STUDY ON THE PATHOPHYSIOLOGICAL MECHANIMS INVOLVED IN EXPERIMENTALLY-INDUCED SEISURES IN ADULT MALE ALBINO RATS

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Abstract

Epilepsy is the most common serious neurological condition affecting 1 to 2 % of world population. Despite extensive progress, there are still many unanswered questions about factors inducing epilepsy. Therefore, this work was carried out in an attempt to study the effect of nitric oxide (NO) modulation on the development of epilepsy. Rats were divided into the following equal groups (8 rats each) according to their treatment; 1. Control non- treated; 2. Pilocarpine-treated; 3. Pilocarpine + Diazepam treated; 4. Pilocarpine + L-arginine (NO precursor)-treated groups. Twenty-four hours' rat observation and biochemical analysis of brain homogenates and serum showed that Pilocarpine induced seizures in all rats withhigh lethality associated with increased brain excitatory transmitter; glutamate, Gamma aminobutyric acid (GABA), malondialdehyde (MDA; oxidative stress marker) and NO levels, along with increased serum level of inflammatory mediator; tumor necrosis factor (TNF-α). Complete protection was observed with Diazepam without reversal of Pilocarpine- induced brain excitatory transmitters changes apart from a significantly higher GABA levels along with reduced brain NO and MDA levels and serum levels of TNF-α. On the other hand, L-arginine pretreatment aggravated the condition and accelerated the induction of seizures with high lethality reaching 100% after 24hrs. Biochemical and tissue parameters showed significantly high serum TNF-α levels as well as significantly high brain Glutamate, NO, MDA along with significantly low brain GABA levels. In conclusion, although the role of NO in the pathophysiology of epilepsy is controversial, our results showed that L-arginine; the NO precursor proved to be proepileptic, and thus opening the way for a new strategy of antiepileptic management.

Keywords:
Epilepsy, Pilocarpine, L-arginine, nitric oxide (NO), malondialdehyde (MDA), tumornecrosis factor (TNF-α).

Introduction

Epilepsy is a neurological disorder characterized by recurrent and unpredictable seizures. Epilepsy is one of the most common chronic disorders affecting around 50 million individuals of all ages worldwide. The incidence of epilepsy is higher in developing countries compared to developed countries (Sculier et al., 2018). Long term sequelae of epilepsy may include neurological, cognitive, behavioral impairments, decline in quality of life and exerts heavy burdens on the patient and the healthcare system. Outcomes depend on type of epilepsy, type of status epilepticus (SE), etiology, duration, and patient's age (Oduah and Iwanowski, 2020).

Epileptogenesis is the process by which the normal neural network is altered into a network of synchronized hyper-excitable neurons (Kuhlmann et al., 2018). It is frequently associate different brain insults characterized by increased reactive oxygen and nitrogen species (ROS/RNS) generation and excitotoxicity mediated by increased glutamate (Inder and Volpe, 2018).

Physiological concentrations of nitric oxide (NO) formed in the brain regulate cerebral blood flow, as well as the activity of dopaminergic, glutaminergic, and GABAergic systems, hormonal release, apoptosis, pain and analgesia, in addition to learning, memory and behavioral circuits (Reis et al., 2017). However, the potential role of nitric oxide in the pathogenesis of epilepsy has not been fully investigated.

Therefore, this work was carried out in an attempt to study the effects of NO modulation in epileptogenesis trying to find new strategies for damping or ameliorating the convulsive effects of epilepsy.

Material and methods

I. Animals:

Thirty two adult male albino rats, weighing (200- 250 gm), were obtained from the National Research Center, Cairo; Egypt. They were housed in stainless steel cages that offered adequate space for free movement and wandering (40 cm x 40 cm x 25 cm) at room temperature with natural dark/light cycles, and allowed free access to water and commercial rat's diet (Nile Company, Egypt) for two weeks for acclimatization. Rats were fed a standard diet of commercial rat chow and tap water *ad libitum* through the time of the study. All experiments were performed according to regulations under the appropriate animal licenses approved by the animal care committee of Faculty of Medicine-Minia University, accordingto the international guidelines.

II. Induction of Seizures:

All experiments were conducted in a quiet lab with constant light condition between 10 a.m. and 3 p.m. Following Pilocarpine injection, rats were put in individual cages, observed closely and continuously for one hour and frequently thereafter for 24 hours when the experiment was terminated. During this period, rats were monitored for the following:

a. The scoring was based on the Racine scale, as described previously ***(Racine et al. 1972)*** with the following stages: stage (0); no abnormality, stage (1); Mouth and facial movements, stage (2); Head nodding, stage (3); Forelimb clonus, stage (4); Rearing, stage (5); Rearing and falling. A full motor seizure, with temporary loss of postural control, is referred to as a Stage 5 motor seizure.

b. Percentage incidence of seizures (rats showing at least clonic spasms of the forelimbs were considered positive): [No. of rats showing seizure / No. of rats per group] x 100.

c. Time of onset of seizures after Pilocarpine; the latent period.

d. Percentage of mortality after one and 24 hours .

III. Experimental design:

Rats were randomly classified into 4 equal groups (8 rats each) as follows:

1. Control group; in which rats were left freely wandering in their cages with free access tofood and water.

2. Pilocarpine treated group; in which rats were administered single intra-peritoneal injection of Pilocarpine (400 mg/kg i.p.) ***(Curia et al. 2008)***.

3. Pilocarpine + Diazepam treated group; in which rats received a single injection of Diazepam as a reference anticonvulsant drug at a dose of 1 mg/kg, i.p., one hour before induction of epilepsy by Pilocarpine as in group 2 ***(Zaeri and Emamghoreishi 2015)***.

4. Pilocarpine + L-arginine treated group*;* in which rats received a single injection of L-argininеas NO precursor at a dose of 500 mg/kg, i.p., one hour before induction of epilepsy by pilocarpine as in group 2 ***(Gulati and Ray 2014).***

IV. Drug protocol:

All chemicals used in the present study were purchased from Bio-diagnostic, Egypt, unless mentioned otherwise.

V. Biochemical analysis:

- Blood samples were withdrawn from the retro-orbital venous plexus either immediately after first seizure or at the end of the first hour for rats that didn't show seizures. Blood was allowed to clot, centrifuged and sera were obtained and stored at -20 ºC for determination of tumor necrosis factor-α (TNF-α) by ELISA method (Prechek Bio, Inc., India).
- At the end of the whole experimental period (24 hrs), the surviving rats were sacrificed by cervical dislocation. The heads of both sacrificed and dead rats were immediately dissected and the brains were gently removed for preparation of brain homogenates.
- Preparation of brain homogenates for biochemical assay: The brains were washed with normal saline to remove blood and brain tissue samples from the hippocampus and temporal lobe were weighed, homogenized in 10 volumes of cold phosphate buffered saline solution (PBS); pH 7.35, using ultrasonic homogenizer (4710 series, Chicago). The homogenate was then centrifuged in cooling

centrifuge at -4 ºC, and the supernatant was used for determination of: MDA as previously described Ohkawa et al. (1979), Nitric oxide (NO) was estimated by ELISA kit (Prechek Bio,Inc., India.), Gamma-Aminobutyric acid (GABA) and glutamate using ELISA kit (BioAssay Systems, USA) according to the manufacturer's instructions.

Statistical analysis

The analysis of the data was carried out using the IBM SPSS 20.0 statistical package software (IBM; Armonk, New York, USA). Analysis of variance (ANOVA) was used for comparison between independent groups for parametric data followed by Tukey post hoc test to assess intergroup differences. A p-value of 0.05 or less was considered significant.

Results

1- Assessment of the effect of Pilocarpine with or without different treatments on the time of onset, % incidence of seizures and % of deaths after 1 and 24 hours:

As shown in Table 1, single intraperitoneal injection of Pilocarpine produced tonic/clonic seizures in all experimental animals (n=8, % incidence 100%) after an average period of 13.17±1.6 minutes with 75% deaths after 1 hour and 12.5% after 24 hours and only one rat survived till the end of experiment (24 hrs). Pilocarpine induced Seizures were completely prevented with the reference anticonvulsive drug; Diazepam pretreatment.

Pretreatment of pilocarpine group with L-arginine, the NO precursor, significantly accelerated the onset of pilocarpine-induced seizures and aggravated the lethality with no rat survival after 24hrs.

2- Assessment of the effect of Pilocarpine with or without different treatments on the different serum and brain parameters:

The results of the present study as shown in Table 2 demonstrated that:

- Intra-peritoneal injection of Pilocarpine produced a significant rise of serum TNF-α level as compared with control group. Pretreatments with Diazepam, 1hr before pilocarpine, produced no significant changes in serum TNF-α level as compared with control group. On the other hand, L-arginine pretreatment, 1hr before pilocarpine, produced significant rise in serum TNF-α level as compared with control and pilocarpine only treated groups.
- Pilocarpine treatment produced higher brain MDA level as compared to the control levels. Pretreatment with Diazepam, 1hr before pilocarpine produced no significant changes in the brain MDA level as compared with control group. L-arginine pretreatment, 1hr before pilocarpine, produced the highest and significant brain MDA levels, among all experimental groups, as compared with control group.
- Pilocarpine treatment produced significant higher glutamate level in the brain as compared to the control group. Diazepam pretreatment, 1hr before Pilocarpine, produced non-significant difference in brain glutamate than that of the Pilocarpine only treated group. L-arginine pretreatment, 1hr before pilocarpine, produced significantly higher brain glutamate level as compared with the pilocarpine group.
- Pilocarpine treatment produced significant higher GABA levels in the brain as compared to the control group. Diazepam pretreatment, 1hr before Pilocarpine, significantly produced higher GABA level than that of the only pilocarpine injected group. L-arginine pretreatment, 1hr before pilocarpine, produced significantly lower brain GABA level as compared with the pilocarpine group.
- Pilocarpine produced a significantly higher brain NO level than the control group; an effect that was partially but significantly reversed by Diazepam. The highest and significant NO level was obtained with L-arginine; the substrate for NO production as compared with control and pilocarpine groups.

Table 1: Effect of pilocarpine with and without different treatments on the onset, %incidence of seizures and % of deaths after 1 and 24 hours:

Groups (n=8)Parameter	Control	Piloc.	Diaz. + Piloc.	L-arg. + Piloc.
Onset of seizures(min)	No seizure	13.17 ±1.6	No seizure	5.46*±1.2
% incidenceof seizures	0 rats 0 %	8 rats 100%	0 rats 0 %	8 rats 100%
No. & % of deathsduring 1st hr.	0 rats 0 %	6 rats 75%	0 rats 0 %	7 rats 87.5%
No. & % of deathsbetween 1 and 24hr	0 rats 0 %	1 rat 12.5%	0 rats 0 %	1 rat 12.5%
No. &% of rats surviving to the end	8 rats 100%	1rat 12.5%	8 rats 100%	0 rats 0 %

Data are expressed as mean ± SEM. of 8 rats in each group. Piloc: Pilocarpine; Diaz: Diazepam; L-arg: L-arginine. * Significant at p value ≤0.05. ANOVA followed by Tukey post-hoc test.

Table 2: Effect of pilocarpine with and without different treatments on different serum and brain parameters:

	Control	Piloc.	Diaz. + Piloc.	L-arg. + Piloc.
	N=8	N=8	N=8	N=8
Serum TNF-α levelMean ± SE (pg / ml)	2.504±0.02	4.022±0.35*	2.641±0.07	8.737±0.48*
Tissue MDA levelMean ± SE (nmol/gm tissue)	23.19±0.52	33.94±0.39*	25.47±0.21*	62.04±0.45*
Tissue glutamate levelMean ± SE (mg/gm tissue)	8.19±0.08	19.88±0.3*	19.72±0.19*	43.59±0.58*
Tissue GABA levelMean ± SE (Pg/gm tissue)	42.22±0.59	124.44±0.87*	37.11±0.99*	81.53±0.64*
Tissue NO level Mean ± SE (µmol/gm tissue)	39.43±0.25	65.88±0.25*	50.32±0.51*	103.57±0.49*

Data are expressed as mean ± SEM. of 8 rats in each group. Piloc: Pilocarpine; Diaz: Diazepam; L-arg: L-arginine. * Significant at p value ≤0.05. ANOVA followed by Tukey post-hoc test.

Discussion

Epilepsy is one of the most common neurologic conditions, with an incidence of approximately 50 new cases per year per 100,000 populations. About 1% of the population suffers from epilepsy, and about one-third of patients have refractory epilepsy (i.e., seizures not controlled by two or more appropriately chosen antiepileptic medications or other therapies).So that, there is an urgent need to find more efficient and safe AEDs based on better understanding of the pathophysiological mechanisms of epilepsy (Ułamek-Kozioł et al., 2019).

The Pilocarpine-induced epilepsy rat model is the most appropriate and experimental model simulating temporal lobe epilepsy in humans; so, it is commonly used to study its patho-physiologic mechanisms and the potency of antiepileptic drugs (AEDs) (Devinsky et al., 2018). That is why we used this model in the present study.

In the present study, intraperitoneal injection of Pilocarpine produced tonic/clonic seizures in all experimental animals with high mortality rate. These data is compatible with other studies reported that Pilocarpine induce epilepsy by acting on brain M_1 muscarinic receptors, especially in the hippocampal region, triggers an imbalance of excitatory and inhibitory transmitter release in favor of the former (Maia et al., 2020).

In the present work, Diazepam pretreatment completely prevented the occurrence of pilocarpine-induced seizure, although it did not reverse the epileptogenic biochemical changes induced by pilocarpine. However, the inhibitory GABA levels were significantly higher. So, diazepam could act through potentiating GABA synthesis, release or attenuating its breakdown to balance glutamate excitotoxicity (Lorenz-Guertin, 2019).

Additionally, in the present study, it was found that pretreatment with L-arginine; the NO precursor, proved to be proepileptic. The incidence of seizures was 100%, the onset was significantly accelerated, and lethality was complete (no survivals after 24 hrs). As far as we know, no previous studies have documented a convulsant effect for NO donors or for NOS modulators, so, NO could not be an inducer of epilepsy as Pilocarpine. However, it could play a permissive role with other inducers. It increases glutamate release by activating presynaptic Ca^{+2} channels (Caviedes et al., 2020). On the other hand, inhibition of glutamine synthase by s-nitrosylation prevents inactivation of glutamate and increases excitotoxicity (Nagel and Eisel, 2021). This could explain why NO precursor, L-arginine,in this work was found proepileptic.

In the present study, Pilocarpine-induced seizure induces oxidative stress that ismanifested by the significant high brain MDA levels than the control levels. In addition, in the present study, Diazepam pretreatment were associated with insignificant high MDA level. On the other hand, L-arginine produced the highest and significant brain MDA levels among all experimental groups.

Reactive oxygen species are generated during epilepsy, as in the present data, through the following mechanisms: a) increased mitochondrial oxidative phosphorylation, and b) increased brain catecholamine transmission, specially dopamine during seizure and their catabolism by monoamine oxidase (MAO) is another mechanism for generating ROS during epilepsy (Shishmanova-Doseva et al., 2021). These mechanisms may explain the data of the current study.

The data of the present work showed significantly higher serum TNF-α level with the Pilocarpine and L-arginine groups than control group that is compatible with other previous studies (Han et al., 2018). Pretreatment with Diazepam, 1hr before Pilocarpine, produced no significant changes in serum TNF-α level as compared with control group. TNF-α enhances the expression of endothelial adhesion molecules and increases capillary permeability, resulting in the infiltration of inflammatory cells to the affected site and eventual tissue necrosis. Elevated TNF-α level also decreases inhibitory transmission which may contribute to the induction of epilepsy (Meng and Yao, 2020).

Based on the results of the present study, it could be concluded that the major mechanisms of seizures and epilepsy development may be attributed to increased neuronal excitability

secondary to oxidative/nitrosative stress and inflammation as evidenced by increased brain excitatory chemical transmitters, MDA, NO and serum TNF-α with pilocarpine and L-arginine treatments while these levels were decreased with the antiepileptic drug; Diazepam. Therefore, future studies may be required to evaluate the possible therapeutic benefit of using NO inhibitors for management of epilepsy.

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