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

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December 2015

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 the statistical significance needed to confirm the trends observed.

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

1. Introduction

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. The mortality rates of Non-Communicable Diseases (NCD) is 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, research and development of therapies for these diseases is crucial. Dr Fred Hersch from SAID Business School of the Oxford University, stated that "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 an 82% increase in mortality and 89% in morbidity [4]. Consequently, 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 amounts of collected MSC are low. 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 [8, 9, 10, 11].

Several studies have been performed using MSC to treat CVD. For instance, a pilot study, where 69 patients (who had suffered an AMI) were injected (via intracoronary injection) with autologous MSC revealing significant improvements of cardiac function when compared to the control patients, who received a saline injection. Furthermore, the results indicated that MSC were still viable 3 months after the transplantation [30].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 [31, 32, 33, 34, 35]. 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 [36]. Therefore, MSC might be used as an "off-the-shelf" product for cardiac problems [29].

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 harvested 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[16].

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.

2. Materials and Methods

2.1. Human Samples

BM samples were previously acquired from healthy donors and were provided by IPO - Instituto Por-tuguês de Oncologia Francisco Gentil, Lisboa, AT samples were acquired from Clínica de Todos os Santos (Lisboa) and 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 human umbilical vein endothelial cells (HUVEC) were purchased from BD and the L929 Fibroblasts were acquired from DSMZ, Germany. MSC from all three sources were cultures in Dulbecco's modified Eagle's medium (DMEM) (Gibco by LifeTechnologies) + 20% MSC-qualified fetal bovine serum (FBS) (Hyclone) and 1%Anti-Anti (Life Technologies) while HUVEC cells were cultured using Endothelial growth medium (EGM)-2 (Lonza).

2.2. Potency Assays

2.2.1 Scratch Wound Assay of HUVEC monolayer by paracrine action

HUVECs were plated at 40,000 cells/well (125,000 cells/cm^2) in 96 well plates and incubated overnight at 37ºC and 5% CO_2 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 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 (Leica) 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.

2.2.2 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 exper-

iment 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 (BM-MSC, UC-MSC, AT-MSC, HUVEC and L929 fibroblasts were used in this experiment). The cells were detached from the T-flasks and plated in 12 well plates (BD falcon) at a cell density of 67,000 cell/wel (around 17,600 $cell/cm^2$) 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 37ºC 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 37ºC 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 37ºC. 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.

3. Results

3.1. Growth Profile

In order to establish a growth profile for the MSC used in this work, the Fold Increase in total cell number, Population Doublings, Cumulative Fold Increase and Cumulative Population Doublings 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. In Figure 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 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 3 and 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 [37]. Furthermore, a study by dos Santos and colleagues (in 2010) also revealed higher Cumulative Fold Increase for the case of hypoxic-cultured human BM-MSC when compared to those cultured in normoxia [40]. 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 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 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 [38]. 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 1 and 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 com-

pared 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) [39].

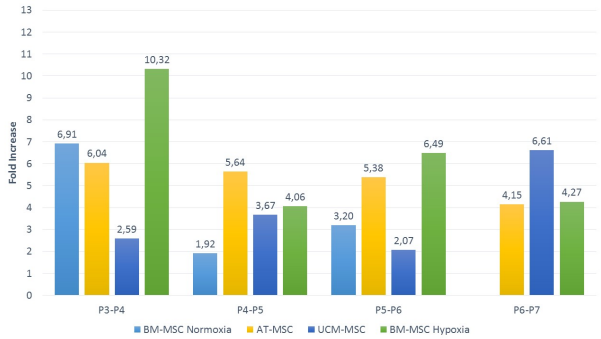

Figure 1: ***Population Doublings of MSC from different Sources BM-MSC, AT-MSC and UCM MSC culture under normoxic (20% O_2) conditions and also BM-MSC cultured under hypoxia (2% O_2),across 3 stages: Passage P3 to P4, P4 to P5 and P5 to P6***

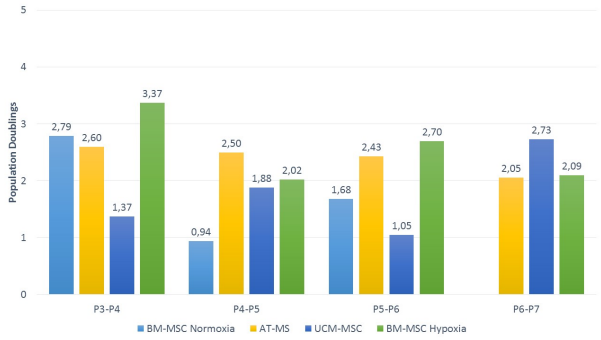

Figure 2: ***Population Doublings of MSC from different Sources BM-MSC, AT-MSC and UCM MSC culture under normoxic (20% O_2) conditions and also BM-MSC cultured under hypoxia (2% O_2),across 3 stages: Passage P3 to P4, P4 to P5 and P5 to P6***

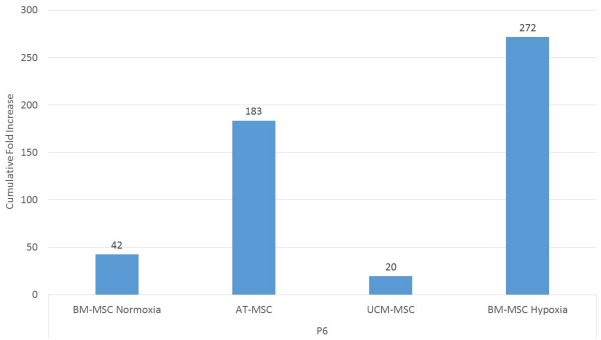

Figure 3: ***Cumulative Fold Increase of MSC from different Sources BM-MSC, AT-MSC and UCM MSC culture under normoxic (20% O_2) conditions and also BM-MSC cultured under hypoxia (2% O_2)***

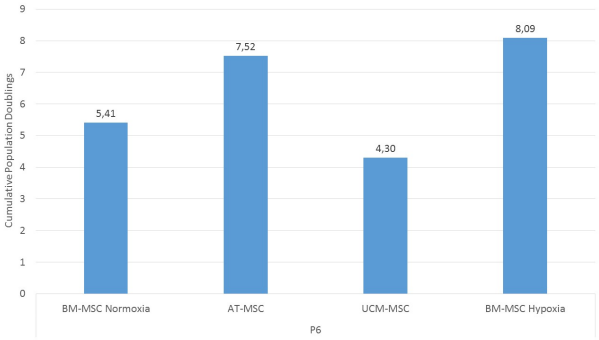

Figure 4: ***Cumulative Population Doublings of MSC from different Sources BM-MSC, AT-MSC and UCM MSC culture under normoxic (20% O_2) conditions and also BM-MSC cultured under hypoxia (2% O_2)***

3.2. Angiogenic Potential

3.2.1 Scratch Wound Assay

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 μL pipette tip resulted in scratches in the adequate range for cells seeded on 96 well plates. Looking at Figure 5, it is possible to see that by the time of Trial 4 and 5, the scratch width values were quite similar between them (average width around 218 μm), improving the high variability obtained in the previous 3 trials (data not shown).

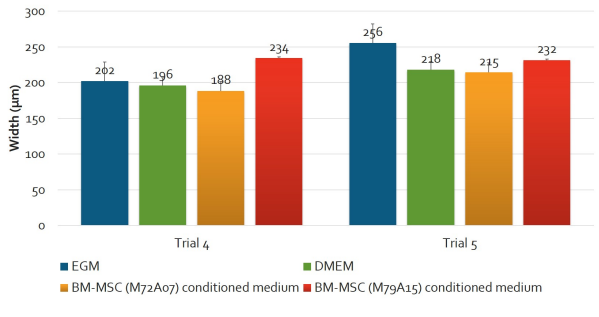

Figure 5: ***Scratch Wound Assay - Average Scratch Width (μm) for each different medium: EGM, DMEM, BM M72A07 Conditioned Medium and BM M79A15 Conditioned Medium. Results are presented as mean $\pm$ SD (standard deviation)***

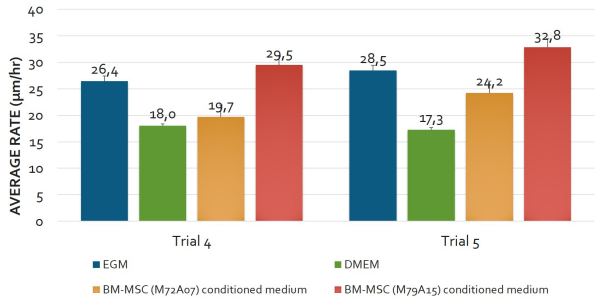

Figure 6: ***Scratch Wound Assay - Average Regeneration Rates (µm/hr) for each different medium: EGM, DMEM, BM M72A07 Conditioned Medium and BM M79A15 Conditioned Medium. Results are presented as mean ± SD (standard deviation)***

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 6. It can also be observed in Figure 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 than EGM-2 medium, albeit higher than those achieved with FBS-supplemented DMEM culture medium. If further trials of this protocol using several donors produce similar results, it will be possible to clarify the effect of biological variability and also to 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 [42]. 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). [43]

3.3. 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 therapy. [44, 45] 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_2O2).

3.3.1 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 UCM) 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 cultured 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 MSC source and culture conditions to be used as cell therapies. In relation to the LDH results presented on Figure 7, there is a difference between MSC from AT, UCM and BM cultured both under normoxic and hypoxic conditions. It seems that AT-MSC registered the lowest levels of cytotoxicity (around 4.0%), followed by UCM-MSC (with 4.7%). Regarding the BM-MSC cultured in hypoxia, toxicity levels 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 (data not shown). BM-MSC cultured under normoxia (donor M72A07) but at a high passage was the one that registered the highest level of cyto-

toxicity, 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.

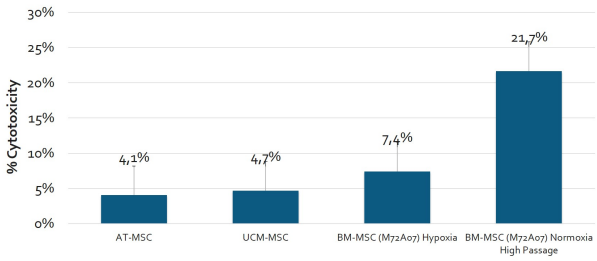

Figure 7: ***Resistance to oxidative stress caused by exposure to 5 mM H_2O_2 for 1 h in a humidified atmosphere at 37º C and 5% CO_2. The LDH produced by BM-MSC (M72A07 under hypoxia, passage 4-7, and under normoxia, passage 10), AT-MSC (passage 4-7) and UCM-MSC (passage 4-7) was measured by absorbance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

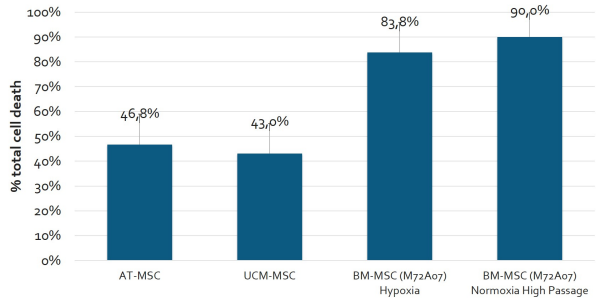

Figure 8: ***Resistance to oxidative stress caused by exposure to 5 mM H_2O_2 for 1 h in a humidified atmosphere at 37º C and 5% CO_2. The total percentage of cell death for BM-MSC (M72A07 under hypoxia, passage 4-7, and under normoxia, passage 10), AT-MSC (passage 4-7) and UCM-MSC (passage 4-7) was measured by flow cytometry and using the Annexin V/PI kit. Results are presented as mean ± 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 $10 \times 10^4 cells/cm^2$ and cultured for 48h. The culture medium was then changed to medium that contained 2 mM of H_2O2. 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. Their results showed that AT-MSC exhibit higher resistance to H_2O2 induced oxidative stress [46], 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: 61% for donor M72A07 and M79A15 and 57% for donor M67A08 (data not shown). 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 [47]. 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_2O2, these MSC would suffer with the cytotoxicity and stop growing [47]. These authors stated that neonatal sources of MSC, such as the UC, only tolerate up to 0.2 mM of H_2O2 concentration [47]. Comparing with the results on Figure 8, UCM-MSC achieved a similar value to the one registered with AT-MSC with 5 mM of H_2O2, which contradicts the information mentioned regarding neonatal sources. Nonetheless, the results presented within the Figures 7 and 8 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.

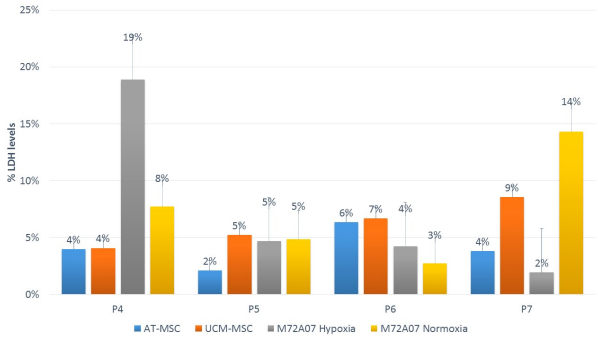

Figure 9: ***Resistance to oxidative stress (presented for each passage) caused by exposure to 5 mM H_2O_2 for 1 h in a humidified atmosphere at 37º C and 5% CO_2. The LDH produced by BM-MSC (M72A07 under hypoxia and under normoxia, passage 4-7), AT-MSC (passage 4-7) and UCM-MSC (passage 4-7) was measured by absorbance and using the Cytotox kit. Results are presented as mean ± SD (standard deviation).***

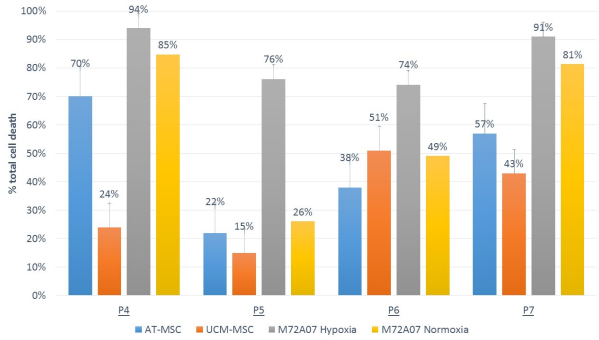

Figure 10: ***Resistance to oxidative stress (presented for each passage) caused by exposure to 5 mM H_2O_2 for 1 h in a humidified atmosphere at 37º C and 5% CO_2. The total percentage of cell death for BM-MSC (M72A07 under hypoxia and under normoxia, passage 4-7), AT-MSC (passage 4-7) and UCM-MSC (passage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

Analyzing Figure 9, 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 with the passage number (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, starting 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 10, 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.

4. Conclusions

During the past decade, MSC clinical applications have been studied in many fields such as autoimmune diseases and other immunity conditions [25, 26], transplantation [28] 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 [25, 26, 27, 29]. 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 [48]. Furthermore, the choice of the right MSC source to be used in a cell therapy to regulate autoimmune responses [49], as well as to improve cardiac regeneration [50, 51, 52] 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: establishment of an assay to study the resistance of cells to oxidative stress, 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-MSC. However, resistance to oxidative stress was also tested with AT-MSC and UCM-MSC, 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-MSC cultured under hypoxia *versus* normoxia, there is a clear difference in terms of fold increase and population doublings with BM-MSC under hypoxia achieving 272 and 18.4 of Cumulative Fold increase and Cumulative Population Doublings, respectively, whereas BM-MSC under normoxia only reached 42.4 and 4.39, correspondingly. This tendency 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 higher values for the case of AT-MSC with values of 183 and 15.7 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 lower proliferation obtained for these cells when compared to BM-MSC was not consistent the literature found.

Although the results still need further confirma-

tion, 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% MSC-qualified FBS as controls, were applied to a Scratch Wound Assay, in which HUVEC were cultured as monolayer. 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 [42, 43].

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 [46], some researchers registered high sensitivity of UCM derived MSC when exposed to oxidative stress [47], 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 this potency assay to test the paracrince action of MSC 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.

Other future studies could include MSC culture under xeno-free and serum-free conditions, which would improve the cell-based therapies. In fact, it avoids the risk of contamination that is inherent culture medium supplemented with FBS (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 [53, 54], which could enhance the results obtained with the Scratch Wound Assay.

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