UNIVERSIDADE PAULISTA

DOUTORADO EM PATOLOGIA AMBIENTAL E EXPERIMENTAL

EFEITOS DA PROPENTOFILINA NO COMPORTAMENTO EM CAMPO ABERTO, NA TRAVE ELEVADA E NA RESPOSTA ASTROCITÁRIA APÓS INJEÇÃO DE DROGA GLIOTÓXICA NO TRONCO ENCEFÁLICO

Tese apresentada ao Programa de Doutorado em Patologia Ambiental e Experimental da Universidade Paulista – UNIP, para obtenção do Título de Doutor em Patologia Ambiental e Experimental.

JOÃO LOPES MARTINS JÚNIOR

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DEDICATÓRIA

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SUMÁRIO

INTRODUÇÃO

Diversos estudos acerca da desmielinização e da remielinização no sistema nervoso central (SNC) têm sido empreendidos baseados no emprego do brometo de etídio (BE), uma droga intercalante gliotóxica (YAJIMA et al., 1979; BONDAN et al., 2004). Nesse modelo, observa-se o desaparecimento oligodendroglial e astrocitário, com consequente perda primária das bainhas de mielina, e ruptura da membrana limitante glial e da barreira hematoencefálica. A ausência dos processos astrocitários, em muitas áreas de lesão induzida pelo BE permite a entrada de linfócitos, de células piais infiltrantes e de células de Schwann, que acabam contribuindo para o reparo mielínico central (BONDAN et al.,1999, 2000).

A remielinização das lesões desmielinizantes tem sido obtida com sucesso por meio da transplantação de células gliais (UTZSCHNEIDER et al., 1994; HONMOU et al., 1996), sugerindo a possibilidade de reparo das lesões observadas em doenças como a esclerose múltipla dos seres humanos e a cinomose dos cães com o uso de enxertos de células mielinogênicas.

A busca de terapias que minimizem os sinais clínicos associados à perda mielínica requerem o desenvolvimento de modelos experimentais, que mimetizem as condições desmielinizantes de ocorrência natural e a avaliação comportamental e clínica minuciosa das deficiências desencadeadas.

Estudos *in vivo* e *in vitro* têm demonstrado que o derivado xantínico propentofilina (PPF) apresenta marcados efeitos neuroprotetores, anti-inflamatórios e antiproliferativos em situações de lesão no SNC e clinicamente a mesma já demonstrou eficácia no tratamento da demência vascular degenerativa e da isquemia cerebral, além de constituir-se em um potencial adjuvante na terapia da esquizofrenia e da esclerose múltipla (SWEITZER; DE LEO, 2011). Possíveis mecanismos de ação, embora não totalmente esclarecidos, incluem modulação direta sobre células gliais para redução da expressão do fenótipo reativo de astrócitos e micróglia, com diminuição da produção e liberação de fatores pró-inflamatórios e aumento *do clearence* astrocitário de glutamato, assim como modulando neurônios pré- e pós-sinápticos no tratamento da dor neuropática (SWEITZER; DE LEO, 2011).

A propentofilina [PPF, 3-metil-1- (5'-oxo-hexil) -7-PPFil xantina] é um derivado de xantina com efeitos farmacológicos distintos dos clássicos das metilxantinas teofilina e cafeína (SWEITZER; DE LEO, 2011).

A PPF mostrou-se capaz de diminuir a ativação de células microgliais e de astrócitos, cujas respostas estão associadas à lesão neuronal durante situações de inflamação e hipóxia,

consequentemente, diminuindo a produção e a liberação glial de fatores pró-inflamatórios prejudiciais (SCHUBERT et al., 1997; SI et al., 1998).

A ativação glial patológica, que induz à conversão de células da micróglia em estado quiescente em células com propriedades citotóxicas e ainda leva à desdiferenciação de astrócitos, está intrinsecamente associada à ocorrência das doenças neurodegenerativas e desmielinizantes. O efeito modulatório da adenosina na regulação dependente de Ca^{++} e de AMPc de tais células pode ser visto com a utilização de agonistas de adenosina, induzindo a diferenciação de astrócitos proliferantes e a expressão de suas propriedades neuroprotetoras, tais como formação de fatores tróficos, regulação iônica etc., assim como pode ser constatado pela inibição das propriedades neurotóxicas potenciais da micróglia, com depressão de sua taxa de proliferação e de conversão em macrófagos e diminuição da liberação de citocinas como TNF-alfa (SCHUBERT et al., 1997).

Efeitos similares foram obtidos com o uso de PPF, que atua como um inibidor de fosfodiesterase, levando ao aumento dos níveis de AMPc, além de conduzir ao aumento da concentração efetiva de adenosina por bloqueio de sua captação celular. Níveis extracelulares aumentados de adenosina, por sua vez, contribuem para a indução de apoptose microglial, dessa forma limitando o dano neural secundário a uma ativação glial patológica (SCHUBERT et al., 1997).

Além disso, a adenosina tem mostrado inibir a atividade sináptica e a liberação de vários neurotransmissores, tais como glutamato, serotonina e acetilcolina, agindo sobre receptores A1 pré-sinápticos (SALIMI et al., 2007).

Yamada et al. (1998) relataram para a PPF um efeito favorável sobre a função cognitiva em pacientes com doença de Alzheimer, sendo os distúrbios de aprendizagem e memória observados nessa doença atribuídos, pelo menos em parte, à perda de neurônios no sistema colinérgico.

Para o tratamento da demência, no entanto, a PPF ainda é considerada como droga especulativa, não sendo comercializada para tal.

A PPF parece ser geralmente bem tolerada, sendo distúrbios gastrointestinais, tonturas, dor de cabeça e azia os eventos adversos mais comuns durante os ensaios clínicos (NOBLE; WAGSTAFF, 1997).

Shimizu et al. (1993) relataram que a PPF inibe a liberação de dopamina (DA) durante uma isquemia transitória, modulando o seu metabolismo no corpo estriado do rato e protegendo as células neuronais por meio da inibição da liberação de glutamato durante tal situação. Tem sido sugerido que a liberação de glutamato induzida pela isquemia cerebral é

capaz de agravar os danos neuronais, acabando por levar à morte tardia dos neurônios, especialmente no hipocampo.

O corpo estriado é uma região abundantemente inervada por neurônios corticoestriatais glutamatérgicos e por vias nigroestriatais dopaminérgicas, que, possivelmente, interagem entre si (SHIMIZU et al., 1993).

Foi relatado que, durante a isquemia transitória, a função dos neurônios dopaminérgicos foi suprimida, sem recuperação após início da reperfusão. A concentração de adenosina extracelular, porém, aumentou significativamente durante a isquemia nos ratos tratados com PPF, sugerindo que seus efeitos protetores poderiam em parte ser atribuídos às ações de adenosina extracelular, e à inibição da liberação de neurotransmissores por meio do bloqueio do influxo de cálcio (SHIMIZU et al., 1993).

Em pacientes com esclerose múltipla (EM), a administração de PPF, em associação a outros dois inibidores de fosfodiesterase, mostrou reduzir e até abolir, em alguns pacientes, o índice de recidivas durante o período de tratamento (SUZUMURA et al., 2000).

No modelo experimental do BE em ratos, a PPF mostrou acelerar o processo de reconstrução das bainhas de mielina perdidas após injeção do gliotóxico no tronco encefálico (BONDAN et al., 2014) e até reverter o atraso encontrado na remielinização de ratos diabéticos (BONDAN et al., 2015).

Enquanto os ratos diabéticos injetados com BE apresentaram maiores quantidades de membranas derivadas de mielina nas áreas centrais das lesões e atraso considerável no processo remielinizante desempenhado por oligodendrócitos sobreviventes e por células de Schwann invasivas, a partir do 15º dia, os ratos diabéticos tratados com PPF apresentaram lesões semelhantes às de animais não diabéticos, com remielinização rápida nas bordas do sítio lesional e rápida remoção de restos de mielina da área central, sugerindo-se que a administração de PPF aparentemente foi capaz de inverter o comprometimento na remielinização induzido pelo estado diabético (BONDAN et al., 2015).

Bondan et al. (2006) sinalizam a necessidade de desenvolver modelos de experimentos que reflitam as condições demielinizantes:

> Muito embora sejam bastante conhecidos os efeitos ultraestruturais da injeção experimental do BE no tronco encefálico, com descrição morfológica detalhada do processo de perda e reparo das bainhas de mielina, o conhecimento exato da repercussão da desmielinização por ele induzida sobre a atividade motora dos animais permanece obscuro. Nesse sentido, é de fundamental importância o estabelecimento de um teste adequado para a detecção de possíveis déficits motores para o

> modelo no tronco encefálico, servindo como base para estudos futuros e para a avaliação precisa da eficácia de estratégias terapêuticas que visem à reabilitação de indivíduos portadores de áreas desmielinizadas no SNC. A busca de terapias que minimizem os sinais clínicos associados à perda mielínica requer o desenvolvimento de modelos experimentais que mimetizem as condições desmielinizantes de ocorrência natural e a avaliação comportamental e clínica minuciosa das deficiências desencadeadas. (p. 497)

O teste de campo aberto foi inicialmente desenvolvido para medir emocionalidade (WALSH; CUMMINS, 1976), mas também tem sido utilizado para medir outras respostas comportamentais, tais como a hiperatividade (FUKUSHIRO et al., 2008), o comportamento exploratório (CRAWLEY, 1985), a atividade locomotora (PATTI et al., 2005) e o comportamento de ansiedade (PRUT; BELZUNG, 2003). Vários estudos têm mostrado que o comportamento em campo aberto é modulado pela dopamina, particularmente no corpo estriado (KULKARNI; DANDIYA, 1975; BERNARDI; PALERMO-NETO, 1984; LAZARINI et al., 2001; PALM et al., 2014). A tendência natural do animal em ambientes novos é de exploração, apesar do medo provocado pela novidade (MONTGOMERY, 1958). Esse aparelho consiste de uma arena circular de madeira pintada de branco. O fundo da arena é dividido por meio de três círculos concêntricos em três partes que, por sua vez, são subdivididas em segmentos de reta aproximadamente iguais.

O teste da travessia da trave elevada (*beam walking test*) foi previamente utilizado para detectar déficits motores em ratos injetados com BE no funículo dorsal da medula espinhal cervical (JEFFERY et al., 1997) e no tronco encefálico (BONDAN et al., 2006), revelando nos animais que receberam o gliotóxico uma diminuição da segurança na colocação das patas ao atravessarem a referida passarela, o que não foi mais observado na quinta semana pós-injeção. Os animais injetados com salina exibiam déficits mínimos e rápida recuperação quanto à habilidade de atravessarem a passarela. Quando impedidos de apresentarem reparo das bainhas de mielina perdidas pela irradiação das lesões com 40 Gy de raio X, foi observada a incapacidade de os animais recuperarem a função perdida (JEFFERY et al., 1997).

Os astrócitos constituem as maiores e mais numerosas células gliais presentes no SNC dos mamíferos, excedendo o número de neurônios na proporção de 10:1 (BENVENISTE, 1992). Apesar de sua pronunciada heterogeneidade morfológica e bioquímica, os astrócitos caracterizam-se pela presença de prolongamentos dotados de filamentos intermediários (fibrilas gliais), cujo componente principal é a proteína glial fibrilar ácida (GFAP - *glial*

fibrillary acidic protein), servindo como meio de identificação desse tipo celular em estudos *in situ* e em cultivo (MONTGOMERY, 1994).

Dentre as inúmeras funções propostas para os astrócitos, destacam-se a manutenção da homeostasia no microambiente neural, exercendo importante papel na detoxificação, na captação de neurotransmissores e na regulação do pH, da osmolaridade e concentração iônica do tecido nervoso. Os astrócitos relacionam-se ainda com a orientação da migração neuronal durante o desenvolvimento do SNC, o suporte mecânico para os oligodendrócitos durante a mielinização, o reparo após agressões no tecido nervoso, a produção e secreção de proteínas da matriz extracelular, e com a síntese de moléculas de adesão, de fatores neurotróficos e promotores do crescimento de neuritos, a indução e manutenção das características de barreira hematoencefálica, a fagocitose de restos celulares e funções imunes, tais como secreção de citocinas (IL-1, IL-3, IL-6, IFN-γ e β, TNF) e expressão de moléculas MHC de classe I e II (PETERS et al., 1991; EDDLESTON; MUCKE, 1993; MONTGOMERY, 1994).

Independentemente da causa da lesão no SNC, o reparo do tecido é sempre realizado em maior ou menor grau com participação astrocitária. A reação dos astrócitos inclui o aumento de seu número (astrocitose) e de suas dimensões (astrogliose), além de várias outras alterações funcionais, como espessamento dos feixes de filamentos gliais e consequente aumento da intensidade de marcação de GFAP (EDDLESTON; MUCKE, 1993; MONTGOMERY, 1994). Esses fenômenos têm sido referidos como gliose astrocitária, astrocitose e astrogliose reativas, cicatriz glial ou simplesmente gliose, podendo ser de dois tipos de acordo com o tipo de dano provocado - isomórfica, em que os processos astrocitários se apresentam orientados pelos elementos teciduais preservados e o arranjo dos feixes de filamentos gliais é uniforme e paralelo, e anisomórfica, em que sua disposição é irregular ao redor de lesão geralmente causadora de dano morfológico grosseiro na estrutura do tecido, com ruptura da barreira hematoencefálica (FERNAUD-ESPINOSA et al., 1993; BIGNAMI; DAHL, 1994).

O presente estudo teve como objetivo investigar se o tratamento com PPF pós-injeção do gliotóxico mostrou-se capaz de afetar ou reverter os déficits locomotores induzidos pelo BE e verificados mediante emprego dos testes do campo aberto e da travessia da trave elevada, comparando os resultados encontrados com aqueles de ratos injetados com BE e não-tratados com PPF. Pretendeu-se observar a evolução das prováveis perdas motoras ao longo de diferentes períodos pós-injeção nos dois grupos (tratados e não-tratados com PPF), a fim de se determinar se existiu recuperação funcional significativa no período analisado e se a referida xantina foi capaz de influenciar positivamente tal recuperação. Ainda, em função da

reconhecida função da PPF de impedir a ativação glial excessiva, o estudo também objetivou avaliar o efeito da droga sobre a resposta astrocitária após a injúria induzida pelo BE, mediante quantificação da expressão da proteína GFAP por imuno-histoquímica.

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Artigo 1

Propentofylline treatment on open field behavior and beam walking test in rats with focal ethidium bromide-induced demyelination in the ventral surface of the brainstem

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Running head: Open field behavior, beam walking test and propentofylline

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ABSTRACT

Propentofylline (PPF) is a xanthine derivative with pharmacological effects that are distinct from those of classic methylxanthines. It depresses the activation of microglial cells and astrocytes, which is associated with neuronal damage during neural inflammation and hypoxia. Our previous studies showed that PPF improved remyelination following gliotoxic lesions that were induced by ethidium bromide (EB). In the present study, the long-term effects of PPF on open field behavior in rats with EB-induced focal demyelination were examined. The effects of PPF were first evaluated in naive rats that were not subjected to EB lesions. Behavior in the beam walking test was also evaluated during chronic PPF treatment because impairments in motor coordination can interfere with behavior in the open field. The results showed that PPF treatment in unlesioned rats decreased general activity and caused motor impairment in the beam walking test. Gliotoxic EB injections increased general activity in rats that were treated with PPF compared with rats that received saline solution. Motor incoordination was also attenuated in PPF-treated rats. These results indicate that PPF reversed the effects of EB lesions on behavior in the open field and beam walking test.

Keywords: beam walking test; demyelination; ethidium bromide; open field behavior; propentofylline; remyelination

Highlights

- ✓ Rats were submitted (EB) or not (NEB) to ethidium bromide-induced focal demyelination.
- ✓ The long-term effects of propentofylline (PPF) were examined on open field behavior and motor coordination
- ✓ In rats of EB group, PPF restored both the open field behavior and motor coordination.
- ✓ In rats of NEB group, PPF decreased both the open field behavior and motor coordination.
- ✓ These contradictory data were explained by the PPF-induced decrease in dopaminergic function.

INTRODUCTION

Important functional roles have been increasingly ascribed to glial cells in states of both health and disease (Barres 2008; Aguzzi et al. 2013; Reissner et al. 2014). Several *in vitro* and *in vivo* studies have shown that propentofylline (PPF; 3-methyl-1-[5'-oxohexyl]-7-propylxanthine), a xanthine derivative, exerts profound neuroprotective, antioxidant, and antiinflammatory effects (Sweitzer and De Leo 2011). It has shown clinical efficacy in degenerative vascular dementia (Kittner et al. 1997) and as a potential adjuvant treatment for Alzheimer's disease (Koriyama et al. 2003), schizophrenia (Salimi et al. 2008), and multiple sclerosis (Suzumura et al. 2000). PPF depresses the activation of microglial cells and astrocytes, which is associated with neuronal damage during inflammation and hypoxia and consequently decreases the glial production and release of damaging proinflammatory factors (Sweitzer and De Leo 2011).

In rats, 7 days of PPF administration significantly decreased both cue- and cocaine-induced reinstatement of cocaine seeking, effects that were attributable to its ability to restore glutamate transporter-1 expression in the nucleus accumbens (Reissner et al. 2014). Systemic treatment with PPF blocked both methamphetamine- and morphine-induced conditioned place preference (Narita et al. 2006). It also improved learning and memory deficits that were induced by β-amyloid protein infusion (1-40) in a rat model of Alzheimer's disease (Yamada et al. 1998). PPF also plays a modulatory role in pain (Zhang et al. 2013) by blocking proinflammatory factors that are related to pain pathways in the central nervous system.

In aged dogs, repeated PPF administration did not affect locomotion in an open field (Siwak et al. 2000). However, in a model of ethidium bromide (EB)-induced gliotoxic injury, PPF significantly increased both oligodendroglial and Schwann cell remyelination at 31 days (Bondan et al. 2014). Previous studies showed that EB-induced demyelination in the

brainstem caused locomotor deficits in the beam walking test in rats 3-31 days post-injection, and remyelination was related to the recovery of function (Bondan et al. 2006).

The open field test was initially developed to measure emotionality (Walsh and Cummins 1976), but it has also been used to measured other behavioral responses, such as hyperactivity (Fukushiro et al. 2008), exploratory behavior (Crawley 1985), locomotor activity (Patti et al. 2005), and anxiety-like behavior (Prut and Belzung 2003). Several studies have shown that open field behavior is modulated by dopamine, particularly in the striatum (Kulkarni and Dandiya 1975; Bernardi and Palermo-Neto 1984; Lazarini et al. 2001; Palm et al. 2014).

Glutamate aggravates neuronal damage associated with injury of the central nervous system, such as damage that is produced by ischemia. The striatum is richly innervated by both corticostriatal glutamatergic neurons and nigrostriatal dopaminergic neurons. The release of both transmitters is somewhat related to ischemic neuronal damage. PPF afforded protection against ischemic damage in striatal dopaminergic neurons (Shimizu et al. 1993).

The present study was performed to investigate the effects of long-term PPF administration on open field behavior in rats with EB-induced gliotoxic injury. The effects of PPF were first examined in naive rats that were not subjected to EB lesions. Behavior in the beam walking test was evaluated during chronic PPF treatment because motor incoordination can interfere with behavior in the open field.

MATERIALS AND METHODS

Ethics Statement

This experiment was approved by the Ethics Commission of the University Paulista (protocol no. 182/13). All efforts were made to minimize suffering of the animals and reduce

the number of animals used. The experiments were performed in accordance with good laboratory practice protocols and quality assurance methods.

Animals, treatments, and experimental design

A total of 35 male Wistar rats, 4-5 months of age, were used. They were housed in polypropylene cages (38 cm × 32 cm × 16 cm; 3-4 rats per cage) at a controlled temperature (22°C ± 2°C) and humidity (55-60%) with artificial lighting (12 h/12 h light/dark cycle, lights on at 6:00 AM). The animals had free access to Nuvilab rodent chow (Nuvital, Sao Paulo, SP, Brazil) and filtered water. Sterilized and residue-free wood shavings were used for animal bedding. All of the experiments, including treatments and behavioral observations, were performed between 9:00 AM and 1:00 PM to minimize the effects of circadian rhythms.

The rats were randomly divided into five groups (n = 7 per group). Two experiments were performed. In the first experiment, 14 rats were divided into two equal groups: PPF group (injected with 12.5 mg/kg PPF daily [20 mg/ml], intraperitoneal [i.p.]; Agener União Quíımica, São Paulo, SP) and control group (injected with 1 ml/kg of 0.9% saline solution, i.p., for the same period of time). The open field test and beam walking test were performed on days 3, 7, 11, 15, 21, and 31 of treatment. In the second experiment, 21 rats were divided into three equal groups: EB+SAL (injected with 10 µl of 0.1% EB solution into the cisterna pontis and treated with 0.9% saline solution, i.p., for 31 days), SAL+PPF (injected with 10 µl of 0.9% saline solution into the cisterna pontis and treated with 12.5 mg/kg PPF daily, i.p., for 31 days), and EB+PPF (injected with 10 µl of 0.1% EB solution into the cisterna pontis and treated with 12.5 mg/kg PPF daily, i.p., for 31 days). These rats were treated and observed similarly to rats in Experiment 1.

Surgical procedure

The rats were anesthetized with thiopental (50 mg/kg, i.p.), and a burr hole was drilled on the right side of the skull, 8 mm rostral to the fronto-parietal suture. They were submitted to a local injection of 10 μl of 0.1% EB into the cisterna pontis, an enlarged subarachnoid space below the ventral surface of the pons, performed freehand using a Hamilton syringe of 10 ml,fitted with a 35° angled polished gauge (26 s) needle.

Open field test

The open field apparatus was previously described by Bernardi and Palermo-Neto (1984). The test was performed in a small room with dim lighting. Each rat was individually placed in the center of the apparatus, and the following parameters were recorded over 5 minutes: total locomotion (one unit was defined as the animal entering one square of the floor with all four paws), peripheral locomotion (one unit was defined as the animal entering the peripheral areas with all four paws), rearing frequency (one unit was defined as the animal standing upright on its hindlimbs), immobility time (time, in seconds, without movement), and number of entries in the central area. The frequencies of locomotion and rearing and duration of immobility were determined to evaluate possible effects of the treatments on motor/exploratory behavior (Bernardi et al. 1981). Peripheral locomotion is considered an index of anxiety (Campos et al. 2013). The apparatus was washed with a 5% alcohol/water solution before placement of the animals to obviate possible bias caused by odor cues left by previous rats.

Beam walking test

Motor coordination was evaluated on a wooden beam as previously described by (Rodrigues-Alve et al. 2009). This model was adapted from the one described by Jeffery and Blakemore (1997). The apparatus was a wooden beam (18 mm width × 18 mm thickness × 2

m length) with a 100 mm^2, 18 mm thick platform at each end. The beam was elevated 20 cm above the floor and painted white with two black vertical marks delimiting 1 m in the central portion. Each rat was trained to walk on the beam in 5 min daily sessions. On the first day, positive reinforcement was employed, in which a small portion of condensed milk was placed on both platforms to habituate the rat to the environment and reinforcement. The next day, the animal was placed on the beam, close to the platform with the reinforcement, with the head facing the local of reinforcement. On subsequent days, the rat was placed on the beam but at progressively farther distances from the platform with the reinforcement, until the animal crossed the entire length of the beam to reach the platform with the reinforcement. It was then returned to the initial platform. The rats always received the reinforcement after each crossing. The training period (7-10 days) was considered complete when each rat reliably crossed the beam without stalling (i.e., four crossings). Few footstep errors were made during this training stage. The animals that were unable to walk the entire length of the beam after 10 days were excluded from the experiment. After training, the rats were subjected to their respective treatment regimens, and observations in the beam walking test were made on days 3, 7, 11, 15, 21, and 31 of treatment. In each observation, a score (Table 1) was attributed for each step of the pelvic member, turned for the observer, when the rat walked in the central portion of beam. The number of steps was also measured. At the end of each session, the scores for each animal for the four crossings were cumulated. The ratio of total score/total number of steps was also calculated. Before each animal was tested, the wooden beam was cleaned with a cloth that was moistened with water. After all of the animals that were housed together in one cage completed the test, the wooded beam was cleaned with a 5% ethanol solution before the next cage of animals was tested. Total scores represent the sum of all scores given to rats from the same group obtained during all periods of observation (day 3 to 31 days of observation).

Statistical analysis

Homogeneity was verified using the F test or Bartlett's test. Normality was verified using the Kolmogorov-Smirnov test. In both experiments, the Two-way ANOVA followed by Bonferroni's multiple-comparison test was used to compare data in the open field test. In experiment 1, the Student's *t*-test was used to analyze differences between two groups for parametric data and for comparisons of total scores and the ratio of total score/total number of steps in the beam walking test between two groups, the Mann-Whitney test was employed. In Experiment 2, one-way analysis of variance (ANOVA) was used to analyze the number of steps. Total scores and the ratio of total score/total number of steps were analyzed using the Kruskal-Wallis test followed by Dunn's multiple-comparison test. The results are expressed as mean ± SEM or median (minimum and maximum). In all cases, the results were considered significant at $p < 0.05$.

RESULTS

Experiment 1. Effects of PPF on open field behavior in rats

No interaction between treatment and days of observation was observed for total locomotion ($F_{5,72} = 0.90$, $p = 0.48$), but significant effects of treatment ($F_{1,72} = 20.34$, $p < 0.0001$) and days of observation ($F_{5,72} = 9.94$, $p < 0.0001$) were found. PPF treatment reduced total locomotion on day 31 of treatment compared with the control group (Fig. 1A). No interaction between treatment and days of observation was observed for peripheral locomotion ($F_{5,72} = 1.53$, $p = 0.19$), but significant effects of treatment ($F_{1,72} = 11.47$, $p = 0.001$) and days of observations ($F_{5,72} = 10.35$, $p < 0.0001$) were found. PPF treatment reduced peripheral locomotion on day 31 of treatment compared with the control group (Fig. 1B). No interaction between treatment and days of observation was observed for rearing frequency ($F_{5,72} = 1.89$, $p = 0.11$), but significant effects of treatment ($F_{1,72} = 71.37$, $p <$

0.0001) and days of observation ($F_{5,72}$ = 7.57, p < 0.0001) were found. PPF treatment reduced rearing frequency from day 11 to 31 of treatment compared with the control group (Fig. 1C). The number of entries in the central area of the open field was affected by PPF treatment ($F_{1,72}$ = 5.22, p = 0.03) but not by days of observation ($F_{5,72}$ = 0.78, p = 0.57), with no interaction between factors ($F_{5,72}$ = 0.95, p = 0.43). A decrease in the number of entries in the central area of the open field was observed on days 7, 11, 21, and 31 of treatment compared with the control group. No differences were found in immobility time between groups (data not shown).

No errors were observed in total scores in the beam walking test in the control group. Rats that were treated with PPF exhibited an increase in the number of errors (U = 0.019; Fig. 2A). The ratio of total score/total number of steps (Fig. 2C) increased in the PPF group compared with the control group. No differences in the number of steps were observed between groups (t = 1.93, df = 12, p = 0.18; Fig. 2B).

Experiment 2. Effects of PPF on open field behavior in rats after EB injection

Total locomotion (Fig. 3A) was affected by both treatment ($F_{2,96}$ = 53.04, p < 0.0001) and days of observation ($F_{5,96}$ = 35.09, p < 0.0001), with a significant interaction between factors ($F_{10,96}$ =10.16, p < 0.0001). On day 3 of treatment, the SAL+PPF and EB+PPF groups exhibited higher total locomotion compared with the EB+SAL group. Total locomotion in the EB+PPF group increased compared with the EB+SAL group on days 3, 7, 11, 15, and 21 of treatment.

Peripheral locomotion (Fig. 3B) was affected by both treatment ($F_{2,96}$ = 40.71, p < 0.0001) and days of observation ($F_{5,96}$ = 34.95, p < 0.0001), with a significant interaction between factors ($F_{10,96}$ = 14.11, p < 0.0001). The SAL+PPF group exhibited an increase in peripheral locomotion on days 3 and 7 of observation compared with the EB+SAL group. The

EB+PPF group exhibited an increase in peripheral locomotion on days 3, 11, 21, and 31 of treatment compared with the EB+SAL group.

Rearing frequency (Fig. 3C) was affected by days of observation ($F_{5,96}$ = 80.21, p < 0.0001) but not by treatment ($F_{2,96}$ = 13.51, p < 0.0001), with a significant interaction between factors ($F_{10,96}$ = 11.14, p < 0.0001). Rearing frequency increased in the SAL+PPF group on days 3, 7, and 31 of treatment compared with the EB+SAL group. The EB+PPF group exhibited a decrease in rearing frequency on day 15 of treatment and an increase in rearing frequency on day 21 of treatment compared with the EB+SAL group.

For immobility time (Fig. 3D), a significant interaction between treatment and days of observation was observed ($F_{5,96}$ = 6.49, p < 0.0001), with significant main effects of treatment ($F_{2,96}$ = 19.02, p < 0.0001) and days of observation ($F_{5,96}$ = 15.79, p < 0.0001). The SAL+PPF group exhibited a decrease in immobility time compared with the EB+SAL group at the end of treatment. A decrease in immobility time was observed in the EB+PPF group on days 7, 11, and 15 of treatment compared with the EB+SAL group.

The frequency of entries in the central area of the open field (Fig. 3E) was affected by treatment ($F_{2,96}$ = 96.17, p < 0.0001) and days of observation ($F_{5,96}$ = 96.17, p < 0.00001), with a significant interaction between factors ($F_{10,96}$ = 11.68, p < 0.0001). The SAL+PPF group exhibited an increase in the number of entries in the central area on day 3 of treatment compared with the EB+SAL group. The EB+PPF group exhibited an increase in the number of entries in the central area on all days of treatment, with the exception of day 21, compared with the EB+SAL group.

The total number of steps ($F_{2,18}$ = 1.21, p = 0.32; Fig. 4A) and ratio between total score/total number of steps (KW = 1.06, p = 0.59; Fig. 4C) in the beam walking test were unaffected by treatment. Total scores decreased significantly in the SAL+PPF and EB+PPF groups compared with the EB+SAL group (KW = 11.67, p = 0.003; Fig. 4B).

DISCUSSION

Chronic PPF treatment gradually reduced general activity in rats until the end of treatment. The most affected parameters were rearing frequency and the number of entries in the central area of the open field. Total and peripheral locomotion gradually decreased, and significant differences were observed only on the last day of treatment. Immobility time was unaffected by chronic PPF exposure. Two hypotheses may explain these effects of PPF on open field behavior: (*i*) PPF increased anxiety-like behavior or (*ii*) PPF decreased exploratory behavior.

Rodents live in social groups and small tunnels. Anxiolytic treatments do not themselves increase exploration in the open field but decrease the stress-induced inhibition of exploratory behavior. An increase in central locomotion or time spent in the central area of the open field, with no changes in total locomotion or vertical exploration, can be interpreted as an anxiolytic-like effect, whereas converse effects (i.e., decreases in these variables) are interpreted as anxiogenic-like effects (Prut and Belzung 2003).

However, a decrease in locomotion was observed only on day 31 of treatment, whereas rearing frequency, associated with a decrease in the number of entries in the central area, occurred on several days of observation. Thus, these data cannot be attributed to an anxiogenic-like effect of PPF.

The present results may also be attributable to an effect of PPF on motor performance. Motor coordination was examined in rats that were treated with PPF, which exhibited an increase in the number of errors in the beam walking test compared with untreated rats. Thus, the decrease in general activity in the open field, particularly in rearing behavior, may be at least partially attributable to interference with motor coordination. Indeed, rearing behavior

needs proper motor skills (Syme 1975) and the impairment of this parameter could be explained by the reduced motor coordination in rats treated with PPF.

Locomotion and rearing frequency in the open field are related to the central dopaminergic system (Bernardi and Palermo-Neto 1984; Oliveira de Almeida et al. 2014). Drugs that reduce dopaminergic activity also decrease locomotion and rearing frequency in the open field (Bernardi et al. 1981). PPF has been reported to inhibit the release of dopamine during transient ischemia and modulate dopamine metabolism in the striatum in rats (Shimizu et al. 1993). Thus, PPF may be responsible for the reduction of rearing frequency in the open field through an action on the dopaminergic system.

As previously reported by our group, both oligodendroglial and Schwann cell remyelination increased 31 days after an injection of the demyelinating agent EB in the brainstem in PPF-treated rats compared with untreated animals (Bondan et al. 2014). We also found a relationship between an increase in remyelination and motor coordination in the beam walking test (Bondan et al. 2006).

In the open field, total locomotion and the frequency of peripheral locomotion increased on day 3 in both the SAL+PPF and EB+PPF groups compared with the EB+SAL group, suggesting a stimulant effect of PPF. A peak of activity occurred on day 11 of treatment in the EB+PPF group. In the remaining sessions, this group also exhibited a general increase in these parameters. Conversely, immobility time in the EB+PPF group decreased compared with the EB+SAL group, suggesting that PPF reversed the effects of EB-induced injury.

Despite some evidence to the contrary, peripheral locomotion has been related to a decrease in anxiety (Lamprea et al. 2008). An increase in the number of entries in the central area of the open field has also been correlated with a decrease in anxiety (Lister 1990). The increase in the number of entries in the central area of the open field and increase in

peripheral locomotion do not agree with an anxiolytic-like effect of PPF but may be a result of an increase in general activity that is induced by the drug.

With regard to scores in the beam walking test, PPF treatment reversed the effects of EB lesions. In both the open field test and beam walking test, PPF appeared to protect rats from demyelinating lesions that were induced by EB. This protection have also been reported to protect neuronal cells through the inhibition of glutamate release during transient ischemia (Shimizu et al. 1993). In rats that were perfused with PPF through a microdialysis probe that was placed in the striatum during 20 min transient ischemia, dopamine release was significantly inhibited and dopamine metabolism presented better recovery compared with unperfused rats.

Rearing behavior is a parameter of vertical exploratory behavior that competes with locomotor behavior in the open field. Increases in locomotion prevent the expression of rearing. The significant decrease in rearing frequency on day 15 of observation may have resulted from an increase in locomotion.

In conclusion, long-term treatment with PPF appeared to rescue open field behavior after EB-induced gliotoxic injury. PPF has been shown to have a wide range of beneficial effects that are related to maintaining the integrity of and repairing nervous tissue. It has also been shown to have antiinflammatory, antioxidant, and neuroprotective actions, inhibit glutamate release, decrease glial activation, and decrease the production of damaging proinflammatory factors that are released from microglia and astrocytes (Sweitzer and De Leo 2011). In unlesioned rats, the decrease in locomotor activity that was induced by PPF may be related to interference with the dopaminergic system (Shimizu et al. 1993).

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Figures

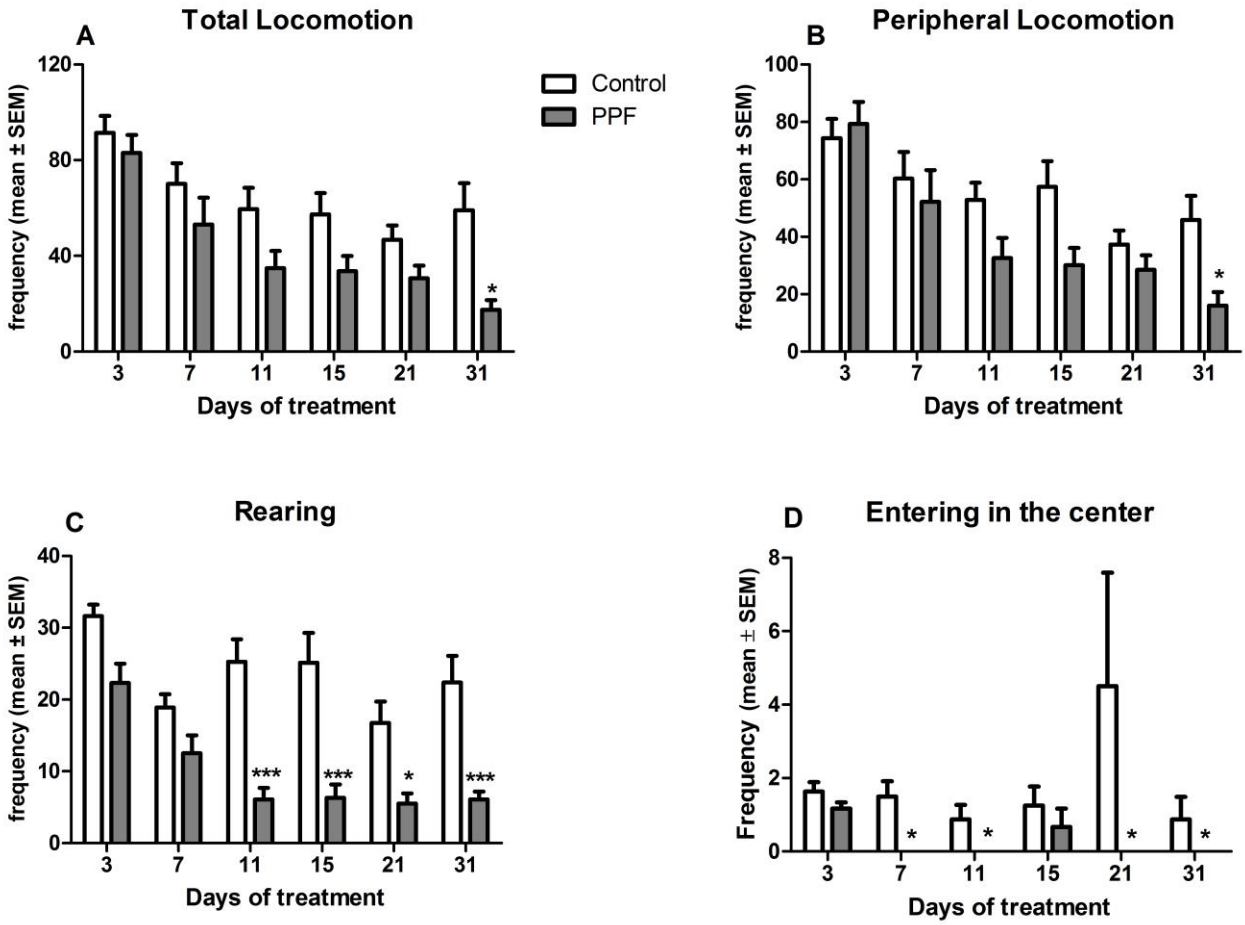

Fig. 1. Effects of long-term propentofylline treatment on general activity in rats treated for 31 days and observed on days 3, 7, 11, 15, 21, and 31. The data are expressed as mean ± SEM. *n* = 7 per group. **p* < 0.05, ****p* < 0.001, compared with control group (two-way ANOVA followed by Bonferroni test).

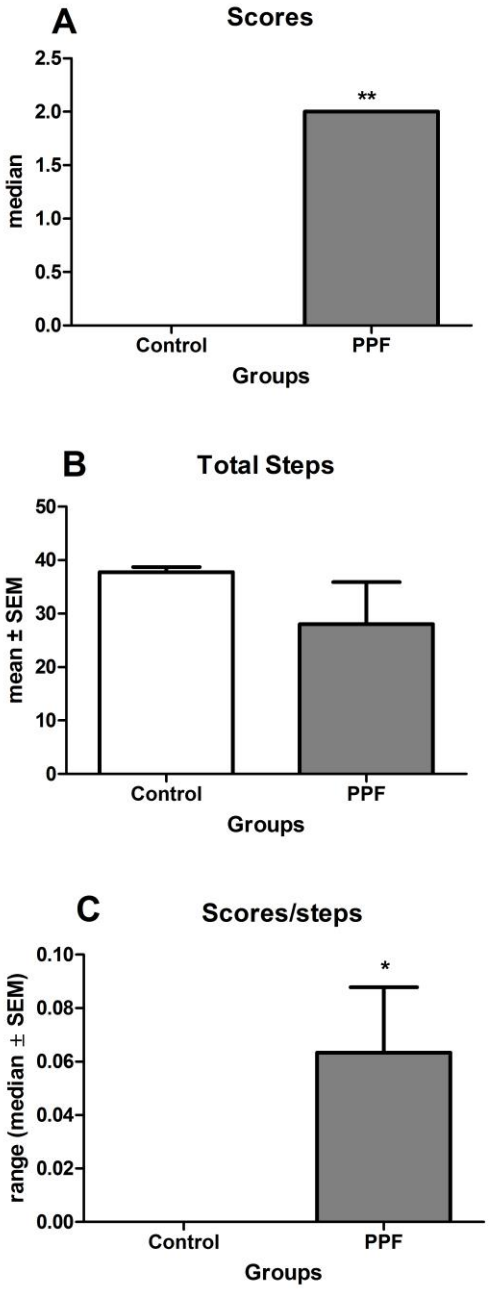

Fig. 2. Total scores for motor coordination, total number of steps, and ratio between total scores for motor coordination and total number of steps in rats treated for 31 days with propentofylline (PPF) and observed on days 3, 7, 11, 15, 21, and 31 of treatment. The data are expressed as mean ± SEM or median. *$p < 0.05$, **$p < 0.019$, compared with control group (Student's *t*-test, Mann-Whitney test).

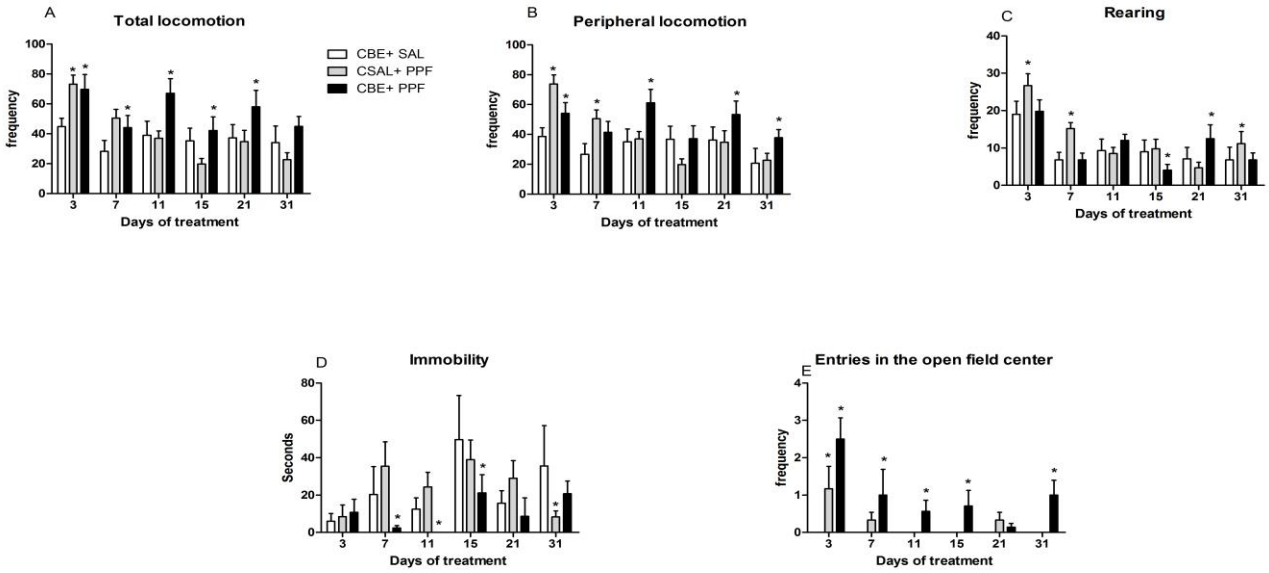

Fig. 3. General activity in rats with focal induction of demyelination using the EB model in the ventral surface of the brainstem, treated or not with propentofylline (PPF) for 31 days, and observed on days 3, 7, 11, 15, 21, and 31. The data are expressed as mean ± SEM. $n = 7$ group. *$p < 0.05$, compared with control group (two-way ANOVA followed by Bonferroni test).

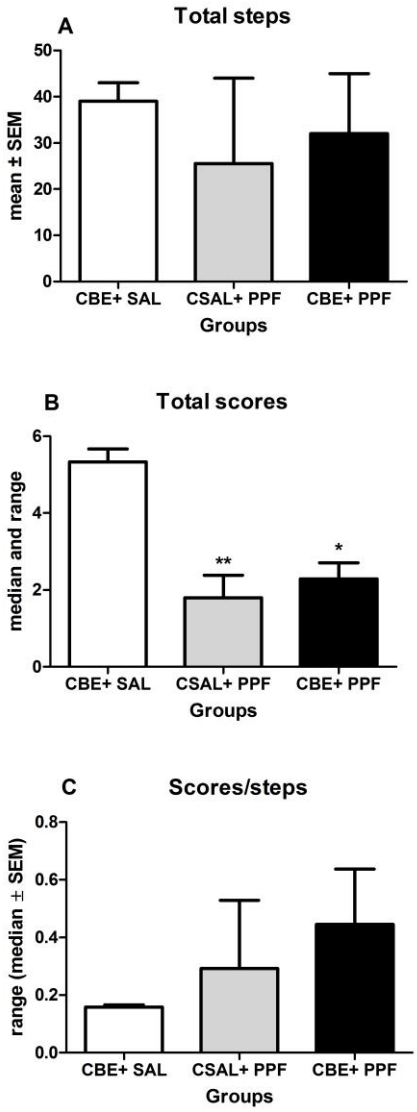

Fig. 4. Total number of steps, total scores for motor coordination, and ratio between total scores for motor coordination and total number of steps in rats with focal induction of demyelination using the EB model in the ventral surface of the brainstem, treated or not with propentofylline (PPF) for 31 days, and observed on days 3, 7, 11, 15, 21, and 31. The data are expressed as mean ± SEM or median and respective limits. The total number of steps was analyzed by one-way ANOVA followed by Tukey's multiple-comparison test. Total scores and the ratio of total score/total number of steps were analyzed by the Kruskal-Wallis test followed by Dunn's multiple-comparison test. *$p < 0.05$, **$p < 0.01$, compared with control group.

Anexos

Table 1. Motor coordination scores in the beam walking test.

Score	Foot position
0	Normal foot position on top of beam, no slippage.
1	Minor error: foot slip so that part of the foot is visible below the lower surface of the beam.
2	Major error: whole foot slip below the lower surface of the beam.

Artigo 2

Effect of propentofylline on astrocyte response and GFAP expression following gliotoxic damage in the rat brainstem

Efeito da propentofilina na resposta astrocitária e na expressão de GFAP após dano gliotóxico no tronco encefálico de ratos

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Running title: Effect of propentofylline on astrocyte response

ABSTRACT

Propentofylline (PPF) is a xanthine derivative that depresses activation of glial cells, whose responses contribute to neural tissue damage during inflammation. It is known that ethidium bromide (EB) injection into the central nervous system induces local oligodendroglial and astrocytic loss, resulting in primary demyelination, neuroinflammation and blood-brain barrier disruption. Surviving astrocytes present a vigorous reaction around the injury site with increased immunoreactivity to glial fibrillary acidic protein (GFAP). This study aimed to evaluate the effect of PPF administration on astrocytic response following gliotoxic injury. Wistar rats were injected with EB into the cisterna pontis and treated or not with PPF (12.5mg/kg/day, intraperitoneal) during the experimental period. Brainstem sections were collected from 15 to 31 days after EB injection and processed for GFAP immunohistochemistry. Results demonstrate that PPF decreased astrocytic activation until the 21st day, suggesting that this drug may have a role in reducing glial scar development following injury.

Key words: astrocytes, ethidium bromide, gliosis, gliotoxin, xanthine

RESUMO

A propentofilina (PPF) é uma xantina que deprime a ativação das células gliais, cujas respostas contribuem para o dano neural durante inflamação. A injeção de brometo de etídio (EB) no sistema nervoso central induz a perda oligodendroglial e astrocitária, resultando em desmielinização, neuroinflamação e ruptura da barreira hematoencefálica. Os astrócitos sobreviventes apresentam vigorosa reação ao redor da lesão com aumento da imunorreatividade à proteína glial fibrilar ácida (GFAP). Este estudo objetivou avaliar o efeito da PPF sobre a resposta astrocitária após injúria gliotóxica. Ratos Wistar foram injetados com EB na cisterna basal e tratados ou não com PPF (12.5mg/kg/dia, intraperitoneal). Amostras do tronco encefálico foram coletadas dos 15 aos 31 dias pós-injeção de EB e processadas para estudo ultraestrutural e imuno-histoquímico para GFAP. Os resultados demonstram que a PPF reduziu a ativação astrocitária até o 21º dia, sugerindo que essa droga pode atuar na redução da cicatriz glial após injúria.

Palavras-chave: astrócitos, brometo de etídio, gliose, gliotoxina, xantina

INTRODUCTION

It is widely described that ethidium bromide (EB) injection in the white matter of the central nervous system (CNS) acts like a gliotoxin causing local oligodendroglial and astrocytic death, with consequent demyelination (although the naked axons remained preserved), blood-brain barrier disruption and Schwann cell invasion due to the glia limitans breakdown[1,2,3]. Surviving astrocytes presented a vigorous reaction around the injury site with increased immunorreactivity to the specific cell marker glial fibrillary acidic protein (GFAP) and reexpression of vimentin (VIM)[2].

Propentofylline [PPF, 3-methyl-1-(5´-oxohexyl)-7-propylxanthine] is a xanthine derivative with pharmacological effects distinct from those of the classical methylxanthines theophylline and caffeine[4]. *In vitro* and *in vivo* studies have demonstrated extensive neuroprotective, antiproliferative and anti-inflammatory effects of PPF in several experimental models in animals[4]. It was sucessfully used in degenerative vascular dementia and as a potential adjuvant treatment to Alzheimer`s disease, schizophrenia and multiple sclerosis[4]. PPF decreases activation of microglial cells and astrocytes, whose responses are associated with neuronal damage during inflammation and hypoxia, and consequently decreases glial production and release of damaging proinflammatory factors[5,6].

In the EB-demyelinating model, PPF administration has showed to significantly increase both oligodendroglial and Schwann cell remyelination following gliotoxic damage[7] and even reversed the impairment in remyelination found in diabetic rats[8]. Despite the beneficial effects of PPF observed on oligodendrocyte remyelinating activity in these investigations, astrocyte behavior was not properly evaluated. Thus, the aim of this study was to evaluate if PPF had the capacity of affecting astrocyte responses during the process of demyelination and remyelination following gliotoxic injury induced by ethidium bromide (EB).

METHOD

The animal procedures were performed in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources and Brazilian Institutional Ethics Committee, Universidade Paulista (protocol number 182/13, CEUA/ICS/UNIP). Seventy-two adult (4-5 month old) male Wistar rats were submitted to a local injection of 10 microlitres of 0.1% EB into the cisterna pontis, an enlarged subarachnoid space below the ventral surface of the pons. All rats were anaesthetized with ketamine and xylazine (5:1; 0.1

ml/100g) and 2.5% thiopental (40 mg/ml) by intraperitoneal (IP) route and a burr-hole was made on the right side of the skull, 8 mm behind the fronto-parietal suture. Injections were performed freehand using a Hamilton syringe, fitted with a 35º angled polished 26 gauge needle into the cisterna pontis. Rats were then distributed into two groups - untreated rats (group I, n=36) and rats treated with 12.5 mg/kg/day of PPF (Agener União Química, São Paulo, SP, 20 mg/ml solution) by IP route during the experimental period (group II, n=36). The animals were kept under controlled light conditions (12 h light-dark cycle) and water and food were given ad libitum during the experimental period.

For ultrastructural investigation, four rats from each group were anaesthetized and were submitted to intracardiac perfusion with 4% glutaraldehyde in 0.1 M Sorensen phosphate buffer (pH 7.4) at each of the following periods - 15, 21 and 31 days post-injection (p.i.). Thin slices of the brainstem (pons and mesencephalon) were collected and post-fixed in 0.1% osmium tetroxide, dehydrated with graded acetones and embedded in Araldite 502 resin, following transitional stages in acetone. Thick sections were stained with 0.25% alkaline toluidine blue. Selected areas were trimmed and thin sections were stained with 2% uranyl and lead acetate and viewed in a JEM -1200 EX2 JEOL transmission electron microscope.

For immunohistochemical study of the expression of the astrocytic marker GFAP, 8 rats were anaesthetized and submitted to intracardiac perfusion with buffered 10% formaldehyde solution at each of the same periods. Their brains were then removed and kept for 3 days in the same fixative. Coronal sections from the brainstem were mounted on silanized slides and submitted to GFAP immunostaining using the avidin-biotin peroxidase complex (ABC) method. Briefly, the sections were desparaffinized in xylene and rehydrated in a crescent graded series of ethanol solutions. Antigen retrieval was done by transferring the slides to 10 mM sodium citrate buffer (pH 6.0) at 95º C for 20 minutes. Endogenous peroxidase was blocked by 3% hydrogen peroxide for 10 minutes at room temperature. Two washes with Tris/HCl buffer pH 6.0 (Wash buffer 10x, S3006, Dako, Glostrup, Danmark) were done between incubations. Polyclonal rabbit anti-GFAP immunoglobulin (Z0334, Dako), at a dilution of 1:1000, was used as primary antibody, for 16 hours, followed by the application of biotinylated secondary antibody (Dako Universal LSAB^{TM} 2 System - HRP, K0690), according to the manufacturer`s instructions. Immunoreactivity was visualized by incubating the sections in a solution containing 0.1% diaminobenzidine (DAB, K3467, Dako). Sections were then counterstained by Harris´ modified hematoxylin solution, dehydrated and mounted in Entellan (Merck, Germany).

Astrocytic evaluation was done in the brainstem of animals from both groups using a computerized image analysis system (Image-Pro-Plus 4.5, Media Cybernetics, Silver Spring, USA), measuring by colorimetry the area stained brown in a total area of 302,952.5 μm^2. Negative controls for immunostaining (sections lacking primary antibody application) were done. Data were analyzed by t test and statistical significance was set at $p < 0.05$.

RESULTS

The general aspect of the EB-induced lesions found in this investigation was similar to that previously described in other studies using this gliotoxin in the rat brainstem (see Pereira et al., 1998 and Bondan et al., 2000). Briefly, they presented extensive demyelinated areas in the ventral surface of the mesencephalon and pons and contained, in the central region, phagocytic cells, myelin debris and naked axons. At the periphery, oligodendrocytes and Schwann cells were observed, the latter occurring in areas of enlarged extracellular spaces devoid of astrocytic extensions. Astrocyte processes were invariably seen near the incipient, but preponderant, oligodendroglial remyelination at the periphery, and Schwann cells also appeared to contribute to myelin repair. Ultrastructural analysis apparently showed that astrocytic processes among oligodendrocyte remyelinated axons were slightly thinner in PPF-treated animals (Fig. 1B) compared to those that had not received the xanthine (Fig. 1A). Although oligodendroglia prevailed in the brainstem myelin repair at the 15th at 31st day, sheaths formed by Schwann cells in astrocyte-free areas were thicker than those produced by oligodendrocytes during the same period. As described earlier in a former investigation (see Bondan et al. 2014), PPF-treated rats presented an increased remyelination from the 15^{th} to the 31^{st} day following EB injection. Some lymphocytes and infiltrating pial cells were occasionally seen, the first contacting phagocytic cells and myelin debris.

By GFAP immunohistochemical staining, it was observed that the EB-induced lesions from group II (PPF-treated rats) apparently presented a decreased astrocytic reaction close to the edges of the injury site, with the observation of fewer and thinner GFAP-stained processes at the periphery at both 15 (Fig. 2A,B) and 21 days (Fig. 2C,D), No astrocytes were observed in the central areas of the lesions even at 31 days after EB injection.

Table 1 presents the mean areas with GFAP staining in μm^2 from both groups at all analyzed periods (15, 21 and 31 days). These results showed that, at 15 days, the mean brown-stained area was significantly smaller in rats treated with PPF (group II - 41,653 ± 7,306.61 μm^2) compared to untreated rats (group I – 55,391.38 ± 5,819.38 μm^2). Similar

finding was seen at 21 days (44,829.38 ± 6,164.66 μm^2 in group II versus 55,381.75 ± 5,785.65 μm^2 in group I), but no statistical difference was seen at 31 days (mean areas of 50,227.38 ± 7,612.02 μm^2 and 50,020.37 ± 6,308.2 μm^2, respectively, in groups I and II).

Table 1. Areas with GFAP staining in μm^2 in a total area of 302,952.5 μm^2 in rats injected with EB, treated (group II) or not (group I) with PPF.

	Group I – EB injection			Group II – EB injection + PPF		
Animal	15 days (μm^2)	21 days (μm^2)	31 days (μm^2)	15 days (μm^2)	21 days (μm^2)	31 days (μm^2)
1	50,231	45,924	39,523	47,292	45,435	46,417
2	60,812	50,125	58,126	39,548	36,021	58,352
3	48,154	54,531	45,132	51,630	43,190	55,325
4	53,824	56,642	44,243	40,226	47,611	45,131
5	57,122	56,134	55,232	34,135	47,163	47,361
6	61,326	57,288	56,785	36,177	56,236	44,372
7	62,451	58,125	44,457	50,723	44,634	44,527
8	49,211	64,915	58,321	33,453	38,345	58,678
Mean	55,391.38[a]	55,381.75[a]	50,227.38[c]	41,653[b]	44,829.38[b]	50,020.37[c]
SD	±5,819.91	±5,785.65	±7,612.02	±7,306.61	±6,164.66	±6,308.2

Distinct letters indicate significant differences between groups I and II ($p < 0.05$).

DISCUSSION

Astrocytes respond to all forms of CNS insults through a process referred to as reactive astrogliosis, which is a finely gradated continuum of progressive changes in gene expression and cell morphology[9,10]. Intermediate filaments of astrocytes are composed mainly of GFAP and this protein has become the best known astrocytic marker[11]. In mild reactive astrogliosis there is variable up regulation of expression of GFAP and other genes as well as hypertrophy of the cell body and processes, but occurring within the domains of individual astrocytes without significant overlap of processes of neighboring astrocytes or loss of individual domains[12]. In this discrete reaction there is little or no astrocyte proliferation, but the increased expression of GFAP can lead to the staining of more cells, giving the false impression of proliferation[12,13]. On the other hand, severe astrogliosis leads to a more pronounced upregulation of GFAP, among other genes, with blurring and disruption of individual astrocyte domains, as usually found in areas surrounding severe focal lesions[12].

Astrocyte precursors and immature astrocytes present principally nestin and vimentin (VIM) and, during development, as astrocytes mature, nestin expression disappears, GFAP becomes increasingly expressed and VIM decreases to undetectable levels[11]. In both mild or severe astrogliosis astrocytes also reexpress VIM and nestin[11]. In the EB demyelinating model, reexpression of VIM and strong astrocytic immunoreactivity to GFAP were described by Bondan et al.[2] in the rat brainstem from the 3rd to the 31st day following gliotoxic injection. This increased GFAP expression around the EB-induced lesions was also confirmed in the present study.

Many different types of signaling molecules are able to trigger and/or regulate astrogliosis and can be released by all cell types of the CNS tissue, including neurons, microglia, oligodendrocyte lineage cells, pericytes, endothelia and other astrocytes, as well as by invasive inflammatory/immune cells . These molecular signals include (a) large polypeptide growth factors and cytokines, such as IL-1, IL-6, TNF-α, IFN-γ, TGF-β, LIF, CNTF, FGF2, among others; (b) mediators of innate immunity such as LPS and other Toll-like receptor ligands; (c) neurotransmitters such as glutamate and noradrenalin; (d) purines (e.g., ATP); (e) reactive oxygen species (ROS); (f) products associated with systemic metabolic activity (e.g., $NH4^{+}$) and (g) regulators of cell proliferation, such as endothelin 1[12,13].

While it was initially thought that astrocyte proliferation was a major component of glial scar it has been repeatedly demonstrated that there are actually few astrocytes

undergoing cell division during glial scar formation[9]. This observation is confirmed by the fact that no astrocytes in mitotic activity were seen in this study and also in previous investigations focusing astrocytic response following gliotoxic lesions[2].

Concerning the mechanisms of PPF action, it has been shown that (i) inhibition of cyclic AMP and GMP-phosphodiesterases (PDE), (ii) inhibition of membrane adenosine transporters and (iii) reinforcement of adenosine A_2 receptor-mediated effects in a synergistic manner are potent pathways responsible for the protective adenosine-mediated actions of this xanthine[4,14]. There is also evidence that PPF is a weak adenosine autoreceptor A_1 antagonist, which can additionally inhibit its reuptake and the activity of the 5´-nucleotidase[14].

Thus, PPF leads to increased intracellular cAMP levels and greater extracellular concentrations of adenosine, stimulating adenosinergic neurotransmission and adenosine 2 (A_2) receptor-mediated cAMP synthesis[5,15].

Intracellular levels of the second messenger cAMP can be elevated by activation of the adenylate cyclase or by inhibition of cAMP-degrading phosphodiesterases (PDE). Eleven PDEs families have been identified with different specificity towards cAMP and cGMP. The PDE4, PDE7 and PDE8 family members are cAMP-specific and PDE5, PDE6 and PDE9 are cGMP-specific. On the other hand, the PDE1, PDE2, PDE3, PDE10 and PDE11 family members hydrolyze both cAMP and cGMP[16].

Regulation of cytokine production includes the adenylate cyclase - cAMP - protein kinase pathway[17]. Yoshiwawa et al.[18] reported that PPF, a type III-IV specific PDE inhibitor, although decreasing in a dose-dependent manner the production of the inflammatory cytokines TNF-α, IL-1 and IL-6 by mouse microglia stimulated by LPS in vitro, increased up to two or three times the production of the inhibitory cytokine IL-10. In turn, IL-10 acts suppressing cytokine release by microglia and macrophages and attenuating astroglial reactivity in vivo[19].

The GFAP is regulated in part by the secretion of factors into the extracellular space. The common pathway for GFAP expression in astrocytes is triggered by the binding of cytokines from the IL-6 family to their receptors. These receptors subsequently activate the JAK/STAT intracellular pathway, leading to the expression of GFAP in astrocytes. Most of the other pathways known to participate in GFAP expression are connected at some point to this pathway. For example, some members of the TGF-β superfamily of cytokines have little or no effect on GFAP synthesis by themselves, but they strongly potentiate GFAP induced by the IL-6 family of cytokines[20]. The PDE inhibitor pentoxifylline is also known to decrease the synthesis of TNF-α, IL-1β and IL-6 through the inhibition of nuclear factor-κB and

stimulation of IL-10 expression in the CNS[21,22]. In the EB demyelinating model, PPF has already showed to decrease the production of TNF-α and IL-1β in the rat brainstem[23].

In the CNS, PPF acts as a glial modulator, with direct actions on microglia, decreasing microglial proliferation and expression of inflammatory cytokines, such as TNF-α and IL-1β, in vitro and in vivo[6,14,18,24].

In the present study, PPF has shown to decrease the astrocytic reaction to the gliotoxic injury as seen through the expression of GFAP and by ultrastructural observation. Morphometric analysis confirmed at 15 and 21 days the initial impression suggested by the observation of semithin and ultrathin sections that PPF treatment decreased the astrocytic reaction to the gliotoxic injection as peripheral GFAP stained areas were significantly greater in EB injected rats that were not treated with PPF compared to rats treated with the xanthine.

Decreased activation of astrocytes and microglia in rats treated with PPF, as shown by reduced GFAP and OX-42 expression, respectively, was also observed *in vivo* by Young et al.[25] after spinal cord injury. PPF also inhibited injury-induced GFAP expression along with enhancement of glutamate transporters GLT-1 and GLAST in the dorsal horn upper laminae in mice submitted to L5-spinal nerve transection[26]. As excessive accumulation of the excitatory neurotransmitter glutamate leads to synaptic dysregulation, its extracellular concentration is usually regulated by a series of sodium-dependent glutamate transporters in astrocytes.

Activated astrocytes may lose their homeostatic functions upon exposure to stressors, decreasing glutamate uptake and increasing the expression of deleterious proinflammatory molecules such as cytokines, nitric oxide, prostaglandins, among others, as an injury response[13]. Thus, reactive astrocytes display decreased glutamate transporters and as a result synaptic glutamate clearance is impaired. In vitro PPF was capable of differentiating astrocytes back to a homeostatic, mature phenotype, competent for glutamate clearance[26].

Both oligodendrocyte and astrocyte loss are hallmarks within the epicenter of an EB lesion while axons remain unaffected. The mechanism of selective glial death has been suggested to occur through EB´s action as a minor-groove DNA intercalator. However, other evidences suggest that while EB does intercalate both chromosomal and mitochondrial DNA, it only affects transcription of mtDNA[27]. So, it is likely that EB injection into the white matter compromises mtDNA in all cells in the lesion site although neurons and endothelial cells appear to be less sensitive than glia in rat models[3].

After trauma, blood-brain barrier dysfunction is immediately observed as well as activation of inflammatory cells including microglia, astrocytes and invading monocytes/macrophages. Activation and recruitment of inflammatory cells into the injured CNS generate proinflammatory cytokines, free radicals and other damaging molecules. The two most important cytokines found in the CNS after trauma are TNF-α and IL-1β, which are highly cytotoxic and regulated by cAMP signaling[16]. The benefits of PDE4 inhibition in reducing inflammation have been well-studied in rodent models of ischemia, stroke and traumatic injury[16]. PDE4 inhibitors have been found to improve neuronal survival, reduce infarct size, and attenuate inflammation and blood-brain barrier breakdown[28]. In experimental autoimmune encephalomyelitis, rolipram, a PDE4 inhibitor, prevent the progression of neurodegeneration and demyelination by increasing cAMP levels[29,30].

It is possible that macrophage and lymphocyte products during the inflammatory response triggered by the EB injection may provide a greater harmful influence to the nervous tissue than the early gliotoxin injection itself. Therefore the anti-inflammatory effects performed by PPF may be possibly beneficial to remyelination.

A Ca^{++}-dependent and excessive activation of glial cells is usually found in neuroinflammation and, in such context, increased levels of adenosine induced by PPF administration may perform a regulatory role on these Ca^{++}- and cAMP-dependent molecular signaling pathways which determine many cell related functions, such as cellular proliferation rate, differentiation state, cytokine production, among others[5].

A strengthening of the cAMP signaling, which can be achieved by adenosine agonists and by PPF, stimulates the production of neurotrophic factors in astrocytes, apparently preventing a deleterious and secondary astrocytic activation caused by previous microglial upregulation[15].

Although not entirely understood, it has been accepted that drugs which elevate extracellular adenosine and/or block the degradation of cyclic nucleotides, like PPF, may be used to counteract glia-related damage in CNS pathological processes[15].

Thus, ultrastructural observation along with morphometric analysis in the present study unequivocally demonstrate that PPF decreased astrocytic activation until the 21st day after gliotoxic lesion, probably by simultaneously suppressing the release of proinflammatory molecules, such as the above mentioned TNF-α and IL-1β, as well as IL-6, which may trigger and promote astrogliosis following CNS injury, and by increasing secretion of the anti-inflammatory cytokine IL-10. Our results clearly indicate that this drug may have a role in preventing or reducing glial scar development following injury.

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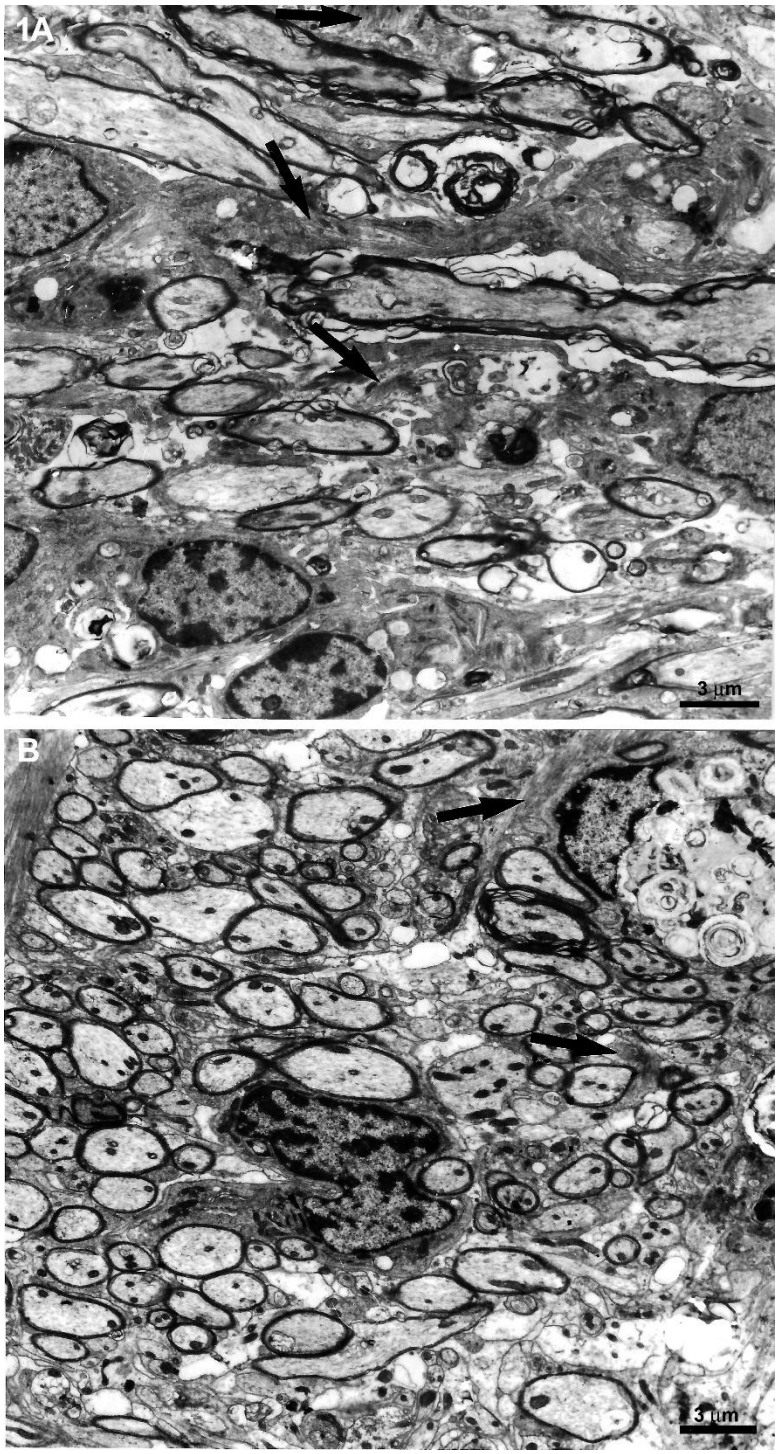

Fig. 1 Electronmicrographs from peripheral areas of the EB-induced lesions in untreated (A) and PPF-treated (B) rats at 21 days. Note the thicker astrocytic processes (arrows) among oligodendrocyte remyelinated axons in A (no treatment) and the greater amount of remyelinated axons in B (PPF treatment). A) Bar= 3 µm; B) Bar= 3 µm.

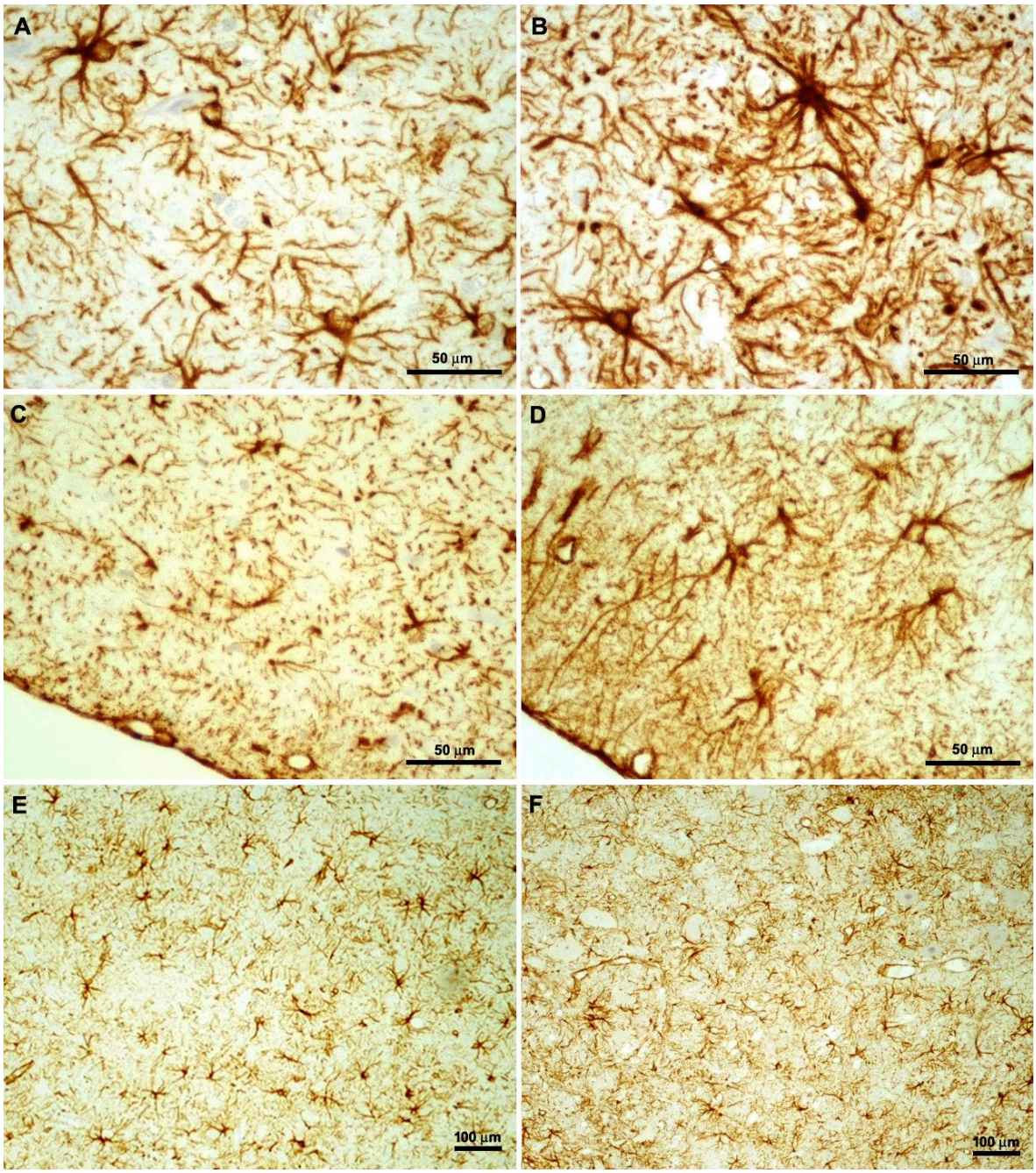

Fig. 2 Peripheral GFAP expression by immunohistochemistry at 15 days (A, B), 21 days (C, D) and 31 days (E, F) in EB-induced lesions from untreated (B, D, F) and PPF-treated (A, C, E) rats. A, B,C, D) Bar = 50 µm, E, F) Bar = 100 µm.