

## Neuroprotective Effects of Low Dose Anandamide in Pentylentetrazole-Induced Kindling in Rats

Omar M.E. Abdel-Salam<sup>1</sup>, Amany A. Sleem<sup>2</sup>,  
Marawan Abd El Baset Mohamed Sayed<sup>2</sup>, Eman R Youness<sup>3</sup>  
and Nermeen Shaffie<sup>4</sup>

<sup>1</sup>Department of Toxicology and Narcotics, <sup>2</sup>Department of Pharmacology, <sup>3</sup>Department of Medical Biochemistry, <sup>4</sup>Department of Pathology, National Research Centre, Cairo, Egypt.

\*Corresponding author E-mail: omasalam@hotmail.com

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Anandamide (*N*-arachidonylethanolamine) is an endogenous cannabinoid receptor CB1 ligand that exhibits neuro protective effects in the brain. In this study, the effect of exogenously given an andamide on pentylentetrazole (PTZ)-induced chemical kindling oxidative stress and brain damage in rats was studied. Rats were intraperitoneally (i.p.) injected with 35 mg/kg PTZ once every 48 hours for 12 times to induce seizures. Anandamide was i.p. given. 30 min prior to PTZ injection at 100 or 200  $\mu$ g/kg. Injections of PTZ induced significant increase in brain lipid peroxidation (malondialdehyde: MDA), and nitric oxide associated with marked decrease in brain reduced glutathione (GSH). There were also significant decrements in acetylcholinesterase (AChE) concentration, butyrylcholinesterase (BChE) and paraoxonase-1 (PON-1) activities in brain tissue of PTZ injected rats. Meanwhile, there was no significant effect for PTZ on the concentration of brain neutrophil elastase. Anandamide administered at 100 and 200  $\mu$ g/kg significantly decreased MDA and increased GSH contents and at 200  $\mu$ g/kg significantly decreased nitric oxide in brain of PTZ-treated rats. The drug also caused significant increments in AChE concentration and PON-1 activity but had no significant effect on BChE or neutrophil elastase in rats treated with PTZ. Anandamide given at the dose of 200  $\mu$ g/kg significantly decreased the mean seizure scores over the study period by 22.3% and the frequency of myoclonic jerks and rearing (stage 3) by 56.7% compared with the vehicle-treated group. Anandamide given at 100 and 200  $\mu$ g/kg completely inhibited the development of generalized tonic-clonic seizures (stage 5). It is concluded that in the PTZ-induced seizures, the cannabinoid receptor CB1 agonist anandamide decreases brain oxidative stress, neuronal injury, and exerts an antiepileptic activity.

**Keywords:** Endocannabinoids; Kindling; pentylentetrazole; Anandamide; Oxidative stress; neurodegeneration.

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*Cannabis sativa* L. (family *Cannabidaceae*) is well known for its recreational uses. The principal psychoactive constituent in cannabis was determined to be the cannabinoid delta<sup>9</sup>-tetrahydrocannabinol (D<sup>9</sup>-THC)<sup>1</sup>. Other cannabinoids eg., cannabidiol, cannabigerol, Ä<sup>9</sup>-tetrahydrocannabivarin, cannabidivarin and

cannabichromene are also present<sup>2</sup>. These are not psychoactive and displays anti-inflammatory<sup>3,4</sup> and neuroprotective effects<sup>5</sup>. Cannabis-based medicines have been advocated for the treatment of a number of neurological disorders such as Parkinson's disease, Alzheimer's disease, Huntington's disease, Tourette's syndrome, multiple sclerosis, and

Chorea<sup>6,7</sup>. Sativex, an oromucosal spray of whole plant extract containing D<sup>9</sup>-THC and cannabidiol (CBD) [THC:CBD=1:1] is in use in the UK, New Zealand, and Spain to treat spasticity in multiple sclerosis<sup>7</sup>. Cannabis preparations are also being used to treat epilepsy. In their study, Hamerle *et al*<sup>8</sup> found that 63/310 (20.3%) of their sample of epilepsy outpatients have consumed cannabis after the diagnosis of epilepsy. In a study by Sulak *et al*<sup>9</sup>, epilepsy patients using cannabis mostly reported the use of cannabidiol-enriched artisanal preparations. D<sup>9</sup>-THC and tetrahydrocannabinolic acid (THCA) might be used in addition. There are also data to suggest a benefit from the use of cannabis to treat refractory epilepsy in children<sup>10</sup>. In surveys on the use of oral cannabis extracts in these children, parents reported a decrease in seizure frequency or even complete absence of seizures on treating their children with cannabidiol-enriched extracts<sup>11,12</sup>. There are in addition case reports suggesting a favorable impact on seizure frequency from using cannabidiol-enriched strain of cannabis in Dravet syndrome<sup>13</sup> or pure cannabidiol in an infant with malignant migrating partial seizures<sup>14</sup>. Cannabidiol-enriched extracts, are however, not without side effects and somnolence, fatigue, convulsions and status epilepticus have been reported among others<sup>11,12</sup>.

In preclinical models of epilepsy, a proconvulsant effect has been reported for cannabis extracts rich in D<sup>9</sup>-THC in PTZ-epileptic seizures in rats<sup>15</sup>. In mice, D<sup>9</sup>-THC increased kindling caused by picrotoxin, PTZ or electroshock<sup>16</sup>. D<sup>9</sup>-THC or the synthetic cannabinoid agonist JWH-018 caused the development of electrographic seizures in mice via CB1-receptor mechanism<sup>17</sup>. In contrast, cannabidiol decreased seizures induced by PTZ in mice<sup>18</sup> while a cannabidivarin-cannabidiol extract showed anticonvulsant effect in PTZ-induced seizures and suppressed the pilocarpine convulsions<sup>19</sup>.

Cannabinoids exert their effects by acting on the cannabinoid CB1 and CB2 receptors. CB1 receptors are found principally in the brain on presynaptic neuronal membranes and modulate the release of neurotransmitters and are also present on brain astrocytes. CB2 receptors on the other hand are primarily found in the periphery on immune cells and tissues<sup>20</sup>. There exist also endogenous cannabinoids eg., arachidonoyl ethanolamide or

anandamide and arachidonoylglycerol. These are produced on demand by enzymatic cleavage of membrane lipid precursors. The endocannabinoid anandamide binds with high affinity to CB1 receptors and thus is expected to mimic the exogenously given D<sup>9</sup>-THC in its pharmacological actions<sup>21</sup>. It also binds to CB2 receptors acting as partial agonist or antagonist<sup>22,23</sup>. Anandamide exerted neuroprotective effect in models of neuronal excitotoxicity<sup>24-26</sup> via CB1 receptor mechanism and showed antioxidant effects in hippocampal neuron cells exposed to hydrogen peroxide *in vitro*<sup>27</sup>. Anandamide is subject to hydrolysis by fatty acid amide hydrolase yielding arachidonic acid and ethanolamine<sup>28</sup>. It has been shown that decreased degradation of the endogenous cannabinoids by inhibition of the enzyme fatty acid amide hydrolase, exerted anti-convulsive effect and reduced EEG epileptiform activity induced by PTZ in rats<sup>29</sup>. Moreover, treatment with fatty acid amide hydrolase inhibitor was found to abolish seizures induced by cocaine in mice<sup>30</sup>. This suggests that endogenous cannabinoids increase seizure threshold.

In this study, the effect of anandamide on brain oxidative stress and the development of seizures in rats subjected to PTZ-kindling was investigated. We in addition examined the effect of anandamide on the histopathological changes induced by PTZ in brain.

## MATERIALS AND METHODS

### Animals

Male Sprague-Dawley rats weighing 180-200 g were used in the study. Rats were group-housed under temperature- and light-controlled conditions and allowed standard laboratory rodent chow and water *ad libitum*. The experiments were done at 9 O'clock to avoid changes in circadian rhythm. The study was done in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) and the institutional ethics committee. Seven to eight rats were used per group.

### Drugs and chemicals

Pentylenetetrazole (PTZ) and anandamide (*N*-arachidonylethanolamine) were purchased from Sigma (St. Louis, MO, USA). Other chemicals were from Sigma.

### Study design

Rats were randomly divided into three groups (7-8 rats each). Rats were treated with saline (group 1), PTZ at 35 µg/kg, i.p once every 48 hours for 12 times alone (group 2) or together with anandamide at 100 and 200 µg/kg i.p given 30 min prior to PTZ injection (groups 3 & 4, respectively). Seizures were recorded for 20 minutes. Two hours after the last PTZ injection, rats were quickly euthanized by decapitation, their brains removed on ice cold glass plate, stored at -80 °C until the biochemical assays. One half of each brain was kept in 10% formal saline for histopathological processing.

### Biochemical assays

#### Determination of lipid peroxidation

Malondialdehyde (MDA), a product of lipid peroxidation was determined in tissue homogenates according to the method of Nair and Turner<sup>31</sup>. In this assay thiobarbituric acid reactive substances (TBA) react with thiobarbituric acid to form TBA-MDA adduct which can be measured colorimetrically at 532 nm.

#### Determination of nitric oxide

Nitric oxide was determined using colorimetric assay where nitrate is converted to nitrite via nitrate reductase. Griess reagent then act to convert nitrite to a deep purple azo compound that can be determined using spectrophotometer [32].

#### Determination of reduced glutathione

Reduced glutathione (GSH) was determined in tissue homogenates using the procedure of Ellman *et al.*<sup>33</sup>. The assay is based on the reduction of Ellman's reagent (DTNB; 5, 5'-dithiobis (2-nitrobenzoic acid)) by the free sulfhydryl group on GSH to form yellow colored 5-thio-2-nitrobenzoic acid which can be determined using spectrophotometer at 412 nm.

#### Determination of acetylcholinesterase

Acetylcholinesterase (AChE) concentration was determined in supernatants using an ELISA kit purchased from NOVA (Bioneovan Co., Ltd, DaXing Industry Zone, Beijing, China) according to the manufacture instructions.

#### Determination of butyrylcholinesterase

Butyrylcholinesterase (BChE) activity in the brain tissue supernatants was determined by a colorimetric method using butyrylcholinesterase

diagnostic kit (Biodiagnostic, Egypt). In this assay, BChE hydrolyzes butyrylcholine to butyrate and thiocholine. The latter reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) forming 5-mercapto-2-nitrobenzoic acid (5-MNBA). The rate of 5-MNBA formation, measured spectrophotometrically, is proportional to the enzymatic activity of BChE in the sample.

#### Determination of paraoxonase-1

The arylesterase activity of PON1 was determined by a colorimetric method using phenyl acetate as a substrate. In this assay, PON1 catalyzes the cleavage of phenyl acetate resulting in phenol formation. The rate of formation of phenol was measured by monitoring the increase in absorbance at 270 nm at 25°C. The working mix consisted of 20 mM Tris/HCl buffer, pH 8.0, containing 1 mM CaCl<sub>2</sub> and 4 mM phenyl acetate as the substrate. Samples diluted 1:3 in buffer were added to the above mix and the changes in absorbance were recorded following a 20 s lag time. One unit of arylesterase activity is equal to 1 imole of phenol formed per minute. The PON1 activity is expressed in kU/L, based on the extinction coefficient of phenol of 1310 M<sup>-1</sup>cm<sup>-1</sup>. Blank samples containing water were used to correct for the spontaneous hydrolysis of phenyl acetate<sup>34</sup>.

#### Determination of neutrophil elastase

The level of neutrophil elastase was determined using an ELISA kit purchased from NOVA (Bioneovan Co., Ltd, DaXing Industry Zone, Beijing, China) according to the manufacture instructions.

#### Chemical kindling

The kindling procedure was induced by repeated i.p. injection of 35 µg/kg pentylenetetrazole (PTZ) (Sigma, St. Louis, USA). Pentylenetetrazole was given once every 48 hours for 12 times. After each PTZ treatment, rats were placed separately under glass funnels, and their convulsive behavior was recorded during individual observations for 20 minutes. The resultant seizure behaviors were classified into 5 stages and scored as follows: 0: no response; 1: ear and facial twitching; 2: convulsive waves through the body; 3: myoclonic jerks, rearing; 4: turn over onto one side position; 5: turn over onto back position, generalized tonic-clonic seizures<sup>35</sup>. The daily average seizure scores and the total scores over the study period were

determined and compared between the PTZ control and anandamide groups. In addition, the mean total scores for each stage, the mean number of incidents of each stage (frequency of events in each stage) as well as the % of seizure free animals in each stage for all groups were determined.

#### Histopathological studies

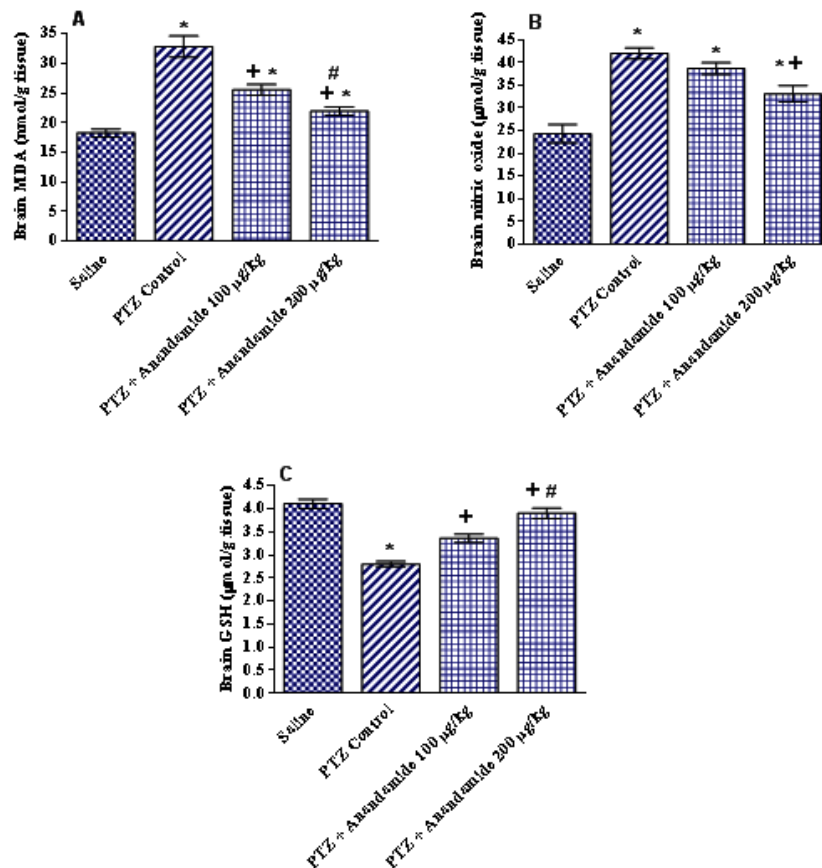
Brain samples of all animals were dissected immediately after death. The specimens were then fixed in 10 % neutral-buffered formalin saline for 72 hours at least. All the specimens were washed in tap water for half an hour and then dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin. Serial sections of 5 $\mu$ m thick were cut and stained with haematoxylin and eosin for histopathological investigation.

#### Quantitative assessment

10 fields for each section were investigated and counted for total cells in field and cells that show degenerative signs such as pyknotic nuclei for cerebral cortex and hippocampus and flattened atrophied cells in substantia nigra. The mean of cells and percentage of damaged cells were calculated.

#### Statistical analysis

Results are expressed as mean  $\pm$  SEM. The results of the biochemical assays analyzed using One Way ANOVA and Duncan's multiple range test. Data of the behavioral study were analyzed by Kruskal-Wallis test using Graphpad Prism software, version 5 (inc., San Diego, USA). A probability value of less than 0.05 was considered statistically significant.



**Fig. 1. A-C.** Effect of anandamide on malondialdehyde (MDA), nitric oxide, and reduced glutathione (GSH) in brain of rats subjected to pentylenetetrazole (PTZ)-induced kindling. \*:  $p < 0.05$  vs. vehicle-treated group. +:  $p < 0.05$  vs. PTZ control group. #:  $p < 0.05$  vs. PTZ + anandamide 100 $\mu$ g/kg group

## RESULTS

### Biochemical results

#### Lipid peroxidation

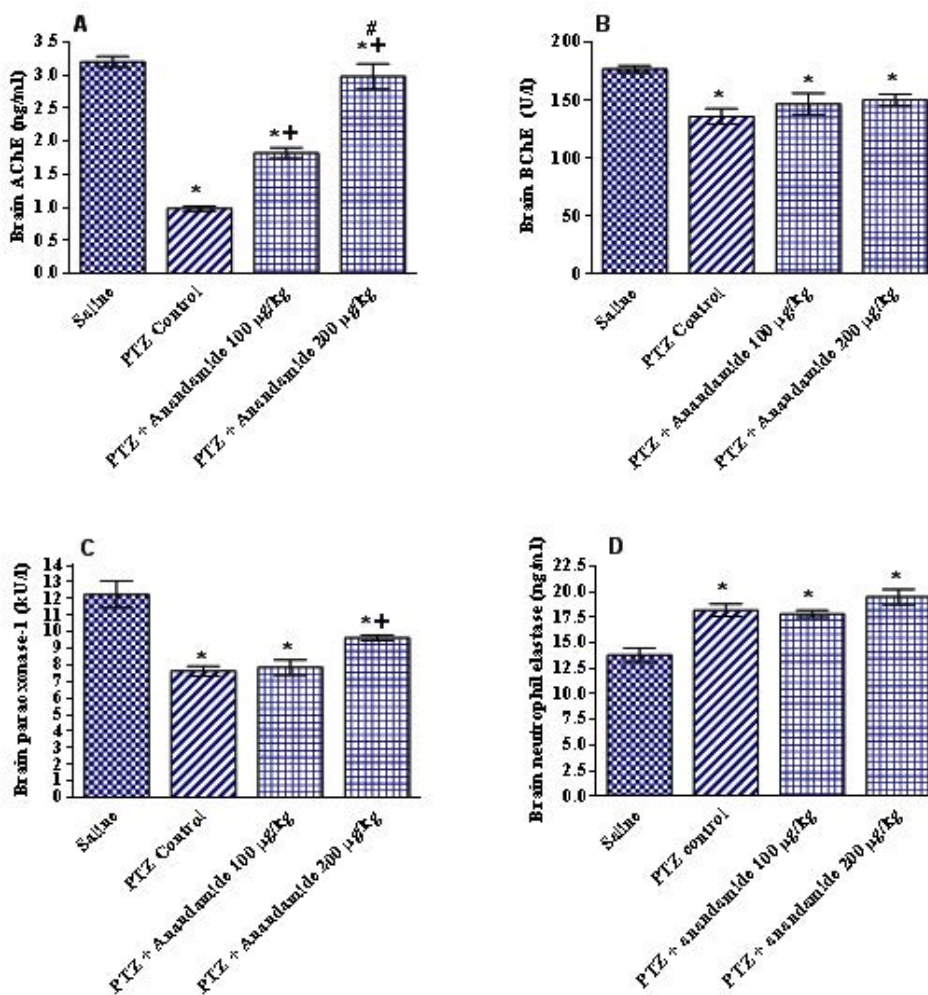
Rats treated with only pentylenetetrazole (PTZ) exhibited significant increase in brain malondialdehyde (MDA) by 79.2% compared with the saline control group ( $32.8 \pm 1.7$  vs.  $18.3 \pm 0.67$  nmol/g.tissue). Malondialdehyde decreased significantly by 21.9% and 33.2% in rats treated with PTZ and anandamide at 100 and 200  $\mu$ g/kg, respectively ( $25.6 \pm 0.9$  and  $21.9 \pm 0.7$  vs.  $32.8 \pm 1.7$  nmol/g.tissue)(Fig. 1A).

#### Nitric oxide

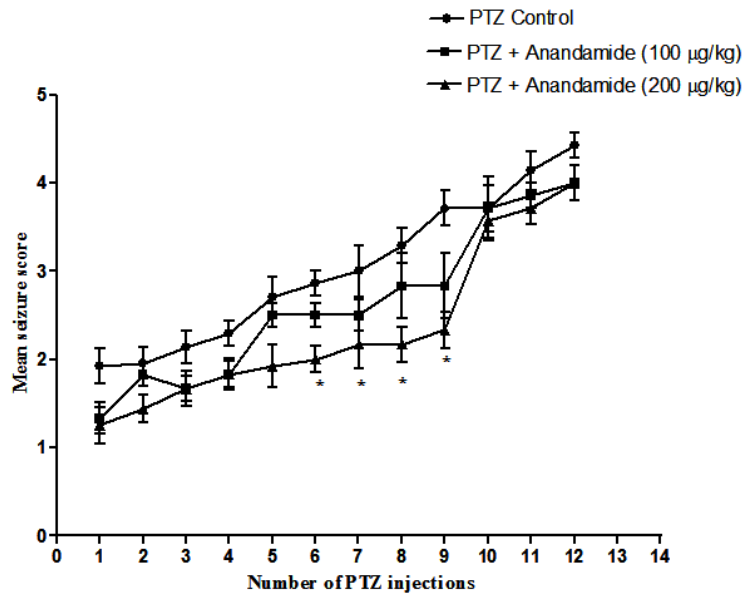
Pentylenetetrazole injections resulted in significantly increased brain nitric oxide content compared to the saline control value ( $41.9 \pm 1.2$  vs.  $24.3 \pm 2.1$   $\mu$ mol/g.tissue). Nitric oxide decreased significantly by 21.0% following treatment with 200  $\mu$ g/kg anandamide ( $33.1 \pm 1.8$  vs.  $41.9 \pm 1.2$   $\mu$ mol/g.tissue) (Fig. 1B).

#### Reduced glutathione

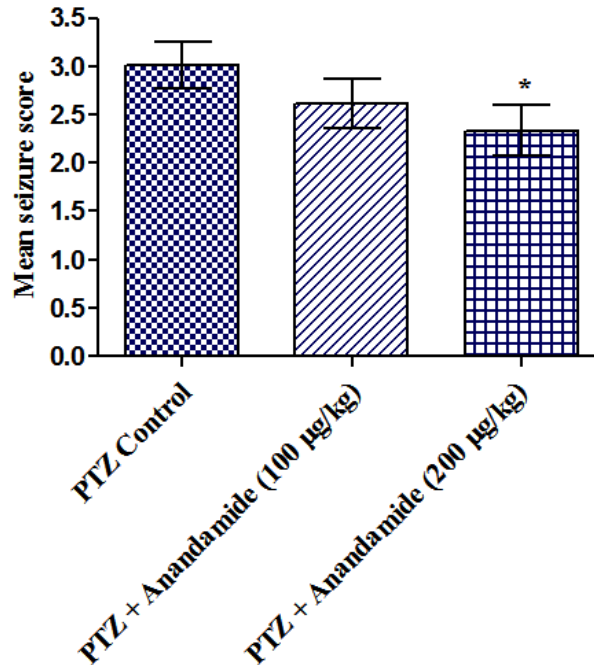
Significant decrease in brain reduced glutathione by 31.9% was observed in PTZ-treated rats compared to the saline control ( $2.8 \pm 0.06$  vs.



**Fig. 2. A-D.** Effect of anandamide on acetylcholinesterase, butyrylcholinesterase, paraoxonase-1 and neutrophil elastase in brain of rats subjected to pentylenetetrazole (PTZ)-induced kindling. \*:  $p < 0.05$  vs. vehicle-treated group and between different groups as shown in the graph. +:  $p < 0.05$  vs. PTZ control group. #:  $p < 0.05$  vs. PTZ + anandamide 100 $\mu$ g/kg group



**Fig. 3.** Effect of anandamide on the mean seizure scores induced by pentylenetetrazole (PTZ) -kindling epilepsy in rats. Rats were i.p. injected with PTZ once every 48 hours for 12 times to induce seizures. Anandamide was i.p. given 30 min before each PTZ injection. Each point in the line represents the mean  $\pm$  S.E. of 7-8 experiments. Data were analyzed by Kruskal-Wallis test. Asterisks indicate significant change from the PTZ control group ( $P < 0.05$ )



**Fig. 4.** The mean total epilepsy scores over the study period in the pentylenetetrazole (PTZ) only, PTZ + anandamide (100 µg/kg) and PTZ + anandamide (200 µg/kg)-treated groups. Rats were injected with i.p. pentylenetetrazole (PTZ) only or given PTZ with anandamide (30 minutes before PTZ). Data represents mean  $\pm$  S.E. of 7-8 experiments. Data were analyzed by Kruskal-Wallis test. \*  $P < 0.05$  vs. PTZ control

4.11 ± 0.1 μmol/g.tissue). Reduced glutathione increased significantly by 20.0% and 39.3% after treatment with 100 and 200 μg/kg anandamide, respectively (3.36 ± 0.09 and 3.9 ± 0.11 μmol/g.tissue) (Fig. 1C).

**Acetylcholinesterase**

Repeated PTZ injections resulted in significant decrease in brain acetylcholinesterase (AChE) concentration by 69.3% relative to the saline control (0.98 ± 0.04 vs. 3.19 ± 0.08 ng/ml). Treatment with anandamide (100 and 200 μg/kg) significantly increased AChE by 85.7% and 203.1%, respectively (1.82 ± 0.08 and 2.97 ± 0.19 vs. 0.98 ± 0.04 ng/ml) (Fig. 2A).

**Butyrylcholinesterase**

Significant inhibition of brain butyrylcholinesterase (BChE) activity by 23.0% was observed after PTZ injections (136.3 ± 6.5 vs. 177.0 ± 3.14 U/l). The administration of anandamide had no significant effect on BChE activity in PTZ-treated rats (Fig. 2B).

**Paraoxonase-1**

Pentylentetrazole resulted in significant inhibition of paraoxonase-1 activity by 37.8%

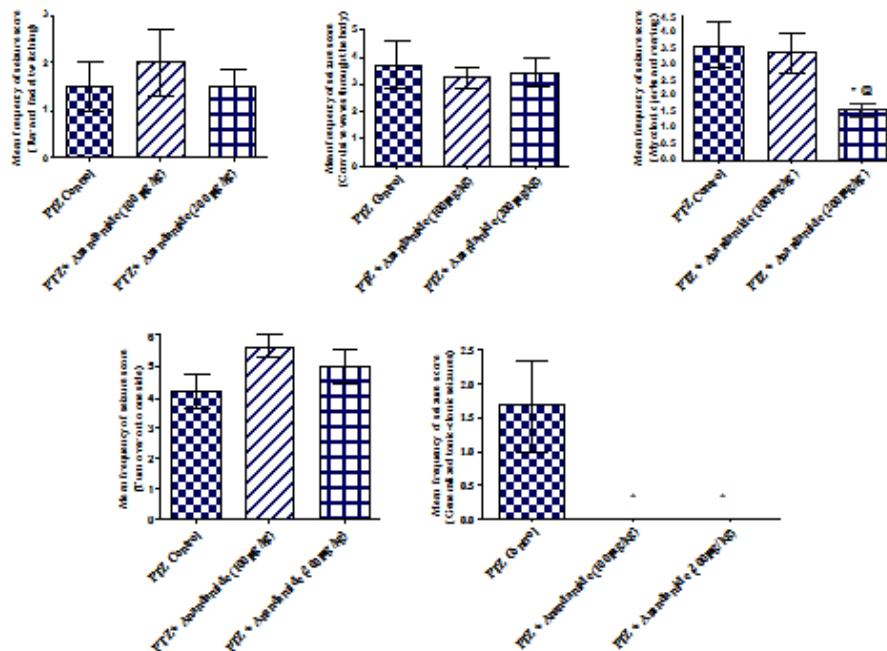
compared with the saline control value (7.62 ± 0.3 vs. 12.26 ± 0.79 kU/l). Paraoxonase-1 activity increased by 26.4% in rats treated with 200 μg/kg anandamide (9.63 ± 0.16 vs. 7.62 ± 0.3 kU/l) (Fig. 2C).

**Neutrophil elastase**

No significant effect was observed for PTZ or PTZ + anandamide on brain neutrophil elastase concentration (Fig. 2D).

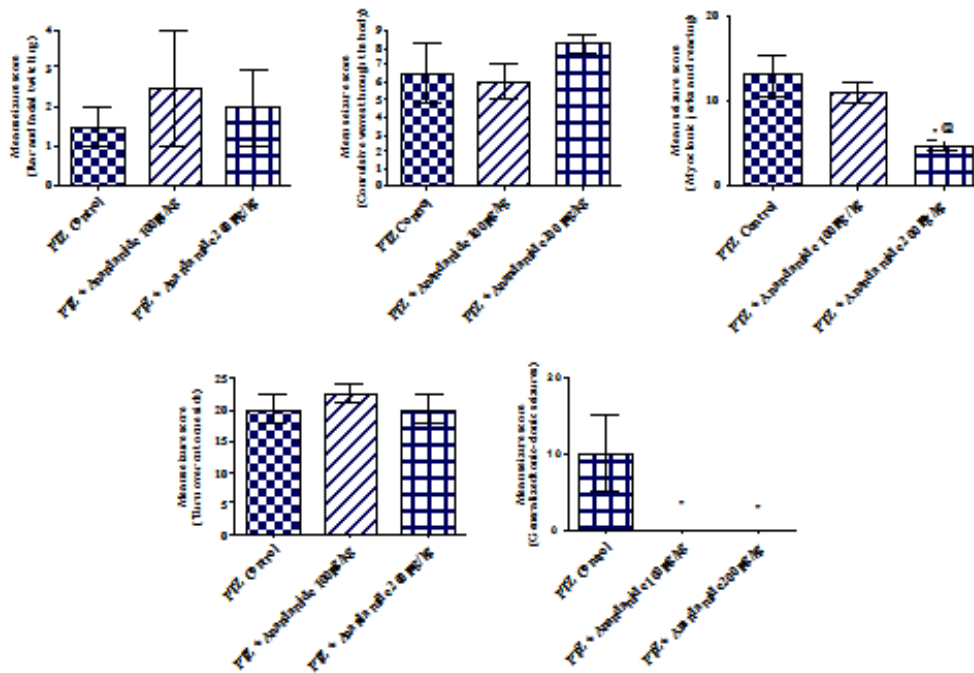
**Kindling results**

Compared with the PTZ only group, treatment with anandamide 200 μg/kg produced significant decrease of the mean seizure scores after the 6<sup>th</sup>, 7<sup>th</sup>, 8<sup>th</sup> and 9<sup>th</sup> PTZ injections during seizure development. This inhibitory effect of anandamide on the development of seizures, however, ceased by the 10<sup>th</sup> injection, suggesting the development of tolerance to some scores (Figure 3). Figure 4 shows the mean total seizure scores over the study period. The mean seizure score was 3.01 ± 0.24 in the PTZ only group, 2.61 ± 0.25 in the PTZ- anandamide (100 μg/kg) group and 2.34 ± 0.26 in the PTZ anandamide (200 μg/kg). The mean seizure score was significantly decreased by

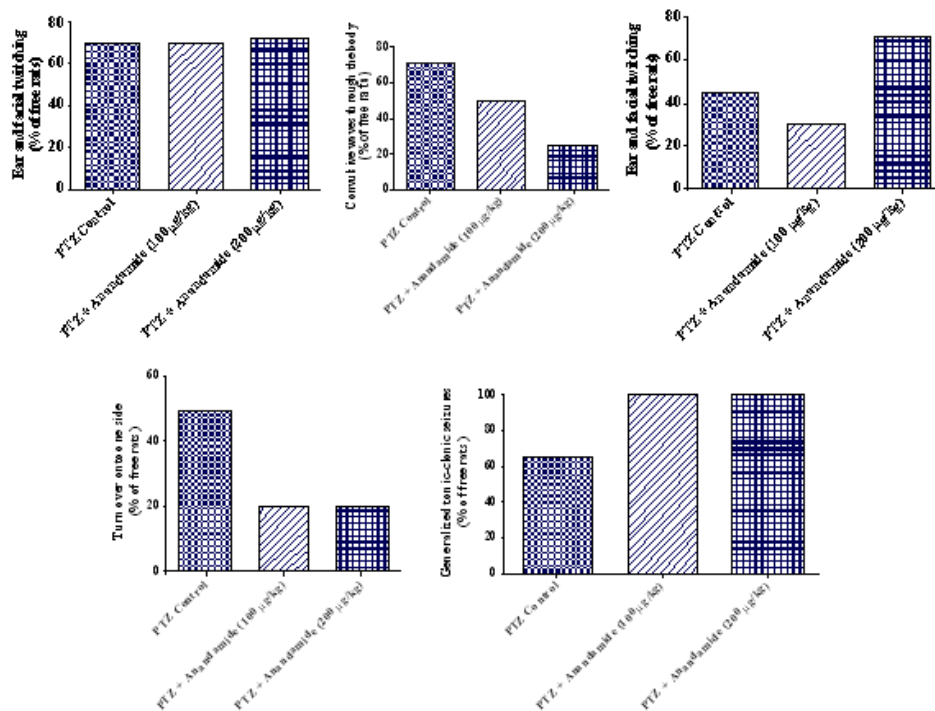


**Fig. 5.** The mean frequency of epilepsy scores (the mean number of occurrences for each animal) over the study period in the pentylentetrazole (PTZ) only, PTZ + anandamide (100 μg/kg) and PTZ + anandamide (200 μg/kg)-treated rats. Each bar represents mean ± S.E. of 7-8 experiments. Kruskal-Wallis test. \* P <0.05 vs. PTZ control. @ P <0.05 vs. PTZ anandamide 100 μg/kg



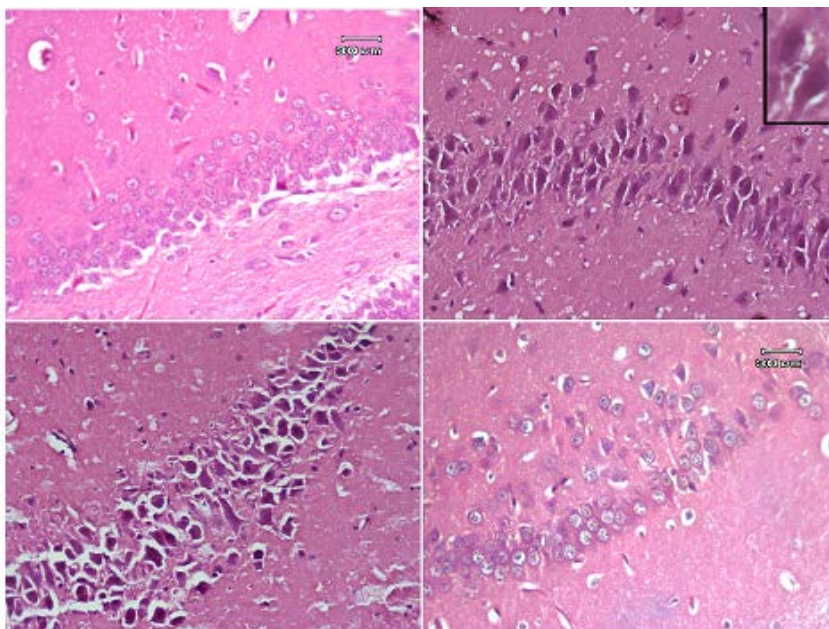


**Fig. 6.** The mean total epilepsy scores over the study period in the pentylenetetrazole (PTZ) only, PTZ + anandamide (100 µg/kg) and PTZ + anandamide (200 µg/kg)-treated rats. Each bar represents mean ± S.E. of 7-8 experiments. Kruskal-Wallis test. \* P < 0.05 vs. PTZ control. @ P < 0.05 vs. PTZ anandamide 100 µg/kg

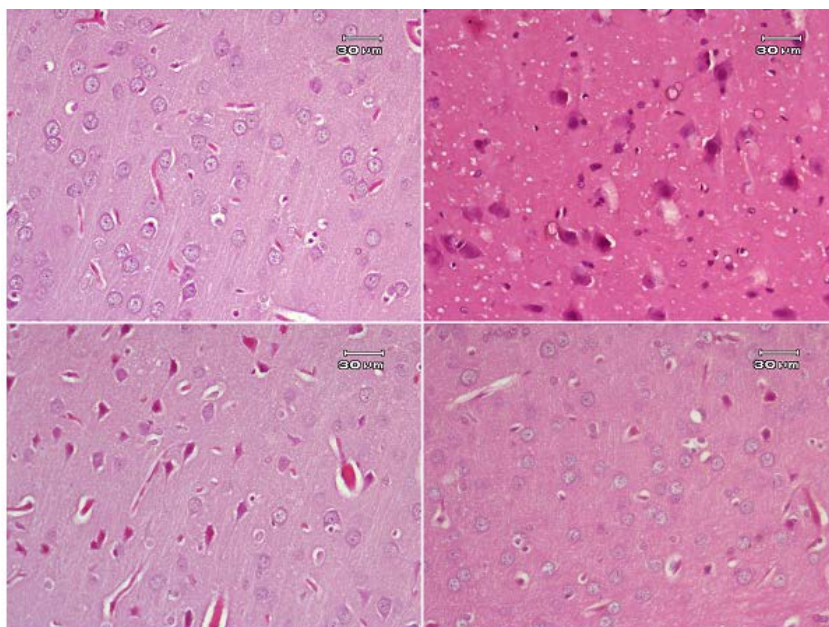


**Fig. 7.** The % of seizure free animals in each stage





**Fig. 8.** Representative photomicrographs of brain tissue sections (hippocampus) stained with Hx & E (A) hippocampus of a control -ve rat. (B) Epileptic rat shows most of the hippocampal neurons are flattened with dark pyknotic nuclei. (C) Epileptic rat treated with anandamide at 100 µg/kg shows no noticeable amelioration of hippocampal area. (D) Epileptic rat treated with anandamide at 200 µg/kg shows most of the neurons appear normal with large rounded vesicular nuclei. Only a very few cells show pyknotic nuclei



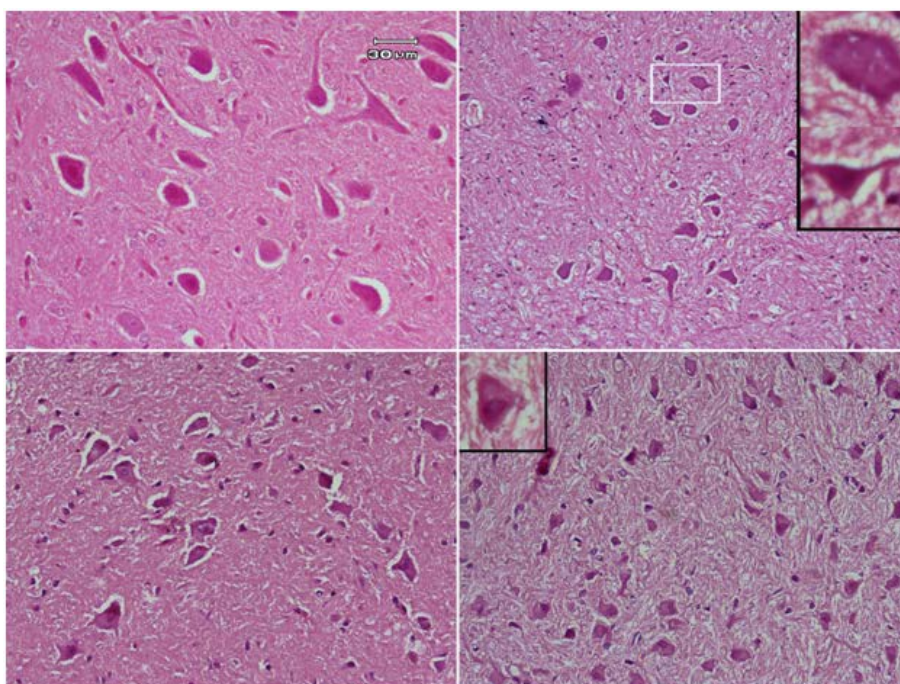
**Fig. 9.** Representative photomicrographs of cerebral cortex sections from (A) Saline control rat. (B) Epileptic (PTZ-treated) rat shows many neurons with dark pyknotic nuclei. (C) Epileptic rat treated with anandamide at 100 µg/kg shows many cells still show signs of degeneration in the form of pyknotic nuclei. (D) Epileptic rat treated with anandamide at 200 µg/kg shows only a few cells with degenerative signs

22.3% in the PTZ + 200  $\mu\text{g}/\text{kg}$  anandamide group with respect to the PTZ control group ( $p < 0.05$ ). Figure 5 shows that anandamide (200  $\mu\text{g}/\text{kg}$ ) significantly decreased the frequency of myoclonic jerks and rearing (stage 3) with respect to both the PTZ control and PTZ + 100  $\mu\text{g}/\text{kg}$  anandamide by 56.75% and 52.85%, respectively. Also, the occurrence of generalized tonic-clonic seizures (stage 5) was completely inhibited by either dose of anandamide. Figure 6 shows that the administration

of 200  $\mu\text{g}/\text{kg}$  anandamide decreased the mean total score of myoclonic jerks and rearing (stage 3), with respect to both PTZ control and PTZ + 100  $\mu\text{g}/\text{kg}$  anandamide by 63.84 % and 42.72 %, respectively. In addition, anandamide given at 100  $\mu\text{g}/\text{kg}$  or 200  $\mu\text{g}/\text{kg}$  completely inhibited the generalized tonic-clonic seizures (stage 5) as compared to the PTZ control group.

#### Histopathological results

Epilepsy induced by PTZ affected the



**Fig. 10.** Representative photomicrographs of substantia nigra sections stained with Hx & E: (A) Saline control rat. shows normal structure of substantia nigra area. (B) Epileptic (PTZ-treated) rat shows decrease in neurons' number. The higher magnified part shows a karyolitic neuron (the upper one), while the lower one is atrophied with pyknotic nucleus. (C) Epileptic rat treated with anandamide at 100  $\mu\text{g}/\text{kg}$  shows noticeable increase in size and number of substantia nigra neurons. (D) Epileptic rat treated with anandamide at 200  $\mu\text{g}/\text{kg}$  shows close to normal structure of the area

**Table 1.** Count and % of neurons with degenerative changes (deg. Neu) in PTZ-treated rats and the effect of anandamide

	Cerebral cortex			Hippocampus			Substantia nigra		
	deg. Neu	total	%	deg. Neu	total	%	deg. Neu	total	%
Saline	7	404	1.7	4	400	1	3	72	4.2
PTZ	176	537	32.8	112	314	35.6	57	131	43.5
PTZ + Anandamide 100 $\mu\text{g}/\text{kg}$	148	592	25	95	584	16	35	110	32
PTZ + Anandamide 200 $\mu\text{g}/\text{kg}$	96	663	14.4	60	643	9.3	21	98	21.4

hippocampal area as many of the neurons in this area became flattened with pyknotic nuclei, although there was no reduction in their number (Fig 8B) if compared with normal control section (Fig. 8A). The administration of anandamide at 100µg/kg did not ameliorate this damaging effect of PTZ (Fig. 8C). The higher dose, however, markedly improved the neurons in this area (Fig. 8D). Examination of cerebral cortex sections from saline-treated rats clarified the normal structure of this area (Fig. 9A). Epilepsy increased number of neurons that showed variable signs of degeneration (Fig. 9B). Low dose of anandamide did not ameliorate the damaged neurons (Fig. 9C) while the high dose markedly decreased the number of cells with signs of degeneration (Fig. 9D). Sections of substantia nigra area showed that injections of PTZ markedly decreased the size and number of pigmented neurons (Fig. 10B) if compared with sections from the saline-treated control (Fig. 10A). Anandamide ameliorated the damaging effect of epilepsy in a dose dependent manner (Fig. 10C & D).

#### Quantitative results

For normal cerebral cortex 7 cells only showed variable levels of damage out of 404 cells counted (1.7%), while in epileptic rat 176 cells showed signs of degeneration out of 537 cells (32.8%). Treating epileptic rats with anandamide at 200 µg/kg decreased the damaged cells to 96 out of 663 (14.4%). The lower dose of 100 µg/kg gave less effect as cells with degenerative signs were 148 out of 592 with (25%).

Results obtained from hippocampus clarified the effect of anandamide in treating epilepsy. Cells with damage signs in normal control rat were 4 out of 400 (1%), while the epileptic rat showed 112 out of 314 neurons (35.6%). Anandamide at 200 µg/kg decreased damaged cells to 60 out of 643 (9.3%). The low dose decreased the damaged cells to 95 out of 584 (16%).

In substantia nigra sections, the cells with signs of degeneration in control normal rat were 3 out of 72 (4.2%), while epileptic rat showed 57 damaged cells out of 131(43.5%). Anandamide at 200 µg/kg decreased the damaged cells to be 21 out of 98 (21.4%), while the low dose decreased the damaged cells to only 34 out of 110 (32%).

#### DISCUSSION

The main findings in this study were that kindling induced in the rat by repeated injection of the epileptogen pentylenetetrazole (PTZ) resulted in brain oxidative stress, inhibition of the enzymes AChE, BChE, and PON-1 in brain. Histologically, PTZ induced neuronal injury in the cerebral cortex, hippocampus and also the substantia nigra, indicating widespread brain injury by the epileptogen. Anandamide, an endocannabinoid, administered prior to PTZ was able to decrease oxidative stress, nitric oxide levels and to increase AChE concentration and PON-1 activity. This occurred along with a suppressive effect for anandamide on the development of seizures by PTZ. Additionally, anandamide exerted marked neuroprotective effect as indicated by the histological examination of the brain tissue.

When injected into rats, PTZ led to oxidative stress as indicated by the increase in the level of the lipid peroxidation end product malondialdehyde (MDA), thereby, suggesting free radicals attack on cell membrane lipids<sup>36</sup>. Significant decrease in reduced glutathione, an important brain antioxidant and a free radical scavenger<sup>37</sup> was also found. These findings agree with previously published observations in the PTZ-induced kindling in the rat<sup>15,38</sup>. Other researchers showed significant increase in lipid peroxidation products and decreased activities of the antioxidant enzymes Cu-Zn superoxide dismutase and catalase as well as decreased reduced glutathione levels in erythrocytes after a single injection of 50 µg/kg PTZ in the rat<sup>39</sup>. Abbott *et al*<sup>40</sup> in addition, reported decreased concentrations of reduced glutathione in the occipital cortex in a genetic mouse model of epilepsy. Moreover, in their study, Pence *et al*<sup>38</sup> found that the increase in MDA in brain of rats treated with PTZ could be reduced by administering glutathione, which would suggest that reduced glutathione decreases in the brain of PTZ-treated rats due to consumption by increased free radicals. Increased brain lipid peroxidation and a decrease in brain reduced glutathione have also been reported in epileptic patients<sup>40,41</sup>. Increased lipid peroxidation has been demonstrated in plasma of epileptic children who showed nuclear magnetic resonance changes<sup>42</sup>. In their study, Mueller *et al*<sup>41</sup>

reported significant reduction in glutathione/water ratio in the parieto-occipital region in both cerebral hemispheres. This finding was independent of seizure activity.

This study in addition demonstrates marked increase in brain nitric oxide content after treatment with PTZ. Other studies indicated that in PTZ-induced seizures in rats, inhibitors of NOS suppressed the generalized tonic extension and delayed the latency to develop myoclonic jerks and clonic seizures<sup>43</sup>. In slices from the hippocampal and entorhinal cortex, the application of non-specific inhibitors of nitric oxide synthase (NOS) or a selective neuronal NOS prevented low  $Mg^{++}$ -induced epileptic activity. Seizure activity was re-induced by the addition of nitric oxide donor [44,45]. It has been suggested that during PTZ-induced seizures, neuronal NOS induces endoplasmic reticulum stress and oxidative tissue damage; this effect being mediated by peroxynitrite<sup>46</sup>.

Our results also provide evidence for an inhibitory effect for PTZ on AChE, BChE in brain of treated rats. The function of the enzymes AChE and BChE is the hydrolysis of acetylcholine (ACh) and thus to terminate its actions at the neuronal synapse, cholinergic nerve endings and motor end-plate<sup>47</sup>. Cholinesterases differ in that AChE hydrolysis ACh faster than BChE and is more specific in this respect whereas BChE is less specific and hydrolysis butyrylcholine and ester containing drugs<sup>48,49</sup>. The inhibition of AChE by PTZ has been previously reported<sup>15</sup>. Increased ACh in brain might have a role in the genesis of seizures. Green *et al.*<sup>50</sup> using immunohistochemical techniques reported loss of AChE fibers in Ammon's horn in resected temporal lobes from epileptic patients. In addition, gene mutation and loss of function of neuronal nicotinic ACh receptors was found in patients with a genetic form of epilepsy<sup>51</sup>. The present study also confirms our previous observations indicating inhibition of PON-1 activity in brain of PTZ kindled rats<sup>15</sup>. The function of PON-1 is to hydrolyse organophosphates and other xenobiotics<sup>52</sup>. This enzyme has important antioxidative and anti-inflammatory actions<sup>53,54</sup> and a decrease in its activity has been found in a number of neurological disorders<sup>55</sup>.

In this study, we have demonstrated that the endocannabinoid anandamide given at doses

as low as 100 and 200  $\mu\text{g}/\text{kg}$  exerted significant antioxidant effect and suppressed the development of seizures induced by PTZ administration in rats. In particular, anandamide completely inhibited the development of generalized tonic-clonic seizures. Other researchers used inhibitors of the enzyme fatty acid hydrolase so as to increase brain anandamide level, and reported an anticonvulsant effect against seizures evoked by PTZ<sup>29</sup> or cocaine<sup>30</sup>. Anandamide (and also 2-arachidonoylglycerol) reduced excitatory postsynaptic currents in hippocampal slices from mice with temporal lobe epilepsy via CB1 receptor mechanism<sup>56</sup>. Several mechanisms are likely to account for the seizure suppressive effect of anandamide. The endocannabinoid acts as a partial agonist at CB1 receptor<sup>21</sup>. This site of action has been reported to account for the neuroprotective effects of anandamide<sup>57</sup>. It also binds to CB2 receptors and acts as a partial agonist or an antagonist<sup>22,23</sup> and binds to transient receptor potential channel vanilloid receptor 1 (TRPV1)<sup>20</sup>. This capsaicin sensitive non-selective cation channel is involved in pain, heat, inflammation<sup>58,59</sup>. The TRPV1 channel, however, has been implicated in increasing the susceptibility to PTZ seizures by hyperthermia in mice<sup>60</sup> and its blockade by capsazepine led to decreased epileptic activity in an acute temporal lobe model of epilepsy in mice<sup>61</sup>. The endocannabinoid might also regulate neuronal excitability by inhibition of T-type calcium channels<sup>62</sup>. Anandamide's ability to inhibit the development of seizures could also be due to an antioxidant effect as shown in the present study. In hippocampal neurons treated with  $\text{H}_2\text{O}_2$ , anandamide was reported to prevent the increase in intracellular reactive oxygen metabolites and the decrease in superoxide dismutase and reduced glutathione<sup>27</sup>.

The endogenous cannabinoid, anandamide, a derivative of arachidonic acid, is synthesized "on demand" from its membrane lipid precursor<sup>28</sup> and as a component of the endocannabinoid system (along with 2-arachidonoyl glycerol) is likely to be involved in maintaining neuronal integrity by protecting neurons from excitotoxic injury<sup>63</sup>. Thus, anandamide (10  $\mu\text{g}/\text{kg}$ , i.p.) was shown to reduce neuronal excitotoxic injury following intracerebral injection of ouabain in neonatal rats<sup>64</sup>. It protected striatal cells in culture cells against quinolinic acid excitotoxicity<sup>24</sup> and retinal amacrine



neurons against AMPA-induced excitotoxicity in vivo via CB1 receptor mechanism<sup>25</sup>. Anandamide (and also 2-arachidonoyl-glycerol) protected prefrontal cortex neurons in culture against excitotoxicity and neuronal death (caused by human immunodeficiency virus type 1 toxin, transactivator of transcription) via CB1 receptor mechanism<sup>26</sup>. In mice subjected to transient middle cerebral artery occlusion, prior treatment with anandamide (i.p. injection of 10 µg/kg ) resulted in a significant decrease in infarct volume<sup>65</sup>. Moreover, inhibition of fatty acid hydrolase which enhanced brain anandamide level protected against neurodegeneration and behavioral deficits in traumatic brain injury in mice<sup>66</sup>. Our data also indicated that anandamide decreased neuronal injury caused by PTZ in different brain regions.

In conclusion, in a model of human epilepsy induced by PTZ injection in the rat, the administration of low doses of the endogenous cannabinoid anandamide reduced brain oxidative stress, seizure development and reduced brain damage.

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