Comprehensive Characterization of Human Mesenchymal Stem/Stromal Cells From Healthy Donors and Acute Myocardial Infarction Patients

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Abstract: Cardiovascular Diseases (CVDs), namely Acute Myocardial Infarction (AMI), are the leading cause of death worldwide. Current treatments of AMI are incapable of regenerating the necrotic tissue resultant from ischemia. Regarding this issue, Mesenchymal Stem/Stromal Cells (MSCs) have shown some promising results in early clinical trials. Autologous MSCs may not represent the best option for a MSC-based therapy since their performance can be impaired by the associated comorbidities, allied to the age, of AMI patients. The use of Bone Marrow (BM)-derived cells can also be potentially limiting since it involves an invasive procedure for cell collection; Adipose Tissue (AT) may represent an alternative promising source of MSCs for AMI settings.
The aim of this work was to establish a comparative study between BM-derived MSCs from AMI patients, and BM-, AT-derived MSCs from healthy donors in terms of proliferative, angiogenic, secretory, and oxidative stress resistance potential, in order to identify the most adequate cell type for the clinical setting. Additionally, two different methods for the isolation of Adipose-derived Stem/Stromal Cells (ADSCs) were studied.
Healthy BM MSCs presented a higher secretory and angiogenic potential, while AMI BM MSCs had the highest proliferative capacity. The incubation of MSCs under hypoxia (2% O_2) boosted the aforementioned results. Further studies should be conducted to investigate the mechanisms underlying the improved *in vitro* proliferative capacity of AMI BM MSCs demonstrated herein. Concerning ADSCs isolation, these cells might be isolated from AT through a non-enzymatic method, albeit the slight lower yield when compared to the standard enzymatic-based protocol.

Keywords: Acute Myocardial Infarction, Mesenchymal Stem/Stromal Cells, Bone Marrow, Adipose Tissue, Angiogenesis, Hypoxia

Introduction

Ischemic Heart Disease (IHD) is a Cardiovascular Disease (CVD)[1] and leads the main causes of death worldwide, having represented 11.8-17.3% of all deaths in 2001[2]. The burden of CVDs is expected to reach 23.0 million deaths in 2030 due to population aging[3].

The Myocardial Infarction (MI) is one of the manifestations of IHD[4], and is defined as the death of Cardiomyocytes (CMCs), induced by a prolonged ischemic episode that is often associated to atheroma plaques in the coronary arteries[5]. The release of some products by the necrotic CMCs, such as Reactive Oxygen Species (ROS), triggers an inflammatory response[6], followed by a fibrotic mechanism[6], in which the necrotic tissue is replaced by a scar that impairs the function of the heart[7]. Amongst the several risk factors for AMI, modifiable risks such as diabetes[8], unhealthy diet[9], obesity[10], and smoking[11], or non-modifiable risks such as age[12], ethnicity[13], gender[14] and heredity[15], might be highlighted.

Contrarily to what was previously thought, the heart has a regenerative potential[16], possibly through a resident Stem Cell (SC) population[17; 18], albeit insufficient to replace the CMCs lost due to an AMI[16]. This humble potential might be used to create a SC-based therapy able to address the gaps of conventional therapies for AMI, like the inability to replenish the dead CMCs[16].

A SC is a cell capable of self-renewal, which produces differentiated cells and presents a clonogenic potential[19]. Several types of adult SCs have been studied in the context of cardiac regeneration[20], such as Cardiac Stem Cells (CSCs)[17; 18], Endothelial Progenitor Cells (EPCs)[21], Hematopoietic Stem Cells (HSCs)[22], and Skeletal Myoblasts (SKMs)[23]. However, these cells present some drawbacks, namely the

reduced number of CSCs in the heart[24], the impaired performance of EPCs from patients with cardiovascular risks[25], the low probability of true differentiation of HSCs into CMCs[26], and the increased risk of arrhythmias for SKMs[27].

Mesenchymal Stem/Stromal Cells (MSCs) are fibroblast-like[28] adherent cells[29] that support haematopoiesis and the growth of HSCs[30], and are capable of giving rise to Colony-Forming Unit-Fibroblast (CFU-F)[28]. These cells differentiate into at least osteocytes[31], chondrocytes[32], and adipocytes[33], and express some surface markers, such as Cluster of Differentiation (CD) 73, CD90 and CD105, while lacking the expression of CD14, CD34, CD45 and HLA-DR[29]. MSCs might be isolated from several tissues[34] based on the adherence to plastic[35]. Bone Marrow (BM) is considered the standard source, despite the low frequency of MSCs, only 0.01-0.001% of nucleated cells[36], and the painful harvesting of the cells[37]. The BM niche is hypoxic, and this low oxygen tension (1-6% O_2[38]) is involved in the increased proliferation capacity, as well as the maintenance of the naive state and the plasticity of MSCs[39]. Adipose Tissue (AT) is an alternative source to BM since it is usually discarded as a medical waste[40], more easily accessible through a less invasive method[36], and richer in SCs, comparing to BM[41].

MSCs have a wide potential for clinical application, from the management of Graft-Versus-Host-Disease (GVHD)[42], to treatment of bone fractures and wound healing[43]. MSCs may also be used in cardiac regeneration due to their features, such as the controversial potential to differentiate into CMCs[44; 45], the angiogenic potential[44-46], the homing property[47; 48], and the secretory profile, which includes anti-apoptotic, anti-fibrotic, and angiogenic factors like Vascular Endothelial Growth Factor (VEGF)[49], basic Fibroblast Growth Factor (bFGF)[50], Interleukin-6 (IL-6)[50], amongst others[50]. MSCs might also exert their beneficial effects by stimulating endogenous CSCs to migrate to the injured site[51], expand and differentiate into CMCs[52]. The preconditioning of MSCs in hypoxic conditions seems to enhance not only their differentiation into CMCs but also their production of some factors and survival rate upon transplantation[53]. Some clinical trials based upon the use of MSCs to treat ischemic cardiomyopathy have shown modest results, namely the PRECISE[54] and the POSEIDON[55] trials, with no signs of calcifications/ossifications as it was seen by Breitbach *et al.* 2007[56].

An autologous setting in the clinical context has some drawbacks, like the time consuming expansion of MSCs *in vitro* to achieve a clinically relevant number[57], and the high probability of impairment of the cells when these are extracted from elderly patients and/or patients who have multiple comorbidities[58-60]. Thanks to the immunomodulatory[61] and hypoimmunogenic[62] properties of MSCs, these may be used in an allogeneic setting without triggering an immune response by the host[62; 63]. Thus, allogeneic MSC, extracted from young and healthy donors, might be used to create a high-quality and consistent off-the-shelf product[57].

The aim of this work was to highlight the *pros* and *cons* of using autologous or allogeneic MSCs in the context of a regenerative treatment for an AMI. To this end, several studies and assays were performed, under normoxic and hypoxic conditions, to ascertain the impact of a low oxygen tension, existing in both the BM and the infarct site, on the performance of MSCs. The cells studied were extracted from BM and AT from healthy donors, or BM from AMI patients. Regarding the AT as a viable source of MSCs, two isolation methods for the Stromal Vascular Fraction (SVF) were compared and the Adipose-derived Stem/Stromal Cells (ADSCs) were then isolated using the usual culture medium or a Xeno(geneic)-, Serum Free (XSF) medium, aiming at the exploration of a non-enzymatic isolation method, free of undefined and animal derived components, and closer to a clinical-grade product.

Materials and Methods

Human Samples

The BM samples from healthy donors and AMI patients were obtained from Instituto Português de Oncologia Francisco Gentil, Lisboa, Portugal, and Hospital de Santa Marta, EPE, Lisboa, Portugal, respectively. These samples were previously processed and the MSCs isolated were cryopreserved and stored at SCBL-RM. ADSCs were obtained from Pennington Biomedical Research Center Institutional Review Board, USA and kept cryopreserved at SCBL-RM. The AT samples that were processed in the scope of this work were obtained from Clínica de Todos-Os-Santos, Lisboa, Portugal. All the samples were obtained after informed consent of the patients and their harvesting and collection was performed in accordance with the protocols of the respective institution. The Human Umbilical Vein Endothelial Cells (HUVEC) used in the present work were a HUVEC cell line from BD Biosciences.

Evaluation of the in vitro Proliferative Potential of MSCs

Cells were expanded on T-25 flasks (BD Falcon™), incubated at 37°C, 5% CO_2, and normoxic or hypoxic conditions, with 20% or 2% of Oxygen (O_2), respectively. Cells were plated at an initial density of 3 000 cells/cm^2 and the medium was changed every 3 to 4 days. The medium used was DMEM+10% MSC FBS. When reaching 70 to 80% confluence, the detachment procedure was performed. Briefly, cells were washed with PBS and harvested with Accutase (Sigma) for 5-7 minutes at 37°C. IMDM+10% FBS was added, in a proportion of 1:2, and the cells were centrifuged at 1250 rpm for 7 minutes. After discarding the supernatant and re-suspending the pellet in the culture medium, the number of cells and their viability was accessed using the Trypan Blue dye (Gibco®) exclusion. The cells were re-plated into new culture flasks at the same initial conditions. The proliferation study was performed from passage number 3 (P3) until P9, and at every passage some parameters were calculated, namely: (i) cell density (ratio between the number of viable cells at the end of the passage and the area in which the cells were plated), (ii) Fold Increase (FI, ratio between the number of viable cells at the end of the passage and the number of viable cells plated at the beginning of the same passage), (iii) Cumulative Fold Increase (CFI, the product of the fold increase of each consecutive passage until the present passage), (iv) Population Doublings (PD, ratio between the base ten logarithm of the fold increase of the passage and the base ten logarithm of 2), and (v) Cumulative Population Doublings (CPD sum of the population doubling of each consecutive passage until the present passage). Other parameter that was considered was the time the cells took to expand from P3 to P9.

MSCs Secretory Potency Assays

The growth factors studied using Enzyme-Linked Immunosorbent Assay (ELISA) kits were IL-6 and VEGF (RayBio®). To evaluate the effect of inflammatory cytokines, the cells were stimulated with Tumour Necrosis Factor Alpha (TNF-α) and Interferon Gamma (IFN-γ).

At the end of P3 or P4, the cells were detached and plated into 4 wells of a 24 well plate, at a density of 3 000 cells/cm^2, using DMEM+10% MSC FBS as medium. After 4 days in culture at 37°C, 5% CO_2 and normoxic or hypoxic conditions, the medium was replaced by fresh DMEM+10% MSC FBS, being also supplemented with 500 U/mL of IFN-γ and 10 ng/mL of TNF-α in 2 wells, which constituted the inducted wells. The non-treated cells were considered the control wells. Cells were further cultured for 4 days, after which the culture medium was collected, centrifuged at 1500 rpm for 10 minutes at room temperature, and stored at -80°C. The determination of cell number and viability for both the stimulated and control wells was done following the detachment procedure previously described. All the kits were prepared according to their respective instructions.

In vitro Angiogenic Assay and Tube Incorporation Assay

The *in vitro* angiogenic potential of MSCs was accessed through three assays: an Angiogenic Assay (AA), where MSCs were seeded alone and compared to HUVEC, a Tube Incorporation Assay (TIA), where MSCs were seeded together with HUVEC, and an Angiogenic Assay with Conditioned Medium (AACM), where the culture medium of MSCs was used to culture HUVEC. All the MSCs used were from P3-P4. The HUVEC cells were expanded on T-75 flasks from P5-P8 and incubated at 37°C, 5% CO_2. These cells were plated at an initial density of 3 000 cells/cm^2 and the medium was changed every 3 to 4 days. The medium used was Endothelial Growth Medium (EGM)-2 (Lonza). When reaching 70 to 80% confluence, the detachment procedure was performed as previously described. In all the assays, wells from a 96 well black/clear tissue culture treated plate flat bottom with lid (BD Falcon™) were coated with Matrigel (10 mg/mL, 0.4 mL, BD Biosciences), that polymerized overnight at 37°C. Each sample was placed in triplicate wells, using a total volume of 50 µL/well of cells suspended in EGM-2, except for the conditioned medium assay.

In the AA, 10 000 cells of HUVEC and MSCs expanded in both normoxia and hypoxia were seeded per each well. In the AACM, 20 000 cells of HUVEC in EGM-2 medium, HUVEC in normoxia conditioned medium and HUVEC in hypoxia conditioned medium were seeded per each well. In the TIA, MSCs were previously labelled with 10 µg/mL DiI-Acetylated-Low-Density Lipoprotein (DiI-Ac-LDL, Biomedical Technologies Inc.) in EGM-2 media at 37°C for 20 minutes. After the incubation, the cells were washed by adding 2 mL of PBS and centrifuging at 1250 rpm for 7 minutes at room temperature. The supernatant was discarded and the pellet was re-suspended in EGM-2 media. In this case, 20 000 HUVEC were seeded in each well, three to form the control group, and in six to form the co-culture groups. From these, three were posteriorly seeded with 5 000 cells per well of MSCs expanded in normoxia or hypoxia.

After seeding, the cells were incubated for approximately 8 hours at 37°C and 5% CO_2, in normoxia conditions. Afterwards, each well was washed with 200 µL of PBS. The cells were then labelled with 100 µl/well of 8 µg/mL of calcein (BD Biosciences) fluorescent dye in PBS and incubated at 37°C and 5% CO_2 for 30 minutes. Each well was again washed with 200 µL of PBS and the tube-like structures were observed under a fluorescence microscope (Leica) and using the software *Nikon ACT-1 2.70*, which was also used to acquire images of each well. The software *ImageJ 1.49b* was used to determine the tube length and the number of tubes.

SVF and ADSCs Isolation

The fresh AT samples were processed at the SCBL-RM, IST, and the SVF was isolated using two different methods, adapted from the literature[64].

Briefly for the enzymatic method, 50-200 mL of aspirate were washed by gentle shaking with an equal volume of pre-warmed Phosphate Buffered Saline solution (PBS, Gibco®) containing 1% of Antibiotic-Antimycotic (AA, Gibco®). The aqueous infra-natant phase was discarded and the tissue was further washed 2-3 times. The resultant volume was mixed with an equal volume of 0.1% collagenase II solution in Hank's Buffered Salt Solution (HBSS, both from Gibco®). The tubes were incubated at 37°C for 30 minutes in a thermomixer (Eppendorf). The digested product was vacuum-filtered and centrifuged at 1250 rotations per minute (rpm) for 7 minutes at room temperature. The SVF pellet was washed by re-suspension in 10 mL of Iscove's Modified Dulbecco's Medium (IMDM, Gibco®) supplemented with 10% Fetal Bovine Serum (FBS, Gibco®) and 1% AA (IMDM+10% FBS). This suspension was centrifuged at 1250 rpm for 7 minutes at room temperature and the pellet was re-suspended in Dulbecco's Modified Eagle's Medium (DMEM, Gibco®) supplemented with 10% MSC qualified Fetal Bovine Serum (Hyclone®) and 1% AA (DMEM+10% FBS).

Briefly for the non-enzymatic method, 25-200 mL of aspirate were washed by vigorous hand-shake for 1-2 minutes with 50 mL of PBS containing 1% of AA. The aqueous infra-natant phase was saved and this wash-step

was repeated 2-3 times. The recovered infra-natant was centrifuged at 1250 rpm for 7 minutes at room temperature and the SVF pellet was washed by re-suspension in 10 mL of IMDM+10% FBS. This suspension was again centrifuged at 1250 rpm for 7 minutes at room temperature and the pellet was re-suspended in DMEM+10% MSC FBS.

For both methods, the number of cells and their viability was accessed using Turk's Reagent Solution (Merck Millipore) dye exclusion method. Then, SVF cells were plated into T-75 or T-175 (BD Falcon™) flasks at a high initial density of 100 000 cells/cm^2 in order to isolate ADSCs, based on the adherence to the plastic surface. Briefly, cells were re-suspended in DMEM+10% MSC FBS or in StemPro® MSC SFM (Gibco®), a Xeno(geneic), Serum-Free (XSF) medium, and cultured at 37°C with 5% Carbon Dioxide (CO_2). After 24 hours the medium was exchanged and subsequently, the medium was changed every 3 to 4 days. When reaching 70-80% confluence, cells were detached, according to the procedure aforementioned, and re-plated at the same initial conditions, except for the initial cell density, which decreased to 3 000 cells/cm^2. When using the XSF medium, the flaks were previously coated with CELLstart™ (Gibco®) for 1 hour at 37°C, diluted in a proportion of 1:100.

Statistical Analysis

The results concerning more than one donor are presented as mean ± Standard Error of the Mean (SEM). Student's unpaired t-test was performed to analyse two sets of data. One-way ANOVA with Tukey *post hoc* test for correction of multiple comparisons was performed to analyse three sets of data. Two-way ANOVA with Bonferroni *post hoc* test or with Tukey *post hoc* test for correction of multiple comparisons was performed to analyse grouped sets of data. A *p* value inferior to 0.05 was considered as statistically significant. The statistical analysis was done through the software *GraphPad Prism 6.05*.

Results and Discussion

Comparison of MSCs from AT and BM (Healthy and AMI Patients)

Proliferative Potential Evaluation

Given the low frequency of MSCs *in vivo*[36], these cells require an *ex vivo* expansion step[37] in order to achieve a clinically relevant number, which motivates a proliferative study of MSCs.

In both the CFI and CPD graphics (Figure 1 a and b, respectively), the results for the cells expanded in hypoxia conditions are always superior to those of cells expanded in normoxia. For the CFI, it is clear that AMI BM-MSCs showed a higher value in both oxygen tensions than the other cells for the same conditions. The ratio of the CPD between hypoxia and normoxia was 1.1, 1.2, and 1.5 for healthy BM-MSCs, ADSCs, and AMI BM-MSCs, respectively. AMI BM-MSCs displayed the lowest (8.2 ± 3.8) and highest (12 ± 2.4) CPD at P9 for normoxia and hypoxia conditions, respectively.

The higher expansion capacity of the cells under hypoxic conditions is supported by several works[65-68], and attributed to an augmented number of MSCs entering cell cycle[65], a smaller accumulation of ROS[67], a minor decrease in the telomere length[67] and less DNA damage[67]. The low oxygen tension, possibly mimicking the niche conditions in the BM, also stimulated the cells to express more adhesion molecules like CXCR4[66], that potentially enhances the engraftment of MSCs to infarcted myocardium[69].

According to the literature[36; 70], ADSCs have a greater[36; 70] and faster[36] proliferative capacity than BM-MSCs due to the presence of more cells in the cell cycle[36]. Herein, both types took the same time to expand, and ADSCs displayed higher CFI and CPD than healthy BM-MSCs only in hypoxia conditions.

The higher CFI and CPD achieved by AMI BM-MSCs in hypoxia when compared to ADSCs and BM-MSCs might be derived from the younger age of the donors of AMI BM-MSCs (with exception for one donor, whose cells showed the most impaired proliferation in this group) and the previous exposure to inflammatory cytokines. In fact, there is a study claiming the inversed correlation between age and the proliferative potential of the cells[71]. Prasanna *et al.* 2010 showed that the stimulation of BM-MSCs with TNF-α and IFN-γ led to higher and smaller population doublings of these cells, respectively[72]. The *in vivo* situation is much more complex, therefore, the interaction of these and other inflammatory cytokines may translate into a higher proliferative potential for BM-MSCs of AMI patients, which would, then, explain the results obtained herein. In the normoxia conditions, the performance of AMI BM-MSCs is no longer better than the other cells, regarding the CPD. This

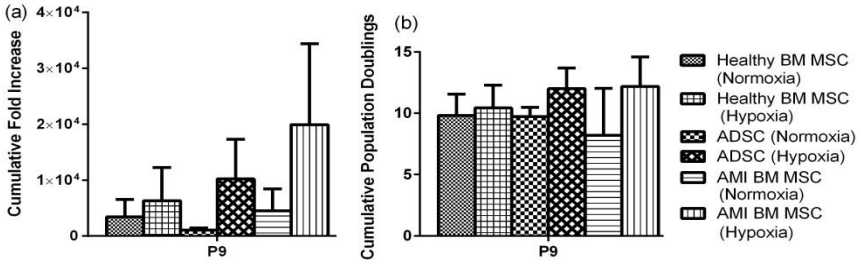

Figure 1. Evaluation of the proliferative potential of healthy BM-MSCs, ADSCs, and AMI BM-MSCs under normoxic (20% O_2) or hypoxic (2% O_2) conditions. (a) CFI; (b) CPD. In all the graphics, n = 3 for every type of cell at every passage.

observation is reverted if the oldest donor is excluded from the analysis, which shows that more samples should be analysed in order to reach more robust values.

Secretory Profile

The induction of the cells with TNF-α and IFN-γ induced statistically significant higher levels of IL-6 when compared to control groups (Figure 2 a), although without differences between oxygen tensions or cell type in both inducted and control situations. The positive effect of stimulation upon BM-MSCs and ADSCs is supported by the studies of Hemeda *et al.* 2010[73] and Lee *et al.* 2010[74]. However, authors reported higher induced values of IL-6 than the ones obtained herein, possibly due to the effect of TNF-α alone[73; 74], higher concentrations of the induction factor[73], or protocol differences[73; 74].

Inducted groups displayed increased concentrations of VEGF than the respective controls, with hypoxia increasing both the basal and inducted levels for all types of cells (Figure 2 b). Though ADSCs had the greater response to the induction with IFN-γ and TNF-α, healthy BM-MSCs were the cells reaching the highest concentration values of VEGF. The study by Li *et al.* 2012 confirms the increased expression of VEGF in BM-MSCs compared to ADSCs[75], while the studies by Kinnaird *et al.* 2004[76] and Rehman *et al.* 2004[49] support the positive effect of hypoxia conditions in the increased levels of VEGF.

Both healthy and AMI cells presented a similar performance in normoxia and hypoxia conditions, in the absence of induction, while the healthy BM secreted higher levels of VEGF upon induction. Possibly, previous exposure of BM-MSCs to inflammatory cytokines after an AMI episode saturates their answer in a posterior *in vitro* situation. However, that would also be supported by a higher VEGF basal expression in these cells, which was not observed.

In vitro Angiogenic Potential and Tube Incorporation Potential

MSCs seeded alone (Figure 3 a) were more prone to form larger junctions from which there was a divergence of many tubes, which were also thicker than the ones present at HUVEC cultures. These differences might be a consequence of the larger size of MSCs when compared to HUVEC, which was observed at the microscope. The tubular network formed in the AACM (Figure 3 a) was much more similar to the one from control, albeit the presence of many incomplete tubes in this setting, more evident in BM-MSCs and hypoxia conditions. The tubular network had also a more regular pattern, similarly to that observed for the control. In the TIA (Figure 3 a), the structures were similar to the ones observed in the AA of MSCs, with thick branch points and larger tubes. The orange spots in the green tubular network correspond to MSCs that successfully integrated into the tubular structure of the HUVEC.

In the **AA** (Figure 3 first group of results in b and c), healthy BM-MSCs and ADSCs presented lower number of tubes, while AMI BM-MSCs had an increase number of tubes, comparatively to control groups, in oxygen tensions. The difference in the number of tubes was statistically significant between AMI BM-MSCs and healthy BM-MSCs (120 ± 5.1 versus 70 ± 12.2 respectively, p = 0.0445). The general trend for this parameter was a crescent number of tubes from healthy BM-MSCs, to ADSCs, to AMI-MSCs, and decreased values in hypoxia, compared to normoxia. Regarding the length of the tubes, HUVEC, ADSCs and AMI BM-MSCs cultured in normoxia presented similar values (50-60 μm), while in hypoxia, the length of MSCs tubes increased to approximately 70 ± 7.1 μm.

Statistical differences were obtained when comparing healthy BM-MSCs to the respective

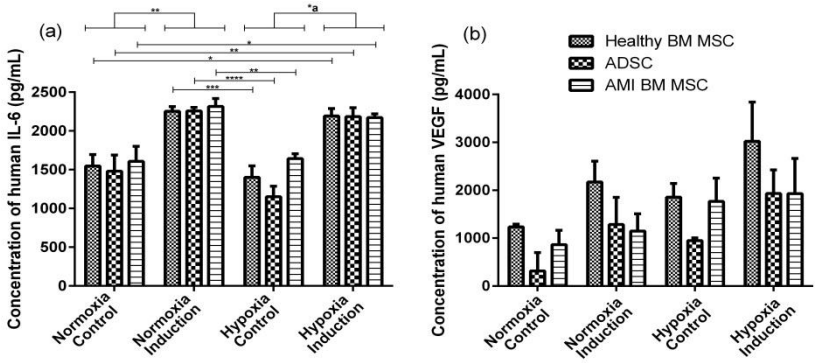

Figure 2. Secretory profile of MSCs under different oxygen tensions, and stimulated or not with TNF-α and IFN-γ. (a) Concentration of human IL-6 (pg/mL); (b) Concentration of human VEGF (pg/mL). In both graphics, n = 2 or 3. * p < 0.05; ** p < 0.01; *** p< 0.001; **** p < 0.0001; *a: p < 0.05 between the groups of AMI BM-MSCs, p < 0.01 between the groups of healthy BM-MSCs, and p < 0.0001 between the groups of ADSCs.

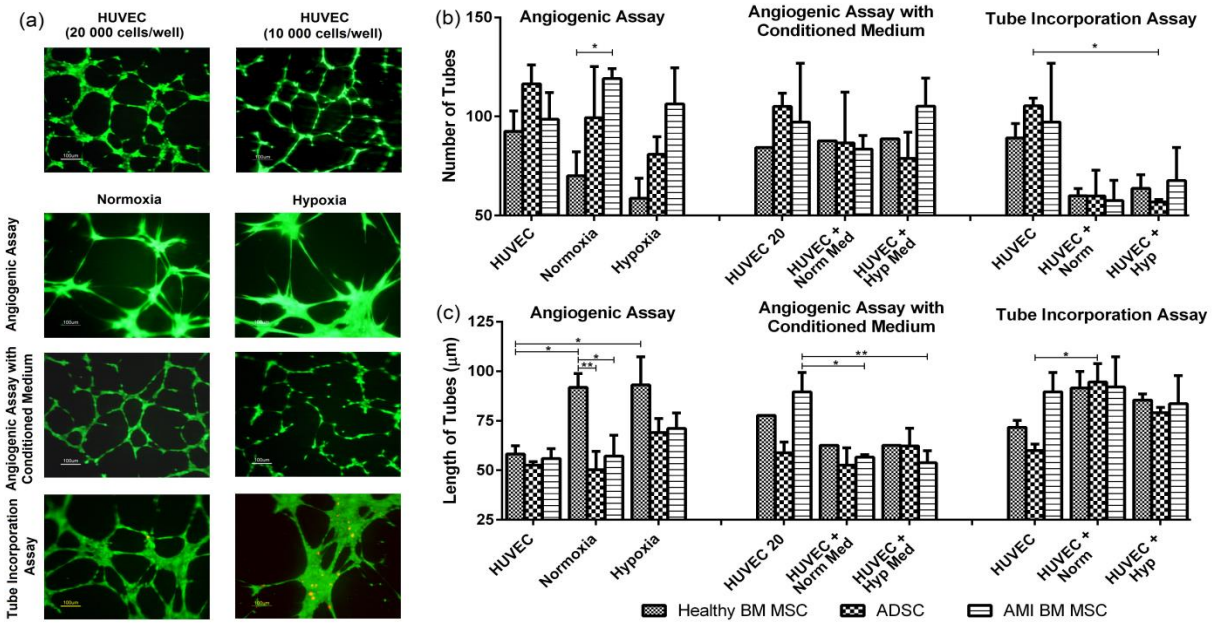

Figure 3. Results of the AA, AACM, and TIA, of healthy BM-MSCs, ADSCs, and AMI BM-MSCs under normoxic or hypoxic conditions. (a) Florescence images from the assays for HUVEC and healthy BM-MSCs; (b) Number of tubes; (c) Length of tubes (µm). In all the graphics and images for all the conditions studied, n = 1-3. * p < 0.05; ** p < 0.01.

HUVEC group and the other cell types in normoxia conditions, and when comparing healthy BM-MSCs from hypoxia conditions to HUVEC. However, healthy BM-MSCs displayed very similar values in both oxygen tensions (90 ± 7.0 µm to 90 ± 7.8 µm). It seems that a lower number of tubes is, thus, linked to longer tubes.

Comparatively to the other assays, the AA was more efficient in highlighting differences between cell types and gave rise to more statistically significant dissimilarities. Nevertheless, the results obtained were not concordant with those from the literature. For example, Janeczek Portalska *et al.* 2012 obtained a higher total length of tubes for HUVEC than for BM-MSCs[77], possibly due to a longer period of incubation of the cells (24 hours against 8 hours)[77], which might have benefited the tubular network of HUVEC. Li *et al.* 2012 obtained longer tubes for ADSCs when compared to BM-MSCs, an opposite trend to that observed herein[75]. Nevertheless, Eto *et al.* 2011 used a cytokine cocktail to induce ADSCs and observed a higher total network length and a more complex capillary-like network structure, with bFGF being the most relevant for the assay, comparing to HUVEC[78]. Although such induction was not performed in the present work, a hypoxic condition stimulates a higher production of bFGF[76]. Making a parallelism between the induction cocktail of the group of Eto (2011)[78] and the hypoxic conditions used herein, both strategies contributed to the great increase of the tube length when compared to HUVEC controls, although herein the difference between hypoxia and normoxia for the healthy BM-MSCs was minimal, since the tubes were already quite long for the normoxic cells.

In the **AACM** (Figure 3, middle group of results in b and c), the results for the number and length of tubes were consistently similar amongst oxygen tensions and the three conditioned medium-exposed HUVEC groups, with no statistically significant differences. HUVEC cultured in the conditioned medium from AMI BM-MSCs were the only group of cells displaying an increased number of tubes than the positive control, but only in hypoxia (110 ± 14.1 versus 100 ± 29.7, respectively). The smaller tubes present in HUVEC cultured in BM-MSCs conditioned medium when compared to control is in agreement with the observations that several tubes from these two conditioned medium seemed incomplete (Figure 3 a). This assay produced the most similar results to the controls, as supported by the images displayed at Figure 3 a. Contrarily to what was observed herein, several studies pointed to an increase in the length of tubes formed by HUVEC or ECs in conditioned medium[79-81], although such difference might be attributed to the use of negative controls (instead of positive, as herein)[79; 80], dissimilarities in the protocols[81], and few samples analysed in this work.

According to Liu *et al.* 2013, the hypoxic conditioned medium of ADSCs induced a higher total network length[82], while Zhang *et al.* 2012 showed that the hypoxic conditioned medium of BM-MSCs produced a higher number of tubes[83], both comparatively to normoxic medium. Herein, the ADSCs-hypoxic conditioned medium induced only slightly longer tubes than the normoxic medium or the controls, while AMI BM-MSCs-hypoxic conditioned medium gave rise to a higher number of tubes, which shows some agreement with the literature. The positive effect of hypoxia was associated to higher levels of VEGF and IL-6[83], which was also observed herein, but only for VEGF (Figure 2 b).

In the **TIA** (Figure 3, later group of results in b and c), a markedly reduction in the number of tubes was observed from the control group to the co-culture groups in either normoxia or hypoxia conditions. This decreasing was statistically significant in the comparison of the co-culture of HUVEC with ADSCs (expanded in hypoxia) and the respective control group ($p = 0.0451$). Associated to this drop of the number of tubes was an increase in the length of the tubes in the co-cultures, statistically significant for the co-culture of ADSCs (from 60 ± 3.3 μm to 100 ± 9.4 μm, $p = 0.0355$). For the co-culture with AMI BM-MSCs, the values between the control group and the co-cultures were very similar, varying from 79-85 μm. The increase in length observed in the co-cultures was supported by the work of Rahbarghazi *et al.* 2013[80] and Duffy *et al.* 2009[84]. According to the latter work, there was an increase in both the thickness of the vessel-structure and the size of the junctions[84], similarly to what was obtained herein (Figure 3 a), and the direct cellular contact was hypothesized to be an important mechanism by which MSCs contributed to the formation and stabilization of the vessels[84].

Processing of MSCs from AT

SVF Isolation

The cell population resultant from the processing of AT is the so-called SVF, which is a heterogeneous cluster of cells encompassing ADSCs, HSCs, ECs, blood cells, fibroblasts, amongst others[40]. Upon seeding on plastic surfaces, the SVF gives rise to a population of adherent cells, similar to MSCs, called ADSCs[40].

The mean number of SVF cells (Figure 4), obtained in the enzymatic and non-enzymatic method per mL of AT was $6.5 \pm 1.9 \times 10^5$ and $5.1 \pm 2.3 \times 10^5$, respectively ($p = 0.6509$). This similitude of values from both methods was also found by Yoshimura *et al.* 2006, although they obtained much higher values than herein[85]. The disparity in the magnitude of the values might be due to different cell counting methods used in both studies, to difficulties in cell counting experienced herein, and to an excessive period between the harvesting of the samples and their processing observed in this work.

The enzymatic process took about 1 hour and 25 minutes to be concluded, while the non-enzymatic required only 35 minutes. Comparing to the tissue processing times reported by Shah *et al.* 2013 (3 hours for the enzymatic and 1 hour for the non-enzymatic[64]), the processes performed in this work were faster, possibly because the incubation time of the AT samples with the collagenase solution was shorter and the volumes of AT processed by the non-enzymatic method were reduced, when comparing to Shah *et al.* 2013[64]. Nevertheless, in both studies there was a tendency of the non-enzymatic method being a faster process[64].

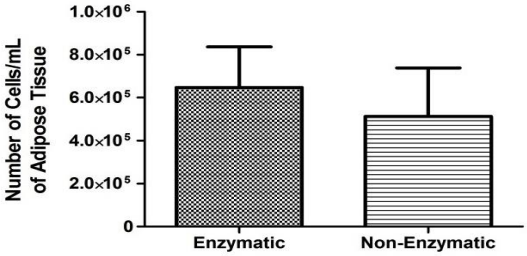

Figure 4. Number of SVF cells isolated per mL of AT. For both graphics, n = 11 and n = 9 for enzymatic and non-enzymatic methods, respectively.

ADSCs Isolation

Table 1. Isolation of ADSCs using two methods and two culture media. Enz – Enzymatic; NEnz – Non-enzymatic.

Conditions		Isolation success rate	Number of cells obtained (P0)/mL of Aspirate (mean ± SEM)
DMEM+10% FBS	**Enz**	100% (7/7)	$2.6 \times 10^5 \pm 1.5 \times 10^5$
	NEnz	50% (2/4)	$4.5 \times 10^4 \pm 0.2 \times 10^4$
StemPro® MSC SFM	**Enz**	62.5% (5/8)	$4.9 \times 10^5 \pm 3.5 \times 10^5$
	NEnz	0% (0/3)	-

Table 1 shows that the enzymatic method was more successful in the isolation of cells than the non-enzymatic, especially using DMEM.

The isolation rate reported in this work for the XSF culture medium using the enzymatic method is similar to the one by Patrikoski *et al.*

2013 (62.5% and 66.7%[86] respectively). The low value for the adhesion may, thus, have led to the complete failure of ADSCs isolation by the non-enzymatic method observed herein.

The enzymatic method had a yield of ADSCs 5.7-fold superior to that of non-enzymatic method, in DMEM (p = 0.5983). Shah *et al.* 2013 reported similar values of the yield relatively to the order of magnitude, however, the enzymatic method was 19-fold more profitable than the non-enzymatic in their work[64]. Differences in the initial density of SVF cells might explain the disparities between the values found in this work and those stated by Shah *et al.* 2013. Even so, the enzymatic method is more efficient than the non-enzymatic when using DMEM in both studies[64].

The yield of ADSCs isolated by the enzymatic method was superior using the XSF culture medium than DMEM, a tendency that complies with the higher proliferation rate of ADSCs cultured in this culture medium, observed by Patrikoski *et al.* 2013[86]. These cells cultured in XSF medium were smaller and sharper than in DMEM, which was also seen by Patrikoski *et al.* 2013 and attributed to the low initial adhesion of the cells in the XSF culture medium[86]. Since both cell cultures were harvested approximately at the same confluence stage, 70-80%, a smaller cell size leads to a higher number of cells and thus translates into a higher yield at the end of the isolation process for the XSF culture medium.

Proliferative Potential Evaluation of ADSCs

Figure 5 shows a trend of ADSCs to have a higher proliferative potential when isolated using the enzymatic method and XSF culture medium, also observed by Patrioski *et al.* 2013[86]. Comparing the enzymatic and non-enzymatic methods, the first resulted in a higher density of cells at P0, as previously stated. However, it appears that ADSCs from the non-enzymatic method were capable of recovering from their isolation process and surpass the performance of the cells from the enzymatic method, which is further supported by a higher fold increase and population doublings at P1 despite the more delayed proliferation (data not shown). The more time-consuming expansion of ADSCs by the non-enzymatic method is confirmed by the studies of Shah *et al.* 2013[64] and Yoshimura *et al.* 2006[85]. It is noteworthy that although these cells take longer time to proliferate, this is not an obstacle for them to reach higher values at the end of the cell passage. However, caution must be taken when interpreting these results since the number of samples for the comparison is neither the same for both conditions nor sufficient to be statistically significant.

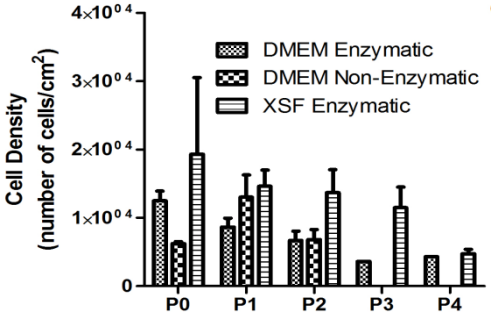

Figure 5. Cell Density (number of cells/cm^2) of ADSCs isolated by two methods, using two culture media. The number of samples varies between methods and passages from 0-7. The proliferative study was only performed until the confirmation of the immunophenotype of the cells, therefore, there is only information from P0-P4.

Conclusions and Future Trends

The clinical application of MSCs has been studied throughout the past years in diverse areas[42; 43; 87], including cardiac regeneration. The fact that IHDs are the leading cause of death[2] has driven the search for the development of a therapy capable of truly regeneration of the heart[16]. MSCs appear to be a great candidate for the development of an efficient therapy due to their intrinsic and unique properties [45; 50; 61; 62] that allow their use in either an autologous or allogeneic setting[63]. Aiming at the comparison of the therapeutic potential of MSCs in each setting, this work focused on the evaluation of key properties of MSCs extracted from BM or AT of healthy donors and of BM-MSCs from AMI patients, expanded in normoxia or hypoxia (a condition shown to enhance cellular proliferation)[68]. Two different methods for the isolation of ADSCs were further explored in order to confirm the AT as a reliable source of MSCs.

In the proliferative study of MSCs, hypoxic conditions enhanced their performance, with AMI BM-MSCs showing the highest CFI and CPD, possibly due to their previous exposure to inflammatory cytokines[72]. The results obtained harein could gain more consistency and suffer less impact from operator errors if performed in fixed time points and using a higher number of samples in all the three groups of cells.

The stimulation of the cells with TNF-α and IFN-γ enhanced the production of IL-6 and VEGF, as well as hypoxic conditions for the latter factor. Healthy BM-MSCs secreted the highest values of VEGF. In future works, a normalization of the concentration levels obtained through the number of cells after the incubation time could eliminate the proliferation/cell number effect on the expression level observed for the different conditions. Thus, a higher expression of a certain cytokine would be linked to a truly improved capacity of a single cell to secret higher levels of it, instead of being possibly linked to an increased number of cells expressing the protein.

The results obtained in the three settings for the angiogenic potential evaluation were quiet different amongst them. The AA was not only capable of showing that MSCs, alone, are able to form tubular structures, but also of differentiating the potential of each cell type, with the AMI BM-MSCs giving rise to a higher number of tubes and healthy BM-MSCs displaying longer tubes, which might be associated to an increased secretion of VEGF. Overall, hypoxia conditions also induced the formation of slightly less but longer tubes, when compared to normoxia. To investigate if MSCs are really capable of acquiring a phenotype close to the one of ECs without their presence, the cells could be previously stained with DiI-Ac-LDL.

The use of conditioned media produced tubes visually more similar to the controls than any other setting, although in less quantity for ADSCs conditioned medium, and smaller for both types of BM MSCs conditioned medium, comparing to controls. The length of the tubes was, however, very similar amongst all the types of cells and oxygen tensions, pointing to the inefficacy of this assay to produce robust and distinctive results according to the cells involved. The assay also produced opposite results to the ones found in the literature, although this is possibly explained by the use of negative controls[79; 80], instead of positive as it was done herein. In the future, both controls should be performed to simplify the comparison of the results with the literature.

The TIA produced results with a greater agreement to the ones reported in the literature. Co-cultures gave rise to considerably less but longer and thicker tubes with larger junctions when compared to control. Despite being incapable of highlighting the effect of oxygen tensions, this setting showed that the presence of MSCs, instead of only their conditioned medium, may be important to induce the formation of more robust and complex tubular structures, possibly with an important effect of direct contact, beyond the paracrine effect[84]. To prove that the co-cultured tubes are, indeed, more robust than tubes formed by HUVEC alone, the network could be analysed throughout time, as the group of Duffy (2009) did[84]. The same could also be studied for the AA.

In future work, the features of BM-MSCs harvested from the same AMI patients at different time points upon infarction should also be investigated and compared to cells obtained from healthy donors. Such study could clarify the source of the differences observed between AMI and healthy cells, as well as show if such differences are transitory or permanent.

Regarding the isolation methods of MSCs from AT (enzymatic and non-enzymatic), both yielded a comparable number of SVF cells, but the latter is simpler, faster, and skips products that could make it less defined, which makes it more economical and closer to generate a current good manufacturing practices product[88]. However, the non-enzymatic method presented a smaller yield of ADSCs at the end of the isolation process of this population. This seems to be overcame during the proliferation, where non-enzymatic isolated cells showed a higher, albeit slower, proliferative potential comparatively to cells isolated by the enzymatic method and expanded in DMEM. In the future, the processing of AT should be performed within 24 hours and more information regarding the local from which the samples were extracted, the applied suction force, amongst others, should be collected since these parameters might affect the cell yield[35].

XSF culture medium demonstrated to be an effective culture medium for the isolation of ADSCs, though it would be desirable to increase the initial adhesion of the SVF cells. Several initial densities of SVF cells should also be tested. These optimization steps could render some successful isolation cases for the non-enzymatic method using this culture medium. XSF medium was associated to an increased proliferation potential of ADSCs isolated by the enzymatic method, when compared to cells expanded in DMEM.

To conclude, the expansion of MSCs in hypoxia conditions should be considered, not

only to provide higher population doublings but also to protect the cells from an excessive oxygen tension[67] and induce the production of important cytokines in the context of an AMI, like IL-6 and VEGF. A pre-stimulation of the cells with inflammatory cytokines may be performed to further increase the secretion of those growth factors and maximize the paracrine action of the cells upon implantation into the infarct site. While healthy BM-MSCs displayed the best secretory and angiogenic potentials, AMI BM-MSCs had the highest proliferative capacity, followed by ADSCs, in hypoxia. Furthermore, 2 out of 3 donors of the AMI group had less than 50 years, which corresponds to a younger age than the one usually seen in AMI patients[89] and might have induced the better proliferative potential seen herein. However, taking into account that the majority of the AMI patients are old[90] and have other diseases[89], which has a negative impact in the features of MSCs[58-60], the autologous setting might not provide the best outcome for these patients. Instead, a cell bank of healthy donors with cells immediately available in relevant numbers and skipping the need for an immunocompatibility-matching is preferred. A higher number of samples in the three groups of cells studied herein should clarify the trends obtained in this work. ADSCs might be an acceptable alternative to BM-MSCs and it can be particularly advantageous if their *ex-vivo* expansion is performed under hypoxic conditions.

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