Neuronal damage and memory deficits after seizures are reversed by ascorbic acid?

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ABSTRACT
The objective of the present study was to evaluate the neuroprotective effects of ascorbic acid (AA) in rats, against the neuronal damage and memory deficit caused by seizures. Wistar rats were treated with 0.9% saline (i.p., control group), ascorbic acid (500 mg/kg, i.p., AA group), pilocarpine (400 mg/kg, i.p., pilocarpine group), and the association of ascorbic acid (500 mg/kg, i.p.) plus pilocarpine (400 mg/kg, i.p.), 30 min before of administration of ascorbic acid (AA plus pilocarpine group). After the treatments all groups were observed for 24 h. Pilocarpine group presented seizures which progressed to status epilepticus in 75% of the animals. Pretreatment with AA led to a reduction of 50% of this rate. Results showed that pretreatment with AA did not alter reference memory when compared to a control group. In the working memory task, we observed a significant day’s effect with important differences between control, pilocarpine and AA plus pilocarpine groups. Pilocarpine and AA plus pilocarpine groups had 81 and 16% of animals with brain injury, respectively. In the hippocampus of pilocarpine animals, it was detected an injury of 60%. As for the animals tested with AA plus pilocarpine, the hippocampal region of the group had a reduction of 43% in hippocampal lesion. Our findings suggest that seizures caused cognitive dysfunction and neuronal damage that might be related, at least in part, to the neurological problems presented by epileptic patients. AA can reverse cognitive dysfunction observed in rats with seizures as well as decrease neuronal injury in rat hippocampus.

Key words: ascorbic acid, hippocampus, neuronal damage, memory, seizures, pilocarpine.

O dano neuronal e o déficit de memória após convulsões são revertidos pelo ácido ascórbico?

RESUMO
O objetivo do presente estudo foi avaliar o efeito neuroprotetor do ácido ascórbico (AA), contra o dano neuronal e o déficit de memória em ratos causados pelas convulsões. Ratos Wistar foram tratados com solução salina a 0,9% (i.p., grupo controle), ácido ascórbico (500 mg/kg, i.p., grupo AA), pilocarpina (400 mg/kg, i.p., grupo pilocarpina), e a associação de ácido ascórbico (500 mg/kg, i.p.) com pilocarpina (400 mg/kg, i.p.), 30 min após a administração de ácido ascórbico (AA + pilocarpina grupo). Após os tratamentos todos os grupos foram observados durante 24 h. O grupo pilocarpina apresentou crises convulsivas que evoluíram para o estado de mal epiléptico em 75% dos animais. O pré-tratamento com AA produz uma redução de 50% nesta taxa. Os resultados mostraram que o pré-tratamento com AA não alterou a memória em relação ao controle. No teste de memória, observou-se um efeito significativo nos dias avaliados entre os grupos controle, pilocarpina e AA + pilocarpina. 81 e 16% dos animais dos grupos AA + pilocarpina e pilocarpina apresentaram danos cerebrais, respectivamente. No hipocampo dos animais do grupo pilocarpina, que foi detectada uma lesão de hipocampal de 60%. Quanto aos animais do grupo AA + pilocarpina, a região do hipocampo apresentou uma redução de 43% na extensão da...
The cholinergic system has an important role in the installation and propagation of limbic seizures, where the free radicals and oxidants systems may be responsible for propagating the brain damage induced by seizures \(^1\). Previous studies have shown that systemic administration of high dose of pilocarpine, a muscarinic cholinergic agonist, induced the development of seizures, status epilepticus (SE) and brain damage in rodents \(^2,3\). Onset of seizures can be blocked by anticholinergic drugs and attenuated by inhibition of acetylcholinesterase activity, suggesting that an endogenous cholinergic activation of the muscarinic cholinergic receptor is responsible for the installation of the convulsive process. Moreover, free radical scavengers can prevent the cerebral damage \(^4,5\).

The areas and the severity of brain damage have been previously described in limbic seizures induced by pilocarpine. In general, the limbic seizures produce neuronal damage in several areas and particularly in limbic structures, causing neuronal loss in various structures (hippocampus, striatum, amygdala, pyriform cortex, entorhinal cortex, frontal cortex, thalamus and substantia nigra) \(^6,7\).

The effects of antioxidant drugs during the temporal evolution of brain damage have not been clarified yet. However, experimental evidence indicates that antioxidants compounds can protect against the neuronal damage observed during the pathophysiology of epilepsy. Studies have demonstrate that ascorbic acid can ameliorate oxidative stress in the hippocampus during seizures \(^8,9\). However, the ascorbic acid effects against the hippocampal lesion produced by seizures are not still established. Based on this fact, the present study attempted to study the ascorbic acid effects on memory deficits and histopathological changes observed in hippocampus of adult rats after pilocarpine-induced seizures. The study design took into consideration the fact that area has been suggested as the installation site of seizures in this model \(^1\). Therefore, the objective of this study was to evaluate the potential effect of ascorbic acid on the attenuation of neuronal damage in the hippocampus and memory deficits in the adult rat during the acute phase of seizures induced by pilocarpine.

**METHOD**

Adult male Wistar rats (250-280 g) were maintained in a temperature controlled room (26±1°C) with a 12-h light/dark cycle and food and water *ad libitum* (Nutrilabor, Campinas SP, Brazil). All experiments were performed according to the Guide for the care and use of laboratory the US Department of Health and Human Services, Washington, DC (1985). The substances used were pilocarpine hydrochloride and ascorbic acid (Sigma, Chemical USA). All doses are expressed in milligrams per kilogram and were administered in a volume of 10 ml/kg injected intraperitoneally (i.p.). In one set of experiments, the animals were divided into four groups and treated with ascorbic acid (500 mg/kg, n=24) or 0.9% saline (n=24) and 30 min later, they received pilocarpine hydrochloride (400 mg/kg). During this 30-min interval rats were observed for the occurrence of any change in their behavior. The treatments previously described represented the ascorbic acid plus pilocarpine (AA plus pilocarpine) and pilocarpine groups, respectively. Other two groups received 0.9% saline (n=24, control group) or ascorbic acid alone (500 mg/kg, n=24, AA group).

After these initial treatments, the animals were placed in 30 cm x 30 cm chambers to record the latency to first seizure (any one of the behavioral indices typically observed after pilocarpine administration such as wild running, clonus, tonus, clonic-tonic seizures) \(^1\) and the number of animals that died after the pilocarpine administration. A previous study had shown that the numbers of convulsions and deaths occurring within one and 24 hours post-injection always followed the same pattern \(^1\). Therefore, in the present study, the animals were observed for 24 h after pilocarpine-induced convulsions at 30-60 min, and deaths were registered within 24 h after the pilocarpine injection. The survivors were killed by decapitation and brains dissected out on ice to collect hippocampus for histopathological determinations. The pilocarpine group comprised those rats that presented seizures; SE for over 30 min, and that survived within 24 h.

It important to clarify that the doses used in this study were determined from dose-response studies and observations of the doses currently used in animal studies in the literature \(^10,11\). The doses used are not equivalent to those used by humans, since rats have different metabolic rates.

At the age of two months, the animals were subjected to behavioral testing. We used the Morris water maze, an
apparatus widely employed to study spatial learning and memory tasks. The water maze consisted of a black round tank, 200 cm in diameter and 100 cm high, containing water 50 cm, which was maintained at constant temperature (23±1°C). The tank was theoretically divided into four equal quadrants for the purpose of analysis. Several distal visual cues were placed on the room walls.

The task consisted of five training sessions, and one test session. In the acquisition phase, the rats had four daily trials to find the platform, being submerged 2 cm under the water surface, and then placed on the center of one of the tank quadrants during all training days. For each trial, the rat was placed in water facing tank wall, in one of the 4 starting locations (N, S, W and E). The order of starting positions varied in every trial and none of the sequences was repeated on acquisition phase days. Rats were allowed to search for the platform during 60 s and in the case of failure to find it, they were gently guided to it. All animals were allowed to remain on the platform for 10 s. Latency to find the platform was measured in each trial. The interval between trials was 15-20 min. In the following day after the last training trial, each rat was subjected to a probe trial in which the platform was removed. Four parameters were measured, namely, the latency to cross onto the location of the platform, the number of target crossings and the time spent in target (the quadrant in which the platform was located in the training sessions) and opposite quadrants. These parameters were taken as a measure for spatial memory.

In order to detect motor impairments that could possibly affect performance in experimental groups, the swimming speed was calculated by taking the distance traveled in the first 15 s of the probe trial. After one week, the working memory version of Morris water maze was performed. The task consisted of four consecutive trials per day, with a 30-s inter-trial interval, when the animals were placed in the tank facing the wall and allowed to search for the submerged platform, positioned on the center of one of the quadrants. Platform position changed every subsequent day during the four testing days. Latencies to find the platform in every first, second, third and fourth trials were calculated considering all testing days so to assess working memory performance.

The task was run in a wooden box measuring 60×40×50 cm with a frontal glass wall, having the floor divided by white lines into 12 equal squares. Animals were placed facing the rear left corner of the arena and observed for 2 min. The number of squares crosses with the four paws from one square to another was indicative of motor activity.

A literature review revealed that there are articles that addressed the role of oxidative stress in neurological disorders, including seizure models induced by pilocarpine in which the modulation of the pro-oxidant / antioxidant balance by seizures per se and by antioxidant agents is discussed. However, the critical role of oxidative stress in this seizure models is not uniform. Studies suggest that high doses of muscarinic cholinergic agonist, pilocarpine, or lithium pre-treatment followed by low doses do pilocarpine result in behavioural changes, seizures and widespread brain damage in adult rats. Based in this fact, we decided to investigate the memory deficits and neuronal damage in the hippocampus of adult rats pretreated with ascorbic acid after pilocarpine-induced seizures. After the observation period of 24 h, all groups were sacrificed by decapitation. Their brains were fixed in 10% formalin for 72 h in order to perform the histopathological analysis. Sagittal cuts, made at intervals of 1 mm, were obtained from a cut near the are the sections were made, 10 µm within the the mamillary bodies. For microscopic studies, cells were stained by Hematoxylin & Eosin (HE) and analyzed by optical microscope. The degree of injury was expressed as a percentage scale from 0 (none) to 100 (total) for hippocampus examined by light microscopy (100×) and previously defined to be reliable for morphological analysis. Animals were defined as having brain damage if hippocampus showed at least 50% involvement. The structure routinely examined was the hippocampus, which was assessed according to Paxinos and Watson.

Results of the latency to first seizure, memory deficits and histopathological alterations were compared using ANOVA and the Student-Newman-Keuls test as post hoc test, since these results show a parametric distribution. The number of animals that seized and the number that survived were calculated as percentages (percentage seizures and percentage survival, respectively), and compared with a nonparametric test ($\chi^2$). In all situations statistical significance was reached at $p \leq 0.05$, slant 0.05. The statistical analyses were performed with the software GraphPad Prism, Version 3.00 for Windows, GraphPad Software (San Diego, CA, USA).

**RESULTS**

Pilocarpine induced the first seizure at 35±0.70 min. The animals presented generalized tonic-clonic convulsions (75%) with SE, and 30% of them survived the seizures. All animals pretreated with the ascorbic acid selected for this study were observed for 24 h before pilocarpine injection. They manifested alterations in behavior, such as peripheral cholinergic signs (100%), tremors (50%), staring spells, facial automatisms, wet dog shakes, rearing and motor seizures (25%), which develop progressively within 1-2 h into a long-lasting SE (25%). Results have shown that when administered before pilocarpine, ascorbic acid (500 mg/kg) reduced seizures in about 50% ($p<0.0001$), increased a latency to the first seizure (259.08±1.02 min) \([T(46)=181.1300; p<0.0001]\) and
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augmented (70%) survival percentage (p<0.0001) when compared to the pilocarpine-treated group. None of the control animals (isotonic saline or ascorbic acid) showed seizure activity (Table 1).

Table 2 shows that administration of ascorbic acid (AA) did not affect the spatial memory acquisition phase. Control and AA groups showed similar ability in finding the platform and learning its location along the five days of training sessions. However, in the pilocarpine group, a decrement of spatial memory was observed for the acquisition phase, when compared to corresponding values of the control group (p<0.0001). On the other hand, the pretreatment with AA 30 min before administration of pilocarpine (AA plus pilocarpine group), reversed those alterations in spatial memory acquisition phase (p<0.0001), when compared to the corresponding values of the pilocarpine group (Table 1). Repeated measures ANOVA (days versus groups) revealed a major daily effect for both groups (p<0.05), without any interaction between days and groups. Four parameters were evaluated in the test session, namely the time spent in target and opposite quadrants, the latency to cross, and the number of crossings onto the platform location (Table 3). It was shown that AA did not affect the time spent in the target, opposite quadrants, the latency to cross onto the location of the platform, and the number of crossings onto the former platform location (p>0.05).

The AA preadministration effects on the performance of rats on the working memory version of Morris water maze were also assessed. Repeated measures ANOVA revealed a significant daily effect with an interaction group versus days (p<0.01). Post hoc independent t tests showed statistical differences in days 2 and 5 among the control, pilocarpine and AA plus pilocarpine groups (p<0.01) (Table 4).

In order to verify whether AA pretreatment would affect motor activity, the animals were submitted to the open field task. In seized animals (pilocarpine group), decreases of 19 and 21% were observed in crossings (p<0.05) and in rearing (p<0.01) (Fig 1), respectively. No motor deficits were found in the animals of the AA or AA plus pilocarpine groups.

Table 1. Effect of pretreatment with ascorbic acid on pilocarpine-induced seizures and lethality in adult rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Latency to first seizures (min)</th>
<th>Percentage seizures</th>
<th>Percentage survival</th>
<th>Number of animals per group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pilocarpine</td>
<td>35±0.70</td>
<td>75</td>
<td>30</td>
<td>24</td>
</tr>
<tr>
<td>AA plus pilocarpine</td>
<td>259.08±1.02*</td>
<td>25*</td>
<td>100*</td>
<td>24</td>
</tr>
<tr>
<td>AA</td>
<td>0</td>
<td>0</td>
<td>100*</td>
<td>24</td>
</tr>
</tbody>
</table>

*p<0.0001 as compared with control group (χ² test); "p<0.0001 as compared with pilocarpine group (ANOVA and Student-Newman-Keuls test); AA: ascorbic acid.

Table 2. Effect of ascorbic acid in adult rats prior to pilocarpine-induced seizures on spatial memory acquisition phase.

<table>
<thead>
<tr>
<th>Groups</th>
<th>1 day</th>
<th>2 days</th>
<th>3 days</th>
<th>4 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>49.60±1.30</td>
<td>33.60±1.54</td>
<td>21.20±1.03</td>
<td>18.30±0.70</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>43.68±1.10*</td>
<td>27.68±1.09*</td>
<td>18.68±1.27*</td>
<td>15.68±1.09*</td>
</tr>
<tr>
<td>AA plus pilocarpine</td>
<td>50.60±1.32**</td>
<td>32.93±1.22**</td>
<td>22.20±1.86**</td>
<td>17.99±0.42**</td>
</tr>
<tr>
<td>AA</td>
<td>48.91±0.84</td>
<td>31.91±0.84</td>
<td>20.94±1.44</td>
<td>17.98±1.84</td>
</tr>
</tbody>
</table>

*p<0.0001 as compared with control group (ANOVA and Student-Newman-Keuls test). **p<0.0001 as compared with pilocarpine group (ANOVA and Student-Newman-Keuls test). AA: ascorbic acid.

Table 3. Effect of ascorbic acid in adult rats prior to pilocarpine-induced seizures on test session parameters namely time spent in the target quadrant, time spent in the opposite quadrant, latency to cross the location of the platform, and number of crossings over platform location.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Pilocarpine</th>
<th>AA plus pilocarpine</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time spent in the target quadrant (s)</td>
<td>28.07±1.90</td>
<td>25.07±1.90</td>
<td>28.74±1.19</td>
<td>28.97±1.84</td>
</tr>
<tr>
<td>Time spent in the opposite quadrant (s)</td>
<td>6.79±1.09</td>
<td>5.99±1.02</td>
<td>7.16±1.47</td>
<td>6.59±0.90</td>
</tr>
<tr>
<td>Time spent to cross the platform (s)</td>
<td>10.13±1.92</td>
<td>8.53±2.22</td>
<td>10.14±1.86</td>
<td>10.13±1.22</td>
</tr>
<tr>
<td>Number of crossing on the platform location</td>
<td>5.41±0.74</td>
<td>3.91±0.84</td>
<td>4.24±0.54</td>
<td>5.11±0.44</td>
</tr>
</tbody>
</table>

AA: ascorbic acid.
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The literature had shown that the systemic administration of ascorbic acid reduced seizures and increases survival rates\(^9\). The effects of ascorbic acid on hippocampal damage during seizures induced by pilocarpine are described in Table 5. Histopathological changes were markedly increased in pilocarpine group. During the acute phase of seizures induced by pilocarpine, an increase (81%) in hippocampal neuronal damage (\(p<0.0001\)) was observed, when compared to the control group. These histopathological alterations were neuronal loss, gliosis and typical vacuolar degeneration. Post hoc comparison revealed that the neuronal damage decreased 65% in those hippocampus of animals pretreated with ascorbic acid (\(p<0.0001\)), when compared to the pilocarpine group. On the other hand, brain histopathological analyses of the controls and ascorbic acid groups, did not show any neuronal changes.

The intensity of the ascorbic acid effects and the hippocampal damage during seizures induced by pilocarpine are presented in Table 5. Post hoc comparison indicated a decrease of 43% in severity of lesions in rats pretreated with ascorbic acid (\(p<0.0001\)) in comparison with the pilocarpine group (Table 5), in which vacuolar degeneration was also observed. Animals treated with saline and ascorbic acid did not show hippocampal damage.

**DISCUSSION**

In present study, ascorbic acid did not produce behavioral changes in animals during seizures induced by pilocarpine. Ascorbic acid may be able to avoid neuronal damage, along with other non enzymatic antioxidants\(^{11}\).
and enzymatic antioxidants, such as glutathione peroxidase, glutathione reductase and others enzymes, suggesting a neuroprotective role for ascorbic acid.

Previous outcomes have shown suppression on glutamate transporter activity in culture of cortical astrocytes caused by H2O2 and peroxynitrite, contributing to the accumulation of glutamate and consequent spread of seizures and production of brain damage induced by this excitatory neurotransmitter. Involved in the defense system against cellular damage induced by free radicals, ascorbic acid is an antioxidant agent that produces beneficial changes in behavior induced by seizures. Results from the present study are in line with the literature data regarding the neuroprotective effects of ascorbic acid during seizures, and SE induced by pilocarpine. However, the effects of ascorbic acid on memory deficits and hippocampal damage caused by seizures had not been fully established yet.

Animal models are useful to understand the pathophysiology of seizures. In this context, the authors have recently evaluated the effects of antioxidant compounds in the pilocarpine model, showing a decrease in nitrite levels in rat hippocampus. Animals exposed to ascorbic acid treatment presented no differences in physical growth and brain weight when compared to the control group, suggesting that ascorbic acid ameliorated metabolic parameters in these rats.

Through this model, the effect of ascorbic acid on spatial navigation tasks in the Morris water maze was investigated. The present results have shown that seized rats did not present impaired performance neither in the acquisition phase, nor on the time spent in target quadrant and in platform location, nor in the latency to cross over the platform location in the reference session of memory task. However, ascorbic acid significantly impaired working memory performance, since there were significant effects on days with an interaction group, and significant differences in days 2 and 5.

The epilepsy model induced by pilocarpine has been extensively studied because it produces spontaneous recurrent seizures, which may explain many of the neurochemical changes in oxidative stress seen in temporal lobe epilepsy in humans. The histopathological changes observed in the hippocampus produces many of these changes observed in humans, including neuronal loss and gliosis, also allowing for the evaluation of the presence of neuronal damage after seizures. The literature shows morphological injuries in several brain structures after seizures and SE. The main changes that may be associated with the convulsive process are the enlargement of the ventricles, the deformation of the dentate gyres with moderate dispersion of the granular cells and neuronal loss in several areas (hippocampus, cerebral cortex, striatum, piriform cortex, amygdala, thalamus, subthalamus, corpus callosum, motor cortex and septal area). Pilocarpine may not be able to produce acute toxicity. Nevertheless, it may induce memory deficits and neurodegeneration after SE and during spontaneous recurrent seizures.

Seizures induced by pilocarpine produce neuronal damage, especially in limbic structures, causing neuronal loss in the hippocampus, amygdala, pyriform cortex, entorinal cortex, lateral septum, thalamus, neocortex and the substantia nigra. In this study, several animals observed for behavioral changes presented vacuolation and gliosis in the groups treated with pilocarpine that were observed for 24 h, suggesting the involvement of cholinergic and oxidants systems in the convulsive process.

The present study has shown a high percentage of animals with memory deficit and brain injury after administration of pilocarpine. In turn, the group that was pre-treated with ascorbic acid before administration of pilocarpine presented memory improvement and a reduction in the number of animals with brain damage. Moreover, only two animals out of those with seizures and SE presented histopathological changes in the hippocampus, suggesting, therefore, that the injuries resulting from the seizures can occur with direct and/or indirect participation of a free radical. Thus, the use of antioxidant drugs may offer a neuroprotective action during seizures in humans, since it may interfere with the mechanisms of seizure spreading, reducing the brain damage induced by them.

The hippocampus has been described as being responsible for the installation of seizures induced by pilocarpine. Studies with ascorbic acid in animals have demonstrated a reduction in the hippocampal lesion. This drug may be able to exert an anticonvulsant effect in the pilocarpine model which presented histopathological changes similar to those of the temporal lobe epilepsy in humans. These results suggest a possible direct and/or indirect interaction between the cholinergic system and the antioxidant defense systems in the development of neuronal injury caused by seizures. In addition, they show that the scavenging free radicals can be useful in reducing hippocampal lesion, although they were not able to prevent the installation of seizures. The present observations in this context showed that the injury produced by pilocarpine in the hippocampus of adult rats can be prevented by the use of ascorbic acid.

In summary, the present findings suggest that seizures induced by pilocarpine are installed by the cholinergic system and possibly propagated by free radicals that are produced during hippocampal oxidative stress, such as that observed after seizures. Free radicals can be responsible for memory deficits and hippocampal damage produced by seizures in adult rats. Ascorbic acid may exert...
neuroprotective effects against memory deficits and hippocampal damage observed during seizures, taking part on the scavenging of free radicals that produce lipid peroxidation, changes in the properties of cell membranes and also alterations in nitrite content in hippocampus. It is believed, therefore, that ascorbic acid may influence the hippocampal damage and lead to memory improvement during seizures induced by pilocarpine. However, further studies should be conducted to fully clarify the mechanism of neuroprotective action of ascorbic acid during the establishment of seizures.

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