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Mesenchymal Stem Cells from Extra-Embryonic Tissues for Tissue Engineering – Regeneration of the Peripheral Nerve

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1. Introduction

Recent advances in Regenerative Biology and Regenerative Medicine are impressive and in the last years the scientific community has witnessed the emergence of many new concepts and discoveries. Until a few years ago, biological tissues were regarded as unable of extensive regeneration, but nowadays organs and tissues like the brain, spinal cord or cardiac muscles appear as capable to be reconstructed, based on "stem cells" [1].

Stem cell research has sparked an international effort due to the variety of possible uses in clinical procedures to treat diseases and improve health and life expectancy. Stem cell research has crossed a century journey and has evolved greatly even in its own defini‐tion. In 1967, Lajtha defined that adult stem cells could only be found in regenerative organs, such as blood, intestine, cartilage, bone and skin. Nowadays, these cells are con‐sidered to exist even in tissues with no commitment to regeneration such as the central nervous system [1, 2].

2. Stem cells

Stem cells are undifferentiated cells, with endless self-renewal sustained proliferation *in vitro* and multilineage differentiation capacity [3]. This *in vitro* multilineage differentiation capacity has targeted these cells with extreme importance for use in tissue and cell-based therapies.

The first stem cell appearance is in the early zygotic cells, which are totipotent and give rise to the blastocyst. They are capable to differentiate into all cell and tissue types. With differentiation, cells become less capable of self-renewal and differentiation in other cell type becomes more limited [1]. Stem cells can be loosely classified into 3 broad categories based on their growth behavior and isolation time during ontogenesis: embryonic, fetal and adult.

Embryonic stem cells (ESCs) were first observed in a pre-implantation embryo by Bongso and colleagues in 1994 [4]. Since then, many cell lines and a multiplicity of tissues have been successfully derived from ESCs and tested in several animal disease models [5-7]. Nevertheless, post-transplantation immune-rejection has been a major problem. Many studies are being conducted to avoid this major issue. This could be resolved by personalizing tissues through somatic nuclear transfer (NT) or induced pluripotent stem cells (iPSC) techniques [8], but the teratoma development in animals is still a concern and a serious problem [9]. In order to overcome the limitations placed by ESCs and iPSCs, a variety of adult stem cell populations have been recently isolated and characterized for their potential clinical use. While still multipotent, adult stem cells have long been considered restricted, giving rise only to progeny of their resident tissues [9]. *In vivo,* adult stem cells exist in a quiescent state, located in almost all tissues, until mediators activate them to restore and repair injured tissues. These cells are surrounded by mature cells that have reached the end line in terms of differentiation and proliferation [10]. Stem cell research focuses on the development of cell and tissue differentiation, so as characterization techniques, for tissue and cell identification with marker patterns. Such protocols are essential for regenerative therapies [11].

2.1. Mesenchymal stem cells

The development of cell-based therapies for cartilage [12] and skin [13] reconstruction marks the beginning of a new age in tissue regeneration. Mesenchymal stem cells (MSCs) have become one of the most interesting targets for tissue regeneration due to their high plasticity, proliferative and differentiation capacity together with their attractive immuno-suppressive properties. MSCs present low immunogenicity and high immunosuppressive properties due to a decreased or even absence of Human Leucocyte Antigen (HLA) class II expression [14]. Research in this field has brought exciting promises in many disorders and therefore in tissue regeneration. Currently the differentiation potential of MSCs in multilineage end-stage cells is already proven, and their potential for treatment of cardiovascular [15], neurological [16], musculoskeletal [17, 18], and cutaneous [19] diseases is now well established. Fibroblast colony-forming units or marrow stromal cells, currently named MSCs,

were first isolated in 1968 from rat bone marrow [20]. These cells were clonogenic, formed colonies when cultured, and were able to differentiate *in vitro* into bone, cartilage, adipose tissue, tendon, muscle and fibrous tissue. Since then many other tissues have been used to isolate these cells. MSCs can be obtained from many different tissues, including bone marrow, adipose tissue, skeletal muscle, umbilical cord matrix and blood, placental tissue, amniotic fluid, synovial membranes, dental pulp, fetal blood, liver, and lung [21]. The concept of MSCs is based on their ability to differentiate into a variety of mesodermal tissues and was first proposed by Caplan in 1991 [22] and further validated by additional research in 1999 [23]. Due to the many different methods and approaches used for MSCs culture, the Mesenchymal and Tissue Stem Cell Committee, of the International Society for Cellular Therapy (ISCT), recommended several standards do define MSCs [24]. Therefore, MSCs are defined as presenting: i) plastic adherent ability; ii) absence of definitive hematopoietic lineage markers, such as CD45, CD34, CD14, CD11b, CD79α, CD19 and class-II Major Histocompatibility Complex (MHC) molecules, specially HLA-DR; and expression of nonspecific markers CD105, CD90 and CD73 iii) ability to differentiate into mesodermal lineage cells, osteocytes, chondrocytes and adipocytes. Along with mesodermal differentiation, it has been demonstrated the capacity of MSCs to differentiate into ectodermal cell lines, as neurons [25, 26], keratocytes [27] and keratinocytes [28], so as endodermal cell line, like hepatocytes [29, 30] and pancreatic β-cells [31]. Moreover, they also possess anti-inflammatory and immunomodulation properties and trophic effects [32, 33]. Increasing evidence now demonstrates that the therapeutic effects of MSCs do not lay only on the ability to repair damage tissue, but also on the capacity of modulating surrounding environment, by secretion of multiple factors and activation of endogenous progenitor cells [34, 35]. Compared with ESCs and other tissue specific stem cells, MSCs are more advantageous. Moreover some studies have demonstrated that MSCs have a higher chromosomal stability and lower tendency to form tumors and teratomas, compared to other stem cells [36, 37].

Although they present similar biological characteristics, it cannot be ignored the existing of some disparities, as differences in, expansion potential under same culture conditions and age-related functional properties [38]. Compared to ESCs, MSCs isolated from the umbilical cord matrix (Wharton's jelly) have many advantages, such as shorter population doubling time, easy culture in plastic flasks, good tolerance towards the immune system, so that transplantation into non-immunesuppressed animals does not induce acute rejection, anticancer properties, [9] and most important absence of tumorigenic activity. As well as ESCs, these cells are originated from the inner cell mass of the blastocyst but with a major difference: they do not raise ethical controversies, since they are collected from tissues usually discarded at birth [39].

2.1.1. MSCs sources and validation of transport and processing protocols

Bone marrow, adipose tissue, umbilical cord blood and umbilical cord matrix have been considered the main sources of MSCs for tissue engineering purposes. Among these sources, bone marrow represents the main source of MSCs for cell therapy. However, the prolifera-

tive capacity [40-43], differentiation potential and clonal expandability [44] of MSCs derived from bone marrow decrease significantly with age, gender and seeding density, and the number of cells per marrow aspirate is usually quite low [3, 45]. It is still a mystery if MSCs ageing is due to factors intrinsic or extrinsic to the cells. Many possible reasons have been described in an attempt to explain MSCs ageing. Possible extrinsic factors include: reduced synthesis of proteoglycans and glycosaminoglycans reducing proliferation and viability [46], and production of glycosylated end products, inducing apoptosis and reactive oxygen species [47]. Intrinsic factors causing MSCs ageing might include: cell senescence-associated β-galactosidase and higher expression of p53 and pathway genes p21 and BAX, resulting in blunted proliferation potential [43]. Regarding seeding density, many authors suggest that lower seeding densities induce faster proliferation rates [48, 49]. This has been explained by contact inhibition in higher seeding densities [49], and higher nutrient availability per cell in lower seeding densities [49]. Use of bone marrow MSCs has disadvantages; donors are submitted to invasive harvest of bone marrow. This raises the need to find alternative sources of MSCs for autologous and allogenic use. Candidate tissue sources should provide MSCs displaying high proliferative and differentiation potency [50].

Extra-embryonic tissues are a good alternative to adult donor. This tissues, such as, amnion, microvillus, Wharton's jelly and umbilical cord perivascular cells, are routinely discarded at child-birth, so little ethical and religious controversy attends the harvesting of the resident stem cell populations. The comparatively large volume of extra-embryonic tissues increases the chance of isolating suitable amounts of stem cells, despite the complex and expensive procedures needed for their isolation. Some protocols use enzymatic digestion while others use enzyme-free tissue explant methods that require longer culture time [51]. There are also MSCs in cord blood (CB), but many studies report low frequency of these cells and unsuccessful isolation. However, Zhang and colleagues were able to isolate MSCs from CB with a 90% successful rate when CB volume was ≥ 90ml and a transport time until storage was ≤ 2 hours [51].

In recent years, MSCs derived from umbilical cord matrix Wharton's jelly, have attracted much interest. Wharton's jelly is a mature mucous tissue and the main component of the umbilical cord, connecting the umbilical vessels to the amniotic epithelium. Umbilical cord derives from extra-embryonic or embryonic mesoderm; at birth it weighs about 40g and measures approximately 30-65cm in length and 1.5cm in width [52]. Anyway, individual differences are observed within newborn babies. Fong and colleagues characterized Wharton's Jelly stem cells and found the presence of both embryonic and MSCs, targeting this source as unique and of valuable use for clinical applications. MSCs from the Wharton's jelly can be cultured with little or even no major loss trough at least 50 passages [53].

CB and more recently, umbilical cord tissue (UCT) have been stored cryopreserved in private and public cord blood and tissue banks worldwide in order to obtain hematopoietic and MSCs and, although guidelines exist (Netcord – Foundation for the Accreditation of Cellular Therapy), standardized procedures for CB and UCT transport from the hospital / clinic to the laboratory, storage, processing, cryopreservation and thawing are still awaited.

These may be critical in order to obtain higher viable stem cells number after thawing and limit microbiological contamination.

Our research group focused in determining whether UCT storage and transport from the hospital / clinical to the laboratory at room temperature (RT) or refrigerated (4-6°C) and immersed in several sterile saline solutions affects the UCT integrity in order to be cryopreserved. The umbilical cord contains two arteries and one vein, which are surrounded by mucoid connective tissue, and this is called the Wharton's jelly. The cord is covered by an epithelium derived from the enveloping amnion. The interlaced collagen fibers and small, woven bundles are arranged to form a continuous soft skeleton that encases the umbilical vessels. In the Wharton's jelly, the most abundant glycosaminoglycan is hyaluronic acid, which forms a hydrated gel around the fibroblasts and collagen fibrils and maintains the tissue architecture of the umbilical cord by protecting it from pressure [54].

One centimeter-long fragments of umbilical cords (N = 12) were collected from healthy donors after written informed consent and following validated procedures according to the clinical and technical guidelines of the Private Bank Biosckin, Molecular and Cell Therapies, SA (authorized for processing and cryopreserving CB and UCT units by the Portuguese Minister of Health, ASST – Autoridade para os Serviços de Sangue e de Transplantação). The 1 cm fragments were immersed for 168 hours in 4 different sterile saline solutions at RT (22-24°C) and refrigerated (4-6°C): NaCl 0.9% (Labesfal, Portugal), AOSEPT®-PLUS (Ciba Vision, Portugal), Dulbecco's Phosphate-Buffered Saline without calcium, magnesium and phenol red (DPBS, Gibco, Invitrogen, Portugal) and Hank's Balanced Salt Solution (HBSS, Gibco, Invitrogen, Portugal). The preservative-free, aqueous AOSEPT® PLUS solution contains hydrogen peroxide 3%, phosphonic acid (stabiliser), sodium chloride, phosphate (buffer system), and poloxamer (surfactant), and is usually used to transport and wash contact lenses. After 168 hours, the fragments were collected in 4% of paraformaldehyde and processed for light microscopy. The samples were fixed in 4% paraformaldehyde for 4 hours and then washed and conserved in phosphate buffer saline (PBS) until embedding. The specimens were dehydrated and embedded in paraffin and cut at 10 μm perpendicular to the main umbilical cord axis. For light microscope analysis, sections were stained with haematoxylin and eosin (HE) and observed with a Leica DM400 microscope equipped with a Leica DFC320 digital camera. The UCT integrity was evaluated through the following parameters:

i.   detachment of vessels and retraction of vascular structures;

ii.   loss of detail and integrity of the endothelium;

iii.   connective tissue degradation;

iv.   autolysis of fat (impossible to assess, due to histological technique); and

v.   loss of detail and integrity of the mesothelium.

It was concluded that the best transport solutions were HBSS or DPBS at a temperature of 4-6°C since those maintained the histological structure of UC evaluated through those 5 parameters previously referred (Figure 1 and Figure 2).

Figure 1. Cross section of an umbilical cord transported immersed in DPBS at the refrigerated temperature of 4-6°C. Samples were stained with haematoxylin and eosin (HE). Magnification: 10X.
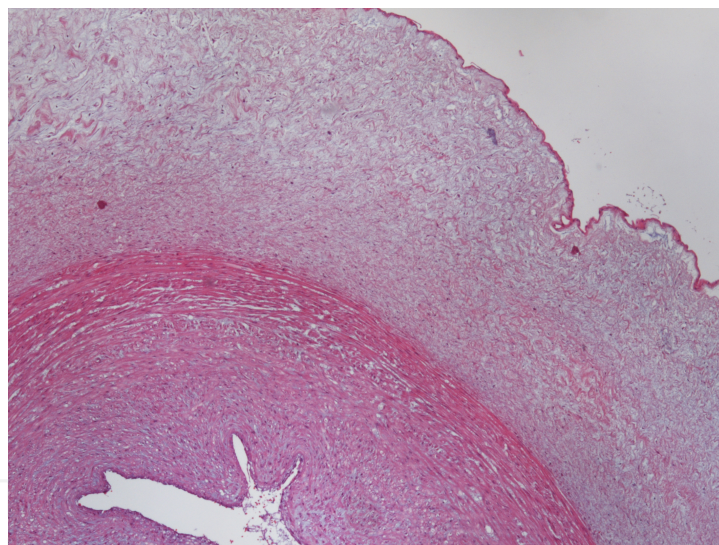

Figure 2. Cross section of an umbilical cord transported immersed in DPBS at the refrigerated temperature of 4-6°C. Samples were stained with haematoxylin and eosin (HE). The UCT integrity was quality evaluated through the following parameters: i) detachment of vessels and retraction of vascular structures; ii) loss of detail and integrity of the endothelium; iii) connective tissue degradation; iv) autolysis of fat (impossible to assess, due to histological technique); and v) loss of detail and integrity of the mesothelium. Magnification: 40X.

As a matter of fact, the UC immersed for 168 hours in DPBS and HBSS at refrigerated temperature presented integrity of the histological structure comparable to a UC collected and processed for histological analysis immediately after birth (Figure 3). With DPBS, a slight retraction of the vessels was noted, which is advantageous since the vessels are stripped and discarded before cryopreservation of the UCT. It was concluded that the transport of the UC

from the hospital / clinic to the cryopreservation laboratory should be performed with the UC immersed in DPBS or HBSS at refrigerated temperatures.
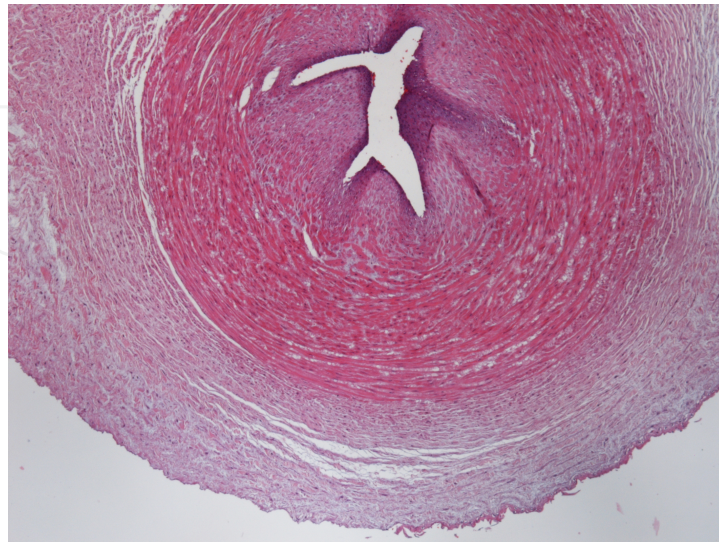

Figure 3. Cross section of an umbilical cord collected and processed for histological analysis immediately after birth under optimal conditions according to Netcord guidelines. Stained with haematoxylin and eosin (HE). Magnification: 40X.

The isolation and culture of MSCs from the Wharton's jelly was performed by our research group in order to obtain undifferentiated MSCs and *in vitro* differentiated into neural-like cells to be tested in axonotmesis and neurotmesis lesions of the rat sciatic nerve. The isolation has been performed by enzyme-free tissue explant and enzymatic isolation. Despite our standard approaches, we are aware that there are still significant variations that exist between laboratory protocols, which must be taken into account when comparing results using other methodologies. There is a wide range of individual differences among donor tissues also and our protocols usually use 15 - 20 cm of UC. While most UC samples will provide a reasonable number of MSCs using the provided protocols, some samples may result in sub-optimal cell isolation and expansion. The reasons behind this phenomenon still remain to be clarified, but as we have previously mentioned, the temperature and the time of transport from the hospital / clinic to the cryopreservation laboratory is crucial.

Irrespective of the specific protocol, the washing procedure of the umbilical cord fragments is crucial in order to avoid microbiological contamination of the cultures. After obtaining the written informed consent from the parents, fresh human umbilical cords are obtained after birth and collected in HBSS or DPBS at 4-6°C, as it was previously described. After washing the umbilical cord unit 4 times in rising DPBS, disinfection is performed in 75% ethanol for 30 seconds. Finally, and before the dissection step, umbilical cord unit is washed in DPBS. The vessels are usually stripped with UC unit still immersed in DPBS. Once washing step in MSCs isolation and culture is essential to achieve good UCT units for cryopreservation and future clinical use, washing protocol was validated. DPBS from the first washing step (used immediately after collection for transportation of the unit to the laboratory – *washing step 1 solution*) and

DPBS used in washing step after disinfection in 75% ethanol (*washing step 6 solution*) from 14 umbilical cord units (N = 14) collected from healthy donors and transported from the hospital/ clinic at 4-6°C in less than 96 hours were tested for microbiological contamination using BacT/ ALERT® (bioMérieux). Each unit was tested for aerobic and anaerobic microorganisms and fungi using 10 ml of the *washing step 1 solution* and *washing step 6 solution* which were aseptically introduced into the BacT/ALERT® testing flasks. All procedures were performed in a laminar flow tissue culture hood under sterile conditions. All the units that presented microbial contamination in DPBS obtained from the first washing step (*washing step 1 solution*) presented no contamination in the analysis performed to DPBS from the last washing step immediately performed before MSCs isolation or UCT cryopreservation (*washing step 6 solution*). The following microorganisms were identified in the DPBS solution from the first washing step: *Staphylococcus lugdunensis* (N = 2); *Staphylococcus epidermidis* (N = 1); *Staphylococcus coagulase* (N = 2); *Escherichia coli* (N = 4); *Enterococcus faecalis* (N = 1); and *Streptococcus sanguinis* (N = 1). The DPBS solution from the first washing step (*washing step 1 solution*) from 3 units was negative for microbial contamination (N = 3). These results permitted us to conclude that the washing protocol was 100% efficient in what concerns microbiological elimination (including aerobic and anaerobic bacteria, yeast and fungi).

Once the transport and washing protocols were validated, it was important to isolate and expand *in vitro* the MSCs from the UCT units for pre-clinical trials.
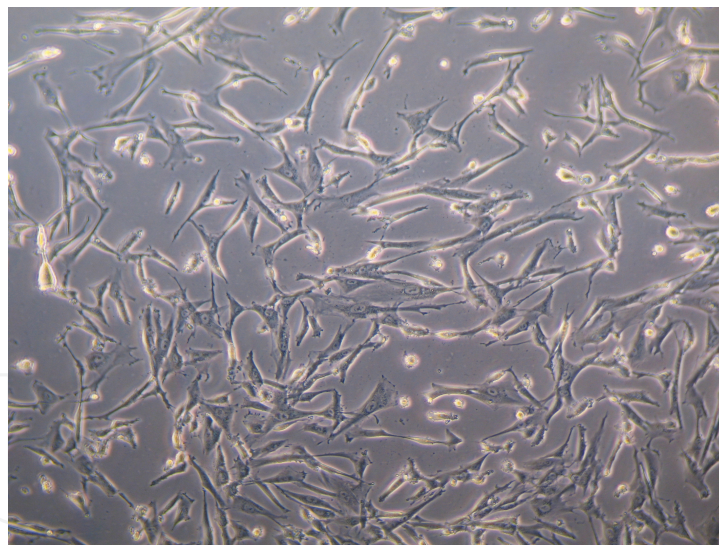

Figure 4. MSCs isolated from Wharton's jelly using the "enzymatic protocol" exhibiting a mesenchymal-like shape with a flat polygonal morphology. Magnification: 100x.

In the "enzymatic procedure" we use collagenase type I (Sigma-Aldrich). With the written informed consent from the parents, fresh human umbilical cords were obtained after birth and stored in HBSS (Gibco, Invitrogen, Portugal) for 1–48 hours before tissue processing to obtain MSCs. After removal of blood vessels, the mesenchymal tissue is scraped off from the Wharton's jelly with a scalpel and centrifuged at 250 g for 5 minutes at room temperature and the pellet is washed with serum-free Dulbecco's modified Eagle's medium (DMEM,

Gibco, Invitrogen, Portugal). Next, the cells are centrifuged at 250 g for 5 minutes at room temperature and then treated with collagenase (2 mg/ml) for 16 hours at 37°C, washed, and treated with 2.5% trypsin-EDTA solution (Sigma-Aldrich) for 30 minutes at 37°C with agitation. Finally, the cells are washed and cultured in DMEM (Gibco, Invitrogen, Portugal) supplemented with 10% fetal bovine serum (FBS), glucose (4.5 g/l), 1% (w/v) penicillin and streptomycin (Sigma), and 2.5 mg/ml amphotericin B (Sigma) in 5% CO_2 in a 37°C incubator (Nuaire). Around 2×10^5 cells are plated into each T75 flask in 10 ml culture medium. Cells are allowed to attach and grow for 3 days. To remove the non-adherent cells or fragments, the flasks are gently washed using pre-warmed DPBS after which 10 ml of pre-warmed culture medium is added. The culture medium is changed every third day (or twice per week). Confluence (80-90%) is normally reached at day 12–16, and the cells are removed with pre-warmed trypsin-EDTA solution (4 ml per flask), for 10 min at 37°C. The cells are plated onto poly-l-lysine coated glass coverslips (in 6- or 24-well tissue culture plates) or on biomaterials used in the nerve reconstruction. Normally, 5000 cells/cm^2 are plated on the coverslips or on the membranes (Figure 4).

In our "enzyme-free tissue explant protocol" for isolation of MSCs, enzymatic digestion is not employed. The mesenchymal tissue (Wharton's jelly) is diced into cubes of about 0.5 cm^3 and the remaining vessels are removed by dissection. Using a sterile scalp, the cubes are diced in 1-2 mm fragments and transferred to a Petri dish pre-coated with poly-l-lysine (Sigma) with Mesenchymal Stem Cell Medium (PromoCell, C-28010) supplemented with 1% (w/v) penicillin and streptomycin (Sigma), and 2.5 mg/ml amphotericin B (Sigma) and cultured in 5% CO_2 in a 37°C incubator (Nuaire). Some tissue fragments will allow cell migration from the explants in 3-4 days incubation. Confluence is normally obtained 15-21 days after.

The laboratory's processing and cryopreservation protocols of the UCT units following the technical procedures of Biosckin, Molecular and Cell Therapies S.A. (BSK.LCV.PT.7) were validated for the ability of isolating and expanding *in vitro* MSCs after cryopreserved UCT thawing. The protocols of processing and cryopreservation of the UCT are protected by a Confidentiality Agreement between Biosckin, Molecular and Cell Therapies S.A. and all the involved researchers. Briefly, the UCT collected from healthy donors (N = 60), and according to Netcord guidelines and following the Portuguese law 12/2009 (*Diário da República, lei 12/2009 de 26 de Março de 2009*) is diced into cubes of about 0.5 cm^3 and the remaining vessels are removed by dissection. In order to ensure the viability of the UCT after parturition and limit the microbiological contamination of the samples, the umbilical cords were transported from the hospital / clinic to the laboratory at refrigerated temperatures monitored by a datalloger in less than 72 hours. The UCT units from 15-20 centimeters-long umbilical cords and after the blood vessels dissection are treated and processed for cryopreservation using a cryoprotective solution (freezing medium). The UCT units are transferred to a computer-controlled slow rate freezer (Sylab, Consensus, Portugal) and a nine-step freezing program is used to set up the time, temperature, and rates specifically optimized for the human umbilical cord-MSCs cooling. To thaw frozen cells, the cryovials are transferred directly to a 37°C water bath. Upon thawing in less than a minute, the cell suspension is centrifuged at 150 × g for 10 min, and the supernatant is gently removed and the cell pellet is resuspended in culture medium. It was possible to obtain MSCs in culture from 52 out of 60 thawed UCT units.

In some UCT cryopreserved units (N = 8) it was not possible to isolate MSCs due to increase number of erythrocytes' lysis or microbiological contamination during cell culture. The MSCs morphology was observed in an inverted microscope (Zeiss, Germany) at different points of expansion. The MSCs exhibited a mesenchymal-like shape with a flat and polygonal morphology. The MSCs obtained were characterized by flow cytometry (FACSCalibur®, BD Biosciences) analysis for a comprehensive panel of markers, such as PECAM (CD31), HCAM (CD44), CD45, and Endoglin (CD105). In the presence of neurogenic medium, the MSCs were able to, became exceedingly long and there was a formation of typical neuro-glial-like cells with multi-branches and secondary branches. These results permitted to conclude that the processing and cooling protocols used for UCT units' cryopreservation were adequate to preserve the UCT viability since it was possible to isolate and expand MSCs after appropriate thaw and in presence of adequate cell culture conditions.

An established and ready-to-use Human MSC cell line was also employed for promoting axonotmesis and neurotmesis lesions regeneration. Human MSCs from Wharton's jelly umbilical cord were purchased from PromoCell GmbH (C-12971, lot-number: 8082606.7). Cryopreservated cells are cultured and maintained in a humidified atmosphere with 5% CO_2 at 37°C. Mesenchymal Stem Cell Medium (PromoCell, C-28010) is replaced every 48 hours. At 80-90% confluence, cells are harvested with 0.25% trypsin with EDTA (Gibco) and passed into a new flask for further expansion. MSCs at a concentration of 2500 cells/ml are cultured on poli-D-lysine coverslips (Sigma) or on biomaterials membranes and after 24 hours cells exhibit 30-40% confluence. Differentiation into neuroglial-like cells is induced with MSC neurogenic medium (Promocell, C-28015). Medium is normally replaced every 24 hours during 3 days. The formation of neuroglial-like cells can be observed after 24 hours in an inverted microscope (Zeiss, Germany) (Figure 5 and Figure 6).
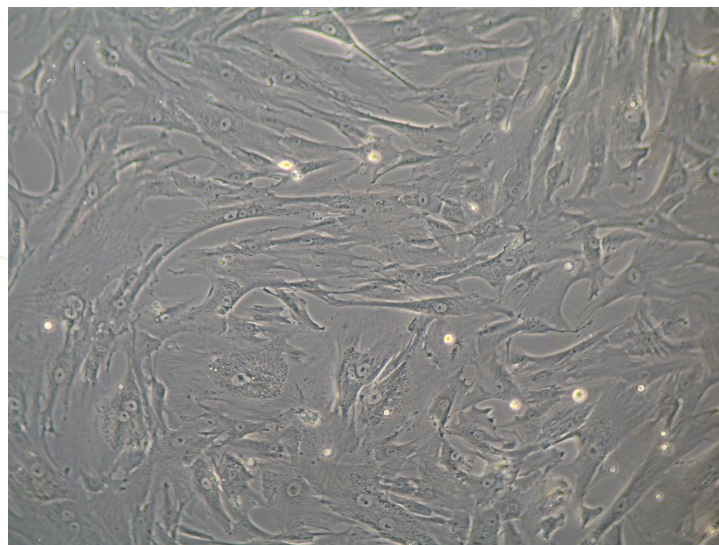

Figure 5. MSC cell line from Wharton's jelly (PromoCell) exhibiting a mesenchymal-like shape with a flat polygonal morphology. Magnification: 100x.
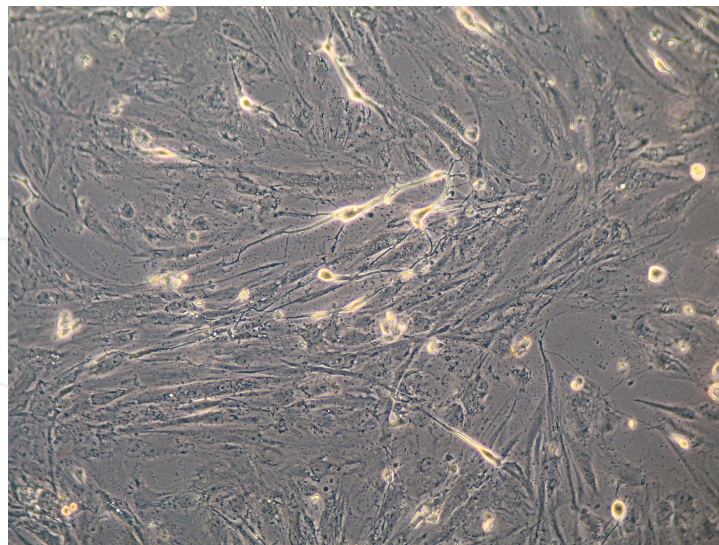
Figure 6. MSC cell line from Wharton's jelly (PromoCell) after 72h of incubation in neurogenic medium. The cells became exceedingly long and there is a formation of typical neuroglial-like cells with multibranches. Magnification: 100x.

This established human MSC cell line is preferred for *in vivo* testing in rats, since the number of MSCs obtained is higher in a shorter culture time, it is not dependent on donors availability and ethic committee authorization, and the protocol is much less time consuming which is advantageous for pre-clinical trials with a large number of experimental animals. As a matter of fact, there is no need of administrating immunosuppressive treatment to the experimental animals during the entire healing period after the surgical procedure. The phenotype of MSCs was assessed by PromoCell. Rigid quality control tests are performed for each lot of PromoCell MSCs isolated from Wharton's jelly of umbilical cord. MSCs are tested for cell morphology, adherence rate and viability. Furthermore, each cell lot is characterized by flow cytometry analysis for a comprehensive panel of markers.

The MSCs isolated with the two protocols described (from fresh and the cryopreserved UCT units) and from the established Promocell cell line exhibited a mesenchymal-like shape with a flat and polygonal morphology. During expansion the cells became long spindle-shaped and colonized the whole culturing surface. After 96 hours of culture in neurogenic medium, cells changed in morphology. The cells became exceedingly long and there was a formation of typical neuroglial-like cells with multi-branches and secondary branches. Giemsa-stained cells of differentiated MSC cell line at passage 5 were analyzed for cytogenetic characterization. However, no metaphases were found, therefore the karyotype could not be established. The karyotype of undifferentiated HMSCs was determined previously and no structural alterations were found demonstrating absence of neoplastic characteristics in these cells, as well as chromosomal stability to the cell culture procedures [55, 56]. The differentiated MSCs karyotype could not be established, since no dividing cells were obtained at passage 5, which can be in agreement with the degree of differentiation. The karyotype analysis of undifferentiated MSCs previously determined, excluded the presence of neoplastic cells,

thus supporting the suitability of our cell culture and differentiation procedures. This concern also resulted from our previous experience with N1E-115 neoplastic cell line and the negative results we obtained in the treatment of axonotmesis and neurotmesis injuries [57-59]. Nevertheless, undifferentiated MSCs from the Wharton's jelly culture (obtained from either protocol or from the Promocell cell line) showed normal morphology when inspected with an inverted microscope (Figure 7).

The differentiation was tested based on the expression of typical neuronal markers such as GFAP, GAP-43 and NeuN by neural-like cells attained from MSCs. Undifferentiated MSCs were negatively labeled to GFAP, GAP-43 and NeuN. After 96 hours of differentiation the attained cells were positively stained for glial protein GFAP and for the growth-associated protein GAP-43. All nucleus of neural-like cells were also labeled with the neuron specific nuclear protein called NeuN showing that differentiation of MSCs in neural-like cells was successfully achieved for MSCs obtained from UCT (fresh and cryopreserved) and for the Promocell MSC cell line (Figure 8) [55].

2.1.2. Differentiation into neuroglial-like cells

MSCs express nestin, a maker for neural and other stem cells [60, 61] and can be differentiated in adipose tissue, bone, cartilage, skeletal muscle cells, cardiomyocyte-like cells, and neuroglial-like cells [54, 55, 60, 62], presenting great potential to biomedical engineering applications. These cells fit into the category of primitive stromal cells and because they are abundant and inexpensive, they might be very useful for regenerative medicine and biotechnology applications.

By employing neuron-conditioned media, sonic hedgehog and fibroblast growth factor 8, MSCs isolated from the Wharton's jelly can be induced toward dopaminergic neurons. These cells have been transplanted into hemiparkinsonian rats where they prevented the progressive degeneration/behavioral deterioration seen in these rats [63]. Rat MSCs isolated from the Wharton's jelly when transplanted into brains of rats with global cerebral ischemia significantly reduced neuronal loss, apparently due to a rescue phenomenon [64]. Neuronal differentiation of human MSCs could also provide cells to replace neurons lost due to neurodegenerative diseases. Recent studies showed that transplanted MSCs-derived neurons become electrophysiologically integrated within the host neural tissue [65]. However, all these therapeutic applications need uniform and reproducible regulation.

A consequence of cell metabolism during *in vitro* expansion is that culture conditions are constantly changing. The comprehension and optimization of the expansion and differentiation process will contribute to maximization of cell yield, reduced need of cell culture, and a decrease in total processing costs [66, 67]. Elucidation of regulatory mechanisms of MSCs differentiation will allow optimization of *in vitro* culture and their clinical use in the treatment of neural-related diseases. Research is being performed to optimize expansion process parameters in order to grow MSCs in a controlled, reproducible, and cost-effective way [68]. Metabolism is certainly one of these parameters.

3. Regeneration and in vivo testing

With the world wide global increase in life expectancy, a variety of disabling diseases with large impact on human population are arising. This includes cardiovascular, neurological, musculoskeletal, and malignancies. Therefore, it is imperative that new and more effective treatment methods are developed to correct for these changes. Further research with experimental animal systems is required to translate to in vivo cell-based therapy that has been extensively investigated in vitro [1]. Stem cell biology is probably the golden key for cell therapies and regenerative medicine. Regeneration is the physical process where remaining tissues organize themselves to replace missing or injured tissues *in vivo* [39].

It has been speculated that once MSCs have the potential to differentiate into several tissues, they might be responsible for turnover and maintenance of adult tissues, just like hematopoietic stem cells have this role in blood cells [69]. First, it was believed that after injection of MSCs, these were able to migrate to the damaged site and to differentiate into ones with the appropriate function for repairing, so MSCs could mediate tissue repair through there multilineage capacity replacing damaged cells. Subsequent studies have suggested that the mechanism used by MSCs for tissue repairing is not really this way. This new idea was reinforced by the confirmation that this cells homed to damaged site, particularly to spots of hypoxia, inflammation and apoptosis [70, 71].

Recent studies demonstrated that transplanted MSCs modified the surrounding tissue microenvironment, promoting repair with functional improvement by secretion factors (known as paracrine effect), stimulation of preexisting stem cells in the original tissue and decreasing of inflammation and immune response [72]. Other studies have demonstrated that MSC-conditioned media by itself could have therapeutic effects. All this data suggest that MSC apply a reparative effect on injured side through its paracrine effects [73].

It is necessary to overcome some barriers before a cell-based therapy becomes routine in clinics, including the cell number and the administration way of treatment. MSCs are difficult to be maintained stable in culture for long time, but due to their short doubling time, if at the outset many cells are harvested they may be properly scaled up in primary culture, never forgetting the ideal seeding number [39].

MSCs are an attractive candidate for cell-based regenerative therapy; the evidence is that currently there are 139 trial registries for MSC therapy 27 of which are based on umbilical cord MSCs [74].

3.1. Nerve regeneration

After Central Nervous System (CNS) lesions, Peripheral Nervous System (PNS) injuries are the ones with minor successes in terms of functional recovery. These kinds of injuries are frequent in clinical practice. About two centuries ago it was assumed that these nerves would never regenerate. Indeed, scientific and clinical knowledge greatly increased in this area. Nevertheless, a full understanding of axonal recovery and treatment of nerve defects,

especially complete functional achievement and organ reinnervation after nerve injury, still remains the principle challenge of regenerative biology and medicine [75, 76].

3.1.1. Nerve repair

Many peripheral nerve injuries can only be dealt through reconstructive surgical procedures. Despite continuous refinement of microsurgery techniques, peripheral nerve repair still stands as one of the most challenging tasks in neurosurgery, as functional recovery is rarely satisfactory in these patients [76]. Direct repair should be the procedure of choice whenever tension-free suturing is possible; however, patients with loss of nerve tissue, resulting in a nerve gap, are considered for a nerve graft procedure. In these cases, the donor nerves used for grafting are commonly expendable sensory nerves. This technique, however, has some disadvantages, with the most prominent being donor site morbidity, that may lead to a secondary sensory deficit and occasionally neuroma and pain. In addition, no donor and recipient nerve diameters often occurs which might be the basis for poor functional recovery. Alternatives to peripheral nerve grafts include cadaver nerve segments allografts, end-to-side neurorrhaphy, and entubulation by means of autologous non-nervous tissues, such as vein and muscles [76]. One advantage of these allografts compared with the autografts is the absence of donor site morbidity and theoretically the unlimited length of tissue available [77]. Experimental work from a number of laboratories has emphasized the importance of entubulation for peripheral nerve repair to manage nerve defects that cannot be bridged without tension (neurotmesis with loss of nerve tissue). Nerves will regenerate from the proximal nerve stump towards the distal one, whereas neuroma formation and ingrowth of fibrous tissue into the nerve gap are prevented [78]. The reliability of animal models is crucial for PN research, including therapeutic strategies using biomaterials and cellular systems. As a matter of fact, rodents, particularly the rat and the mouse, have become the most frequently used animal models for the study of peripheral nerve regeneration because of the widespread availability of these animals as well as the distribution of their nerve trunks which is similar to humans [79]. Because of its PN size, the rat sciatic nerve has been the most commonly experimental model used in studies concerning the PN regeneration and possible therapeutic approaches [80]. Functional recovery after PN injury is frequently incomplete, even with adequate microsurgery, so, many research and clinical studies have been performed including biomaterials for tube-guides. Since the 80's, Food and Drug Administration (FDA) has approved a variety of these biomaterials both natural and synthetic. The ideal biomaterial nerve graft should increase number, length and speed of axon regeneration [77]. It should be:

i.   biocompatible, not toxic neither present undesired immunologic response;

ii.   permeable enough to permit nutrient and oxygen diffusion and allows cell support systems;

iii.   flexible and soft to avoid compression;

iv.   biodegradable, the ideal rate is to remain intact during axon regeneration across nerve gap and after degrade softly and

v.   technically reproducible, transparent, easy to manipulate, and sterilize [81].

Currently 3 types of materials are available for nerve reconstruction: non-resorbable, natural resorbable and synthetic resorbable. Polyvinil alcohol hydrogel (PVA) is an example of a non-absorbable biomaterial. It combines water in similar proportions to human tissue, with PVA providing a stable structure easy to sterilize, which is a main advantage of this materials, but has some limitations such as: nerve compression and suture tension after regeneration due to its non-resorbable nature [77]. Collagen type I from humans or animals, is an example of a natural resorbable device, which has some advantages such as:

i.   easy to isolate and purify,

ii.   good adhesiveness for supporting cell survival an proliferation,

iii.   has been proven to be highly biocompatible and support nerve regeneration *in vivo*.

On the other hand, offers some immune response requiring the use of immunosuppressive drugs or pre-treatment of the material before clinical use [77]. Poly (DL-lactide-ε-caprolactone) (PLC) a synthetic resorbable material is the only transparent device approved by FDA, important characteristic for the surgeon that facilitates the insertion of the nerve stumps across the nerve gap, but, on the other hand it is not flexible [77]. Chitosan, PLC, collagen, poly(L-lactide) and poly(glycolide) copolymers (PLGA) and others, some of them, previously studied by our group [57, 58, 82] were associated to cellular systems, which are able to differentiate into neuroglial-like cells or capable of modulating the inflammatory process, improved nerve regeneration, in terms of motor and sensory recovery, and also shortening the healing period after axonotmesis and neurotmesis, avoiding regional muscular atrophy [57, 58, 82].

Researches with acellular nerve allografts, as alternative for repairing peripheral nerve defects have been reported. These nerve allografts remove the immunoreactive SCs and myelin however preserve the internal structure of original nerve, containing vital components such as collagen I, laminin and growth factors essential for repairmen of the lesions [83]. Acellular grafts remain insufficient, due to the increasing extent of nerve damages. Also, viable cells are necessary for debris removal and environmental regeneration reestablishment [83].

Cell transplantation, such as Schwann cells (SCs) transplantation has been proposed as a method of improving peripheral nerve regeneration [84]. SCs are peripheral glial cells that enwrap axons to form myelin with a central role in neuronal function. When there is damage in PNS, SCs are induced to mislay myelin, proliferate and segregate numerous factors, including cytokines responsible for reproducing a microenvironment suitable for supporting axon regeneration [85, 86]. They also have a vital participation in endogenous repair, reconstructing myelin, which are essential for functional recovery [85, 86]. SCs, MSCs, ESCs, marrow stromal cells are the most studied support cells candidates. SCs transplantation enhance axon outgrowth both *in vitro* [87] and *in vivo* [88]. Although to achieve an adequate amount of autologous SC, a donor nerve is necessary and a minimum of 4-8 weeks for *in vitro* expansion. Umbilical cord MSCs may be the perfect cell model as supplement for nerve grafts, once they are easily obtained, with no ethical controversy and can differentiate into

neuglial-like cells [83]. Matuse and collaborators induced MSCs from the umbilical cord into SCs capable of supporting peripheral nerve regeneration and myelin reconstruction *in vivo*. They transplanted these SCs into injured sciatic nerve, and proved that these cells maintained their differentiated phenotype *in vivo*, and contributed for axonal regeneration and functional recovery [89].

In our studies we aimed to explore the therapeutic value of human umbilical cord matrix (Wharton's jelly) derived MSCs, undifferentiated and differentiated in neuroglial-like cells, both *in vitro* and *in vivo*, associated to a variety of biomaterials such as, Poly (DL-lactide-ε-caprolactone) PLC (Vivosorb®) membrane, and Chitosan type III on rat sciatic nerve axonotmesis and neurotmesis experimental model. For cell transplantation into injured nerves (with axonotmesis and neurotmesis injuries), there are two main techniques. The cellular system may be directly inoculated into the neural scaffold which has been interposed between the proximal and distal nerve stumps or around the crush injury (in neurotmesis and axonotmesis injuries, respectively); or the cells can be pre-added to the neural scaffold via inoculation or co-culture (in most of the cellular systems, it is allowed to form a monolayer) and then the biomaterial with the cellular system is implanted in the injured nerve [82].

Our PLC studies [55] demonstrated that this biomaterial does not interfere negatively with the nerve regeneration process, in fact, the information on the effectiveness of PLC membranes and tube-guides for allowing nerve regeneration was already provided experimentally and with patients [82]. PLC becomes hydrophilic by water uptake, which increases the permeability of the polymer. This is essential for the control of nutrient and other metabolite transportation to the surrounding healing tissue. A few weeks after implantation, the mechanical power gradually decreases and there is a loss of molecular weight as a result of the hydrolysis process. Nearly in 24 months, PLC degrades into lactic acid and hydroxycaproic acid which are both safely metabolized into water and carbon dioxide and/or excreted through the urinary tract. In contrast to other biodegradable polymers, PCL has the advantage of not creating an acidic and potentially disturbing micro-environment, which is favorable to the surrounding tissue [90]. Chitosan has attracted particular attention in medical areas due to its biocompatibility, biodegradability, and low toxicity, low cost, improvement of wound-healing and antibacterial properties. Moreover, the potential use of chitosan in nerve regeneration has been demonstrated both *in vitro* and *in vivo* [57, 91]. Chitosan is a partially deacetylated polymer of acetyl glucosamine obtained after the alkaline deacetylation of chitin [57, 82]. While chitosan matrices have low mechanical strength under physiological conditions and are unable to maintain a predefined shape after transplantation, their mechanical properties can be improved by modification with a silane agent, namely γ-glycidoxypropyltrimethoxysilane (GPTMS), one of the silane-coupling agents which has epoxy and methoxysilane groups. The epoxy group reacts with the amino groups of chitosan molecules, while the methoxysilane groups are hydrolyzed and form silanol groups. Finally, the silanol groups are subjected to the construction of a siloxane network due to the condensation. Thus, the mechanical strength of chitosan can be improved by the cross-linking between chitosan, GPTMS and siloxane network. By adding GPTMS and employing a freeze-drying technique, we have previously obtained chitosan type III membranes (hybrid

chitosan membranes) with pores of about 110 μm diameter and about 90% of porosity, and which were successful in improving sciatic nerve regeneration after axonotmesis and neurotmesis [56, 57, 82].

The induction of a crush injury in rat sciatic nerve provides a very realistic and useful model of damage for the study of the role of numerous factors in regenerative processes [57]. Focal crush causes axonal interruption but preserves the connective sheaths (axonotmesis). After axonotmesis injury regeneration is usually successful, after a short (1-2 day) latency, axons regenerate at a steady rate towards the distal nerve stump, supported by the reactive SCs and the preserved endoneural tubules enhance axonal elongation and facilitate adequate re-innervation [92]. Our research group has been testing the efficacy of combining biomaterials and cellular systems in the treatment of sciatic nerve crush injury [57-59, 82, 90, 91, 93-95]. Following transection, axons show staggered regeneration and may take substantial time to actually cross the injury site and enter the distal nerve stump [60]. Although delayed axonal elongation might be caused by growth inhibition originating from the distal nerve itself, growth-stimulating influences may overcome axons stagger. More robust and fast nerve regeneration is expected to result in better reinnervation and functional recovery. As a potential source of growth promoting signals, MSCs transplantation is expected to have a positive outcome. Our results showed that the use of either undifferentiated or differentiated HMSCs enhanced the recovery of sensory and motor function in axonotmesis lesion of the rat sciatic nerve [56]. Neurotmesis must be surgically treated by direct end-to-end suture of the two nerve stumps or by a nerve graft harvested from elsewhere in the body in case of tissue loss. To avoid secondary damage due to harvesting of the nerve graft, a tube-guide can be used to bridge the nerve gap. Acutely after sciatic nerve transection there is a complete loss of both motor and thermal sensory function. Sensory and motor deficit then progressively decrease along the post-operative. From a morphological point of view, nerve regeneration occurs if Wallerian degeneration is efficient and is substituted by re-growing axons and the accompanying viable SCs [96, 97]. The axon regeneration pattern is improved by using appropriate biomaterials for the tube-guide design, like chitosan type III and PLC and cellular systems like MSCs from the Wharton jelly [57, 90, 91, 95]. The surgical technique and the time for the reconstructive surgery is also crucial for the nerve regeneration after neurotmesis [57, 90, 91, 95].

3.2. Assessment of nerve regeneration in the sciatic nerve rat model

Although both morphological and functional data have been used to assess neural regeneration after induced crush injuries, the correlation between these two types of assessment is usually poor [94, 98-100]. Classical and newly developed methods of assessing nerve recovery, including histomorphometry, retrograde transport of horseradish peroxidase and retrograde fluorescent labeling [79] do not necessarily predict the reestablishment of motor and sensory functions [100-103]. Although such techniques are useful in studying the nerve regeneration process, they generally fail in assessing functional recovery [100]. In this sense, research on peripheral nerve injury needs to combine both functional and morphological assessment. The use of biomechanical techniques and rat's gait kinematic evaluation is a prog-

ress in documenting functional recovery [104]. Indeed, the use of biomechanical parameters has given valuable insight into the effects of the sciatic denervation/reinnervation, and thus represents an integration of the neural control acting on the ankle and foot muscles, which is very useful and accurate to evaluate different therapeutic approaches [103-105].

3.2.1. Functional Assessment

After injury and treatment of animals, follow-up results are very important for analysis of functional recovery. Animals are tested preoperatively (week 0), and every week during 12 and 20 weeks, for axonotmesis and neurotmesis of the rat sciatic nerve, respectively. Motor performance and nociceptive function are evaluated by measuring extensor postural thrust (EPT) and withdrawal reflex latency (WRL), respectively [55, 58, 94]. For EPT test, the affect‐ ed and normal limbs are tested 3 times, with an interval of 2 minutes between consecutive tests, and the 3 values are averaged to obtain a final result. The normal (unaffected limb) EPT (NEPT) and experimental EPT (EEPT) values are incorporated into an equation (Equa‐ tion (1)) to derive the percentage of functional deficit, as described in the literature [106]:

$$\% \text{ Motor deficit } = \left[\left(\text{NEPT} - \text{EEPT} \right) / \text{NEPT} \right] \times 100 \tag{1}$$

The nociceptive withdrawal reflex (WRL) was adapted from the hotplate test developed by Masters et al. [107]. Normal rats withdraw their paws from the hotplate within 4s or less. The cutoff time for heat stimulation is set at 12 seconds to avoid skin damage to the foot.

For Sciatic Functional Index (SFI), animals are tested in a confined walkway that they cross, measuring 42 cm long and 8.2 cm wide, with a dark shelter at the end. Several measure‐ ments are taken from the footprints:

i. distance from the heel to the third toe, the print length (PL);

ii. distance from the first to the fifth toe, the toe spread (TS); and

iii. distance from the second to the fourth toe, the intermediary toe spread (ITS).

In the static evaluation (SSI) only the parameters TS and ITS, are measured. For SFI and SSI, all measurements are taken from the experimental (E) and normal (N) sides. Prints for meas‐ urements are chosen at the time of walking based on precise, clear and completeness of foot‐ prints. The mean distances of three measurements are used to calculate the following factors (dynamic and static):

$$\text{Toe spread factor } \left(\text{TSF} \right) = \left(\text{ETS} - \text{NTS} \right) / \text{NTS} \tag{2}$$

$$\text{Intermediate toe spread factor } \left(\text{ITSF} \right) = \left(\text{EITS} - \text{NITS} \right) / \text{NITS} \tag{3}$$

$$\text{Print length factor } (\text{PLF}) \ = \ (\text{EPL} - \text{NPL}) \ / \ \text{NPL} \tag{4}$$

SFI is calculated as described by Bain et al. [108] according to the following equation:

$$\begin{aligned}
\text{SFI} &= -38.3(\text{EPL} - \text{NPL}) \ / \ \text{NPL} + 109.5(\text{ETS} - \text{NTS}) \ / \ \text{NTS} + 13.3(\text{EIT} - \text{NIT}) \ / \ \text{NIT} - 8.8 \\
&= (-38.3 \times \text{PLF}) + (109.5 \times \text{TSF}) + (13.3 \times \text{ITSF}) - 8.8
\end{aligned} \tag{5}$$

For SFI and SSI, an index score of 0 is considered normal and an index of -100 indicates total impairment. When no footprints are measurable, the index score of -100 is given [109]. In each walking track 3 footprints are analyzed by a single observer, and the average of the measurements is used in SFI calculations.

Ankle kinematics analysis is carried out prior nerve injury, at week-2 and every 4 weeks during the 12 or the 20-week follow-up time, for axonotmesis and neurotmesis lesions, respectively. The motion capture is performed with 2 digital high speed cameras (Oqus, Qualysis®) at a rate of 200 images per second, and Qualisys Track Manager software (QTM, Qualysis®). The cameras operate on a infra-red light frequency ensuring a high level of accuracy on the determination of reflective marker position and a position residual of less than 2.7 mm was obtained. Cameras are usually positioned to not recorder significant signal deflection during the test and four reflective markers were placed at the skin of the rat right hindlimb at the proximal edge of the tibia, the lateral malleolus and the fifth metatarsal head. Advanced analysis of the 2-D movement (sagittal plan) data is performed with Visual3D software (C-Motion®, Inc). The rats' ankle angle is determined using the scalar product between a vector representing the foot and a vector representing the lower leg. With this model, positive and negative values of position of the ankle joint (θ°) indicate dorsiflexion and plantarflexion, respectively. For each step cycle the following time points are identified: midswing, midstance, initial contact (IC) and toe-off (TO) [104, 109-113] and are time normalized for 100% of step cycle. The normalized temporal parameters are averaged over all recorded trials. Angular velocity of the ankle joint (Ω °/s) is also determined where negative values correspond to dorsiflexion. A total of 6 walking trials for each animal with stance phases lasting between 150 and 400 ms are considered for analysis, since this corresponds to the normal walking velocity of the rat (20–60 cm/s) [104]. Animals walk on a Perspex track with length, width and height of respectively 120, 12, and 15 cm. In order to ensure locomotion in a straight direction, the width of the apparatus is adjusted to the size of the rats during the experiments.

3.2.2. Morphologic Assessment

Nerve samples are processed for quantitative morphometry of myelinated nerve fibers [114]. Fixation is usually carried out using 2.5% purified glutaraldehyde and 0.5% saccarose in 0.1M Sorensen phosphate buffer for 6-8 hours and resin embedding is obtained following Glauerts' procedure (Scipio et al., 2008). Series of 2-μm thick semi-thin transverse sections are cut using a Leica Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany)

and stained by Toluidine blue. Stereology is carried out on a DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Wetzlar, Germany). Systematic random sampling and D-disector is always adopted using a protocol previously described [115, 116]. Fiber density and total number of myelinated fibers is estimated together with fiber and axon diameter and myelin thickness.

3.3. Results

3.3.1. Differentiation and metabolism of MSCs from Wharton's jelly

In our experimental studies we expanded undifferentiated MSCs from human umbilical cord Wharton's jelly that exhibited a normal star-like shape with a flat morphology in culture (Figures 4 and 5). To prevent the possibility of eventual mutations due to expansion artifacts, a total of 20 Giemsa-stained metaphases of these cells, were analyzed for numerical aberrations. Sporadic, non-clonal aneuploidy was found in 3 cells (41-45 chromosomes). The other 17 metaphases had 46 chromosomes (Figure 7). The karyotype was determined in a completely analyzed G-banding metaphase. No structural alterations were found. The karyotype analysis to the MSCs cell line derived from Human Wharton jelly demonstrated that this cell line has not neoplastic characteristics and is stable during the cell culture procedures in terms of number and structure of the somatic and sexual chromosomes [55].

Figure 7. Selected metaphases from undifferentiated MSC cells isolated from Wharton's jelly, showing the normal number of chromosomes (46, XY). Magnification: 1000X.

We differentiated MSC from Wharton's Jelly into neuroglial-like cells. After 96 hours of incubation in neurogenic medium, we observed a morphological change. The cells became exceedingly long and there was a formation of typical neural-like cells with multi-branches and secondary branches (Figure 6). The differentiation was tested based on the expression of typical neuronal markers such as GFAP, GAP-43 and NeuN by neural-like cells attained

from HwMSCs. Undifferentiated MSCs were negatively labeled to GFAP, GAP-43 and NeuN (Figure 8A,C,E). After 96 hours of differentiation the attained cells were positively stained for glial protein GFAP (Figure 8B) and for the growth-associated protein GAP-43 (Figure 8D). All nucleus of neural-like cells were also labeled with the neuron specific nuclear protein called NeuN (Figure 8F) showing that differentiation of MSCs in neural-like cells were successfully achieved [55].
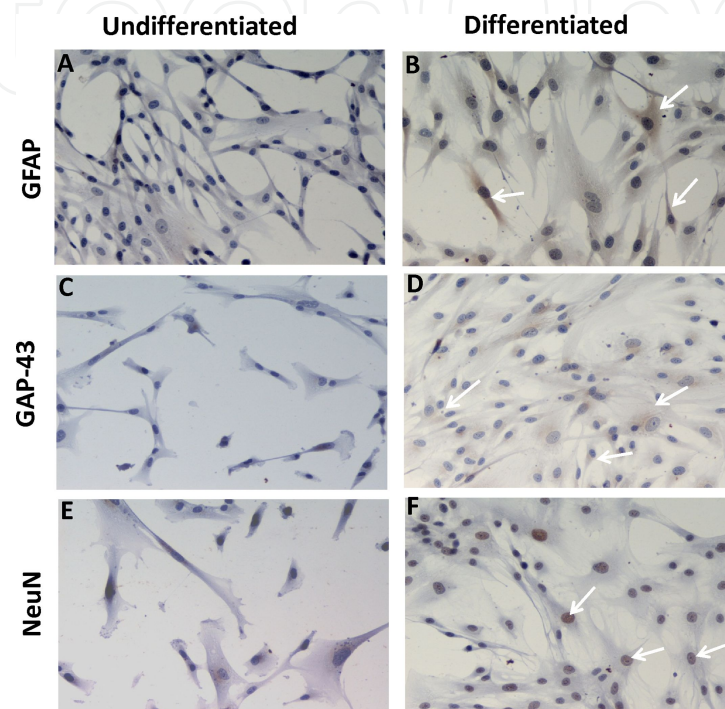

Figure 8. Undifferentiated MSC cells from the Wharton's jelly presenting a negative staining for: (A) GFAP which is a glial cell marker; (C) GAP-43 which is related with axonal outgrowth and (E) NeuN which is a marker for nucleus of neurons. Neuroglial-like cells obtained from HMSCs in vitro differentiated with neurogenic medium exhibiting a positive staining for: (B) GFAP; (D) GAP-43 and (F) NeuN. Magnification: 200x [55].

The *in vitro* expansion and differentiation of MSCs for clinical cell-based therapy is a very expensive and long process that needs standardization. Although pre-clinical and clinical data demonstrated the safety and effectiveness of MSCs therapy in some pathologies such as neurological, there are still questions surrounding the mechanism of action. In our research work we aimed to disclose the possible role of metabolism not only in the MSCs maintenance and expansion but also during the differentiation in neural-like cells [55]. MSCs maintenance and differentiation, to neural-like cells, depends on metabolic modulation. *In vitro*, glucose is the most widely used substrate for the generation ATP which is essential for cell growth and maintenance. It has been proposed that cells undergoing high proliferation rates depend on glycolysis to generate ATP, known as Warburg effect, although this pathway is less effective than the oxidative phosphorylation in terms of ATP production [117]. Our results showed that during expansion, the undifferentiated MSCs consume glucose and produce high concentration of lactate as a metabolic sub product which is consistent with the

Warburg effect and glycolysis stimulation. MSCs do not require oxidative phosphorylation to survive as alternative, hypoxia extends the lifespan, increases their proliferative ability and reduces differentiation [118]. The morphologic and biochemical characteristics of neural-like cells are already described but the mechanism by which stem cells differentiate into neural-like cells is still unknown. In our research work, MSCs that undergone differentiation into neural-like cells, consumed significantly less glucose and produced significantly less lactate than MSCs that undergone only expansion. These major differences allow us to conclude that during MSCs differentiation in neural-like cells the glycolytic process, which proved to be the crucial metabolic mechanism during MSCs expansion, is switched to oxidative metabolism [55].

Our results show clear evidences that MSCs expansion is dependent of glycolysis while their differentiation in neural-like cells requires the switch of the metabolic profile to oxidative metabolism. Also important may be the role of oxidative stress during this process. This work is a first step to identify key metabolic-related mechanisms responsible for human MSCs from the Wharton's jelly expansion and differentiation [55].

The lack of standardization of MSCs isolated from the Wharton's jelly culture conditions has limited some progress in scientific and clinical research. Understanding these MSCs metabolism during expansion, as well as determining molecular and biochemical mechanisms for differentiation is of great significance to develop new effective stem cell-based therapies.

4. Biomaterial and cellular system association – discussion and final remarks

Using the rat model, we recently tested *in vivo* the efficacy of biomaterials and cellular system association in treatment of sciatic nerve axonotmesis and neurotmesis injury. Following transection, axons show staggered regeneration and may take substantial time to cross the injured site and enter the distal nerve stump [119]. However delayed axonal elongation might be caused by growth inhibition originated from the distal nerve itself, growth-stimulating influences may overcome axons stagger. As a potential source of growth promoting signals, MSCs transplantation is expected to give a positive outcome. Our results showed that the use of either undifferentiated or differentiated MSCs in axonotmesis lesion boosted the recovery of sensory and motor function. In both cell-enriched experimental groups we observed that the myelin sheath was thicker, this suggests that MSCs might apply their positive effects on SCs, the key element in Wallerian degeneration and the following axonal regeneration [120]. Also results from *in vivo* testing previously performed by our research group showed that infiltration of MSCs from the Wharton's jelly, or the combination of chitosan type III membrane enwrapment and MSCs enrichment after nerve crush injury provide an advantage to post-traumatic nerve regeneration [56, 57]. Chitosan type III was developed as a hybrid of chitosan by adding GPTMS. A synergistic effect of an extra permeability and physicochemical properties of chitosan type III and the presence of silica ions

may be responsible for the good results in post-traumatic nerve regeneration promotion observed in the sciatic nerve after axonotmesis and neurotmesis [57, 91]. The substantial improvement of axonal regeneration found in sciatic nerve crush enwrapped by chitosan type III membranes and for bridging nerve gaps after neurotmesis [57, 91], suggests that this biomaterial may not just work as a simple mechanical device but instead may induce nerve regeneration. The neuroregenerative properties of chitosan type III may be explained by the effect on SCs proliferation, axon elongation and myelinization [55, 91]. Our data also showed that PLC does not deleteriously interfere with the nerve regeneration process, as a matter of fact, the information on the effectiveness of PLC membranes and tube-guides for allowing nerve regeneration was already provided experimentally and with patients [82]. The MSCs from the Wharton's jelly may be a valuable source in the repair of the peripheral nervous system with capacity to differentiate into neuroglial-like cells. The transplanted MSCs are also able to promote local blood vessel formation and release the neurotrophic factors brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) [55]. Previous results obtained by our research group using N1E-115 cells *in vitro* differentiated into neuroglial-like cells to promote regeneration of axonotmesis and neurotmesis lesions in the rat model showed that there was no significant effect in promoting axon regeneration and, when N1E-115 cells were cultured inside a PLGA scaffold used to bridge a nerve defect, they can even exert negative effects on nerve fiber regeneration. The presence of transplanted N1E-115 cells in nerve scaffolds competing for the local blood supply of nutrients and oxygen and by space-occupying effect could have hindered the positive effect of local neurotrophic factor release leading a negative outcome on nerve regeneration. Thus, N1E-115 cells did not prove to be a suitable candidate cellular system for treatment of nerve injury after axonotmesis and neurotmesis and their application is limited only to research purposes as a basic scientific step for the development of other cell delivery systems, due to its neoplastic origin [57-59, 91, 93]. The MSCs isolated from the Wharton´s jelly through PLC and chitosan type III membranes might be a potentially valuable tool to improve clinical outcome especially after trauma to sensory nerves, such as digital nerves. The results from our experimental work [55, 56] showed that the use of either undifferentiated or neuroglial-like differentiated MSCs enhanced the recovery of sensory and motor function of the rat sciatic nerve. The observation that in both cell-enriched experimental groups myelin sheath was thicker, suggest that MSCs might exert their positive effects on SCs, the key element in Wallerian degeneration and the following axonal regeneration [120]. In addition, these cells represent a non-controversial source of primitive mesenchymal progenitor cells that can be harvested after birth, cryogenically stored, thawed, and expanded for therapeutic uses, including nerve injuries like axonotmesis and neurotmesis. The time and temperature of the transport (and the saline solution used) of the UC units from the hospital / clinic to the laboratory is crucial for a successful outcome considering MSCs isolation and proliferation from fresh and cryopreserved UCT. It is highly recommend that the transport from the clinic or hospital to the laboratory should be refrigerated, and the UC units should be immediately immersed in a sterile saline solution like HBSS or DPBS.

Acknowledgements

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