Relatório Final de Estágio

Mestrado Integrado em Medicina Veterinária

APPLYING CELL THERAPIES IN EQUINE CHONDROARTICULAR DISORDERS – MESENCHYMAL STEM CELLS CHARACTERIZATION AND OPTIMIZATION

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Abstract

Regenerative medicine evolved in the past decades, both for human and veterinary medicine, demonstrating promising outcomes regarding diseases with ineffective conventional treatments, such as osteoarthritis in equine patients.

Mesenchymal stem cells (MSCs) are applied *in vivo*, and cell tracking is commonly used to assess cells path. One of the purposes of this study is to characterize *in vitro* MSCs transfected with green fluorescence protein (GFP) as tracking method, to infer whether these cells maintain their characteristics and properties *in vitro*. The GFP helps tracking the cells, thus allowing to track cell migration.

Equine bone marrow mesenchymal stem cells (BM-MSCs) already transfected with GFP were cultured and induced to differentiate. Further, real time quantitative polymerase chain reaction (RT-qPCR) and specific stainings were performed to confirm cell differentiation. RT-qPCR and qualitative staining results showed cellular differentiation towards the desired lineages. However, GFP transfected cells did not overexpress specific markers as much as non-transfected cells compared to undifferentiated control. One possible explanation could be the greater passage number GFP transfected cells suffered and not because of the transfection process itself. To overcome this situation, further works should apply the same passage number for each cell.

Other sources of MSCs are the umbilical cord and the dental pulp due to their interesting therapeutic potential. Both can be acquired in a non-invasive way and banking options are in crescent development.

The other purpose is regarding the xeno-free (absence of components derived from other species) approach so that ethical and economical restrains can be avoided. Cellular viability and senescence assays were performed to study the behavior of canine/equine synovial-membrane derived MSCs (SM-MSCs) cultured in different supplemented media. Results proved that cSM-MSCs presented good viability values using xeno-free media, however, eSM-MSCs did not present so promising results. One possible explanation could be the allogenic serum preparation.

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My family, my support, my reason of who I am today. To my parents, not only you are the brightest persons I have ever met as you are the strongest, the most loyal and lovable. I know from the deeps of my soul that everything I achieved was because you would never leave me. You were there for me all the way, every day, in the easy and in the hardest. You told me to follow my dreams, and here I am. But my biggest dream is to grow up to be like you. You are my biggest inspiration, and I am so grateful. For my little brother, that will always be little for me, my love, although our differences you make me want to be a better person and you make me better. I love you and I will always here for you. To my grandparents, uncles, cousins, for everyone that helped me. Forever in my heart.

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List of abbreviations

ADSCs	Adipose-derived MSCs
ALP	Alkaline phosphatase
BM-MSCs	Bone marrow derived MSCs
B-2M	β-2- macroglobulin
COL1A1	Collagen type I
DJD	Degenerative joint diseases
DMSO	Dimethyl sulfoxide
GAPDH	Gliceraldehído-3-fosfato dehydrogenase
GFP	Green fluorescent protein
GMP	Good manufacture practices
IL-1β	Interleukin 1 beta
IA	Intra-articular
LPL	Lipoprotein lipase
MHC	Major histocompatibility complex
MSCs	Mesenchymal stem cells
MHC	Major Histocompatibility Complex
OA	Osteoarthritis
PPARγ	Peroxisome proliferator-activated receptor gamma
PRP	Platelet-rich plasma therapy
PB	Presto Blue™ Viability Assay
RQ	Relative Quantification
RT-qPCR	Reverse transcription quantitative PCR
RUNX2	Runt-related transcription factor 2
RT	Room temperature
SMSCs	Synovium-derived MSCs
TNFα	Tumor Necrosis Factor alpha
UCB	Umbilical cord blood

Introduction

Regenerative medicine

Regenerative medicine is an important field in medicine that can transform clinical practice [1]. Diseases with difficult outcomes now have a different approach that can help reaching better results. This field is based on the use of biological therapies such as tissue engineering, stem cells and growth factor-based products like platelet-rich plasma therapy (PRP) and secretome. Regarding tissue engineering, biomaterials used as *scaffolds* are an important component in regenerative medicine since they can simulate the native extracellular matrix of tissues contributing to a better function and structure of the new tissue [2, 3].

The first therapeutic application of stem cells in horses was performed by Herthel to treat an equine desmitis of the suspensory ligament [4]. Nowadays, stem cells are attracting attention because they can be easily isolated, differentiate into different cell lines and therefore presenting characteristics that make them competent biomedical candidates [5].

In humans, mesenchymal stem cells (MSCs) are considered potential and promising therapies for example cardiac injuries and immunologic diseases [6].

In veterinary medicine, MSCs are commonly applied in musculoskeletal lesions, mainly in horses and dogs [4]. Nowadays, chronic or debilitating diseases such as osteoarthritis in horses have a different option of therapy that is developing good outcomes [7, 8].

Despite all the benefits, there are some challenges related to the use of MSCs. Firstly, there are some limitations about the methods available to perform an adequate identification and characterization. For instance, the surface antigen expression criteria used in human MSCs may not be sufficient to characterize MSCs for all the different species. So, because of this lack of knowledge there is the necessity of evaluating the factors that could affect safety, quality and effectiveness of veterinary MSCs [9]. Additionally, it is important to highlight how important the bridge created between veterinary medicine and human medicine is because of the “One Health” concept – small and large animals serve as models for preclinical evaluation of the application of MSCs in humans [7].

Degenerative joint diseases in horses

Osteoarthritis (OA) is the most common and prevalent degenerative joint disease (DJD) in both humans and equine/canine species [8, 10]. Musculoskeletal disorders have reduced the well-being of animals and normally are characterized as chronical healing processes that end

up in permanent lesions [11]. These articular diseases have a great impact in the daily life and it is considered the most prevalent cause of diminishing the performance of athlete horses [12]. Clinically, the degenerative disease is defined as the progressively loss of cartilage, synovitis, formation of osteophytes, remodeling of the subchondral bone and eventually loss of articular function [12]. The main problem is when there is no longer a positive feedback with the use of corticosteroids or rest of the animal [12]. Therefore, a successful treatment is a challenge and it is important to focus therapies on diminishing pain, avoiding greater deterioration of the articular joint and attenuating the disorder [8, 13-16].

OA can appear for various reasons such as trauma and subsequent synovitis. This mainly happens because of the overuse of the joint or conformational problems that can influence the inappropriate biomechanical forces on the cartilage [17]. The main factor in pathophysiology of this condition is the inflammation, because of the release of proinflammatory molecules such as Interleukin 1 beta (IL-1β) and Tumor Necrosis Factor alpha (TNFα). The damage in the cartilage takes place because of the complex molecular cascade induced by these inflammatory cytokines [15]. These cytokines lead to the degradation of the cartilage mainly through the activation of proteinases such as matrix metalloproteases (MMP), aggrecanases, free radicals and prostaglandins [18]. In consequence, cartilage products from its disintegrations are released into the synovial fluid, increasing the synovitis. In response, the activated synovial cells produce more proinflammatory mediators that conduct to a higher proteolytic enzymes production, which are the responsible for the cartilage breakdown, thus creating a positive feedback loop [17]. Besides, it is common to observe subchondral bone sclerosis associated with the cartilage breakdown to compensate its abnormal function and the boundary between hyaline and calcified articular cartilage is infiltrated with blood vessels [17].

The mediators of inflammation detected in the synovial fluid in OA appear from different tissues such as the cartilage, synovium and subchondral bone. For that reason, the ideal therapeutic approach may reduce the inflammation, subsequently diminishing the cartilage breakdown and promoting the regeneration of the articular structures [16].

Early treatments are an attempt to inhibit the progression of the disease and can help treat inflammation and diminish the changes in the joint. However, it is not simple to detect OA in early stages, so some treatments are not effective in subacute and chronic patients. Some of the treatments that can modulate the synovial inflammation and help reducing the pain are the non-steroid anti-inflammatory drugs, articular washes, surgery and intra-articular (IA) corticoids or hyaluronic acid [18]. Because of the prementioned conventional treatment limitations the application of mesenchymal stem cells (MSCs) IA have been developing, both for human and veterinary medicine, special regarding equine and canine patients, showing promising results [8, 13-16]. Trophic, immunoregulatory ability, regeneration and anti-inflammatory potential,

proliferation, and differentiation properties are some of the main reasons why these cells are gaining such importance.

Mesenchymal Stem Cells

The first to identify MSCs was Friedenstein and collaborators, in guinea pig bone marrow and they were further characterized taking into account the self-renew ability, the possibility of differentiating into different lineage pathways and contribute to tissue regeneration [6]. Furthermore, it is important to refer that these cells exist in virtually all adult tissues. In 2006 the International Society for Cellular Therapy settled essential criteria for labeling a cell as a MSC, which includes the potential of differentiation into three lineages (adipogenic, osteogenic and chondrogenic) (Figure 1), being plastic adherent in standard culture conditions, expressing specific cell-surface markers (CD90, CD105 and CD73) and lacking expression of hematopoietic markers (CD45, CD34) and CD14, CD79 and HLA – DR [19].

However, MSCs that are expanded in culture are a heterogeneous population that exhibit different properties depending on the tissue from which they are collected, the donor (including its species), and the laboratory techniques such as isolation, culture, media and passage number [19]. Yet, standards to define the critical attributes of MSCs based products that derive from veterinary medicine do not exist. So, human MSCs guidelines were adopted to veterinary medicine, however different species have antibody cross reactivity and specific cell surface marker expression [9]. Therefore, MSCs characterization still remains challenging and there is the arising need to establish specific criteria for safety and therapeutic use.

The primary mechanism which promotes a regenerative environment that can help producing healthy tissue is the secretion of paracrine factors, such as growth factors, cytokines and chemokines. Besides, some *in vivo* studies indicated that using therapies with MSCs can promote angiogenesis, differentiation and growth of the local cells [20]. Furthermore, MSC can help preventing apoptosis and fibrosis (which helps to heal the damage tissue because these processes delay healing), contribute with attracting immune cells to the injury and modulate immune responses [21]. Moreover, when there is an inflammation as in OA, the proinflammatory cytokines released by the inflammatory process regulate the expression of immunoregulatory molecules by MSCs [22].

The inflammatory environment that MCSs face in the injury site may not activate the cells completely provided that the quantity of proinflammatory cytokines can be insufficient to result in the liberation of immunoregulatory molecules. That is the main reason why some studies use *in vitro* MSC priming containing the proinflammatory molecules before IA administration, so that it is possible to enhance their therapeutic profit [16].

These potential properties are some of the reasons that make these cells so attractive to use in regenerative medicine. For example, clinical trials have proven the efficacy of MSCs administration in some horse diseases, such as OA – administration in cartilage repair to treat the degenerative cartilage disease [13, 23, 24]. Considering these characteristics, adult MSCs are progressively more used in regenerative therapies. However, the isolation and expansion of these cells are time-consuming, so it has been proposed the use of previously banked MSCs from allogeneic donors. Therefore, when administrating the cells, it is important to distinguish between autologous and allogeneic. Autologous means that the cells used come from the own patient. On the other hand, allogenic means that cells are obtained from a donor that is different than the recipient (patient), from the same species. As aforementioned, autologous cells have some limitations such as delay in the treatment and limitation of the own cells, not only concerning the immune-mediated disorders but also because of factors related to the donor, as advanced age (elderly patients), genetic or even metabolic disorders (less efficacious than the ones collected from healthy donors). So, if the treatment is needed at the time the diagnosis is made it is indicated the use of banked allogenic MSCs because the use of autologous ones requires several weeks to culture [25]. For that reason, the use of allogeneic has huge potential since they can be readily isolated and expanded *in vitro* to use promptly in treatments [22]. However, immunogenicity of allogenic MSCs must be considered. One factor that should be considered when using these cells is the major histocompatibility complex (MHC) matching or mismatching between the donor and the recipient, as well as the degree of MHC expression. Furthermore, allogenic therapy involves the risk of transmitting infectious diseases, and for that reason the donors have to be well chosen and clinically examined before using these cells [21].

The previously mentioned reasons make it so important to study and compare the use of autologous and allogenic cells in different species and different diseases, so there is a clearer idea of the benefits and limitations of each one [22].

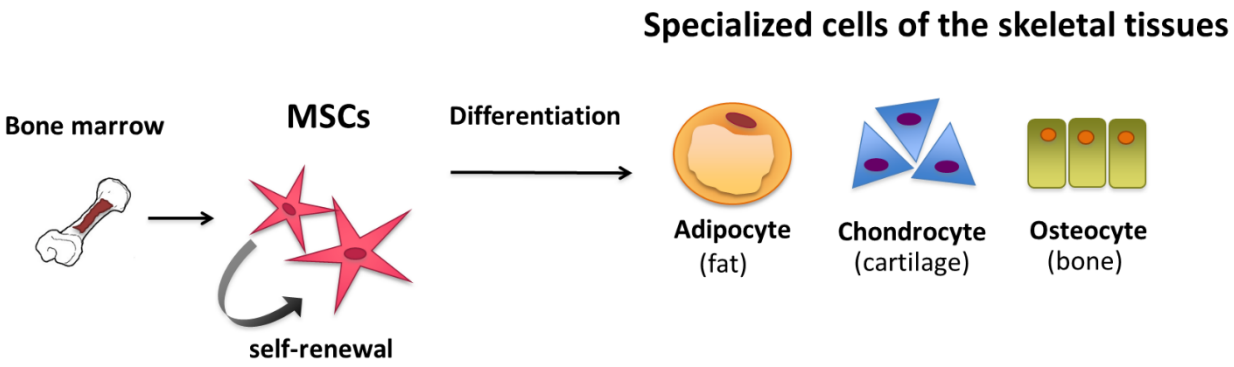


Figure 1: Scheme of the three-lineage differentiation (adipocyte, chondrocyte and osteocyte) of bone marrow derived MSCs (image obtained from: https://mediland.clinic/about-mesenchymal-stem-cells/).

Major Complex of Histocompatibility

To better understand the immune system and the role of MHC in antigen presentation it is important to be aware that there are two kind of MHC, class I and class II. As mentioned, allogenic MSCs would be appropriated to an immediate treatment and therefore eliminate the necessity of getting bone marrow/adipose tissue or another source from the patient. However, the main problem is related to the immunogenicity of these cells *in vivo* because the term allogeneic is used to describe MSCs that belong to a different animal, but most of the times, it is not mentioned if these MSCs were MHC matched/mismatched. This factor is relevant since previous studies have proved that if cells are MHC-mismatched certain humoral and memory T cell response can happen *in vivo* [21].

There is information concerning other species which prove that donor MHC – mismatched cells are targeted for destruction by the immune system of the recipient. For that reason, allogeneic mismatched MSCs cannot persist as long as allogeneic MHC-matched or autologous ones. The problem is that cells need to linger throughout the phase of inflammation into the phase of remodeling so that they can achieve maximal therapeutic benefit and success [21]. So, studies about this subject are increasing recently because of the raising awareness of this concern and its importance to achieve good therapeutic results.

Practical considerations

Since the application of MSCs is getting more common in various treatments it is important to be aware of the cells characteristics as well as the precautions that should be considered in the laboratory and in the administration.

The most largely investigated sources for clinical reasons are bone marrow and adipose tissue. However, to develop techniques and harvest in a non-so invasive way, there are other sources being studied, such as peripheral blood, synovial membrane, dental pulp, perinatal sources and others. Most types of cells have been thoroughly studied so that protocols of differentiation, expansion and culture are optimized to allow a best use of the different sources of MSCs [25]. Besides, some tissues require a more invasive way of harvesting while other sources like the dental pulp (extraction of a teeth for external reasons) and the umbilical cord are not invasive [26].

After, the harvested tissue is sent to the laboratory, where some techniques are applied to isolate and expand the MSCs [25]. In the laboratory, MSCs are isolated and a few days after, colonies of adherent cells appear and will progressively cover the plate until they reach confluence. Normally, it takes around 2-3 weeks to acquire autologous stem cells for use in

therapy, and cells with a passage beyond 6 should be avoided [25]. Furthermore, the time of expansion depends on the patient, since in elderly donors the proliferation potential is lower [25].

Bone marrow and synovia derived mesenchymal stem cells

Bone marrow derived MSCs (BM-MSCs) evidence greater capacities for differentiation into osteoblasts and is the most widely studied source for equine locomotor injuries. However, there is reports of improper differentiation during cartilage regeneration [11].

Synovial membrane-derived MSCs (SM-MSCs) as well as BM-MSCs are promising sources for musculoskeletal therapies. In comparison to the previous cells, these present higher proliferative capacity and greater capacity to chondrocyte differentiation. Besides, they maintain their characteristics regardless of the donor, inclusive the donor age, cryopreservation or cell passage number. Therefore, under specific conditions they can produce hyaline-like cartilage tissue and be a promising approach to cartilage injury therapies [11, 15].

Umbilical cord and dental pulp

Nowadays, hematopoietic progenitor cells obtained from the umbilical cord blood (UCB) are normally used for transplantation. This source is mainly used in patients with hematologic disorders and for further investigation of the utility of these cells in other treatments and in the earlier detection of babies illnesses [27]. However, there are other perinatal tissue that are normally discarded as medical waste and contain MSCs, such as placental tissue, amniotic fluid and umbilical cord tissue. Only the amniotic fluid is obtained in an elective amniocentesis, but the other tissues are collected in a noninvasive procedure [28]. Therefore, there are several advantages in this procedure, such as lower risk of transmitting viral infections, fastened availability of banked units and diminish incidence of graft versus host disease compared to the cells obtained from other sources as bone marrow [29]. However, there are some limitations as well, such as slower engraftment rates, deficient quantity and quality of one only UCB to engraft in adults and the probability to transfer premalignant or abnormal cells [30]. Furthermore, the therapeutic value, the lack of ethical problems and all the other benefits make this technique a good approach, so numerous cord blood banks are expanding the process and are cryopreserving additional tissue such as umbilical cord stroma, placental tissue, amniotic fluid and membrane alongside with the UCB [28]. Besides, these cellular lines can be used in other pathologies, as osteoarthritis or wound healing in horses, however equine MSCs are less well characterized than human MSCs [31].

Another source of MSCs are the dental tissues derived stem cells, normally the dental pulp stem cells. These are an accessible way of obtaining autologous adult stem cells that can be acquired from extracted wisdom teeth or if for any orthodontic reason another teeth has to be removed [32]. Furthermore, dental autologous stem cells have an interesting therapeutic potential and are easy to harvest in adults, so the idea of developing tooth banking is promising [33]. In order to obtain viable cells, it is important to isolate and cryopreserve the samples following good manufacture practices (GMP). The purpose of the isolation process is to collect a huge amount of MSCs after the expansion *in vitro* because they grow fast and normally are viable at higher number of passages [34]. About the cryopreservation, it is a critical process involving an adequate cryoprotectant and where the rate of the controlled freezing procedure is crucial. Furthermore, it is important to optimize these methods to achieve the best results reconstituting viable stem cells from the frozen cord blood. To this purpose of cryopreservation, the most used cryoprotectant is dimethyl sulfoxide (DMSO) [35].

Potential risk of implanting MSCs

MSCs are applied in both clinical and preclinical trials (please consult www.clinicaltrials.gov) but its safety continues to represent a significant challenge, as there are risks associated such as fibrosis, proinflammation and tumorigenicity when applied in several pathologies and tissues [5].

The most critical risk is tumorigenicity, since MSCs can produce an excess of cytokines, such as growth factors and chemokines, which act directly on the cancer cells receptors and regulate tumor growth. Besides, MSCs can also produce fibrotic reactions, because these cells can differentiate into myofibroblasts [5]. The therapeutic effect of MSCs can be improved and consequently diminish the risks by taking some measures into consideration. Such as, settle specific biosafety rules, explore in detail the MSCs effects in the immune system and assure that the quantity of cytokines is not harmful. Luckily, there are not many adverse effects according to most of the studies and clinical trials, but since the application of these cells is getting more and more common, there should be additional precautions [5].

Importance of labelling and tracking

Because of all the aforementioned, before applying MSCs, it is important to ensure their safety and efficacy, independently of their source, so further studies and investigation are needed to collect more data about how dose and distribution can affect safety [36].

More studies are needed to have a further knowledge about these interactions between allogeneic MSCs and the immune system *in vivo*. For that purpose, it is necessary to label MSCs and learn how long MSCs can remain viable in the site where they are administered or whether they can be targeted by the immune system. There are many techniques of labelling cells which normally involve labeling receptors or antigens with high affinity, such as lectins, antibodies, toxins, or other specific ligands that in a direct or indirect way are attached to markers. However, there are some limitations such as low duration, stain passing to resident cells and attenuation of signal [36]. For that purpose, one of the good strategies is to transfect cells with the green fluorescent protein (GFP) gene, which encodes a protein that is present in the jellyfish "*Aequorea Victoria*" and exhibits green fluorescence when exposed to light. It is an adaptable biological marker, commonly used to monitor physiological processes and to detect *in vivo* transgenic expression. GFP has longer duration and stability than other techniques such as membrane or intracellular dyes [36]. However, there are some problems regarding this technique such as auto-fluorescence of some tissues and unspecific expression because of the re-uptake of the protein, for example by the macrophages [37].

Concerning the clinical use of MSCs, the animal models and the route of administration should be appropriate. Furthermore, transfection is the more stable way of marking the cells, although potential changes in the MSCs properties must be accounted since it is a genetic manipulation [36, 38] .

Importance of assays about viability and senescence

Presto Blue™ Viability Assay (PB) is used to detect cell-mediated cytotoxicity *in vitro.* It is a compound based in resazurin (water soluble dye) that is converted into its reduced form by the enzymes present in the mitochondrial system, therefore only by viable cells. Consequently, the reduction leads to a change in color by the reagent and a consequent shift in its fluorescence, therefore being possible to quantify this conversion using spectrophotometer or fluorometric techniques. Also, PB is a ready to use solution and very sensitive [39]. Trypan Blue Exclusion Assay allows cell counting, distinguishing between dead and viable cells, as it only stains dead cells (compromised cell membrane). Cellular senescence is defined as the phase where cells can no longer divide. Besides, senescence cells express β-Galactosidase activity, so this enzyme is used for the detection of senescent cells in culture.

Main purpose of the study

Part of this work is an ongoing project to assess how allogeneic MSCs interact with the immune system under different conditions: inflammatory priming, differentiation, and

matching/mismatching MHC. For that, one of the procedures is to place the cells in a certain site inside the organism where they would be exposed to the immune system and that is relatively low invasive and easy to recover (for example, under the skin of the neck and encapsulated in alginate so they 'remain' there) in horses which were previously selected based on their MHC-haplotype to stablish matched and mismatched groups. Along with other assessments, it is important to check whether allogenic MSCs are able to evade recipient's immune response under these conditions and remain viable, for what it is necessary to track these cells by transfecting them with GFP.

The main purpose of this study is to assess whether equine BM-MSCs transfected with GFP maintain the same characteristics as the non-transfected cells in terms of differentiation potential (assessed by both specific staining and specific markers gene expression by reverse transcription quantitative PCR (RT-qPCR)), as a previous step for using GFP-MSCs to study MSC immunogenicity *in vivo*. So, in this case, GFP is important to mark the cells because the alginate implants will be recovered after 3 weeks, 6 weeks and 3 months to assess whether cells remain viable in the implants and to differentiate them from possible immune cell infiltration. Furthermore, this knowledge is helpful to be more aware of the benefits of using the alginate capsules of MSCs in other cases, as the degenerative joint diseases previously mentioned.

The other studied cells were from the umbilical cord and dental pulp because of their therapeutic value and arising use. Both sources have several advantages and uses in diverse pathologies that were previously mentioned [27, 28, 32]. For this reason, the process to extract the cells from the umbilical cord blood and the umbilical cord itself is described. Additionally, some studies mention the use of these cells in other pathologies [29].

Besides, xeno-free assays were performed to study cell viability and senescence using alternative media supplementation, rather than FBS. FBS is one of the mainly used medium supplementation to expand and culture MSCs and involves the use of bovine serum, the use of this substance poses a potential risk of transmitting zoonotic agents, unknown viruses and mycoplasma. Besides, there are ethical questions related to the use of animals to produce the serum since it also implies a huge number of young animals sacrifice for collecting blood, which is a problem of animal welfare [40].

So, Presto Blue™ viability assay, Trypan Blue Exclusion Assay and ß-galactosidase senescence assay were performed to evaluate equine and canine SM-MSCs behavior in culture, with different media supplementation, at different time points (24, 72 and 120h). Therefore, to corroborate if equine and canine SM-MSCs can grow in horse serum (HS) and canine serum (CS) supplemented media, respectively. Besides, these media were used in two different concentrations (10 and 20%) so that it was possible to assess if there was any remarkable difference in the same medium but at different supplement concentrations.

Materials and Methods

Study 1

Osteogenic differentiation

Equine BM-MSCs were seeded at 20.000 cells/cm^2 with 500 μL of medium in each well in two 24-well plate, as presented in Figure 2, one for isolating RNA to perform RT-qPCR and the other to dye the cells with specific staining. In each plate, 6 wells had normal medium (undifferentiated control) and the other 6 had differentiation medium, and in each one of these groups 3 wells had transfected cells (GFP) and the other were non-transfected (from the same animal).

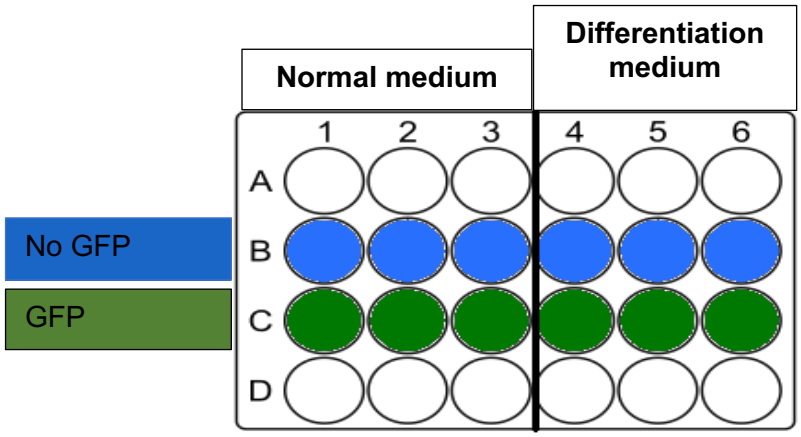


Figure 2: Distribution of equine BM-MSCs (GFP/no GFP) in normal and differentiation media.

These cells were cultured under osteogenic conditions for 7 days. The osteogenic differentiation medium contained basal medium (low glucose DMEM, 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin) supplemented with 10 nmol/l dexamethasone, 10 mmol/l b-glycerophosphate and 100mmol/l ascorbate-2-phosphate (all from Sigma-Aldrich)3 [26, 41]. The culture was maintained at 37ºC, 5% CO_2 and 95% humidity for 21 days. The medium had to be changed each 48h. On day 7, one of the plates was washed with PBS and frozen to perform the extraction of RNA and later cDNA synthesis. The other one was used to dye cells with Alizarin Red S, to assess their osteogenic differentiation. So, in this procedure cells were fixed with 70% ethanol for one hour at room temperature (RT) and then were stained with 2% Alizarin Red S for 30 minutes RT. Finally, cells were washed with PBS and observed under phase contrast inverted microscope.

Adipogenic differentiation

Cells were plated at a density of 5,000 cells/cm^2 with 500 μL of medium in two P24 plates following the same distribution than for the osteogenic differentiation protocol. In this case, the medium used was specifically to differentiate cells into adipocytes. So, the medium was made with basal medium supplemented with 1 mmol/l dexamethasone, 500 mmol/l 3-isobutyl-1-methylxanthine[3], 200 mmol/l indomethacin[3] and 15% rabbit serum[3]. The cultures were maintained for 14 days in the same conditions than for osteogenic differentiation [26]. After the 14 days, the RNA plate was washed with PBS and frozen. The other plate was used to dye the cells with oil red-o. In this protocol cells were fixed with 10% formalin for 15 minutes RT and then stained with 0,3% of oil red o for 30 minutes at 37ºC. Afterwards, cells were washed with distilled water [26] and observed under phase contrast inverted microscope.

Chondrogenic differentiation

Four alginate capsules were created in total in four wells of a 24 well plate: non-transfected MSCs with basal medium or with differentiated medium, GFP transfected-MSCs with differentiated medium and an acellular control. To make the alginate capsules at 1,5%, it is necessary to prepare alginate 3% (300 mg of alginate in 10 ml PBS) and then mix 0,5 mL of this solution with 0,5 mL of PBS containing the corresponding MSCs (at concentration of $10 \cdot 10^6$ MSCs/ml). Therefore, each implant has 1 ml (0,5 ml of MSCs and 0,5 of alginate, with a total of $5x10^6$ MSCs).

The protocol begins by detaching adherent MSCs with 0,5% Trypsin-EDTA and counting and separating 10 $x10^6$ MSCs for each condition. Then, the cells of each condition were pelleted by centrifugation and resuspended in 1200 μL PBS. The suspensions of alginate and MSCs were mixed in a 5ml Eppendorf (1200 μl of alginate 3% + 1200 μl MSCs). 500 μl $CaCl_2$ 100 mM was added to each correspondent well, following the addition of 1 ml of the previous cell suspension and alginate at 1,5% (previously pre-formed in a spoon by depositing the solution with a syringe). Finally, the well preparation is established with the addition of another 500 μl $CaCl_2$ in top of the alginate. The P24 with the implants was incubated at room temperature for 30 minutes and then the $CaCl_2$ is removed and the wells washed 3 times with 1 ml PBS. Although, in the third wash the cells that are undergoing chondrogenesis are washed with incomplete differentiation medium (without TGF-β3). After the washing procedure, 1 ml of the respective medium is added in each well. The differentiation medium consists in high glucose DMEM supplemented with 10% FBS, 10ng/mL TGFβ-3 (R&D Systems), ITS + premix (BD), 40 μg/mL proline (Sigma), 50 μg/mL ascorbate-2-phosphate and 0,1 μM dexamethasone [26]. Undifferentiated and acellular controls were cultured with basal medium. The culture was

maintained in 37ºC, 5% CO_2 and 95% humidity for 21 days. The medium had to be changed every 48h. After 21 days, each implant was divided into two pieces and one half of each one was evaluated histologically (H&E and Alcian Blue staining), and the other half was frozen at -80ºC to study the genic expression.

RNA extraction and cDNA synthesis

The protocol used to study the expression of genes related with each cell lineage begins with the extraction of RNA and then with the transcription to cDNA. To do so, RNA isolation should be performed carefully and with all the materials really clean and free of nucleases to achieve good results and avoid contaminations and degradation.

Cells to cDNA II (Ambion, Thermofisher) commercial kit was used for this purpose with the cells undergoing osteogenesis and adipogenesis following manufacturer's instructions.

Afterwards a Turbo DNAse kit (Ambion, Thermofisher was employed for elimination of genomic DNA (cells-to-cDNA). RNA retro transcription was done with the kit qScript cDNA super mix (Quanta Biosciences), both according to manufacturer's guidelines.

Real time quantitative PCR (RT-qPCR) analysis

For this experiment RT-qPCR was important to confirm the increased gene expression of specific markers of each lineage to be sure, along with the visualization of specific staining, that GFP transfected cells maintained their characteristic differentiation profile as MSCs. For that purpose, in the osteogenic differentiation the markers that were analyzed are the alkaline phosphatase (ALP) and runt-related transcription factor 2 (RUNX2). For the adipogenic study, peroxisome proliferator-activated receptor gamma (PPARγ) and lipoprotein lipase (LPL) were the studied genes [41]. The chondrogenic markers are collagen type II alpha I (COL2A1) and aggrecan (ACAN). In this case, the housekeeping genes used are β-2- microglobulin (B-2M) and GAPDH.

The obtained cDNA was analyzed using RT-qPCR and the amplification was performed using SYBR Green Master Mix (Applied Biosystems). All the reactions were performed in triplicate using 10 μl of volume from which 2 μl is cDNA previously obtained, 5μl is SYBR Green Master Mix, 0,3μl is specific primers (300nM) and the rest nuclease-free water. The protocol consisted in 20 seconds at 95 ºC to the initial activation and denaturalization. Then, it was followed by 40 cycles of 3 seconds at 95 ºC and 30 seconds at 60 ºC. Subsequently, a melting curve or dissociation curve was performed to identify any "false" PCR amplicon or high levels of primer

dimers. This was carried out by progressively increasing the temperature at 0.5ºC per minute until 90ºC.

Furthermore, the gene expression was determined by the comparative method using ΔCt, which uses a normalization factor (this value is calculated by the geometric mean of the two-housekeeping used in this protocol quantities) obtained by the expression of the housekeeping. RQ was calculated by $RQ = 2^{-(\Delta\Delta Ct)}$ [42].

Study 2

Processing umbilical cord blood

First, the blood transfusing / collecting bag transported from the hospital/clinic to the laboratory under refrigerated temperatures around 4-6ºC is weighed and homogenized for 10 minutes. The minimum of umbilical cord blood (UCB) to process by AXP method, for separation of plasma, buffy coat and erythrocytes is 15ml. So, after confirming the volume, the UCB is attached to the processing AXP kit (Thermogenesis) in the laminar flow hood (to avoid contamination) and drained outside to the kit. In this part it is important to label each part of the kit so there are no mistakes between samples. All the UCB present in the bag is squeezed into the kit and the blood clots are retained in the filter. After this transference, the UCB is agitated manually horizontally for about 2 minutes. Then, the initial volume is removed (1-2 ml of blood) and disconnected from the kit so that this volume is weighed separately. The UCB from the initial volume is divided in tubes, the first one is stored in the parallel bank and the second is to perform the hemogram. The first tube is used to determine the blood type of the newborn, but sometimes it is not possible because of the presence of maternal blood. The control tube is to confirm the values from the platelets and the hematocrit. For this procedure, first we use the control tubes (abnormal low, normal and abnormal high) and after we agitate the samples (initial volume) so that they are warmer to make the desired measures. If there is not enough volume, hydroxyethyl starch solution (HES) is added to the sample and it is included while agitating the sample. After opening this substance, it can be used only till 8 days after. Afterwards, the kit without the initial volume is allocated in the thermogenesis so that it can be centrifuged. The first centrifugation is for 20 minutes at 10ºC and 1400rpm and the second one is for 10 minutes at 10ºC and 1000rpm. The final volume is withdrawn after the centrifugation (1-2 ml as in the initial volume) to analyze the hematocrit and platelets. A recuperation of 70% is a very good value [29].

In the laminar flow hood the plasma is removed (20 ml) to do the BacT/ALERT which consists in two flasks, one anaerobic and the other aerobic to confirm that there was not contamination during the process of aerobic or anaerobic microorganisms. First, 10 ml are allocated in the anaerobic flask and then another 10 ml in the aerobic. If in some cases, there is

not enough plasma the erythrocytes can be used as well. The samples stay at 35ºC for 14 days and if the UCB present microbial contamination the microorganism has to be identified and an antibiogram is performed to detect the antibiotic sensitivity.

To cryopreserve the samples, the freezing process is a controlled freezing, which means that in the hood 7 ml of DMSO is attached in a syringe to the blood kit and it is introduced in the kit until reaching the entrance of the blood. Then it goes to the COOLMIX at 4ºC so the other part of the DMSO is slowly introduced in the sample. To complete the freezing process, the samples go to the Sylab to diminish the temperature gradually (starts in the 4ºC till reaching the temperature of -120ºC). After freezing, these units are transferred to a liquid nitrogen tank and stored in the gaseous phase [43].

After this procedure, the kit returns to the hood and all the air is removed from the buffy coat section so that it can be sealed. Meanwhile, in the extension of the kit some marks are made and sealed so that it is possible to test the viability of the sample each 5 years. This section is removed from the rest of the kit and it goes correctly identified to a bag and into a cassette.

The maternal blood is centrifuged, and the plasma is removed to use for microbiological tests. So, it is screened for some pathologies as Venereal disease/Syphilis, Cytomegalovirus, HIV-I and II, Hepatitis B and C, Toxoplasmosis and Human T-Cell Lymphotropic Virus Types I and II (only if relevant) [44].

To perform flow cytometry analysis, the sample from the final volume is used as well as two tubes, one control and the other to the sample. The markers used are CD34 and CD45, 10 μl in each tube and 50 μl of the sample (not in the control). Then they are agitated and kept for 20 minutes in the dark for incubation. After this time, a marker of dead cells (7AAD) is added to the sample tube and incubates again for 10 minutes. In the end, flow cytometry analysis is performed in the same day of the processing, in order to access viability of the cells cryopreserved.

Processing umbilical cord

The materials needed to process the umbilical cord are: 2 plates, 2 flasks (one to put alcohol and make washes and the other to collocate the material after the washes), scalpel and clamp. The umbilical cord is received in PBS and washed for 30 seconds with alcohol, being finally washed with PBS to eliminate the alcohol. This procedure is repeated two times. After, the umbilical cord is measured and cut in 5 cm long sections, facilitating the process of choosing the best area, less clots, less curled and with more Wharton's jelly tissue. After sectioning it is washed in PBS to keep the sample clean. Images from this process are present in attachment, Figure 1 A, B and C.

Cell viability assay with PrestoBlue™

The purpose was to test xeno-free (absence of components originated from other species) media supplementation. So, SM-MSCs from equine and canine species were cultured and expanded. The experimental design included, for each cellular population (eSM-MSCs and cSM-MSCs) two groups cultured with medium supplemented with FBS at 10% and 20%, and two other groups (xeno-free groups) supplemented with horse/canine serum at 10% and 20%. DMSO 10% supplementation was used as negative control.

For the serum inactivation, autologous blood was harvested into citrate tubes. The harvested blood goes into a 15 ml falcon tube and is centrifuged at 2500-3000 rpm for 10 minutes. After this process, the serum is carefully transferred, and the precipitate is discarded. Afterwards, it stays in a water bath at 56ºC for 30 minutes for inactivation by heat. Immediately after, it is allocated in ice until it stays cool. The serum is then filtrated with a 0,22 µm syringe filter and frozen.

Assessment of cell viability was performed by seeding both cellular populations in 24 well plates in triplicates, at 6000 cells/cm ^{2} and were maintained in the original standard medium for 24 hours so that they could adhere to the plate.

Culture media was removed, and adequate culture media (corresponding to the specific test group) was added at each time point (24, 72 and 120 hours) to each well. In addition, 10% (v/v) of PrestoBlue™ cell viability reagent was added, and plates were incubated for 1 hour at 37ºC, 5% CO_2 and 95% humidity. After the incubation period, supernatant absorbance was read at two different absorbances (570 nm and 595 nm) in a Thermo Scientific Multiskan FC plate reader. Absorbance readings were normalized, and data corrected to unseeded control wells' readings (blanks) [45].

Trypan Blue Exclusion Assay

eSM-MSCs and cSM-MSCs were seeded in 24 wells plate at 6000 cells/cm ^{2}, considering triplicates for each time point. At each time point (24, 72 and 120 hours) and using Trypsin-EDTA to detach the cells, samples were harvested and cells counted with Trypan Blue exclusion dye assay using an automated counter, allowing differentiation of dead and viable cells [44].

Cell senescence – Beta-galactosidase senescense assay

The β-Galactosidase activity was assessed with the aim to evaluate signs of senescence in the cells and for this purpose, cells were seeded in 96 well plates. At defined timepoints (0,

72 and 120 hours), culture media was removed and consequently the cells were washed one time with PBS. Then, they were incubated with 100 µL β-Galactosidase Assay Reagent for 30 minutes at 37ºC and the supernatant absorbance was read at 405 nm with the Thermo Scientific Multiskan FC plate reader [45].

Statistical analysis

The statistical analysis was performed with the GraphPad Prism 8.00 version, GraphPad Software, La Jolla California USA. The analysis was made using One-Way ANOVA with the Tukey's multiple comparisons test. It was considered statistically significant $p \leq 0.05$. The results are presented with the * symbol and indicate $0.01 < p \leq 0.05$, $0.001 < p \leq 0.01$, $0.0001 < p \leq 0.001$ and $p \leq 0.0001$ with *, **, *** and **** respectively.

Results

Study 1

Osteogenic differentiation

Osteogenic differentiation was achieved in both cells with and without GFP. Alizarin Red-S stained multiple calcium deposits, consistent with bone differentiation, can be observed in both cases (Figure 3). The analyses achieved from the staining's are qualitative and the quantitative results will be presented in the PCR section.

The undifferentiated cells do not have extracellular calcium deposits, so they are colorless or slightly purple. On the other hand, the differentiated cells since they have calcium deposits stain with Alizarin Red S.

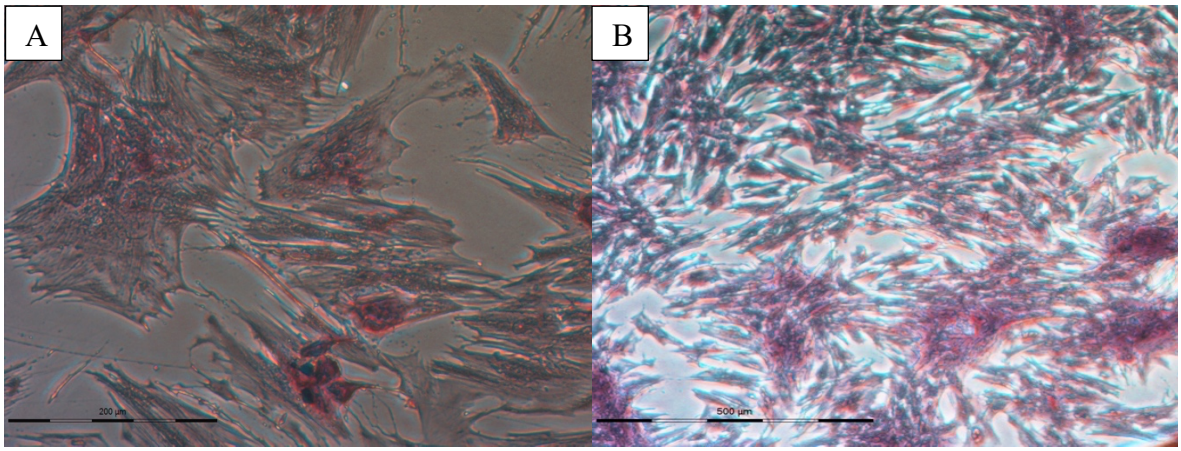


Figure 3: osteogenic differentiation of cells with GFP (A) and cells without GFP (B).

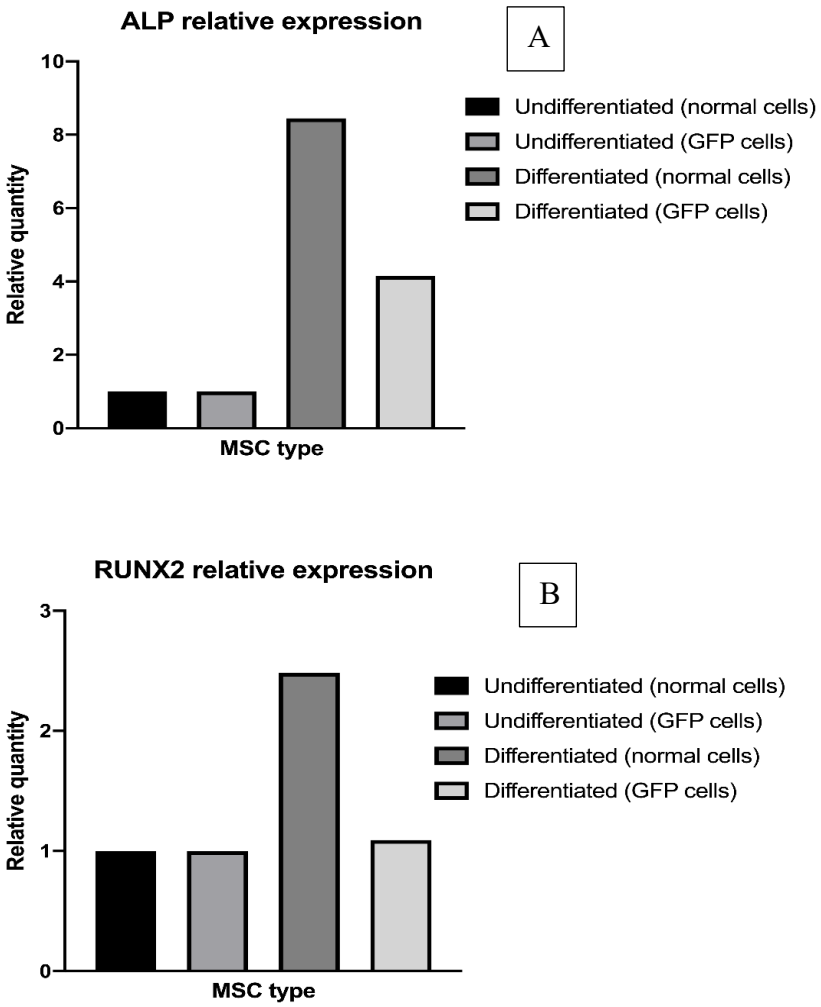


Figure 4: Graphic A – ALP marker from osteogenic differentiation in undifferentiated normal/GFP cells and differentiated normal/GFP; Graphic B – RUNX2 marker from osteogenic differentiation in undifferentiated normal/GFP cells and differentiated normal/GFP.

RQ means the number of increases of the expression regarding the reference sample. So, in Figure 4 (A) it is possible to observe that normal cells differentiated upregulated the expression of ALP. However, in GFP-cells, the ALP marker was overexpressed, but the value was not so high in comparison with normal cells. Besides, in Figure 4 (B), differentiated normal MSCs have the highest expression of RUNX2. GFP-cells differentiated only increased a little their expression of this marker in comparison with undifferentiated (GFP cells). Statistical analysis was not assessed since the result are based in only one Δ CT value.

Adipogenic differentiation

Differentiated cells from the adipogenic lineage were stained using Oil Red and the differentiation was achieved in both cell groups, with and without GFP. Qualitative assessment allowed visualization of lipid vesicle accumulation in the adipocytes stained by bright red.

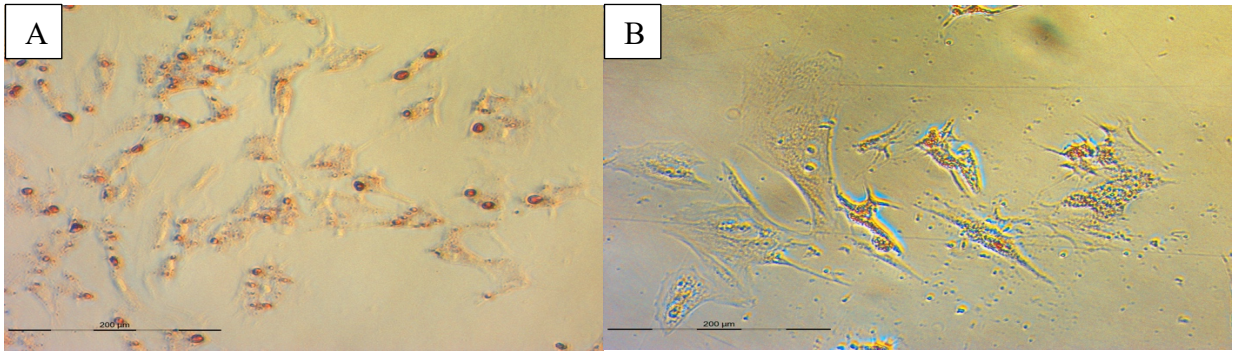


Figure 5: Adipogenic differentiation in control cells (A) and in cells with GFP (B).

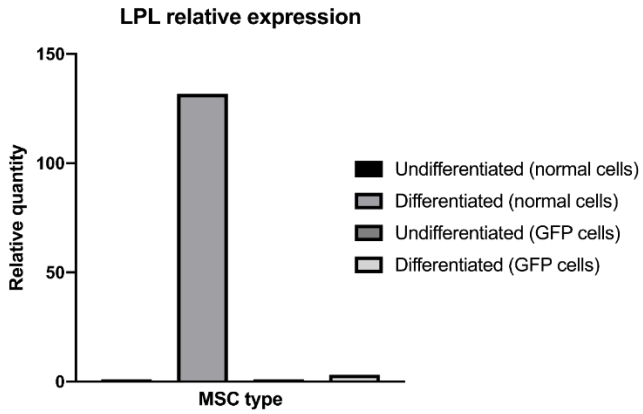


Figure 6: PCR results representing LPL marker expression from adipogenic differentiation in undifferentiated normal/GFP cells and differentiated normal/GFP cells.

In Figure 6 it is possible to observe that differentiated normal MSCs have the highest values of RQ, indicating higher levels of LPL expression. However, GPF-cells differentiated only increased a little their expression of this marker. Statistical analysis was not assessed since the result are based in only one Δ CT value.

Chondrogenic differentiation

Chondrogenic lineage has blank, undifferentiated normal MSCs, differentiated normal MSCs and differentiated GFP-MSCs both using two different methods hematoxylin and eosin stain and alcian blue.

In the images presented in Figure 7 it is possible to observe the lacunae formation and higher blue intensity in the edge of the lacunae, as it is expected in the differentiated cells. However, in the GFP-cells the lacunae are not so evident.

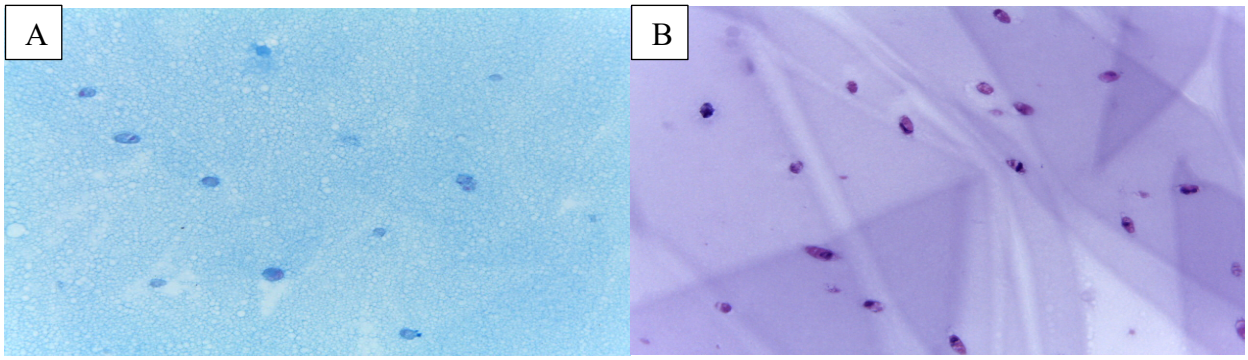


Figure 7: Chondrogenic differentiation with GFP (A) HE and Alcian Blue (B).

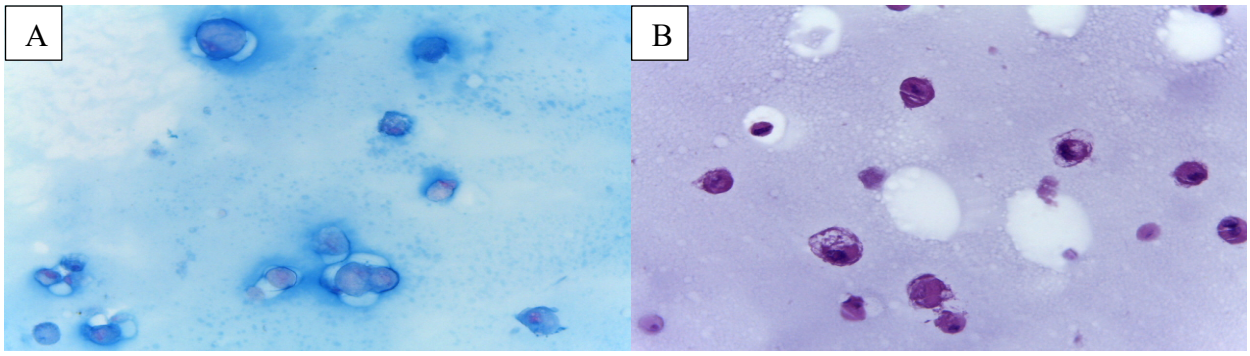


Figure 8: Normal chondrogenic differentiation (A) HE and Alcian Blue (B).

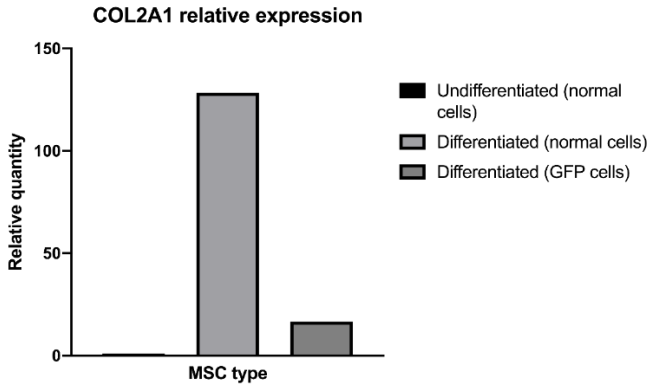


Figure 9: Graphic with COL2A1 marker expression from adipogenic differentiation (COL2A1) in undifferentiated normal cells and differentiated normal/GFP cells.

In Figure 9, differentiated normal cells have the highest expression of this COL2A1 marker. Besides, differentiated GFP also have higher expression in comparison with undifferentiated (control) cells, but lower than non-GFP differentiated cells. Statistical analysis was not assessed since the result are based in only one Δ CT value.

Study 2

Presto Blue™ Viability Assay – canine SM-MSCs

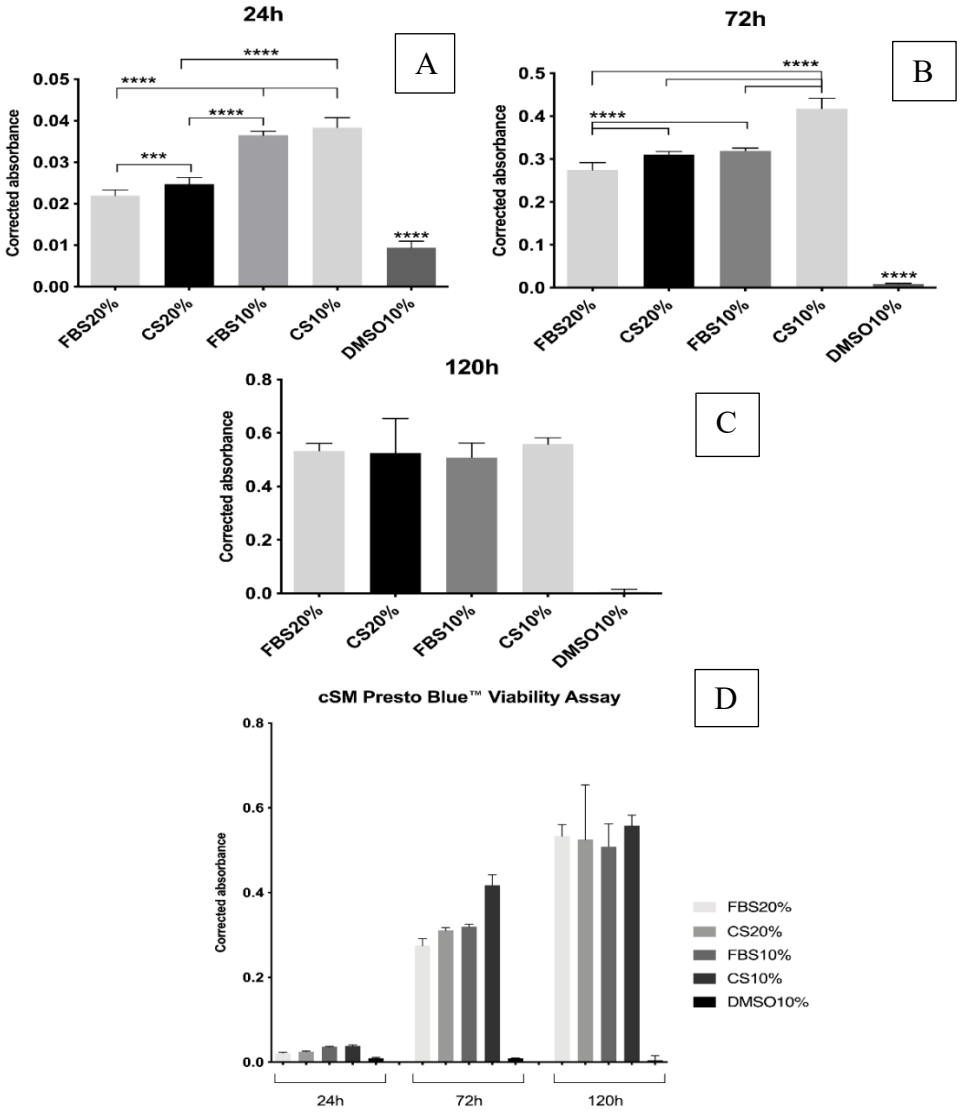


Figure 10: Graphic A – cSM-MSCs Presto Blue™ Viability Assay™ at 24h; Graphic B – cSM-MSCs Presto Blue™ Viability Assay at 72h; Graphic C – cSM-MSCs Presto Blue™ Viability Assay at 120h; Graphic D – cSM-MSCs Presto Blue™ Viability Assay™ at all the time points (24, 72 and 120h). The results are presented with the * symbol and indicate $0.01<p\leq0.05$, $0.001<p\leq0.01$, $0.0001<p\leq0.001$ and $p\leq0.0001$ with *, **, *** and **** respectively.

The 24h and 72h graphic show that cell viability was higher in CS10% comparing to the other media supplementation, with statistical significant differences, as shown in graphic A and B, respectively. Besides, FBS20% supplementation group showed less viability at early timepoints, 24 and 72h, as shown in graphic A and B. At 120h no statistically significant differences were detected between groups (except for the negative control group, as expected).

Presto Blue™ Viability Assay– equine SM-MSCs

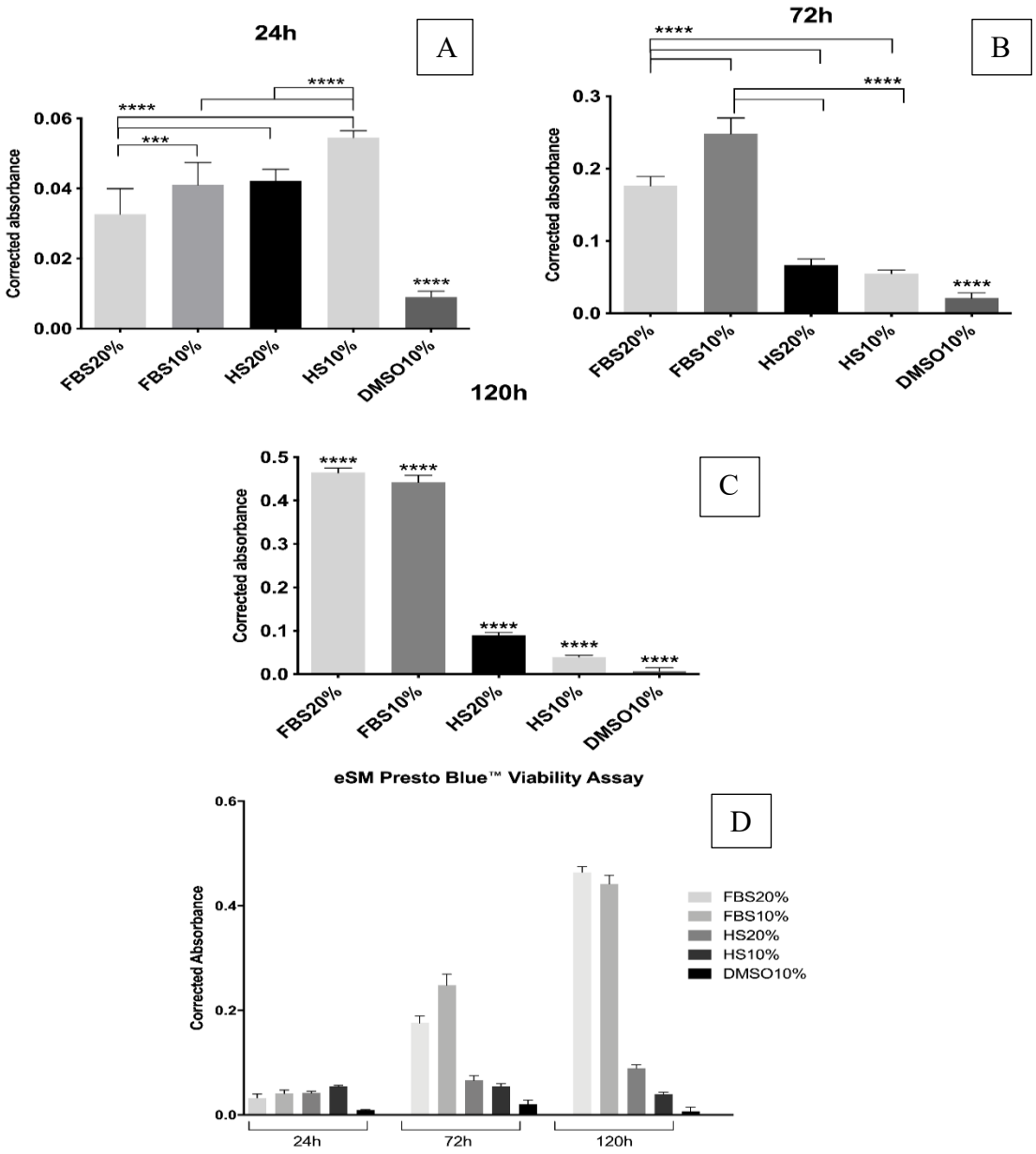


Figure 11: Graphic A – eSM-MSCs Presto Blue™ Viability Assay™ at 24h; Graphic B – eSM Presto Blue™ Viability Assay at 72h; Graphic C – eSM-MSCs Presto Blue™ Viability Assay at 120h; Graphic D – eSM-MSCs Presto Blue™ Viability Assay™ at all the time points (24, 72 and 120h). The results are presented with the * symbol and indicate $0.01<p\leq0.05$, $0.001<p\leq0.01$, $0.0001<p\leq0.001$ and $p\leq0.0001$ with *, **, *** and **** respectively.

At 24h the medium with higher viability measures was the HS10% supplemented medium. However, both HS supplemented media presented low viability values for longer timepoints, when comparing to the FBS supplemented groups, as shown in Figure 11 (B) and 11 (C). Between both FBS supplemented groups, FBS10% presented better results than FBS20% with statistical difference at early timepoints, 24 and 72h, graphic A and B

respectively. At 120h time point (Figure 11 C) there were statistical significant differences but FBS20% presented better results than FBS10%.

Trypan Blue Exclusion Assay – canine SM-MSCs

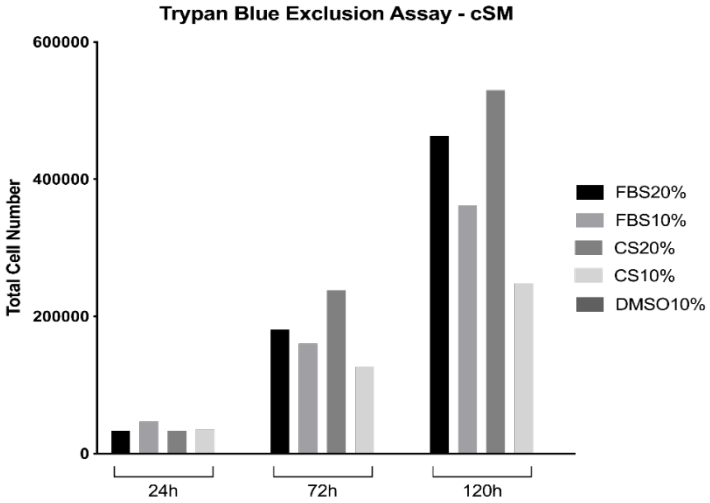


Figure 12: Graphic representing Trypan Blue exclusion Assay cSM-MSCs at different time points (24, 72 and 120h).

Trypan Blue Exclusion Assay – equine SM-MSCs

Figure 12 shows the Trypan Blue Exclusion Assay – cSM-MSCs. At 24h the FBS10% supplemented medium presented the highest number of total cells. In the intermedium time point CS20% was the medium with the highest value. In the last time point, at 120h, CS20% maintains the highest value of total cell number, showing more similarities to FBS20% than to the other concentration of CS (10%).

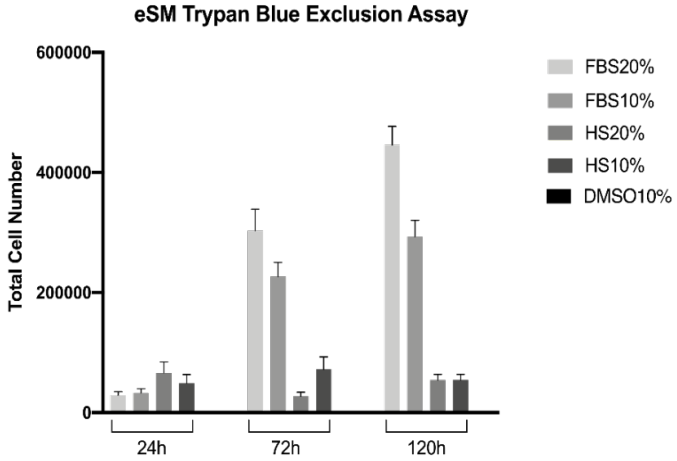


Figure 13: Trypan Blue Exclusion Assay eSM-MSCs at different time points (24, 72 and 120h).

Figure 13 shows the Trypan Blue Exclusion Assay – eSM-MSCs. At 24h the HS20% was the medium with the highest number of viable cells. At 72 and 120h time points, FBS20% supplemented medium presented the highest total cell number values. As observed in the Presto Blue™ viability assay, HS supplemented media presented very poor results, when comparing to the FBS supplemented media.

Beta-galactosidase senescence assay – canine SM-MSCs

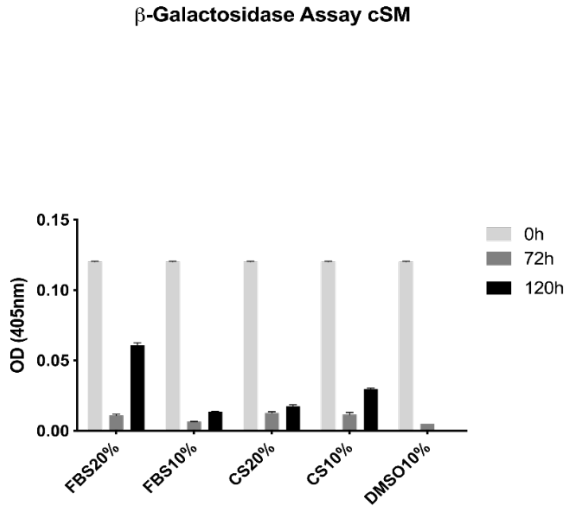


Figure 14: ß- Galactosidase senescence assay cSM-MSCs at different time points (24, 72 and 120h).

In Figure 14, at 0h the values are higher than the expected. A possible explanation could be cell viability loss for these plate wells. For that reason, 0h time point value was not considered. At early time points 72h all the media have similar senescence values. At later time-points there are more accentuated differences and the medium which provokes more cell senescence is the FBS20% supplemented medium.

The table with the statistical significances at each time point is present in attachment (Table 1).

Beta-galactosidase senescence assay – equine SM-MSCs

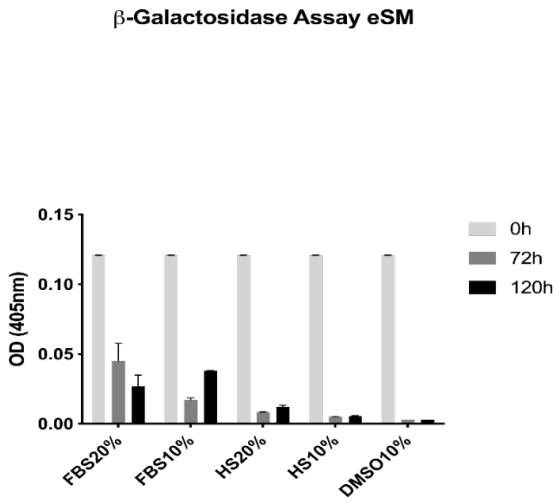


Figure 15: ß- Galactosidase senescence assay eSM-MSCs at different time points (24, 72 and 120h).

In Figure 15, at 0h the values are higher than the expected. A possible explanation could be cell viability loss for these plate wells. For that reason, 0h time point values were not considered. At 72h FBS20% supplemented medium has the highest senescence values and the HS10% supplemented group the lowest. DMSO10% has very low values because cells are all dead, so beta galactosidase activity is not detected. Besides, at the last time point (120h) FBS10% supplemented group presented the highest senescence value followed by the FBS20% supplemented group and as in the early stages HS10% supplemented group maintains the lowest value.

The table with the statistical significances at each time point is present in attachment (Table 2).

Discussion and Conclusion

MSC isolation and differentiation was performed to confirm if GFP cells maintain MSCs characteristics. The GFP helps tracking the cells, thus allowing too further track cell migration *in vivo*. This technique is especially relevant when applied to equine patients suffering from chondroarticular disorders. GFP was chosen due to its longer duration and stability than other techniques such as the use of membrane or intracellular dyes for *in vivo/in vitro* cell tracking.

Histological qualitative analysis proved that cells differentiate in both cases (transfected and non-transfected cells) but less in the transfected cells. So, to corroborate these results a

quantitative RT-qPCR was performed to check if gene expression of specific markers for each lineage was overexpressed [8, 41].

RT-qPCR was used due to its accuracy, sensitivity and faster results. Specific genes of reference were applied to normalize the results from the mRNA quantification.

Concerning the osteogenic differentiation, ALP genes is overexpressed in differentiated normal group and in the transfected cells differentiated group. However, RUNX2 is not overexpressed in GFP-MSCs, only in the normal differentiated group. This was not expected since the osteogenic differentiation was confirmed by staining the cells (Alizarin Red S). Probably, the labelling can have some negative influence in the osteogenic differentiation capacity and the results are somehow contradictory [8, 41].

Besides, in the adipogenic differentiation, the PPARγ marker was not analyzed since there was not enough sample, so only the results from LPL are illustrated. In Figure 7 it is noticeable that differentiated normal MSCs overexpressed LPL and so did GFP differentiated cells. In this case, GFP cells maintained the normal cells characteristics as expected. For the last differentiation group, chondrogenic differentiation, COL2A1 is overexpressed in differentiated normal and in GFP cells (lower in GFP cells). There was not enough sample, so the gene ACAN only had results to undifferentiated/differentiated normal cells, since the purpose was to confirm differentiation in transfected cells this graphic is not in the results since it is not relevant [8, 41]. About these results, probably the main reason for such differences between the differentiated GFP/normal cells and the lower differentiation potential of GFP-MSCs is the higher passage number (10 times for GFP cells versus 3-4 for the normal cells) rather than the transfection process. The cells might be entering senescence, so they did not perform very well.

Regarding the xeno-free approach to in vitro cultures of SM-MSCs, results obtained regarding cSM-MSCs suggested that xeno-free supplementation is a valuable alternative regarding *in vitro* cultures. Presto Blue™ Viability assay, showed that CS10% supplementation presented good viability results in early time-points, comparing to the other supplemented groups. Also, in general, 10% serum supplementation, regardless of bovine or allogenic, presented better viability result when compared to 20% serum supplementation groups. However, at later time point, differences between groups showed no statistically significant differences (except for the negative control group, as expected). Trypan Blue Exclusion Assay is expressed in Total Cell Number, allowing to compare cell viability assessed by Presto Blue™ viability assay with the total cell number. Results show that at later time points, 20% serum supplemented media presented higher total cell numbers, when compared to the 10% serum supplemented media. However, cell viability between these groups demonstrated no statistical significant differences at this time-point, showing that total cell number does not mean cell viability, especially when considering 72h time-point, where total cell number is greater for the

20% supplemented media, but cell viability on the other hand, is greater in the 10% supplemented media, specifically in the 10%CS supplemented group. To better understand these results, the beta-galactosidase senescence assay allows to assess cellular senescence between groups, and present higher senescence values for the 20% supplemented group at 72 hours' time-point. Thus, representing why, even with a higher total cell number, these groups presented lower viability results, when comparing to the 10% supplemented groups. Overall, results suggest xeno-free supplementation with canine serum as a valid alternative to FBS supplementation, but further assays should be performed, in order to select the best concentration of the supplementation to achieve the same or even better outcomes, when comparing to the standard FBS supplementation [40, 44].

Regarding e-SM-MSCs results presented rather worse results, especially when referring to the xeno-free supplemented groups.

At early stages, 10%HS supplemented group presented better viability outcomes, as assessed by the Presto Blue™ Viability Assay. However, at 72- and 120-hours' time points, HS supplemented groups showed significant loss of viability. Regarding the FBS supplemented group, overall 20% supplementation presented better outcomes. These results are corroborated by the Trypan Blue Exclusion Assay and by the Beta-galactosidase senescence assay. The latter presented lower senescence values for HS supplemented groups, however, these results can be explained as cell death result in lower cell number and thus in lower cell expressing senescence, as most of them are already dead. These results suggest that xenofree supplementation may not be citocompatible *in vitro*, however, a possible explanation for these poor outcomes can be the serum quality employed for this study. Further assays are envisioned, employing fresh horse serum collected from a pool of healthy selected donors, and adequately processed *in vitro* for inactivation and sterility [44].

To sum up, regenerative medicine is bringing a broad of new treatments and options. However, more studies are needed to understand better how to improve and optimize the use of these cells. The main purpose of this study was to learn more about regenerative medicine and its importance in nowadays therapeutic options. Therefore, it is possible to conclude that stem cells are unequivocally very important and with lot of potential.

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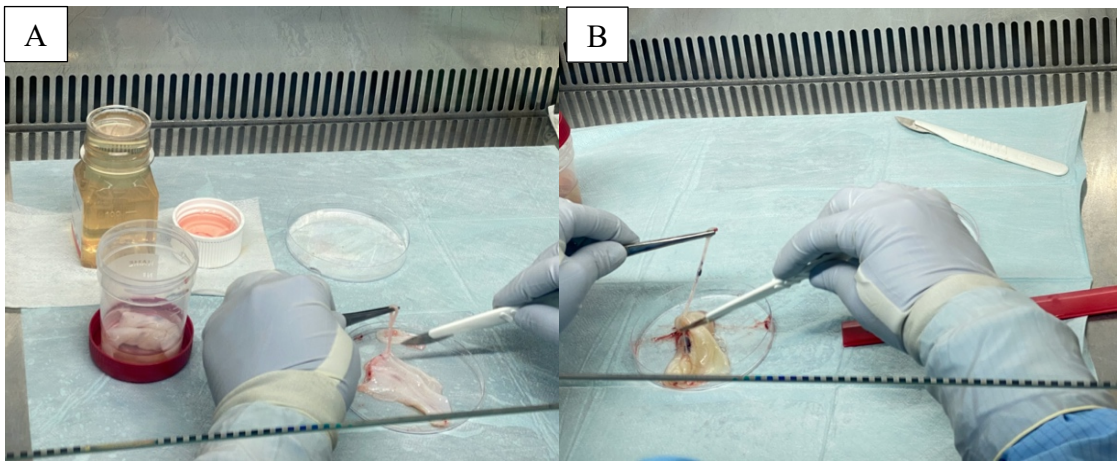
Annex

Table 1: Statistical significances about ß- Galactosidase senescence assay cSM-MSCs at different time points (24, 72 and 120h).

	0 horas					72 horas					120 horas				
	FBS20%	FBS10%	CS20%	CS10%	DMSO10%	FBS20%	FBS10%	CS20%	CS10%	DMSO10%	FBS20%	FBS10%	CS20%	CS10%	DMSO10%
FBS20%		ns	ns	ns	ns		***	ns	ns	****		****	****	****	****
FBS10%			ns	ns	ns			****	****	ns			**	****	****
CS20%				ns	ns				ns	****				****	****
CS10%					ns					****					****
DMSO 10%															

Table 2: Statistical significances about ß- Galactosidase senescence assay eSM-MSCs at different time points (24, 72 and 120h).

	0 horas					72 horas					120 horas				
	FBS20%	FBS10%	HS20%	HS10%	DMSO10%	FBS20%	FBS10%	HS20%	HS10%	DMSO10%	FBS20%	FBS10%	HS20%	HS10%	DMSO10%
FBS20%		ns	ns	ns	ns		ns	ns	**	***		ns	ns	**	***
FBS10%			ns	ns	ns			****	****	****			****	****	****
HS20%				ns	ns				ns	ns				ns	ns
HS10%					ns					ns					ns
DMSO 10%															



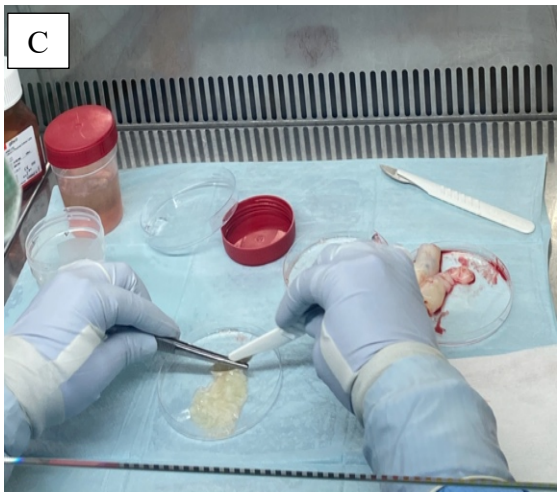


Figure 1: A, B and C - umbilical cord stroma tissue isolation.