Int Cardiovasc Res J.2022;**16**(3):103-110.icrj.129983

International Cardiovascular Research Journal
www.ircrj.com

The Effect of Human Cardiac Myocyte-Derived Conditioned Medium on Differentiation of Mesenchymal Stem Cells

Nadia Rousta [1], MSc; Sirous Naeimi [1], PhD; Shahrokh Zare [2], MD; Mahintaj Dara [2], MD; Iman Razeghian-Jahromi [3,*], PhD

[1] Department of Genetics, College of Science, Kazerun Branch, Islamic Azad University, Kazerun, IR Iran
[2] Stem Cells Technology Research Center, Shiraz University of Medical Sciences, Shiraz, IR Iran
[3] Cardiovascular Research Center, Shiraz University of Medical Sciences, Shiraz, IR Iran

ARTICLE INFO

Article Type:
Research Article

Article History:
Received: 16 Jul 2022
Revised: 17 Aug 2022
Accepted: 5 Sep 2022

Keywords:
Mesenchymal Stem Cells
Cardiac Myocytes
Differentiation
Conditioned Medium

ABSTRACT

Background: Mesenchymal stem cells (MSC) possess specific properties that make them good candidates for cell therapy, especially in organs like the heart with limited regeneration capacity. Generating cardiac myocytes in vitro is an important step toward restoring lost function in cardiovascular diseases.

Objective: In this study, we investigated the potential of a human cardiomyocytes-conditioned medium to induce cardiogenic differentiation (indicated by *GATA4* expression) of rat bone marrow MSCs.

Methods: Rat bone marrow MSCs were extracted and cultured. They were characterized based on morphology, adipogenic and cardiogenic differentiation potential, and expression of mesenchymal markers. MSCs were exposed to the conditioned medium (case group) or normal saline for 1, 3, and 7 days. The growth curve, population doubling time, and *GATA4* expression were compared between the groups.

Results: MSCs of the case group showed accelerated growth with a lower population doubling time. *GATA4* expression as a specific cardiac marker was significantly higher in the case group compared with the controls on day 1. By days 3 and 7, *GATA4* expression remained higher, albeit the difference with the control group became less.

Conclusions: Our findings demonstrated that possible cardiac myocyte-derived factors instigate the proliferation and cardiogenic differentiation of MSCs. This study provides a novel bone marrow MSC model for investigating early cardiomyocyte generation, which can open new avenues in the treatment of heart diseases.

1. Background

A key yet challenging goal of medicine is to restore injured cells and tissues to normal functional status (1). Myocardial infarction (MI), the main culprit of cardiovascular hospitalization and mortality, causes death in a localized area of cardiomyocytes, increasing the workload of the remaining cardiomyocytes and leading to tissue dysfunction. This situation elevates the risk of further infarctions and cardiac failure (2, 3). Unfortunately, the heart's capacity to replace dysfunctional cells with functional ones is inadequate, especially in major events like myocardial infarction (4). Moreover, conventional therapies only focus on relieving symptoms without altering necrotic tissues. All these limiting factors have become the driving forces to seek alternative remedies for regenerating injured cardiac tissues (2).

In this regard, cell-based therapeutics are interesting tools (5). In particular, outstanding characteristics of stem cells provide a unique opportunity for use in infarcted myocardium (6, 7). Both unfractionated bone marrow mononuclear cells and autologous bone marrow mesenchymal stem cells (MSCs) can significantly improve cardiac function (7). Myocyte-derived MSCs considerably reduced infarct size in transplantation experiments in animal models (9-11). Transplantation of the heart from female donors to male recipients showed footprints of Y-chromosome-positive cardiomyocytes. Findings showed that such cardiomyocytes result from cardiogenic differentiation of circulating bone marrow MSCs (12). All such evidence verify that host tissue microenvironment signals trigger MSCs to commence

*Corresponding author: Iman Razeghian-Jahromi Cardiovascular Research Center, 3rd floor, Mohammad Rasoolallah Research Tower, Nemazee Hospital, Shiraz, Iran. Tell: +98-7136122235,
Email: razejahromi@yahoo.com.

differentiation toward mature cells. In fact, the most efficacious factor in the cardiogenic differentiation of stem cells is assumed to be the paracrine activity received from the microenvironment (13, 14).

Although harboring very few MSCs (15), bone marrow is considered a suitable source for isolating these stem cells (16). The prominent properties of bone marrow MSCs, including ease of *ex vivo* expansion and low risk of rejection, make them promising candidates for heart regeneration (17). Also, they are immune-privileged cells that can be isolated from patients themselves or their family members (18). Cardiac markers are spontaneously expressed by human MSCs, showing an intrinsic propensity toward cardiac lineage (17). The multipotency and differentiation ability of MSCs into a variety of cell types, including cardiac myocytes, were previously reported. The functionality of differentiated cells is evidenced by the expression of certain markers like myosin heavy chain, β-actin, and troponin T (19).

The ability of MSCs to differentiate into cardiac cells is promising, but requirements for such transformation are not fully elucidated. It was revealed that *in vitro* cardiogenic differentiation requires appropriate factors. Different inducers like chemical substances (20-22), growth factors (23-25), and coculturing with primary cardiomyocytes (26) have been tested. However, some concerns, including toxicity, imperfect collection of required factors, and ethical hurdles, restrain such approaches.

The expression of an early cardiac gene such as *GATA4* indicates that cells have commenced the differentiation process. *GATA4* is fundamental in regulating endoderm and mesoderm development in the postgastrula embryo (27). In particular, this protein regulates the expression of critical genes during cardiomyogenesis. Cardiac differentiation is promoted in pluripotent or embryonic stem cells by induction of *GATA4* overexpression (28). Intriguingly, *GATA4* possesses self-regulation via suppression of its own expression (27).

2. Objectives

In this study, we aimed to investigate the potential of a human cardiomyocytes-conditioned medium to induce cardiogenic differentiation of rat bone marrow MSCs.

3. Materials and Methods

This study conformed to the Declaration of Helsinki and was approved by the institutional ethics committee. Three male adult rats of 200 - 250 g weight were obtained from the institutional animal house, and after deep anesthesia and cervical dislocation, bone marrow cells were extracted from the femurs and tibias of both sides. Centrifugation of cell suspension yielded cell pellet that was subsequently seeded into culture flasks (SPL, Cat. No.: 70075) containing Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) (Shellmax, Cat. No.: D6050; BIO-IDEA, Cat. No.: BI-1201). Following 24 h incubation in humidified 5% CO_2 incubator at 37 ˚C, floating cells were discarded, and adherent cells were nourished with fresh culture medium. After the cells reached 80% confluence, they were detached by trypsin/EDTA (Shellmax, P3790) treatment and were split into new flasks. Passaging of the cells was continued until reaching P3.

- Osteogenic and adipogenic differentiation of MSCs

To confirm the identity of MSCs at P3, they were subjected to adipogenic and osteogenic differentiation. Cells were seeded in 6-well plates (SPL, Cat. No.: 30006) in three groups: control, osteogenic differentiation, and adipogenic differentiation. Each group was exposed to a specific medium. The control medium consisted of DMEM/FBS, the osteogenic group medium consisted of low glucose DMEM/FBS/dexamethasone/ascorbate 2-phosphate/b-glycerophosphate, and the adipogenic medium was Ham's F-12/FBS/glutamine/dexamethasone/ascorbic acid/indomethacin (29). After 20 days of exposure, cells were washed with PBS, and following fixation, they were stained with alizarin red and oil red.

- Expression of mesenchymal-specific markers

In a T75 flask, cells at P3 were washed with PBS (Shellmax, Cat. No.: P1430), and following treatment with trypsin/EDTA, they were subjected to centrifugation to yield cell pellets. RNA was extracted from the pellet by RNAx-plus solution (Cinagen-Iran, Cat. No: EX6101) according to the instructions. RNA concentration was measured by Nanodrop (NanoDrop™ 2000/2000c Spectral photometer) at 260 nm wavelength to assure the quality of the extraction procedure and quantity of the RAN molecules for downstream steps. cDNA synthesis was carried out (AddScript cDNA Synthesis Kit, Adbio, Cat. No.: 22701) from the RNA template, before PCR reagents (Taq DNA Polymerase 2x Master Mix, Ampliqon, Cat. No.: A180301) were mixed with cDNA and specific mesenchymal primers (CD73 and CD90). After applying predefined cycles of time and temperature by 96-well thermal cycler (Applied Biosystems™ Veriti™), the PCR products were visualized with the aid of a DNA-safe stain (Cinaclone, Cat. No.: PR881603) on 1.5% agarose (Sigma-Aldrich, Cat. No.: 9012-36-6) gel. The primer sequences were CD73 (F: TGCATCGATATGGCCAGTCC, R: AATCCATCCCCACCGTTGAC) and CD90 (F: GACCCAGGACGGAGCTATTG, R: TCATGCTGGATGGGCAAGTT).

- Human cardiomyocyte (HCM)-conditioned medium

A confluent T25 culture flask (SPL, Cat. No.: 70025) of human cardiomyocytes was purchased from Pasteur Institute, Tehran, Iran. After storage in the incubator for 24 hours, cells were passaged with a specific HCM culture medium. After reaching 80% confluence, the culture medium was collected and regarded as the HCM-conditioned medium. This medium was air-dried at -56˚C under negative pressure (vacuum degree < 10 pas) by a lyophilizer for 48 hours (BIOBASE BK-FD10PT freeze dryer).

- MTT assay

In order to evaluate the viability of MSCs after exposure to the HCM-conditioned medium, the MTT assay was performed. In a 96-well plate (SPL, Cat. No.: 30096), MSCs (5,000 cells/200 µl/well) were seeded and exposed to different concentrations of dried conditioned medium from 1 ng/ml to 10×10^6 ng/ml at three time points (days 1, 3, and 7). The MTT assay was carried out according to the vendor protocol (https://www.sigmaaldrich.com/NL/en). Light absorption was measured at a wavelength of

570 nm on a microplate reader (FLUOstar Omega, BMG LABTECH, Germany).

- Exposing MSCs to HCM-conditioned medium

The P3 passage of MSCs was prepared in T25 flasks (400,000 cells/flask) in four groups. Groups 1, 2, and 3 contained 100 ng/ml of the conditioned medium and were evaluated after 1, 3, and 7 days, respectively. Group 4 was the control group with no HCM-conditioned medium. Also, similar groups were prepared in 24-well plates (SPL, Cat. No: 30024) for cell counting on seven consecutive days.

- *GATA4* expression

Following the treatment of cells with the conditioned medium, cells in both control and treatment groups were detached from the flasks on days 1, 3, and 7, as previously described. The cell pellets were subjected to RNA extraction, cDNA synthesis, and real-time PCR (RealQ Plus 2x Master Mix Green, Ampliqon, Cat. No.: A325402) in a 48-well thermal cycler (Applied Biosystems™, StepOne™) with *GATA4* specific marker. The thermal process started at 95 ˚C (15 min), followed by 95 ˚C (10 sec; 40 cycles) and 52˚C (15 sec). In the end, the PCR products went through the melt curve process: one cycle of 95 ˚C (15 sec.), then 52 ˚C (1 min), increased by 0.1˚ increments up to 95 ˚C afterward.

The β-actin gene was considered the endogenous control. Primer sequences were as follows: *GATA4* (F: TGATGGATGGAAGAAGAT, R: GTGATGAAGACAAGGAAG). Data were analyzed using the $2^{\hat{}-\Delta\Delta ct}$ method.

4. Results

In this study, bone marrows of three male adult rats were used to establish an MSC pool. In the first days of *in vitro* culture, cells appeared in different shapes: spindle, flat, star, and round. However, the majority of adherent cells were spindle-shaped. The round floating cells were discarded upon changing the culture medium. During P0, the cell density increased, some colonies formed, and gradually, cells covered nearly the entire surface of the culture flask. Treatment of the confluent flask with trypsin/EDTA and seeding the cells into new flasks made P1, and these procedures were continued up to P3. The proliferation of MSCs was accelerated at P1 and the following passages in a way that MSCs covered about 80-90% of the flask surface during only 3 - 4 days. At P3, almost all the cells acquired a spindle-shaped or fibroblastic appearance.

MSCs were characterized after induction of differentiation to osteocytes and adipocytes. Staining of the induced cells by specific dyes (alizarin red and oil red) confirmed such differentiation (Figure 1). In order to further confirm the identity of cells at P3, they were assessed with respect to the expression of mesenchymal-specific markers. Following treatment of cultured cells at P3 with trypsin/EDTA, centrifugation of the cell suspension produced the pellet and supernatant phases containing mesenchymal and hematopoietic cells, respectively. Both phases were subjected to RNA extraction, cDNA synthesis, and PCR using primers of mesenchymal-specific markers, CD73 and CD90. As shown in Figure 2, the cells in the pellet expressed CD73 (208 bp) and CD90 (197 bp), while the expression of these markers was absent in the supernatant.

During the first days of culture, HCMs mostly appeared round, some with short projections toward different directions. However, they became more fibroblast-like at P1, which is the usual appearance of these cells (Figure 3). They need a specific culture medium containing various ingredients including Ham's F12 Nutrient Mix, DMEM, FBS, insulin, and basic fibroblast growth factor. It is recommended to subculture the cells in poly-L-lysin-coated flasks.

HCM was passaged once, and after reaching confluence, the conditioned medium was collected and refrigerated at -76 ˚C until use.

Different lyophilized HCM-conditioned medium concentrations were exposed to MSCs at P3 at three time points (day 1, day 3, and day 7). The MTT assay was performed to evaluate the impact of the HCM-conditioned medium on the viability of MSCs during the 7-day interval. It was revealed that none of the concentrations had significant toxicity on MSCs. Eventually, a concentration of 100 ng/ml, at which MSCs demonstrated close viability to the control group, was chosen for upcoming experiments. In fact, this concentration imposed neither remarkable proliferation nor significant apoptosis.

The number of MSCs exposed to 100 ng/ml of the HCM-conditioned medium was enumerated for eight consecutive

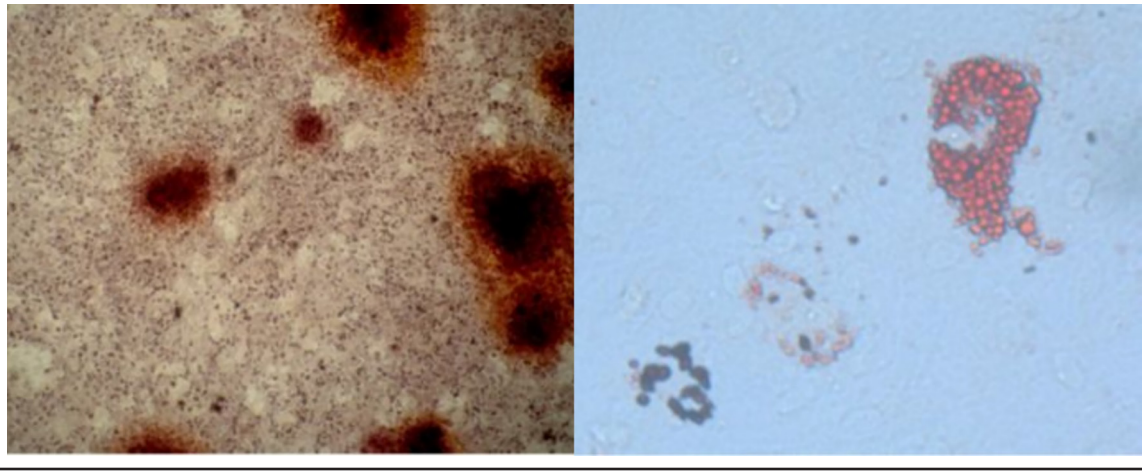

Figure 1. Induction of Osteogenesis and Adipogenesis in Mesenchymal Stem Cells. The Mesenchymal Nature of the Cultured Cells Was Approved by Differentiation Induction. Left: Red Appearance of the Oeteocytes' Cytoplasm Due to Staining with Alizarin Red Dye. Right: Red Appearance of the Adipocytes' Cytoplasm Due to Dtaining with Oil Red Dye.

days. Thereafter, growth curves and population doubling time (PDT) were compared between the treated and control groups, revealing that the trend of growth and proliferation was not similar between the two groups. MSCs in the control group started the proliferation phase after a lag period of three days, while MSCs in the treated group showed sharp proliferation after only one day, followed by mild proliferation. Initially, the HCM-conditioned medium significantly increased the proliferation of MSCs compared with the control group. However, this effect remained unchanged for the following days in the treated group (Figure 4).

Also, the PDT was calculated as 50 and 58 hours on day 8 for the treated and control groups, respectively (Table 1).

There was no considerable difference in the appearance of MSCs between the treated and control groups. However, the density of the cells, which represents the proliferation rate, was obviously higher in the treated group (Figure 5).

Expression of *GATA4* is assumed as a sign that reveals the commencing of cardiac differentiation. In fact, one of the earliest molecular markers associated with the initiation of cardiac gene expression is *GATA4* (29). *GATA4* expression was considerably higher in the treated group compared with the control peers after one day, while no remarkable differences were seen between the groups after three and seven days (Figure 6).

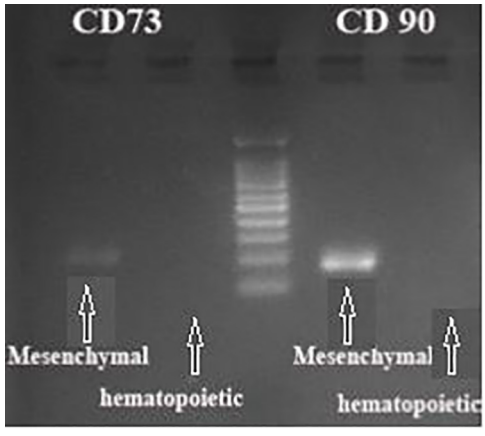

Figure 2. Expression of Mesenchymal-Specific Markers by Adherent Cells at P3. The Figure Shows that the Cell Pellet (Containing Mesenchymal Cells) Expressed CD73 (208 bp) and CD90 (197 bp), While the Supernatant (Containing Hematopoietic Cells) Did not Express These Markers.

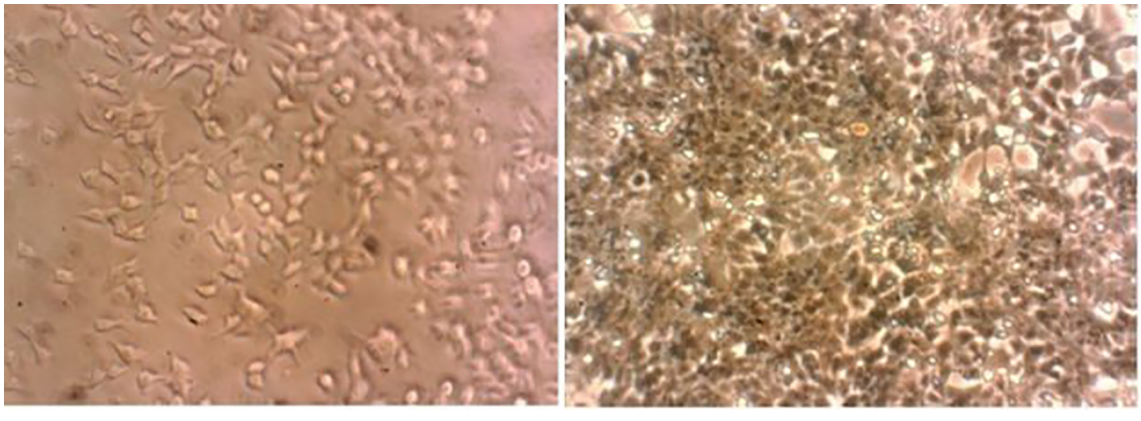

Figure 3. Human Cardiac Myocytes During the Early Days of Culture (Right) and at the First Passage (Left). They Mostly Appeared in Round Shapes on the First Days of Culture; Later, Fibroblast-Like Cells Became Dominant.

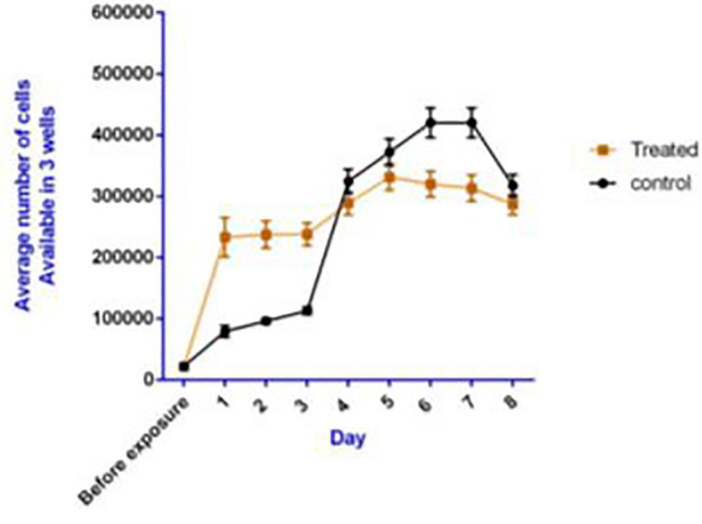

Figure 4. The Growth Curve of Mesenchymal Stem Cells in the Treatment Group Compared to the Control Group in Eight Consecutive Days. On the First Days, the Cell Count Was Significantly Higher in the Treatment Group, While Cells in the Control Group Outnumbered the Ones in the Treatment Group in the Following Days.

Table 1. Population Doubling Time in Treated and Control Groups

Days (Post-Exposure)	Treated Group (Hours)	Control Group (Hours)
1	7	13
2	14	22.5
3	21	30.5
4	28.5	24.5
5	34	29.5
6	42	34
7	47.5	39.5
8	58	50

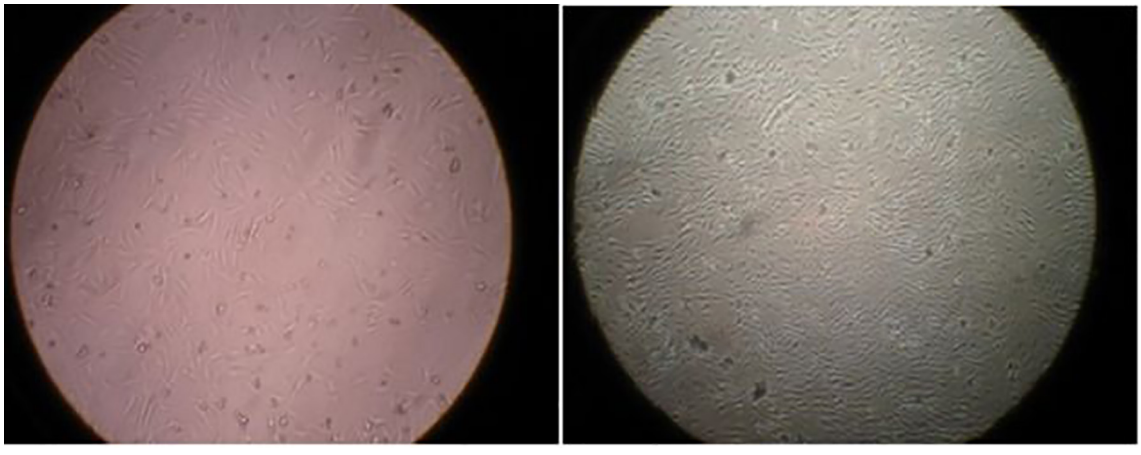

Figure 5. Mesenchymal Stem Cells in the Treatment Group (Right) and the Control Group (Left). The Density of Cells Was Significantly Higher in the Treatment Group due to the Existence of the Conditioned Medium Derived from Human Cardiac Myocytes.

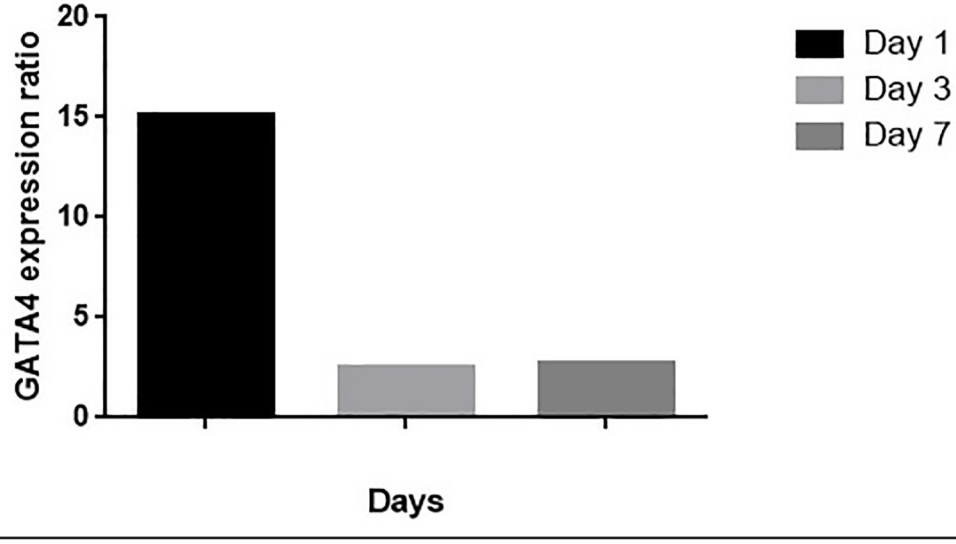

Figure 6. Comparison of *GATA4* Expression Ratio in the Treatment/Control Groups at Three Time Points. At Day 1, Expression of *GATA4* Was Remarkably Higher in the Cells Treated with Human Cardiac Myocyte-Conditioned Medium Compared with the Control Group. However, the Groups Had no Considerable Differences at Days 3 and 7.

5. Discussion

The present study evaluated cardiogenic differentiation of rat bone marrow MSCs after exposure to a defined concentration of human HCM-conditioned medium. To begin with, three rats were used for the extraction of bone marrow MSCs in order to omit the biological differences between donors and to establish a homogenous cell pool. The spindle- or fibroblastic-shaped cells at P3 demonstrated some features, including plastic adherence, expression of specific mesenchymal markers, and ability to differentiate into mesodermal cell lineage. Accordingly, these cells clearly met the minimal criteria for defining MSCs. Following exposure to the HCM-conditioned medium, MSCs growth was considerably augmented up to day three compared with the controls. In fact, the HCM-derived microenvironment promoted the proliferation of MSCs. However, it seems that induction of proliferation was lost in the next days, and that is why the cells in the control group overtook those in the treated group during days 4 to 8. This was marked by higher PDT by day three and lower PDT by day eight in the treated cells compared with the controls. Regarding cardiogenic differentiation, treated

cells expressed a remarkably higher level of *GATA4* than the control group after one day. After 3 and 7 days, the level of *GATA4* expression remained higher in the treated cells, but the difference with the control group became low.

The major challenge for restoration of the lost function in the cardiac apparatus is that the heart possesses little regeneration potential after injuries. Also, lack of cytokinesis in cardiac myocytes and unwanted cell death following induction of cell cycle activation hamper the regeneration potential. After major cell loss (e.g., a myocardial infarction), the heart's inherent response is compensatory hypertrophy. This condition, accompanied by increasing myocytes' size, ultimately leads to heart failure (31). Therefore, researchers seek therapies that restore the lost function by increasing the number of myocytes rather than their size. The interesting promise of cellular therapy, especially the formation of functional cardiac myocytes, has been elaborated as an alternative remedy for cardiac disease (32). In this regard, understanding the contributors involved in the process of precursor differentiation into cardiac cells is an important step.

Adult stem cells may be assumed as an intermediate cell type that, as progenitor precedents, have no prominent physiological role and, simultaneously, do not show a fully functional character (33). As one type of adult stem cells, MSCs have shown differentiation potential into cardiomyocytes (15, 34), and bone marrow MSCs generate a highly pure population with superb growth kinetics (2).

The fate of a progenitor/stem cell is mainly determined by its microenvironment (35). Some researchers declared that differentiation of bone marrow MSCs into myocytes needs contact with neighboring myocytes, and direct cell-to-cell contact was assumed essential in this process (16). Early expression of connexin 43, a gap junction protein, prior to transdifferentiation of bone marrow MSCs into myocytes supports this belief (36). This connection mediates intercellular signaling, resulting in the expression of differentiation genes like *GATA4* (16).

On the other hand, the differentiation of rat bone marrow MSCs into cardiomyocyte-like cells is feasible in a cardiac environment independent of physical contact with myocytes (14). Injection of bone marrow MSCs into the infarcted heart showed great cardiac recovery in an animal model. Injected cells reach a mature phenotype in less than two weeks and last in the injured site for approximately two months (37, 38). Seemingly, myocyte-secreted factors are in charge of bone marrow MSCs differentiation toward the myocardial phenotype (39, 40). However, the exact molecular mechanisms that control the cardiac differentiation of MSCs are not fully understood.

The differentiation of transplanted MSCs to cardiomyocytes showed that the microenvironment of adult heart tissue provides sufficient signals for cardiogenic transformation (41). Soluble factors, besides growth factors, are continuously released by cardiomyocytes, creating a suitable microenvironment for cardiac differentiation (42). A microenvironment generated by embryonic cardiomyocytes was shown to activate the cardiac genetic program in MSCs. It was demonstrated in one study that the expression of cardiac genes reached the highest level after five days of co-culture, and was downregulated continuously afterward (43). cAMP, neuregulin-1, and bFGF are among the signaling molecules involved in myocardial cell fate. These three are also implicated in the induction of the expression of cardiac transcription factors like *GATA4* in embryonic stem cells (23, 44, 45).

Cardiogenic differentiation is approved by the expression of cardiac markers, both at the protein and mRNA levels (2). *GATA4*, as a cardiomyocyte transcription factor, is expressed during the transdifferentiation of bone marrow MSCs (16). MSCs co-cultured with myocytes highly expressed *GATA4* and acquired a myocyte phenotype (16). Cardiac transcription factors such as *GATA4* and *MEF-2* regulate the gene expression of cardiac-specific genes, controlling the complex process of cardiogenesis. In particular, the regulation of cardiac structure and gene expression is governed by *GATA4*. Expression of *GATA4* activates transcription of further key myocardial structural genes required for the formation of sarcomeric α-actinin, cardiac MHC, and troponin I (42). Also, *GATA4* significantly contributes to heart development and cardiac specification (18). The lack of a clear and consensus definition for cardiogenic differentiation with respect to the expression of specific markers is one of the underlying reasons for controversies in different studies.

Various induction agents are used to differentiate MSCs to cardiac lineage. Chemicals such as 5-azacytidine induce the cardiogenic differentiation of MSCs (22, 46, 47). However, the potential carcinogenic hazard of such chemicals hinders the clinical translation of differentiated cells. Cocktails of growth factors have also been used to differentiate MSCs into cardiomyocytes (48). Not only is one growth factor incapable of developing cardiomyocytes, but the exact combination of growth factors required for this action is not fully elucidated (2). Accordingly, in the present study, we tried to provide a microenvironment with a cardiac-conditioned medium of human cardiac myocytes similar to that of natural ones. Cardiac myocytes are fully differentiated cells that are unable to divide (49).

Another feature of our study was the stimulation of MSCs from one species by a microenvironment derived from another species. This shows that the microenvironment of human cardiac myocytes contains at least a minimum of factors to promote rat MSCs toward cardiac lineage, which demonstrates a conservative induction repertoire between these two species. Co-culturing of human bone marrow MSCs and rat neonatal cardiomyocytes led to xenogenic transdifferentiation, indicated by the expression of *GATA4*. However, sarcomeric structures were not formed. Even the co-culturing of MSCs with embryonic cardiomyocytes for 15 days did not reveal electrical transdifferentiation toward the cardiomyocyte phenotype. This was called partial differentiation, which happened because of altered and incomplete genetic programming (43). On the other hand, the formation of organized sarcomeres and the generation of action potentials indicate true differentiation (50, 51).

5.1. Limitations

We did not refresh the conditioned medium every day, and the differentiation potential of the conditioned medium

might decrease over time. Characterization of myocytes relied only on the expression of *GATA4*. More in-depth characterizations are essential. It is possible to concentrate the conditioned media to further investigate its effect on the cardiac differentiation of MSCs. The true identity and regenerative potential of such in vitro-generated cells remain to be ascertained in future experiments. Different MSC sources, such as bone marrow versus umbilical cord blood, do not necessarily yield the same results. Also, the development stage of the MSC donor is a determining factor for their multipotency potential.

5.2. Conclusion

The conditioned medium of human cardiac myocytes promotes cardiogenic differentiation of rat bone marrow MSCs. This study provides a novel bone marrow MSC model for investigating early cardiomyocyte generation that could be used for designing new avenues in the treatment of heart diseases in the future.

5.3. Ethical Considerations

This study is approved by regional ethics committee (IR.IAU.KAU.REC.1400.054).

Acknowledgements

There is no acknowledgement.

Authors' Contribution

IRJ contributed substantially to the design and concept of the study. NR, SZ, and MD had roles in data acquisition. SN had a role in the analysis and interpretation of data. IRJ wrote the initial draft. All the authors reviewed the manuscript critically and approved it.

Funding/Support

This study was funded by Azad University, Kazerun branch [grant number: 25961].

Financial Disclosure

The authors confirm that there are no conflicts of interest. The funder was not involved in the manuscript writing, editing, approval, or decision to publish.

References

1. Boyle AJ, Schulman SP, Hare JM, Oettgen PJC. Is stem cell therapy ready for patients? Stem cell therapy for cardiac repair. Ready for the next step. 2006;**114**(4):339-52.
2. Hafez P, Jose S, Chowdhury SR, Ng MH, Ruszymah B, Abdul Rahman Mohd RJCbi. Cardiomyogenic differentiation of human sternal bone marrow mesenchymal stem cells using a combination of basic fibroblast growth factor and hydrocortisone. 2016;**40**(1):55-64.
3. Razeghian-Jahromi I, Karimi Akhormeh A, Razmkhah M, Zibaeenezhad MJJIJoI, Pharmacology. Immune system and atherosclerosis: Hostile or friendly relationship. 2022;**36**:03946320221092188.
4. Hatzistergos KE, Quevedo H, Oskouei BN, Hu Q, Feigenbaum GS, Margitich IS*, et al.* Bone marrow mesenchymal stem cells stimulate cardiac stem cell proliferation and differentiation. 2010;**107**(7):913-22.
5. Behfar A, Terzic AJJom, cardiology c. Optimizing adult mesenchymal stem cells for heart repair. 2007;**42**(2):283-4.
6. Dimmeler S, Zeiher AM, Schneider MDJTJoci. Unchain my heart: the scientific foundations of cardiac repair. 2005;**115**(3):572-83.
7. Murry CE, Field LJ, Menasché PJC. Cell-based cardiac repair: reflections at the 10-year point. 2005;**112**(20):3174-83.
8. Laflamme MA, Murry CEJNb. Regenerating the heart. 2005;**23**(7):845-56.
9. Kudo M, Wang Y, Wani MA, Xu M, Ayub A, Ashraf MJJom*, et al.* Implantation of bone marrow stem cells reduces the infarction and fibrosis in ischemic mouse heart. 2003;**35**(9):1113-9.
10. Orlic D, Kajstura J, Chimenti S, Limana F, Jakoniuk I, Quaini F*, et al.* Mobilized bone marrow cells repair the infarcted heart, improving function and survival. 2001;**98**(18):10344-9.
11. Kocher A, Schuster M, Szabolcs M, Takuma S, Burkhoff D, Wang J*, et al.* Neovascularization of ischemic myocardium by human bone-marrow–derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. 2001;**7**(4):430-6.
12. Deb A, Wang S, Skelding KA, Miller D, Simper D, Caplice NMJC. Bone marrow–derived cardiomyocytes are present in adult human heart: a study of gender-mismatched bone marrow transplantation patients. 2003;**107**(9):1247-9.
13. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR*, et al.* Pluripotency of mesenchymal stem cells derived from adult marrow. 2002;**418**(6893):41-9.
14. Li X, Yu X, Lin Q, Deng C, Shan Z, Yang M*, et al.* Bone marrow mesenchymal stem cells differentiate into functional cardiac phenotypes by cardiac microenvironment. 2007;**42**(2):295-303.
15. Gnecchi M, Cervio EJMsct. Mesenchymal stem cell therapy for heart disease. 2013:241-70.
16. Xu M, Wani M, Dai Y-S, Wang J, Yan M, Ayub A*, et al.* Differentiation of bone marrow stromal cells into the cardiac phenotype requires intercellular communication with myocytes. 2004;**110**(17):2658-65.
17. Gallo MP, Ramella R, Alloatti G, Penna C, Pagliaro P, Marcantoni A*, et al.* Limited plasticity of mesenchymal stem cells cocultured with adult cardiomyocytes. 2007;**100**(1):86-99.
18. Leong YY, Ng WH, Umar Fuaad MZ, Ng CT, Ramasamy R, Lim V*, et al.* Mesenchymal stem cells facilitate cardiac differentiation in Sox2-expressing cardiac C-kit cells in coculture. 2019;**120**(6):9104-16.
19. Rangappa S, Entwistle JW, Wechsler AS, Kresh JYJTJot, surgery c. Cardiomyocyte-mediated contact programs human mesenchymal stem cells to express cardiogenic phenotype. 2003;**126**(1):124-32.
20. Banfi A, Muraglia A, Dozin B, Mastrogiacomo M, Cancedda R, Quarto RJEh. Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: Implications for their use in cell therapy. 2000;**28**(6):707-15.
21. Antonitsis P, Ioannidou-Papagiannaki E, Kaidoglou A, Papakonstantinou CJIc, surgery t. In vitro cardiomyogenic differentiation of adult human bone marrow mesenchymal stem cells. The role of 5-azacytidine. 2007;**6**(5):593-7.
22. Hsu C-P, Wang J-S, Hung S-C, Lai S-T, Weng Z-C, Chiu RC-JJACS. Human sternal mesenchymal stem cells: isolation, characterization and cardiomyogenic differentiation. 2010;**26**(4):242-52.
23. Khezri S, Valojerdi MR, Sepehri H, Baharvand HJSmj. Effect of basic fibroblast growth factor on cardiomyocyte differentiation from mouse embryonic stem cells. 2007;**28**(2):181.
24. Hahn J-Y, Cho H-J, Kang H-J, Kim T-S, Kim M-H, Chung J-H*, et al.* Pre-treatment of mesenchymal stem cells with a combination of growth factors enhances gap junction formation, cytoprotective effect on cardiomyocytes, and therapeutic efficacy for myocardial infarction. 2008;**51**(9):933-43.
25. Franco D, Lozano-Velasco E, Aránega A. Transcriptional Networks of Embryonic Stem Cell-Derived Cardiomyogenesis. *Embryonic Stem Cells-Differentiation and Pluripotent Alternatives*: IntechOpen; 2011.
26. Labovsky V, Hofer E, Feldman L, Vallone VF, Rivello HG, Bayes-Genis A*, et al.* Cardiomyogenic differentiation of human bone marrow mesenchymal cells: Role of cardiac extract from neonatal rat cardiomyocytes. 2010;**79**(2):93-101.
27. Rossi JM, Dunn NR, Hogan BL, Zaret KSJG, development. Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. 2001;**15**(15):1998-2009.
28. Yilbas AE, Hamilton A, Wang Y, Mach H, Lacroix N, Davis DR*, et al.* Activation of GATA4 gene expression at the early stage of cardiac specification. 2014;**2**:12.
29. Mehrabani D, Rabiee M, Tamadon A, Zare S, Razeghian Jahromi I, Dianatpour M*, et al.* The growth kinetic, differentiation properties, karyotyping, and characterization of adipose tissue-derived stem cells in hamster. 2016;**25**(5):1017-22.

30. Välimäki MJ, Ruskoaho HJJIl. Targeting GATA4 for cardiac repair. 2020;**72**(1):68-79.
31. Ahuja P, Sdek P, MacLellan WRJPr. Cardiac myocyte cell cycle control in development, disease, and regeneration. 2007;**87**(2):521-44.
32. Rogers TB, Pati S, Gaa S, Riley D, Khakoo AY, Patel S*, et al.* Mesenchymal stem cells stimulate protective genetic reprogramming of injured cardiac ventricular myocytes. 2011;**50**(2):346-56.
33. Belema Bedada F, Technau A, Ebelt H, Schulze M, Braun TJM, biology c. Activation of myogenic differentiation pathways in adult bone marrow-derived stem cells. 2005;**25**(21):9509-19.
34. Qayyum AA, Haack-Sørensen M, Mathiasen AB, Jørgensen E, Ekblond A, Kastrup JJRm. Adipose-derived mesenchymal stromal cells for chronic myocardial ischemia (MyStromalCell Trial): study design. 2012;**7**(3):421-8.
35. Fuchs E, Tumbar T, Guasch GJC. Socializing with the neighbors: stem cells and their niche. 2004;**116**(6):769-78.
36. Chedrawy EG, Wang J-S, Nguyen DM, Shum-Tim D, Chiu RCJTJot, surgery c. Incorporation and integration of implanted myogenic and stem cells into native myocardial fibers: anatomic basis for functional improvements. 2002;**124**(3):584-90.
37. Beltrami AP, Urbanek K, Kajstura J, Yan S-M, Finato N, Bussani R*, et al.* Evidence that human cardiac myocytes divide after myocardial infarction. 2001;**344**(23):1750-7.
38. Hosoda T, D'Amario D, Cabral-Da-Silva MC, Zheng H, Padin-Iruegas ME, Ogorek B*, et al.* Clonality of mouse and human cardiomyogenesis in vivo. 2009;**106**(40):17169-74.
39. Chen J, Wang C, Lü S, Wu J, Guo X, Duan C*, et al.* In vivo chondrogenesis of adult bone-marrow-derived autologous mesenchymal stem cells. 2005;**319**(3):429-38.
40. Li H, Yu B, Zhang Y, Pan Z, Xu W, Li HJB*, et al.* Jagged1 protein enhances the differentiation of mesenchymal stem cells into cardiomyocytes. 2006;**341**(2):320-5.
41. Vincentelli A, Wautot F, Juthier F, Fouquet O, Corseaux D, Marechaux S*, et al.* In vivo autologous recellularization of a tissue-engineered heart valve: are bone marrow mesenchymal stem cells the best candidates? 2007;**134**(2):424-32.
42. Huang YS, Li IH, Chueh SH, Hueng DY, Tai MC, Liang CM*, et al.* Mesenchymal stem cells from rat olfactory bulbs can differentiate into cells with cardiomyocyte characteristics. 2015;**9**(12):E191-E201.
43. Rose RA, Jiang H, Wang X, Helke S, Tsoporis JN, Gong N*, et al.* Bone marrow-derived mesenchymal stromal cells express cardiac-specific markers, retain the stromal phenotype, and do not become functional cardiomyocytes in vitro. 2008;**26**(11):2884-92.
44. Chen Y, Shao JZ, Xiang LX, Guo J, Zhou QJ, Yao X*, et al.* Cyclic adenosine 3′, 5′-monophosphate induces differentiation of mouse embryonic stem cells into cardiomyocytes. 2006;**30**(4):301-7.
45. Liu X, Gu X, Li Z, Li X, Li H, Chang J*, et al.* Neuregulin-1/erbB-activation improves cardiac function and survival in models of ischemic, dilated, and viral cardiomyopathy. 2006;**48**(7):1438-47.
46. Antonitsis P, Ioannidou-Papagiannaki E, Kaidoglou A, Charokopos N, Kalogeridis A, Kouzi-Koliakou K*, et al.* Cardiomyogenic potential of human adult bone marrow mesenchymal stem cells in vitro. 2008;**56**(02):77-82.
47. HassanHeidari M, Noruzian M, Namjo Z, Frahani R, Ebrahimi A, Bandepour M*, et al.* Human mesenchymal stem cells differentiation to skeletal muscle cells in vitro study. 2013;**3**(8):156-64.
48. Behfar A, Yamada S, Crespo-Diaz R, Nesbitt JJ, Rowe LA, Perez-Terzic C*, et al.* Guided cardiopoiesis enhances therapeutic benefit of bone marrow human mesenchymal stem cells in chronic myocardial infarction. 2010;**56**(9):721-34.
49. Peter AK, Bjerke MA, Leinwand LAJMbotc. Biology of the cardiac myocyte in heart disease. 2016;**27**(14):2149-60.
50. Nishiyama N, Miyoshi S, Hida N, Uyama T, Okamoto K, Ikegami Y*, et al.* The significant cardiomyogenic potential of human umbilical cord blood-derived mesenchymal stem cells in vitro. 2007;**25**(8):2017-24.
51. Pijnappels DA, Schalij MJ, Ramkisoensing AA, van Tuyn J, de Vries AA, van der Laarse A*, et al.* Forced alignment of mesenchymal stem cells undergoing cardiomyogenic differentiation affects functional integration with cardiomyocyte cultures. 2008;**103**(2):167-76.