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Adipose-derived stem cells decrease pain in a rat model of oxaliplatin-induced neuropathy: Role of VEGF-A modulation

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ABSTRACT

Oxaliplatin therapy of colorectal cancer induces a dose-dependent neuropathic syndrome in 50% of patients. Pharmacological treatments may offer limited relief; scientific efforts are needed for new therapeutic approaches. Therefore we evaluated in a preclinical setting the pain relieving properties of mesenchymal stem cells and its secretome. Rat adipose stem cells (rASCs) were administered in a rat model of oxaliplatin-induced neuropathy.

A single intravenous injection of rASCs reduced oxaliplatin-dependent mechanical hypersensitivity to noxious and non-noxious stimuli taking effect 1 h after administration, peaking 6 h thereafter and lasting 5 days. Cell-conditioned medium was ineffective. Repeated rASCs injections every 5 days relieved pain each time with a comparable effect. Labeled rASCs were detected in the bloodstream 1 and 3 h after administration and found in the liver 24 h thereafter. In oxaliplatin-treated rats, the plasma concentration of vascular endothelial growth factor (pan VEGF-A) was increased while the isoform $VEGF_{165}b$ was upregulated in the spinal cord. Both alterations were reverted by rASCs. The anti-VEGF-A monoclonal antibody bevacizumab (intraperitoneally) reduced oxaliplatin-dependent pain. Studying the peripheral and central role of $VEGF_{165}b$ in pain, we determined that the intraplantar and intrathecal injection of the growth factor induced a pro-algesic effect. In the oxaliplatin neuropathy model, the intrathecal infusion of bevacizumab, anti-rat $VEGF_{165}b$ antibody and rASCs reduced pain.

Adult adipose mesenchymal stem cells could represent a novel approach in the treatment of neuropathic pain. The regulation of VEGF-A is suggested as an effective mechanism in the complex response orchestrated by stem cells against neuropathy.

1. Introduction

Oxaliplatin is an antineoplastic drug primarily used in the treatment of metastatic colorectal cancer alone or in combination with other agents (De Gramont et al., 1997; Diaz-Rubio et al., 1998). Unlike other platinum compounds, oxaliplatin infusion is associated with mild nephron-and hematological toxicity with no grade 3–4 anemia, thrombocytopenia or neutropenia (Diaz-Rubio et al., 1998; Holmes et al., 1998; Souglakos et al., 2002). Nevertheless, oxaliplatin administration induces the development of neuropathic syndrome in 50% of patients receiving a total cumulative dose higher than 1000 mg/m^2 over four cycles or more of therapy (De Gramont et al., 2000; Kannarkat et al., 2007). Symptoms of oxaliplatin neurotoxicity include altered pain threshold, paresthesia and dysesthesia that affect both lower and upper extremities in a characteristic “glove and stocking” distribution (Kannarkat et al., 2007; Wolf et al., 2008) resulting in impaired quality of life in cancer survivors since symptoms do not remit after chemotherapy

Abbreviations: CMTMR, 5-(and 6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine; DRG, dorsal root ganglion; i.p., intraperitoneal; i.t., intrathecal; i.v., intravenous; MSCs, mesenchymal stem cells; rASCs, rat adipose stem cells; PAG, periaqueductal gray; VEGF-R1, VEGF receptor 1.

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in many patients. Currently, there are no effective therapies (Hershman et al., 2014). Therefore, efforts are still in progress to study the basis of oxaliplatin-induced neuropathic pain in preclinical experimental models. In animals, neuropathic syndrome induced by oxaliplatin is characterized by important alterations to the peripheral and central nervous systems resulting in aberrant somatosensory processing (Cavaletti et al., 2001; Di Cesare Mannelli et al., 2013).

There is increasing interest in adult mesenchymal stem cells (MSCs) for the treatment of different degenerative and ischemic pathologies as well as neuropathic pain. Adult MSCs are not only a pluripotent cellular source for replacing injured tissues but also represent a source of neuroprotective and anti-inflammatory mediators. MSCs are able to modulate the inflammatory cascade associated with immune-related diseases, such as rheumatoid arthritis (Gonzalez-Rey et al., 2010), but also painful neuropathies consequent to nerve trauma (Sacerdote et al., 2013; Watanabe et al., 2015), or to metabolic alterations (Shibata et al., 2008). MSCs can be isolated from fetal liver, umbilical cord blood, bone marrow and stromal vascular cell fraction of adipose tissue which is an abundant and easily accessible source. Adipose derived stem cells (ASCs) are characterized by a phenotypic profile and differentiating capability similar to bone marrow or embryonal derived MSCs (Kern et al., 2006; Baglioni et al., 2009). Several studies have demonstrated that ASCs are able to efficiently differentiate toward adipogenic- chondrogenic- neurogenic- osteogenic-like cells (Quirici et al., 2010; Choudhery et al., 2013), and to modulate biomolecular signals (Gonzalez-Rey et al., 2010; Sacerdote et al., 2013; Nasef et al., 2008; Yoo et al., 2009). In particular, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and the transforming growth factor-beta 1 (TGF-β1) belong to the stem cell secretome (Ribeiro et al., 2012; Salgado et al., 2010) and these growth factors have been related to pain modulation and neuroprotection (Kiguchi et al., 2014; Maiese, 2015; Wang et al., 2015).

The purpose of this study was to evaluate the effect of rat ASCs (rASCs) in a rat model of oxaliplatin-induced neuropathy by measuring their capability of relieving pain. We also evaluated the rASCs fate in the body and the concentration of typical stem cell-derived mediators as possible explanation of cell effectiveness.

2. Materials and methods

2.1. Animals

For all the experiments described below, male Sprague-Dawley rats were used. Rats weighting approximately 550–600 g were employed for rASCs isolation while for functional and behavioral experiments animals weighed 200–250 g. All animal manipulations were carried out according to the Directive 2010/63/EU of the European parliament and of the European Union council on the protection of animals used for scientific purposes. Experiments involving animals have been reported according to ARRIVE guidelines (McGrath and Lilley, 2015).

2.2. Cell preparation and characterization

Retrosternal, thighs and aortic fat pads from Wistar rats weighing approximately 550–600 g, were harvested and digested with collagenase type I. The sample was filtered through a 100 μm mesh filter to remove debris and at 700 × g for 10 min. The pellet was suspended and plated in Nutrient mixture F-12 Ham supplemented with 20% FBS and 1% penicillin-streptomycin and incubated at 37 °C in 5% CO_2 atmosphere. Immunophenotypical analysis of cultured cells was performed by using the FITC-, PE-, APC or eFluor 450- conjugated monoclonal antibodies (mAbs) against CD90, CD79a, CD29, CD45 respectively. In all experiments, only cells at P2 of culture were used. Detailed procedures used for rASCs, rat coronary endothelial cells (Failli et al., 2000) and rat aortic smooth muscle cells (Mazzetti et al., 2003) were described in the Supplementary material.

2.3. Oxaliplatin-induced neuropathic pain model

Neuropathic pain was induced by i.p. injection of 2.4 mg kg^{-1} oxaliplatin dissolved in a 5% glucose and administered i.p. for 4 consecutive days every week for 2 weeks (Cavaletti et al., 2001; Di Cesare Mannelli et al., 2013 with minor modifications). Control rats received an equal volume of 5% glucose. During treatments, general conditions of animals were evaluated. On day 13, body weight was decreased in comparison to control (264 ± 8 *vs* 301 ± 10), as well the number of red blood cells ($3.8 \times 10^6 \pm 0.6 \times 10^6$ *vs* $6.8 \times 10^6 \pm 1 \times 10^6$, $P < .05$).

2.4. rASCs administration

For i.v. administration, cells were suspended in 400 μL of DMEM without phenol red and with 500 I.U. heparin and injected into the tail vein in the amount of 2×10^6 (5×10^3 cells/μL) per rat; control rats received the same amount of vehicle or rASCs conditioned medium (i.p., see the next chapter for conditioned medium preparation).

Animals were randomly divided into the following experimental groups of 10–12 rats each, receiving according to Fig. 1:

- control + DMEM: control (oxaliplatin vehicle i.e. 5% glucose), DMEM (rASCs medium);
- oxaliplatin + DMEM: oxaliplatin, DMEM at day 13 (single administration) or DMEM at days 6, 13, 17 and 20 (repeated administrations).
- control + rASCs: control (oxaliplatin vehicle), 2×10^6 rASCs at day 13 (single administration),
- oxaliplatin + rASCs: oxaliplatin, 2×10^6 rASCs at day 13 (single administration) and 2×10^6 rASCs at day 6, 13, 17 and 20 (repeated administrations); oxaliplatin + conditioned medium: oxaliplatin, conditioned medium (3.5 mL i.p., see below) at day 13 (single administration)
- control + labeled rASCs: control (oxaliplatin vehicle), 2×10^6 labeled rASCs at day 13 (single labeled administration)
- oxaliplatin + labeled rASCs: oxaliplatin, 2×10^6 labeled rASCs at day 13 (single labeled administration).

In a separated experiment rASCs (75×10^3/15 μL; 5×10^3 cells/μL in saline) were intrathecally infused in control and oxaliplatin-treated rats (on day 13 after the beginning of oxaliplatin treatment).

rASCs conditioned medium treatment and rASCs labeling were performed as described in the Supplementary material.

2.5. $VEGF_{165}b$, bevacizumab and anti-rat-$VEGF_{165}b$ antibody administration

Human recombinant $VEGF_{165}b$ in physiological solution was administered intraplantarly in the right paw. Bevacizumab (saline) was injected i.p. in control and oxaliplatin treated groups (on day 13 after the beginning of oxaliplatin treatment). In separated experiments $VEGF_{165}b$, bevacizumab and an anti-rat-$VEGF_{165}b$ antibody were injected i.t. according to Mestre et al. (1994) (more information in the Supplementary material).

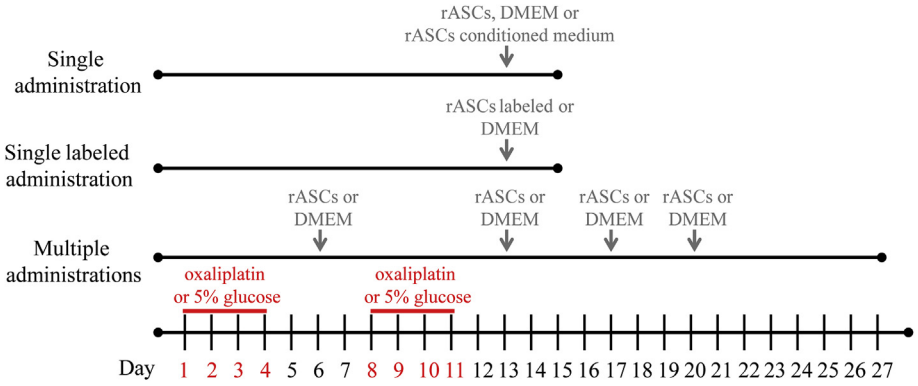

Fig. 1. Rat ASCs administrations in a neuropathy model induced by oxaliplatin. Representative scheme of single (on day 13), single labeled (on day 13) and repeated (on day 6, 13, 17 and 20) rASCs or DMEM administrations in rats repeatedly treated with oxaliplatin. rASCs (2×10^6) were injected into the tail vein in 400 μL of DMEM containing 500 I.U. heparin. Oxaliplatin (2.4 mg kg^{-1}) was administered intraperitoneally (i.p.) for 4 consecutive days every week for 2 weeks. Control rats received 5% glucose.

2.6. Paw-pressure, Von Frey and incapacitance tests

The hypersensitivity to a noxious mechanical stimulus of rats was determined with an analgesimeter, according to the method described by Leighton et al. (1998). An electronic Von Frey hair unit was used to evaluate the response to non noxious mechanical stimulus accordingly with Di Cesare Mannelli et al. (2013) and Sakurai et al. (2009). Weight bearing changes were measured using an incapacitance apparatus detecting changes in postural equilibrium after a hind limb injury (Bove et al., 2003; Di Cesare Mannelli et al., 2014). Data were expressed as the difference between the weight applied on the limb contralateral to the injury and the weight applied on the ipsilateral one (Δ Weight).

2.7. Ex vivo analyses

Blood and organ were collected as described in the Supplemental Experimental Procedures. Cytokines, growth factors, pain related peptides were measured into plasma samples of groups using quantitative Milliplex Luminex. Data were collected and analyzed using a BioPlex 200 instrument equipped with Bio-Manager analysis software. Nerve growth factor (NGF) was measured by ELISA kit. Western blot analysis were performed on DRGs, lumbar spinal cord, PAG, thalamus, and primary somatosensory (S1) cortex or in samples obtained from cell cultures of rASCs or rat coronary endothelial cells (Failli et al., 2000). Ponceau stained membranes were used as loading control for plasma sample analysis. α-tubulin or GAPDH normalization was performed for nervous tissue samples, rASCs and rat coronary endothelial cells. Values were reported as percentages of controls arbitrarily fixed at 100%.

2.8. Statistical analysis

Results were expressed as means ± S.E.M. and the analysis of variance was performed by ANOVA test. A Bonferroni's significant difference procedure was used as a post hoc comparison. P values less than 0.05 were considered significant. Data were analyzed using the "Origin 8.1" software.

3. Results

3.1. Cell cytocharacterization

Different sources of rASCs were evaluated by processing rat thighs, retrosternal and aortic fat pads. The stemness of isolated cells was assessed in terms of percentage of $CD29^-$, CD90-positive cells, as markers for mesenchymal stem cells, and $CD45^-$, CD79α-positive cells, as markers for hematopoietic stem cells. All cell lines obtained from different anatomical fat pads exhibited the typical phenotype of mesenchymal stem cells ($CD90^+$ $CD29^+$ $CD45^-$ $CD79\alpha^-$) (Supplementary material, Table S1). The retrosternal fat pad held a higher percentage of $CD90^-$and CD29-positive cells (83.3% and 92.5%, respectively) than thighs (86.7% and 87.6% respectively) and aortic fat pads (59.9% and 88.7% respectively). The expression of CD45 and CD79α was lower in retrosternal fat-derived cells (7%) than in thighs and aortic fat-derived cells (12% and 11% respectively) (Supplementary material, Table S1). Based on these results retrosternal-derived rASCs were chosen to perform all experiments. Several cell lines at different serial passages (from P1 to P4) and after thawing and culture until confluence were cyto-characterized by flow cytometry (Supplementary material, Table S2). Rat ASCs at P2 showed the better stemness phenotype and cell cryopreservation did not change it. On these bases, retrosternal fat pad rASCs at P2 were used for *in vivo* experiments. Supplemental information reported in Fig. S1 shows the surface positive CD90/CD29 and negative CD79α/CD45 markers as indexes of mesenchymal phenotype of a representative rASCs preparation (P2). There were 91% CD90 positive cells which also expressed CD29 (Supplementary information, Fig. S1, panel A) and 99% of the CD79α and CD45 negative cells (Supplementary material, Fig. S1, panel B). In order to highlight mesenchymal characteristics, rASCs were cultured *in vitro* and differentiated under specific conditions to mesenchymal cell lineages such adipocytes and osteocytes (Supplementary material, Fig. S1, panel C and D).

3.2. Neuropathic pain measurements

Neuropathic pain was induced in rats by repeated administrations of oxaliplatin (Fig. 1). On day 13, the response of oxaliplatin-treated rats to a noxious mechanical stimulus (Paw pressure test) was altered and the weight tolerated on the posterior paw significantly decreased from the control value of 64.1 ± 1.6 g (control + DMEM) to 43.4 ± 0.6 g for oxaliplatin-treated rats (oxaliplatin + DMEM) (Fig. 2). On the same day, 2×10^6 rASCs were injected into the caudal vein of control + rASCs and oxaliplatin + rASCs groups. Rat ASCs induced a significant reduction of oxaliplatin-induced hypersensitivity 1 h after injection which peaked at 6 h (Fig. 2). The effect of rASCs lasted at least 48 h. In control rats the caudal vein rASCs administration (2×10^6) did not alter pain sensitivity. Moreover, the administration of rASCs conditioned medium was ineffective in reducing pain in

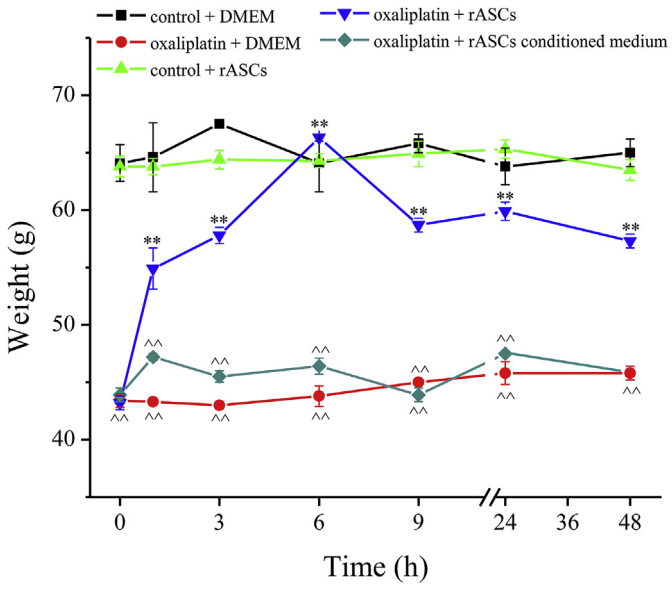

Fig. 2. Effect of rASCs administration on pain threshold due to noxious stimulus in oxaliplatin-induced neuropathic rats. Oxaliplatin (2.4 mg kg^{-1} i.p.) was administered for 4 consecutive days on weeks 1 and 2. On day 13, 2×10^6 rASCs (suspended in 400 μL of DMEM containing 500 U.I. heparin), were injected into the tail vein of the control + rASCs and oxaliplatin + rASCs groups. Other groups received DMEM (i.v.) or rASCs conditioned medium (i.p.) as indicated. For more details, see methods. The pain threshold was evaluated through the paw pressure test (measuring the weight tolerated on the paw) at different time (1–48 h). Each value represents the mean ± SEM of 10 rats per group, performed in 2 different experimental sets. ^^P < .01 vs control + DMEM; **P < .01 vs oxaliplatin + DMEM.

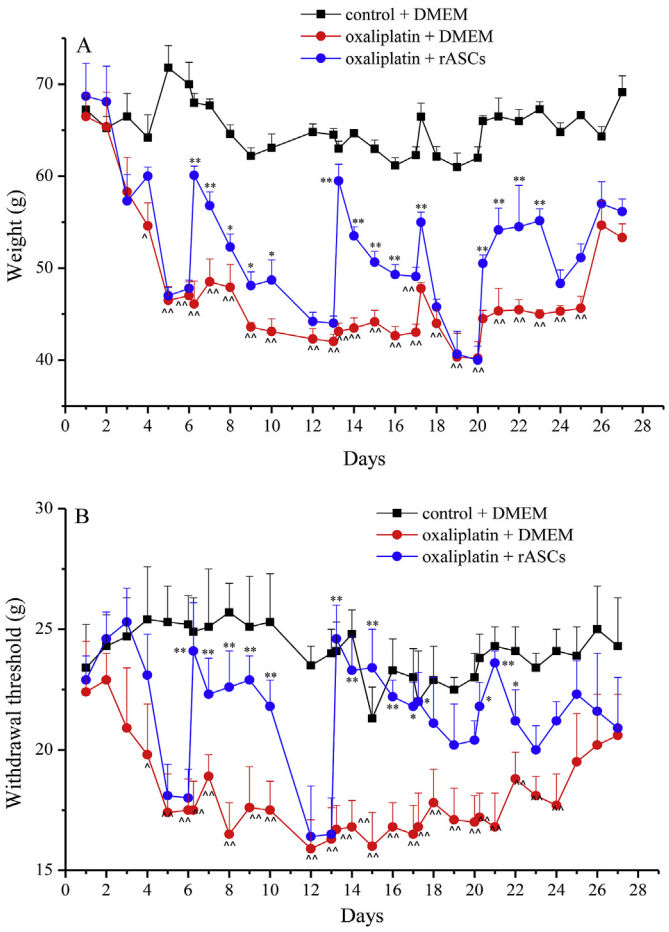

Fig. 3. The effect of repeated rASCs administration on the pain threshold of noxious and non-noxious stimuli in oxaliplatin-induced neuropathic rats. A) Paw pressure and B) Von Frey tests. Oxaliplatin (2.4 mg kg^{-1} i.p.) was administered for 4 consecutive days on weeks 1 and 2. On day 6, 13, 17 and 20, 2×10^6 rASCs (suspended in 400 μL of DMEM containing 500 U.I. heparin) were injected into the tail vein. Each value represents the mean ± SEM of 12 rats per group, performed in 2 different experimental sets. Values obtained in oxaliplatin + DMEM treated rats were significantly reduced as compared to those of control + DMEM animals starting from the 4th day until the 27th day (A, Paw pressure) and until the 21st (B, Von Frey). The significance in the oxaliplatin + rASCs group is shown with stars. *P < .05 and **P < .01 vs oxaliplatin + DMEM.

oxaliplatin-treated rats, suggesting that stem cells are necessary to exert the antineuropathic effect.

In order to better understand the effect of rASCs on neuropathic pain repeated administrations of rASCs were performed (scheme in Fig. 1). As shown in Fig. 3, rASCs determined a significant, comparable reduction of hypersensitivity induced by mechanical noxious (Paw pressure test; Fig. 3A) and non-noxious (Von Frey test; Fig. 3B) stimuli each time that cells were administered. This analgesic effect lasted for 5 days after injection. To validate the specificity of the stem cellular therapy, 2×10^6 rat aortic smooth muscle cells were i.v. infused in oxaliplatin-treated rats. No effects were revealed (data not shown).

3.3. Fate of rASCs and cytokines and growth factors measurement

In order to track the injected rASCs, CMTMR-labeled cells were identified by flow cytometry analysis in the blood and organs of oxaliplatin-treated (oxaliplatin + labeled rASCs) or control rats (control + labeled rASCs). CMTMR-labeled rASCs were detectable 1 and 3 h after cell administration in the blood of both groups, whereas 6 h later labeled cells were at the lowest limit of flow cytometry sensitivity (Supplementary material Table S3). After 24 h, only a small percentage of cells were detectable in the liver of both groups (0.02 ± 0.007%), whereas no CMTMR-labeled rASCs were revealed in the lung, DRG or spinal cord. Mechanical hypersensitivity (Paw pressure test) of animals receiving labeled rASCs was very similar to that of rats receiving unlabeled cells.

To evaluate the biomolecular response evoked by rASCs, plasmatic concentrations of IL-1α, IL-6, IP-10, TNF-α, pan VEGF-A, EGF, α-MSH, β-Endorphin, Substance P, TGF-β1 and NGF were assayed. As shown in Fig. 4A, pan VEGF-A significantly increased in oxaliplatin-treated rats compared to control animals. Twenty four h after rASCs administration to oxaliplatin-treated rats, VEGF concentrations significantly decreased. The administration of rASCs to control animals did not influence VEGF concentrations (Fig. 4A). TGF-β1 and EGF concentrations were not found to be altered in any group when compared to the control one (data not shown). No other analyzed peptides were detectable in the plasma.

3.4. VEGF-R1 expression in rASCs

The study of the relevance of VEGF in rASCs activity begun by the evaluation of the protein expression of the VEGF specific receptor VEGF-R1. As shown in Fig. 4B, rASCs were characterized by the expression of VEGF-R1 in a quantity comparable to that detected in rat coronary endothelial cells.

3.5. Bevacizumab effects on oxaliplatin-treated rats

In the described rat model of oxaliplatin-induced neuropathy, increasing i.p. doses of bevacizumab (1–15 mg kg^{-1}), reduced mechanical hypersensitivity in a dose dependent manner starting 15 min after administration (Fig. 5). The effect of bevacizumab

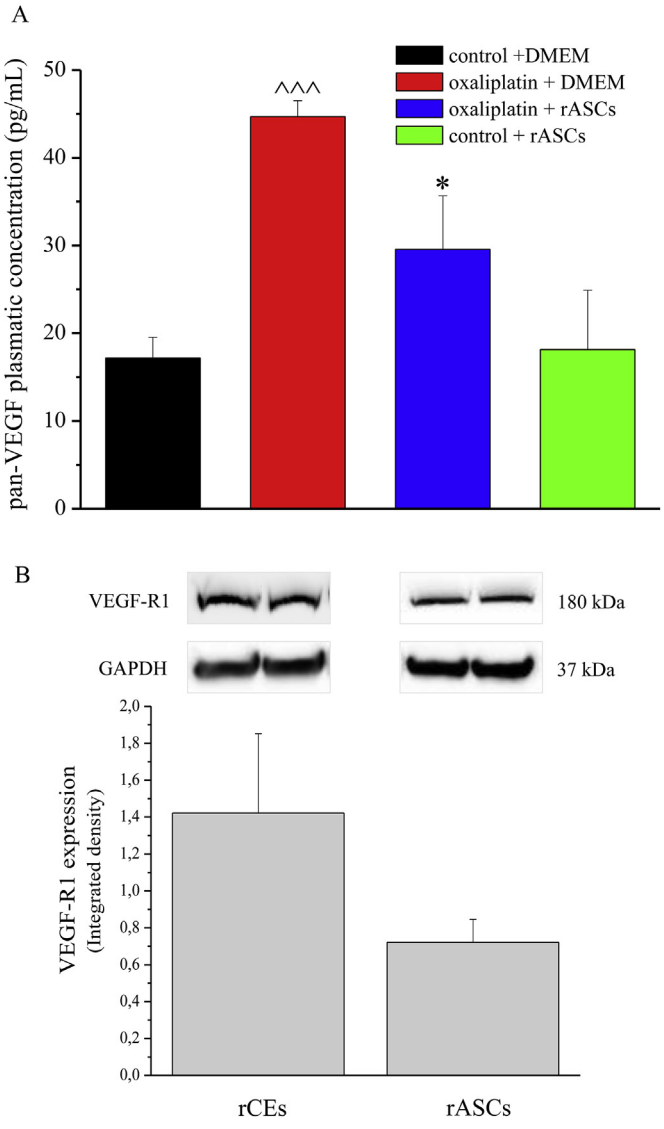

Fig. 4. A) Pan VEGF-A plasma concentration. Oxaliplatin (2.4 mg kg^{-1} i.p.) was administered for 4 consecutive days on weeks 1 and 2. On day 13, 2×10^6 rASCs (suspended in 400 μL of DMEM containing 500 I.U. heparin) were injected into the tail vein of rats of the control + rASCs and oxaliplatin + rASCs groups. Before, and at 3, 6, 9, 24 and 48 h after i.v. cell administration, the blood was collected. VEGF was measured in plasma by Bioplex kits. Each value represents the mean ± SEM of 4 rats per group, performed in 2 different experimental sets. °P < .05, °°P < .01 and °°°P < .001 vs control + DMEM at the same time. B) VEGF-R1 expression in rASCs. Protein fractions (40 μg) from rat endothelial (rCEs) or adipose stem cells (rASCs) were analyzed in duplicate by western blot in order to evaluate VEGF-R1 level. Representative blot are shown, as well as their respective densitometric analysis. GAPDH normalization was performed. Each value represents the mean of 4 preparations, performed in two different experimental sets.

(15 mg kg^{-1}) peaked 2 h after treatment and it was still significant at 3 h. Lower doses of bevacizumab showed a similar time-course with a lower efficacy, peaking 1 h after administration and lasting up to 3 h. Comparable results were obtained with a non noxious mechanical stimulus (Von Frey test; Fig. 5B), all dosages showing effectiveness in a time span of between 30 min and 3 h (Fig. 5B). In control animals, bevacizumab (15 mg kg^{-1}, i.p.) did not alter the pain threshold (Fig. 5, panels A and B).

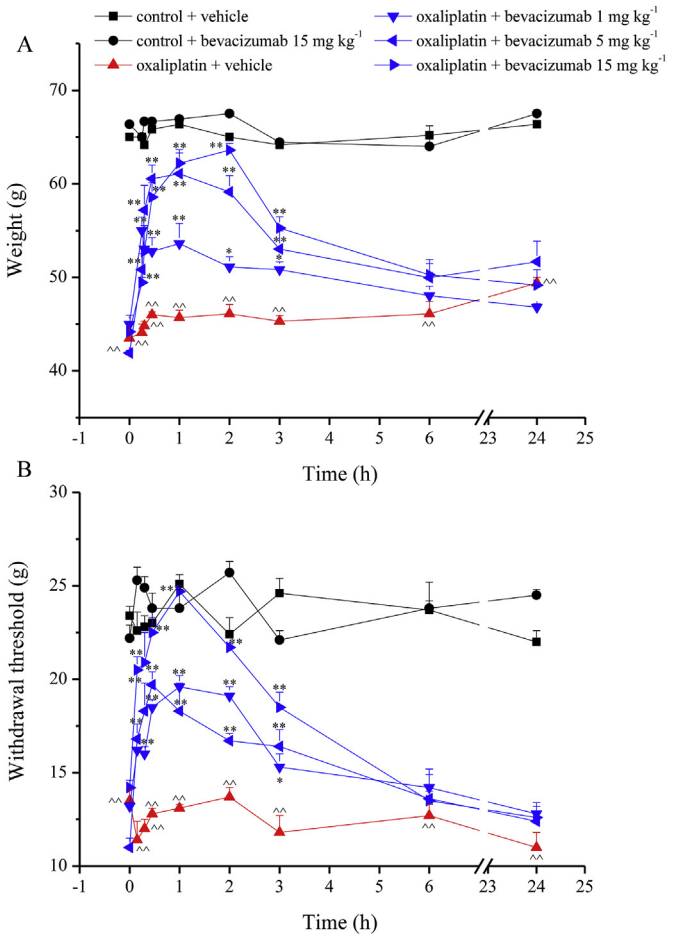

Fig. 5. Effect of bevacizumab against oxaliplatin-induced hypersensitivity. Oxaliplatin (2.4 mg kg^{-1} i.p.) was administered for 4 consecutive days on weeks 1 and 2. On day 13, bevacizumab (1, 5 and 15 mg kg^{-1}) was injected i.p. in control and oxaliplatin treated groups. Other groups received saline. A) The response to mechanical noxious stimulus was evaluated by the paw pressure test over time (0 min–24 h). B) The response to mechanical non noxious stimulus was evaluated by the Von Frey test over time (0 min–24 h) Each value represents the mean ± SEM of 10 rats per group, performed in 2 different experimental sets. °°P < .01 vs control + vehicle, **P < .01 vs oxaliplatin + vehicle.

3.6. *Intraplantar recombinant $VEGF_{165}b$ effects on normal pain threshold*

Aimed to evaluate the pro-nociceptive potential of VEGF, we injected $VEGF_{165}b$, a splice variant of VEGF-A involved in pain modulation (Hulse et al., 2014), into the right paw of naïve rats. Fig. 6A shows the decrease of the pain threshold evaluated by the stimulation with a mechanical insult (Paw pressure test). Thirty min after the intraplantar administration, recombinant $VEGF_{165}b$ (10, 30 or 100 ng) dose-dependently reduced the weight tolerated on the paw. The maximal pain perception was reached after 3 h, 24 h thereafter it returned to a level comparable to control value. Similarly, $VEGF_{165}b$ evoked spontaneous pain as measured by hind limb weight bearing alterations (Incapacitance test; Δ weight is the difference between the weight burdened on the contralateral and the ipsilateral paw, Fig. 6B). Bevacizumab (15 mg kg^{-1} i.p.) fully prevented the algesic effect and significantly reduced the achiness induced by $VEGF_{165}b$ (Fig. 6A and B).

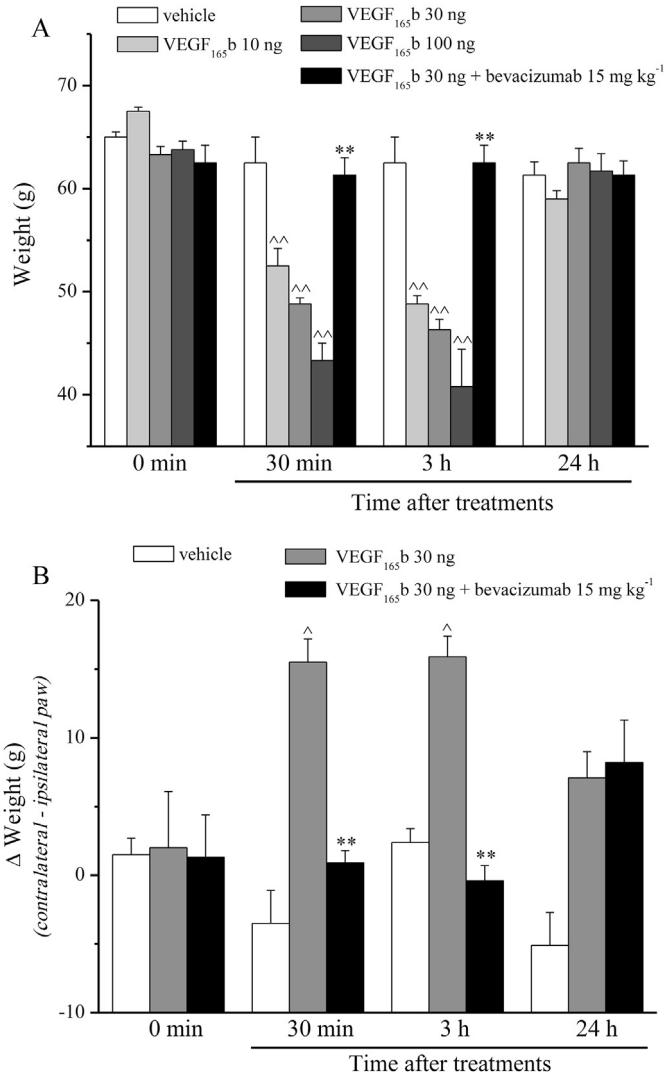

Fig. 6. Effect of recombinant $VEGF_{165}b$ on pain threshold. Intraplantar injection. Naïve rats were treated with 10, 30 or 100 ng of $VEGF_{165}b$ in comparison to vehicle (saline). A final volume of 50 μl was administered by intraplantar injection in the right paw. Bevacizumab (15 mg kg^{-1} i.p.) was administered 15 min before $VEGF_{165}b$. A) The response to mechanical noxious stimulus was evaluated by the paw pressure test over time (30 min–24 h). B) Spontaneous pain was assessed by hind limb weight bearing alterations using an Incapacitance test that measures the postural imbalance related to pain; data are expressed as the difference between the weight applied on the contralateral and ipsilateral limbs to the intraplantar injection (Δ Weight). Each value represents the mean ± SEM of 10 rats per group, performed in 2 different experimental sets. ^P < .05 and ^^P < .01 *vs* the same group before treatment (0 min); **P < .01 *vs* 30 ng $VEGF_{165}b$ in the absence of bevacizumab.

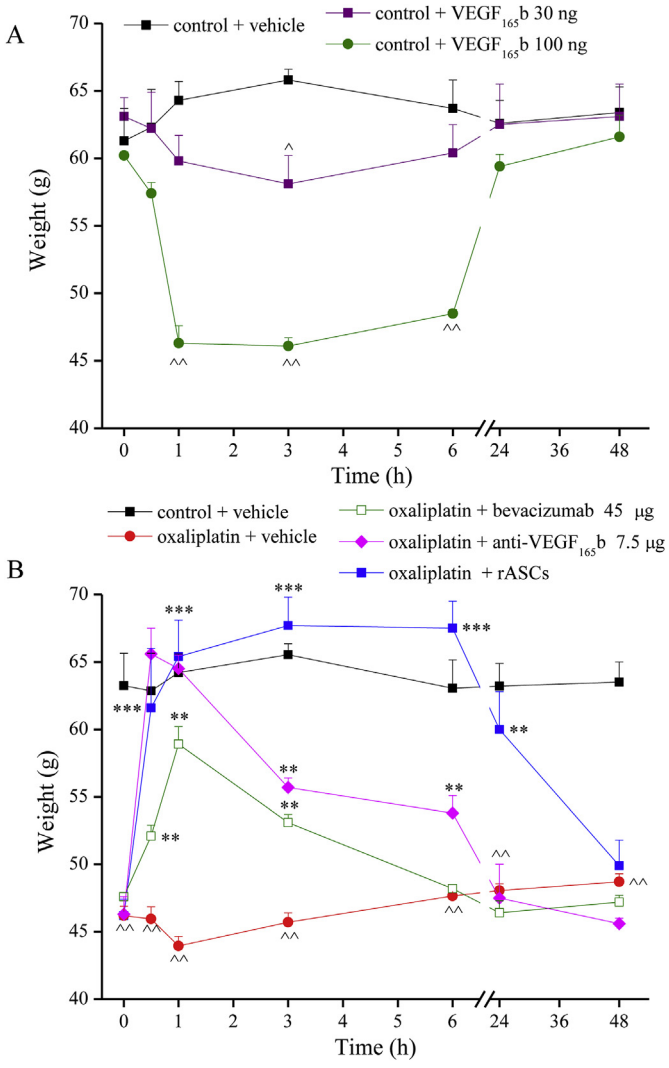

Fig. 7. Spinal modulation of pain: effects of $VEGF_{165}b$, VEGF-A antibodies and rASCs. Intrathecal injection. A) Naïve rats were treated with 30 or 100 ng of $VEGF_{165}b$ in comparison to vehicle (saline). B) Effect of bevacizumab (45 μg) and a rat specific antibody against $VEGF_{165}b$ (7.5 μg) in comparison to the effect of 75×10^3 rASCs after intrathecal injection in control or oxaliplatin-treated rats (day 13). The response to a mechanical noxious stimulus was evaluated by the paw pressure test over time (1–48 h). Each value represents the mean ± SEM of 10 rats per group, performed in 2 different experimental sets. ^P < .05 and ^^P < .01 vs control + vehicle, **P < .01 vs oxaliplatin + vehicle.

3.7. Spinal modulation of pain: effects of $VEGF_{165}b$, VEGF-A antibodies and rASCs

The central component of the VEGF role in pain signaling was evaluated injecting $VEGF_{165}b$ intrathecally (i.t., 30 and 100 ng). Fig. 7A shows the dose-dependent decrease of pain threshold (Paw pressure test) 1–6 h after treatment. The relevance of central VEGF was confirmed by the efficacy of bevacizumab i.t. (45 μg) in reducing oxaliplatin-dependent hypersensitivity (Fig. 7B). Moreover, higher efficacy was obtained injecting i.t. 7.5 μg of the specific antibody against $VEGF_{165}b$ (anti-rat-$VEGF_{165}b$ antibody). The effect peaked between 30 min–1 h after injection and was lost at 24 h (Fig. 7B). To extend the study of the central regulation of oxaliplatin-dependent pain, 75×10^3 rASCs were intrathecally injected in oxaliplatin-treated rats. The onset was similar to that shown by both VEGF antibodies but the effect was more long lasting since the pain threshold was yet significantly enhanced 24 h after treatment (Fig. 7B). Control animals treated i.t. with bevacizumab, anti-$VEGF_{165}b$ or rASCs showed a pain threshold similar to controls treated with vehicle (data not shown).

3.8. Evaluation of $VEGF_{165}b$ isoform in plasma and peripheral and central nervous system

$VEGF_{165}b$ was evaluated in plasma and in the peripheral and central nervous system of control + DMEM, oxaliplatin + DMEM and oxaliplatin + rASCs groups by western blot analysis (Fig. 8).

In the plasma of the oxaliplatin + DMEM rat group, $VEGF_{165}b$ protein levels were not modified as compared to control (control + DMEM), whereas in oxaliplatin + rASCs they were decreased by 63% as compared to control + DMEM and

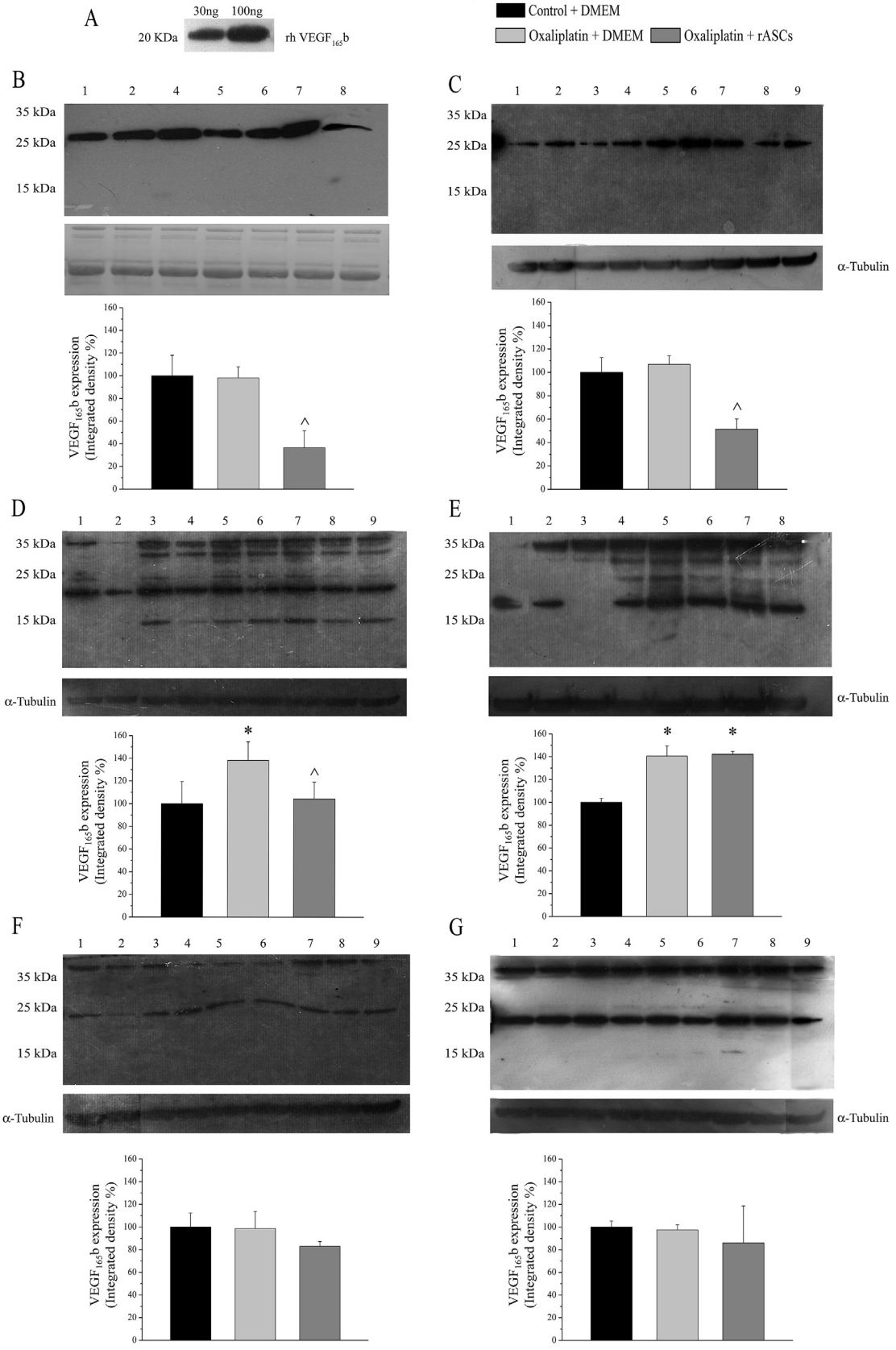

Fig. 8. $VEGF_{165}b$ expression in plasma and nervous tissue homogenates. A) Two different concentrations (30 and 100 ng) of recombinant human (rh) $VEGF_{165}b$ protein were probed with the anti-$VEGF_{165}b$ antibody. B-F) Oxaliplatin (2.4 mg kg^{-1} i.p.) was administered for 4 consecutive days on weeks 1 and 2. On day 13, 2×10^6 rASCs (suspended in 400 μL of DMEM containing 500 I.U. heparin) were injected into the tail vein of rats of the oxaliplatin + rASCs group. Twenty-four hours after i.v. administration the rats were sacrificed, tissue explanted and blood collected in order to evaluate $VEGF_{165}b$ level. B) Plasma, C) DRG, D) spinal cord, E) PAG, F) thalamus and G) S1 cortex representative Western blot are shown, as well as their respective densitometric analysis. Ponceau stained membranes were used as loading control for plasma quantification. α-tubulin normalization was performed on nervous tissue samples. In the panels, 1, 2 and 3 represent the three repetitions of control + DMEM group; 4, 5 and 6 represent the three repetitions of oxaliplatin + DMEM group; 7, 8 and 9 represent the three repetitions of oxaliplatin + rASCs group. Each value represents the mean of 10 rats per group, performed in two different experimental sets. *P < .05 versus oxaliplatin + DMEM; ^P < .05 versus oxaliplatin + rASCs.

oxaliplatin + DMEM (Fig. 8B). In the DRG, a similar trend of $VEGF_{165}b$ level was measured (Fig. 8C). In the spinal cord, $VEGF_{165}b$ was increased in oxaliplatin + DMEM rats by 43% whereas administration of rASCs restored control values (Fig. 8D). In the PAG oxaliplatin increased $VEGF_{165}b$ (about 40%) and rASC administration was ineffective (Fig. 8E). In the thalamus and cortex, $VEGF_{165}b$ was not ever altered by oxaliplatin or rASC treatments (Fig. 8, panels F and G).

To confirm antibody specificity, the anti-$VEGF_{165}b$ antibody was used to probe a blot of an SDS–polyacrylamide gel loaded with increasing quantities of recombinant human $VEGF_{165}b$. Fig. 8A shows that the antibody concentration-dependently recognized $VEGF_{165}b$. The $VEGF_{165}b$-specific antibody detected a protein with a molecular weight of about 23 kDa which was consistent with the monomeric form of the growth factor. In plasma and DRG this band was the only one detected; in other tissues it was prominent but the antibody revealed other bands coherent with the presence of $VEGF_{165}b$ homodimer (about 40 kDa) or other VEGF-A sister isoforms (Woolard et al., 2004; Ergorul et al., 2008; Manetti et al., 2011).

4. Discussion

The present data describe the pain relieving efficacy of rASCs in a rat model of oxaliplatin-induced neuropathy. The ability to modulate the VEGF is suggested as a possible mechanism of rASC-mediated pain control.

Adipose-derived stem cells are a class of mesenchymal stem cells isolated from the stromal vascular cell fraction of the adipose tissue. In ischemic, neurodegenerative and traumatic pain, their beneficial effect seems to be dependent on their modulatory and stimulating properties of mechanisms involved in tissue repair (Chan et al., 2014). In several anatomical localized neuropathic pain models (chronic constriction injury (Franchi et al., 2012); spared nerve injury (Siniscalco et al., 2011; spinal cord injury (Watanabe et al., 2015)), the administration of stem cells by i.v. or spinal injection can reduce pain sensitivities with different time-courses and intensities. However, to our knowledge this is the first time that i.v. rASCs have been reported to reduce chemotherapy-induced neuropathic pain.

As already described (Cavaletti et al., 2001; Di Cesare Mannelli et al., 2013), repeated oxaliplatin administration evokes mechanical hypersensitivity (hyperalgesia- and allodynia-like measurements) starting from the 4th day of treatment. Rat ASCs administration significantly reduces the pain threshold alteration starting after 1 h and lasting up to 4–5 days after injection. Repeated cell administrations determine the effect of pain relief with the same time-course and intensity as the first one. Even if rASCs are detected in the bloodstream from 1 to 3 h and in the liver 24 h after cell injection, their effect can last for several days, suggesting that rASCs could activate long-lasting antihyperalgesic mechanisms.

Mesenchymal stem cells can be primed to express a “medicinal phenotype” (Caplan and Correa, 2011) following a lesion, and secrete bioactive molecules that establish a microenvironment sustaining a regeneration of injured tissues. Secreted bioactive molecules can stimulate angiogenesis (Sorrell et al., 2009), inhibit apoptosis and activate progenitor tissue-intrinsic stem cells to divide and differentiate appropriately (Rehman et al., 2004; Wagner et al., 2009). Evidence indicates that the modulation of pro- and anti-inflammatory cytokines and growth factors is an important way of explaining a possible mechanism of action of stem cells (Gonzalez-Rey et al., 2010; Sacerdote et al., 2013; Franchi et al., 2012; Siniscalco et al., 2011).

The implication of VEGF in nociception has been recognized in several forms of pain even if conflicting data in literature suggest both pro- and anti-nociceptive effects for this growth factor. Verheyen and colleagues (Verheyen et al., 2012) demonstrate that neutralization of all endogenous VEGF isoforms, or VEGF receptor antagonism increase pain sensitivity in paclitaxel-induced neuropathy. Conversely, in chronic constriction injury rats anti-VEGF receptor treatment alleviates neuropathic pain by decreasing the expression of VEGF and purinergic P2X2/3 receptors in DRG neurons (Lin et al., 2010); the VEGF increase in synovial fluid is correlated with the degree of osteoarthritis pain (Hamilton et al., 2016). Moreover, the plantar injection of VEGF-A dose-dependently increases hypersensitivity to Von Frey mechanical and noxious heat stimuli applied to the ipsilateral paw (Selvaraj et al., 2015). The effect of VEGF-A is mediated by the activation of VEGF type 1 receptor (VEGFR1) as demonstrated using VEGFR1 antagonist, VEGFR1 signal transduction blockers and specific DRG conditionally VEGFR1 lacking mice. Furthermore, VEGFR1 is involved in tumor-associated pain in mice models of cancer-associated pain (i.e. osteolytic sarcoma, femur implanted breast carcinoma, lung carcinoma, pancreatic carcinoma) (Selvaraj et al., 2015). VEGF-A is extensively alternatively spliced into two families of splice variants (Woolard et al., 2004) and an opposite effect of $VEGF_{165}a$ (pro-nociceptive) and $VEGF_{165}b$ (anti-nociceptive) has been noticed in rats with saphenous nerve injury (Hulse et al., 2014) where $VEGF_{165}a$ is overexpressed. $VEGF_{165}b$ exerts anti-nociceptive and protective effects in steptozotocin-induced diabetic rats (Hulse et al., 2015). On the other hand, both splice variants ($VEGF_{165}a$ and $VEGF_{165}b$) can prevent cisplatin induced sensory neurodegeneration and cell death (Vencappa et al., 2015). Also the transplantation of neural stem cells expressing VEGF cells in a rat sciatic nerve injury model enhances functional recovery and remyelination and induces pain reduction (Lee et al., 2015).

In the present work, the plasmatic concentration of pan VEGF-A undergoes a significant increase in oxaliplatin-treated rats that could be justified by hypoxic condition (Schoch et al., 2002) here shown as a significant decrease of red blood cells induced by the repeated treatment with the drug (see Methods). Rat ASCs reduce pan VEGF-A plasmatic concentration. Supporting the hypothesis that VEGF increase plays a relevant role in the development of neuropathic pain, our data show that the systemic administration of the anti-VEGF antibody bevacizumab decreases oxaliplatin-dependent pain sensitivity. $VEGF_{165}b$ protein expression is unchanged in the plasma and DRG of oxaliplatin-treated rats and administration of rASCs strongly reduces it. A different and specific pattern is observed in peripheral and central nerve system areas. $VEGF_{165}b$ increases in the spinal cord and PAG of neuropathic rats. The treatment with rASCs reverts this $VEGF_{165}b$ enhancement in the spinal cord and decreases $VEGF_{165}b$ level under control values. The relevance of $VEGF_{165}b$ in pain perception is confirmed in naïve rats since the intraplantar administration of the recombinant protein induces a dose-dependent, long lasting hypersensitivity to noxious stimuli as well as spontaneous pain. The pro-nociceptive effects of $VEGF_{165}b$ are prevented by systemic bevacizumab. Interestingly, the VEGF-A role in pain possesses also a central component since the i.t. administration of $VEGF_{165}b$ evokes a long lasting hypersensitivity, confirmed by the efficacy in reducing oxaliplatin-dependent pain-threshold alteration after i.t. injection of both bevacizumab (anti-human-panVEGF-A antibody) and the specific anti-rat-$VEGF_{165}b$ antibody. Interestingly, the i.t. injection of rASCs evokes a complete reversion of oxaliplatin hypersensitivity for 24 h. These results allow us to hypothesize a pharmacodynamic relevant role of VEGF-A in oxaliplatin-induced neuropathy and in rASCs efficacy. Since VEGF-R1 is well expressed in rASCs, the elevated concentration of VEGF-A in plasma of oxaliplatin-treated rats could sensitize rASCs which can influence the environment

and execute their beneficial role after stimulation.

On the other hand, a quick regulation of VEGF concentrations is physiologically envisaged. For instance, VEGF-A signaling can be inhibited by an endogenously produced soluble VEGF receptor 1 (sFlt1) which lacks the transmembrane portion and the receptor tyrosine-kinase domain but binds to VEGF-A (Shibuya, 2013; Ferrara et al., 2003; Keyt et al., 1996). Local administration of sFlt1 blocks VEGF-A-induced hyperalgesia and decreases tumor-induced hyperalgesia (Selvaraj et al., 2015). Moreover, VEGF can be degraded in endothelial cells through the internalization of the VEGF-VEGF receptor complex and through alternate pathways such as via low density lipoprotein receptor-related protein-1 in conjunction with thrombospondin-1 (Greenaway et al., 2007). In chronic wounds and some tumors, VEGF loss appears to be related to proteolytic degradation (Lauer et al., 2000, 2002; Mineur et al., 2007) initially dependent on plasmin cleavage (Lauer et al., 2000; Lee et al., 2005). Adipose SCs promote the utilization of the plasminogen activator-plasmin axis by endothelial cells (Kachgal and Putnam, 2011). VEGF degradation could also explain the rapid VEGF decrease measured in our experimental conditions in relation to the decreased pain sensitivity induced by rASCs. The regulatory role of VEGF-A in oxaliplatin-induced pain and rASC relieving mechanism deserves further studies.

Other growth factors, such as EGF and TGF-β, are not modified in plasma of oxaliplatin-treated rats or after rASCs administration, while several cytokines and pain-related peptides are not detectable. It is important to note that chemokines are locally produced by damaged, inflamed areas and can induce stem cells migration and activation at the site of injury (Caplan and Dennis, 2006). Oxaliplatin-induced neuropathy is a widespread model of lesions and alterations affecting the peripheral and central nervous system, with low inflammatory components, whereas other models of neuropathic pain are characterized by severely damaged restricted areas (Di Cesare Mannelli et al., 2013). Therefore, it is not surprising that in our model, only few cytokines are involved. Further studies will clarify these biochemical features.

In conclusion, our data demonstrate for the first time that rASCs administration rapidly and long-lastingly decreases pain due to oxaliplatin neurotoxicity and that rASCs pain relieving efficacy involves the modulation of VEGF-A. Moreover, we prove that an increase in VEGF-A is involved in oxaliplatin-induced neuropathic pain that could be mitigated by VEGF-A antibodies. These experimental results open a new therapeutic horizon for the use of mesenchymal stem cells in the treatment of neuropathic pain.

Author contributions

All listed authors have contributed substantially to the research or manuscript preparation. In particular, FC, BT and AV performer cell isolation, characterization and culture; LDCM, LM and MZ carried out the *in vivo* experiments. BT, AL and AMC performed molecular measurements about protein expression. LDCM, PF and CG conceived the study, planned its design, and drafted the paper. All authors read and approved the final paper.

Conflicts of interest

The authors declare no conflict of interest. The work described has not been submitted elsewhere for publication, in whole or in part.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuropharm.2017.12.020.

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