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Use of Mometasone furoate in prolonged treatment of experimental spinal cord injury in mice: A comparative study of three different glucocorticoids

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ABSTRACT

Traumatic spinal cord injury (SCI) represents one of the most disabling injuries of the human body causing temporary or permanent sensory and/or motor system deficit, particularly hind limb locomotor function impairment. At present, steroidal inflammatory drugs, in particular methylprednisolone sodium succinate (MPSS) are the first line choice treatment of acute SCI. Despite progress in pharmacological, surgical and rehabilitative treatment approaches, SCI still remains a very complex medical and psychological challenge, with no curative therapy available. The aim of the present study was to compare the efficacy of MPSS in respect to other GCs such as dexamethasone (Dex) and mometasone furoate (MF) in an *in vitro* suitable model of LPS-induced inflammation in J774 cells as well as in an *in vivo* experimental mouse SCI (compression model). In both the *in vitro* and *in vivo* experiments, MF resulted surprisingly more potent than Dex and MPSS. In detail, mice sacrificed seven days after induction of SCI trauma resulted not only in tissue damage, cellular infiltration, fibrosis, astrocyte activation, iNOS expression, extracellular signal regulated kinase 1/2 phosphorylation in injured tissue, poly (ADP-ribose) polymerase 1 (PARP-1) activation but also apoptosis (Bax and Bcl-2 expression). All three GCs demonstrated the ability to modulate inflammatory, oxidative as well as apoptotic pathways, but MF demonstrated the best efficacy, while Dex and MPSS showed alternative potency with a different degree of protection. Therefore, we can conclude that MF is the best candidate for post-traumatic chronic treatment, since it ameliorates different molecular pathways involved in the damage's propagation to the surrounding areas of the injured spinal cord.

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1. Introduction

In reference to thoracolumbar spinal trauma, better known as Spinal Cord Injury (SCI), we mean all those events of traumatic and non-traumatic origin that affect central nervous system (CNS) and create disability with temporary or permanent deficit of sensory and/or motor system, in particular hind limb locomotor function [1]. Car and motorcycle accidents (50%), falls (43%) and sporting injuries (7%) are the main causes of trauma [2], while malignant spinal cord compression due to cancer, arthritis, osteoporosis and inflammation represent the etiology for non-traumatic SCI that results overall in 54% of spinal stenosis and 26% of tumors [3]. In any cases, SCI affects style and quality of life as well as survival [4]. For this reason, SCI can be considered a widespread problem with high costs for national health systems.

The first steps in SCI management consist of patient immobilisation, spinal cord decompression and stabilization. Surgery in early stages is used to remove bone fragments, foreign objects, herniated disks or fractured vertebrae compressing the spine but it can also be deemed necessary in preventing pain or deformities. However, case by case evaluation is necessary because these procedures are at high risk of further complications [5]. Physiotherapy is frequently associated with pharmacological treatment.

SCI management mainly consists in avoiding progressive degeneration and extension of damage, counteracting the secondary

Abbreviations: SCI, Spinal cord injury; MPSS, methylprednisolone sodium succinate; Dex, dexamethasone; MF, mometasone furoate; CNS, central nervous system; GCs, glucocorticoids; GR, glucocorticoid receptor; pERK1/2, extracellular signal regulated kinase 1/2; GILZ, glucocorticoid-induced leucine zipper gene; GFAP, glial fibrillary acidic protein; PARP-1, poly (ADP-ribose) polymerase 1.

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injury at the perilesional area, in order to relieve pain and symptoms, and restore functional motor ability [6]. Furthermore, secondary injury observed in SCI is sustained by an inflammatory response through the activation of the innate immune response and, in particular, through the activation of macrophages [7,8].

Glucocorticoids (GCs) are steroid hormones regulated by the hypothalamic-pituitary-adrenal axis [9]. By binding to glucocorticoid receptor (GR), almost ubiquitously expressed, GCs become pleiotropic regulators of multiple cell types, with a wide range of roles in both healthy and diseased individuals [10], assuming a critical role in physiological systems [11].

Acute SCI involves the adoption of steroidal anti-inflammatory drugs. In particular, to date, methylprednisolone sodium succinate (MPSS) is considered the first line treatment.

MPSS is a synthetic glucocorticoid, the only FDA-approved therapeutic agent recognized as standard care in acute SCI treatment. Early treatment with MPSS within an 8 hour time period after injury [12], is demonstrated to reduce severe edema development as well as to preserve spinal cord architecture at damage site [13]. Pharmacological management prescribes 30 mg/kg MPSS intravenously as a bolus, followed by 5.4 mg/Kg/hour intravenous infusion for 24–48 h [14,15]. Nevertheless, side-effects, such as sepsis, bronchopneumonia, gastrointestinal haemorrhage [16], wound infection and psychological neurosis following high doses of MPSS administration can occur [17]. Today, the main goal of researchers is an effective pharmacological treatment useful in the repair of spinal damage together with the prevention of secondary effects.

The aim of the present work was to test the efficacy of three different GCs performing both an *in vitro* suitable model of inflammation which characterizes SCI (specifically, J774 macrophages stimulated with lipopolysaccharide) and an *in vivo* model of SCI in mice.

In detail, the study was designed to compare the conventionally used MPSS efficacy with the possible therapeutic properties of mometasone furoate (MF), a synthetic GC typically prescribed for topical applications in chronic hand eczema [18] and airway inflammation management of asthma [19], and dexamethasone (Dex), another synthetic corticosteroid commonly used as anti-inflammatory, immunosuppressive as well as anti-shock drug [20] in SCI lesions.

Achieved results have shown the peculiarity of each pharmacological treatment. Surprisingly, MF was the most effective in counteracting chronic degeneration at level of the perilesional area, demonstrating the capability to modulate different signalling pathways.

2. Materials and Methods

2.1. GC treatments

Both *in vitro* and *in vivo* studies were performed testing the concentration of three different GCs, in particular:

Glucocorticoid	Chemical name	*in vivo* tested Dosage
MPSS	$C_{22}H_{30}O_5$ 1S,2R,8S,10S,11S,14R,15S,17S)-14,17-didroxi-14-(2-idroxiacetil)-2,8,15-trimetitetraciclo[8.7.0.02,7.011,15]eptadeca-3,6-dien-5-one	6 mg/Kg, 5% DMSO, ip
Dex	$C_{22}H_{29}FO_5$ (9-fluoro-11β,17,21-tridrossi-16a-metilpregna-1,4-diene-3,20-dione)	1 mg/Kg, ip
MF	$C_{27}H_{30}Cl_2O_6$ 9α,21-dichloro-11β,17-dihydroxy-16α-methylpregna-1,4-diene-3,20-dione 17-(2-furoate)	0.1 mg/Kg,5% DMSO, ip

The dose of MPSS used in the clinical practice is currently 30 mg/Kg [14,15], that results to high for *in vivo* mice chronic treatment. For this reason, we have set experimentally and empirically the effective dosage of MPSS defining the concentration of 6 mg/Kg.

The dose of other GCs has been scaled based on their relative potency compared to MPSS.

2.2. In vitro study

2.2.1. J774 cells

The murine monocyte/macrophage J774 cell line was grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 2 mM glutamine, 25 mM Hepes, penicillin (100 U/ml), streptomycin (100 μg/ml), 10% foetal bovine serum (FBS) and 1.2% Na pyruvate. Cells were plated in 24-well culture plates at a density of 2.5 ± 10^5 cells/ml or in 60 mm-diameter culture dishes (3 ± 10^6 cells per 3 ml dish) and allowed to adhere at 37 °C in 5% CO_2. After 24 h, cells were pre-treated (for 2 h) with increasing concentration of old (Dex and MET) and new (MF), and stimulated with LPS from *Escherichia coli*, Serotype 0111:B4, (10 μg/ml). After 24 h of treatment, the supernatants were collected for nitrite measurement.

2.2.2. Nitrite measurement

The nitrite concentration in the samples was measured by the Griess reaction, by adding 100 μl of Griess reagent (0.1% naphthylethylenediamide dihydrochloride in H_2O and 1% sulphanilamide in 5% concentrated H_2PO_4; vol. 1:1) to 100 μl samples. The optical density at 540 nm (OD_{540}) was measured using microplate reader (Multiskan 60, Thermo Scientific). Nitrite concentration was calculated by comparison with OD_{540} of standard solutions of sodium nitrite prepared in culture medium.

2.2.3. iNOS and COX-2 expression

The analysis of iNOS, COX-2, α-tubulin and β-actin in J774 macrophages were performed on whole cell lysates. After stimulation with LPS for 24 h, cells were washed with cold PBS and lysed for 10 min at 4 °C with a lysis buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 400 mM NaCl, 1 mM EDTA, 1 mM ECTA, 1 mM DTT, 0.5 mM PMSF, 15 μg/ml soybean trypsin inhibitor, 3 μg/ml pepstatin, 2 μg/ml leupeptin, 40 μM benzamidine, 50 mM NaF, 1% Nonidet P-40 and 20% glycerol). Lysates from adherent cells were collected by scraping and centrifuged at 12,000 × g for 15 min at 4 °C. The supernatants were collected and protein concentration in cell lysates was determined by Bio-Rad Protein Assay (Bio-Rad).

Equal amounts of proteins were mixed with gel loading buffer (250 mM Tris, 8% SDS, 40% glycerol, 20% 2-mercaptoethanol, 1% bromophenol) in a ratio of 1:4, boiled and centrifuged at 10,000 × g for 10 min. Each sample was loaded and electrophoresed on a 10% SDS-polyacrylamide gel. The proteins were transferred on to nitrocellulose membranes (Protran Amersham). The membranes were blocked with 0.1% TBS-Tween containing 5% BSA for iNOS, with 1% PBS-Tween containing 3% non-fat dry milk for COX-2 and with 0.1% PBS-Tween containing 5% non-fat dry milk for α-tubulin and β-actin. After the blocking, the membranes were incubated with the relative primary antibody overnight at 4 °C. Rabbit monoclonal antibody anti iNOS (Cell Signaling) was diluted 1:1000 in 0.1% TBS-Tween, containing 5% BSA; mouse monoclonal antibody anti COX-2 was diluted 1:1000 in 0.1% PBS-Tween containing 3% non-fat dry milk; mouse monoclonal antibodies anti β-actin and α-tubulin (Santa Cruz Biotecnology) were diluted 1:1000 in 0.1% PBS-Tween containing 5% BSA. After incubation, the membranes were washed six times with 0.1% TBS-Tween or 0.1% PBS-Tween and incubated for 1.5 h at room temperature with horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies (Santa Cruz Biotecnology) diluted 1:5000 and 1:2000 in 0.1% TBS/Tween containing 5% BSA and 0.1% PBS-Tween containing 5%

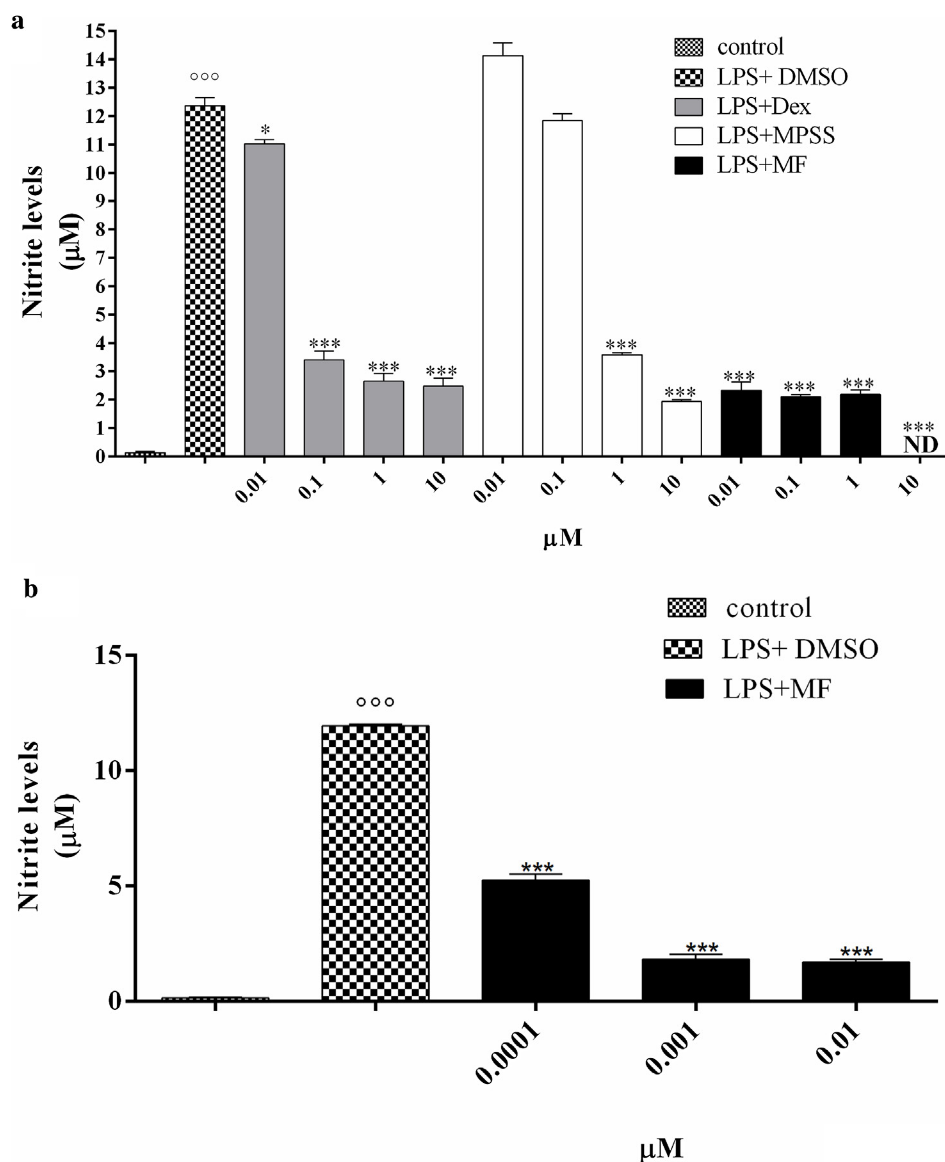

Fig. 1. Dex, MPSS and MF effects on nitrite production in LPS-treated J774 macrophages. Cells were pre-treated with Dex, MPSS and MF: a) (0.01, 01, 1 and 10 μM) and MF: b) (0.0001, 0.001 and 0.01 μM) or vehicle (DMSO 0.5%) for 2 h and further incubated for 24 h with LPS (10 μg/ml). At the end of incubation (24 h), the supernatant were collected for the nitrite measurement by the Griess reaction. Data are expressed as means ± SEM from $n = 3$ independent experiments performed in triplicates, each. °°°$p < 0.001$ *vs* unstimulated cells (control); *$p < 0.05$ and ***$p < 0.001$ *vs* LPS + DMSO.

non-fat dry milk, respectively. Following incubation, membranes were washed and developed using ImageQuant-400 (GE Healthcare, USA).

2.3. In vivo study

2.3.1. Animals

Male adult CD1 mice (25–30 g weight) were purchased from Harlan (Milan, Italy). Animals were housed in individually ventilated cages with food and water *ad libitum*. The room was maintained at a constant temperature and humidity on a 12 h/12 h light/dark cycle.

Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116/92) as well as with the EEC regulations (O.J. of E.C.L 358/1 12/18/1986).

2.3.2. SCI induction

After anaesthesia, induced with an anesthetic cocktail composed of tiletamine (100 mg/Kg) plus xylazine (15 mg/Kg; 10 ml/Kg, i.p), mice were subjected to SCI, according to the model described by Rivlin and Tator [21].

A longitudinal incision was made on the midline of the back, exposing the paravertebral muscles. These muscles were dissected exposing T5–T8 vertebrae. The spinal cord was exposed via a four-level T5–T8 laminectomy and SCI was produced by extradural compression of the spinal cord at level T6–T7 using an aneurysm clip with a closing force of 24 g. In all injured groups, the spinal cord was compressed for 1 min.

Following surgery, 1 ml of saline was administered subcutaneously in order to replace the blood volume lost during the surgery. The induced damage was verified, after the animals were awaked, evaluating the mobility of the hind limbs on a flat surface.

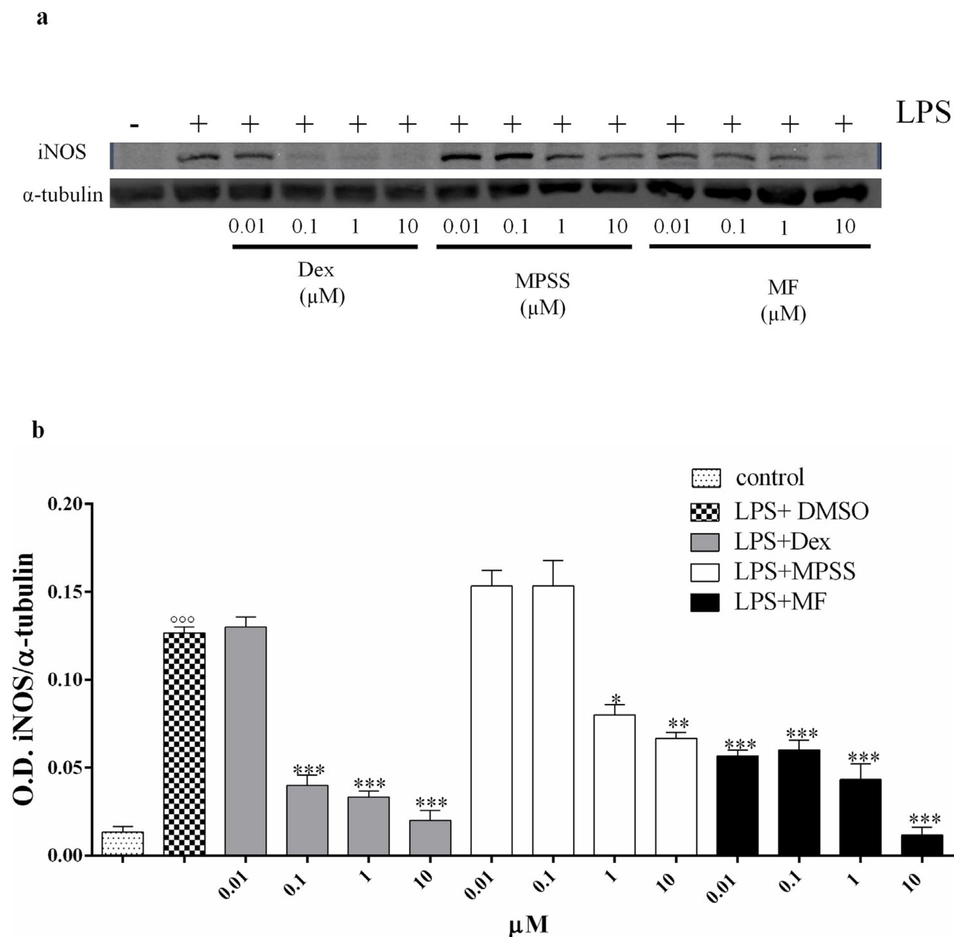

Fig. 2. Dex MPSS and MF effects on iNOS expression in LPS-treated J774 macrophages. Cells were pre-treated with Dex, MPSS and MF (0.01, 01, 1 and 10 μM) or vehicle (DMSO 0.5%) for 2 h and further incubated for 24 h with LPS (10 μg/ml). Equal amounts of whole lysates were fractionated by 10% SDS–PAGE and subjected to Western blot analysis. Data are expressed as means ± SEM from $n = 2$ independent experiments performed in triplicates, each. $^{\circ\circ\circ}p < 0.001$ *vs* unstimulated cells (control); $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs* LPS + DMSO.

2.3.3. Experimental design

Mice were randomly allocated into the following groups ($n = 60$ total animals):

Sham group ($n = 5$): Mice not subjected to SCI but only to T5–T8 vertebrae exposition, sacrificed as control.

SCI group ($n = 10$): Mice were subjected to surgical operations to induce SCI and injected with saline;

SCI **+** MF ($n = 10$): Mice were subjected to surgical operations to induce SCI and treated with MF 0.1 mg/Kg;

SCI **+** Dex ($n = 10$): Mice were subjected to surgical operations to induce SCI and treated with Dex 1 mg/Kg;

SCI **+** MPSS ($n = 10$): Mice were subjected to surgical operations to induce SCI and treated with MPSS 6 mg/Kg.

In another experimental set, investigated drugs were preliminary tested in order to evaluate a more efficient dosage. Moreover, control groups (not included in the present work) designed as "Sham + MF" ($n = 5$), "Sham + Dex" ($n = 5$), "Sham + MPSS" ($n = 5$), "Sham + vehicle (5% DMSO)" ($n = 5$) were tested to evaluate toxicity of GC as well as vehicle (DMSO) injection.

Mice were treated i.p. 30 min following trauma induction and once a day for 7 days until sacrifice.

Spinal cord area, corresponding to the thoracic spine, was sampled, in order to evaluate the various parameters.

2.3.4. May-Grunwald Giemsa staining

Spinal cord biopsies, taken at 7 days following trauma, were fixed in 10% (w/v) PBS-buffered formaldehyde. Tissue segments containing the lesion were paraffin-embedded and cut into 7 μm-thick sections. Tissue sections were deparaffinized with xylene, stained with May-Grunwald Giemsa solution for tissue sections (Bio Optica, Milan, Italy) according to manufacturer's protocol and studied using light microscopy (LEICA ICC50HD microscope).

2.3.5. Immunohistochemical localization

Seven-μm sections, prepared from paraffin-embedded tissue, were deparaffinated and endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. Nonspecific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin (DBA, Milan, Italy), respectively. Sections were incubated overnight with: anti-glial fibrillary acidic protein (GFAP) mouse monoclonal Ab (Cell Signaling Technology, 1:100); anti-iNOS rabbit polyclonal Ab (Santa Cruz Biotechnology, 1:100); anti-poly (ADP-ribose) polymerase 1 (PARP-1) rabbit polyclonal Ab (Santa Cruz Biotechnology, 1:100); anti-Bax rabbit polyclonal Ab (Santa Cruz Biotechnology, 1:100); anti-Bcl-2 rabbit polyclonal Ab (Santa Cruz Biotechnology, 1:100). Sections were washed with PBS and incubated with secondary antibody. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA, Milan, Italy). The counter-stain was developed with diaminobenzidine (brown color) and ematossilin (blue background) or nuclear fast red (red background). All images were acquired using in a LEICA ICC50HD

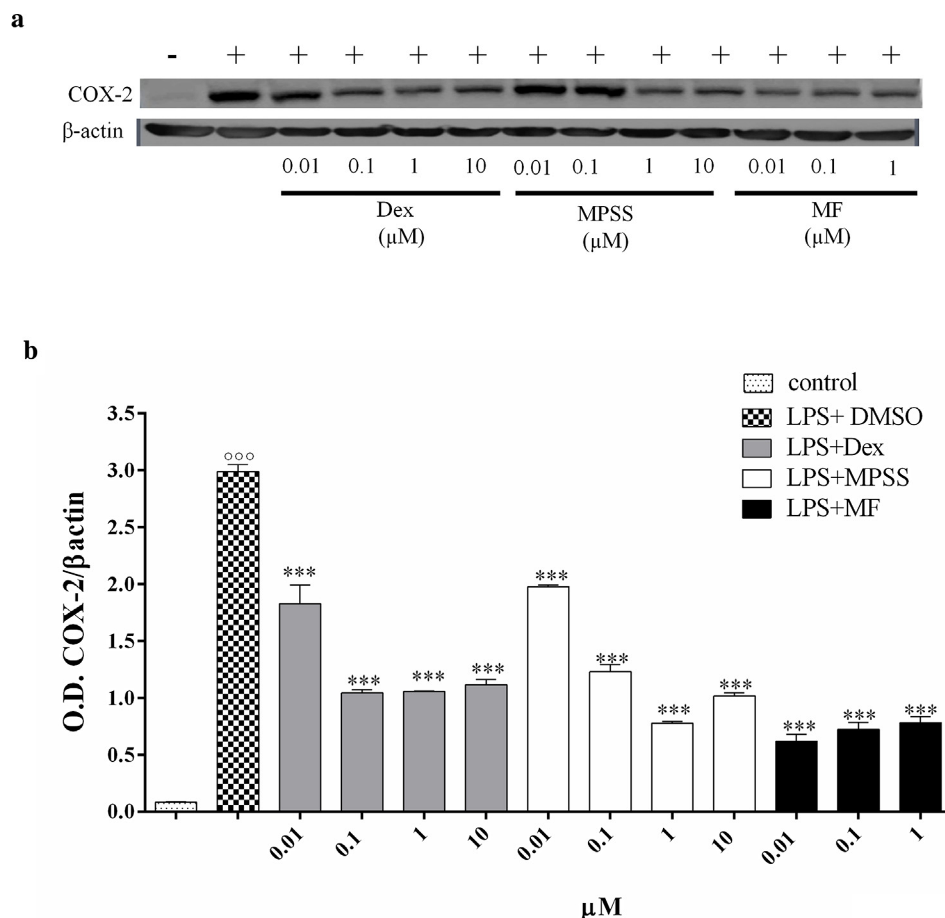

Fig. 3. Dex MPSS and MF effects on COX-2 expression in LPS-treated J774 macrophages. Cells were pre-treated with Dex, MPSS (0.01, 01, 1 and 10 μM) and MF (0.01, 01 and 1 μM) or vehicle (DMSO 0.5%) for 2 h and further incubated for 24 h with LPS (10 μg/ml). Equal amounts of whole lysates were fractionated by 10% SDS–PAGE and subjected to Western blot analysis. Data are expressed as means ± SEM from $n = 2$ independent experiments performed in triplicates, each. °°°$p < 0.001$ *vs* unstimulated cells (control); ***$p < 0.001$ *vs* LPS + DMSO.

light microscope. Quantitative data obtained through densitometric analysis were acquired using Leica Application Suite V4.2.0 software

2.3.6. Extracellular signal regulated kinase 1/2 (pERK1/2), PARP-1 and Bax expression

Protein content for specific antibodies was evaluated in the spinal cord by SDS page electrophoresis and blotting according to standardized protocol for spinal cord tissues [22].

Membranes were probed at 4 °C overnight with rabbit phospho-p44/42 MAPK (pERK1/2) (Cell Signaling Technology, 1:1000), rabbit PARP-1 (Santa Cruz Inc, 1:200), rabbit Bax (Cell Signaling Technology, 1:500). Signal was developed following incubation with horseradish peroxidase-conjugated anti-rabbit (1:5000; Santa Cruz Biotecnology) for 1 h at room temperature and using an enhanced chemiluminescence system (Luminata Western HRP Substrates, Millipore). p44/42 MAPK (ERK1/2) (Cell Signaling Technology, 1:1000) was used to normalize the signals of phospho-p44/42 MAPK, while β-actin and GAPDH were used to normalized PARP-1 and Bax expression, respectively.

The protein bands were acquired with ChemiDoc™ MP System (Bio-Rad) and quantified using a computer program (ImageJ).

2.4. Statistical evaluation

The results are expressed as mean ± standard error (SEM) of the mean of n observations, where n represents the number of experiments performed in different days or the number of animals. For *in vitro* study triplicate wells were used for the various treatment conditions. Data were analyzed in GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA). The results were analysed by one-way ANOVA followed by a Bonferroni *post-hoc* test for multiple comparisons. A p value of <0.05 was considered to be statistically significant.

3. Results

3.1. In vitro study

3.1.1. Nitrite production and iNOS expression are strongly inhibited by MF in LPS-stimulated J774 macrophages

NO pathway is activated during the inflammatory process that characterizes the secondary injury of SCI. Although it is well known that GCs inhibit NO pathway, there is insufficient data in support of MPSS, the reference drug for SCI treatment, and Dex and MF. For this reason, we evaluated the effects of these GCs on nitrite production and iNOS expression in LPS-treated J774 macrophages.

LPS (10 μg/ml, 24 h) treatment of J774 macrophages induced a significant increase of nitrite production in the cell medium ($p < 0.001$, $n = 3$) in respect to unstimulated macrophages (control). When Dex, MPSS (0.01, 0.1, 1 and 10 μM; Fig. 1a) and MF (0.01, 0.1, 1 and 10 μM in Fig. 1a and 0.0001, 0.001, 0.01 in Fig. 1b) were added to J774 macrophages, 2 h before LPS stimulation, a significant and concentration-dependent decrease of nitrite production

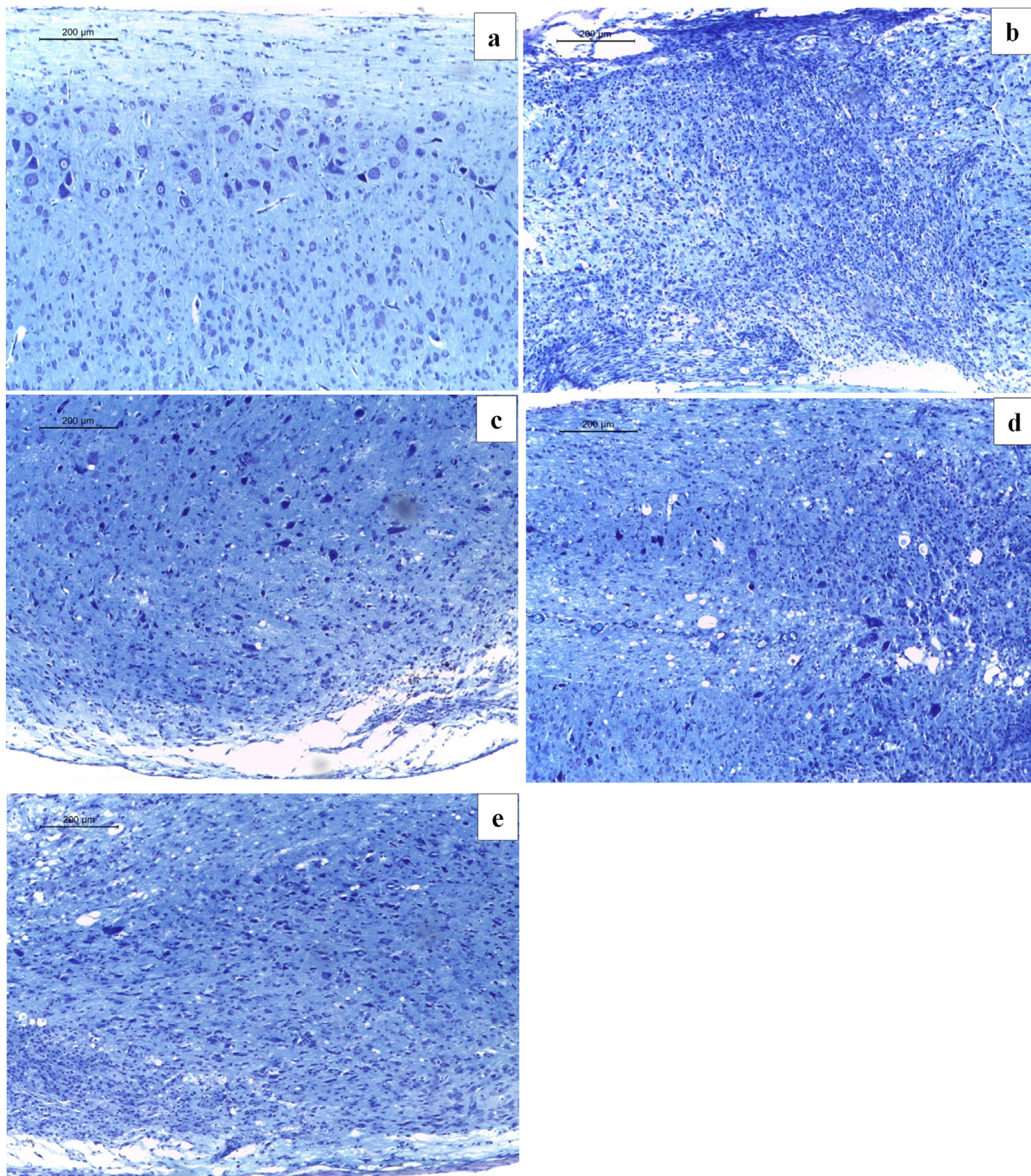

Fig. 4. WBC infiltration in spinal cord tissue. May Grunwald Giemsa staining reveals that sham animals do not show presence of WBC infiltrate (a). Conversely, after seven days, untreated SCI evoke lymphocyte and monocytic infiltration accompanied by fibrotic tissue indicating (b). Total protection is evident in SCI-damage mice treated with MF (c), while a lower capability to counteract cellular infiltrate and fibrosis is assessed following treatments with Dex (d) and MPSS (e).

in cell medium was observed (Fig. 1a and b). In particular, MF was more potent than Dex and MPSS. In fact, the IC_{50} were 0.061 μM, 0.53 μM and 0.00024 μM, for Dex, MPSS and MF, respectively.

To determine whether the different potency of these GCs on LPS-induced nitrite production was mediated by the regulation of iNOS protein expression, we evaluated the levels of iNOS protein in whole lysates from J774 cells, stimulated with LPS and pre-treated with test compounds, as described above. As shown in the Fig. 2, MF was more potent since it significantly inhibited iNOS expression already at a 0.01 μM concentration whereas Dex became active at 0.1 μM and MPSS at 1 μM.

3.1.2. *COX-2 expression are strongly inhibited by MF in LPS-stimulated J774 macrophages*

Since COX-2 expression is another enzyme activated in the inflammatory response to secondary injury, its effects were also compared to those of MPSS, Dex and MF. As observed for iNOS expression, Dex, MPSS (0.01, 0.1, 1 and 10 μM) and MF (0.01, 0.1 and 1 μM) inhibited COX-2 expression in LPS-treated J774 macrophages (Fig. 3a, see densitometric analysis b). In particular, MF was more potent than Dex and MPSS. In fact, the inhibition of protein expression at 0.01 μM was 79% for MF and 39–34% for Dex and MPSS, respectively.

3.2. *In vivo study*

3.2.1. *GC treatments protect by white blood cell (WBC) infiltration in SCI*

SCI causes severe damage in mice both for mechanical injury and for infiltrating cells at the site of injury.

While sham animals do not show presence of WBC (Fig. 4a), seven days after spinal trauma induction, this condition is completely reversed in SCI damaged animals (Fig. 4b). Despite no presence of eosinophil cells (pinkish–red basophile cytoplasm) is observed in this animals, on the contrary, a marked and massive CNS lymphocyte and monocyte infiltration, distributed homoge-

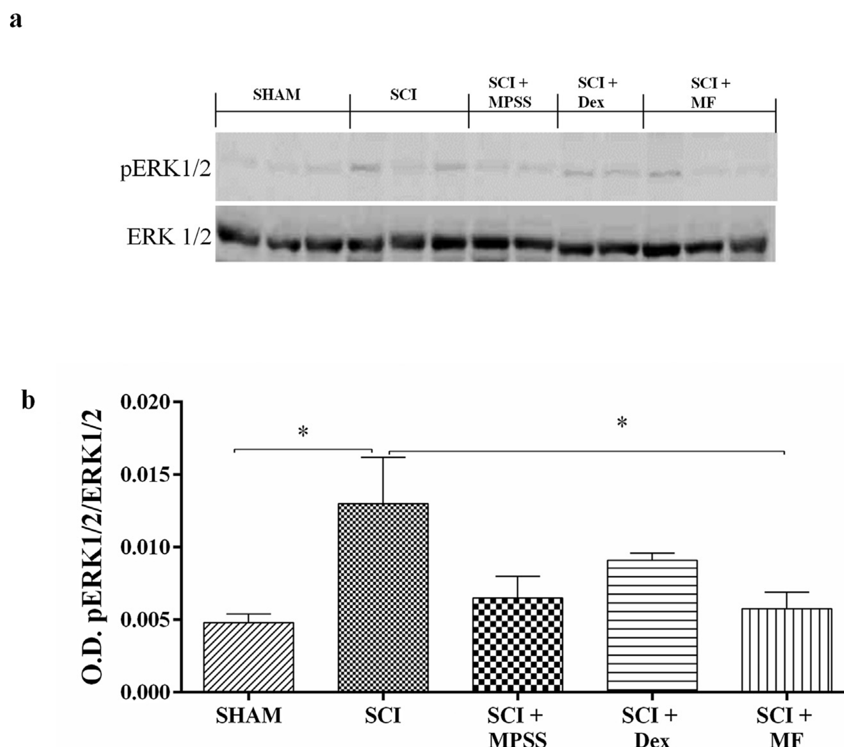

Fig. 5. pERK1/2 is modulated by GC treatment, better than other by MF. pERK1/2 expression is signifintly upregulated in SCI untreated mice comparing to sham animals (a). Conversely, all GCs inhibit pERK1/2 activation, but only the MF inhibition results significant ($p < 0.05$) (a). (b) Normalized pERK1/2 expression on ERK 2 was graphed in the showed densitometric analysis.*$p < 0.05$.

neously in the perilesional tissue and accompanied by fibrotic tissue indicating damage in the adjacent areas has been seen. Although treatment with Dex (Fig. 4d) and MPSS (Fig. 4e) protect the spinal cord tissue by cellular infiltration and fibrosis, there is a complete and more evident protection in SCI-damage mice treated with MF (Fig. 4c).

In control groups, samples of spinal cord were designed to evaluate the possible toxic side-effects of GC injection. No pathological condition was assessed following May-Grunwald Giemsa staining when mice administered with MF, Dex and MPSS were compared with sham animals (data not shown).

3.2.2. pERK1/2 expression is inhibited by MF treatment following SCI

To investigate mechanisms of spinal cord degeneration, MAPK pathway was evaluated.

Tissue samples of SCI untreated mice displayed a significative ($p < 0.05$) increase of pERK1/2 expression in respect to sham animals (Fig. 5a, see densitometric analysis b). Conversely, GCs inhibited pERK1/2 activation, but only MF inhibition resulted significant ($p < 0.05$) (Fig. 5a, see densitometric analysis b).

3.2.3. GFAP increased expression is strongly inhibited by MF treatment following SCI

GFAP is considered a marker of astrocyte cells as well as of neuronal damage.

Immunostaining for GFAP clearly demonstrated that SCI-damaged mice (Fig. 6b) have an enhanced and significant high GFAP expression when compared to sham animals ($p < 0.0001$, Fig. 6a, see densitometric analysis Fig. 6f). Interestingly, all GC tested inhibited GFAP expression (Fig. 6, see densitometric analysis Fig. 6f). Overall, comparing the ability of GCs to avoid tissue expression of this indirect marker of astrocyte activation, we established that SCI mice treated with MF (Fig. 6c, see densitometric analysis Fig. 6f) displayed lower positivity for GFAP, followed by Dex (Fig. 6d, see densitometric analysis Fig. 6f) and finally by MPSS (Fig. 6e, see densitometric analysis Fig. 6f). The remarkable efficacy of MF was further confirmed by a significant difference between this GC and SCI + Dex ($p < 0.01$) as well as SCI + MPSS groups ($p < 0.01$, for both see densitometric analysis Fig. 6f).

3.2.4. GCs differently modulate iNOS expression following SCI

Radicalic species production has a key role in development of severe spinal cord damage, especially following SCI.

During investigation of oxidative pathways, sham mice resulted negative for iNOS staining (Fig. 7a, see densitometric analysis Fig. 7f). Analysis of samples of SCI mice demonstrated that vehicle treated-animals displayed a significant increase of iNOS ($p < 0.01$, Fig. 7b, see densitometric analysis Fig. 7f), whereas iNOS expression in GC treatment was the same as that observed in the *in vitro* model. In particular, the grading scale of protection *vs* untreated SCI mice was given by MF ($p < 0.01$) > MPSS ($p < 0.01$) > Dex (non significant, ns) (Fig. 7c–d, respectively. See densitometric analysis Fig. 7f).

3.2.5. GCs counteract SCI-induced PARP-1 activation

Oxidative stress can result in DNA damage, in turn leading to PARP-1 activation, an enzyme involved in cell death processes as well as strictly linked to proinflammatory mechanisms [23].

Sham mice resulted negative for PARP-1 (Fig. 8a, see densitometric analysis Fig. 8f). On the contrary, compared to this group, SCI mice group displayed both an increased and significant ($p < 0.01$) expression of this marker (Fig. 8b, see densitometric analysis Fig. 8f). In regards to SCI–GC treated mice, we found that MF demonstrated higher protection capability which avoided PARP-1 activation ($p < 0.01$) followed by MPSS ($p < 0.05$) and finally Dex (not significant) (Fig. 8c–d, respectively. See densitometric analy-

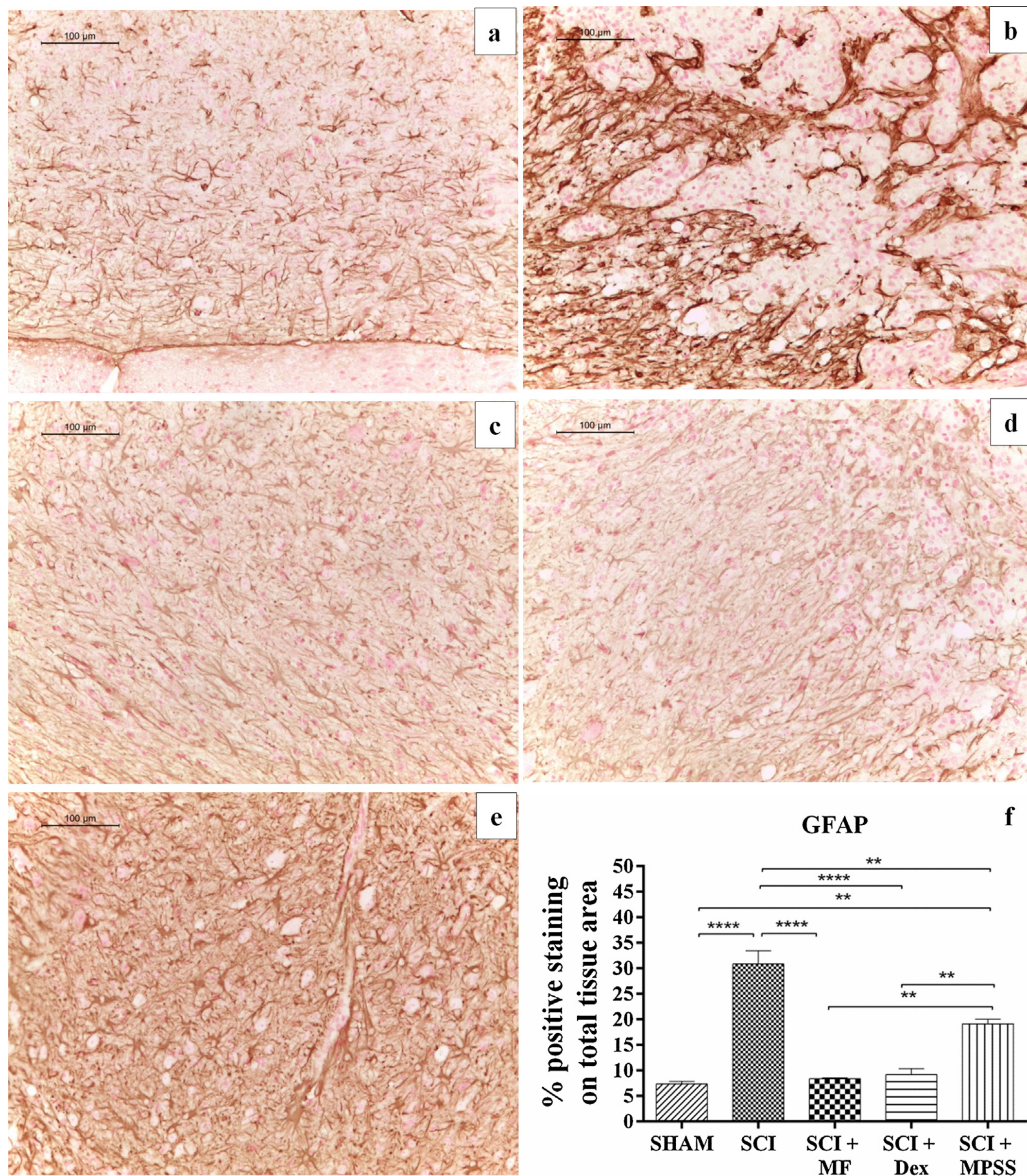

Fig. 6. IHC staining for GFAP. SCI-damaged mice (b) have an enhanced and significant high GFAP expression when compared with sham animals (a) as well as with SCI + MF group (c). SCI mice treated with MF (c) display the lower positivity for GFAP, followed by Dex (d) and finally by MPSS (e). Densitometric quantitative analysis is provide in f. $^{**}p < 0.01$, $^{****}p < 0.0001$

sis Fig. 8f). The best efficacy of MF was confirmed by tissue protein detection in spinal cord extracts (Fig. 8g).

3.2.6. Bax/Bcl-2 unbalanced expression following SCI is modulated by GC treatments

Apoptotic mechanisms are triggered by traumatic insults to the spinal with subsequent tissue degeneration.

In the study of the apoptotic pathway through IHC analysis, we assessed a completely negative staining for Bax (Fig. 9a, see densitometric analysis Fig. 9f) and a marked positivity for Bcl-2 (Fig. 10a, see densitometric analysis Fig. 10f) in sampled sections of sham animals. Conversely, SCI mice resulted strongly positive for Bax ($p < 0.0001$, Fig. 9b, see densitometric analysis Fig. 9f) and negative for Bcl-2 ($p < 0.001$, Fig. 10b, see densitometric analysis Fig. 10f). Among the different pharmacological treatments, MF demonstrated a significant capacitance in protecting the unbalance between Bax ($p < 0.0001$)/Bcl2 ($p < 0.001$) (Figs. 9 and 10c, see densitometric analysis Figs. 9 and 10f, respectively), while Dex ($p < 0.0001$) and MPSS ($p < 0.001$) possessed a lower, although still significant, power against Bax increase (Fig. 9 d and e, see densitometric analysis Fig. 9f) and Bcl-2 degradation (Fig. 10d and e, see densitometric analysis Fig. 10f). Also western blot detection confirmed a significant tissue protection against Bax overexpression (Fig. 9g).

4. Discussion

Following the acute phase, it is important to underline the effectiveness of MPSS as the elective choice in the treatment of inflammatory conditions associated with SCI even though side effects such as depression of the immune system as well as skin problems must be taken into consideration. However, there is a gap in the possible chronic management of the disease that at the moment takes into account baclofen as muscle relaxer and antispastic agent [24]. Nevertheless, although this therapy provides a reduction of the spasticity, it does not provide protection against

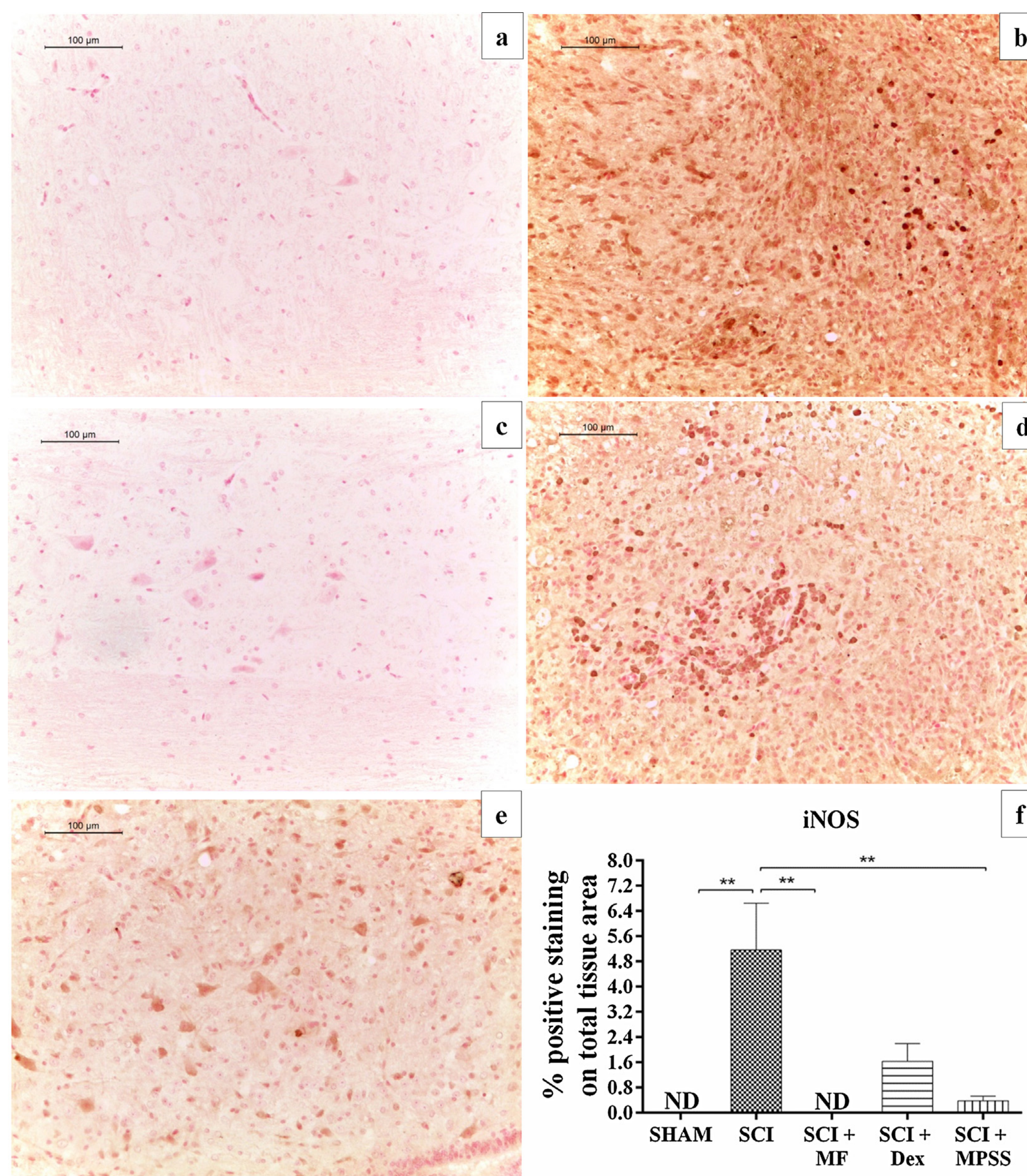

Fig. 7. IHC staining for iNOS. Sham mice resulted negative for iNOS staining (a). Analyzing sections sampled by SCI mice, we demonstrated that untreated animals display a significant iNOS increasing (b), while there is a differentially modulated iNOS expression by GC treatments. In particular, the grading scale of protection vs untreated SCI mice is given by MF (c) >MPSS (e) >Dex (d). Densitometric quantitative analysis is provide in f. $^{**}p < 0.01$.

the propagation of secondary damage affecting tissue which surrounds the traumatized area.

The aim of our study consisted in the evaluation of the best possible treatment for chronic SCI treatment by means of comparison of different GCs (MPSS, MF and Dex).

Commercially, MF is a topical corticosteroid widely used to relieve inflammation characterized by scaly eruptions and accompanied by stinging and itching, such as psoriasis [25] and several types of dermatitis [26]. Moreover, it is also prescribed in the treatment of asthma [27].

On the another hand, Dex is normally prescribed in combined therapy with other drugs in the treatment of CNS tumors (mainly lymphoma) [28,29]. In this study, the *in vitro* and *in vivo* comparison of the efficacy of these GCs demonstrated that MF is notably more effective in the contrasting the diffusion of the so-called perilesional secondary damage. Our conclusions were based on the evaluation of the different pathways that inclusively represent an indication for long-term therapy with MF. At present, all three treatments have demonstrated the ability to modulate inflammatory, oxidative as well as apoptotic pathways. However, all the investigated parameters and evaluated markers demonstrated that MF was the most effective, whereas Dex and MPSS showed alternative results with a factor-by-factor assessable protection degree. To this end, evidence has proven quite convincing.

Preliminary *in vitro* experiments performed on LPS-stimulated murine J774 macrophages have been a useful tool in verifying whether there could be a differentially modulated efficacy of tested GCs on inhibition of the enzymes mainly involved in the inflammatory reaction, such as iNOS and COX-2. Convincing data concerning the efficacy of MF more than the other GCs were achieved not only by evaluating its ability in limiting iNOS induction but also, as a consequence, nitrite production at all tested concentrations. Moreover, MF revealed the higher efficacy not only at lower doses but also in the control of COX-2 expression. In regards to the histological evaluation provided by May Grunwald Giemsa staining, that gives a measure of tissue infiltrate, the data report a clearly evident

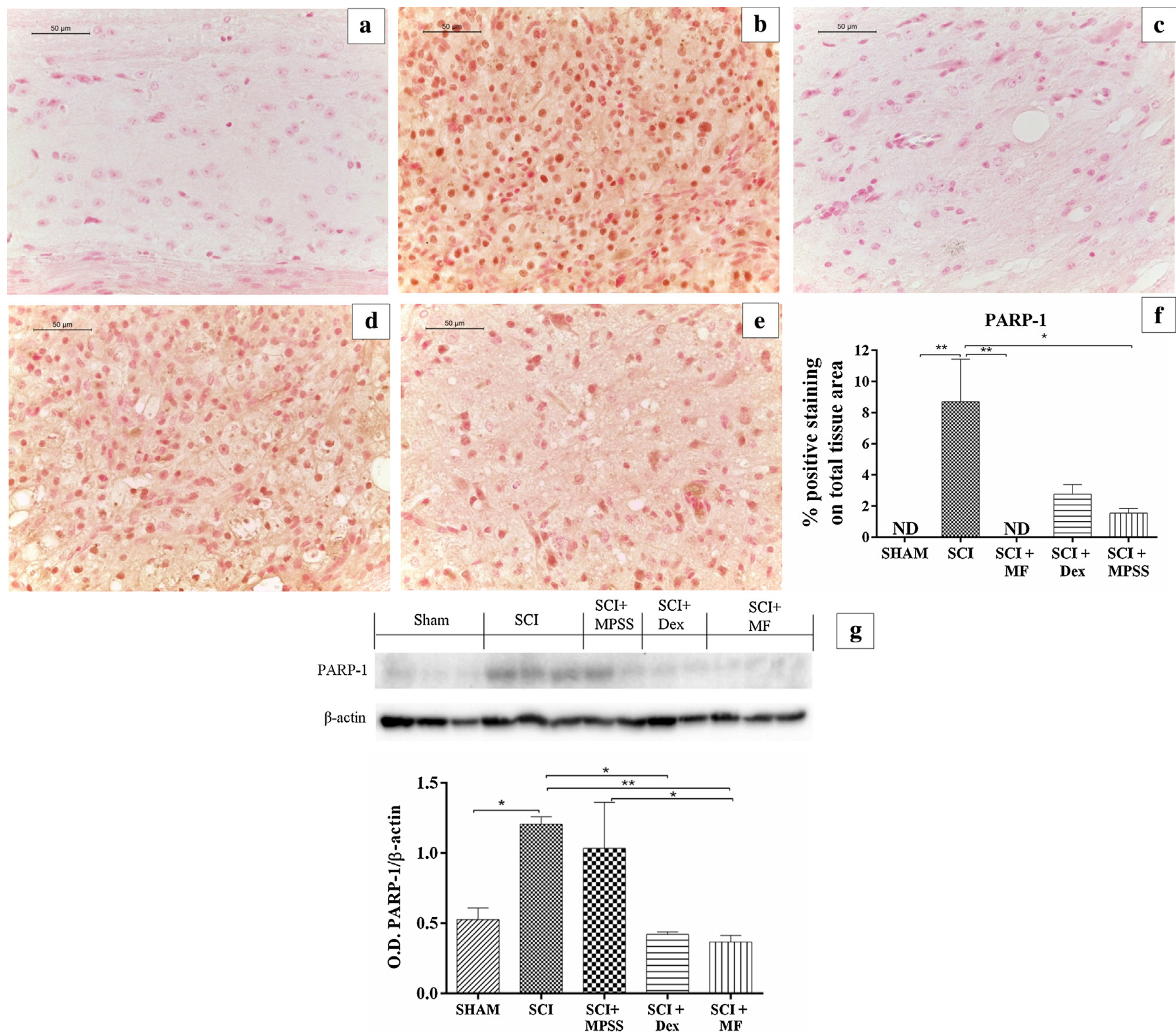

Fig. 8. GCs counteract SCI-induced PARP-1 activation. Sham mice resulted negative for PARP-1 (a). On the contrary, comparing to this group SCI mice group display an increased and significant expression for this marker (b). About SCI-GC treated mice, we revealed that MF (c) has the best protection capability avoiding PARP-1 activation followed by MPSS (e) and finally Dex (d). Densitometric quantitative analysis is provide in f. Western blot analysis of spinal cord protein extracts confirmed the best efficacy of MF in to counteracting PARP-1 overexpression (g). $^{*}p < 0.05$, $^{**}p < 0.01$, ND = Not Detectable.

and total protection against WBC infiltrates by MF treatment, and a lower efficacy by Dex or MPSS treatment.

Moreover, we have demonstrated the important role and involvement of MAPK in the pathogenesis of SCI, as a signaling pathway of pivotal importance for the downstream mechanisms in disease progression [30].

Under our experimental conditions, it is interesting to note that MF significantly inhibited ERK1/2 activation, an important pathway in the pathophysiology of spinal cord cell and tissue injury following trauma [30]. Moreover, the ERK1/2 signaling pathways have been found to be involved in microglial/macrophage activation [31–33] and in free radical production, such as NO [33,34]. This also translates into a tissue protection from the detrimental action of oxidative mediators.

In this regard, the role of GFAP in CNS injuries is clear and well established in literature [35] as marker of astroglial activation and gliosis. Our evidence shows how the role of GCs, in counteracting oxidative pathway mediated by GFAP, arise from a reduced expression of this marker during SCI neurodegeneration induced by MF treatment in comparison to Dex and MPSS. Moreover, it is also demonstrated that GFAP is strongly promoted by NO release [36]. Therefore, it was not a surprise to find a correspondent upstream total absence of iNOS induction particularly following MF treatment.

Moreover, the evaluation of the apoptosis pathway confirmed the high MF ability in protecting the tissue surrounding the damaged area. It is well known from the literature that PARP-1 inhibitors ameliorate experimental SCI [37].

Chan et al. reports that inhibiting upregulation of PARP-1 in the nucleus, in turn, inhibits nuclear translocation of apoptosis-inducing factors that are related with mitochondrial translocation of Bax and cytochrome c release [38].

Here we have confirmed that MF, despite probably it does not act directly on PARP-1 activity, is able to reduce the related mechanisms of damage that in not pharmacologically treated SCI animals produce its activation. Lower activity has been shown by Dex and MPSS that, however, reduce PARP-1 release in comparison with untreated SCI. Finally, by avoiding the activation mechanisms of reparation triggered by serious injury, we investigated whether MF activity could be related to the nerve tissue protection from the

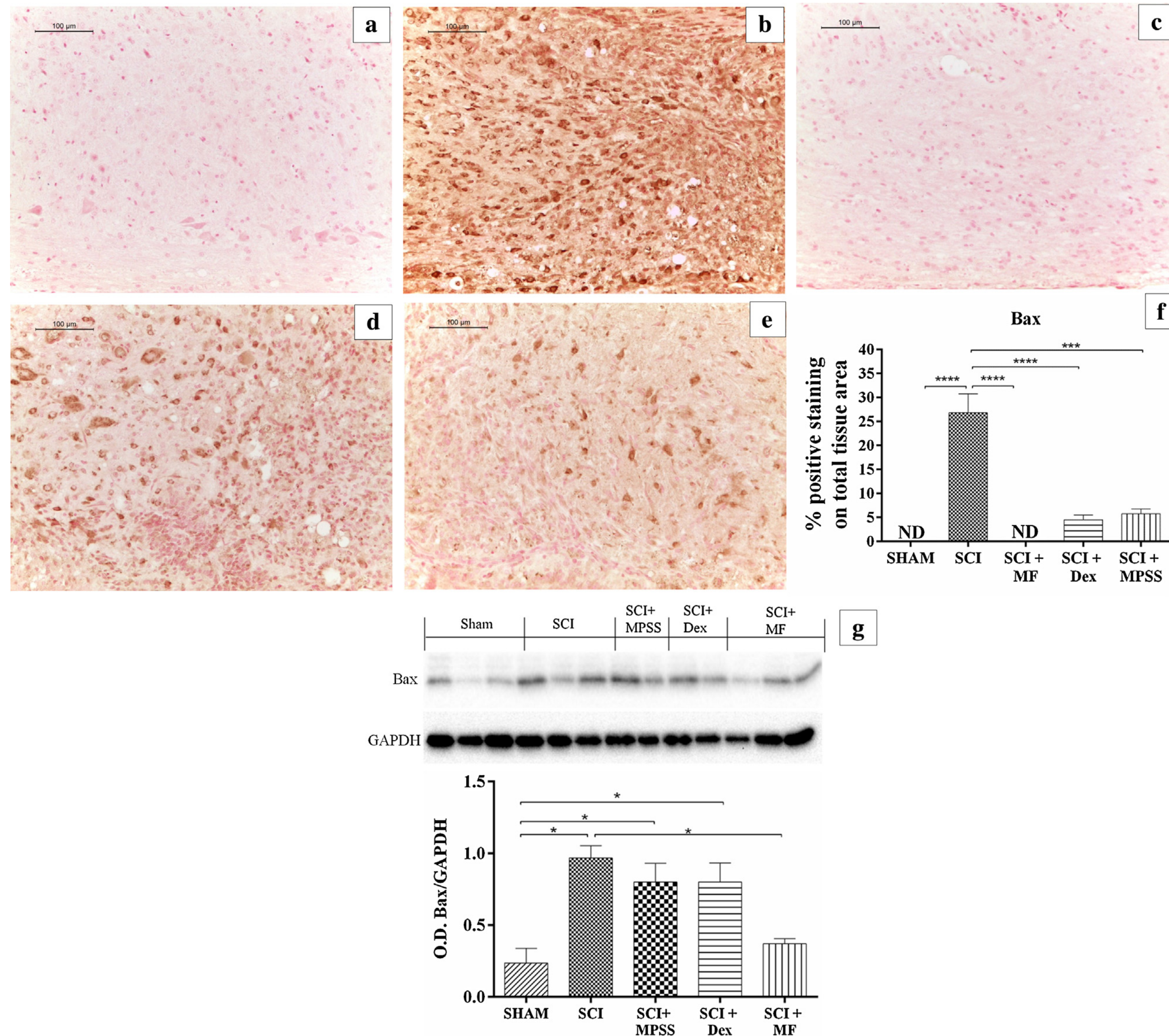

Fig. 9. Bax expression following SCI is modulated by GC treatments. A completely negative staining for Bax results in sections sampled by sham animals (a). Conversely, SCI mice are strongly positive (b). MF resulted able to completely and significantly protect tissue by Bax overexpression (c), while Dex (d) and MPSS (e) possess a lower, although still significant power. Densitometric quantitative analysis is provide in f. Western blot analysis of spinal cord protein extracts confirmed the best efficacy of MF in to counteracting Bax overexpression (g). $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$, ND = Not Detectable.

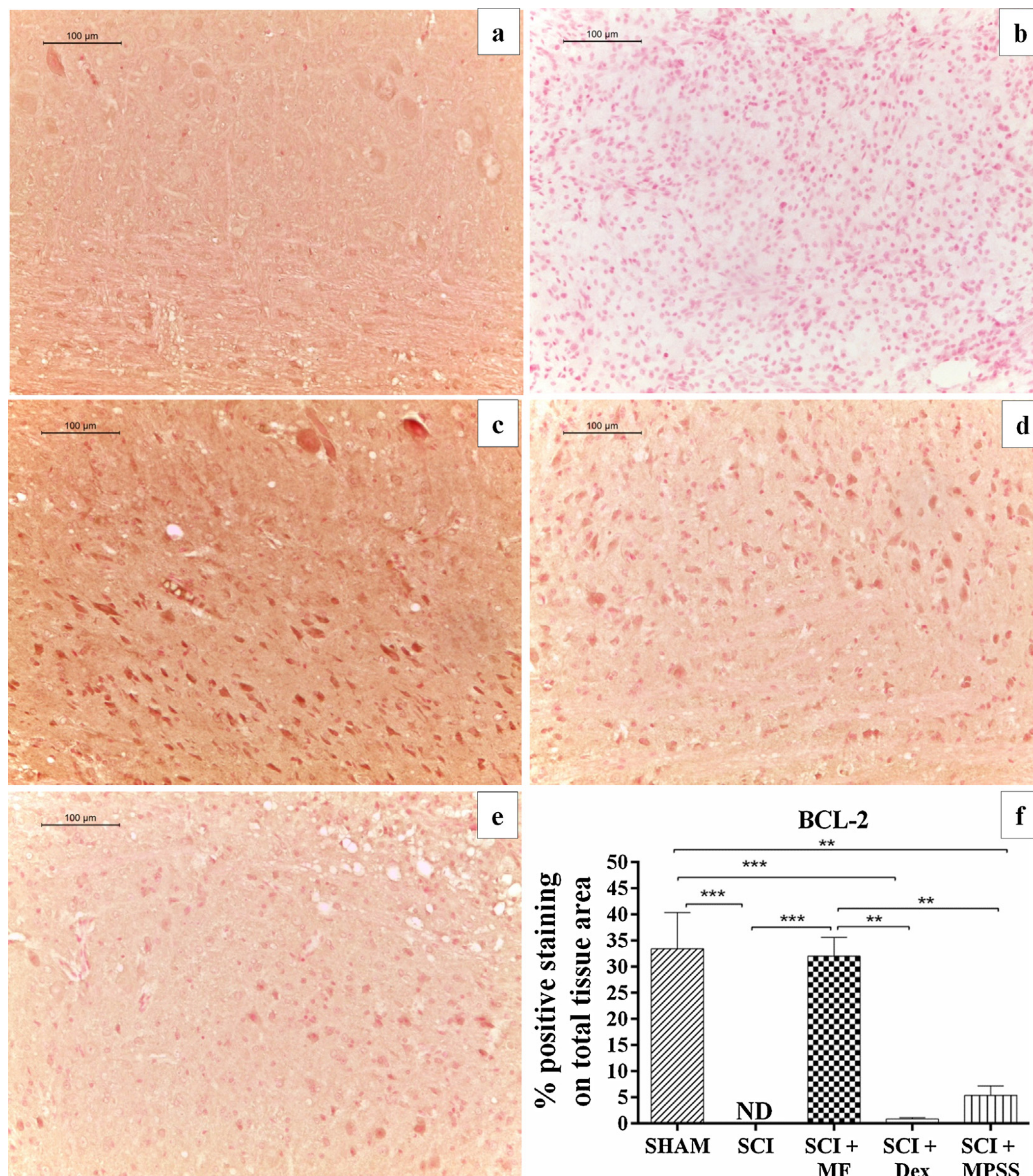

Fig. 10. Bcl-2 expression following SCI is modulated by GC treatments. A marked positivity for Bcl-2 is evident in sections sampled by sham animals (a). Conversely, SCI mice are negative for Bcl-2 (b), while MF treatment completely and significantly protect the tissue by Bcl2 downregulation (c). Dex (d) and MPSS (e) possess a lower, although still significant, power to protect against Bcl-2 degradation.Densitometric quantitative analysis is provide in f. $^{**}p < 0.01$, $^{***}p < 0.001$, ND = Not Detectable.

altered mitochondrial permeability observed in SCI. Effectively, by considering the Bax/Bcl-2 balance, MF was better able than Dex and MPSS in preserving mitochondrial homeostasis.

5. Conclusions

The present study was designed to evaluate a new therapy in the management of secondary damage following SCI and its eventual use in post-traumatic therapy alone or in association with the currently used emergency protocol with MPSS. For this purpose we have compared the efficacy of MPSS, Dex and MF.

After evaluation of the results, we can conclude that, more than MPSS and Dex, MF is a better therapy for post-traumatic chronic treatment of SCI, since it ameliorates different molecular pathways at the basis of the damage's propagation to the surrounding areas of the injured spinal cord. Since these events are the cause of severe invalidating consequences in affected patients, the discovery of new treatments becomes of pivotal importance for a more adequate clinical management of SCI.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2015.07.013

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