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MASTER'S DEGREE FINAL PROJECT

MOLECULAR BIOLOGY AND BIOMEDICINE

EFFECT OF HUMAN MSCs-DERIVED EXOSOMES UNDER HYPOXIC CONDITIONS ON OSTEOBLASTOGENESIS AND ADIPOGENESIS. APPLICATIONS IN BONE REGENERATION

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1. INTRODUCTION

1.1 MESENCHYMAL STEM CELLS (MSCs)

Mesenchymal stem cells, also known as stromal stem cells or MSCs (Mesenchymal Stem Cells or Mesenchymal Stromal Cells) are multipotent cells, progenitors of connective tissues (osteoblast, chondrocytes and adipocytes) as well as endothelial, cardiovascular and neurogenic lineages. (Kim et al. 2018; Rani et al. 2015). Recently, it has been also advised to use the term Medicinal Signalling cells (MSC), since, although the MSCs can be induced to differentiate in culture, they do not do this *in vivo* (Caplan 2019). MSCs have a fibroblast–like morphology, behaving as colony-forming unit-fibroblast (CFU-Fs). These cells can be isolated from different tissues, including bone marrow, fatty tissue, hair follicle, synovium, umbilical cord, placenta and periodontal ligament, among others (Casado-Díaz, Quesada-Gómez, and Dorado 2020). Since MSCs isolation from bone marrow is one of the best-documented isolation procedures to date, MSCs obtained from this source have been widely used in both cell therapy and research.

MSCs capacity to differentiate into diverse cellular lineages, as well as their anti-inflammatory and immunosuppressive activities, have made them an interesting therapeutic tool in cellular therapy and regenerative medicine. MSCs are involved in maintaining cellular homeostasis in the organism through tissue regeneration and repair. Its important therapeutic potential is mainly due to multiple features such as the fact that they are easy to culture and to cryopreserve once isolated, that they are susceptible to *in vitro* manipulation, possess strong immunomodulatory activity, are hypo-immunogenic (due to its intermediate and low levels of MHC class I and II) and finally, for their ability to undergo multilineage differentiation. Recent studies suggest that these therapeutic effects are mainly linked to their paracrine effectors, being the exosomes and extracellular vesicles (EVs) secreted by these cells, key players in these effects (Joo et al. 2020; Rani et al. 2015).

MSCs can differentiate towards different cell lineages, including adipocytes, the cells responsible for fatty tissue formation. The main transcription factor regulating adipocyte differentiation is PPARγ (Peroxisome proliferator-activated receptor γ). Its activation in response to adipogenic stimuli induces the expression of adipogenic genes involved in lipid and glucidic metabolism, giving rise to the typical adipocytic phenotype. Furthermore, PPARγ plays a primary role in the regulation of insulin sensitivity, survival and adipocyte function, as well as in the lipogenesis process (Lefterova et al. 2014). MSCs can also differentiate to produce osteoblasts or bone-forming cells. Bone formation is determined by the speed and efficiency of these precursor cells to differentiate into mature osteoblasts and produce a mineralized matrix. This process is influenced by vitamin D and parathyroid hormone (PTH). Osteoblasts produce a matrix consisting

of type I collagen, osteocalcin, osteopontin, and osteonectin, among other molecules (Rachner, Khosla, and Hofbauer 2011) that later on undergoes mineralization. Osteoblastic differentiation is controlled by several signalling pathways, among which the canonical Wnt/β-catenin pathway and the activation of the RUNX2 transcription factor stand out. Osteoblasts mature into osteocytes, which are inmerserd in the mineralized extracellular matrix and lose their ability to divide. Osteocytes influence the mineralization process by expressing factors that regulate phosphate metabolism, being esclerostine, enconded by the SOST gene, the most important. Sclerostine exerts an inhibitory effect on the Wnt/β-catenin pathway and thus, also on osteoblast differentiation and bone formation (Naji et al. 2019; Rachner et al. 2011).

MSCs differentiation towards the adipose and osteoblastic lineage is carefully balanced. However, during the aging process or in certain bone diseases, MSCs tend to increase their differentiation towards adipocytes in detriment of the osteoblast (Moerman et al. 2004; Singh et al. 2016). This produces an increase in the adiposity of the bone marrow and a decrease in bone forming cells, which favors the loss of bone mass and the appearance of pathologies such as osteoporosis and the risk of fractures (Qadir et al. 2020; Rosen and Bouxsein 2006). Therefore, therapeutic strategies that activate osteoblast differentiation from MSCs and inhibit adipocyte differentiation, can be used to promote bone regeneration and the treatment of bone pathologies (Chen et al. 2016).

1.2 EXTRACELLULAR VESICLES (EVs)

Despite the potential therapeutic applications of MSCs, current technology faces several hurdles, such as the low viability of the transplanted cells, their innate heterogenicity, unidentified factors related to aging of the donor, and the potential tumorigenicity of these cells. For those reasons, the utilization of EVs secreted by MSCs has been recognized as an optimal alternative (Kim et al. 2018).

EVs are heterogeneous lipid bilayer membrane-enclosed vesicles, released by cells in response to the micro-environmental cues.

The first observations of the existence of circulating vesicles were reported in 1946 by Chargaff and West (Randolph and Service 1946) and consisted on the identification of pro-coagulant platelet-derived particles in normal blood plasma. Later on, these particles were defined as “platelet dust” (Wolf 1967). Subsequent studies at the beginning of the 1980s determined the first insights into the subcellular origin of the released vesicles generated after the formation of multi-vesicular bodies (MVB) in reticulocytes. Soon afterwards, the term exosome was coined to design these endosomal vesicles (Harding, Heuser, and Stahl 1984; Pan and Johnstone 1983). Evidence that EV may contain ribonucleic acids (RNA) and in particular microRNA (miRNA),

and that vesicular RNA could be translated by acceptor cells (Ratajczak et al. 2006; Valadi et al. 2007), initiated an exponential growth in the field since vesicles were identified as a major tool in cell-to-cell communication. Later on, it was noted that this role was exerted in a paracrine or endocrine manner (Angulo, Royo, and Falcón-Pérez 2019).

There are three main types of EVs based on their size and mechanism of formation: exosomes, microvesicles (MVs) and apoptotic bodies. They are perceived as mediators for intercellular communication, allowing the exchange of DNA, RNA, proteins and lipids between cells (Ren et al. 2019). Among the identified subpopulations of EVs, exosome is gaining an increasing interest in immune modulation and regenerative therapy since recent reports have established that exosomes mediate much of the therapeutic properties of MSCs (Kusuma et al. 2017).

1.3 EXOSOMES

Exosomes are extracellular membrane vesicles with a size ranging from 30 to 150 nm in diameter. They have a lipid bilayer and a small cytosol devoid of any cellular organelles. They are derived from endocytic membranes and play significant roles in various biological functions, including transfer of biomolecules and regulation of physiological and pathological processes (Gurunathan et al. 2019). Exosomes contain a specific mix of proteins, lipids, RNA and DNA, depending on the producer cell type and its physiological conditions (van Niel, D'Angelo, and Raposo 2018). Due to this characteristic and also to their biocompatible nature, exosomes are considered to be promising biomarkers for the diagnosis and prognosis of various diseases, as well as a likely therapeutic tool in medicine.

Before we discuss the therapeutic use of exosomes, it is essential that we understand the basic molecular mechanisms behind the transport and function of these EVs.

1.3.1 Exosomes Biogenesis

Exosome biogenesis is a tightly regulated process that can be divided into three different stages (**Figure 1**), including the formation of endocytic vesicles by invagination of the plasma membrane; the formation of Micro Vesicles Bodies (MVBs) by inward budding of the endosomal membrane; and finally, the fusion of MVBs with the plasma membrane and release of the vesicular contents.

Cell to cell communication mediated by exosomes requires their docking and fusion to the membrane of the recipient cell. This process takes place with the aid of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNAREs) complexes and the Endosomal Sorting

Complex Required for Transport (ESCRT), that regulate the MVBs and exosome formation and release (Gurunathan et al. 2019). Tetraspanin complexes are also involved in target cell selection *in vitro* and *in vivo* (Casado-Díaz et al. 2020).

Although endosome-dependent pathway is considered the main mechanism for exosome biogenesis, some studies suggest that direct budding from the plasma membrane, as well as an ESCRT-independent pathway can also account for exosome biogenesis (Joo et al. 2020).

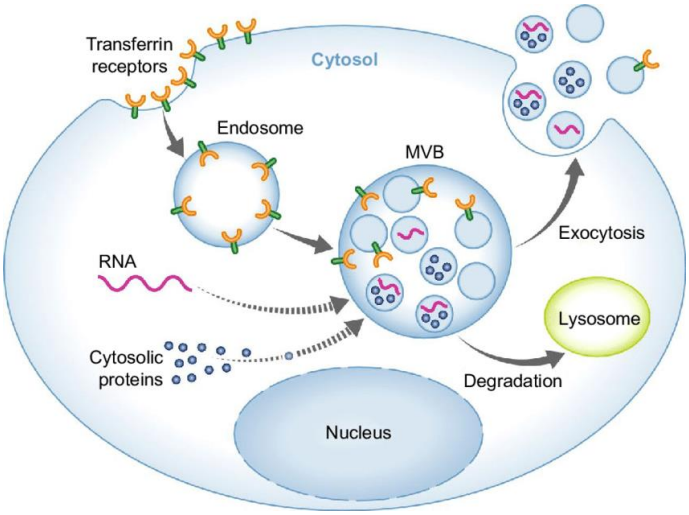


***Figure 1**. Exosome biogenesis (Vakhshiteh and Ostad 2019).*

1.3.2 Exosome Components (paracrine effectors)

The exosomal cargos depend on the cell of origin and its status at the time of exosome generation. The main components of the exosomes are proteins, lipids and nucleic acids (RNA and DNA) (**Figure 2**). Regarding the lipid composition, exosomes mainly contain components of the plasma membrane including a large amount of cholesterol, sphingomyelin, and ceramide.

Regarding the protein composition of exosomes, as exosomal formation and MVB transportation are regulated by ESCRT proteins, these proteins are expected to be found in exosomes. Besides, the most common proteins in exosomes are members of the tetraspanin family, a group of scaffolding membrane proteins that include CD63, CD81 and CD9 (Jeppesen et al. 2019); they are transmembrane proteins localized in the surface of exosome and thus, this set of proteins are often termed “exosomal marker proteins”. Other common proteins include membrane transporter and fusion proteins such as GTPases, annexins and flotillin, heat shock proteins such as HSP70, MVB biogenesis proteins such as Alix and TSG101, lipid-related proteins, and phospholipases. Exosomes also tend to be enriched in glycoproteins. Some studies indicate that there is another biogenesis mechanism independent of ESCRT. In this case, exosome

release is thought to depend on sphingomyelinase enzyme (Doyle and Wang 2019; Joo et al. 2020).

As previously mentioned, exosomes also contain mRNAs and miRNAs (Ratajczak et al. 2006; Valadi et al. 2007). ExoCarta, an exosome curated database of exosomal proteins, lipids and RNA contains 41,860 protein, 1116 lipid molecules, 3408 mRNAs and 2838 miRNAs entries, derived from studies of exosomes in several species (Joo et al. 2020).

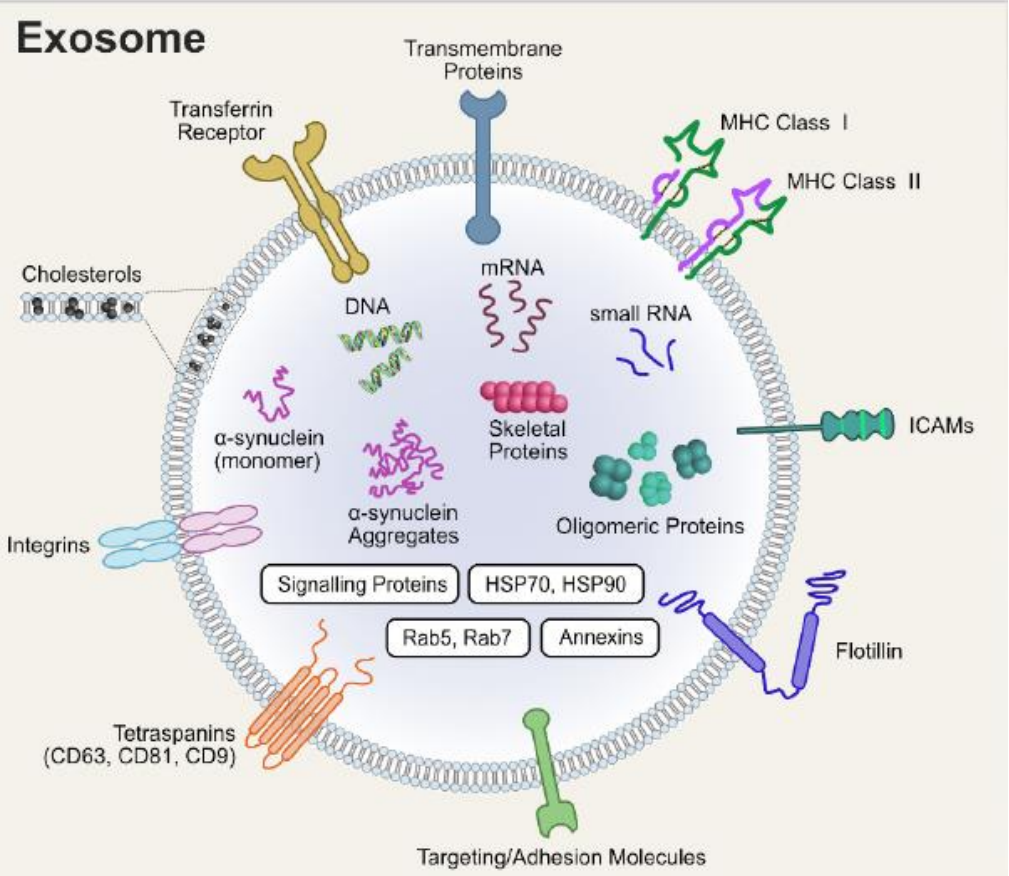


***Figure 2**. Exosomes composition and structure (Gurunathan et al. 2019).*

1.3.3 Biological functions of Exosomes

Exosomes were originally thought to be a source of cellular dumping, or a way for cells to get rid of unneeded or unwanted material, however in recent studies it has been found that exosomes participate in cell-to-cell communication, cell maintenance and tumour progression. For instance, in the nervous system, exosomes haven been found to help promote myelin formation, neurite growth, and neuronal survival, thus playing a role in tissue repair and regeneration. Also it has been found that exosomes stimulate immune response by acting as antigen-presenting vesicles (Doyle and Wang 2019).

Altogether, these features and the ones mentioned before, support the idea that exosomes play significant roles in various biological processes, such as angiogenesis, antigen presentation, apoptosis, coagulation, cellular homeostasis, inflammation, differentiation, proliferation, and intercellular signalling (Casado-Díaz et al. 2020).

Within these multiple roles, the ones that have been researched in this project are cell proliferation, differentiation and migration. Exosomes play a significant role in the development and differentiation of stem cells. MSCs have the potential to generate many different cell types that could differentiate into different cell types to replace injured or dead tissue. Exosomes are inducers of differentiation in a variety of stem cells, which could be used for tissue repair (Gurunathan et al. 2019). Specifically it has been shown that MSC-derived exosomes can induce osteogenic differentiation (depending on the stage), through modulation of the Wnt signalling pathway and endocytosis via modification of miRNA profiles, in a subpopulation of hMSCs (Wang et al. 2018).

1.3.4 Isolation of Exosomes

Exosomes have been isolated from a variety of body fluids, such as blood, semen, saliva, plasma, urine, cerebrospinal fluid, epididymal fluid, amniotic fluid, malignant and pleural effusions of ascites, broncho alveolar lavage fluid, synovial fluid, and breast milk. Some of the methods employed to isolate exosomes include differential and density gradient ultracentrifugation, ultrafiltration, size exclusion, precipitation, immunoaffinity separation and the most recently one, microfluidics-based isolation (**Figure 3**) (Gurunathan et al. 2019).

The most conventional exosome isolation method is differential ultracentrifugation, which separates exosomes by density and size. This method, however, lacks specificity, and the exosomes could be contaminated with other extracellular vesicles with similar diameter. This limitations can be overcome in part by using the combining methods that improve both yield and purity. Exosomes can be isolated also by filtration to eliminate small contaminants, or by immunoaffinity chromatography, in which antibodies in the chromatography column capture exosomes by binding to specific surface (Joo et al. 2020).

A comparative analysis of the different methods shown in **Figure 3**, based on a recent study (Patel et al. 2019) showed that the precipitation-based technique, named polymer-based in the table produced the maximum yield, followed by the gel filtration technique (a size exclusion technique), ExoCap kit and the differential ultracentrifugation. On the other hand, the affinity-based technique, consisting of purification with magnetic beads, produced the least amount of exosomes.

In our research we have combined ultrafiltration with Size-Exclusion Chromatography (SEC) techniques, isolating the exosomes by fractionation. By ultrafiltration with Amicon®Ultra-15 devices, particles < 100KDa are excluded, meanwhile SEC allows the separation of exosomes from proteins and smaller contaminants, getting high-purity preparations of exosomes.

Although this technique is not included in the table, ultrafiltration technique consists on the separation of particles based on the size and molecular weight cut off (MWCO) of the membrane being used, at the same time that it allows particle concentration. Particles larger than the MWCO of the filter (in our case was 100KDa) are retained, and the smaller ones pass through the filter into the filtrate. The main problem of this technique is the possibility of particle deformation and lysis of exosomes due to shear force. While the SEC technique consist on a column packed with a porous stationary phase in which small particles can penetrate, slowing down it movement, causing them to elute later in the gradient, after the large particles (Doyle and Wang 2019).

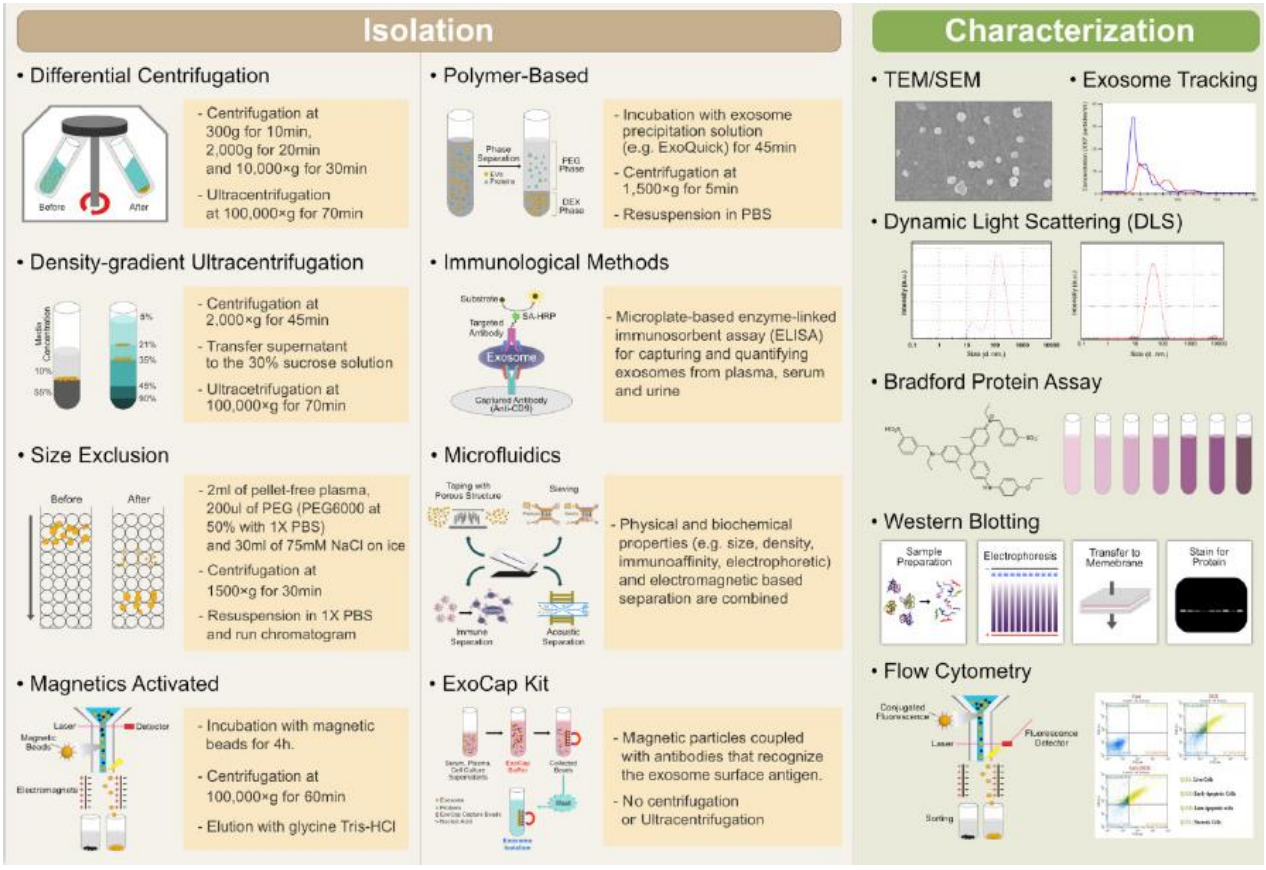


***Figure 3**. Various techniques used for isolation and characterization of exosomes. Gurunathan et al., 2019.*

1.3.5 Characterization of Exosomes

Recently, several characterization methods have been developed to analyse exosomes purity and cargo. These methods include transmission electron microscopy (TEM), scanning electron microscopy (SEM), atomic force microscopy (AFM), nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), tunable resistive pulse sensing (TRPS), enzyme-linked immunosorbent assay (ELISA), flow cytometry, fluorescence-activated cell sorting (FACS), and microfluidics and electrochemical biosensors (Gurunathan et al. 2019). Some of this techniques are shown in **Figure 3**.

In this research, exosomes have been characterized by TEM and DLS. TEM has been used to characterize the structure, morphology and size, and its working principle consist on the creation of images as a beam of electrons passes through a sample, where the electrons that do not interact with the particles, pass through the sample and are detected. These electrons are collected and magnified using special lenses. Whereas DLS, also known as photon correlation spectroscopy, is an alternative technique for measuring the size of exosomes. Its working principle is that a monochromatic coherent laser beam passes through a suspension of particles. This technique uses the fluctuations in the intensity of the scattered light due to Brownian motion of particles to estimate particle size and concentration.

Western-Blot has also been used here for the characterization of exosomes. This technique is based on the affinity binding of and antigen (target proteins such as CD9 and CD63) to an antibody that specifically recognizes this antigen. Unlike TEM, this method does not allow the observation of intact vesicles, since vesicles are lysed and the proteins are denatured in the sample preparation.

1.3.6 Applications of exosomes in medicine

Depending on their specific characteristics, exosomes can be used in disease diagnosis (as biomarkers), for drug delivery and as therapeutic agents (**Figure 4**). Exosomes could be used as diagnostic biomarkers for cancer and neurodegenerative, metabolic, and infectious diseases.

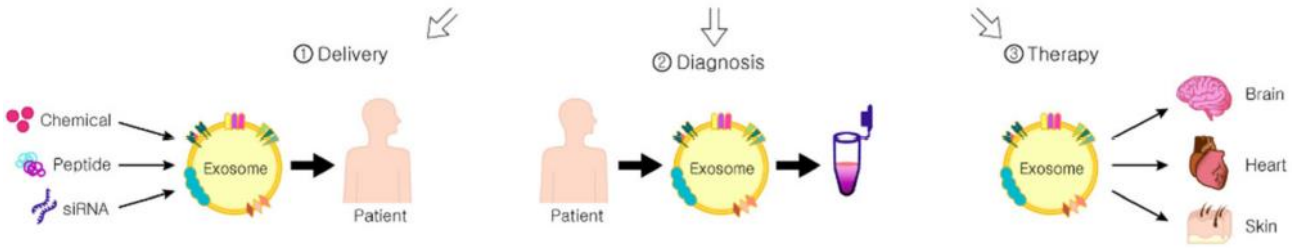


***Figure 4**. Applications of exosomes. Joo et al., 2020.*

Other features make exosomes a potential drug delivery tool, due to their ability to mediate intercellular communication, moving throughout the body via the blood and even being able to penetrate the blood-brain barrier without inducing an immune response. Moreover, exosomes can be used as an alternative cell therapy in a safer and easier manner, because in contrast to cells, exosomes cannot mutate or proliferate (Joo et al. 2020). For that reason, in this work we are going to focus on therapeutic application (**Figure 5**), described in detail below.

1.3.7 Therapeutic applications of Exosomes

Exosomes therapeutics are finally entering human testing (**Figure 5**). Extracellular vesicles are able to produce the same paracrine factors and tissue regeneration effects as stem cells, but with much better features. Exosomes are not expected to have toxicity issues, can be engineered and functionalized and are incapable of division, so there is no risk of malignancy. They are also more robust than cells, because are more stable *in vivo* and also can be lyophilized and stored without loss of efficacy. And last but not least, both autologous and allogeneic exosomes are non-immunogenic. With all these features, companies have started to research and develop exosomes as a new treatment. For instance, the company Aegle is raising funds to enter the clinic in 2020 with a mixture of exosomes and microvesicles-derived from MSCs to treat skin diseases (Zipkin 2019).

Recent studies have demonstrated that exosomes are effective therapeutic reagents. In particular, exosomes derived from MSCs have been tested in various disease models, such as respiratory, musculoskeletal, cardiovascular, neurological, hepatic, gastrointestinal, dermatological, and renal disease (Willis, Kourembanas, and Mitsialis 2017). MSC-derived exosomes inhibit the expression of pro-inflammatory cytokines, and promote tissue regeneration by enhancing extracellular matrix remodelling. They also recapitulate the immunomodulatory and cytoprotective activities of their parent cells (Gurunathan et al. 2019). Since exosomes have the ability to cross the blood-brain barrier, this vesicles may also represent a new strategy for treating neural injuries and neurodegenerative diseases. In fact, it has been reported that exosomes exhibit neuroprotective effects after stroke, increasing nerve regeneration and neurological recovery, regulating neurogenesis, angiogenesis and axonal plasticity (Joo et al. 2020).

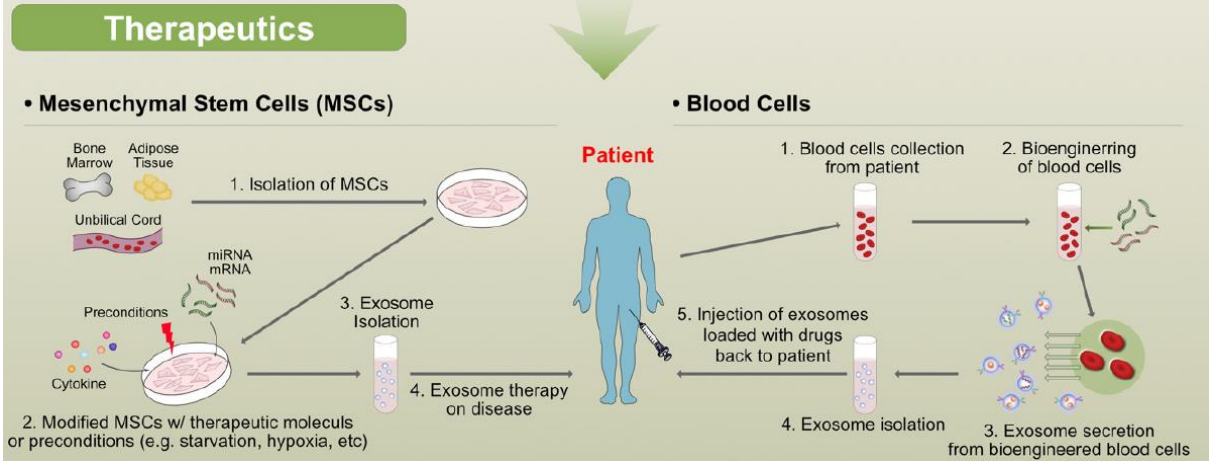


***Figure 5**. Therapeutic applications of exosomes. (Gurunathan et al. 2019).*

1.3.8 Pre-conditioning approaches to increase exosomes efficacy

Stem cells live in niches, that is, a microenvironment that plays an important role in directing their functions, differentiation and division. Some studies suggest that exosome yield, the cargo they carry and their characteristics, can vary substantially depending on the status of the MSCs from which they are derived and are influenced by these microenvironment conditions. For that reason, it has been shown that different pre-conditioning approaches can stimulate and increase the efficacy of exosomes. In particular, those conditions include cytokines, hypoxia, trophic and physical factors, and chemical and pharmacological agents (Ding et al. 2019). Confluence of cell cultures also seems to play a critical role, as confluent cultures produce more exosomes than preconfluent ones, however, contact inhibition between cells can reduce the release of exosomes, since these cells are entering into quiescence and are not actively dividing (Gurunathan et al. 2019).

Exosomes derived from MSCs cultured in priming culture conditions that mimic the *in vivo* microenvironmental niche (primed MSCs) are packaged with a distinct profile of metabolites than those of the classically cultured MSC. The majority of these metabolites have been associated with immunomodulation (Showalter et al. 2019). Hypoxia preconditioning for MSCs can be used to elevate the levels of paracrine effectors in exosomes and for promoting neovascularization (Han et al. 2019). Hypoxic preconditioning could also increase angiogenesis, neuroprotection, proliferation and migration of MSCs, by using deferoxamine (DFO) as a hypoxic-mimetic in the cell culture (Ding et al. 2019). At the cellular level, hypoxia induces activation of the hypoxia-inducible factor-1α (HIF1α). This transcription factor under normoxia conditions is hydroxylated, ubiquitous, and degraded by the cytoplasmic proteasome. However, in the face of tissue damage, ischemic processes or exposure to hypoxia, the decrease in oxygen levels inhibits the hydroxylation of HIF1α, which causes its accumulation and translocation to the nucleus, where it

induces the expression of genes involved in adaptation to hypoxia and related to angiogenesis, scarring, anaerobic glucose metabolism, erythropoiesis, proliferation, differentiation and apoptosis, among others. It has been shown that hundreds of genes can be transcriptionally regulated by HIF1α (Mole et al. 2009), including SDF-1α and vascular endothelial growth factor (VEGF), which in endothelial cells induces angiogenesis and improves endothelial function.

In our research, MSCs have been cultured in both normoxia and hypoxia, to be able to compare the effects of these two conditions in characteristics such as migration, differentiation and proliferation.

2. OBJETIVES

This TFM has been carried out in group GC17 "Pathophysiology of the endocrine system of vitamin D. Biotechnology and aging" of the Maimonides Institute for Biomedical Research of Córdoba (IMIBIC). Among the lines they develop, there is a project whose objective is the evaluation of the effects of exosomes derived from mesenchymal stem cells grown in hypoxia, on tissue regeneration.

As explained previously, exosomes are considered the ultimate therapeutic tool in biomedicine. For this reason, the main objective of this research is:

- Study the effect of exosomes derived from MSCs cultured in hypoxia on MSC differentiation towards the osteoblastic and adipocytic lineages.
- As previously explained, preconditioning in hypoxia could increase the effectiveness of exosomes to induce angiogenesis and regulate cell differentiation. Therefore, potentially, they could be used for the treatment of bone pathologies and tissue regeneration.

To undergo this project, different secondary objectives have had to be carried out, such as:

1. Exosomes isolation from human bone marrow MSCs, grown in hypoxia or normoxia.
2. Characterization and quantification of these exosomes by Western Blot, Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS).
3. Study the effect of different concentrations of exosomes obtained in hypoxia and normoxia on the proliferation, migration and chemotaxis of MSCs and Human Umbilical Vein Endothelial Cells (HUVECs).
4. Study in HUVEC the effect of the two types of exosomes on angiogenesis.
5. Evaluate the effect of exosomes obtained in hypoxia and normoxia on the differentiation of MSC into osteoblast and adipocytes.

3. MATERIALS AND METHODS

3.1 Culture and Expansion of MSC and HUVEC

The MSCs used in this work come from the collection at the IMIBIC GC-17 research group. These MSCs were previously isolated from the bone marrow samples of healthy individuals, provided by the hematology service of the Reina Sofía University Hospital in Córdoba.

After isolation, the cells were cryopreserved in liquid nitrogen in α-MEM culture medium (Capricorn Scientific GmbH) supplemented with 30% FBS (fetal bovine serum) (Gibco) and 5% DMSO, until thawed for completion of the different studies carried out in this work. Thawed MSCs were seeded in 75 cm^2 culture bottles (Nalgene-Nunc-Thermo Fisher Scientific), in α-MEM culture medium supplemented with 10% FBS, 1% ultra-glutamine, 30µl/100ml streptomycin, 40 µl /100 ml of penicillin and 1 ng / ml of FGF-2 (Fibroblast growth factor-2).

The culture medium was replaced by fresh media every 3-4 days. Upon reaching 80-90% confluency, cells were detached with trypsin-EDTA (Gibco) and replated into new 75 cm^2 culture bottles at a split ratio of 1:2,5. In the last passage, the final split ratio was 1:6.

Human umbilical-vein endothelial cells (HUVEC) were obtained from Lonza. After isolation, cells grown in endothelial basal-medium (EBM), with 10% FBS and supplemented with growth factors, known as endothelial growth medium (EGM) from the same manufacturer (EGM BulletKit). Cultures were maintained at 37°C with 5% CO_2 and 95% humidity. Cells were detached from culture surface with trypsin (Gibco) and replated into new culture bottles, for maintenance, expansion and required experiments.

3.2 Collection of Exosomes

Upon reaching around 70% confluency, the culture media was replaced with fresh media supplemented with exosome-free FBS (5%). After the media change, cells were subsequently cultured for additional 48 h in different conditions: hypoxia (5% CO_2, 3% O_2 and 37ºC) and normoxia (5% CO_2, 16% O_2 and 37ºC), to stimulate the secretion of exosomes into the medium.

After incubation, MSC media was harvested and centrifuged with Amicon® Ultra-15 Centrifugal Filter Devices to concentrate the medium and exclude the particles <100KDa. Later, to exclude smaller particles, were used a Size Exclusion Chromatography columns for Exosome and Microvesicle isolation (HansaBioMed) getting 10 fractions of 750 µL in which exosomes will be between fraction 2-6.

This fractions are the ones that will be centrifuge again with Amicon® Ultra-15 Centrifugal Filter Devices to be concentrated 15 times more.

3.3 Characterization of Exosomes

The morphology of exosomes was analysed by TEM. Briefly, samples were applied to carbon-coated copper grids (20 µL was applied on top). The solution was allowed to evaporate at room temperature with no humidity control. The samples on the grids were stained with 2% (w/v) uranyl acetate (UrAc) for 1 min. Excess of UrAc was blotted-off. Results were recorded with the High Resolution Transmission Electron Microscope JEOL JEM 1400 (SCAI, University of Córdoba) at an acceleration voltage between 80 to 200 keV.

Nanoparticle analysis was conducted to determine the size and number of particles/ml, using Nanosight NS300 (Department of Analytical Chemistry, University of Cordoba).

The amount of exosomal protein was estimated by the Nanodrop Lite method at 280 nm of absorbance.

3.4 Western Blotting

For the extraction of total proteins from different cell cultures, after removing the culture medium and washing the wells with physiological serum, cells were lysed with "Cell Extraction Buffer" (Thermo Fisher Scientific) supplemented with 1 mM of phenylmethylsulfonyl fluoride (PMSF) and 50 µl/ml protease inhibitor cocktail (PIC) (both from Sigma-Aldrich). The lysate, once collected, was incubated on ice for 30 minutes, with vortex stirring every 10 minutes. Finally, the lysate was centrifuged for 10 minutes at 13000 g at 4ºC, the precipitate of cellular remains was discarded and the supernatant was transferred to a new tube that was stored at -20ºC until its use. Protein concentration was quantified with the "Bio-Rad DC Protein Assay" kit (Bio-Rad) according to the manufacturer's protocol. For protein extraction from exosomes, the protocol was the same. The vesicles were lysed with "Cell Extraction Buffer" supplemented with 1 mM PMSF and 50 µl/ml PIC, and de protein concentration quantified with the Bio-Rad DC Protein Assay.

Subsequently, between 2 and 10 µg of protein from each sample were loaded into an 8-16% acrylamide gel (nUView Tris-Glycine Precast Gels, NuSeP) under denaturing conditions and run on a "Mini-Protean" electrophoresis system (Bio-Rad) at 200V for 40-50 min. After electrophoresis, proteins were transferred to a Polyvinylidine Difluoride (PVDF) membrane (Bio-Rad) using a Trans-Blot Turbo Transfer System (Bio-Rad). After the proteins were transferred, the membrane was blocked with a 5% solution of skimmed milk powder in T-TBS buffer (20 mM

Tril-HCl pH 7.6, 150 mM NaCl, 0.05% Tween) for 1 hour at room temperature. Subsequently, the membrane was incubated overnight at 4°C with the primary antibodies, anti-CD9 (1:700), anti-CD63 (1:700) (both from Invitrogen, ThermoFisher Scientific) or anti-calnexin (1:1000) from Sigma-Aldrich, in 1% milk in T-TBS. Following incubation, the antibody solution was removed and the membrane washed with T-TBS 3 times and incubated with the secondary antibody, anti-mouse IgG H & L-HRP (1:5000) (Invitrogen, ThermoFisher Scientific) for CD9 and CD63 and anti-Rabbit IgG H & L-HRP (1:3000) (Abcam) for calnexin in 1% milk on T-TBS for 1 hour. Finally, after washing with T-TBS, the membrane was developed using the "Clarity Western ECL Substrate" (Bio-Rad) and visualized in a Bio-Rad ChemiDoc ™ XRS + gel documentation system. The acquisition and analysis of the images was carried out through ImageLab software from the same commercial house.

3.5 Quantification of gene expression by PCR

RNA from the different samples was isolated using the "NZY total RNA isolation kit" (NZYTech), following manufacturer's instructions. Nucleic acids were quantified with a NanoDrop ND-1000 Spectrophotometer from Thermo Fisher Scientific at 260 nm. Next, up to 900 ng of RNA was retrotranscribed into cDNA using "iScript cDNA Synthesis Kit" (Bio-Rad), according to the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR) was carried out on a LightCycler 96 Instrument from Roche Applied Science. Each PCR reaction was performed in a 10 ul volume containing 1 µl of cDNA, 1 µM of primers (**Table 1**) and 1X of "SensiFAST Sybr No-Rox Mix" (BIOLINE). The PCR amplification program included one cycle at 95ºC for 2 minutes (DNA denaturation and activation of DNA polymerase) and 40-45 cycles of 95°C for 5 seconds (DNA denaturation) and 65ºC for 30 seconds (hybridization of the primers and extension of the product). The results were analysed with LightCycler 1.1 software from the same manufacturer. POLR2A Polymerase (RNA; DNA directed) II polypeptide A (POLR2A) was used as the constitutive gene.

Gene	Forward and reverse primer-sequence (5' → 3')	Product size (bp)
Runt-related transcription factor 2 (***RUNX2***)	TGGTTAATCTCCGCAGGTCAC ACTGTGCTGAAGAGGCTGTTTG	143
Osterix (***SP7***)	AGCCAGAAGCTGTGAAACCTC AGCTGCAAGCTCTCCATAACC	163
Collagen, type I, alpha 1 (***COL1A1***)	CGCTGGCCCCAAAGGATCTCCTG GGGGTCCGGGAACACCTCGCTC	263
Integrin-binding sialoprotein (***BSP***)	AGGGCAGTAGTGACTCATCCG CGTCCTCTCCATAGCCCAGTGTTG	171

Peroxisome proliferator-activated receptor gamma 2 (***PPARG2***)	GCGATTCCTTCACTGATACACTGGAG TGGGAGTGGTCTTCCATTAC	136
Lipoprotein lipase (***LPL***)	AAGAAGCAGCAAAATGTACCTGAAG CCTGATTGGTATGGGTTTCACTC	113
Fatty-acid-binding protein 4 (***FABP4***)	TCAGTGTGAATGGGGATGTGAT TCTGCACATGTACCAGGACACC	162
Fatty acid synthase ***(FASN***)	AAGCTGAAGGACCTGTCTAGG CGGAGTGAATCTGGGTTGATG	146
Polymerase (RNA; DNA directed) II polypeptide A ***(POLR2A***)	TTTTGGTGACGACTTGAACTGC CCATCTTGTCCACCACCTCTTC	125

Table 1. Primer sequences and amplicon sizes.

3.6 Cell viability assay

Cell viability was determined using 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich). MSCs and HUVEC were seeded in 96-well plates at a density of 4000 and 8000 cells per well, respectively, in culture medium and incubated for 24 hours prior to treatment. Subsequently, cells were treated in culture medium supplemented with exosome free FBS, with different concentrations of normoxia or hypoxia MSC-derived exosomes ($3x10^7$, $9x10^7$ and $15x10^7$ part/ml). At 48 hours, the culture medium was removed and was added 100µl of DMEM (Dulbecco's Modified Eagle Medium) (Capricorn Scientific GmbH) with 1 mg/ml MTT, without FBS or phenol red. Cell cultures were incubated at 37°C for 2 hours. The medium was then removed and the formazan crystals formed during the incubation were dissolved in 100% isopropanol. Finally, in the resulting solution, the absorbance was measured at 570nm and corrected at 650nm, with a PowerWave XS microplate spectrophotometer (BioTek Instruments).

3.7 Cell migration assay

MSCs migration was evaluated using the "Oris™ Cell Migration Assay" (Platypus technologies). MSCs were plated in 96-well plate (15000cells/well) and incubated at 37ºC with cell seeding stoppers in each well, until 90% confluence. Then the stoppers were removed leaving a 2 mm halo in the middle of the well, as a scratch. Meanwhile, HUVEC were plated in 24-well plate (20000 cells/well) and incubated at 37ºC until 90% confluence. The confluent monolayer was scratched using 200 µl pipette tip and were visualized in the microscope to verify the correct scratch. Each well was washed twice with PBS.

Then, for MSCs, α-MEM + 2% FBS (exosome free) was added containing normoxia MSC-derived exosomes (MSC-ExoN) or derived from hypoxia (MSC-ExoH), in a concentration of

$3x10^7$, $9x10^7$ and $15x10^7$ part/ml, or PBS for the control. In HUVEC the medium used was EBM + 10% FBS (exosome free) with MSC-ExoN or MSC-ExoH in a concentrations of $3x10^7$ and $6x10^7$ part/ml.

Images for 3 fields of view per scratch were taken at 0h, 12h and 18h (just in MSC), using a phase-contrast microscope Incucyte® Systems for Live-Cell Imaging. The level of migration was measured by the ratio of closure area to initial wound area (t=0), as follows: migration area (%) = (A0-A12h)/A0 x 100, where A0 represents the area of initial wound area, and A12h represents the residual area at the metering point, that's to say, after 12 hours. The wound area was measured using *ImageJ* software.

3.8 Cell differentiation assay

MSCs were seeded in P12 or P24 culture plates (Nalgene-Nunc-Thermo Fisher Scientific) and once a confluence of between 60 and 80% was reached, they were induced to differentiate into adipocytes or osteoblasts in presence or absence of $3x10^7$ part/ml of MSC-ExoN or MSC-ExoH. To induce adipocyte differentiation, the culture medium without FGF, was supplemented with $5x10^{-7}$ M dexamethasone (DXT), 50 µM indomethacin and 0.5 mM isobutylmethylxanthine. On the other hand, the differentiation to osteoblasts was carried out by supplementing the culture medium with 10^{-8} M dexamethasone, 10 mM β-glycerolphosphate and 0.2 mM ascorbic acid. These cells were allowed to grow for 3-4 weeks to allow mineralization of the osteoblasts. All the inducers and supplements of the cultures were from Sigma-Aldrich.

After 13 days from the start of differentiation, culture samples from the different treatments were collected for RNA extraction and analysis of gene expression of osteoblastic or adipocytes gene markers.

3.8.1 Mineralization staining

In MSC induced to osteoblast, at 15 days, the matrix mineralization was evaluated by alizarin-red staining. Cultures were fixed with 3.7% formaldehyde for 10 min and stained with a solution of alizarin red 40 mM, pH 4,1. All reagents were from Sigma-Aldrich. Then, wells were washed with isopropanol 60%, dried and visualized under the microscope. Alizarin-red deposits were eluted with 10% acetic acid and neutralized with 10% ammonium hydroxide. Elutes were quantified by absorbance at 405 nm, using a PowerWave XS microplate spectrophotometer from BioTek Instruments.

3.8.2 Fat vesicles staining

Fat-droplet formation in cultures induced into adipocytes was evaluated by oil-red O staining at 13 days of differentiation. Cultures were fixed with 3.7% formaldehyde for 20 minutes and stained with a solution made by mixing 8.2 ml of 0.3% oil red (w/v in isopropanol) with 6.8 ml of distilled water. After 15 to 20 min incubation, cells were washed with distilled water, stained with hematoxylin and visualized by optical microscopy. Nine pictures (200x) were obtained from each well (three well in total) and the oil-red O staining was quantified with *ImageJ* software and normalized with the cell number of each picture

3.9 Statistical analysis

The comparison between the different treatments was performed using the ANOVA test to detect significant changes followed by a Tukey test to identify significant differences between treatment pairs. Significant changes have been considered for $p < 0.05$. At least 3 data per studied parameter have been obtained. Data are shown as the mean plus standard error of the mean (Mean ± SEM).

4. RESULTS

4.1 Characterization of Exosomes

Exosomes were isolated from the supernatant of human MSCs culture, grown under normoxia or hypoxia (3% O_2) conditions, by Size Exclusion Chromatography. We collected ten fractions of 750 µL and the absorbance of each fraction was measured at 280 nm, as an estimate of the amount of protein. As shown in the **Figure 6A**, the protein concentration increases after fraction 7. This indicates that the exosomes free of soluble protein contained in the FBS of the medium are found in the previous fractions.

To ensure correct isolation of exosomes in samples, we performed a Nanoparticle Tracking Analysis (NTA) showing the mean sizes of exosomes derived from normoxia MSCs (MSC-ExoN) and hypoxic MSCs (MSC-ExoH) to be around 180 nm (**Figure 8**). Therefore, we collect fractions from 2 to 6 to mix them, concentrate them by ultrafiltration and characterize the exosomes.

The transmission electron microscopy showed the classic native spherical morphology and small vesicle structure of exosomes (**Figure 6B**).

Western blot analysis (**Fig. 7**) demonstrated that these vesicles displayed exosomal surface markers (CD9 and CD63) and that they are negatives for Calnexin, an endoplasmic reticulum protein not present in extracellular vesicles.

A **B**

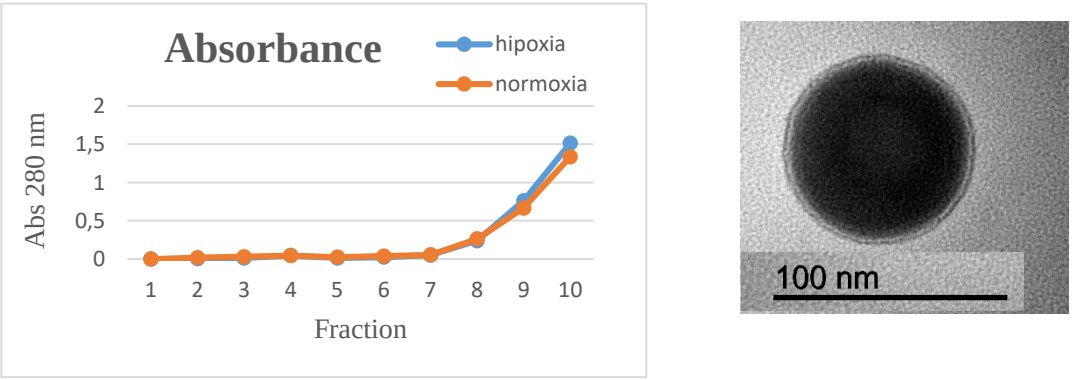


***Figure 6. Characterization of exosomes derived from MSC-N and MSC-H**. (A) Absorbance of fractions obtained from Size Exclusion Chromatography, measured at a wavelength of 280 nm. (B) TEM analysis of exosomes. Scale bar is 100 nm.*

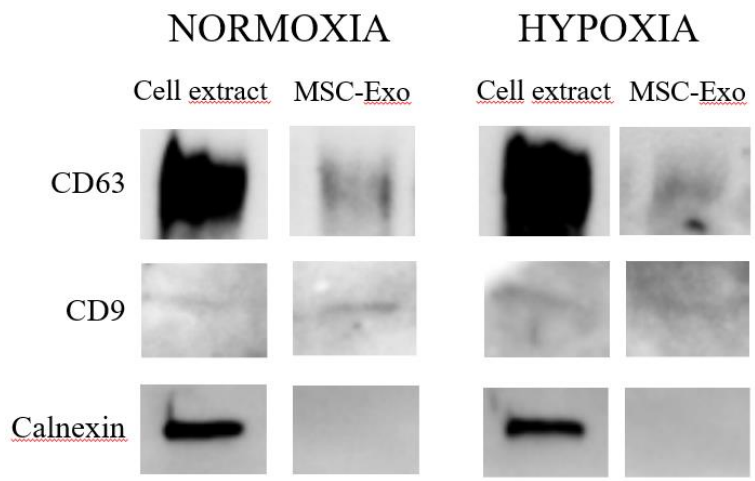


Figure 7. Western Blot*. Western Blot analysis of positive (CD63 and CD9) and negative (Calnexin) exosomal markers in exosome and cell protein extracts, derived from MSC cultured under normoxia and hypoxia.*

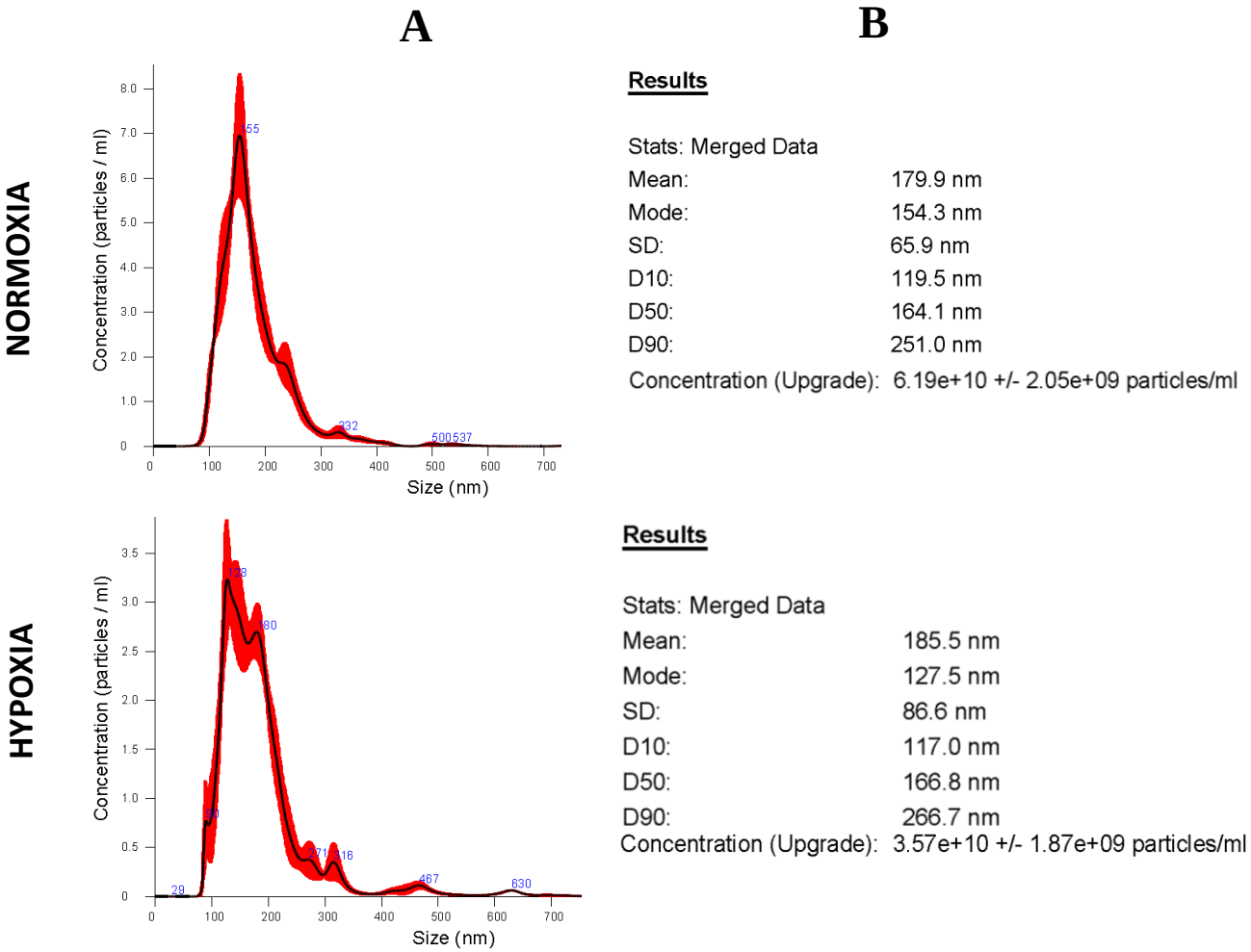


Figure 8. The size of exosomes measured by Nanosight analysis. *(A) The curves showed the particles size distribution of MSC-ExoN and MSC-ExoH. The x-coordinate represents the particle diameter. (B) Concentration analysis of MSC-ExoN and MSC-ExoH. The mean diameter and other parameters were shown.*

4.2 Effect of Exosomes on Viability

For a functional analysis, we investigated whether different concentrations of MSC-ExoN and MSC-ExoH affect the growth of MSCs and HUVEC. Interestingly, in MSC the higher concentrations of exosomes increased the viability, in both cases (**Figure 9A**), being slightly higher in the case of MSC-ExoH. But in contrast, in HUVECs, higher concentrations of exosomes inhibit viability (**Figure 9B**).

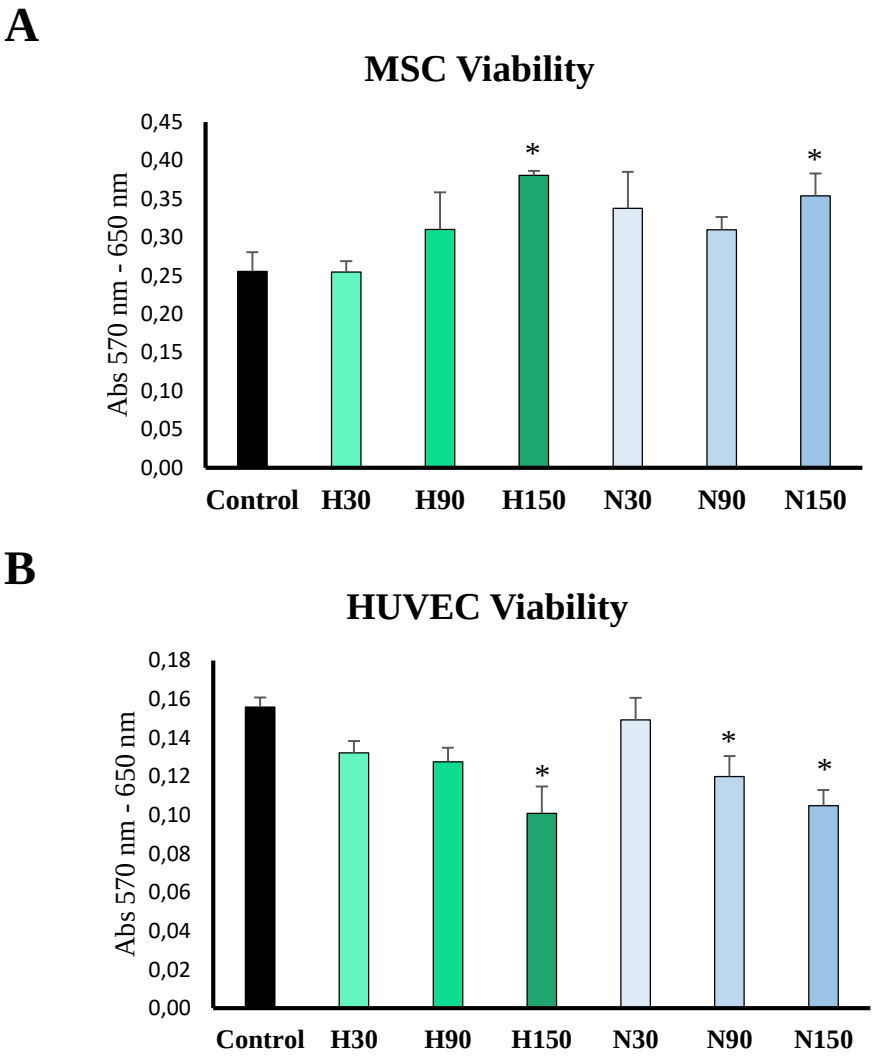
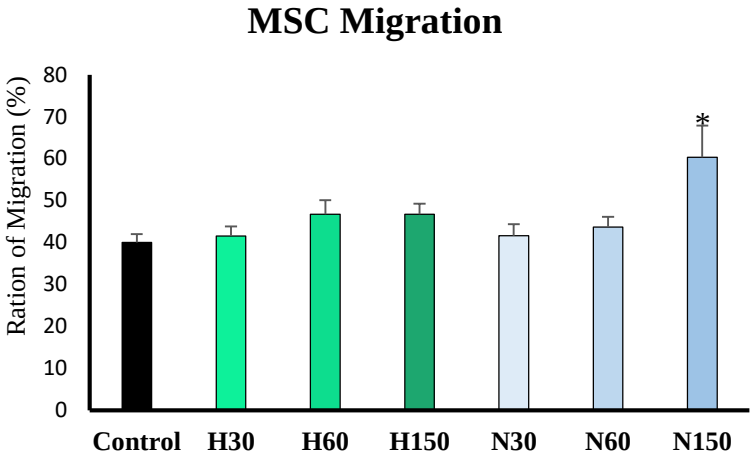


Figure 9. MSCs (A) and HUVEC (B) viability analysis by MTT assay*. At 48 h of exosomes treatment, the viability was measured by MTT assay. Treatment legend: Exosomes derived from MSC or HUVEC, cultured under hypoxic (H) and normoxia (N) conditions, in concentrations of $3x10^7$ part/ml (30), $9x10^7$part/ml (90) and $15x10^7$part/ml (150). * p< 0.05 vs Control (cells not treated).*

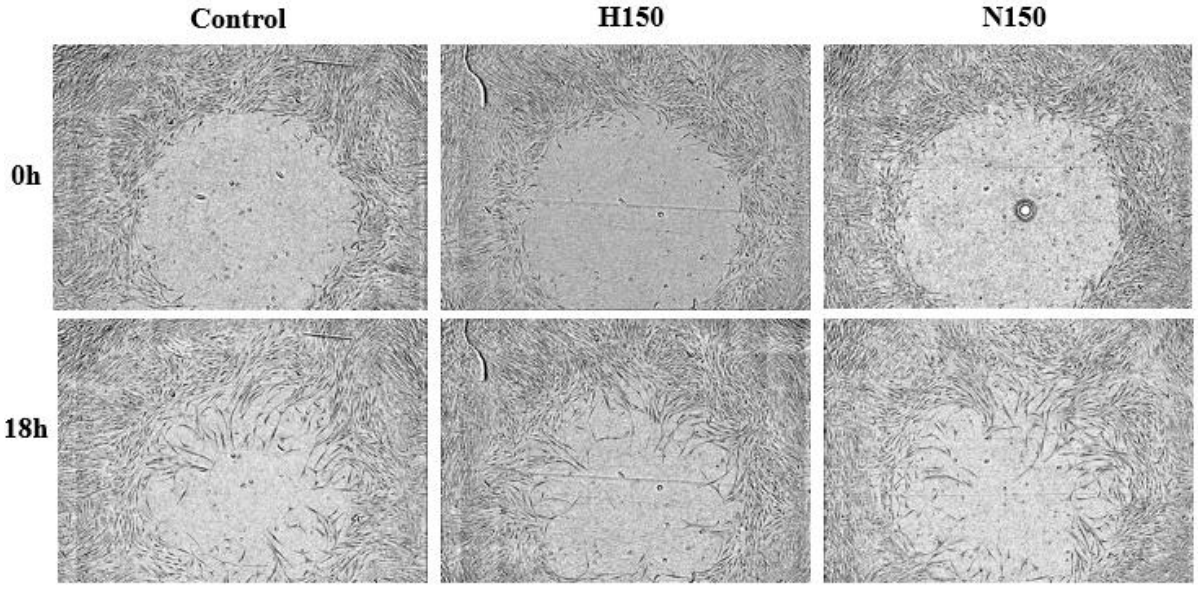
4.3 Effect of Exosomes on Migration

To test the effect of MSC-ExoN and MSC-ExoH on MSCs and HUVEC migration, scratch test assay was employed. As shown in **Figure 10A and 10B**, in MSC migration, although an increase is observed with the exosome concentration, it is only significant with MSC-ExoN at a concentration of $15x10^7$ part/ml ($p<0.05$ vs. Control). In HUVEC (**Figure 10C and 10D**), we have not seen significant changes, since migration was similar to the cultures not treated with exosomes.

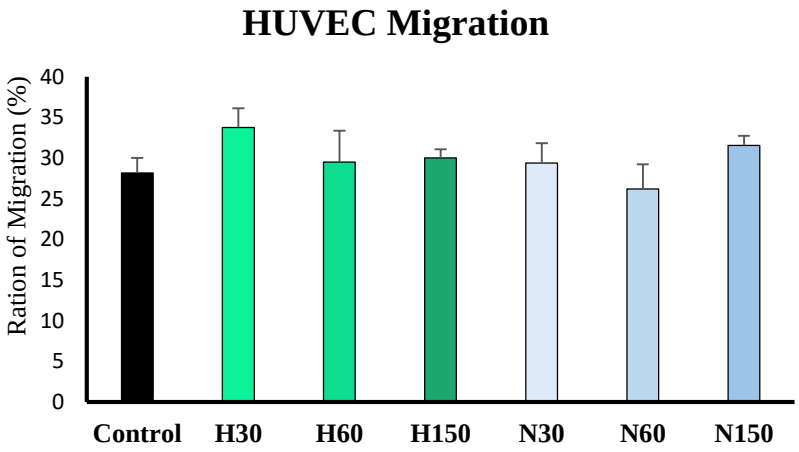
A



B



C



D

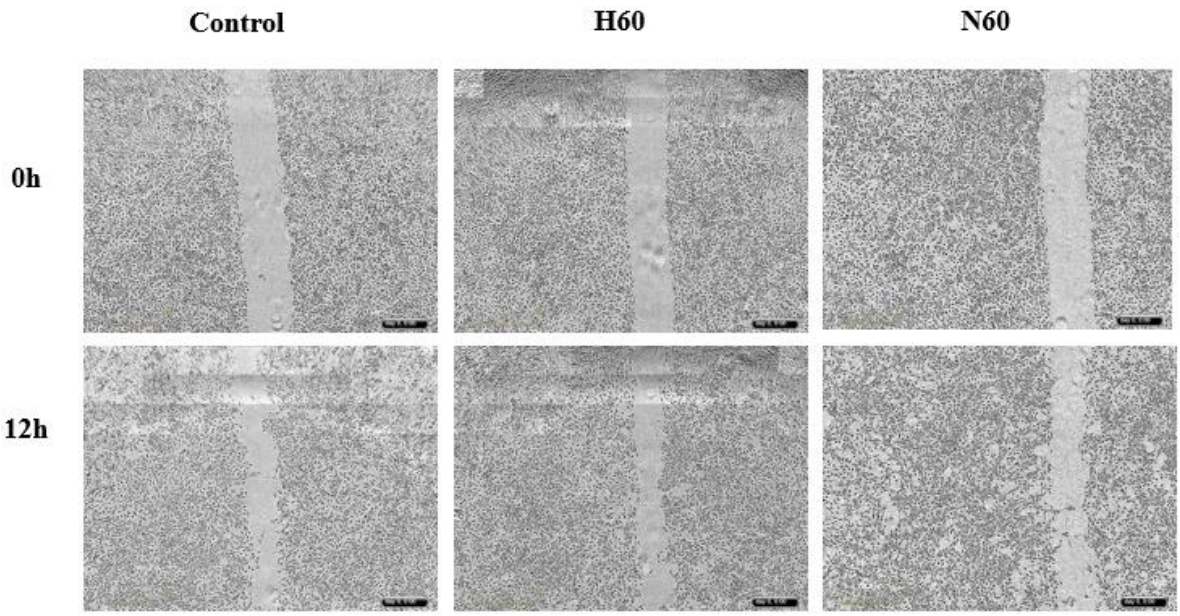


Figure 10. Migratory effects of MSC-ExoH and MSC-ExoN on MSCs (A,B) and HUVEC (C,D). *(A,C) Quantification of the closure area was presented as the ratio of closure area to the initial wound area. The diagram illustrates the mean ± SEM of 3 independent experiments (*p<0.05). (B,D) Scratch assay of MSCs (B) or HUVEC (D) with MSC-ExoN (N) or MSC-ExoH (H) at $3x10^7$ part/ml (30), $6x10^7$ part/ml (60) and $15x10^7$ part/ml (150) concentration, or equal volume of PBS in the control.*

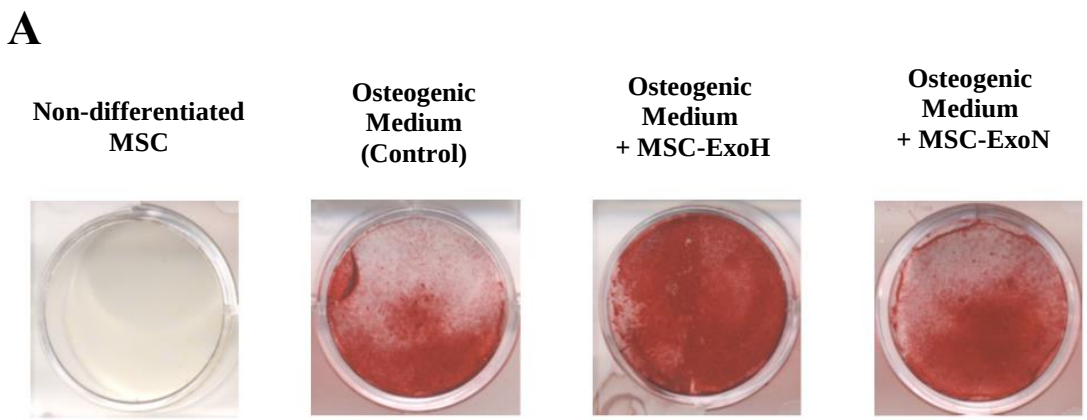
4.4 Effect of Exosomes on MSCs Differentiation

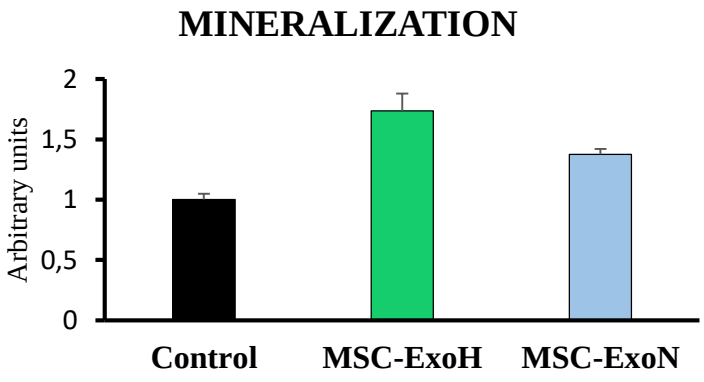
4.4.1 Osteoblasts: Mineralization and Gene Expression

Once having confirmed that exosomes treatment can augment the viability and the migration in MSCs, it was time to start to study whether this treatments with a concentration of $3x10^7$ part/ml of MSC-ExoN and MSC-ExoH could regulate the MSC differentiation into osteoblast.

Phenotypic analysis were carried out by the measurement of the mineralization in MSCs not differentiated and in differentiated ones, with MSC-ExoN or MSC-ExoH. The results obtained are that mineralization augment with MSC-ExoH and MSC-ExoN. This is also reflected in the photograph after staining the wells with alizarin red (**Figure 11A**).

Regarding the genotype, different osteoblast genes marker have been analysed. In both, the genes coding for the transcription factor RUNX2 and the extracellular matrix protein collagen type 1 alpha 1 (COL1A1), there are no significant changes, but there is a significant increase in gene expression for the transcription factor SP7 with MSC-ExoH, as well as in the integrin-binding sialoprotein gene (IBSP), with both, MSC-ExoN and MSC-ExoH (**Figure 11B**).





B

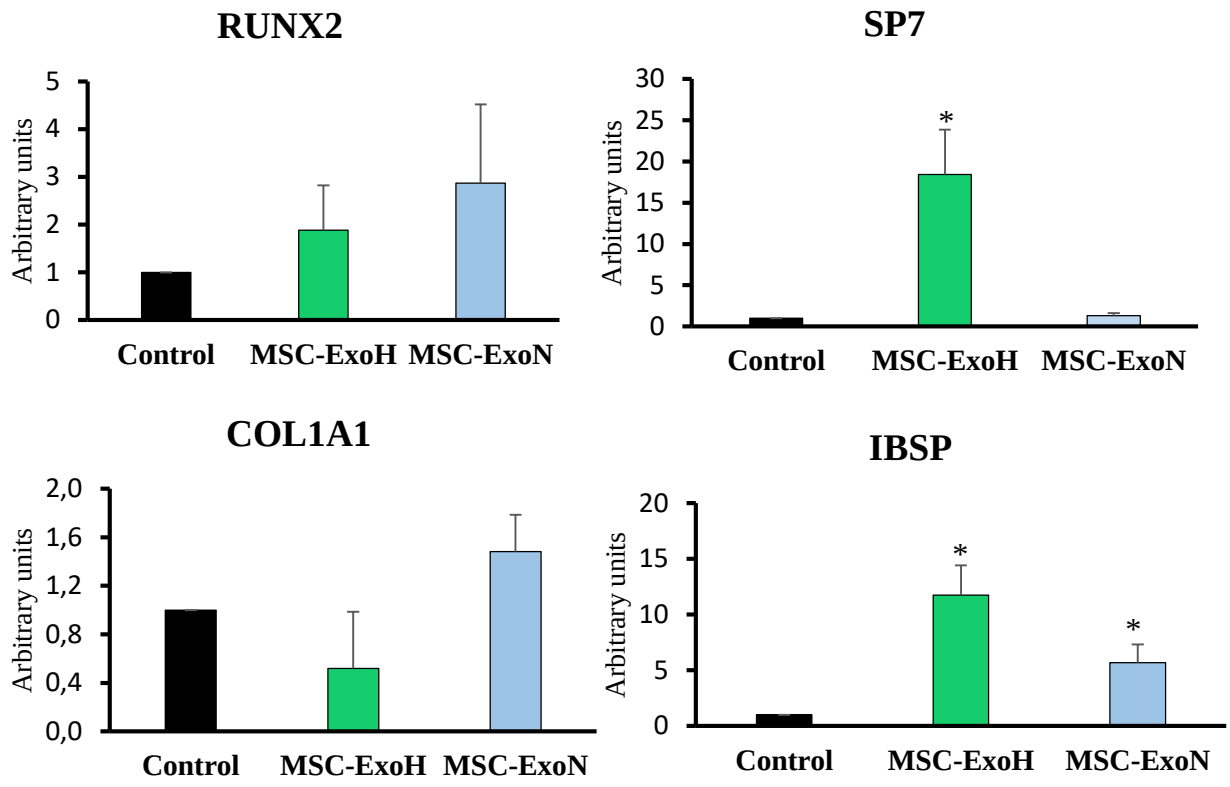
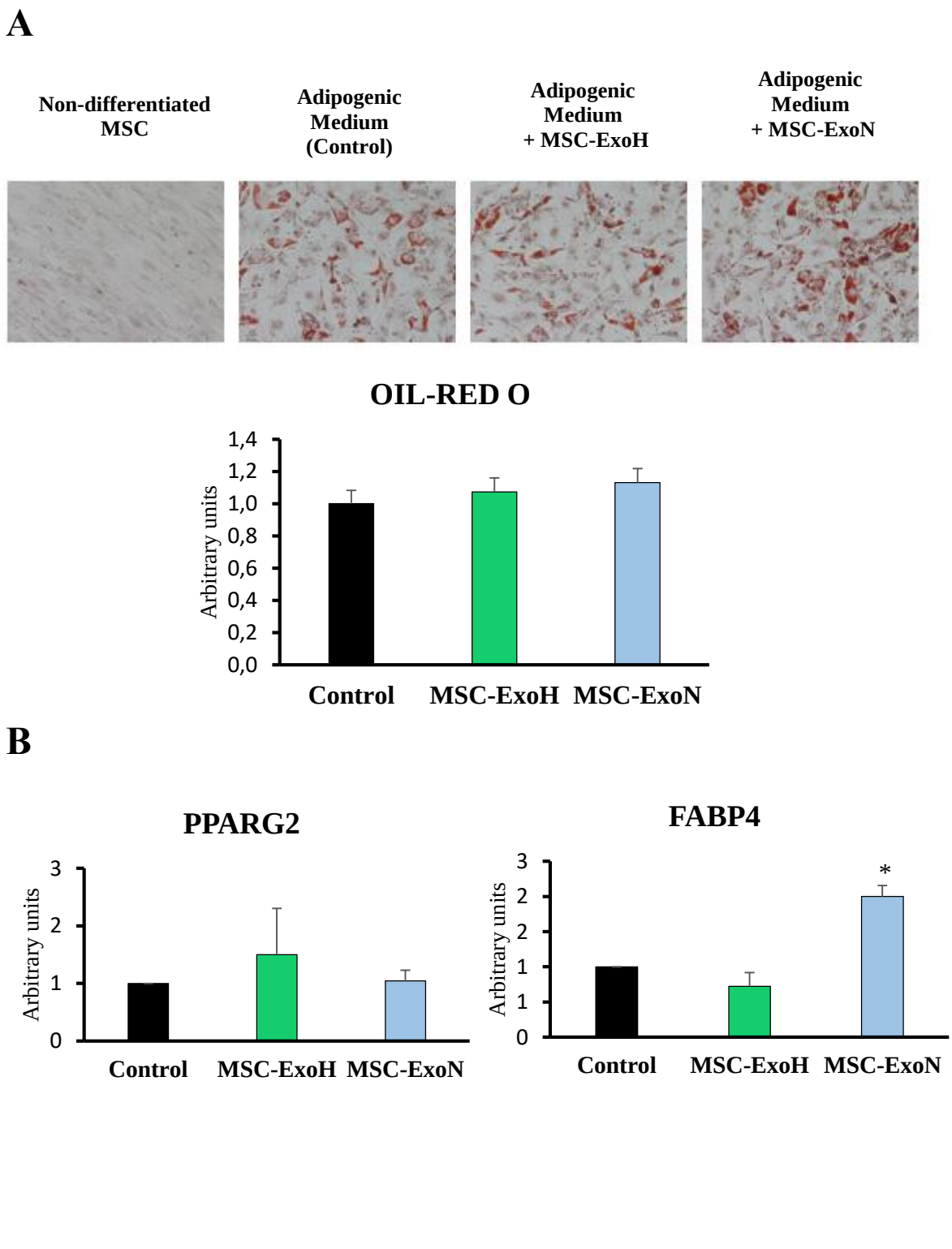


Figure 11. Effect of exosomes derived from MSC on osteogenic differentiation. *(A) MSCs were stained with alizarin red after 15 days of osteogenic induction. The images shown the mineralization of MSC cultures in P12 plates, not induced to differentiate or differentiated to osteoblasts in the presence or absence of MSC-ExoH or MSC-ExoN. Quantification of the staining of the cultures shown in the images is shown below. (B) qRT-PCR analysis of osteoblastic genes including RUNX2, SP7, COL1A1 and IBSP, 13 days after osteogenic induction of MSC cultures not treated, or treated with 3x10^7 part/ml of exosomes derived from MSC grown in hypoxia (MSC-ExoH) or normoxia (MSC-ExoN). Gene expression was normalized to control. Data are presented as the mean ± SD (n = 3 independent experiments). * p <0.05 vs. Control.*

4.4.2 Adipocytes: Fat formation and Gene Expression

In the same way, it was also studied the effect of MSC-ExoN and MSC-ExoH at a concentration of $3x10^7$ part/ml on MSC adipogenesis. The results obtained were not really significative.

In phenotype analysis, there were no change in fat vesicles formation in MSC induced to adipocyte and treated with MSC-ExoH or MSC-ExoN, respect cultures not treated (**Figure 12A**). Regarding the gene expression of the adipogenic genes PPARG2, LPL, FABP4 and FASN; the only gene whose expression increase significative with MSC-ExoN was FABP4 (**Figure 12B**).



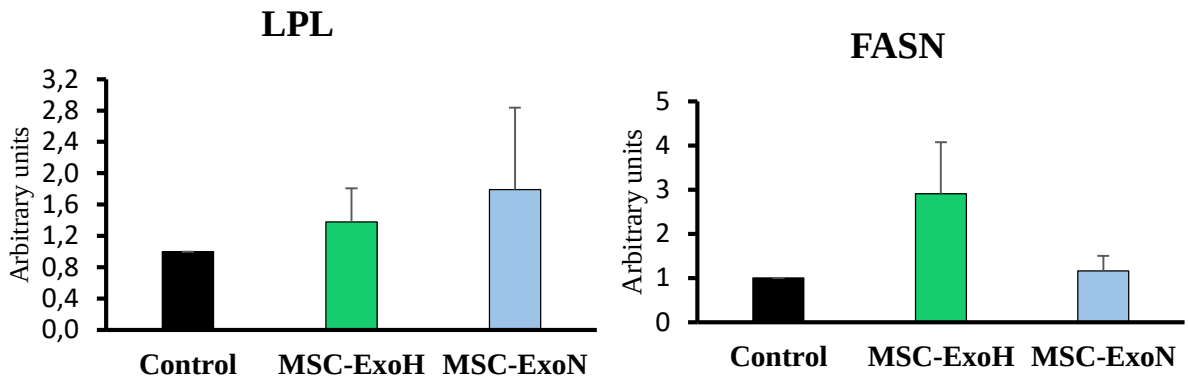


Figure 12. Effect of exosomes derived from MSC on adipogenic differentiation. *(A) Representative images of oil red O staining of MSC cultures differentiated to adipocytes for 13 days in the presence or absence of MSC-ExoH or MSC-ExoN (images at 200x). Quantification of the staining of the cultures is shown below. (B) qRT-PCR analysis of adipogenic genes including PPARG2, FABP4, LPL and FASN, 13 days after the adipogenic induction of MSC cultures not treated (control) or treated with at $3x10^7$ part/ml of exosomes derived from MSCs grown in hypoxia (MSC-ExoH) or normoxia (MSC-ExoN). Gene expression was normalized to control. Data are presented as the mean ± SD (n = 3 independent experiments). * p <0.05 vs Control.*

5. DISCUSSION

The number of exosome-related manuscripts, and the funding to support this work, are exponentially growing, representing enthusiasm about this area of research. In particular, MSC-derived exosomes are being extensively investigated as potential treatments for various intractable diseases, because they carry most of the therapeutic effect of the MSCs themselves. Moreover, exosomes are a cell-free therapy, which would minimize the safety concerns linked to the injection of live MSCs (Joo et al. 2020).

Recent studies suggest that cells transmit biomolecules to other cells through the luminal cargo and are recognised by membrane molecules present in the exosomes (Katsuda and Ochiya 2015). From a translational viewpoint, exosomes secreted from MSCs have shown promising therapeutic effects in a wide range of preclinical models of tissue repair, including kidney, liver, lung, myocardial infarction, cerebral artery occlusion, and skin wound healing (Rani et al. 2015).

The exosomes used have been obtained by concentration of the culture medium with ultrafiltration and purification by size exclusion chromatography. This methodology has the advantage that it does not need large devices such as ultracentrifuges and that microvesicles obtained have a high purity with little contamination of soluble proteins. This is evident in our results on estimating the protein concentration of the different fractions obtained after chromatography and is in line with that described by different authors (Gámez-Valero et al. 2016).

In MSCs, treatment with exosomes obtained from both normoxia and hypoxic cultures increased their viability when applied at a concentration of $15x10^7$ part/ml. The increase in cell viability by exosomes derived from MSCs has been described by different studies (Shabbir et al. 2015). However, some authors have described that exosomes derived from MSCs grown in hypoxia have more capacity to increase viability than those obtained in normoxia (Almeria et al. 2019). The discrepancy regarding these results may be due to the conditions of the cultures, the treatments, as well as the isolation and concentration of the exosomes, since there is great heterogeneity.

Also regarding the migration of MSCs, treatment with MSC-ExoH and MSC-ExoN tended to increase it, although just significantly did the higher concentration of MSC-ExoN ($15x10^7$ part/ml). Increased induction of migration is associated with increased regenerative capacity of exosomes (Cooper et al. 2018). Hypoxic conditions simulate those found on the tissue after an injury that affects vascular system. In these conditions cells produce specific factors that can stimulate tissue regeneration (Nauta, van Hinsbergh, and Koolwijk 2014). Our results suggest that

the migration of the MSCs has not been influenced by these possible factors included in the exosomes obtained in hypoxia.

Regarding the effect of MSC-ExoH and MSC-ExoN treatments on HUVEC proliferation and migration, our results show that they do not affect migration, but they do affect viability, which is inhibited with the highest concentrations of exosomes. Several studies suggest that exosomes derived from MSC on endothelial cells favours their proliferation and migration capacity (Ren et al. 2019; Wang et al. 2018). Furthermore, hypoxic conditions are characterized by inducing angiogenic factors, such as VEGF, that favour endothelial cell migration and growth (Nauta et al. 2014). Taking these considerations into account, our results seem contradictory. However, exosomes derived from MSC have also been reported to have antiangiogenic capacity in tumours (Shojaei et al. 2019). In our case, the MSC cultures were approximately 80% confluent before being maintained for an additional 48 hours in culture under the conditions tested for the isolation of the microvesicles. Thus, at the end of those 48 hours, the crops had reached 100% confluence in advance. In this state, contact between MSCs has been described to inhibit proliferation and also secrete factors that can also negatively affect their angiogenic properties (Ren et al. 2019). Therefore, our results suggest that the culture conditions used to obtain exosomes may be the main reason why the viability of endothelial cells is negatively affected by the treatment of these microvesicles. This result should be taken into account in future studies and it would be interesting to study the effect on HUVEC of exosomes derived from MSC cultures at different degrees of confluence.

Osteoporotic defects are common clinical complications of postmenopausal osteoporosis. Currently, the prevention and therapeutic approaches for osteoporosis are mainly focused on medical treatment. Meanwhile, autologous bone grafting is considered the gold standard for repairing bone defects, but some serious limitations such as donor site morbidity and limited bone supplies cannot be overcome. Thus, it is necessary to develop new approaches to stimulate bone regeneration and reverse bone loss and thus treat systemic bone loss without detrimental side effects (Qi et al. 2016). In our present study, we demonstrated that MSC-ExoN and MSC-ExoH effectively stimulated the proliferation and osteogenic differentiation of MSCs. Moreover, the effect was more noticeable with increasing concentrations of exosomes. This is in line with recent studies carried out *in vivo* with exosomes derived from MSC maintained in hypoxia (Liu et al. 2020). We also showed that exosomes do not produce significant changes in adipocytes genes markers or fat-droplets formation.

In recent years, the implantation of stem cells has emerged as an option for the treatment of bone defects. MSCs are the preferred stem cells for use in autologous transplantation therapy. Besides, their use in bone regenerative techniques is even more promising due to its ability to

undergo osteogenic differentiation. However, harvesting adult MSCs requires suitable donors and invasive procedures. In addition, the extraction and the limited cell quantity of MSCs requires long times of in vitro expansion which, in turn, leads to a decrease in the differentiation capacity as they enter replicative senescence, limiting their therapeutic efficacy. Interestingly, the ability of MSCs to undergo osteoblastic differentiation under osteoporotic conditions is significantly weakened, which ultimately leads to a delay in bone formation.

In this study the results obtained by qRT-PCR suggested that both MSC-ExoN and MSC-ExoH increase the expression of important osteogenic genes such as RUNX2, SP7 and IBSP. IBSP and COL1A1 are bone matrix proteins synthesized by osteoblasts, while RUNX2 and SP7 are key transcription factors required for bone formation; COL1A1 and RUNX2 act as early indicators of osteogenesis, while IBSP and SP7 act later in the signalling pathway. Thus, the increase of these genes clearly suggests an activation of the osteogenic pathway.

The role of MSC-ExoN and MSC-ExoH is not significative, since the results from qRT-PCR show that the effect in adipogenic genes PPARG2, LPL and FASN did not increase. Just the FABP4 gene, which encodes a Fatty Acid-Binding Protein, significantly increased its expression with MSC-ExoN treatment. FABP4 is involved in different cellular functions as a lipid transporter and it has also been assigned various extracellular functions, (Hotamisligil and Bernlohr 2015) therefore the application of MSC-ExoN may affect aspects related to fatty acid metabolism during adipogenesis.

PPARG2 is a key transcription factor required for adipocytes formation; LPL and FASN are proteins implicated in lipids metabolism.

In summary, all the above data demonstrated that MSC-ExoN and MSC-ExoH can improve bone regeneration by promoting MSCs osteogenesis. Nevertheless, the detailed mechanism by which MSC-Exos modulate MSCs remains incompletely understood and is being further investigated by our team.

6. CONCLUSSION

In this study, we investigated the effect of exosomes derived from human MSC cultured under hypoxic conditions on osteoblastogenesis and adipogenesis. The results showed that high concentrations ($15x10^7$ part/ml) of MSC-ExoH and MSC-ExoN treatments significantly promoted proliferation and migration of MSCs. However, in HUVEC occur that proliferation decreases and migration remains unchanged. This may be due both to improper maintenance of the HUVECs, or to the fact that they have been obtained from a culture with too much confluence, and that therefore the exosomes have cargos that inhibit proliferation.

We have demonstrated that both MSC-ExoN and MSC-ExoH increase the expression of the osteogenic markers RUNX2, SP7 and IBSP, the latter two being the more significant ones since they are the genes expressed later in the osteogenic signalling pathway. Therefore, its expression would verify the correct differentiation of osteoblasts to the latter stages, what would favour an earlier maturation and thus, also an enhanced mineralization.

Regarding adipocytes, the results obtained show that exosomes do not produce significant changes in adipocytes gene markers or fat-droplets formation.

Thus, the results of this study suggest that exosomes could increase bone mineralization, without favouring adipogenesis, thus avoiding bone fragility. Our study suggest a possible use of exosomes as therapeutic treatment for systemic bone loos such as in osteoporosis or local bone loss as the one seen in non-union fractures.

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