Effect of L-Carnitine on Pilocarpine-Induced Seizures in Rats

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Abstract: Reactive oxygen species have been implicated in seizure-induced neurodegeneration and there is a correlation between free radical level and scavenger enzymatic activity in epilepsy. It has been suggested that pilocarpine-induced seizures is mediated by an increase in oxidative stress. Current research has found that antioxidant may provide, in a certain degree, neuroprotection against the neurotoxicity of seizures at the cellular level. L-Carnitine has a powerful antioxidant action. The objective of the present study was to evaluate the neuroprotective effects of L-Carnitine (L-CAR) in rats, against oxidative stress caused by pilocarpine-induced seizures. 30 min prior to behavioral observation, rats were treated with (0.9% saline i.p., control group), L-CAR 300 mg/kg alone. (L-CAR 300 mg/kg i.p., L-CAR group), pilocarpine (400 mg/kg, i.p., P400 group) and the combination of L-CAR (300 mg/kg, i.p.) and pilocarpine (400 mg/kg, i.p.). After the treatments all groups were observed for 6 hrs. The enzymatic activities, lipid peroxidation and nitrite concentrations were measured using spectrophotometric methods and these data were assayed. In P400 group rat there was a significant increase in lipid peroxidation and nitrite levels. However, no alteration was observed in superoxide dismutase (SOD) and catalase activities. In the L-CAR and pilocarpine co-administered rat, antioxidant treatment significantly reduced the lipid peroxidation level and nitrite content, as well as increased the SOD and catalase activities in rat hippocampus after seizures. Our findings strongly support the hypothesis that oxidative stress occurs in hippocampus during pilocarpine-induced seizures, indicate that brain damage induced by the oxidative process plays a crucial role in seizures pathogenic consequences, and imply that strong protective effect could be achieved using L-carnitine. **In conclusion**: L-carnitine could enhance activities of SOD and reduce the MDA level and could reduce seizure period, inhibit neuronal damage as free radical scavenger.
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1. Introduction

Seizures and status epilepticus (SE) induced by pilocarpine in animal models are similar to human temporal lobe epilepsy in semiology and electrographic appearance. The epileptic model induced by pilocarpine is very useful for us to study the development and neuropathology of temporal lobe epilepsy (Treiman, 1995). Neurochemical as well as enzymatic activities studies suggest that excitotoxic stimulation in SE induces excess production of reactive oxygen species, and finally leads to oxidative stress (Barros et *al.,* 2007). In these circumstances, the production of reactive oxygen species may have a very important role in the development of seizures itself (Ayyildiz et *al.,* 2006).

Reactive oxygen species have been implicated in the development of seizures and SE induced by pilocarpine (Andreoli and Mallett, 1997). The mechanism underlying seizures-induced oxidative stress is not well understood yet, but several interpretations have been proposed, which include excitotoxicity associated with excessive neurotransmitter release, oxidative stress leading to free radical damage (Andreoli and Mallett , 1997; Liang *et al.,*2007). Several studies have examined the role of oxidative stress on pilocarpine induced seizures which was thought possibly via the formation of free radicals (Heaton et *al.,*2000). Free radicals are generated during oxidative stress. It's attractive as a possible mechanism to pilocarpine-induced seizures for many reasons. The brain processes large amounts of O2 in relatively small mass and has a high content of substrates available for oxidation in conjunction with low antioxidant activities, making it extremely susceptible to oxidative damage (McCord, 1989; Walz *et al.,*2000).

The brain is more vulnerable to oxidative damage than other tissues because it contains large quantities of oxidizable lipids and metals, and has comparatively less antioxidant capacities. Then, the free radicals formed have a great probability to react with non-radical molecules and transform them into secondary free radicals which is normally observed during the lipid peroxidation producing hydroperoxides. The lipid peroxidation and nitrite are increased in hippocampus rats during pilocarpine-induced seizures (Freitas, 2009) Thus, it is worth while assessing the role of antioxidant compounds in prevention of these neurochemical alterations on oxidative stress during seizures.

Free radicals have been implicated in development of seizures and status epilepticus (SE) induced by pilocarpine (Castagne *et al.,*1999). The mechanism

behind seizures-induced oxidative stress is not well understood, but several explanations have been proposed. These include excitotoxicity associated with excessive neurotransmitter release, oxidative stress leading to free radical damage (Andreoli and Mallett, 1997). Several studies have examined the role of oxidative stress on pilocarpine-induced seizures, possibly via the formation of free radicals.

Free radicals and reactive oxygen species (ROS) are generated during oxidative metabolism and can inflict damage on all classes of cellular macromolecules, eventually leading to cell death (Walz *et al.,*2000). Oxidative stress is attractive as a possible mechanism for the pilocarpine-induced seizures for many reasons. The brain processes large amounts of O_2 in relatively small mass, and has a high content of substrates available for oxidation in conjunction with low antioxidant activities, making it extremely susceptible to oxidative damage (Bergamini *et al.,*2004).

Pilocarpine, a muscarinic cholinergic agonist, is able to elicit seizures and status epilepticus (SE) in rodents, which is characterized as an experimental model frequently used to study spontaneous recurrent seizures (Costa-Lotufo *et al.,*2002; Freitas *et al.,*2003). This experimental model can be used to study the pathophysiology of seizures and to identify potential therapeutic agents to treat epilepsy. The pilocarpine-induced seizures produce SE and lead to acute damage to the brain. In this acute phase, compounds can be identified with efficacy against refractory epilepsy and/or their neuroprotection against further damage induced by SE (Xavier *et al.,*2007).

Seizure activity with a broad variety of local biochemical changes affects several neurotransmitters, such as dopamine (DA), serotonin (5-HT), norepinephrine (NE), glutamate and aminobutyric acid (GABA), which is similar to those observed in human temporal lobe epilepsy . The seizures also appear to be affected by the antioxidant enzymatic activities in the hippocampus of adult rats (Freitas *et al.,*2004).

L-Carnitine (L-CAR) plays an important regulatory role in the mitochondrial transport of long chain free fatty acids (FFA). 3-Nitropropionic acid (3-NPA) is known to induce cellular energy deficit and oxidative stress-related neurotoxicity via an irreversible inhibition of mitochondrial succinate dehydrogenase (SDH) (Binienda *et al.,* 2001). The current study was undertaken to investigate the effect of L-carnitine on pilocarpine-induced seizures in rats.

2. Material and Methods:

Drugs and chemicals:

1. Pilocarpine hydrochloride (Sigma Chemical Company , Egypt).
2. L-carnitine (Carnitol) (Global Napi Pharmaceuticals , Egypt).
3. Thiobarbeturic acid (Sigma chemical co., USA)
4. Nitric oxide kits (R&D System, Inc., USA)
5. Superoxide dismutase kits (Diamond Biodiagnosis, Egypt)
6. Catalase kits (Diamond Biodiagnosis, Egypt)

Animals and Procedures:

Adult male rats (250–280 g) were maintained in a temperature controlled room and food and water *ad libitum*. The dosages of pilocarpine hydrochloride and L-carnitine (Carnitol®) are expressed at milligrams per kilogram of body weight, and were administered in a volume of 10 ml/kg b.wt. injected intraperitoneally (i.p.). A total of 96 rats were treated with either 200 mg/kg L-carnitine (i.p., L-CAR) or 0.9% saline (i.p.). 30 min after the treatments 24 rats from each above group were randomized to pilocarpine hybrochloride administration (400 mg/kg b.wt., i.p., P400). Thus there was 4 subgroups of rats in this set of experiments: group 1, saline treatment served as control group (n = 24) ; group 2, P400 plus saline treatment (n = 24); group 3, L-CAR alone administration (n = 24); and group 4, L-CAR 300 and P400 co-administration (n = 24) .

After the treatments, the animals were recorded with:

Latency to first seizure (any one of the behavioral indices typically observed after pilocarpine administration: wild running, clonuses, tonus , clonic-tonic seizures).

Number of animals that died for (6-24) hrs after pilocarpine administration.

At the end of observations, the survivors were killed by decapitation and brains were dissected to remove hippocampus for determinations of :

Lipid peroxidation level in hippocampus of all experimental groups by measuring the thiobarbituric-acid-reacting substances in homogenates and expressed as nmol of malondialdehyde (MDA)/g wet tissue , as previously described by Draper and Hadley, 1990 .

Nitrite content in hippocampus of all experimental groups. The supernatants were collected, and nitric oxide production was determined and expressed as nM. based on the Griess reaction (Green *et al.,* 1981).

Superoxide Dismutase and Catalase activities in hippocampus of all experimental groups. The supernatants were collected and SOD activity was assayed by using xanthine and xanthine oxidase to generate superoxide radicals and expressed as U/mg of protein (Flohe and Otting, 1984). Catalase activity was measured by the method that uses H_2O_2 to generate H_2O and O_2 and expressed as mmol/min/mg of protein (Chance and Maehly , 1955).

Histopathological investigation of hippocampus of all experimental groups. The brains were dissected out and fixed in formalin 10% (Marinho *et al.,* 1998;

Szyndler *et al.,* 2005). and stained with Hematoxylin & Eosin (HE) for light microscopy. The degree of hippocampal damage severity was defined by a scale ranging from 0 (none) to 100 (total) by light microscopy. Brain damage presence was confirmed if hippocampus showed at least 50% involvement (Paxinos and Watson, 1986).

Statistical Analysis:

Results of latency to first seizure, histopathological abnormalities and neurochemical alterations were compared by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test ($p \leq 0.05$) (Graphpad program Intuitive, Software for Science, San Diego, CA). The number of animals that seized and the number that survived were calculated as percentages (seizures percentage and survival percentage, respectively), and compared with a nonparametric test (v2).

3. Results:

Pilocarpine induced the first seizure at 35.00 ± 0.70 min. All the animals studied showed generalized tonic-clonic convulsions with status epilepticus (SE), and 30% survived the seizures. All animals pretreated with L-carnitine were observed for 6 hrs before pilocarpine injection and their manifested alterations in behavior, such as peripheral cholinergic signs (100%), tremors (50%), wild running, wet dog shakes, rearing and motor seizures (60%) developed progressively within 1–2 h into a long-lasting SE (60%). Table1 showed that L-carnitine (300 mg/kg) administration before pilocarpine treatment reduced by 25% the percentage of animals that seized ($p<0.0001$), increased latency (212%) to the first seizure (109.09 ± 1.05 min) ($p<0.0001$) and increased (40%) the survival ($p<0.0001$) when compared to the pilocarpine only group. None of the control animals (saline or L-carnitine) showed seizures (Table 1).

Effects of L-carnitine in lipid peroxidation and nitrite concentrations during seizures induced by pilocarpine are presented in Figs. 1 and 2. Lipid peroxidation was markedly increased in pilocarpine group in comparison with the values of the saline group. During acute phase of seizures induced by pilocarpine a significant increase (90%) in thiobarbituric-acid-reacting substances ($p<0.0001$) was observed. Seizures induced by pilocarpine produced a significant increase in hippocampal nitrite content (94%, $p<0.0001$, Fig. 2). Rats pretreated with L-carnitine showed decrease in lipid peroxidation level (79%, $p<0.0001$) and nitrite content (56%, $p<0.0001$) when to compared with the pilocarpine group (Fig. 1). In addition, the pretreatment with L-carnitine , 30 min before administration of pilocarpine also reduced lipid peroxidation level (60%, $p<0.0001$) and nitrite content (15%, $p<0.005$) when compared to the control group (Figs. 1, 2). On the other hand, none of the control animals (saline or L-carnitine) showed alterations in lipid peroxidation level and nitrite content (Figs. 1, 2).

Superoxide dismutase and catalase activities in the hippocampus during acute phase of seizures were not markedly altered in pilocarpine group, when compared to corresponding values to the control saline group. By the contrary, it was found a significant increase in hippocampal superoxide dismutase (40 and 43%) and catalase (51 and 53%) activities of rats pretreated with L-carnitine in comparison to the pilocarpine and saline groups, respectively ($p<0.0001$) (Figs. 3, 4). However, there were no enzyme alterations in L-CAR group (Figs. 3, 4).

Brain tissue examinations of the control (saline 0.9%), L-carnitine groups (L-CAR group) did not reveal hippocampal histopathological changes. On the other hand, P400 group presented neuronal loss, gliosis, and typical vacuolar degeneration in hippocampus region (Fig. 5).

Table 1: Effect of pretreatment with l-carnitine on pilocarpine-induced seizures and lethality in adult rats.

Groups Parameters	Latency to first seizures (min)	Percentage seizures	Percentage survival	Number of animals/group
P400	**35.00 ± 0.70**	**60**	**30**	**24**
L-CAR plus P400	**109.09 ± 1.05 c**	**35 a**	**70 a**	**24**
L-CAR	**00**	**00**	**100 a,b**	**24**

ap< 0.05 as compared with P400 group (v2-test). **b**p< 0.05 compared with L-CAR plus P400 group (v2-test). **c**p< 0.05 as compared with P400 group (ANOVA and Student–Newman–Keuls test)

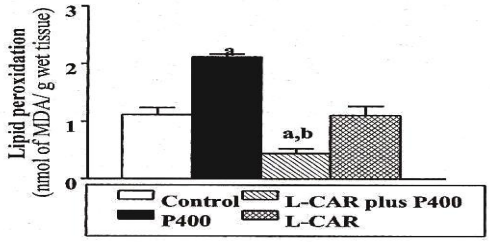

Fig. 1 Effects of l-carnitine (L-CAR) on status of lipid peroxidation level in hippocampus of adult rats prior to seizures induced by pilcarpine

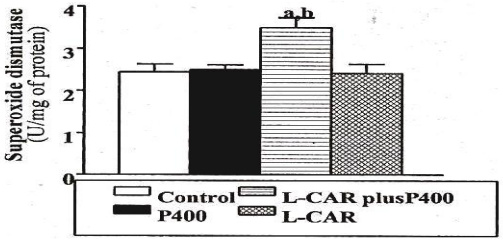

Fig. 3 Effects l-carnitine (L-CAR) on the superoxide dismutase activities in hippocampus of adult rats prior to seizures induced by pilcarpine

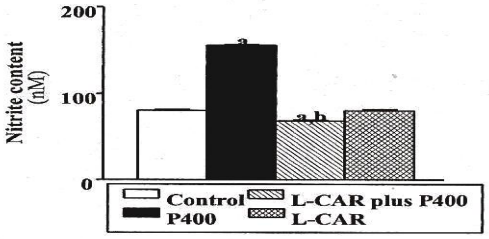

Fig. 2 Effects of l-carnitine (L-CAR) on the nitrite content in hippocampus of adult rats prior to seizures induced by pilocarpine.

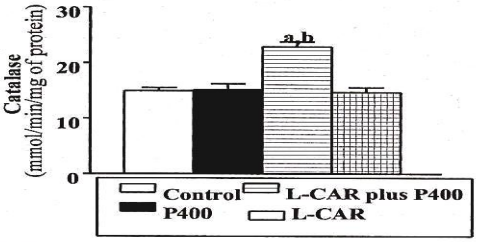

Fig. 4 Effects of l-carnitine (L-CAR) on catalase activities in hippocampus of adult rats prior to seizures induced by pilocarpine.

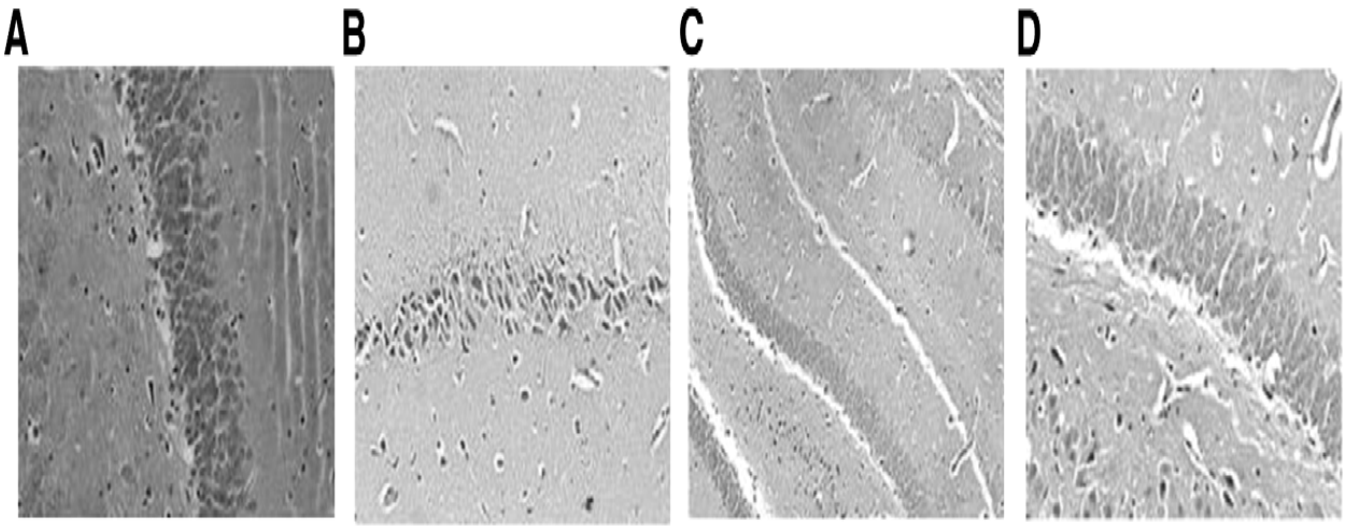

Fig. 5: Histopathological alterations in hippocampus of all rat groups

A: Control group

B: P400 group

C: L-CAR group

D: L-CAR plus P400 group

4. Discussion:

Epilepsy is one of the most common neurologic problems all over the world, being associated with paroxysmal discharge of cerebral neurons and is characterized by several symptoms including alterations of behaviors and consciousness (Godlevskii *et al.,* 2004). The molecular observations of epilepsy include the temporal correlation between free radical generation and the development of seizures in some pathological conditions, and the protective efficacy of antioxidative treatments against some types of seizures.

L-Carnitine (trimethylamino-b-hydroxybutyrate) (L-CAR) is present in cells and tissues as both free carnitine and acylcarnitines, including acetyl L-carnitine. L-CAR is a naturally occurring, endogenous compound in all mammalian species and its most widely known function is as an important transporter of long chain fatty acids into mitochondria for b-oxidation (Bieber , 1988). Humans obtain carnitine from their diet, predominately from meat and dairy, and through endogenous biosynthesis. L-CAR is synthesized in vivo from L-lysine and L-methionine, mostly in liver and kidney.

In this study, we investigated the influence of L-carnitine on the level of lipid peroxidation, nitrite content and enzymatic activities of superoide dismutas and catalase in the rat hippocampus during pilocarpine-induced seizures. Generation of reactive oxygen species is currently viewed as one of the process through which epileptic activity exert their deleterious effects on brain (Rauca *et al.,* 2004). These reactive oxygen species in the absence of an efficient defense mechanism cause peroxidation of membrane polyunsaturated fatty acids (Castagne *et al.,* 1999).

Brain is particularly susceptible to peroxidation due to simultaneous presence of high levels of polyunsaturated fatty acids which was the target of free radical damage. We showed that the lipid peroxidation was rising in hippocampus homogenate of rats after 6 hours of acute phase of seizures. The increase of lipid peroxidation was reflected by the rise of thiobarbituric-acid-reacting substances level which may be related to its intermediate free radicals formed during seizures induced by pilocarpine (Halliwell and Gutteridge, 1999).

Moreover, pilocarpine-induced seizures was led to changes in nitric oxide metabolism, and increased the production of its metabolites (nitrite and nitrate). The reduction in nitrite content, after pretreatment with L-carnitine, is most readily explained as a consequences of radical formation inhibiting, scavenges reactive oxygen species and lipid peroxidation products (Tejada *et al.,* 2006).

Histopathological studies of animals pretreated with L-carnitine thirty min before pilocarpine injection showed that a decrease of 60% in the number of animals that presented hippocampal damage after seizures. We also observed that, none of the animals which received L-carnitine presented hippocampal damage. However, 80% of the animals which had seizures and that developed SE presented hippocampal damage. On the other hand, the hippocampus of rats pretreated with L-carnitine presented a small damage extension.

Moreover, these results suggested that oxidative stress mediated by pilocarpine exerts its pathologic effects during seizures and also that the neuroprotective and anticonvulsive role of L-carnitine can be mediated by a reduction in lipid peroxidation levels and nitrite content (Santina *et al.,* 2005). Possibly, this reduction is due to the modulatory activity of L-carnitine in the antioxidant enzymes (superoxide dismutase and catalase) in the hippocampus of adult rats. Superoxide dismutase and catalase activities do not protect against seizures induced by pilocarpine. However, there no changes in hippocampal superoxide dismutase activity during acute phase of seizures induced by pilocarpine.

On the other hand, the catalase activity augmented in those animals presenting seizures, which suggests that H_2O_2 generated during superoxide dismutation would not be sufficiently removed from the hippocampus by catalase during acute phase of seizures (Savitha and Panneerselvam , 2006). The scavenging of O_2 - produces a decrease in the H_2O_2 levels generated by superoxide dismutation hippocampus, causing increase of the activities of the enzymes superoxide dismutase and catalase as neuroprotective action mechanism of L-carnitine.

Free radical formation elevations are frequently accompanied by an immediate compensatory increase in the activities of the free radical scavenging enzymes. Previous studies have showed that an increase in catalase activity in the hippocampus during a 24 h period of acute phase of seizures (Freitas *et al.,* 2005). Moreover, during the convulsive process, the neuronal changes are accompanied by alterations in the cerebral metabolic rate evidenced by modifications in the regional cerebral blood flow (Tran *et al.,* 2005).

In conclusion, we were founded that L-carnitine pretreatment was able to inhibit pilocarpine induced seizures, SE and mortality of adult rats. The capability of L-carnitine to increase antioxidant enzymes activities, and decrease free radical formation will finally lead to a significant decrease in the susceptibility to seizures induced by pilocarpine. These observations suggest L-carnitine has promising anticonvulsant effect on pilocarpine induced seizures. Further investigations of the effects of L-carnitine against necrosis and apoptosis observed during the acute phase of this epilepsy model are in progress to confirm its neuroprotective effects.

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