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Pentylenetetrazole kindling-induced epilepsy rat models: Insight on the severity state, a comparative study

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ABSTRACT

This study aimed to carry out a comparative study of the main models of chronic epilepsy induced by pentylenetetrazole (PTZ)-kindling method and to assess the efficacy of sodium valproate, one of the main antiepileptics, on the best epilepsy-inducing kindling model. Two sets of 24 animals were divided into 4 groups of 6 animals and treated as follow: Set 1 included: group 1, control; group 2, the classic kindling PTZ group (UKEOD); group 3, PTZ kindling every other day group with challenge (CKEOD); group 4, PTZ kindling every day group, with challenge (CKED); Set 2 included: group 1, control; group 2, CKEOD group; group 3 and 4, receiving either valproate 200 mg/kg or valproate 300 mg/kg + CKEOD procedure. Results show that CKEOD group significantly reduced the number of injections necessary to reach the fully-kindled state, increased the severity of seizures and improved the stability of seizures. In addition, the CKEOD group significantly increased the level of malondialdehyde and GABA transaminase, reduced the level of reduced glutathione, catalase and GABA. Furthermore, it had no impact on plasma levels of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT). Valproate 300 mg/kg significantly protected animals against kindling induced by CKEOD. The kindling model with a challenge dose administered on day 1 (CKEOD) thus allows to induce more severe, more stable chronic epilepsy and in a shorter period of time, and could thus contribute to a better understanding of epilepsy, as well as its uses in drug discovery.

1. Background

Epilepsy is a neurological disease characterized by the recurrence of paroxysmal clinical episodes, resulting from abnormal and hypersynchronous discharge from one or many groups of brain neurons (Aliyu et al. 2014; Stafstrom and Carmant, 2015). It affects nearly 1 % of the world's population and remains a major global public health problem (WHO, 2020). Faced with this concern, numerous studies have been undertaken for many years in order to better understand this pathology and to find out therapeutic solutions for the people suffering from it. As experimental studies require adequate and reliable experimental models to be achieved, many methods for the experimental studies of epilepsy have been developed over the years both in vitro and in vivo. Regarding the methods of in vivo studies of epilepsy, these are based on three main modes of induction: surgery, aimed at inducing epileptiform lesions, electrical induction using electrodes, and induction via chemical substances (Erkeç and Arihan, 2015; Samokhina and Samokhin, 2018).

Of interest, pentyletetrazole (PTZ) kindling is recognized as one of the preeminent methods of inducing chronic epilepsy (Goel et al. 2015). The full mechanism of action by which PTZ induces epilepsy is not yet fully understood. However, PTZ has been shown to induce epilepsy by antagonizing GABA receptors. Numerous studies have revealed that PTZ acts on both $GABA_A$ receptors and $GABA_B$ receptors. However, its effect is more pronounced and more elucidated on the $GABA_A$ receptor (Erkeç and Arihan, 2015; Samokhina and Samokhin, 2018). It thus acts on the $GABA_A$ receptor complex, by interacting with 3 sites namely: the benzodiazepines site, the picrotoxin site and the GABA site. By blocking these three sites, it thus prevents activation of the $GABA_A$ receptor, thereby preventing the entry of chloride ions into neurons, resulting in the lifting of GABAergic inhibition. This lifting of GABAergic inhibition will result in progressive hyperexcitation, notably by overactivation of glutamatergic signaling, leading to the progressive development of

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epileptogenesis, ultimately leading to epilepsy (Erkeç and Arihan, 2015; Samokhina and Samokhin, 2018).

Since the development of the kindling method by Goddard (1967), then the first PTZ kindling model by Mason and Cooper (1972), many studies have been carried out on this last model, on the one hand for the evaluation of new antiepileptic drugs (AEDs), but also for deepen the understanding of the pathophysiology of epilepsy, and finally to improve the kindling PTZ model. Thus, several variants have been developed over time, including: the consecutive injection of subconvulsive doses of PTZ (every day PTZ kindling model), without a challenge dose, until the development of the fully-kindled state, which requires an induction extending over 6–8 weeks and requiring between 29 and 36 injections before reaching the fully kindled state (Corda et al. 1991; Seghatoleslam et al. 2016; Zhen et al. 2016); the 48 h interval injection model of subconvulsive doses of PTZ (every other day PTZ kindling model), with or without challenge dose at the end, which requires an induction extending over 4–6 weeks and requiring between 13 and 19 injections before reaching the fully kindled state (Pahuja et al. 2013; Taiwe et al. 2016; Hassanzadeh et al. 2017); and the Win-PTZ model, consisting of an administration of 4 subconvulsive doses on the 1st day, then a period of 22 days without injection, and finally one injection per day for the last three days for a total of 25 days before the kindle state (Davoudi et al. 2013; Erkeç and Arihan, 2015). Among these different models, the most widely used is the injection of subconvulsive doses (30 or 35 mg/kg) every 48 h, with administration of a challenge dose at the end (Pahuja et al. 2013) or without administration of a challenge dose at the end for a total average of 15 injections (Taiwe et al. 2015).

The existence of such a diversity of models of kindling-PTZ raises the concern of which model suits best to induce chronic epilepsy and what are the advantages and limitations of each model? Indeed, since Corda et al. (1991), no study to our knowledge has attempted to compare the different models of kindling with a view to highlighting their advantages and limitations at both the behavioral and biochemical levels. Furthermore, the literature reveals that some authors administer a challenge dose at the end of the induction of kindling while others do not (Pahuja et al. 2013; Taiwe et al. 2015). Let's take for example the case of the subconvulsive dose of PTZ 30 or 35 mg/kg injected every other day, which has shown to induce kindling after 12–19 injections (Corda et al. 1991; Pahuja et al. 2013; Taiwe et al. 2015; Erkeç and Arihan, 2015) and presented all the typical phenotypic characteristics as describe by the Racine'scale, attesting the good development of epileptogenesis and epilepsy; the same model was later modified with the administration of a challenge dose (70 mg/kg) at the end of the study, in order to assess the stability of induced epilepsy over time, but also to assess the effectiveness of a potential antiepileptic drug on tonic-clonic seizures appearing following the administration of the challenge dose (Pahuja et al. 2013).

Although the challenge injected at the end increased the severity of the epilepsy, it however also resulted in the death of many experimental subjects who had nevertheless reached the end of the induction cycle of kindling (up to 60 % of subjects), which made it impossible to use their biochemical data (Pahuja et al. 2013). The deaths observed do not question the role and the necessity to have the challenge in this model, but rather the right period to have that challenge, and for which outcomes? We therefore hypothesize that given the ability of the challenge dose to trigger tonic-clonic seizures which are normally only seen at the end of the epileptogenesis process, challenging the animal at the beginning of the induction could increase the severity of the epileptogenesis and improve the number of animals undergoing kindling and reducing or cancelling the number of animals dying at the end of the experiment.

Thus the present study sought first to compare the effect of the administration of the challenge dose at the beginning of the experiment, on the development of kindling in the model of PTZ-kindling frequently encountered in the literature, namely the kindling every other day (KEOD) versus the kindling every day (KED) and later to verify the effectiveness of sodium valproate on the model that meets best the kindling's severity.

2. Materials and methods

2.1. Animals

Albinos Wistar rats *(Ratus norvegicus)* from both sexes aged between 2 and 3 months weighing between 150 and 200 g were used for this study. They were raised at room temperature and subjected to a natural light-dark cycle at the animal house of the Department of Animal Biology of University of Dschang, Cameroon. Rats received water and food ad libitum. Animals were treated in accordance with the guidelines of the Cameroonian bioethics committee (reg N. FWA IRB00001954) and in accordance with NIH-Care and Use of Laboratory Animals manual. Efforts were also made to minimize animal suffering and to reduce the number of animal used in the experiment.

2.2. Drugs and treatments

The work spanned two experiments I and II: **Experiment I** aimed to determine the kindling model that best induces epilepsy; **Experiment II** aimed to determine the dose of valproate which protects the most effectively against the kindling model inducing the best epilepsy which will have been highlighted in the first part.

For the first part of the work, pentyletetrazole (PTZ) (Sigma-Aldrich, St. Louis, USA) was used to induce kindling. PTZ was injected intraperitoneally (ip) either at a dose of 70 mg/kg (challenge dose) or 35 mg/kg (subconvulsive dose). Distilled water was administered by oral gavage (p.o) (10 mL/kg). A total of 24 rats was divided into 4 groups of 6 rats (3 males and 3 females) and treated as follow: **Group 1:** Control, receiving only distilled water (10 mL/kg); **Group 2:** Usual kindling every other day (UKEOD) group, receiving distilled water followed by administration of PTZ 35 mg/kg every alternate day; **Group 3:** Challenged kindling every other day (CKEOD) group, receiving a first dose of PTZ 70 mg/kg (challenge dose) at day one, and by day 3, distilled water followed by administration of PTZ 35 mg/kg every alternate day; **Group 4:** Challenged kindling every day (CKED) group, receiving a first dose of PTZ 70 mg/kg (challenge dose) at day one, and by day 2, distilled water followed by administration of PTZ 35 mg/kg every day. The challenged kindling every day group was used here as second control of the challenged kindling every other day. Since our aims was to see the impact of the challenge on the PTZ kindling model most used from the literature review, we did not therefore use a group for kindling every day without challenge.

Chronic epilepsy was induced by the kindling method (Pahuja et al. 2013; Erkeç and Arihan, 2015; Zhen et al. 2016). Kindling was induced by i.p. injections of subconvulsive doses (35 mg/kg) of PTZ to the animals. However, based on the different groups the sub-convulsive PTZ dose was given a day later following a single administration of a convulsive PTZ dose of 70 mg/kg (challenge dose) (CKED) or two days following the challenge (CKEOD). Subconvulsive PTZ dose injections were made consecutively, at 48 h interval for the UKEOD and CKEOD models and 24 h interval for the CKED model. After each injection, animals were placed individually in cages and observed for 30 min. The intensity of the convulsions was characterized using the 6-point grid of the Racine scale as described below (Pahuja et al. 2013):

- Stage 0: no response;.
- Stage 1: contraction of the ears, face, tail;.
- Stage 2: tilt of the head, clonies of the head and myoclonic shaking of the body;.
- Stage 3: unilateral front paw clonies;.
- Stage 4: training of the body with bilateral clonies of the front legs;.
- Stage 5: generalized tonic-clonic seizures with loss of normal reflexes.

Kindling was considered successful on the appearance of stage 4 or

stage 5 characteristics in PTZ-treated animals during 02 consecutive trials. In order to appreciate the stability of the kindling model over time, 7 additional injections were administered to the animals after they were fully-kindled. The parameters evaluated were: the speed of progression of the stages (evaluated by the number of injections necessary to reach stages 4 and 5), the severity of seizures (evaluated by the percentage of animals having reached stage 4 and stage 5, and the onset time to reach these different stages) and the stability of seizures (evaluated by the percentage of animals having repeated stages 4 and 5 during the 7 additional injections after the fully-kindled state) (Fig. 1).

Experiments II: Sodium valproate (Sanofi-aventis, Paris, France) was administered to the animals in order to verify the dose of the latter which best protects against the model which best induces kindling. The two doses of valproate most frequently used in the literature to protect against kindling (200 mg/kg and 300 mg/kg) were thus administered to different groups of animals (Löscher et al. 1984; Sefil et al. 2015; Taiwe et al. 2016; Kumar et al. 2016; Pahwa and Goel, 2016). A total of 24 rats was divided into 4 groups of 6 rats (3 males and 3 females) and treated as follow: **Group 1:** Control (neutral control), receiving only distilled water (10 mL/kg); **Group 2:** receiving distilled water (10 mL/kg); **Group 3**: receiving valproate 200 mg/kg (VAL 200); **Group 4:** receiving valproate 300 mg/kg (VAL 300). Animals in groups 2, 3 and 4 were subjected to kindling induced by the CKEOD method, 30 min after having received the various treatments (distilled water or valproate). Immediately after each PTZ injection, animals were observed for 30 min during which the intensity of the convulsions was noted according to the above-mentioned racine scale grid. Each fully-kindled animal was subjected to 7 additional injections as described in experiments I of the work (Fig. 1).

2.3. Biochemical analysis

At the end of each part of the work, twenty-four hours (24 h) after the end of the kindling induction period, animals were sacrificed by decapitation. The blood was collected in EDTA tubes, and then centrifuged at 3500 rpm for 15 min (MF-80–1D centrifuge, Medifield equipment, England) and the plasma was collected and frozen for assessment of Alanine amino transferase (ALAT) and Aspartate amino transferase (ASAT) levels. The hippocampus and the pre-frontal cortex were also removed, weighed and frozen at − 20 °C to perform the assays for oxidative stress markers such as malondialdehyde (MDA), reduced glutathione (GSH) and Catalase activity, and to evaluate the GABA level and GABA transaminase (GABA-T) activity.

2.3.1. Homogenates preparation

Hippocampus and pre-frontal cortex were grind to homogenate (10 % w/v) in a porcelain mortar. Each of the homogenates was prepared using a solution of 0.1 M phosphate buffer containing 1 % Triton-100X (pH 7.4). These grinded structures were individually centrifuged at room temperature for 15 min at 3000 rpm using a MF-80–1D centrifuge (Medifield equipment, England) and the supernatant were collected and served as homogenate for various assays (Ngoupaye et al. 2017).

2.3.2. Determination of malondialdehyde level

A volume of 250 µl of the homogenate was introduced in tests tubes. 250 µl of 20 % trichloroacetic acid (TCA), 500 µl of 0.67 % thiobarbituric acid (TBA) and 10 µl of 0.1 % BHT (Butylated hydroxytoluene) were added to each tube. The blank solution consisted of all the above stated elements except the homogenate. The tubes were sealed and incubated for 10 min at 90 °C and then cooled with tap water. The result was centrifuged at 3000 rpm for 10 min at room temperature using a MF-80–1D centrifuge (Medifield equipment, England). The supernatant was pipetted and the absorbance read at 532 nm on a BIORAD spectrophotometer, SMART SPEC 3000 (USA) against the blank and results were expressed in nmol/mg of wet tissue (Ngoupaye et al. 2020).

2.3.3. Determination reduced glutathione level

A volume of 1500 µl of the Ellman reagent DTNB (0.1 mM 5,5'-dithio bis-2-nitrobenzoic acid in 0.3 M phosphate buffer with 1 % of sodium citrate solution) was introduced into tubes previously containing 100 µl of homogenate (test tube) and 100 µl of phosphate buffer (PBS) then the mixtures were incubated for 1 h at room temperature. The blank solution consisted of all the above stated elements except the homogenate. The absorbance was read after incubation using a BIORAD spectrophotometer SMART SPEC 3000 (USA) at 412 nm against the blank and results were expressed in nmol/mg of wet tissue (Ngoupaye et al. 2020).

2.3.4. Determination of catalase activity

A volume of 375 µl of phosphate buffer (PBS) was introduced into the test tube previously containing 25 µl of tissue homogenates. Subsequently, 100 µl of 50 mM hydrogen peroxide (H_2O_2) was introduced into the tube and the reaction proceeded for 1 min, after which 1000 µl of the solution of 5 % potassium dichromate + acetic acid was introduced into the reaction medium to stop the reaction. The tubes were then brought to a boil for 10 min in a boiling water bath. After cooling, the reading was done using a BIORAD spectrophotometer SMART SPEC 3000 (USA) at 570 nm against the blank. Activity was established from an H_2O_2 calibration curve and results were expressed in µmol of H_2O_2 decomposed/ min/ mg of proteins (Dimo et al. 2006).

2.3.5. Determination of GABA transaminase activity

The activity of GABA transaminase (GABA-T) was evaluated according to the method described by Moto et al. (2018) with little modification and is based on the coloration formed by succinic semialdehyde acid resulting from the degradation of GABA by GABA-T in the presence of $FeCl_3$. Briefly, the reaction medium consisted of 15 µmol of

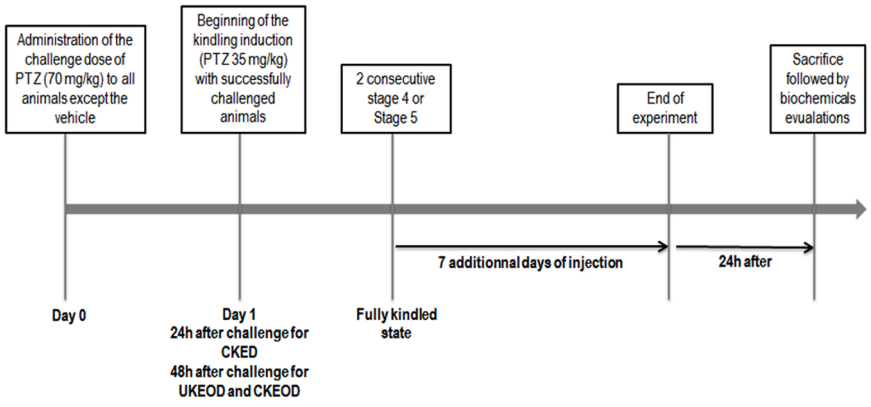

Fig. 1. Experimental procedure for kindling evaluation.

α-oxoglutarate, 15 µmol of GABA, 10 µg of pyridoxal phosphate. To this reaction medium was added either 100 µl of homogenate (test tubes) or 100 µl of 5 % methanol (white tube). Then the volume of each tube was made up to 3 mL using tris-HCl buffer (50 mM, pH 7.4). The tubes were then incubated at 37 °C for 30 min. After incubation, 500 µl of 20 % TCA was introduced into each tube, and 30 s later, 1000 µl of 12 % $FeCl_3$ was introduced into each tube and the absorbance was immediately read with a spectrophotometer at 610 nm against blank, then a second reading was taken 60 s after the first reading. The absorbance was proportional to the activity of GABA-T in the sample. GABA-T activity was expressed as nmol of GABA decomposed/min/mg protein.

2.3.6. Determination of GABA level

GABA was evaluated according to the method described by Moto et al. (2018) with little modification and is based on the coloration formed by the reaction of GABA and ninhydrin in an alkaline medium, in the presence of glutamate. Briefly, in the reaction medium consisting of 200 µl of 0.14 M Ninhydrin [prepared in carbonate-bicarbonate buffer (0.5 M, pH 9.9)], and 100 µl of glacial tricloroacetic acid (TCA) at 10 %, 100 µl of homogenate was introduced, and the whole was incubated at 60 °C for 30 min. After cooling, all the contents of each tube were introduced into tubes containing 5000 µl of copper tartrate. The tubes were then incubated at 25 °C for 10 min. The absorbance was then read on a spectrophotometer at 451 nm against the blank, and was proportional to the concentration of GABA in the sample.

The concentration of GABA in each sample was expressed in µg/mg of tissue and was determined from a GABA standard curve. The reaction medium for the standard samples contained in addition to the ninhydrin and the TCA mentioned above, 1.5 mg of glutamate prepared in 100 µl of 10 % TCA.

2.3.7. Determination of ALAT level

The plasma level of ALAT was determined using a commercial kit with reagents already prepared (Dutch Diagnostics, Netherlands). The absorbance was read using a BIORAD spectrophotometer SMART SPEC 3000 (USA) at 340 nm against the blank and results were expressed in U/L.

2.3.8. Determination of ASAT level

The plasma level of ASAT was determined using a commercial kit with reagents already prepared (Dutch Diagnostics, Netherlands). The absorbance was read using a BIORAD spectrophotometer SMART SPEC 3000 (USA) at 340 nm against the blank and results were expressed in U/L.

2.4. Statistical analysis

The results were analyzed using Graph Pad Prism software version 5.03. They were presented as mean ± Standard Error on Mean (SEM). The one-way ANOVA followed by Newman-keuls post hoc test was used for the multiple comparisons of groups and two-way ANOVA followed by the Bonferonni test compared the averages for bivariate tests. The tests were significant when $p < 0.05$.

3. Results

3.1. Effect of different kindling models on the induction of epilepsy

3.1.1. Effect of each kindling model on stages progression

Fig. 2 depicts the effect of each kindling model on the stage progression. There was a treatment effects within the groups as shown Two-way ANOVA followed by Bonferroni test [$F(3,20) = 12.13$; $p < 0.0001$]. The CKEOD showed a significant increase in the epileptogenesis compared to UKEOD, as seen by the reduction of the number of injections in stage 3, from 8.83 ± 1.33 in the UKEOD group to 3.67 ± 0.64 in the CKEOD group [$F(1,10) = 7.114$; $p = 0.0236$], stage 4 from 13.67 ± 2.04 in the UKEOD group to 6.33 ± 1.84 in the CKEOD group [$F(1,10) = 5.559$; $p = 0.0076$], stage 5 from 17.17 ± 1.80 in the

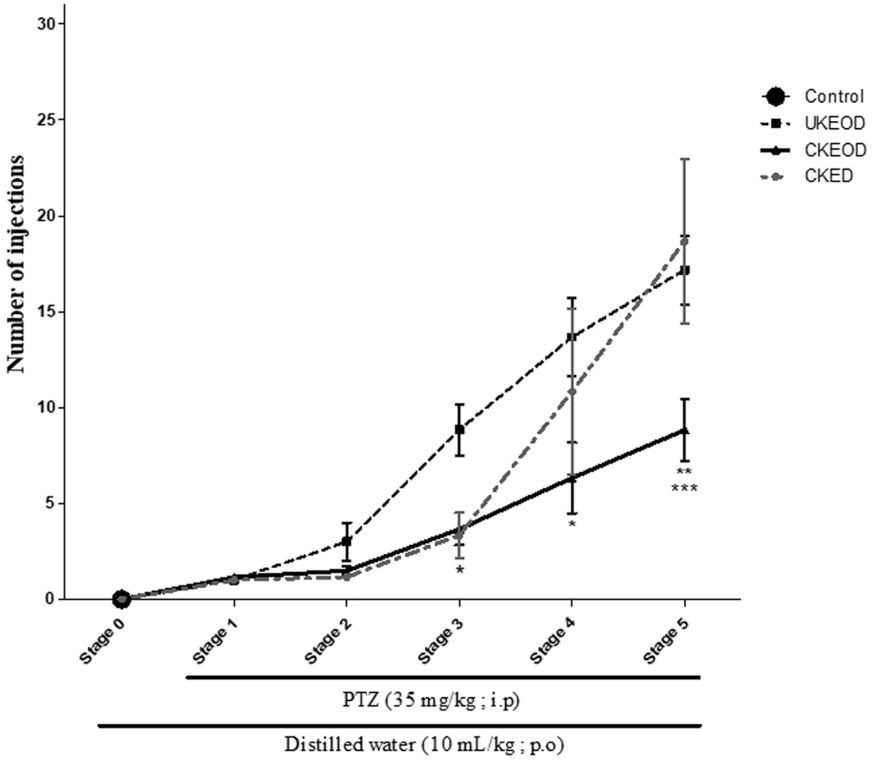

Fig. 2. Effect of each kindling model on stages progression Data expressed as Mean ± SEM; n = 6; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.01$ when compared to CKEOD; two-way ANOVA followed by Bonferonni post-test; Control= neutral control; UKEOD = usual kindling every other day; CKEOD = challenged kindling every other day; CKED = challenged kindling every day.

UKEOD group to 8.83 ± 1.62 in the CKEOD [F (1,10)= 11.11; p = 0.0076]. Likewise, the reduction of injections was seen in CKEOD compared to CKED at the stage 3 [F (1,10)= 8.000; p = 0.0179], and at the stage from 5 18.67 ± 4.31 in the CKED group to 8.83 ± 1.62 in the CKEOD group [F (1,10)= 31.75; p < 0.00001] (Fig. 2).

3.1.2. Effect of each kindling model on seizure severity

Animals from all treated groups reached stage 4. However, the animals in the CKEOD group significantly reduced the onset time of stage 4 compared to the UKEOD group, which dropped from 12.67 ± 3.73 min in the UKEOD group to 5.24 ± 0.35 min in the CKEOD group [F (2,17)= 3.896; p = 0.0434] (Table 1). In addition, the CKEOD group reduced the onset time of stage 4 by 46.42 % compared to the CKED group, which dropped from 9.78 ± 1.25 min in the CKED group to 5.24 ± 0.35 min in the CKEOD group [F (2,17)= 11.46; p = 0.0010] (Table 1).

Five animals out of six animals (83.33 %) reached the stage 5 in the CKEOD group while in the UKEOD and CKED groups two animals out of six (33.33 %) reached the stage 5 (Table 1). There was a challenge effect in the UKEOD group as the CKEOD showed a reduce onset time of stage 5 in CKEOD versus UKEOD from 23.92 ± 4.17 min in the UKEOD group to 15.44 ± 4.66 min at the CKEOD group [F (2,17)= 6.946; p = 0.0073] and from 28.46 ± 1.17 min in the CKED group to 15.44 ± 4.66 min in the CKEOD group [F (2,17)= 6.946; p = 0.0073] (Table 1).

3.1.3. Effect of each kindling model on seizure stability

Table 2 depicts the impacts of the challenge dose given during the kindling on seizure stability assessed on stage 4 and 5, after 7 post injections. CKEOD group showed an increase in the number and percentage of repetition of the stage 4 during the 7 days of injections post kindled, from 16.66 % in UKEOD and CKED to 83.33 % every single day of injection (Table 2). There was a treatment effect as 7 additional injections induced significant increase in the number of seizure repetition at stage 5 in CKEOD group compared to UKEOD and CKED groups [F (2,17)= 8.238; p = 0.039] and the control [F (2,17)= 11.43; p = 0.0010]. This is seen as the challenge given on the first day has risen the percentage of animal which reached the stage 5 after every single injection post kindle stage from 0 % in UKEOD and CKED to 33.33 % in CKEOD (Table 2).

3.1.4. Effect of different kindling models on oxidative stress

Fig. 3 depicts the oxidative stress activities in the hippocampus and the prefrontal cortex. CKEOD showed a significant increase in MDA level in the hippocampus [F (2,11)= 11.66; p = 0.0032] and the prefrontal cortex [F (2,11)= 5.392.46;p = 0.0289] compared to UKEOD and CKED. Likewise it showed a significant increase in the hippocampus [F (2,11)= 5.361; p = 0.0293] and the prefrontal cortex [F (2,11)= 4.520; p = 0.011] compared to the control (Fig. 3A and 3B).

CKEOD showed a significant decrease in the level of GSH in the hippocampus [F (2,11)= 5.392.46;p = 0.0289] and a tendency in the prefrontal cortex [F (2,11)= 3.618;p = 0.0703]compared to CKED and [F (2,11)= 21.43; p = 0.0004] compared to control in the prefrontal cortex. The UKEOD decreased significantly the level of GSH in the hippocampus [F (2,11)= 5.692; p = 0.0253] and the prefrontal cortex as compared to the control [F (2,11)= 7.761; p = 0.0110] (Fig. 3C and 3D). The catalase level vas significantly decreased in the hippocampus of UKEOD group compared to the control [F (2,11)= 6.774; p = 0.0160] (Fig. 3E and 3F).

Table 2
Effect of each kindling model on seizure stability.

	Stage 4			Stage 5		
	7/7	6/7	5/7	7/7	6/7	5/7
Control	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)
UKEOD	1 (16.66 %)	3 (50 %)	2 (33.33 %)	0 (0 %)	0 (0 %)	0 (0 %)
CKEOD	5 (83.33 %)	0 (0 %)	1 (16.66 %)	1 (16.66 %)	2 (33.33 %)	0 (0 %)
CKED	1 (16.66 %)	2 (33.33 %)	2 (33.33 %)	0 (0 %)	0 (0 %)	0 (0 %)

Table shows the number and the percentage of animals having exhibited the stage 4 or stage 5 during all the 7 days of post-kindling injections (7/7), during 6 days over the 7 (6/7), and during 5 days over the 7 (5/7).

3.1.5. Effect of different kindling models on GABAergic signaling

Fig. 4 depicts the Gabaergic signaling pathways assessed on the levels of GABA and GABA transaminase (GABA-T) activity in the hippocampus and the prefrontal cortex. The kindling induced a significant decrease in the hippocampal GABA level in the UKEOD [F (2,11)= 9.263; p = 0.0065]and CKEOD compared to the control [F (2,11)= 7.585; p = 0.0117]. Whereas there was a significant increase in the GABA-T activity in the UKEOD and CKEOD groups compared to control [F (2,11)= 11.18; p = 0.0036] (Fig. 4A and 4C).

The GABA content in the prefrontal cortex was significantly increased in the CKED group compared to the control [F (2,11)= 7.41; p = 0.0008], the UKEOD group [F (2,11)= 18.99; p = 0.0006], and The CKEOD group [F (2,11)= 8.923; p = 0.0073]. There was no change in the GABA-T activity [F (4,15)= 0.8120; p = 0.05114] (Fig. 4B and D).

3.1.6. Effect of different kindling models on liver toxicity

No significant difference was recorded between the different groups in either the plasma level of ALAT (Fig. 5A) or the plasma level of ASAT (Fig. 5B).

3.2. Effect of different doses of valproate on the protection against epilepsy induced by the CKEOD model

3.2.1. Effect of different doses of valproate on the rate of progression of seizures

Fig. 6 depicts the effect of the different doses of valproate on seizure progression. The kindling induced stage 4 in animal of the negative control group after 6.33 ± 1.84 injections [F (2,15)= 10.31; p = 0.0015]. This number of injection was significantly increased when animals were treated with Valproate, from 6.33 ± 1.84 in the negative control to 15.17 ± 3.29 at the 200 mg/kg dose [F (2,15)= 4.107; p = 0.0378], and to 16.17 ± 3.12 at 300 mg/kg [F (2,15)= 7.004;

Table 1
Effect of each kindling model on seizure severity.

	Stage 4			Stage 5		
	Number/6	Percentage (%)	Onset time (Min)	Number/6	Percentage (%)	Onset time (Min)
Control	0	0	30 ± 0.00	0	0	30 ± 0.00
UKEOD	6	100	12.67 ± 3.73	2	33,33	23.92 ± 4.17
CKEOD	6	100	5.24 ± 0.35 *	5	83,33	15.44 ± 4.66 * *
CKED	6	100	9.78 ± 1.25 * *	2	33,33	28.46 ± 1.17 * *

Data expressed as Mean ± SEM; n = 6; * p < 0.05; ** p < 0.01 when compared to UKEOD, one-way ANOVA followed by Newman keuls post-test; Control= neutral control; UKEOD = usual kindling every other day; CKEOD = challenged kindling every other day; CKED = challenged kindling every day.

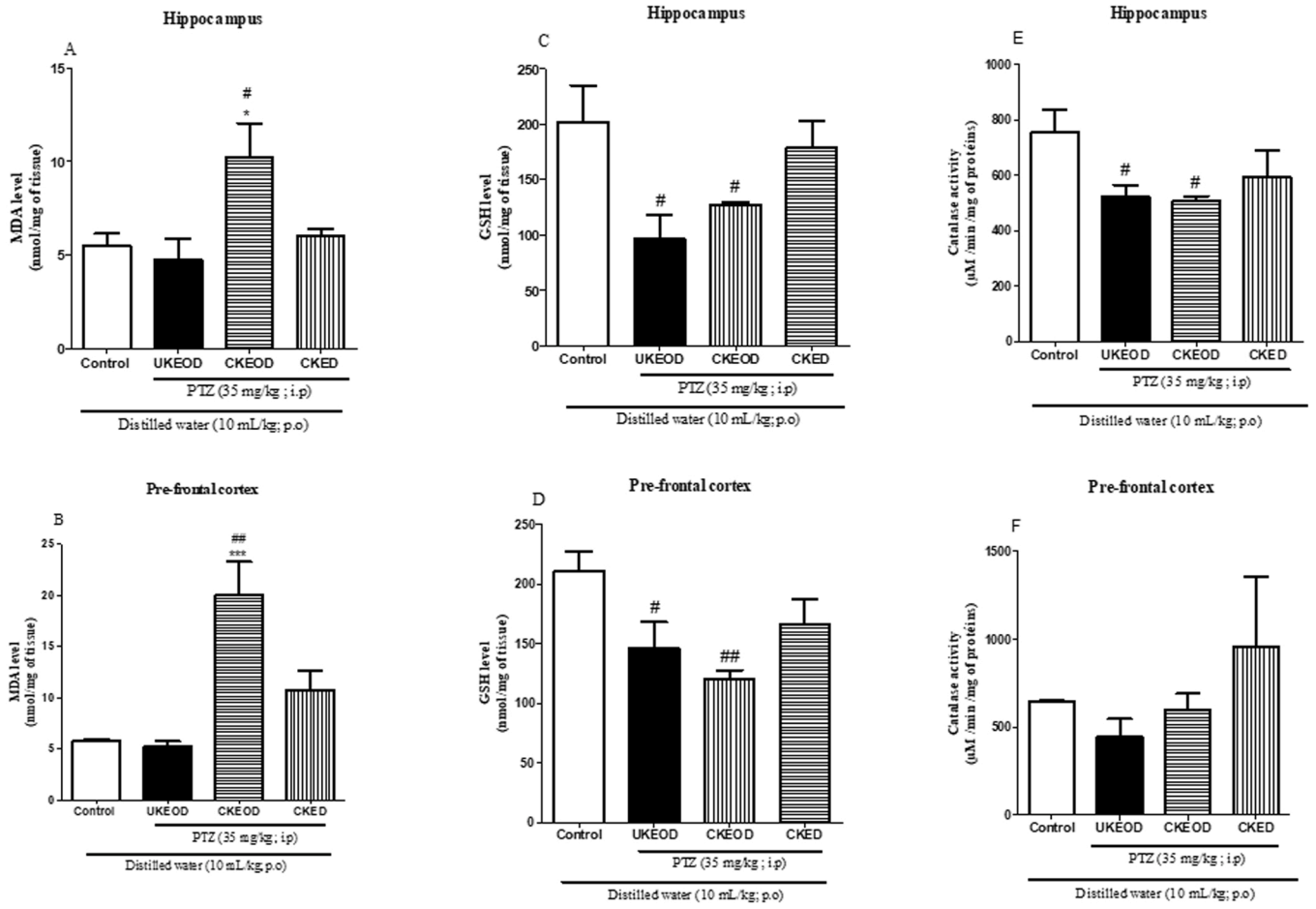

Fig. 3. Effect of each kindling model on MDA, GSH and Catalase in the hippocampus and the prefrontal cortex Data expressed as Mean ± SEM; n = 4; * $p < 0.05$, *** $p < 0.001$ when compared to CKEOD; # $p < 0.05$, ## $p < 0.01$ when compared to control, one-way ANOVA followed by Newman keuls post-test; Control = neutral control; UKEOD = usual kindling every other day; CKEOD = challenged kindling every other day; CKED = challenged kindling every day.

p = 0.0071]. The kindling induced stage 5 in animal of the negative control group after 8.83 ± 1.62 injections [F(2,15)= 24.50; p < 0.0001]. This number of injection was significantly increased when animals were treated with Valproate, from 8.83 ± 1.62 in the negative control to 17.17 ± 2.43 at the dose of 200 mg/kg [F (2,15)= 5.774; p = 0.0138], and to 18.67 ± 2.33 at the dose of 300 mg/kg [F (2,15)= 20.85; p < 0.0001]. Valproate 300 mg/kg has a marked increase compare to valproate 200 mg/kg in the number of injection at stage 4 and 5 (P = 0.0071 vs p = 0.0378) and (p < 0.0001 vs p = 0.0138) (Fig. 6).

3.2.2. *Effect of different doses of valproate on seizure severity*

Fig. 7 depicts the effect of Valproate on seizure severity and stability induced by the CKEOD model.

Valproate at both doses significantly altered the seizure severity by significantly decreasing the percentage of animal that reached the stage 4 [F (2,17)= 3.824; p = 0.0455] and 5 [F (2,17)= 5.333; p = 0.0178] respectively at the doses 200 mg/kg and 300 mg/kg as compared to the animal that received only distilled water (H_2O) (Fig. 7A and 7B). Likewise, the onset time to reach stage 4 was significantly decreased when animal treated with valproate 200 mg/kg and 300mk/kg. It shifted from 5.24 ± 0.35 min in the negative control to 20.43 ± 4.51 min at the 200 mg/kg dose [F (2,17)= 11.20; p = 0.0011] and to 22.28 ± 4.89 min at a dose of 300 mg/kg [F (2,17)= 12.02; p = 0.0008] as compared to H_2O group. Only Valproate at the dose 300 mg/kg was able to significantly delay the onset time of stage 5 as compared to H_2O group, from 15.44 ± 4.66 min in the negative control to 29.28 ± 0.72 min at the 300 mg/kg dose [F (2,17)= 8.420; p = 0.0035] (Fig. 7C and 7D).

Regarding the effects of the different doses of Valproate on the seizure stability during the 7 additional injections, assessed on the percentage of repetition to reach the stage 4 and stage 5; Valproate at the dose 200 mg/kg as well as the dose 300 mg/kg significantly decrease the percentage of repetition from 95.24 % in the negative control to 38.10 % at the dose 200 mg/kg [F (2,17)= 8.889; p = 0.0028], and to 28.57 % the dose 300 mg/kg [F (2,17)= 11.53; p = 0.0009], with a marked effect at the dose 300 mg/kg(p = 0.0009). The percentage of repetition of stage 5 was significantly reduced at both doses as compared to H_2O group, from 57.14 % in the negative control (H_2O group) to 4.76 % [F (2,17)= 9.030; p = 0.0027] and [F (2,17)= 8.288; p = 0.0038] respectively at the doses 200 mg/kg and 300 mg/kg (Fig. 7E and 7F).

3.2.3. *Effect of different doses of valproate on oxidative stress*

Fig. 8 depicts effects of the differents doses of Valproate on oxidative stress parameters such as Malondialdehyde (MDA), reduced Glutathione (GSH) and Catalase (CAT). The kindling has significantly increased the MDA levels in both the hippocampus [F (2,11)= 5.554; p = 0.0269]and prefrontal cortex [F (2,11)= 9.641; p = 0.0058]compared to the control. This level was significantly decreased by only the valproate 300 mg/kg [F (2,11)= 11.46; p = 0.0034] as compare to the negative control in the hippocampus. Whereas in the prefrontal cortex Valproate at the doses 200 mg/kg [F (2,11)= 12.34; p = 0.0026] and 300 mg/kg [F (2,11)= 11.78; p = 0.0031] significantly decreased the MDA levels as compared to the negative control (Fig. 8A and B).

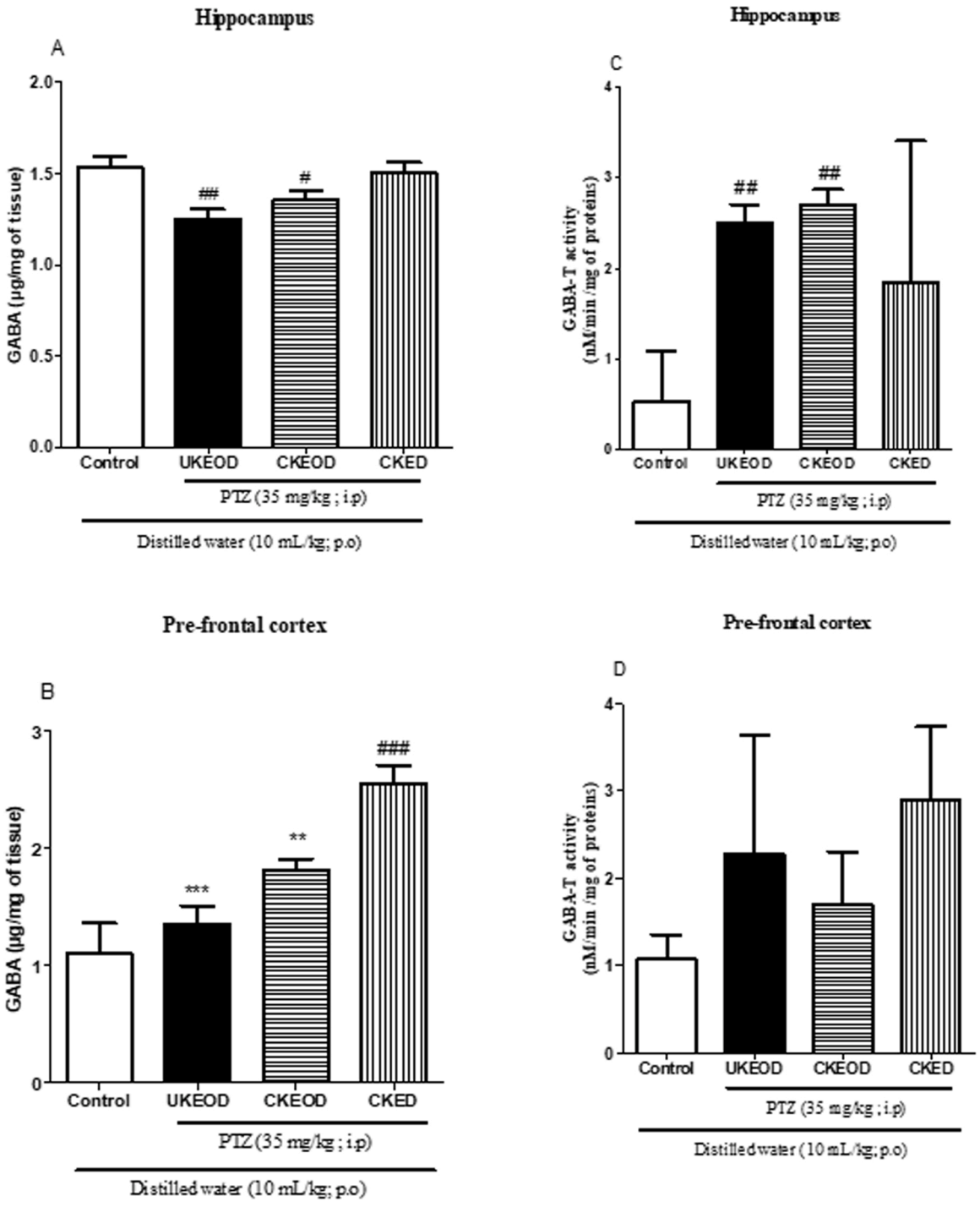

Fig. 4. Effect of different kindling models on GABAergic signaling in the hippocampus and the prefrontal cortex. A, C=GABA; B,D=GABA transaminase. Data expressed as Mean ± SEM; n = 4; # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$, when compared to control;; ** $p < 0.01$; *** $p < 0.001$ when compared to CKED, one-way ANOVA followed by Newman keuls post-test; Control = neutral control; UKEOD = usual kindling every other day; CKEOD = challenged kindling every other day; CKED = challenged kindling every day.

The Reduced GSH was significantly reduced in the negative control group in the hippocampus [F (2,11)= 5.424; p = 0.0285] and the prefrontal cortex [F (2,11)= 13.55; p = 0.0019] as compared to the control. This level was significantly increased at the doses 200 mg/kg [F (2,11)= 8.318; p = 0.0090] and 300 mg/kg[F (2,11)= 23.07; p = 0.0003] in the hippocampus. This level was further increased in the prefrontal cortex at the doses 200 mg/kg [F (2,11)= 4.643; p = 0.0412] and 300 mg/kg [F (2,11)= 14.01; p = 0.0017] as compared to the negative control, with a marked effect at the dose 300 mg/kg (p = 0.0017).

Fig. 8 E and F depicts the levels of the catalase in the hippocampus (Fig. 8E) and the prefrontal cortex (Fig. 8F). The Catalase level was significantly reduced in the negative control group only in the hippocampus [F (2,11)= 4.771; p = 0.0387] as compared to the control. This level was significantly increased at the doses 200 mg/kg [F (2,11)= 41.23; p < 0.0001] and 300 mg/kg[F (2,11)= 10.97; p = 0.0039] in the hippocampus. There was no significant catalase activity in the prefrontal cortex [F (3,15)= 2.273; p = 0.1323].

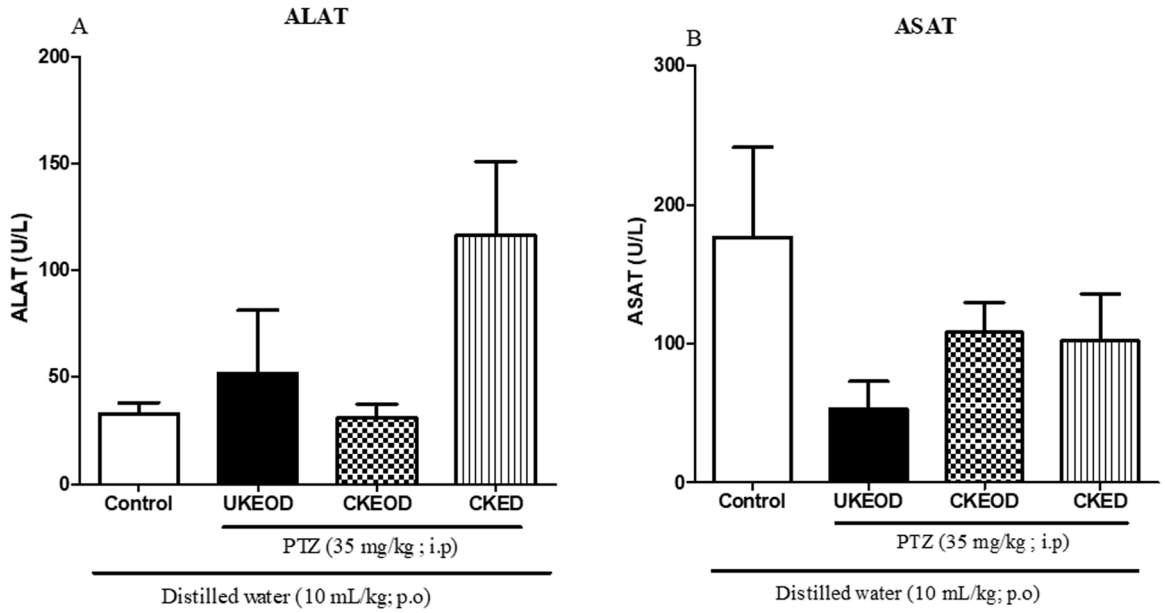

Fig. 5. Effect of each kindling model on plasma level of ALAT and ASAT Data expressed as Mean ± SEM; n = 4; one-way ANOVA followed by Newman keuls post-test; Control = neutral control; UKEOD = usual kindling every other day; CKEOD = challenged kindling every other day; CKED = challenged kindling every day.

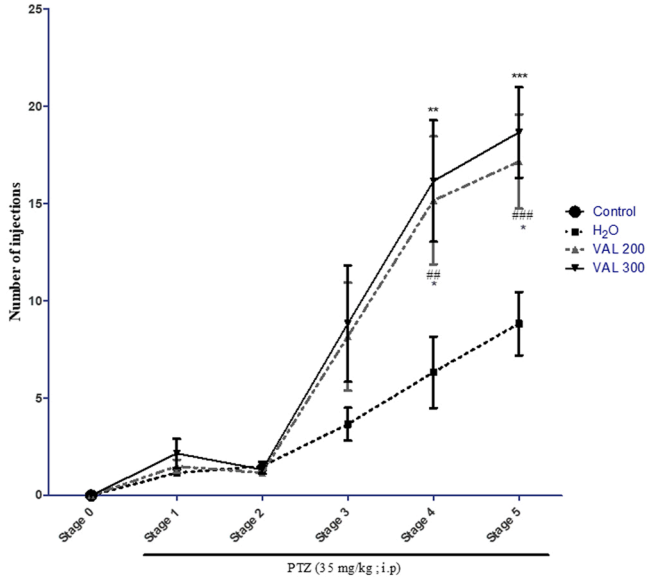

Fig. 6. Effect of various doses of valproate on stages progression in CKEOD model. Data expressed as Mean ± SEM; n = 6; *** $p < 0.001$ when compared VAL 300 and VAL 200 to H_2O; ## $p < 0.01$, ### $p < 0.001$ when compared to Control, two-way ANOVA followed by Bonferonni post-test; Control= neutral control; H_20 = distilled water; VAL 200 = Valproate 200 mg/kg; VAL 300 = Valproate 300 mg/kg.

3.2.4. *Effect of different doses of valproate on GABAergic signaling*

Fig. 9 depicts effects of the different doses of Valproate on Gabaergic signalling assessed on GABA level and GABA-T. There was a tendency to decrease in the negative control GABA level in the hippocampus [F (2,11)= 4.171; p = 0.0523] and a significant increase in the prefrontal cortex [F (2,11)= 5.678; p = 0.0254] as compared to the control. Valproate (200 mg/kg) significantly decreased the GABA level previously increased by the kindling in the prefrontal cortex (Fig. 9A and 9B).

The GABA-T activity was significantly increased in the negative control in the hippocampus [F (2,11)= 7.316; p = 0.0095] as compared to the control. The valproate significantly decreased the level of GABA-T respectively at the dose 200 mg/kg [F (2,11)= 29.55; p = 0.0001] and 300 mg/kg [F (2,11)= 20.20; p = 0.0005].

3.2.5. *Effect of different doses of valproate on liver toxicity*

Fig. 10 depicts the effect of Valproate toxicity biomarkers. There was a significant increase in serum ALAT level in Valproate 200 mg/kg [F (2,11)= 21.87; p = 0.0004] and 300 mg/kg [F (2,11)= 10.26; p = 0.0048] as compared to the negative control (Fig. 10A). There was no significant change in the plasma ASAT level [F (2,11)= 4.171; p = 0.0523] as compared to the negative control (Fig. 10 B).

Data expressed as Mean ± SEM; n = 4; * *p < 0.01, * **p < 0.001, when compared to H_20, one-way ANOVA followed by Newman keuls post-test; Control = neutral control; H_20 = distilled water; VAL 200 = Valproate 200 mg/kg; VAL 300 = Valproate 300 mg/kg.

4. Discussion

The aim of the present study was to evaluate the effect of the administration of the challenge dose on day 1, on the development of kindling in the two models of PTZ-kindling frequently encountered in the literature, namely kindling every day and kindling every other day, this in comparison to a classic model of kindling every other day without challenge. Kindling PTZ is one of the most widely used models for inducing chronic epilepsy and is recognized to mimic many features of human epilepsy (Erkeç & Arihan, 2015). The comparison was made on 3 main parameters, namely: the number of injections necessary to reach the fully kindled state, the severity of the induced chronic epilepsy and the stability of this epilepsy over time.

Regarding the number of injections necessary to reach the fully kindled state the CKEOD model significantly reduced the number of injections necessary to reach this state, compared to the UKEOD and CKED. Many studies show that kindling is considered successful when the animals develop the characteristics of Stage 4 or Stage 5 for at least 2 consecutive trials (Pahuja et al. 2013; Erkeç and Arihan, 2015; Samokhina and Samokhin, 2018). The CKEOD model thus allows reducing the time required for the development of chronic epilepsy, and consequently reduces the duration of studies. It is also important to note that no significant difference was found between the UKEOD and CKED groups in terms of the number of injections needed.

The reduction in the number of injections necessary for the

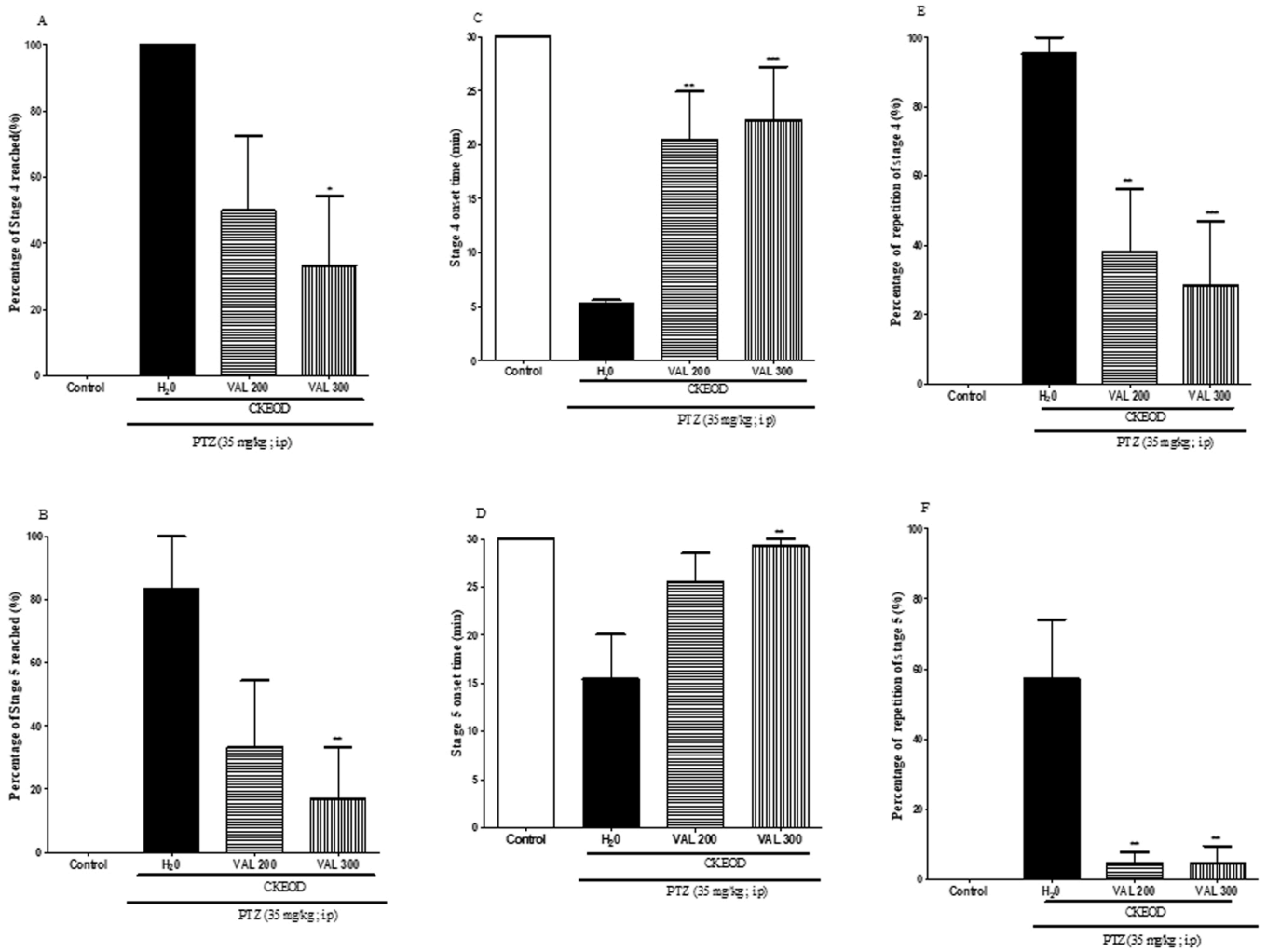

Fig. 7. Effect of various doses of valproate model on seizure severity and stability in CKEOD mode. Data expressed as Mean ± SEM; n = 6; * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$ when compared to H_2O, one-way ANOVA followed by Newman keuls post-test; Control = neutral control; H_20 = distilled water; VAL 200 = Valproate 200 mg/kg; VAL 300 = Valproate 300 mg/kg.

development of the fully kindled state observed with the CKEOD group could be due to the fact that the challenge dose would have strongly sensitized the cerebral neurons, resulting in a reduction of their threshold of excitability, in particular in the pre-frontal cortex, which is known to have overall low excitability thresholds compared to deep brain structures such as the hippocampus (Samokhina & Samokhin, 2018). This reduction in the threshold of excitability would have been significant enough to surpass the natural compensation process that would have taken place in the event that the administered dose was subconvulsive (Samokhina and Samokhin, 2018). The reduction of this threshold of excitability would therefore have facilitated the development of the epileptogenesis process in these animals, leading to a more rapid development of kindling in the latter (De Souza et al. 2019).

The seizures severity was also assessed through the percentage of animals having reached stages 4 and 5, as well as the latency at which these stages appeared in the animals. Indeed, the Racine's scale clearly established that stages 4 and 5 are those that characterize successful kindling (Pahuja et al. 2013; Erkeç and Arihan, 2015; Samokhina and Samokhin, 2018). The present study shows that the CKEOD group significantly increased the percentage of animals reaching stage 5 compared to the UKEOD and CKED groups. In addition, the CKEOD group significantly reduced the onset time of stages 4 and 5 compared to the UKEOD and CKED groups. This increase in the severity of the seizures observed with the CKEOD group could be explained by the lowering of the threshold of excitability induced by the starting challenge dose (PTZ 70 mg/kg). Indeed, the lowering of the threshold of excitability would have favored on, a desensitization of the GABAergic neurons, and a glutamatergic synaptogenesis, this reinforcing the glutamatergic signaling, with the consequence of reducing their activation time, hence an occurrence of more abundant and earlier seizures (Erkeç and Arihan, 2015; Samokhina and Samokhin, 2018; Taspinar et al. 2021). These results corroborate those of Ekonomou et al. (2001) who demonstrated that kindling PTZ amplifies glutamatergic signaling.

In order to appreciate the stability of each kindling model, 7 additional injections were made after the animals reached the fully kindled state. Although in general the fully kindled state was stable in all groups, the CKEOD group significantly maintained animals in a severe state of epilepsy throughout the duration of the 7 additional injections compared to the UKEOD groups and CKED. These results are thought to be due to the fact that greater glutamatergic synaptogenesis would be developed in the CKEOD group, thus allowing maintaining a much more severe epileptic state over time (Ekonomou et al. 2001; Samokhina and Samokhin, 2018).

Since an increase in cerebral excitatory activity leads to an acceleration of the metabolism, resulting in an increased production of free radicals which can be harmful to neurons(Liu et al. 2012; Vezzani et al. 2016), it was a question of verifying the state of oxidative stress in the different models of kindling. The results show that the CKEOD group

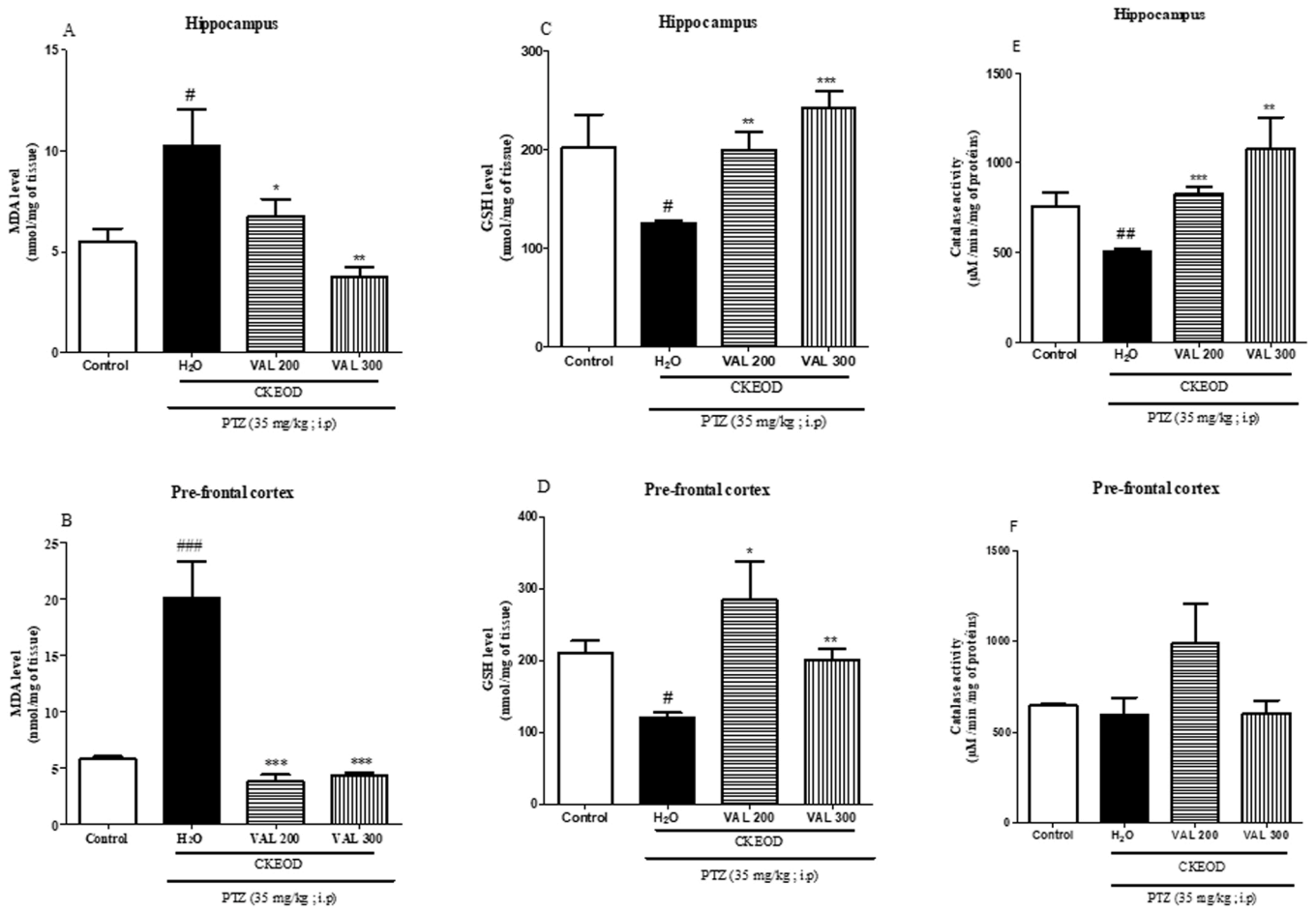

Fig. 8. depicts effects of the differents doses of Valproate on oxidative stress parameters such as Malondialdehyde (MDA) (A and B), reduced Glutathion (GSH) (C and D) and Catalase (CAT) (E and F) in the hippocampus and prefrontal cortex. Data expressed as Mean ± SEM; n = 6; * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$ when compared to H_2O, one-way ANOVA followed by Newman keuls post-test; Control = neutral control; H_20 = distilled water; VAL 200 = Valproate 200 mg/kg; VAL 300 = Valproate 300 mg/kg.

exhibits a more pronounced state of oxidative stress than the other groups, resulting in a significant increase in the level of malondialdehyde and a significant reduction in the level of reduced glutathione and catalase compared to the UKEOD group as well as to the control group. In fact, the free radicals produced as a result of cerebral hyperactivity such as that generated by kindling, interact with the lipids of the neuronal membrane, in particular at the level of the axon, which leads to peroxidation of lipids, one of its main derivatives being the MDA (Liu et al. 2012; Vezzani et al. 2016). In addition, GSH is a cofactor of glutathione peroxidase, an enzyme which contributes to the neutralization of free radicals in order to protect neurons. A decrease in reduced glutathione leads to an inability of glutathione peroxidase to neutralize free radicals, which exposes neurons to lipid peroxidation, one of the results of which is a reduction in the threshold of excitability of neurons, thus making them hyperexcitable, hence leading to the development of epilepsy (Liu et al. 2012; Vezzani et al. 2016). Catalase is an antioxidant enzyme that converts hydrogen peroxide (H_2O_2), a free radical, into water and oxygen, helping to protect the brain from potential damage from this free radical. Thus, a decrease in catalase activity contributes to amplifying brain oxidative stress, thereby exposing neurons to damage such as lipid peroxidation (Liu et al. 2012; Vezzani et al. 2016). The fact that the CKEOD group exhibited a greater increase in the level of MDA as well as a greater decrease in the level of GSH, and a decrease in catalase activity, compared to other groups thus corroborates the behavioral observations which demonstrate that the CKEOD model induces more severe epilepsy than the other models. Moreover, these significant alterations in the CKEOD group were observed in both the pre-frontal cortex and the hippocampus; and it is clearly established that the pre-frontal cortex and the hippocampus are among the most vulnerable structures during epileptogenesis (Roganovic et al. 2019). In addition, many hypotheses suggest that the oxidative stress created during epileptogenesis would contribute to the development of refractory forms of epilepsy, which do not respond to currently available antiepileptic drugs (Fokoua et al. 2021).

Since GABAergic signaling is the main inhibitory signaling in the central nervous system, and in particular in the brain (Taiwe et al. 2016; Moto et al. 2018), it was important to assess the impact of the different models on this signaling pathway. This was done by measuring GABA-T activity and brain GABA concentration. It appears that the CKEOD group, as well as the UKEOD group, significantly increased the activity of GABA-T, and significantly reduced the concentration of GABA in the hippocampus. It is clearly established that one of the main pathophysiological components of epilepsy is the inability of the inhibitory system to compensate the neuronal hyperexcitability induced by epileptogenesis (Taspinar et al. 2021). This physiopathological characteristic is thus reproduced in the CKEOD model accordingly, as shown by the reduction in the cerebral concentration of GABA. In addition, a measure of GABA-T activity, an enzyme that degrades GABA, to produce succinic semialdehyde acid and glutamate (Taiwe et al. 2016; Moto et al. 2018), revealed a significant increase of the latter, which would therefore explain the decrease in the concentration of GABA, and would thus reinforce the fact that the CKEOD model reproduces the disturbance of

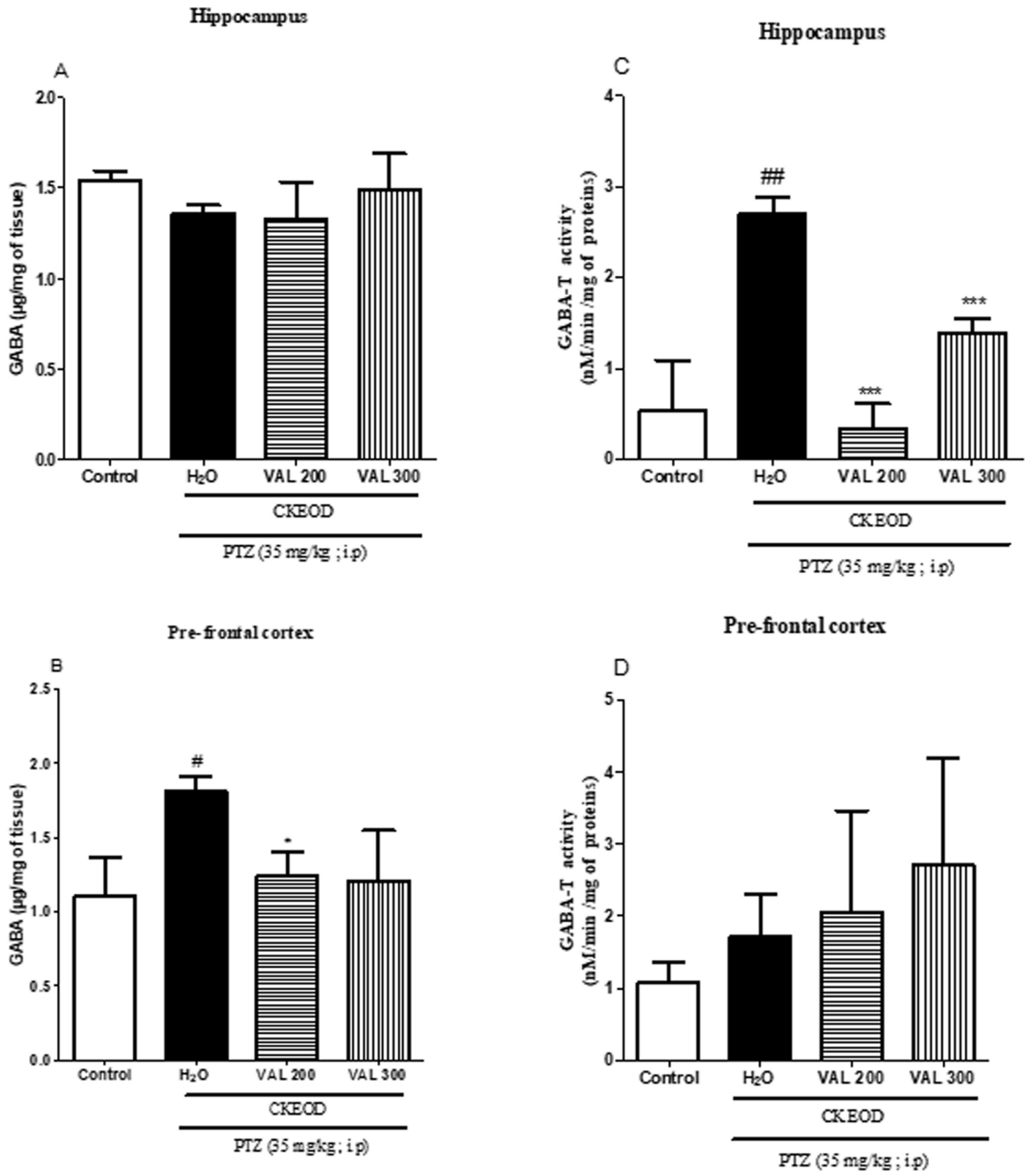

Fig. 9. Effects of the different doses of Valproate on Gabaergic signalling assessed on GABA levels (A and B) and GABA-T (C and D). Data expressed as Mean ± SEM; n = 4; # $p < 0.05$, ## $p < 0.01$ when compared to control; * $p < 0.01$, *** $p < 0.001$ when compared to H_20, one-way ANOVA followed by Newman keuls post-test; Control = neutral control; H_20 = distilled water; VAL 200 = Valproate 200 mg/kg; VAL 300 = Valproate 300 mg/kg.

GABAergic signaling which is found in subjects with epilepsy (Taiwe et al. 2016; Moto et al. 2018; Taspinar et al. 2021).

In order to verify the impact of the different models of kindling on the systemic toxicity of animals, two markers of the activity of the liver, which is the main purifying organ of the organism, were evaluated, in particular the ALAT and the ASAT (Hussein et al. 2013). It was found that the CKEOD model, like the other models, did not affect liver function. In fact, during its functioning, the liver produces many enzymes, one of the main ones being ALAT (Hussein et al. 2013). Under normal operating conditions, ALAT is mainly found in liver cells and at this level helps neutralize toxins. It is only when the liver cells are destroyed that ALAT leaks into the blood and ends up in the plasma. The fact that the ALAT rate in the CKEOD group remained relatively the same as that in the control group thus reveals that the CKEOD model does not present a risk of systemic toxicity that could hinder its use. Moreover, unlike the kindling model where the challenge dose is administered at the end and which causes the death of many subjects at the end of the experiment, thus making it impossible to use their data, particularly biochemical data (Pahuja et al. 2013), successfully challenged animals when subjected to the kindling procedure no longer die during the rest of the induction period, which makes the CKEOD model even more beneficial. Therefore, out of the three kindling models assessed, the CKEOD seems to be the model that best induced chronic epilepsy, reducing on top of that the length of epileptogenesis development.

Sodium valproate is one of the most widely used antiepileptic drugs in the world (Srivastava and White, 2013; Sefil et al. 2015; Romoli et al.

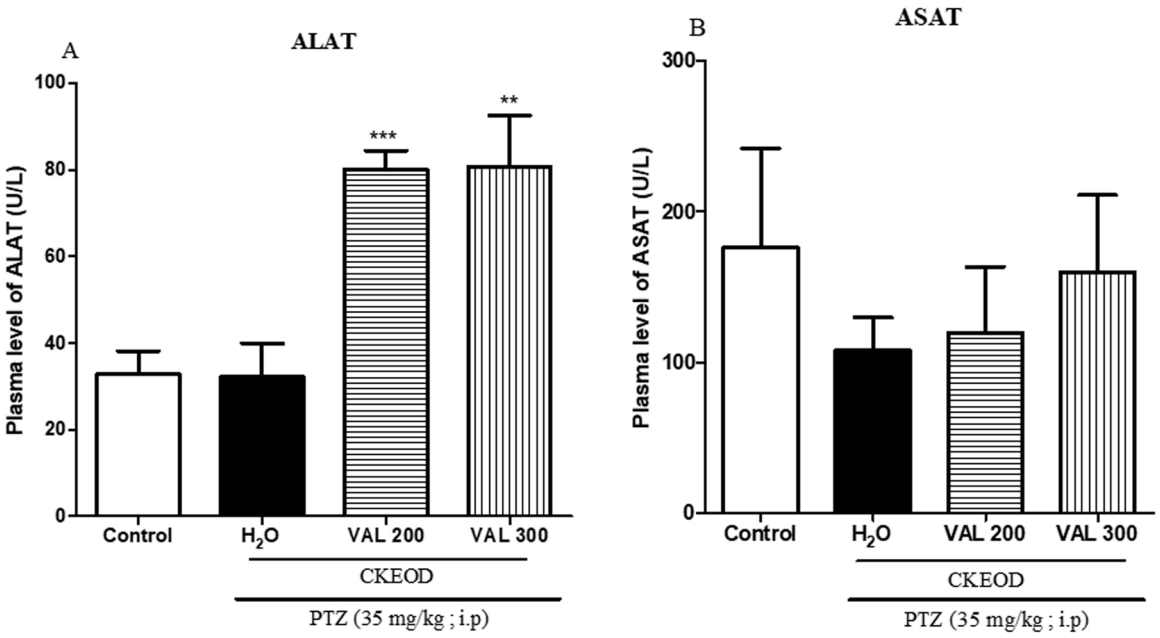

Fig. 10. Effect of Valproate doses on plasma ALAT and ASAT level in CKEOD model.

2019). This motivated us to verify the response provided by sodium valproate on the model that best induced chronic epilepsy, namely the CKEOD model. The doses most frequently used in the literature to conduct the studies were selected to be tested on the CKEOD model (Löscher et al. 1984; Sefil et al. 2015; Taiwe et al. 2016; Kumar et al. 2016; Pahwa and Goel, 2016). At the behavioral level, although both doses protected the animals against CKEOD-induced epilepsy, the 300 mg/kg dose was more effective than the 200 mg/kg dose. It is known that valproate is an antiepileptic drug which acts by various mechanisms, some of which are not fully understood (Löscher et al. 1984; Romoli et al. 2019). Valproate would act on the one hand by inhibiting the voltage-dependent channels, thus slowing down neuronal depolarization; on the other hand, it would also stimulate GABAergic receptors, thus potentiating GABAergic inhibition, which would significantly slow down the development of epileptogenesis as well as the appearance of paroxysmal discharges (Romoli et al. 2019), hence the large number of injections necessary to reach stage 4, the low severity of the seizures, and almost an absence of stability of the convulsive seizures following the 7 additional injections were observed with the different doses of valproate. These results corroborate those of Pahwa and Goel (2016) who demonstrated that valproate protects against epilepsy induced by kindling PTZ.

Biochemically, valproate significantly inhibited oxidative stress, resulting in particular in a significant reduction in the level of MDA and a significant increase in the level of GSH and catalase. In addition, valproate significantly reduced GABA-T activity. Indeed, the action of valproate in particular on voltage-dependent channels as well as on GABAergic signaling results in an inhibition of neuronal depolarization, thus protecting neurons from hyperexcitability (Sefil et al. 2015; Romoli et al. 2019). This results in limiting the production of free radicals, thus preventing the development of oxidative stress (Taiwe et al. 2016). These biochemical results thus corroborate the behavioral observations highlighting the protection exerted by valproate against the development of chronic epilepsy.

Regarding the systemic toxicity, it appeared that valproate significantly increased plasma ALAT levels at all doses. This therefore implies that chronic administration of valproate has a harmful effect on the liver. These results are similar to those obtained by many authors who have shown that valproate has a harmful effect on the liver, but which is however outweighed by its beneficial effects (Hussein et al. 2013; Fu et al. 2019). However, it appears that no difference was recorded between the two doses tested, which suggests that the two doses present an identical level of risk to the body. Therefore, since the valproate 300 mg/kg has more protective effect than valproate 200 mg/kg, and since the both doses have the same level of toxicity, the risk/benefit ratio is in favor of valproate 300 mg/kg and this dose is thus more suitable to be used as positive control.

5. Conclusion

The present study highlights the fact that the model of kindling PTZ by repeated injection of subconvulsive doses every 48 h is the best indicated for the induction of chronic epilepsy, and that the administration of a challenge dose at the beginning of the this process allows to develop chronic epilepsy in a shorter period of time due to the reduction in the number of injections, and exhibiting more severe characteristics over time than a conventional induction. In addition, this model prevents the death of animals during experiments, thus allowing the exploitation of all the necessary data. On the other hand, sodium valproate has been shown to be effective against this model, in particular at the dose of 300 mg/kg; sodium valproate 300 mg/kg could thus be used as a positive control in this model. The CKEOD model could thus be beneficial for conducting various studies on the different aspects of epilepsy and drug discovery.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Data Availability

The datasets generated during this study are available from the corresponding author on reasonable request.

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Author Contributions

Ngoupaye designed and supervised the experiments; Adassi and Foutsop conducted the laboratory trials as part of their PhD's thesis; Yassi gave material and human support for experiments; Ngo Bum gave general advices concerning the work.

Ethics approval

Animals were treated in accordance with the guidelines of the Cameroonian bioethics committee (reg N. FWA IRB00001954) and in accordance with NIH-Care and Use of Laboratory Animals manual. Efforts were also made to minimize animal suffering and to reduce the number of animal used in the experiment.

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