

**EVALUATION OF THE CARDIAC REGENERATIVE POTENTIAL OF
MESENCHYMAL STEM/STROMAL CELLS FROM DIFFERENT
HUMAN SOURCES**

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ABSTRACT

As one of the main death causes worldwide, Cardiovascular Diseases, namely Acute Myocardial Infarction, have been the target of many intensive studies in order to develop new therapies capable of regenerating the damage caused by ischemia. Mesenchymal Stem/Stromal Cells (MSC) have been considered a prime candidate for cell therapy based treatments and have shown encouraging results in several early clinical trials. Despite presenting several promising capabilities, namely the production of molecules with angiogenic and immunomodulatory properties, the invasive harvesting process of these cells from Bone Marrow (BM) still poses as a problem. Alternative MSC sources such as Adipose Tissue (AT) and Umbilical Cord Matrix (UCM) have been studied. This work had the intent of evaluating the performance of BM derived MSC in terms of paracrine action on regeneration (through a Scratch wound assay with the use of MSC-based conditioned medium), as well as to establish an in vitro assay to measure oxidative stress resistance to study MSC from each source (BM, AT and UC) by the addition of H_2O_2 to the culture medium. When compared to regular Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, BM-derived MSC conditioned medium presented enhanced pro-regeneration rates. However, further studies should be performed in order to statistically support these results. In terms of oxidative stress resistance, AT and UC derived MSC cultured under normoxia (20% O_2) showed more resistance than BM derived MSC cultured in normoxic and hypoxic (2% O_2) conditions. Since this protocol was implemented and optimized during this thesis, further studies should be performed in order to acquire enough data to confirm this tendency.

Keywords: Acute Myocardial Infarction, Mesenchymal Stem/Stromal Cells, Bone Marrow, Adipose Tissue, Umbilical Cord Matrix, Angiogenesis, Paracrine Action, Oxidative Stress

RESUMO

Como uma das principais causas de morte no mundo, as doenças cardiovasculares (principalmente o enfarte agudo do miocárdio) têm sido alvo de estudos intensivos a fim de desenvolver novas terapias que visam a regeneração dos tecidos com danos resultantes da isquémia. Neste contexto, as células estaminais/estromais mesenquimais (MSC) têm sido consideradas um dos principais candidatos a terapias celulares e têm demonstrado resultados bastante promissores em vários ensaios clínicos. Apesar de apresentarem diversas capacidades com um enorme potencial terapêutico, nomeadamente a produção de moléculas que promovem angiogénese e modulam o sistema imunitário, o processo de colheita de células da medula óssea ainda é um problema, uma vez que se trata de um processo invasivo. Consequentemente, alternativas tais como o isolamento de MSC do tecido adiposo e da matriz do cordão umbilical têm sido investigadas. Este trabalho teve por objectivo avaliar o desempenho das MSC isoladas da medula óssea em termos da produção de moléculas que melhoram a regeneração, bem como estabelecer um ensaio *in vitro* que permita medir a resistência ao stress oxidativo das MSC de cada fonte através da adição de H_2O_2 ao meio de cultura. Quando comparado ao Dulbecco's Modified Eagle's Medium suplementado com 10% soro fetal bovino, o meio condicionado por MSC derivadas da medula óssea apresentou uma taxa de regeneração melhorada. No entanto, mais replicados são necessários para que estes resultados possuam mais relevância estatística. Em termos de resistência ao stress oxidativo, MSC derivadas do tecido adiposo e do cordão umbilical mostraram mais resistência que MSC isoladas da medula óssea cultivadas em condições de normóxia (20% O_2) e hipóxia (2% O_2). Uma vez que este protocolo foi implementado e optimizado durante esta tese, mais ensaios devem ser realizados para se obterem dados estatisticamente significativos que confirmem esta tendência.

Palavras-chave: enfarte agudo do miocárdio, as células estaminais mesenquimais, medula óssea, tecido adiposo, matriz do cordão umbilical, angiogénese, stress oxidativo, acção parácrina

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LIST OF ABBREVIATIONS

AMI	Acute Myocardial Infarction
AT	Adipose Tissue
AT-MSC	Adipose-derived stem/stromal cells
BIGH3	Transforming Growth Factor, Beta Induced, 68 kDa
BM	Bone-Marrow
CVD	Cardiovascular Disease
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
EC	Endothelial Cells
ECG	Electrocardiogram
EGM	Endothelial Growth Medium
FBS	Fetal Bovine Serum
FCT	Fundação para a Ciência e Tecnologia
FI	Fold Increase
FITC	Fluorescein Isothiocyanate
G-CSF	Granulocyte-Colony Stimulating Factor
GRP78	78 kDa Glucose-Regulated Protein
HGF	Hepatocyte Growth Factor
HSC	Hematopoietic Stem Cells
IGF-1	Insulin-like Growth Factor
LDH	Lactate Dehydrogenase
M-CSF	Macrophage Colony Stimulating Factor
MI	Myocardial Infarction
MSC	Mesenchymal Stem/Stromal Cells
NCD	Non-Communicable Diseases
NK	Natural Killer
NO	Nitric Oxide
P	Cell Passage
PBS	Phosphate Buffered Saline
PAI-1	Plasmin Activator Inhibitor - 1

PEDF Pigment Epithelium Derived Factor

PGE2 Prostaglandin E2

PTX3 Pentraxin Related Protein 3

ROS Reactive Oxygen Species

tPA Tissue Plasminogen Activator

TGF- β Transforming Growth Factor β

UC Umbilical Cord

UCM Umbilical Cord Matrix

VEGF Vascular Endothelial Growth Factor

WHO World Health Organization

1

INTRODUCTION

1.1. FRAMEWORK AND MOTIVATION

The 21st century has been largely dominated by the collaboration of technology and medicine in order to find new approaches to diseases that are lacking ideal medical solutions or even a solution at all. Although rates of mortality of Infectious Diseases are decreasing (by 47% globally since 2000 for the case of Malaria a [1]), the problem regarding Non-Communicable Diseases (NCD) has risen to the level of being the number one cause of death nowadays. According to World Health Organization (WHO), in 2008, from 57 million deaths that occurred globally, 63% were due to NCD, mainly, cardiovascular diseases (CVD) (48%), cancers (21%), chronic respiratory diseases (12%) and diabetes (3.5%) [2]. Therefore, we can conclude that NCD are a real problem and research and development on this topic is crucial for this century. As it was said by Dr Fred Hersch from SAID Business School of the Oxford University, "Globally the challenge of providing quality, affordable health care has never been so urgent. (...) There has never been a greater need for innovation in health care" [3].

Within CVD, Acute Myocardial Infarction (AMI) is expected to see an increase in mortality and morbidity rates between 1990 and 2020 [4]. Roughly, in developing countries, it is expected a 82% increase in mortality and 89% in morbidity [4]. Taking into consideration this expected growth in AMI incidence, it is imperative that the medical and engineering community investigate other therapies and treatments for this condition. Mesenchymal Stem/Stromal Cells (MSC) are proving to be a key player for cell-based therapies due to their

regenerative properties. A randomized study to evaluate the cardiac regenerative potential of bone marrow (BM)-derived MSC was made by Luciano C. Amado and his team, from the Cardiology Division of Johns Hopkins Hospital. The authors reported that, in pigs, allogeneic MSC can be administered to a region on the damaged myocardium without an adverse response of the body. Furthermore, engrafted MSC greatly reduce the necrotic myocardium and promote regeneration of new heart muscle [5].

MSC can be isolated from several sources exist, such as: BM, umbilical cord matrix (UCM) and adipose tissue (AT). Being the most studied, BM-derived MSC are the first candidate for harvesting. However, BM collection is an invasive process and the low amounts of collected MSC are a disadvantage. Due to those facts, the above mentioned two sources are starting to be explored since the UCM obtained from umbilical cord units is easily accessible as well as the lipoaspirates, which are discarded after liposuction procedures [45–48].

When harvesting MSC, important questions arise: Do MSC from different sources have the same characteristics and capabilities? What are the differences? In order to try to answer these questions, this thesis was focused on the development of a characterization platform of MSC from different cell sources, especially in terms of proliferation, pro-regenerative capacity and resistance to oxidative stress.

1.2. CARDIOVASCULAR DISEASES - ACUTE MYOCARDIAL INFARCTION

According to WHO, a CVD is the name given to the group of disorders of the heart and blood vessels. The most common are heart attacks and strokes. Although these two are related to different body parts (heart and brain, respectively), both result from a fat build-up on the inner wall of the blood vessels that supply the heart or brain. In terms of behavioral risk factors, they include unhealthy diet, physical inactivity, tobacco use and excessive alcohol consumption. The practice of the mentioned behaviors can lead to increased blood pressure, raised levels of glucose in the blood, overweight and obesity. These are high risk indicators of developing a CVD. As it was reported by WHO, 12.2% of worldwide deaths were caused by ischemic heart disease. More specifically, it was the leading cause of death in high or middle-income countries. Although AMI death rate has been decreasing, it was still responsible for one in three of USA total deaths in 2008 [2].

1.2.1. PATHOPHYSIOLOGY

A living cell needs a constant flow of nutrients, oxygen and a removal of metabolites to be able to survive. These processes are assured by the blood, which delivers all the nutrients and oxygen the cell needs and helps with the removal of substances that are the result of the cell's metabolism. During an AMI, the occlusion of the blood flow of the coronary arteries occurs. A prolonged occlusion can result in irreversible myocardial damage and cell death due to lack of nutrient and oxygen supply. This obstruction is normally caused by the atherosclerotic plaque, which increases the possibility of intra-coronary thrombus formation. The erosion of

that plaque can sometimes create a thrombus large enough to block the coronary blood flow and initiate a myocardial infarction (MI). The severity level of a MI is described by 3 factors: level of occlusion, duration of the occlusion and presence/absence of collateral circulation [6].

1.2.2. DIAGNOSIS

Taking into consideration the information made available by the WHO, a patient can be diagnosed with MI if two or three of the following criteria are met [11]:

1. Clinical history of ischemic type chest pain lasting more than 20 minutes.
2. Changes in serial ECG tracings.
3. Rise and fall of serum cardiac biomarkers such as creatine kinase and troponin.

Relatively to a general examination of the patient, the physician needs to account for several symptoms, among which are included: pain in breathing (known as dyspnea) [12] or chest discomfort during rest or exercise that lasts more than 20 minutes [13]. Furthermore, the patient can also suffer from nausea, syncope (brief loss of consciousness) or diaphoresis (excessive production of sweat) [12, 13]. Since these represent just some symptom guidelines, and their presence can be indicator of other condition besides AMI, it is of vital importance to take further testing using methods that allow a confirmation of the existence/possibility of infarction.

In terms of specific examination, the most common is the Electrocardiogram (ECG). Depending on the characteristics of the patients' ECG, they are divided into one of the following 3 groups ([14, 15] (Figure 1.1) :

- with ST segment elevation (suspicious for acute injury)
- with ST segment depression (suspicious for ischemic injury)
- with a normal ECG. However it does not discard the possibility AMI.

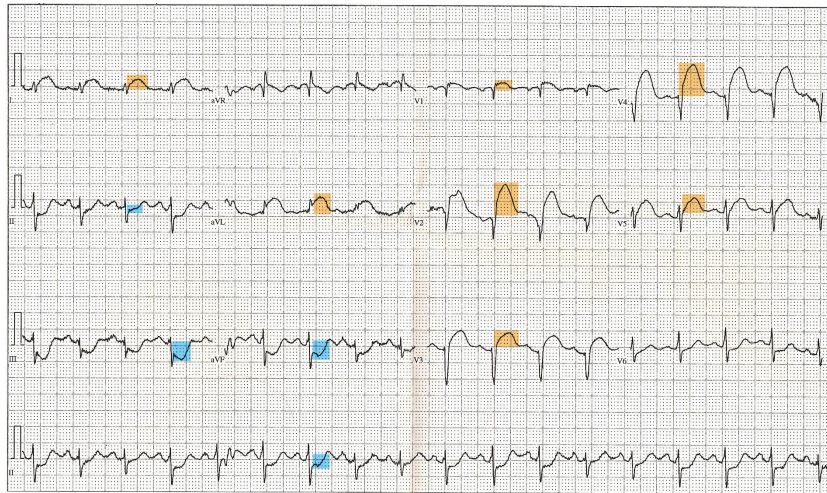


Figure 1.1: **Pathological ECG (with ST segment elevation in orange and ST depression in blue)**. Source: http://en.wikipedia.org/wiki/Electrocardiography_in_myocardial_infarction, Consulted on April 2nd, 2015

In combination with the fulfillment of the mentioned criteria , general diagnosis and specific examination, there is another important aid for an accurate diagnosis: Risk Factors, which are going to be explored in the next section.

1.2.3. RISK FACTORS

A risk factor is a variable association with an increased probability of a certain disease or infection. According to the World Heart Federation, there are several risk factors associated with the possibility of coronary heart disease which are divided into two clusters: Modifiable (which are possible to correct) and Non-Modifiable Factors (are not possible to change) [16].

Within the group of Modifiable Risk Factors, Hypertension is the one of the most important. It is usually related to a hazardous lifestyle in terms of unhealthy diet and lack of exercise. Through a change of routine, medication and a specific management plan, this risk factor can be minimized [16].

Another factor that is on top of the list is smoking. Whether smoked or chewed, tobacco increases the risk of CVD, especially if it is a habit you develop in a young age [16].

There are several more modifiable factors such as physical inactivity, unhealthy diet and diabetes type 2 which greatly increase the probability of developing CVD. However, a serious approach in controlling these conditions is an important step in decreasing the chances of being affected by CVD [16].

In terms of the second group, Non-modifiable Risk Factors, these are the ones that can not be changed. The usual factors are age, medical family history of CVD, gender and ethnic origin. For instance, the risk of CVD increases with age, as well as the presence of family relatives who were affected by these diseases. More-

over, men have greater risk of heart disease than women and people with African or Asian ancestry also have increased risk [17].

1.2.4. PREVENTION, TREATMENT AND MANAGEMENT

Prevention of heart disease has its basis in the correction of old behaviors that can increase the risk of developing an AMI and the adoption of new ones such as, a healthy diet. This is the most effective way to avoid this disease, hence the importance of living a healthy life.

In terms of Treatment and Management, patients that suffer an AMI usually receive a first set of Emergency Care. Its goal is to minimize the first symptoms characteristic of a heart attack. Usually morphine is used to treat chest pain in order to minimize vasoconstriction and heart workload. There is also the possibility of using β - blockers for the same purpose. Moreover, the patient also receives oxygen to compensate for any breathing problems as well as a tranquilizer to minimize anxiety [18].

Since during an AMI episode, time is crucial, the patient needs to arrive at the hospital as soon as possible so, to avoid any kind of delays and to have the chance of receiving medical treatment. Within 1/2 hours of the onset of symptoms, patients might be advised to take Aspirin and Nitroglycerin to trigger a pharmacological reperfusion therapy. This might help in avoiding necrosis of the myocardium and possibly leading to a better prognosis [19–21].

PHARMACOLOGICAL REPERFUSION THERAPY

Reperfusion therapy is the name given to the treatment that restores the blood flow after an AMI which comprises the administration of specific drugs. The process that serve as basis for this treatment is called thrombolysis and the drugs administered activate enzymes which remove the blockage and reestablish the blood flow [22]. This thrombolytic therapy has its highest efficiency rate in the first 2 hours. The reason behind this fact is that, after 2 hours, irreversible injury occurs. So, in order to preserve the highest extant of functional myocardium, this treatment should be applied before that time window. Although there are several different thrombolytic agents, the effective ones have similar general characteristics: high sustained patency rate, specific for recent thrombi, create a low risk for intra-cerebral and systemic bleeding, easily and rapidly administered, have no antigenicity, adverse hemodynamic effects, or clinically significant drug interactions, and be cost effective [23]. The available agents consist of streptokinase and other similar examples like tissue plasminogen activator (tPA). All of these belong to group of fibrinolytic medications. These increase the production of plasmin which is responsible for the degradation of fibrin, the major component of blood thrombi. Based on the agent used, adjuvant anticoagulation medication may be used in order to maintain coronary artery patency (openness) [24, 25].

PERCUTANEOUS CORONARY INTERVENTION - PCI

This procedure is the one with highest efficiency rate, restoring the blood flow in around 95% of the patients [26].

It implicates performing a coronary angiogram, which allows the physician to determine the anatomical location of the blocked vessel, followed by a balloon angioplasty of the thrombotic segment. An attempt to aspirate the thrombus is often performed prior to the angioplasty [27].

Additional therapy during PCI includes medications such as heparin, aspirin and clopidogrel. Glycoprotein IIb/IIIa inhibitors are also used to reduce the risk of ischemic problems throughout the procedure [28, 29].

CORONARY ARTERY BYPASS SURGERY

A coronary artery bypass surgery consists on the implantation of an artery or vein to bypass narrowing or blockages on the coronary arteries. However, this intervention is applied only in situations that, besides the infarction, mechanical problems are also involved that lead to cardiogenic shock. One of the drawbacks of this procedure is the high initial costs associated, albeit being cost-effective in the long term [30, 31].

1.2.5. EPIDEMIOLOGY

CVDs are the cause of around 4 million deaths annually in Europe. More specifically, in Portugal, 2011 registered a death rate caused by CVD's of 174.7 per 100 thousand people for males and 126.8 for females. These values are comparable to those registered in 2010 in countries like the UK, where the death rate was 205.2 per 100 000 people for males and 129.0 for females [32]. Generally speaking, the incidence of AMI is still increasing, and there are some countries that are a reflection of those numbers, such as Japan. Indeed, probably due to the increasing acceptance and adoption of western lifestyle and diet, increasing smoking levels and higher incidence of conditions like diabetes and obesity, Japan is suffering a rise in AMI cases. Despite the increasing incidence of AMI, cardiovascular problems are being tackled and there are some important improvements worth mentioning [33–36, 38–41]:

- Higher number of informed people in terms of what are the main risk factors for AMI and how to control them. The need for physical exercise, the dangers of tobacco smoking and the problems associated with high cholesterol have been some of the points targeted.
- Improved detection of AMI milder cases with more sensitive biomarkers
- Higher quality treatments such as Percutaneous Coronary Intervention, Coronary Artery Bypass and the use of the thrombolysis effect result in much better prognosis.

Data from 2012 revealed a percentage of death caused by CVD of 30.4% in Portugal. This value is less than the 45% registered in 1999, but it is still too high. One of the explanations possibly resides on the fact that Portuguese population still shows high percentage of certain risk factors such as: hypertension (42%), high blood cholesterol for people over 18 (63.7%) as well as overweight (59.1%) and physical inactivity (53.9%) [41].

1.3. CARDIAC REGENERATION AND REPAIR

One of the main problems after an AMI occurs is that the necrotic tissue is not replaced by functional cardiac muscle. Instead, fibroblasts are called to the injury site where they form a collagen-rich scar tissue without any contractile ability, which will weaken the heart making it more susceptible to additional AMI and organ failure [42]. Due to this fact, the development of a cell-based therapy that can reestablish functionality to the damaged heart muscle after an AMI is of major importance.

1.4. CELLULAR THERAPY AND TREATMENTS

Although there is evidence of the regenerative potential of the heart, it is indeed limited when applied to the regeneration of the cardiomyocytes that were affected after an AMI [43]. Due to that fact, alternative strategies are being explored in order to improve heart regeneration, not only through the administration of innovative and more suitable drugs, but more recently, through the use of cell based therapies [44].

1.4.1. MESENCHYMAL STEM/STROMAL CELLS

DEFINITION AND IDENTIFICATION

Around 1960, Ernest A. McCulloch and his partner, James E. Till, identified the first MSC as clonal bone marrow stromal cells, which were further investigated by Friedenstein in the 70s [7]. Their capability of self-renewal and multi-lineage differentiation was also tested by Friedenstein around 1980 [8] and, during the last couple of decades, their possible therapeutic applications have been target of extensive research due to their regenerative potential and immunomodulatory capacities (which makes them prime candidates for therapies regarding tissue regeneration and immune-mediated conditions) [63].

Although there is still no consensus amongst the scientific community regarding the characteristics that define a MSC [9, 10], according to The International Society for Cellular Therapy, a cell needs to fulfill a minimum criteria to be defined as a multi-potent human MSC: (i) capability of adhesion to plastic under standard culture conditions; (ii) expression of CD105, CD73 and CD90 and absence of expression of the haematopoietic cell surface markers CD34, CD45, CD11a, CD19 or CD79a, CD14 or CD11b and histocompatibility locus antigen (HLA)-DR; (iii) ability to differentiate into osteocytes, adipocytes and chondrocytes in vitro, under

standard *in vitro* differentiating conditions [53]. In terms of specific identification of MSC, a sole surface marker is yet to be found. In this field, recent researchers have been looking into potential options and are paying special attention to the following 4: Stro-1, CD271, SSEA-4 and CD146 [10].

SOURCES

MSC can be extracted from several tissues, being the most popular BM (Figure 1.2 on the left) [45], AT (Figure 1.2 on the center) [46] and UC matrix (Figure 1.2 on the right) [47, 48]. Although MSC are present in these three types of tissue, they register some differences between them. The gold standard MSC source is BM. It was the first discovered and it is the most used, albeit only around 0.01% of nucleated cells are recovered from BM harvesting [45]. The harvesting process is another drawback of this source. It is a painful procedure and requires anesthesia. Moreover, the number BM-MSC that can be obtained decreases with the age of the patient [46]. Due to all these problems, researchers are exploring alternative sources for MSC. AT is reported as a possible good alternative since it requires a less invasive harvesting process (it is usually discarded as medical waste from interventions such as lipoaspirations) and higher frequency of MSC has been collected when compared to BM [49, 50, 106]. In terms of isolation processes, the general base of them is the ability of plastic adherence characteristic of MSC. For BM, mononucleated cells are isolated from the BM aspirate by centrifugation on a density gradient [51]. When isolating MSC from AT, there is a first processing step that involves an enzymatic reaction, in order to isolate the Stromal Vascular Fraction, which is a heterogeneous cell population (it includes HSC, Endothelial Cells (EC), adipose stromal cells, fibroblasts, among others). Upon culture initiation, the MSC population or Adipose-derived Stem/Stromal Cells (ADSC) will adhere to the plastic culture surface and can be collected after expansion [50]. The process of MSC isolation from the UCM involves an enzymatic digestion or an explant culture. The isolation process of UCM-MSC is explained in the Materials and Methods section.

Taking into consideration the availability of biological samples from BM, AT and UCM sources, one of the goals of this work was to study the potential of these cell sources and further characterize its similarities and differences. More specifically, the work was more incisive in evaluating the resistance of MSC from different sources to oxidative stress. The importance of this relies on the fact that there is formation of oxygen reactive species upon an AMI. These are toxic species for MSC and lead to their premature senescence which impair their regenerative potential [52].

Choosing the MSC source that presents more resistance to this environment will, possibly, increase the efficiency of the graft upon administration of the cell therapy for the treatment of patients that suffered an AMI.

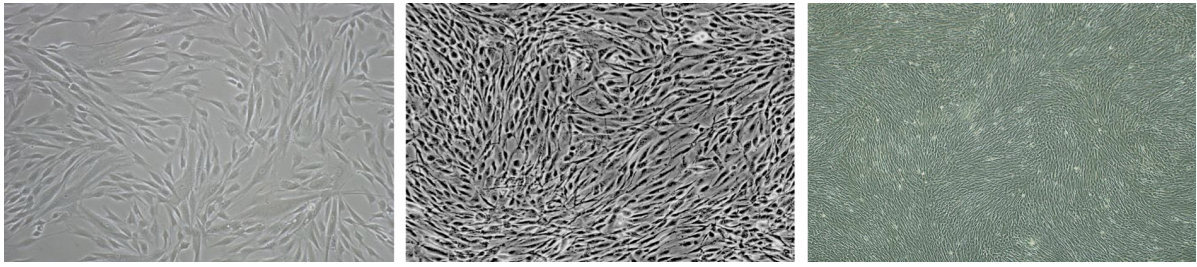


Figure 1.2: Human MSC from BM (left), AT (center) and UCM (right)

CHARACTERISTICS

- **Regenerative Properties**

In the last couple of years, the suggestion that MSC have important influence in regeneration upon injury has been supported by both clinical and preclinical trials [63]. From wound healing to cell replacement, research has been exploring MSC effect on tissue injury and degenerative diseases. For instance, the administration of autologous BM-MSC in cirrhosis patients showed improved clinical values of liver function as well as in patients with hepatitis B [63]. BM-MSC have been also showing promising results in regeneration of other tissues such as bone, skin [64–66], cornea [67], spinal cord and lungs [68–70]. Furthermore, it has also been shown that MSC can improve the effect of HSC in the treatment of radiation victims [71].

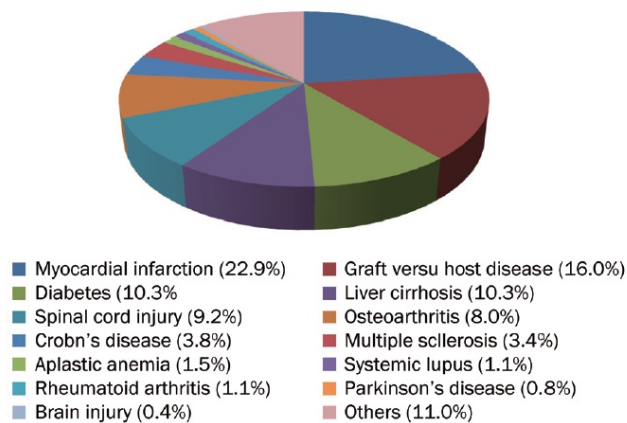


Figure 1.3: Several diseases studied with mesenchymal stem/stromal cells therapies. Data from ClinicalTrials.gov and [63] (accessed on March 20th 2015)

Immunomodulation

- T Cells

In relation to T cells, these are a type of lymphocyte with an extremely important role within the immune system. Responsible for cell-mediated immunity (immunological reaction with the use of cells instead of antibodies), these cells can subdivide themselves into several subtypes according to their tasks, for example: Helper T Cells, which assist other white blood cells in immunological processes, Cytotoxic T Cells, which destroy virus-infected cells and tumor cells and Memory T Cells which, after being used for a certain disease, persist in the body providing the individual with long-term immunity to that disease [72]. Regarding T cells-*MSC* interaction, *MSC* act as inhibitors to their proliferation by preventing their entrance into the S phase of the cell cycle and by creating a phase arrest at the G0/G1 stage [54]. The modulation effects of *MSC* are based on the secretion of soluble factors (in the case of human *MSC*) and cell-to-cell interaction (in the case of rodent *MSC*) [55–58]. For instance, there are some studies that claim that the nitric oxide (NO) secreted by *MSC* is also a way used by these cells to inhibit T cells' division (NO suppresses the phosphorylation of signal transducer and activator of transcription-5 (STAT5), a transcription factor essential for T cell activation and proliferation, thus inhibiting the proliferation) [59].

– B Cells

B cells (or B lymphocytes) are part of the adaptive immune system. Their role is to secrete antibodies (that act against body threats) to a specific type of antigen (which is the marker of a certain disease). Furthermore, they also secrete cytokines (proteins) that control some signaling immune regulatory functions [73]. Similarly to T cells, inhibition of B cells by *MSC* is made by blocking G0/G1 phases of the cell cycle. Although the mechanisms behind this inhibition are not yet totally known, the suppression is attributed to the contact between *MSC* and B cells as well as to the secretion of several soluble factors. This results in an arrest of the cell cycle with no cell apoptosis [53]. Moreover, *MSC* also have an inhibitory action in the expression of chemokine receptors, as well as IgG production stimulated by B cells [54].

– Natural Killer (NK) Cells

NK cells are a type of lymphocyte crucial to the innate immune system. Based on the concept of cytotoxicity (i.e. cell toxicity), NK cells detect and infect viral-infected cells as well as tumor cells and promote their apoptosis [74]. Regarding NK cells inhibition, many studies showed that *MSC* inhibit proliferation of NK cells as well as IFN gamma production (through the production of IL-2 and IL-15). However, that inhibition is only partial in the case of activated NK cells [53]. On the other hand, some studies also showed that activated NK cells can kill *MSC*. More specifically, IL-2-activated NK cells can lyse *MSC* due to the fact that *MSC* exhibit ligands that are recognized by NK

activated cells' receptors [60, 61].

– Dendritic Cells

Dendritic cells are cells from the immune system with the function of presenting antigens to the T cells. These antigens enclose the information regarding certain types of diseases and this information is given to the T lymphocytes to gain immunity to several medical conditions (for example chicken pox). Therefore dendritic cells are the connection between the innate (nonspecific component of the immune system that provides general protection against foreign organisms) and adaptive immune system (which is specific to the foreign microorganism in question) [75]. MSC act on the modulation of these cells by inhibiting the maturation signals and consequently hindering the differentiation of monocytes into dendritic cells [53, 54].

Differentiation Potential

Being multipotent stem cells, MSC have the capability of differentiating into several cells types, amongst which are included: adipocytes, osteoblasts, chondrocytes and myoblasts. Furthermore, it has been shown that MSC can differentiate into cardiomyocytes and other tissues after their *in vivo* infusion during the treatment of conditions like myocardial infarction, both in animals and humans. Besides this ability, MSC can also be delivered to injury sites together with biomaterial-based scaffolds. This kind of approach has already been applied to cartilage and bone repair. There is a great potential for this technology, and the development of better delivery vehicles and better compatibility between "scaffold-*MSC-tissue*" will certainly increase the number of possible clinical applications [63].

Homing Properties

It has been found that MSC have the tendency of directing themselves to the injured sites upon *in vivo* administration. In several animal and human trials, it was verified that these cells migrate to damaged tissues where inflammation is happening. This kind of inflammation-directed MSC homing has been demonstrated to involve several different types of molecules such as chemokines (like CXCR4) [76, 77] and adhesion molecules (such as P-selectin and VCAM-1) [78]. This capability is seen as a potential starting point for achieving breakthroughs also in cancer targeted therapies. For instance, MSC are being developed to work as vehicles to specific nanoparticles that enhance tumoricidal effects [79, 80].

Secreted Factors

Regarding the secretion of certain factors with therapeutic potential, MSC are known for being a great source of trophic factors (substances that promote cell growth, differentiation and survival. When the MSC arrive to the injury site, they interact with the environment and those local stimuli can induce the MSC to produce more growth factors, which are of paramount importance to tissue regeneration. More specifically, many of these factor have an important role in angiogenesis and in preventing cell apoptosis, such as vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1) and hepatocyte growth factor (HGF) [81–83].

MESENCHYMAL STEM/STROMAL CELLS NICHE

According to the concept of "niche", introduced by Schofield in 1978 [84], the MSC "niche" refers to the microenvironment that immediately surrounds the MSC cells when they are in their naive state. It includes the extracellular matrix, the soluble molecules and the non-stem cells with which the MSC might be in contact. All these components interact with each other and are crucial in maintaining the MSC in their undifferentiated state [85]. When MSC are needed in order to promote the regeneration of a tissue, external cues are directed to those MSC, inducing their differentiation [86]. Since MSC can be isolated from BM, it is pertinent to analyze some of the characteristics of its microenvironment. The BM niche is characterized by a low oxygen percentage (varying from below 1% in the niche itself and 6% in sinusoidal cavity [87], which is intimately related to an increased proliferation and maintenance of a naive state of MSC [85]. Furthermore, it has been shown by Rosová and co-workers, that a preconditioning of MSC based on a low oxygen tension can enhance their therapeutic capabilities [88]. Therefore, in order to establish cellular therapies that are more efficient, it is of great importance to have detailed knowledge of how MSC behave when subjected to hypoxic microenvironments. Santos and co-workers developed, in 2010, an analysis of proliferation kinetics and metabolism of MSC under hypoxic conditions (2% O₂) and showed that, not only the hypoxic conditions favored cell proliferation, but also increased metabolic function [89]. Thus, supporting the rationale of also applying the protocols develop during this thesis to hypoxic-cultured MSC.

KINETICS OF CYTOKINE SECRETION

Like it was mentioned above in this section, MSC have been shown to be able to secrete several molecules that influence their surroundings. For example, MSC are able to produce TGF- β and PGE₂ (prostaglandin E₂) which suppress the inflammatory response, VEGF, which mediates endothelial and epithelial cell proliferation and cytokines such as G-CSF and M-CSF [90–93]. However, when undergoing differentiation, MSC can suffer some changes in terms of secreted molecules [94]. Chiellini and colleagues studied the secretome of human mesenchymal stem cells at early steps of differentiation towards adipocytes and osteoblasts. Their results showed a couple of differences, not only when comparing day 0 and day 3 of adipogenesis and osteogenesis, but also between day 3 of adipogenesis and day 3 of osteogenesis. For instance: when compared

to day 0, PEDF (pigment epithelium derived factor), GRP78 (78 kDa glucose-regulated protein precursor), BIGH3 (transforming growth factor-beta-induced protein) and PTX3 (pentraxin 3) showed increased expression at day 3 of differentiation towards adipocytes and osteoblasts. Furthermore, PAI-1 was present towards all osteogenesis, whereas it was not detected after day 3 of adipogenesis. [94].

MESENCHYMAL STEM/STROMAL CELLS SUBSETS

Not only MSC can be isolated from different sources [45–48], subpopulations can also be identified inside a MSC population [95]. Tormin and colleagues published in 2009 a study in which they tackled this subject. Although they stated that the standard MSC markers did not allow for an identification of subpopulations within the cultured BM-MSCs, they performed a distinction of those subpopulations based on proliferation characteristics of the cells [95]. Tormin and co-workers were able to distinguish a slow and non-dividing cell (SDC and NDC, respectively) subpopulation and a rapidly-dividing cell subpopulation (RDC). Their results showed several differences between these subpopulations, for instance, NDC subpopulation cells were large and cuboid shaped, whereas RDC cells were small and spindle-shaped and there was also a difference in terms of adipogenic differentiation: NDC cells revealed impaired adipocyte differentiation when compared to the RDC cells. Furthermore, the NDC subpopulation showed the lowest concentration of clonogenic progenitors while RDC showed the highest [95].

1.4.2. CHALLENGES TO OVERCOME IN CELL THERAPY CLINICAL TRIALS FOR CARDIOVASCULAR DISEASES

In terms of clinical trials, several studies have been made regarding the use of MSC as treatment to cardiovascular diseases. For instance, a pilot study, where 69 patients (who had suffered an AMI) were injected (via intracoronary injection) with autologous MSC revealed that significant improvements were obtained when compared to the control patients, who received a saline injection. Furthermore, this study included MSC viability measurement and cardiac function evaluation through cardiac electromechanical mapping. The results indicated that MSC were still viable 3 months after the transplantation [97]. After this study, many others followed using MSC as a therapy for acute and chronic myocardial infarction, revealing many improvements in patient's heart function [98–102]. Allogeneic MSC were also tested as an alternative to autologous therapy, showing a good performance when compared to the patients that received the placebo treatment [103]. Taking into consideration these results, there is the possibility of developing an "off-the-shelf" MSC treatment for cardiac problems [96].

Despite the fact that important developments have been made regarding therapies with MSC, there are still plenty of aspects that need to be further studied. In fact MSC do not raise ethical issues, they can be har-

vested from different sources and do not present risk of teratoma formation. However, their use in a clinical setting is still not fully matured. Under debate are questions that need to be clarified such as which source of MSC should be used for a specific disease, which is the best way to administer the cells and which are the contraindications of these treatments[63].

2

AIM OF THE STUDY

The main purpose of this thesis was, firstly, to establish an *in vitro* assay to measure oxidative stress resistance of MSC and then, perform a comprehensive study regarding the different properties showed by MSC from different sources - bone marrow, adipose tissue and umbilical cord matrix. Several cell features were studied, namely proliferative capacity but specially in terms of angiogenic potential and resistance to oxidative stress.

In summary, *ex-vivo* expansion of BM-derived MSC and AT-MSC was performed, as well as the isolation and expansion of UCM-MSC. The cells were studied and compared in order to provide important clues on the cardiac regenerative properties of MSC from different human sources and, ultimately, potentially defining the *pros* and *cons* of each MSC source.

In terms of collaborations, the main partners were IPO - Instituto Português de Oncologia Francisco Gentil, Lisboa, who kindly provided us with the BM samples, Clínica de Todos os Santos who provided the AT samples and finally Hospital São Francisco Xavier, who contributed with the samples of umbilical cord units.

3

MATERIALS AND METHODS

This section has the purpose of providing an inside view of all the protocols used and developed in order to achieve the results presented in section 4. It starts with basic cell processing techniques and then moves to more specific experiments and assays that are intimately related to the work developed during this thesis on the characterization of the potential of MSC from different cell sources.

3.1. CELL CULTURING

The first step was to thaw the vials containing the cell suspension in a water bath (Concessus) at 37°C and add it to 5mL of low-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco by LifeTechnologies) + 20% MSC-qualified fetal bovine serum (FBS) (Hyclone) and 1%Anti-Anti (Life Technologies). The cell suspension was then centrifuged (ScanSpeed) for 7 min, at 1250 rpm (349.375 g's), the supernatant was removed and the cell pellet was re-suspended in the appropriate medium (DMEM + 10% FBS + 1% Anti-Anti for MSC culture or Endothelium Growth Medium (EGM) - 2 (Lonza) for Human Umbilical Cord Vein Endothelial Cells (HUVEC) culture). The cells were plated on T-flasks at a cell density of 3.000 cell/ cm^2 .and incubated in a humidified atmosphere at 37°C with 5% CO_2 . The media was changed every 3 or 4 days.

3.2. CELL PASSAGING

As a standard protocol, the cell passaging was performed when cells reach around 70-80% confluency. Firstly, the spent medium was removed and a wash with Phosphate Buffered Saline (PBS) (Gibco) was made. Afterwards, Trypsin 0,05% (Gibco) was added to dissociate the cells (and incubated for 7 min at 37°C). The enzymatic reaction was stopped with culture media (DMEM + 10% FBS not MSC grade (Gibco)) by adding two times the volume of trypsin. The cell suspension was then centrifuged for 7 min, 1250 rpm (349.375 g's), and resuspended using the desired culture medium.

3.3. CELL FREEZING

The cells were frozen in 90% FBS with 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich). The Cryo Tube vials (Thermo Scientific) were filled with 1 mL of the cell suspension (which was previously suspended in freezing media). The cells were frozen overnight at -80°C in freezing containers to promote a slow cooling. Afterwards, the cells were transferred to liquid nitrogen.

3.4. UCM ISOLATION

Next it is described the two methods used for the isolation of the MSCs from UCM, the enzymatic digestion method and a non-enzymatic method.

Enzymatic Digestion Method

Firstly, the UC was transferred to a 250mL shot glass and washed with PBS EDTA (EDTA from Fluka Analytica) with 1% Anti-Anti. Afterwards, the UC was dissected, the 2 arteries and 1 vein were removed and the tissue around them was collected. The tissue was then minced and digested in a solution of 0.1% Collagenase Type II (Sigma-Aldrich) in DMEM + 1% Anti-Anti (up to 6 g per 40 mL of enzymatic solution) for 4 hours (750rpm at 37 ° C, Termomix from Eppendorf). Using a steriflip (Millipore), the digested solution was filtered and centrifuged at 1500 rpm (503.1 g's) for 10 min. After that, the top viscous solution was carefully discarded, DMEM + 1% Anti-Anti was added to the Falcon tube until to reach the 50 ml of total volume, and the solution was once again centrifuged, 1250 rpm (349.375 g's) for 7 min. Following this step, the supernatant was discarded, the cell pellet re-suspended in DMEM + 1% Anti-Anti and the cells were counted using Turk's Reagent (Merck Millipore) (dilution 1:10). As a final step, the mononuclear cell fraction was frozen or plated at a density of 10000 cell/cm^2 , in a humidified atmosphere at 37°C and 5% CO_2 , for *ex-vivo* expansion.

Non-Enzymatic Method (Explants)

The first part of the protocol that involves washing the UC and collecting the desired tissue was identical

to the one used for Enzymatic Digestion. However, in this case, after mincing the tissue, the pieces were placed in a 6 well plate or Petri Dish and left in the incubator for 5 to 10 min in order for them to adhere to the bottom of the surface. Subsequently, the cells were plated in the desired medium and incubated in a humidified atmosphere at 37 ° C, 5% CO₂. The culture medium was changed every 3 to 4 days. The explants were removed after around 8 to 10 days and the plate left incubating until the wells reached confluency.

3.5. HUMAN SAMPLES

BM samples were previously acquired from healthy donors (Table 3.1) and were provided by IPO - Instituto Português de Oncologia (Lisboa), AT samples were acquired from Clínica de Todos os Santos (Lisboa) and the samples from the umbilical cord units were provided by Hospital São Francisco Xavier. All these samples were obtained with informed consent and were processed and cryopreserved at the SCBL-RM Laboratory. The HUVEC also used during the experiments were a cell line purchased from BD and the L929 Fibroblasts were acquired from DSMZ, Germany.

Table 3.1: BM Donors used in this work

Donor	Donor Birth Year	BM Harvesting Year	Donor Age	Donor Gender
M72A07	1972	2007	35	Male
M79A15	1979	2015	36	Male
M67A08	1967	2008	41	Male

3.6. POTENCY ASSAYS

3.6.1. ANGIOGENIC/TUBE REMODELING ASSAY

In order to evaluate the effects that MSC can have regarding the Angiogenesis phenomena, the interaction between MSC and HUVEC by direct contact and paracrine action was tested. The co-culture system, intends to mimic in vivo conditions of vascular regeneration processes. The effect of direct contact of cells was tested in a tube formation assay using Matrigel and consequently a wound healing assay by chemical disruption of the tubes formed. On the other hand, the paracrine action was studied by using the MSC conditioned media to promote wound healing in HUVEC cultured in monolayer.

TUBE FORMATION AND INCORPORATION/CHEMICAL TUBE DISRUPTION WITH DMSO

As a first attempt to evaluate the MSC angiogenic potential, three protocols were used. Tube formation assay and Tube incorporation, which were previously developed in the group, and Tube disruption which was developed during this project. In summary:

- For tube formation: 25,000 HUVEC ([104]) were cultured in Matrigel (Corning) coated wells (50 μ L of Matrigel per well and incubate for 1 hour) in 96 well plates (BD). This assay was also tested MSC, however, 10,000 cells per well were used. Duplicates were used.
- For tube incorporation: 20,000 HUVEC ([104]) were co-cultured, in EGM-2 media (Lonza) with 5,000 MSC ([104]) in Matrigel coated wells of 96 well plates (50 μ L of Matrigel per well). The MSC were previously labeled with 10 μ g/ml Dil-Acetylated-Low-Density Lipoprotein in EGM-2 media at 37 ° C for 20 min . After the incubation period, the cells were washed with 2 mL of PBS and centrifuged at 1250 rpm (349.375 g's) for 7 min at room temperature. The supernatant was then discarded and the pellet resuspended in EGM-2 media. Labeled MSC were mixed with HUVEC and plated on top of the Matrigel coating (200 μ L of cell suspension per well). The co-culture of cells was incubated in a humidified atmosphere at 37°C and 5% CO₂, first during 16 h and afterward during 8 h). After the incubation time, the cells were washed with PBS (200 μ L/well) and incubated with 100 μ L of calcein solution (8 μ g/mL) for 30min in a humidified atmosphere at 37°C and 5% CO₂. Then, the cells were washed with PBS (200 μ L/well) and the fluorescence was pictured under a fluorescent microscope (Leica) and using the software Nikon ACT-1 2.70. Duplicates were used.
- Chemical tube disruption: The tubes were allowed to form as described in the tube formation protocol. The disruption was performed chemically by the addition of DMSO in (EGM-2). Incubation time and DMSO concentration (0.1, 1, 2, 5, 10, 30, 50, 70, and 100%) were optimized. After the incubation time, fresh culture media was added and the cells were incubated in a humidified atmosphere at 37°C and 5% CO₂ to allow tube remodeling.
- Mechanical tube disruption: The tubes were allowed to form as described in the tube formation protocol. The disruption was using both syringes of different sizes and pipet tips and the 200 μ L pipette tip was chosen. The wound was performed perpendicularly to the surface of the well. Afterwards, the culture medium was removed, the cells washed with PBS and replaced by fresh culture media. Consequently, the cells were incubated at 37°C and 5% CO₂ for 7 to 8 hours, during which pictures were taken every hour in order to register the remodeling of the tubes.

SCRATCH WOUND ASSAY OF HUVEC MONOLAYER BY PARACRINE ACTION

To assess the regenerative potential of MSC by paracrine action, MSC conditioned medium was collected and incubated with HUVEC cultured in monolayer after mechanical disruption to promote wound healing.

HUVECs were plated at 40,000 cells/well in 96 well plates) and incubated overnight at 37°C and 5% CO₂ in order to have a cell monolayer. Afterwards, using a pipet tip vertically to the cell culture surface, a scratch was made on the monolayer. The goal was to achieve a wound with around 100-200 μ m of width. The next

step was to remove exhausted medium, wash each well twice with EGM-2 medium, and then add 200 μL of conditioned media (from donors BM M72A07 and BM M79A15) per well. EGM-2 and DMEM + 10% MSC-qualified FBS were used as controls. Wound remodeling was pictured every hour by using the fluorescent microscope and the software Nikon ACT-1 2.70. As a final step, the average wound width at each time point was calculated by measuring the wound width of each picture (4 to 5 images per condition and per replicate and a total of 20 to 40 measurements of scratch width were done) using the imaging analysis software ImageJ.

3.6.2. OXIDATIVE STRESS

The oxidative stress was performed incubating the cells with a H_2O_2 solution. First the cell number, H_2O_2 concentration in MSC culture medium and time of incubation was optimized using the viability assays with Alamar Blue (Invitrogen) following the suppliers guidelines. After narrowing the conditions under optimization, each trial of this experiment was composed of two read outs of cell viability. One using the supernatants for LDH (Lactate Dehydrogenase) measurements using the CYTOTOX 96® NON-RADIOACTIVE CYTOTOXICITY ASSAY kit and the other is the labeling of cells with fluorescent markers for flow cytometry analysis using the FITC Annexin V/PI staining kit (cells used were BM-MSC, UC-MSC, AT-MSC, HUVEC and L929 fibroblasts). The cells were detached from the T-flasks and plated in 12 well plates (BD falcon) at a cell density of 17,632 cell/cm^2 (67,000 cell/well) using DMEM with 10% MSC-qualified FBS and 1% Anti-Anti for MSC and fibroblasts or EGM-2 for HUVEC. (1mL per well) and then incubated overnight at 37°C and 5% CO_2 . Duplicates for the cells incubated with H_2O_2 and cells without treatment conditions were used. On the next day, the medium was changed to fresh MSC culture medium or for H_2O_2 solutions (500 μL per well) and incubated at 37°C and 5% CO_2 for 1h. As a positive control for LDH measurement one extra well was incubated with a lysis solution (37.5 μL lysis solution per 500 μL of DMEM culture medium) for 45 min at 37°C. Afterwards, the supernatants were collected for LDH measurement and the cells were collected by trypsin detachment (400 μL per well) and stained with FITC Annexin V/PI. One extra well was used as a control for flow cytometry, in which the cells had no H_2O_2 treatment neither Annexin V/PI staining.

CYTOX 96® NON-RADIOACTIVE CYTOTOXICITY ASSAY

The CYTOTOX 96® NON-RADIOACTIVE CYTOTOXICITY ASSAY (Promega) was performed according to the manufacture guidelines. In summary, after incubation with H_2O_2 , lysis or fresh culture medium, the supernatants of each well were collected (supernatants of duplicates were mixed), centrifuged at 1500 rpm (HERMLE Z300K) (2000 G) for 10 min and 50 μL of each supernatant were transferred into wells of a black 96 well plate with transparent bottom (BD) (triplicates per condition were done). It was followed by the addition of 50 μL of a substrate solution to each well and incubation for 30 min at room temperature, protected from light. After the incubation, 50 μL of stop solution were added per well in order to stop the enzymatic reaction.

Right after, the absorbance was read at 490 nm using a spectrophotometer (Infinite® 200 PRO, Tecan) and the software Tecan i-control 1.8. Besides the negative control (cell without H_2O_2 treatment condition), a positive control was also used, which was the cells incubated with a lysis solution, included in the kit.

ANNEXIN V-FITC STAINING

Regarding the Annexin V-FITC/PI staining assay (BD Pharmingen), the protocol was followed as indicated by the manufacturer. In summary, the cells of each well were detached using trypsin 0.05% (except the well treated with lysis solution), counted and resuspended in 100 μ L of 1x Binding Buffer in FACS tubes (BD). Following this step, the antibodies were added to the wells, 2,5 μ L of FITC Annexin V and 5 μ L of PI, and the tubes were incubated at room temperature for 15 min, light protected. After that time, 200 μ L of 1x Binding Buffer was added per tube and the samples were analyzed by flow cytometry within one hour. The conditions of H_2O_2 solution and without H_2O_2 treatment were in duplicate, therefore, for the cells from the duplicated were mixed before the staining. Additionally, one extra well was plated in which no treatment was performed, the cells were collected, centrifuge and resuspended in 300 μ L of 1x binding buffer to serve as a control of cells without staining and to allow the definition of the cell population when measuring the samples in the flow cytometer. The software FlowJo vX.0.7 was used to determine the percentage of early apoptotic cells, which are positive for Annexin V and negative for PI, late apoptotic cells, that are positive for both Annexin V and PI and necrotic, which are positive for Annexin V and PI.

4

RESULTS AND DISCUSSION

In this chapter, the results related to the cell characterization of different MSC cell types used in this project are presented and discussed, namely, growth profile, angiogenic potential and resistance to oxidative stress. As referred in Chapter 3 (Material and Methods), the protocols used in this project were previously established in the SCBL-RM group with the exception of resistance to oxidative stress and wound healing, which were developed in this project.

4.1. GROWTH PROFILE

In order to establish a growth profile for the MSC used in this work, the Fold Increase in total cell number (eq. 4.1), Population Doublings (eq. 4.2), Cumulative Fold Increase (eq. 4.3) and Cumulative Population Doublings (eq. 4.4) were calculated across the cell passages (P), more specifically, P3 to P6. In this section, the results are presented for BM-MSC cultured under normoxia (20% O_2) and hypoxia (2% O_2) (all from the same donor - M72A07), AT-MSC and UCM-MSC. Firstly, in Figure 4.1, across all three stages (P3 to P4, P4 to P5 and P5 to P6), BM-MSC cultured under hypoxic conditions had higher fold increase than BM-MSC cultured in normoxia especially in the early passages (10.3 to 6.91 respectively). Regarding the population doublings (Figure 4.2), the same trend is verified, with the maximum values attained in the early stage as well for both BM-MSC cultured in hypoxia and normoxia (3.37 and 2.79 respectively). This trend is also verified in Figures 4.3 and 4.4, with BM-MSC cultured under hypoxia reaching 272 and 8.09 for Cumulative Fold Increase and Cumulative Population Doublings, respectively, whereas BM-MSC cultured under normoxia only achieved

42.4 and 5.41. This pattern is in accordance to the literature found. In 2006, Ren published a study where the effect of hypoxia and normoxia in mouse BM-MSC expansion was evaluated. Their results showed that, after 8 days of culture, the BM-MSC cultured in hypoxia showed a 2.8-fold increase in cell number, when compared to normoxic cultured BM-MSC [105]. Furthermore, a study by Dos Santos and colleagues (in 2010) also revealed higher Cumulative Fold Increase for the case of hypoxic human BM-MSC when compared to those cultured in normoxia [89]. Although more replicates and BM donors should be tested to have additional support to these results, this data suggests that BM-MSC cultured under hypoxia might be able to achieve higher proliferation rates than when under normoxic conditions.

Comparing the three different sources of MSC (BM-MSC, AT-MSC and UCM-MSC) cultured under normoxic conditions, Figure 4.1 shows that AT-MSC have higher fold increase than the other MSC sources. Although in the first stage (P3-P4), BM-MSC has the highest value of the three (6.91), it was surpassed by the AT-MSC in the following stages, with AT-MSC achieving 5.64 and 5.38 at P4-P5 and P5-P6 whilst BM-MSC only reached 1.92 and 3.20 in the mentioned last two stages. The same trend is present in Figure 4.2, where AT-MSC reached Population Doublings of 2.60, 2.50 and 2.43 across the three stages, while BM-MSC achieved 2.79, 0.94 and 1.68. These results are then translated into a wide difference in terms of cumulative data: AT-MSC registered 183 and 7.53 in terms of Cumulative Fold Increase and Cumulative Population Doublings while for BM-MSC 42.4 and 5.41 was obtained, respectively. This trend is also in agreement with the literature, more specifically, with a comparative study made by Peng and co-workers, in 2008, where they showed that, not only AT-MSC have greater proliferative potential, but these cells could also grow faster than BM-MSC [106]. Although, only a true systematic comparison of BM and AT cells from the same donor, in the same medium and passage would be conclusive. In relation to the comparison with UCM-MSC, the results obtained in Figures 4.1 and 4.2 illustrate a superiority of BM-MSC in normoxia, with higher results in all stages apart from P4-P5 where BM-MSC registered 1.92 and 0.94 for Fold Increase and Population Doublings respectively, whereas UCM-MSC achieved 3.67 and 1.88. However, looking at the cumulative results, BM-MSC reached 42.49 and 5.41 in Cumulative Fold Increase and Cumulative Population Doublings, respectively, while UCM-MSC measured only 19.7 and 4.30, respectively. When comparing with the studies found, this trend is not verified. In a research made by Baksh and colleagues, they compared MSC derived from BM and UC, and found that MSC derived from the UC achieved higher levels of cumulative population doublings, being the difference between values more pronounced between day 7 and 14 (2.8 for UC and 1.82 for BM) [107].

$$\text{Fold Increase} = \frac{\text{number of viable cells at the end of the passage } n}{\text{number of viable cells initially plated}} \quad (4.1)$$

$$\text{Population Doublings} = \frac{\log_{10} \text{fold increase in passage } n}{\log_{10} 2} \quad (4.2)$$

$$Cumulative\ Fold\ Increase = \prod_{i=1}^n \text{fold increase of passage } i \tag{4.3}$$

$$Cumulative\ Population\ Doublings = \sum_{i=1}^n \text{population doubling of passage } i \tag{4.4}$$

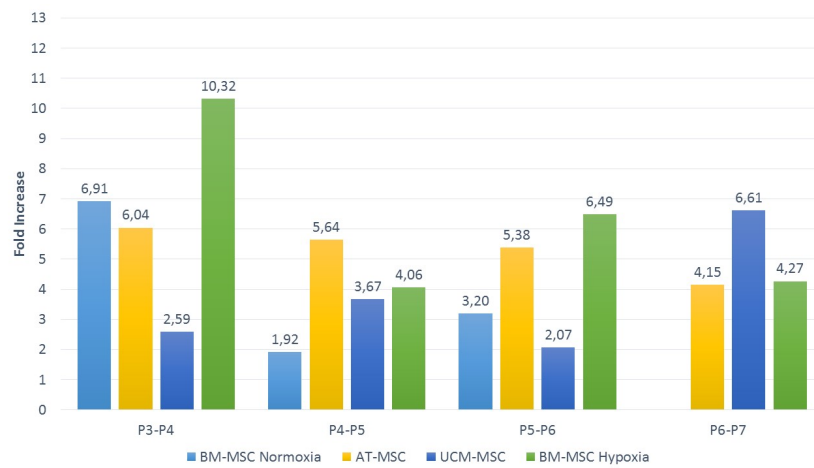


Figure 4.1: Population Doublings of MSC from Different Sources BM-MSC, AT-MSC and UCM MSC culture under normoxic (20% O₂) conditions and also BM-MSC cultured under hypoxia (2% O₂), across 3 stages: Passage P3 to P4, P4 to P5 and P5 to P6

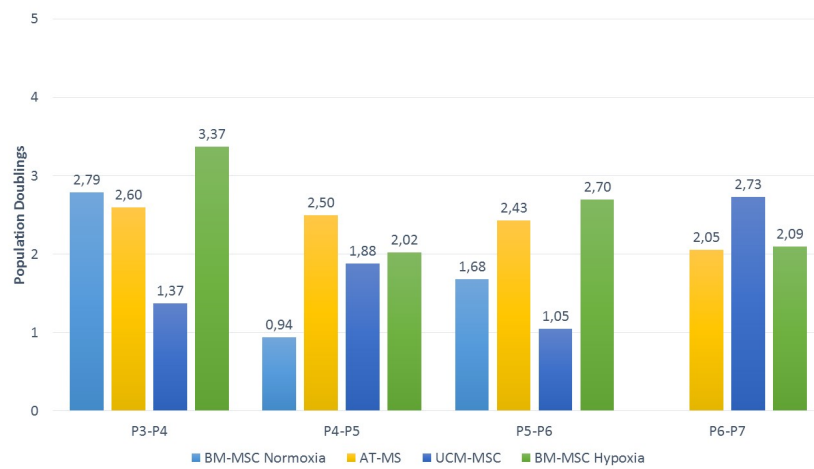


Figure 4.2: Population Doublings of MSC from Different Sources BM-MSC, AT-MSC and UCM MSC culture under normoxic (20% O₂) conditions and also BM-MSC cultured under hypoxia (2% O₂), across 3 stages: Passage P3 to P4, P4 to P5 and P5 to P6

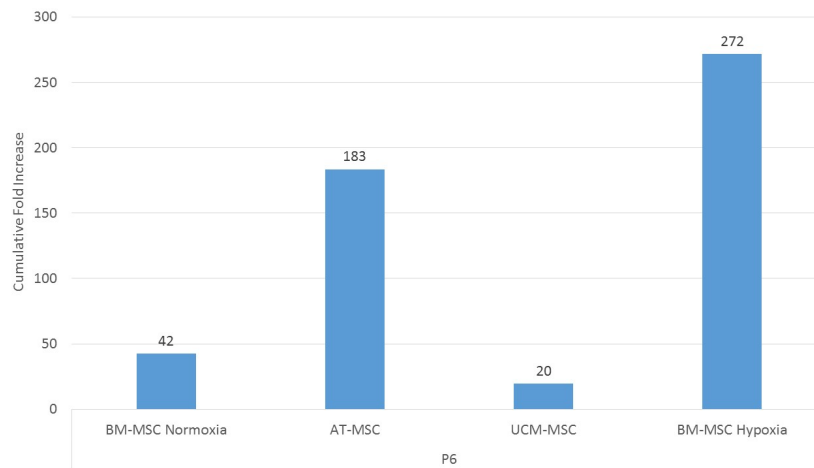


Figure 4.3: Cumulative Fold Increase of MSC from Different Sources BM-MSC, AT-MSC and UCM MSC culture under normoxic (20% O_2) conditions and also BM-MSC cultured under hypoxia (2% O_2)

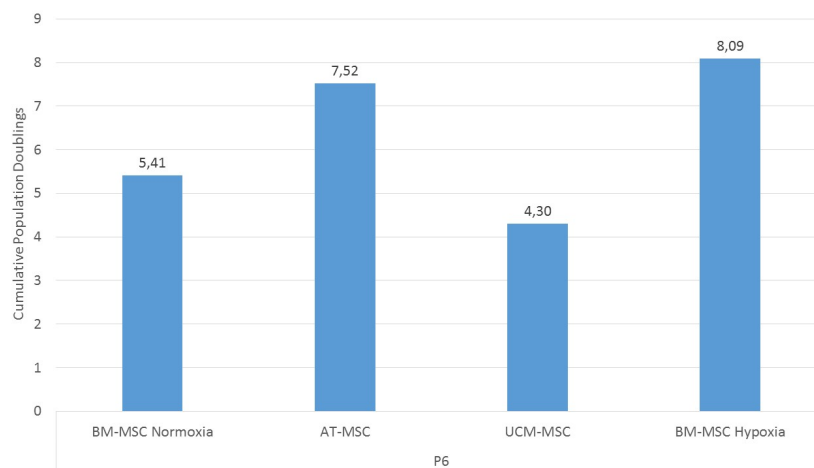


Figure 4.4: Cumulative Population Doublings of MSC from Different Sources BM-MSC, AT-MSC and UCM MSC culture under normoxic (20% O_2) conditions and also BM-MSC cultured under hypoxia (2% O_2)

4.2. ANGIOGENIC POTENTIAL

4.2.1. TUBE REMODELING BY CHEMICAL DISRUPTION WITH DMSO

As it is mentioned in section 3, in the beginning of this project, bDMSO was chosen as a tube disruption agent based on the article published by Wang and colleagues [108]. The authors used 0.5% of DMSO concentration in MSC Basal Medium (Mouse) (StemCell Technologies, Inc., British Columbia, Canada) for mouse BM-MSC (incubation with DMSO for 48h, with 50.000 cells per well (25,000 cells/ cm^2) of a 24 well plate. An attempt to replicate those conditions was pursued in this thesis, although a 96 well plate was used, and it was not possible to obtain significant disruption. Then, it was decided to test several increasing concentrations: 1, 2, 5, 10, 30, 50, 70, 100% DMSO; different incubation times (30 min, 1h and 2h) and cellular concentration

(31,250 cell/cm², 78,125 cell/cm², 125,000 cell/cm² and 250,000 cell/cm²). Summarizing the results: above 10% there was total loss of cell viability, below 2% there was no significant disruption and, between 2% and 10% there was partial disruption of the tubes. Despite this conclusion, there was a certain lack of consistency in the results since the first couple of trials of this experiment did not produce disruption with 10% DMSO. Maybe due to pipetting inaccuracy (the volumes of DMSO that were added directly to the wells were really small, which increased the possibility of introducing errors). Therefore, it was not possible to evaluate the angiogenic potential of BM-MSC conditioned medium on tube remodeling and it was aimed to develop a mechanical disruption assay instead of using a chemical. Following the protocol described in section 3, the tube disruption was made and the evolution of the wells was carefully followed for 7-8h (pictures were taken every hour). Although some tubes presented a certain level of regeneration, these reformed along the wound, and not across. To try to correct this problem, additional Matrigel was added to each well after making the scratch. The results did not change and so the tube remodeling assay was adapted to a wound healing assay using HUVEC monolayer. Some important remarks about this protocol: the value of 5,000 MSC for seeding in each well (15,625 cells/cm²) was chosen for the tube incorporation due to the fact that, using a higher value, would create aggregates which would compromise the assay.

4.2.2. SCRATCH WOUND ASSAY OF HUVEC MONOLAYER BY PARACRINE ACTION

The first stage of protocol optimization was the mechanical disruption of the HUVEC monolayer. Several sizes of needles and pipette tips were tested for the scratch assay and the 200 μ L pipette tip resulted in scratches in the adequate range for cells seeded on 96 well plates. Looking at Figure 4.5, it is possible to see by the time Trial 4 and 5 (a high level of variability was obtained in the previous 3 trials, which was then corrected for trial 4 and 5) were performed, the scratch process was optimized with scratch width values quite similar between them (average width around 218 μ m).

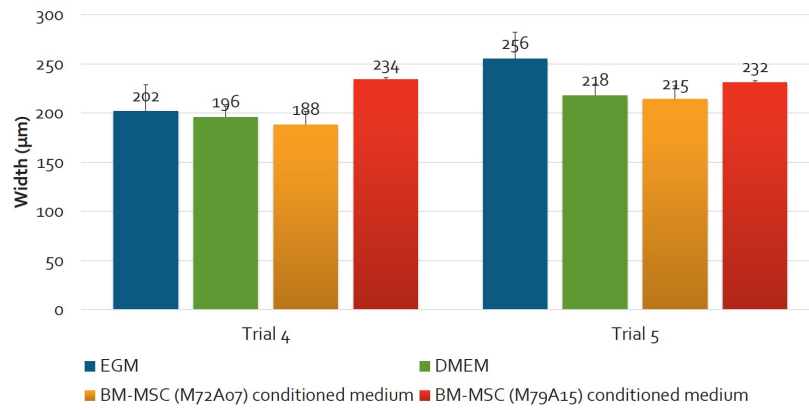


Figure 4.5: Scratch Wound Assay - Average Scratch Width (μm) for each different medium: EGM, DMEM, BM M72A07 Conditioned Medium and BM M79A15 Conditioned Medium. Duplicates per each condition. Results are presented as mean \pm SD (standard deviation)

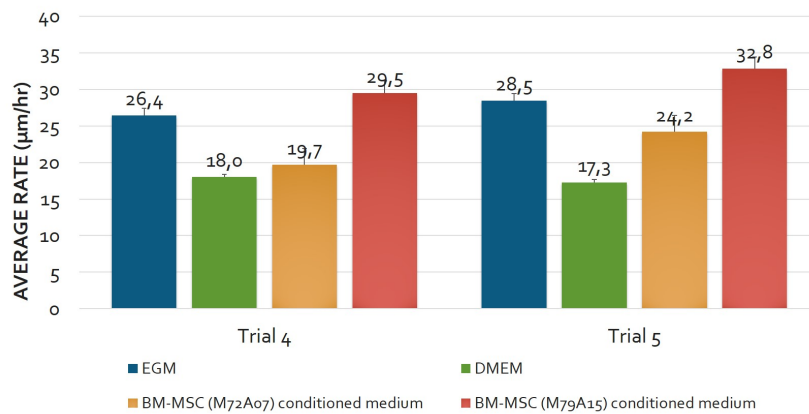


Figure 4.6: Scratch Wound Assay - Average Regeneration Rates ($\mu\text{m/hr}$) for each different medium: EGM, DMEM, BM M72A07 Conditioned Medium and BM M79A15 Conditioned Medium. Duplicates per each condition. Results are presented as mean \pm SD (standard deviation)

Analyzing the regeneration rate in Figure 4.6, there are some inferences and possible interpretations that can be made using the results. However, their accuracy would need to be evaluated through more trials of this assay with more replicates and donors. Furthermore, this would also allow for the assessment of the biological variability which is inherent to the existence of different donors.

EGM-2 medium was used as positive control through which the regeneration of the monolayer would be faster as it is supplemented with several growth factors such as VEGF and bFGF that promote angiogenesis. DMEM supplemented with 10% MSC qualified FBS was used as a negative control, as it was seen during cell culture that HUVEC grow slower (data not shown) and also the lowest regeneration rate was achieved for the two trials performed, as it is represented in Figure 4.6). It can also be observed in Figure 4.6 that conditioned medium produced by BM -MSC from the M79A15 donor, resulted in a higher rate of regeneration (when comparing to the controls) being close to the values of EGM-2 medium. On the other hand, conditioned medium

of BM-MSC donor M72A07 resulted in a lower migration rate of cells than when using EGM-2 medium, albeit higher than the one achieved with FBS-supplemented DMEM culture medium. If further trials of this protocol produce similar results, it will be possible to use this information as an additional support to the hypothesis that MSC produce paracrine factors that improve angiogenesis.

In summary, the results showed that both conditioned medium from M79A15 and M72A07 donors lead to higher wound regeneration rate when compared to FBS-supplemented DMEM culture medium. This trend, although needing further studies, revealed the same improved results for BM-MSC as the ones obtained by the Chen and colleagues, where BM-MSC showed improved migration of HUVEC when compared to the control media (serum-containing complete culture medium) in mice [109]. The obtained results are also partially in agreement to other studies, such as the one by Shen and co-workers. In this study the authors reported increased regeneration during migration/scratch wound regeneration assay, although the MSC used were derived from UC. That assay was performed, not only with HUVEC monolayer, but also with Fibroblast and MSC. Although the MSC cells were from different sources, their results also showed increased wound regeneration when the monolayer of all three cell lines was subjected to UC derived MSC conditioned medium (60%, 75% and 90% of wound closure percentage after 6h respectively), in comparison to FBS-supplemented DMEM culture media (30%, 20%, 50% respectively). [110]

4.3. OXIDATIVE STRESS RESISTANCE POTENTIAL

The reasoning behind this set of experiments and the need for the establishment of an assay to measure oxidative stress resistance potential resides in the intimate relation that exists between an AMI and the oxidative stress. When the blood flow is blocked and ischemia arises, an inflammation process is also associated. This, allied to the medical procedures used to reestablish the blood flow, cause a great rise in the production of oxygen reactive species, which are toxic to the cardiomyocytes and to the MSC that are transplanted in a case of a cell-based scenario. [111, 112] Therefore, it is of crucial importance to study the behavior of MSC when exposed to oxidative stress (which in this work was induced by using H_2O_2).

4.3.1. OPTIMIZATION

The optimization of this protocol was made throughout several assays where 3 main variables were evaluated for the final protocol: cell density in each well, H_2O_2 concentration and incubation time with H_2O_2 . In order to measure cell death, Alamar Blue was used. Table 4.1 translates each of the 7 optimization trials with every condition tested. Furthermore, it also presents the main observations that lead to the final protocol, more specifically: in Trial 1, 30 minutes did not result in enough cell death to be measured, so the best incubation

time found would be 1 hour. Across Trial 2, 3, 4 and 5, the cell densities were adjusted due to the fact that they were not high enough for the measurements with Alamar Blue. Moreover, the concentrations of H_2O_2 were fixed at 1, 3 and 5 mM. 10 mM was discarded since it caused 100% of cell death for all cell types studied which did not allow for measuring the differences of oxidative stress resistance between different cells.

Table 4.1: Oxidative Stress Assay Optimization

Trial	Donor	Initial Cell Density (cells/cm ²)	Initial Cell Density (cells/well)	H ₂ O ₂ Concentration (mM)	H ₂ O ₂ incubation time	OBSERVATIONS
1	M79A15 P3	375, 750, 1500, 2500, 3750, 9375, 18750, 37500	120, 240, 480, 800, 1200, 3000, 6000, 12000	0.1, 1, 5	30min, 1h	30 min was not enough to cause cell death, cell density values were too low for detection with Alamar Blue.
2	M79A15 P4	4687, 9375, 18750, 37500, 75000	1500, 3000, 6000, 12000, 24000	1, 5, 10	1h	Cell densities still needed adjustments
3	M72A07 P5	4687, 9375, 18750, 37500, 56250	1500, 3000, 6000, 12000, 18000	1, 3, 5, 10	1h	10 mM caused 100% cell death
4	M79A15 P4	4687, 9375, 18750, 37500, 56250	1500, 3000, 6000, 12000, 18000	1, 3, 5	1h	Similar results to the previous trial, with values in the same intervals, but this time 10 mM was discarded.
5	M72A07 P6, M79A15 P5	9375, 18750, 37500, 56250	3000, 6000, 12000, 18000	1, 3, 5	1h	Results from Trial 3, 4 and 5 seemed to indicate that 3 mM could be the best option for the H ₂ O ₂ concentration (cell death averaging 60%)
6	M72A07 P7, M79A15 P6, HUVEC P5, L929 P9	9375, 18750, 37500, 56250	3000, 6000, 12000, 18000	1, 3, 5	1h	Assay was repeated with donors M72A07 and M79A15 to assess biological variability, HUVEC and L929 cells (fibroblasts) were tested to evaluate the possibility of using them as negative or positive control for the oxidative stress.
7	M72A07 P8, M79A15 P7, HUVEC P6, L929 P10	9375, 18750, 37500, 56250	3000, 6000, 12000, 18000	1, 3, 5	1h	Trial 6 and 7 results showed some variability. Lower passages were thawed for the following trials.

4.3.2. OXIDATIVE STRESS ASSAY - OPTIMIZED PROTOCOL

Using the data from the previous trials, a more standardized protocol was planned. This time, the H_2O_2 concentration chosen was 3 mM, for the first couple of trials of this new protocol, and then 5 mM was reintroduced. With this protocol, Alamar Blue staining was not used (flow cytometry delivers more accurate measurements), and it was replaced by the Annexin V/PI and CytoTox kits. Furthermore, to have enough cells for flowcytometry measurement, a 12 well plate was used instead of the 96 well plate used in the previous trials. Finally, there was also the addition of a new condition: the inclusion of a Lysis Solution (prepared in FBS-supplemented DMEM culture medium). The goal of this was to have an expression of 100% cytotoxicity. This way, it would be able to compare the cytotoxicity of H_2O_2 with the Lysis solution. Towards the end of this experiment, 10mM was also reintroduced in the protocol in order to acquire additional data regarding an even higher level of H_2O_2 concentration.

As Figure 4.7 shows, HUVEC were the cell type that revealed the lowest percentage of cell death for 5 mM and 10 mM concentrations of H_2O_2 . However, for the concentration of 3 mM, it was the cell line that registered highest cell death percentage. This trend was the opposite for the case of MSC cells. They showed higher level of cell death for 10mM and 5mM concentrations, and lower percentage of cytotoxicity for 3mM. Relatively to the L929 cell line, LDH measurements also showed higher toxicity for the higher concentrations of H_2O_2 . As a general analysis, both MSC and L929 are more susceptible to the toxicity of H_2O_2 (in this case, higher concentrations of H_2O_2 , result in higher cell death). However, HUVEC cells seem to be more resistant to this oxidative stress when the concentration of H_2O_2 rises (like it was said, HUVEC showed higher cell death levels for the lowest concentration of H_2O_2 , 3mM respectively). In order to acquire more support to the results, flow cytometry measurements were also performed. On Figure 4.8, the average percentage of cell death for each cell line is registered. In this case, MSC and HUVEC tested with 10mM of H_2O_2 showed the highest levels of cell death (between 80 and 86%). Regarding the MSC, the trend observed with the LDH measurements seems to hold, although HUVEC showed a totally different results. In terms of cell death levels for 5mM of H_2O_2 , all cell lines achieved similar results (between 55 and 61%). Finally, 3 mM concentration of H_2O_2 produced the lowest level of cell death for MSC donors and L929 (as it was also seen during the LDH measurements). Moreover, HUVEC registered the same trend with the flow cytometry measurements regarding 3mM of H_2O_2 concentration. It was the cell line with highest levels of cell death in this case.

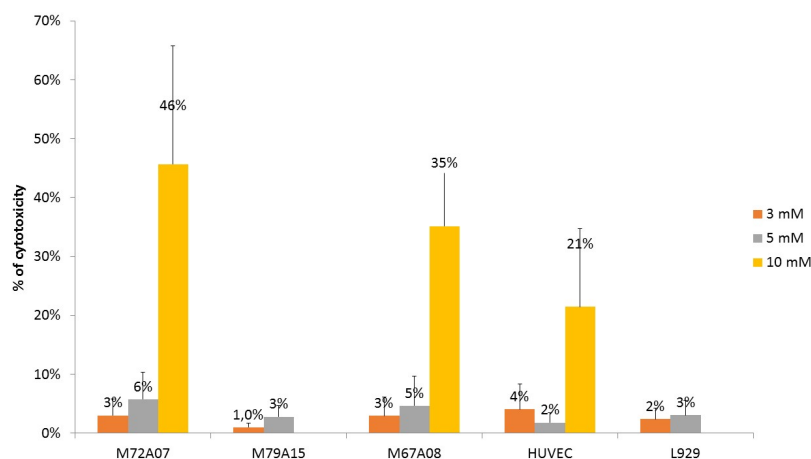


Figure 4.7: Resistance to oxidative stress caused by exposure to 3, 5 and 10 mM H_2O_2 for 1 h in a humidified atmosphere at 37° C and 5% CO_2 . The LDH produced by BM-MSc from three donors (M72A07 passage 3-7, M79A15 passage 3-6, M67A08 passage 4-7), HUVEC (passage 8-11) and fibroblasts L929 (passage 10-16) was measured by absorbance and using the Cytotox kit. Triplicates per each condition. Results are presented as mean \pm SD (standard deviation).

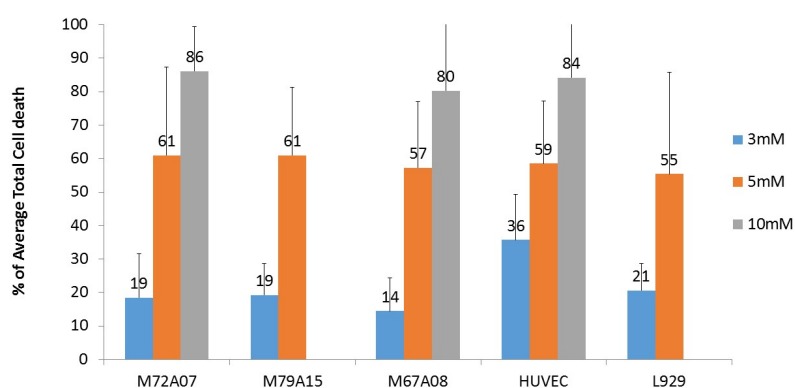


Figure 4.8: Resistance to oxidative stress caused by exposure to 3, 5 and 10 mM H_2O_2 for 1 h in a humidified atmosphere at 37° C and 5% CO_2 . The percentage of total cell death for BM-MSc from three donors (M72A07 passage 3-7, M79A15 passage 3-6, M67A08 passage 4-7), HUVEC (passage 8-11) and fibroblasts L929 (passage 10-16) was measured by flow cytometry and using the Annexin V/PI kit. Duplicates per each condition. Results are presented as mean \pm SD (standard deviation).

Despite all these results, it is crucial to mention that this is an analysis of average results. Although this protocol was optimized and standardized as much as possible, there were some adjustments made along the trials and that fact, allied to the already existent biological variability among different donors, affected the final results.

4.3.3. OXIDATIVE STRESS WITH DIFFERENT MSC SOURCES

After the protocol optimization and results on BM-MSc, the next step was to evaluate the behavior of MSC from other sources (more specifically AT and UC) when exposed to oxidative stress. There was also the addition of a condition of BM-MSc cultured under hypoxic conditions (2% O_2) as well as BM-MSc cells under

normoxia (20% O_2) at a high passage (more specifically, in P10) to study how both these conditions would affect the performance of BM-MSc when exposed to oxidative stress. Ultimately, this would provide useful information to help when deciding the best source of MSC for use in cell therapies. In relation to the LDH results presented on Figure 4.9, there is a difference between MSC from AT, UC, BM and those cultured under hypoxic conditions. It seems that AT-MSc registered the lowest levels of cytotoxicity (around 4.0%), followed by UCM-MSc (with 4.7%). When comparing to the LDH levels in for BM-MSc under normoxia (20% O_2) present in Figure 4.7, the three MSC donors (M72A07, M79A15 and Fernando) achieved similar results (between 3 and 6%). Regarding the BM-MSc in hypoxia results, these achieved 7.4% of toxicity levels, which is not much higher than the maximum value of 6.0% registered from BM-MSc cultured under normoxia (Figure 4.7. BM-MSc cultured under normoxia (donor M72A07) but at a high passage was the one that registered the highest level of cytotoxicity, reaching 21.7%. From this information, a possible conclusion can be that MSC at high passages (in the case of BM-MSc) have less resistance to the presence of oxygen reactive species. However, further experiments with these conditions are needed to confirm these results.

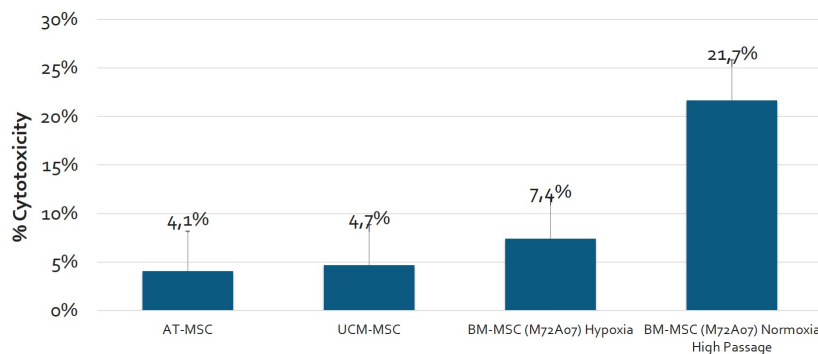


Figure 4.9: Resistance to oxidative stress caused by exposure to 5 mM H_2O_2 for 1 h in a humidified atmosphere at 37° C and 5% CO_2 . The LDH produced by BM-MSc (M72A07 under hypoxia, passage 4-7, and under normoxia, passage 10), AT-MSc (passage 4-7) and UCM-MSc (passage 4-7) was measured by absorbance and using the Cytotox kit. Triplicates per each condition. Results are presented as mean \pm SD (standard deviation)

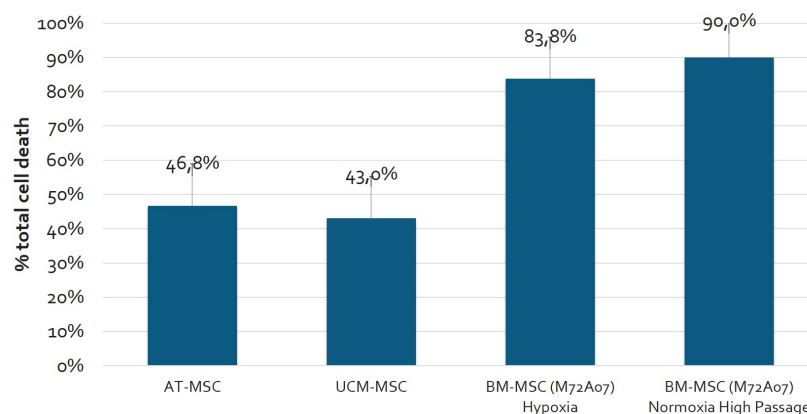


Figure 4.10: Resistance to oxidative stress caused by exposure to 5 mM H_2O_2 for 1 h in a humidified atmosphere at 37° C and 5% CO_2 . The total percentage of cell death for BM-MSC (M72A07 under hypoxia, passage 4-7, and under normoxia, passage 10), AT-MSC (passage 4-7) and UCM-MSC (passage 4-7) was measured by flow cytometry and using the Annexin V/PI kit. Duplicates per each condition. Results are presented as mean \pm SD (standard deviation)

When comparing to the literature, Ertas and colleagues developed a study in 2012, where human AT-MSC and human BM-MSC were subjected to similar oxidative stress conditions. The authors seeded MSC onto 6-well plates, at $10 \times 10^4 \text{ cells/cm}^2$ and cultured for 48h. The culture medium was then changed to medium that contained 2 mM of H_2O_2 . The cells were in contact with this medium for 60 min and were then analyzed using Annexin-V-FITC Apoptosis Detection Kit, which was the same kit used in this project for apoptosis detection. Their results showed that AT-MSC exhibit higher resistance to H_2O_2 induced oxidative stress [113], which is in accordance to the trend verified in this data, in which AT-MSC registered lower values of cytotoxicity (46.8% of total cell death) when compared to MSC derived from BM (presented in Figure 4.8: 61% for donor M72A07 and M79A15 and 57% for donor M67A08). Furthermore, UCM-MSC also showed lower cell death (43%) when compared to the same BM-MSC mentioned. However, it is reported that due to the lack of antioxidant enzymes activity, neonatal sources are prone to be highly sensitive to oxidative stress [114]. For example, Choo and colleagues presented a study on Wharton's Jelly MSC (which are derived from UC) and found that, under concentrations higher than 0.2 mM of H_2O_2 , these MSC would suffer with the cytotoxicity and stop growing [114]. These authors stated that neonatal sources of MSC, such as the UC, only tolerate up to 0.2 mM of H_2O_2 concentration [114]. Comparing with the results on Figure 4.10, UCM-MSC achieved a similar value to the one registered with AT-MSC with 5 mM of H_2O_2 , which contradicts the information mentioned regarding neonatal sources. Nonetheless, the results presented within the Figures 4.9 and 4.10 are average results from only one set of experiments throughout cell passages. Further replicates and more donors should be evaluated in order to have statistical significant results.

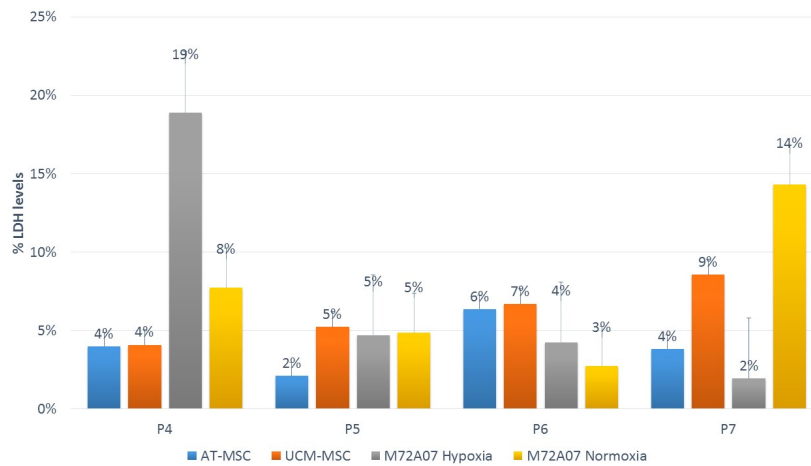


Figure 4.11: Resistance to oxidative stress (presented for each passage) caused by exposure to 5 mM H_2O_2 for 1 h in a humidified atmosphere at 37° C and 5% CO_2 . The LDH produced by BM-MSC (M72A07 under hypoxia and under normoxia, passage 4-7), AT-MSC (passage 4-7) and UCM-MSC (passage 4-7) was measured by absorbance and using the Cytotox kit. Triplicates per each condition. Results are presented as mean \pm SD (standard deviation).

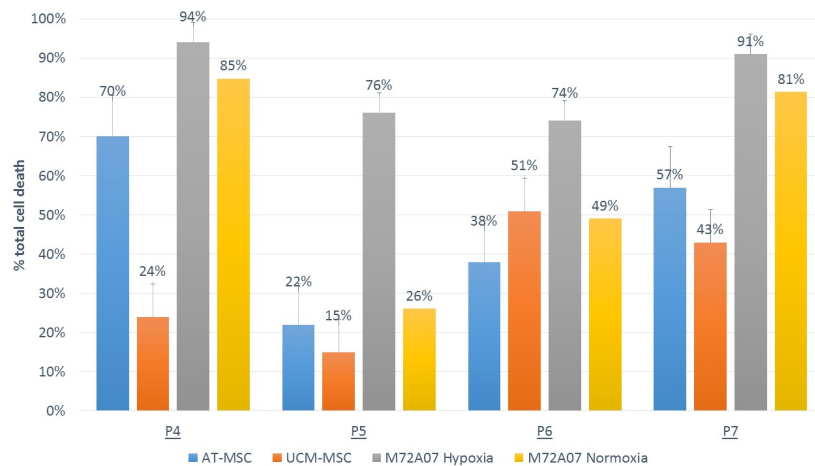


Figure 4.12: Resistance to oxidative stress (presented for each passage) caused by exposure to 5 mM H_2O_2 for 1 h in a humidified atmosphere at 37° C and 5% CO_2 . The total percentage of cell death for BM-MSC (M72A07 under hypoxia and under normoxia, passage 4-7), AT-MSC (passage 4-7) and UCM-MSC (passage 4-7) was measured by flow cytometry and using the Annexin V/PI kit. Duplicates per each condition. Results are presented as mean \pm SD (standard deviation)

Analyzing Figure 4.11, AT-MSC registered the lowest levels of cytotoxicity at the earliest passages, with 4 and 2.10% for P4 and P5, respectively. However, the toxicity levels raised at P6, lowering again at P7, thus showing some inconsistency. For the case of UCM-MSC, the values registered followed a constant increase as the passage number raised (4.09, 5.23, 6.70 and 8.56 for P4, P5, P6 and P7 respectively). Regarding the BM-MSC (donor M72A07) cultured under hypoxia, there was a regular decrease in cytotoxicity as the passages increased. They started at 18.90% for P4, and then 4.68, 4.23 and 1.95% for P5, P6 and P7 respectively. Comparing the AT-MSC and UCM-MSC with BM-MSC (donor M72A07) cultured under normoxia, P4 and P7 were the passages where AT-MSC and UCM-MSC displayed lower toxicity. However, when in P5 and P6, BM-MSC

reached lower cytotoxicity than the AT-MSC and UCM-MSC. In terms of M72A07 under hypoxia *versus* normoxia, there is not a discernible pattern as well. For passages P5 and P7, BM-MSC under normoxia appear to be more susceptible to the toxicity, whereas for passages P4 and P6, the opposite is true with BM-MSC cultured under hypoxia, which registered higher toxicity. In Figure 4.12, results using Annexin-V-FITC Apoptosis Detection Kit show that BM-MSC cultured under hypoxia seem to be the more sensitive to the oxidative stress than BM-MSC cultured in normoxia, having registered higher cytotoxicity levels across all passages. In terms of AT-MSC and UCM-MSC, the cells achieved lower levels of cytotoxicity when compared to BM-MSC cultured under normoxia in all passage numbers . These results must further be confirmed by evaluating more donors and performing more replicates of this assay.

5

CONCLUSION AND FUTURE TRENDS

During the past decade, MSC clinical applications have been studied in many fields such as autoimmune diseases and other immunity conditions [53, 54], transplantation [56] and myocardial regeneration [5]. Due to the fact that CVD are the leading cause of death worldwide [2], these conditions have been target of an intensive study in order to develop more effective treatments. Taking into consideration all the therapeutic features of MSC, these cells are a prime candidate for establishing these novel therapies [53–55, 96]. However, there are still several open questions related with MSC function and mechanisms of action that need further studies. More specifically, still there is not an agreement among the scientific community in relation to a standardized definition of what is a MSC [115]. Furthermore, the choice of the right MSC source to be used in a cell therapy to regulate autoimmune responses [116], as well as to improve cardiac regeneration [117–119] also need further study. The present work was focused on extending the knowledge on the regenerative properties of MSC that can be important in a clinical setting, such as: resistance to oxidative stress (its protocol was established for the first time during this thesis), to which the cells are exposed during an AMI, and angiogenic potential (more specifically related to the production of angiogenic factors that enhance regeneration). These properties were tested mainly for BM-MS. However, resistance to oxidative stress was also tested with AT-MS and UCM-MS, two important alternative sources to BM-derived cells.

In terms of the growth profile, several differences were observed between the cell types studied. In summary, comparing BM-MS cultured under hypoxia *versus* normoxia, there is a clear difference in terms of fold increase and population doublings with BM-MS under hypoxia achieving 272 and 18.4 of Cumulative Fold in-

crease and Cumulative Population Doublings, respectively, whereas BM-MSC under normoxia only reached 42.4 and 4.39, correspondingly. This trend is supported by other studies made on the topic of BM-MSC expansion in hypoxia and normoxia. Regarding the comparison between different MSC sources (BM-MSC, AT-MSC and UCM-MSC), results revealed superior values for the case of AT-MSC with values of 183 and 15.73 for Cumulative Fold Increase and Cumulative Population Doublings when compared to those achieved by BM-MSC in normoxia. In what regards to UCM-MSC, the inferior performance of these cells when compared to BM-MSC was not observed in the literature found.

Although the results still need further confirmation, it was possible to formulate some hypothesis. Firstly, the production of paracrine molecules by MSC was tested with the conditioned media from two BM-MSC donors, M72A07 and M79A15 (35 and 36 years old, respectively). These two conditioned media, along with EGM-2 and DMEM-10% FBS as controls, were applied to a Scratch Wound Assay, where a monolayer of HUVEC was cultured on several wells from a 96 well plate, a scratch was made on this layer, and then the medium of each well was changed to EGM-2, DMEM-10% FBS, DMEM conditioned medium from BM-MSC M72A07 and M79A15. The results showed improved regeneration when the HUVEC were cultured with BM-MSC conditioned medium compared to DMEM culture medium. EGM-2 achieved a high migration rate, however the conditioned medium of the BM-MSC donor M79A15 resulted in an even higher regeneration rate. These results, are similar to the ones presented in other studies where the research teams obtained increased migration of HUVEC when using medium that was conditioned by MSC, suggesting the fact that MSC can increase wound healing via paracrine effect [109, 110].

The next part of this work consisted in the evaluation of the response of BM, AT and UCM derived MSC to oxidative stress (which was established at the SCBL-RM Laboratory during this thesis). MSC cultured in hypoxic conditions were also tested. The results showed higher resistance for the case of AT and UCM MSC when compared to BM-MSC. Although there are studies that present similar conclusions regarding the performance of AT-MSC [113], some researchers registered high sensitivity of UCM derived MSC when exposed to oxidative stress [114], which is not in agreement to some of the data obtained in this work. Due to that fact, there is still the need to perform more trials of this protocol in order to have additional support for the attained results.

Now that the protocols are standardized, besides increasing the number of each experiment in order to acquire statistical significant data, it would be interesting to apply the Scratch Wound Assay protocol with the conditioned medium from other sources of MSC, like UC and AT. Moreover, testing this protocol with media conditioned by MSC cultured under hypoxic conditions would also be an interesting study. In addition to changing the MSC source for producing conditioned medium, it would also be interesting to quantify by ELISA the soluble factors present in each type of conditioned medium (focusing on identifying the differences

between them). In relation to the oxidative stress studies, the main point for improvement would be further tests of this assay. This would allow for a statistical relevant analysis of the dissimilarities that exist between this work and the literature. Hopefully, this will provide important information that can help in the choice of MSC source and culture conditions.

In terms of other future studies, the use of xeno-free culture medium could also be a possibility since it would improve the safety of using MSC for cell-based therapies due to the fact that it does not have the risk of contamination that is inherent to the case culture medium supplemented with fetal bovine serum (which may be a source of animal proteins, bacteria and virus). In what regards MSC paracrine action, a modulation of the secretion of soluble factor could possibly be achieved through the use of a bioreactor that could control the levels of shear stress and oxygen tension. This could be attractive since hypoxia and shear stress upregulate the production of VEGF [120, 121], which could enhance the results obtained with the Scratch Wound Assay.

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