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Mesenchymal stem cells reduce the oxaliplatin-induced sensory neuropathy through the reestablishment of redox homeostasis in the spinal cord

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Abstract

Aims: The present study was designed to investigate whether the antinociceptive effect of bone marrow-derived mesenchymal stem/stromal cells (MSC) during oxaliplatin (OXL)-induced sensory neuropathy is related to antioxidant properties. **Main methods:** Male mice C57BL/6 were submitted to repeated intravenous administration of OXL (1 mg/kg, 9 administrations). After the establishment of sensory neuropathy, mice were treated with a single intravenous administration of MSC ($1x10^6$), vehicle or gabapentin. Paw mechanical and thermal nociceptive thresholds were evaluated through von Frey filaments and cold plate test, respectively. Motor performance was evaluated in the rota-rod test. Gene expression profile, cytokine levels, and oxidative stress markers in the spinal cord were evaluated by real-time PCR, ELISA and biochemical assays, respectively. **Key findings:** OXL-treated mice presented behavioral signs of sensory neuropathy, such as mechanical allodynia and thermal hyperalgesia, which were completely reverted by a single administration of MSC. Repeated oral treatment with gabapentin (70 mg/kg) induced only transient antinociception. The IL-1β and TNF-α spinal levels did not differ between mice with or without sensory neuropathy. MSC increased the levels of anti-inflammatory cytokines, IL-10 and TGF-β, in the spinal cord of neuropathic mice, in addition to increasing the gene expression of antioxidant factors SOD and Nrf-2. Additionally, nitrite and MDA spinal levels were reduced by the MSC treatment. **Significance:** MSC induce reversion of sensory neuropathy induced by OXL possibly by activation of anti-inflammatory and antioxidant pathways, leading to reestablishment of redox homeostasis in the spinal cord.

Keywords: Neuropathic pain; chemotherapy; Mesenchymal Cells; Oxidative Stress; Cytokines.

Introduction

Neuropathic pain is a type of chronic pain which is triggered by injury or dysfunction of the somatosensory system [1]. Chemotherapy-induced sensory neuropathy, manifested clinically as sensory symptoms and neuropathic pain, is a frequent clinical problem which stands out for its high refractoriness to available pharmacological treatments. In fact, the prevalence of chemotherapy-induced sensory neuropathy has been increasing due to advances in postoperative adjuvant therapy, which provides longer survival for cancer patients [2]. Despite not being a cause of death, this neuropathic syndrome has a strong negative impact on the patients' quality of life and, thus, influences their adherence to antineoplastic treatment [3].

Oxaliplatin (OXL) is a third generation platinum derivative with a broad antitumor activity, currently used to treat ovarian cancer, cervical cancer and especially colorectal cancer [4,5]. However, neurotoxicity is the most debilitating side effect of this chemotherapy [6,7]. Several mechanisms have been proposed to explain oxaliplatin-induced neurotoxicity, among which the most well-accepted are: (1) interference with ion channels [8,9]; (2) generation of reactive oxygen species (ROS) [10]; (3) mitochondrial lesions with increased oxidative stress [11] and (4) glial cell activation [12-14]. Interestingly, even though OXL does not have access to the CNS as it does not cross the blood-brain barrier [15], oxidative damage to the spinal cord of rats treated with this chemotherapy has been described, associated with the development of neuropathic pain [16].

Therapeutic approaches to chemotherapy-induced painful neuropathy control have been based on evidence from other neuropathies treatment such as diabetes and post-herpetic [17,18], but these practices have been found to be unsatisfactory. The pharmacological treatment of choice is based on the use of drugs that reduce neural excitability, such as gabapentin, which has low efficacy in reducing pain [19,20]. No drugs currently available are considered effective in the clinical management of the painful neuropathy associated with chemotherapy, especially that induced by OXL.

To establish more effective approaches to neuropathic pain syndromes treatment, the therapeutic potential of stem cells has been investigated in different stages of sensory neuropathy. Stem cells transplantation induces beneficial effects during experimental sensory neuropathy associated with diabetes [21], sciatic nerve ligation [22] and spinal cord injury [23,24]. This approach was initially based on the neuroprotective and neuroregenerative properties of stem cells, which has been widely demonstrated [25-28]. More recently, the analgesic properties of MSC have been shown both in experimental [29,30] and clinical [31,32] conditions, highlighting the potential of these cells to neuropathic pain control. In this context, MSC have been the most studied cell type due to their broad therapeutic potential and low tumorigenesis, besides being easily isolated and expanded *in vitro* [33]. The mechanisms involved in the therapeutic properties of MSC have not yet been elucidated, but these effects are probably due to multiple mechanisms, reflecting the broad spectrum of biological effects of these cells [21,22,29,30]. The present study was designed to investigate whether the antioxidant properties contribute to the antinociceptive effect induced by MSC during OXL-induced sensory neuropathy, restoring the redox balance in the spinal cord.

Materials and methods

Animals: Experiments were performed on male C57BL/6 mice (20–25 g) obtained from the Animal Facilities of Instituto Gonçalo Moniz/FIOCRUZ (Brazil). Animals were housed in temperature-controlled rooms (22±1° C), under a 12:12-h light-dark cycle, with access to water and food *ad libitum*. All behavioral tests were performed between 7:00 a.m. and 5:00 p.m., and animals were not reused in different experimental protocols. Animal care and handling procedures were in accordance with the Institutional Animal Care and Use Committee FIOCRUZ (IGM 025/2011) and the International Pain Study Association [34]. Every effort was made to minimize the number of animals used and to avoid any unnecessary discomfort.

Bone marrow-derived MSC culture and transplantation: MSC were obtained from bone marrow of femurs and tibiae of C57BL/6 mice as described [35]. A purified population of MSC with spindle-shaped morphology were maintained at 37 °C with 5% CO_2 and expanded in DMEM medium supplemented with 2 mM L glutamine, 1 mM sodium piruvate, 50 µg/mL gentamycin, and 10% fetal bovine serum (all reagents were acquired from Sigma). The cells were expanded during 5-6 passages, when 80-90% confluence was reached, cells were detached using 0.25% trypsin, (Invitrogen/Molecular Probes, Eugene, OR, USA), washed in 0,9% Sodium Chloride solution and then used for transplant. The number of MSC administered in each animal was $1x10^6$ in sterile saline solution with 10% heparin. The cells were slowly administered intravenously by the tail vein. The identity of MSC was confirmed on the basis of morphological criteria, plastic adherence, and specific surface antigen expression evaluated by flow cytometry. The analysis of cell surface markers showed that MSC expressed low levels of hematopoietic cell lineages markers (1.9% CD45, 2.1% CD34 and 1.1% CD11b) and expressed common MSC-specific cell surface markers suchs as CD90 (90%), CD44 (96%) and Sca-1 (88%). Additionally, the differentiation capacity into osteocyte, adipocyte and chondrocyte was evaluated after induction using specific media, as previously described [21]. After induction, MSC differentiated into osteocytes (mineralizing cells stained with alizarin red), adipocytes (stained with oil red) or chondrocytic lineage cells stained with Alcian blue.

Oxaliplatin-induced sensory neuropathy (OISN) model and experimental design: sensory neuropathy was induced by the alternate day model, as previously described [36]. OXL (1 mg/kg) administrations were performed through the lateral tail vein of the mouse twice a week (on Mondays and Thursdays) for four and a half weeks, for a total of 9 administrations. Therefore, each mouse received 9 mg/kg OXL. In the control group, the mice were submitted to the same administration protocol, but received vehicle, 5% dextrose in distilled water, replacing OXL. The experimental period of this study was 10 weeks, being the first 4.5 weeks for the chemotherapy cycle with OXL (induction of the chronic neuropathy model), 1.5 week between the end of induction and the treatments, and 4 weeks of post-treatment follow-up. Sensorial parameters of neuropathic pain were assessed throughout the experimental period by using the established behavioral assays that evaluate mechanical (von Frey filaments) and thermal (cold plate) nociceptive thresholds [37,38]. Throughout the experimental period, the nociceptive threshold readings were taken once a week. Only in the week following treatment with MSC, nociceptive threshold readings were taken daily. Behavioral tests were performed in blind fashion. Animals were randomized and divided into experimental groups (n = 6): non-neuropathic group (Control), neuropathy plus vehicle treatment (OXL), neuropathy plus MSC treatment (MSC), neuropathy plus gabapentin (GBP) and neuropathy plus gabapentin treatment control (GBP-control). In the week

(6^{th} week) of gabapentin treatment (70 mg/kg, oral route, every 12 hours for 6 consecutive days) nociceptive threshold readings were taken before and 2 hours after drug administration in this group. Tissue analyzes were performed 4 weeks after MSC treatment and 24 hours after gabapentin administration period.

Determination of mechanical nociceptive threshold with von Frey filaments: Withdrawal threshold to mechanical stimulation was measured with von Frey filaments (Stoelting; Chicago, IL, USA). In a quiet room, mice were placed in acrylic cages (12 × 10 × 17 cm) with wire grid floor, allowing full access to the ventral aspect of the hind paws, 40 min before the beginning of the test. A logarithmic series of nine filaments were applied to the plantar surface of the ipsilateral hind paw to determine the threshold stiffness required for 50% paw withdrawal according to the non-parametric method of Dixon, as described by Chaplan and collaborators [37]. A positive response was characterized by the removal of the paw followed by clear flinching movements. The development of neuropathic pain was characterized by mechanical allodynia, indicated by the reduction of the paw withdrawal threshold (in grams).

Determination of thermal nociceptive threshold - cold plate: In order to evaluate the nociceptive threshold to cold thermal stimulation the cold plate test (TECA®) was used. In this test, the nociceptive threshold is represented by the total number of nociceptive behaviors, as described by Ta et al. [38]. The mice were placed on the plate at a controlled temperature of -2.5ºC ± 0.2ºC, remaining for 5 minutes under observation, when total nociceptive behaviors such as licking, shaking and hind paw elevation were counted and recorded. The animals were placed on the cold plate on the day before the test, and each animal remained two minutes on the plate at the above temperature. Three latency measurements were obtained on different days to determine baseline thresholds. The increase of nociceptive behavior in relation to the basal profile of the animals characterizes the development of cold thermal hyperalgesia.

Motor function assay – rota-rod: To evaluate the motor performance, mice were submitted to the rota-rod test, as previously described [39]. The rota-rod apparatus (Insight, Ribeirão Preto, Brazil) consisted of a bar with a diameter of 3 cm, subdivided into five compartments. On the day of test, mice from different experimental groups were placed on the rotating rod (eight revolutions per min) and the falling avoidance was measured for up to 120 seconds. Mice treated with diazepam (10 mg/kg; Cristália, Itapira, SP, Brazil), the test reference drug, were placed on a rotating rod 1 hour after treatment. The results were analyzed as the average time(s) the animals remained on the rota-rod in each group. Rota-rod was performed before, during and after administration of the treatments.

Cytokine measurement by ELISA: At the end of the experimental time or at the end of the 6^{th} week for gabapentin groups, animals were sacrificed for spinal cord collection (L4-L5). Tissues were homogenized in 500 µl PBS buffer containing 0.05% Tween 20, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM benzethonium chloride, 10 mM ethylenediaminetetraacetic acid (EDTA) and 0.01 mg/ml aprotinin A. Then, the homogenate remained for 1 hour at 4°C and the samples were centrifuged at 10,000 rpm for 25 minutes and the supernatant collected. Samples were then used to quantify pro-inflammatory cytokines, IL-1β and TNF-α, and anti-inflammatory cytokines, IL-10 and TGF-β, by ELISA enzyme immunoassay using Duoset ELISA Development System kits (R&D Systems, Minneapolis, USA) for each cytokine. Plates 96 well (NUNC –

Immuno Plate MaxisorpSurface) were sensitized with 50 µl of capture antibody (purified anti-cytokine monoclonal antibody), diluted in 1x PBS and kept at 4°C overnight. The plates were then washed 3X with 0.05% PBS - Tween® 20, and 100 µL of the 5% Tween® 20 and 0.05% NaN3 PBS solution was added. They were then kept at room temperature for 2 hours to block non-specific sites. Soon after, the plates were washed 3x and incubated for 2 hours at room temperature with 50 µL of the sample (per well, in duplicate) and their respective standard curves. After further washing, biotinylated anti-cytokine detection antibody was added and the plates were incubated again for 2 hours at room temperature. After washes, 50 µl / well of 1:200 diluted streptavidin were added and incubated for 20 minutes at room temperature. After further washes, the reaction was developed with 50 µl / well developer solution containing 10 mL 1M citrate phosphate buffer, 2 µL H_2O_2 and one TMB (tetramethylbenzidine) pellet. The reaction was blocked by the addition of 50 µL / well of 1:20 phosphoric acid. The plates were read on a spectrophotometer (Spectra Max 190-Molecular Devices, California, USA), at 450 nm wavelength and the data analyzed in Softmax 4.3.1 software (Molecular Devices). The results are expressed as picograms of cytokine per milligram of protein. The total protein concentration of the samples was determined using the Bradford protein assay [40].

Real-time PCR: At the end of the experimental time or at the end of the 6th week for gabapentin groups, animals were sacrificed for spinal cord collection (L4-L5). Plastic materials and RNA-free solutions were used throughout the procedure. At the time of RNA extraction, tissues were homogenized in 1 mL Trizol reagent (Gibco - BRL Life Technologies, Grand Island, N.Y.), and total RNA extraction was performed according to manufacturer's instructions. Concentration was determined by photometric measurement using the NanoDrop 2000c apparatus (Thermo Scientific). Then total RNA was transcribed by reverse transcriptase enzyme using the High Capacity Reverse Transcription kit (Applied Biosystems). The cycling conditions used were as follows: 1st cycle (25ºC - 10 minutes), 2nd cycle (37ºC - 1 hour), 3rd cycle (37ºC - 1 hour) and 4th cycle (4ºC - ∞) on the Profle. thermal cycler (Biosystems, USA). The cDNA obtained was used for analysis of quantitative expression of the *nrf-2* (Mm 00477784_m1) and *sod-1* (Mm 01344233_g1) genes in Real Time PCR reactions. To the qRT-PCR amplification reaction containing 4.5 µL cDNA sample and 5.5 µL MIX formed by Taqman Master Mix (Applied Biosystems), above Taqman hydrolysis probes (Applied Biosystems) and ultrapure water were performed in duplicate in the ABIPrism7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) under standard thermal cycling conditions. The mean values of Cycle threshold (Ct) were used to calculate the expression of the studied gene, with normalization to internal control (*gapdh* - Mm 9999915_g1) using the formula $2 - \Delta Ct$. Samples with coefficient of variation greater than 5% were excluded. One control without sample (NTC) and one without reverse transcription enzyme (No-RT) were also included.

Estimation of nitrite and lipid peroxidation: At the end of the experimental time or at the end of the 6th week for gabapentin groups, animals were sacrificed for spinal cord collection (L4-L5). L4–L5 spinal segments were rinsed with ice-cold saline and homogenized in chilled phosphate buffer (pH 7.4), then used to determine lipid peroxidation and nitrite estimation. The malondialdehyde (MDA) content, a marker of lipid peroxidation, was assayed in the form of thiobarbituric acid-reactive substances, as previously described [41]. Briefly, 0.5 ml of homogenate and 0.5 mL of Tris– HCl were incubated at 37 °C for 2 hours. After incubation, 1 ml of 10% trichloroacetic acid was

added and centrifuged at 1000 g for 10 min. To 1 mL of supernatant, 1 mL of 0.67% thiobarbituric acid was added and the tubes were kept in boiling water for 10 min. After cooling, 1 mL double distilled water was added and absorbance was measured at 532 nm. Thiobarbituric acid-reactive substances were quantified using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and were expressed as micromoles of malondialdehyde per milligram of protein (μmol/mg). Nitrite was estimated in the spinal cord homogenate using the Griess reagent and served as an indicator of nitric oxide production [21]. Next, 500 μL of Griess reagent (1:1 solution of 1% sulphanilamide in 5% phosphoric acid and 0.1% napthaylamine diamine dihydrochloric acid in water) was added to 100 μL of homogenate, and absorbance was measured at 546 nm. Nitrite concentration, expressed as micrograms of nitrite per milligram of protein (μg/mg), was calculated using a standard curve for sodium nitrite.

Statistical analyses: Results were expressed as mean ± standard error of the mean of 6 animals. The comparison between the groups was made using one-way ANOVA followed by Tukey's test. In repeated measures studies, the two-way ANOVA was used followed by the Bonferroni test. Data were analyzed using GraphPad Prism v.5.0 software (GraphPad Inc., San Diego, CA), and differences were considered statistically significant for p values < 0.05.

Results

MSC transplantation reverts OXL-induced sensory neuropathy: Behavioral testing was performed at baseline (before OXL administration) and weekly to evaluate the effects of MSC transplantation on measurable sensorial parameters of OISN. OXL induced sensory neuropathy, associated with cold hyperalgesia (Fig. 1) and mechanical allodynia (Fig. 2), evident 2 weeks and 3 weeks after the mouse model induction, respectively ($p < 0.05$). To determine whether MSC induce therapeutic effects in OISN, mice were treated with MSC (1×10^6, 100 μL) 1.5 week after chemotherapy cycle, when sensorial neuropathy was fully established. Four and seven days after treatments, neuropathic mice treated with MSC demonstrated antinociceptive effect to cold stimuli (Fig. 1a, $p < 0.001$) and mechanical stimuli (Fig. 2a, $p < 0.001$), respectively. The antinociceptive effect of MSC was progressive and persisted throughout the experimental period ($p < 0.001$). Unlike cell treatment, oral gabapentin (70 mg/kg), used as the gold standard, induced short-term antinociceptive effect ($p < 0.05$). Gabapentin induced an increase of nociception threshold 1 hour after administration (Figures 1b and 2b, $p < 0.05$), but this effect was reverted 12 hours after treatment. After the gabapentin treatment period, mechanical allodynia and cold hyperalgesia were fully reestablished.

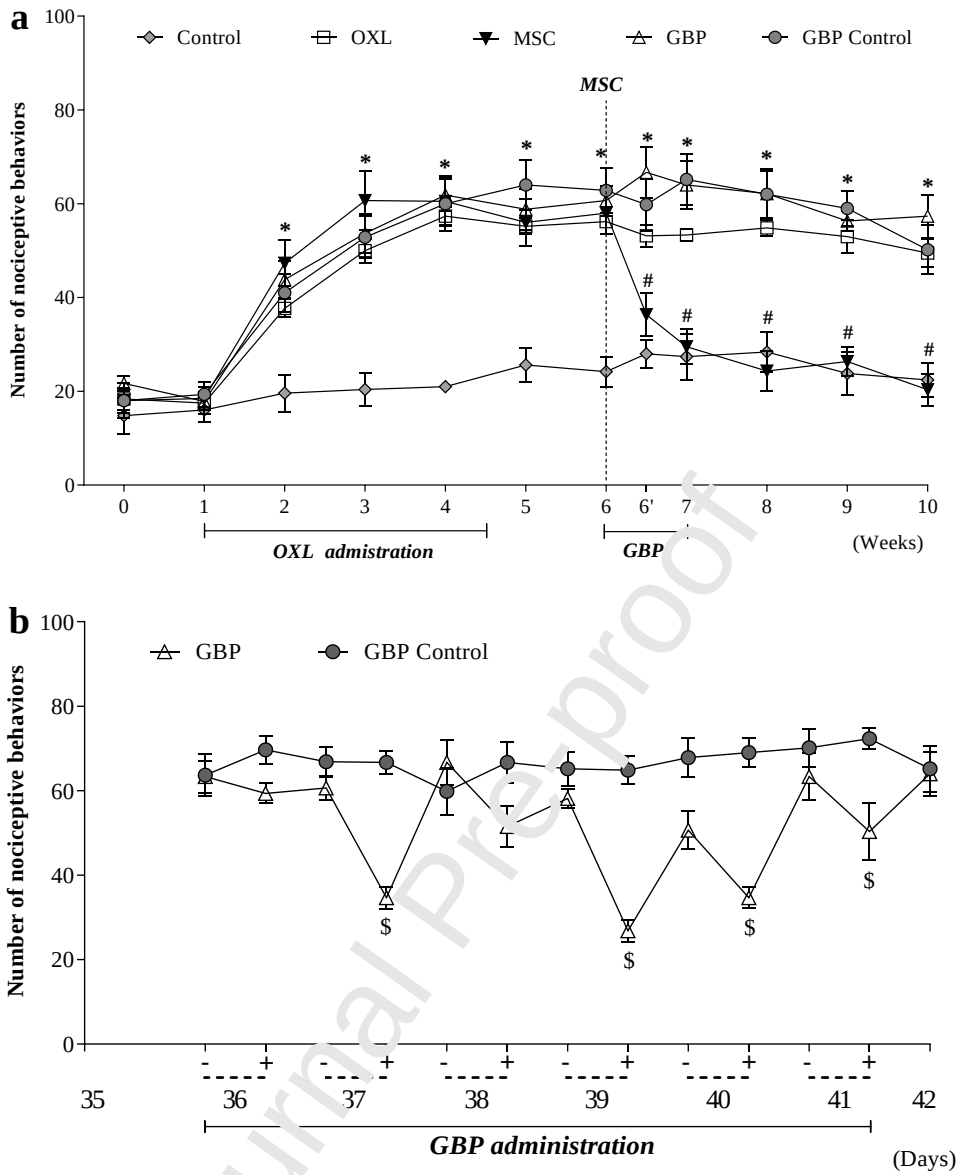

Figure 01: Effect of MSC and gabapentin on cold nociceptive threshold of mice with OXL-induced sensory neuropathy. **a**: thermal nociceptive threshold in weeks during the experimental period (10 weeks). Time zero corresponds to basal thresholds of mice prior to performing any procedure. The chemotherapy cycle with OXL (1 mg / kg / i.v.) was indicated on the graph (OXL administration). The control group represents mice that received vehicle (5% dextrose) to replace OXL in the same administration protocol. The dotted line at week six indicates the day of intravenous administration of MSC ($1x10^6$; MSC group) or saline (200 µL; OXL group) in neuropathic animals. Time 6' corresponds to day 4 after intravenous treatment. The y axis represents the total of nociceptive behaviors in the cold plate. **b**: shows the week 6 represented in days, which corresponds to the administration period of gabapentin (GBP group; 70 mg / kg, oral route, every 12 hours for 6 consecutive days) or its vehicle (GBP Control group). * Statistical significance relative to the control group ($p < 0.05$); # statistical significance relative to the remaining groups, except control group ($p < 0.05$); $ statistical significance relative to GBP-control ($p < 0.05$), as determined by two-way ANOVA followed by Bonferroni test. Data are represented as the average of 6 (six) animals per group ± SD.

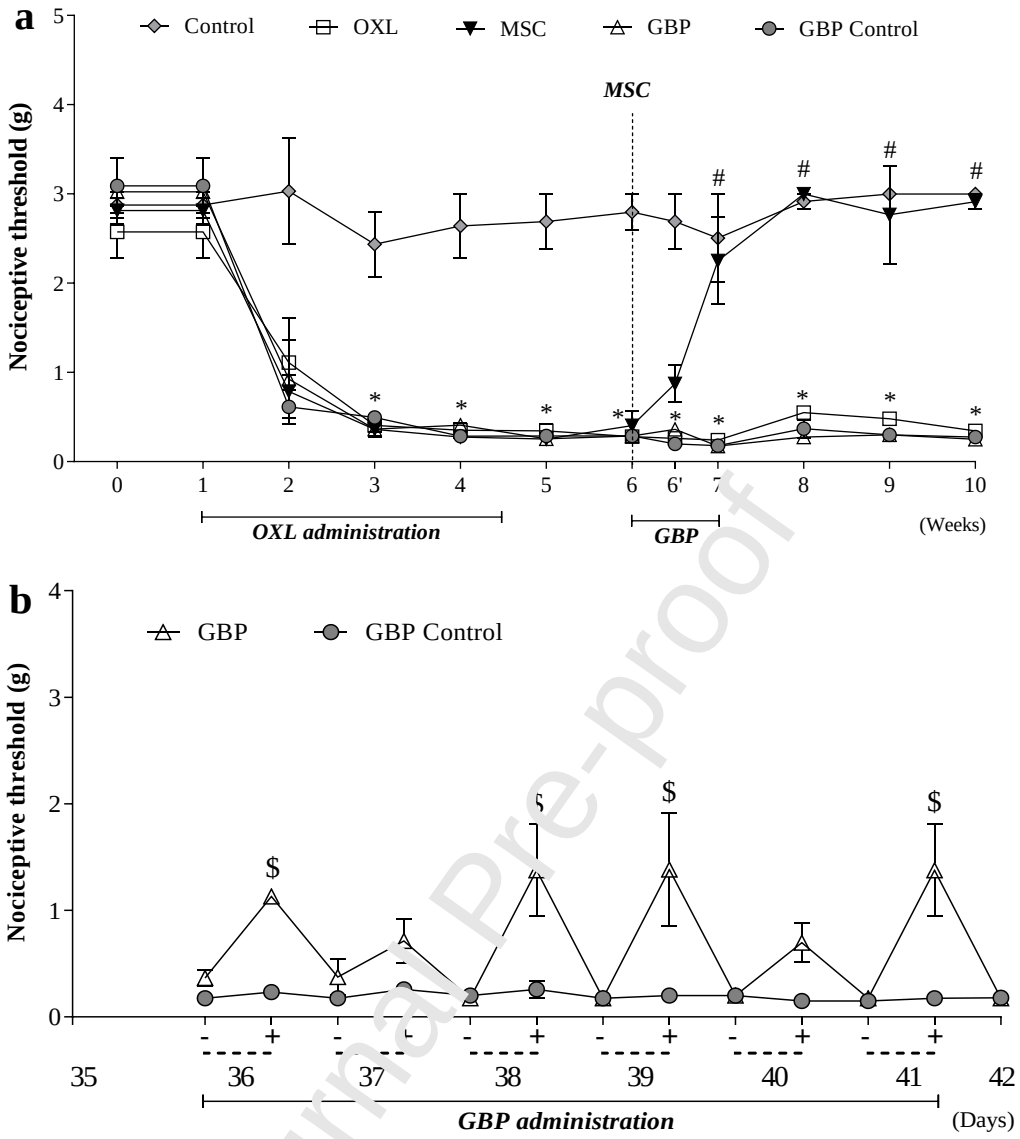

Figure 02: Effect of MSC and gabapentin on mechanical nociceptive threshold of mice with OXL-induced sensory neuropathy. **A:** mechanical nociceptive threshold in weeks during the experimental period (10 weeks). Time zero corresponds to basal thresholds of mice prior to performing any procedure. The chemotherapy cycle with OXL (1 mg / kg / i.v.) was indicated on the graph (OXL administration). The control group represents mice that received vehicle (5% dextrose) to replace OXL in the same administration protocol. The dotted line at week six indicates the day of intravenous administration of MSC ($1x10^6$; MSC group) or saline (200 µL; OXL group) in neuropathic animals. Time 6' corresponds to day 4 after intravenous treatment. The y axis represents the filament weight (g) in which the animal responds in 50% of presentations. **b**: shows the week 6 represented in days, which corresponds to the administration period of gabapentin (GBP group; 70 mg / kg, oral route, every 12 hours for 6 consecutive days) or its vehicle (GBP Control group). * Statistical significance relative to the control group ($p < 0.05$); # statistical significance relative to the remaining groups, except control group ($p < 0.05$); $ statistical significance relative to GBP-control ($p < 0.05$), as determined by two-way ANOVA followed by Bonferroni test. Data are represented as the average of 6 (six) animals per group ± SD.

MSC transplantation does not cause gross motor impairment: Considering that the behaviors measured in nociceptive tests are motor responses, the motor performance was monitored throughout the experimental period in the rota-rod test (Fig 3). CNS depressant diazepam (10 mg/kg/ip) was used as a reference drug for positive control of the test. Figure 3 shows that diazepam induced a strong reduction in the time spent on the rod-rod ($p < 0.0001$), as expected. For the remaining groups, no changes in the length of stay in the device over time were observed, indicating that OXL-induced neuropathy, as well as the treatments performed in this study, did not cause motor impairment detectable in this test. In addition, it is important to note that all mice survived until the end of the study.

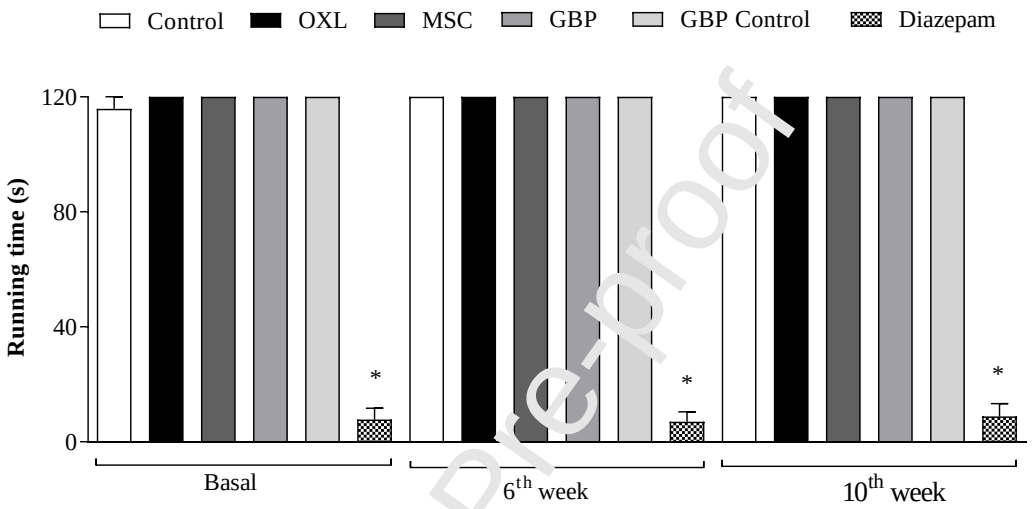

Figure 03: Motor function of mice in the rota-rod test throughout the experimental period. The control group represents animals that received vehicle (5% dextrose) instead of oxaliplatin. The other groups represent oxaliplatin-treated mice treated with MSC ($1x10^6$), vehicle (OXL), gabapentin (GBP group; 70 mg / kg, oral route, every 12 hours for 6 consecutive days) or gabapentin control (GBP Control). Diazepam (10 mg / kg / i.p.) administered 30 minutes before the test was used as the reference drug in the test. * Statistical significance relative to the control group ($p < 0.0001$) as determined by one-way ANOVA followed by Tukey test. Data are represented as the average of 6 (six) animals per group ± SD.

MSC transplantation induces the production of anti-inflammatory cytokines in the spinal cords of OISN mice: The dysregulation of cytokines at the spinal cord is a key event in the development and maintenance of neuropathic pain. Therefore, a possible modulatory action of MSC on spinal cytokines production during OISN was evaluated. Levels of IL-1β, TNFα, IL-10 and TGF-β in the spinal cord (L4-L5) were evaluated 4 weeks after treatments (Fig. 4). The spinal levels of the pro-inflammatory cytokine IL-1β and TNF-α did not differ between mice with or without sensory neuropathy, indicating that these cytokines probably do not contribute to the OISN maintenance (Fig. 4a and Fig. 4b, $p < 0.001$). In addition, ELISA data demonstrated that the MSC transplantation increased the levels of anti-inflammatory cytokines, IL-10 and TGF-β, in the spinal cord of mice with OXL-induced neuropathy (Fig. 4c and Fig. 4d, $p < 0.001$). In contrast, gabapentin treatment did not alter the expression pattern of these cytokines in the spinal cord.

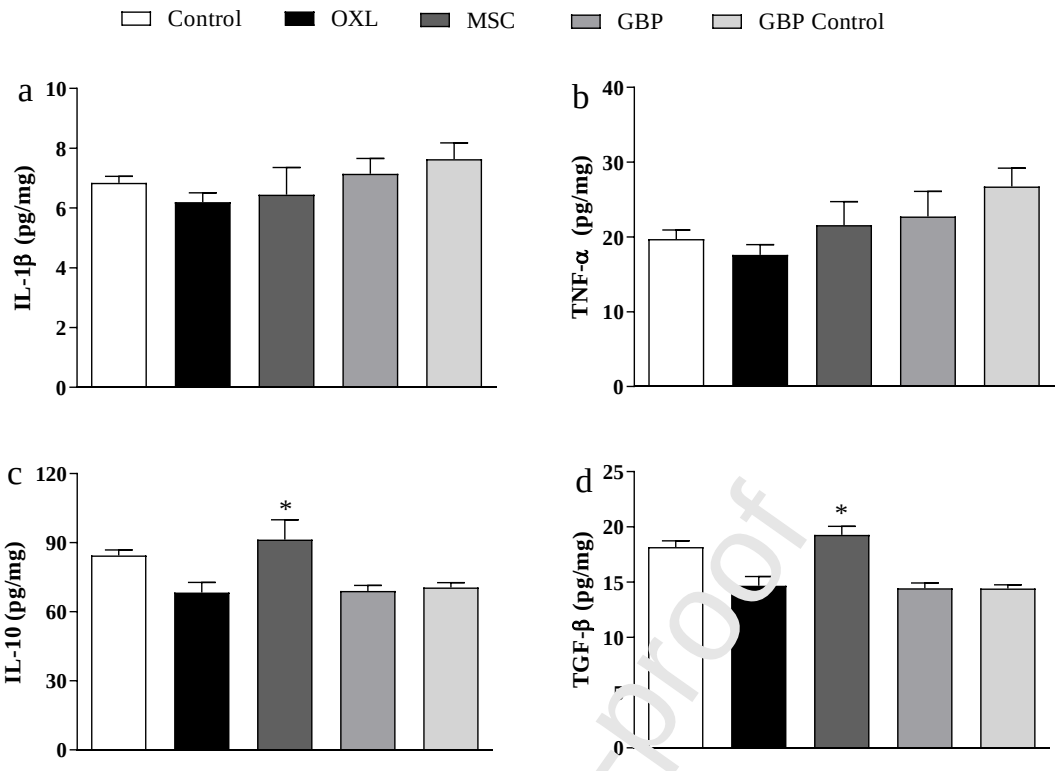

Figure 04: Effect of MSC treatment on anti-inflammatory (TGF-β and IL-10) and pro-inflammatory (IL-1β and TNF-α) cytokine levels in the spinal cord of mice with OXL-induced sensory neuropathy. The y-axis shows the spinal levels of pro-inflammatory, IL-1β (**a**) and TNF-α (**b**), and anti-inflammatory, IL-10 (**c**) and TGF-β (**d**), cytokines. Spinal cytokine levels were evaluated 4 weeks after treatments (10th week). The results are expressed as picograms of cytokine per milligram of protein. The control group represents animals that received 5% dextrose instead of oxaliplatin. The remaining groups received oxaliplatin and were treated with saline (OXL group), MSC (1 x 10^6; MSC group), gabapentin (GBP group; 70 mg / kg, oral route, every 12 hours for 6 consecutive days) or vehicle (GBP Control group). * Statistical significance relative to the OXL group ($p < 0.05$) as determined by one-way ANOVA followed by Tukey test. Data are represented as the average of 6 (six) animals per group ± SD.

MSC treatment activates the antioxidant defense system in the spinal cords of OISN mice: RT-qPCR analysis for selected key genes showed an antioxidant profile in the spinal cord of mice with OXL-induced sensory neuropathy and treated with MSC. Four weeks after transplantation, neuropathic mice treated with MSC showed higher values of superoxide dismutase (SOD; Fig. 5a) and Nrf-2 (Fig. 5b) mRNA in the spinal cord compared to vehicle-treated neuropathic mice ($p < 0.01$). Gabapentin treatment did not modifiy the spinal mRNA levels of these antioxidant agents.

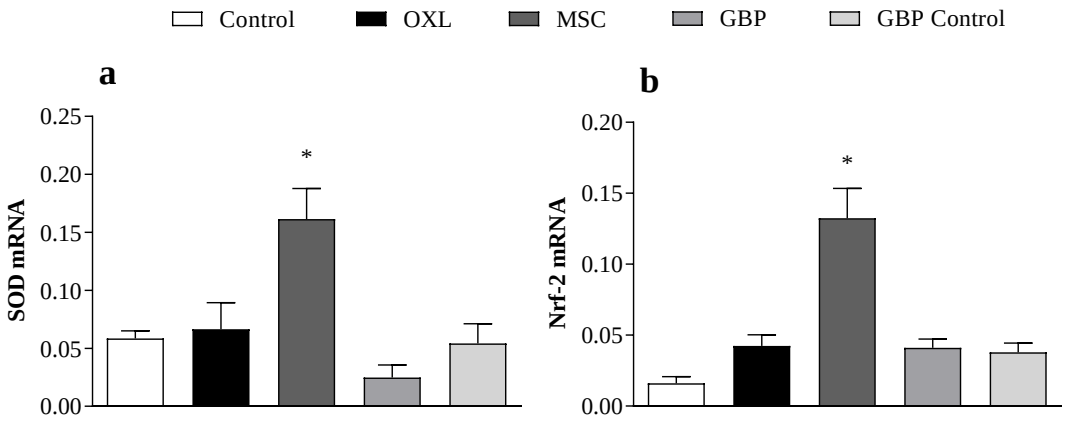

Figure 05: Effect of MSC on SOD and Nrf-2 mRNA levels in the spinal cord of neuropathic mice. The spinal levels of mRNA were measured by RT-qPCR 4 weeks after transplantation (10th week). The y-axis shows the mRNA levels of SOD (**a**) and Nrf-2 (**b**) in the spinal cord normalized by GAPDH. The control group represents animals that received vehicle (5% dextrose) instead of oxaliplatin. Additional groups represent OXL-treated mice treated with MSC ($1x10^6$; MSC group), saline (200 µL; OXL group), gabapentin (GBP group; 70 mg / kg, oral route, every 12 hours for 6 consecutive days) or their vehicle (GBP Control). Data are represented as the average of 6 (six) animals per group ± SD.* Statistical significance relative to the remaining groups ($p < 0.01$), as determined by one-way ANOVA followed by Tukey test.

MSC treatment reduces oxidative stress biomarkers in the spinal cords of mice with OISN: Based on the RT-qPCR data, the effects of MSC on nitrosative stress and lipid peroxidation were investigated by measuring the tissue levels of nitrite and MDA in the spinal cord of mice 4 weeks after transplantation. Nitrite (Fig. 6a) and MDA (Fig. 6b) levels were significantly elevated in the spinal cord of OXL-induced neuropathic mice when compared to control group ($p < 0.01$). The spinal levels of nitrite and MDA were significantly reduced in MSC treated neuropathic mice. Gabapentin treatment did not change nitrite and MDA levels in OISN mice.

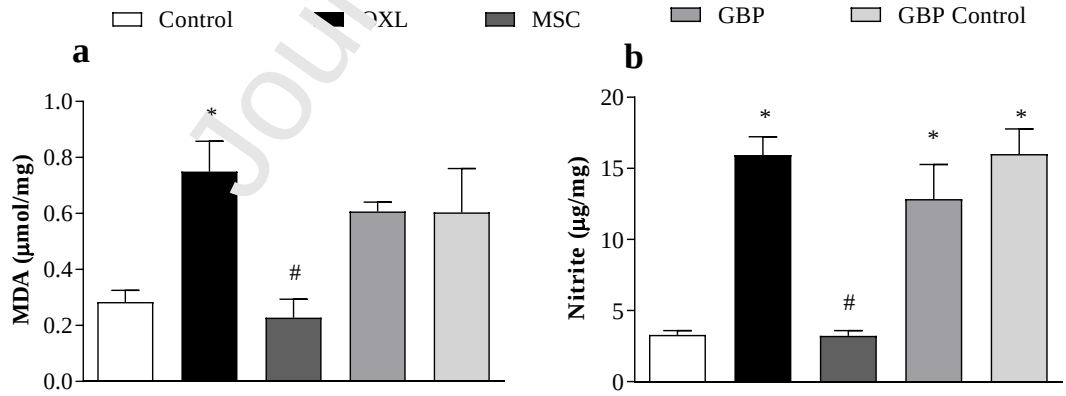

Figure 06: Effect of MSC treatment on MDA and nitrite levels in the spinal cord of mice with OXL-induced sensory neuropathy. The y-axis shows MDA (µmol / mg; panel a) and nitrite (µg / mg; panel b) levels in the spinal cord four weeks after transplantation. The control group represents mice that received 5% dextrose instead of oxaliplatin. Additional groups represent OXL-treated mice treated with MSC ($1x10^6$; MSC group), saline (200 µL; OXL group), gabapentin (GBP group; 70 mg / kg, oral route, every 12 hours for 6 consecutive days) or their vehicle (GBP Control). Data are represented as the average of 6 (six) animals per group ± SD. *Statistical significance relative to the control group ($p < 0.001$); # Statistical significance relative to the OXL group, as determined by one-way ANOVA followed by Tukey test.

Discussion

Although OXL has been shown to be effective in the treatment of some types of cancer [5], chronic sensory neuropathy induced by this drug is refractory to analgesics, reducing patients' adherence to treatment, which limits its clinical use [42,43]. In recent years, new therapeutic approaches have been identified as promising to improve the treatment of painful neuropathies, including cell therapy [44,45]. The potential of MSC for the treatment of OXL-induced neuropathic pain was recently evidenced in a pre-clinical study [30], but the mechanisms involved in this antinociceptive property are not well established. The present data corroborate the analgesic potential of MSC during neuropathy induced by OXL and establish a new possible mechanism of action of MSC via redox balance regulation in the spinal cord.

The repeated administration of OXL in mice induced an increase in thermal sensitivity and mechanical nociceptive stimuli for up to 10 weeks, which evidences the development of cold thermal hyperalgesia and mechanical allodynia, characterizing sensory neuropathy. Indeed, changes in sensitivity to cold thermal stimulus are the most common sensory symptoms described by patients treated with OXL [46]. Thus, the model used in this study was able to reproduce the symptoms that occur clinically during or after chronic use of OXL [36,47], indicating good clinical equivalence. Besides sensory evaluations, motor function of mice with OXL-induced sensory neuropathy was assessed. Neuropathy development was not associated with detectable motor deficits. In fact, no motor alteration has been demonstrated in mice treated with OXL [48,38], which corroborates the findings of the present work, and indicates that OXL-induced neurotoxicity appears to occur primarily in sensory fibers.

Single-dose of MSC completely reverted the cold thermal hyperalgesia and mechanical allodynia in mice with OXL-induced neuropathic pain, an effect that persisted until the end of the experimental period. Importantly, comparative analysis of the antinociceptive profile induced by MSC and GBP showed that this drug, a gold standard in the treatment of neuropathic pain, only temporarily reduced the sensory signs in neuropathic mice. This data is corroborated by clinical practice, which shows modest analgesic efficacy of GBP in patients with neuropathic pain induced by OXL [49,50], indicating that cell therapy opens the prospect for a more effective therapeutic approach for these conditions. Actually, the antinociceptive effects of MSC have been demonstrated in experimental sensory neuropathy of different origins, such as peripheral nerve ligation [22], diabetes [21] and spinal cord injury [23,24]. The analgesic properties of MSC have also been demonstrated in clinical studies, with efficacy in patients with pain of neuropathic [51] and non-neuropathic [52-55] origin.

The long-lasting effect profile may indicate that antinociception induced by MSC during OISN results from a disease-modifying action rather than an analgesic action. Furthermore, considering that the MSC transplantation was able to revert the established sensory neuropathy, this treatment should regulate events involved in maintaining pain. To investigate such hypothesis, effects of MSC transplantation on pathophysiological events commonly involved in the maintenance of painful neuropathies were evaluated. The role of cytokines in the pathophysiology of sensory neuropathy has been widely demonstrated [56-59]. Thus, the present study evaluated whether MSC transplant modulates the pattern of cytokine expression in the spinal cord of mice with OXL-induced sensory neuropathy. The levels of pro-inflammatory cytokines in the spinal cord did not differ between mice with or without sensory neuropathy. These data suggest that, unlike neuropathies of diabetic origin and nerve

damage, spinal production of IL-1β and TNF-α probably does not contribute to the maintenance of OXL neuropathy.

On the other hand, MSC transplantation increased the levels of the anti-inflammatory cytokines IL-10 and TGF-β in the spinal cord of mice with OISN. These results corroborate the literature data suggesting the contribution of IL-10 to the therapeutic effects of MSC, including during neuropathic syndromes [60-64]. According to Hanisch [65], IL-10 exerts neuroprotective action, interacting with glial cells and causing a reduction in inflammatory mediators and/or attenuating their effects. In addition, IL-10 is able to stimulate axonal regeneration [66,67] and has been considered a viable option to treat painful neuropathies [68]. Besides IL-10, TGF-β may be involved in the antinociceptive effect of MSC during neuropathic pain. In a study by Chen et al. [69], MSC has been shown to alleviate signs of allodynia and hyperalgesia for several weeks in a nerve constriction model in rats. This effect was attributed to TGF-β, which modulates the excitability of dorsal root ganglion neurons and spinal cord via TGF-β receptor-1. Thus, the increased levels of TGF-β and IL-10 in the spinal cord, showed here, may be associated with the antinociceptive effect of MSC during the OXL-induced sensory neuropathy.

The neuronal damage due to oxidative stress has been considered a central pathophysiological mechanism of OXL-induced neuropathic pain [70], and, therefore, the impact of MSC transplantation on redox homeostasis in the spinal cord was next investigated. Oxidative stress may represent a key event for apoptosis, metabolic disorders and bioenergetic failure in sensory neurons, triggering and perpetuating sensory neuropathy [70-72]. The mechanism by which oxaliplatin provokes ROS increase is not completely established and it could be due to a characteristic cell damage. A mitochondrial alteration has been suggested as a mechanism of oxaliplatin-mediated oxidation [73,16]. The activation of the cellular antioxidant defenses is fundamental for the maintenance of redox homeostasis [74]. The high production of reactive species is controlled by the activation of cell antioxidant defense system, which includes a range of antioxidant enzymes [75]. In the present study, MSC treatment has been shown to increase Nrf-2 and SOD mRNA in the spinal cord of OISN mice. Nrf-2 is a key transcription factor in regulating the body's antioxidant defenses, which acts by activating the expression of antioxidant enzymes such as SOD, catalase, GST, among others [75]. Nrf-2 has been revealed as a coordinator for maintaining redox homeostasis in healthy cells. A decline in Nrf-2 activity may lead to increased oxidative stress, which further results in the development of sensory neuropathy [76]. Conversely, agents capable of increasing Nrf-2 levels or activity can be promising to prevent or treat sensory neuropathies [77]. The fact that MSC increased the expression of Nrf2 and SOD in the spinal cord evidenced the antioxidant action of these cells during neuropathy induced by OXL. In fact, the antioxidant properties of MSC have already been demonstrated [78-83], and can contribute for its therapeutic uses. Recent studies using an acute liver failure model have demonstrated the potentiality of MSC to reduce oxidative stress by increasing Nrf-2 activity [84,85]. In line with this hypothesis, the present study provides evidence, for the first time, of a correlation between the antioxidant effect of MSC mediated by Nrf-2 and its antinociceptive effect during the sensory neuropathy.

To corroborate the antioxidant properties of MSC during OXL-induced neuropathic pain, oxidative stress biomarkers were also evaluated. The data consistently indicated that OXL-induced sensory neuropathy is associated with enhanced spinal levels of MDA and nitrite, indicating the presence of oxidative stress in the spinal cord microenvironment of neuropathic mice. MDA is one of the aldehydes originated from

the decomposition of polyunsaturated fatty acid peroxides from membranes. Cellular lipids are frequent targets of reactive species formed during oxidative stress [86]. In oxaliplatin neuropathy, the role of lipid peroxidation has already been proposed. In an OXL-induced neuropathy model, increased levels of lipid peroxidation products in the spinal cord have been demonstrated, resulting in redox imbalance and alteration of peroxisome function [87]. Data presented here support this hypothesis, since spinal cord MDA levels in OISN mice were much higher than in non-neuropathic mice. In neuropathic mice treated with MSC, the spinal levels of MDA and NO were reduced, confirming the antioxidant properties of these cells. MSC was able to enhance the expression of Nrf-2 and SOD and reduce MDA and NO levels, suggesting that a single MSC administration is able to promote the reestablishment of redox homeostasis in the spinal cord of mice with OISN. According to these data, it is possible to propose as a mechanistic hypothesis that, via their antioxidant properties, MSC restore the redox balance in the spinal cord, inducing long-lasting antinociceptive effects during the OXL-induced sensory neuropathy.

Conclusion

In summary, the presented dataset allows the following hypothesis to be elaborated: MSC enhance the levels of anti-inflammatory cytokines IL-10 and TGF-β and upregulate the Nrf-2 expression, activating the cells' antioxidant defense system and restoring the redox balance in the spinal cord. The precise mechanisms through which MSC exerts its antinociceptive effects during OXL-induced neuropathy are currently under investigation, but they may possibly correlate to the return to redox balance in the spinal cord. This study does not end with the use of MSC to treat sensory neuropathies, but opens the search for the understanding of its underlying mechanisms of action.

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Statement of Ethics

Animal care and handling procedures were in accordance with the National Institutes of health guide for the care and use of laboratory animals (NIH, 8023) and the Institutional Animal Care and Use Committee FIOCRUZ (CPqGM 025/2011).

Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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