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Research Article

Superior Therapeutic Potential of Young Bone Marrow Mesenchymal Stem Cells by Direct Intramyocardial Delivery in Aged Recipients with Acute Myocardial Infarction: *In Vitro* and *In Vivo* Investigation

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Introduction. Bone-marrow-derived mesenchymal stem cells (MSCs) have been studied for treatment of myocardial infarction (MI). Since MSCs from older donors show quantitative and qualitative senescent changes, we hypothesized that a better outcome may be achieved if aged recipients are given MSCs obtained from young donors, rather than using their own autologous MSCs. *Methods. In vitro* studies compared properties of young and old MSCs. Aged rats randomized into 3 groups underwent coronary artery ligations and were then injected with either old (O) or young (Y) MSCs, or ligation alone. Echocardiography evaluated ejection fractions (EF). At 16 weeks, scar deposition was analyzed. *Results.* Old MSCs exhibited decreased cell viability, proliferation, and differentiation potentials. EF significantly improved early in both cell therapy groups ($P < .05$). However, at later stages of the study, group Y showed significantly better function which correlated with decreased scar deposition. *Conclusions.* The significant difference between young and old cells indicates the possible advantage for allotransplanting MSCs from young donors to elderly patients with MI.

1. Introduction

Coronary artery disease remains a major cause of morbidity and mortality in developed nations. Recently, several animal models and clinical trials have demonstrated the therapeutic effect of mesenchymal stem cells (MSCs) to improve cardiac function following an acute myocardial infarction (MI) [1, 2]. These cells are able to differentiate into a variety of cell phenotypes [3], including cardiomyocytes, and can upregulate anti-inflammatory cytokines in the host microenvironment [4]. Furthermore, our lab and others have demonstrated that MSCs are immune tolerant such that they can be used in allogenic and even xenogenic transplants [1, 2, 5–7].

Since patients most likely to benefit from cell therapy for the treatment of acute MI are the elderly, it is important to consider the effects of aging on donor MSCs. In vitro studies involving MSCs have shown that an increased donor age is associated with decreased proliferation rates [8, 9]. Furthermore, studies that compared aged and young MSCs have shown an age-related decrease in differentiation potential [10–12]. These changes in the cellular characteristics are due to intrinsic changes within the cell as well as the effects of the aged microenvironment. Considering the effects of aging on MSCs and the target population of cell therapy, a better strategy for treatment of an acute MI could therefore involve isolating these cells from young donors for allotransplanation into the elderly, rather than using their own aging autologous cells.

Based on this background, our objective was to compare the in vitro properties and in vivo therapeutic effect of young

versus old MSCs in an aging rodent model of acute MI. We hypothesized that old MSCs will have a decrease in their potential to improve cardiac function following an acute MI when compared with MSCs harvested from younger donors.

2. Materials and Methods

2.1. Animals. Immunocompetent female syngenic Sprague Dawley (SD) rats (400–450 g, Charles River, QC, Canada) were used. All procedures were in compliance with the Guide for the Care and Use of Laboratory Animals (NIH publication number 85—23) and the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care.

2.2. Isolation, Labelling and Culture of Rat MSCs. MSCs harvested from young (6-weeks-old) and old rats (54-weeks-old) were cultured and prepared according to Caplan's method. Briefly, bone marrow aspirates were passed through a density gradient, and hematopoietic cells, fibroblasts, and other nonadherent cells were washed away during medium changes. As has been done in previous studies, the remaining purified MSC population was further expanded in culture using 10% fetal bovine serum supplemented DMEM media to form a clonal homogenous population of cells [6, 7]. The resulting MSCs were expanded for transplantation.

2.3. In Vitro Cell Proliferation Assay. The cell proliferation was evaluated using the Cell Titer 96 Aqueous Non radioactive Cell Proliferation MTS Assay (Promega, Madison, WI). MTS is chemically reduced by viable cells into formazan, which is soluble in tissue culture medium. The dehydrogenase enzyme activity found in the metabolically active cells is measured in terms of light absorbance values in this assay. The measurement of the absorbance of the formazan was carried out using the plate reader (Victor3 Multilabel Counter from Perkin Elmer) at 490 nm. Since the production of formazan is directly related to the number of living cells, the intensity of the produced color is an indicator of the viable cells. For this, the cells were seeded in a 96-well plate with 2×10^4 cells per well using serum-supplemented DMEM. After 4 days, the proliferation test was done by MTS assay as per the manufacturer's instructions mentioned elsewhere [13]. The experiments were performed in triplicates.

2.4. In Vitro Hypoxic Induction Assay. For the hypoxia experiment, the cells were seeded in 96-well plates as mentioned earlier with 2×10^4 cells per well and incubated overnight. The cells were then washed twice with PBS and replaced with serum-free DMEM media. The hypoxic condition was then generated using a hypoxia modular incubator chamber (Billups-Rothenburg Inc., Del Mar, CA) according to the protocol mentioned elsewhere [14]. Briefly, the cell culture plates were enclosed in the chamber and flushed with a mixture of gases (95% N_2 and 5% CO_2) for 5 minutes. At the end of the flushing period, the chamber was closed to prevent free flow of exogenous air into the chamber. The final level of hypoxia was around 1%. The cells were maintained under this hypoxic serum-free condition for 16 hours inside the 37°C incubator before they were taken out for proliferation studies.

2.5. Adipogenic and Osteogenic Differentiation of MSCs. To induce differentiation, young and old MSCs were first seeded in 24-well plates at a high confluency using serum supplemented DMEM media. After 24 hours of culture, the media were replaced with either osteogenesis induction media or adipogenesis induction media (Millipore, Billerica, MA) to induce differentiation [15, 16]. During this period, the induction media were replaced every alternate day with fresh induction media and later, differentiation media according to the manufacturer's protocol. After an induction period of 3 weeks, the cells were stained with either Oil Red O stain, which stains the lipid molecules, to assess adipogenic differentiation or Alizarin Red stain, which stains mineralized extracellular matrix, to assess osteogenic differentiation.

2.6. In Vivo Experimental Design. Immunocompetent SD rats, approximately 56-weeks-old, underwent proximal left anterior descending coronary artery ligations and were randomly assigned into three groups (group L, $n = 5$; group Y, $n = 8$; group O, $n = 7$). In group L, coronary arteries were ligated without cell injections as control. In group Y, young rat MSCs (3×10^6 cells) suspended in 150 μL of culture medium were injected transmurally around the peri-infarct area of the rat left ventricle (LV), 10 minutes after coronary ligation. In group O, rat MSCs (3×10^6 cells) from old donors were similarly injected as above after coronary ligation. All rats survived for the duration of the study which was predetermined to be 16 weeks.

2.7. Functional Assessment Using Echocardiography. Transthoracic echocardiography was performed on all rats at baseline and biweekly for 16 weeks after ligation and stem cell therapy. All animals were sedated in a quiet room and positioned in a right lateral decubitus fashion to ensure similar views in all echocardiographic assessments. An echocardiographic system was obtained commercially using a 12 MHz probe with small footprint (SonoSite, Seattle, WA) and performed as previously described [7]. The heart was scanned in longitudinal view in 2D and M-mode where it is usually possible to see a two-chamber view. Then, the probe was turned 90 degrees to get the best possible cross-sectional view of the left ventricle just above the papillary muscles. To minimize the intraobservational error in measurements, three consecutive measurements were made and averaged. Left ventricular end-diastolic (LVEDD) and end-systolic (LVESD) diameters were measured. This was done according to the American Society of Echocardiology leading-edge method [17]. Fractional shortening was determined as [(LVEDD-LVESD)/LVEDD] $\times$ 100 (%). The ejection fraction (EF) was estimated as [(LVEDV-LVESV)/LVEDV] $\times$ 100 (%). All pre- and post-intervention measurements were performed by one experienced observer blinded to the treatment groups to ensure reproducibility.

2.8. Heart Specimen Harvesting and Histopathological Analysis. Upon sacrifice at 16 weeks, hearts from all groups were harvested and dissected from surrounding tissue. Five consecutive 5 μm sections were prepared from the mid-portion of the infracted area in all samples. Sections were stained with collagen-specific dye Sirius red 3BA in saturated picric acid solution to allow a clear discrimination between cardiomyocytes and extracellular collagen matrix (ECM). The slides were scanned at 2400 dpi with 4x magnification, and the pictures were RGB split to green color layer for measurement. In the digital images, scar area and the total area of myocardium were traced manually by a blinded observer and measured automatically by the computer by means of Image J-1.41 software. Infarct size, expressed as a percentage, was calculated by dividing the sum of infarct areas by the total sum of LV areas including those without infarct scar and multiplying by 100.

2.9. Statistical Analysis. All data are expressed as mean values ± standard deviation. Analysis was carried out by using SPSS 16.0 software (SPSS Inc, Chicago, IL). Comparisons between the three groups were made using one-way analysis of variance (ANOVA) with a Tukey's post hoc. Comparisons between two groups were done using Student's t-test. Statistical significance was defined as P-value less than .05 for all tests.

3. Results

3.1. In Vitro Assessment

3.1.1. Stem Cell Proliferation Capability. Cell proliferation assays were performed on passages 1, 5 and 10 of both cell types. Cells were seeded with 2×10^4 cells per well and after 4 days MTS cell proliferation assay was done. Both cell types had rapid and highest proliferation in passage 5, while with higher passages, such as at passage 10, the proliferation potential decreased (Figure 1(a)). Young MSCs had significantly higher ($P < .05$) proliferation potential than old MSCs at every passage.

3.1.2. Stem Cell Response to Hypoxic Condition. Sixteen hour hypoxia under serum free conditions had no major effect on cell viability of young MSCs in passages 1 and 5 (Figure 1(b)). However, this was not the case with old MSCs, where there was a marked reduction in cell viability (Passage 5: Group Y 97.0% ± 1.2% versus Group O 78.0% ± 2.1%, $P < .05$). Young MSCs maintained a significantly higher viability in passage 10 under serum free hypoxic conditions as compared to that of old MSCs. This data showed that young MSCs were much more suitable for cell therapy after an acute MI than their older counterpart.

3.1.3. Stem Cell Differentiation to Osteogenic and Adipogenic Lineages. The osteogenic differentiation of old and young MSCs were examined using the osteogenesis and adipogenesis induction media. Both cell types were able to differentiate to the induced lineages, although qualitatively it was demonstrated that young MSCs tended to differentiate readily and better than the older ones (Figure 2). These results confirmed the multipotency of the cells studied, an important property of stem cells.

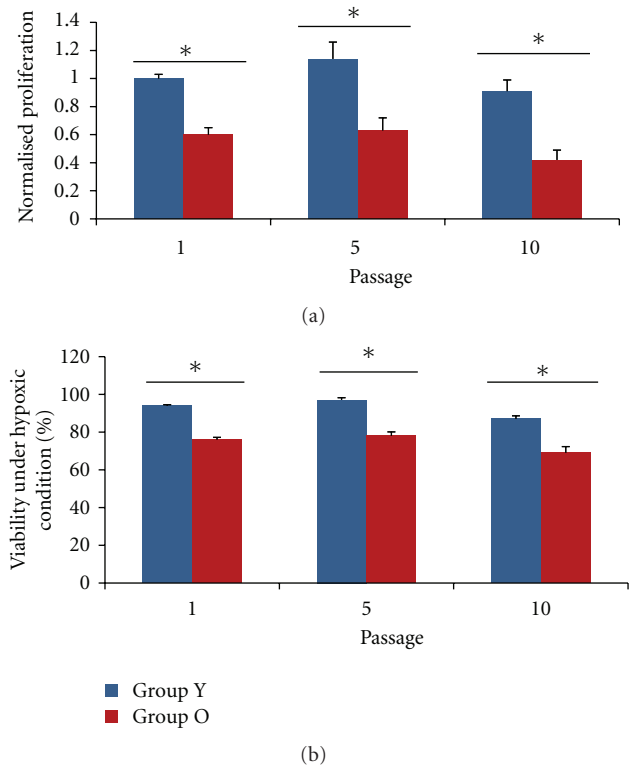


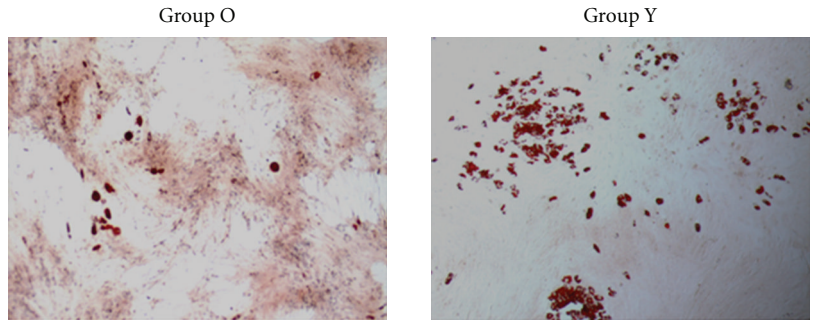
Figure 1: (a) The normalized data (young MSCs at passage 1 has been taken as 1.0, and other values are normalized to it) demonstrates highest proliferation in passage 5, compared to passages 1 and 10, with group Y having significantly higher proliferation rates than group O at all passages (*$P < .05$). (b) The data demonstrates that there is no major effect of hypoxic condition on young MSCs in all the passages compared to old cells (*$P < .05$).

3.2. In Vivo Assessment

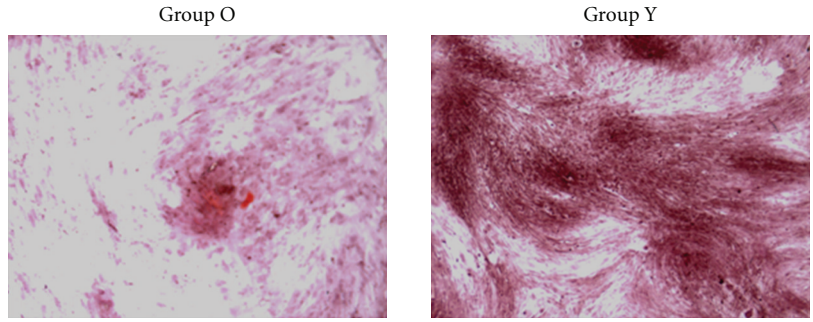
3.2.1. Functional Improvement of MSC-Treated Hearts. The left ventricular ejection fraction (LVEF) was not significantly different among the groups preoperatively. Overall, there was a significant improvement in LVEF in the two cell therapy groups compared to the control group L, indicating a beneficial effect of MSC therapy after MI (Figure 3). However, at a later stage beyond 10 weeks of follow-up, group Y demonstrated much more significant improvement in LVEF, while the old MSC group assumed a similar decline pattern as control group L.

Preoperative fractional shortening was also similar among the 3 groups prior to cell therapy. A rather similar pattern was also observed between the cell therapy groups versus control group, although the separation of the young donor group occurred at a slightly later time (Figure 4).

3.2.2. Left Ventricular Scar Area Analysis. Histopathological analysis of myocardial tissues showed significantly reduced scar extracellular matrix deposition in group Y at 16 weeks compared to group O and group L (Figure 5).



(a) Adipogenic differntiation



(b) Osteogenic differntiation

FIGURE 2: The cells, from passage 5, were seeded at high confluency for osteogenic and adipogenic differentiation using serum-supplemented DMEM. After overnight culture, the media was replaced with respective induction media. After 21 days of osteogenic induction, the cells were stained with alizarin red to detect the osteogenically differentiated cells. Similarly the cells were stained with oil red O to detect the mature adipocytes. Both cell types showed differential potential, with young cells being more efficient.

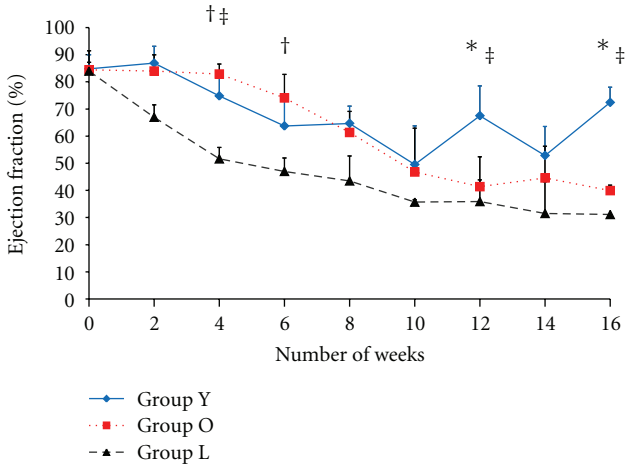


FIGURE 3: Echocardiography demonstrated that left ventricular ejection fractions improved in both cell therapy groups compared to the control group. Furthermore, there was an overall observed trend that the group that received cells from young donors experienced the greatest improvement in ejection fraction following acute MI (*Group O versus Group Y, $P < .05$; †Group O versus Group L, $P < .05$; ‡Group Y versus Group L, $P < .05$).

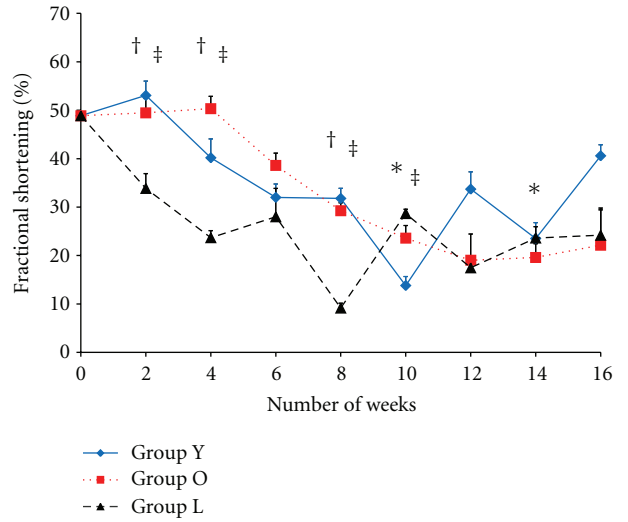


FIGURE 4: There was an observed trend that left ventricular fractional shortening improved in both cell therapy groups compared to the control and when group O was compared to group Y, group Y had an overall greater improvement in fractional shortening (*Group O versus Group Y, $P < .05$; †Group O versus Group L, $P < .05$; ‡Group Y versus Group L, $P < .05$).

4. Discussion

Many studies have demonstrated that MSCs isolated from older donors undergo senescence and display several qualitative and quantitative changes compared to young donors [8–12]. These observed age-associated changes may affect the therapeutic impact of cell therapy in an acute MI setting. Since the clinical application of cell therapy is often

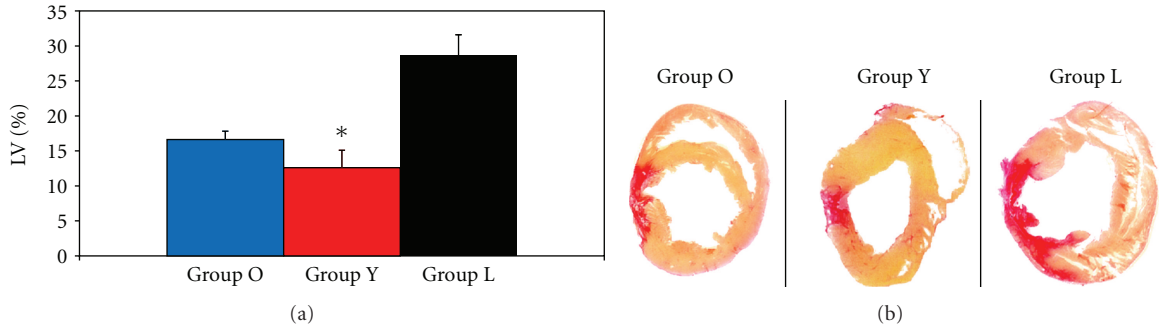


FIGURE 5: (a) At 16 weeks, group Y experienced significantly ($*P < .01$) decreased scar formation. (b) Sirius red staining shows collagen deposition (scar) in red against cardiomyocytes and intramyocardial vessels in orange (original magnification x4).

relevant to the elderly patients, senescent stem cell donors may provide less efficient functional improvement following cellular cardiomyoplasty. Our lab and others have shown that MSCs are immune tolerant [1, 2, 7] and using MSCs isolated from young donors for allogenic transplantation to older patients would provide a better alternative. The current in vitro data suggested that MSCs from young donors had increased differentiation potential compared to older donors. In addition, the proliferation rates were also higher in the young donors; therefore, expanding MSCs to large therapeutic doses after initial harvesting is much more feasible when isolated from younger donors [18]. The present study also provided an additional in vivo rodent model comparing the therapeutic effect of MSCs isolated from young versus old donors to treat acute MI in aged recipient animals and demonstrated that cells isolated from young donors resulted in greater improvement of cardiac function and decreased scar formation when compared to MSCs from older donors. Both cell-treated groups also experienced an increased ejection fraction compared to the control group, demonstrating a therapeutic effect of MSC implantation. However, this beneficial effect gradually faded off over longer-term follow-up in the aging MSC donor group. The histopathological analysis showed that this functional deterioration in rats treated with MSCs from aged donors (group O) was associated with greater ECM deposition at 16 weeks compared to the recipient rats with young donor MSCs. This suggests that the local effect of MSCs from older donors is not long lasting compared with the younger counterparts. The mechanism as of why the beneficial effects of MSCs at different ages only separated many weeks after implantation is not well understood. A recent study by Chen et al. from our lab has shown that the early functional improvement of LV function after acute myocardial injury and stem cell therapy was attributed to the anti-inflammatory effect of cytokine that was significantly upregulated immediately after MSC implantation, a mechanism that may explain the early functional improvement before cell differentiation has time to take place [6]. It is possible that the decline in paracrine and differentiation potential of aging MSCs may be temporally different and may explain the time-related decline in MSC benefit. For this reason, an end point of 16 weeks was chosen prior to the start of the experiment to compare both short-term and long-term effects of transplanting young and old stem cells.

The functional and histopathological results of age-related decline in therapeutic effect of MSCs are consistent with studies that have looked at different cell types. Edelberg et al. used bone-marrow-derived endothelial progenitor cells to treat the aging-associated decline in cardiac angiogenesis. They showed that transplantation of endothelial progenitor cells from young mice maintained and restored the angiogenic function of exogenous cardiac tissue but aged donor endothelial progenitor cells failed to do so [19]. The ability of hematopoietic stem cells to reconstitute the immune cells of irradiated mice in allogenic stem cell transplantations has also been evaluated. It has been shown that transplants from young donors significantly affected lymphocyte reconstitution when compared to aged donors [20]. Similarly, the efficacy of treating atherosclerosis with hematopoietic and stromal-enriched cells derived from young and aged marrow has been tested in a murine model. It has been shown that the atherosclerotic burden was significantly greater in mice that received treatment from old donor cells compared with that of the young donor cells [21]. On the other hand, this age-related decline in tissue repair ability may be cell specific such that certain cell types are less prone to the effects of aging and retain their therapeutic potential better than other cell types. Zhang et al. reported in an ischemic cardiomyopathic heart model that aged donor smooth muscle cells from 2-year-old rats were associated with significantly improved cardiac function compared with aged donor MSCs from the same donor age which had very little benefit [22].

There are different possible explanations as to why MSCs isolated from young donors resulted in greater functional improvement compared to old donor MSCs. These may be related to the mechanisms by which MSCs improve cardiac function after cell therapy including transdifferentiation into cardiomyocytes and/or paracrine effects through secretion of various cytokines [23]. The current study demonstrates that with increasing donor age, the differentiation potential of MSCs declines significantly. MSCs from older donors may therefore be less capable of regenerating neomyocytes resulting in functional deterioration and increased scar over time. In addition, MSCs from old donors may exhibit a different cytokine profile compared to young MSCs. In

vitro and animal studies have shown that MSCs implanted in infracted myocardium resulted in increased gene expression of interleukin-10 [6], a cytokine that has been demonstrated to reduce infarct size in animals subjected to myocardial ischemia/reperfusion [24]. Furthermore, our lab has previously demonstrated that MSCs affected the expression of tissue inhibitor of metalloproteinase-1 and matrix metalloproteinase-2, both of which influence ventricular remodeling following MI [25]. A reduction of the paracrine effect of aging MSCs may conceivably have resulted in greater scar formation and decreased LV function as observed in the current study. Nevertheless, other mediators or yet unknown mechanisms may be responsible for the differences observed between young and aged MSCs.

While increasing evidence suggests an age-dependent decrease in the therapeutic potential of multipotent MSCs, it would be important to determine what donor age would provide the best therapeutic effect. Markel et al. have demonstrated an age threshold in which MSCs were able to provide superior myocardial protection [26]. They compared neonatal MSCs, approximately 2.5-weeks-old, with adult MSCs, approximately 9-weeks-old, in a rodent model of ischemia/reperfusion and showed that adult MSCs were able to improve cardiac function following ischemia/reperfusion while neonatal MSCs provided no protective effect, similar to the control group.

Although we did not compare the effect of recipient age on MSCs in an acute MI, it has recently been shown that the age of the recipient is also a critical factor in determining the host's response to cell therapy [27, 28]. In a study by Kan et al. that used an MI model of young and aged rats that received skeletal myoblasts isolated from young donor rats, they found that although cardiac function improved in all the myoblasts recipients in comparison to the control group, the improvement in young rats was significantly greater than that of the old rats when using donors of the same age [28]. Therefore, it is possible that after a certain age, the recipient will benefit less from cell therapy than younger recipients and therefore optimizing the age of MSC donors will potentially benefit this compromised situation.

There are some limitations to the current study that merit further investigations. First, correlating rodent age to human age is an imprecise estimate that can be based on various factors such as life expectancy, the age of puberty, weaning, and musculoskeletal maturity [29]. Depending on which of these factors are used as reference, our 56-week-old rat may correlate to a human ranging from 9-years-old to over 100-years-old. On the other hand, from the rodent's life span point of view, they exceeded over two-thirds of their average life span. In addition, they had signs of aging such as tumour growth that is rarely seen in the younger rats. Therefore, although the precise age is still difficult to confirm in our study, it is likely that our findings reflected the effects of aging MSCs on aging recipients.

The current rodent model is not exactly a common clinical scenario because injecting MSCs into the myocardium 10 minutes after coronary ligation may not necessarily simulate the clinical setting. However, the use of small rodents is a well-accepted model for MI and is preferable to study the mechanisms of stem cell therapy. It provides useful evidence to form a basis for additional mechanistic studies. Further investigation into the mechanisms of stem cell therapy will allow for future studies in larger-animal models and provide information for evidence-based clinical research trials.

In general, the standard procedure for isolating bone marrow is invasive and can be fatal for the donor. Thus their clinical applications are normally limited by the logistic, economic, and timing issues when harvesting autologous cells from elderly sick patients. From a clinical perspective, this study implicates that young allogeneic donor's stem cells would be preferable to using autologous stem cells isolated from an aged recipient. Young allogeneic MSCs can be harvested and mass-produced well in advance, tested for their functional capabilities, and stored as a standardized cell population for immediate off-the-shelf use on any patient without delay after an acute myocardial infarction. Such logistic advantages are not available with the use of autologous MSCs that are currently the cell source of choice. Moreover, because such allogeneic MSCs can be obtained from young healthy donors, allogeneic MSCs could be of great value in patients with genetic cardiomyopathies and in elderly patients with advanced diabetes, heart failure, or cachexia whose own MSCs could be dysfunctional.

5. Conclusions

The current study demonstrated that MSCs isolated from young donors resulted in improved cardiac function and reduced scar deposition and myocardial injury compared to MSCs from aged donors when used to treat acute MI in an aging recipient model. These findings are clinically relevant since most of the recipients in the clinical setting may be elderly patients receiving their own autologous MSCs. Alternatively, it has been reported that MSCs were immune tolerant and MSC transplant from human donor to allogenic patients showed no evidence of rejection even without administration of immunosuppression [2]. The current study supports the role of universal donor cells, and with the use of younger MSC donors, we may optimize the full potential of cellular cardiomyoplasty.

Acknowledgments

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