*Research Article*

# The Role of Gasotransmitters; Nitric Oxide, Carbon Monoxide, and Hydrogen Sulphide in Pilocarpine-Induced Epilepsy in Adult Male Albino Rats

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## Abstract:
Forty two adult male albino rats were used to study the role of gasotransmitters; nitric oxide (NO), carbon monoxide (CO), and hydrogen sulphide ($H_{٢}S$) in the pathogenesis of pilocarpine-induced epilepsy using a model simulating the human disease. Rats were divided into the following equal groups (٦ rats each) according to treatment; ١) control non treated, ٢) pilocarpine treated, ٣) diazepam+pilocarpine treated, ٤) L-arginine (a substrate for NO synthesis)+pilocarpine, ٥) aminoguanidine (a selective inhibitor of inducible nitric oxide synthase; (iNOS)+pilocarpine, ٦) Hemin (inducer of CO synthesis)+pilocarpine, and ٧) sodium hydrogen sulphide (NaHS; an $H_{٢}S$ donor)+pilocarpine. Twenty four hours rat observation and biochemical analysis of brain homogenates and serum showed that pilocarpine induced seizures in all rats with high lethality associated with high levels of malondialdehyde; (MDA) signifying oxidative stress, as well as high NO, glutamate and Gamma aminobutyric acid (GABA) levels. Complete protection was observed with diazepam without reversal of pilocarpine-induced biochemical changes apart from a significantly higher GABA levels. Aminoguanidine was protective and reversed the pilocarpine induced biochemical changes, while L-arginine was proepileptic and accelerated its onset. Although it significantly reduced brain NO, yet hemin, had no significant effect on epilepsy development, while NaHS offered protection through ameliorating oxidative stress. **In conclusion**: Although the role of gasotransmitters in the pathophysiology of epilepsy is controversial, multifactorial, and intermingled, our results showed that blocking iNOS with aminoguanidine, or the $H_{٢}S$ donor; NaHS proved protective, opening the way for a new strategy of antiepileptic management.
**Keywords:**
Pilocarpine, epilepsy, nitric oxide (NO), carbon monoxide (CO), hydrogen sulphide ($H_{٢}S$)

## Introduction
Epilepsy is the most common serious neurological condition affecting ١ to ٢٪ of the world population. Among the epileptic patients, ٣٠٪ of them are currently refractory to the current antiepileptic drugs (AEDs). Even combined therapy is not always effective and has serious adverse effects including serious neurodegenerative, behavioral and psychiatric changes, in addition to metabolic disorders and bone marrow affection. Therefore, there is an urgent need to find more efficient and safe AEDs based on better understanding of the pathophysiologic mechanisms of Epilepsy[١,٢].

Epileptogenesis is the process by which a normal neural network is altered into a network of synchronized hyperexcitable neurons. This involves an imbalance between the inhibitory circuits particularly gamma aminobutyric acid (GABA) transmission and the excitatory circuits represented mainly by glutamate transmission, in favor of the latter. The mechanism of its initiation and propagation still requires intensive investigations [٣]. Epileptogenesis frequently associate different brain insults characterized by increased reactive oxygen and nitrogen species (ROS/RNS) generation and excitotoxicity mediated by increased glutamate[٤]. Oxidative stress targets mitochondrial DNA with consequent ATP

depletion, produces lipid peroxidation of nerve cell membranes that facilitates development of abnormal hyperexcitable circuits. In addition, disruption of blood brain barrier and free movement of excitatory molecules between blood and the extracellular brain fluid could also occur[٢, ٥].

Gasotransmitters include NO, CO, and $H_{٢}S$. They are small molecules freely permeable to membranes, endogenously and enzymatically generated in the brain as well as in most tissues of the body *via* enzymatic processes that can be regulated. Under physiological conditions, they modulate synaptic transmission in the brain, control cerebral blood flow, possess cytoprotective, antiapoptotic, and anti-inflammatory effects and control the release and modulate the function of other brain transmitters[٦].

Three heme-containing isoforms of nitric oxide synthases (NOS) catalyze NO formation from L- arginine. Both neuronal (nNOS) and endothelial (eNOS) forms are $Ca^{+٢}$- dependent and form the physiological levels of NO, while the inducible isoform (iNOS) is $Ca^{+٢}$- independent and induced under pathological conditions of ischemia, inflammation, degeneration and necrosis producing high NO concentrations with the risk of generating the toxic free radical; peroxynitrite [٧]. On the other hand, two isoforms of heme oxygenases; the contitutive; HO-٢, and the inducible; HO-١ catalyse the catabolism of heme to produce CO and bilirubin. In addition to the importance of CO as a cell signaling molecule, both CO and bilirubin are efficient endogenous free radical scavengers. Both CO and NO may partly share a common cyclic guanosine monophosphate (cGMP)-dependent cascade of functions in the body [٨]. The third gasotransmitter, $H_{٢}S$ is produced by the catalytic activity of cystathionine beta synthase (CBS) and cystathionine gamma lyase (CSE) on cysteine and homocysteine. It plays an important role in regulating cerebral blood flow. Furthermore, all these enzymes are expressed in the neurons as well as in astrocytes of the brain [٩].

Clinically, altered formation of gasotransmitters has been associated with many neurodegenerative disorders including Alzheimer's disease, Parkinsonism, multiple sclerosis, and ischemia[١٠,١١]. Controversial studies described their involvement in epilepsy, and the exact role of each one in its pathogenesis; whether proepileptic or antiepileptic requires thorough investigation to open the way for a new strategy of antiepileptic drugs targeting their enzyme synthesis or degradation or acting as donors [١٢, ١٣].

The aim of the present work is to study the effects of modulating gasotransmitters on the development of epileptic seizures in the pilocarpine model of epilepsy in adult male albino rats.

## Material and methods

**Chemicals:**

All chemicals used in the present study were purchased from Sigma chemical co., U.S.A., unless mentioned otherwise.

**Animals:**

Forty two adult male albino rats (Sprague dawley strain) of average weight ١٥٠ ± ١٥ g were obtained from the National Research Center, Cairo; Egypt. They were housed in groups of six in stainless steel cages that offered adequate space for free movement and wandering (٤٠ cm x ٤٠ cm x ٢٥ 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. Experimental procedures and care of the rats were carried out according to the guidelines of the animal care and use committee of Minia Faculty of Medicine which coincided with international guidelines. Rats were randomly assigned into seven equal groups (٦ rats each) as follows:

١- **Control group;** in which rats were left freely wandering in their cages with free access to food and water.

٢- **Pilocarpine-induced epileptic seizure group;** in which rats were administered single intraperitoneal injection of pilocarpine (٤٠٠ mg/kg i.p.)[١٤]. Alpha methyl scopolamine was given subcutaneously in a dose of ١ mg/kg as

antimuscarinic drug ٣٠ minutes before pilocarpine to block its peripheral cholinergic side effects; salivation, dacryorrhea, diarrhea, bradycardia and hypotension. It does not cross the BBB and does not interfere with the central effects of pilocarpine on seizure induction[١٥].

٣- **Pilocarpine + Diazepam treated group;** in which rats received a single injection of diazepam as a reference anticonvulsant drug at a dose of ١ mg/kg, i.p., one hour before induction of epilepsy by pilocarpine as in group ٢ [٥].

٤- **Pilocarpine + L-arginine treated group;** in which rats received a single injection of L-arginine as NO precursor at a dose of ٥٠٠ mg/kg, i.p., one hour before induction of epilepsy by pilocarpine as in group ٢ [١٠].

٥- **Pilocarpine + Aminoguanidine treated group;** in which rats received a single injection of aminoguanidine; a selective inhibitor of iNOS at a dose of ٢٠٠ mg/kg i.p. one hour before induction of epilepsy by pilocarpine as in group ٢[١٦].

٦- **Pilocarpine + Hemin treated group;** in which rats received a single injection of hemin; a HO-١ inducer at a dose of ٥٠ mg/kg i.p. twelve hours before induction of epilepsy by pilocarpine as in group ٢[١٧].

٧- **Pilocarpine + Sodium hydrosulphide (NaHS) treated group;** in which rats received a single injection of NaHS; as H٢S donor at a dose of ١٠٠ µmol/kg, one hour before induction of epilepsy by pilocarpine as in group ٢ [١٨].

All experiments were conducted in a quiet lab with constant light condition **between** ١٠ a.m. and ٣ p.m. Following pilocarpine injection, rats were put in individual cages, observed closely and continuously for one hour and frequently thereafter for ٢٤ hours when the experiment was terminated. During this period, rats were monitored for the following according to Roy et al., [١٩]

**(a)** 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 ١٠٠.

**(b)** Time of onset of seizures after pilocarpine injection; the latent period,

**(c)** Percentage of mortality after one and ٢٤ hours.

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 [٢٠]. Blood was allowed to clot, centrifuged and sera were obtained and stored at -٢٠ ºC for determination of serum MDA levels. At the end of the whole experimental period (٢٤ 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 ١٠ volumes of cold phosphate buffered saline solution (PBS); pH ٧.٣٥, using ultrasonic homogenizer (٤٧١٠ series, Chicago). The homogenate was then centrifuged in cooling centrifuge at -٤ ºC, and the supernatant was assayed for:

١- **Malondialdehyde (MDA);** was determined as indicator of lipid peroxides using thiobarbituric acid colorimetric method described by Okawa et al., [٢١].The absorbance was read at ٥٣٢ nm using spectrophotometer (Spectronic ٢٠٠٠, Baush and Lumb). ١,١,٣,٣-tetramethoxypropane was used to prepare a standard curve for MDA.

٢- **NO**; was determined using a colorimetric nitrite assay kit (Bio-diagnostic, Egypt). It is based on the conversion of nitrate to nitrite; the stable metabolite of NO by nitrate reductase, followed by the addition of Griess reagent to convert nitrite to a deep purple azo compound proportionate to NO concentration. The

absorbance was read at ٥٢٠ nm using spectrophotometer (Spectronic ٢٠٠٠), and was compared with a standard curve that was simultaneously prepared using sodium nitrite of different concentrations.

٣- **Glutamate;** brain glutamate level was determined colorimetrically using EnzyChromTM Glutamate Assay Kit (BioAssay Systems, USA) for Quantitative Colorimetric Determination of Glutamate. It is based on glutamate dehydrogenase catalyzed oxidation of glutamate, in which the formed NADH reduces a formazan (MTT) Reagent. The intensity of the product color, measured at ٥٦٥ nm, is proportionate to the glutamate concentration in the sample [٢٢].

٤- **GABA;** brain GABA levels was measured by Enzyme-linked Immuno-sorbent Assay (ELISA) Kit (Biomatik, USA). It depends on a competitive inhibition reaction between biotin labeled GABA and unlabeled GABA (Standards or samples) with a pre-coated antibody specific to GABA. Then, avidin conjugated to Horseradish Peroxidase (HRP) is added. The amount of bound HRP conjugate and the intensity of color developed is inversely proportional to the concentration of GABA in the sample, at ٤٥٠ ± ١٠nm.

## Statistical analysis:

Statistical analysis was performed using Graph and Prism software and significant difference between groups was done by one way ANOVA followed by Tukey-Kramer post hoc test for multiple comparisons with a value of p≤٠.٠٥ considered statistically significant.

## Results:

Figure (١) shows two rats in two different stages of seizure. Rat ١ shows the fully developed picture of seizure with tonic contraction of tail muscles with straightening of the tail and clonic convulsions of the limbs, while rat ٢ is still in the prodroma being motionless, showing frequent oro-facial movements, eye blinking, twitching of vibrissae, and yawning.

**Figure (١): two rats in different stages of seizure.**

**I. Effect of pilocarpine with and without different treatments on the time of onset, % incidence of seizures and % of deaths after ١ and ٢٤ hours:**

**Table (١)** shows that single intraperitoneal injection of pilocarpine produced tonic/clonic seizures in all experimental animals (n=٦, % incidence ١٠٠٪) after an average period of ١٤.١٧ ± ١.٥٤ minutes with ٦٦.٦٧٪ deaths after ١ hour and ١٦.٦٧٪ after ٢٤ hours and only one rat survived till the end of experiment (٢٤ hrs). These seizures were completely prevented with the reference anticonvulsive drug; diazepam

pretreatment. **NO** modulation showed that, while L-arginine; the precursor of NO accelerated the onset of pilocarpine-induced seizures and aggra-vated its lethality with no rat survival after ٢٤hrs, blocking iNOS with aminoguanidine offered protection, manifested by a delay in seizure onset, a lowered number of deaths within the first ٢٤ hrs (only one rat) with higher survival number (٥ rats). **CO** modulation by induction of HO-١ enzyme with hemin pretreatment did not significantly alter the pilocarpine-induced seizure parameters, while, the **H٢S** donor; NaHS significantly delayed the onset of seizures, and decreased mortality (٥ out of ٦ rats survived)**.**

**Table ١: Effect of pilocarpine with and without different treatments on the onset (min), % incidence of seizures and % of deaths after ١ and ٢٤ hours:**

| **Groups (n=٦)** **Parameter** | **Control** | **Piloc.** | **Piloc + Diaz.** | **Piloc + L-arg.** | **Piloc + Aminog.** | **Piloc + Hemin** | **Piloc + NaHS** |
|---|---|---|---|---|---|---|---|
| Onset of seizures (min) | **No seizure** | **١٤.١٧ ±١.٥٤** | **No seizure** | **٦.٦٧* ±١.٠٥** | **٣٢.٥٠* ±٢.٥٠** | **٩.٠٠ ±١.٨٧** | **٢٦.٢٥ * ±٢.٣٩** |
| % incidence of seizures | **٠ rats ٠ %** | **٦ rats ١٠٠٪** | **٠ rats ٠ %** | **٦ rats ١٠٠٪** | **٢ rats ٣٣.٣٪** | **٥ rats ٨٣.٣٪** | **٤ rats ٦٦.٧٪** |
| No. & % of deaths during ١st hr. | ٠ rats **٠ %** | **٤ rats ٦٦.٧٪** | **٠ rats ٠ %** | **٥ rats ٨٣.٣٪** | **٠ rats ٠ %** | **٤ rats ٦٦.٧٪** | **١rat ١٦.٧٪** |
| No. & % of deaths between ١ and ٢٤ hrs | **٠ rats ٠ %** | **١rat ١٦.٧٪** | **٠ rats ٠ %** | **١rat ١٦.٧٪** | **١rat ١٦.٧٪** | **٠ rats ٠ %** | **٠ rats ٠ %** |
| No. & % of rats surviving to the end | **٦ rats ١٠٠٪** | **١rat ١٦.٧٪** | **٦ rats ١٠٠٪** | **٠ rats ٠ %** | **٥ rats ٨٣.٣٪** | **٢ rats ٣٣.٣٪** | **٥ rats ٨٣.٣٪** |

Data represent means± standard errors of the means (M±S.E.).
*: significant from pilocarpine-induced seizure group.
Piloc: pilocarpine; Diaz: diazepam; L-arg: L-arginine; Aminog: aminoguanidine; NaHS: sodium hydrosulphide

**II. Effect of pilocarpine with and without different treatments on serum and brain MDA levels:**

**Figures (٢) and (٣) show that:**

- Pilocarpine treatment produced significant higher serum and brain MDA levels (١٧.٧٧ ± ١.١١ nmol/ml and ٣٣.٩٢ ± ١.٢٦ nmol/gm tissue) as compared to the control (١٢.٤٨ ± ٠.٦٦ nmol/ml and ٢٤.٦٧ ± ١.١٣ nmol/gm tissue) levels, respectively.
- Pretreatment with either diazepam, aminoguanidine or NaHS significantly attenuated the pilocarpine-induced rise in both serum and brain MDA levels with mean serum levels of ١١.٩٤ ± ٠.٨٧, ١٢.٥٩ ± ٠.٥٧ and ١١.٣١ ± ٠.٤٢ nmol/ml, while the mean brain levels were ٢٥.٣٢ ± ١.٢٢, ١٩.٨٤ ± ١.٩٣ and ٢١.٩٩ ± ٢.٤٤ nmol/gm tissue respectively. These levels were insigni-ficant from the corresponding control levels.
- L-arginine pretreatment, produced the highest and significant MDA levels in both serum and brain (٢١.٧٣ ± ٠.٦٠ nmol/ml and ٦١.٧٢ ± ٢.٨٤ nmol/gm tissue, respectively) among all experimental groups.

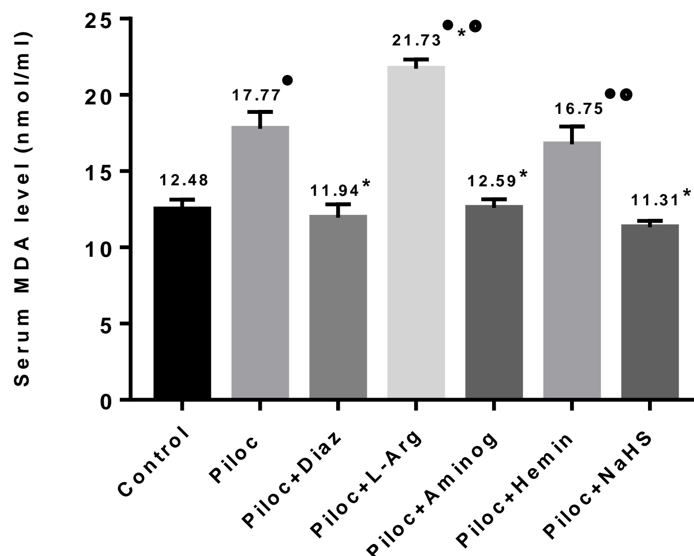


**Fig. ٢: Effect of pilocarpine with and without different treatments on serum MDA levels**
Data represent means ± standard errors of the means (M±S.E.).
•: significant from control *: significant from pilocarpine non-treated group.
O: significant from diazepam group MDA: malondialdehyde; Piloc: pilocarpine; Diaz: diazepam; L-arg: L-arginine; Aminog: aminoguanidine; NaHS: sodium hydrosulphide

- Hemin pretreatment failed to produce any significant effect on the elevated serum and brain MDA levels induced by pilocarpine injection. They were ١٦.٧٥ ±١.١٧ nmol/ml and ٣٤.٦٢ ± ١.٣٨ nmol/gm tissue, respectively and were significantly higher than those of the control group.

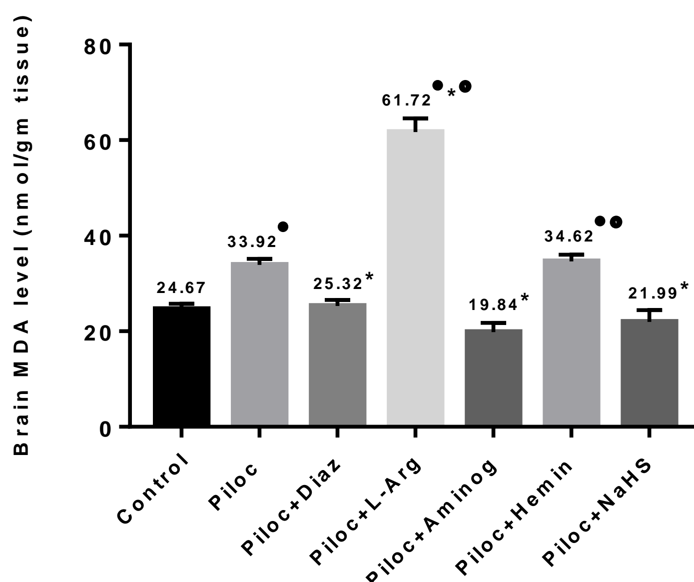


**Fig. ٣: Effect of pilocarpine with and without different treatments on brain MDA levels**
Data represent means and standard errors of the means (M±S.E.).
•: significant from control *: significant from pilocarpine non-treated group.
O: significant from diazepam group MDA: malondialdehyde; Piloc: pilocarpine; Diaz: diazepam; L-arg: L-arginine; Aminog: aminoguanidine; NaHS: sodium hydrosulphide

**III. Effect of pilocarpine with and without different treatments on brain NO levels:**

Table (٢) shows that pilocarpine produced a significantly higher brain NO level than the control group; an effect that was partially but significantly reversed by diazepam. Hemin (HO-١ inducer) and NaHS ($H_٢S$ donor) on the other hand, completely reversed the effect of pilocarpine and produced lower brain NO levels insignificant from control rats. The highest NO level was obtained with L-arginine; the substrate for NO production, while the selective NOS inhibitor; aminoguanidine completely blocked the effects of pilocarpine and decreased brain NO levels to the control level.

**Table (٢): Effect of pilocarpine with and without different treatments on brain NO levels**

| Groups (n=٦) Parameter | Control | Piloc. | Piloc + Diaz. | Piloc + L-arg. | Piloc + Aminog. | Piloc + Hemin | Piloc + NaHS |
|---|---|---|---|---|---|---|---|
| NO level (µmol/gm brain tissue) | ٣٨.٨٥ ±١.٠٨ | ٦٥.٦٣• ±١.٧٧ | ٥٠.٣٦•* ±٢.٨٣ | ١٠٢.٠٤•*O ± ٣.٩٨ | ٣٩.٧١* ±١.٥٦ | ٤٧.٧٧* ± ١.٨٤ | ٣٢.٩٤*O ±١.٧٣ |

Data represent means ± standard errors of the means (M±S.E.).
•: significant from control *: significant from pilocarpine non-treated group.
O: significant from diazepam group NO: nitric oxide; Piloc: pilocarpine; Diaz: diazepam; L-arg: L-arginine; Aminog: aminoguanidine; NaHS: sodium hydrosulphide

**IV. Effect of pilocarpine with and without different treatments on brain glutamate and GABA levels:**

**Figures (٤) and (٥) show that:**

- Pilocarpine treatment produced significant higher glutamate and GABA levels in the brain (١٩.٩٦ ± ١.٦٩ mg/gm tissue & ١٢٢.٤ ± ٤.٦٥ pg/gm tissue) as compared to the control group (٨.٤١ ± ٠.٦٤ mg/gm tissue & ٤٢.١٤ ± ٢.٨٣ pg/gm tissue, respectively).
- Diazepam pretreatment significantly produced higher GABA level (٢٣٦.٤ ± ٤.٨٨ pg/gm tissue) than that of the only pilocarpine injected group with no significant difference in brain glutamate between them.
- L-arginine pretreatment produced significantly higher brain glutamate level (٤٢.٠٤ ± ٣.١٩ mg/gm tissue), with a significantly lower brain GABA level (٨١.٣٩ ± ٣.٧٩ pg/gm tissue) as compared with the pilocarpine group. On the other hand blocking NO synthesis with aminoguanidine pretreatment counteracted the effects of pilocarpine and produced brain glutamate and GABA levels (٧.٧٤ ± ٠.٥٨ mg/gm tissue & ٥٨.٠٣ ± ٣.٤٩ pg/gm tissue, respectively) that were insignificant from the control group.
- Finally, neither hemin nor NaHS pretreatments produced any significant change on the elevated brain glutamate and GABA levels induced by pilocarpine injection which remained insignificantly different from the pilocarpine group.

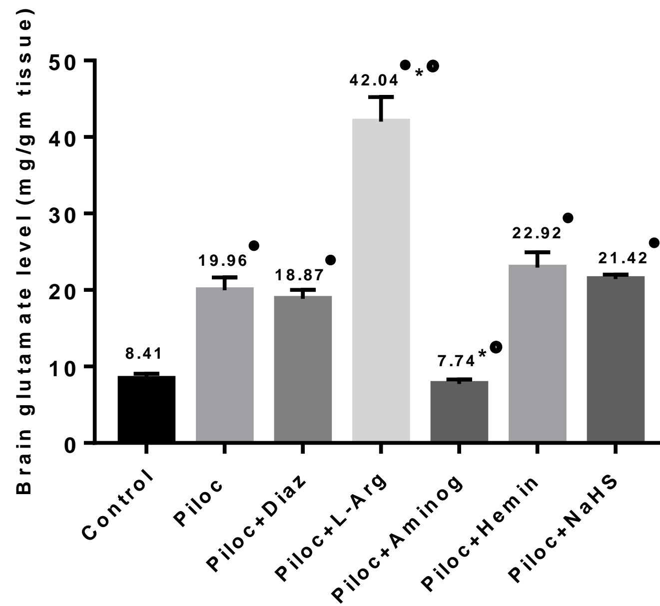


**Fig. ٤: Effect of pilocarpine with and without different treatments on brain glutamate levels**

Data represent means ± standard errors of the means (M±S.E.).
•: significant from control *: significant from pilocarpine non-treated group.
O: significant from diazepam group Piloc: pilocarpine; Diaz: diazepam; L-arg: L-arginine; Aminog: aminoguanidine; NaHS: sodium hydrosulphide

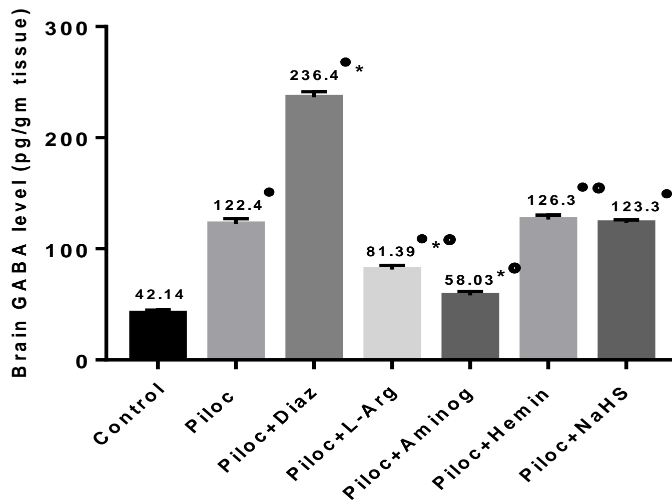


**Fig. ٥: Effect of pilocarpine with and without different treatments on brain GABA levels**

Data represent means and standard errors of the means (M±S.E.).
•: significant from control *: significant from pilocarpine non-treated group.
O: significant from diazepam group. GABA: gamma amino-butyric acid; Piloc: pilocarpine; Diaz: diazepam; L-arg: L-arginine; Aminog: aminoguanidine; NaHS: sodium hydrosulphide.

## Discussion:

Epilepsy is one of the major health problems and, despite the extensive research to find an adequate therapy, epilepsy remains poorly controlled in ٣٠ % of the patients. So, a better understanding of epileptogenesis is the only way for development of new antiepileptic treatments[٣]. 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)[٢٣]. That is why we used this model in the present study. Muscarinic receptor stimulation by pilocarpine, or carbachol[١٥] injections or locally applied acetylcholine[٢٤] in the brain, trigger an imbalance of excitatory and inhibitory transmitter release in favor of the former.

Pilocarpine induced epilepsy, in the present study and in other studies, by acting on brain $M_١$ muscarinic receptors, especially in the hippocampal region, to activate phospholipase C with increased production of diacylglycerol (DAG) and inositol triphosphate ($IP_٣$). Both synchronize to increase mobilization of free cytoplasmic $Ca^{+٢}$ that triggers the release of the excitatory transmitter glutamate and decreases the adenosine triphosphatase (ATPase) activities. The later effect increases further neuronal excitability through the consequent failure of repolarization and/or $Ca^{+٢}$ extrusion, thus maintaining a high cytoplasmic $Ca^{+٢}$ with more glutamate release. Glutamate excite-toxicity starts by acting on N-methyl-D-aspartate (NMDA) receptors to increase $Ca^{+٢}$ influx into postsynaptic neurons that triggers apoptosis and/or cell necrosis and a vicious circle is created[٢٥]. On the other hand, Pilocarpine acts on brain $M_٢$ muscarinic receptors to decrease adenyl cyclase activity and hence increases the inhibitory GABA transmitter release. However, its inhibition is overridden by the excitotoxic glutamate, hence the induction of seizure[١]. This is compatible with the significantly higher glutamate and GABA levels found in this work in the pilocarpine group and with temporal lobe degeneration found by Schartz et al., [٢٦]

In the present study, oxidative stress during pilocarpine-induced seizure is manifested by the significantly higher MDA levels than the control levels. The brain is susceptible to oxidative stress because of its high content of unsaturated fatty acids, high oxygen consumption rate (٢٠٪ of the inspired oxygen at rest), and large number of mitochondria. On the other hand, the antioxidant defense system is relatively poor. ROS are generated during epilepsy through the following mecha-nisms: a) increased mitochondrial oxidative phosphorylation, b) activation of nNOS and eNOS by the excitotoxicity-induced $Ca^{+٢}$ surge, c) activation of iNOS by glutamate-induced NMDA receptor stimulation and/or by the neurodegeneration-induced inflame-matory process with consequent increase of peroxinitrite (RNS) formation, and d) increased brain catecholamine transmission, specially dopamine during seizure has been observed by Cifelli and Grace [٢٧] and their catabolism by monoamine oxidase (MAO) is another mechanism for generating ROS during epilepsy. Not only the brain, but also epilepsy associated generalized oxidative stress condition is manifested in our study by the increased serum MDA level. This is compatible with Menon et. al. [٢٨] in epileptic patients and could be ascribed to the extensive muscular effort, cardio-vascular and respiratory changes affecting tissue oxygenation and metabolism. Also, alpha tocopherol administration; a free radical scavenger was found to decrease the severity of pilocarpine-induced seizure in rats[٢٩]. During epileptogenesis, increased free radicals produces lipid peroxidation, reactive glial cells, hippocampal neurodegeneration and reorganization of neural networks to form hyperexcitable circuits.

The high mortalities during seizures found in this work could be due to irradiation of impulses from the ectopic epileptic foci to the autonomic and vital brain centers producing cardiac arrhythmia, hypertension, pulmonary edema, and even

sudden apnea as previously monitored by Scorza et al., [١].

In the present work, diazepam pretreatment completely prevented (٠ % incidence) 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. In addition, diazepam acts also as an agonist of $GABA_A$ receptor linked to chloride channels and by prolonging chloride influx and/or increasing the frequency of chloride channel opening, it increases the amplitude or duration of GABA-mediated inhibitory post-synaptic potential providing antiepileptic effect according to Greenfield Jr. [٣٠]

Physiological concentrations of 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 [٣١]. When formed in excess, by iNOS under pathological conditions, it becomes toxic due to formation of toxic peroxynitrite radical with extensive nitrosylation of functional proteins. Due to this double faced coin of functions, contradictory results have been obtained *in vivo*, in which manipulation of the brain NO level was either proepileptic or antiepileptic [٣٢]. NO has been found to be antiepileptic in convulsions induced by C-methyl-D-aspartate, penicillin, kainic acid (KA), picrotoxin, and pentylenetetrazole [٣٣, ٣, ٣٢]. On the other hand, some investigators demonstrated that a decline in NO production by treatment with NOS inhibitors in rats led to inhibition of convulsions evoked by pentylenetetrazole (PTZ) pretreatment [٣٤]. Also, L-arginine reduced the incidence and severity of homocysteine thiolactone-induced seizure in rats, but aggravated lindane-induced seizures. These effects were reversed by L-NAME administration [٣, ١٢].

In the present study, it was found that pretreatment with L-arginine; the NO precursor, proved to be proepileptic. The incidence of seizures was ١٠٠٪, the onset was significantly accelerated, and lethality was complete (no survivals after ٢٤ hrs). This was associated with significantly higher levels of MDA, NO, and glutamate with a lower GABA level. On the contrary, the selective iNOS inhibitor; aminoguanidine, was found to be antiepileptic. The incidence was reduced to ٣٣.٣٪ and a significantly longer delay of onset was observed with minimal lethality (only one rat). The biochemical changes induced by pilocarpine were completely reversed with no statistical differences with the control.

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 $Ca^{+٢}$ channels. S-nitrosylation of $Na^+$ channels increases $Na^+$ influx with increased excitability in hippocampal neurons, allowing persistent cycling of impulses in the ectopic foci, while s-nitrosylation of NMDA receptors increases their sensitivity to glutamate [٧]. On the other hand, inhibition of glutamine synthase by s-nitrosylation prevents inactivation of glutamate and increases excitotoxicity[٣٥]. This explains why selective blocking of iNOS with amino-guanidine in this work was found protective.

In the present work, Hemin (a HO-١ inducer) pretreatment did not produce a significant protection against pilocarpine-induced seizure. Furthermore, it did not alter the pilocarpine-induced biochemical changes apart from a significant reduction in NO. HO-١ induction during epilepsy has been found by Parfenova et al., [٨] and is produced by both increased glutamate and ROS. The cerebrovascular dilatation produced by the increased CO ensures adequate blood flow to meet the increased neuronal activity. This is mediated by both

a cGMP-dependent and a direct smooth muscle relaxing effect. Although, the products of heme oxygenase catalyzed reaction; CO and bilirubin are strong ROS scavengers during seizure, yet increased glucose and oxygen flow to the brain due to increased blood flow stimulate mito-chondrial oxidative mechanisms with more generation of ROS [٢]. So, from the present results, it is probable that HO-١ is maximally induced during epilepsy and therefore pretreatment with hemin had no additional effect, or the prooxidant effect of increased blood flow nullified any additional antioxidant effect.

The colocalization of NOS and HO in the neurons allow them to exert mutual inhibition. NO inhibits HO by nitrosylation of its protein, and HO inhibits NOS by reacting with its heme group [٣٦]. This may explain the significantly lower NO level found with hemin pretreatment. CO also, partly share a common secondary messenger pathway with NO through sti-mulation of guanylyl cyclase and cGMP, so they may interfere with each other [٣٧].

In the present work, it was found that pretreatment with the H٢S donor; NaHS proved protective with delayed onset, decreased incidence and lethality of seizures. NaHS completely reversed the increased MDA and NO levels to control levels, but did not affect the increased GABA or glutamate. These results support a protective effect of CO mediated through a powerful antioxidant effect (reduced MDA). Reduced ROS consequently decreased iNOS induction, neural degeneration and lethality during seizure. These results are compatible with Gupta et al.,[٩] who reported that NaHS, Panax ginseng herb extract and S- adeno-sylmethionine; a stimulant of endogenous H٢S production could ameliorate different models of experimental seizures. Liu et al., [٣٨] also, found that H٢S is an endogenous cytoprotective in most tissues specially the brain and improves cerebral circulation. In contrast to our results, Gupta et al.,[٩] reported that overproduction of H٢S in Down's syndrome leads to neuronal degeneration via inhibition of cytochrome oxidase or overstimulation of NMDA receptors. Also, in cerebrovascular strokes, H٢S levels correlated with the infarct size. Luo et al., [١٣] found that NaHS exacerbated both PTZ-induced and pilocarpine-induced seizures in *in vivo* rat models as well as in *in vitro* hippocampal slices. They proved that this aggressive effect is mediated by increasing the sensitivity of NMDA receptors and/or by increasing potential-gated $Na^+$ channel conductance.

**In conclusion,** the role of gasotransmitters in epilepsy is controversial due to multiple mechanisms of regulation, shared mechanisms of action, mutual interaction between different enzymes of synthesis and the possible interaction between a given gasotransmitter and the synthesizing enzyme(s) of another one. The present work showed that induction of CO synthesis had no significant effect while, NO is proepileptic and H٢S is antiepileptic. So, inhibitors of iNOS or H٢S donors could be a new antiepileptic strategy. Future research should try combinations of this new strategy with ordinary AEDs to increase their potency, minimize side effects and overcome refractoriness.

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