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Peripheral nerve regeneration after experimental section in ovine radial and tibial nerves using synthetic nerve grafts, including expanded bone marrow mesenchymal cells: morphological and neurophysiological results

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ABSTRACT

The standard treatment of peripherical nerve injuries with substance gap is to introduce the nerve free extremes in a biodegradable tube which, as a biocamera, allows the continuity of the nerve, promote the neuroconduction and save the lesion from the surrounding fibrosis. However, this procedure has not any direct effect on the neuroregeneration nor to resolve high severe lesions.
The mesenchymal stem cells (MSC) can derivate "*in vitro*" in different lineages, including Schwann cells. Different studies have shown MSC can promote the nerve
regeneration in rodents, dogs and primates. Moving to the human clinical application requires the procedure standardization, including the optimal cell dose which we have to use.
In the sheep model animal we performed a study of 1 cm. nerve section-ressection and repair with a Neurolac™ biocamera, in whose gap we applied between 30 to 50×10^6 MSC from cancellous bone, all of them selected and cultured with GMP procedures. The results were compared with controls (saline serum ± platelet-rich plasma).
We used radial nerve (sensitive) and tibial nerve (motor) from 7 sheep. In the first step we performed the surgical lesion and bone marrow aspiration, and in 3 weeks we performed the surgical repair. 3 sheep were sacrificed in 3 months, and 4 sheep in 6 months. In all surgeries we performed a neurophysiological register.
When we obtained the tissue samples, we performed an histological, immunohistiquimical and morphometrical study. The recovery percentage was defined comparing the axonal density from the proximal and distal lesion margins.
The 3 months samples results were wrong. In 6 months samples results we observed a significative myelined nervous fibers and conduction increasing, in front of controls, both radial and tibial nerves. These results suggest the MSC application in biodegradable scaffold in nerve injuries promotes good results in terms of regeneration and functional recovery.

Introduction

Peripheral nerve lesions are a common cause of pain and disability [1]. Microsurgery and nerve grafts are often used to treat peripheral nerve injury, but regeneration of injured nerves following microsurgery is limited, and nerve allografts are hampered by immunorejection. Autologous grafting is the gold standard for peripheral nerve repair because it satisfactorily repairs damaged nerves and there are no immunorejection issues; however, nerve sources are limited, so it is not possible to treat extended or multiple nerve lesions [2].

Platelet-rich plasma (PRP) and biodegradable nerve conduits (BNC) are often used to complement these techniques. PRP is an endogenous therapy prepared from the patient's own blood; it forms scaffolds that facilitate cell adhesion and regeneration [3,4,5], and is rich in growth factors that can induce differentiation of progenitor cells into Schwann cells [6]. The positive effects of PRP on nerve repair have been shown in animal studies and are thought to be the result of remyelination and axon regeneration [7,8]. PRP is often used to supply growth factors to supplement biomaterials [9]: it is mainly used as a complement as PRP alone does not repair extended nerve damage or sectioning. BNCs are

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effective for repair of small nerve gaps and are less abrasive than non-biodegradable scaffolds as there is no need for surgical extraction; however, conduits do not promote nerve growth to fill larger gaps [10]. Other biological approaches, including tissue cell therapy, have been developed as alternatives in recent years.

Tissue cell therapy using stem cells that can differentiate into appropriate cell types in the damaged area has developed rapidly in the last decades. Neural and mesenchymal stem cells regenerate damaged nerves. Mesenchymal stem cells (MSC) are the preferred option for the simplicity of their harvesting and differentiating protocols. They have been used successfully for the treatment of cardiovascular, neurological and musculoskeletal disorders and are used in 46% of cellular tissue therapies [11,12]. MSC are multipotential cells that differentiate *in vitro* into osteoblasts, chondrocytes, adipocytes, cardiomyocytes and hepatocytes [13] and into neural lineages, including neurones, astrocytes, oligodendrocytes, microglia and Schwann cells [12]. Nerve regeneration after injury is mainly conducted by Schwann cells [14]. MSC enhance generation and survival of Schwann Cells and can differentiate into Schwann cells *in vitro* in the presence of specific inductors [15]. MSC can be obtained from bone marrow, blood, adipose tissue, synovium, periosteum and cartilage [16,17]. Bone marrow is the preferred source because of the abundance of bone marrow MSC (BM-MSC) and these cells have been used successfully to promote regeneration of peripheral nerves in primates [18]. Several studies have shown that differentiated BM-MSC are superior to undifferentiated MSC in the production of growth factors and nerve regeneration [15]. In addition, BM-MSC-derived Schwann cells are more stable and less likely to revert to MSC phenotypes after transplantation into the affected area [15]. A promising study about regeneration after human and autologous stem cell transplantation in a nerve injury animal model has recently been published [19]. Finally, several studies have proved that a combination of PRP and MSC contributes to the regeneration of damaged bone tissues and peripheral nerves [20,21,22]. Further standardisation of the protocols for BM-MSC subpopulation selection and harvesting, differentiation, optimal cell concentration for transplant, and healing times, among others, is required, however, before the clinical application of MSC therapy. Also, most studies have been conducted in rodents, and need to be adapted for use in humans [23].

Objective

We propose to investigate the therapeutic efficacy of a novel protocol combining differentiated BM-MSC and PRP, using a biodegradable scaffold, for the repair of extended peripheral nerve injury in sheep. Nerve regeneration will be evaluated using comparative clinical evolution, near nerve action potentials (NAPs) recorded intraoperatively, and morphological study, which will facilitate translation of the study treatments for clinical use in humans.

Material and methods

Animal surgery

Seven healthy female sheep aged two years were used in the study. All sheep were normal on physical examination and had normal blood test parameters. Animals were anaesthetised using a standardised procedure, and 1 cm transection on the radial nerve (sensory) and on the tibial nerve (motor) was performed surgically in the anterior and posterior left extremities, respectively. NAPs were recorded immediately before and after the transection. All animal work was conducted in the Department of Animal Medicine and Surgery (Facultat de Veterinària, Universitat Autònoma de Barcelona, Bellaterra, Spain). This study received ethical approval from the *"Comité de Ética en Experimentación Animal y Humana de la Universitat Autònoma de Barcelona"*.

Isolation and culture of bone marrow mesenchymal stem cells (BM-MSC)

Bone marrow was extracted after general anaesthesia during the nerve transection surgery. A total of 30-35 mL of bone marrow was extracted by aspiration from the sternum using 10 mL syringes and collected in four Falcon tubes. Samples were sent to the cell producer laboratory (Laboratorio de Genética Bioquímica, Facultad de Veterinaria, Universidad de Zaragoza, Spain), where the samples were homogenised and centrifuged at 1750 rpm for 20 minutes. The layer containing the nucleated cells was collected and washed in phosphate-buffered saline (PBS, 1:1 volume) and centrifuged at 1,750 rpm for 5 minutes. The supernatant was discarded and the pellet washed in PBS (1:1 volume) for a further spin at 1,750 rpm for 5 minutes. The resulting pellet was diluted in 10 mL of culture medium containing 1×Dulbecco's Modified Eagle's Medium [DMEN], Sigma), 10% foetal bovine serum (Gibco), 1% penicillin-streptomycin solution (Sigma) and 1% L-Glutamine. An aliquot of 100 µL was mixed with 50 µL of erythrocyte lysis buffer and, after 15 minutes incubation, plated out at a concentration of 2×10^6 nucleated cells/cm^2. When 80% confluence was reached, cultured cells were tryptinised and re-plated. An aliquot was frozen in liquid nitrogen for later use.

Platelet-rich plasma (PRP)

We prepared the PRP using the double centrifugation technique. A volume of 40 mL of fresh blood was obtained by puncture from each study animal using sodium citrate (3.2% v/v) vacutainer tubes (Venoject, Terumo). Blood samples were centrifuged at 1,750 rpm for 30 minutes. The plasma layer directly over the buffy coat was collected in 10 mL siliconised tubes, and centrifuged at 1,800 rpm for 5 minutes to concentrate the platelets. The platelet pellet was resuspended in siliconised tubes containing 500 µL of plasma. This technique produces 4 mL of PRP for every 40 mL of blood. PRP quality and quantity were analysed by cytometry (ADVIA 120TM).

Biodegradable scaffold

The biodegradable scaffold, Neurolac® (Polyganics BV, The Netherlands) is a fully synthetic, 100% biologically safe, resorbable, transparent nerve guide that has been tested successfully in humans. Neurolac® is indicated for reconstruction of peripheral nerve discontinuity of up to 20 mm in patients with a complete division of a peripheral nerve.

This product loses its strength and the majority of its mass by approximately 10 weeks after implantation, and has been completely absorbed by the body after approximately 24 months. The degradation process occurs in two steps: the first step is the breakdown of the polymers into smaller fragments via hydrolysis; and the second step is the complete degradation of the smaller fragments by enzymatic degradation.

Surgical intervention for nerve regeneration

Three weeks after the initial nerve transection, animals were anaesthetised and damaged nerves were covered with 3-4 mm Neurolac® scaffolds. The scaffolds were supplemented with PRP