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Conditioned Medium from Bone marrow-derived Mesenchymal Stem Cells improves recovery after Spinal Cord Injury in rats: an original strategy to avoid cell transplantation.

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| Abstract: | Spinal cord injury triggers irreversible loss of motor and sensory functions. Numerous strategies aiming at repairing the injured spinal cord have been studied. Among them, the use of bone marrow-derived mesenchymal stem cells (BMSCs) is promising. Indeed, these cells possess interesting properties to modulate CNS environment and allow axon regeneration and functional recovery. Unfortunately, BMSC survival and differentiation within the host spinal cord remain poor, and these cells have been found to have various adverse effects when grafted in other pathological contexts. Moreover, paracrine-mediated actions have been proposed to explain the beneficial effects of BMSC transplantation after spinal cord injury. We thus decided to deliver BMSC-released factors to spinal cord injured rats and to study, in parallel, their properties in vitro. We show that, in vitro, BMSC-conditioned medium (BMSC-CM) protects neurons from apoptosis, activates macrophages and is pro-angiogenic. In vivo, BMSC-CM administered after spinal cord contusion improves motor recovery. Histological analysis confirms the pro-angiogenic action of BMSC-CM, as well as a tissue protection effect. Finally, the characterization of BMSC-CM by cytokine array and ELISA identified trophic factors as well as cytokines likely involved in the beneficial observed effects. In conclusion, our results support the paracrine-mediated mode of action of BMSCs and raise the possibility to develop a cell-free therapeutic approach. |
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Liege, June 4 2013.

Dear Editor,

Dear Dr Rameshwar,

We are pleased to send you the revised version of our manuscript "PONE-D-13-15700" entitled "*Conditioned medium from bone-marrow derived mesenchymal stem cells improves recovery after spinal cord injury in rats: an original strategy to avoid cell transplantation*, for which a minor revision was required.

Thanks to the very good quality of the review, changes have been made, all in accordance with both Reviewer's insightful comments and suggestions. A "response to Reviewers" file is joined, in which we provide point-to-point answers to all issues raised by both Reviewers. Please note that all modifications made within the text of this new version are highlighted in clear blue, as recommended.

We believe and hope that this improved version of our manuscript now reaches the criteria for publication in *PLoS ONE*.

We thank you for considering our revised manuscript,

Sincerely yours,

A handwritten signature in blue ink, appearing to read 'Rachelle Franzen', written over a horizontal line.

Rachelle Franzen, PhD

Conditioned Medium from Bone marrow-derived Mesenchymal Stem Cells improves recovery after Spinal Cord Injury in rats: an original strategy to avoid cell transplantation.

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Abstract

Spinal cord injury triggers irreversible loss of motor and sensory functions. Numerous strategies aiming at repairing the injured spinal cord have been studied. Among them, the use of bone marrow-derived mesenchymal stem cells (BMSCs) is promising. Indeed, these cells possess interesting properties to modulate CNS environment and allow axon regeneration and functional recovery. Unfortunately, BMSC survival and differentiation within the host spinal cord remain poor, and these cells have been found to have various adverse effects when grafted in other pathological contexts. Moreover, paracrine-mediated actions have been proposed to explain the beneficial effects of BMSC transplantation after spinal cord injury. We thus decided to deliver BMSC-released factors to spinal cord injured rats and to study, in parallel, their properties *in vitro*. We show that, *in vitro*, BMSC-conditioned medium (BMSC-CM) protects neurons from apoptosis, activates macrophages and is pro-angiogenic. *In vivo*, BMSC-CM administered after spinal cord contusion improves motor recovery. Histological analysis confirms the pro-angiogenic action of BMSC-CM, as well as a tissue protection effect. Finally, the characterization of BMSC-CM by cytokine array and ELISA identified trophic factors as well as cytokines likely involved in the beneficial observed effects. In conclusion, our results support the paracrine-mediated mode of action of BMSCs and raise the possibility to develop a cell-free therapeutic approach.

I. INTRODUCTION

Spinal cord injury (SCI) is characterized by the primary lesion, rapidly followed by a cascade of cellular and molecular events that trigger the development of the secondary lesion, known to be deleterious for axonal regeneration and functional recovery. This worsening of the primary lesion is characterized by inflammatory reactions [1,2], free radical production [3], glutamate excitotoxicity [4], neuronal death and oligodendrocyte apoptosis [5-7]. With time, necrosis spreads to adjacent tissues and a cystic cavity appears [8-10]. Moreover, the initiated spontaneous axonal regeneration is repressed by the inhibitory environment composed of astroglial scar, and myelin-derived inhibitory molecules [11].

In this context, numerous experimental studies have been performed to improve functional recovery, focusing on various parameters: control of inflammation [12,13], rescue of neural tissue [14,15], stimulation of axonal regeneration by modulation of the lesioned environment [16-18] or promotion of remyelination [19,20]. Among the developed strategies, cell transplantation aims at replacing lost cells or producing beneficial effects at the lesion site. Among them, olfactory ensheathing cells, schwann cells, macrophages, fibroblasts, neural stem cells or BMSCs have been transplanted in various spinal cord injured contexts [21-29].

BMSCs are adult stem cells easily isolated from the bone marrow. BMSC transplantations have been widely studied in the context of SCI, and have proven beneficial effects on various aspects: inflammation, apoptosis, axonal regrowth, angiogenesis, tissue sparing, astroglial scar, and motor recovery [21,23,24,30-34]. Nevertheless, BMSC transplantations have some disadvantages: they do not persist within the host injured spinal cord after transplantation [35,36], and have a limited ability to replace lost cells [23,37,38]; finally, when used in other pathological contexts, BMSC transplants can have various adverse effects [39-44].

It is well known that BMSCs secrete a large variety of molecules and many studies have shown beneficial impact of these BMSC-released factors in different models [45-48]. Thus, we decided to study the effects of molecules secreted by BMSCs in an adult rat SCI model, using rat BMSC-conditioned medium (BMSC-CM). This original strategy constitutes a multifactorial treatment that could act on many aspects of SCI pathophysiology, avoiding all disadvantages and ethical problems related to the use of cells.

The aim of this study is to evaluate the effect of BMSC-CM on secondary processes involved in lesion extension after SCI. *In vitro* experiments were run in parallel to assess the potential beneficial properties of BMSC-CM on apoptosis, angiogenesis and inflammation.

II. MATERIALS & METHODS

II.1. BMSC culture and BMSC-CM preparation

BMSCs were obtained from the bone marrow of femurs and tibias of adult Wistar rats and characterized as previously described [21]. After having pooled cells from different donors, BMSCs were expanded in DMEM (Invitrogen) containing 4.5 g/L glucose, 0.58 g/L L-glutamine and 0.11 g/L pyruvate, supplemented with 10% non-heat inactivated fetal bovine serum (FBS, Invitrogen) and 100 I.U./ ml penicillin and 100 µg/ml streptomycin (Invitrogen). Medium was changed twice a week, and cells were passaged when 90% of confluence was reached.

BMSC identity is confirmed on the basis of morphological criteria, plastic adherence and specific surface antigen expression: CD90(+), CD271(+), CD45(-) and CD11b(-). Differentiation ability of BMSCs was also evaluated after induction using specific media as previously described [97]. Oil Red O and Alizarin Red S stainings (Sigma-Aldrich) were used to assess adipogenic and osteogenic differentiation.

BMSC-CM was generated as follows: 90% confluent, passage 2-4 BMSCs in T75 tissue culture flask, were washed 3 times with phosphate-buffered saline (PBS) and transferred to a serum-free DMEM culture medium without phenol red during 48 h. CM from different flasks were harvested and pooled. Then, CM were concentrated 40 times by centrifugation at 4000g for 15 min at 13°C, using 10-kDa MW cut-off filter units (Millipore). We consistently obtained, from 10 ml starting volumes, filtrate volumes of about 250 µl. Filter units were used only one time to avoid membrane saturation. Concentrated CM were then sterilized on 0.22 µm filters (Millipore), and stored at -80°C until use. The mean protein concentration of BMSC-CM is of 1.2-1.5 mg/ml. BMSC-CM was divided into small aliquots (500 µl-1 ml) before freezing to avoid repeated freeze/thaw cycles. Each aliquot was visually inspected before use to verify absence of precipitate (meaning a possible loss of protein function). There was no difference in protein concentration between fresh and freezed CM. As additional control, we also tested the stability of BMSC-CM by keeping it for 7 days at 37°C before assessing its anti-apoptotic property using the protocol described below. Serum-/Phenol red-free DMEM, centrifuged and filtered, was used as control medium.

II.2. Cerebellar granule neuron culture and apoptosis

CGNs were obtained from 4- to 7-day-old Wistar rats, and dissociated as previously described [98]. Cells were seeded at a density of 125,000 cells/well. A purity of 95% was obtained and confirmed by double GFAP/ β 3-tubulin immunostaining. Three to four hours after seeding, CGNs were treated overnight with one of the following serum free media to induce apoptosis: (1) CGN culture medium, (2) CGN culture medium + 25% BMSC-CM, (3) CGN culture medium + 50 ng/ml TNF α (Invitrogen), (4) CGN culture medium + 50 ng/ml TNF α + 25% BMSC-CM. This proportion of BMSC-CM was chosen because pilot experiments showed the same effect when 25% or 50% of BMSC-CM were used. 25% was thus the better compromise to obtain an effect without neither affecting neuronal survival nor wasting BMSC-CM. Cells were then fixed in buffered 4% paraformaldehyde (PFA) for 10 min. Apoptosis was evaluated with the TUNEL method, according to the manufacturer's protocol (Roche). Cells were then counterstained with DAPI and mounted on glass slides. Photomicrographs of 20 random fields per experimental condition were taken (Olympus AX70) at 40x magnification.

For each condition, the total number of apoptotic cells was reported to the total number of cells within the 20 fields. Experiment was repeated 3 or 4 times. The results are expressed as a mean apoptotic rate in percent.

II.3. Ex-vivo aortic ring assay

Rat aortic rings were cultured in three dimensional type-I collagen gels as described by Sounni *et al.* [99]. The following culture conditions were tested: 5% BMSC-CM, 5% control medium, 20 ng/ml rat VEGF with or without 1 μ g/ml anti-rat VEGF (PeproTech). This proportion of BMSC-CM was chosen to limit medium dilution that could affect neovessel regrowth and because it already triggered a strong response. Aortic rings were cultured for 9 days at 37°C in 5% CO₂. Photomicrographs were captured, with a Zeiss phase contrast microscope at 2.5x magnification (Axiovert 25). A minimum of 3 aortic explants were used per experimental condition and experience was repeated at least 3 times. Quantification was performed following the algorithm described elsewhere [100].

II.4. Macrophage culture and activation

Rat macrophage cell line (CRL2192) from ATCC was expanded in Ham's F-12K medium (Invitrogen) supplemented with 15% heat inactivated FBS. Cells were transferred to Petri dishes (Falcon) at a density of 2×10^5 cells/ml in the presence of Ham's F-12K/FBS supplemented with: (1) 25% control medium, (2) Lipopolysaccharides (LPS) + Interferon- γ (IFN γ), (3) LPS + IFN γ + 25% BMSC-CM and (4) LPS + IFN γ + IL-4 + IL-13. LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich) and IFN γ (Millipore) were respectively used at a final concentration of 10 and 2 ng/ml. IL-4 and IL-13 (R&D Systems) were used at a final concentration of 10 ng/ml each. Proportion of 25% BMSC-CM was chosen for the same reason as for apoptosis experiment. After 24 h at 37°C, adherent macrophages were detached and cells and supernatants were collected. Cells were counted to normalize the cytokine concentration. Supernatants were centrifuged at 200g to eliminate cells and the cytokine contents were analysed by ELISA. Media without macrophages were used as controls to remove cytokines that could come from serum and/or BMSC-CM. Experiment was repeated 2 to 4 times.

II.5. ELISA and antibody array

ELISA were used to assess *i*) the amounts of NGF, BDNF, IL-1 β , IL-6, TNF α and IL-10 in BMSC-CM, as well as *ii*) the amounts of IL-1 β , IL-6, TNF α and IL-10 in the macrophage culture supernatants, according to the manufacturer's recommendations (R&D Systems). For macrophage supernatant, values were related to 100,000 cells. Samples were run in duplicate for each experiment, and macrophage experiment was repeated 2 or 4 times. For BMSC-CM, samples were run in duplicate for each experiment, and experiment was repeated 2 to 13 times, according to the factor. Final results are expressed as a mean concentration in cytokine.

In parallel, a biotin label-based rat antibody array (RayBiotech) coated with 90 antibodies was used to obtain a broad view of molecules present in BMSC-CM. Three conditioned media from passage 3 BMSC cultures were tested.

II.6. Animal model

A total of 40 adult female Wistar rats (200g) from the Animal Facility of the University of Liege were used. Rats were treated in strict accordance with protocols approved by the Ethical Commission for animal use of the University of Liege, and all efforts were made to minimize

suffering. Rats were housed in individual cages from 7 days before the experiment, in a 12 h light/dark cycle, and in a temperature-controlled environment with access to food and water *ad libitum*. Animals were randomly divided into 4 experimental groups: BMSC-CM treated and control animals, sacrificed 1 or 6 weeks after SCI. 10 animals were studied in each group.

II.7. Contusion injury and BMSC-CM/control medium administration

Contusion SCI was induced with the « Infinite Horizons Spinal Cord Impactor IH-0400 » from *Precision Systems and Instrumentation*, LLC, Version 5.0., USA. Rats were deeply anaesthetized by inhalation of a mixture O₂ (N25) with 5% isoflurane, anaesthesia being maintained with 2-3% isoflurane during surgery (Forene, Abbott).

The skin was incised to expose the thoracic vertebral column and a T10 laminectomy was performed. After stabilization of the spinal column, contusion lesion was performed using a 250 kilodynes force of impact. Dura was opened at the level of the lesion and 10 µl of BMSC-CM or control medium were directly dropped with a Hamilton syringe onto the lesion. A mini-osmotic pump (model 1007D, ALZET, flow rate: 0.5 µl/ h/ 7 days) filled with BMSC-CM or control medium and linked to a catheter was then implanted to deliver the treatment at the lesion site. Muscles and skin were sutured. After 1 week, rats allowed to survive for 42 days were briefly re-anaesthetized to remove the empty pump, in order to avoid inflammation. Bladder function was assessed every day and manually emptied until rats regained control.

II.8. Neurological function evaluation

Two behavioural tests were used after SCI to assess motor performances of rats: the Basso, Beattie and Bresnahan (BBB) open-field test [101] consisting of a 21 point rating scale, and the grid navigation test with a 5 point scale [102]. All rats were accustomed to both tests 1 week before SCI. Two independent experienced individuals carried out all evaluations on a blind basis.

II.9. Euthanasia and histological procedures

After a deep anaesthesia with intraperitoneal injection of pentobarbital (200mg/kg, Nembutal, CEVA Santé Animale), rats were transcardially perfused with 300 ml cold saline containing 0.01% heparine (LEO Pharma), then with 500 ml cold buffered 4% PFA in 0.1M PBS (pH 7.4), 1 or 6 weeks post-injury. Spinal cords were dissected out, post-fixed for 24 h in 4% PFA at 4°C and cryoprotected for 48 h in a 30% sucrose buffer at 4°C. Serial, longitudinal (20µm thick) and transverse (25µm) sections were performed on, respectively, 15 mm-long

and 10 mm-long tissue blocks centred on the lesion site. 1 out of 5 transversal sections were preserved. Inflammation and angiogenesis were studied on rats sacrificed 1 week after SCI (N=10/group), using respectively CD11b and RECA-1 (Rat Endothelial Cell Antigen) immunostainings, on 3 longitudinal sections/animal. Axonal regeneration, astroglial scar formation and cavitation were studied on rats sacrificed 6 weeks after SCI. Axonal regeneration and astrogliosis were evaluated by immunohistology using antibodies against Neurofilaments (NF) and Growth-associated protein 43 (GAP-43) for axons and Glial fibrillary acidic protein (GFAP) for astrocytes, on 3 longitudinal sections/animal (N=4/group). Finally, cystic cavity extension was measured after Luxol-fast blue/ periodic acid - Schiff / hematoxylin staining on 6 transverse sections/animal (N= 6/group).

The following antibodies were used: *Primary antibodies*: mouse anti-NF200 (1:400, Sigma-Aldrich); rabbit anti-GAP-43 (1:1000, Millipore); mouse anti-GFAP (1:400, Sigma-Aldrich), mouse anti-CD11b (1:1000, AbD Serotec); mouse anti-RECA-1 (1:200, AbCam). *Secondary antibodies*: biotinylated horse anti-mouse IgG and biotinylated goat anti-rabbit IgG (1:500, Vector Laboratories). For anti-NF/GAP-43/GFAP/CD11b/RECA-1, sections were processed as previously described [21], except fixation in acetone and incubation in 0.5% H₂O₂ for RECA-1 antibody.

II.10. Image analysis

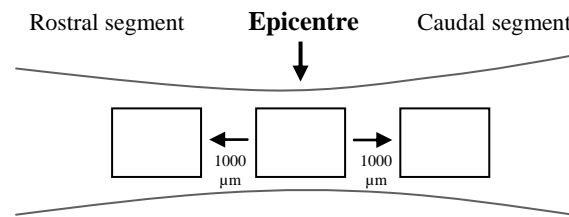
II.10.1. Cystic cavity

To quantify cavitation along the ventro-dorsal axis, 6 stained sections/rat (N=6/group), covering a distance of 1500 µm on either side of the lesion epicentre, were analyzed. Each section was observed under microscope (Zeiss, AxioImager Z1) to measure the height of the ventro-dorsal extent of the cyst relative to the one of the spinal cord midline (Mercator Pro software, Explora Nova). The mean ratio cyst extent / spinal cord extent was calculated for each experimental group and expressed as percentages.

II.10.2. Immunostaining quantification

To quantify GFAP, NF, GAP-43 and CD11b, 3 longitudinal sections per animal were immunostained for each antigen. Bright field images of successive fields at 5x magnification were captured to perform a full reconstruction of each section (Leica DM6000B). On these photomicrographs, the immunostained area was measured in 3 boxed areas of 3 mm² placed as follows: one at the lesion epicentre, one more rostrally (1000 µm from the epicentre) and one more caudally (1000 µm from the epicentre) (see diagram). Using analySIS software (Olympus), reconstructed photomicrographs were transformed in grey scales. A threshold

intensity of staining was applied. The immunostained surface was then measured in each of the 3 boxed areas. Data are presented as the averaged percentage of total stained areas (μm^2) / boxed area (μm^2).



To quantify the blood vessel diameters, RECA-1 immunostained sections were used. 3 longitudinal sections per animal were used and 3 fields per section were randomly chosen at the lesion epicentre. Bright field images at 10x magnification were captured with Zeiss microscope (AxioImager Z1). On each field, diameter of 10 blood vessels was measured with analySIS software. The blood vessel mean diameter for both experimental groups is considered.

II.11. Statistical analysis

All data were expressed as means \pm standard error of the mean (SEM). The improvement of motor recovery was analyzed by Generalized Linear Mixed Model (GLMM), which evaluates the global evolution of the two experimental groups. Other data were analyzed by unpaired Student's t-test. P-value (p) $<$ 0.05 was considered statistically significant. All the tests were conducted using Statistica software (StatSoft), except for neovessel growth in aortic ring model (Aphelion (Adcis) and Matlab 7.9 (MathWorks)).

III. RESULTS

III.1. Characterization of rat BMSCs

BMSCs were expanded in plastic dishes in classical *in vitro* conditions. Cells were characterized by their plastic adherence, the expression of specific antigens and their multipotency was checked by their differentiation capacity. Cells were used at low passages P2, P3 or P4, to work with young cells and avoid senescence-associated effects progressively acquired with passages [49]. P4 BMSCs express surface antigens CD90 and CD271

(p75NGFr) but didn't express CD45 and CD11b. Under specific induction media, cells differentiated into adipocytes and osteocytes (figures 1A-F).

III.2. BMSC-CM protects neurons from apoptosis *in vitro*

Cerebellar granule neurons (CGNs) were cultured and submitted to pro-apoptotic conditions: serum free medium or tumor necrosis factor alpha (TNF α) (50 ng/ml). BMSC-CM was added in each condition at a final proportion volume of 25%. After overnight treatment, cells were fixed and apoptosis rates evaluated using the Tunel method and a DAPI counterstain. Incubation with BMSC-CM significantly decreased apoptotic rates of neurons whatever the experimental condition (figure 2): in serum deprivation condition, BMSC-CM decreased apoptotic rate by 10% (from 14.8% \pm 1.1 to 4.9% \pm 0.7) (p=0.0002, Student's *t*-test) and by more than 12% in TNF α -induced apoptosis (from 21.2% \pm 2.8 to 8.6% \pm 0.6) (p=0.0038, Student's *t*-test). Results were identical with the use of BMSC-CM kept for 7 days at 37°C before performing the experiment (data not shown).

III.3. BMSC-CM is pro-angiogenic

As vessel growth favours axonal regrowth after SCI, we assessed the effect of BMSC-CM on angiogenesis. Rat aortic rings were incubated 9 days in a specific medium supplemented with 5% BMSC-CM, 5% control medium (negative control) or recombinant rat vascular endothelial growth factor (VEGF, 20 ng/ml; positive control) (figure 3A). Anti-VEGF functional antibody (1 μ g/ml) was also added to assess a possible role of VEGF in observed effects. The number of intersections (N_i) of neovessels as a function of the distance from the ring is illustrated on figure 3B. Areas below curves were calculated in order to include both parameters: N_i and the maximal distance from the ring reached by the neovessels. BMSC-CM induced a strong angiogenic response in comparison to the control (270.5 \pm 18.3 vs 119.3 \pm 11) to reach approximately the same level as with VEGF treatment. Anti-VEGF administration abolished the pro-angiogenic effect of BMSC-CM (270.5 \pm 18.3 vs 125.3 \pm 25.2) and, as expected, of VEGF. The differences were statistically significant between BMSC-CM and control medium (p < 0.0001, Student's *t*-test) and between BMSC-CM and BMSC-CM + anti-VEGF (p=0.0003, Student's *t*-test; figure 3C). This indicates that VEGF is involved in pro-angiogenic effects and must be present in BMSC-CM.

III.4. BMSC-CM favours a pro-inflammatory state of macrophages *in vitro*

In order to study the effects of BMSC-CM on macrophage phenotype, rat macrophages were activated with LPS (10ng/ml) and IFN γ (2 ng/ml) to simulate the *in vivo* inflammation that follows SCI. Activated macrophages were further incubated with 25% control medium, 25% BMSC-CM, or anti-inflammatory cytokines: interleukin 4 and 13 (IL-4 and IL-13) (10 ng/ml). After 24h, culture media were harvested to assess their content in IL-1 β , IL-6, TNF α and IL-10 by ELISA. Each value was normalized for 100,000 cells, and the concentration of cytokines present in BMSC-CM or serum was deduced. Without LPS/IFN γ stimulation, 100,000 macrophages secreted 3.7 pg/ml \pm 0.4 of IL-1 β , while activated cells secreted 115.5 pg/ml \pm 31.2. Incubation with BMSC-CM significantly increased IL-1 β concentration in supernatant of activated macrophages from 115.5 pg/ml \pm 31.2 to 206 pg/ml \pm 19 (p=0.048, Student's *t*-test). The use of IL-4 and IL-13 on activated macrophages reduced slightly the secretion of IL-1 β to 95.6 pg/ml \pm 10.2 (figure 4A). IL-6 and TNF α values also show a tendency to increase after BMSC-CM treatment, without reaching significant levels of differences (figures 4B-C). On the contrary, no secretion of IL-10 was observed (data not shown). Thus, BMSC-CM is globally pro-inflammatory on macrophages, as it exacerbates their secretion of pro-inflammatory cytokines.

III.5. BMSC-CM characterization

In order to further characterize BMSC-CM, we performed large antibody arrays (90 proteins) as well as ELISA assays. Antibody arrays, performed on 3 distinct BMSC-CM samples (figure 5A), reveal the presence of 23 factors involved in either apoptosis, inflammation, angiogenesis, oxidative stress, excitotoxicity, all involved in the modulation of the spinal cord injured environment (Table 1).

ELISA were performed on 4 cytokines: IL-6, IL-1 β , TNF α , IL-10 and 2 neurotrophins: brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF). The analysis of different BMSC-CM demonstrates that NGF and BDNF are present at 356 pg/ml \pm 117 and 208 pg/ml \pm 57 respectively, as well as IL-6 at 427 pg/ml \pm 168 (figure 5B). IL-1 β , TNF α and IL-10 were not detected in these samples.

III.6. BMSC-CM improves functional recovery after SCI

Spinal cord injured animals were submitted to 2 complementary behavioural tests. The Basso, Beattie and Bresnahan (BBB) open-field test was used to assess motor skills of rats until 6

weeks post-injury (figure 6A). The difference between treated and control groups was statistically significant (*Injured + BMSC-CM vs Injured + control* $p=0.0003$, Generalized Linear Mixed Model). From day 4 after SCI, BMSC-CM treated animals show better scores than control animals and this difference was maintained during the study. Score 9 is crucial as it indicates the weight support step. The figure shows that this stage is reached by treated animals 4 days before control animals. Moreover, control animals never recovered their ability to separate toes and to maintain hindpaws in a parallel position at lift off during stepping (score of 16). These parameters, successfully performed by treated animals, bear witness to a fine recovery of motricity.

The grid navigation test was used to assess fine motor coordination and movement accuracy (figure 6B). Mean scores over time were significantly higher in BMSC-CM treated group compared to control group (*Injured + BMSC-CM vs Injured + control* $p=0.0001$, Generalized Linear Mixed Model). We noticed that difference between both groups started from day 4 post-injury. Score 4, testifying a correct placement on grid bars, was reached only by BMSC-CM treated animals.

III.7. BMSC-CM reduces cystic cavity

The improved motor recovery observed in BMSC-CM treated animals could be explained by a reduced extension of the lesion. To investigate this aspect, spinal cord injured transversal sections from both experimental groups were performed 6 weeks after injury and stained with Luxol-fast-blue / periodic acid - Schiff / hematoxylin. Serial sections were then analyzed to quantify the ventro-dorsal extent of the cystic cavity (figures 7A-B). As spinal cord volume can vary over the lesion epicentre, cystic height was related to spinal cord height on the same dorso-ventral axis, and ratio expressed in percentages. BMSC-CM treated group shows reduced mean values compared to control group (*Injured + BMSC-CM: 67.72% \pm 3.4 vs Injured + control: 83.4% \pm 2.1; figure 7C). Difference between BMSC-CM treated and control groups was statistically significant ($p=0.0029$, Student's *t*-test).*

III.8. BMSC-CM is pro-angiogenic *in vivo*

Longitudinal spinal cord injured sections from treated and control groups were performed 1 week post-injury and immunostained for RECA-1. Diameters of blood vessels were measured at the lesion epicentre. Blood vessels exhibit larger diameters in BMSC-CM treated animals compared to controls (*Injured + BMSC-CM: 14.4 μ m \pm 1.2 vs Injured + control: 6.9 μ m \pm*

0.8; figures 8A-B). Difference between both groups was statistically significant ($p < 0.0001$, Student's *t*-test) .

III.9. *In vivo*, BMSC-CM has no effect on astrogliosis, macrophage invasion and axonal regrowth

To study astrogliosis, longitudinal sections performed 6 weeks after injury were immunostained for GFAP. Quantification of the immunostained surface was carried out in 3 defined areas. This value was related to the total area of each of the 3 zones to express a proportion of stained area. We didn't detect any difference between BMSC-CM treated and control animals (Student's *t*-test). After CD11b immunostaining, quantifications were performed as described above. Values obtained for BMSC-CM treated and control groups were not significantly different (Student's *t*-test). In order to assess the ability of BMSC-CM to favour axonal regrowth after SCI, anti-NF and anti-GAP-43 immunostainings were performed. Quantifications didn't reveal any difference between groups, whatever the delay (Student's *t*-test) (Table 2).

IV. DISCUSSION

Our results show that, *in vitro*, BMSC-CM provides protection against neuronal apoptosis, is pro-angiogenic and confers a pro-inflammatory phenotype to macrophages. *In vivo*, we demonstrate for the first time that BMSC-released molecules are able to reduce cystic cavity size along the ventro-dorsal axis at the lesion epicentre, to favour large blood vessel growth and to improve locomotor recovery in a spinal cord contusion injury model.

Many studies suggest that beneficial effects obtained after BMSC transplantation in SCI were more likely due to paracrine actions than to effective integration and differentiation of the cells within the host tissue [21,23,36,38,50-53]. Our study confirms this hypothesis.

We decided to deliver factors secreted by BMSCs in the form of concentrated conditioned medium (CM), to optimize potential effects. We also chose to use a fraction of this BMSC-CM that contains factors of a molecular weight > 10 kDa, as this fraction was earlier described as beneficial on myocardial infarct size, oxidative stress and apoptosis [54]. Moreover, we eliminated serum from the medium because of its poorly defined composition.

Two complementary behavioural tests were used to assess recovery of motor functions: BBB as a global recovery test, and grid navigation to evaluate more precisely fine motor movements. In both tests, control animals show spontaneous recovery after spinal cord contusion. The great spontaneous recovery obtained here can also be explained by the type of the lesion, which, despite the use of a 250 kdyn force, doesn't severely affect the ventral part of the spinal cord, thus preserving white matter tracts [55]. According to the literature, 200 kdyn already corresponds to a severe injury, but this parameter is also known to vary upon the animal weight or strain [56,57]. Despite this fact, BMSC-CM treated rats obtained significantly higher scores in both tests compared to control animals. Difference between groups started from days 4 to 7, indicating a rapid effect after administration, as also described after BMSC transplantation in a model of spinal cord contusion injury [24]. Later on, BBB scores continue to improve, with treated rats displaying at each delay higher scores compared to controls. In their study, Namiki *et al.* showed an improvement of inclined plane scores one week after BDNF infusion by osmotic pump in a SCI clip model. However, this evolution stopped very early after the end of the BDNF treatment [58]. The prolonged effect that we obtain may be due to the combined action of several factors present in our CM, with maybe redundant molecules with variable bioavailabilities. It is also possible that the cocktail of administered factors favours the survival of endogenous cells preserving the tissue from further lesion extension.

In other studies, after BMSC transplantation in contusion or compression models of rat SCI, locomotor improvements were also observed during weeks following the graft, with BBB scores reaching similar levels. For example, 4 weeks after spinal contusion and BMSC graft, Karaoz and colleagues obtain a BBB score of 15 while our treated rats reach the score of 16 at the same delay. Also, 3 weeks after BMSC graft in a compression injury model, Quertainmont *et al.* show that rats reach a BBB score of 12 which is even lower to the score of 15 reached in the present study [21,24]. This reinforces our hypothesis that BMSC-secreted factors are beneficial after SCI and that they improve functional recovery as do BMSC transplants.

Improved recovery can be a consequence of neural tissue protection, which is favoured by angiogenesis. Angiogenesis is indeed known to induce axonal regrowth via the supply of oxygen and nutritional factors that helps preserving injured spinal tissue from further degradation [59,60]. Moreover, it has been shown that the pro-angiogenic VEGF molecule possesses neuroprotective properties [61-63]. This factor is present in BMSC-CM and our *in vitro* data on aortic rings confirm that it is involved in the pro-angiogenic effect. Other factors which are known to promote angiogenesis were also detected in BMSC-CM: osteopontin

[64,65], matrix metalloproteinase-13 (MMP-13) [66] and fibroblast growth factor-binding protein (FGF-BP) [67,68]. *In vivo*, blood vessels observed at the lesion epicentre of treated animals, even if not higher in number, have larger diameters compared to controls, and could thus provide, via increased blood flow, more nutritional substances and oxygen to damaged tissue, contributing to tissue protection, as already described after thoraco-abdominal aneurysm in pigs [69]. Lu *et al.* have also measured this parameter and their data suggest that atorvastatin treatment elicits larger vascular diameter, thus contributing to enhanced regional blood flow perfusion and neuron rescue [70]. So, larger diameters of blood vessels seem to be associated with a better tissue perfusion, protecting neuronal cells from degeneration. Yet, we didn't observe increased axon regeneration in treated spinal cords compared to control ones. In the same context, Dray's study suggests that even if some axons benefit from vascular support to accelerate their growth, this support is only transient and limited in time [60].

Tissue protection could also be related to a reduced apoptosis rate within the injured cord. Our data show that BMSC-CM protects neurons from apoptosis *in vitro*. Neuronal death and apoptosis rapidly followed by oligodendrocyte apoptosis are parts of secondary processes making the lesion worse. BMSC transplantations have been successfully used to reduce apoptotic death in the context of SCI, and associated to a better motor recovery [32]. In this study, we demonstrate that BMSC-CM treated spinal cords have a reduced depth of cystic cavity, protecting white matter tracts. You *et al.* demonstrated a positive correlation between spared ventral white matter and the final BBB scores of rats [55]. Also, the rubrospinal tract in the dorsolateral part and the corticospinal tract located in the dorsal part of the spinal cord white matter in rats are particularly important for precise limb movements, and can be assessed by grid navigation test [71]. Based on our behavioural data, we can thus conclude that the better motor recovery observed in BMSC-CM treated animals is a direct consequence of improved tissue sparing.

Among factors identified in BMSC-CM by cytokine arrays and ELISA, some may also contribute to tissue preservation. NGF stimulates the survival of sympathetic and sensory neurons, while TIMP-1 (tissue inhibitor of metalloproteinase-1) and CINC-3 (cytokine-induced neutrophil chemoattractant-3) are neuroprotective [72,73]. BDNF administration decreases apoptosis and demyelination in a spinal cord compression model [74] and reduces astroglial scar formation [75]. Also, other factors, thus not described here, are known to be secreted by rat BMSCs: IGF-1, HGF, TGF- β 1, EGF, SDF-1, MIP-1 α/β , GM-CSF or FGF-2 [76,77]. The fact that we didn't find those factors is first due to the fact that some of them were not included in our 90-protein array assay. Moreover, BMSC culture conditions may

influence their secretome, which would explain why factors described in other studies were not detected here and conversely.

In our model, BMSC-CM doesn't affect astroglial reactivity. This result could be considered as unexpected, as both NGF and BDNF, present in BMSC-CM, are known to reduce reactive astrogliosis [75,78]. This discrepancy is likely due to the variable concentrations of these two neurotrophins. Also, according to the literature, few studies report any effect of BMSC transplantation on astroglial scar development after SCI. This is also the case for axonal regeneration, which is rarely reported as associated to improved recovery after BMSC transplants [24,79-82].

In vitro data on IFN γ /LPS-activated macrophages show that BMSC-CM further favours their pro-inflammatory state, as assessed by their significant increased IL-1 β secretion and their obvious but non-significant increased IL-6 and TNF α production. In parallel, we also show that BMSC-CM contains IL-6, which possesses pro-inflammatory properties as well [83,84]. *In vivo*, we didn't detect any difference between treated and control groups, in the number of macrophages that invaded the lesion site 1 week post-injury, as assessed by the total area stained for CD11b. Microglia/macrophages present within the lesion exhibited in both groups round cell bodies without branching processes, characteristic of "amoeboid" cells, and indicative of an activated status. This reactive cell form is associated with neuroprotective effects via trophic factor delivery and with phagocytosis that is essential to remove cell and myelin debris [23,85-87]. In this context, we hypothesize that BMSC-CM has further favoured the activated state of resident microglia and invading macrophages, conferring a phenotype beneficial for tissue preservation, without affecting the number of inflammatory cells within the lesion. This hypothesis is in accordance with the fact that IL-1 β and IL-6 do indirectly promote axonal outgrowth [88,89] and protect neurons from death [90,91]. In the literature, contradictory results have been described concerning the effect of BMSC transplantation on post-injury inflammatory reaction, going from reduced inflammation [92] to enhanced macrophage/microglia response [81]. Some authors also suggest that microglial activation after SCI is linked to the reduction of the lesion size [23,93]. Indeed, microglia can be involved in neuroprotection via secretion of factors such as NGF, IGF-1 (insulin-like growth factor-1) or via up-regulation of FGF-2 (fibroblast growth factor-2) in neurons [94-96].

In conclusion, our data show that the use of BMSC-CM in the context of SCI is beneficial and not deleterious, and leads to improved motor recovery. This treatment constitutes a novel

promising therapeutic perspective in SCI context. More investigations are needed to evaluate the potential of this treatment in chronic lesion models, and its future clinical application.

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VI. REFERENCES

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FIGURE LEGENDS

Figure 1. BMSC characterization. P4 BMSCs *in vitro* are immunostained for (A) CD90 and (B) CD271 (p75NGFr), but are negative for (C) CD45 and (D) CD11b. Nuclei are labelled with Vectashield-DAPI mounting medium. (E) Adipogenic differentiation of P4 BMSC revealed with Oil Red O and (F) osteocyte differentiation revealed by Alizarin Red S.

Figure 2. BMSC-CM protects neurons from apoptosis.

Percentage of apoptotic CGN in serum-free medium or with TNF α (50 ng/ml), cultured with or without 25% BMSC-CM. BMSC-CM significantly decreases apoptosis. Experiment was repeated 3 to 4 times. *** p <0.001; ** p <0.01.

Figure 3. BMSC-CM promotes angiogenesis *in vitro*.

(A) Representative photomicrographs of rat aortic rings after 9 days in culture medium containing 5% control medium, 5% BMSC-CM or 20 ng/ml of rat VEGF.

(B) Quantification of number of intersections (N_i) of neovessels treated with 5% control medium, 5% BMSC-CM and 20 ng/ml VEGF, with or without 1 μ g/ml anti-VEGF.

(C) Area below curves generated for each condition by the semi-assisted computerized quantification. Presence of BMSC-CM significantly favours angiogenesis and this effect is mediated by VEGF. Experiment was repeated 3 to 15 times. *** p <0.001.

Figure 4. BMSC-CM stimulates macrophages to secrete pro-inflammatory cytokines.

Concentration of IL-1 β (A), IL-6 (B) and TNF α (C) in the supernatant of macrophages cultured in various conditions: non-activated, activated with LPS and IFN γ with or without 25% BMSC-CM, or with IL-4 and IL-13. BMSC-CM increases significantly IL-1 β secretion

by macrophages, and tends also to increase their IL-6 and TNF α secretion. Experiment was repeated 2 to 4 times. * p <0.05.

Figure 5. BMSC-CM characterization by antibody arrays and ELISA.

(A) Multiple array membrane incubated with BMSC-CM reveals the presence of hormones, proteases, cytokines and trophic factors. Experiment was repeated 3 times.

(B) Concentrations of NGF, BDNF and IL-6, measured by ELISA, within BMSC-CM. Experiment was repeated 2 to 13 times.

Figure 6. Behavioural analysis.

Evolution of motor scores for BMSC-CM treated and control rats over 6 weeks, as assessed (A) by BBB scale and (B) by grid navigation scale. BMSC-CM treated rats obtained significantly higher scores compared to control animals. 10 animals per group were studied. *** p <0.001.

Figure 7. BMSC-CM reduces lesion extension.

(A) Illustration of an injured spinal cord transversal section invaded by a cyst. For each section, cystic cavity extent (H1) and spinal cord extent (H2) were measured on the median axis. Both values were related to express a ratio in percents.

(B) Photomicrographs of Luxol-fast-blue / periodic acid - Schiff / hematoxylin stained sections of BMSC-CM treated and control animals, taken at the lesion epicentre, 6 weeks after injury. Cyst extent is shown by an arrow.

(C) Ratio cyst extent / spinal cord extent of BMSC-CM treated and control animals, 6 weeks after injury. BMSC-CM treated animals obtained a ratio significantly reduced compared to control group. 6 animals per group were studied. ** p <0.01.

Figure 8. BMSC-CM increases blood vessel diameter within the lesion.

(A) Mean blood vessel diameter at the lesion epicentre measured on RECA-1 immunostained sections from BMSC-CM and control groups 1 week after SCI, illustrated in (B). Blood vessels in BMSC-CM treated animals have larger diameters in comparison to control animals. 10 animals per group were studied. *** p <0.001.

Table 1. Factors detected in BMSC-CM.

| <i>Phenomena</i> | <i>Specific properties</i> | <i>Factors</i> |
|----------------------------|-----------------------------------------------------|------------------------------------------------------------------------------------------------------|
| Apoptosis | Anti-apoptotic | VEGF-A, TIMP-1, CINC-3, TIMP-2, Osteopontin, Growth Hormone + GHR, EG-VEGF/PK1, TAL1A*, FGF-BP, BDNF |
| | Pro-apoptotic | TROY, TRAIL, Fas ligand/TNFSF6 |
| Inflammation | Pro-inflammatory | TLR4 , CINC-3, Osteopontin, EG-VEGF/PK1, CINC-2, MMP-13, MDC, Fas ligand/TNFSF6 |
| | Immunomodulator | Osteopontin, CXCR4, MDC |
| Angiogenesis | Pro-angiogenic | VEGF-A, VEGF-C, Osteopontin, EG-VEGF/PK1, TAL1A*, MMP-13, FGF-BP |
| | Anti-angiogenic | TIMP-2 |
| Neuronal modulation | Neuroprotective and Neuro-/neurito-trophic | TIMP-1, CINC-3, Growth Hormone + GHR, BDNF |
| | Anti-neuritotrophic | TROY |
| | Neuronal differentiation | TIMP-2 |
| | Modulation of neuronal activity | BDNF |
| Stress | Protection from excitotoxicity and oxidative stress | Osteopontin, FGF-BP |
| Other | Cell fate regulation | TIMP-1, TIMP-2, Hepassocin, Growth Hormone + GHR, EG-VEGF/PK1 |
| | Cell survival | TIMP-1, TIMP-2, Growth Hormone + GHR, BDNF |
| | ECM modulation | TIMP-1, MMP-13 |
| | Metabolism | Insulin degrading enzyme, Insulin, Growth Hormone + GHR |

Antibody arrays against 90 proteins were performed on 3 different samples of BMSC-CM and identified 23 factors likely involved in the modulation of the spinal cord injured environment.

*protein not known to be secreted.

Table 2. Immunostaining quantifications.

| Studied parameters | Studied regions | Proportion of stained area (%) in BMSC-CM treated animals | Proportion of stained area (%) in control animals | P-value and significance (treated vs control) | N / group |
|-------------------------------------------|-----------------|-----------------------------------------------------------|---------------------------------------------------|-----------------------------------------------|-----------|
| Astroglial scar (GFAP) | Epicentre | 7.4 ± 0.4 | 6.2 ± 1 | 0.29 - NS | 4 |
| | Rostral | 5.8 ± 1.4 | 7.1 ± 1.7 | 0.6 - NS | 4 |
| | Caudal | 5.4 ± 0.9 | 7.2 ± 2 | 0.46 - NS | 4 |
| Inflammation (CD11b) | Epicentre | 32.1 ± 2 | 32.2 ± 2.7 | 0.98 - NS | 10 |
| | Rostral | 13.1 ± 1.3 | 15 ± 3.2 | 0.57 - NS | 10 |
| | Caudal | 12.1 ± 1.5 | 17 ± 3.9 | 0.25 - NS | 10 |
| Axonal regrowth 1 week post SCI (GAP-43) | Epicentre | 8.2 ± 0.9 | 7.3 ± 1.4 | 0.57 - NS | 10 |
| | Rostral | 8 ± 1 | 7.5 ± 1.6 | 0.79 - NS | 10 |
| | Caudal | 7.3 ± 1.1 | 6.9 ± 1.3 | 0.81 - NS | 10 |
| Axonal regrowth 6 weeks post SCI (GAP-43) | Epicentre | 5.1 ± 1.2 | 6.2 ± 0.7 | 0.48 - NS | 4 |
| | Rostral | 3.1 ± 1.3 | 4.3 ± 0.6 | 0.44 - NS | 4 |
| | Caudal | 1.9 ± 0.6 | 3.1 ± 0.5 | 0.21 - NS | 4 |
| Axonal regrowth 6 weeks post SCI (NF) | Epicentre | 5.4 ± 1.6 | 6.3 ± 1.4 | 0.71 - NS | 4 |
| | Rostral | 6.9 ± 2.5 | 4.8 ± 1.3 | 0.48 - NS | 4 |
| | Caudal | 8.9 ± 3.1 | 6.7 ± 2 | 0.59 - NS | 4 |

After specific immunostainings (GFAP, CD11b, GAP-43, NF), quantifications of proportion (%) of stained area were performed in 3 defined areas. No statistical differences between treated and control rats were found.

Figure 1
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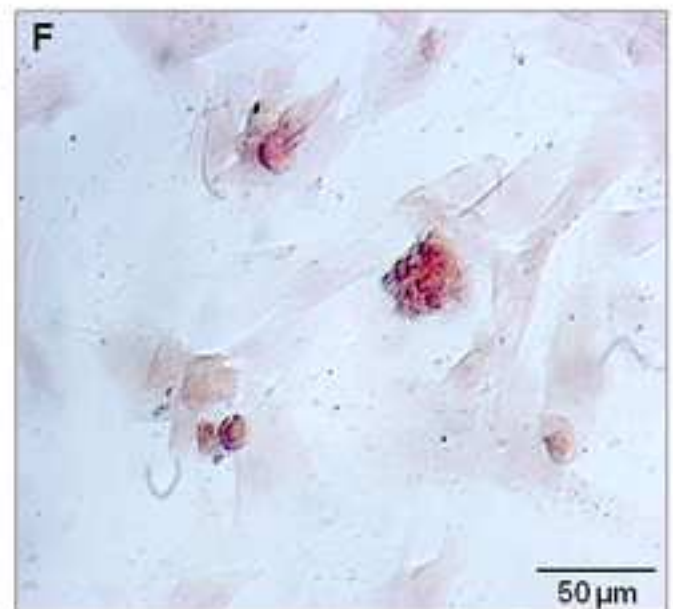
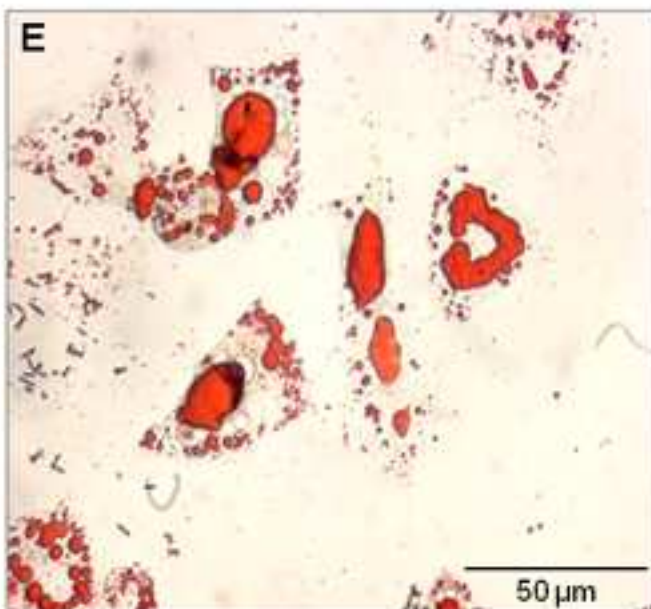
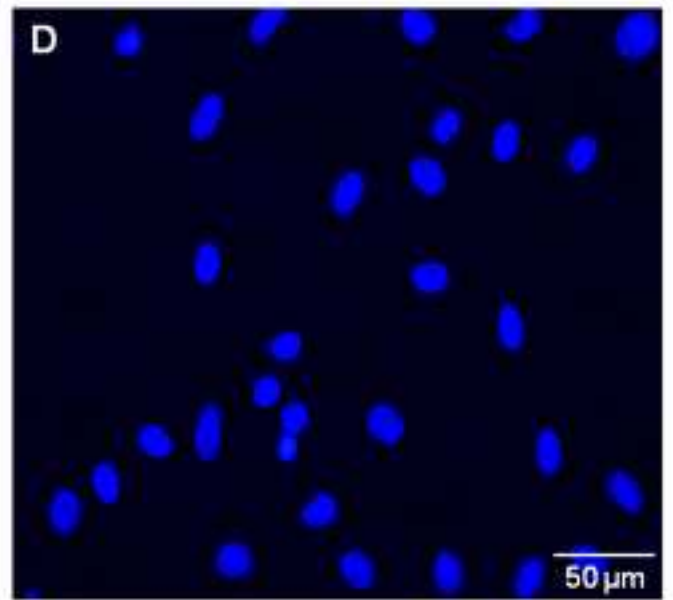
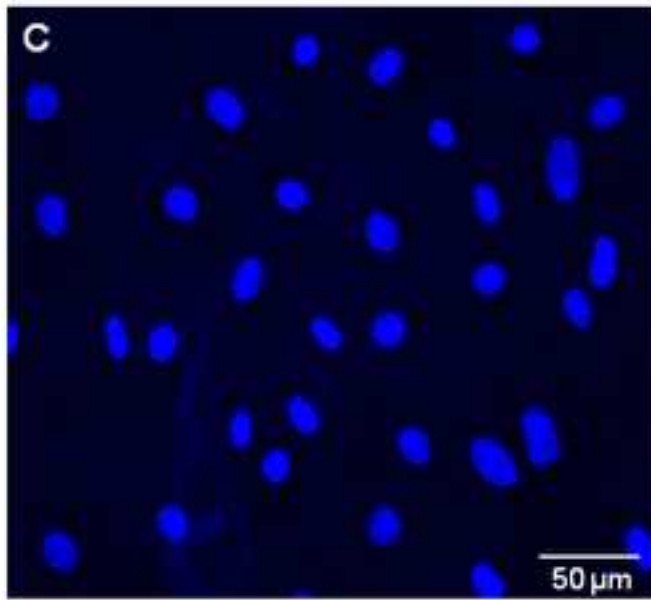
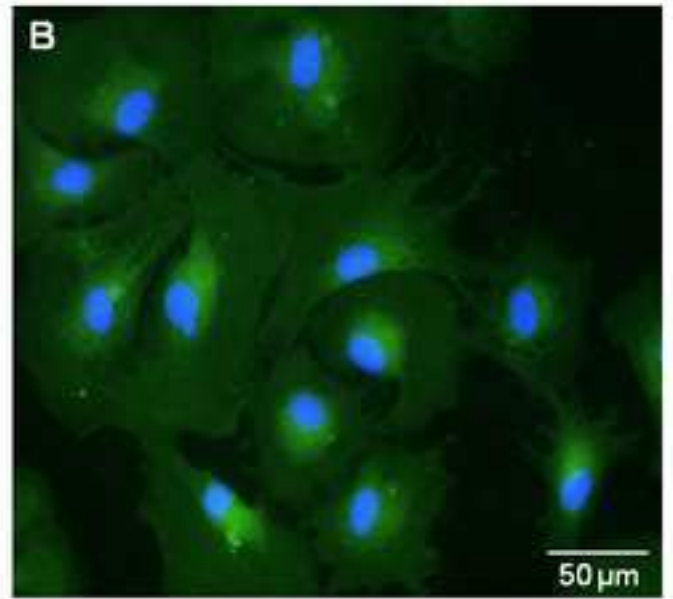
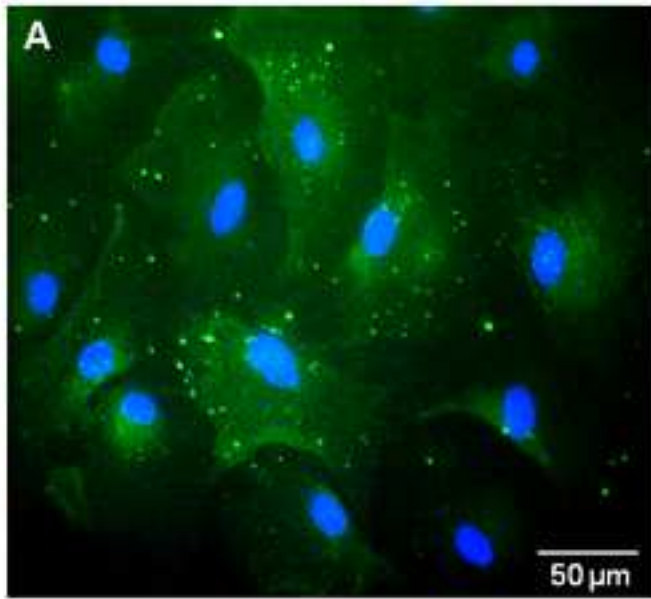
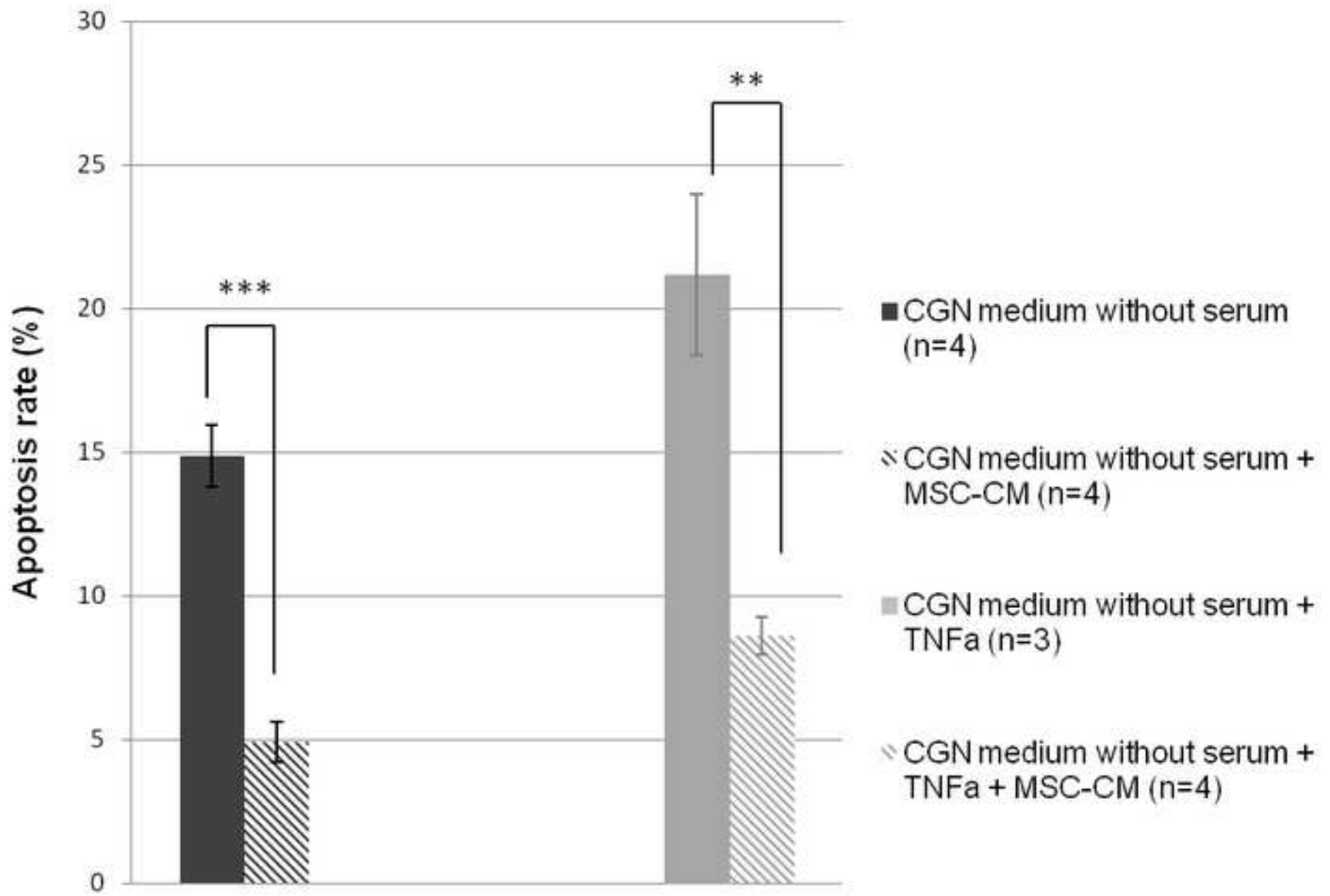
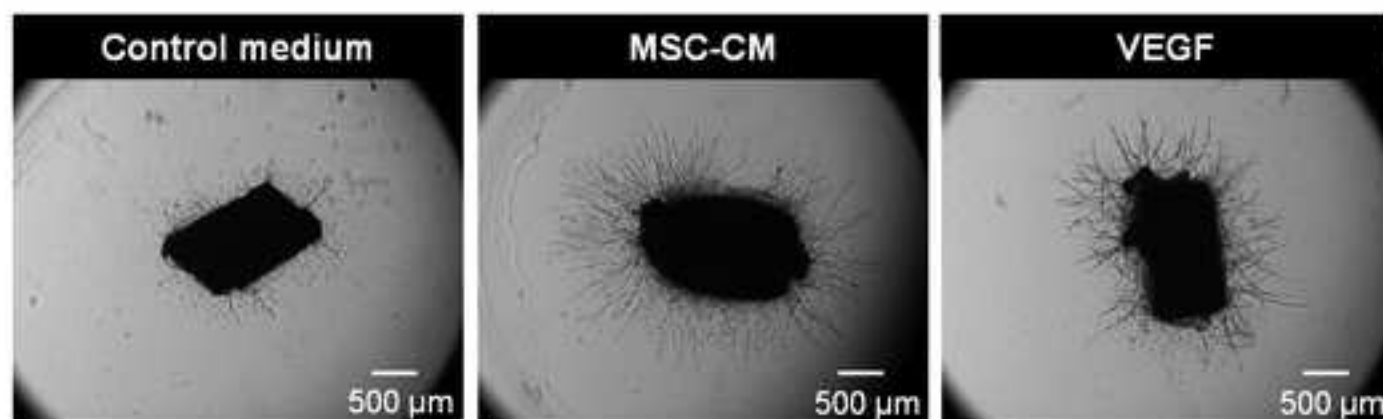


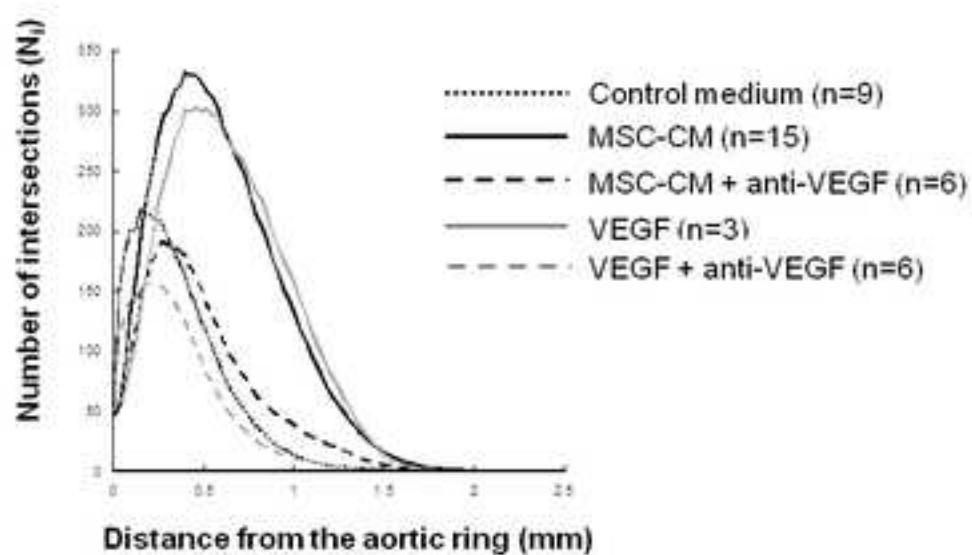
Figure 2
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A



B



C

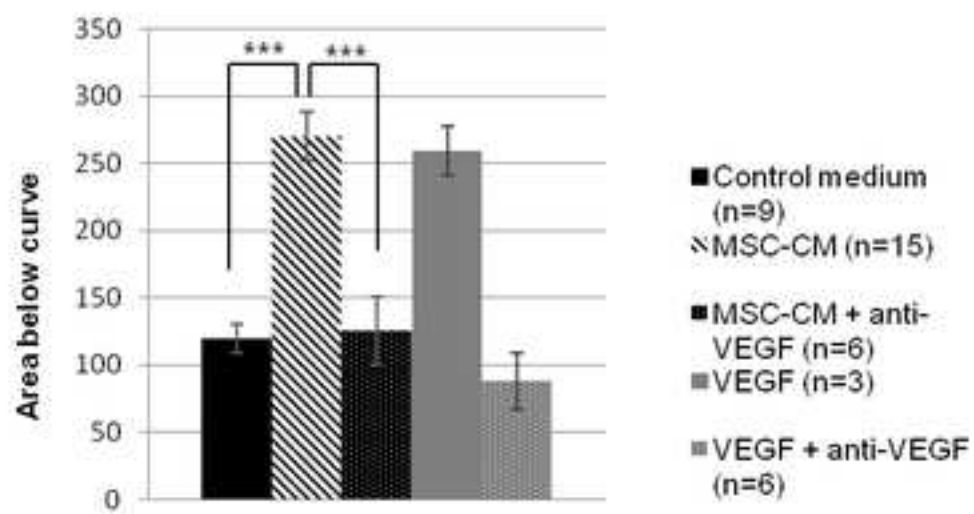


Figure 4

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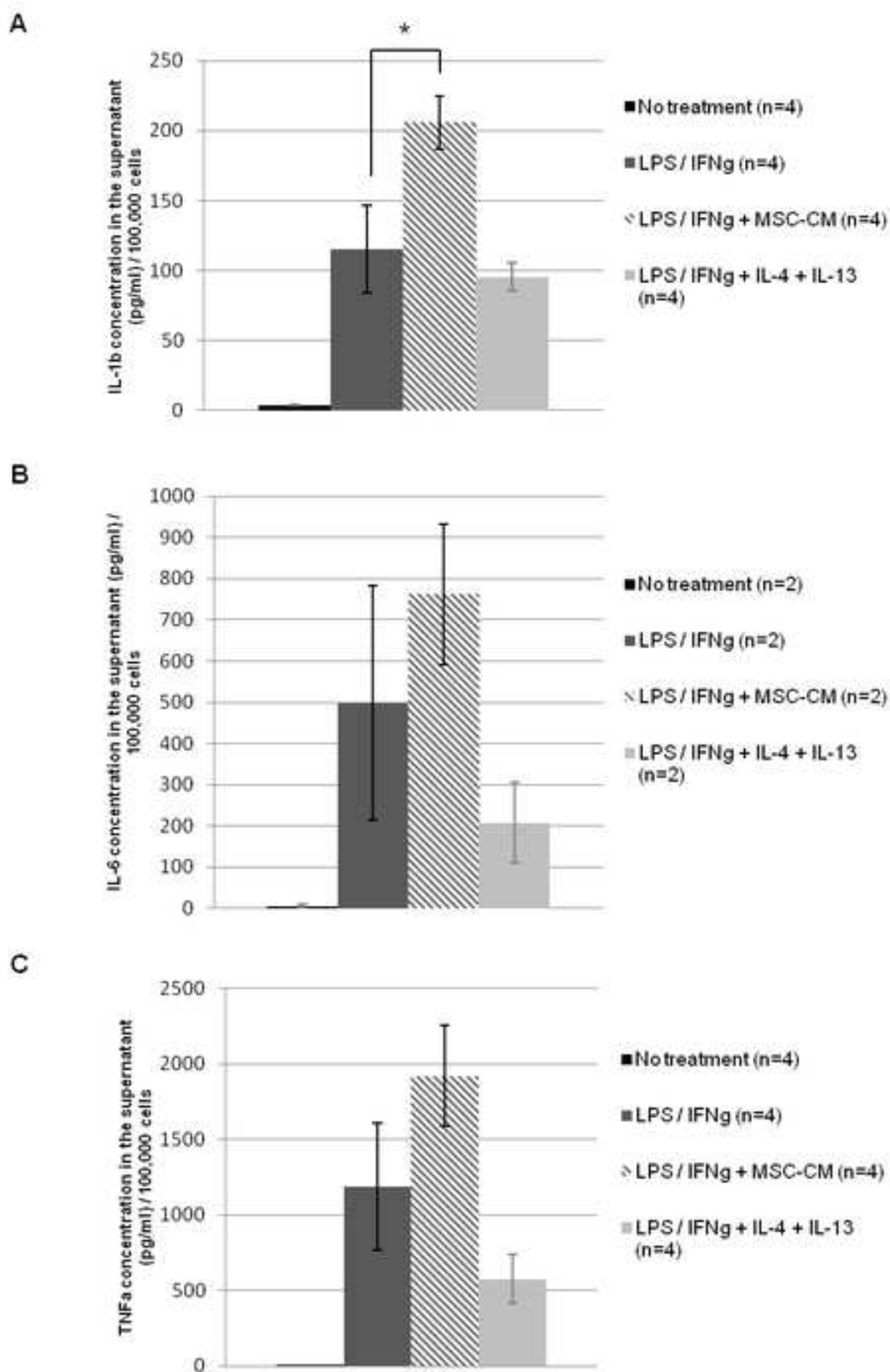
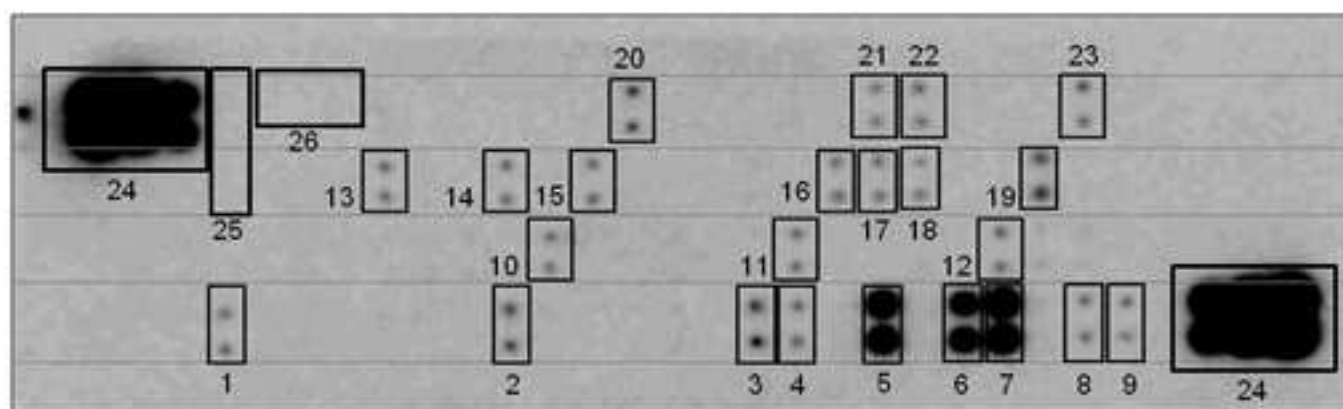


Figure 5

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A



- | | | | |
|----------------|-----------------|------------------------------|-----------------------|
| 1- Osteopontin | 8- VEGF-A | 15- FGF-BP | 22- CINC-3 |
| 2- TAL1A | 9- VEGF-C | 16- Growth Hormone (GH) | 23- CXCR4 |
| 3- TIMP-1 | 10- Insulin | 17- GH receptor | 24- Positive controls |
| 4- TIMP-2 | 11- MDC | 18- Hepassocin | 25- Blanks |
| 5- TLR4 | 12- MMP-13 | 19- Insulin degrading enzyme | 26- Negative controls |
| 6- TRAIL | 13- EG-VEGF/PK1 | 20- BDNF | |
| 7- TROY | 14- Fas ligand | 21- CINC-2 | |

B

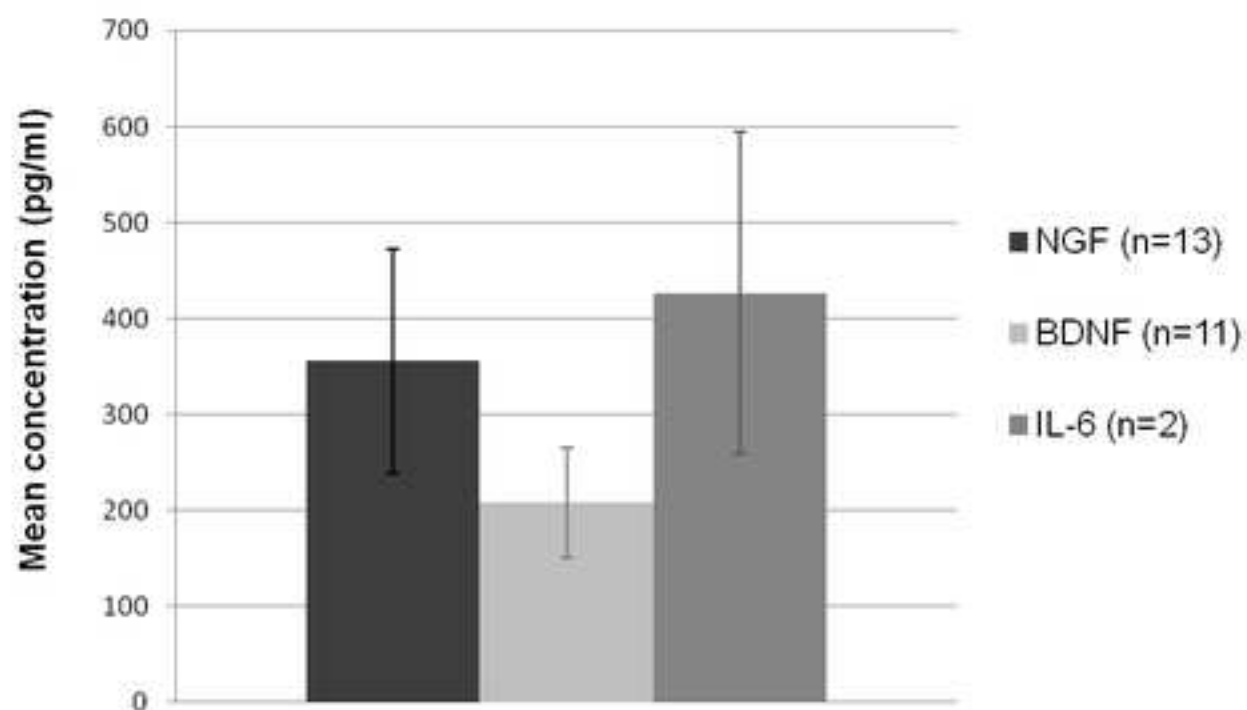


Figure 6
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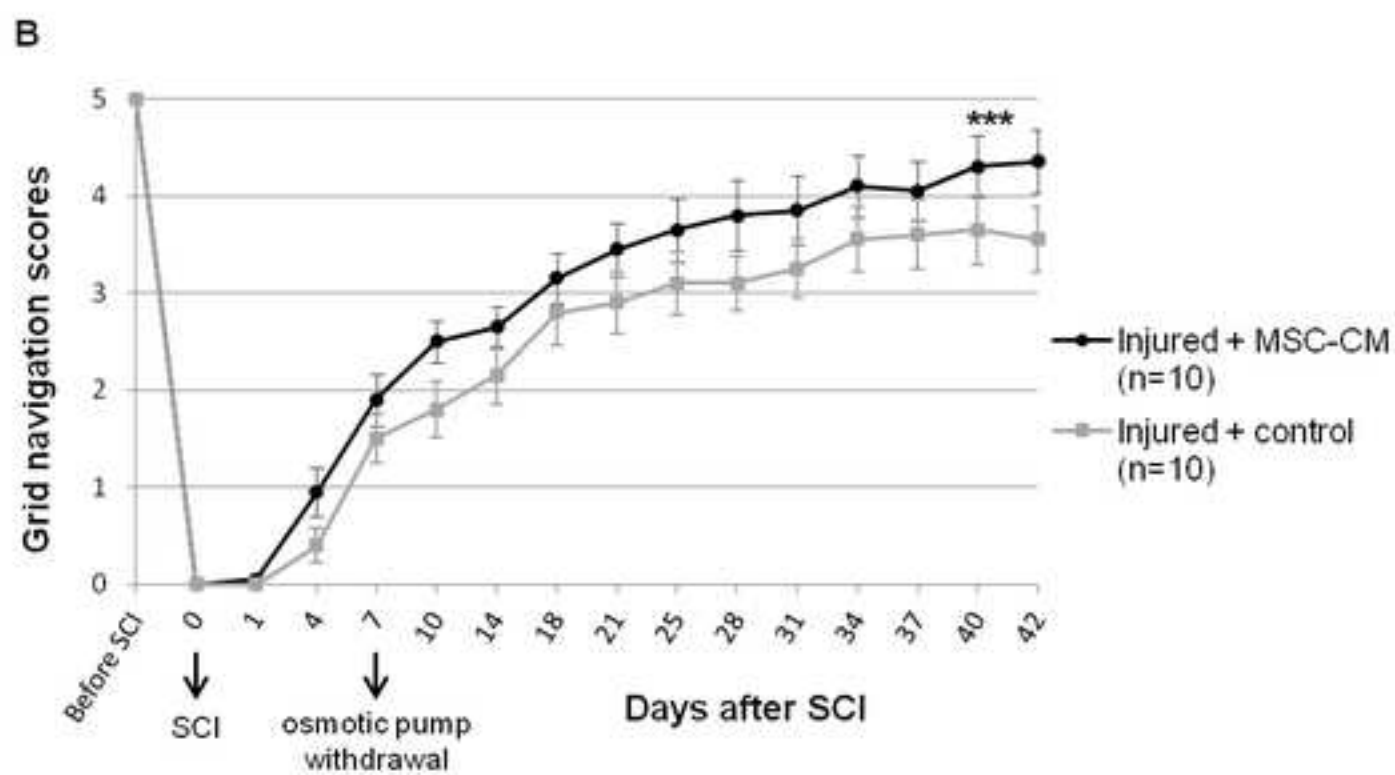
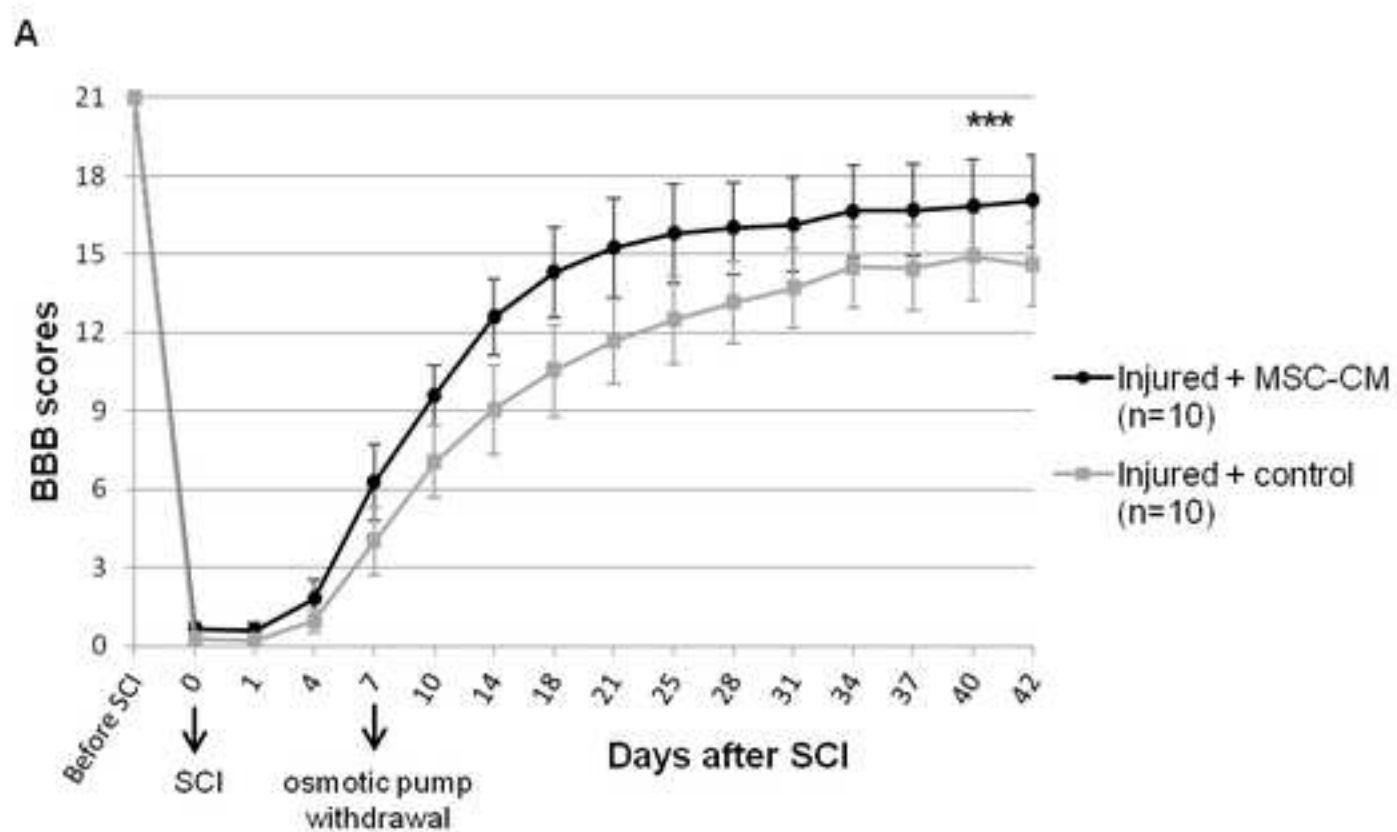


Figure 7

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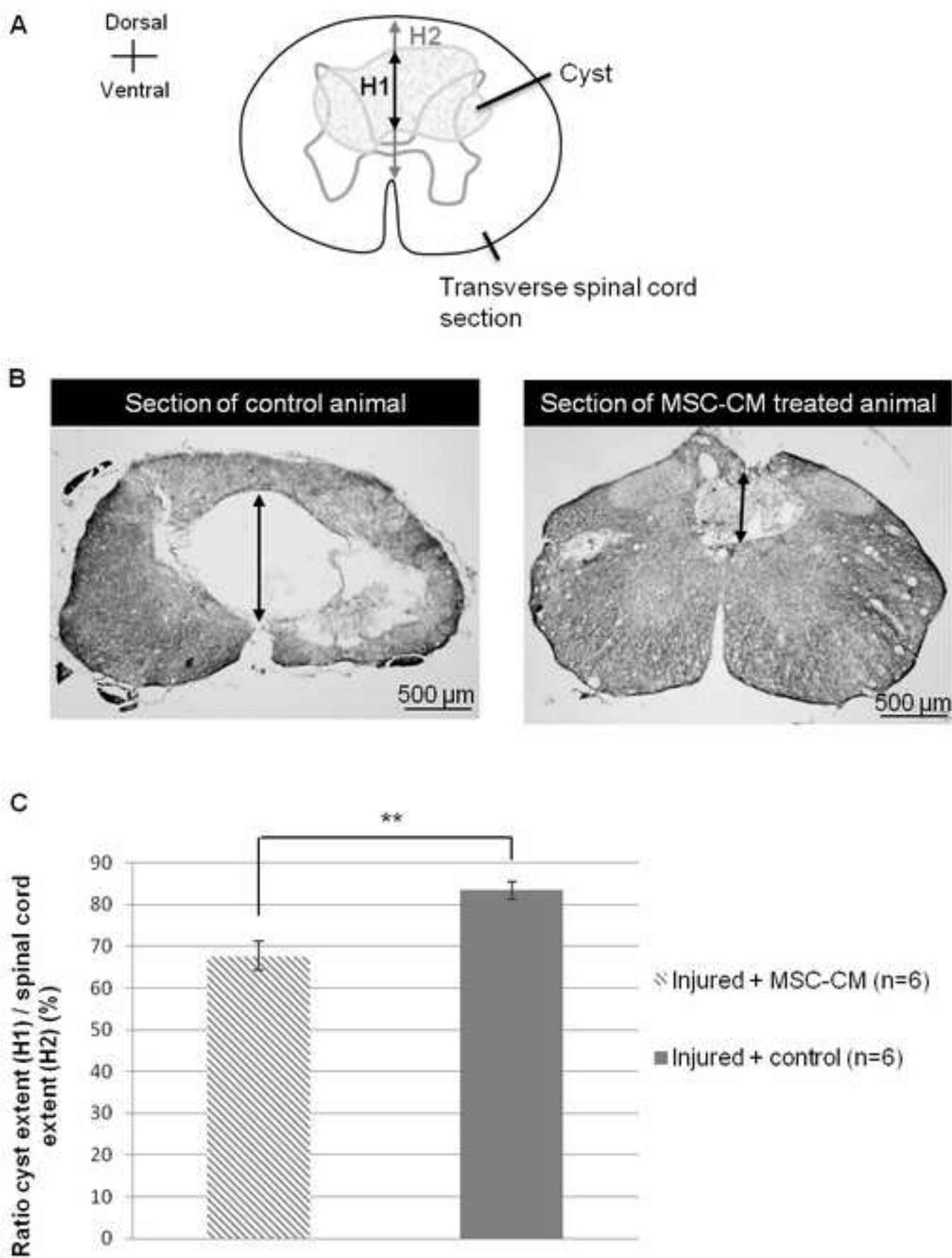
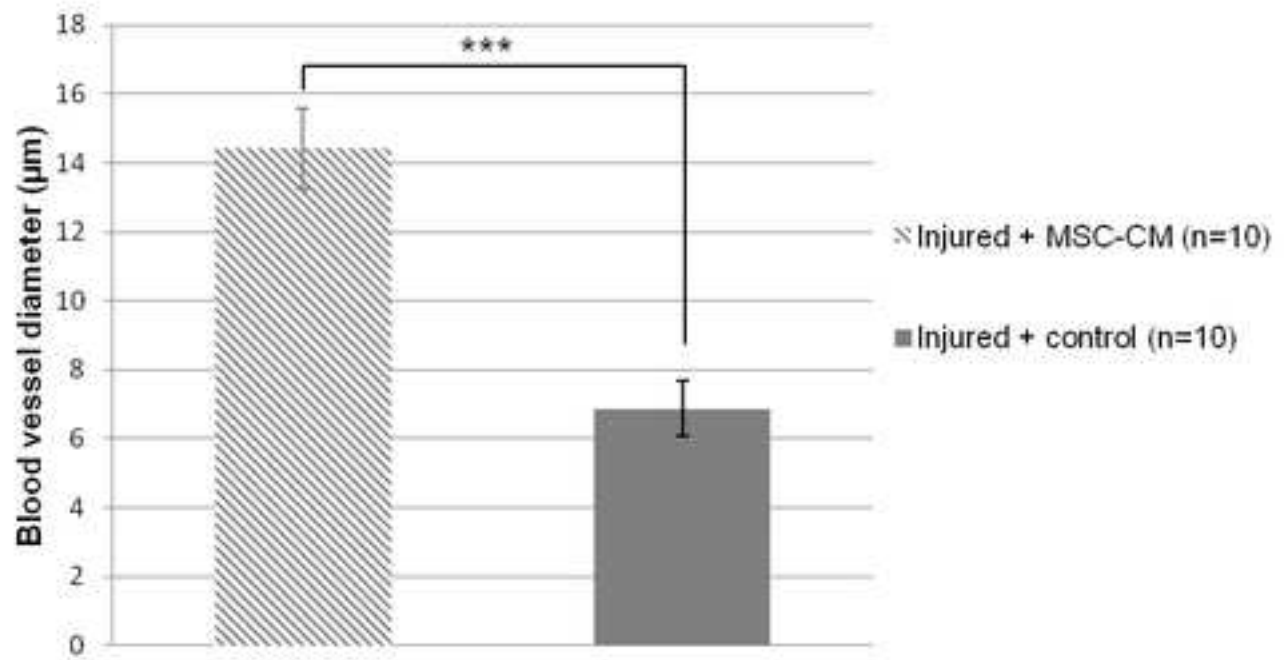
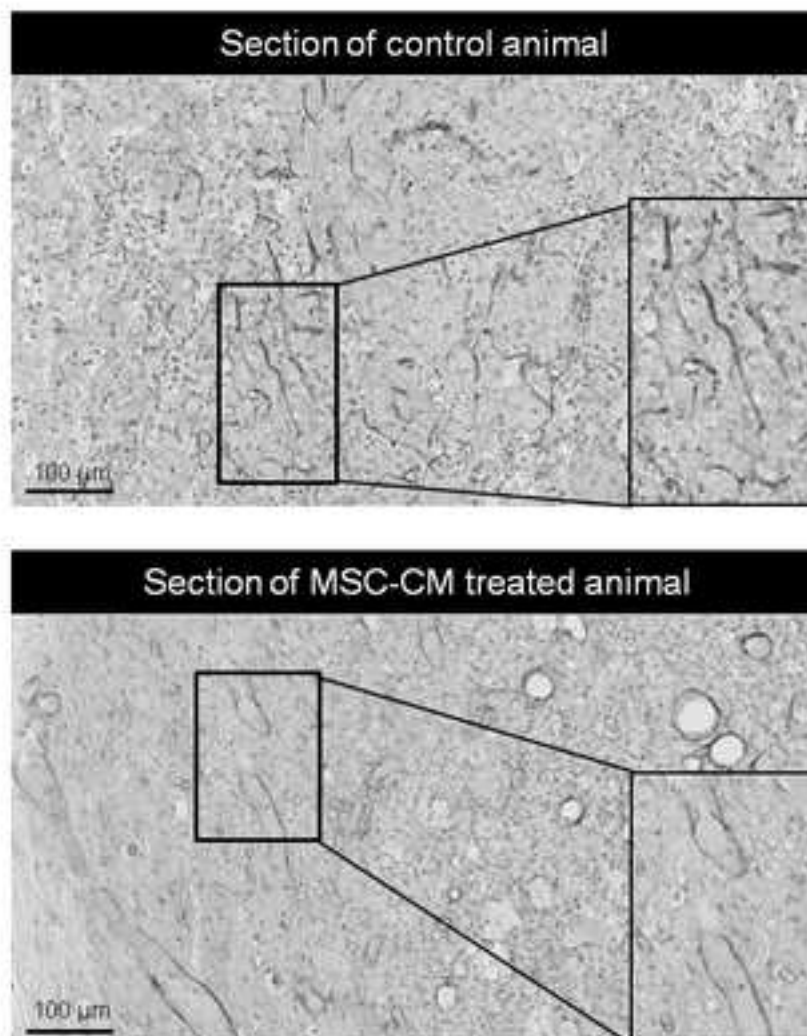


Figure 8
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A



B



Conditioned Medium from Bone marrow-derived Mesenchymal Stem Cells improves recovery after Spinal Cord Injury in rats: an original strategy to avoid cell transplantation.

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Abstract

Spinal cord injury triggers irreversible loss of motor and sensory functions. Numerous strategies aiming at repairing the injured spinal cord have been studied. Among them, the use of bone marrow-derived mesenchymal stem cells (BMSCs) is promising. Indeed, these cells possess interesting properties to modulate CNS environment and allow axon regeneration and functional recovery. Unfortunately, BMSC survival and differentiation within the host spinal cord remain poor, and these cells have been found to have various adverse effects when grafted in other pathological contexts. Moreover, paracrine-mediated actions have been proposed to explain the beneficial effects of BMSC transplantation after spinal cord injury. We thus decided to deliver BMSC-released factors to spinal cord injured rats and to study, in parallel, their properties *in vitro*. We show that, *in vitro*, BMSC-conditioned medium (BMSC-CM) protects neurons from apoptosis, activates macrophages and is pro-angiogenic. *In vivo*, BMSC-CM administered after spinal cord contusion improves motor recovery. Histological analysis confirms the pro-angiogenic action of BMSC-CM, as well as a tissue protection effect. Finally, the characterization of BMSC-CM by cytokine array and ELISA identified trophic factors as well as cytokines likely involved in the beneficial observed effects. In conclusion, our results support the paracrine-mediated mode of action of BMSCs and raise the possibility to develop a cell-free therapeutic approach.

I. INTRODUCTION

Spinal cord injury (SCI) is characterized by the primary lesion, rapidly followed by a cascade of cellular and molecular events that trigger the development of the secondary lesion, known to be deleterious for axonal regeneration and functional recovery. This worsening of the primary lesion is characterized by inflammatory reactions [1,2], free radical production [3], glutamate excitotoxicity [4], neuronal death and oligodendrocyte apoptosis [5-7]. With time, necrosis spreads to adjacent tissues and a cystic cavity appears [8-10]. Moreover, the initiated spontaneous axonal regeneration is repressed by the inhibitory environment composed of astroglial scar, and myelin-derived inhibitory molecules [11].

In this context, numerous experimental studies have been performed to improve functional recovery, focusing on various parameters: control of inflammation [12,13], rescue of neural tissue [14,15], stimulation of axonal regeneration by modulation of the lesioned environment [16-18] or promotion of remyelination [19,20]. Among the developed strategies, cell transplantation aims at replacing lost cells or producing beneficial effects at the lesion site. Among them, olfactory ensheathing cells, schwann cells, macrophages, fibroblasts, neural stem cells or BMSCs have been transplanted in various spinal cord injured contexts [21-29].

BMSCs are adult stem cells easily isolated from the bone marrow. BMSC transplantations have been widely studied in the context of SCI, and have proven beneficial effects on various aspects: inflammation, apoptosis, axonal regrowth, angiogenesis, tissue sparing, astroglial scar, and motor recovery [21,23,24,30-34]. Nevertheless, BMSC transplantations have some disadvantages: they do not persist within the host injured spinal cord after transplantation [35,36], and have a limited ability to replace lost cells [23,37,38]; finally, when used in other pathological contexts, BMSC transplants can have various adverse effects [39-44].

It is well known that BMSCs secrete a large variety of molecules and many studies have shown beneficial impact of these BMSC-released factors in different models [45-48]. Thus, we decided to study the effects of molecules secreted by BMSCs in an adult rat SCI model, using rat BMSC-conditioned medium (BMSC-CM). This original strategy constitutes a multifactorial treatment that could act on many aspects of SCI pathophysiology, avoiding all disadvantages and ethical problems related to the use of cells.

The aim of this study is to evaluate the effect of BMSC-CM on secondary processes involved in lesion extension after SCI. *In vitro* experiments were run in parallel to assess the potential beneficial properties of BMSC-CM on apoptosis, angiogenesis and inflammation.

II. MATERIALS & METHODS

II.1. BMSC culture and BMSC-CM preparation

BMSCs were obtained from the bone marrow of femurs and tibias of adult Wistar rats and characterized as previously described [21]. After having pooled cells from different donors, BMSCs were expanded in DMEM (Invitrogen) containing 4.5 g/L glucose, 0.58 g/L L-glutamine and 0.11 g/L pyruvate, supplemented with 10% non-heat inactivated fetal bovine serum (FBS, Invitrogen) and 100 I.U./ ml penicillin and 100 µg/ml streptomycin (Invitrogen). Medium was changed twice a week, and cells were passaged when 90% of confluence was reached.

BMSC identity is confirmed on the basis of morphological criteria, plastic adherence and specific surface antigen expression: CD90(+), CD271(+), CD45(-) and CD11b(-). Differentiation ability of BMSCs was also evaluated after induction using specific media as previously described [97]. Oil Red O and Alizarin Red S stainings (Sigma-Aldrich) were used to assess adipogenic and osteogenic differentiation.

BMSC-CM was generated as follows: 90% confluent, passage 2-4 BMSCs in T75 tissue culture flask, were washed 3 times with phosphate-buffered saline (PBS) and transferred to a serum-free DMEM culture medium without phenol red during 48 h. CM from different flasks were harvested and pooled. Then, CM were concentrated 40 times by centrifugation at 4000g for 15 min at 13°C, using 10-kDa MW cut-off filter units (Millipore). We consistently obtained, from 10 ml starting volumes, filtrate volumes of about 250 µl. Filter units were used only one time to avoid membrane saturation. Concentrated CM were then sterilized on 0.22 µm filters (Millipore), and stored at -80°C until use. The mean protein concentration of BMSC-CM is of 1.2-1.5 mg/ml. BMSC-CM was divided into small aliquots (500 µl-1 ml) before freezing to avoid repeated freeze/thaw cycles. Each aliquot was visually inspected before use to verify absence of precipitate (meaning a possible loss of protein function). There was no difference in protein concentration between fresh and freezed CM. As additional control, we also tested the stability of BMSC-CM by keeping it for 7 days at 37°C before assessing its anti-apoptotic property using the protocol described below.

Serum-/Phenol red-free DMEM, centrifuged and filtered, was used as control medium.

II.2. Cerebellar granule neuron culture and apoptosis

CGNs were obtained from 4- to 7-day-old Wistar rats, and dissociated as previously described [98]. Cells were seeded at a density of 125,000 cells/well. A purity of 95% was obtained and confirmed by double GFAP/ β 3-tubulin immunostaining. Three to four hours after seeding, CGNs were treated overnight with one of the following serum free media to induce apoptosis: (1) CGN culture medium, (2) CGN culture medium + 25% BMSC-CM, (3) CGN culture medium + 50 ng/ml TNF α (Invitrogen), (4) CGN culture medium + 50 ng/ml TNF α + 25% BMSC-CM. This proportion of BMSC-CM was chosen because pilot experiments showed the same effect when 25% or 50% of BMSC-CM were used. 25% was thus the better compromise to obtain an effect without neither affecting neuronal survival nor wasting BMSC-CM. Cells were then fixed in buffered 4% paraformaldehyde (PFA) for 10 min. Apoptosis was evaluated with the TUNEL method, according to the manufacturer's protocol (Roche). Cells were then counterstained with DAPI and mounted on glass slides. Photomicrographs of 20 random fields per experimental condition were taken (Olympus AX70) at 40x magnification.

For each condition, the total number of apoptotic cells was reported to the total number of cells within the 20 fields. Experiment was repeated 3 or 4 times. The results are expressed as a mean apoptotic rate in percent.

II.3. Ex-vivo aortic ring assay

Rat aortic rings were cultured in three dimensional type-I collagen gels as described by Sounni *et al.* [99]. The following culture conditions were tested: 5% BMSC-CM, 5% control medium, 20 ng/ml rat VEGF with or without 1 μ g/ml anti-rat VEGF (PeproTech). This proportion of BMSC-CM was chosen to limit medium dilution that could affect neovessel regrowth and because it already triggered a strong response. Aortic rings were cultured for 9 days at 37°C in 5% CO₂. Photomicrographs were captured, with a Zeiss phase contrast microscope at 2.5x magnification (Axiovert 25). A minimum of 3 aortic explants were used per experimental condition and experience was repeated at least 3 times. Quantification was performed following the algorithm described elsewhere [100].

II.4. Macrophage culture and activation

Rat macrophage cell line (CRL2192) from ATCC was expanded in Ham's F-12K medium (Invitrogen) supplemented with 15% heat inactivated FBS. Cells were transferred to Petri dishes (Falcon) at a density of 2×10^5 cells/ml in the presence of Ham's F-12K/FBS supplemented with: (1) 25% control medium, (2) Lipopolysaccharides (LPS) + Interferon- γ (IFN γ), (3) LPS + IFN γ + 25% BMSC-CM and (4) LPS + IFN γ + IL-4 + IL-13. LPS from Escherichia coli 0111:B4 (Sigma-Aldrich) and IFN γ (Millipore) were respectively used at a final concentration of 10 and 2 ng/ml. IL-4 and IL-13 (R&D Systems) were used at a final concentration of 10 ng/ml each. Proportion of 25% BMSC-CM was chosen for the same reason as for apoptosis experiment. After 24 h at 37°C, adherent macrophages were detached and cells and supernatants were collected. Cells were counted to normalize the cytokine concentration. Supernatants were centrifuged at 200g to eliminate cells and the cytokine contents were analysed by ELISA. Media without macrophages were used as controls to remove cytokines that could come from serum and/or BMSC-CM. Experiment was repeated 2 to 4 times.

II.5. ELISA and antibody array

ELISA were used to assess *i*) the amounts of NGF, BDNF, IL-1 β , IL-6, TNF α and IL-10 in BMSC-CM, as well as *ii*) the amounts of IL-1 β , IL-6, TNF α and IL-10 in the macrophage culture supernatants, according to the manufacturer's recommendations (R&D Systems). For macrophage supernatant, values were related to 100,000 cells. Samples were run in duplicate for each experiment, and macrophage experiment was repeated 2 or 4 times. For BMSC-CM, samples were run in duplicate for each experiment, and experiment was repeated 2 to 13 times, according to the factor. Final results are expressed as a mean concentration in cytokine.

In parallel, a biotin label-based rat antibody array (RayBiotech) coated with 90 antibodies was used to obtain a broad view of molecules present in BMSC-CM. Three conditioned media from passage 3 BMSC cultures were tested.

II.6. Animal model

A total of 40 adult female Wistar rats (200g) from the Animal Facility of the University of Liege were used. Rats were treated in strict accordance with protocols approved by the Ethical Commission for animal use of the University of Liege, and all efforts were made to minimize

suffering. Rats were housed in individual cages from 7 days before the experiment, in a 12 h light/dark cycle, and in a temperature-controlled environment with access to food and water *ad libitum*. Animals were randomly divided into 4 experimental groups: BMSC-CM treated and control animals, sacrificed 1 or 6 weeks after SCI. 10 animals were studied in each group.

II.7. Contusion injury and BMSC-CM/control medium administration

Contusion SCI was induced with the « Infinite Horizons Spinal Cord Impactor IH-0400 » from *Precision Systems and Instrumentation*, LLC, Version 5.0., USA. Rats were deeply anaesthetized by inhalation of a mixture O₂ (N25) with 5% isoflurane, anaesthesia being maintained with 2-3% isoflurane during surgery (Forene, Abbott).

The skin was incised to expose the thoracic vertebral column and a T10 laminectomy was performed. After stabilization of the spinal column, contusion lesion was performed using a 250 kilodynes force of impact. Dura was opened at the level of the lesion and 10 µl of BMSC-CM or control medium were directly dropped with a Hamilton syringe onto the lesion. A mini-osmotic pump (model 1007D, ALZET, flow rate: 0.5 µl/ h/ 7 days) filled with BMSC-CM or control medium and linked to a catheter was then implanted to deliver the treatment at the lesion site. Muscles and skin were sutured. After 1 week, rats allowed to survive for 42 days were briefly re-anaesthetized to remove the empty pump, in order to avoid inflammation. Bladder function was assessed every day and manually emptied until rats regained control.

II.8. Neurological function evaluation

Two behavioural tests were used after SCI to assess motor performances of rats: the Basso, Beattie and Bresnahan (BBB) open-field test [101] consisting of a 21 point rating scale, and the grid navigation test with a 5 point scale [102]. All rats were accustomed to both tests 1 week before SCI. Two independent experienced individuals carried out all evaluations on a blind basis.

II.9. Euthanasia and histological procedures

After a deep anaesthesia with intraperitoneal injection of pentobarbital (200mg/kg, Nembutal, CEVA Santé Animale), rats were transcardially perfused with 300 ml cold saline containing 0.01% heparine (LEO Pharma), then with 500 ml cold buffered 4% PFA in 0.1M PBS (pH 7.4), 1 or 6 weeks post-injury. Spinal cords were dissected out, post-fixed for 24 h in 4% PFA at 4°C and cryoprotected for 48 h in a 30% sucrose buffer at 4°C. Serial, longitudinal (20µm thick) and transverse (25µm) sections were performed on, respectively, 15 mm-long

and 10 mm-long tissue blocks centred on the lesion site. 1 out of 5 transversal sections were preserved. Inflammation and angiogenesis were studied on rats sacrificed 1 week after SCI (N=10/group), using respectively CD11b and RECA-1 (Rat Endothelial Cell Antigen) immunostainings, on 3 longitudinal sections/animal. Axonal regeneration, astroglial scar formation and cavitation were studied on rats sacrificed 6 weeks after SCI. Axonal regeneration and astrogliosis were evaluated by immunohistology using antibodies against Neurofilaments (NF) and Growth-associated protein 43 (GAP-43) for axons and Glial fibrillary acidic protein (GFAP) for astrocytes, on 3 longitudinal sections/animal (N=4/group). Finally, cystic cavity extension was measured after Luxol-fast blue/ periodic acid - Schiff / hematoxylin staining on 6 transverse sections/animal (N= 6/group).

The following antibodies were used: *Primary antibodies*: mouse anti-NF200 (1:400, Sigma-Aldrich); rabbit anti-GAP-43 (1:1000, Millipore); mouse anti-GFAP (1:400, Sigma-Aldrich), mouse anti-CD11b (1:1000, AbD Serotec); mouse anti-RECA-1 (1:200, AbCam). *Secondary antibodies*: biotinylated horse anti-mouse IgG and biotinylated goat anti-rabbit IgG (1:500, Vector Laboratories). For anti-NF/GAP-43/GFAP/CD11b/RECA-1, sections were processed as previously described [21], except fixation in acetone and incubation in 0.5% H₂O₂ for RECA-1 antibody.

II.10. Image analysis

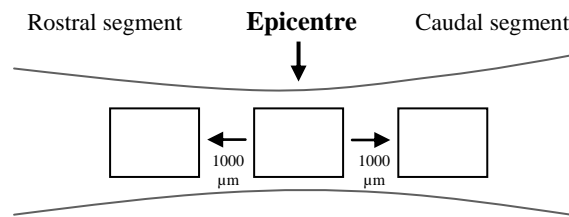
II.10.1. Cystic cavity

To quantify cavitation along the ventro-dorsal axis, 6 stained sections/rat (N=6/group), covering a distance of 1500 µm on either side of the lesion epicentre, were analyzed. Each section was observed under microscope (Zeiss, AxioImager Z1) to measure the height of the ventro-dorsal extent of the cyst relative to the one of the spinal cord midline (Mercator Pro software, Explora Nova). The mean ratio cyst extent / spinal cord extent was calculated for each experimental group and expressed as percentages.

II.10.2. Immunostaining quantification

To quantify GFAP, NF, GAP-43 and CD11b, 3 longitudinal sections per animal were immunostained for each antigen. Bright field images of successive fields at 5x magnification were captured to perform a full reconstruction of each section (Leica DM6000B). On these photomicrographs, the immunostained area was measured in 3 boxed areas of 3 mm² placed as follows: one at the lesion epicentre, one more rostrally (1000 µm from the epicentre) and one more caudally (1000 µm from the epicentre) (see diagram). Using analySIS software (Olympus), reconstructed photomicrographs were transformed in grey scales. A threshold

intensity of staining was applied. The immunostained surface was then measured in each of the 3 boxed areas. Data are presented as the averaged percentage of total stained areas (μm^2) / boxed area (μm^2).



To quantify the blood vessel diameters, RECA-1 immunostained sections were used. 3 longitudinal sections per animal were used and 3 fields per section were randomly chosen at the lesion epicentre. Bright field images at 10x magnification were captured with Zeiss microscope (AxioImager Z1). On each field, diameter of 10 blood vessels was measured with analySIS software. The blood vessel mean diameter for both experimental groups is considered.

II.11. Statistical analysis

All data were expressed as means \pm standard error of the mean (SEM). The improvement of motor recovery was analyzed by Generalized Linear Mixed Model (GLMM), which evaluates the global evolution of the two experimental groups. Other data were analyzed by unpaired Student's t-test. P-value (p) $<$ 0.05 was considered statistically significant. All the tests were conducted using Statistica software (StatSoft), except for neovessel growth in aortic ring model (Aphelion (Adcis) and Matlab 7.9 (MathWorks)).

III. RESULTS

III.1. Characterization of rat BMSCs

BMSCs were expanded in plastic dishes in classical *in vitro* conditions. Cells were characterized by their plastic adherence, the expression of specific antigens and their multipotency was checked by their differentiation capacity. Cells were used at low passages P2, P3 or P4, to work with young cells and avoid senescence-associated effects progressively acquired with passages [49]. P4 BMSCs express surface antigens CD90 and CD271

(p75NGFr) but didn't express CD45 and CD11b. Under specific induction media, cells differentiated into adipocytes and osteocytes (figures 1A-F).

III.2. BMSC-CM protects neurons from apoptosis *in vitro*

Cerebellar granule neurons (CGNs) were cultured and submitted to pro-apoptotic conditions: serum free medium or tumor necrosis factor alpha (TNF α) (50 ng/ml). BMSC-CM was added in each condition at a final proportion volume of 25%. After overnight treatment, cells were fixed and apoptosis rates evaluated using the TUNEL method and a DAPI counterstain. Incubation with BMSC-CM significantly decreased apoptotic rates of neurons whatever the experimental condition (figure 2): in serum deprivation condition, BMSC-CM decreased apoptotic rate by 10% (from 14.8% \pm 1.1 to 4.9% \pm 0.7) (p=0.0002, Student's *t*-test) and by more than 12% in TNF α -induced apoptosis (from 21.2% \pm 2.8 to 8.6% \pm 0.6) (p=0.0038, Student's *t*-test). Results were identical with the use of BMSC-CM kept for 7 days at 37°C before performing the experiment (data not shown).

III.3. BMSC-CM is pro-angiogenic

As vessel growth favours axonal regrowth after SCI, we assessed the effect of BMSC-CM on angiogenesis. Rat aortic rings were incubated 9 days in a specific medium supplemented with 5% BMSC-CM, 5% control medium (negative control) or recombinant rat vascular endothelial growth factor (VEGF, 20 ng/ml; positive control) (figure 3A). Anti-VEGF functional antibody (1 μ g/ml) was also added to assess a possible role of VEGF in observed effects. The number of intersections (N_i) of neovessels as a function of the distance from the ring is illustrated on figure 3B. Areas below curves were calculated in order to include both parameters: N_i and the maximal distance from the ring reached by the neovessels. BMSC-CM induced a strong angiogenic response in comparison to the control (270.5 \pm 18.3 vs 119.3 \pm 11) to reach approximately the same level as with VEGF treatment. Anti-VEGF administration abolished the pro-angiogenic effect of BMSC-CM (270.5 \pm 18.3 vs 125.3 \pm 25.2) and, as expected, of VEGF. The differences were statistically significant between BMSC-CM and control medium (p < 0.0001, Student's *t*-test) and between BMSC-CM and BMSC-CM + anti-VEGF (p=0.0003, Student's *t*-test; figure 3C). This indicates that VEGF is involved in pro-angiogenic effects and must be present in BMSC-CM.

III.4. BMSC-CM favours a pro-inflammatory state of macrophages *in vitro*

In order to study the effects of BMSC-CM on macrophage phenotype, rat macrophages were activated with LPS (10ng/ml) and IFN γ (2 ng/ml) to simulate the *in vivo* inflammation that follows SCI. Activated macrophages were further incubated with 25% control medium, 25% BMSC-CM, or anti-inflammatory cytokines: interleukin 4 and 13 (IL-4 and IL-13) (10 ng/ml). After 24h, culture media were harvested to assess their content in IL-1 β , IL-6, TNF α and IL-10 by ELISA. Each value was normalized for 100,000 cells, and the concentration of cytokines present in BMSC-CM or serum was deduced. Without LPS/IFN γ stimulation, 100,000 macrophages secreted 3.7 pg/ml \pm 0.4 of IL-1 β , while activated cells secreted 115.5 pg/ml \pm 31.2. Incubation with BMSC-CM significantly increased IL-1 β concentration in supernatant of activated macrophages from 115.5 pg/ml \pm 31.2 to 206 pg/ml \pm 19 (p=0.048, Student's *t*-test). The use of IL-4 and IL-13 on activated macrophages reduced slightly the secretion of IL-1 β to 95.6 pg/ml \pm 10.2 (figure 4A). IL-6 and TNF α values also show a tendency to increase after BMSC-CM treatment, without reaching significant levels of differences (figures 4B-C). On the contrary, no secretion of IL-10 was observed (data not shown). Thus, BMSC-CM is globally pro-inflammatory on macrophages, as it exacerbates their secretion of pro-inflammatory cytokines.

III.5. BMSC-CM characterization

In order to further characterize BMSC-CM, we performed large antibody arrays (90 proteins) as well as ELISA assays. Antibody arrays, performed on 3 distinct BMSC-CM samples (figure 5A), reveal the presence of 23 factors involved in either apoptosis, inflammation, angiogenesis, oxidative stress, excitotoxicity, all involved in the modulation of the spinal cord injured environment (Table 1).

ELISA were performed on 4 cytokines: IL-6, IL-1 β , TNF α , IL-10 and 2 neurotrophins: brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF). The analysis of different BMSC-CM demonstrates that NGF and BDNF are present at 356 pg/ml \pm 117 and 208 pg/ml \pm 57 respectively, as well as IL-6 at 427 pg/ml \pm 168 (figure 5B). IL-1 β , TNF α and IL-10 were not detected in these samples.

III.6. BMSC-CM improves functional recovery after SCI

Spinal cord injured animals were submitted to 2 complementary behavioural tests. The Basso, Beattie and Bresnahan (BBB) open-field test was used to assess motor skills of rats until 6

weeks post-injury (figure 6A). The difference between treated and control groups was statistically significant (*Injured + BMSC-CM vs Injured + control* $p=0.0003$, Generalized Linear Mixed Model). From day 4 after SCI, BMSC-CM treated animals show better scores than control animals and this difference was maintained during the study. Score 9 is crucial as it indicates the weight support step. The figure shows that this stage is reached by treated animals 4 days before control animals. Moreover, control animals never recovered their ability to separate toes and to maintain hindpaws in a parallel position at lift off during stepping (score of 16). These parameters, successfully performed by treated animals, bear witness to a fine recovery of motricity.

The grid navigation test was used to assess fine motor coordination and movement accuracy (figure 6B). Mean scores over time were significantly higher in BMSC-CM treated group compared to control group (*Injured + BMSC-CM vs Injured + control* $p=0.0001$, Generalized Linear Mixed Model). We noticed that difference between both groups started from day 4 post-injury. Score 4, testifying a correct placement on grid bars, was reached only by BMSC-CM treated animals.

III.7. BMSC-CM reduces cystic cavity

The improved motor recovery observed in BMSC-CM treated animals could be explained by a reduced extension of the lesion. To investigate this aspect, spinal cord injured transversal sections from both experimental groups were performed 6 weeks after injury and stained with Luxol-fast-blue / periodic acid - Schiff / hematoxylin. Serial sections were then analyzed to quantify the ventro-dorsal extent of the cystic cavity (figures 7A-B). As spinal cord volume can vary over the lesion epicentre, cystic height was related to spinal cord height on the same dorso-ventral axis, and ratio expressed in percentages. BMSC-CM treated group shows reduced mean values compared to control group (*Injured + BMSC-CM: 67.72% \pm 3.4 vs Injured + control: 83.4% \pm 2.1; figure 7C). Difference between BMSC-CM treated and control groups was statistically significant ($p=0.0029$, Student's *t*-test).*

III.8. BMSC-CM is pro-angiogenic *in vivo*

Longitudinal spinal cord injured sections from treated and control groups were performed 1 week post-injury and immunostained for RECA-1. Diameters of blood vessels were measured at the lesion epicentre. Blood vessels exhibit larger diameters in BMSC-CM treated animals compared to controls (*Injured + BMSC-CM: 14.4 μ m \pm 1.2 vs Injured + control: 6.9 μ m \pm*

0.8; figures 8A-B). Difference between both groups was statistically significant ($p < 0.0001$, Student's *t*-test).

III.9. *In vivo*, BMSC-CM has no effect on astrogliosis, macrophage invasion and axonal regrowth

To study astrogliosis, longitudinal sections performed 6 weeks after injury were immunostained for GFAP. Quantification of the immunostained surface was carried out in 3 defined areas. This value was related to the total area of each of the 3 zones to express a proportion of stained area. We didn't detect any difference between BMSC-CM treated and control animals (Student's *t*-test). After CD11b immunostaining, quantifications were performed as described above. Values obtained for BMSC-CM treated and control groups were not significantly different (Student's *t*-test). In order to assess the ability of BMSC-CM to favour axonal regrowth after SCI, anti-NF and anti-GAP-43 immunostainings were performed. Quantifications didn't reveal any difference between groups, whatever the delay (Student's *t*-test) (Table 2).

IV. DISCUSSION

Our results show that, *in vitro*, BMSC-CM provides protection against neuronal apoptosis, is pro-angiogenic and confers a pro-inflammatory phenotype to macrophages. *In vivo*, we demonstrate for the first time that BMSC-released molecules are able to reduce cystic cavity size along the ventro-dorsal axis at the lesion epicentre, to favour large blood vessel growth and to improve locomotor recovery in a spinal cord contusion injury model.

Many studies suggest that beneficial effects obtained after BMSC transplantation in SCI were more likely due to paracrine actions than to effective integration and differentiation of the cells within the host tissue [21,23,36,38,50-53]. Our study confirms this hypothesis.

We decided to deliver factors secreted by BMSCs in the form of concentrated conditioned medium (CM), to optimize potential effects. We also chose to use a fraction of this BMSC-CM that contains factors of a molecular weight > 10 kDa, as this fraction was earlier described as beneficial on myocardial infarct size, oxidative stress and apoptosis [54]. Moreover, we eliminated serum from the medium because of its poorly defined composition.

Two complementary behavioural tests were used to assess recovery of motor functions: BBB as a global recovery test, and grid navigation to evaluate more precisely fine motor movements. In both tests, control animals show spontaneous recovery after spinal cord contusion. The great spontaneous recovery obtained here can also be explained by the type of the lesion, which, despite the use of a 250 kdyn force, doesn't severely affect the ventral part of the spinal cord, thus preserving white matter tracts [55]. According to the literature, 200 kdyn already corresponds to a severe injury, but this parameter is also known to vary upon the animal weight or strain [56,57]. Despite this fact, BMSC-CM treated rats obtained significantly higher scores in both tests compared to control animals. Difference between groups started from days 4 to 7, indicating a rapid effect after administration, **as also described after BMSC transplantation in a model of spinal cord contusion injury [24]**. Later on, BBB scores continue to improve, with treated rats displaying at each delay higher scores compared to controls. In their study, Namiki *et al.* showed an improvement of inclined plane scores one week after BDNF infusion by osmotic pump in a SCI clip model. However, this evolution stopped very early after the end of the BDNF treatment [58]. The prolonged effect that we obtain may be due to the combined action of several factors present in our CM, with maybe redundant molecules with variable bioavailabilities. It is also possible that the cocktail of administered factors favours the survival of endogenous cells preserving the tissue from further lesion extension.

In other studies, after BMSC transplantation in contusion or compression models of rat SCI, locomotor improvements were also observed during weeks following the graft, with BBB scores reaching similar levels. For example, 4 weeks after spinal contusion and BMSC graft, Karaoz and colleagues obtain a BBB score of 15 while our treated rats reach the score of 16 at the same delay. Also, 3 weeks after BMSC graft **in a compression injury model**, Quertainmont *et al.* show that rats reach a BBB score of 12 which is even lower to the score of 15 reached in the present study [21,24]. This reinforces our hypothesis that BMSC-secreted factors are beneficial after SCI and that they improve functional recovery as do BMSC transplants.

Improved recovery can be a consequence of neural tissue protection, which is favoured by angiogenesis. Angiogenesis is indeed known to induce axonal regrowth via the supply of oxygen and nutritional factors that helps preserving injured spinal tissue from further degradation [59,60]. Moreover, it has been shown that the pro-angiogenic VEGF molecule possesses neuroprotective properties [61-63]. This factor is present in BMSC-CM and our *in vitro* data on aortic rings confirm that it is involved in the pro-angiogenic effect. Other factors which are known to promote angiogenesis were also detected in BMSC-CM: osteopontin

[64,65], matrix metalloproteinase-13 (MMP-13) [66] and fibroblast growth factor-binding protein (FGF-BP) [67,68]. *In vivo*, blood vessels observed at the lesion epicentre of treated animals, even if not higher in number, have larger diameters compared to controls, and could thus provide, via increased blood flow, more nutritional substances and oxygen to damaged tissue, contributing to tissue protection, as already described after thoraco-abdominal aneurysm in pigs [69]. Lu *et al.* have also measured this parameter and their data suggest that atorvastatin treatment elicits larger vascular diameter, thus contributing to enhanced regional blood flow perfusion and neuron rescue [70]. So, larger diameters of blood vessels seem to be associated with a better tissue perfusion, protecting neuronal cells from degeneration. Yet, we didn't observe increased axon regeneration in treated spinal cords compared to control ones. In the same context, Dray's study suggests that even if some axons benefit from vascular support to accelerate their growth, this support is only transient and limited in time [60].

Tissue protection could also be related to a reduced apoptosis rate within the injured cord. Our data show that BMSC-CM protects neurons from apoptosis *in vitro*. Neuronal death and apoptosis rapidly followed by oligodendrocyte apoptosis are parts of secondary processes making the lesion worse. BMSC transplantations have been successfully used to reduce apoptotic death in the context of SCI, and associated to a better motor recovery [32]. In this study, we demonstrate that BMSC-CM treated spinal cords have a reduced depth of cystic cavity, protecting white matter tracts. You *et al.* demonstrated a positive correlation between spared ventral white matter and the final BBB scores of rats [55]. Also, the rubrospinal tract in the dorsolateral part and the corticospinal tract located in the dorsal part of the spinal cord white matter in rats are particularly important for precise limb movements, and can be assessed by grid navigation test [71]. Based on our behavioural data, we can thus conclude that the better motor recovery observed in BMSC-CM treated animals is a direct consequence of improved tissue sparing.

Among factors identified in BMSC-CM by cytokine arrays and ELISA, some may also contribute to tissue preservation. NGF stimulates the survival of sympathetic and sensory neurons, while TIMP-1 (tissue inhibitor of metalloproteinase-1) and CINC-3 (cytokine-induced neutrophil chemoattractant-3) are neuroprotective [72,73]. BDNF administration decreases apoptosis and demyelination in a spinal cord compression model [74] and reduces astroglial scar formation [75]. Also, other factors, thus not described here, are known to be secreted by rat BMSCs: IGF-1, HGF, TGF- β 1, EGF, SDF-1, MIP-1 α/β , GM-CSF or FGF-2 [76,77]. The fact that we didn't find those factors is first due to the fact that some of them were not included in our 90-protein array assay. Moreover, BMSC culture conditions may

influence their secretome, which would explain why factors described in other studies were not detected here and conversely.

In our model, BMSC-CM doesn't affect astroglial reactivity. This result could be considered as unexpected, as both NGF and BDNF, present in BMSC-CM, are known to reduce reactive astrogliosis [75,78]. This discrepancy is likely due to the variable concentrations of these two neurotrophins. Also, according to the literature, few studies report any effect of BMSC transplantation on astroglial scar development after SCI. This is also the case for axonal regeneration, which is rarely reported as associated to improved recovery after BMSC transplants [24,79-82].

In vitro data on IFN γ /LPS-activated macrophages show that BMSC-CM further favours their pro-inflammatory state, as assessed by their significant increased IL-1 β secretion and their obvious but non-significant increased IL-6 and TNF α production. In parallel, we also show that BMSC-CM contains IL-6, which possesses pro-inflammatory properties as well [83,84]. *In vivo*, we didn't detect any difference between treated and control groups, in the number of macrophages that invaded the lesion site 1 week post-injury, as assessed by the total area stained for CD11b. Microglia/macrophages present within the lesion exhibited in both groups round cell bodies without branching processes, characteristic of "amoeboid" cells, and indicative of an activated status. This reactive cell form is associated with neuroprotective effects via trophic factor delivery and with phagocytosis that is essential to remove cell and myelin debris [23,85-87]. In this context, we hypothesize that BMSC-CM has further favoured the activated state of resident microglia and invading macrophages, conferring a phenotype beneficial for tissue preservation, without affecting the number of inflammatory cells within the lesion. This hypothesis is in accordance with the fact that IL-1 β and IL-6 do indirectly promote axonal outgrowth [88,89] and protect neurons from death [90,91]. In the literature, contradictory results have been described concerning the effect of BMSC transplantation on post-injury inflammatory reaction, going from reduced inflammation [92] to enhanced macrophage/microglia response [81]. Some authors also suggest that microglial activation after SCI is linked to the reduction of the lesion size [23,93]. Indeed, microglia can be involved in neuroprotection via secretion of factors such as NGF, IGF-1 (insulin-like growth factor-1) or via up-regulation of FGF-2 (fibroblast growth factor-2) in neurons [94-96].

In conclusion, our data show that the use of BMSC-CM in the context of SCI is beneficial and not deleterious, and leads to improved motor recovery. This treatment constitutes a novel

promising therapeutic perspective in SCI context. More investigations are needed to evaluate the potential of this treatment in chronic lesion models, and its future clinical application.

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FIGURE LEGENDS

Figure 1. BMSC characterization. P4 BMSCs *in vitro* are immunostained for (A) CD90 and (B) CD271 (p75^{NGFr}), but are negative for (C) CD45 and (D) CD11b. Nuclei are labelled with Vectashield-DAPI mounting medium. (E) Adipogenic differentiation of P4 BMSC revealed with Oil Red O and (F) osteocyte differentiation revealed by Alizarin Red S.

Figure 2. BMSC-CM protects neurons from apoptosis.

Percentage of apoptotic CGN in serum-free medium or with TNF α (50 ng/ml), cultured with or without 25% BMSC-CM. BMSC-CM significantly decreases apoptosis. Experiment was repeated 3 to 4 times. *** p <0.001; ** p <0.01.

Figure 3. BMSC-CM promotes angiogenesis *in vitro*.

(A) Representative photomicrographs of rat aortic rings after 9 days in culture medium containing 5% control medium, 5% BMSC-CM or 20 ng/ml of rat VEGF.

(B) Quantification of number of intersections (N_i) of neovessels treated with 5% control medium, 5% BMSC-CM and 20 ng/ml VEGF, with or without 1 μ g/ml anti-VEGF.

(C) Area below curves generated for each condition by the semi-assisted computerized quantification. Presence of BMSC-CM significantly favours angiogenesis and this effect is mediated by VEGF. Experiment was repeated 3 to 15 times. *** p <0.001.

Figure 4. BMSC-CM stimulates macrophages to secrete pro-inflammatory cytokines.

Concentration of IL-1 β (A), IL-6 (B) and TNF α (C) in the supernatant of macrophages cultured in various conditions: non-activated, activated with LPS and IFN γ with or without 25% BMSC-CM, or with IL-4 and IL-13. BMSC-CM increases significantly IL-1 β secretion

by macrophages, and tends also to increase their IL-6 and TNF α secretion. Experiment was repeated 2 to 4 times. * p <0.05.

Figure 5. BMSC-CM characterization by antibody arrays and ELISA.

(A) Multiple array membrane incubated with BMSC-CM reveals the presence of hormones, proteases, cytokines and trophic factors. Experiment was repeated 3 times.

(B) Concentrations of NGF, BDNF and IL-6, measured by ELISA, within BMSC-CM. Experiment was repeated 2 to 13 times.

Figure 6. Behavioural analysis.

Evolution of motor scores for BMSC-CM treated and control rats over 6 weeks, as assessed (A) by BBB scale and (B) by grid navigation scale. BMSC-CM treated rats obtained significantly higher scores compared to control animals. 10 animals per group were studied. *** p <0.001.

Figure 7. BMSC-CM reduces lesion extension.

(A) Illustration of an injured spinal cord transversal section invaded by a cyst. For each section, cystic cavity extent (H1) and spinal cord extent (H2) were measured on the median axis. Both values were related to express a ratio in percents.

(B) Photomicrographs of Luxol-fast-blue / periodic acid - Schiff / hematoxylin stained sections of BMSC-CM treated and control animals, taken at the lesion epicentre, 6 weeks after injury. Cyst extent is shown by an arrow.

(C) Ratio cyst extent / spinal cord extent of BMSC-CM treated and control animals, 6 weeks after injury. BMSC-CM treated animals obtained a ratio significantly reduced compared to control group. 6 animals per group were studied. ** p <0.01.

Figure 8. BMSC-CM increases blood vessel diameter within the lesion.

(A) Mean blood vessel diameter at the lesion epicentre measured on RECA-1 immunostained sections from BMSC-CM and control groups 1 week after SCI, illustrated in (B). Blood vessels in BMSC-CM treated animals have larger diameters in comparison to control animals. 10 animals per group were studied. *** p <0.001.

Table 1. Factors detected in BMSC-CM.

| <i>Phenomena</i> | <i>Specific properties</i> | <i>Factors</i> |
|----------------------------|-----------------------------------------------------|------------------------------------------------------------------------------------------------------|
| Apoptosis | Anti-apoptotic | VEGF-A, TIMP-1, CINC-3, TIMP-2, Osteopontin, Growth Hormone + GHR, EG-VEGF/PK1, TAL1A*, FGF-BP, BDNF |
| | Pro-apoptotic | TROY, TRAIL, Fas ligand/TNFSF6 |
| Inflammation | Pro-inflammatory | TLR4 , CINC-3, Osteopontin, EG-VEGF/PK1, CINC-2, MMP-13, MDC, Fas ligand/TNFSF6 |
| | Immunomodulator | Osteopontin, CXCR4, MDC |
| Angiogenesis | Pro-angiogenic | VEGF-A, VEGF-C, Osteopontin, EG-VEGF/PK1, TAL1A*, MMP-13, FGF-BP |
| | Anti-angiogenic | TIMP-2 |
| Neuronal modulation | Neuroprotective and Neuro-/neurito-trophic | TIMP-1, CINC-3, Growth Hormone + GHR, BDNF |
| | Anti-neuritotrophic | TROY |
| | Neuronal differentiation | TIMP-2 |
| | Modulation of neuronal activity | BDNF |
| Stress | Protection from excitotoxicity and oxidative stress | Osteopontin, FGF-BP |
| Other | Cell fate regulation | TIMP-1, TIMP-2, Hepassocin, Growth Hormone + GHR, EG-VEGF/PK1 |
| | Cell survival | TIMP-1, TIMP-2, Growth Hormone + GHR, BDNF |
| | ECM modulation | TIMP-1, MMP-13 |
| | Metabolism | Insulin degrading enzyme, Insulin, Growth Hormone + GHR |

Antibody arrays against 90 proteins were performed on 3 different samples of BMSC-CM and identified 23 factors likely involved in the modulation of the spinal cord injured environment.

*protein not known to be secreted.

Table 2. Immunostaining quantifications.

| Studied parameters | Studied regions | Proportion of stained area (%) in BMSC-CM treated animals | Proportion of stained area (%) in control animals | P-value and significance (treated vs control) | N / group |
|-------------------------------------------|-----------------|-----------------------------------------------------------|---------------------------------------------------|-----------------------------------------------|-----------|
| Astroglial scar (GFAP) | Epicentre | 7.4 ± 0.4 | 6.2 ± 1 | 0.29 - NS | 4 |
| | Rostral | 5.8 ± 1.4 | 7.1 ± 1.7 | 0.6 - NS | 4 |
| | Caudal | 5.4 ± 0.9 | 7.2 ± 2 | 0.46 - NS | 4 |
| Inflammation (CD11b) | Epicentre | 32.1 ± 2 | 32.2 ± 2.7 | 0.98 - NS | 10 |
| | Rostral | 13.1 ± 1.3 | 15 ± 3.2 | 0.57 - NS | 10 |
| | Caudal | 12.1 ± 1.5 | 17 ± 3.9 | 0.25 - NS | 10 |
| Axonal regrowth 1 week post SCI (GAP-43) | Epicentre | 8.2 ± 0.9 | 7.3 ± 1.4 | 0.57 - NS | 10 |
| | Rostral | 8 ± 1 | 7.5 ± 1.6 | 0.79 - NS | 10 |
| | Caudal | 7.3 ± 1.1 | 6.9 ± 1.3 | 0.81 - NS | 10 |
| Axonal regrowth 6 weeks post SCI (GAP-43) | Epicentre | 5.1 ± 1.2 | 6.2 ± 0.7 | 0.48 - NS | 4 |
| | Rostral | 3.1 ± 1.3 | 4.3 ± 0.6 | 0.44 - NS | 4 |
| | Caudal | 1.9 ± 0.6 | 3.1 ± 0.5 | 0.21 - NS | 4 |
| Axonal regrowth 6 weeks post SCI (NF) | Epicentre | 5.4 ± 1.6 | 6.3 ± 1.4 | 0.71 - NS | 4 |
| | Rostral | 6.9 ± 2.5 | 4.8 ± 1.3 | 0.48 - NS | 4 |
| | Caudal | 8.9 ± 3.1 | 6.7 ± 2 | 0.59 - NS | 4 |

After specific immunostainings (GFAP, CD11b, GAP-43, NF), quantifications of proportion (%) of stained area were performed in 3 defined areas. No statistical differences between treated and control rats were found.

Response to Reviewers

Reviewer #1

Specific comments

1: p-values and statistical tests used in experiments

The new version of the manuscript takes into account the Reviewer's advice and each "Results" section contains now a clear description of the statistical tests used and p-values obtained.

2: Conclusion about the increase of IL-6 secretion by macrophages

The Reviewer suggests being more cautious about the conclusion of an IL-6 increase because only 2 samples were analysed (Fig 4B). We mention now clearly that IL-6 shows only a tendency to increase, as does TNF-a (Fig 4C), conferring to MSC-CM a global pro-inflammatory action, finding reinforcing the significant increase of IL1b (Fig 4A). (see "Results" and "Figure legends" sections).

Reviewer #2

Specific comments

1: Absence of references supporting tumour development in spinal cord after MSC transplantation and lack of precision.

Text has been modified as requested by the Reviewer (see abstract and introduction). We now describe the disadvantages of BMSC grafts in the specific context of SCI (lack of differentiation and integration within the host) and we just mention that in other pathological contexts, BMSCs transplants have various adverse effects. Some references were removed, while one has been added, a review describing both the beneficial and the adverse effects of BMSC transplantation in various pathological models (Wong R, 2011).

2: Differences between our previous published study and this work.

- The Reviewer noticed that the control group in the present study has a better spontaneous recovery than the BMSC-transplanted group from our previous study (Quertainmont et al., 2012). We agree and can explain this difference between studies by the fact that the lesion models used in each study are not the same: compression injury in Quertainmont's study, and contusion injury in the present study. In the new version of the manuscript, we are more cautious in our comparisons and text has been adapted in accordance with this (see Discussion).
- The Reviewer asked if the observers performing behavioural tests were blinded to the type of therapy. This was the case, and was already mentioned in the "Material & Methods" section of the original version (point II.8).

- The Reviewer noticed a divergence between grid navigation scales described in "Materials and Methods" section (mentioned as a 6-point scale) and the graph presented in the "Results" where a 5-point scale is illustrated. Indeed, there is a mistake in "Materials and Methods" section, and correction has been made. (See Material & Methods, point II.8).
- Moreover, Reviewer asked that we explain why, in the grid navigation test, we use in the current study a 5-point scale and in our previous study a 4-point scale. The 5-point scale is a little bit more precise and has been published later than the scale used in our previous work (4-point scale). Both scales have been used in the literature.

Details of each score for 4-point scale (from Gaviria et al., 2002):

0: hindlimb drag; no foot placement

1: consistent foot faults; foot misses wire and falls through mesh

2: consistent foot faults; foot slips off wire after accurate step and falls through mesh

3: accurate foot placement on wire but toes do not grip

4: normal course over the grid with toes gripping wire

Details of each score for 5-point scale (from Perrin et al., 2010):

0: no foot placement

1: feet miss and fall in the hole

2: feet try a placement on the grid but can't perform it

3: after accurate step, feet slip off grid and fall

4: accurate foot placement but toes do not grip

5: normal course

- No data from injured-only rats were mentioned, because this control has not been done in the study, as no differences between our 2 controls were found in the previous study by Quertainmont. This is also justified by the fact that our aim was to assess the effect of MSC-CM after SCI. The more appropriate control was thus to compare treated rats with a group of animals submitted exactly to the same experimental protocol (anaesthesia, injury, mini-osmotic pump placement, solution delivery during 7 days and pump withdrawal) with the sole difference being the content of the solution delivered by the pumps: MSC-CM or control medium (same composition as the medium used for MSC cultures). Thus, if differences between groups were observed, they could only be attributed to the conditioned medium.

3: Factors known to be secreted by BMSCs are not detected in the present study.

The Reviewer advised us to contrast our findings with similar studies since we do not detect in the BMSC-CM some factors already described as being secreted by BMSCs.

We have followed the suggestion of the Reviewer and have adapted the text in the discussion section.

4: Stability of factors continuously delivered during 1 week *in vivo*

The Reviewer suggests that we discuss the stability of growth factors that are continuously delivered during one week, and how we can prove that their effects are maintained when implanted *in vivo*.

In the first version of the manuscript, we didn't mention that we assessed the stability of our MSC-CM in order to be sure that it would keep its effectiveness when used *in vivo* for 7 days. In this purpose, we have compared the anti-apoptotic effect of MSC-CM (i) freshly defrosted or (ii) kept for 7 days at 37°C, on neurons using the protocol described in the manuscript. No differences were seen between the 2 MSC-CM, comforting us in the fact that MSC-CM preserves its properties at least 7 days at 37°C. This is now explained in "Material & Methods" and "Results" sections.

5: Description of BMSC-CM preparation and justification of proportion used in *in vitro* experiments

The Reviewer would like that we describe more precisely the generation of conditioned medium as it is the primary material investigated in our study. He also asked to explain why different concentrations of BMSC-CM were used among *in vitro* experiments.

We have followed the suggestions of the Reviewer and more details are given in the "Materials and Methods" section (point II.1).

- The size of culture flask is T75 (75cm²).
- Cell number used to generate CM were not counted, but protein concentration assays of different aliquots of BMSC-CM were performed. We always obtained a concentration of 1.2 to 1.5 mg/ml.
- CM-concentration is performed by centrifugation: starting volume of 10 ml (pooled CM) to obtain a final volume of 250 µl.
- Effect of freezing was limited by defrosting aliquot of BMSC-CM only once. Also, we never observed any precipitate in defrosted BMSC-CM. Also, as already mentioned earlier, BMSC-CM keeps its properties if used after being defrosted or kept for 7 days at 37°C.
- The use of 25% or 5% BMSC-CM proportions for our *in vitro* experiments is now justified in "Materials and Methods" section. This choice is based on results obtained in pilot studies and not shown in the manuscript. We tested 25% and 50% BMSC-CM proportions to assess effects on neuronal apoptosis, and obtained same results for both conditions. We were also concerned to keep the highest possible proportion of nutrient medium (75%), to avoid apoptosis due to lack of nutrients and/or ions (KCl must be highly concentrated in cerebellar granule neuron medium for neuron survival). 25% BMSC-CM was chosen for the same reasons in macrophage experiment. 5% BMSC-CM was chosen in aortic ring experiment because this proportion already induced a strong angiogenic answer that would have been unquantifiable if more important.

6: Description of passage number of BMSCs on panels C and D of figure 1

The Reviewer asked to give the passage number of BMSCs on panels C and D for CD45 and CD11b in BMSC characterization. Passage 4 BMSCs were used for this characterization by immunostaining for CD90, CD271, CD45 and CD11b. This has been clarified in the figure 1 legend.

7: Incomplete description of multipotency in the context of SCI

The Reviewer claims that figures 1E-F are incomplete because they do not support multipotentiality of our BMSCs in spinal cord injury context. The purpose of this figure is not to demonstrate the potentiality of cells to differentiate into cell types beneficial in

SCI context but to characterize cells and prove that we are really in presence of BMSCs. Indeed, to prove that cells are actually MSCs, it is generally accepted that cells must (1) adhere to plastic in standard culture conditions, (2) express some markers and be negative for others as shown in this study and (3) have the ability to differentiate *in vitro* into osteoblasts and adipocytes. In this purpose, quantification was not necessary.

8: Origin of the BMSC-CM

The Reviewer is interested to know if BMSCs used to prepare CM derive from different donors. It is actually the case, and we did it to insure absence of donor-specific effects. BMSCs collected from different rats were pooled before seeding in T75 flasks. So BMSC-CM harvested from these cells derived from various donors. Moreover, CM harvested from different flasks were pooled before concentration on centrifugal units.

These details have been added in the first point of "Materials and Methods" section.

9: Tissue sparing and remyelination

The Reviewer writes it is difficult to assert from the figure 8B that there is a tissue sparing. He advised us to examine electron microscopic images to conclude to a sparing of the tissue and a better remyelination.

- Figure 8B shows lesioned spinal cord sections immunostained for RECA-1 to reveal blood vessels. This figure is thus not useful to assess for any remyelination or tissue sparing.
- Probably, the Reviewer made reference to figure 7B that shows microphotographies of spinal cord tissue sections stained with Luxol-fast-blue / periodic acid - Schiff / hematoxylin. This figure shows clearly that the ventro-dorsal extension of the lesion is significantly lower in treated animals compared to controls. It is important to note that these quantifications were made on sections from the lesion epicentre, where the injury is the worse, and thus, the more tissue preserved, the better recovery will be possible.
Finally, remyelination is a parameter that we didn't mention nor quantify in our manuscript. This is why electron microscopy was not used.

10: Number of replicates.

The Reviewer suggests indicating more clearly the number of replicates performed in all experiments. This has been made in "Materials and Methods" and "Figure legends" sections.

Minor comments

- The sentence "and which constitutes the called" in the "Introduction" section has been rephrased.