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Anticonvulsive effect of vitamin C on pentylenetetrazol-induced seizures in immature rats ☆

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ABSTRACT

Vitamin C helps to prevent brain oxidative stress and participate in the synthesis of progesterone. It also possesses a progesterone-like effect and acts synergistically with progesterone on the brain. Progesterone and its metabolites, but also vitamin C have been associated with anticonvulsant effects. We evaluated the progesterone concentration 30 min and 24 h after the last administration of vitamin C (500 mg/kg, i.p. for five days). We also evaluated how vitamin C altered pentylenetetrazol (PTZ)-induced seizures by measuring the onset latency of seizures, percentage of incidence and mortality as well as amino acid levels after seizures. Vitamin C treatment alone increased basal progesterone concentrations to 531% after 30 min compared to 253% after 24 h. Furthermore, vitamin C significantly increased the latency to the first myoclonic, clonic and tonic seizure induced by PTZ (80 mg/kg, i.p.) and decreased the percentage of incidence of clonic and tonic seizures as well as the mortality rate. Changes in tissue concentration of amino acids were primarily observed at 24 h after vitamin C treatment. Our results suggest that vitamin C together with progesterone and/or its metabolites are involved in the protection against PTZ-induced seizures in immature rats.

1. Introduction

Epilepsy is a neurological disorder characterized by spontaneous and recurring seizures (Burnham, 1998) occurring more frequently in children than in adults. Actually, it has been shown that the immature brain is more susceptible to the development of seizures than the mature brain (Moshé et al., 1983). Approximately 60–70% of patients respond to conventional anticonvulsant drug treatment, but there are 30–40% of patients, who do not respond to one therapy alone, and they usually need two or more anticonvulsant drugs combined (Vining, 1999). Nevertheless, there are epilepsies especially resistant to the pharmacologic treatment where preclinical and clinical studies are required to look for new alternative therapies to help those patients with drug-resistant seizures.

Vitamin C or L-ascorbate is an essential nutrient required for a range of essential metabolic reactions in all animals and plants. The pharmacophore of vitamin C is the ascorbate ion. In living organisms, ascorbate is an antioxidant, which protects the body against oxidative stress (Sebrel and Harris, 1967; Padayatti et al., 2003). Oxidative stress can arise through the increased production of reactive oxygen species or through deficiencies that are developed due to a decreased intake of antioxidant substances, such as vitamin C (Sinclair et al., 1990). It has been shown that vitamin C participates in many reactions because it is an electron donor; and therefore a reducing agent, and all its known physiological and biochemical properties are due to its action as an electron donor. Besides the biological functions of vitamin C as a reducing agent, it is required for the biosynthesis of steroids and peptide hormones and for the prevention or reduction of the oxidation of biomolecules (Sebrel and Harris, 1967; Wilson, 2002; Asard et al., 2004). For instance, vitamin C and other antioxidative substances help to prevent oxidative stress and the production of free radicals that interfere with progesterone production (Henmi et al., 2003; Roscetti et al., 1998). In fact, vitamin C possesses a progesterone-like effect and acts synergistically with progesterone (Sharaf and Gomaa, 1972). Even though the widespread importance of vitamin C as a significant reductive force in many tissues is well known (Carr and Frei, 1999; Frei et al., 1990; May et al., 1998), but, this function has

☆ All experimental procedures were carried out according to a protocol approved by the Local Animal Ethics Committee and in compliance with national (NOM-062-ZOO-1999) and international rules stated in the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

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not been experimentally exploited to a major degree in reference to the brain (Asard et al., 2004).

One of the anticonvulsant properties of vitamin C that has been observed as an increase in latency to the onset of seizures and a decrease in mortality rate after administering pilocarpine in rats (Xavier et al., 2007). Furthermore, the neuroprotective effect of vitamin C on seizures and status epilepticus induced by pilocarpine is associated with the prevention of the accumulation of polyunsaturated fatty acids in the brains of rats during the convulsive process (Santos et al., 2008; Xavier et al., 2007). In the CNS, progesterone, a neurosteroid hormone (Baulieu et al., 1996), is catalyzed promptly to 5α-dihidroprogesterone by the action of the 5α-reductase; subsequent reduction to 5α-dihidroprogesterone is catalyzed by the action of 3α-HSOR (3α-hydroxysteroid oxydoreductase) producing 5α-pregnane-3α-ol-20-one (allopregnanolone) (Akwa et al., 1992; Jung-Testas et al., 1989; Robel and Baulieu, 1994). Several studies have shown that progesterone and its metabolites can exert anticonvulsant effects (Beyenburg et al., 2001). The administration of allopregnanolone protected rats against seizures produced by pentylenetetrazol (PTZ), bicuculline, kainic acid and picrotoxine (Frye and Scalise, 2000; Grosso et al., 2003). Since allopregnanolone acts in the brain as a modulator of the γ-amynobutiric acid A ($GABA_A$) receptor (Harrison and Simmonds, 1984; Majewska, 1992), its effect might be mediated by GABAergic neurotransmission. Considering that the number of published reports relating to the functional implications of vitamin C in the CNS and specifically on epilepsy is certainly not voluminous and that the reports that do exist, it is in many cases, isolated findings. The aim of the present study was to investigate if vitamin C administration increases; progesterone production, inducing anticonvulsive activity on the PTZ-induced seizures in immature rats, as well as, it modifies levels GABA, glutamine (Gln), glutamate (Glu) and aspartate (Asp) amino acids.

2. Materials and methods

2.1. Animals

Ten-day-old male and female Sprague–Dawley rats were used for these experiments. At birth, the baby rats were mixed and assigned to eight mothers in groups of 10 (5 males and 5 females). All the animals were housed in a temperature and light controlled room under a 12 h light–dark cycle (lights on at 7:00 a.m.) with water and food *ad libitum*. All experimental procedures were carried out according to a protocol approved by the local Animal Ethics Committee and in compliance with national (NOM-062-ZOO-1999) and international rules stated in the National Institutes of Health Guide for Care and Use of Laboratory Animals. The number of experimental animals was kept to a minimum, and they were used only once and sacrificed immediately after the experiment. For each experiment, the animal groups consisted of at least ten rats.

2.2. Drugs

Vitamin C (Sigma, St. Louis MO, USA) was dissolved in 0.9% saline solution (SS). Pentilenetetrazole (PTZ, Sigma, St. Louis MO, USA) was dissolved in distilled water, and both were freshly prepared. GABA, Gln, Glu and Asp were purchased from Sigma. Progesterone levels were analyzed using a solid phase radioimmunoassay (Siemens Medical Solution Diagnostic, Los Angeles, CA, USA).

2.3. Experimental design

VITC30min and VITC24h groups (n = 10 per group). Immature rats from these groups were administered a 500 mg/kg dose of vitamin C intraperitoneally (i.p.) for 5 days as previously described (Huang et al., 2002). Control groups SS30min and SS24h received only saline solution (SS) the same period of time. Thirty minutes or 24 h after the last injection of vitamin C or SS, rats were sacrificed by decapitation, and their blood was collected to examine progesterone concentrations via radioimmunoassay analysis.

SS+PTZ30min and SS+PTZ24h groups (n = 10 per group). Thirty minutes or 24 h after the last SS administration, rats were injected i.p. with 80 mg/kg PTZ in a volume of 0.1 ml/10 g of body weight.

VITC+PTZ30min and VITC+PTZ24h groups (n = 10 per group). Thirty minutes or 24 h after the last vitamin C administration, rats from these groups received PTZ (80 mg/kg, i.p.).

Immediately after PTZ injection, rats were individually observed in a transparent plastic box to register latency onset and incidence of myoclonic (whole-body twitch), clonic (clonic spasms often followed by stupor or unusual posturing) and tonic (tonic hindlimb extension) seizures for 1 h after PTZ injection (Yonekawa et al., 1980).

Animals from the SS+PTZ30min, SS+PTZ24h, VITC+PTZ30min and VITC+PTZ24h groups that received PTZ were sacrificed by decapitation, and their brains were dissected to obtain cortex, amygdala and hippocampus cerebral areas to quantify the amino acids GABA, glutamate (Glu), aspartate (Asp) and glutamine (Gln) by using high performance liquid chromatographic (HPLC) analysis after derivatization with o-phthaldialdehyde (Geddes and Wood, 1984). With the purpose of reducing post-mortem effects, the cerebral structures were immediately frozen in liquid nitrogen and stored at −70 °C until their analysis.

2.4. Radioimmunoassay for progesterone

Progesterone concentrations were analyzed by using a solid phase radioimmunoassay (Siemens Medical Solution Diagnostic, Los Angeles, CA, USA). Briefly, the 125I-labeled progesterone competes for a fixed time with progesterone in the rat sample for antibody sites. Because the antibody is immobilized to the wall of a polypropylene tube, simply decanting the supernatant is suffices for to determine the competition and to isolate the antibody-bound fraction of the radiolabeled progesterone. Counting the tube in a gamma counter then yields a number that converts by way of a calibration curve to a measurement of the progesterone present in the rat sample (Kubasik et al., 1984). The progesterone concentration was calculated as ng/ml, in relation to intraassay and interassay precision was expressed as coefficient of variation (CV) and were less than 10%.

2.5. Aminoacid analysis by high performance liquid chromatography (HPLC)

Cerebral regions were weighed and homogenized under dark conditions in a perchloric acid (0.1 M) and sodium methabisulphyte 4 mM solution at 30 μl/10 mg of tissue using a Thomas homogenizer with teflon emboli at 1000 rpm for 60 s at 4 °C. Samples were centrifuged at 10,000 *g* for 4 min and processed to quantify the protein concentration (Lowry et al., 1951). The supernatant was used for analysis of tissue content of amino acids.

The off-line derivatization procedure was performed as previously described (Kendrick et al., 1988). The derivatization reagent (6 μl) was prepared as follows: 15 mg of o-phtaldialdehyde was dissolved in 300 μl of methanol and 2.8 ml of 0.4 M potassium tetraborate buffer plus 25 μl of 2-β-mercapthoetanol were added. The HPLC system consisted of Millenium 32 (Waters) with a fluorescence detector model 474 (Waters) operated at an excitation wavelength of 360 nm and an emission wavelength of 450 nm. Separations were achieved using a Novapack C18 conventional column (particle size 60 Å). Ternary gradient elution was used. The mobile phase A consisted of 40 mM sodium acetate buffer in 10% methanol adjusted to a pH of 5.7. Mobile phase B consisted of 8 mM sodium acetate buffer in 80% methanol, adjusted to a pH of 6.7 with acetic acid, both degassed using an ultrasonic bat. The elution profile was as follows: at time 0, 77% A, 23% B; 1 min 55% A, 45% B; 7 min 30% A, 70% B; 11 min 3% A, 97% B;

18–20 min 77% A, 23% B. The flow-rate was 500 µl min^{-1} and the calibration curves were adjusted from chromatograms with standard solutions that contained 0.1, 0.3 and 0.5 ng $µl^{-1}$ of GABA, Gln, Glu and Asp. Peak area ratios of the standard concentration were adjusted by least-squares linear regression analysis using the system manager (Waters).

The cerebral tissue was resuspended with 200 µl bidistilled water and diluted 40 times. From this dilution, 100 µl were taken for analysis. The reaction began by adding 500 µl of the reagent A, which contained 500 µl of 1% $CuSO_4$ and 500 µl, 2% tartrate of sodium and potassium in 50 ml of Na_2CO_3 2% diluted in NaOH (1 N). The mixture was vigorously shaken and left to rest at room temperature. After 10 min, 50 µl of the reactive B was added (i.e., reactive of Folin diluted 1:2 with bidistilled water). Samples were incubated for 30 min and the colorimetric reaction was analyzed in a spectrophotometer Perkin Elmer lambda 25 UV/VIS to 700 nm equipment. This procedure was associated with the values obtained by HPLC. The concentrations of amino acids are expressed in ng/mg of protein.

2.6. Statistical analysis

The tissue content of amino acids and progesterone levels were analyzed using a one way analysis of variance (ANOVA) followed by Bonferrioni test. The percentages of change were analyzed using a chi-squared test. Statistical analysis was carried out using SPSS 18.0. A *P value <0.05 was considered significant.

3. Results

3.1. PTZ-induced seizures

Administration of PTZ induced seizures in all control animals. The SS+PTZ30min group showed seizure activity 100% of the time and 57% of them died. The SS+PTZ24h group showed myoclonic and clonic seizures 100% of the time and tonic seizures 86% of the time. Death occurred in 72% of animals (Fig. 1). The SS+PTZ30min and SS+PTZ24h groups showed myoclonic seizures at 0.94 ± 0.03 min and 1.13 ± 0.14 min, respectively, clonic seizures at 1.96 ± 0.14 min and 2.60 ± 0.34 min, respectively, and tonic seizures at 24.6 ± 2.11 min and 21.70 ± 0.69 min, respectively. Death occurred at 26.67 ± 0.88 min and 26.34 ± 1.22 min, respectively (Fig. 2).

3.2. PTZ-induced seizures on vitamin C treatment

The rats from the VITC+PTZ30min group exhibited myoclonic seizures 100% of the time, but the number of animals exhibiting clonic and tonic seizures was significantly reduced (57% and 60%, respectively; $p<0.05$ for both). The animals of the VITC+PTZ24h group had myoclonic seizures 100% of the time and showed a significantly reduction in clonic (72% $p<0.05$) and tonic (72%, $p<0.05$) seizures

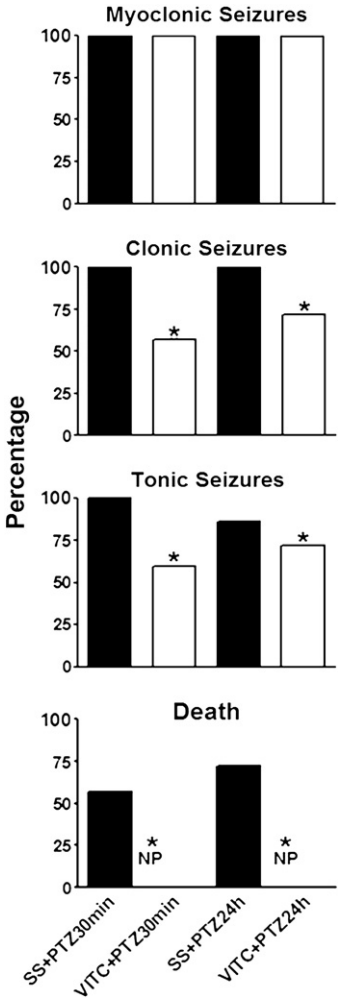

Fig. 1. The percentage of animals presenting with myoclonic (upper panels), clonic (medium panels), tonic seizures (medium panels) and death (lower panels) induced by PTZ (80 mg/kg, i.p.) administration 30 min (SS + PTZ30min and VITC + PTZ30min) and 24 h (SS + PTZ24h and VITC + PTZ24h) after the last vitamin C or SS administration, respectively. Bars represent the mean ± SEM of 10 animals per group. X^2 test, *$p<0.05$.

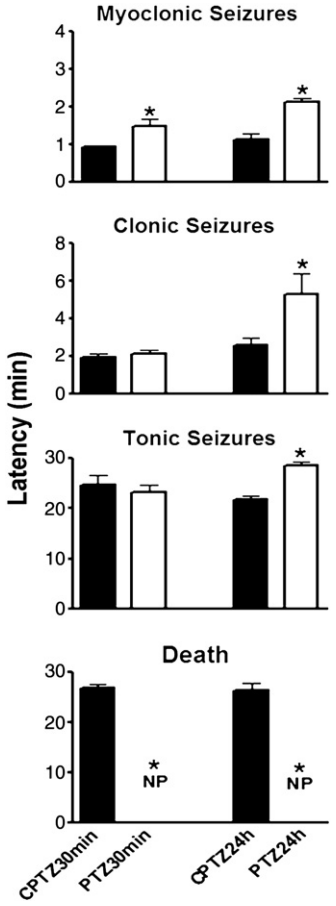

Fig. 2. Latency of the onset of myoclonic (upper panels), clonic (medium panels), tonic seizures (medium panels) and death (lower panels) induced by PTZ (80 mg/kg, i.p.) administration 30 min (SS + PTZ30min and VITC + PTZ30min) and 24 h (SS + PTZ24h and VITC + PTZ24h) after the last vitamin C or SS administration, respectively. Bars represent the mean ± SEM of 10 animals per group. ANOVA followed by Bonferroni test, *$p<0.05$.

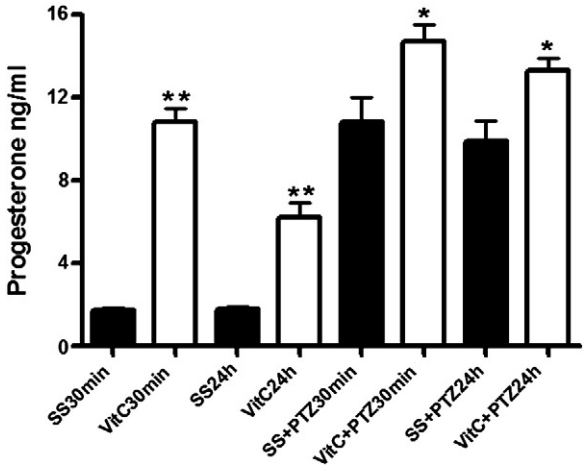

Fig. 3. Bars represent serum progesterone concentrations (ng/ml) in the brain of immature rats analyzed 30 min after the last vitamin C (VITC30min) or solution saline (SS30min) administration, as well as after 24 h (VITC24h or SS24h) alone and after PTZ-induced seizures. Values represent the mean ± SEM of 10 animals per group. ANOVA followed by Bonferroni test, *$p<0.05$.

(Fig. 1). In contrast with the control group, the VITC + PTZ30min group had a significantly increased latency to show myoclonic seizures (57%, $p<0.05$) and the mortality rate was reduced to 0% ($p<0.05$). The VITC + PTZ24h group showed a significantly augmented latency with myoclonic (86% compared to controls, $p<0.05$), clonic (104%, $p<0.05$) and tonic seizures (31%, $p<0.05$) after PTZ administration. Mortality rate was reduced to 0% ($p<0.05$) (Fig. 2).

3.3. Progesterone concentrations

The basal progesterone concentrations observed for the control groups (SS30min and SS24h) were 1.71 ± 0.13 ng/ml and 1.76 ± 0.14 ng/ml, respectively. Rats receiving vitamin C administration in VITC30min and VITC24h groups showed significantly increased progesterone concentrations of 10.79 ± 0.69 ng/ml ($p<0.01$), and 6.22 ± 0.69 ng/ml ($p<0.01$), respectively. This progesterone increase was duplicated when measured in the VITC30min group (529%) in comparison to the one observed in the VITC24h group (253%) (Fig. 3). Progesterone concentrations were significantly increased 1 h and 24 h after the PTZ-induced seizures (Fig. 3). These concentrations were also significantly enhanced when rats were treated with vitamin C (Fig. 3).

3.4. Amino acid analysis

Rats from the VITC+PTZ30min group exhibited a decrease in amino acid concentrations in comparison to the respective SS+PTZ30min control group for all amino acids analyzed in both the cerebral cortex and amygdala. These results were significant ($p<0.05$) for GABA (46%) and Gln (43%) in the cortex (Table 1). In contrast, a slight increase was found for amino acid concentrations for GABA, Gln, and Asp analyzed in the hippocampus, and a small decrease was observed for Glu (2699.2 ± 426.5 vs 1983.3 ± 245.2) (Table 1). These changes, however, were not significant.

Compared to the SS+PTZ24h control group, rats from the VITC+PTZ24h group showed a significant increase in concentrations of all amino acids examined in the cerebral cortex (Gln: 77%, Glu: 275% and Asp: 109%; $p<0.05$ for all). In contrast, a significant decrease in the amino acid concentrations was observed in the hippocampus (GABA: 72%, Gln: 62%, Glu: 55%, and Asp: 66%; $p<0.001$ for all). A significant reduction was also observed in the amygdala (Gln: 78%, Glu: 55% and Asp: 61%; $p<0.05$ for all) (Table 2).

4. Discussion

In the present study, the administration of vitamin C produced a delay on the PTZ-induced seizures as well as a reduction in the percentage of incidence of seizures and mortality in immature rats. Basal progesterone concentrations also increased after vitamin C treatment, which influenced amino acids levels after the seizures.

Both vitamin C and progesterone (or its metabolites) have been associated with anticonvulsant effects (Beyenburg et al., 2001; Frye and Scalise, 2000; Grosso et al., 2003; Xavier et al., 2007), most likely by acting in the brain as powerful positive modulators of $GABA_A$ receptors (Majewska, 1992; Stell et al., 2003). The non-specific antagonist of the $GABA_A$ receptor PTZ excites neurons and produces tonic–clonic seizures and death depending on the tested dose (Yonekawa et al., 1980), and it is the first choice for anticonvulsive drug screening (Huang et al., 2002; Pineau et al., 1999). In this study, immature rats pretreated with vitamin C (500 mg/kg) for 5 days and examined for PTZ-induced seizures 24 h after the last administration of vitamin C significantly delayed the appearance of myoclonic, clonic, and tonic seizures. Rats injected with PTZ 30 min after the last administration of vitamin C only delayed myoclonic seizures. The percentage of rats manifesting clonic and tonic seizures was also reduced in all the rats treated with vitamin C. These results give evidence of the anticonvulsant effects produced by presence of vitamin C.

Regarding, vitamin C and progesterone concentrations, it has been described that vitamin C plays a role in the modulation of neurosteroidogenesis (Roscetti et al., 1998), facilitating progesterone (Henmi et al., 2003) and pregnanolone production in a concentration-dependent manner (Roscetti et al., 1998), and it also possesses a progesterone-like effect and acts synergistically with this hormone (Sharaf and Gomaa, 1972). Consistent with previous findings (Henmi et al., 2003), we found that administering vitamin C to immature rats at a 500 mg/kg dose for 5 days produced an increase in progesterone synthesis that after 24 h was lower than at 30 min. Hormones, including gonadal, adrenal and thyroid hormones, can alter the excitability of neurons in the brain (Woolley and Schwartzkroin, 1998). The dynamic relationship between hormones and neuronal excitability is most clearly established for the ovarian sex steroid hormones: estrogen and progesterone (Herzog and Frye, 2003; Herzog, 2009). Menstrually related hormonal fluctuations in estrogen and progesterone underlie the patterns of catamenial seizure exacerbation.

Table 1
Tissue concentration of amino acids (ng/mg of protein) in cerebral areas of immature rats pretreated with saline solution 0.9% (SS) or vitamin C (VITC, 500 mg/kg, i.p.) 30 min after the last administration and after PTZ-induced seizures.

	Cortex		Hippocampus		Amygdala	
	SS + PTZ30min	VITC + PTZ30min	SS + PTZ30min	VITC + PTZ30min	SS + PTZ30min	VITC + PTZ30min
GABA	214.9 ± 31.1	115.2 ± 20.8[a]	601.9 ± 80.2	1 097.8 ± 343.4	465.9 ± 47.0	383.4 ± 97.7
Gln	596.5 ± 55.2	339.6 ± 30.6	786.4 ± 82.2	1 222.9 ± 377.9	1 142.8 ± 259.2	738.6 ± 295.3
Glu	583.7 ± 54.9	427.4 ± 31.0	2699.2 ± 426.5	1983.3 ± 245.2	1315.9 ± 127.7	1466.4 ± 186.6
Asp	233.9 ± 44.1	198.1 ± 18.0	695.7 ± 155.8	1 161.7 ± 371.8	614.9 ± 48.7	536.1 ± 77.9

GABA: γ-amynobutiric acid; Gln: glutamine; Glu: glutamate; Asp: aspartate. Values are the mean ± SEM from 10 animals per group. Anova followed Bonferroni test, [a]$p<0.05$.

Table 2
Tissue concentration of amino acids (ng/mg of protein) in cerebral areas of immature rats pretreated with saline solution 0.9% (SS) or vitamin C (VITC, 500 mg/kg, i.p.) 24 h after the last administration and after PTZ-induced seizures.

	Cortex		Hippocampus		Amygdala	
	SS + PTZ24h	VITC + PTZ24h	SS + PTZ24h	VITC + PTZ24h	SS + PTZ24h	VITC + PTZ24h
GABA	166.9 ± 14.5	253.1 ± 51.7	2 228.5 ± 335.3	628.3 ± 98.0 [aa]	465.9 ± 47.0	383.4 ± 97.7
Gln	421.1 ± 33.6	744.3 ± 35.9 [a]	3 406.6 ± 97.7	1293.7 ± 127.8 [aa]	5 094.9 ± 582.7	1 129.5 ± 238.9 [aa]
Glu	356.9 ± 13.2	1 338.2 ± 264.2 [a]	3 998.4 ± 740.3	1 799.9 ± 60.1 [aa]	4 566.3 ± 220.8	2 047.0 ± 534.8 [aa]
Asp	183.4 ± 16.2	368.5 ± 57.4 [a]	1 733.3 ± 306.7	584.7 ± 34.7 [aa]	2 617.5 ± 158.9	1 024.3 ± 331.7 [a]

GABA: γ-amynobutiric acid; Gln: glutamine; Glu: glutamate; Asp: aspartate. Values are the mean ± SEM from10 animals per group. Student´s t test, [a]$p<0.05$; [aa]$p<0.01$.

Estrogens facilitate seizures, whereas progesterone protects against seizures (Herzog, 2009). In this study, progesterone concentrations were increased after 1 h and 24 h PTZ-induced seizures and these levels were significantly augmented in presence of vitamin C. It has been reported that progesterone decreases ictal activity induced by PTZ in ovariectomized rodents (Frye et al., 2002; Reddy et al., 2004). Furthermore, some evidence suggests that the anti-seizure effect of progesterone may be due to part to its 5α-reduced metabolite allopregnanolone (Rhodes and Frye, 2005). Because the anticonvulsant effect observed in rats pretreated with vitamin C evaluated 24 h after the last administration was better compared to the effect 30 min after the last injection, this suggests that progesterone and its metabolites may participate in the protection against PTZ-induced seizure activity. It is known that PTZ-induced myoclonic and clonic seizures result from activation of forebrain structures, whereas the tonic extension is mediated by diencephalon and brainstem structures (Ben-Ari et al., 1981; Browning, 1985; Miller et al., 1987). Seizures that were evaluated were delayed in rats pretreated with vitamin C (principally, after 24 h of the last administration). These findings suggest that more than one cerebral area is involved in its effect.

We observed major changes in amino acid levels 24 h after the last administration of vitamin C compared to 30 min after in animals with PTZ-induced seizures. Vitamin C plus PTZ showed significant changes in all cerebral areas examined, but primarily in the hippocampus as well as the amygdala. Concentrations of all amino acid levels obtained 1 h after PTZ-induced seizures were reduced in rats that received pretreatment with vitamin C assessed 24 h after the last vitamin C administration. These results suggest that the anticonvulsant action of vitamin C is better at 24 h after its last administration, as the seizure activity was either absent or delayed. Moreover, these results are consistent with reports describing that the amygdala and hippocampus are structures involved in the expression and propagation of seizures, where tissue levels of amino acids, mainly the inhibitory ones, are modulators (Bear and Lothman, 1993). In fact, the hippocampus is one of the brain areas that show the most vulnerability in epilepsy (Hass et al., 2001). In the hippocampus, neurosteroids such as allopregnanolone have blocked induction of seizures (Martin-García and Pallares, 2005). Furthermore, an increased sensitivity to neurosteroids on GABAergic neurotransmission has been considered for immature rats (Mitchedlishvili et al., 2003). Significant changes on the amino acid levels in these regions after the ictal phase have been associated with an increase in the synthesis (Bikjdaouene et al., 2003; Walton et al., 1990) and release of those amino acids, which are induced by seizures in both the hippocampus and amygdala (Ding et al., 1998; Ueda and Tsuru, 1995).

Our findings also suggest that other structures in addition to the hippocampus participate in the generation and propagation of convulsive seizures (Bernasconi et al., 1999; Piredda and Gale, 1985). These areas, namely, the piriform cortex (Doherty et al., 2000) and the entorhinal cortex, represent regions that are anatomically and functionally different from hippocampus. The entorhinal cortex is extensively interconnected with neocortical sensory areas and multimodal associations as well as with subcortical regions. It is the most direct route of the afferents to the hippocampus and receives important connections from the amygdala (Insausti et al., 1987; Van Hoesen and Pandya 1975). This most likely explains the increase of glutamate and aspartate in the cortex observed at different points in time. In addition, this increment suggests that the seizures were not completely blocked.

On the other hand, the oxidative stress has been associated with neuronal damage induced by seizures and status epilepticus (Bellissimo et al., 2001; Xavier et al., 2007). Previous studies demonstrated antioxidant and anticonvulsant effects in adult rats acutely treated with 250 mg/kg i.p of vitamin C (Xavier et al., 2007). In our present study, an anticonvulsant effect of vitamin C was also observed on pentylenetetrazole-induced seizures in immature rats receiving 500 mg/day during 5 days. It has been described that vitamin C readily scavenges reactive oxygen and nitrogen species and may thereby prevent oxidative damage to important biological macromolecules such as DNA, lipids, and proteins and protect from several diseases (Sebrel and Harris, 1967; Padayatti et al., 2003; González et al., 2002). Nevertheless, it has been reported that lipid hydro peroxide can react with ascorbic acid to form products that could potentially damage DNA, suggesting that it may form genotoxic metabolites from lipid hydroperoxides implicating that ascorbic acid at high concentrations may enhance mutagenesis and risk of cancer (Shambeger, 1984; Anderson et al., 1997; Nowak et al., 1991; Podmore et al., 1998; Lee et al., 2001). Because up to 10 g/day of vitamin C may interfere with the healthy antioxidant-prooxidant balance in the body and considering that cells that have been damaged by high concentrations of H_2O_2 during seizures, they may be more susceptible to the potential cytotoxic effects in presence of high dose ascorbate. Thus, the dose of vitamin C and duration of administration are important factors to consider in control of seizures.

In conclusion, our results suggest that vitamin C interacts with progesterone synthesis and could be acting synergistically on the $GABA_A$ receptor to produce neuronal protection against PTZ action in immature rats. Additional studies are required to fully clarify the anticonvulsant mechanisms of action of vitamin C, and also about the safety in the chronic use of vitamin C.

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References

Akwa Y, Morfin RF, Robel Pe, Baulieu EE. Neurosteroid metabolism. Biochem J 1992;288:959–64.
Anderson D, Phillips BJ, Yu YW, Edwards AJ, Ayesh R, Butterworth KR. The effects of vitamin C supplementation on biomarkers of oxygen radical generated damage in human volunteers with 'low' or 'high' cholesterol levels. Environ Mol Mutagen 1997;30:161–74.
Asard H, May JM, Smirnoff M. Vitamin C. Functions and biochemistry in animals and plants. Taylor & Francis; 2004.

Baulieu EE, Schumacher M, Koenig H, Jung-Testas I, Akwa Y. Progesterone as a neurosteroid: actions within the nervous system. Cell Mol Neurobiol 1996;16: 143–54.
Bear J, Lothman EW. An in vitro study of focal epileptogenesis in combined hippocampal-para hippocampal slices. Epilepsy Res 1993;14:183–93.
Bellissimo MI, Amado D, Abdalla DS, Ferreira EC, Cavalheiro EA, Naffah-Mazzacoratti MG. Superoxide dismutase, glutathione peroxidase activities and the hydroperoxide concentration are modified in the hippocampus of epileptic rats. Epilepsy Res 2001;46:121–8.
Ben-Ari Y, Tremblay E, Riche D, Ghilini G, Naquet R. Electrographic, clinical and pathological alterations following systemic administration of kainic acid, bicuculline or pentetrazole: metabolic mapping using the deoxyglucose method with special reference to the pathology of epilepsy. Neurosci 1981;6:1361–91.
Bernasconi N, Bernasconi A, Andermann F, Dubeau F, Feindel W, Reutens DC. Entorhinal cortex in temporal lobe epilepsy. A quantitative MRI study. Neurology 1999;52: 1870–6.
Beyenburg S, Stoffel-Wagener B, Bauer J, Watzka M, Blumcke I, Bidlingmaier F, et al. Neuroactive steroids and seizure susceptibility. Epilepsy Res 2001;44:141–53.
Bikjdaouene L, Escames G, León J, Ferrer JMR, Khaldy H, Acuña-Castroviejo Vive F, et al. Changes in brain amino acids and nitric oxide after melatonin administration in rats with pentylenetetrazole-induced seizures. J Pineal Res 2003;35:54–60.
Browning RA. Role of the brain-stem reticular formation in tonic–clonic seizures: lesion and pharmacological studies. Fed Proc Nat Acad 1985;44:2425–31.
Burnham WM. Principles of medical pharmacology. 6a. ed. Oxford University Press; 1998.
Carr A, Frei B. Does vitamin C act as a pro-oxidant under physiological conditions? FASEB J 1999;13:1007–24.
Ding R, Asada H, Obata K. Changes in extracellular glutamate and GABA levels in the hippocampal CA3 and CA1 areas and the induction of glutamic acid decarboxilase-67 in dentate granule cells of rats treated with kainic acid. Brain Res 1998;800: 105–13.
Doherty J, Gale K, Eagles DA. Evoked epileptiform discharges in the rat anterior piriform cortex: generation and local propagation. Brain Res 2000;861:77–88.
Frei B, Stocker R, England L, Ames BN. Ascorbate: the most effective antioxidant in human blood plasma. Adv Exp Med Biol 1990;264:155–63.
Frye CA, Rhodes ME, Walf AA, Harney JP. Progesterone reduces pentylenetetrazol-induced ictal activity of wild-type mice but not those deficient in type I 5α-reductase. Epilepsia 2002;43:14–7.
Frye CA, Scalise TJ. Anti-seizure effects of progesterone and 3α, 5α-THP in kainic acid and perforant pathway models of epilepsy. Psyconeuroendocrinology 2000;25(4): 407–20.
Geddes JW, Wood JD. Changes in the amino acid content of nerve endings (synaptosomes) induced by drugs that alter the metabolism of glutamate and γ-aminobuttyric acid. J Neurochem 1984;42:16–24.
Gonzalez MJ, Riordan HD, Miranda-Massari JR. Vitamin C and oxidative DNA damage revisited. J Orthomolecular Medicine 2002;17:225–8.
Grosso S, Luisi S, Mostardini R, Farnetani M, Cobellis L, Morgese G, et al. Inter-ictal and post-ictal circulating levels of allopregnanolone, an anticonvulsivant metabolite of progesterone, in epileptic children. Epilepsy Res 2003;54:29–34.
Harrison NL, Simmonds MA. Modulation of the GABA receptor complex by a steroids anaesthetic. Brain Res 1984;323:287–92.
Hass KZ, Sperber EF, Opanashuk LA, Stanton PK, Moshé SL. Resistance of immature hippocampus to morphologic and physiologic alterations following status epuilepticus or kindling. Hippocampus 2001;11:615–25.
Henmi H, Endo T, Kitaiima Y, Manase K, Hata H, Kudo R. Effects of ascorbic acid supplementation on serum progesterone levels in patients with a luteal phase defect. Fertil Steril 2003;80:459–61.
Herzog AG, Frye CA. Seizure exacerbation associated with inhibition of progesterone metabolism. Ann Neurol 2003;53:390–1.
Herzog AG. Hormonal therapies: progesterone. Neurotherapeutics 2009;6:383–91.
Huang LT, Yang SN, Liou CW, Hung PL, Lai MC, Wang CL, et al. Pentylenetetrazol-induced recurrent seizures in rat pups: time course on spatial learning and long-term effects. Epilepsia 2002;43:567–73.
Insausti R, Amaral DG, Cowan WM. The entorhinal cortex of the monkey. II. Cortical afferents. J Comp Neurol 1987;264:356–95.
Jung-Testas I, Hu ZY, Baulieu EE, Robel P. Neurosteroids: biosynthesis of pregnenolone and progesterone in primarycultures of rat glial cells. Endocrinology 1989;125: 2083–91.
Kendrick KM, Keverne EB, Chapman C, Baldwin BA. Microdialysis measurement of oxytocin, aspartate, γ-aminobutyric acid and glutamate release from the olfactory bulb of the sheep during vaginocervical stimulation. Brain Res 1988;442:171–4.
Kubasik NP, Hallaver GD, Brodows RG. Evaluation of a direct solid-phase radioimmunoassay for progesterone. Clin Chem 1984;30:284–6.
Lee SH, Oe T, Blair IA. Vitamin C-induced decomposition of lipid hydroperoxides to endogenous genotoxins. Science 2001;292:2083–6.
Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951:265–75.
Majewska MD. Neurosteroids: endogenous bimodal modulators of the GABA-A receptor. Mechanism of action and physiological significance. Prog Neurobiol 1992;38:379–95.
Moshé SL, Albala BJ, Ackermann RF, Engel Jr J. Increased seizure susceptibility of the immature brain. Dev Brain Res 1983;7:81–5.
Martin-García E, Pallares M. The intrahippocampal administration of the neurosteroid allopregnanolone blocks the audiogenic seizures induced by nicotine. Brain Res 2005;1062:144–50.
May JM, Qu ZC, Mendiratta S. Protection and recycling of alpha-tocopherol in human erythrocytes by intracellular ascorbic acid. Arch Biochem Biophys 1998;349:281–9.
Miller JW, McKeon AC, Ferrendelli JA. Functional anatomy of pentylenetetrazol and electroshock seizures in the rat brainstem. Ann Neurol 1987;22:615–21.
Mitchedlishvili Z, Zun CS, Harrison MB, Kapur J. Increased neurosteroid sensitivity of hippocampal GABAa receptors during postnatal development. Neurosci 2003;18: 655–66.
Nowak D, Piasecka G, Pietras T, Antczak A. Effect of ascorbic acid on killing of lymphocytes and macrophages by hydrogen peroxide. Biochim Biomed Acta 1991;50:1077–86.
Padayatti SJ, Katz A, Wang Y, Eck P, Kwon O, Lee J, et al. Vitamin C as an antioxidant: evaluation of its role in disease prevention. J Am Coll Nutr 2003;22:18–35.
Pineau N, Charriaut-Marlangue M, Jacques NA. Pentylenetetrazol seizures induce cell suffering but not death in the immature rat brain. Dev Brain Res 1999;112:139–44.
Piredda S, Gale K. A crucial epileptogenic site in the deep prepiriform cortex. Nature 1985;317:623–5.
Podmore ID, Griffiths HR, Herbert KE, Mistry N, Mistry P, Lunec J. Vitamin C exhibits pro-oxidant properties. Nature 1998:392:559.
Reddy DS, Castaneda DC, O´Malley BW, Rogawski MA. Anticonvulsant activity of progesterone and neurosteroids in progesterone receptor knockout mice. J Pharmacol Exp Ther 2004;310:230–9.
Rhodes ME, Frye CA. Attenuating 5α-pregnane-3α-ol-20-one formation in the hippocampus of female rats increases pentylenetetrazole-induced seizures. Epilepsy Behav 2005;6(2):140–6.
Robel P, Baulieu EE. Neurosteroids: biosynthesis and function. Trends Endocrinol Metab 1994;5:1–8.
Roscetti G, Del Carmine R, Trabucchi M, Massotti M, Purdy RH, Barbaccia ML. Modulation of neurosteroid synthesis/accumulation by L-ascorbic acid in rat brain tissue: inhibition by selected serotonin antagonists. J Neurochem 1998;71:1108–17.
Santos LF, Freitas RL, Xavier SM, Saldahna GB, Freitas RM. Neuroprotective actions of vitamin C related to decreased lipid peroxidation and increased catalasa activity in adult rats after pilocarpine-induced seizures. Pharmacol Biochem Behav 2008;89: 1–5.
Sebrel WH, Harris RS. The vitamins: chemistry, physiology, pathology and methods. New York: Academic Press; 1967.
Shambeger RJ. Genetic toxicology of ascorbic acid. Mutat Res 1984;133:135–59.
Sharaf A, Gomaa A. Progesterone-like effect of vitamins. Plant Foods Hum Nutr 1972;21: 159–64.
Sinclair AJ, Barnett AH, Lunec J. Free radicals and antioxidant systems in health and disease. Br J Hosp Med 1990;43:334–44.
Stell MB, Brickley GS, Tang YC, Farrant M, Mody I. Neuroactive steroids reduce neuronal excitability by selectively enhancing tonic inhibition mediated by δ subunit-containing $GABA_A$ receptors. Proc Natl Acad Sci 2003;100:14439–44.
Ueda Y, Tsuru N. Simultaneous monitoring of the seizure-related changes in extracellular glutamate and γ-aminobutyric acid concentration in bilateral hippocampi following development of amygdaloid kindling. Epilepsy Res 1995;20:213–9.
Van Hoesen GW, Pandya DN. Some connections of the entorhinal (area 28) and perirhinal (area 36) cortices of the rhesus monkey. I. Temporal lobe afferents. Brain Res 1975;95:1-24.
Vining EPG. Clinical efficacy of the ketogenic diet. Epilepsy Res 1999;37:181–90.
Walton NY, Gunawan S, Treiman DM. Brain amino acid concentration changes during status epilepticus induced by lithium and pilocarpine. Exp Neurol 1990;108:61–70.
Wilson JX. The physiological role of dehydroascorbic acid. FEBS Lett 2002;527:5–9.
Woolley CS, Schwartzkroin PA. Hormonal effects on the brain. Epilepsia 1998;39(Suppl 8): 2–8.
Xavier SM, Barbosa CO, Barros DO, Silva RF, Oliveira AA, Freitas RM. Vitamin C antioxidant effects in hippocampus of adult rats after seizures and status epilepticus induced by pilocarpine. Neurosci Lett 2007;420:76–9.
Yonekawa DW, Kupferberg JH, Woodbury MD. Relationship between pentylenetetrazol-induced seizures and brain pentylenetetrazol levels in mice. J Pharmacol Exp Ter 1980;214:589–93.