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Piperine decreases pilocarpine-induced convulsions by GABAergic mechanisms

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ABSTRACT

Piperine, an alkaloid present in the *Piper* genus, was shown to have an anticonvulsant activity, evaluated by the pilocarpine-induced model, in mice. Pilocarpine (350 mg/kg, i.p.) was administered 30 min after piperine (2.5, 5, 10 and 20 mg/kg, i.p.) which significantly increased latencies to 1st convulsion and to death, and percentage of survivals. These parameters were also increased in the pilocarpine groups pretreated with atropine plus piperine (10 and 2.5 mg/kg, respectively), as related to the pilocarpine group. However, they were not altered in the pilocarpine groups pretreated with memantine (a NMDA-type glutamate receptors blocker, 2 mg/kg, p.o.) or nimodipine (a calcium channel blocker, 10 mg/kg, p.o.), both associated with piperine (1 or 2.5 mg/kg), as compared to the piperine plus pilocarpine group. Moreover, the pilocarpine group pretreated with diazepam (which binds to the $GABA_A$ receptor, 0.2 and 0.5 mg/kg, i.p.) plus piperine (1 and 2.5 mg/kg) significantly increased latency to the 1st convulsion, as related to the pilocarpine group, suggesting that the GABAergic system is involved with the piperine action. Furthermore, the piperine effect was blocked by flumazenil (2 mg/kg, i.p.), a benzodiazepine antagonist. Untreated P350 animals showed decreased striatal DA and increased DOPAC and HVA levels that were not affected in the piperine plus pilocarpine groups. Piperine increased striatal levels of GABA, glycine and taurine, and reversed pilocarpine-induced increases in nitrite contents in sera and brain. Hippocampi from the untreated pilocarpine group showed an increased number of TNF-α immunostained cells in all areas, as opposed to the pilocarpine group pretreated with piperine. Taken together, piperine anticonvulsant effects are the result of its anti-inflammatory and antioxidant actions, as well as TNF-α reduction. In addition, piperine effects on inhibitory amino acids and on the GABAergic system may certainly contribute to the drug anticonvulsant activity.

1. Introduction

Piperine (PPR), a piperidine alkaloid, is a major component of plants from the *Piper* genus (Piperaceae family), as *Piper nigrum*, *Piper longum* and *Piper tuberculatum*. It is known to present several pharmacological properties, as anti-inflammatory, antifertility and stimulator of serotonin synthesis in the central nervous system, among others (Daware et al., 2000; Kim and Lee, 2009; Vijayakumar and Nalini, 2006). Piperine is an inhibitor of the microsomal system for drug metabolization, and was shown to be a selective noncompetitive inhibitor of CYP3A4. The family of the cytochrome P450 (CYP450) enzyme system is the major catalyst of oxidative biotransformation reactions involved in drug metabolism, and the subtype CYP3A4 is the most prevalent cytochrome, accounting for 30–50% of drugs metabolized by type I enzymes (Haddad et al., 2007; Volak et al., 2008). Thus, there are several studies focusing on piperine bioavailability enhancing properties (Shaikh et al., 2009; Srinivasan, 2007). On the other hand, only a few and very earlier studies are related to piperine anticonvulsant effects and its possible mechanism of action (D'Hooge et al., 1996).

Epilepsy is the commonest neurological condition, characterized by spontaneous recurrent seizures, triggered by abnormal electrical activity in the brain cortex. The involvement of hyperexcitable neurons links the pathogenesis of epilepsy and the generation of synchronized neuronal activity with an imbalance between inhibitory GABA-mediated and excitatory (glutamate-mediated) neurotransmission (Dalby and Mody, 2001).

Currently, there is a great availability of drugs in clinics for epilepsy management. However, most of them are alternatives or 2nd choices, pointing out to the need for new safer and more efficacious drugs for that neurologic condition. Thus, drug-resistant seizures pose a great

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challenge for drug development and, despite available therapeutic interventions for seizure disorders, the incidence of epilepsies and mortality associated to *status epilepticus* (SE) remain elevated (Marchi et al., 2011). Acute and chronic toxicities complicate all antiepileptic drugs (AEDs) and are idiosyncratic ones. An earlier study shows that three major chronic toxicities of AEDs have been noted, such as soft tissue and gum hypertrophy, progressive ataxia, and peripheral neuropathy. New AEDs require careful post-marketing surveillance, since all of them have some adverse effects which differ in type and severity (Cramer et al., 2010).

Inflammation is known to participate in the mediation of a growing number of acute and chronic neurological disorders, including epilepsy. Thus, inflammatory processes, as activation of microglia and astrocytes, production of proinflammatory cytokines and related molecules, have been described in human epilepsy as well as in experimental models of epilepsy (Choi and Koh, 2008).

The objectives of the present study were to evaluate the effects of piperine, alone and associated to atropine (ATRP), memantine (MEMT), nimodipine (NIMO), diazepam (DZP) and flumazenil (FLUM), on the model of pilocarpine-induced convulsions in mice, attempting to clarify the PPR anticonvulsive mechanism of action. Besides, considering the relationship of this drug effect with the anti-inflammatory and antioxidant properties, its action on brain monoamines, amino acids and cytokines (TNF-alpha) were also pursued.

2. Material and methods

2.1. Drugs and reagents

Piperine, pilocarpine, cremophor, atropine, standard monoamines and amino acids were purchased from Sigma-Aldrich, USA. Memantine was from Apsen, Brazil; nimodipine from EMS S/A, Brazil; diazepam from Hipolabor, Brazil; and flumazenil, morphine and naloxone from Laboratório Cristália, Brazil. All other reagents were of analytical grade.

2.2. Animals and experimental protocol

Male Swiss mice (25–30 g) were provided by the Animal House of the Faculty of Medicine Estácio of Juazeiro do Norte (FMJ), Ceará, Brazil. The animals were housed into plastic cages with sawdust as beddings, and kept in a room with controlled temperature (25 ± 2 °C) under a 12 h/12 h light/dark cycle, and food and water supplied *ad libitum*. The animals were acutely administered with piperine (PPR: 2.5, 5, 10 and 20 mg/kg, i.p.), followed by the pilocarpine injection (P: 350 mg/kg) 30 min later. PPR was suspended in a 2% Cremphor aqueous solution, immediately before use. For the associations protocols, the following drugs were administered 15 min before PPR (i.p.), therefore 45 min before P350 (i.p): ATRP (i.p.), MEMT (p.o.), DZP (i.p.) and NIMO (p.o.). In the FLUM (i.p.) association, PPR (i.p) and FLUM (i.p) were administered 45 and 30 min, respectively, before P350. The experiments were carried out according to the Guide for the Care and Use of Laboratory Animals of the U.S. Department of Health and Human Services. The project was previously approved by the Animal Ethics Committee of the Faculty of Medicine of the Federal University of Ceará, Barbalha, Ceará, Brazil.

2.3. Behavioral testing: pilocarpine-induced convulsions model

The systemic injection of a high dose of pilocarpine is one of the most common models for evoking SE and subsequent epilepsy in rodents, and results in severe brain damage (Cavalheiro et al., 2006). Mice (8–27 animals in PPR-treated groups and 39 animals in the P350 group) were distributed into the following groups: pilocarpine (P: 350 mg/kg, i.p.) and PPR + P350 (PPR: 1, 2.5, 5, 10 or 20 mg/kg, i.p.) followed by pilocarpine, 30 min later. The association experiments were: 1. ATRP1 + PPR1; ATRO10 + PPR2.5; 2. MEMT2 + PPR2.5; 3. NIMO10 + PPR2.5; 4. DZP0.2 + PPR1; DZP0.5 + PPR2.5; 5. FLUM2 + PPR5. The observed parameters were: latency to the first convulsion and latency to death (up to 2 h). Besides, the number of survival animals was also determined (for this, the animals were observed for 24 h). For monoamine and amino acid experiments, as well as immunohistochemistry assays for TNF-alpha, the animals were sacrificed by decapitation after the 1st convulsion (usually 30 min in the P350 group and 2 h in PPR + P350 groups). These two times were chosen since the animals from P350 groups did not survive longer than 30 min. They were thus decapitated immediately after their 1st convulsion or death. On the other hand, groups pretreated with PPR prior to P350 were sacrificed after their 1st convulsion (2 h cut off time). The brains were dissected for isolation of striata and preparation of homogenates, in the cases of monoamine and amino acid determinations. For TNF-alpha immunohistochemistry assays, sections of hippocampi were processed, as described below.

2.4. Monoamine levels determination

For measurements of dopamine (DA) and their metabolites (3,4 dihydroxyphenylacetic acid — DOPAC and homovanilic acid — HVA), *striata* from all groups after pilocarpine-induced convulsions, untreated (P350) or pretreated with PPR (PPR + P350), were used to prepare 10% homogenates. These were sonicated in 0.1 M $HClO_4$, for 30 s, centrifuged at 4 °C for 15 min, at 15,000 rpm, and the supernatants were filtered (0.2 μm, Millipore). Twenty-microliter samples were then injected into a high-performance liquid chromatograph (HPLC) column (C18, 250 × 4.6 mm, 5 μm) and a 0.6 mL/min flow rate. The mobile phase was 0.163 M citric acid, pH 3.0, containing 0.02 mM EDTA with 0.69 mM sodium octanesulfonic acid (SOS), as an ion pairing reagent, 4% v/v acetonitrile and 1.7% v/v tetrahydrofuran. The monoamines were electrochemically detected, using an amperometric detector (Shimadzu, Japan), by oxidation on a glassy carbon electrode at 0.85 V, relatively to the Ag–AgCl reference electrode. Their concentrations were determined by comparison with standards injected into the HPLC column at the day of experiment, and the values expressed as ng/g tissue.

2.5. Amino acid determinations

Amino acid concentrations were determined by reversed-phase high performance liquid chromatography (RP-HPLC), involving pre-column derivatization with orthophthalaldehyde (OPA). The Shimadzu RP-HPLC system (Japan) consisted of a spectrofluorimeter detector (excitation and emission wavelengths of 350 and 450 nm, respectively) coupled with an integrator. The chromatographic column used was a C18 (250 × 4.6 mm, 5 μm) with a 1 mL/min flow rate, and the mobile phase A had the following composition: 50 mM NaH_2PO_4 solution in 20% methanol, pH 5.5; the mobile phase B was 100% methanol. Both 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. For 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.

2.6. Nitrite determinations in the blood and brain cortex of mice treated with PPR and submitted or not to pilocarpine-induced convulsions

The blood was collected from the retro-orbital plexus and centrifuged (3000 rpm, 10 min) for the separation of sera, after the animal's 1st

convulsion (around 15 min in the P350 group, and between 1 and 2 h in the PPR + P350 groups). For the brain cortex, a 10% homogenate (in saline) was centrifuged (5000 rpm, 20 min). Then, 100 μL of 10% picric acid were added to the supernatants for protein precipitation, followed by centrifugation (14,000 rpm for 30 min) whose supernatants were then used for nitrite determination by the method of Green et al. (1981). For this, 100 μL Griess reagent (1% sulphanilamide/0.1% N-(1-naphthyl)-ethylenediamine hydrochloride/1% H_3PO_4/distilled water, 1:1:1:1) were added to 100 μL serum or brain cortex supernatants, followed by 10 min incubation at room temperature. The standard curve was made with concentrations of $NaNO_2$, ranging from 0.75 to 100 μM, under the same conditions. Blanks were prepared by the addition of 100 μL Griess reagent to 100 μL serum, and the absorbance determined at 560 nm.

2.7. Immunohistochemistry assay for TNF-alpha in hippocampi from mice submitted to pilocarpine-induced convulsions

For TNF-α immunohistochemistry assays, three groups of 3 mice each were treated with P350 or with PPR (5 and 10 mg/kg, i.p.) followed by P350, 30 min later. The animals were sacrificed after the 1st convulsion (usually 30 min in the P350 group, and 2 h in the PPR + P350 groups), for brain dissection. Then, hippocampal sections were immersed in buffered formalin solution for 24 h, followed by immersion in 70% ethanol. The sections were then deparaffinized, dehydrated in xylol and ethanol, and immersed in 0.1 M citrate buffer (pH 6) under microwave heating (18 min), for antigen recovery. After cooling at room temperature (20 min), sections were washed with a phosphate buffered saline (PBS), followed by a 15 min blockade of endogenous peroxidase with a 3% H_2O_2 solution. The sections were incubated overnight (4 °C) with rabbit primary antibodies (anti-TNF-α, 1:200 dilutions in PBS-BSA). At the next day, the sections were washed in PBS, and incubated for 30 min with the secondary biotinylated rabbit antibody (anti-IgG, 1:200 dilution in PBS-BSA). After washing in PBS, the sections were incubated for 30 min with the conjugated streptavidin peroxidase complex (ABC Vectastain® complex, Vector Laboratories, Burlingame, CA, USA). After another washing with PBS, the sections were stained with 3,3′diaminobenzidine-peroxide (DAB) chromophore, counter-stained with Mayer hematoxylin, dehydrated and mounted in microscope slides for analyses.

2.8. Statistical analyses

The data for latencies to the 1st convulsion and to death (pilocarpine and piperine + pilocarpine groups) were analyzed by the non-parametric Kruskal–Wallis test, followed by the Dunn's as a *post hoc* multiple comparisons test, and the frequency to death by the Log-rank (Mantel–Cox) test. Chi-square and p values were estimated for each two survival curves. Two-way ANOVA (figures from GraphPad Prism 5), followed by the Mack–Skillings statistical test (a non-parametric Two-way ANOVA used for incomplete block designs) and by the Dwass–Steel–Critchlow–Fligner test for multiple comparisons were used (XLSTAT), for the association experiments with two or more drugs. All other cases were analyzed with One-way ANOVA, followed by the Student–Newman–Keuls as the test *post hoc*. The results were considered significant at $p<0.05$.

3. Results

3.1. Behavioral testing

3.1.1. PPR effects on the development of pilocarpine-induced convulsions

Piperine, at the doses of 2.5, 5, 10 and 20 mg/kg, administered (i.p.) 30 min before pilocarpine (PPR + P350), showed an anticonvulsant effect reducing the percent of mice that convulsed from 97% (P350) to 81, 85, 76 and 80%, respectively (Table 1). At the same doses, PPR significantly increased by 31, 65, 55 and 109%, respectively, the latency to the 1st convulsion, as compared to the P350 group (Fig. 1A). The PPR1 + P350 showed no significant difference from the P350 group (Table 1).

3.1.2. PPR effects on mice mortality rate after pilocarpine-induced convulsions

Ninety seven percent of mice that developed convulsions, died, up to 30 min after P350 administration. All piperine doses, increased from 17 to 63% the percentage of survivals 24 h after the 1st convulsion in a dose-dependent manner (Table 1). Among the animals that had convulsed and died, piperine at all doses, but not at 1 mg/kg, increased the latency to death from 58 to 138% (Fig. 1B).

3.1.3. PPR effects after its association to atropine, memantine, nimodipine, diazepam and flumazenil

In order to clarify the mechanism for the PPR anticonvulsive effect, these behavior tests were further carried out with the P350 group pretreated with atropine (ATRP, a muscarinic antagonist), memantine (MEMT, a NMDA-type glutamate receptor blocker), nimodipine (NIMO, a Ca^{2+} channel blocker), diazepam (DZP, a drug which binds to a specific subunit of the $GABA_A$ receptor) and flumazenil (FLUM, a benzodiazepine antagonist) plus PPR (1 or 2.5 mg/kg).

Association to atropine (*ATRP*): although not significant (probably due to the fact that a large number of animals did not convulse), a trend for an increase in the latency to the 1st convulsion was seen in P350 groups pretreated either with PPR2.5, ATRP10 or the association of PPR2.5 + ATRP10 as related to the untreated P350 group. Besides, in the last two groups, convulsing animals did not die, and these data were

Table 1
Ratios and percentages of development of convulsions and survivals, after pilocarpine-induced (P350) convulsions, in mice pretreated with piperine (PPR).

	Treatments before P350					
	P350	PPR 1	PPR 2.5	PPR 5	PPR 10	PPR 20
Number of convulsed animals up to 90 min after P350	38/39 (97%)	18/18 (100%)	17/21 (81%)	23/27 (85%)	16/21 (76%)	16/20 (75%)
Survivors up to 24 h after P350-induced convulsions	1/38 (3%)	3/18 (17%)	6/17 (35%)[a]	9/23 (39%)[b]	7/16 (44%)[c]	10/16 (63%)[d]
Latency to 1st convulsion (seconds)	700.2 ± 20.1	701.6 ± 52.5	921.0 ± 63.8 **	1156.0 ± 72.6 ***	1088.0 ± 51.8 ***	1460.0 ± 97.5 ***
Latency to death (seconds)	1130 ± 45.3	1171 ± 109.3	1791 ± 225.4 *	2220 ± 254.0 ***	2695 ± 336.0 ***	2070 ± 237.7 **

The animals were treated with distilled water or piperine (PPR: 1, 2.5, 5, 10 or 20 mg/kg, i.p.) and, 30 min later, administered with pilocarpine (P: 350 mg/kg, i.p.). Latencies to the 1st convulsion and to death are represented by means ± S.E.M. (seconds), after P350 administration, and analyzed by Kruskal–Wallis, followed by the Dunn's multiple comparison test. Latency to 1st convulsion: **p<0.01 and ***p<0.001 *vs.* P350. Latency to death: *p<0.05, **p<0.01 and ***p<0.001 *vs.* P350. Data on Survivors were analyzed by the nonparametric Log-rank (Mantel–Cox) test (comparison of each survival curve to P350): a. $\chi^2 = 11.08$, p<0.0009; b. $\chi^2 = 13.7$, p<0.0002; c. $\chi^2 = 14.8$, p<0.0001; d. $\chi^2 = 24.42$, p<0.0001; all *versus* P350.

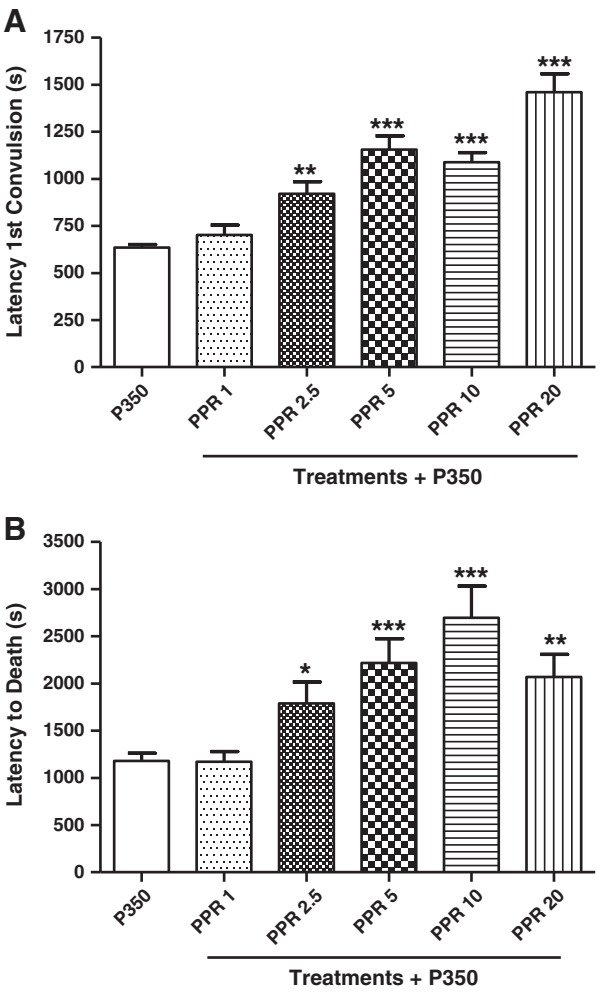

Fig. 1. Piperine (PPR: 5, 10 and 20 mg/kg, i.p.) significantly and dose-dependently increases latencies to the first convulsion (A) and to death (B) of pilocarpine-induced convulsions in mice. Pilocarpine (P: 350 mg/kg, i.p.) was administered to mice, 30 min after their pretreatments with PPR. The data are means ± S.E.M. for latencies to the 1st convulsion and to death (20 to 39 animals per group). Latency to the 1st convulsion: *p<0.05 and ***p<0.001 *vs.* P350. Latency to death: **p<0.01 and ***p<0.001 *vs.* P350 (Kruskal–Wallis followed by Dunn's multiple comparison test).

not submitted to statistical analysis. Similarly, there were no significant differences in the latency to the 1st convulsion among PPR1, ATRP2 and PPR1 + ATRP2 groups and no potentiation of the PPR anticonvulsive action by ATRP (Fig. 2).

Association to memantine (*MEMT*): The memantine pretreatment was not able to avoid pilocarpine-induced convulsions and, thus, no significant increase in the latency to the 1st convulsion was observed in the P350 group pretreated with the association of PPR2.5 + MEMT2, as related to P350 or MEMT2 + P350 groups. A similar result was observed in the latency to death, suggesting no interference of MEMT with PPR anticonvulsive action (Fig. 3).

Association to diazepam (*DZP*): significant increases in the latency to the 1st convulsions were observed in PPR2.5, DZP0.5, PPR1 + DZP0.2 and PPR2.5 + DZP0.5 groups pretreated with P350, as related to the untreated P350 group, suggesting a potentiation of the PPR effect by DZP. However, no significant differences were noticed in the latency to death among groups, probably due to the fact that most of the animals did not convulse nor die after the association of PPR with DZP (Fig. 4).

Association to flumazenil (*FLUM*): flumazenil alone or associated to PPR5 (*i.e.*, FLUM2 or PPR5 + FLUM2, drugs administered prior to

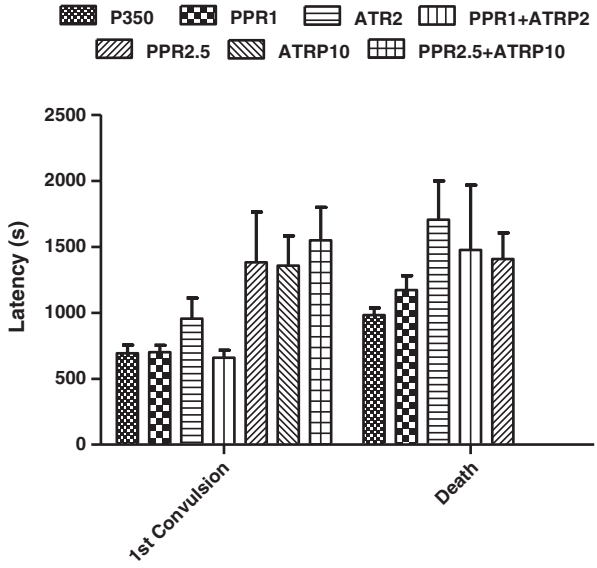

Fig. 2. Effects of piperine (PPR: 1 and 2.5 mg/kg, i.p.) isolated or in association with atropine (ATRP: 2 and 10 mg/kg, i.p.), respectively, on the latency to pilocarpine (P: 350 mg/kg, i.p.)-induced 1st convulsion and death. ATRP was administered 15 min before PPR and 45 min prior to P350. The data represent means ± S.E.M. for latency to the 1st convulsion and death (6 to 18 and 3 to 14 animals per group, respectively). No significant differences among treatments were observed (Skillings–Mack non-parametric Two-way ANOVA, followed by the Dwass–Steel–Critchlow–Fligner test for multiple comparisons).

P350) did not alter the latency to the 1st convulsion or to death, as related to the untreated P350 group. On the other hand, the P350 group pretreated with PPR5 significantly increased the latency to the 1st convulsion, as compared to the untreated P350 and to P350 groups pretreated with FLUM2 and PPR5 + FLUM2. These results suggest that flumazenil blockades the anticonvulsant effect of PPR. Furthermore,

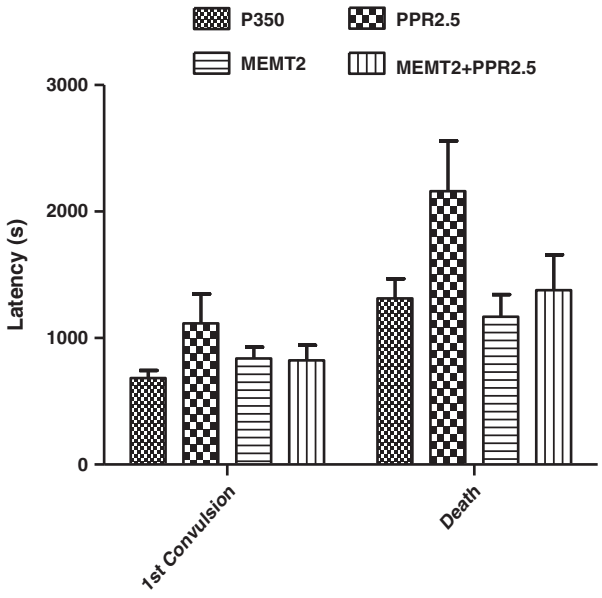

Fig. 3. Effects of piperine (PPR: 2.5 mg/kg, i.p.) isolated or in association with memantine (MEMT: 2 mg/kg, p.o.), on latencies to pilocarpine (P: 350 mg/kg, i.p.)-induced 1st convulsion and death. MEMT2 was administered 15 min before PPR2.5 and 45 min prior to P350. The data represent means ± S.E.M. for latencies to the 1st convulsion and to death (6 to 18 animals per group). No significant differences among treatments were observed (Skillings–Mack non-parametric Two-way ANOVA, followed by the Dwass–Steel–Critchlow–Fligner test for multiple comparisons).

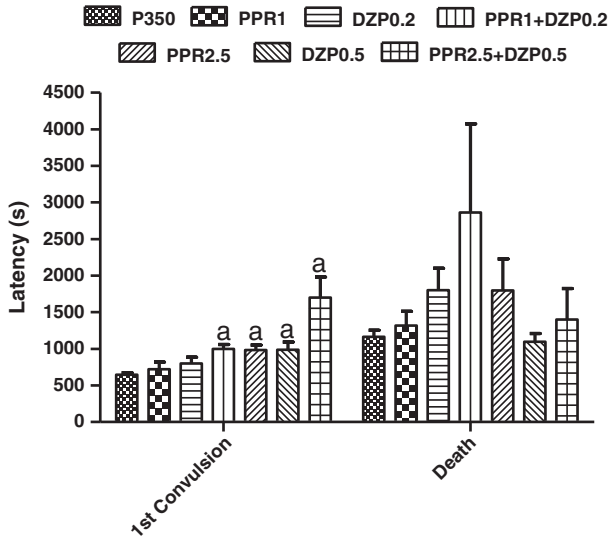

Fig. 4. Effects of piperine (PPR: 1 and 2.5 mg/kg, i.p.) isolated or in association with diazepam (DZP: 0.2 and 0.5 mg/kg, i.p.), respectively, on the latency to pilocarpine (P: 350 mg/kg, i.p.)-induced 1st convulsion and death. DZP was administered 15 min before PPR and 45 min prior to P350. The data represent means ± S.E.M. for latency to 1st convulsion and death (5 to 21 and 2 to 18 animals per group, respectively). Latency to the 1st convulsion: a. $p<0.004$ to 0.010 *vs.* P350 (Skillings–Mack non-parametric Two-way ANOVA, followed by the Dwass–Steel–Critchlow–Fligner test for multiple comparisons).

the P350 group pretreated with PPR5 showed a significant increase in the latency to death, as related to the FLUM2 group (Fig. 5).

Association to nimodipine (*NIMO*): in order to assess the participation of the Ca^{2+} channel in the anticonvulsive effect of PPR, nimodipine (NIMO, 10 mg/kg) was administered alone or associated to PPR2.5, previously to the pilocarpine injection. An increase in the latency to the 1st convulsion was observed in the P350 group pretreated with the association of PPR2.5 + NIMO10, as compared to the untreated P350 group. In addition, the latency to death was not significantly altered in any of the studied groups, suggesting that the Ca^{2+} channel does not have an important role in the PPR anticonvulsive action (Fig. 6).

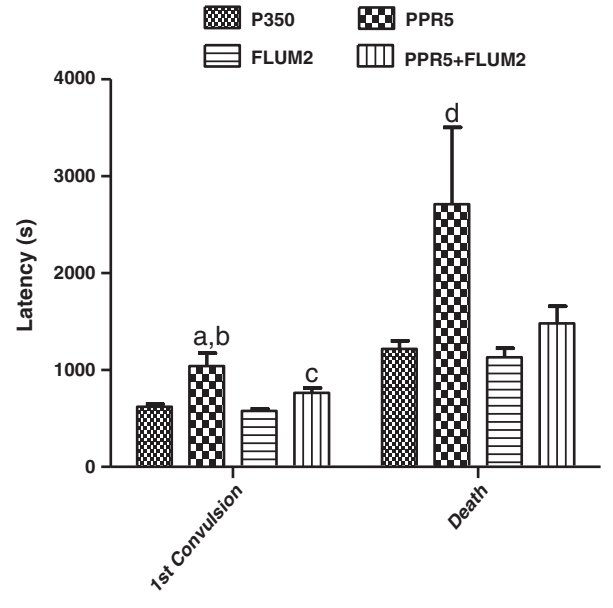

Fig. 5. Effects of piperine (PPR: 5 mg/kg, i.p.) isolated or in association with flumazenil (FLUM: 2 mg/kg, i.p.), on the latency to pilocarpine (P: 350 mg/kg, i.p.)-induced 1st convulsion and death. FLUM2 was administered 15 min after PPR5, and 30 min prior to P350. The data represent means ± S.E.M. for latency to the 1st convulsion and to death (14 to 20 and 5 to 18 animals per group, respectively). Latency to 1st convulsion: a. $p<0.001$ PPR5 *vs.* P350; b. $p<0.000$ PPR5 *vs.* FLUM2; c. $p<0.036$ PPR5 + FLUM2 *vs.* FLUM2; Latency to death: d. $p<0.047$ PPR5 *vs.* FLUM2 (Skillings–Mack non-parametric Two-way ANOVA, followed by the Dwass–Steel–Critchlow–Fligner test for multiple comparisons).

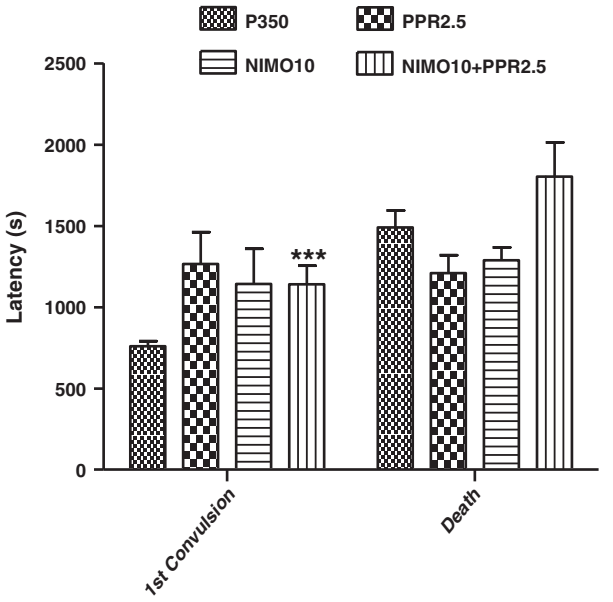

Fig. 6. Effects of piperine (PPR: 2.5 mg/kg, i.p.) isolated or in association with nimodipine (NIMO: 10 mg/kg, p.o.), on the latency to pilocarpine (P: 350 mg/kg, i.p.)-induced 1st convulsion and death. NIMO10 was administered 15 min before PPR2.5, and 45 min prior to P350. The data represent means ± S.E.M. for latency to the 1st convulsion and to death (9 to12 and 4 to 11 animals per group, respectively). ***$p<0.009$ *vs.* P350 (Skillings–Mack non-parametric Two-way ANOVA, followed by the Dwass–Steel–Critchlow–Fligner test for multiple comparisons).

3.2. Determination of monoamines in mice striata

The untreated P350 group showed a significant decrease of 84% in DA contents, as related to normal controls. This effect persisted in the

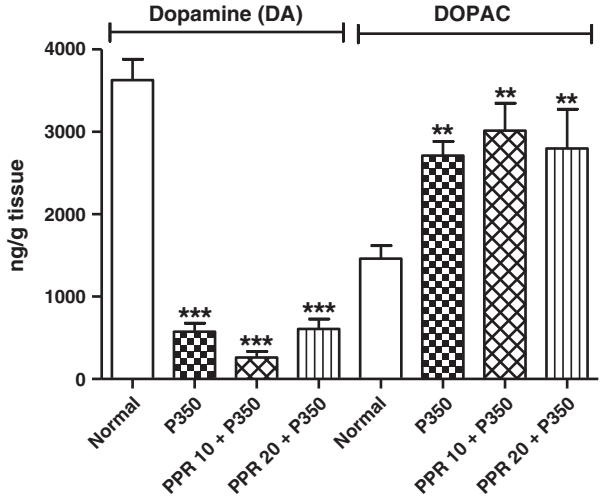

Fig. 7. Piperine (PPR: 10 and 20 mg/kg, i.p.) did not reverse the decreases in striatal DA and their metabolite contents, observed after the pilocarpine-induced convulsions in mice. Pilocarpine (P: 350 mg/kg, i.p.) was administered, 30 min after D-water (distilled water, i.p.) or PPR treatments, and the striata were dissected (for determination of monoamines) 15 to 60 min afterwards. The columns are means ± S.E.M. from 4 to 6 animals per group, analyzed by One-way ANOVA with the Student–Newman–Keuls as a *post-hoc* test. DA: ***$p<0.001$ *vs.* Normal controls; DOPAC: **$p<0.01$ *vs.* Normal control.

P350 group pretreated with PPR, at the doses of 10 and 20 mg/kg. On the other hand, an opposite effect was observed in DOPAC contents. Thus, not only in the untreated P350 group but also in the PPR groups, significant increases ranging from 1.8 to 2.1 times were observed (Fig. 7). Similar results were shown with HVA levels. However, these data were significant only in the untreated P350 group that showed an increase of 2.1 times in HVA levels, as related to normal controls. The values of NE remained unchanged in all groups (data not shown).

3.3. Determination of amino acid contents in mice brain areas from P350 groups untreated or treated with PPR

Amino acids determinations were performed in mice striata from P350 groups untreated or treated with PPR (5, 10 and 20 mg/kg, i.p.). In the untreated P350 group or in pretreated PPR5 and PPR10 groups, there were no significant differences in GLU contents, when compared to normal controls. Despite GLU levels in the PPR20 + P350 falls 65% when compared to normal control, there were not different relatively to the untreated P350 group. Except for a significant increase in the PPR5 + P350 group, as related to the P350 and normal controls, no other change was noticed in ASP contents (Fig. 8). While P350 did not change GABA levels, these were significantly increased in the PPR5 + P350 and PPR10 + P350 groups, as related to untreated P350 group. Interestingly, in untreated P350 group GLY contents was decreased in 64%, as related to normal control. Pretreatment with PPR, completely reversed this reduction (Fig. 9). Although P350 significantly decreased by 71% TAU contents, in relation to normal controls, this effect was partially reversed in PPR5 + P350 and PPR10 + P350 groups, whose values were brought towards those of the normal controls. However, in the PPR20 + P350 group, TAU values were similar to those of the untreated P350 group (Fig. 10).

3.4. Nitrite determination in the blood and brain cortex of mice treated with PPR and submitted or not to pilocarpine-induced convulsions

The P350 group showed increases of serum nitrite concentrations around 2.2 fold, as related to normal controls, and these values were significantly lower (ranging from 1.6 to 1.3 fold) in the PPR pretreated

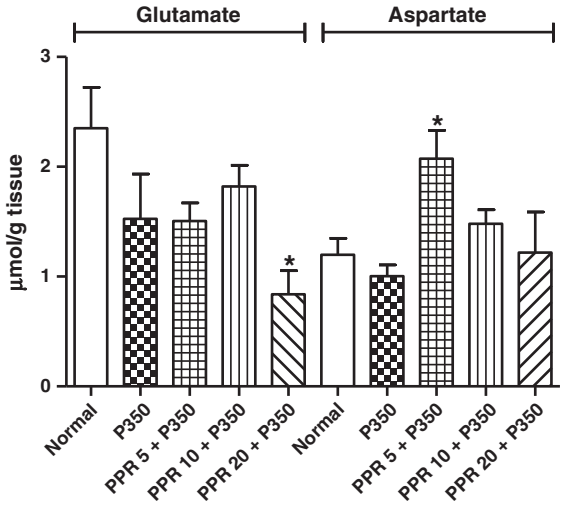

Fig. 8. Piperine (PPR: 5, 10 and 20 mg/kg, i.p.) did not reverse the glutamate (GLU) decrease, and only PPR 5 increased aspartate (ASP) contents, both observed in mice submitted to pilocarpine-induced convulsions. Pilocarpine (P: 350 mg/kg, i.p.) was administered, 30 min after the D-water (distilled water, i.p.) or PPR treatments, and the striata were dissected (for amino acid determinations) 15 to 60 min afterwards. The data are means ± S.E.M. for 4 to 6 animals per group, analyzed by One-Way ANOVA with the Student–Newman–Keuls as a *post-hoc* test. GLU: *p<0.05 *vs.* Normal control; ASP: *p<0.05 to 0.01 *vs.* all other groups.

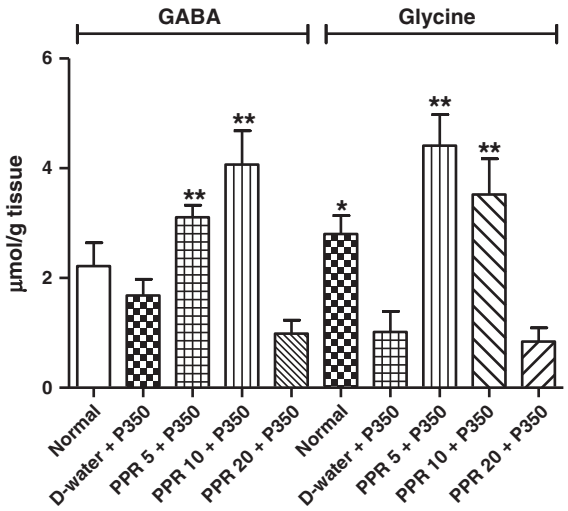

Fig. 9. Piperine (PPR: 5 and 10 mg/kg, i.p.) significantly increased GABA and glycine contents, in mice submitted to pilocarpine-induced convulsions. P350 was administered, 30 min after PPR treatments, and the striata were dissected (for amino acid determinations) 15 to 60 min afterwards. The data are means ± S.E.M. for 4 to 6 animals per group, analyzed by One-way ANOVA with the Student–Newman–Keuls as a *post-hoc* test. GABA: **p<0.01 *vs.* D-water + P350. GLY: *p<0.05 *vs.* Normal controls and **p<0.01 *vs.* D-water + P350.

P350 group. Interestingly, in the all doses of PPR tested, nitrite levels were close and not significantly different from those of normal controls. Similar results were observed for nitrite contents in the brain cortex. Thus, while a 1.4 fold increase in nitrite levels was shown in the P350 group, lower values were observed in the PPR + 350 groups. Here, only nitrite values in the PPR10 + P350 group were similar to those of normal controls (Fig. 11).

3.5. Immunohistochemistry assays for TNF-α in mice hippocampi, from animals submitted to the pilocarpine-induced convulsions, without (P350 group) and after piperine pretreatments (PPR + P350 groups)

In Fig. 12 (left column), an intense immunostaining was observed in all areas (CA1, CA3, DG hippocampus and cortex), indicating the

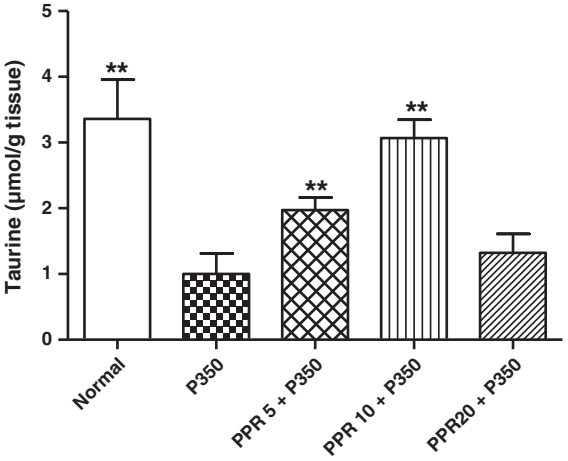

Fig. 10. Piperine at the lower doses (PPR: 5, and 10 mg/kg, i.p.), but not at 20 mg/kg, reversed the taurine contents decrease, as showed in the D-water (distilled water, i.p.) + pilocarpine (P: 350 mg/kg, i.p.) group. P350 was administered to mice, 30 min after the PPR treatments, and the striata were dissected (for amino acid determinations) 15 to 60 min afterwards. The data are means ± S.E.M. for 4 to 6 animals per group, analyzed by One-way ANOVA with the Student–Newman–Keuls as a *post-hoc* test. **p<0.01 *vs.* D-water + P350.

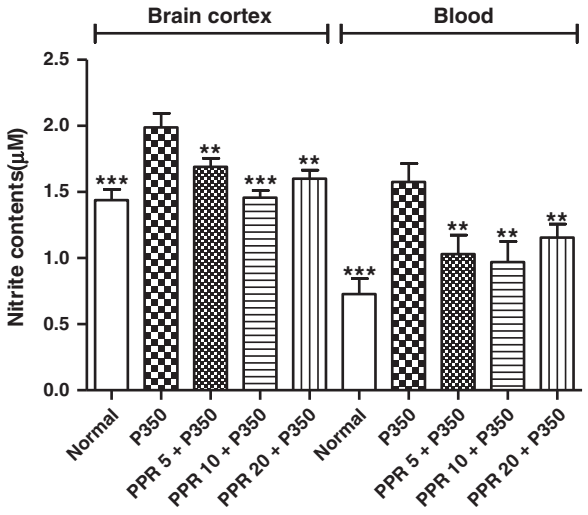

Fig. 11. Piperine (PPR: 5, 10 and 20 mg/kg, i.p.) reversed the nitrite content increases in mice sera and brains, due to pilocarpine-induced convulsions. Pilocarpine (P: 350 mg/kg, i.p.) was administered to mice, 30 min after distilled water, i.p., or PPR treatments, and the blood was collected 15 (P350 group) and 60 min (P350 group pretreated with PPR), after the 1st convulsion. The data are means ± S.E.M. for 4 to 7 (sera) or 10 to 12 (brains) animals per group, analyzed by One-way ANOVA with the Student–Newman–Keuls as a *post-hoc* test. Brains and sera: $^{**}p<0.01$ and $^{***}p<0.001$ *vs.* D-water + P350, respectively.

activity and expression of TNF-α in pilocarpine injected mice (P350 group). TNF-α is a pro-inflammatory cytokine known to be involved with convulsions and epilepsy. The piperine pretreatments at both doses (10 and 20 mg/kg, i.p.) reduced immunostainings for TNF-α (PPR10 + P350 and PPR20 + P350 groups, middle and right columns, respectively), as related to the P350 group. The PPR effect was observed mainly in hippocampal CA1, CA3 and DG areas (three upper rows, from top to bottom, respectively). In the cortex, the TNF-α immunostained cells were still present in the PPR10 + P350 group (fourth row).

4. Discussion

Epilepsy is a neurological disorder characterized by recurrent spontaneous seizures and, according to WHO data (Scott et al., 2001), its incidence rates are very high in the elderly population. Most importantly it is to know that up to 40% of epileptic patients respond poorly to conventional pharmacotherapy, and impaired drug uptake into the brain is considered to be an important contributor to therapeutic failure (Kwan and Brodie, 2006; Löscher and Potschka, 2005). This makes the issue of looking for some new, safer and more efficacious therapeutic agents an urgent need.

Experimental models of seizures and epilepsy have been of great value for understanding the basic mechanisms underlying ictogenesis and epileptogenesis, and for contributing to the development of therapeutic alternatives (Marchi et al., 2009). One of the most frequently used models to evoke SE and subsequent epilepsy is the systemic injection of pilocarpine in rodents (Cavalheiro et al., 2006). In the present paper, we studied for the 1st time the anticonvulsant effect of piperine (PPR) on pilocarpine-induced convulsions in mice.

We showed that PPR significantly increases up to 2.3 times both the latency to the 1st convulsion and to death, as compared to the pilocarpine untreated group (P350). Furthermore, PPR significantly increased percentages of animals' survivals, as well as of animals showing no convulsion. Although the anticonvulsant action of PPR in mice was demonstrated in earlier studies (D'Hooge et al., 1996), those models used genetic E1 mice or the intra-ventricular injection of kainate, both associated with higher PPR doses.

Even if the direct activation of the cholinergic system by pilocarpine in the brain is believed to be the initiating factor for triggering seizures, this question is not completely solved as yet. Thus, novel findings (Marchi et al., 2009) raise the possibility that pilocarpine-induced seizures may also derive from primary proinflammatory actions of pilocarpine in the periphery. Previously (Marchi et al., 2007), pilocarpine has been shown to cause acute peripheral proinflammatory changes, leading to blood brain barrier (BBB) leakage, prior to the onset of SE. The anti-inflammatory action of PPR, as already demonstrated by us in acute models of inflammation in the periphery (Cruz et al., 2011), as well as by others (Bae et al., 2010; Bang et al., 2009; Kumar et al., 2007) is certainly involved with the anticonvulsant effect of the drug.

In order to clarify the PPR anticonvulsive mechanism of action, the association of this drug with atropine, a muscarinic antagonist, was administered to mice before pilocarpine-induced convulsions. The activation of cholinergic neurons is recognized as the only factor triggering pilocarpine SE, since the pretreatment with methylscopolamine counteracts most cholinergic effects of systemically injected pilocarpine (Marchi et al., 2007). Although we showed that atropine, as expected, offered some protection against convulsions, no potentiation of the PPR effect was demonstrated after the PPR + ATRP association. Previously (Maslanki et al., 1994), atropine was shown to be a potent inhibitor of pilocarpine-induced seizures, acting through M_1 receptors. And later, Bymaster et al. (2003) reported that muscarinic M_1 receptors appear to be the only muscarinic receptor subtype mediating seizures. Thus, under our experimental conditions, other and more important factors seem to be involved with PPR effects.

Evidences (Moldrich et al., 2003; Kong et al., 2012) indicate that glutamate plays a crucial role in seizures initiation and propagation, and abnormal glutamate release causes synchronous firing of large populations of neurons, leading to seizures. It has been hypothesized that changes in glutamatergic transmission, in the perforant path, promote the epileptogenic process and seizure generation. Enhanced glutamatergic transmission, as evaluated by patch-clamp recordings in rat hippocampal slices after pilocarpine-induced SE, was shown to contribute to lowering the seizure threshold (Scimemi et al., 2006). We showed that the memantine (a NMDA-type glutamate receptor blocker) pretreatment was not able to prevent pilocarpine convulsions, and its association to PPR did not potentiate the PPR anticonvulsive action, suggesting that the glutamatergic transmission does not have an important role in this drug effect.

It is widely accepted that voltage-gated calcium channels contribute to the control of excitability, at both cellular and neuronal network levels. Thus, alterations in the expression or biophysical properties of subtypes of calcium channels are implicated in the pathophysiology underlying epileptic seizures, constituting important players in idiopathic generalized epilepsies (Cain and Snutch, 2010; Khosravani and Zamponi, 2006). Both T-type and P/Q-type channels appear to be involved in seizure genesis, modulation of network activity and genetic seizure susceptibility (Zamponi et al., 2010). In order to assess the participation of the Ca^{2+} in the anticonvulsive effect of PPR, nimodipine, a calcium channel blocker, was administered alone or associated to PPR2.5 previously to the pilocarpine injection. Our results showed that increases in latency to the 1st convulsion and latency to death were not significantly different among P350 groups, pretreated with nimodipine or PPR, alone or associated, suggesting that calcium channels do not play an important role in the PPR anticonvulsive action.

In recent years, the dysfunction of inhibitory GABAergic circuits has been proposed as a cause for epilepsy, and studies performed on both animal models and *postmortem* human samples indicate that GABAergic neurons are altered in epilepsy (Sgadò et al., 2011). These data suggest that the excitation/inhibition imbalance resulting from defects in GABAergic circuitry might represent a pathogenic mechanism for epilepsy. Considering that pilocarpine-induced limbic seizures in rodents are terminated by benzodiazepines, which are $GABA_A$-receptor positive modulators (Feng et al., 2008), we evaluated the possible

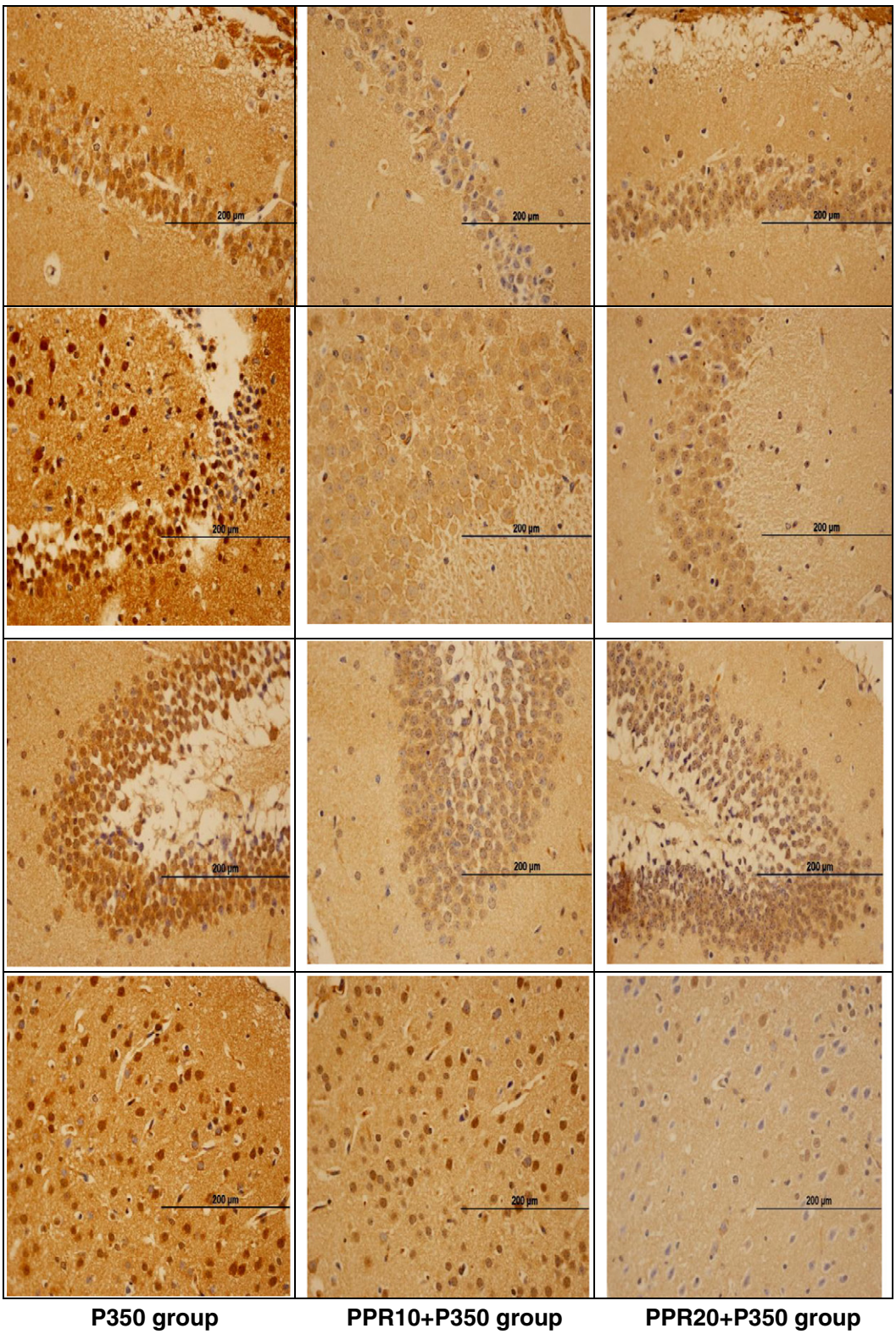

Fig. 12. Representative photomicrographs (400×) showing that piperine treatments (PPR: 10 and 20 mg/kg, i.p.) reduced the number of hippocampal immunostained TNF-α cells, in mice submitted to pilocarpine-induced convulsions. In the cases of PPR pretreatments, the animals were administered with PPR, followed by the pilocarpine injection (P: 350 mg/kg, i.p.), 30 min later. Hippocampi were dissected up to 30 min (or immediately after death) after the 1st convulsion, in the P350 group, and sacrificed 2 h later in the PPR + P350 groups. Piperine pretreatments at both doses (10 and 20 mg/kg, i.p.) reduced immunostainings for TNF-α (PPR10 + P350 and PPR20 + P350 groups, middle and left columns), as related to the P350 group (right column). The PPR effect was mainly observed in hippocampal CA1, CA3 and DG areas (three upper rows, from top to bottom, respectively). In the cortex, the TNF-α immunostained cells were still present in the PPR5 + P350 group (fourth row).

contribution of the GABA transmission for PPR anticonvulsive effects. For that, P350 groups pretreated with the association of PPR with DZP showed a further increase in the latency to the 1st convulsion, as compared to the effects of each drug separately. Besides, in the associated group, 50% of the animals did not convulse and 100% survived, suggesting that DZP potentiates the PPR anticonvulsive effect.

Furthermore, while flumazenil, a benzodiazepine receptor antagonist, in association with PPR did not alter latency to the 1st convulsion or latency to death, it significantly blocked increases in these parameters, as observed with the PPR5 + P350 group. It has been shown that the systemic administration of flumazenil completely reverses the diazepam-evoked anticonvulsant action, in the model of pilocarpine-induced seizures in rats (Khan et al., 2000). These authors conclude that the diazepam anticonvulsant action is mediated by the benzodiazepine–$GABA_A$-receptor complex, what could also be the case under our experimental conditions.

Furthermore, while in the untreated P350 group there was a significant decrease in striatal DA contents and increase in its DOPAC and HVA metabolites, as related to normal controls, these effects were not altered by the PPR pretreatment. In addition, changes in DA induced by PPR alone were much smaller. Previously (Nascimento et al., 2005), we showed that striatal contents of DA decreased while HVA increased, in pilocarpine-induced SE in rats. Additionally, decreases in DA and HVA contents were observed in the cortex of epileptic rats (Freitas et al., 2006). Evidences (Clinckers et al., 2005) demonstrated that DA and 5-HT exert anticonvulsant effects against limbic seizures in rats, and the anticonvulsant activity was always accompanied by significant increases in DA and 5-HT levels in the hippocampus. Other findings (D'Hooge et al., 1996) suggest that DA differentially modulates the seizure threshold in the forebrain, acting *via* D_1 mechanisms in the *substantia nigra* and *via* D_2 mechanisms in the striatum. Since PPR did not reverse P350-induced decreases in striatal DA contents, dopaminergic mechanisms are probably not involved with the PPR anticonvulsant action.

Seizures are evoked in freely moving rats by intra-hippocampal micro-perfusion of 10 mM pilocarpine, increasing hippocampal glutamate, GABA, DA and 5-HT concentrations during seizures (Meurs et al., 2008). DA and 5-HT have been shown to exert anticonvulsant effects against limbic seizures in rats, mediated by D_2 and 5-HT_{1A} receptors stimulation. For exogenously administered monoamines, the anticonvulsant activity was only observed following high increases in baseline levels (Clinckers et al., 2005). In pilocarpine-evoked seizures in rats, the anticonvulsant activities of oxcarbazepine and its metabolite were always accompanied by significant increases in DA and 5-HT.

Excitatory amino acids such as glutamate and aspartate are involved in the generation and expression of epileptic seizures in the mammalian brain. After its interaction with the NMDA-subtype of glutamate receptor, glutamate induces the Ca^{2+} influx, increasing the neuronal nitric oxide synthase (nNOS) activity and nitric oxide (NO) production. On the other hand, inhibitory amino acids such as GABA and glycine counteract the neuronal excitation. GABA, acting through the $GABA_A$ receptor, increases the chloride influx, leading to hyperpolarization of the cell membrane and antagonism of epileptic seizures.

In the present work, we showed that although P350 decreased striatal glutamate levels, it did not alter aspartate contents, and these effects were maintained in the PPR pretreated group. While no changes were seen in GABA levels in the untreated P350 group, these values were increased by more than twice in P350 groups treated with the lower PPR doses. P350 decreased glycine levels, as compared to normal controls, and these values were more than doubled in the PPR + P350 groups. Our data suggest that inhibitory but not excitatory amino acids are involved in the anticonvulsant action of PPR. An earlier study postulated that the GABA antagonism exerted by GCs could underlie their epileptogenic character (D'Hooge et al., 1996). Others found that the excitatory effects of GCs on the CNS may be explained by the activation of NMDA receptors and concomitant inhibition of GABA receptors (Meurs et al., 2008).

Furthermore, while in the P350 group significant increases in nitrite contents were seen in the blood and the brain cortex, as related to normal controls, the PPR pretreatment decreased these values towards those of normal controls. Evidences have associated the use of piperine with a reduction of both inflammation and oxidative stress, in several experimental models (Diwan et al., 2011; Pathak and Khandelwal, 2007; Vijayakumar and Nalini, 2006; Vijayakumar et al., 2004). On the other hand, excessive free radicals production has been implicated in the pathogenesis of various neurological disorders, including epilepsy. Thus, the ability of antioxidants for reducing seizure manifestations, and the accompanying biochemical changes further support a role for free radicals in seizures (Devi et al., 2008; Shin et al., 2011). Therefore, the antioxidant property of PPR shows its potential for neuroprotection, and highlights its possible role as an adjunct to antiepileptic drugs for better seizure control.

We showed that, while pilocarpine significantly increased the number of hippocampal TNF-α immunostained cells, indicating the presence of inflammation, this effect was blocked in groups pretreated with piperine. Inflammation is known to be an important factor in the pathophysiology of seizure generation and epileptogenesis (Riazi et al., 2010). In addition, a variety of factors influence the incidence and prevalence of seizures and, among those, cytokines play an important role. Thus, cytokines as TNF-α are known to be rapidly induced in rodent brains, during epileptic activity (Rao et al., 2009). An earlier study (Bock et al., 1996) showed the release of TNF-α in the rat hippocampus, following epileptic seizures, while another (Lim et al., 2006) demonstrated that seizure activities cause the elevation of proinflammatory cytokines, and activate inflammatory reactions.

Epileptic seizures were shown to induce an inflammatory response within the brain, through activation of microglia and production of proinflammatory cytokines (Vezzani and Granata, 2005; Vezzani et al., 2008). Furthermore, considering that piperine presents an anti-inflammatory property and decreases the number of brain TNF-α-producing cells, these two actions are certainly involved with its anticonvulsant activity. As already observed by us, other drugs also exert anticonvulsant actions and neuroprotection against pilocarpine-induced convulsions, by means of their antioxidant effect and modulation of brain amino acids (Nogueira et al., 2011).

In conclusion, piperine anticonvulsant effects do not have a single mechanism of action, but instead seem to be the result of the association of many factors. Among those are its anti-inflammatory and antioxidant properties, manifested by *in vivo* and *in vitro* models. Furthermore, PPR may also act by modulating/activating the GABA neurotransmission, as well as increasing brain inhibitory amino acid levels in the striatum, making the participation of these events in the PPR action a strong possibility.

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