

Expansion and Phenotypic Changes of Mouse Bone Marrow Mesenchymal Cells Cultured with FGF-2 and Facial Nerve-Conditioned Medium

Expansión y Cambios Fenotípicos de Células Mesenquimales de Médula Ósea de Ratón Cultivadas con FGF-2 y Medio Condicionado por el Nervio Facial

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SUMMARY: Mesenchymal cells (MCs) exhibit great regenerative potential due to their intrinsic properties and ability to restore tissue function, either directly through transdifferentiation or indirectly through paracrine effects. This study aimed to evaluate morphometric and phenotypic changes in MCs grown with facial nerve-conditioned medium in the presence or absence of fibroblast growth factor 2 (FGF-2). For quantitative phenotypic analysis, the expression of GFAP, OX-42, MAP-2, β -tubulin III, NeuN, and NF-200 was analyzed by immunocytochemistry. Cells cultured with facial nerve-conditioned medium in the presence of FGF-2 expressed GFAP, OX-42, MAP-2, β -tubulin III, NeuN, and NF-200. On average, the area and perimeter of GFAP-positive cells were higher in the group cultured with facial nerve-conditioned medium compared to the group cultured with conditioned medium and FGF-2 ($p=0.0001$). This study demonstrated the plasticity of MCs for neuronal and glial lineages and opens up new research perspectives in cell therapy and trans.differentiation.

KEY WORDS: Mesenchymal stem cells; Facial nerve injury; Fibroblast growth factor 2; Conditioned medium; Plasticity; Trans-differentiation.

INTRODUCTION

The plasticity or ability of cells to transdifferentiate has raised great interest in regenerative medicine and this ability is directly related to the potential use of these cells in tissue engineering. In this respect, mesenchymal cells (MCs) have shown promising potential. Bone marrow contains two distinct populations of stem cells (SCs): hematopoietic SCs and stromal progenitors, which can give rise to different cell types including bone, cartilage, tendons, muscles, and adipose tissue (Caplan, 1994). Stem cells have been used in combination with artificial scaffolds and acellular grafts, which contributed to the improvement of

electrophysiological, morphometric and/or behavioral recovery in animal models (Vitry *et al.*, 2003). Although their potential to produce functional myelin *in vivo* has been questioned, it has been shown that Schwann cells derived from bone marrow SCs are able to produce myelin *in vitro* (Keilhoff *et al.*, 2006).

Numerous mechanisms have been proposed to explain the regulation of cell division and self-renewal. The expansion mechanism can be symmetric or asymmetric. In asymmetric division, each SC undergoes division in which

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one cell remains at rest (original stage of rest) and the other cell, called the parent, acquires the ability to differentiate. In symmetric division, two identical daughter cells or two different cells are formed from each SC. The environment influences the biochemical and morphological properties of SCs, which adapt to the surroundings and select specific lines according to the stimuli received by their niche (Fuchs & Segre, 2000). In general, cell division and differentiation are influenced by extrinsic and intrinsic factors. Neurotrophic factors can temporarily influence these processes.

Neurotrophic factors are polypeptides that play a role in the regeneration of the peripheral nervous system. These proteins essentially comprise a set of molecules and their receptors responsible for maintaining the growth and survival of sensory and motor axons and neurons after tissue damage. Trophic factors, also known as growth factors, have been used and tested *in vitro* and *in vivo* for the regeneration of peripheral nerves. These proteins act directly on the proliferation and differentiation of different cell types, promoting tissue repair and functional recovery (Boyd & Gordon, 2003).

Several neurotrophic factors are released and act together after peripheral nerve injury to stimulate neural regeneration, including nerve growth factor (NGF), a brain-derived neurotrophic factor (Boyd & Gordon), neurotrophin 3 (NT-3) (Markus *et al.*, 2002), insulin growth factors (IGF-I and IGF-II) (Perlson *et al.*, 2004), and fibroblast growth factor 2 (FGF-2) (Terenghi, 1999). FGF-2 is a pleiotropic growth factor that is involved in several cellular pathways during embryogenesis and in adult life (Eswarakumar *et al.*, 2005; Thisse & Thisse, 2005). Although described as an inhibitor of cellular senescence, FGF-2 is also a potent mitogen for different cell types (Coutu *et al.*, 2011).

Although the implementation of SC-based strategies in clinical settings is promising, the understanding of the differentiation and proliferative capacity of SCs *in vitro*, the optimization of cell inoculation and the careful investigation of the outcome of transplanted cells are needed to ensure safety and maximum effectiveness of these therapies. The control of plasticity is a complex event that requires the transformation of cells in an undifferentiated state to a given state of development. Cell determination is viewed as a static event that is induced by intrinsic factors and aided by extrinsic factors.

According to Muñoz-Elias *et al.* (2004), MCs exhibit typical changes in neuronal morphology after 3 hours of exposure to neural induction medium. Caddick *et al.* (2006) and Mahay *et al.* (2008) demonstrated that the biochemical and functional characteristics of MCs differentiated *in vitro*

are similar to those of Schwann cells. Caddick *et al.* observed that most of the cells exhibited stretching and bipolarity after 14 days of differentiation. Similarly, Lin *et al.* (2008) demonstrated that MCs exhibit neuronal morphology after 3 days when suitably induced.

In recent years, extensive research has been done on the therapeutic efficacy of mesenchymal SCs (Matsushita, 2016). Owing to their unique abilities of self-renewal and multilineage differentiation, including neuronal, glial, and mesodermal cells (Bae *et al.*, 2011; Zeng *et al.*, 2011; De Luca *et al.*, 2013), numerous preclinical studies have reported the protective role of mesenchymal SCs in regenerative medicine (D'Souza *et al.*, 2015).

The manipulation of environmental signals can induce cell differentiation in certain lineages and can increase our understanding of the mechanisms of neural and glial development. Therefore, the aim of this study was to evaluate morphometric and phenotypic changes in MCs grown with facial nerve-conditioned medium in the presence or absence of FGF-2.

MATERIAL AND METHOD

Male Wistar rats weighing approximately 250 g were used. The study was approved by the Ethics Committee on Animal Experiments of Rio Grande do Norte State University (Protocol number 007/2012) and was conducted in accordance with the ethical principles adopted by the Brazilian Society for Laboratory Animal Science. The animals were separated from their mothers after weaning to avoid any suffering or stress and were kept in separate cages in an animal house under appropriate housing conditions, with free access to food and water, until they reached the appropriate weight and age.

Mesenchymal bone marrow SCs were collected from six 3-month-old Wistar rats. The animals were killed with an overdose of the anesthetic (ketamine and xylazine, Agener União®) and the femur and tibia were removed under aseptic conditions. Under laminar flow, 60-mm culture plates were prepared with cell culture medium. The culture medium used was KnockOut Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum, 10 U/ml penicillin G, 10 µg/ml streptomycin, and 25 µg/ml amphotericin B (Gibco®).

In animals from which MCs were extracted after euthanasia, the facial regions were shaved. The buccal and mandibular branches of the facial nerve were identified and

isolated through a preauricular approach in both antimeres. The dissected nerves were cut into 1-mm long explants. Under laminar flow, the nerve fragments were transferred to 60-mm plates containing DMEM medium supplemented with 10 % fetal bovine serum and 0.1 % gentamicin. On average, 18 explants of the facial nerve were plated on each P60 plate. Viability of the facial nerve explants was evaluated by a slight migration of fibroblastoid morphology of cells around the explants (Fig. 1).

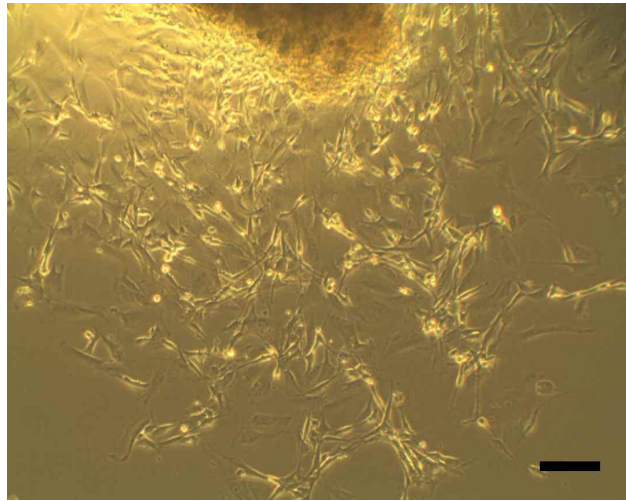


Fig. 1. Viability of facial nerve explants and cellular migration (10x). Scale bar: 100 μm .

Aliquots containing 1×10^6 cells were plated onto 48 P60 plates, which were divided into two groups: group 1, bone marrow MCs + facial nerve-conditioned medium; group 2, bone marrow MCs + facial nerve-conditioned medium + FGF-2. In groups 1 and 2, one ml of medium conditioned with facial nerve that had been transferred at least once to another plate was added. In group 2, 1 ml FGF-2 (Sigma[®]) was added at a concentration of 1:10 (Guzen *et al.*, 2012).

The cells were observed under a CKX41 inverted phase-contrast microscope (Olympus[®]) coupled to a Moticam 3.0 (Motic[®]) digital camera at three culture intervals (24, 48, and 72 hours) to monitor cell adhesion and proliferation. After 72 hours of observation, the plates were used for the immunocytochemistry procedures.

On the third day (72 hours), the medium was removed and cells adhered to the plates were washed twice for 5 minutes in 0.1 M phosphate-buffered saline (PBS), pH 7.4, fixed in paraformaldehyde (4 %) for 30 minutes, and washed three times in PBS (5 minutes each). The cells were then treated with 0.5 % Triton (Sigma[®]) for 10 minutes and washed in PBS. Next, nonspecific sites were blocked

for 30 minutes in a solution of 0.1 M PBS containing 0.2 % Triton and 1 % bovine serum albumin. The cells were then incubated for 2 hours at room temperature with the following primary antibodies: anti-mouse GFAP (Sigma, 1:400), anti-mouse OX-42 (Millipore, 1:500), anti-mouse MAP-2 (Abcam, 1:2000), anti-rabbit β -tubulin III (Millipore, 1:500), anti-mouse NeuN (Millipore, 1:500), and anti-mouse NF-200 (Abcam, 1:1200). The cells were washed in 0.1 M PBS (pH 7.4) for 5 minutes, incubated for 1 hour with anti-mouse and anti-rabbit secondary antibodies produced in donkey (Jackson, USA) and conjugated to AlexaFluor 488, AlexaFluor 594, FITC or TRITC fluorophore, and kept under refrigeration protected from light. After incubation with the secondary antibodies, the cells were washed in PBS for 5 minutes and immediately examined under a fluorescence microscope (Eclipse E200, Nikon[®]) and later under another fluorescence microscope (Eclipse Ni, Nikon[®]).

The presence of fluorescent staining in MCs was recorded, with careful examination of immunoreactivity in the subcellular, cytoplasmic, and nuclear compartments. Two previously calibrated independent investigators ($\kappa = 0.94$) measured the perimeter (μm) and area (mm^2) of each cell in nonoverlapping random fields, using cell images from three different plates at 10x magnification. The Motic Images Plus 2.0 software (Motic[®]) was used for morphological/morphometric analysis, the ImageJ software for cell count, and the Adobe Photoshop CS6.0 software (Adobe) to adjust minimum brightness and contrast of the photomicrographs.

The database was constructed using the SPSS 22.0 software (Statistical Package for the Social Sciences), followed by verification of data consistency. Descriptive statistics were used for analysis of the data (area and perimeter of immunostained cells). The results were compared by the Mann-Whitney U test and t-test for independent groups and differences were considered significant when $p < 0.05$.

RESULTS

After 72 hours of observation, immunocytochemistry was performed in the two experimental groups and different fluorescent microscope filters were used to verify the staining in MCs. Cells of group 1 (conditioned medium) expressed GFAP and OX-42 (Fig. 2 A and B, respectively). Cells of group 2 (conditioned medium + FGF-2) expressed GFAP, OX-42, MAP-2, β -tubulin III, NeuN, and NF-200 (Figs. 3 A, B, C, D, E and F, respectively).

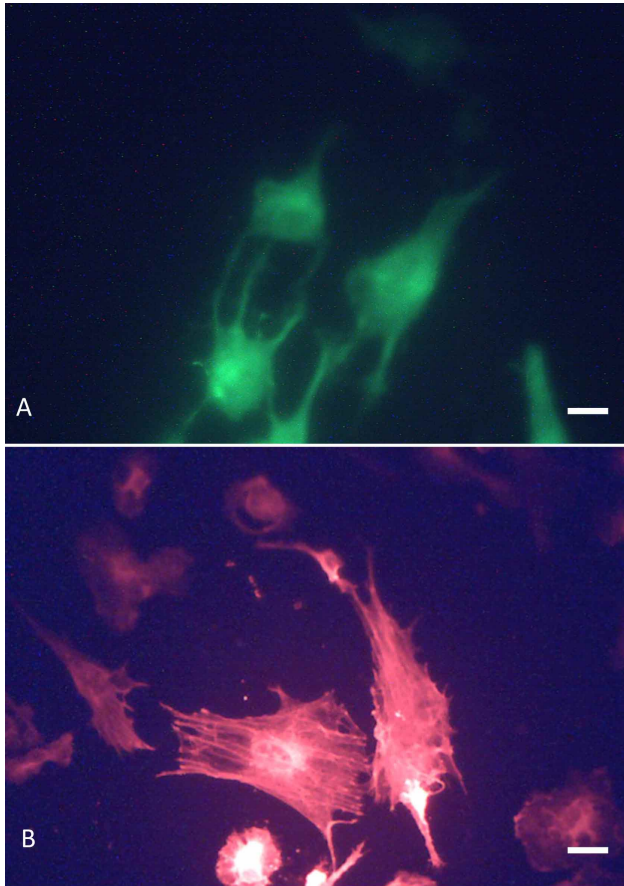


Fig. 2. Immunofluorescent staining in mesenchymal cells of group 1 treated with the GFAP/Alexa 488 (A) and OX-42/TRITC (B) antibodies (20x) on day 3. Scale bar: 100 μ m.

In group 1, migration of GFAP and OX-42 immunoreactivity from the cytoplasm to the nuclear compartment of the cells was observed, along with a higher intensity of staining. In addition, lower and narrower staining was found in the cytoplasmic compartment. In group 2, several cells with the characteristic bipolar shape were identified, as well as cells with typical mesenchymal morphology. Brighter uniform staining for b-tubulin III could be observed throughout the cytoplasm, as well as staining for OX-42 and MAP-2 in the nuclear compartment.

The average area and perimeter of GFAP-positive cells were greater in group 1 than in group 2 ($p=0.0001$) (Fig. 4).

DISCUSSION

Mesenchymal cells derived from bone marrow are considered a promising source of SCs because of their

accessibility, plasticity potential and differentiation capacity. The techniques used for the purification, expansion and osteogenic, chondrogenic and adipogenic differentiation of MCs can address the challenges of tissue engineering (Liu *et al.*, 2013). Since they are multipotent, MCs meet the requirements of an ideal cell transplant, including rapid in vitro expansion and low immunogenicity. Furthermore, MCs can differentiate into Schwann cells (Keilhoff *et al.*).

For appropriate function, cells must organize themselves in space and interact mechanically with the surrounding environment. They must have a correct conformation, be physically robust, and be appropriately structured internally. Many cells must also be able to change their shape and to migrate to other locations. In addition, every cell must be able to reorganize its internal components in response to growth processes and division and adapt to changes in the environment (Alberts *et al.*, 2010).

The mechanisms that govern the in vivo differentiation and migration of MCs to the sites of injury are still unclear; however, neurotrophic factors, cytokines and local SCs are likely to play a role. Understanding the environment in which these cells are cultured permits to improve cell expansion methods and to develop different plasticity protocols. Adult SCs are influenced by the environment that surrounds them, i.e., by epigenetic factors. Once these factors are removed, SCs are free to take a different path (Rieske *et al.*, 2009).

An important finding of the present study were the morphological changes that occurred in the cells as the days progressed. Cells grown with conditioned medium (groups 1 and 2) exhibited rapid morphological changes, resembling neurons and glial cells. Fibroblastoid and fusiform (group 2) features were also identified.

The biological functions of mesenchymal SCs (also referred to as mesenchymal stromal cells) range from tissue repair/regeneration to immunomodulatory activity (Salem & Thiemermann, 2010; Le Blanc & Mougiakakos, 2012; Ma *et al.*, 2014). Furthermore, different culture conditions or additional treatment can regulate or enhance the biological functions of mesenchymal SCs. Their multifunctional properties have made mesenchymal SCs the subject of numerous animal studies and clinical trials investigating their use to treat a wide range of diseases.

The most important factor determining cell survival in a recipient tissue is the microenvironment. Initially, this aspect is related to the expression of cell surface adhesion markers, which interact with extracellular matrix components. Added to the paracrine effects of growth factors

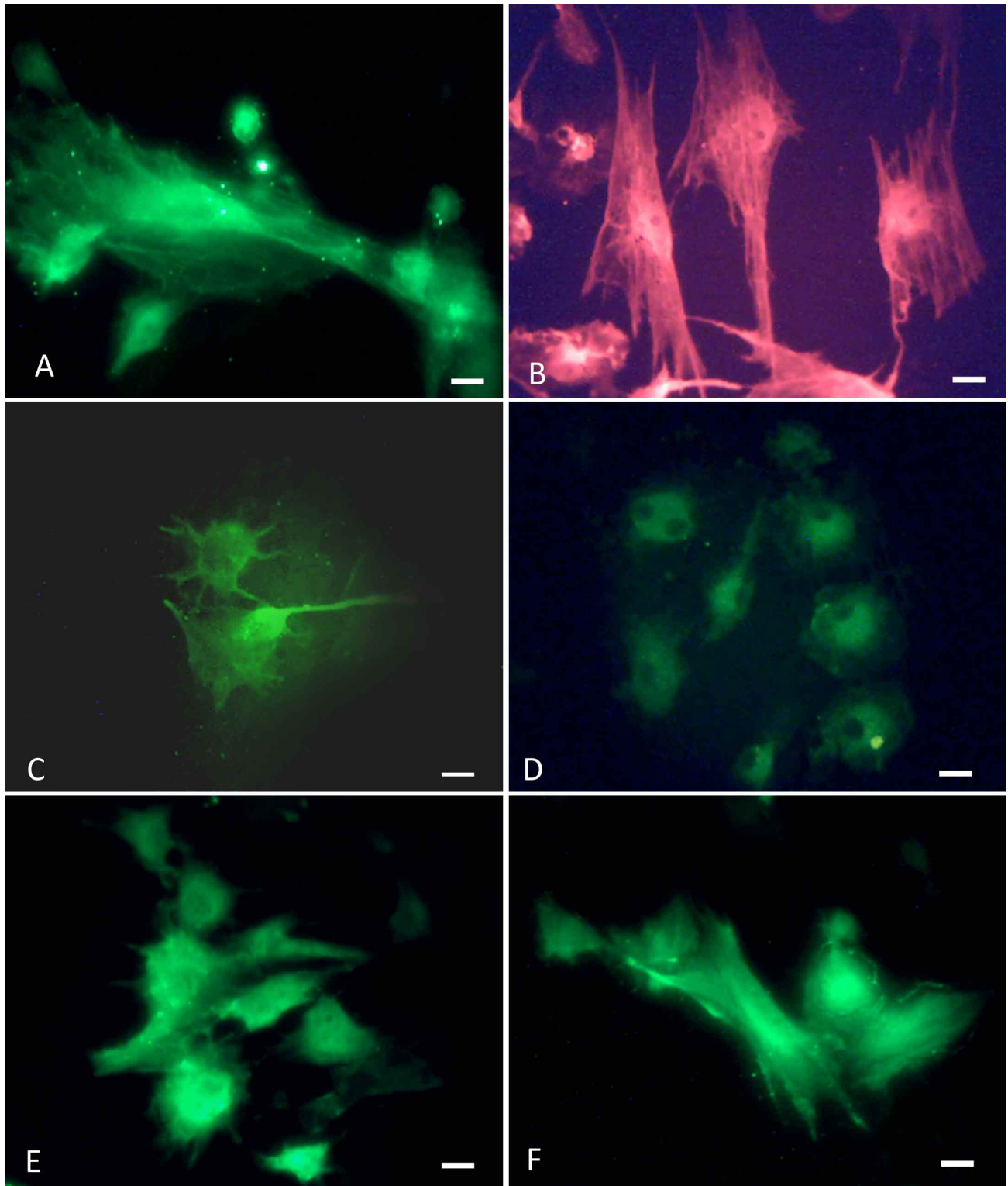


Fig. 3. Immunofluorescent staining in mesenchymal cells of group 2 treated with the GFAP/Alexa 488 (A), OX-42/TRITC (B), MAP-2/FITC (C), b-tubulin III/FITC (D), NeuN/Alexa 488 (E), and NF-200/Alexa 488 (F) antibodies (20x) on day 3 (72 hours). Scale bar: 100 μ m.

secreted by surrounding cells, the microenvironment provides conditions for survival, migration, invasion, and

tissue differentiation (Caddick *et al.*). Neuronal differentiation can currently be achieved in four ways: use

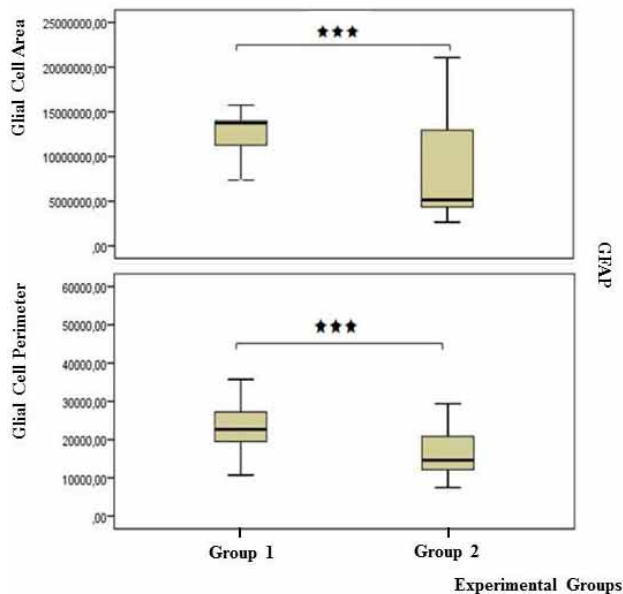


Fig. 4. Area and perimeter of GFAP-positive mesenchymal cells in groups 1 and 2 observed on day 3 (72 hours) (**p=0.0001, Mann-Whitney U test).

of neurotrophic factors or cytokines, exposure to chemical inducers, or a combination of these nerve cell co-cultures (Yang *et al.*, 2014).

In our study, the medium created from nerve fragments simulated a nerve injury situation. Thus, the explants became reactive and naturally secreted factors that would provide a favorable environment for nerve regeneration after trauma. As the properties of neurotrophic factors are quite discussed, the use of FGF-2 could enhance the phenotypic effects of cells and increase their proliferative activity.

Within this context, the exquisite specificity of antibodies makes them valuable reagents for the detection, quantification and purification of antigens and enables the use of protocols for the investigation of cell differentiation or plasticity. Thus, this study used immunocytochemistry as the technique of choice to identify the plasticity of MCs in different culture media. The antibodies used were neuronal lineage markers (MAP-2, b-tubulin III, NeuN and NF-200) and glial lineage markers (GFAP and OX-42). Our experiments showed that cells grown with facial nerve-conditioned medium in the presence of FGF-2 expressed MAP-2, b-tubulin III, NeuN, and NF-200. It was hypothesized that FGF-2 enhances the phenotypic expression of group 2 cells over a short period of time after induction (3 days). Such behavior becomes important, as it can induce a "transdifferentiation" in vitro when inoculating these cells

in order to induce regeneration of the nervous system. In fact, the histological organization of the facial nerve may contribute to the rapid release of neurotrophic factors into the medium. Thus, the microenvironment simulated by medium conditioned with facial nerve explants could explain the speed of neuronal and glial differentiation. Alternative non-anatomical explanations such as a difference in the degenerative and/or regenerative capacity of the nerves after trauma are also possible (Barghash *et al.*, 2013).

Furthermore, Zainal Ariffin *et al.* (2013) examined the direct differentiation of dental pulp-derived neurons in the absence of an inducing chemical. To confirm the differentiation of dental pulp cells into a neuronal phenotype and to demonstrate that this stage was not an artifact, the authors performed three analyses: observation of morphological changes, expression of MAP-2, and expression of neuronal markers. Two days after induction, the majority of cells resembled neurons. However, some fibroblastoid cells were still observed. After 5 days, the cells differentiated into neurons when cultured in a medium free of growth factors.

Huang *et al.* (2007) evaluated the plasticity of fat cells. For induction of cell differentiation, the cells were washed in PBS and DMEM containing 10 mM forskolin, 200 mM 0.5 % ethanol, 5 mM KCl, 2 mM valproic acid, 1 mM hydrocortisone, and 5 mg/mL insulin. After neuronal induction, adipocytic SCs exhibited changes in cell morphology, including cytoplasmic retraction and the formation of axons and dendritic projections. In addition, SCs expressed adipocytic MAP-2 and b-tubulin III along the cell body and neuritic processes similar to those observed in neurons.

Krampera *et al.* (2007) treated bone marrow MCs with different molecules and growth factors and observed rapid morphological changes typical of neuronal cells, as well as the expression of neural markers such as nestin, neurofilament, NeuN, and MAP-2. For neural induction, the cells were grown in basal medium containing DMEM and fetal bovine serum supplemented with 5 ng/mL fibroblast growth factor for 24 hours. After this pre-induction, the cells were washed in PBS and supplemented with KCl, valproic acid, and phospholine.

The control of size is essential for the development and differentiation of organs and tissues. The size of a cell depends on the cell type and its stage in the cell cycle (Kharitonova & Vasiliev, 2008). In culture, changes occur in the number, size, and area of cells during growth (Vasiliev *et al.*, 2004). The shape of the cell is controlled by internal cytoskeletal structures and adhesion molecules fixed to the

cell surface (Moizhess & Vasiliev, 2001; Rovinsky *et al.*, 2001). The control of cell shape involves the organization within the plasma membrane of a contact surface with the extracellular environment (surrounding cells, extracellular matrix, culture medium) (Levayer & Lecuit, 2008). Integrins are versatile signaling receptors that permit the exchange between the external and internal environment. Many processes such as cell morphology, motility, proliferation, differentiation, and cell death are possible because of this pathway (Docheva *et al.*, 2007).

Mesenchymal cells have been proposed as promising candidates for tissue regeneration based on their easy accessibility, rapid expansion, low immunogenicity, and multipotency. When exposed to classical chemical cocktails, MCs adopt specific phenotypes, express cell-specific markers, and exhibit functional characteristics. However, the expression of markers and functional characteristics are transient after the inducing agent is withdrawn. Within this context, it is necessary to optimize the culture conditions for differentiation in order to produce cells with stable features that can be used in clinical therapeutic procedures.

CONCLUSION

The area and perimeter of GFAP-positive cells were greater in group 1 (conditioned medium) than in group 2 (conditioned medium + FGF-2). These findings suggest that FGF-2 promotes the expression of neuronal proteins, which could contribute to peripheral nerve regeneration.

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RESUMEN: Las células mesenquimales (CM) exhiben un gran potencial regenerativo debido a sus propiedades intrínsecas y la capacidad de restaurar la función del tejido, ya sea directa-

mente, a través de la transdiferenciación, o indirectamente, a través de efectos parácrinos. Este estudio tuvo como objetivo evaluar los cambios morfométricos y fenotípicos en CM cultivadas con medio condicionado por nervio facial en presencia o ausencia de factor de crecimiento de fibroblastos 2 (FGF-2). Para el análisis fenotípico cuantitativo, se analizó la expresión de GFAP, OX-42, MAP-2, β -tubulina III, NeuN y NF-200 mediante inmunocitoquímica. Las células cultivadas con medio condicionado por el nervio facial en presencia de FGF-2 expresaban GFAP, OX-42, MAP-2, β -tubulina III, NeuN y NF-200. En promedio, el área y el perímetro de las células positivas para GFAP fueron mayores en el grupo cultivado con medio condicionado por el nervio facial en comparación con el grupo cultivado con medio acondicionado y FGF-2 ($p = 0,0001$). Este estudio demostró la plasticidad de CM para linajes neuronales y gliales y abre nuevas perspectivas de investigación en terapia celular y transdiferenciación.

PALABRAS CLAVE: Células madre mesenquimales; Lesión del nervio facial; Factor de crecimiento de fibroblastos 2; Medio condicionado; Plasticidad; Trans-diferenciación.

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