent in the open arm and also a significantly ($P<0.01$) decrease in the number of open arm entries. The treatment groups valproate, TMP 30 mg/kg, and 60 mg/kg did not significantly increase the % of time spent in the open arm and the number of open arm entries. The anxiety index showed a significant increase ($P<0.01$) in the PTZ control group of animals compared to the normal control group of animals. The treatment group, valproate showed a significant decrease ($P<0.05$) in the anxiety index than the PTZ control group of animals. In contrast, TMP 30 & 60 mg/kg did not significantly decrease the anxiety index than the PTZ control group of animals (**Figures 3A, 3B & 3C**).

The elevated plus-maze test was also performed to evaluate the memory dysfunction in animal models. In the present study, the PTZ control group of animals showed a significant ($P<0.001$) increase in the retention transfer latency compared to the control group of animals. The treatment group valproate, showed a significant ($P<0.01$) amelioration of memory dysfunction by decreasing the retention transfer latency as compared to the PTZ control group of animals. The TMP treatment at both doses also noticeably decreased the retention transfer latency (**Figure 4**).

Effect of TMP on Y-maze test: The spatial memory was significantly ($P<0.001$) decreased in the PTZ control group of animals by reducing the % spontaneous alternations compared to the control group of animals. The treatment group valproate showed a significant ($P<0.01$) increase in the % spontaneous alternations when compared to the PTZ control group of animals, which indicates the improvement of spatial memory. The TMP treatment also showed the fairly substantial increase in the % spontaneous alternations when compared to the PTZ control group of animals (**Figure 5**).

Effect of TMP on novel object recognition test: In the memory phase of the NOR test, almost all groups of animals except PTZ group showed a considerable (but not significant) increase in exploration behaviour at the novel object rather than the familiar object. The PTZ control group of animals not at all significantly explored the novel and familiar objects. Accordingly, the exploratory ratio of the PTZ control group of animals was significantly ($P<0.01$) decreased when compared to the control group of animals. However, the treatment group's valproate and TMP 30 & 60 mg/kg didn't show a significant increase in exploratory ratios compared to the PTZ control group of animals (**Figures 6A & 6B**).

Effect of TMP on novel place recognition test: The PTZ control group of animals relatively spent less time at the novel place than in the familiar place compared to the control animals ($P<0.05$). The treatment group valproate alone showed a considerable (but not significant) increase in novel place exploration rather than the familiar place. The PTZ control group of animals showed a significant ($P<0.01$) inhibition of the exploratory ratio compared to the control group of animals. The treatment group valproate showed a significant ($P<0.05$) increase in the exploratory ratio compared to the PTZ control group of animals. The TMP treatment did not show a significant increase in the exploration ratio compared to the PTZ control group of animals (**Figures 7A & 7B**).

Effect of TMP on forced swim test: The PTZ control group of animals showed a significant ($P<0.001$) increase in the immobility time compared to the control group of animals. The

249 treatment groups valproate, and TMP 60 mg/kg showed significant ($P<0.05$) decrease in the
250 immobility time when compared to the PTZ control group of animals (**Figure 8**).

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252 **Effect of TMP on oxidative stress parameters:** The MDA and nitrite levels were significantly
253 increased in the PTZ control group of animals compared to the normal control group of animals
254 in the hippocampus [$F (4, 25) = 5.26$, $P<0.01$ & $F (4, 25) = 15.2$, $P<0.001$] and cortex [$F (4, 25)$
255 $= 4.76$, $P<0.01$ & $F (4, 25) = 7.86$, $P<0.001$] respectively [$F (4, 25) = 5.26$. The standard VPA
256 treatment group showed significant alleviation in MDA ($P<0.01$ & $P<0.05$) and nitrite levels
257 ($P<0.01$) in the hippocampus and cortex when compared to the PTZ control group of animals.
258 The TMP 60 mg/kg showed a significant ($P<0.01$) decrease in MDA and nitrite levels in the
259 hippocampus and the cortex. The TMP 30 mg/kg did not significantly decrease MDA and nitrite
260 levels compared to the PTZ control group of animals.

261 The endogenous antioxidant parameters like GSH [$F (4, 25) = 10.3$, $P<0.001$ & $F (4, 25)$
262 $= 7.21$, $P<0.01$], CAT [$F (4, 25) = P<0.001$ & $F (4, 25) = 19.3$, $P<0.001$] and SOD [$F (4, 25) =$
263 5.34, $P<0.01$ & $F (4, 25) = 5.29$, $P<0.01$] were significantly diminished in the PTZ control group
264 of animals in the hippocampus and cortex, respectively as compared to the normal control group
265 of animals. The VPA treatment group significantly attenuated the PTZ induced alteration in the
266 levels of GSH ($P<0.01$), CAT ($P<0.01$ & $P<0.001$), and SOD ($P<0.05$) in the hippocampus and
267 cortex, respectively, as compared to the PTZ control group of animals. The TMP 60 mg/kg
268 significantly improved the levels of GSH ($P<0.01$), CAT ($P<0.01$) and SOD ($P<0.05$) in the
269 hippocampus and cortex, respectively, as compared to the PTZ control group of animals. The
270 TMP 30 mg/kg showed a significant elevation in the CAT ($P<0.05$) levels in the cortex alone as
271 compared to the PTZ control group of animals (**Table 2**).

Effect of TMP on AChE activity: The administration of a chronic sub-convulsive dose of PTZ significantly raised the activity of AChE [$F(4, 25) = 12.6$, $P<0.001$ & $F(4, 25) = 13.4$, $P<0.01$] in the hippocampus and cortex, respectively, compared to the normal control group of animals. The VPA treatment significantly inhibited the AChE ($P<0.001$ & $P<0.01$) activity in the hippocampus and cortex, respectively, compared to the PTZ control group of animals. The TMP treatment groups 30 mg/kg ($P<0.05$) and 60 mg/kg ($P<0.01$ & $P<0.05$) attenuated the activity of AChE in the hippocampus and cortex, respectively when compared to the PTZ control group of animals, whereas significance was not found in the cortex of TMP 30 mg/kg treatment group (**Figure 9**)

Effect of TMP on GABA and glutamate: The administration of chronic sub-convulsive doses of PTZ significantly ($P<0.01$) altered the neurotransmitters in the brain by escalating the glutamate levels and declining the GABA levels in the hippocampus [$F(4, 25) = 5.32$ & $F(4, 25) = 5.19$] and cortex [$F(4, 25) = 5.58$ & $F(4, 25) = 4.13$] when compared to the control group of animals respectively. The VPA treatment significantly ($P<0.05$) diminished the neurotransmitter alterations in the hippocampus and cortex induced by the PTZ compared to the PTZ control group of animals. The TMP 60 mg/kg showed a significant ($P<0.05$) decrease in the glutamate levels in the hippocampus and cortex when compared to the PTZ control group of animals. GABA levels were also significantly ($P<0.05$) elevated in the hippocampus but not in the cortex than in the PTZ control group of animals. At the same time, the low dose of TMP 30 mg/kg did not significantly differ from the PTZ control group of animals in both hippocampus and cortex (**Figures 10A & 10B**).

Effect of TMP on pro-inflammatory cytokines: The administration of PTZ significantly ($P<0.001$) elevated the pro-inflammatory cytokines (IL-1β & TNF-α) in the hippocampus [$F(4, 25) = 30.80$ & $F(4, 25) = 9.864$] and cortex [$F(4, 25) = 41.51$ & $F(4, 25) = 14.73$] when compared to the control group of animals. The standard VPA treatment group significantly reduced the levels of IL-1β and TNF-α in both the hippocampus ($P<0.001$ and $P<0.01$) and cortex ($P<0.001$ and $P<0.05$), respectively when compared to the PTZ control group of animals. The TMP 60 mg/kg treatment group was significantly decreased the IL-1β and TNF-α levels in both the hippocampus ($P<0.001$) and cortex ($P<0.05$), whereas the TMP 30 mg/kg has not significantly decreased the levels of pro-inflammatory cytokines, except IL-1β ($P<0.05$) in the hippocampus when compared to the PTZ control group of animals (**Figures 11A & 11B**).

Effect of TMP on histological alterations in the hippocampus and cortex: The present study observed histological alterations in the hippocampus (CA1, CA3, and DG) and cortex. The PTZ treatment group significantly ($P<0.001$) decreased the viable cell count in the different regions of the hippocampus (CA1, CA2, and DG) [$F(4, 25) = 18.7$, $F(4, 25) = 23.8$, and $F(4, 25) = 14.1$] and cortex [$F(4, 25) = 14.1$]. The treatment group VPA significantly attenuated the changes induced by PTZ in the hippocampus CA1 ($P<0.01$), CA3 ($P<0.001$), and DG ($P<0.01$) along with the cortex ($P<0.01$) as compared to the normal control group of animals. The TMP of 30 mg/kg increased the viable neuronal cell count in all the regions, but significance ($P<0.05$) was only found in the CA3 and DG regions of the brain compared to the PTZ control group of animals. The high dose of TMP 60 mg/kg attenuated PTZ induced neuronal loss significantly ($P<0.05$) in the hippocampus (CA1, CA3, and DG) and cortex as compared to the PTZ control group of animals (**Figure 12 & Table 3**).

Discussion:

The PTZ induced model was well established to explore the epileptogenesis pattern and the behavioural, biochemical, and neurochemical alterations affected by epilepsy.[36] Further, the kindling model is a pivotal and indistinguishable model of pharmacoresistant epilepsy, interfering with the current antiepileptic drug therapy.[23] So, in the present study, we investigated the effect of TMP on the development of PTZ induced kindling and its associated behavioural despair in rats.

According to the previous reports,[5,23,37] repetitive administration of sub-convulsive doses of PTZ on alternative days results in kindling, as evidenced by the seizure score adapted from Racine's scale and % of animals kindled. Our finding in the present study revealed that the treatment with TMP 60 mg/kg diminished seizure score and the % of animals kindled at the end of the 4th week of kindling with PTZ. A recent study demonstrated that the TMP 20 mg/kg and 50 mg/kg significantly reduced the seizure score in electrical kindled mice. In contrast, TMP did not reverse the generalized seizures induced by both maximal electroshock (MES) and pentylenetetrazole (PTZ) models in the same study.[38] Our study results are consistent with their research in the kindling model, but the significant reversal of seizure score was observed only in the late stage of the treatment protocol (3rd and 4th week). We hypothesized that the chronic treatment with TMP reduced the progression of seizure score in the late stage of PTZ kindling instead of the initial stages of the kindling process.

Recurrent seizures are associated with emotional imbalance,[39] psychological problems,[40] and cognitive impairment.[41] Many patients with epilepsy have been diagnosed

with emotional disorders like anxiety and depression.[42] In the present study, the open field test and elevated plus maze test were performed to evaluate the anxiety-like behaviour in the PTZ induced kindled animals. There were no significant differences between the groups in the open field test. In line with the previous literature,[43] in the present study, PTZ induced kindling did not increase the time spent in the periphery of the open field test, which is not anxiogenic. VPA and TMP treatment groups were also not significantly different from the PTZ alone treatment group. In contrast, in the elevated plus-maze test, the PTZ control group increased the anxiety index more than the control animals. In rodents showing repugnance towards the open and elevated spaces, they spent more time in the dark and enclosed areas, indicating an anxiogenic nature of the animals. In the present study, the PTZ induced kindling associated anxiety in the elevated plus-maze test is in line with the previous reports.[6,44] Our study results showed that the treatment group VPA showed a significant decrease in the anxiety index in the elevated plus-maze test. Additionally, TMP 60 mg/kg treated animals showed a fairly substantial decrease in the anxiety index. A recent study[45] has demonstrated that the TMP treatment decreased the anxiety index and reduced the grooming behaviour in the single prolonged stress (SPS) animal model. Further, in the same study, it was also proved that TMP administration reduced anxiety-like behaviour, which is indicated by an increase in the central zone exploration during the open field test. Lee et al. (2018)[45] hypothesized that the anti-anxiety effect of TMP is related to its inhibitory effect on serotonergic dysregulation. Our results of the elevated plus-maze test are in line with the previous literature.[45]

Experimental and clinical evidence has proved that epileptic patients suffer from cognitive impairment.[46] It has been shown that the memory deficit caused by chronic administration of PTZ is a result of excessive generation of free radicals and subsequent neuronal

damage in several regions of the brain.[47] Some other factors may contribute to kindling-induced cognitive impairments, such as a decline in the acetylcholine levels and increased neuronal death in the hippocampal regions of the brain.[23] In the present study, we found that the PTZ induced kindling results in cognitive impairment, which is evident in the elevated plus-maze test, Y-maze test, novel object recognition test, and novel place recognition test. The PTZ kindling-induced cognitive impairment results are in good agreement with the previous literature.[23,43] With the exception of the novel object recognition test, treatment with VPA in the current investigation alleviated cognitive deficits. The TMP treatment demonstrated dose-dependent therapeutic effectiveness even though it did not significantly address cognitive impairments. Several reports supported the protective effect of TMP on memory impairment. TMP proved to be a potent pharmacological agent in improving cognitive performance by restoring cAMP/PKA/CREB signalling pathway deficit against scopolamine-induced memory impairment.[48] In another study, TMP mitigated the short-term and long-term memory impairment induced by intracerebral administration of streptozotocin by inhibiting the GSK-3β and restoring the cholinergic function.[49] Our recent study on the effect of TMP against 3-nitropropionic acid-induced neurotoxicity showed a significant improvement in cognitive performance by protecting the neuronal cells in the hippocampus and restoring the cholinergic neurotransmission in the brain.[16] The present study results are in good agreement with the previous literature.[16,23]

Another most frequent comorbidity associated with epilepsy is depression, with a prevalence of 25-55% in epileptic patients.[50] The present study assessed the depression in kindled animals by the forced swim test. A considerable increase in the immobility time indicates depression in animal studies. PTZ control group of animals showed depression-like behaviour, as revealed by longer immobility time in the forced swim test. Imbalance in the

central monoaminergic levels has been considered the major contributing factor in the development of depression.[51] Studies showed that a decline in the monoamines in PTZ induced kindled rats results in a longer immobility time in the forced swim test.[1] Our study also showed a significant increase in the immobility time in the PTZ control group of animals. Whereas treatment with VPA and TMP (60 mg/kg) reduced the immobility time significantly, suggesting the potential of TMP in mitigating the PTZ induced depression. The results are in tune with the earlier reports, demonstrated in several animal models. Alteration in the hippocampal neuronal cells is the hallmark in the animal models of depression. In addition, scientific reports also indicated the role of oxidative stress in depression-like symptoms and the therapeutic benefits of several antioxidants in dealing with depression.[52] Our study results proved that the treatment with TMP restored the antioxidant defence in different brain regions, which may be the contributing factor in decreasing the immobility time in kindled rats. Some studies stated that the TMP treatment promoted the BDNF signalling pathway, and phosphorylation of CREB proteins in the hippocampus may be attributed to its anti-depressant activity.[21] The present study results showed the anti-depressant effect of TMP, which may be attributed to its neuronal protection in the hippocampus.

In the previous literature, it was reported that a significant increase in the brain/blood concentration ratio of TMP[22] and a lower brain/blood concentration ratio to the VPA indicates a higher penetration of TMP into the brain rather than the VPA.[53] Contrary to the pharmacokinetic data, in the present study, the behavioural alterations induced by the PTZ kindling were significantly mitigated by the standard drug VPA rather than the TMP administration. However, treatment with the TMP substantially reduced the behavioural alterations dose dependently. Hence, the possible reason for an insignificant improvement in

behavioural alterations with the TMP might be due to its modest dose administration than the VPA.

Studies have demonstrated that redox homeostasis is essential for the brains' normal functioning. The excessive generation of ROS and RNS contributes to impairment in the brain's redox state, which appears to be involved in the pathogenesis of epilepsy.[54,55] Accumulating evidence indicated that the administration of PTZ increases the generation of ROS and RNS, which may play an essential role in neuronal damage.[56] Moreover, currently using conventional antiepileptic drugs disrupt the redox homeostasis by increasing the oxidative stress, thereby worsening the brain's antioxidant status that may hinder the antiepileptic activity.[2,55] So, it may prove worthwhile to use an alternative antiepileptic agent with potent antioxidant properties in modulating the process of epileptogenesis. MDA is an end product of lipid peroxidation, an indicator of oxidative damage. The innate antioxidant defence system like SOD, CAT, and GSH acts as scavengers against oxidative stress.[57] Our study results confirm with the earlier studies that the PTZ kindled rats showed a significant increase in the MDA levels and a significant decline in the SOD, CAT, and GSH levels in different regions of the brain. The treatment with VPA and TMP restored the antioxidant defence system and reduced MDA levels in the brain. The study findings confirm the antioxidant potential of TMP and its neuronal protection. Further, TMP and its derivatives proved to be a potent activator of the Nrf2 signalling pathway. They were responsible for enhancing the antioxidant defence system, inhibiting the excitotoxicity, and inhibiting the apoptotic process in the neuronal cells.[58,59]

Maintaining a balance between the excitatory and inhibitory neurotransmitters in the CNS plays a crucial role in preventing neuronal disorders, especially epilepsy. It has been reported that the elevated levels of NO in the striatum modulate the release of the excitatory

neurotransmitter glutamate in the chemically induced neurotoxic models.[60] On the other hand, studies proved that the elevation of nitrotyrosine promoted the peroxidation of lipids. Observations in the PTZ kindled animals lend additional confirmation to the involvement of nitrosative stress in the seizure-mediated hippocampal neurodegeneration.[56] In the present study, NO levels were indirectly measured by estimating the nitrite levels in the brain. The PTZ control group showed elevated nitrite levels in the hippocampus and cortex in tune with the literature. The treatment with VPA and TMP reduced the levels of nitrite induced by PTZ in the hippocampus and cortex. TMP proved its inhibitory response on iNOS to reduce the nitrosative stress and inflammatory response in the retinal capillary endothelial cells.[61] Thus, reducing nitrosative stress might be a prominent therapeutic approach to mitigating epileptogenesis in kindles rats.

Studies have documented the cholinergic dysfunction in the PTZ kindled animals.[23] In tune with the literature, the present study also showed a significant decline in the acetylcholine levels in the hippocampus and cortex, which was evident by an indirect measure of increased AChE activity. Furthermore, elevated AChE may be a significant contributing factor in kindling-induced cognitive impairment.[23] Since the dysfunction in the cholinergic system of the epileptic brain has been reported in the literature, we hypothesized that the enhancement of cholinergic activity in the brain is a potential target in mitigating epilepsy and its related comorbidities.[62] Treatment with the VPA and TMP showed significant mitigation in cholinergic dysfunction. A recent study on the effect of TMP against 3-NP induced Huntington's disease-like symptoms in rodents also proved the role of TMP in increasing the Ach levels in the brain.[16] So, the results proposed TMP's role in ameliorating epilepsy and its associated comorbidities by declining the AChE in the hippocampus and cortex.

The alterations of excitatory and inhibitory neurotransmission are implicated in the pathophysiology of epileptic patients and animal models.[60] The previous literature proved that the administration of PTZ induced kindling elevated the levels of glutamate and decreased GABA levels in the hippocampus and cortex of the brain.[23] Studies showed that elevated levels of glutamate and aspartate were observed in the brain's extracellular regions following the administration of PTZ in rats.[36] Further, the PTZ- induced kindled rats showed decreased neuronal uptake of glutamate, which results in neuronal excitotoxicity.[63] Indeed, the present study results showed elevated levels of glutamate and reduced GABA levels in the PTZ kindled rats. The cell cultures of glioma treated with the TMP reduced the glutamate levels in the culture media. It was speculated that it might be due to inhibition of glutamate biosynthesis and enhancement of glutamate uptake.[64] The TMP also proved its neuroprotective activity against 3-nitropropionic acid-induced neurotoxicity by elevating the GABA levels in the brain.[16] In line with the previous literature, the TMP 60 mg/kg treatment significantly opposed the kindling-induced neurochemical alteration by decreasing the glutamate levels and elevating the GABA levels in both hippocampus and cortex. Thus, the present study results suggest a firm link between the neurochemical balance in treating epilepsy and its associated comorbidities with TMP.

In addition, the experimental and clinical evidence indicates that the inflammation in the brain might be a consequence of epilepsy or its cause.[65] The animal models of epilepsy also showed increased pro-inflammatory cytokines like IL-1β, IL-6, and TNF-α in the brain tissue.[2,66] Similar observations were also found in the serum and cerebrospinal fluid samples of epileptic patients.[67] The overexpression of inflammatory cytokines like IL-6 and TNF-α in the astrocytes demonstrated the decreased seizure threshold, increased frequency of spontaneous

seizures, and neuronal loss in animal models.[68,69] On the other hand, IL-1β regulates neuronal excitability by decreasing the production of the inhibitory neurotransmitter GABA[70] and may promote other cytokines IL-6 TNF-α.[71] In the present study, the pro-inflammatory cytokines were significantly elevated in the PTZ control groups of animals, consistent with the previous literature.[2,66] TMP attenuated the production of pro-inflammatory cytokines in the activated microglial cells and effectively reduced NF-kB activation.[35] In another study, TMP attenuated the neuroinflammation via the miR-150/AKT3 pathway regulation and mitigated the cognitive impairment induced by anaesthetics.[72] TMP treatment groups decreased the pro-inflammatory cytokines dose-dependently in the current study in parallel with the literature. So, the neuroprotective potential of TMP might be strongly associated with the inhibition of neuroinflammation in the brain.

Further, the neuroprotective role of TMP against PTZ induced kindling was assessed by histological studies in the hippocampus (CA1, CA3, and DG) and cortex. Recurrent seizures lead to neuronal death.[73] Studies have shown that the PTZ kindling induces neuronal damage in the hippocampus and cortex, resulting in cognitive impairment.[23,74] Literature proved that oxidative stress, mitochondrial dysfunction, and excitotoxicity promote the neuronal cells to neurodegeneration.[57] In line with the literature, study results showed a significant decrease in the viable neuronal count in the hippocampus (CA1, CA3, and DG) and cortex. VPA and TMP's treatment groups significantly protected the neuronal cells from neurodegeneration and increased the viable neuronal count in the hippocampus (CA1, CA3, and DG) and cortex. These results are in good agreement with the earlier reports, stating that the attenuation of 3-NP induced Huntington's like symptoms and cerebral ischemic conditions by the administration of TMP in rodents.[16,75]

As a multi-target product, TMP is promising and deserves more intensive research to establish an appropriate dose for its therapeutic benefit. Several studies were done to examine its clinical effectiveness; the findings are too good to be true. There is no definitive information on the average dosage, therapeutic duration, or adverse event reporting, all of which are crucial for clinical trials. The establishment of standard TMP dose use would be more beneficial. The outcomes of a clinical study would thus be more convincing and provide trustworthy support for practical decision-making. Academics still face several challenges, such as toxicity, efficacy, and pharmacological effects, despite the overwhelming interest in therapeutic usage.

Conclusion and future perspectives:

The current study results revealed that TMP's administration attenuated the development of PTZ induced kindling, but it was not significantly mitigated the behavioural alterations except depression behaviour. Whereas, VPA administration significantly attenuated the behavioral alterations when compared to the TMP treatment. However, treatment with the TMP substantially reduced the behavioural alterations dose dependently. The observed effects may be attributed to decreasing oxidative-nitrosative stress, excitotoxicity, neuroinflammation, and increasing the AChE levels in the brain. The neuroprotection against PTZ-induced kindling was confirmed by the histological observation in the hippocampus and cortex. Further pharmacokinetic and dynamic studies are warranted in preclinical and clinical scenarios to establish an appropriate dose and the development of TMP as a therapeutic regimen for the management of epilepsy and associated behavioural alterations. In conclusion, the

multifunctionality of TMP make it a good option for future investigation in the quest to attain the highest possible therapeutic effectiveness with the lowest possible toxicity.

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References:

1. Kumar M, Kumar P. Protective effect of spermine against pentylenetetrazole kindling epilepsy induced comorbidities in mice. *Neurosci Res* 2017; 120. doi:10.1016/j.neures.2017.02.003.

2. Singh N *et al.* Effect of dimethyl fumarate on neuroinflammation and apoptosis in pentylenetetrazol kindling model in rats. *Brain Res Bull* 2019; 144. doi:10.1016/j.brainresbull.2018.11.013.

3. Walker LE *et al.* Personalized medicine approaches in epilepsy. *J Intern Med* 2015; 277(2). doi:10.1111/joim.12322.

4. Kola PK *et al.* Naringin in a combined therapy with phenytoin on pentylenetetrazole-induced kindling in rats. *Epilepsy Behav* 2018; 89. doi:10.1016/j.yebeh.2018.10.006.

5. Ahmadian SR *et al.* Arbutin attenuates cognitive impairment and inflammatory response in pentylenetetrazol-induced kindling model of epilepsy. *Neuropharmacology* 2019; 146. doi:10.1016/j.neuropharm.2018.11.038.

6. de Souza AG *et al.* Prevention of pentylenetetrazole-induced kindling and behavioral comorbidities in mice by levetiracetam combined with the GLP-1 agonist liraglutide: Involvement of brain antioxidant and BDNF upregulating properties. *Biomed Pharmacother* 2019; 109. doi:10.1016/j.biopha.2018.10.066.

7. Aguiar CCT *et al.* Oxidative stress and epilepsy: Literature review. *Oxid Med Cell Longev* 2012. doi:10.1155/2012/795259.

8. Rana A, Musto AE. The role of inflammation in the development of epilepsy. *J*

564 *Neuroinflammation* 2018; 15(1). doi:10.1186/s12974-018-1192-7.

565 9. Shao L-R *et al.* Pediatric Epilepsy Mechanisms: Expanding the Paradigm of
566 Excitation/Inhibition Imbalance. *Children* 2019; 6(2). doi:10.3390/children6020023.

567 10. Zhao Y *et al.* Mechanisms and Clinical Application of Tetramethylpyrazine (an
568 Interesting Natural Compound Isolated from Ligusticum Wallichii): Current Status and
569 Perspective. *Oxid Med Cell Longev* 2016; 2016. doi:10.1155/2016/2124638.

570 11. Gao HJ *et al.* Ligustrazine monomer against cerebral ischemia/ reperfusion injury. *Neural*
571 *Regen Res* 2015; 10(5). doi:10.4103/1673-5374.156991.

572 12. Kao TK *et al.* Tetramethylpyrazine reduces cellular inflammatory response following
573 permanent focal cerebral ischemia in rats. *Exp Neurol* 2013; 247: 188–201.
574 doi:10.1016/J.EXPNEUROL.2013.04.010.

575 13. Lu C *et al.* Neuroprotective effects of tetramethylpyrazine against dopaminergic neuron
576 injury in a rat model of parkinson's disease induced by MPTP. *Int J Biol Sci* 2014; 10(4).
577 doi:10.7150/ijbs.8366.

578 14. Huang X *et al.* Tetramethylpyrazine Improves Cognitive Impairment and Modifies the
579 Hippocampal Proteome in Two Mouse Models of Alzheimer's Disease. *Front Cell Dev*
580 *Biol* 2021; 9: 469. doi:10.3389/FCELL.2021.632843/BIBTEX.

581 15. Zhang G *et al.* Tetramethylpyrazine Nitrone Improves Neurobehavioral Functions and
582 Confers Neuroprotection on Rats with Traumatic Brain Injury. *Neurochem Res* 2016;
583 41(11): 2948–2957. doi:10.1007/S11064-016-2013-Y/METRICS.

584 16. Danduga RCSR *et al.* Neuroprotective activity of tetramethylpyrazine against 3-

585 nitropropionic acid induced Huntington's disease-like symptoms in rats. *Biomed*
586 *Pharmacother* 2018; 105. doi:10.1016/j.biopha.2018.06.079.

587 17. Shih YH *et al.* Protective effects of tetramethylpyrazine on kainate induced excitotoxicity
588 in hippocampal culture. *Neuroreport* 2002; 13(4). doi:10.1097/00001756-200203250-
589 00032.

590 18. Zhang W *et al.* Inactivation of Macrophage Scavenger Receptor Class B Type I Promotes
591 Atherosclerotic Lesion Development in Apolipoprotein E-Deficient Mice. *Circulation*
592 2003; 108(18). doi:10.1161/01.CIR.0000093189.97429.9D.

593 19. Chang Y *et al.* Tetramethylpyrazine suppresses HIF-1α, TNF-α, and activated caspase-3
594 expression in middle cerebral artery occlusion-induced brain ischemia in rats. *Acta*
595 *Pharmacol Sin* 2007; 28(3). doi:10.1111/j.1745-7254.2007.00514.x.

596 20. Xu Q *et al.* Tetramethylpyrazine ameliorates high glucose-induced endothelial
597 dysfunction by increasing mitochondrial biogenesis. *PLoS One* 2014; 9(2).
598 doi:10.1371/journal.pone.0088243.

599 21. Jiang B *et al.* Tetramethylpyrazine produces antidepressant-like effects in mice through
600 promotion of BDNF signaling pathway. *Int J Neuropsychopharmacol* 2015; 18(8).
601 doi:10.1093/ijnp/pyv010.

602 22. Tsai TH, Liang CC. Pharmacokinetics of tetramethylpyrazine in rat blood and brain using
603 microdialysis. *Int J Pharm* 2001; 216(1–2): 61–66. doi:10.1016/S0378-5173(01)00572-5.

604 23. Kola PK *et al.* Protective effect of naringin on pentylenetetrazole (PTZ)-induced kindling;
605 possible mechanisms of antikindling, memory improvement, and neuroprotection.

606 *Epilepsy Behav* 2017; 75. doi:10.1016/j.yebeh.2017.07.011.

607 24. Lee B *et al.* L-Tetrahydropalmatine Ameliorates Development of Anxiety and
608 Depression-Related Symptoms Induced by Single Prolonged Stress in Rats. *Biomol Ther*
609 *(Seoul)* 2014; 22(3). doi:10.4062/biomolther.2014.032.

610 25. Bagheri S *et al.* Effect of probiotic supplementation on seizure activity and cognitive
611 performance in PTZ-induced chemical kindling. *Epilepsy Behav* 2019; 95.
612 doi:10.1016/j.yebeh.2019.03.038.

613 26. Lee B *et al.* Effects of systemic administration of ibuprofen on stress response in a rat
614 model of post-traumatic stress disorder. *Korean J Physiol Pharmacol* 2016; 20(4).
615 doi:10.4196/kjpp.2016.20.4.357.

616 27. Pearson JN *et al.* Specific alterations in the performance of learning and memory tasks in
617 models of chemoconvulsant-induced status epilepticus. *Epilepsy Res* 2014; 108(6).
618 doi:10.1016/j.eplepsyres.2014.04.003.

619 28. Mortazavi F *et al.* Spatial learning deficits and emotional impairments in
620 pentylenetetrazole-kindled rats. *Epilepsy Behav* 2005; 7(4).
621 doi:10.1016/j.yebeh.2005.08.019.

622 29. Ohkawa H *et al.* Assay for lipid peroxides in animal tissues by thiobarbituric acid
623 reaction. *Anal Biochem* 1979; 95(2). doi:10.1016/0003-2697(79)90738-3.

624 30. Green LC *et al.* Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal*
625 *Biochem* 1982; 126(1). doi:10.1016/0003-2697(82)90118-X.

626 31. Moron MS *et al.* Levels of glutathione, glutathione reductase and glutathione S-

transferase activities in rat lung and liver. *BBA - Gen Subj* 1979; 582(1). doi:10.1016/0304-4165(79)90289-7.

32. MARKLUND S, MARKLUND G. Involvement of the Superoxide Anion Radical in the Autoxidation of Pyrogallol and a Convenient Assay for Superoxide Dismutase. *Eur J Biochem* 1974; 47(3). doi:10.1111/j.1432-1033.1974.tb03714.x.

33. Ellman GL *et al.* A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 1961; 7(2). doi:10.1016/0006-2952(61)90145-9.

34. Polopalli S *et al.* A prognostic study on the effect of post-traumatic stress disorder on cerebral ischaemia reperfusion-induced stroke. *World J Biol Psychiatry* 2021. doi:10.1080/15622975.2021.1935318.

35. Kim M *et al.* Tetramethylpyrazine, a natural alkaloid, attenuates pro-inflammatory mediators induced by amyloid β and interferon-γ in rat brain microglia. *Eur J Pharmacol* 2014; 740. doi:10.1016/j.ejphar.2014.06.037.

36. Rocha L *et al.* Pentylenetetrazol-induced kindling: Early involvement of excitatory and inhibitory systems. In: *Epilepsy Research*, Vol 26., 1996. doi:10.1016/S0920-1211(96)00046-0.

37. Corda MG *et al.* Pentylenetetrazol-induced kindling in rats: Effect of GABA function inhibitors. *Pharmacol Biochem Behav* 1991; 40(2). doi:10.1016/0091-3057(91)90562-G.

38. Jin Y *et al.* Tetramethylpyrazine Reduces Epileptogenesis Progression in Electrical Kindling Models by Modulating Hippocampal Excitatory Neurotransmission. *ACS Chem Neurosci* 2019; 10(12). doi:10.1021/acschemneuro.9b00575.

39. Dodrill CB. Correlates of Generalized Tonic-Clonic Seizures with Intellectual, Neuropsychological, Emotional, and Social Function in Patients with Epilepsy. *Epilepsia* 1986; 27(4). doi:10.1111/j.1528-1157.1986.tb03559.x.

40. Levin R *et al.* Psychosocial Dimensions of Epilepsy: A Review of the Literature. *Epilepsia* 1988; 29(6). doi:10.1111/j.1528-1157.1988.tb04238.x.

41. Dodrill CB. Progressive cognitive decline in adolescents and adults with epilepsy. In: *Progress in Brain Research*, Vol 135., 2002. doi:10.1016/S0079-6123(02)35037-4.

42. Ettinger AB *et al.* Fatigue and depression in epilepsy. *J Epilepsy* 1998; 11(2). doi:10.1016/S0896-6974(97)00139-4.

43. Jiang Y *et al.* Ketogenic diet attenuates spatial and item memory impairment in pentylenetetrazol-kindled rats. *Brain Res* 2016; 1646. doi:10.1016/j.brainres.2016.06.029.

44. Sharma P *et al.* Apigenin reverses behavioural impairments and cognitive decline in kindled mice via CREB-BDNF upregulation in the hippocampus. *Nutr Neurosci* 2020; 23(2). doi:10.1080/1028415X.2018.1478653.

45. Lee B *et al.* Tetramethylpyrazine reverses anxiety-like behaviors in a rat model of post-traumatic stress disorder. *Korean J Physiol Pharmacol* 2018; 22(5). doi:10.4196/kjpp.2018.22.5.525.

46. Kaur H *et al.* Curcumin attenuates inflammatory response and cognitive deficits in experimental model of chronic epilepsy. *Neurochem Int* 2015; 89. doi:10.1016/j.neuint.2015.07.009.

47. Kumar A *et al.* Possible nitric oxide mechanism in the protective effect of hesperidin

against pentylenetetrazole (PTZ)-induced kindling and associated cognitive dysfunction in mice. *Epilepsy Behav* 2013; 29(1). doi:10.1016/j.yebeh.2013.06.007.

48. Wu W *et al.* Tetramethylpyrazine protects against scopolamine-induced memory impairments in rats by reversing the cAMP/PKA/CREB pathway. *Behav Brain Res* 2013; 253. doi:10.1016/j.bbr.2013.07.052.

49. Lu F *et al.* Tetramethylpyrazine reverses intracerebroventricular streptozotocin-induced memory deficits by inhibiting GSK-3β. *Acta Biochim Biophys Sin (Shanghai)* 2017; 49(8). doi:10.1093/abbs/gmx059.

50. Cramer JA *et al.* The influence of comorbid depression on quality of life for people with epilepsy. *Epilepsy Behav* 2003; 4(5). doi:10.1016/j.yebeh.2003.07.009.

51. Singh G *et al.* From seizures to epilepsy and its substrates: Neurocysticercosis. *Epilepsia* 2013; 54(5). doi:10.1111/epi.12159.

52. Rojas P *et al.* Antidepressant-like effect of a Ginkgo biloba extract (EGb761) in the mouse forced swimming test: Role of oxidative stress. *Neurochem Int* 2011; 59(5). doi:10.1016/j.neuint.2011.05.007.

53. Eskandari S *et al.* Brain delivery of valproic acid via intranasal administration of nanostructured lipid carriers: in vivo pharmacodynamic studies using rat electroshock model. *Int J Nanomedicine* 2011; 6: 363. doi:10.2147/IJN.S15881.

54. Rowley S *et al.* Mitochondrial respiration deficits driven by reactive oxygen species in experimental temporal lobe epilepsy. *Neurobiol Dis* 2015; 75. doi:10.1016/j.nbd.2014.12.025.

690 55. Williams S *et al.* Status epilepticus results in persistent overproduction of reactive oxygen
691 species, inhibition of which is neuroprotective. *Neuroscience* 2015; 303.
692 doi:10.1016/j.neuroscience.2015.07.005.

693 56. Tawfik KM *et al.* Neuroprotective mechanisms of sildenafil and selenium in PTZ-kindling
694 model: Implications in epilepsy. *Eur J Pharmacol* 2018; 833.
695 doi:10.1016/j.ejphar.2018.05.035.

696 57. Singh N *et al.* Neuroprotective effect of Nrf2 activator dimethyl fumarate, on the
697 hippocampal neurons in chemical kindling model in rat. *Epilepsy Res* 2018; 143.
698 doi:10.1016/j.eplepsyres.2018.02.011.

699 58. Chen H *et al.* A novel tetramethylpyrazine derivative protects against glutamate-induced
700 cytotoxicity through PGC1α/Nrf2 and PI3K/Akt signaling pathways. *Front Neurosci*
701 2018; 12(AUG). doi:10.3389/fnins.2018.00567.

702 59. Qing L *et al.* Tetramethylpyrazine improved the survival of multiterritory perforator flaps
703 by inducing angiogenesis and suppressing apoptosis via the Akt/Nrf2 pathway. *Drug Des*
704 *Devel Ther* 2019; 13. doi:10.2147/DDDT.S195090.

705 60. Bogdanov MB, Wurtman RJ. Possible involvement of nitric oxide in NMDA-induced
706 glutamate release in the rat striatum: An in vivo microdialysis study. *Neurosci Lett* 1997;
707 221(2–3). doi:10.1016/S0304-3940(96)13278-X.

708 61. Zhu X *et al.* Tetramethylpyrazine protects retinal capillary endothelial cells (TR-iBRB2)
709 against IL-1β-induced nitrative/oxidative stress. *Int J Mol Sci* 2015; 16(9).
710 doi:10.3390/ijms160921775.

62. Gnatek Y *et al.* Acetylcholinesterase loosens the brain's cholinergic anti-inflammatory response and promotes epileptogenesis. *Front Mol Neurosci* 2012; (MAY 2012). doi:10.3389/fnmol.2012.00066.

63. Schünzel G *et al.* Pentylenetetrazol kindling and factors of glutamate transmitter metabolism in rat hippocampus. *Neuroscience* 1992; 49(2). doi:10.1016/0306-4522(92)90102-8.

64. Fu YS *et al.* Tetramethylpyrazine inhibits activities of glioma cells and glutamate neuro-excitotoxicity: Potential therapeutic application for treatment of gliomas. *Neuro Oncol* 2008; 10(2). doi:10.1215/15228517-2007-051.

65. Vezzani A *et al.* The role of inflammation in epilepsy. *Nat Rev Neurol* 2011; 7(1). doi:10.1038/nrneurol.2010.178.

66. Hoda U *et al.* Resveratrol suppressed seizures by attenuating IL-1β, IL1-Ra, IL-6, and TNF-α in the hippocampus and cortex of kindled mice. *Nutr Neurosci* 2017; 20(9). doi:10.1080/1028415X.2016.1189057.

67. Youn Y *et al.* The role of cytokines in seizures: Interleukin (IL)-1β, IL-1ra, IL-8, and IL-10. *Korean J Pediatr* 2013; 56(7). doi:10.3345/kjp.2013.56.7.271.

68. Campbell IL *et al.* Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin 6. *Proc Natl Acad Sci U S A* 1993; 90(21). doi:10.1073/pnas.90.21.10061.

69. Probert L *et al.* TNF-α transgenic and knockout models of CNS inflammation and degeneration. In: *Journal of Neuroimmunology*, Vol 72., 1997. doi:10.1016/S0165-

5728(96)00184-1.

70. Serantes R *et al.* Interleukin-1β enhances GABAA receptor cell-surface expression by a phosphatidylinositol 3-kinase/Akt pathway: Relevance to sepsis-associated encephalopathy. *J Biol Chem* 2006; 281(21). doi:10.1074/jbc.M512489200.

71. Alomar SY *et al.* IL-1β (interleukin-1β) stimulates the production and release of multiple cytokines and chemokines by human preadipocytes. *Arch Physiol Biochem* 2016; 122(3). doi:10.3109/13813455.2016.1156706.

72. Cui H *et al.* Tetramethylpyrazine ameliorates isoflurane-induced cognitive dysfunction by inhibiting neuroinflammation via miR-150 in rats. *Exp Ther Med* 2020. doi:10.3892/etm.2020.9110.

73. Sutula TP *et al.* Do epileptic seizures damage the brain? *Curr Opin Neurol* 2003; 16(2). doi:10.1097/01.wco.0000063770.15877.bc.

74. Hashemian M *et al.* Curcumin-loaded chitosan-alginate-STPP nanoparticles ameliorate memory deficits and reduce glial activation in pentylenetetrazol-induced kindling model of epilepsy. *Prog Neuro-Psychopharmacology Biol Psychiatry* 2017; 79. doi:10.1016/j.pnpbp.2017.07.025.

75. Kao TK *et al.* Neuroprotection by tetramethylpyrazine against ischemic brain injury in rats. *Neurochem Int* 2006; 48(3). doi:10.1016/j.neuint.2005.10.008.

Table 1

Effect of TMP on seizure score and seizure latency

Groups	Week 1	Week 2	Week 3	Week 4	% of animals Kindled	Number of animals died/used
PTZ	1.33 ± 0.211 *b	1.67 ± 0.333 *c	3.17 ± 0.477 *c	4.00 ± 0.365 *c	66.66	2/15
Valproic acid +PTZ	0.500 ± 0.224	0.833 ± 0.307	1.17 ± 0.167@c	1.67 ± 0.333 @c	0	0/15
TMP 30 mg/kg + PTZ	0.667 ± 0.333	1.17 ± 0.167	1.67 ± 0.494#c	3.00 ± 0.365	33.33	1/15
TMP 60 mg/kg + PTZ	0.500 ± 0.224	1.17 ± 0.401	1.33 ± 0.211$c	2.00 ± 0.447$c	16.66	0/15

Results are expressed as mean ± SEM (n=12); *b, *c indicates P<0.01, P<0.001, respectively PTZ Vs control; @c indicates P<0.001 PTZ Vs VPA; #b, #c indicates P<0.01, P<0.001, respectively PTZ Vs TMP 30 mg; $c indicates P<0.001 PTZ Vs TMP 60 mg. Data were analyzed by two-way ANOVA, followed by Bonferroni's post hock test using Graph pad prism software. Statistical significance was set at $P \leq 0.05$.

763 **Table 2**

764 ***Effect of TMP on PTZ induced oxidative stress parameters in the hippocampus and cortex.***

	Hippocampus					Cortex				
Treatment	MDA nmol/gm wet weight tissue	Nitrite Nmol/gm wet weight tissue	GSH µmol/gm wet weight tissue	CAT µmol/min/gm wet weight tissue	SOD Units/gm wet weight tissue	MDA nmol/gm wet weight tissue	Nitrite Nmol/gm wet weight tissue	GSH µmol/gm wet weight tissue	CAT µmol/min/gm wet weight tissue	SOD Units/gm wet weight tissue
Normal control	132 ± 38.60	47.4 ± 6.08	2.02 ± 0.28	16.2 ± 1.69	19.9 ± 2.36	Normal control	123 ± 20.4	82.8 ± 4.46	2.76 ± 0.53	18.9 ± 1.45
PTZ	371 ± 58.50**	100 ± 4.97***	0.56 ± 0.08***	6.15 ± 0.96***	8.60 ± 1.38**	PTZ	328 ± 67.0**	132 ± 4.91***	0.37 ± 0.05**	6.21 ± 1.05***
VPA	149 ± 32.10$^{\#\#}$	68.5 ±3.91$^{\#\#}$	1.72 ± 0.21$^{\#\#}$	13.4 ± 1.24$^{\#\#}$	18.0 ± 1.41$^{\#}$	VPA	133 ± 15.1$^{\#}$	96.1 ± 7.93$^{\#\#}$	2.77 ± 0.61$^{\#\#}$	14.7 ± 0.97$^{\#\#\#}$
TMP 30 mg/kg	202 ± 45.60	87.3 ± 5.11	0.91 ± 0.07	9.98 ± 0.67	13.7 ± 2.17	TMP 30 mg/kg	178 ± 35.0	112 ± 6.27	1.16 ± 0.23	11.0 ± 0.94$^{\#}$
TMP 60 mg/kg	174 ± 28.40$^{\#}$	74.1 ± 5.25$^{\#}$	1.53 ± 0.18$^{\#\#}$	12.9 ± 1.27$^{\#\#}$	16.7 ± 2.00$^{\#}$	TMP 60 mg/kg	164 ± 28.6$^{\#}$	103 ± 8.54$^{\#}$	2.48 ± 0.30$^{\#\#}$	12.9 ± 0.77$^{\#\#}$

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Results are expressed as mean ± SEM (n=6); **, *** indicates P<0.01, P<0.001, respectively, Vs control; #, ## and ### indicates P<0.05, P<0.01, and P<0.001 respectively, Vs PTZ. Data were analyzed by one-way ANOVA followed by Tukey's test using Graph pad prism software. Statistical significance was set at $P \leq 0.05$.

Table 3

Effect of TMP on the hippocampus (CA1, CA3, and DG) and cortex neuronal count in PTZ induced kindled rats.

Treatment	Hippocampus			Cortex
	CA1	CA3	DG	
Normal control	33.5 ± 2.64	29.8 ± 2.26	52.5 ± 3.43	30.5 ± 2.88
PTZ	11.8 ± 1.58***	9.83 ± 1.25***	22.5 ± 2.47***	11.7 ± 1.89***
VPA	23 ± 1.81##	21.5 ± 1.26###	43.0 ± 4.11##	22.3 ± 1.73##
TMP 30 mg/kg	15.3 ± 1.41	14.8 ± 1.76#	27.0 ± 2.78	16.3 ± 1.28
TMP 60 mg/kg	20.5 ± 1.93#	17.5 ±0.76#	38.5 ± 3.10#	20.5 ± 1.06#

Results are expressed as mean ± SEM (n=6); *** indicates P<0.001, Vs control; #, ##, and ### indicate P<0.05, P<0.01, and P<0.001, respectively, Vs PTZ. Data were analyzed by one-way ANOVA followed by Tukey's test using Graph pad prism software. Statistical significance was set at $P \leq 0.05$.