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Research Report

Neuroprotection by erythropoietin administration after experimental traumatic brain injury

Giovanni Grasso[a,*], *Alessandra Sfacteria*[b], *Francesco Meli*[a], *Vincenzo Fodale*[c], *Michele Buemi*[d], *Domenico G. Iacopino*[e]

[a]*Department of Clinical Neurosciences, Neurosurgical Clinic, University of Palermo, Italy*
[b]*Department of Veterinary Pathology, University of Messina, Italy*
[c]*Department of Anesthesiology, University of Messina, Italy*
[d]*Department of Internal Medicine, University of Messina, Italy*
[e]*Department of Clinical Neurosciences, Neurosurgical Clinic, University of Palermo, Italy*

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ABSTRACT

A large body of evidence indicates that the hormone erythropoietin (EPO) exerts beneficial effects in the central nervous system (CNS). To date, EPO's effect has been assessed in several experimental models of brain and spinal cord injury. This study was conducted to validate whether treatment with recombinant human EPO (rHuEPO) would limit the extent of injury following experimental TBI. Experimental TBI was induced in rats by a cryogenic injury model. rHuEPO or placebo was injected intraperitoneally immediately after the injury and then every 8 h until 2 or 14 days. Forty-eight hours after injury brain water content, an indicator of brain edema, was measured with the wet–dry method and blood–brain barrier (BBB) breakdown was evaluated by assay of Evans blue extravasation. Furthermore, extent of cerebral damage was assessed. Administration of rHuEPO markedly improved recovery from motor dysfunction compared with placebo group ($P<0.05$). Brain edema was significantly reduced in the cortex of the EPO-treated group relative to that in the placebo-treated group (80.6±0.3% versus 91.8%±0.8% respectively, $P<0.05$). BBB breakdown was significantly lower in EPO-treated group than in the placebo-treated group (66.2±18.7 μg/g versus 181.3±21 μg/g, respectively, $P<0.05$). EPO treatment reduced injury volume significantly compared with placebo group (17.4±5.4 mm3 versus 37.1±5.3 mm3, $P<0.05$). EPO, administered in its recombinant form, affords significant neuroprotection in experimental TBI model and may hold promise for future clinical applications.

1. Introduction

Traumatic brain injury (TBI) is a major cause of morbidity and mortality in the United States. It is estimated that each year, on average, TBIs associated with 1.1 million emergency department visits, 235,000 hospitalizations, and 50,000 deaths (2007). Despite improvements in medical interventions, there are still currently no neuroprotective agents available to counteract secondary or delayed damage to the traumatically injured human brain or to promote its repair. Many researches have long explored agents with proved *in vitro* and *in vivo* neuroprotective effect in order to assist in repairing damaged

* *Corresponding author*. Neurosurgical Clinic, Policlinico P. Giaccone, Via del Vespro 127, 90100, Palermo, Italy. Fax: +39 0916553227.
E-mail address: ggrasso@unipa.it (G. Grasso).

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nervous tissue. Although several compounds have been demonstrated to be neuroprotective in preclinical models, only a part of these have entered clinical development and some of those that survived early safety trials have been studied in controlled efficacy trials. Despite these efforts, all phase III trials have so far failed in demonstrating efficacy of neuroprotective agents.

Progress in the development and synthesis of new drugs represents the first step in tailoring a potential successful neuroprotective agents. In this scenario, a large body of evidence has pointed out the efficacy of the hormone erythropoietin (EPO) as neuroprotectant. Although peripherally administered recombinant human EPO (rHuEPO) has shown to penetrate the blood–brain barrier (BBB) and reduce brain injury following a variety of insults (Brines et al., 2000; Digicaylioglu and Lipton, 2001; Grasso et al., 2004), its potential neuroprotective efficacy in an *in vivo* model of experimental TBI has been scarcely investigated (Brines et al., 2000; Lu et al., 2005; Ozturk et al., 2005; Shein et al., 2005; Siren et al., 2006; Verdonck et al., 2007; Yatsiv et al., 2005). Evidence shows widespread efficacy of rHuEPO in injury models of spinal cord (Celik et al., 2002; Gorio et al., 2002; Grasso et al., 2006), subarachnoid hemorrhage (Buemi et al., 2002a,b; Catania et al., 2002; Grasso, 2001; Grasso et al., 2002a,b; Springborg et al., 2002), retina, and the heart damage (Calvillo et al., 2003; Junk et al., 2002).

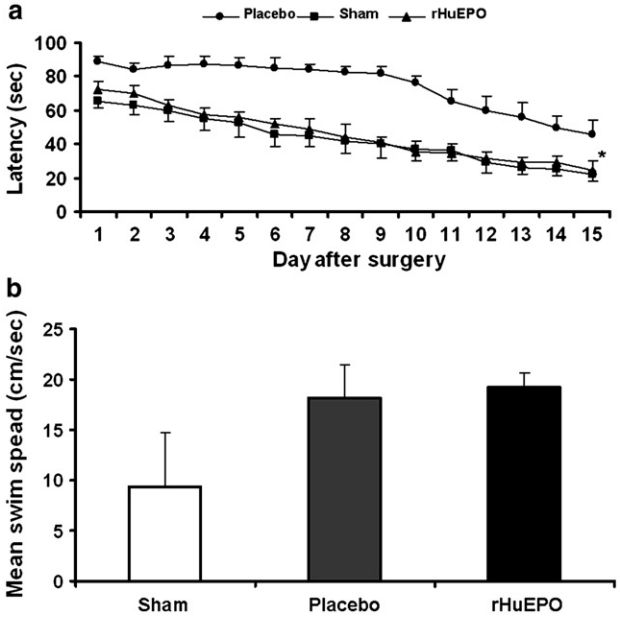

Fig. 1 – The effect of rHuEPO treatment on cognitive behavior and swim speed in a Morris water maze after TBI. (a) Sham-injured control animals demonstrated an ability to learn a cognitive task as demonstrated by a reduced latency to find the platform over time, whereas placebo-treated injured animals showed a significantly impaired ability to complete this task compared to shams ($P<0.05$). rHuEPO treatment significantly reversed this impairment in injured animals (* $P<0.05$). (b) Mean swim speeds generated from all trials in the experiment. While there was an overall significant effect of treatment, no significance was found between any of the treatment groups.

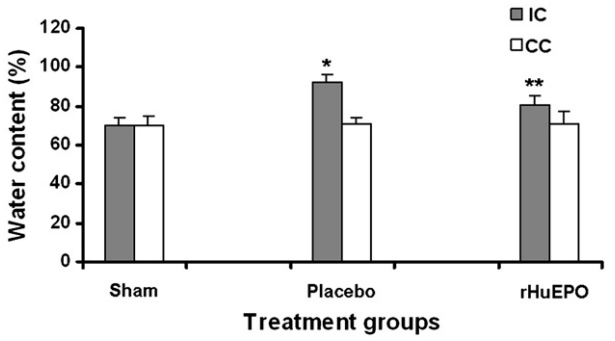

Fig. 2 – Graph showing the effect of rHuEPO administration on tissue water content, an indicator of brain edema after TBI. By 48 h after TBI, the injured cortex showed significant brain edema, which was notably reduced in the ipsilateral cortex after treatment with rHuEPO as compared with placebo. Data are presented as mean ± standard deviation; IC, ipsilateral cortex; CC, contralateral cortex; *, $P<0.05$, as compared with contralateral cortex; **, $P<0.05$, as compared with placebo treatment.

The mechanisms by which EPO exerts its beneficial effects are incompletely understood. Available evidence suggests that EPO acts in a coordinated fashion at multiple levels to limit the production of tissue-injuring molecules such as glutamate (Kawakami et al., 2001), reverse vasospasm (Grasso, 2001), attenuate apoptosis (Celik et al., 2002; Digicaylioglu and Lipton, 2001), modulate inflammation (Brines et al., 2000), and recruit stem cells (Shingo et al., 2001). Furthermore, a recent phase II clinical trial has demonstrated significant improvement in outcome of ischemic stroke patients administered rHuEPO intravenously within 8 h of the onset of symptoms (Ehrenreich et al., 2002).

These data support the role of EPO as an essential mediator of protection in the CNS. Based on this evidence we designed the present study to investigate further the role of EPO following experimental TBI. Specific purpose of this research was

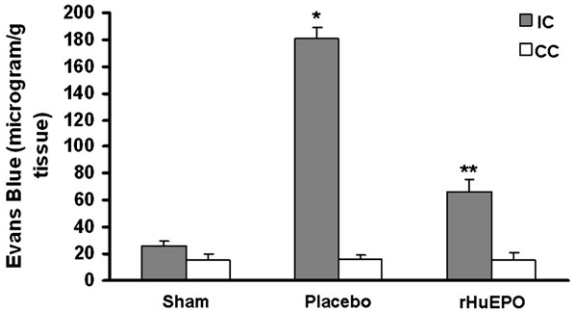

Fig. 3 – Graph showing the effect of rHuEPO on Evans blue extravasation, an indicator of BBB permeability, after TBI. By 48 h after TBI, the injured cortex showed significant BBB permeability, which was notably reduced after treatment with rHuEPO. Data are presented as mean ± standard deviation; IC, ipsilateral cortex; CC, contralateral cortex; *, $P<0.05$, as compared with sham-operated rats; **, $P<0.05$ as compared with placebo treatment.

to determine the effectiveness of EPO administration on neurological impairment, brain edema formation, blood–brain barrier (BBB) breakdown, and injury volume extent after TBI. Brain injury was induced by a cryogenic injury model, a paradigm well known to produce brain lesions resembling some of the aspects of TBI in patients (Amorini et al., 2003; Fukui et al., 2003; Zhao et al., 2003).

2. Results

2.1. Neurological findings

Cerebral injury induced significant neurological deficits in placebo-treated subjects when compared to sham animals ($P<0.05$). rHuEPO administration reduced the deficit observed in placebo-treated animals by significantly shortening the latency required to complete the task over time ($P<0.05$). In addition, animals given rHuEPO did not present significantly different latencies from sham-injured animals. There was an overall significant effect of treatment on the mean swim speeds displayed throughout the behavioral experiment of the subjects ($P<0.05$) (Fig. 1).

2.2. Brain water content quantification

Overall, TBI caused a significant increase in the percentage of water content in the injured ipsilateral cortex compared with the contralateral cortex (92.3±0.4% 71.1±0.3%, respectively, $P<0.05$). Treatment with rHuEPO reduced the percentage of water content in the ipsilateral cortex compared with the contralateral cortex (80.6±0.3% and 71.1±0.3%, respectively, $P<0.05$) and compared with placebo-treated group (80.6±0.3% versus 91.8%±0.8%, $n=6$, $P<0.05$) (Fig. 2).

2.3. BBB permeability assessment

In both treatment groups Evans blue extravasation in the ipsilateral cortex was significantly higher than that of the contralateral cortex. However, rHuEPO treatment reduced dye extravasation in the ipsilateral cortex for about 60% as compared with placebo treatment (66.2±18.7 μg/g versus 181.3±21 μg/g, respectively, $n=6$ for both groups, $P<0.05$) (Fig. 3).

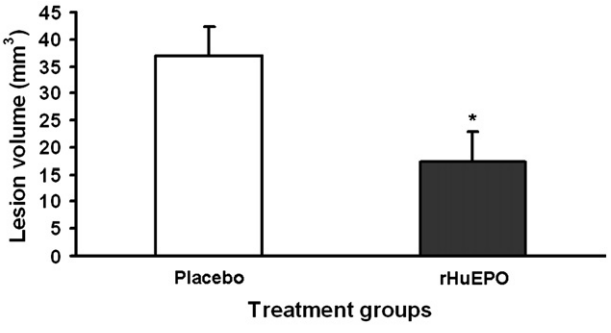

Fig. 4 – Graph showing the lesion volume assessed 48 h after TBI. Placebo-treated rats had larger lesion volumes than rHuEPO-treated rats, as measured by TTC staining (* $P<0.05$). Data are expressed as mean±standard deviation.

2.4. Lesion volume quantification

TBI produced a region of necrotic tissue in the right parietal cortex. Treatment with rHuEPO significantly reduced injury volume by 53% as compared with placebo treatment (from 37.1±5.3 mm^3 in placebo-treated animals to 17.4±5.4 mm^3 in rHuEPO-treated animals, $n=6$ for both groups, $P<0.05$) (Fig. 4).

3. Discussion

The present study demonstrates that rHuEPO confers neuroprotection in an *in vivo* model of TBI and emphasizes its beneficial effect on neurological dysfunction, brain edema formation, BBB dysfunction, and cerebral tissue injury extent following a cryogenic model of brain damage. Such a model mimics several pathophysiological characteristics of human focal cortical contusion (Nag, 1996) and produces a reproducible, demarcated lesion to the neocortex that allows evaluation of the efficacy of compounds with a neuroprotective potential (Hortobagyi et al., 2000).

The present findings extend previous work by showing that rHuEPO significantly reduces brain injury and improves neurological recovery following traumatic insults (Brines et al., 2000; Lu et al., 2005; Ozturk et al., 2005; Shein et al., 2005; Siren et al., 2006; Verdonck et al., 2007; Yatsiv et al., 2005). In particular, rHuEPO administration improved both functional and cognitive recovery of the rats with an effect that was significantly shown since the early stage after TBI and lasted for 15 days. Beside an improved neurobehavioral recovery, rHuEPO-treated rats presented with less brain edema than placebo-treated group, as demonstrated by the water content assessment, with a better BBB integrity and a reduced cerebral injury volume as compared with placebo treatment group.

Although the role of EPO as a neuroprotectant has been studied extensively in a wide range of *in vitro* and *in vivo* models of brain injury, up to date, there are only few studies evaluating the effect of rHuEPO after experimental TBI (Brines et al., 2000; Lu et al., 2005; Ozturk et al., 2005; Shein et al., 2005; Siren et al., 2006; Verdonck et al., 2007; Yatsiv et al., 2005) and none has used a cryogenic injury paradigm. Neurobehavioral improvement after EPO administration has been reported in other studies where different models of brain injury were used. In this regard, it has been demonstrated that rat pups given rHuEPO had a significantly better performance in the Morris water maze test compared with untreated animals (Kumral et al., 2004). Others subjected rats to bilateral transection of the fimbria-fornix and rHuEPO treatment allowed a better posttraumatic functional recovery (Mogensen et al., 2004). We have previously demonstrated that rHuEPO prevented cognition impairment in a gerbil transient brain ischemia model (Catania et al., 2002) and neurological dysfunction following experimental subarachnoid hemorrhage in rabbits (Grasso et al., 2002a). These studies and the present findings suggest that the neuroprotective effects exerted by rHuEPO administration in models of brain damage are associated with the maintenance of neurological functions.

In the present study we have also demonstrated that EPO can limit the BBB dysfunction after TBI. Our findings are in agreement with previous studies where treatment with EPO

was shown to protect BBB breakdown after experimental seizure (Uzum et al., 2006) and to reduce infarct size after ischemia/reperfusion (Liu et al., 2006). An *in vitro* study has also shown that EPO protects against the VEGF-induced permeability of the BBB, decreases the levels of endothelial nitric oxide synthase, and restores junction proteins (Martinez-Estrada et al., 2003).

The modality by which rHuEPO acts in the CNS across the BBB remains a matter of controversy. Expression of EPOR in brain capillary endothelial cells and the ability of systemically administered EPO to cross the BBB have been reported *in vivo* (Brines et al., 2000; Grasso et al., 2002a). Furthermore, it has been suggested that, after systemic administration, rHuEPO may be transported across the BBB by a specific receptor-mediated mechanism (Brines et al., 2000).

The basis of EPO-mediated neuroprotection depends upon expression of a cognate receptor where the drug can be bound at the time of exposure. EPO exerts its effects through the activation of its receptor (EPOR), part of the cytokine-receptor type I superfamily. Recent evidence suggests that the hematopoietic and tissue-protective activities could be separated and that the hormonal and neuroprotective actions of EPO can occur via different signaling systems (Leist et al., 2004). Specifically, the receptor complex mediating the neuroprotective effects of EPO has been reported to be associated with the common receptor (cR) subunit, also known as CD131, which is the signal-transducing component used by the granulocyte-macrophage colony stimulating factor (GM-CSF), IL-3, and IL-5 receptors (D'Andrea and Gonda, 2000).

Although the mechanisms by which EPO acts as neuroprotectant are still a matter of controversy, an increasing number of evidence suggests that EPOR activation following EPO binding inhibits neuronal apoptosis (Celik et al., 2002; Digicaylioglu and Lipton, 2001). Prevention of neuronal apoptosis involves the activation of JAK-2 and nuclear factor (NF)-kB signaling pathways (Digicaylioglu and Lipton, 2001). In addition, EPO also appears to prevent apoptotic injury through an Akt dependent mechanism (Bao et al., 1999).

EPO-mediated neuroprotection after TBI includes other triggering events such as restoration and maintenance of vascular autoregulation and BBB integrity. In this regard, it is well known that TBI is associated with an early loss of vascular autoregulation leading to the development of vascular hyperpermeability and tissue edema that ultimately cause neuronal degeneration (Unterberg et al., 2004). In preclinical injury models of cerebral vasospasm induced by subarachnoid hemorrhage (Grasso et al., 2002a; Springborg et al., 2002) it has been shown that EPO can reverse vascular spasm thus providing neuroprotection. The mechanism of this vascular effect of rHuEPO appears to depend on the modulation of inducible nitric oxide synthase activity (Squadrito et al., 1999). Because one mechanism explaining the neuroprotective effect of rHuEPO has been shown to depend on inhibition of nitric oxide production (Calapai et al., 2000), it is reasonable to hypothesize that similar mechanisms may be relevant after TBI. Furthermore, inflammatory cells are involved in the late damage that occurs to the oligodendrocytes that provide brain degeneration (Leinhase et al., 2006). rHuEPO appears to reduce the inflammatory infiltrate and in this manner likely reduces the contribution of late injury to the neurological deficit (Gorio et al., 2002). However, antiapoptotic action and reduced neuroinflammatory response by rHuEPO (Brines et al., 2000; Siren et al., 2001; Yatsiv et al., 2005) are based on mechanisms needing several hours, after the insult, to be effective. Processes occurring in the early stage after TBI, such as release of glutamate, lactate, potassium, calcium, free oxygen radicals, histamine, and kinins by injured cells, seem to be more relevant in the pathophysiological mechanisms underlying brain damage following TBI (Unterberg et al., 2004). In this regard, EPO administration has been associated with protection from glutamate toxicity by activation of calcium channels (Sakanaka et al., 1998), production of antioxidant enzymes in neurons (Koshimura et al., 1999), and reduction of the NO toxicity in neurons (Calapai et al., 2000; Sakanaka et al., 1998).

In conclusion, our results confirm the positive effects exerted by rHuEPO in treating the post-TBI pathogenic cascade because rHuEPO significantly reduced brain edema formation, BBB dysfunction, and cerebral tissue injury following the trauma, and consequently, the neurological outcome in this experimental setting. The present findings extend the work of previous studies in several ways. First, we have demonstrated that systemic administration of rHuEPO can significantly attenuate TBI related injuries in a well-characterized and reproducible experimental model. Second, we have documented that the neuroprotective effect of rHuEPO is evident even 48 h later the induction of the trauma. Finally, we have observed an improvement of the neurological outcome since the early stage of the injury lasting for almost 2 weeks. Such an observation strongly supports further studies in order to assess additional information about a possible clinical use of EPO after the onset of TBI.

4. Conclusions

In the present study conducted in rats, our results indicate that EPO provides tissue protection after experimental TBI. This finding, consistent with beneficial effect on neurological dysfunction, brain edema formation, BBB dysfunction, and cerebral tissue injury following a cryogenic injury model of brain damage, has clear relevance for treatment of TBI.

5. Experimental procedures

5.1. *Animal preparation*

Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with Italian and international laws and policies.

Rats were anesthetized with isofluorane (2%) in oxygen (0.8 l/min) and nitrous oxide (0.4 l/min). Cryogenic cerebral injury was induced as previously described (Gorlach et al., 1998). Briefly, a cryogenic lesion was produced by application of a precooled copper cylinder (∅ 5 mm, −78 °C) on the dura mater. Under surgical microscope the skull was exposed by a midline incision and a circular hole (approximately 5 mm in diameter) was drilled on the skull over the right temporo-parietal cortex leaving the dura intact. The precooled cylinder was lowered onto the dural surface and kept in place for 60 s.

Sham surgeries (no vehicle or drug treatment) were performed using the same technique, but without producing cryogenic injury.

After the operation, rats were housed in pairs to reduce isolation-induced stress. Animals were maintained in a 12-hour light/dark cycle with water and food freely available at an ambient temperature of 25 to 27 °C. No prophylactic antibiotics were given.

5.2. *Treatment groups and drug administration*

Sixty-six male Sprague–Dawley rats, each weighing 250 to 350 g, were assigned to one of three groups: Group 1, sham operated; Group 2, TBI plus placebo; or Group 3, TBI plus rHuEPO; each group contained twenty-two animals.

All injections were administered 5 min after induction of TBI and were continued every 8 h up to 14 days in animals which underwent neurological examination (4 animals from each groups), and 48 h in animals undergoing brain edema, BBB integrity, and lesion volume quantification (6 animals from each group). All doses of placebo or rHuEPO were administered intraperitoneally. Rats in Group 2 received the vehicle used to administer rHuEPO (serum albumin [2.5 mg/ml], sodium chloride [5.84 mg/ml], sodium citrate [5.8 mg/ml], anhydrous citric acid [0.057 mg/ml], and water), at a dose of 1 ml/kg of body weight as placebo. Rats in Group 3 were given rHuEPO at a dose of 1000 IU/kg. The dose of 1000 IU/kg was based on results obtained in our previous studies (Grasso, 2001; Grasso et al., 2002a,b).

5.3. *Neurological function evaluation*

To examine cognitive deficits, a Morris water maze paradigm was used (Morris, 1984). Briefly, the task involved rat repeatedly locating within 90 s a clear platform submerged in one of four locations in a fiberglass pool. If unsuccessful in a trial, the subjects were gently guided to the platform. Once on the platform, subjects were allowed to remain there for 30 s. Extra visual cues were placed around the room and the platform was kept in a fixed position for each rat. The experiment extended for 14 consecutive days, beginning on day 1 after injury, and consisted of two sessions per day (four trials per session), with an inter-trial interval of 30 min and an inter-session interval of 120 min. Each trial was recorded and movement was tracked using a commercially available software. The length of time taken to reach the platform and swim speeds of the animals could then be calculated.

5.4. *Brain edema assessment*

Brain water content, an indicator of brain edema, was measured with the wet–dry method 48 h after injury (Dogan et al., 1997; Gove et al., 1997). After the animals were killed by decapitation under anesthesia, their brains were removed and the ipsilateral and contralateral cortical tissues were dissected and weighed immediately to assess wet weight. After drying in a desiccating oven for 48 h at 70 °C, the tissues were re-weighed to yield dry weight. The percentage of water in the tissues was calculated according to the formula: %water = 100 × (wet weight − dry weight)/wet weight.

5.5. *Evaluation of BBB integrity*

The integrity of the BBB was investigated by assessing extravasation of Evans blue dye as previously described (Iseki et al., 1996). Briefly, Evans blue dye (2% in saline) was injected intravenously (3 mg/kg) 48 h after TBI and allowed to circulate 60 min. To remove the intravascular dye, we perfused the animals with saline through the left ventricle at 100 cm of water pressure until clear perfusion fluid was obtained from the right atrium. After the animals were decapitated, the brains were removed and the ipsilateral and contralateral cortical tissues were dissected. Each tissue sample was weighed, homogenized in 2 ml of 50% trichloroacetic acid (w/v), and centrifuged for 20 min. The supernatant was then diluted with solvent (one part 50% trichloroacetic acid to three parts ethanol). Tissue levels of Evans blue dye were assessed using a spectrofluorometer at an excitation wavelength of 620 nm and an emission wavelength of 680 nm. Sample values were compared with those of Evans blue dye standards mixed with the solvent (100–1000 ng/ml).

5.6. *Lesion volume quantification*

Forty-eight hours after surgery, the rats were perfused with 10% paraformaldehyde under anesthesia and brains rapidly removed and sliced into 1-mm slices in a rat brain matrix. To quantify lesion volume, brain slices were incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St Louis, MO, USA) in 0.9% saline for 30 min at room temperature in the dark and stored at 4 °C in neutral 10% formalin for up to 7 days prior to analysis. Digital images of each slice were taken using a scanner, and lesion volume, defined as the area of unstained tissue, calculated using ScionImage software (Frederick, MD, USA).

5.7. *Statistical analysis*

Data were expressed as the mean ± standard deviation and were analyzed by using one-way analysis of variance (ANOVA) followed by Dunett's post hoc multiple comparison test. A P value of <0.05 was considered statistically significant.

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