SOCIETÀ ITALIANA DI FARMACOLOGIA

MODELLO PER INVIO RELAZIONE DI METÀ E FINE PERIODO

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TIPOLOGIA DI BORSA RICEVUTA: Borsa SIF-Fondazione Ferro

TIPOLOGIA DI RELAZIONE: metà periodo

TITOLO DELLA RELAZIONE: *Adipose stem cells for neuropathic pain relief: study of mechanisms and safe applications of secretome*

RELAZIONE:

Background

Regenerative medicine based on stem cells (SCs) is a powerful therapeutic intervention potentially suitable for ameliorate several pathological states, including ischemic, traumatic and degenerative diseases. Unfortunately, although many studies utilize SCs for regenerative medicine, the rarity and fragility of autologous progenitor cell populations from adult tissue and poor integration upon transplantation into host limit the widespread use of SC therapies in the clinic. Moreover, the optimum dose, source, and route of administration of SCs are outstanding questions which remain to be addressed. In many experiments, the common route of SC administration is intramuscular/intra-arterial infusion, but other delivery methods may reduce the number of cells and concentrate locally the SC therapeutic promise.

SCs can be differentiated into several cell phenotypes to regenerate damaged tissues (Chen et al., 2012, Biotechnol Adv 30:658–672). Nevertheless, in recent years, it is becoming increasingly accepted that the regenerative effects promoted by SCs and in particular for the mesenchymal population, are mainly associated with the secretion of bioactive molecules (secretome; Meyerrose et al., 2010 Adv Drug Deliv Rev 62:1167–1174). The undifferentiated SCs secrete cytokines and growth factors which promote angiogenesis, and are immunosuppressive and restorative (Gir et al., 2012 Plast Reconstr Surg 129:1277–1290). Therefore,

the maintenance of SC multipotency and the consequent secreting ability could be preferable to the SC differentiation vs specific tissues.

One of the obstacle in the clinical use of SCs lies within the ability to recreate the microenvironment in which SCs naturally proliferate in the undifferentiated state maintaining their regenerative potential (Muscari et al., 2013 J Biomed Sci 20:63-76). Adult tissue SCs generally reside within specialized microenvironments, known as SC niches, where specific local conditions play a part in maintaining SCs in a quiescent state, which is essential to preserve their self-renewal capacity (Ito and Suda, 2014 Nat Rev Mol Cell Biol 15:243-256). SC niches are the optimal microenvironment for cell survival and proliferation in the secretory, undifferentiated state.

Therefore, the use of SCs into an artificial niche mimicking the wild environment could improve SC regenerative potential (Furth et al., 2007 Biomaterials 28:5068–5073). Noteworthy the segregation of cells in a constructed, synthetic niche avoids the direct contact of SCs with the degenerated tissue. The physical localization of SCs could limit the uncontrolled cell proliferation and their alteration vs a tumoral phenotype. Indeed concerns whether adult SCs themselves harbor cancer-promoting mutations are just beginning to be addressed (Mishra et al., 2009 Cancer Res 69:1255-1258).

Recently, a relevant role of mesenchymal stem cells in relieving neuropathic pain has been shown (Ghelardini et al., 2018 Neuropharmacology 131:166-175). A single injection of rat adipose stem cells (rASCs) reduced chemotherapy-dependent pain taking effect 1h after administration and peaking 6h 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 3h after administration and found in the liver 24h thereafter. In oxaliplatin-treated rats, the plasma concentration of vascular endothelial growth factor (pan VEGF-A) was increased whereas the isoform $VEGF_{165}b$ was upregulated in the spinal cord. Both the alterations were reverted by rASCs suggesting the regulation of VEGF-A as an effective mechanism in the complex response orchestrated by stem cells against neuropathy. Nevertheless, a further analysis of stem cell secretome in neuropathic conditions could offer powerful opportunities to individuate pain modulatory mechanisms and novel pharmacological targets.

The aim of the present research proposal is:

1) to test an engineered niche with characteristics of biocompatibility but not biodegradability. In this system, SCs would preserve their renewal and secretory potential avoiding the evolution into cancer cells. The artificial niches would permit a bidirectional flux of solutes from the inner and outer environment without a direct delivery of SCs in the surrounding tissue. Autologous SCs isolated from adipose tissue will preferentially be used in order to minimize non-self rejection. The anti-hyperalgesic effect of this system will be tested in a rat model of oxaliplatin-induced neuropathy.
2) to deep inside the modulation of VEFG-A levels as a target for novel pain killer treatments. The role of increased concentration of VEGF-A in nociception will be studied; the possibility to decrease VEGF-A by specific antibodies or other direct or indirect modulators will be evaluated

3) to improve knowledge about the stem cell secretome analyzing the proteic components by a proteomic approach

Results

The efficacy of stem cells seems to be linked to their ability to turn into "medicinal" stem cells when stimulated by pathological environment (Caplan and Correa, 2011 Cell Stem Cell 9(1): 11-15). "Medicinal" stem cells secrete several immunomodulatory and trophic factors able to counteract tissue damage and pathological processes. In order to set up a biocompatible device that let the release of rASCs secretome when implanted we were inspired to the physiological niches, that generally support cell survival and proliferation in the secretory, undifferentiated state. Aimed to set up an artificaial niche able to promote rASCs proliferation, to ensure the maintenance of stemness and meanwhile permit a bidirectional flux of solutes from the inner and outer environment without a direct delivery of cells, the artificial and biocompatible commercial AlgiMatrix® sponges were tested. Lyophilized AlgiMatrix® sponges were reconstituted using $3x10^4$ and $12.5x10^4$ rASCs suspended in DMEM serum free or with 10% FBS. Cells were cultured for 24 (Figure 1A) and 72h (Figure 1B). At the end of incubation cell viability was measured with Alamar blue assay. AlgiMatrix® sponges ensured cell viability and the percentage of viable cells increase proportionally to the inoculated number of ASCs. In sponges with $12.5x10^4$ ASCs, cell viability was about 40% more than the sponges with $3x10^4$ cells, both in the presence or in the absence of FBS and after both times of incubation.

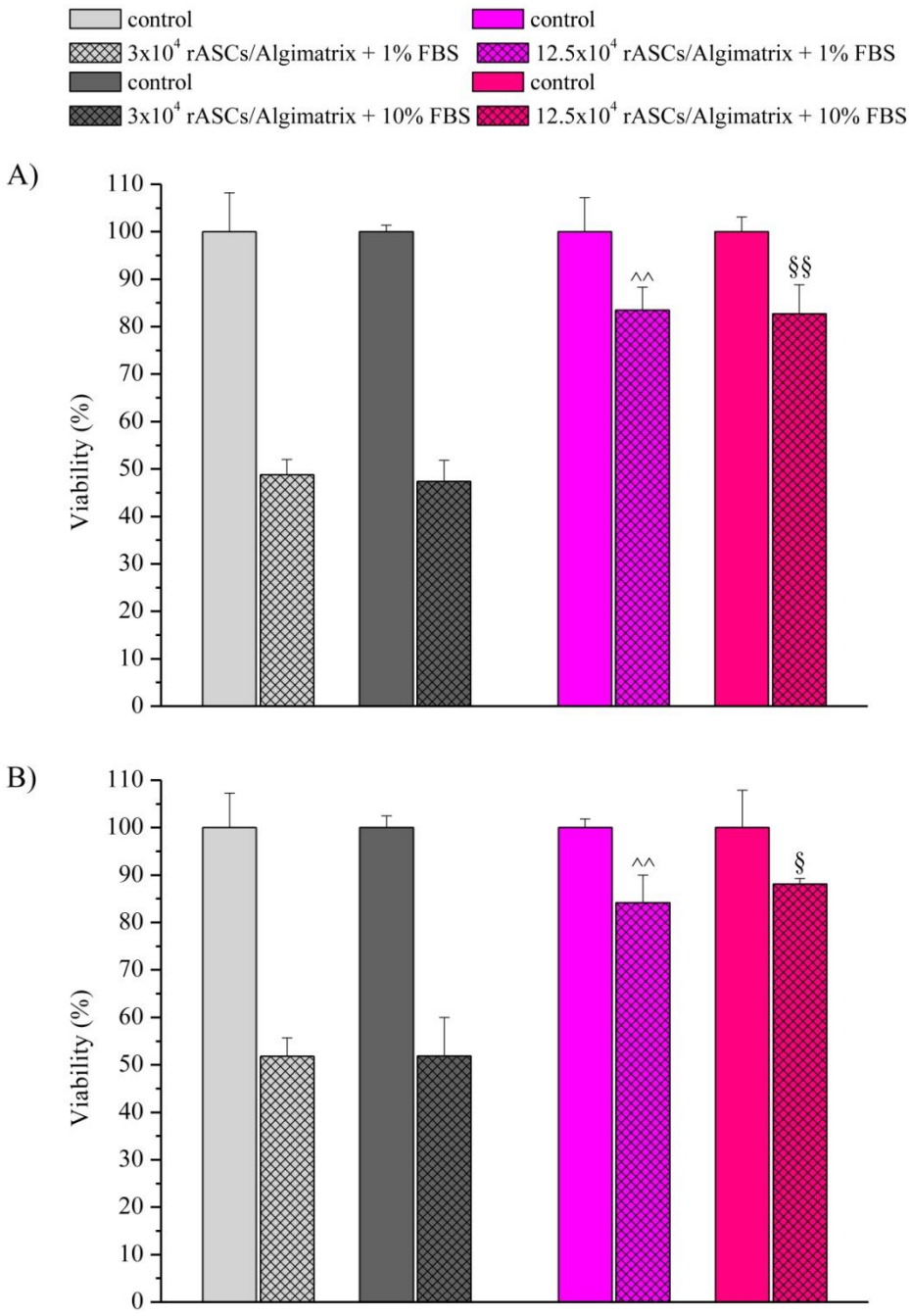

Figure 1. Cell viability assay. *Lyophilized AlgiMatrix® sponges were reconstituted using 3x10^4 and 12.5x10^4 rASCs suspended in DMEM serum free or with 10% foetal bovine serum (FBS). Cells were cultured for A) 24 and B) 72h. At the end of incubation cell viability was measured with alamar blue assay. After 3 h of incubation at 37°C the absorbance was read at 570 and 600 nm with a standard spectrophotometer. The number of viable cells correlates with the magnitude of dye reduction and is expressed as a percentage of alamar blue reduction with respect to positive control. Positive control contained not encapsulated rASCs cultured in DMEM serum free or with 10% FBS. Each value represents the mean ± SEM of four experiments. ^^P<0.01 vs 3x10^4 rASCs/Algimatrix w/o FBS; §P<0.05 and §§P<0.01 vs 3x10^4 rASCs/Algimatrix + 10% FBS.*

Neuropathic pain was induced in rats by repeated administrations of oxaliplatin (Figure 2). On day 13, when neuropathy was established, $4x10^6$ ASCs were inoculated into each AlgiMatrix® sponges and cultured for 24 h. The day after, AlgiMatrix® sponges were implanted s.c. in the back of neuropathic rats (two sponges *per* rat). A statistically significant reduction in oxaliplatin-induced hypersensitivity was observed starting from 6h and peaking at 2 days after the surgery when the weight tolerated on the posterior paw was increased from the control (vehicle + sham group) value of 45.5 ± 0.5 g to 63.3 ± 0.8 of the rats implanted with encapsulated rASCs (oxaliplatin + encapsulated rASCs group) (Figure 2). The effect on hypersensitivity remained constant for 7 days after AlgiMatrix® sponges implantation and disappeared at 8th day.

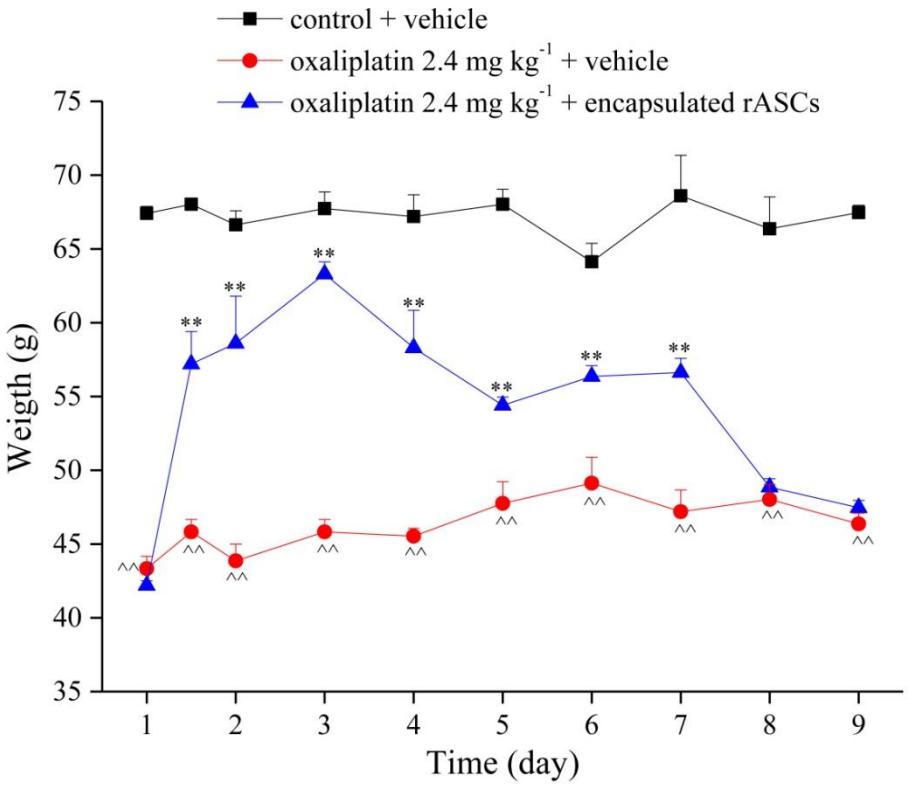

Figure 2. Effect of encapsulated rASCs in reducing oxaliplatin-induced hyperalgesia. *Oxaliplatin (2.4 mg kg^{-1} i.p.) was administered for 4 consecutive days on weeks 1 and 2. On day 13, 4x10^6 rASCs were encapsulated into each AlgiMatrix® sponge. The day after AlgiMatrix® sponges were implanted s.c. in the back of neuropathic rats (two sponges per rat). The pain threshold was evaluated through the paw pressure test at different time (6 h – 8 days). Each value represents the mean ± SEM of 10 rats per group, performed in 2 different experimental sets. ***P<0.001 vs vehicle + DMEM treated animals; ^^^P<0.001 vs oxaliplatin + DMEM treated animals.*

Conclusions

Stem cell encapsulation technique holds significand promise. With this procedure, stem cells can be encapsulated in a semipermeable membrane that allows the influx of oxygen and nutrition and the efflux of growth factors. The implantation of encapsulate stem cells have been effective in

significantly suppressing seizure activities and protecting hippocampal neurons in an animal model of temporal lobe epilepsy (TLE) (Huber et al., 2001 Proc Natl Acad Sci USA 98: 7611–7616; Kuramoto et al., 2011 Brain Res 1368: 281–289; Kanter-Schlifke et al., 2009 Exp Neurol216: 413–419) but also are efficacy in reducing amyloid load, stabilizing synaptic integrity, attenuating cholinergic degeneration and improving cognitive function in an animal model of Alzheimer disease (Emerich et al., 1994 J Comp Neurol 349: 148–164; Garcia et al., 2010 J Neurosci 30: 7516–7527; Spuch et al., 2010 Biomaterials 31: 5608–5618). In our model of oxaliplatin-induced neuropathy rASCs, encapsulated in a 3D porous alginate scaffold named AlgiMatrix® that ensures cell viability. The implantation of encapsulate cells subcutaneously in the back of the neuropathic rats were efficacy in counteracting the hypersensitivity induced by repeated oxaliplatin administration. These data further support the importance of stem cell secretome as mediator of their efficacy, moreover make MSCs very suitable for use as therapeutic agents in vivo.

Materials and Methods

Animals

For all the experiments described below, male Sprague-Dawley rats (Harlan, Varese, Italy) were used. Rats weighting approximately 550-600 g were employed for rASCs isolation while for functional and behavioral experiments animals weighed 200-250 g. Animals were housed in CeSAL (Centro Stabulazione Animali da Laboratorio, University of Florence) and used at least one week after their arrival. Four rats were housed per cage (size 26 × 41 cm) kept at 23 ± 1°C with a 12 h light/dark cycle, light at 7 a.m; were fed a standard laboratory diet and tap water ad libitum. All animal manipulations were carried out according to the Directive 2010/63/EU of the European parliament and of the European Union council (22 September 2010) on the protection of animals used for scientific purposes. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines (McGrath and Lilley, 2015 Br J Pharmacol 172:3189-3193). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Rat ASCs preparation

Retrosternal, thighs and aortic fat pads from Wistar rats weighing approximately 550–600 g, were harvested in a sterile fashion, washed with phosphate-buffered saline (PBS), minced and digested

with 2 mg mL^{-1} *Clostidium histolyticum* collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) in PBS at 37°C in a shaking water bath. The sample was filtered through a 100 µm mesh filter to remove debris. Enzyme activity was neutralized with medium containing 20% fetal bovine serum (FBS; GIBCO, Life Technologies – Thermo Fisher Scientific, Waltham, MA, USA) and samples were centrifuged at 700xg for 10 min. The pellet was suspended and plated in Nutrient Mixture F-12 Ham (Sigma-Aldrich, Milan, Italy) supplemented with 20% FBS and 1% penicillin-streptomycin (P/S, Sigma-Aldrich, Milan, Italy) and incubated at 37°C in 5% CO_2 atmosphere. After 4 h, non-adherent cells were removed, and adherent cells were washed twice. The medium was replaced every 2-3 days, and cells with 80-90% confluence were detached with the use of trypsin/ethylenediaminetetraacetic acid (EDTA) (0.5%) and passed.

Evaluation of cell viability in AlgiMatrix® sponges: Alamar Blue assay

Rat ASCs were inoculated in AlgiMatrix® sponges as previously described. For this assay AlgiMatrix® sponges conteined in 24-well plates were used.

The Alamar blue assay assesses mitochondrial ability to reduce resazurin into the fluorescent product resorufin. Before performing the assay, Alamar Blue medium was prepared by mixing medium with Alamar Blue solution (Bio Rad, Berkeley, CA, USA) in a 10:1 ratio. After the incubation of rASCs with AlgiMatrix® sponges for 24 and 72 h at 37°C in 5% CO^2 atmosphere, the medium was discarded, and Alamar Blue medium (500 µL) was transferred to the wells. The plates were incubated at 37 °C for 3 h, and fluorescence was measured using a fluorescence spectrometer (PerkinElmer) with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Cell viability was calculated using the ratio of the fluorescence of treated cells to the fluorescence of control cells, and the data were expressed as percentages.

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 Eur J Cancer 2457-2463; Di Cesare Mannelli et al., 2013 J Pain 1585-1600 with minor modifications). Control rats received an equal volume of 5% glucose.

Preparation of encapsulated rASCs

For *in vitro* experiments, lyophilized AlgiMatrix® sponges were rehydrate using DMEM with 10% v/v AlgiMatrix® Firming Buffer. After 5 minutes of incubation, $3x10^4$ and $12.5x10^4$ rASCs suspended in 500 µL DMEM serum free or with 10% foetal bovine serum (FBS) were inoculated in

AlgiMatrix® sponges. Cells were cultured for 24 and 72h at 37°C in 5% CO_2 atmosphere. At the end of incubation cell viability was measured with Alamar Blue assay.

For *in vivo* experiments, AlgiMatrix® sponges were initially rehydrate as described above. After 5 minutes of incubation $4x10^6$ rASCs, suspended in 500 µL DMEM serum free or with 10% foetal bovine serum (FBS), were inoculated into each AlgiMatrix® sponges and cultured for 24h. The day after AlgiMatrix® sponges were subcutaneously (s.c.) implanted in the back of neuropathic rats (two sponges *per* rat). Behavioural measurements were performed starting 6h after the surgery.

Paw-pressure test

The nociceptive threshold of rats was determined with an analgesimeter (Ugo Basile, Varese, Italy), according to the method described by (Leighton et al., 1988 Br J Pharmacol 93: 553-560). In single administration experiments, before and 1, 3, 6, 9, 24 and 48 h after rASCs injection and in multiple administration experiments, before and 6, 24, 48, 72, 96 h after rASCs injections, a constantly increasing pressure was applied to a small area of the dorsal surface of the hind paw using a blunt conical probe by a mechanical device. Mechanical pressure was increased until vocalization or a withdrawal reflex occurred while rats were lightly restrained. Vocalization or withdrawal reflex thresholds were expressed in grams. Rats scoring below 40 g or over 75 g during the test before drug administration were rejected (25%). For analgesia measures, mechanical pressure application was stopped at 120 g.

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.

Data 02/05/2018 Firma