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Neuroprotective actions of vitamin C related to decreased lipid peroxidation and increased catalase activity in adult rats after pilocarpine-induced seizures

Lúcia F.L. Santos, Rizângela L.M. Freitas, Sarah M.L. Xavier, Gláucio B. Saldanha, Rivelilson M. Freitas *

Curso de Farmácia da Faculdade Católica Rainha do Sertão, Rua Juvêncio Alves, 660, Centro, Quixadá, 63900-00, CE, Brazil

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Abstract

In the present study, we examined the neuroprotective effects of vitamin C in adult rats after pilocarpine-induced seizures. Vitamin C is an exogenous antioxidant that can be used in treatment of seizures. It can alter oxidative stress and damage neuronal induced by seizures. Its antioxidant properties can be proved in epilepsy models, such as pilocarpine-induced seizures in adult rats. In order to investigate neuroprotective effects of vitamin C, adult male rats (2 months-old) were pretreated with vitamin C (VIT C 250 mg/kg, i.p.) 30 min before receiving pilocarpine (400 mg/kg, s.c., P400 group). The other three groups were treated with vitamin C (VIT C group) and saline 0.9 (control group) alone. The pretreatment with vitamin C increased the latency to first seizures and reduced mortality rate after pilocarpine-induced seizures. Pretreatment with vitamin C alone decrease lipid peroxidation levels when compared to pilocarpine group and P400+VIT C. In P400, P400+VIT C and VIT C groups were observed an increased hippocampal catalase activity when compared to control group. Our results can suggest that neuroprotective effects of vitamin C in adult rats can be the result of reduced lipid peroxidation levels and increase of catalase activity after seizures and status epilepticus induced by pilocarpine.

Keywords: Vitamin C; Pilocarpine; Hippocampus; Lipid peroxidation; Catalase; Seizures

1. Introduction

Status epilepticus is a neurologic emergency which has an associated mortality rate of 10–12%. This condition is characterized by prolonged or repetitive epileptic discharges, resulting in persistent clinically alterations of normal brain function and cognitive state (Treiman, 1995). Pilocarpine-induced seizures have demonstrated behavioral and electroencephalographic characteristics similar to those in human temporal lobe epilepsy (Turski et al., 1983).

Recent studies suggested that seizures and status epilepticus can be associated with oxidative stress (Barros et al., 2007). Another study showed the oxidative stress role with seizures-induced neuronal death (Walz et al., 2000). The membrane lipid peroxidation, which is due to an increase in free radicals and/or decrease in antioxidant defense mechanisms, has been suggested to be accidentally involved in some forms of epilepsy as such as pilocarpine model (Simmet and Peskar, 1990; Freitas et al., 2004a). The brain is a preferential target for the peroxidative process because it has a high content of polyunsaturated fatty acids. Central nervous system (CNS) has systems that prevent hazardous effects of free radicals such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-pX) and reduced glutathione (GSH) (Costa, 1994; Naffah-Mazzacoratti et al., 2001). Freitas et al. (2004a) showed that during the establishment of convulsive process in adult rats antioxidant enzymatic activity changes occur.

Previous studies have demonstrated that exogenous antioxidant like vitamin E and C, can inhibit the neuronal damage produced by lipid peroxidation during seizures (Xavier et al., 2007, Barros et al.,

* Corresponding author. Rua Monte Verde 1443, Paupina, Fortaleza, 60870-420, Brazil. Tel./fax: +55 88 3412 2209.
E-mail address: rivmendes@bol.com.br (R.M. Freitas).

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2007). Barros et al. (2007) showed that vitamin E exerted antioxidant and neuroprotective effects in seizures induced by pilocarpine. It also has been reported to prevent the increase in brain free fatty acid levels in epileptic rats, suggesting that the protection induced by vitamin E may be mediated by antioxidant enzymatic activity such as increase of hippocampal catalase activity in adult rats pretreated with vitamin E (Barros et al., 2007).

The objective of the present work was to analyse the effects produced by pretreatment of vitamin C, as assessed by the behavioral studies, lipid peroxidation levels and catalase activity in order to evaluate the antioxidant and neuroprotective effects of vitamin C, attempting to clarify their mechanism of action.

2. Materials and methods

2.1. Animals

Experiments were conducted using 2-month-old male Wistar rats weighing 250–280 g. Animals were housed in cages with free access to food and water. All animals were kept with standard light–dark cycle (lights on 07:00h a.m.). All experiments were performed according to the Guide for the Care and Use of Laboratory Animals, from the US Department of Health and Human Services, Washington, DC, 1985.

2.2. Drugs

Pilocarpine hydrochloride was purchased from Sigma Chem. Co. (St. Louis, MO, USA). Vitamin C (ascorbic acid) was purchased from ICN (CA, USA).

2.3. Treatment

Experiments were conducted at 7:00 a.m. in an experimental room. In a set of experiments, the animals were pretreated with vitamin C 250 mg/kg, i.p. (VIT C 250) or 0.9% saline, i.p., and 30 min later, they received pilocarpine hydrochloride, 400 mg/kg, s.c. (P400 group) alone. Other two groups received VIT C 250 or 0.9% saline (control). Animals were closely observed for behavioral changes (appearance of peripheral cholinergic reactions, such as miosis, piloerection, chromodacryorrhea, diarrhea, masticatory and stereotyped movements), latency to the development of the first seizure, status epilepticus and mortality rate, after the administration of pilocarpine during 24 h. The animals that survived were killed by decapitation and their brains were dissected on ice to remove hippocampus for determination of lipid peroxidation level and catalase activity. The pilocarpine group (P400) was constituted by those rats that presented seizures; SE for a period longer than 30 min and that did not die during 24 h.

2.4. Measurements of lipid peroxidation

Immediately after decapitation of animals, the hippocampus was dissected for the preparation of homogenates 10% (w/v). The formation of lipid peroxides during lipid peroxidation process was analysed by measuring the thiobarbituric-acid-reacting substances (TBARS) in cells, as previously described by Huong et al. (1998). Briefly, the samples were mixed with 50 mM potassium phosphate monobasic buffer pH 7.4 and catalytic system of formation of free radicals ($FeSO_4$ 0.01 mM and ascorbic acid 0.1 mM), and then incubated at 37 °C for 30 min. The reaction was stopped with 0.5 ml of trichloroacetic acid 10%, then the samples were centrifuged (3000 rpm/15 min), the supernatants were retrieved and mixed with 0.5 ml of thiobarbituric acid 0.8%, then heated in a boiling water bath for 15 min and after this period, immediately cold in bath of ice. Lipid peroxidation was determined by absorbance at 532 nm. The results above were expressed as μmol of malondialdehyde (MDA)/ g wet tissue.

2.5. Evaluation of catalase activity

Immediately after decapitation of animals, the hippocampus was dissected and ultrasonically homogenized in 1 ml of 0.05 M phosphate buffer, pH 7.0; the protein concentration was measured according to the method described by Lowry et al. (1951) and used for catalase activity determinations. Catalase activity was measured by method that employs hydrogen peroxide to generate H_2O and O_2 (Maehly and Chance, 1954). The activity was measured by degree of this reaction. The standard assay substrate mixture contained 0.30 ml of hydrogen peroxide in 50 ml of 0.05 M phosphate buffer, pH 7.0. The sample aliquot (20 μl) was added to 980 μl of substrate mixture. After 1 min, initial absorbance was recorded and final absorbance was read after 6 min. The reaction was followed at 230 nm. A standard curve was established using purified catalase (Sigma, MO, USA) under identical conditions. All samples were diluted with 0.1 mmol/l phosphate buffer (pH 7.0) to provoke an inhibition 50% of diluent rate (i.e. the uninhibited reaction) and results expressed as μM/min/μg protein (Maehly and Chance, 1954; Chance and Maehly, 1955).

Table 1
Effects of vitamin C after seizures and status epilepticus induced by pilocarpine, in behavioral study

Groups	Dose (mg/kg)	Latency to first seizures (min)	Latency to SE (min)	% Seizures	% Survival	Number of animals/group
P400 group	400	35±0.7	67.00±3.50	75	27	80
P400+VIT C 250	250	80±1.3 [c]	198.20±3.75 [c]	33 [a]	100 [a]	12
VIT C 250	250	00	00 [a, b]	00 [a, b]	100 [a]	12

Experiments were performed as described in Materials and methods. Animals were pretreated with vitamin C 250 mg/kg i.p., 30 min before treatment with pilocarpine 400 mg/kg, s.c. Values for latency to first seizure and latency to status epilepticus are expressed as means+SEM of animal number used in experiments shown in table. Values for % seizures and % survival are expressed as percentages of animal number from each experimental group.

[a] $p<0.0001$ as compared to pilocarpine group (χ^2 test).

[b] $p<0.0001$ as compared to h P400+VIT C 250 (χ^2 test).

[c] $p<0.05$ as compared TO P400+VIT C 250 (ANOVA and Student–Newman–Keuls test as the *post-hoc* test).

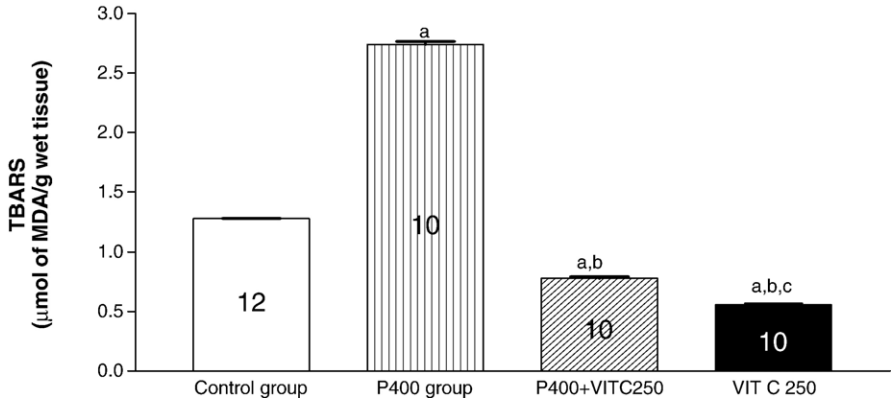

Fig. 1. Effects of vitamin C 250 in the TBARS content determinations in hippocampus of adult rats after pilocarpine-induced seizures and status epilepticus. Experiments were performed as described in Materials and methods. Each bar represents the mean ± S.E.M., from 10–12 animals per group. [a]$p<0.05$ as compared to control group, [b]$p<0.05$ as compared to P400 group and [c]$p<0.05$ as compared to P400 + VIT C 250 group (ANOVA and Student–Newman–Keuls as the *post hoc* test). Abbreviations: TBARS — thiobarbituric-acid-reacting substances; P400 — pilocarpine group; P400 + VIT C 250 — pretreated with vitamin C 30 min before administration of pilocarpine and VIT C 250 — vitamin C group.

2.6. Statistics analyses

Results of the latency to first seizure, status epilepticus, lipid peroxidation levels and catalase activity were compared using ANOVA and the Student–Newman–Keuls test as *post-hoc* test, because these results show a parametric distribution. The number of animals that seized and the number that survived were calculated as percentages (% seizures and % survival, respectively) and compared with a nonparametric test (χ^2). In both situations statistical significance was reached at p less-than-or-equals, slant 0.05. The statistical analyses were performed with the software Graph Pad Prism, Version 3.00 for Windows, Graph Pad Software (San Diego, CA, USA).

3. Results

3.1. Behavioral alterations in adult rats pretreated with vitamin C

All animals treated with P400 presented peripheral cholinergic signs (miosis, piloerection, chromodacriorrhea, diarrhea, masticatory), and stereotyped movements (continuous sniffing, paw licking and rearing) followed by motor limbic seizures in 75% of tested animals ($p<0.0001$). The convulsive process persisted and built up to a status epilepticus in 75% of these rats, leading to death of 63% of the animals ($p<0.0001$) (Table 1). The animals pretreated with VIT C and 30 min before treated with pilocarpine (P400 group) developed cholinergic reactions, 33% had seizures, 25% built up to status epilepticus ($p<0.0001$) and no one animal died (Table 1). The latency to the first seizure in animals pretreated with vitamin C was increased in 128% ($p<0.0001$) and in latency of status epilepticus was observed an increase of 196% when compared to control group ($p<0.0001$) (Table 1). No one animal that received injections of isotonic saline (control) or VIT C (250) alone showed seizure activity.

3.2. Hippocampal lipid peroxidation levels in adult rats pretreated with vitamin C

Vitamin C, at the dose 250 mg/kg, i.p., showed neuroprotective effects as assessed by the lipid peroxidation determinations,

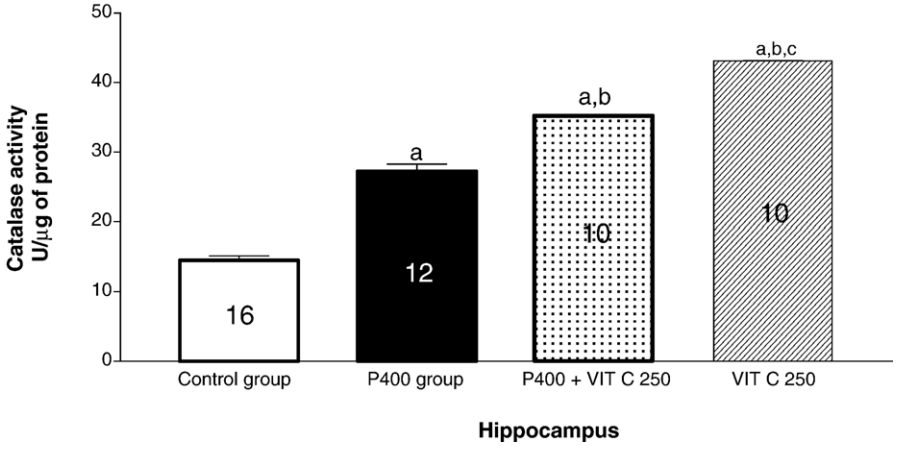

Fig. 2. Effects of vitamin C in catalase activities in hippocampus of adult rats after seizures and status epilepticus induced by pilocarpine. Experiments were performed as described in Materials and methods. Each bar represents the mean ± S.E.M., from 10–16 animals per group. [a]$p<0.05$ as compared to control group, [b]$p<0.05$ as compared to P400 group and [c]$p<0.05$ as compared to P400 + VIT C 250 group (ANOVA and Student–Newman–Keuls as the *post hoc* test).

in rats (Fig. 1). The treatment of animals with P400 (2.8±0.05) resulted in elevated MDA levels when compared to control group (1.29±0.02). However when the groups were administered with the association of vitamin C and pilocarpine, the pretreatment with VIT C 250 mg/kg (0.80±0.06) decreased the lipid peroxidation levels when compared to P400 group alone (2.8±0.05). The animals treated with vitamin C 250 alone (0.60±0.03) showed a reduction on MDA levels when compared control group (1.29±0.02) (Fig. 1).

3.3. Determination of hippocampal catalase activity of adult rats pretreated with vitamin C

Results of catalase activity determinations in hippocampus in 2-months-old rats are presented in Fig. 2. The treatment with single dose of pilocarpine (27.25±1.03) produced an increase in catalase activity when compared with the control group (14.50± 0.65). The catalase activity determined in animals treated with VIT C 250 alone (43.10±0.94) showed an increase of 197% when compared with control group (14.50±0.65). The group pretreated with VIT C 250 mg/kg and 30 min before received pilocarpine (35.30±0.38) showed a decrease in relation to pilocarpine group (27.25±1.03).

4. Discussion

In the present work, the central effects of vitamin C were studied. Vitamin C was firstly evaluated in behavioral study which gives a good indication in reduction of seizures' mortality rate. The results showed that vitamin C was able to significantly decrease the lipid peroxidation content after seizures and status epilepticus induced by pilocarpine.

Vitamin E have anticonvulsivant properties in seizures and status epilepticus induced by pilocarpine (Barros et al., 2007) and vitamin C, in particular, when compared to others antioxidant drugs, exert potent anticonvulsant and neuroprotective effects (Xavier et al., 2007). Our results confirm the vitamin C anticonvulsant activity on pilocarpine-induced seizures and status epilepticus, as revealed by increases in latency to the onset of seizures, in latency of status epilepticus, but also by seizures' mortality rate reduction.

Although the exact pathophysiological mechanism still needs to be clarified, the whole process can be related to the decrease of lipid peroxidation content. A variety of biochemical processes, including the activation of membrane phospholipases, proteases and nucleases which cause degradation of membrane phospholipids, proteolysis of cytoskeleton proteins and protein phosphorylation (Costa, 1994) are showed during seizures (Walz et al., 2000). In particular, polyphosphoinositides play an important role in convulsive process. Several alterations in membrane phospholipids metabolism result in liberation of free fatty acids (FFA), particularly free arachidonic acid, diacylglycerols, eicosanoids, lipid peroxides and free radicals in brain. These lipid metabolites along with abnormal ion homeostasis and lack of energy generation may contribute to neuronal injury and death (Costa, 1994). Barros et al. (2007) showed that the vitamin E, at the dose of 200 mg/kg, i.p.; increase hippocampal catalase activity of adult rats, but not one change was observed in hippocampal superoxide dismutase activity of adult rats after pilocarpine-induced seizures and status epilepticus.

The pilocarpine, a cholinergic agonist, is widely used in epilepsy experimental model (Cavalheiro, 1995). Freitas et al. (2004b) showed that lipid peroxidation levels are increased during the acute period of pilocarpine-induced seizures and status epilepticus in adult rats. Our results also showed that pilocarpine administration produced an increased lipid peroxidation in hippocampus of adult rats after seizures and status epilepticus, and, therefore, demonstrated and confirmed the possible role of free radicals brain injury after seizures induced by pilocarpine. The increase in free radical levels may be responsible for the establishment of neuropathology of seizures and status epilepticus. However, cerebral antioxidant enzymatic systems against free radical induced neuronal damage involve a cooperative action of these enzymes: SOD, CAT and GSH-Px. The seizures and status epilepticus also alter oxidative stress by activation of free radicals scavenging enzymes as such as SOD and CAT, indicating their neuroprotective effects (Erakovic et al., 2002, Ferreira and Matsubara, 1997). Furthermore, we observed an increase of antioxidant catalase activity in hippocampus of adult rats, 24 h after pilocarpine-induced seizures, suggesting antioxidant and neuroprotective actions of this enzyme in seizures. Indeed, vitamin C pretreatment decrease hippocampal lipid peroxidation content. The results showed the vitamin C was able to significantly increase not only the catalase activity, indicative of a possible antioxidant effect, but also decrease lipid peroxidation levels.

The mechanisms underlying the vitamin C neuroprotective effects are not fully understood, however, our results are in agreement with neuroprotective actions of vitamin C reported in previous studies in epilepsy model induced by pilocarpine. The pretreatment with vitamin C induced radical free content changes during epileptic activity in adult rats (Xavier et al., 2007).

Our results give support to the idea that vitamin E interacts with the catalase activity, probably at the activation of this enzyme that mediate antioxidant effects, to produce neuronal protection, and also acts to decrease the lipid peroxidation level that is the main factor responsible for neuronal damage after seizures and seizures and status epilepticus induced by pilocarpine. Additional studies, however, are needed to fully clarify the mechanism of antioxidant and neuroprotective effects of vitamin C. Furthermore, vitamin C could manifest these effects suggesting their potential use in clinical practice.

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