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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.

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%