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Analgesic activity of piracetam: Effect on cytokine production and oxidative stress

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ABSTRACT

Piracetam is a prototype of nootropic drugs used to improve cognitive impairment. However, recent studies suggest that piracetam can have analgesic and anti-inflammatory effects. Inflammatory pain is the result of a process that depends on neutrophil migration, cytokines and prostanoids release and oxidative stress. We analyze whether piracetam has anti-nociceptive effects and its mechanisms. Per oral pretreatment with piracetam reduced in a dose-dependent manner the overt pain-like behavior induced by acetic acid, phenyl-*p*-benzoquinone, formalin and complete Freund's adjuvant. Piracetam also diminished carrageenin-induced mechanical and thermal hyperalgesia, myeloperoxidase activity, and TNF-α-induced mechanical hyperalgesia. Piracetam presented analgesic effects as post-treatment and local paw treatment. The analgesic mechanisms of piracetam were related to inhibition of carrageenin- and TNF-α-induced production of IL-1β as well as prevention of carrageenin-induced decrease of reduced glutathione, ferric reducing ability and free radical scavenging ability in the paw. These results demonstrate that piracetam presents analgesic activity upon a variety of inflammatory stimuli by a mechanism dependent on inhibition of cytokine production and oxidative stress. Considering its safety and clinical use for cognitive function, it is possible that piracetam represents a novel perspective of analgesic.

1. Introduction

Acute inflammatory pain is characterized by sensitization of nociceptors resulting in hyperalgesia and allodynia, which is exacerbated pain intensity in response to painful stimuli, and pain to stimuli that is not normally painful, respectively (Millan, 1999). Inflammatory stimuli such as carrageenin induce a cascade of inflammatory cytokines resulting in inflammatory hyperalgesia. For instance, carrageenin induces the production of TNF-α, which triggers IL-1β production, which in turn, activates the synthesis of PGE_2 (Cunha et al., 2005). These inflammatory mediators are responsible for sensitization of nociceptors and activation of second messenger pathway (cAMP, PKA, and PKC) which reduce the nociceptor threshold and increase neuronal membrane excitability, facilitating the primary nociceptor activation and impulse transmission, resulting in hyperalgesia (Cury et al., 2011; Verri et al., 2006a; Villarreal et al., 2009).

Neutrophils are great contributors to acute inflammatory hyperalgesia. There is evidence that neutrophils recruited by cytokines, endothelin-1, complement component C5a and leukotriene B_4 contribute to hyperalgesia by further producing nociceptive mediators such as PGE_2 (Cunha et al., 2008; Guerrero et al., 2008; Ting et al., 2008; Verri et al., 2009). Therefore, blockade of neutrophil recruitment reduces inflammatory hyperalgesia (Cunha et al., 2008). Another important component of inflammatory pain is the oxidative stress with the generation of molecules such as hydrogen peroxide, superoxide anion, and peroxinitrite, which are produced in response to stimuli and can promote hyperalgesia (Keeble et al., 2009; Ndengele et al., 2008; Wang et al., 2004). Under normal conditions, oxidative stress products are counteracted by endogenous antioxidant systems, which include enzymes (superoxide dismutase, glutathione reductase, and catalase) and other molecules (GSH, bilirubin, and uric acid) (Limón-Pacheco and Gonsebatt, 2009). During inflammatory processes, the levels of oxidative stress products exceed endogenous antioxidant system overwhelming their capacity and producing an imbalance that mediates tissue injury and inflammatory pain (Keeble et al., 2009; Ndengele et al., 2008; Wang et al., 2004).

Nootropic drugs improve cognitive functions such as learning and memory (Genkova-Papazova and Lazarova-Bakarova, 1996; Winblad, 2005). Piracetam, 2-oxo-1-pyrrolidine acetamide, is the prototype of nootropic drug derived from GABA, however its mechanism of action does not seem to be related to GABA and the precise mechanism of

Abbreviations: ABTS, 2,2V-azinobis (3-ethylbenzothiazoline 6-sulfonate); AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; cAMP, cyclic adenosine monophosphate; CFA, complete Freund's adjuvant; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); FRAP, ferric reducing/antioxidant power; GABA, γ-aminobutyric acid; GSH, reduced glutathione; HTAB, hexadecyltrimethylammonium bromide; MPO, myeloperoxidase; PBQ, phenyl-*p*-benzoquinone; PKA, proteins kinase A; PKC, protein kinase C.

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action remains unknown (Winblad, 2005). There are evidences that piracetam acts by restoring the membrane fluidity and by promoting AMPA receptor allosteric modulation (Winblad, 2005).

To our knowledge, the only evidence that piracetam inhibits nociceptive behavior demonstrated that it reduces acetic acid-induced writhing response by mechanisms involving adenosine receptors, and that blockade of muscarinic, adrenergic and opioid receptors potentiates the antinociceptive effect of piracetam (Abdel-Salam, 2006). In contrast, levetiracetam, an antiepileptic drug α-etil analogue of piracetam, did not affect formalin-induced nociceptive response (Munro et al., 2007) while it reduced carrageenin-induced hyperalgesia by mechanisms mediated at least in part by direct/indirect activation of $GABA_A$, opioid, serotoninergic and adrenergic receptors (Micov et al., 2010). It is noteworthy to mention that these studies applied models of inflammatory pain with differences in the nociceptive endpoint and nociceptive mechanisms, which explain why the antinociceptive spinal/central mechanisms of piracetam (Abdel-Salam, 2006) contrast with the results demonstrated for levetiracetam (Micov et al., 2010). It is also important to mention that levetiracetam reduces the IL-1β production in astrocytes culture (Haghikia et al., 2008) and decreases oxidative stress in hippocampus (Oliveira et al., 2007). These results suggest that levetiracetam and possibly piracetam can modulate additional mechanisms such as the production of inflammatory/nociceptive molecules that eventually would contribute to their analgesic mechanism. Furthermore, piracetam inhibits the exudation induced by formalin injection in the peritoneal cavity and cell proliferation induced by cotton pellet (Nikolova et al., 1984), indicating that it reduces peripheral inflammation. Thus, the effect of piracetam seems not restricted to the central nervous system.

Therefore, considering that the spinal/central antinociceptive effect of piracetam was demonstrated in only one study (Abdel-Salam, 2006) and that it can also reduce peripheral inflammation (Nikolova et al., 1984) as presented above, we propose to investigate whether piracetam has antinociceptive effects in models of inflammatory pain and address its peripheral antinociceptive mechanisms of action focusing on cytokines and oxidative stress.

2. Materials and methods

2.1. Animals

Male Swiss mice, from Universidade Estadual de Londrina, Londrina, Paraná, Brazil, weighing approximately 25 ± 5 g served as experimental animals. Mice were housed in standard clear plastic cages with free access to food (Nuvilab® from Nuvital Nutrientes, Colombo, PR, Brazil) and water, with a light/dark cycle of 12:12 h, at 21 °C. All behavioral testing was performed between 9 a.m. and 5 p.m. in a temperature-controlled room. Mice were placed in a quiet room 15–30 min before the start of testing. The mice were used only once. Animals' care and handling procedures were in accordance with the International Association for Study of Pain (IASP) guidelines and with the approval of the Ethics Committee of the Universidade Estadual de Londrina. All efforts were made to minimize the number of animals used and their suffering. It is noteworthy that different experimenters prepared the solutions, made the administrations and performed the evaluation of pain-like behavior.

2.2. Drug and reagents

The following materials were obtained from the sources indicated: piracetam (Nootropil®) from Sanofi-Aventis (Suzano, SP, Brazil); acetic acid from Mallinckrodt Baker S.A (Mexico City, Mexico); Trolox® (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid), PBQ and CFA from Sigma Aldrich Co. (St. Louis, MO, USA); carrageenin from FMC Corp (Philadelphia, PA, USA); TNF-α and ELISA kits from e-Bioscience Inc (San Diego, CA, USA); and formaldehyde from Merck (Rio de Janeiro, RJ, Brazil).

2.3. Writhing response tests

PBQ and acetic acid-induced writhing models were performed as previously described (Collier et al., 1968; Valério et al., 2007; Verri et al., 2008). In brief, PBQ (diluted in DMSO 2%/saline, 1890 μg/kg), acetic acid (0.6% v/v, diluted in saline, 10 mL/kg), or vehicle was injected into the peritoneal cavities of mice. Each mouse was placed in a large glass cylinder and the intensity of nociceptive behavior was quantified by counting the total number of writhings (contractions of abdominal muscles accompanied by an elongation of the body and extension of the hind limbs) occurring between 0 and 20 min after stimulus injection.

2.4. The formalin and CFA tests

The time spent licking the paw was determined between 0 and 30 min after intraplantar (i.pl.) injection of 2.5% formalin diluted in saline 0,9% (25 μL/paw) or CFA (10 μL/paw), in similar ways and as previously described (Dubuisson and Dennis, 1977; Mizokami et al., 2012; Valério et al., 2009). Results were presented at 5 min interval up to 30 min for formalin test and total time spent licking the paw between 0 and 30 min in CFA test. CFA (10 μL/paw) was also used as stimulus to induce mechanical and thermal hyperalgesia (Mizokami et al., 2012; Valério et al., 2009).

2.5. Electronic pressure-meter test

Mechanical hyperalgesia was tested in mice, as previously reported (Cunha et al., 2004). Briefly, mice were placed in acrylic cages (12 × 10 × 17 cm) in a quiet room 15–30 min before the start of testing. The test consisted of evoking a hindpaw flexion reflex with a hand-held force transducer (electronic anesthesiometer, Insight Equipamentos, Ribeirao Preto, SP, Brazil) adapted with a 0.5 mm^2 polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hindpaw with a gradual increase in pressure. The end point was characterized by the removal of the paw and flinching movements. The stimulation of the paw was repeated until the animal presented two similar measurements. The results are expressed by delta (Δ) withdrawal threshold (in g) calculated by subtracting the zero-time mean measurements from the mean measurements of 1, 3, or 5 h after stimulus. Withdrawal threshold was 9.4 ± 0.2 g (mean ± SEM; n = 6) before injection of the hyperalgesic agents (e.g. carrageenin, TNF-α or CFA).

2.6. Hot plate test

Thermal hyperalgesia was evaluated before and after stimulus. The test was performed as previously reported (Verri et al., 2005). In brief, mice were placed in a 10 cm wide glass cylinder on a hot plate (Hot Plate HP-2002, Insight Equipamentos, Ribeirao Preto, SP, Brazil) maintained at 55 °C. The reaction time was scored when the animal jumped, flinched or licked its paws. The normal latency (reaction time) was 12 ± 0.7 s (mean ± SEM; n = 6). A maximum latency (cutoff) was set at 30 s to avoid tissue damage.

2.7. Neutrophil migration to the paw skin tissue (myeloperoxidase activity)

The neutrophil migration to paw was indirectly evaluated by the MPO activity kinetic-colorimetric assay (Bradley et al., 1982; Casagrande et al., 2006). Briefly, paw skin sample was collected in 50 mM K_2PO_4 buffer (pH 6.0) containing 0.5% HTAB and were homogenized using Ultra-Turrax® (IKA T10 Basic, CQA Química, Paulínea, SP). Then the homogenates were centrifuged at 16100 *g* for 2 min at 4 °C. 15 μL of resulting supernatant was mixed with 200 μL of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/mL o-dianisidine dihydrochloride and 0.05% hydrogen peroxide and was assayed spectrophotometrically for MPO activity determination at 450 nm (BEL SP2000UV, Photonics, São

Paulo, SP, Brazil). MPO activity of samples was compared with a standard curve of neutrophils and presented as MPO activity (number of neutrophils $\times 10^4$/mg of tissue).

2.8. Cytokine measurement

For cytokine measurement in paw skin, mice were terminally anesthetized and the skin tissues were removed and homogenized in 500 μL of buffer containing protease inhibitors (Verri et al., 2006b). The samples were centrifuged and IL-1β and TNF-α levels were determined in the supernatants by enzyme-linked immunosorbent assay (ELISA) using eBioscience kit according to the manufacturer's instructions. The results are expressed as pg of cytokine/100 mg of paw skin.

2.9. GSH measurement

Paw skin sample was collected and maintained at −80 °C for at least 48 h. The sample was homogenized with 200 μL of 0.02 M EDTA. The homogenate was mixed with 25 μL of 50% trichloroacetic acid and was homogenized three times during 15 min. The mixture was centrifuged (15 min × 1500 *g* × 4 °C). The supernatant was added to 200 μL of 0.2 M TRIS buffer, pH 8.2, and 10 μL of 0.01 M DTNB. After 5 min, the absorbance was measured at 412 nm against a reagent blank with no supernatant. A standard curve was performed with standard GSH. The results are expressed as GSH per mg of protein (Sedlak and Lindsay, 1968). To protein determination, 60 μL of supernatant was mixed with 60 μL of copper reagent freshly prepared. After 10 min, 180 μL of Folin solution was added. The resulting solution was incubated at 50 °C for 10 min. The absorbance was measured at 660 nm and the results equated to a standard curve of bovine serum albumin (Lowry et al., 1951).

2.10. FRAP and ABTS assays

Paw skin sample was collected and immediately homogenized with 500 μL of 1.15% KCl, and was centrifuged (10 min × 200 *g* × 4 °C). The ability of sample to resist oxidative damage was determined as ferric reducing ability by FRAP assay, and as free radical scavenging ability by ABTS assay (Katalinic et al., 2005; Re et al., 1999). For FRAP assay, 50 μL of supernatant was mixed with 150 μL of deionized water and 1.5 mL of FRAP reagent freshly prepared. The reaction mixture was incubated at 37 °C for 30 min and the absorbance was measured at 595 nm. For ABTS assay, ABTS solution was diluted with phosphate buffer saline pH 7.4 (PBS) to an absorbance of 0.80 at 730 nm. Then, 1.0 mL of diluted ABTS solution was mixed to 20 μL of supernatant. After 6 min, the absorbance was measured at 730 nm. The results were equated against a Trolox standard curve (1.5–30 μmol/L, final concentrations). The results are expressed as Trolox equivalents per gram of tissue weight in both assays.

2.11. Experimental procedures

For this study, mice received per oral (p.o.) pretreatment with piracetam (10–300 mg/kg/10 mL diluted in saline) 60 min before inflammatory stimulus or post-treatment 30 min after carrageenin or starting 24 h after CFA injection. Local treatment with piracetam (10, 30, 100 and 300 μg/paw/25 μL diluted in saline) was also performed. The doses of inflammatory stimuli were determined in our laboratory and based on previous studies (Cunha et al., 2005; Mizokami et al., 2012; Pavão-de-Souza et al., 2012; Valério et al., 2007, 2009; Verri et al., 2008; Zarpelon et al., 2012, 2013). The writhing response was evaluated for 20 min after i.p. injection of PBQ (1890 μg/kg) or acetic acid 0.6% (10 mL/kg). The nociceptive behavior of licking the paw was quantified for 30 min after formalin 2.5% (25 μL/paw) or CFA (10 μL/paw) injection. Mechanical hyperalgesia was evaluated 1–5 h after carrageenin (100 μg/25 μL/paw) or TNF-α (100 pg/25 μL/paw) injection, and 1–7 days after injection of CFA (10 μL/paw). All inflammatory stimuli induced only ipsilateral (in the paw in which the stimulus was injected) mechanical hyperalgesia. Thermal hyperalgesia was evaluated 1–5 h after carrageenin (100 μg/25 μL/paw) and 1–7 days after injection of CFA (10 μL/paw). Neutrophil migration was evaluated in paw skin by myeloperoxidase activity assay 5 h after carrageenin (100 μg/25 μL/paw) or TNF-α (100 pg/25 μL/paw) injection. IL-1β and TNF-α levels were measured in paw skin 3 h after carrageenin injection, and IL-1β level was also determined 3 h after TNF-α injection. The GSH level and FRAP and ABTS assay were evaluated 3 h after carrageenin injection.

2.12. Statistical analysis

Results are presented as means ± SEM of measurements made on 6 mice in each group per experiment. Experiments were performed twice. Two-way analysis of variance (ANOVA) was used to compare the groups and doses at all times (curves). The analyzed factors were treatments, time and time versus treatment interaction. When there was a significant time versus treatment interaction, one-way ANOVA followed by Bonferroni's t-test was performed for each time. On the other hand, when the nociceptive responses were presented as total values at indicated time period, the differences between responses were evaluated by one-way ANOVA followed by Bonferroni's t-test. Statistical differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Piracetam reduces pain-like behavior induced by varied stimuli

In the first series of experiments, the analgesic effect of piracetam was evaluated in acetic acid-, PBQ-, formalin- and CFA-induced pain-like behaviors. Mice were treated with piracetam (10–300 mg/kg, p.o.) 1 h before the i.p. stimulus with acetic acid (Fig. 1A). The dose of 10 mg/kg of piracetam did not affect while the doses of 30, 100 and 300 mg/kg of piracetam reduced in a significant manner the abdominal contortion induced by acetic acid (Fig. 1A). The effect of 100 and 300 mg/kg of piracetam was significant when compared to the dose of 10 mg/kg of piracetam. Therefore, the dose of 100 mg/kg was selected for the following experiments on overt pain-like behavior. Piracetam inhibited PBQ-induced abdominal contortions with up to 41% inhibition (Fig. 1B). In the formalin test, piracetam reduced the first (5 min) and second phases (20 and 25 min) of licking response (Fig. 1C). In the CFA test, the treatment with piracetam also reduced the licking response (Fig. 1D).

3.2. Piracetam reduces carrageenin-induced mechanical and thermal hyperalgesia and myeloperoxidase activity

Mice were treated with piracetam (10–100 mg/kg, p.o.) 1 h before i.pl. stimulus with carrageenin (100 μg) (Fig. 2). Piracetam reduced in a dose-dependent manner the mechanical hyperalgesia induced by carrageenin (Fig. 2A). There was a tendency of reduction of mechanical hyperalgesia by 10 mg/kg of piracetam that was not significant (Fig. 2A). The dose of 30 mg/kg of piracetam reduced carrageenin-induced mechanical hyperalgesia at 3 h. The dose of 100 mg/kg of piracetam reduced the mechanical hyperalgesia induced by carrageenin at 3 and 5 h. At 3 h the effect of piracetam 100 mg/kg was significant compared to the lower doses of piracetam and at 5 h compared to the dose of 10 mg/kg (Fig. 2A). In the hot plate test, the dose of 100 mg/kg of piracetam reduced the thermal hyperalgesia induced by carrageenin at 3 and 5 h without alterations by the doses of 10 and 30 mg/kg of piracetam (Fig. 2B). At 5 h, mice were sacrificed and samples of paw plantar skin were collected for MPO activity assay (Fig. 2C). Only the dose of 100 mg/kg of piracetam

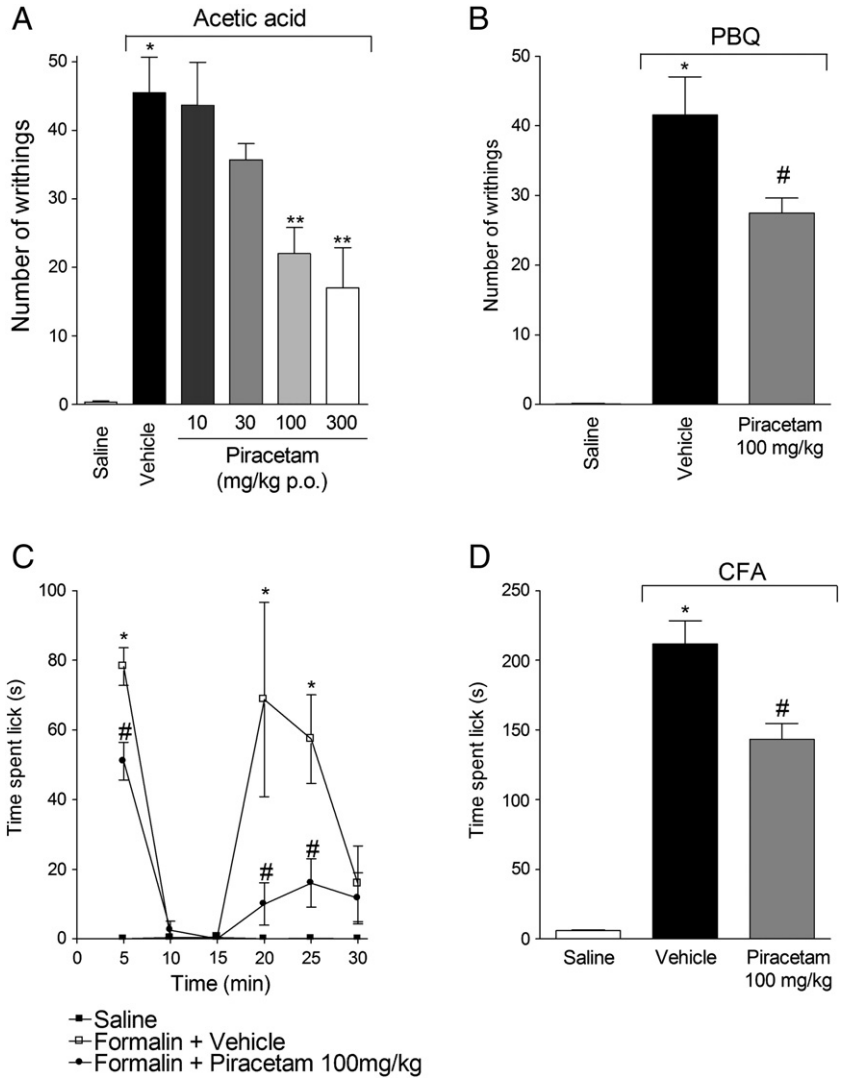

Fig. 1. Piracetam reduced overt pain-like behavior induced by acetic acid, PBQ, formalin and CFA. Mice were treated with piracetam p.o. (10, 30, 100 or 300 mg/kg) 60 min before i.p. injection of acetic acid 0.6% (panel A) or PBQ 1890 μg/kg (panel B), or i.pl. injection of 25 μL of formalin 2.5% (panel C) or 10 μL of CFA (panel D). The total number of writhing was evaluated for 20 min (A and B) and the time spent licking the paw for 30 min (C and D). $n = 6$. * $P < 0.05$ compared with the saline group, # $P < 0.05$ compared with the stimulus group, ** $P < 0.05$ compared with the stimulus group and the dose of 10 mg/kg of piracetam.

reduced the MPO activity (Fig. 2C). Considering the results of Fig. 2, 100 mg/kg of piracetam was the dose selected for the next experiment.

3.3. Post-treatment with piracetam reduces carrageenin- and CFA-induced mechanical and thermal hyperalgesia

Mice were treated with piracetam (100 mg/kg, p.o.) 30 min after the i.pl. injection of carrageenin (100 μg/paw), and mechanical (Fig. 3A) and thermal (Fig. 3B) hyperalgesia were evaluated 1, 3 and 5 h after treatment. Piracetam reduced mechanical hyperalgesia at 1, 3 and 5 h, and thermal hyperalgesia at 3 and 5 h after treatment with piracetam. In another experimental setting, mice received i.pl. injection of CFA (10 μL/paw), and mice were treated daily during 7 days with piracetam (30, 100 and 300 mg/kg, p.o.) starting 24 h after CFA stimulus. Five hours after each treatment, mechanical and thermal hyperalgesia were evaluated (Fig. 3C and D). Piracetam reduced in a dose-dependent manner the mechanical hyperalgesia induced by CFA, with significant effects at 30, 100 and 300 mg/kg. There were statistical differences comparing the dose of 100 and 300 mg/kg to the CFA control group and piracetam at 30 mg/kg (Fig. 3C). The dose of piracetam of 30 mg/kg did not reduce CFA-induced thermal hyperalgesia while the doses of 100 and 300 mg/kg were effective in all 7 days. Piracetam at 100 mg/kg dose was also effective compared to 30 mg/kg at days 1 and 2, and the dose of 300 mg/kg was effective compared to the dose of 30 mg/kg between 1 and 5 days (Fig. 3D).

3.4. Local treatment with piracetam reduces carrageenin-induced mechanical and thermal hyperalgesia

Mice were treated with piracetam (10, 30, 100 and 300 μg/paw) 30 min before i.pl. injection of carrageenin (100 μg/paw), and at indicated time points, mechanical (Fig. 4A) and thermal (Fig. 4B) hyperalgesia were evaluated. Piracetam reduced in a dose-dependent manner the mechanical hyperalgesia induced by carrageenin (Fig. 4A). There was significant reduction of mechanical hyperalgesia by piracetam at the doses of 30, 100 and 300 μg/paw at 3 and 5 h. Additionally, the dose of 100 μg/paw presented statistical differences with the dose of 10 μg/paw at 3 h and with the doses of 10 and 30 μg/paw at 5 h. Piracetam at 300 μg/paw dose also presented statistical differences compared to the doses of 10 and 30 μg/paw at 3 and 5 h. Piracetam also inhibited thermal hyperalgesia at doses of 30, 100 and 300 μg/paw compared to control carrageenin and lower dose of piracetam at 3 and 5 h (Fig. 4B). In addition, piracetam at 300 μg/paw also reduced carrageenin-induced

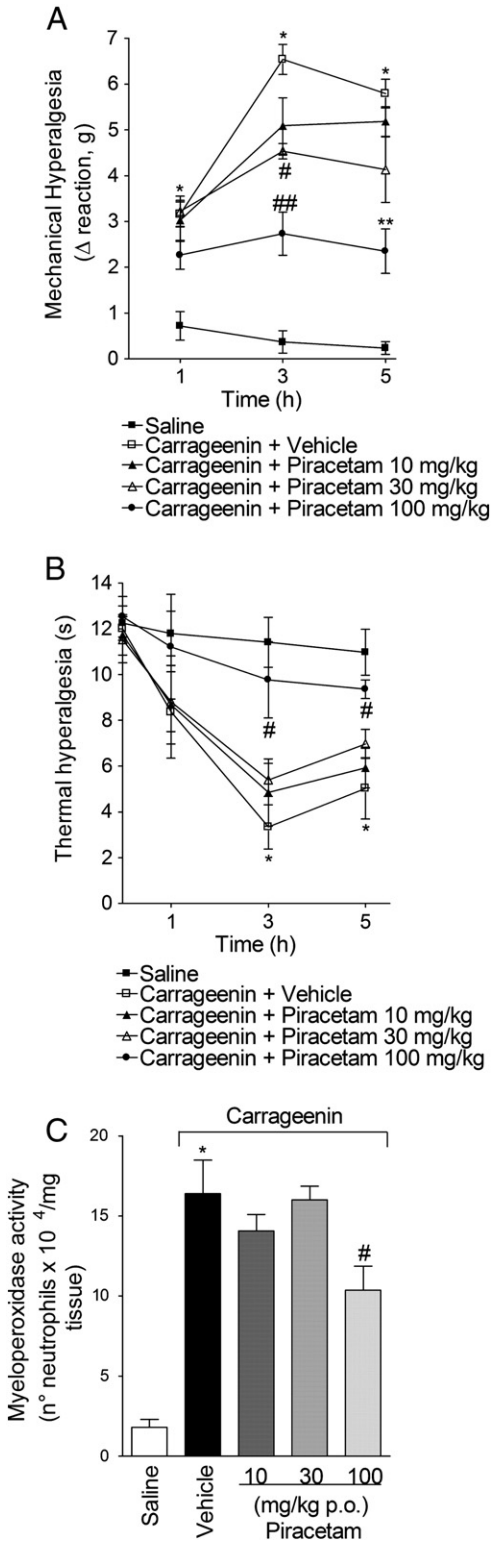

Fig. 2. Piracetam reduced carrageenin-induced mechanical hyperalgesia, thermal hyperalgesia and myeloperoxidase activity. Mice were treated with piracetam p.o. (10, 30 or 100 mg/kg) 60 min before carrageenin (100 μg/paw) injection. The mechanical and thermal hyperalgesia were evaluated 1, 3 and 5 h after carrageenin injection (panels A and B respectively). The neutrophil migration (myeloperoxidase activity) was measured 5 h after the carrageenin injection (panel C). $n = 6$. * $P < 0.05$ compared with the saline group, # $P < 0.05$ compared with the carrageenin group, ** $P < 0.05$ compared with the carrageenin group and the dose of 10 mg/kg of piracetam, ## $P < 0.05$ compared with the carrageenin group, the dose of 10 and 30 mg/kg of piracetam.

thermal hyperalgesia at 1 h. Considering that there were no statistical differences between the doses of piracetam of 100 and 300 μg/paw, the dose of 100 μg/paw was selected for next experiments (Fig. 4C and D). The contra-lateral (CL) treatment with piracetam did not affect the mechanical (Fig. 4C) and thermal (Fig. 4D) hyperalgesia induced by carrageenin. Furthermore, the local treatment with piracetam did not alter the mechanical (Fig. 4C) or thermal (Fig. 4D) threshold per se.

3.5. Piracetam reduces carrageenin-induced IL-1β without affecting TNF-α production in paw skin

Mice were treated with piracetam as described in Fig. 2 and received i.pl. injection of carrageenin (100 μg) and samples were collected after 3 h. Piracetam significantly inhibited the carrageenin-induced production of IL-1β (Fig. 5A) in the paw (41%) without affecting TNF-α production (Fig. 5B).

3.6. Piracetam reduces TNF-α-induced mechanical hyperalgesia, MPO activity and IL-1β production

It has been demonstrated that in the carrageenin-induced paw inflammation, TNF-α is responsible for inducing IL-1β production (Cunha et al., 2005). Therefore, considering that piracetam inhibited carrageenin-induced IL-1β production without affecting TNF-α production, it was evaluated whether piracetam would inhibit TNF-α-induced mechanical hyperalgesia, MPO activity and IL-1β production (Fig. 6). Mice were treated with piracetam as described above and after 1 h received i.pl. injection of TNF-α (100 pg/paw). Piracetam reduced TNF-α-induced mechanical hyperalgesia at 1, 3 and 5 h (Fig. 6A), MPO activity at 5 h (Fig. 6B) and IL-1β production at 3 h (Fig. 6C).

3.7. Piracetam reduces carrageenin-induced decrease in antioxidant defenses

Mice were treated with piracetam as described above before carrageenin i.pl. injection and paw skin samples were collected after additional 3 h for GSH, FRAP and ABTS assays (Fig. 7). Carrageenin reduced the endogenous GSH skin levels (Fig. 7A), ferric reducing ability (FRAP assay; Fig. 7B) and free radical scavenging ability (ABTS assay; Fig. 7C), and piracetam treatment prevented these reductions of antioxidant defenses in the skin.

4. Discussion

Piracetam is a nootropic drug that has been used to treat cognitive impairment. The present study provides evidence that piracetam exerts analgesic effects upon varied inflammatory stimuli by mechanisms related to reduction of MPO activity, IL-1β production, and oxidative stress.

There is one previous evidence that piracetam could present analgesic effects in the acetic acid writhing test (Abdel-Salam, 2006). Herein, we confirmed and extended this evidence to additional models of overt pain-like behavior including acetic acid- and PBQ-induced writhing response and formalin- and CFA-induced paw licking. Furthermore, it was observed that piracetam also reduced mechanical and thermal hyperalgesia induced by carrageenin and CFA, and mechanical hyperalgesia induced by TNF-α. Importantly, piracetam exerted analgesic effect as post-treatment in the mechanical and thermal hyperalgesia induced by carrageenin and CFA. The importance of these data is that the analgesic effect of piracetam was observed in varied models and post-treatment, which increases the probability of its possible clinical applicability. In agreement, piracetam has been used as a nootropic drug for about 40 years with low adverse reaction in clinical settings, thus, reducing the steps needed to use piracetam as an analgesic. Moreover, according to the package insert of Nootropil®, the treatment with piracetam ranges from 2.4 to

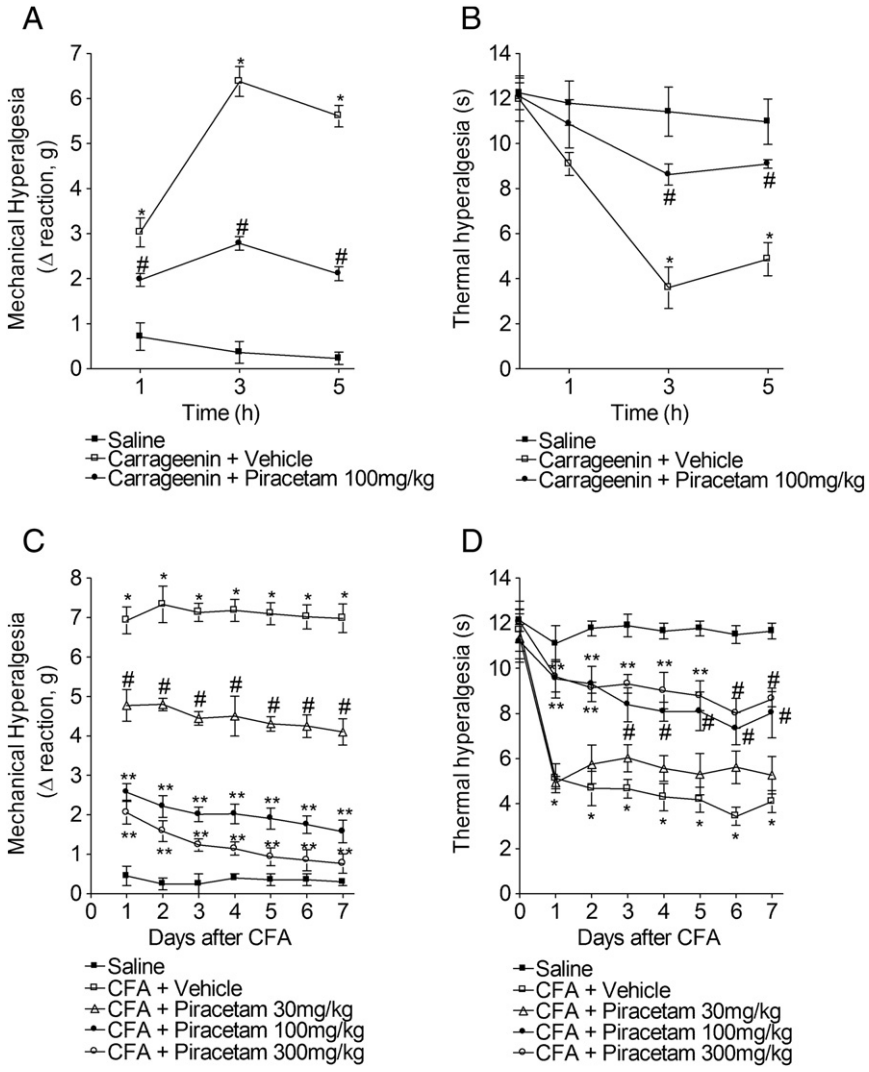

Fig. 3. Post-treatment with piracetam diminished carrageenin-induced mechanical and thermal hyperalgesia. Mice were treated with piracetam (30–300 mg/kg, p.o.) 30 min after carrageenin (100 μg/paw) (panels A and B) and daily starting at 24 after CFA (10 μg/paw; panels C and D). Mechanical and thermal hyperalgesia were evaluated 1–5 h after treatment (panels A and B, respectively) or 5 h after each daily treatment with piracetam (panels C and D, respectively). $n = 6$. * $P < 0.05$ compared with the saline group, # $P < 0.05$ compared with the stimulus group, ** $P < 0.05$ compared with the CFA group and the dose of 30 mg/kg of piracetam.

12 g per day. Taking into account an adult of 70 kg, the consequent dose range would be 34 to 171 mg/kg. In the present study, the piracetam optimal analgesic dose of 100 mg/kg was selected over a dose–response curve. Therefore, the analgesic dose of piracetam would be within the present dose range in clinical use.

The thermal hyperalgesia analyzed in the hot plate test in naïve animals is considered to involve supraspinal nociceptive mechanisms (Le Bars et al., 2001). Previous study reported that piracetam alone does not alter the basal latency in hot plate test (Abdel-Salam, 2006), indicating that piracetam does not present supraspinal analgesic mechanisms in thermal hyperalgesia. In the present study, piracetam reduced carrageenin- and CFA-induced thermal hyperalgesia, suggesting that piracetam acts mainly in the peripheral inflammatory component of pain, which is responsible for nociceptor sensitization in this model. The peripheral injection of carrageenin also induces the spinal microglia activation and peripheral inhibition of inflammation will result in diminished spinal activation (Hua et al., 2005). Corroborating the importance of peripheral action of piracetam, it was also observed that local i.pl. treatment with piracetam diminished carrageenin-induced mechanical and thermal hyperalgesia. Moreover, confirming the peripheral effect of the i.pl. treatment with piracetam, the treatment of the contra-lateral paw to the stimulus did not present analgesic effect in the ipsilateral paw to the stimulus.

The varied models of inflammatory pain used in the present study share some pain mechanisms such as the production of nociceptive cytokines including IL-1β in the acetic acid, PBQ, second phase of formalin test, CFA, carrageenin and administration of TNF-α models (Chichorro et al., 2004; Cunha et al., 2005; Ribeiro et al., 2000; Verri et al., 2006a, 2008). Therefore, a reasonable mechanism of action to be investigated was whether piracetam would reduce pain-like behavior by reducing the production of nociceptive cytokines. In fact, piracetam reduced IL-1β production induced by carrageenin administration. However, piracetam did not affect carrageenin-induced TNF-α production. A possible explanation for this selective inhibition of IL-1β production could rely on the nociceptive mechanisms triggered by carrageenin in which IL-1β production depends on TNF-α (Cunha et al., 2005). In fact, piracetam reduced TNF-α-induced mechanical hyperalgesia and IL-1β production. To our knowledge, this is the first demonstration that piracetam decreases IL-1β production.

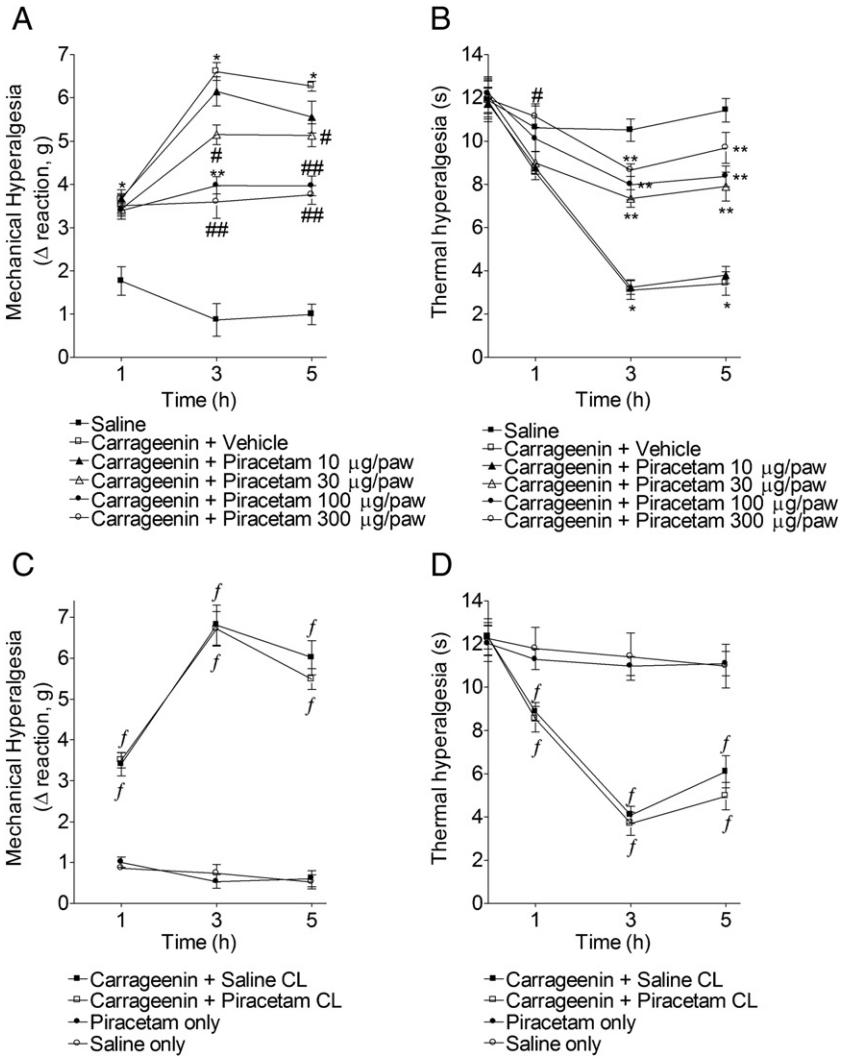

Fig. 4. Local treatment with piracetam diminished carrageenin-induced mechanical and thermal hyperalgesia. Mice were pretreated with piracetam i.pl. (10–300 μg/paw) 30 min before carrageenin injection (100 μg/paw). Mice received the treatment and stimulus in the ipsilateral paw (panels A and B) or treatment in the contra-lateral (CL) paw to the stimulus (panels C and D). Mechanical and thermal hyperalgesia were evaluated 1–5 h after stimulus. $n = 6$. * $P < 0.05$ compared with the saline group, # $P < 0.05$ compared with the carrageenin group, ** $P < 0.05$ compared with the carrageenin group and the dose of 10 μg/kg of piracetam, ## $P < 0.05$ compared with the carrageenin group and the dose of 10 μg/kg and 30 μg/kg of piracetam, *f* $P < 0.05$ compared with the piracetam only group and the saline only group.

In agreement, an analogue of piracetam, levetiracetam, also reduced the immunoreactivity to IL-1β and IL-1R1 by activated astrocytes and microglia of epileptic rats (Kim et al., 2010). Levetiracetam reduced the mRNA expression for TNF-α in a rat model of temporal lobe epilepsy (Christensen et al., 2010), which would contribute to explain an analgesic effect of piracetam, and is not in contradiction to the present study since we analyzed TNF-α protein levels and not mRNA expression.

Another important component of carrageenin-induced hyperalgesia is the recruitment of neutrophils, which may contribute to mechanical hyperalgesia by further producing nociceptive molecules including PGE_2 (Cunha et al., 2008). Piracetam also reduced the MPO activity in the paw skin induced by carrageenin and TNF-α, suggesting that reduction of neutrophil recruitment could be part of the analgesic effect of piracetam. Corroborating the result on MPO activity in paw skin samples, piracetam reduced carrageenin-induced neutrophil recruitment to the peritoneal cavity (unpublished observation of Navarro SA et al.). These data are also in line with the inhibition of carrageenin- and TNF-α-induced IL-1β production, since IL-1β is a chemoattractant cytokine for neutrophils (Verri et al., 2010). The inhibition of IL-1β production diminishes chemoattraction of neutrophils due to reduced expression of adhesion molecules and production of IL-1β-dependent mediators (Verri et al., 2010). Furthermore, reactive oxygen species are also contributing molecules to the recruitment of neutrophils and the inhibition of oxidative stress observed herein is also an explanation for reduced neutrophil chemotaxis (Hattori et al., 2010).

In fact, cytokines are also closely related to oxidative stress. TNF-α and IL-1β activate NADPH oxidase inducing the production of superoxide anion starting the generation of reactive oxygen species (Lambeth, 2004; Drummond et al., 2011). In turn, superoxide anion can induce cytokine production by activation of NFκB (Verri et al., 2012). Oxidative stress is a contributing factor to inflammatory hyperalgesia together with inflammatory cytokines (Verri et al., 2012). Further corroborating an intricate analgesic mechanism of action of piracetam, in the present study it prevented the carrageenin-induced decrease of endogenous GSH, ferric reducing ability (FRAP assay) and free radical scavenging ability (ABTS assay). Furthermore, the present data on reduction of oxidative stress by piracetam are in accordance with the demonstration that it inhibited ethidium bromide-induced acute demyelination of the rat brain that depends on GSH depletion

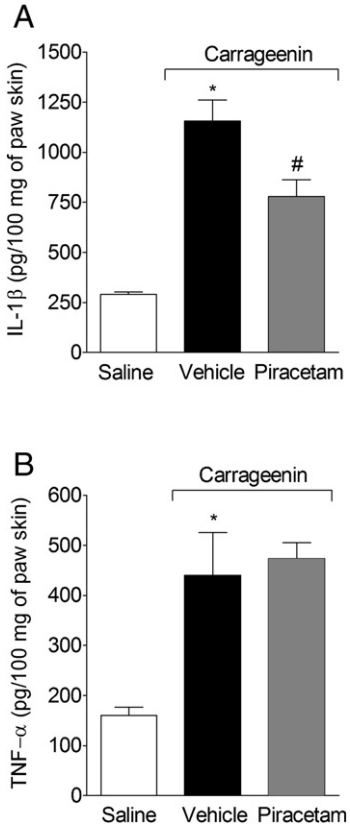

Fig. 5. Piracetam reduced carrageenin-induced IL-1β production without affecting TNF-α production. Mice were treated with piracetam p.o. (100 mg/kg) 60 min before i.pl. injection of carrageenin (100 μg/paw) and 3 h after stimulus IL-1β (panel A) and TNF-α (panel B) levels were determined by ELISA. $n = 6$. * $P < 0.05$ compared with the saline group, # $P < 0.05$ compared with the carrageenin group.

(Abdel-Salam et al., 2011), and attenuated the propoxur- and phosphamidon-induced increase in brain malondialdehyde levels (e.g. lipid peroxidation) (Gupta et al., 2009; Kosta et al., 2013). Additionally, the reduction of leukocyte recruitment could account for impairing oxidative stress since leukocytes such as neutrophils that are recruited in the initial hours of inflammation produce reactive oxygen species (Lambeth, 2004; Drummond et al., 2011).

In addition to the effects of piracetam on cytokine production, oxidative stress and MPO, it was noticeable that it reduced the first phase of formalin-induced licking response, which suggests a possible neuronal effect of piracetam. Previous evidence demonstrates that the first phase of the formalin test depends, at least in part, on serotonin release and action on nociceptors (Parada et al., 2001). Considering that the analgesic effects of levetiracetam were inhibited by serotonin receptor antagonist (Micov et al., 2010), and piracetam reduces the release of serotonin (Nalini et al., 1992), it is possible that inhibition of serotonin release explains, at least in part, the inhibition of the first phase of the formalin test by treatment with piracetam.

Concluding, piracetam reduces inflammatory pain by mechanisms related to reduction of peripheral cytokine production, MPO activity and oxidative stress, demonstrating previously unrecognized analgesic mechanisms of piracetam. Therefore, the present study suggests the potential use of piracetam as an analgesic, which merits further clinical investigation.

Conflict of interest

The authors declare that they have no conflict of interest.

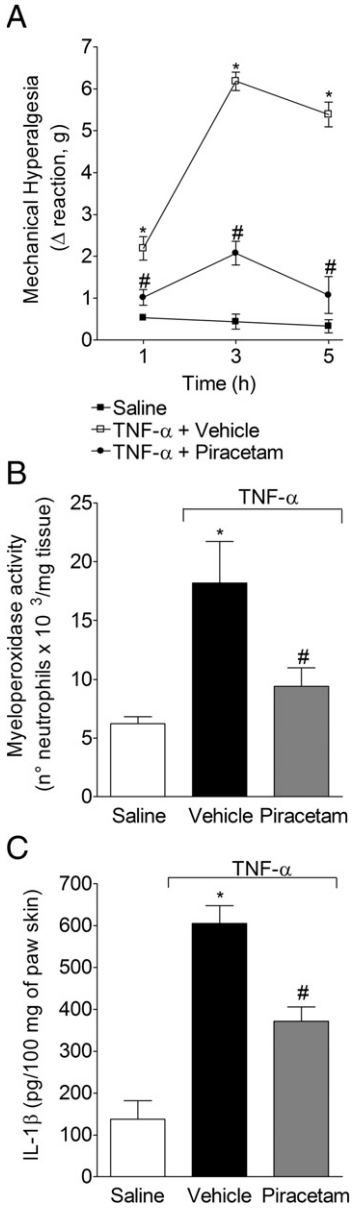

Fig. 6. Piracetam reduced TNF-α-induced mechanical hyperalgesia, myeloperoxidase activity and IL-1β production. Mice were treated with piracetam p.o. (100 mg/kg) 60 min before TNF-α (100 pg/25 μL/paw) injection. Mechanical hyperalgesia was evaluated 1–5 h after TNF-α injection (panel A), neutrophil migration was evaluated by MPO assay 5 h after TNF-α injection (panel B), and IL-1β level was determined by ELISA 3 h after TNF-α injection (panel C). $n = 6$. * $P < 0.05$ compared with the saline group, # $P < 0.05$ compared with the TNF-α control group.

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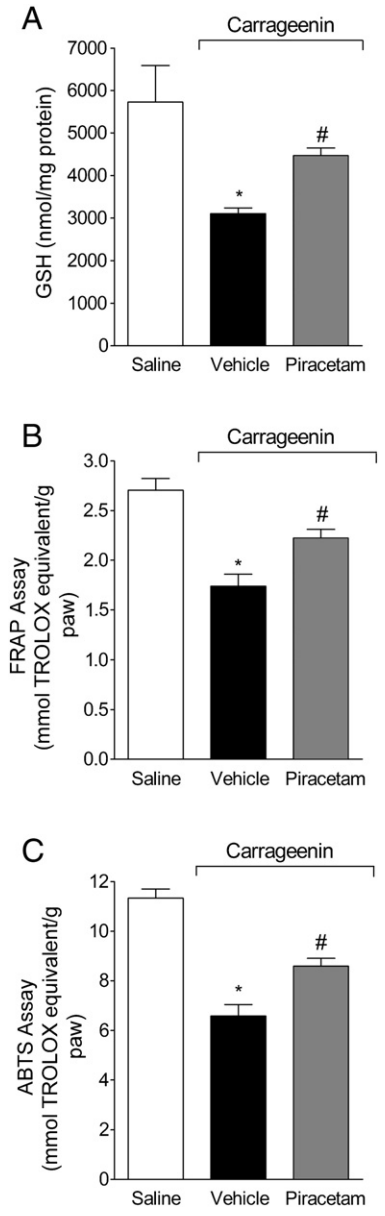

Fig. 7. Piracetam reduced carrageenin-induced decrease of GSH level and anti-oxidative capacity in FRAP and ABTS assays. Mice were treated with piracetam p.o. (100 mg/kg) 60 min before carrageenin (100 μg/paw) injection. Samples for GSH level measurement (panel A), FRAP (panel B, ferric reducing ability) and ABTS (panel C, free radical scavenging ability) assays were collected 3 h after stimulus injection. $n = 6$. * $P < 0.05$ compared with the saline group, # $P < 0.05$ compared with the carrageenin group.

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