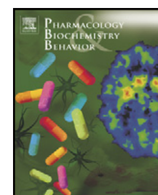
Pharmacology, Biochemistry and Behavior 98 (2011) 525–532

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh



Doxycycline protects against pilocarpine-induced convulsions in rats, through its antioxidant effect and modulation of brain amino acids

Carlos Renato Alves Nogueira [a], Flávio Maia Damasceno [a], Manuel Rufino de Aquino-Neto [a], Geanne Matos de Andrade [a], Juvênia Bezerra Fontenele [a], Thales Augusto de Medeiros [b], Glauce Socorro de Barros Viana [a,b,*]

[a] Department of Physiology and Pharmacology, Faculty of Medicine, Federal University of Ceará, Rua Cel. Nunes de Melo, 1127, CEP 60430-270, Fortaleza, Brazil
[b] Faculty of Medicine of Juazeiro do Norte, Avenida Tenente Raimundo Rocha s/n, CEP 63040-360, Juazeiro do Norte, Brazil

ARTICLE INFO

Article history:
Received 12 November 2010
Received in revised form 18 February 2011
Accepted 25 February 2011
Available online 5 March 2011

Keywords:
Pilocarpine-induced convulsions
Epilepsy
Neuroprotection
Doxycycline
Amino acid concentrations
Antioxidant activity

ABSTRACT

This work evaluated doxycycline (2nd generation tetracycline) protection against pilocarpine-induced convulsions in rats. The animals were treated with doxycycline (Dox: 10 to100 mg/kg, i.p., 7 days), 30 min before the pilocarpine injection (P: 300 mg/kg, i.p.) and observed for cholinergic signs, latencies to the first convulsion and death. Amino acid concentrations, lipid peroxidation and nitrite levels in temporal cortices were determined as well as the radical scavenging activity. Doxycycline increased latencies to the first convulsion and death as compared to the untreated P300 group. It also decreased glutamate and aspartate, increased GABA, blocked nitrite formation, reduced TBARS contents and showed a radical scavenging activity. Finally, doxycycline decreased the number of degenerating neurons (evaluated by fluoro-jade staining) and increased the number of viable neurons (assessed by cresyl violet staining) as compared do the P300 group. The antioxidant effect associated with decreased levels of excitatory and increased levels of inhibitory amino acids could explain the neuroprotective effect of doxycycline.

© 2011 Elsevier Inc. All rights reserved.

1. Introduction

The pilocarpine model of temporal lobe epilepsy in rodents reproduces behavioral features, electroencephalographic characteristics and the pattern of neuroanatomical damage observed in humans. The early phase of the response involves akinesia, ataxia, facial automatisms and head tremor, followed by motor limbic seizures, with rearing, forelimb clonus, salivation, masticatory movements and loss of posture. These episodes can lead to *status epilepticus* (SE) and to high lethality rate increases during the first 24 h (Turski et al., 1984). The initial induced toxicity is followed by a distinct non-cholinergic phase produced by excessive release of excitatory amino acids such as glutamate and aspartate (Solberg and Belkin, 1997). Furthermore, the release of intracellular glutamate from dying cells can raise to toxic levels the concentration of glutamate around neighboring neurons (Lipton and Roseberg, 1994). Thus, intervention in the glutamate excitotoxic cascade may contribute to the disease modification.

Neurochemical studies performed after pilocarpine-induced convulsive processes (Erakovic et al., 2000) show that it affects not only neurotransmitters (adenosine, norepinephrine, dopamine, serotonin, glutamate, and GABA) but also muscarinic or dopaminergic receptor densities (Freitas et al., 2003). However, the mechanism of pilocarpine-induced SE causing a subsequent neuronal damage is still unclear. Second generation tetracyclines, such as minocycline and doxycycline, have attracted interest as inhibitors of caspases and matrix metalloproteinases, MMP (Chen et al., 2000), and evidences indicate doxycycline to be beneficial in inflammatory diseases associated with excessive MMP activity (Brown et al., 2004; Pasquale and Tan, 2005). These drugs also cause the reduction of cytokine releases such as TNF-alpha (Brown et al., 2004; Roach et al., 2002). Although the contribution of the anti-inflammatory effects of these tetracyclines to neuroprotection has been investigated in models of stroke and neurodegenerative diseases, the results are still conflicting (Yrjanheikki et al., 1999; Du et al., 2001; Kriz et al., 2002; Smith et al., 2003).

Furthermore, inflammatory processes in the brain, such as the production of pro-inflammatory cytokines and related molecules, have been described in experimental models of seizures and in clinical cases of epilepsy (Vezzani, 2005). Disturbances in the brain metabolism of some amino acids especially glutamate and GABA may lead to seizures (Szyndler et al., 2008). In addition, the glutamate release by activated microglia induces excitotoxicity and may contribute to neurodegeneration in numerous neurological diseases, including epilepsy (Takeuchi et al., 2008).

Recently (Yilmaz et al., 2006), minocycline but not doxycycline has been shown to decrease the loss of hippocampal neurons and the

* Corresponding author at: Rua Barbosa de Freitas, 130/1100, CEP 60170-020 Fortaleza, Brazil. Tel./fax: 55 85 3242 3064.
E-mail address: gbviana@live.com (G.S.B. Viana).

0091-3057/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2011.02.025

motor incoordination in penicillin-epileptic rats. According to that work, minocycline could protect against neurological insults, including epilepsy. However, another study (Jantzie et al., 2005) showed that in vulnerable brain regions doxycycline significantly decreased caspase-3-immunoreactivity, promoted neuronal survival, inhibited microglial activation, and reduced reactive astrocytosis in animal models of cerebral ischemia.

Both tetracyclines, due to their anti-inflammatory properties, could act as neuroprotective agents by avoiding microglial activation, decreasing inductions of interleukin 1beta-converting enzyme mRNA and of iNOS mRNA, as well as preventing nitric oxide synthase (NOS) protein expression (Villareal et al., 2003). Despite the wealth of data in the literature, most of them were carried out with minocycline. Thus, the objective of the present work was to study the effects of repeated doxycycline administration on pilocarpine-induced seizures in rats, attempting to clarify its mechanism of action and potential benefit as a neuroprotective agent. The work focuses on behavioral, neurochemical and histological aspects of the drug effects *in vivo* and *in vitro*.

2. Materials and methods

2.1. Drugs

Pilocarpine, orthophthaldehyde, 2-mercaptoethanol, aspartate (Asp), glutamate (Glu), glycine (Gly), tyrosine (Tyr), taurine (Tau), gama-aminobutyric acid (GABA), tryptophan (Trp) and other standard amino acids were purchased from Sigma Chemical Co., USA. Doxycycline was from Galena-Brazil and dissolved in distilled water before use. All other drugs were of analytical grade.

2.2. Animals and treatments

Male Wistar rats (200 g) from the Animal House of the Federal University of Ceará (5 to 6 per cage) were maintained in a 12 h light/dark cycle, with free access to water and standard food. All the experiments were performed according to the Guide for the Care and Use of Laboratory Animals, from the US Health and Human Services Department. The animals were pretreated with doxycycline (10 to 100 mg/kg, i.p.) for 7 days. At the 7th day and 30 min after the last doxycycline (Dox) administration, they received a single pilocarpine injection (300 mg/kg, i.p.). Positive controls were injected with pilocarpine only (P300), while negative controls were injected with distilled water. The research was submitted and approved by the Research Committee for Animal Experimentation of the Faculty of Medicine of the Federal University of Ceará, Brazil.

All animals were observed for the following parameters: peripheral cholinergic signs (miosis, chromodacryorrhea, piloerection, tremors, salivation and diarrhea), stereotyped movements (increase of masticatory movements and paw licking), latency to the first convulsion, latency to death and number of deaths. Behavioral experiments were performed in the morning (at 9 a.m.). Surviving animals from all groups, that were still alive 7 h later and developed SE (acute period), had their temporal cortices dissected on ice and stored at −70 °C, for further biochemical determinations (nitrite/nitrate concentrations, antioxidant activity and lipid peroxidation) and neurochemical assays (amino acid analyses). Under the same experimental conditions, other groups were decapitated and sections from their brain cortices used for histological studies.

2.3. Amino acid determinations

Amino acid concentrations were determined by reversed-phase high performance liquid chromatography (RP-HPLC), involving pre-column derivatization with orthophthaldehyde (OPA). The Shimadzu RP-HPLC system (Japan) consisting of a spectrofluorimeter detector (excitation and emission wavelengths of 350 and 450 nm respectively) coupled with an integrator was used. The chromatographic column was a C18 (250 × 4.6 mm, 5 μ) and a 1 ml/min flow rate. The mobile phase A consisted of a 50 mM NaH_2PO_4 solution in 20% methanol, pH 5.5, and the mobile phase B was 100% methanol. The phases were prepared in ultra pure water (Milli-Q system) and filtered through 0.22 μm filters from Millipore.

A 2.5 mM stock solution of standard amino acids was prepared in the mobile phase A. Brain homogenates (at 10%) were prepared in 0.1 M perchloric acid, centrifuged (25,000 ×*g*, 30 min) and the supernatants collected and filtered. To make the derivatization solution, 13.5 mg OPA were dissolved in 250 μl ethanol followed by the addition of 10 μl 2-mercaptoethanol, and the volume was completed to 2.25 ml with borate buffer, pH 9.3. The solution was then filtered through a 0.22 μm filter (Millipore) and used after 24 h. For derivatizations, 20 μl amino acids or samples were diluted with 20 μl OPA, and injected into the HPLC column, 1 min later.

2.4. Lipid peroxidation

The content of malondialdehyde (MDA) formation in homogenates of temporal cortices was determined by the thiobarbituric acid (TBA) method. Briefly, 500 μl tissue homogenate (10% w/v in 1.15% KCl) were firstly mixed with 1% phosphoric acid (3 ml), followed by the addition of 0.6% TBA (1 ml). The tubes containing the assay mixture were vortexed. After boiling for 45 min, samples were cooled, extracted with 4 ml n-butanol, vortexed vigorously, and centrifuged (3500 ×*g*, 10 min). The MDA content in the n-butanol layer was then spectrophotometrically determined at 535 nm (Mihara and Uchiyama, 1978).

2.5. Nitrite concentration

The nitrite contents were determined in 10% homogenates from temporal cortices by the Griess method. Briefly, homogenates were centrifuged (3500 ×*g*, 15 min) and 100 μl supernatants were incubated with 100 μl Griess reagent (1% sulfanilamide in 5% H_3PO_4:0.1% N-(1-naphthyl)-ethylenediamine dihydro-chloride:5% H_3PO_4/distilled water, 1:1:1:1), at room temperature for 5 min. The absorbance was measured at 560 nm in a microplate reader.

2.6. In vitro determination of the antioxidant activity of doxycycline (Dox) by the diphenylpicryl-hydrazyl (DPPH) assay

The antioxidant activities of Dox and alpha-tocopherol (reference drug) were determined by the DPPH assay (Saint-Cricq de Gaulejac et al., 1999). Briefly, 0.1 ml alpha-tocopherol (75 μg/ml) or Dox (50, 100 and 500 μg/ml) were placed in test tubes, followed by the addition of 0.3 mM DPPH (3.9 ml in 1:1 methanol:H_20). Doxycycline, alpha-tocopherol and vehicle (30% DMSO in 1:1 methanol:ethanol) were vigorously shaken with DPPH, and left standing for 60 min in the dark. For the blank, 0.1 ml methanol:ethanol solution was used. The reduction of DPPH was spectrophotometrically determined at 517 nm. The radical scavenging activity (RSA) was calculated as the percentage of the DPPH discoloration, by the equation: $\% \ RSA = [(A_{DPPH} - A_s)/A_{DPPH}] \times 100$, where A_s is the absorbance of the test solution when the compound is added, and A_{DPPH} is the absorbance of the DPPH solution.

2.7. Fluoro-jade staining

Fluoro-jade is an anionic fluochrome capable of selectively staining degenerating neurons in brain slices. The technique used was that previously described (Schmued et al., 2005). Temporal cortex sections were dissected from normal animals, P300 treated animals and P300 animals pretreated with Dox (50 mg/kg, i.p., for 7 days). The animals from the two last groups developed SE, and their brains were

dissected 7 h latter. Brain sections were fixed in formalin and mounted into a block of paraffin to prepare 10 μm slices. Afterwards, the paraffin was removed by immersions in xylol (twice, 5 min each) and the slices were mounted onto gelatin coated slides. The tissue was re-hydrated by immersion in ethanol for 3 min, followed by 3 min changes in 70 and 50% ethanol and finally in distilled water. The slides were then transferred to a 0.06% potassium permanganate solution for 15 min, rinsed in distilled water, and transferred to the fluoro-jade staining solution where they were gently shaken for 30 min. After staining, the sections were rinsed with distilled water (three times, 1 min each). The excess water was drained off, and the slides air dried and mounted on the Fluoromount medium. The sections were then examined with an epifluorescence microscope.

2.8. Cresyl violet staining

The method is used for the detection of Nissl body on formalin-fixed and paraffin embedded tissue sections. This staining is commonly used for identifying the basic neuronal structure in the brain. Hippocampus sections from normal, P300 and Dox + P300 groups were used. The animals were treated for 7 days with Dox (50 mg/kg, i.p.) before the pilocarpine-induced convulsions. The hippocampus was chosen since it is known to be the brain area most sensitive to insults and critical for maintaining epileptic seizures in temporal lobe epilepsy, the most common type in adults.

2.9. Statistical analyses

The data are presented as means ± SEM. Data on the latency to the first convulsion and latency to death were analyzed by a non-parametric Kruskal–Wallis test followed by the Dunn's multiple comparison test. Other data were analyzed by One-Way ANOVA, followed by the Student–Newman–Keuls for multiple comparisons as the *post hoc* test. The results were considered significant at $p<0.05$.

3. Results

3.1. Neurologic profile and autonomic signs

Two to five minutes after the pilocarpine (P300) administration, the animals presented behavioral changes, including akinesia, ataxia, peripheral cholinergic signs (miosis, piloerection, chromodacryorrhea, diarrhea and masticatory automatisms), stereotyped movements (sniffing, paw licking and rearing), clonic movements of the forelimbs, head bobbing, tremors and convulsions (generally followed by SE and death). Except for convulsions and SE (not shown in some animals), all those changes were also observed, although with less intensity in the pilocarpine group after the pre-treatment with doxycycline, Dox + P300 group (Table 1).

3.2. Effect of doxycycline (Dox) on the 1st convulsion and latency to death in pilocarpine-treated rats

Fig. 1A shows that the latency time to the 1st convulsion was significantly increased (1.6 to 5 times) in all pilocarpine groups previously treated with doxycycline (Dox + P300) as compared to controls (P300). However, the highest effect (423%) was observed with the 25 mg/kg dose, and the dose–response curve tended to be bell shaped. No significant alteration of pilocarpine effects was observed after doxycycline, at the dose of 10 mg/kg. A similar curve was shown with the death latency which increased from 1.9 to 9.9 times, at doses ranging from 25 to 100 mg/kg, i.p. (Fig. 1B). Interestingly, the highest effect in this parameter was seen with the dose of 50 mg/kg. However, not only the latency to the 1st convulsion but also the latency to death were not significantly altered, at the dose of 10 mg/kg, as compared to the P300 group.

Table 1
Signs and symptoms of overstimulation, number of deaths and percentage of survival, after pilocarpine injection, in the absence and presence of doxycycline (Dox), in rats.

Group	Peripheral signs	Animals with tremors and convulsions	Stereotyped movements	No. deaths/ No. animals	Survival %
P300	Within 3 min	5/18[a] 18/18[b]	2/18 (wet dog)	12/12	0
Dox25 + P300	Within 3 min	0/18[a] 18/18[b]	2/18 (wet dog)	2/12	80
Dox50 + P300	Within 3 min	0/18[a] 16/18[b]	3/18 (paw licking)	7/12	40
Dox100 + P300	Within 3 min	5/12[a] 2/12[b]	–	7/14	50

Male Wistar rats (12 to 18 per group) were treated with doxycycline (Dox, 25, 50 and 100 mg/kg, i.p.) or distilled water, daily for 7 days. Thirty minutes after the last administration, the animals received a single injection of pilocarpine (P, 300 mg/kg, i.p.), and were observed for up to 24 h.
[a] Tremors.
[b] Convulsions.

Furthermore, the survival percentage of the pilocarpine group was also significantly higher after the doxycycline pretreatment. Thus, while no animal survived in the P300 group, the percentage of surviving animals was around 80 in the doxycycline pretreated (Dox25 +

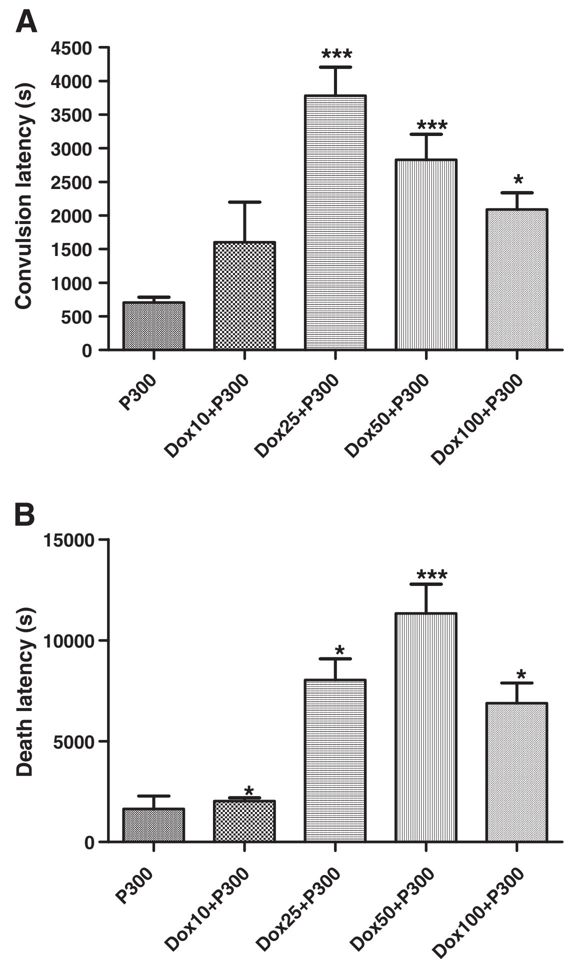


Fig. 1. Doxycycline (Dox) increased the latency to the first convulsion (A) and the latency to death (B) in the model of pilocarpine-induced convulsions in rats. The animals (6–12 per group) were treated with Dox (10 to 100 mg/kg, i.p.) or distilled water, daily for 7 days. Thirty minutes after the last injection, they were administered with pilocarpine (P, 300 mg/kg, i.p.). A: ***$p<0.001$ (P300 vs. Dox25 + P300 and vs. Dox50 + P300); *$p<0.05$ (P300 vs. Dox100 + P300). B: ***$p<0.001$ (P300 vs. Dox50 + P300); *$p<0.05$ (Dox10 + P300 vs. Dox50 + P300) and (P300 vs. Dox25 + P300 and vs. Dox100 + P300).

P300) group (Table 1). The animals pretreated with doxycycline and surviving after the pilocarpine administration usually did not develop SE.

3.3. Amino acid levels after doxycycline (Dox) pretreatment, in the temporal cortices of pilocarpine-treated rats

The cortical levels of amino acids in normal controls and in the P300 groups (untreated and pretreated with Dox at the doses of 50 and 100 mg/kg, i.p., daily for 7 days) are presented in Table 2. The results (expressed as μmol/g wet tissue) show that P300 increased more than 7 times aspartate and more than 6 times glutamate levels, as related to normal controls. Surprisingly, the levels of glycine (4 times) and GABA (2 times) were also significantly higher in the P300 group. On the other hand, the doxycycline pretreatment significantly decreased glutamate concentrations by 28 and 33%, and increased GABA by 112 and 91% with the doses of 50 and 100 mg/kg respectively, as compared to the P300 group. The increase in GABA levels (near 4 times) was even higher in the doxycycline pretreated group, when compared to normal controls. While the higher dose significantly decreased aspartate concentrations by 61%, only the lower dose increased glycine levels by 131%. The levels of tyrosine and taurine were unchanged.

3.4. Effect of doxycycline (Dox) on the nitrite formation

Our results show that pilocarpine increased 3.7 times nitrite contents, as compared to controls. Doxycycline presented no effect *per se*, but totally blocked the pilocarpine effect (Dox50 + P300 group). While significant differences were detected between Dox50 and P300 groups, no significant differences were observed between Dox50 and Dox50 + P300 groups, as compared to normal controls (Fig. 2).

3.5. Effect of doxycycline (Dox) on the lipid peroxidation

Pilocarpine administration doubled the thiobarbituric acid reacting substances (TBARS) concentration, as compared to normal controls injected with distilled water. Surprisingly, a similar profile was observed in the group treated with doxycycline only (50 mg/kg, for 7 days). On the other hand, a partial blockade of the increase in TBARS contents was seen in the Dox50 + P300 group, as compared to the P300 or Dox50 groups (Fig. 3).

3.6. Free radical scavenging activity (RSA) of doxycycline

The free radical scavenging activity (RSA) of doxycycline (Dox) was studied *in vitro* and measured by the DPPH assay. Values of RSA for Dox were expressed as the percent decrease in the absorbance of DPPH at 517 nm, as related to the absorbance of DPPH in the absence of Dox. The results showed that Dox (50, 100 and 500 μg/ml) has a dose-dependent radical scavenging activity with effects of 26, 36 and 60% respectively, as related to controls. The RSA percentage for alpha-tocopherol (75 μl/ml) used as standard was 94.5 (T: 0.17 ± 0.0165) (Table 3).

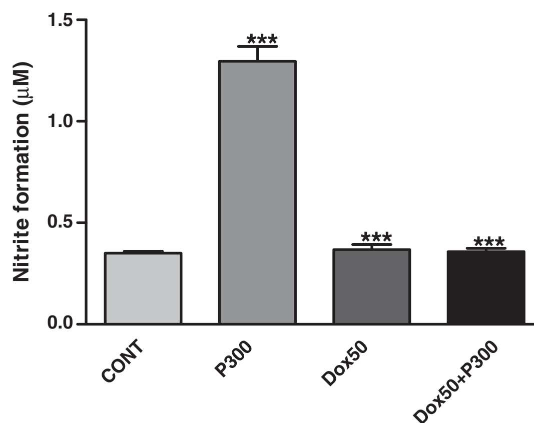


Fig. 2. Doxycycline (Dox) reversed the increase in nitrite formation, in the cortex of rats subjected to pilocarpine-induced convulsions. The animals (5–6 per group) were treated with Dox (50 mg/kg, i.p.) or distilled water, daily for 7 days. Thirty minutes after the last injection, the animals were administered with pilocarpine (P, 300 mg/kg, i.p.), sacrificed 7 h later, and had their temporal cortices dissected, as described in Materials and methods. ***$p < 0.001$ (P300 vs. control; vs. Dox50 and vs. Dox50 + P300).

3.7. Fluoro-jade staining of temporal cortices

Fluoro-jade stains cell bodies, dendrites and axons of degenerating neurons, but does not stain healthy neurons. Degenerating neurons appear bright green against a dark background. Our results (Fig. 4A) show that, in frontal cortices from normal control animals, there are almost no fluorescent cells but instead a high predominance of a dark background, while a great number of bright green and fluorescent cells are visualized in the temporal cortices from the P300 group (Fig. 4B). The number of fluorescent cells (expressing degenerating neurons) was much smaller in the Dox50 + P300 group (Fig. 4C). Fig. 4D shows the percentage of cell death in the P300 and Dox50 + P300 groups.

3.8. Cresyl violet staining of hippocampus

The percentage of viable neurons was counted in three sections from the cortex, CA1, CA3 and dentate gyrus of different animals. The results showed that doxycycline (Dox) protected the brain against the pilocarpine-induced convulsions injury in all areas studied. While in the brain from normal controls the percentage of viable neurons ranged from 90 to 99, these values decreased from 66 to 75% in the P300 group. In the P300 group pretreated with doxycycline (Dox50 + P300) the number of viable neurons was significantly higher and

Table 2
Effects of Doxycycline (Dox) pretreatment on amino acid concentrations in rat temporal cortices, after pilocarpine-induced seizures.

Amino acid concentration (μmol/g wet tissue)							
Group	Aspartate	Glutamate	Glycine	Tyrosine	Taurine	GABA	Tryptophan.
Control	0.92 ± 0.11	1.71 ± 0.35	0.46 ± 0.05	n.d	n.d.	1.7 ± 0.09	n.d.
P300	7.0 ± 0.36 (a)	10.7 ± 0.48 (a)	1.9 ± 0.07 (a)	6.5 ± 0.53	2.3 ± 0.13	3.3 ± 0.28 (a)	0.6 ± 0.11
Dox50 + P300	7.4 ± 1.50 (a)	7.7 ± 0.35 (a,b)	4.4 ± 0.25 (a,b)	6.4 ± 0.47	2.6 ± 0.18	7.0 ± 0.46 (a,b)	0.9 ± 0.13
Dox100 + P300	2.7 ± 0.010 (a,b)	7.2 ± 0.33 (a,b)	2.2 ± 0.19 (a)	6.1 ± 0.21	3.1 ± 0.17	6.3 ± 0.30 (a,b)	0.8 ± 0.10

Male Wistar rats were treated with doxycycline (Dox, 50 and 100 mg/kg, p.o.) daily for 7 days, and 30 min after the last administration received a single injection of pilocarpine (P, 300 mg/kg, i.p.). The P300 group received distilled water instead, at the same conditions. Normal controls also received distilled water (n.d. = not determined). Immediately after death, the animals had their temporal cortices dissected on ice for the preparation of homogenates and amino acid determinations, as described in Materials and methods. a. and b.: vs. Control (normal animals) and P300, respectively.

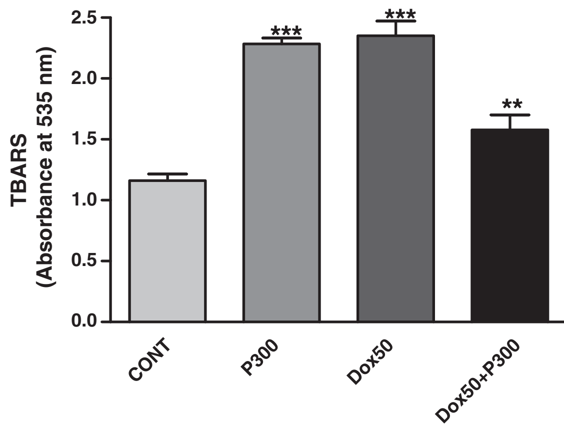


Fig. 3. Doxycycline (Dox) reversed the increase in lipid peroxidation, in the cortex of rats subjected to pilocarpine-induced convulsions. The animals (3–6 per group) were treated with Dox (50 mg/kg, i.p.) or distilled water, daily for 7 days. Thirty minutes after the last injection, the animals were administered with pilocarpine (P, 300 mg/kg, i.p.), sacrificed 7 h later, and had their temporal cortices dissected, as described in Materials and methods. ***$p<0.001$ (control vs. P300; vs. Dox50); **$p<0.01$ (Dox50 vs. Dox50 + P300; vs. P300).

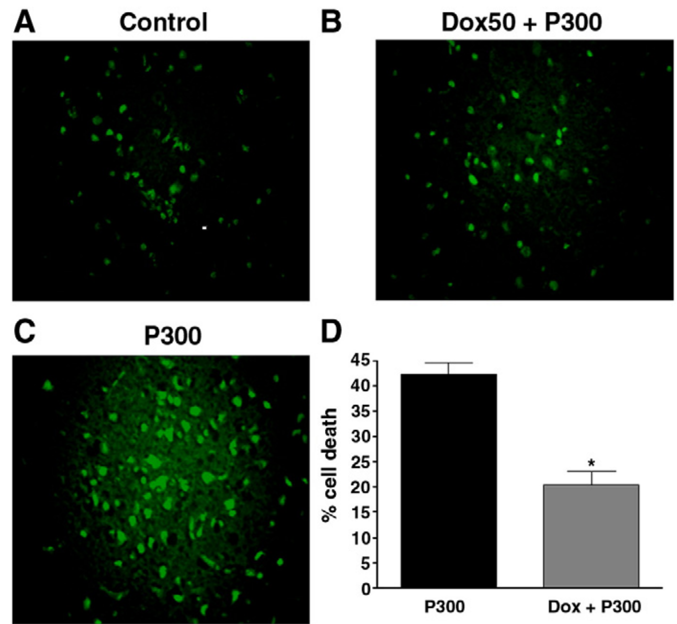


Fig. 4. Doxycycline (Dox) pretreatment decreased the number of degenerating neurons, in the cortex of rats subjected to pilocarpine-induced convulsions. The animals were treated with Dox (50 mg/kg, i.p.) or distilled water (controls), daily for 7 days, and 30 min after the last injection were administered with pilocarpine (P, 300 mg/kg, i.p.). Seven hours later, two animals from each group (normal controls, P300, and Dox + P300) were decapitated, and their temporal cortex sections were processed for fluoro-jade staining, as described in Material and methods. Photomicrographies are representative sections from each group (A: Control; B: Dox50 + P300; and C: P300), where 3 fields per slice were counted (around 60 cells each). D: shows the percentages of cell death in the P300 and Dox + P300 groups. *$p<0.05$ (P300 vs. Dox + P300).

ranged from 77 to 91%. Surprisingly, the highest protection was seen in the dentate gyrus followed by the CA1 and CA3 hippocampus areas (Figs. 5A, B, C and D). The right-hand side of each figure shows the percentage of viable neurons in controls, P300 and Dox50 + P300 groups.

4. Discussion

Doxycycline is a semi-synthetic, second generation tetracycline presenting anti-inflammatory effects unrelated to its antimicrobial action (Pasquale and Tan, 2005). Pro-inflammatory cytokines are known to be rapidly induced in rodent brains during the epileptic activity (Vezzani, 2005; Rao et al., 2010). Furthermore, seizures and brain damage in a rat model of bacterial meningitis were shown to be attenuated by doxycycline, an inhibitor of MMP and TNF-alpha-converting enzyme (Meli et al., 2006). We demonstrated that doxycycline, besides presenting a potent anti-inflammatory activity *in vivo* (data not shown), significantly blocked myeloperoxidase activity, a biomarker for inflammation, in PMA-stimulated human neutrophils (Viana et al., 2009). Although the mechanism of epileptogenesis is not well established, a higher incidence of seizures is reported to occur among patients with chronic inflammatory diseases (Rao et al., 2008).

Inflammatory responses, notably interleukin-1beta signaling, have been shown to be associated with SE and seizure frequency (Marchi et al., 2009). As shown in experimental models and in tissue from epileptic patients, seizures evoke the release of cytokines not only from neurons but also from glial and endothelial cells (Ravizza et al., 2008). As a matter of fact, a brief period of doxycycline treatment was shown to have a profound but selective effect on vascular inflammation, reducing aortic wall neutrophil and cytotoxic T-cell content (Lindeman et al., 2009). Despite the wealth of data in the literature, mechanisms underlying immune and inflammatory responses in temporal lobe epilepsy remain largely elusive (Fabene, 2010). However, it is widely accepted that the seizure initiation is related to increased neuronal excitation, decreased neuronal inhibition or both. Recent studies demonstrated a protective action of doxycycline in models of diabetic cardiomyopathy (Yaras et al., 2009), global cerebral ischemia (Lee et al., 2009) and nigral dopaminergic degeneration (Cho et al., 2009), conditions where the inflammatory component is a common link. Systemic inflammation and damage of the blood-brain barrier (BBB) are etiologic cofactors in the pathogenesis of pilocarpine-induced SE, and animals pre-treated with IL-1 receptor antagonists were reported to exhibit significant reduction of SE onset and BBB damage (Marchi et al., 2009).

In the present work, we showed that doxycycline protected against pilocarpine-induced seizures, significantly increasing not only the latency time to the 1st convulsion but also latency to death. A higher percentage of surviving animals was also observed in the pilocarpine group after the doxycycline pretreatment. This neuroprotective effect decreased in intensity at higher doses, possibly due to some degree of toxicity presented by doxycycline at doses equal or higher than 100 mg/kg. Neuroprotection has been considered as a promising therapy for preventing and treating temporal lobe epilepsy (Acharya et al., 2008). Thus, the pilocarpine model is a valuable tool not only to study the pathogenesis of temporal lobe epilepsy in humans, but also to evaluate potential antiepileptogenic drugs (Scorza et al., 2009).

It is known that GABA and glutamate can exert anti- and proconvulsive effects respectively, in seizures and SE induced by pilocarpine (Solberg and Belkin, 1997; Treiman, 1995). An earlier study (Carlson et al., 1992) showed elevations of aspartate, glycine, glutamate and serine concentrations in the onset of seizures in epileptic patient. Others (Engstrom et al., 2001) also reported elevations of aspartate and glutamate levels in the brain tissue of epileptic rats. It has been shown

Table 3
Radical scavenging activity (RSA) of doxycycline on DPPH radicals *in vitro*.

Compound	Concentration (μg/ml)			
	50	75	100	500
Doxycycline	3.073 ± 0.0418	–	2.801 ± 0.1212	1.227 ± 0.0763*
α-Tocopherol		0.170 ± 0.0165		

Assay performed as described in Materials and methods. Values are absorbance at 517 nm of 6 to 9 samples. The absorbance of DPPH in the absence of doxycycline or alpha-tocopherol was 3.073 ± 0.0417 (Controls). * vs. Controls, $p<0.05$.

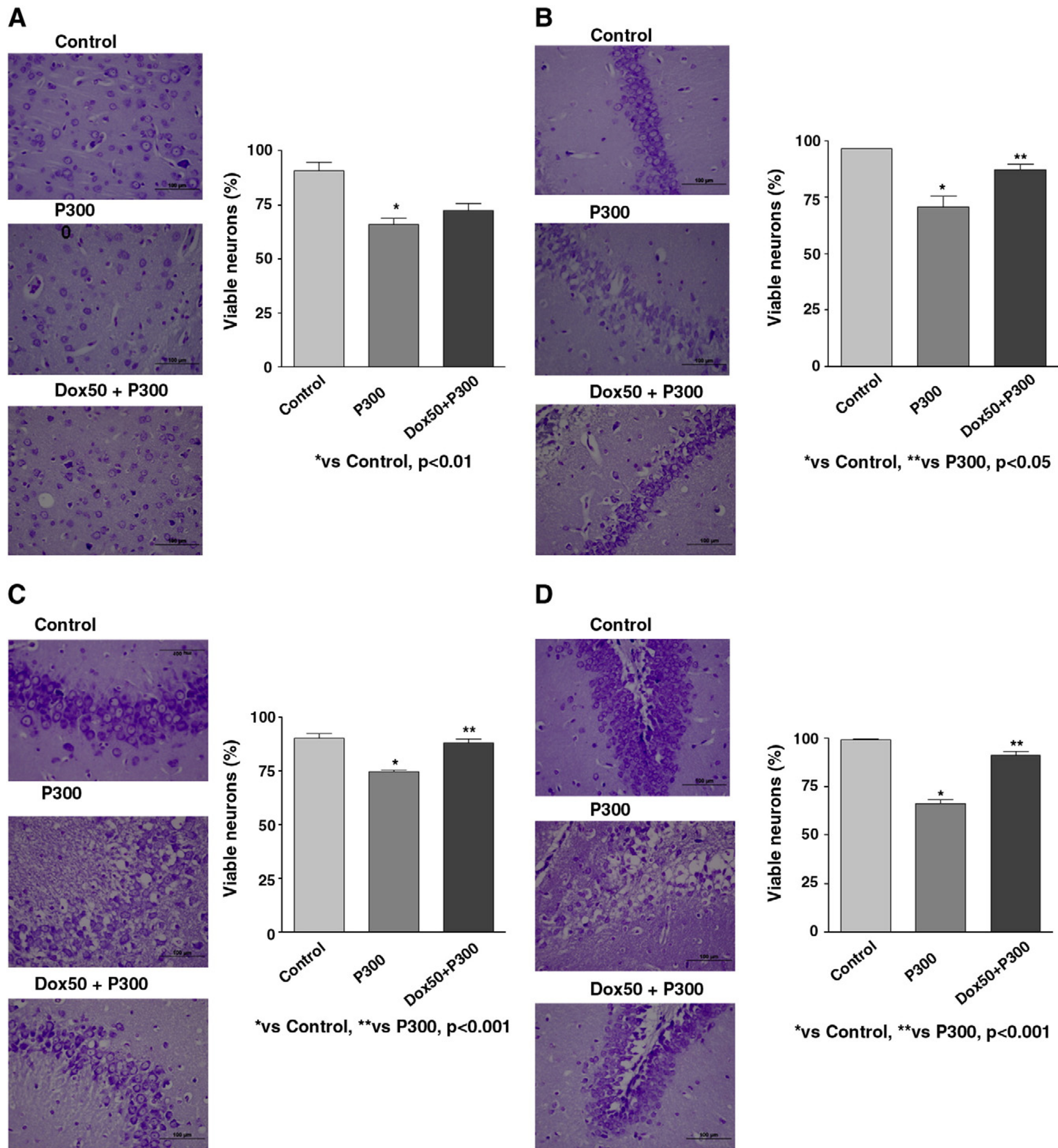


Fig. 5. Doxycycline (Dox) pretreatment increases the number of viable neurons in the brain cortex and hippocampus of rats subjected to pilocarpine-induced convulsions. The animals were treated with Dox (50 mg/kg, i.p.) or distilled water (normal controls), daily for 7 days, and 30 min after the last injection they were administered with pilocarpine (P, 300 mg/kg, i.p.). Seven hours later, 2 animals from each group (normal controls, P300 and Dox + P300) were decapitated, and their hippocampus sections were processed for cresyl violet staining, as described in Material and methods. Left-hand side: representative photomicrographies (400×) of sections from each group. Right-hand side: percentages of cell death. A: cortex (*$p<0.05$, P300 vs. control); B: CA1 hippocampus (*$p<0.05$, P300 vs. control; **$p<0.05$, Dox50 + P300 vs. P300); C: CA3 hippocampus (*$p<0.001$, P300 vs. control; **$p<0.001$, Dox50 + P300 vs. P300); and D: dentate gyrus (*$p<0.001$, P300 vs. control; **$p<0.001$, Dox50 + P300 vs. P300).

that the temporal lobe epilepsy is associated with a smaller hippocampal volume and with elevated extracellular glutamate levels (Cavus et al., 2008). Thus, the glutamate neurotoxicity is a contributing factor in acute neuronal damages, and overstimulation of glutamate receptors is a key event in excitotoxicity.

Our data showed that doxycycline significantly decreased aspartate and glutamate levels, and increased GABA concentrations, as compared to pilocarpine-treated rats in the absence of doxycycline. Similarly, doxycycline could also protect neurons against neuronal death due to pilocarpine-induced seizures, by decreasing glutamate and aspartate levels in the temporal cortex. A pronounced glutamate release has been related to neuronal death due to SE, in several brain structures (Cavalheiro et al. 1994; Costa et al., 2004). Interestingly, we recently showed that doxycycline afforded a significant protection to cortical cells against glutamate cytotoxicity *in vitro* (Viana et al., 2009).

Microdialysis studies in rodents after limbic seizures showed an increase in the extracellular concentration of glutamate and aspartate

before or during the seizure onset (Meldrum, 1994). In addition, pups treated with doxycycline had lower hippocampal glutamate, as demonstrated in a model of neonatal hypoxia–ischemia (Jantzie et al., 2006). The mechanism of action of some anticonvulsant drugs is related to the blockade of glutamatergic neurotransmission. Thus, by decreasing cortical glutamate (and aspartate) levels, doxycycline could behave as a glutamate antagonist in the pilocarpine-induced convulsion model. Furthermore, doxycycline is known to inhibit microglia activation which certainly plays a role in brain excitotoxicity.

In the present work, significant increases in the cortical levels of GABA were observed in the pilocarpine group after doxycycline pretreatment. The pilocarpine treatment induced downregulation of GABAergic receptors in several brain regions, including the frontal cortex (Freitas et al., 2004), and different receptors have been implicated in the mechanism of pilocarpine-induced seizures. Thus, activation of M_1 muscarinic receptors is involved in the first step of seizure activity, while serotonin, glutamate, dopamine and GABA systems appear to mediate the propagation and/or maintenance of seizure activity (Cavalheiro et al., 1994). Furthermore, evidences (Bernard et al., 2000; Cossart et al., 2001; Calcagnotto et al., 2005) have shown that impaired neuronal inhibition is involved with epilepsy. Kirchner et al. (2003) reported that glycine receptor agonists could serve as potential anticonvulsants, and suggested an important role for glycine receptors in cortical function and dysfunction. We also demonstrated that the neuroprotective effect of doxycycline is in part due to increased glycine levels.

Neuronal hyperexcitability and excessive production of free radicals have been implicated in the pathogenesis of several neurological disorders, including epilepsy (Devi et al., 2008). Thus, oxidative stress and mitochondrial dysfunction are acute consequences of SE (Jarret et al., 2008) what is supported by the ability of antioxidants for reducing seizure manifestations and SE biochemical changes, such as the increase in free radicals. We previously reported (Freitas et al., 2005) significant increases in lipid peroxidation and nitrite concentrations in the hippocampus of pilocarpine-treated rats. According to some studies, oxidative stress may play a role in the initiation and progression of epilepsy (Costello and Delanty, 2004; Sudha et al., 2001).

We showed that the doxycycline pretreatment caused a complete blockade of the nitrite formation induced by pilocarpine in the temporal cortex. Doxycycline and minocycline are known to cause inhibition of the inducible nitrite oxide synthase (iNOS), a putative mediator of inflammation (Amin et al., 1996; Amin et al., 1997). Furthermore, these drugs also cause inhibition of the protein tyrosine nitration by scavenging peroxynitrites (Whiteman and Halliwell, 1997), an important event that may occur in free radical formation and oxidative stress. In the present work, we showed that the doxycycline pretreatment significantly reduced the increase in lipid peroxidation induced by pilocarpine. Unexpectedly, doxycycline alone increased lipid peroxidation, an effect also observed for older tetracyclines (Skakun and Vysotskii, 1982). Others (Gnanasoundari and Pari, 2006) observed that the administration of oxytetracycline (at the dose of 200 mg/kg, i.p., for 15 days) caused a significant elevation in lipoperoxidation markers. It seems that doxycycline *per se* presents not only an antioxidant effect at lower doses, but also a pro-oxidant effect at higher doses.

As a matter of fact, the doxycycline antioxidant property could be responsible for its neuroprotection property, as observed in the present study. In addition, this neuroprotection was confirmed by the fluoro-jade staining of cortical cells when the number of degenerating neurons in the pilocarpine groups pretreated with doxycycline decreased, as compared to the untreated pilocarpine group. A similar protection against pilocarpine-induced convulsions was observed after cresyl violet staining of hippocampus sections in the pilocarpine group pretreated with doxycycline. Recurrent seizures may cause structural and functional changes in the hippocampus (Tasch et al., 1999), and neuronal loss is a major neurobiologic abnormality in the epileptic brain.

In conclusion, we demonstrated that the onset to the 1st convulsion was postponed, the intensity of pilocarpine-induced seizures reduced, and the percentage of animals survival increased, after the doxycycline pretreatment. The protective mechanism of action of doxycycline seems to be mediated by the decrease of excitatory amino acids, such as glutamate and aspartate, and by alterations in inhibitory amino acid levels, evidenced by the increase in glycine and GABA. Additionally, doxycycline by its antioxidant effect could antagonize the increase of free radicals content, lipid peroxidation and glutamate excitotoxicity, observed in pilocarpine-induced convulsions.

Acknowledgements

The authors are grateful to Prof. M.O.L. Viana for the manuscript orthographic revision and the technical assistance of Ms. M. Vilani R. Bastos. The work had the financial support from the Brazilian National Research Council (CNPq).

References

Acharya MM, Hattiangady B, Shetty AK. Progress in neuroprotective strategies for preventing epilepsy. Prog Neurobiol 2008;84:363–404.

Amin AR, Attur MG, Thakker GD, Patel PD, Vyas PR, Patel RN, et al. A novel mechanism of action of tetracyclines: effects on nitric oxide synthases. Proc Natl Acad Sci USA 1996;93:14014–9.

Amin AR, Patel RN, Thakker GD, Lowenstein CJ, Attur MG, Abramson SB. Post-transcriptional regulation of inducible nitric oxide synthase mRNA in murine macrophages by doxycycline and chemically modified tetracyclines. FEBS Lett 1997;410:259–64.

Bernard C, Cossart R, Hirsch JC, Esclapez M, Ben-Ari Y. What is GABAergic inhibition? How is it modified in epilepsy? Epilepsy 2000;Suppl 6:S90–5.

Brown DL, Desai KK, Vakili BA, Nouneh C, Lee HM, Golub LM. Clinical and biochemical results of the metalloproteinase inhibition with sub-antimicrobial doses of doxycycline to prevent acute coronary syndromes (MIDAS) pilot trial. Atheroscl Thromb Vasc Biol 2004;24:733–8.

Calcagnotto ME, Paredes MF, Tihan T, Barbaro NM, Baraban SC. Dysfunction of synaptic inhibition in epilepsy associated with focal cortical dysplasia. J Neurosci 2005;25: 9649–57.

Carlson H, Ronne-Engstrom E, Ungerstedt U, Hillered L. Seizure related elevations of extracellular amino acid in human focal epilepsy. Neurosci Lett 1992;140:30–2.

Cavalheiro EA, Fernandes MJ, Turski L, Naffah-Mazzacoratti MG. Spontaneous recurrent seizures in rats: amino acid and monoamine determination in the hippocampus. Epilepsia 1994;35:1-11.

Cavus I, Pan JW, Hetherington HP, Abi-Saab W, Zaveri HP, Vives KP, et al. Decreased hippocampal volume on MRI is associated with increased extracellular glutamate in epilepsy patients. Epilepsia 2008;49:1358–66.

Chen M, Ona VO, Li MKB, Zhu S, Bian J, Guo L, et al. Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. Nat Med 2000;6:797–801.

Cho Y, Son HJ, Kim E-M, Choi JH, Kim ST, Ji IJ, et al. Doxycycline is neuroprotective against nigral dopaminergic degeneration by a dual mechanism involving MMP-3. Neurotox Res 2009;16:361–71.

Cossart R, Dinocourt C, Hirsch JC, Merchant-Perez A, Felipe JD, Ben-Ari Y. Dendritic but not somatic GABAergic inhibition is decreased in experimental epilepsy. Nature Neurosci 2001;4:52–62.

Costa MS, Rocha JB, Perosa SR, Cavalheiro EA, Naffah-Mazzacoratti MG. Pilocarpine-induced status epilepticus increases glutamate release in rat hippocampal synaptosomes. Neurosci Lett 2004;356:41–4.

Costello DJ, Delanty N. Oxidative injury in epilepsy: potential for antioxidant therapy? Expert Rev Neurother 2004;4:541–53.

Devi PU, Manocha A, Vohora D. Seizures, antiepileptics, antioxidants and oxidative stress: an insight for researchers. Expert Opin Pharmacother 2008;9:3169–77.

Du Y, Ma Z, Lin S, Dodel RC, Gao F, Bales KR, et al. Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. Proc Nat Acad Sci 2001;98:14669–74.

Engstrom ER, Hillered L, Flink R, Kihlstrom L, Lindquist C, Nie JX, et al. Extracellular amino acid levels measured with intracerebral microdialysis in the model of post-traumatic epilepsy induced by intracortical iron injection. Epilepsy Res 2001;43: 135–44.

Erakovic V, Zupan G, Varljen J, Laginja J, Simonic A. Lithium plus pilocarpine induced status epilepticus-biochemical changes. Neurosci Res 2000;36:157–66.

Fabene PF. Non-neuronal cells, inflammation and epilepsy. Eur J Neurosci 2010;31: 1098–9.

Freitas RM, Bezerra Felipe CF, Nascimento VS, Oliveira AA, Viana GS, Fonteles MM. Pilocarpine-induced seizures in adult rats: monoamine content and muscarinic and dopaminergic receptor changes in the striatum. Com Biochem Physiol C Toxicol Pharmacol 2003;136:103–8.

Freitas RM, Sousa FCF, Vasconcelos SMM, Viana GSB, Fonteles MM. Pilocarpine-induced *status epilepticus* in rats: lipid peroxidation level, nitrite formation, GABAergic and

glutamatergic receptor alterations in the hippocampus, striatum and frontal cortex. Pharmacol Biochem Behav 2004;78:327–32.
Freitas RM, Vasconcelos SMM, Souza FCF, Viana GSB, Fonteles MMF. Oxidative stress in the hippocampus after pilocarpine-induced *status epilepticus* in Wistar rats. FEBS J 2005;272:1307–12.
Gnanasoundari M, Pari L. Impact of naringenin on oxytetracycline-mediated oxidative damage in kidney of rats. Ren Fail 2006;28:599–605.
Jantzie LL, Cheung PY, Todd KG. Doxycycline reduces cleaved caspase-3 and microglial activation in an animal model of neonatal hypoxia–ischemia. J Cereb Blood Flow Metab 2005;25:314–24.
Jantzie LL, Rauw GA, Todd KG. The effects of doxycycline administration on amino acid neurotransmitters in an animal model of neonatal hypoxia–ischemia. Neurochem Int 2006;49:717–28.
Jarret SG, Liang LP, Hellier JL, Staley KJ, Patel M. Mitochondrial DNA damage and impaired base excision repair during epileptogenesis. Neurobiol Dis 2008;30: 130–8.
Kirchner A, Breustedt J, Rosche B, Heinemann UF, Schmieden V. Effects of taurine and glycine on epileptiform activity induced by removal of Mg^{2+} in combined rat entorhinal cortex-hippocanpal slices. Epilepsia 2003;44:1145–52.
Kriz J, Nguyen MD, Julien JP. Minocycline slows disease progression in a mouse model of amyotrophic lateral sclerosis. Neurobiol Dis 2002;10:268–78.
Lee H, Park Jong-Wook, Kim Sang-Pyo, Lo Eng H, Lee Seong-Ryong. Doxycycline inhibits matrix-9 and laminin degradation after transient global cerebral ischemia. Neurobiol Dis 2009;34:189–98.
Lindeman JHN, Abdul-Hussein H, van Bockel JH, Wolterbeek R, Kleemann R. Clinical trial of doxycycline for matrix metalloproteinase-9 inhibition in patients with an abdominal aneurysm: doxycycline selectively depletes aortic wall neutrophils and cytotoxic T cells. Circulation 2009;119:2209–16.
Lipton SA, Roseberg PA. Excitatory amino acids as a final common pathway for neurological disorders. N Engl J Med 1994;330:613–22.
Marchi N, Fan Q, Ghosh C, Fazio V, Bertolini F, Betto G, et al. Antagonism of peripheral inflammation reduces the severity of *status epilepticus*. Neurobiol Dis 2009;33: 171–81.
Meldrum BS. The role of glutamate in epilepsy and other CNS disorders. Neurology 1994;44:S14–23.
Meli DN, Coimbra RS, Erhart DG, Loquet G, Bellac CL, Tauber MG, et al. Doxycycline reduces mortality and injury to the brain and cochlea in experimental pneumococcal meningitis. Infect Immun 2006;74:3890–6.
Mihara M, Uchiyama M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal Biochem 1978;86:271–8.
Pasquale TR, Tan JS. Non-antimicrobial effects of antibacterial agents. Clin Infect Dis 2005;40:127–35.
Rao RS, Medhi B, Saikia UN, Arora SK, Toor JS, Khanduja KL, et al. Experimentally induced various inflammatory models and seizure: understanding the role of cytokine in rat. Eur Neuropsychopharmacol 2008;18:760–7.
Rao RS, Medhi B, Khanduja KL, Pandhi P. Correlation of seizures and biochemical parameters of oxidative stress in experimentally induced inflammatory rat models. Fund Clin Pharmacol 2010;24:325–31.
Ravizza T, Gagliardi B, Noe F, Boer K, Aronica E, Vezzani A. Innate and adaptive immunity during epileptogenesis and spontaneous seizures: evidence from experimental models and human temporal lobe epilepsy. Neurobiol Dis 2008;29: 142–60.
Roach DR, Bean AG, Demangel C, France MP, Briscoe H, Britton WJ. TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection. J Immunol 2002;168:4620–7.
Saint-Cricq de Gaulejac NS, Provost C, Vivas N. Comparative study of polyphenol scavenging activities assessed by different methods. J Agric Food Chem 1999;47: 425–31.
Schmued LC, Stowers CC, Scallet AC, Xu L. Fluoro-Jade C results in ultra high resolution and contrast labeling of degenerating neurons. Brain Res 2005;1035:24–31.
Scorza FA, Arida RM, Naffah-Mazzacoratti MG, Screni DA, Calderazzo L, Cavalheiro EA. The pilocarpine model of epilepsy: what have we learned? Ann Acad Bras Cienc 2009;81:345–65.
Skakun NP, Vysotskii IIu. Effect of tetracycline antibiotics on lipid peroxidation. Antibiotiki 1982;27:684–7.
Smith DL, Woodman B, Majal A, Sathasivam K, Ghazinoori S, Lowden PA, et al. Minocycline and doxycycline are not beneficial in a model of Huntington's disease. Ann Neurol 2003;54:186–96.
Solberg Y, Belkin M. The role of excitotoxicity in organophosphorous nerve agents central poisoning. Trends Pharmacol Sci 1997;18:183–5.
Sudha K, Rao AV, Rao A. Oxidative stress and antioxidants in epilepsy. Clin Chem Acta 2001;303:19–24.
Szyndler J, Maciejak P, Turzynska D, Sobolewska A, Lehner M, Taracha E, et al. Changes in the concentration of amino acids in the hippocampus of pentylenetetrazole-kindled rats. Neurosci Lett 2008;439:245–9.
Takeuchi H, Jin S, Suzuki H, Doi Y, Liang J, Kawanokuchi J, et al. Blockade of microglial glutamate release protects against ischemic brain injury. Exp Neurol 2008;214:144–6.
Tasch E, Cendes F, Li LM. Neuroimaging evidence of progressive neuronal loss and dysfunction in temporal lobe epilepsy. Ann Neurol 1999;45:568–76.
Treiman DM. Electroclinical features of *status epilepticus*. J Clin Neurophysiol 1995;12: 343–62.
Turski WA, Cavalheiro EA, Bortolotto ZA, Moraes Mello LE, Schwarz M, Turski L. Seizures produced by pilocarpine in mice: a behavioural, electroencephalographic and morphological analysis. Brain Res 1984;321:237–53.
Vezzani A. Inflammation and epilepsy. Epilepsy Curr 2005;5:1–6.
Viana GB, Nobre-Jr HV, Calou IB, Garcia FA, Leal LKA, Andrade GM. Doxycycline, a 2nd generation tetracycline, protects neuronal cells against glutamate but not against 6-OHDA-induced cytotoxicity. 9th International Conference on ADPD Prague; 2009.
Villareal FJ, Griffin M, Omens J, Dillmann W, Nguyen J, Covell J. Early short-term treatment with doxycycline modulates post infarction left ventricular remodeling. Circulation 2003;108:1487–92.
Whiteman M, Halliwell B. Prevention of peroxynitrite-dependent tyrosine nitration and inactivation of alpha 1-antiproteinase by antibiotics. Free Rad Res 1997;26: 49–56.
Yaras N, Sariahmetoglu M, Bilginoglu A, Koksoy-Aydemir A, Onay-Besikci A, Turan B, et al. Protective action of doxycycline against diabetic cardiomyopathy in rats. Brit J Pharmacol 2009;155:1174–84.
Yilmaz I, Adiguzel E, Akdogan I, Kaya E, Hatip-Al-Khatib I. Effects of second generation tetracyclines on penicillin-epilepsy-induced hippocampal neuronal loss and motor incoordination in rats. Life Sci 2006;79:784–90.
Yrjanheikki J, Tikka T, Keinanen R, Goldsteins G, Chan PH, Koistinaho J. A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. Proc Nat Acad Sci USA 1999;96:13496–500.