Original Article

Cardiogenic Differentiation of Murine Bone Marrow-Derived Mesenchymal Stem Cells by 5-Azacytidine: A Follow-up *In vitro* Study

Heba E. M. Sharaf Eldin, Marwa A. A. Ibrahim, Amany M. I. Mousa, Hala G. Metwaly[1], Nadia F. E. Abo-Hassan

Department of Histology and Cell Biology, Faculty of Medicine, Tanta University, Tanta, [1]Department of Clinical Pathology, Faculty of Medicine, Cairo University, Cairo, Egypt

Abstract

Background: Cell-based therapy is a promising tool in the management of myocardial infarction. **Aim of the Work:** The aim of this study is to examine the *in vitro* potential differentiation of murine bone marrow (BM)-derived stem cells into cardiomyocytes using 5-azacytidine after 1, 3, and 5 weeks and follow it up after 8 weeks. **Materials and Methods:** BM-derived mesenchymal stem cells (MSCs) were extracted from the bones of adult albino rats. MSCs were induced with 10 µM 5-azacytidine for 24 h. The cells were examined after 1, 3, 5, and 8 weeks. Cell characterization with immunocytochemistry for detection of CD105, desmin, and T-troponin and transmission electron microscopy was performed. **Results:** The 5-azacytidine-induced MSCs showed light and electron microscopic histological characteristics resembling cardiomyocytes and progressively expressed the cardiac muscle-specific markers over the 1st, 3rd, and 5th weeks, yet by the 8th week, these parameters were significantly downregulated. **Conclusion:** Prolonged survival of 5-azacytidine-induced MSCs in culture beyond the 8th week resulted in loss of the newly acquired cardiomyocyte characteristics. It is not recommended to prolong the maintenance of 5-azacytidine-induced MSCs in culture on the hope of increasing its cardiogenic potentiality beyond 5 weeks.

Keywords: 5-Azacytidine, cardiogenic differentiation, *in vitro*, mesenchymal stem cells

Introduction

Ischemic heart disease is a leading cause of death in developed countries. Myocardial infarction (MI) is caused by the obstruction of coronary arteries leading to the degeneration of cardiomyocytes which do not regenerate, as they are withdrawn from cell cycle soon after birth and are no longer able to proliferate. Cardiomyocytes can only undergo hypertrophy but not hyperplasia; therefore, the heart has a limited capacity for self-renewal resulting in a depressed left ventricular function and heart failure.[1,2]

Stem cell therapy represents a potentially effective tool for the restoration of contractile function after MI. In comparison with the current therapies that focus on the restoration of the blood flow, stem cells not only could improve neovascularization but also might replace the lost cardiomyocytes, since they have the capacity to differentiate into cardiomyocytes.[3]

Regenerative medicine applies the basic stem cell knowledge to develop specific cells or tissue to replace the original cells or tissue that has been degenerated, injured, or damaged by different processes. This is the basic concept of the promising cell- and tissue-based therapy that could potentially cure many chronic diseases, such as MI, insulin-dependent diabetes mellitus, and other conditions.[4]

Stem cells are undifferentiated resting cells waiting for a division signal to start proliferation. They are found in embryos and the later stages of animal and human life.[5] The differentiation process of the stem cells usually goes through several stages which acquire specialization at each step. The differentiation process involves two types of signals: internal

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Address for correspondence: Prof. Marwa A. A. Ibrahim,
Department of Histology and Cell Biology,
Faculty of Medicine, Tanta University, Tanta, Egypt.
E-mail: marwa.ibrahim@med.tanta.edu.eg

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signals that are controlled by the cell's genes and the external signals that include chemicals secreted by other cells, physical contact with neighboring cells, and certain molecules in the microenvironment.[6]

Mesenchymal stem cells (MSCs) represent a rare population of cells in bone marrow (BM) – about 0.001%–0.01% of the nucleated cells. It was reported that the greatest number of MSCs is found in neonates and then reduced during the lifespan to about the half. However, MSCs may not be detected in circulating blood, but it is suggested that all tissues have MSC reservoirs localized in the perivascular niche.[7,8] MSCs have been studied in both basic cardiovascular research and preclinical studies. They have several attractive characteristics including the easy culture, an obtainable high number needed for transplantation, an apparent potential for mediating both myocardial and vascular repair, and immunoregulatory properties, which may enable their use as an allogeneic treatment.[9]

Cultured MSCs secrete various bioactive molecules which have antiapoptotic, immunomodulatory, angiogenic, chemoattractant, and antiscarring properties, enabling them to create a local regenerative environment *in vivo,*[10,11] thus representing a promising tool for the restoration of contractile function of the heart after MI.[3]

5-Azacytidine is a potent inducer of cardiomyogenic differentiation in both embryonic and adult stem cells. The demethylating activity of 5-azacytidine may play a nonspecific role in upregulating the differentiation-promoting factors of stem cells. Moreover, 5-azacytidine can hypomethylate a myogenic-determinant locus resulting in the transcriptional activation of this locus and thereby allowing the induction of myogenic differentiation. However, the mechanism by which 5-azacytidine promotes cardiomyogenic differentiation remains to be clarified.[12-15]

In the current work, we hypothesized that prolonged survival of MSCs in culture with the aim of introducing them *in vivo* afterward could improve their differentiation potentiality. Therefore, this work aimed to examine the *in vitro* potential differentiation of murine BM-derived stem cells into cardiomyocytes using 5-azacytidine after 1, 3, and 5 weeks and follow up that differentiation after 8 weeks.

Materials and Methods

Isolation and culture of rat mesenchymal stem cells

MSCs were obtained from the BM of the femurs and tibias of 60 adult male albino rats, each weighing 150–200 g, according to Zhang and Chan.[16] Briefly, both ends of the femur and tibia were cut with sharp scissors. The BM was flushed out of the bones using complete culture medium composed of Dulbecco's Modified Eagle Medium (DMEM) (B12-604F, Lonza, Switzerland) containing 10% fetal bovine serum (10270-106, Gibco, Invitrogen, USA) and 1% penicillin/streptomycin (17-602E, Lonza, Switzerland). The flushed BM was centrifuged at 1200 rpm for 10 min at 20°C. The cell pellets were resuspended with complete DMEM and seeded into 75 cm^2 cell culture flasks (690170, Greiner Bio-One, Germany) and incubated at 37°C in a 5% CO_2 humidified incubator.

The cultured cells were examined daily under a phase-contrast microscope (Axiovert 200M, Zeiss, Germany) to check for adherence. Culture medium was first changed after 3–4 days to remove the nonadherent cells and then every 2–3 days. Cells were subcultured using trypsin/EDTA (CC-5012, Lonza, Switzerland) giving Passage 1 cells (P_1), which were again subcultured into Passage 2 (P_2) until becoming 70%–80% confluent.

Cardiogenic differentiation of rat mesenchymal stem cells *in vitro*

MSCs of Passage 2 (P_2) were divided into two main groups: control group (I) and 5-azacytidine-induced group (II). MSCs from Group II (at a frequency of 1×10^5 cells) were incubated with a freshly prepared 10 μM 5-azacytidine (A1287, Sigma-Aldrich, USA) at 37°C in a 5% CO_2 humidified incubator for 24 h.[17] Thereafter, the induced cells were washed twice with a phosphate-buffered saline (PBS), and fresh complete culture medium was then added and changed every 2–3 days. Groups I and II were further divided into four subgroups: a, b, c, and d, where the cells were examined under phase-contrast microscope for morphological characterization after 1, 3, 5, and 8 weeks, respectively. Cells were then processed for immunocytochemical and transmission electron microscopy characterization.

Immunocytochemical staining

The cultured cell pellets were thinly spread over positively charged slides, left to air-dry, and fixed in ice-cold ethanol 100% for 10 min. The cells were washed twice with PBS, incubated with 3% H_2O_2 in methanol for 10 min, and then washed twice with PBS. The cells were incubated overnight with the primary antibodies (rabbit polyclonal anti-CD105, cat# RB-9291-P; mouse monoclonal anti-desmin, cat# MS-376-S0; or mouse monoclonal anti-T-troponin; cat# MS-295-P0, Thermo Fisher Scientific, MA, USA). After washing with PBS, the cells were incubated with biotinylated goat anti-polyvalent IgG secondary antibody (TP-060-HL, Thermo Fisher Scientific, MA, USA) for 30 min. The slides were then incubated with streptavidin-peroxidase for another 30 min. Antigen–antibody reaction was visualized by applying freshly prepared diaminobenzidine as a chromogen. Finally, immunostained cells were counterstained with Mayer's hematoxylin, dehydrated in ascending grades of ethanol then xylol, and then mounted.[3] The positive reaction appeared as a brownish coloration of the cytoplasm.

Transmission electron microscopy

The cell pellets were washed twice with PBS and prefixed with 2.5% glutaraldehyde in 0.1 ml phosphate buffer (pH 7.4) for 2 h at room temperature and then washed with 1 ml of phosphate buffer. This was followed by a postfixation with 1% osmium tetroxide for 1 h and then washed with distilled water. The cells were then dehydrated in ascending grades of

alcohol and cleared in acetone. Infiltration was then done using equal volumes of acetone and epoxy resin overnight. Finally, the cells were embedded in gelatin capsules filled with freshly prepared epoxy resin and kept in oven at 70°C for 24 h to allow polymerization. Semithin sections (1 μm) were cut on an ultramicrotome (Leica Microsystems, Austria) and stained with 1% toluidine blue for evaluation by light microscope. Sections were re-embedded for further ultrathin sectioning (50–70 nm) on an ultramicrotome and picked up on copper grids. The grids were then stained with 2% uranyl acetate for 10 min, followed by lead citrate for another 10 min. The grids were then rapidly washed with distilled water to remove excess stain.[18] Images were captured using a Jeol JEM-100 TEM (Jeol, Tokyo, Japan) at the Electron Microscopic Unit, Faculty of Medicine, Tanta University.

Morphometric analysis

An Olympus light microscope (BX53, Tokyo, Japan) coupled to an Olympus digital camera (E-420, Tokyo, Japan) was used for image acquisition and the software "ImageJ" (version 1.48v, National Institute of Health, Bethesda, Maryland, USA) was used for image analysis. Ten nonoverlapping fields from each slide were examined at a magnification of 400-fold.

To quantitatively evaluate CD105-, desmin-, or troponin -immunostained cells, the number of positive immunostained cells was counted and expressed as a percentage out of the total cells counted ([number of labeled cells/total cell number] ×100).

Statistical analysis

The data were analyzed using one-way analysis of variance, followed by Tukey's test as a postcomparison test between the groups. All values were reported as mean ± standard deviation. Differences were regarded as significant if probability value $P < 0.05$ and highly significant if $P < 0.001$.[19]

Results

Morphological characterization with phase-contrast microscopy

On the 1st day of the primary culture of BM-MSCs, Passage 0 (P0) revealed rounded, crowded, and floating cells, while 3–4 days later, most of the cells were adherent in the form of spindle and triangular cells with processes, yet few cells appeared rounded [Figure 1a]. Six to seven days from the primary culture, the MSCs reached 50%–60% confluency. The cells appeared spindle, triangular, and star shaped with many cytoplasmic processes and eccentric vesicular nuclei, in addition to some rounded nonadherent cells [Figure 1b]. Seven to nine days from the primary culture, the MSCs reached about 70%–80% confluency. Most of them were spindle in shape with multiple long processes and vesicular nuclei with prominent nucleoli [Figure 1c]. MSCs of P_2 showed the same morphology, and most of the cells were positive for CD105 (89.32% ± 1.02%) in the form of a brown cytoplasmic coloration [Figure 1d].

Examination of control MSCs of P_2 after 1 week (subgroup Ia) depicted their characteristic spindle-shaped cells with well-developed interdigitating cytoplasmic processes, granular cytoplasm, and eccentric vesicular nuclei [Figure 2a]. Both subgroups Ib and Ic examined after 3 and 5 weeks, respectively, showed spindle-shaped cells along with broad flattened cells, and some of them were aggregated forming colonies [Figure 2b]. Subgroup Id examined after 8 weeks showed that MSCs were mainly large and flattened in shape [Figure 2c].

Examination of cells treated with 5-azacytidine after 1 week (subgroup IIa) showed that the cells appeared larger in size compared to its corresponding control group. They possessed multiple cytoplasmic processes having central and vesicular nuclei with prominent nucleoli. Some cells had two central nuclei and others appeared elongated with cytoplasmic processes joining the processes of adjacent cells [Figure 2d]. After 3 weeks of intervention (subgroup IIb), some cells were assembled together in clusters or ball-like structures, while other cells were elongated and appeared stick-like [Figure 2e]. In addition, some large cells showed prominent cytoplasmic striations, and disc-like structures began to appear between adjacent cells [Figure 2f]. Moreover, examination of the cells of subgroup IIc showed that most of them attained an elongated appearance and connected with each other forming myotube-like structures with string-bead-like nuclei [Figure 2g]. Most of the cultured cells showed extensive cytoplasmic striations [Figure 2h]. On the other hand, by the

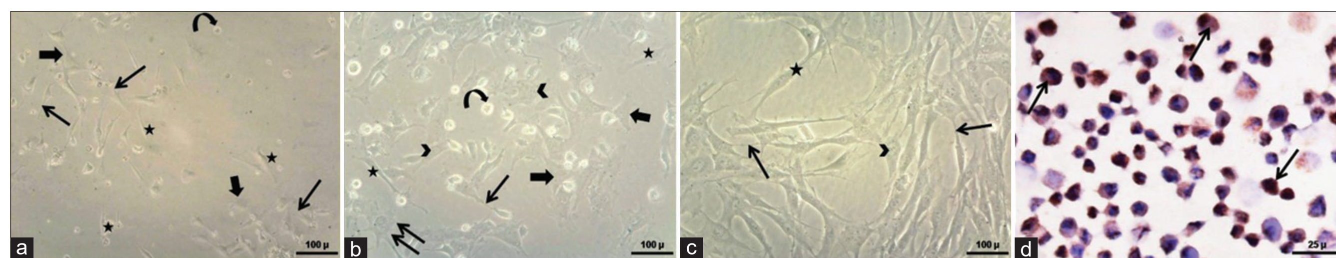

Figure 1: Phase-contrast microscopy of the rat bone marrow mesenchymal stem cells primary culture: (a) 3 days: Most cells are adherent, spindle (stars) or triangular (thick arrows) with processes (thin arrows), some rounded refractile cells (curved arrow). (b) 7th day: Cells are larger with vesicular nuclei (arrow heads), star in shape (double arrows). (c) 9th day: Spindle cells (star) with well-developed interdigitating cytoplasmic processes (thin arrows), granular cytoplasm and eccentric vesicular nuclei (arrow head). (d) CD105 immunostaining: Most mesenchymal stem cells are positive for CD105 (thin arrows)

end of the 8th week (subgroup IId), the cells became smaller in size compared to the previous subgroup. They became spindle in shape with central vesicular nuclei and no cytoplasmic striations compared to the previous subgroups [Figure 2i].

Immunocytochemical, morphometrical, and statistical analysis

MSCs of the control group were negative for the immunocytochemical staining for detection of both desmin and cardiac troponin-T by the end of the 1st, 3rd, 5th, and 8th weeks [Figure 3a]. Whereas, subgroup IIa revealed that a few induced cells were positive for desmin (10.65 ± 0.21) [Figure 3b] while some cells were positive for cardiac troponin-T (16.46 ± 2.84) [Figure 3c]. After 3 weeks (subgroup IIb), some of the induced cells were positive for both desmin (31.81 ± 1.7) [Figure 3d] and cardiac troponin-T (34.96 ± 1.77) [Figure 3e] with a significant increase compared to subgroup IIa. Moreover, subgroup IIc showed a significant increase in the cells positive for both desmin (34.64 ± 2.57) [Figure 3f] and cardiac troponin-T (40.11 ± 2.52) [Figure 3g] compared to both subgroups IIa and IIb. On the other hand, subgroup IId showed a significant decrease in the positive immune reaction for both desmin (24.32 ± 3.8) [Figure 3h] and cardiac troponin-T (30.19 ± 4.93) [Figure 3i] compared to subgroups IIb and IIc yet with a significant increase compared to subgroup IIa [Table 1].

Morphological identification with transmission electron microscopy

Toluidine blue-stained semithin sections of both primary culture MSCs and the control group at any time point showed rounded cells with many pseudopodia and large, oval, and eccentric euchromatic nuclei [Figure 4a].

Semithin sections from subgroup IIa showed apparently large cells with few pseudopodia. Some cells had central or eccentric nuclei [Figure 4b]. Some induced cells of subgroup IIb were large with scanty pseudopodia. Moreover, some cells were

Table 1: Mean percentage of immunostained positive cells of the different study groups

Antibodies	Control	Subgroup			
		IIa	IIb	IIc	IId
Desmin	Negative	10.65±0.21^{b,c,d}	31.81±1.7^{a,c,d}	34.64±2.57^{a,b,d}	24.32±3.8^{a,b,c}
Troponin-T	Negative	16.46±2.84^{b,c,d}	34.96±1.77^{a,c,d}	40.11±2.52^{a,b,d}	30.19±4.93^{a,b,c}

Data are expressed as mean±SD. Superscript letters denote significant differences between subgroups. SD: Standard deviation

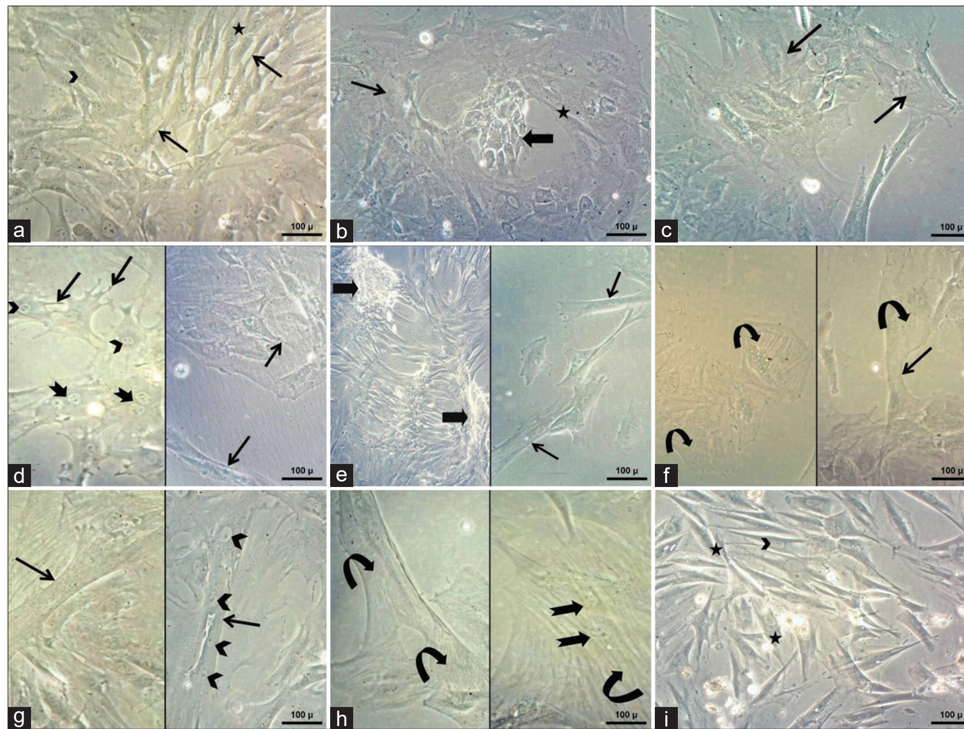

Figure 2: Phase-contrast microscopy: (a) Subgroup Ia: Spindle cells (star) with processes (thin arrows) and vesicular nuclei (arrow head). (b) Ib and Ic: Spindle cells (star), flattened cells (thin arrow) and cell colonies (thick arrow). (c) Id: Flattened cells (thin arrows). (d) IIa: Large cells with processes (thin arrows) and nucleoli (arrow head). Binucleated cells (notched arrows). (e and f) IIb; Cells clusters (thick arrows), stick-like cells (thin arrows), striations (curved arrows) and disc-like structures (thin arrow). (g) IIc: Myotube-like cells (thin arrows) with string-bead nuclei (arrow heads). (h) IIc: Striations (curved arrows), binucleated cells (notched arrows). (i) IId: Spindle cells (stars) with vesicular nuclei (arrow head)

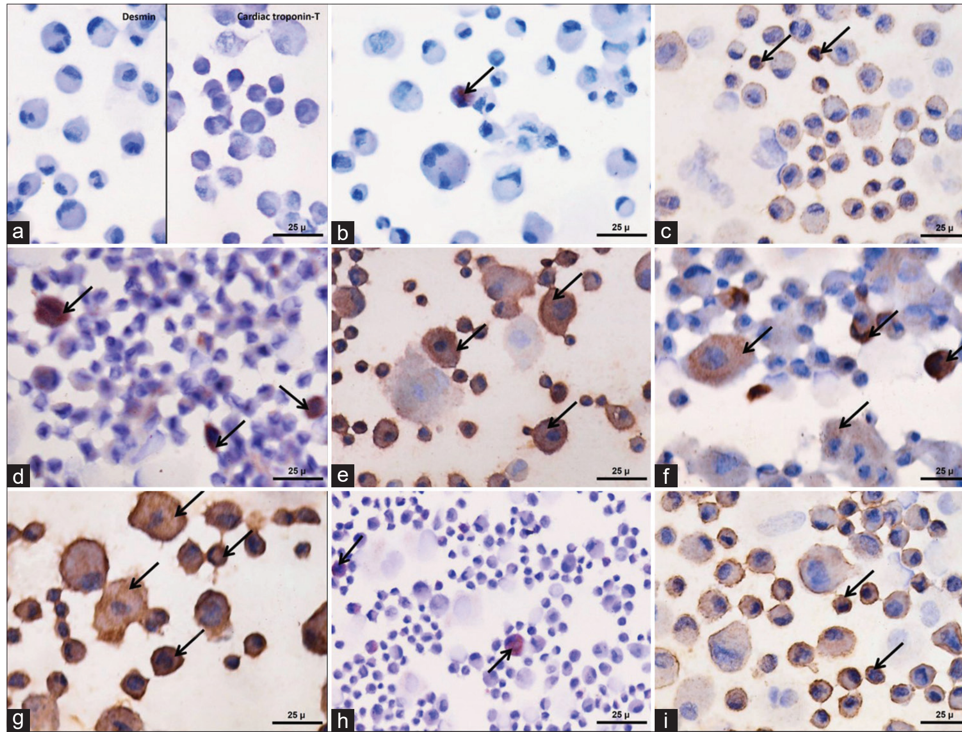

Figure 3: Immunocytochemical analysis: (a) Control mesenchymal stem cells are negative for both desmin and cardiac troponin-T. (b and c) Subgroup IIa: Few desmin-positive cells and some cardiac troponin-T-positive cells (thin arrows), respectively. (d and e) Subgroup IIb: Some desmin-positive cells and many cardiac troponin-T-positive cells (thin arrows), respectively. (f and g) Subgroup IIc: Increase in the number of desmin- & cardiac troponin-T-positive cells (thin arrows), respectively. (h and i) Subgroup IId: Few desmin- & cardiac troponin-T-positive cells (thin arrows), respectively.

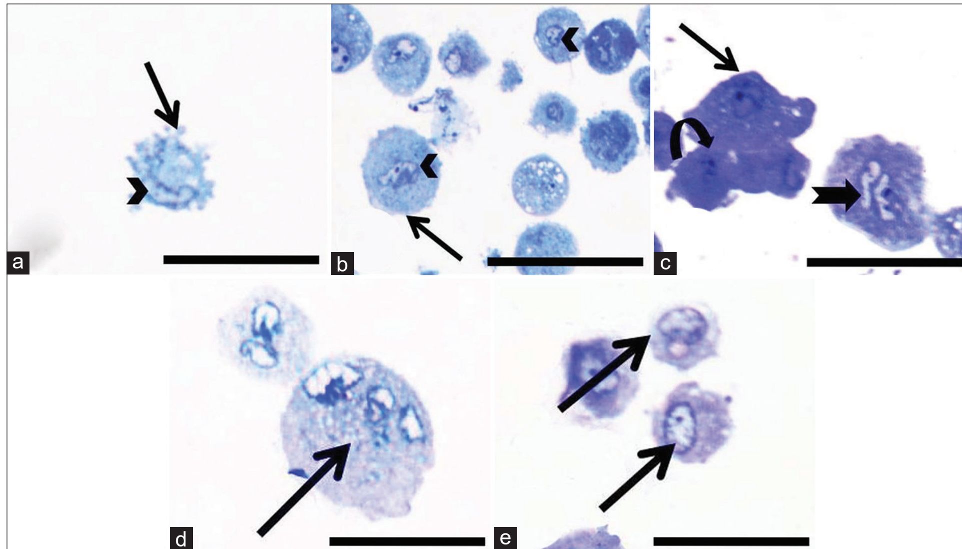

Figure 4: Toluidine blue staining: (a) Primary culture mesenchymal stem cells and control group; rounded cells with pseudopodia (thin arrow) and eccentric euchromatic nucleus (arrow head). (b) Subgroup IIa: Large cells with few pseudopodia (thin arrow), cells with central nuclei (arrow heads). (c) Subgroup IIb: Large cells with scanty pseudopodia (thin arrow), some attached cells (curved arrow) and binucleated cells (notched arrow). (d) Subgroup IIc: Multinucleated cells (thin arrow). (e) Subgroup IId: Small cells with vesicular nuclei (thin arrows). (Scale bar=25 micron)

attached to each other [Figure 4c]. Cells from subgroup IIc were large and multinucleated [Figure 4d], whereas subgroup IId cells were small with vesicular nuclei [Figure 4e].

Ultrathin sections of primary culture MSCs showed rounded cells with pseudopodia all around. They had large eccentric nuclei with euchromatin and prominent nucleoli. The cytoplasm of the cells was granular, rich in free ribosomes, many rounded to oval mitochondria and rough endoplasmic reticulum (rER) [Figures 5a and b]. Control subgroup Ia was almost similar to the primary culture MSCs [Figure 5c]. Subgroup Ib showed some binucleated

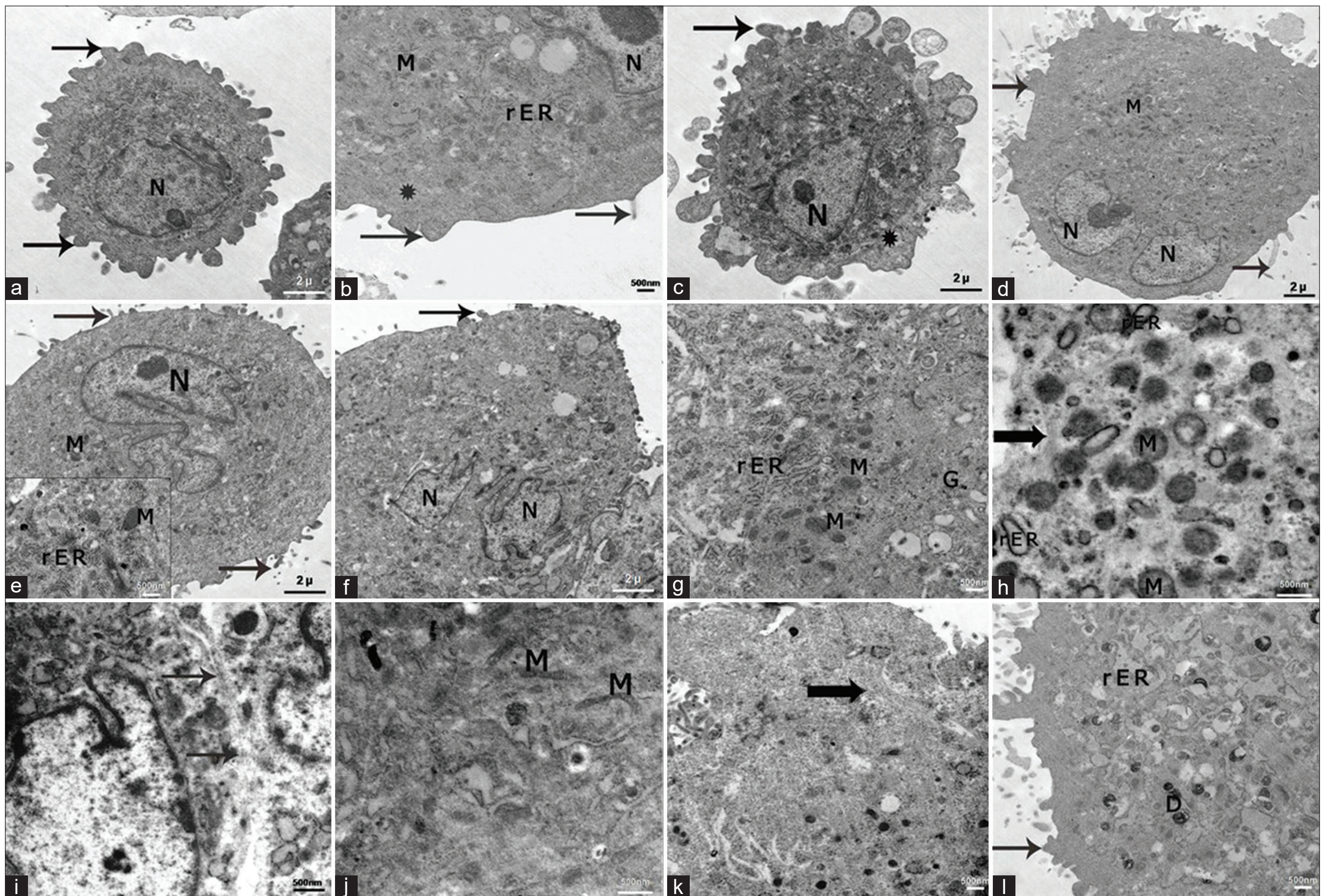

Figure 5: Transmission Electron Microscopy: (a and b) Mesenchymal stem cells: Pseudopodia (thin arrows), nucleoli (N), granular cytoplasm (asterisk), mitochondria (M), rER (rER). (c) Subgroup Ia: Nucleus (N), granular cytoplasm (asterisk), pseudopodia (thin arrow). (d) Ib: Two nuclei (N), mitochondria (M), pseudopodia (thin arrow). (e) Ic and Id: Nucleus (N), rough endoplasmic reticulum (rER), mitochondria (M), pseudopodia (thin arrows). (f) IIa: Few pseudopodia (thin arrow), two central nuclei (N). (g-i) IIb: Dilated cisternae of rough endoplasmic reticulum (rER), mitochondria (M), glycogen granules (G). Myofilaments (thick arrow), desmosome-like structures (thin arrows). (j and k) IIc: Mitochondria (M), myofilaments (thick arrow). (l) IId: Electron-dense material (D), rough endoplasmic reticulum (rER), pseudopodia (thin arrow)

cells with euchromatic and peripheral nuclei [Figure 5d]. Both subgroups Ic and Id showed larger cells compared to the previous two control subgroups. The cells had peripheral irregular-shaped nuclei, several cisternae of rER, and mitochondria [Figure 5e].

Ultrathin sections from subgroup IIa revealed large cells with few pseudopodia, and some cells showed two central nuclei [Figure 5f]. Cells from subgroup IIb showed several dilated cisternae of rER, large and elongated mitochondria, and numerous glycogen granules [Figure 5g]. Myofilaments could be detected in the cytoplasm of some cells [Figure 5h]. Plasma membranes of adjoining cells were very closely approximated at several points with the appearance of desmosome-like structures [Figure 5i]. In subgroup IIc, the induced cells showed large and elongated mitochondria with closely packed long cristae [Figure 5j]. Some cells expressed numerous bundles of myofilaments without forming typical sarcomeres [Figure 5k]. On the other hand, cells of subgroup IId expressed many electron-dense materials and dilated rER. In addition, cell pseudopodia could be observed [Figure 5l].

Discussion

Cell-based strategies have emerged over the past decade as a potential therapy for MI. The role of cardiac stem cells in MI repair appears minimal and functionally inadequate.[20] It is now accepted that BM-MSCs are a promising therapy for MI as they exhibit low immunogenicity with the ability to proliferate and differentiate into cardiomyocytes.[21,22]

In the current study, phase-contrast microscopic characterization of cultured cells of control subgroup Ia revealed spindle-shaped cells, which was consistent with a previous study.[23] Whereas, subgroup IIa showed large cells with multiple processes as was similarly reported by other scientists.[24] On the other hand, subgroup IIb showed that some cells were assembled in clusters, while others were elongated and stick-like. These findings agreed with a previous work.[25] Moreover, some cells of this group depicted cytoplasmic striations and disc-like structures which could be attributed to the expression of cardiac troponin and connexin proteins.[26] In addition, cells of subgroup IIc appeared elongated forming myotube-like

structures with string-bead-like nuclei which could be due to the fusion of more primitive myoblasts. On the other hand, cells of subgroup IId regressed in size with no cytoplasmic striations in contrary to other researchers who reported that the cultured cells retained the cardiogenic phenotype until the 8th week.[27]

In this work, immunocytochemical characterization of MSCs was performed by the end of the 1st, 3rd, 5th, and 8th weeks of intervention by 5-azacytidine to detect the cardiac muscle-specific markers of myogenic differentiation: desmin and cardiac troponin-T. It was reported that both desmin and cardiac troponin-T are important components of muscle tissue that play an essential role in contraction of muscle cells. Troponin-T is particularly important for an effective cardiomyocyte's contraction, as it regulates the force and velocity of myocardial contraction.[28]

In the current study, cells of the control subgroups were negative for both desmin and cardiac troponin-T; this was in accordance with previous studies which declared that noninduced MSCs were negative for both desmin and cardiac troponin-T.[25,29] By the end of 1st week (subgroup IIa), few cells were positive for desmin, which agreed with the work of others.[3] Some cells were positive for cardiac troponin-T, which was also consistent with other researchers who detected positive cells for cardiac troponin-T as early as day 5 after BM-MSCs were induced.[30] On the contrary, another research reported that the expression of cardiac troponin-T was detected only after 3 weeks of induction of rat BM-MSCs by 5-azacytidine.[31] By the end of the 3rd week (subgroup IIb), there was a statistically significant increase in the number of desmin-positive cells as was similarly reported by previous studies.[3,31,32] Moreover, there was a significant increase in the number of cardiac troponin-T-positive cells, which was consistent with the finding of another study.[3] By the end of the 5th week (subgroup IIc), there was a significant increase in the number of both desmin- and cardiac troponin-T-positive cells, which agreed with the work of other researchers.[33] The expression of these specific markers may be associated with activation of the myogenic gene MyoD.[34] On the other hand, by the end of the 8th week from the day of induction (subgroup IId), both desmin- and cardiac troponin-T-positive cells were significantly decreased.

These immunocytochemical findings confirmed the phase-contrast microscopy observations of the current work. These results could be explained by the report of other researchers who suggested that the addition of exogenous growth factors or cytokines as co-inducers might be required in combination with 5-azacytidine to promote differentiation of MSCs into cardiomyocyte-like cells.[15] In this work, it was found that almost 30% of the cells were transformed into cardiomyocyte-like cells, which coincided with the work of others.[27] Moreover, another study reported that about 30% of BM-MSCs induced by cardiotrophin-1 were positive for α-cardiac actin and cardiac troponin-T.[35]

Toluidine blue-stained semithin sections from the present work showed that the cultured MSCs revealed no difference from cells of the control subgroups throughout the experiment. However, by the end of 1st week, cells appeared large with few pseudopodia. Whereas, by the end of 3rd week, the cells were attached to each other, and by the 5th week, large multinucleated cells were observed. These findings agreed with previous works.[31,36] However, by the 8th week, the cells were observed to decrease in size.

In this research, electron microscopic examination revealed rounded MSCs with pseudopodia, euchromatic nuclei, and cytoplasm rich in free ribosomes, mitochondria, and rER. These results confirmed the previous researches.[23,37] The presence of many small pseudopodia was attributed to their capacity for migration within tissues.[38] Cells of control subgroup Ia were similar to MSCs, while in subgroup Ib, binucleated cells were observed. These findings coincided with previous researches.[39,40] Whereas, in both subgroups Ic and Id, cells became large with irregular-shaped nuclei, several cisternae of rER, and elongated mitochondria. These observations agreed as well with other scientists.[26,36]

By the end of the 1st week of induction (subgroup IIa), the induced cells appeared large with few pseudopodia with one or two nuclei. These observations were in accordance to those found by other researchers.[31] By the end of the 3rd week (subgroup IIb), the cells revealed dilated rER, numerous glycogen granules, and some large elongated mitochondria. These findings were consistent with those obtained by another work.[41] Researchers attributed the presence of mitochondria and numerous glycogen granules in the induced cells to the increasing need for energy production for the contractile activity of cardiomyocytes.[42] They also related the protein synthesis, by the abundant free ribosomes and dilated rER, to the demand of cardiomyogenic cells to form myofilaments. Furthermore, in the present work, myofilaments were detected in the cytoplasm of some cells of subgroup IIb. This was consistent with the observations of a previous study which reported that 5-azacytidine-treated cells expressed abundant parallel myofilaments aligned in the long axis of cells but did not exhibit the characteristic sarcomeric organization.[43] The presence of cytoplasmic myofilaments might be due to the synthesis of cytoskeleton proteins involved in cell proliferation and differentiation through signaling pathways.[38,39] In addition, in subgroup IIb, the plasma membranes of adjoining cells revealed desmosome-like structures as was similarly detected in previous researches.[42,43] By the end of the 5th week (subgroup IIc), the induced cells contained large elongated mitochondria with closely packed long cristae, which agreed with a previous study.[44] Many cells expressed numerous bundles of myofilaments without forming typical sarcomeres. This observation confirmed the findings of other researchers who reported numerous cytoplasmic myofilaments aligned in a parallel fashion yet without forming typical striated sarcomeres after 4 weeks from induction of human BM-MSCs with 5-azacytidine.[37]

On the other hand, by the end of the 8th week from the day of induction (subgroup IId), the cells showed many

electron-dense materials and dilated rER, but no cytoplasmic myofilaments were observed. These findings agreed with an earlier study.[17] Yet, it came in contrast to the findings of other scientists who reported the appearance of more organized myofilaments within the cytoplasm of the cells by the 8th week.[36] It was proposed that after demethylation of MSCs with 5-azacytidine, they undergo a commitment to differentiate into cardiomyocytes. First, MSCs enter a transient state of rapid proliferation leading to their exhaustion and withdrawal from the cell cycle and terminally differentiate into cardiomyocytes with a subsequent switching off of the telomerase gene.[27] Moreover, it was suggested that the treatment of embryonic stem cells by 5-azacytidine could increase the expression of transforming growth factor-β and bone morphogenetic protein (BMP-2 and BMP-4) resulting in cardiomyogenic differentiation.[45] On the other hand, it was reported that 5-azacytidine may promote cell proliferation and viability instead of their differentiation into cardiomyocytes and that the mechanism of action of 5-azacytidine is nonspecific as it induces osteogenesis, adipogenesis, and chondrogenesis.[46]

Conclusion

Taken altogether, it could be concluded from the present study that MSCs derived from the BM of adult albino rat could be potentially induced by 5-azacytidine to differentiate into cardiomyocyte-like cells *in vitro*. The induced cells showed morphological characteristics resembling cardiomyocytes and expressed the cardiac muscle-specific markers. Nevertheless, their prolonged survival in culture beyond the 8th week did not result in an improved differentiation yet resulted in a loss of the newly acquired cardiomyocyte characteristics. Therefore, it is not recommended to prolong the maintenance of 5-azacytidine-induced MSCs in culture on the hope of increasing their cardiogenic potentiality beyond 5 weeks.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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