ROMANIAN ACADEMY

Institute of Cellular Biology and Pathology

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THESIS

Study of progenitor cells differentiation into cardiomyocytes in order to improve cardiac cellular transplant

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KEYWORDS

Cardiomyocytes

Apoptosis

Ischaemia/Reperfusion

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Mesenchymal stem cells

Adipogenic differentiation

Osteogenic differentiation

Chondrogenic differentiation

Myogenic differentiation

Hypoxia

Percoll gradient

Cardioprotective effect

Conditioned medium

microRNA

SUMMARY

Introduction

Ischaemic and non ischaemic myopathies have as a primary consequence the dysfunction of the left ventricle, which finally leads to heart failure. This impairment of heart function decreases the quality of life, life expectancy and increases medical costs. Thus, it represents one of the major health issues all over the world (McMurray and Pfeffer, 2005). Current therapies improve in some extent the survival of the patients and relieve the symptoms, but until now, an efficient method of reversing the damaged condition of the myocardium to a normal state has not been found (Kuraitis *et al.*, 2011). The recent development of stem cell research could provide an efficient treatment of heart failure, by replacing damaged tissue with healthy and functional myocardium, which would increase the quality of life and survival of patients with various cardiomyopathies.

Mesenchymal stem cells (MSC) isolated from adult bone marrow have been intensively investigated due to their regenerative and immunomodulatory properties. Various studies on animal models showed that the transplantation of MSC in an infracted aria of the myocardium improves the cardiac function by decreasing the apoptotic process and by releasing angiogenic factors (Tang *et al.*, 2005). Nevertheless, the efficiency of differentiation into cardiomyocytes (CMC) is extremely low and it cannot explain the transitory beneficial effect observed in preclinical and clinical studies of MSC transplantation in an infracted myocardium (Rose *et al.*, 2008). Therefore, this effect was attributed to the paracrine action of MSC (Gnecchi *et al.*, 2008). These cells are able to release into the extracellular environment soluble factors (citokines, chemokines and growth factors) which have a protective effect on the injured cells of the ischemic myocardium. Moreover, recently it has been shown that MSC are able to communicate with neighbouring cells through circular membrane fragments called microvezicles. In this way, MSC can secrete various soluble components such as receptors, growth factors, biologically active lipids and nucleic acids (mRNA and microRNA) which are protected against aggressive extracellular environment (Morel *et al.*, 2004; Gatti *et al.*, 2011).

MSC are considered to be used in regenerative medicine because they have several advantages, such as:

(i) these cells can be easily isolated and propagated in culture in order to obtain a high amount of cells, needed in cellular therapy;

(ii) they have low immunogenicicy, which makes them suitable for allogeneic transplant (Dai *et al.*, 2005);

(iii) ethical issues adjacent to other types of stem cells such as embryonic stem cells are avoided.

In this general context, the aim of this thesis was to study the differentiation of progenitor cells, such as MSC isolated from adult bone marrow, into cardiac muscle cells, in order to improve the efficiency of cellular therapy of myocardial infarction. Thus, over the entire period of the doctoral stage, the studies have been focused on understanding the mechanisms by which the heart muscle is damaged during and after myocardial infarction and finding an efficient method of tissue repair. This thesis is composed of two main sections. The first part, structured in four chapters, describes the current status of scientific knowledge on the subject of this thesis, while the second part contains the original contributions of the PhD student.

I. Molecular alterations leading to cardiomyocytes apoptosis in ischaemia-reperfusion

In the first chapter of the original contributions section, the focus of the study was on answering the question whether the ischemia or reperfusion is responsible for inducing the apoptotic process in CMC. Knowing the main stimulus responsible for inducing apoptosis in ischemic myocardium is necessary in order to achieve a therapy able to slow down the progression of heart failure due to loss of contractile tissue. Thus, this study aimed to determine the individual contribution of ischemia, reperfusion and exogenous oxidants to the induction of CMC apoptosis.

The results showed that, reperfusion, rather than ischemia *per se*, was the main inductor of apoptosis in CMC. Exogenous oxidants, brought by reperfusion from the surrounding medium, are responsible for triggering the cell death process. 25-hydroxicholesterol, a component of oxidized LDL, promoted CMC apoptosis by a caspase-3 dependent mechanism. This implied the increased expression of the pro-apoptotic protein Bax, which was regulated at transcriptional level and the post-translational degradation of the anti-apoptotic protein Bcl-2 (Figure 2). The impairment of the pro-and anti-apoptotic protein ratio created favourable conditions for the initiation of the apoptotic process, which could only occur during reperfusion, when the ATP and glucose reserves were restored. The data obtained by performing this study indicated that an antioxidant therapy could play an important role in preventing the loss of viable myocardium through programmed cell death of CMC.

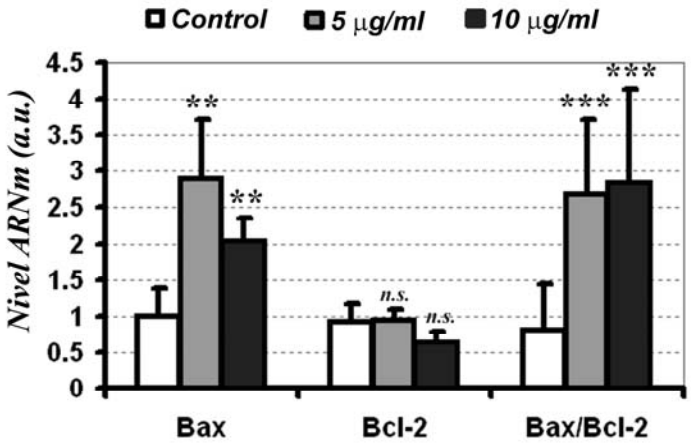


Figure 1: Quantitative RT-PCR illustrating the modification of bax and bcl-2 gene expressions and bax/bcl-2 ratio in CMC after 72 hours of treatment with 25-hydroxicholesterol.

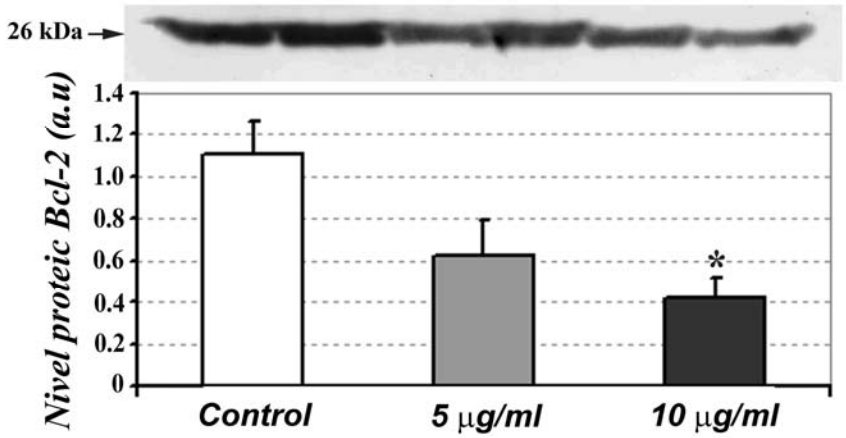


Figure 2: Quantification of the Bcl-2 protein level assessed by Western Blot after 72 hours of treatment with 25-hydroxicholesterol.

II. Isolation of mesenchymal stem cells

An important step of the doctoral stage was the development of a reliable method of isolation mesenchymal stem cells (MSC) for further use in *in vitro* differentiation studies. This presented a special interest because mouse MSC are more difficult to isolate and propagate *in vitro* than human or rat cells, because of the extensive contamination of the adherent culture with cells of haematopoietic origin. For this reason, obtaining a pure mouse MSC culture represented a challenge which resulted in the simultaneously isolation of such cells by two methods. The

homogenous MSC culture was consequently characterized as suggested by the International Society for Cellular Transplant.

II.1. Isolation of stem cells by centrifugation on a discontinuous Percoll gradient

Centrifugation of cells on a discontinuous Percoll gradient (ranging from 1.050 to 1.083 g/cm3) resulted in the recovery of six cell fractions (I–VI), corresponding to the six densities of the gradient: 1.050, 1.057, 1.067, 1.070, 1.076 and 1.083 g/ml (figure 3). Progenitor cells, characterized by the expression of Sca-1 and c-kit markers were found in fractions III-V.

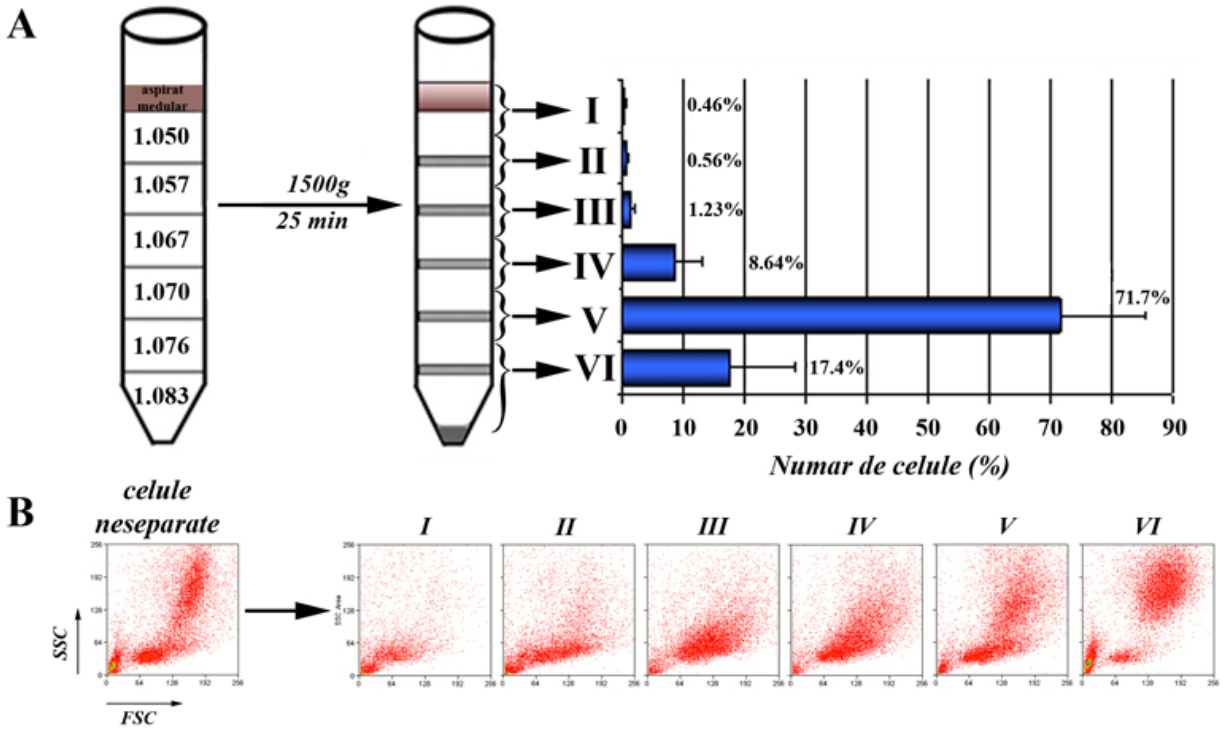


Figure 3: Bone marrow cells separation by centrifugation of Percoll discontinuous gradient. (A) Schematic illustration of the discontinuous Percoll gradient used for fractionation and the percentage of cells segregated in each fraction after centrifugation. (B) Dot-plot histograms resulting from FACS analysis illustrating the heterogeneity of cells before separation and the morphology of cells in each fraction.

The cells in fraction IV, even though they were still heterogeneous, contained a superior amount of haematopoietic progenitors, namely 45% of the population ($2x10^6$ progenitor cells from a total of $4.3x10^6$ cells, figure 4). In addition to c-kit and Sca-1 positive cells, this fraction included endothelial cells, which could play an important role in restoring function of the injured myocardium, by contributing to tissue revascularization by angiogenesis. In culture, this fraction

generated CD45^{-}/c-kit^{-}/Sca-1^{+}/PECAM^{-}/VEGFR2^{-}/osteocalcin^{-}/colagen II^{-} cells. By incubating these cells in specific differentiation media, adipocytes and osteoblasts were obtained, demonstrating their multipotent capacity (figures 5 and 6).

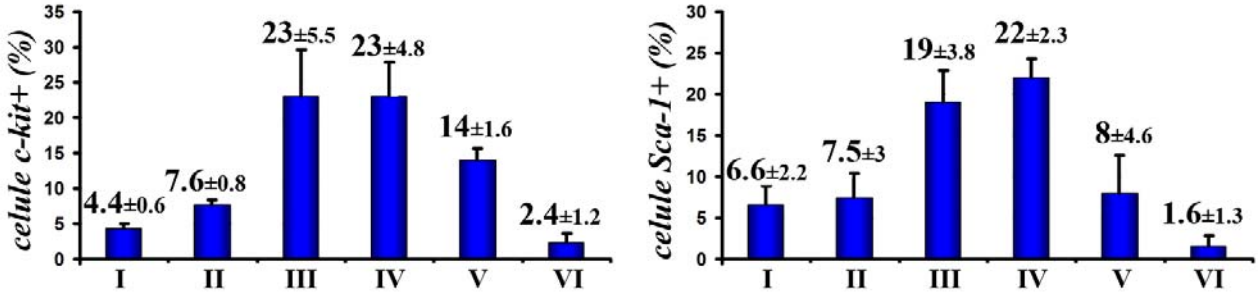


Figure 4: Overall representation of flow cytometry data showing the average percentages of c-kit+ and Sca-1+ cells within each fraction obtained after Percoll gradient separation of bone marrow aspirate.

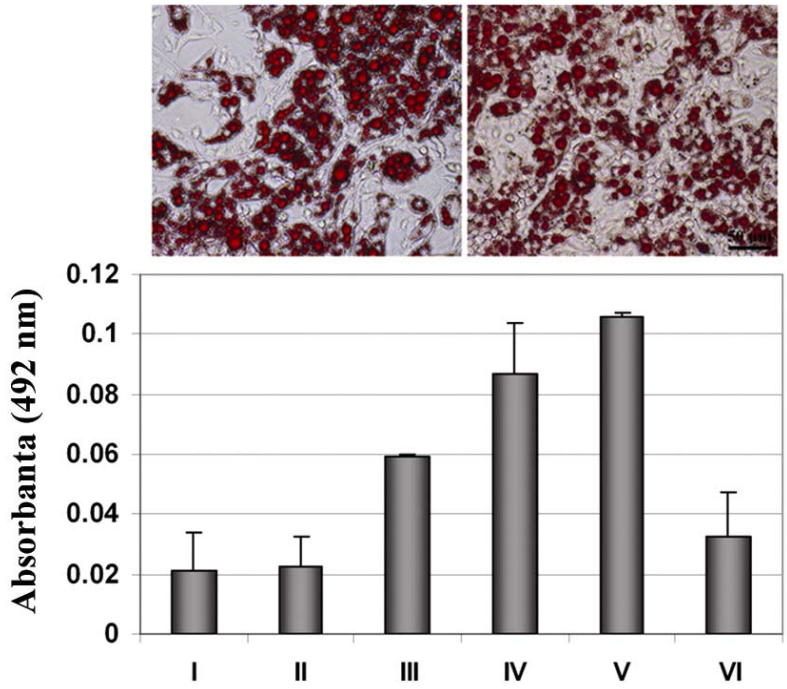


Figure 5: Spectrophotometric quantification of lipid accumulation in the six fractions after culturing the cells in adipogenic medium. The upper panel depicts a representative picture of Oil Red staining in fractions III and IV. Fraction V, despite the massive lipids accumulation, is not enriched in progenitor cells; the staining is most likely due to the presence of preadipocytes.

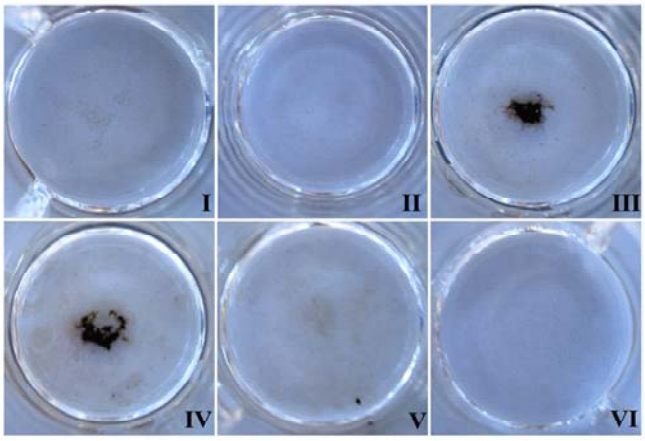


Figure 6: Light microscopy of von Kossa staining in the six fractions after cultivation in osteogenic medium. It can be noticed that fractions III and IV, but not fraction V (that contained the majority of the cells), were able to generate osteoblasts in appropriate conditions.

II.2. Isolation of mesenchymal stem cells form haematogenous bone marrow

In parallel with the method described above, a homogenous population of mouse MSC was obtained by maintaining the cells in culture for a long period of time. After approximately eight weeks, the cells a homogeneous culture with fibroblast-like appearance and highly proliferative. RT-PCR (Figure 7a) showed the presence of expression of Sca-1 and CD105 (endoglin) stem cell markers and the lack of c-kit marker, which suggested the depletion of haematopoietic progenitor cells. Another characteristic of these cells was the presence of vimentin and nestin intermediate filaments. Knowing that the presence of nestin filaments is specific to early progenitor cells with migratory capacity, the expression of nestin, associated with vimentin expression (Figure 7b) suggested for these cells a possible role in tissue regeneration.

Characterization of these cells was complemented by studies of differentiation into multiple cell types, such as adipocytes, osteoblasts and chondrocytes (Figure 8). These experiments showed their multipotent character, which was unaltered over several passages. This culture was subsequently used in studies of differentiation towards muscle cells. The data were presented in Chapter III of the thesis.

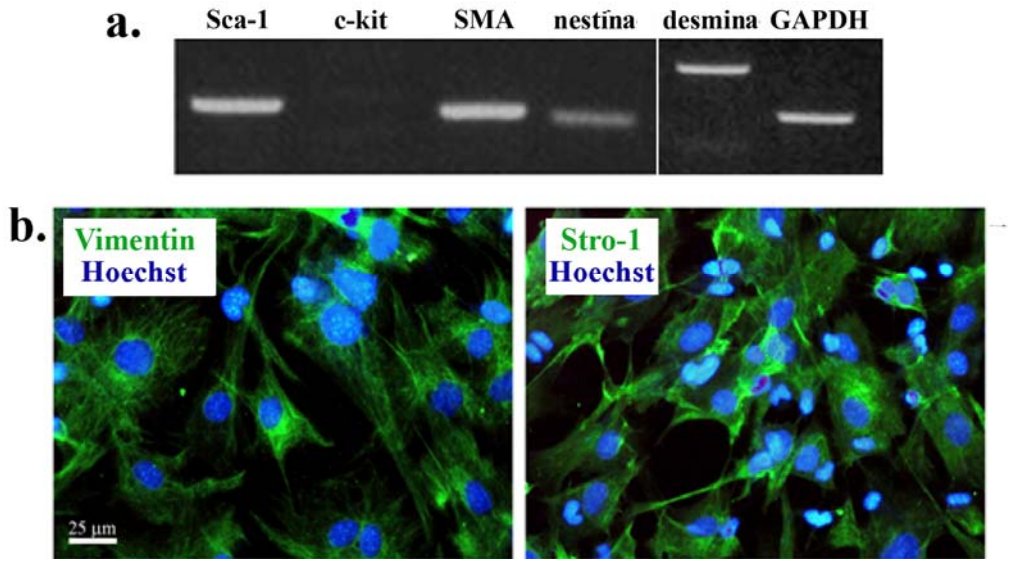


Figure 3. (a) RT-PCR showing the presence of Sca-1, smooth muscle actin (SMA), nestin, and desmin, and the lack of the hematopoietic stem cells marker c-kit; (b) Imunocitochemistry depicting the presence of the intermediary filament vimentin and of the stromal cells marker Stro-1 in all cells in the culture of MSC.

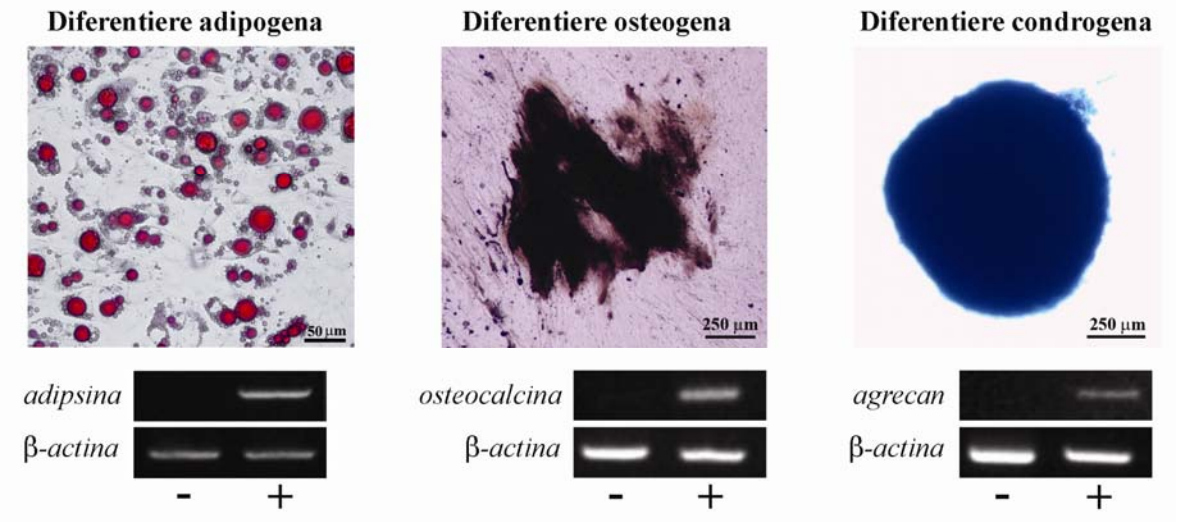


Figure 8: Characterization of MSC multipotency: Adipogenic differentiation (left panel) is shown by Oil Red staining and activation of adipsin gene expression. Osteogenic differentiation (middle panel) was confirmed by von Kossa staining and activation of osteocalcin gene expression. Chondrogenic differentiation (right panel) was confirmed by Alcian Blue staining and activation of aggrecan gene expression in differentiated cells.

III. Studies of mesenchymal stem cells differentiation

In order to assess the differentiation capacity toward CMC of MSC, the demethylating compound 5-azacytidine, known for its ability to enhance the differentiation of embryonic stem cells towards CMC was used.

A point of interest was the action of 5-azacytidine on multipotent stem cells, namely the ability of this compound to affect the ability of MSC to differentiate into multiple cell lines. The results showed that cells treated with 5-azacytidine gradually lost their ability to differentiate towards adipocytes and osteoblasts when they were incubated in specific differentiation conditions. An unexpected result was the increase of condrogenic differentiation potential induced by 5-azacytidine treatment. The conclusion of this study was that MSC keep their multipotency after the first exposure to 5-azacytidine, but their capacity to differentiate into multiple cell lines is gradually reduced with each exposure, while their capacity to generate chondrocytes is increased (Figure 9).

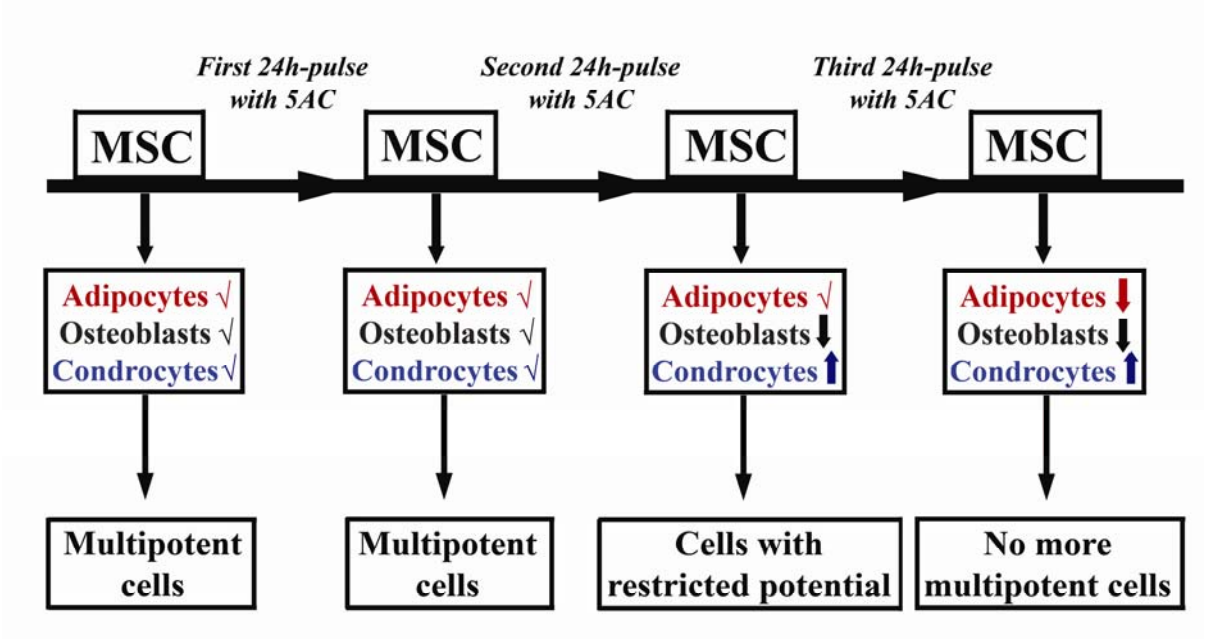


Figure 9: The effect of 5-azacytidine pulses on the multipotency of MSCs. The multipotency of MSC becomes restricted after exposure to more than one pulse of 5-azacytidine. After two or three pulses with 5-azacytidine, their capacity to differentiate into chondrocytes is considerably increased.

In the same time, the ability of 5-azacytidine to induce differentiation of MSC towards a cardiac muscle phenotype was followed. As shown in Figures 10 and 11, except for some muscle-specific genes (α-actin cardiac and L-type calcium channel), the expression of any of the genes involved in differentiation of cardiac muscle induced by this treatment. However, 5-azacytidine induced the expression of some skeletal muscle specific genes, which promoted the differentiation of MSC into myoblasts when incubated in appropriate conditions (Figure 12). In conclusion, 5-azacitidine treatment could favour MSC differentiation into cardiac cells, if specific factors that could affect the completion of the differentiation process into CMC mature and functional differentiation would be provided.

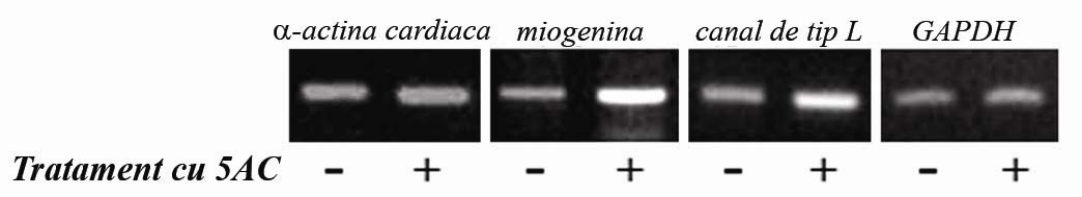


Figure 10: RT-PCR illustrating the expression of several muscle-specific genes that are elevated in MSC after one pulse with 5-azacytidine.

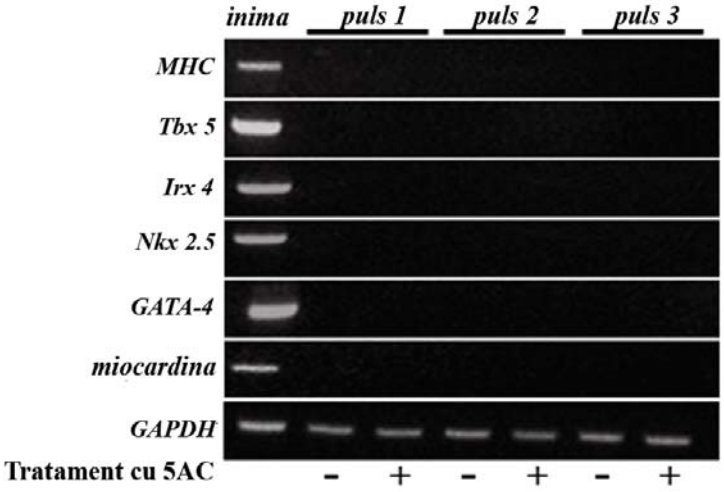


Figure 11: RT-PCR illustrating that the expression levels of genes specifically involved in cardiac differentiation are not increased in MSC by 5-azacytidine treatment.

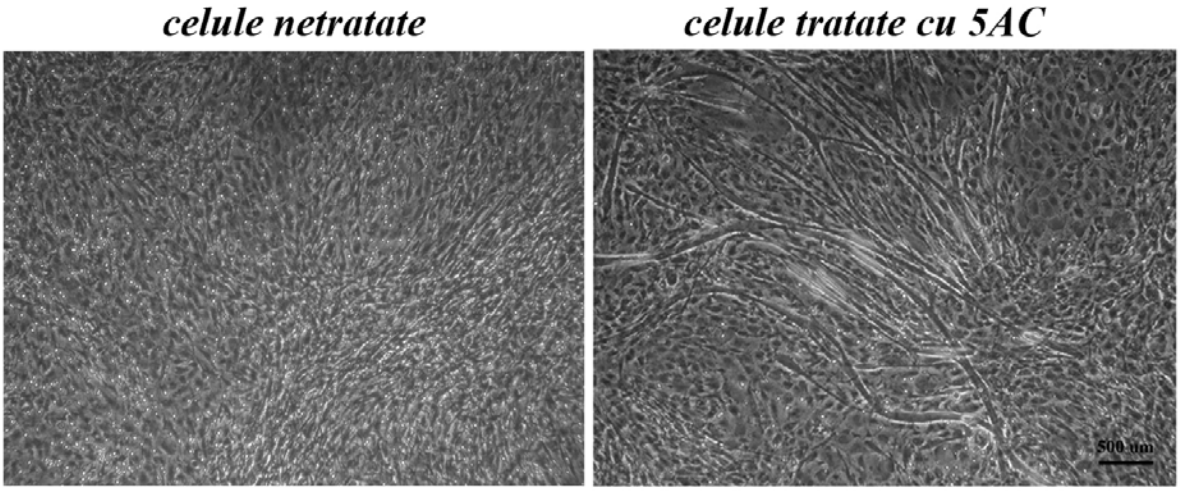
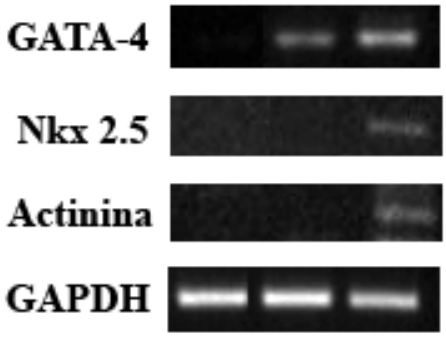


Figure 12: Phase-contrast microscopy image illustrating the appearance of myotubes in cultures of MSC incubated under serum deprivation conditions after three previous pulses with 5-azacytidine.

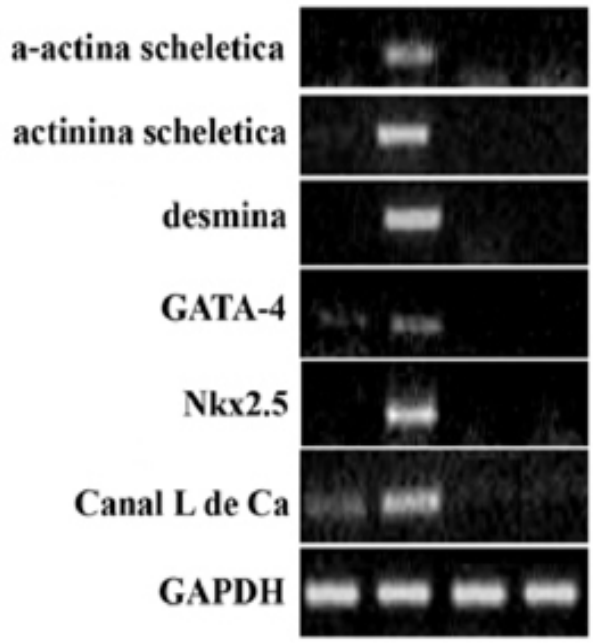
Following these observations, we found that 5-azacitidine treatment associated with direct contact with cardiac muscle cells may have a beneficial effect on differentiation into CMC of a stromal cell population. Thus, the changes in marrow stromal cells through direct contact with cardiac muscle cells combined with 5-azacitidine treatment were studied. The results showed that the two factors resulted in triggering the expression of cardiac specific transcription factors in mouse bone marrow stromal cells such as GATA-4 and Nkx2.5 (Figure 13).



CSA	+	+	+
CMC	-	+	+
5AC	-	-	+

Figure 13: RT-PCR showing induction of cardiac markers gene expression in cells treated with 5-azacitidine after 1 week of coculture with CMC. It can be noticed that the cumulative effect of the two conditions (treatment with 5-azacitidine and direct contact between stromal cells and CMC) is superior to simple contact between the two cell types.

Moreover, it was observed that expression of the latter was induced only if both differentiation stimuli were applied. In addition, actinin expression was induced, indicating differentiation towards to a striated muscle phenotype. Furthermore, the results of double chambers coculture experiments showed that combining 5-azacitidine treatment and soluble factors released from ischemic myocardium resulted in increased differentiation of bone marrow cells towards a cardiac phenotype. Thus, addition of exposure to 5-azacytidine to signals from the ischemic myocardial tissue triggered the transcription of key factors involved in cardiac differentiation, such as: muscle specific actin and striated type actinin, desmin, GATA-4, Nkx2.5 and L-type calcium channel (figure 14). Thus, it can be concluded that the initiation of differentiation induced by demethylation of certain genes is supported by the effect of some cardiac soluble factors that are able to complete this process.



Inima	+	+	-	-
CSA	+	+	+	+
5AC	-	+	-	+

Figure 14: RT-PCR showing the induction of gene expression of several cardiac markers in the presence of 5-azacytidine and local factors released from ischemic myocardium.

IV. Identification of some paracrine factors secreted by normal and hypoxic mesenchymal stem cells

Recent reports on clinical trial results showed that the beneficial effects of stem cell transplantation in myocardial infarction is transient, suggesting a paracrine effect exerted by the transplanted cells through the secretion of cardioprotective and angiogenic factors. Thus, in the last part of the thesis the anti-apoptotic effect of soluble factors released by MSC on cardiac muscle cells was studied and some factors released by MSC in normal and hypoxic conditions were identified. A particularly important observation was the MSC capacity to adapt and survive in hypoxic conditions, as demonstrated by the resistance of these cells to the hypoxic conditions induced apoptosis (Figure 15).

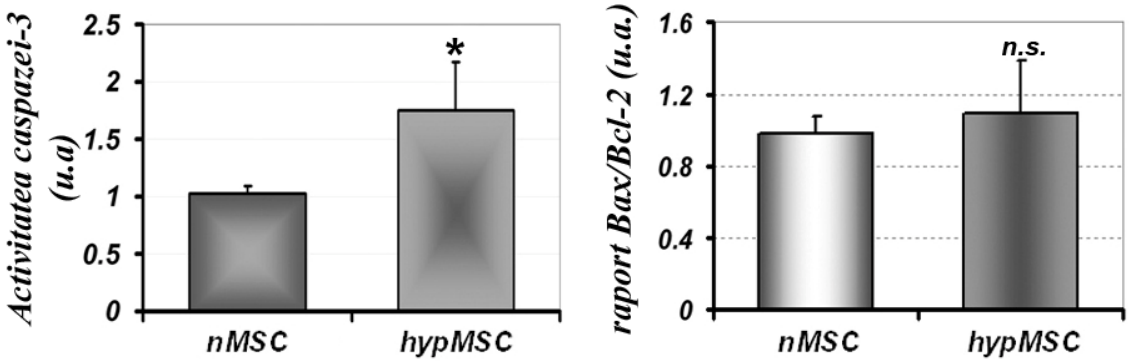


Figure 15: MSC in ischemic conditions: the graph on the left side of the figure shows a slight increase in the activity of caspase-3, while the ratio of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 gene expression, as determined by quantitative PCR, remained unchanged (chart on the right).

MSC resistance to hypoxia-induced apoptosis is particularly important for their survival in the ischemic myocardium, where the blood vessels needed to ensure adequate oxygenation are lacking.

Our results sowed that MSC derived conditioned medium had cardioprotective effects on hypoxic CMC. Thus, the presence of MSC derived conditioned medium prevented the trigger of the apoptotic process induced by hypoxia in CMC (Figure 16) by preserving the integrity of mitochondrial membrane. By concluding, MSC transplanted in hypoxic myocardium *in vivo* might have an important contribution to the survival of the CMC affected by hypoxia and oxidative stress during reperfusion.

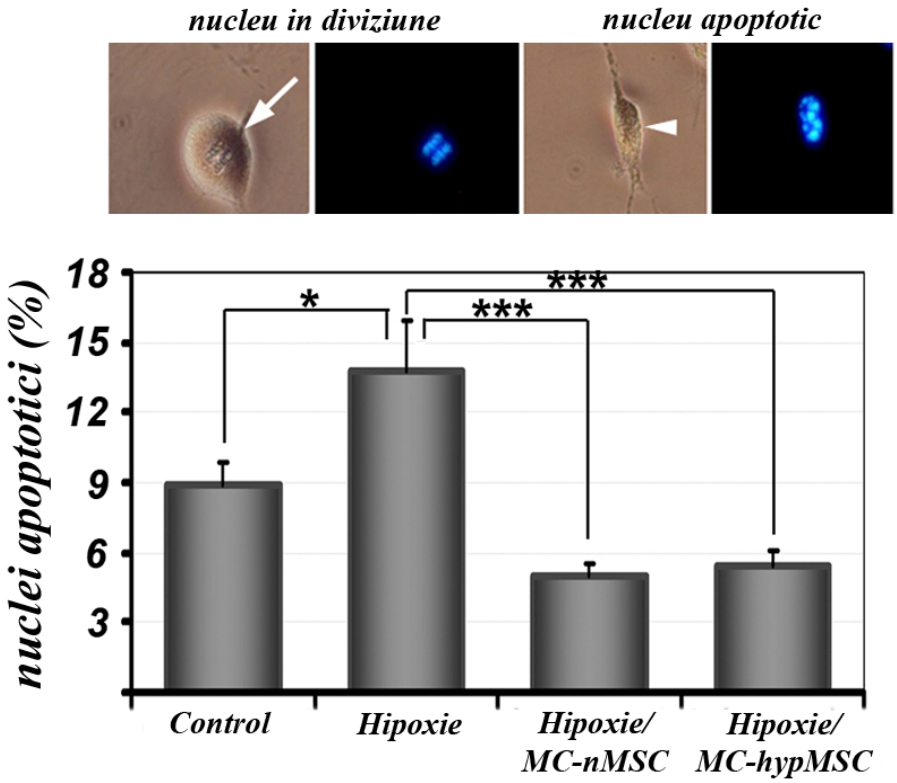


Figure 16: Quantitative determination of apoptosis in cardiac myocytes after exposure to hypoxia in the presence and absence of conditioned medium collected from MSC. The chart shoes the percentages of apoptotic nuclei determined by Hoechst staining. Images of apoptotic versus mitotic nuclei can be seen above.

Moreover, conditioned medium harvested from both normal and hypoxic MSC had similar effects, which suggested that MSC maintain their cardioprotective properties in hypoxic conditions.

Assessing the composition of MSC-conditioned medium showed that these cells are able to retain their ability to synthesize and release some cardioprotective factors such as cytokines and microRNAs. Several cytokines secreted by both normal and hypoxic MSC were identified, four of which were found in similar quantities the MSC incubated in hypoxia compared to the control: TIMP-1, MCP-1, SDF-1α and IL1ra (Figure 17). According to the literature, these factors could be responsible, at least in partially, of the cardioprotective effect exerted by the presence of MSC in the ischemic myocardium, either due to their anti-apoptotic action or by inducing angiogenesis, which is important for restoring the oxygen and nutrients supply.

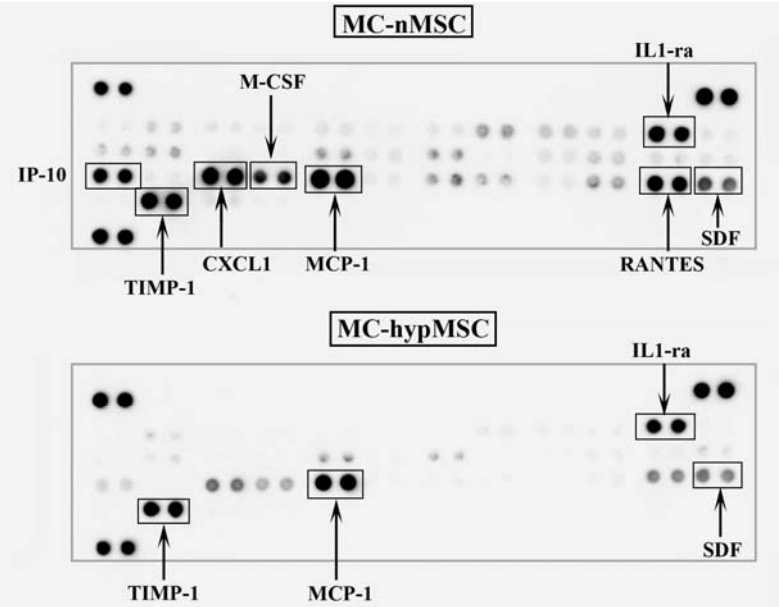


Figure 17: Citokine array showing the composition of normoxic MSC conditioned medium (upper panel) comparing to hypoxic MSC conditioned medium (lower panel).

In addition to these molecules, it appears that the transfer of genetic information between MSC and damaged cells is an important mechanism, which might have a repairing effect on injured myocardium. Thus, the hypoxic MSC derived conditioned medium contained an increased quantity of microRNAs 199a and 125b, known for their anti-apoptotic action (Figures 18 and 19).

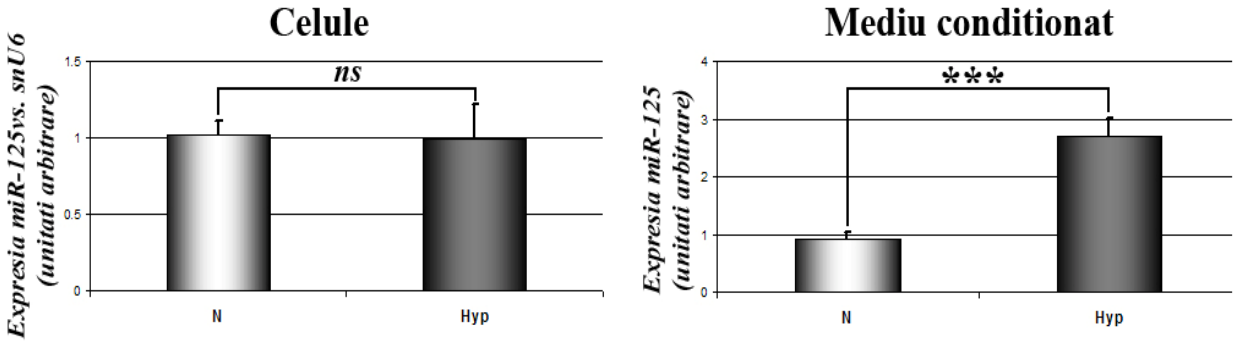


*Figure 18: Quantitative PCR showing microRNA-125 expression in normal versus hypoxic MSC (left chart) and the extracellular medium harvested from cells maintained in normal comparing to hypoxic conditions (right chart) (*** p <0.0005).*

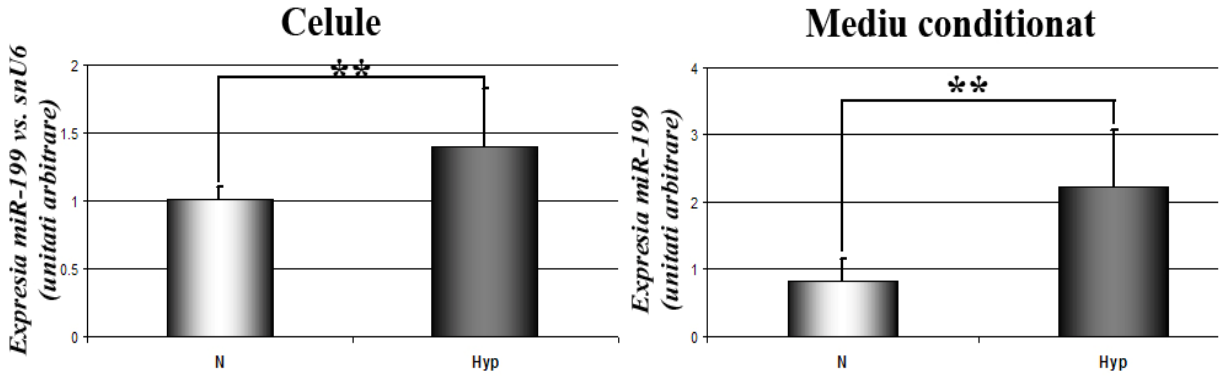


*Figura 19: Quantitative PCR showing microRNA-199a expression in normal versus hypoxic MSC (left chart) and the extracellular medium harvested from cells maintained in normal comparing to hypoxic conditions (right chart)(** $p<0.005$).*

In conclusion, the composition of MSC conditioned medium consists in a mixture of factors with either anti-apoptotic effect (TIMP-1, MCP-1, miR-199a, miR-125b), inhibitory for fibroblasts proliferation (IL1ra) or angiogenic (SDF-1α). The presence of these cardioprotective factors in MSC secretion may be responsible for anti-apoptotic effect on CMC hypoxic conditions observed in our experiments.

Conclusions

The data obtained during the doctoral stage were published in international journals with ISI impact factor and were presented at national and international scientific meetings as oral presentations or posters. Briefly, the main results were as follows:

- It has been shown that the main factor that triggers apoptosis in CMC after myocardial infarction is represented by the exogenous oxidants brought at the site of injury during reperfusion;
- A method for obtaining a population of progenitor cells obtained from bone marrow aspirate by centrifugation on Percoll discontinuous gradient was developed;
- Homogenous MSC cultures meeting the standards required by the International Society for Cellular Transplant were obtained;
- The effect of 5-azacytidine induced demethylation on the multipotency of MSC was pointed out;
- The beneficial cumulative effect of 5-azacytidine and soluble factors released by ischemic myocardium on MSC cardiac differentiation was demonstrated;

- The anti-apoptotic effect of factors released from normal and hypoxic MSC on hypoxic CMC was shown;
- Anti-apoptotic, anti-fibrotic and pro-angiogenic factors were identified in normal and hypoxic MSC derived conditioned medium.

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