Differentiation of Bone Marrow-derived Mesenchymal Stem Cells into Cardiomyocytes Using Different Regimens of 5- Azacytidine

Original Article

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ABSTRACT

Background: Conventional therapeutic approaches for ischemic heart disease have limited benefit in preventing post-ischemic heart failure. Therefore, stem cell therapy emerges as a hopeful tool for treatment of ischemic heart disease following myocardial infarction.

Aim: To standardize a protocol ensuring the best differentiation of bone marrow mesenchymal stem cells (BM-MSCs) into cardiomyocytes by using 5-azacytidine with minimum toxicity.

Materials and Methods: BM-MSCs were isolated from the bone marrow of albino rats, characterized by flow cytometry and then divided into five groups: Group I: control group, group II: exposed to differentiating medium containing 10 μmol/L of 5-azacytidine for 24 h, group III: exposed to the differentiating medium for 72 h, group IV: the differentiating medium was renewed twice weekly, group V: daily renewal of the differentiating medium was done. The experiment was terminated after 3 weeks from the induction time. Morphological changes of the cultured cells were examined under a phase contrast microscope. The effect of different duration of 5- aza exposure on BM-MSCs viability was evaluated by cell counting Kit 8 assay. Expression of cardiac troponin I and connexin 43 was measured using Quantitative reverse transcription-PCR. Transmission electron microscope was used to observe ultrastructural features of the induced cells. Morphometric study for comparing the length of the formed myofilaments in the four treated groups was done.

Results: The obtained results from groups II and III showed initial limited signs of differentiation towards cardiac lineage. Groups IV and V showed optimal signs of differentiation with better results regarding group V, yet, this was at the expense of the viability of the cultured cells.

Conclusion: The use of 5-aza showed a valuable differentiating effect on MSCs towards cardiomyocytes. Prolonged use resulted in promotion of the differentiation. Yet, it was at the expense of the viability of these cells.

INTRODUCTION

Ischemic heart diseases have been increasing year by year and considered the principal cause of cardiovascular burden. Globally,17.5 million people die each year from cardiovascular diseases[1]. Compensation for myocytes loss is very difficult as cardiomyocytes are static cells, having a limited proliferative capacity, and only 1% of these cells can proliferate[2].

Following insult to the myocardium, remodeling of the left ventricle results in a decrease in myocardial function and efficiency. Formation of myocardial scar tissue that replaces the necrotic myocardium is the fundamental driving force of this process that often progresses into congestive heart failure (HF)[3].

HF remains a deadly disease, despite advances in therapy that merely serve as transient "delayers" of an inevitably progressive disease process that carries a significant risk of morbidity and mortality[4].

Heart transplantation is currently the only therapeutic tool for a failing heart. However, due to the ever-present shortage of donor organs and high cost, it is important to search for alternatives[5].

Recently, stem cell-based myocardial regenerative therapy has become a promising beneficial alternative for dealing with ischemic myocardial diseases[6] Mesenchymal stem cells (MSCs) are particularly suitable for cell replacement therapy. They are multipotent, low immunogenic, easily isolated and purified with high expansion potential. Therefore, MSCs are the most popularly used in researches nowadays[7].

Azacytidine (Aza; 5-Aza), a DNA demethylation agent, is an analogue of cytidine. The US Food and Drug Administration approved the drug for the treatment of myelodysplastic syndrome, chronic myelomonocytic leukemia, and acute myeloid leukemia[8]. Moreover, many studies have shown that 5- aza can induce stem cells to

differentiate into cardiomyocytes, but some issues still remain to be settled such as the cytotoxicity of that drug[9,10].

Therefore, the present work was designed in a trial to establish an optimum protocol for in vitro cardiomyocyte differentiation using azacytidine starting from bone marrow-derived mesenchymal stem cells (BM-MSCs).

MATERIALS AND METHODS

This study was carried out on ten male albino rats, as a source of stem cells, weighing 20 -25 grams and aged about 2 weeks. The animals were purchased from the Animal House Center, Faculty of Medicine, Alexandria University. The whole experiment was done at Center of excellence for research in regenerative medicine and its applications (CERRMA).

Isolation and culture of BM-MSCs

The animals were sacrificed under general anesthesia. The MSCs were collected from the femoral and tibial bone marrow of 2-weeks-old albino rats. The isolated cells were cultured in Low Glucose Dulbecco's Modified Eagle's Medium (L-DMEM Sigma, USA), supplemented with 10% fetal bovine serum (Sigma), 1% penicillin/ streptomycin (Lonza) and 1% l-glutamine (Lonza). The cells were incubated in a CO_2 incubator at 37 º C in a humidified atmosphere with 5% CO_2 and 95% O_2. Daily follow-up of cultured cells was done using a phase contrast inverted microscope (Olympus Bx41). The medium was changed every 3 days and the cells were subcultured to increase purity when the BM-MSCs had reached about 80% confluency[11,12].

Characterization of MSCs by flow cytometry

MSCs at the third passage were characterized for CD90 as one of the surface markers known to be associated with MSCs (positive marker), in addition to CD34, a hematopoietic surface marker that is expected to be absent (negative marker). Cells were detached with 0.25% trypsin-EDTA solution, washed with phosphate buffer saline (PBS), and incubated at room temperature for 30 minutes in the dark, with monoclonal phycoerythrin(PE)-conjugated antibody for CD34 (Abcam, UK) and monoclonal fluorescein allophycocyanin (APC)-conjugated antibody for CD90 (Abcam, UK). The cells were subsequently washed three times with PBS and suspended in 500 µL FACS buffer[13].

Material for induction of differentiation into cardiomyocytes

- 5-azacytidine, 100 mg, purchased from Sigma -Aldrich (St Louis, MO, USA) and stored at -20°C.

- Cell Counting Kit 8 (CCK-8) assay

This assay was obtained from Dojindo molecular technologies inc, CA, USA for the determination of cell viability and cytotoxicity assays. It is supplied as a one-bottle solution.

Experimental design

BM-MSCs were divided into five main experimental groups:

Group I: control group, received 5- aza-free complete culture medium (CCM).

Group II: MSCs received CCM supplemented with 5-aza for 24 hours, followed by substitution with 5-aza-free CCM. Changing the medium was done every three days till the end of the experiment.

Group III: MSCs received CCM supplemented with 5-aza daily for 72 hours, then aza- free CCM medium was added. Changing the medium was done every three days till the end of the experiment.

Group VI: received CCM supplemented with 5-aza) with every medium change, twice weekly, till the end of experiment (continuous exposure).

Group V: received CCM containing 5- aza daily for 3 weeks (continuous exposure) till the end of the experiment.

The concentration of 5-aza was standard in all the treated groups which was 10 µmol/L.(10) The whole experiment was terminated after 3 weeks from the induction time[14].

Cytotoxicity assay using CKK8

The metabolic activity was determined on the basis of tetrazolium (WST-8) production. An orange colored product (formazan) is produced due to reduction of tetrazolium by cellular dehydrogenases. This product is soluble in the tissue culture medium. The color is then read with a microplate reader. The number of the living cells is proportional to the amount of generated formazan dye[15,16].

a. In the 96 well plate (12 columns and 8 rows), columns (2-6) were seeded with BM-MSCs. Cell suspensions were seeded as 5000 cells in a volume of 100 / µL well and incubated in 5% CO_2 at 37 °C.

b. On day 1 post- seeding, the wells were monitored by the inverted phase contrast microscope to check the confluency.

c. Column 1 was considered the blank column (media only) in all trials. This was performed in order to subtract the effect of the color of the culture medium (phenol red color) from the final reading.

d. Column 2: the wells were considered as a control group in all trials and were not loaded by the drug (only media and cells).

e. The rest of the columns were loaded with the drug for different duration, the same as previously mentioned groups.

f. At the end of the experiment, CCK-8 reagent was applied as 10% of the total volume of media to all the wells and incubated for 4 h in 5% CO_2, at 37 °C.

g. After 4 h incubation, the color absorbance was read at 450 nm using a Stat Fax 2100 microplate reader. All values were corrected with the reference wavelength at 630 nm and normalized against the mean value of blank wells (containing only media).

Characterization of differentiating cells

A- Morphological changes of the differentiating cells

After applying the protocol of differentiation, daily observation of the cultured cells was done regarding its viability and shape.

B- Real-time RT-PCR-based detection of cardiac troponin I (cTnI) and connexin 43 (Cx43) expression at the end of the experiment:[17-19]

Total RNA, including mRNA, was extracted from BM-MSCs samples using Gene JET RNA Purification Kit and following the manufacturer's protocol (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Reverse transcription was next performed in 20 μL reaction volume using100 ng of total RNA and high capacity cDNA reverse transcription Kit (Invitrogen, USA). The real-time PCR measurement of cTnI and Cx43 cDNA was performed using the StepOne PCR system (Applied Biosystems, USA).

Amplification of the synthesized cDNAs was performed in duplicates in a 25 μL reaction volume containing 2X Maxima SYBR Green qPCR Master Mix (Applied Biosystems, USA).

The specific primer pair for cTnI was the sense primer TGAAGTTTTCTGGAGGCGGAG and the antisense primer TCTCTACCTCTGGAGATCAGCATGG (Gen Bank accession number NM_017144.2).

As for Cx43, specific primer pair was the sense primer CAGAACAAGTCTGCACGA and the antisense primer TCTAACTTGGAGCGCAGA (Gen Bank accession number AH003191.2).

The amplification consisted of one cycle at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 sec, a 60°C annealing step for 30 sec, and an extension step at 72°C for 30 sec.

PCR amplification was followed by a melting curve analysis where the identity of the PCR product was confirmed. Furthermore, to confirm the specificity of the reaction, a negative control was included with each PCR run.

Data were analyzed by comparative CT method for gene expression using Step One™ Software v2.3. Results were normalized to the housekeeping gene glyceraldehyde 3 phosphate dehydrogenase (GAPDH) and were expressed as fold change from the control group.

C- Ultrastructural characterization of the induced cells:

At the end of the experiment, the cells in each group were harvested by trypsinization, centrifuged at 1200 rpm for 5 minutes, the resulting pellet from each group was fixed in 3% glutaraldehyde, post-fixed in 1% osmium tetroxide, embedded in epoxy resin, and double-stained with uranyl acetate and lead citrate[20,21]. The cellular ultrastructure was observed using a Jeol 100 CX transmission electron microscope at the Electron Microscopy Unit, Faculty of Science, University of Alexandria.

Morphometric study

The electron micrographs were obtained. The length of the formed myofilaments in the four treated groups was measured. For each group, five randomly selected electron micrographs were analyzed using the NIH Fiji© program photos[22,23].

Statistical analysis

All numerical data were shown as mean±SD. Differences were considered statistically significant at $p < 0.05$ calculated by the ANOVA and the Student's t-test.

RESULTS

Isolation, propagation, and characterization of MSCs

A. Microscopic appearance of BM-MSCs using the phase contrast inverted microscopy: MSCs were isolated from the bone marrow of albino rats by direct plating depending on their adherence property. The initially seeded cells were floating rounded cells of variable sizes. Seventy two hours later, the cells adhered to the culture plate and some cells started to extend short processes to acquire a spindle shape resembling fibroblasts, (Figure 1a). These cells began to proliferate at about day 7 and gradually grew to form small colonies, (Figure 1b) to reach 80% confluency on day 10. Subsequent passages resulted in rapid proliferation of the cultured MSCs, (Figure 2a-d), a: primary culture (p 0), b: p 1, c: p 2, d: p 3.

B. Immunophenotyping of BM-MSCs by flow cytometry: The flow cytometric analysis of cell-surface markers of BM-MSCs at p3 using antibody against MSC marker (CD90-APC) and another antibody against hematopoietic antigen (CD34-PE), showed that 87.50% of cells were positive for anti CD90, while only 0.14 % of cells were positive for anti CD34, (Figure 3).

5-aza cytotoxicity assay results

At the end of the experimental period, in group I which received 5-aza-free CCM and group II which received CCM supplemented with 10μmol/L of 5-aza for 24 hours, the viability was nearly 100%.

Group III, after MSCs exposure to 5-aza for three consecutive days showed 86.3% viable cells

Group IV, MSCs which were continuously exposed to 5- aza with every medium change, showed a viability of 61.7%.

In group V, after daily exposure of MSCs to 5- aza, the cell viability was decreased to 27.1%.

There was no statistically significant difference between the control (group I) and group II. However, in between group I and the rest of the groups there was a significant difference and the viability percentage decreased in proportion to the time of exposure to 5- aza, (Table 1, Figure 4).

Characterization of the differentiating cells

a. Morphological alterations after induction with 5-aza

In group I which received 5-aza-free CCM, no change in the shape of the cultured MSCs was noticed. On the other hand, in the other experimental groups, after the application of the differentiation protocol, some adherent cells died and detached. The surviving cells began to proliferate for 2 days then the proliferation stopped. On day 7, the morphology of the MSCs changed.

In groups II and III, some cells acquired an elongated appearance and were parallel to each other, forming a stick- like morphology. Other cells remained spindle and polygonal in shape, (Figure 5a, b).

In groups IV and V, areas of contact between adjacent cells were observed and some of the elongated cells were seen forming myotube-like structures, (Figure 5c, d). By continuously applying the 5- aza on group V, marked decrease in the number of attached cells was observed, as many cells died.

b. Electron microscopic appearance of the differentiating cells

The pre-induced MSCs (Group I) showed an irregular outline with many cell processes extending from the cell body. The nucleus was large euchromatic with prominent marginated nucleolus. The cytoplasm contained multiple arrays of the rough endoplasmic reticulum, mitochondria, and vacuoles, (Figure 6).

After short term exposure to 5- aza, some induced cells started to show primitive myofilaments in the cytoplasm in both group II exposed to 5- aza for 24 h, (Figure 7a) and group III exposed to 5- aza for three consecutive days, (Figure 7 b).

With long term exposure to 5- aza in both group IV and V; an increase in the number of cells containing myofilaments was observed. The formed myofilaments were evident and more developed occupying larger areas in the cytoplasm (Figures 8a, 9). Well evident electron dense lines appeared in between adjacent cells with disappearance of intercellular spaces. (Figures 8b, 9)

Morphometric analysis results

There was no significant difference in length mean of the formed myofilaments between the cells of group II exposed to a single dose of 5- aza (361.90 nm) and those of group III exposed to 5- aza for three consecutive days (421.99 nm).

With long term exposure to 5- aza, the morphometric results showed a significant increase in the length means of the formed myofilament in both group IV and V in comparison to the two previously mentioned groups. In group V, the myofilaments were the most prominent in comparison to all the treated groups as the mean of this group was about (1540.18nm), (Table 2, Figure 10).

Detection of specific cardiac proteins (cTnI and Cx43) by real time PCR

Concerning the effect of the exposure duration to 5-aza, short term exposure to 5-aza, whether single exposure for 24 h (group II) or exposure to 5-aza for 72 h (group III), had a weaker differentiating effect on MSCs towards cardiomyocytes, as a single exposure to 5- aza, had raised the expression of cTnI by 2.10 folds and of Cx43 by1.20 folds in comparison to the untreated cells, while repeating the dose of 5- aza for three consecutive days raised the cTnI expression 2.50 folds from the control group and the Cx43expression to 2.20 folds.

There was no significant difference between 5-aza single exposure for 24 h (group II) and exposure to aza for 72 h (group III).

Continuous exposure to 5- aza, whether twice weekly (group IV) or daily (group V), showed a highly significant differentiating effect on MSCs (enhancing the mRNA expression of cTnI and CX43). In group IV, continuous exposure to 5- aza twice weekly caused increased mRNA expression of cTnI by 3.45 folds and Cx43 by 14.77 folds compared to the untreated control. In group V, continuous daily exposure to aza showed the utmost effect on enhancing the expression of both cTnI mRNA (5.26 folds) and Cx43 (18.44 folds) compared to the untreated control, (Table3, Figure 11).
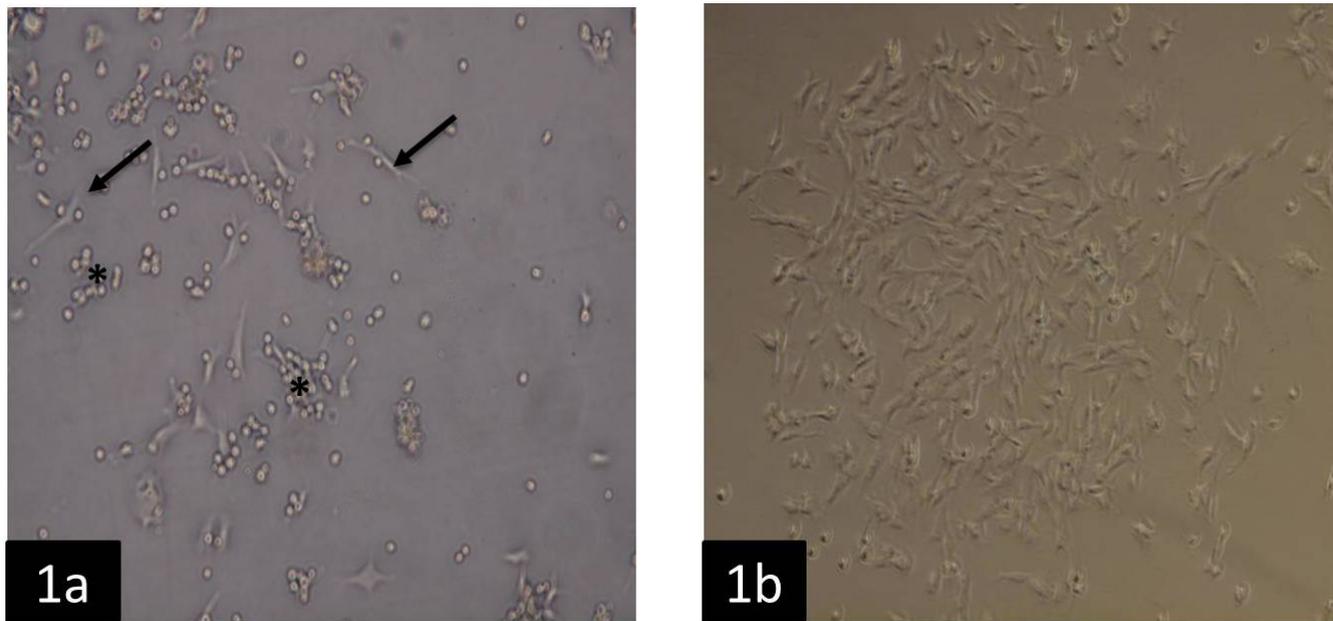
Fig. 1: Light photomicrographs of cultured BM-MSCs showing: a- adherent BM- MSCs acquiring spindle shaped (arrow) and many non- adherent cells appear rounded in shape (asterisks), b- One colony formed by the proliferating spindle shaped cells. (Phase contrast inverted microscope, unstained, Mic.Mag. x 100)
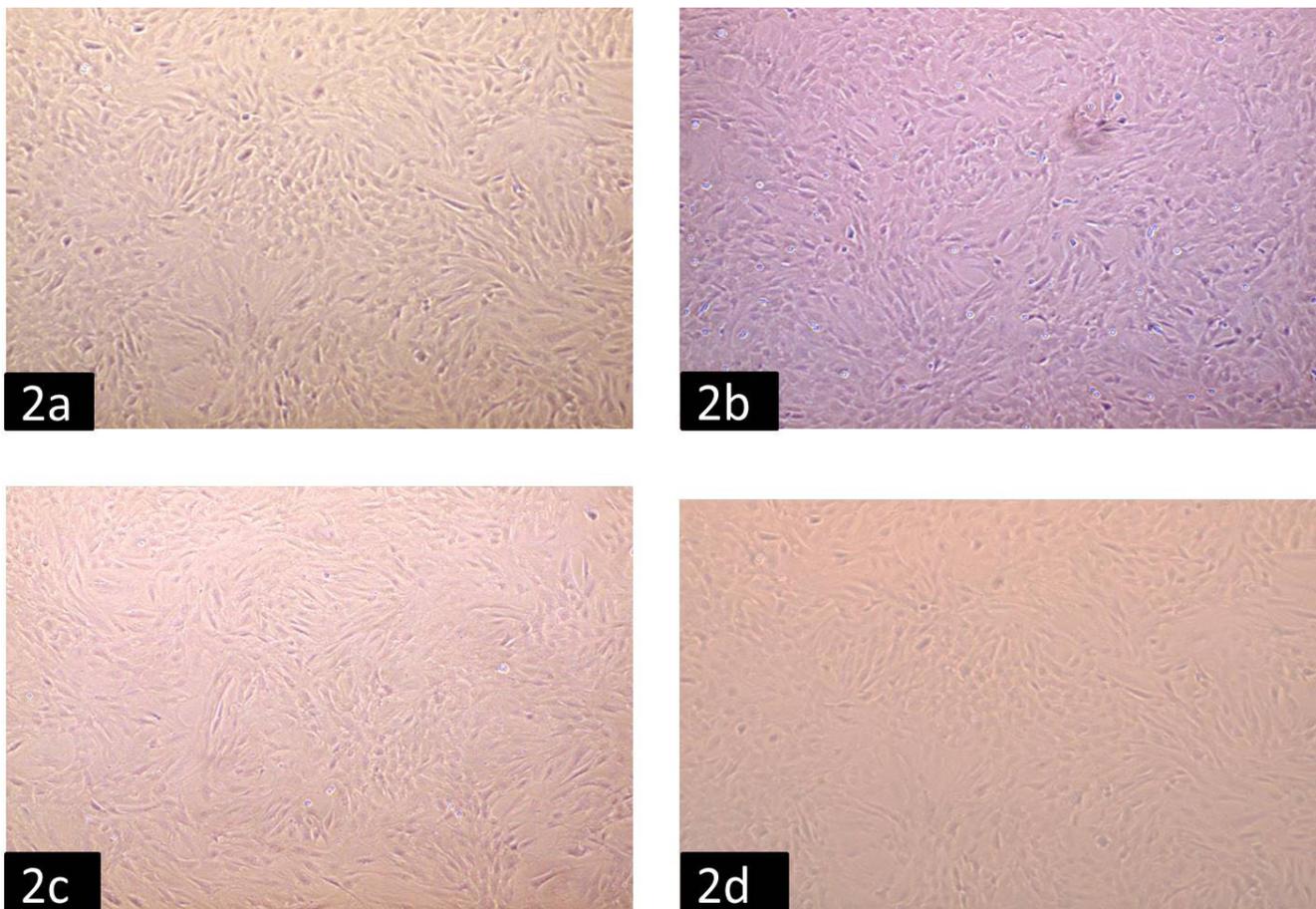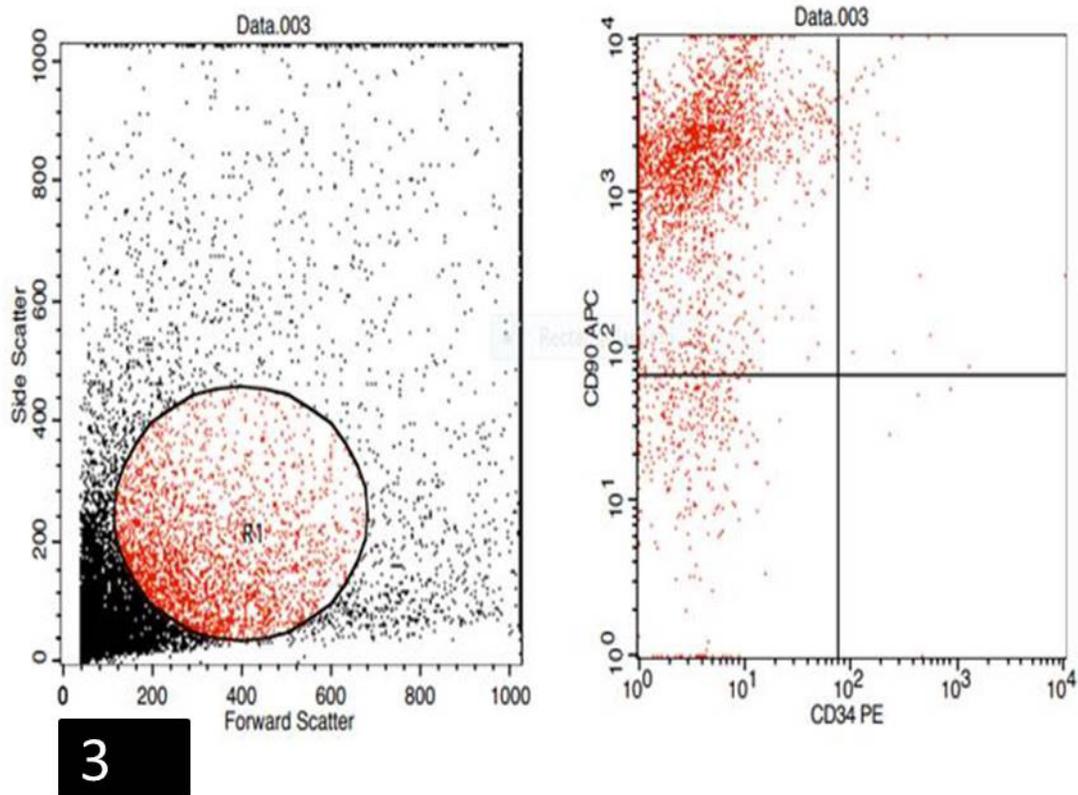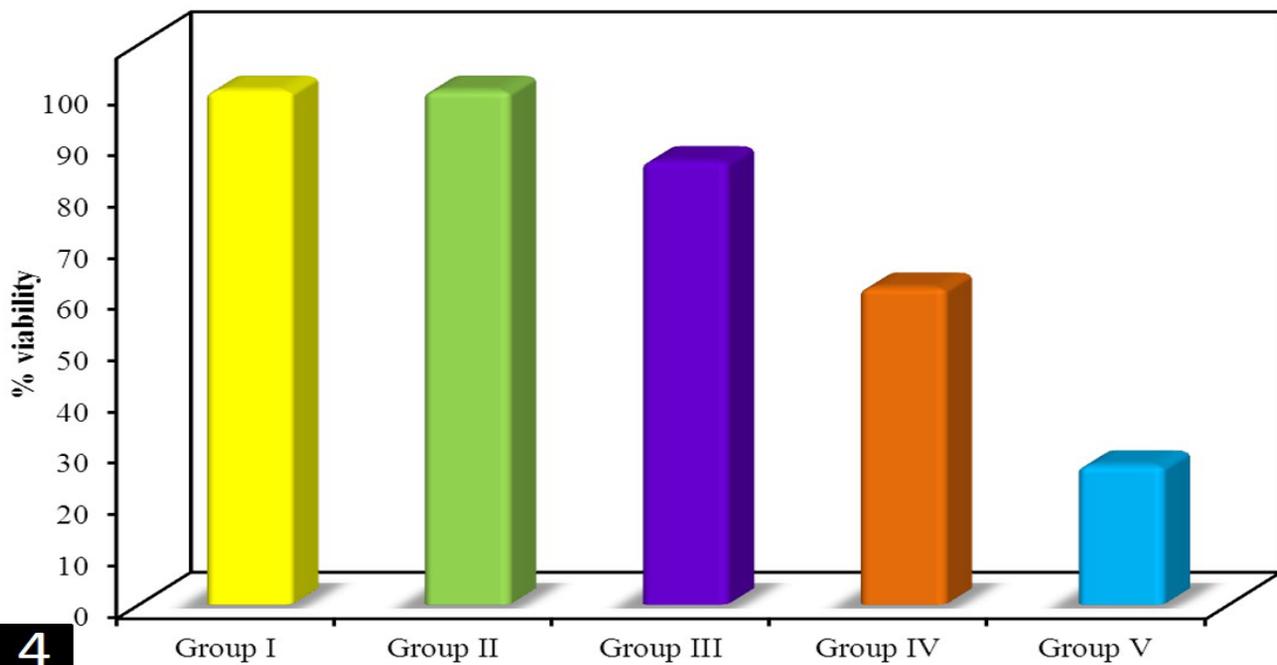

Fig. 2: Light photomicrographs showing confluent spindle shaped BM- MSCs at different passages: a- Day 10 primary culture (passage 0), b-   passage 1, c- passage 2, and d- passage 3. (Phase contrast inverted microscope, unstained, Mic.Mag x 100)

Fig. 3: A representative flow cytometric analysis of cell-surface markers of BM-MSCs at passage 3. Anti CD90 cells (MSCs) appear representing 87.50 % (Upper left quadrant). Cells positive for anti CD34 are only forming 0.14 % of the dot plot (Lower right quadrant)

Fig. 4: A bar chart showing comparison between the studied groups according to the percentage of the cell viability after exposure to 5-aza for different durations.
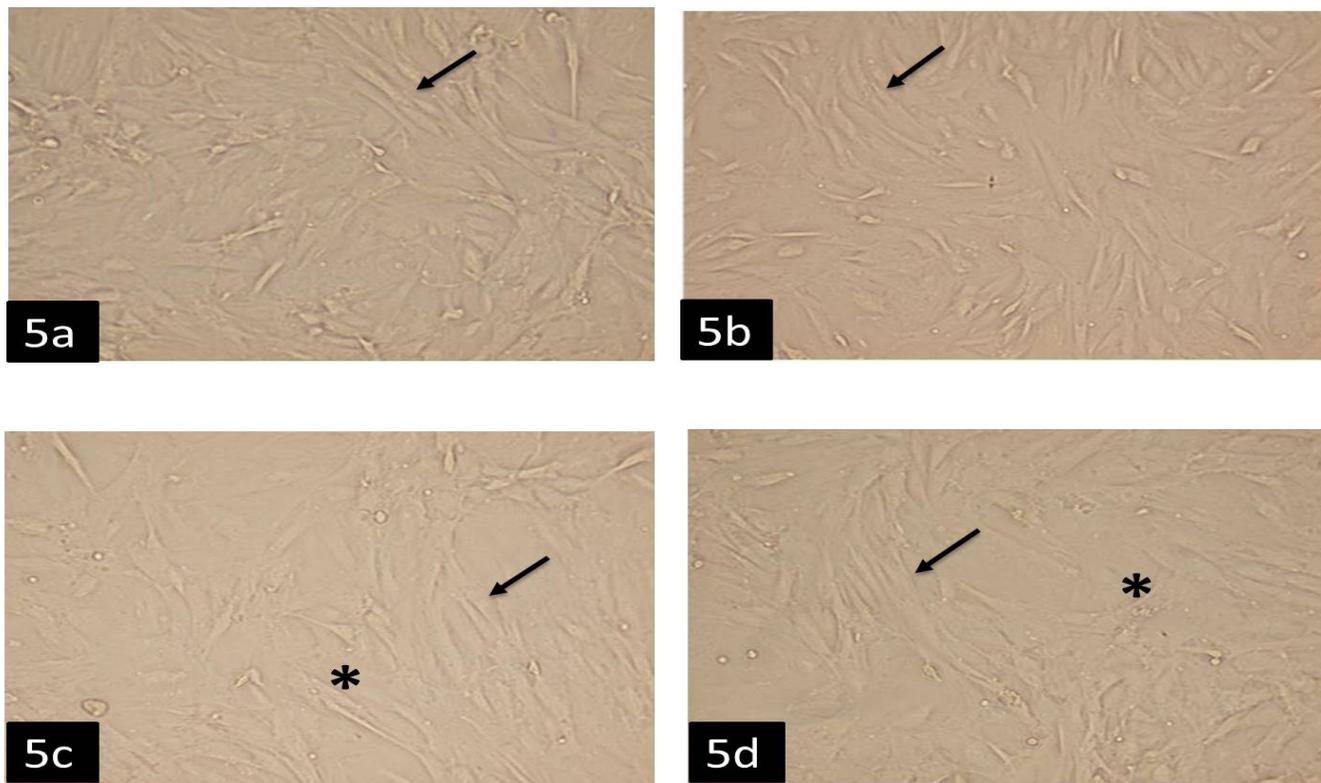
Fig. 5: Light photomicrographs of day 21 after applying the differentiation protocol. a: Group II, b: Group III, c: Group IV, d: Group V. In all these groups, some of the differentiating cells acquired an elongated appearance and were parallel to each other, forming a stick-like morphology (arrows). In groups IV and V, Some of the elongated cells form myotube-like structure with areas of contact between them (asterisks). (Phase contrast inverted microscope, unstained, Mic.Mag. x 200)
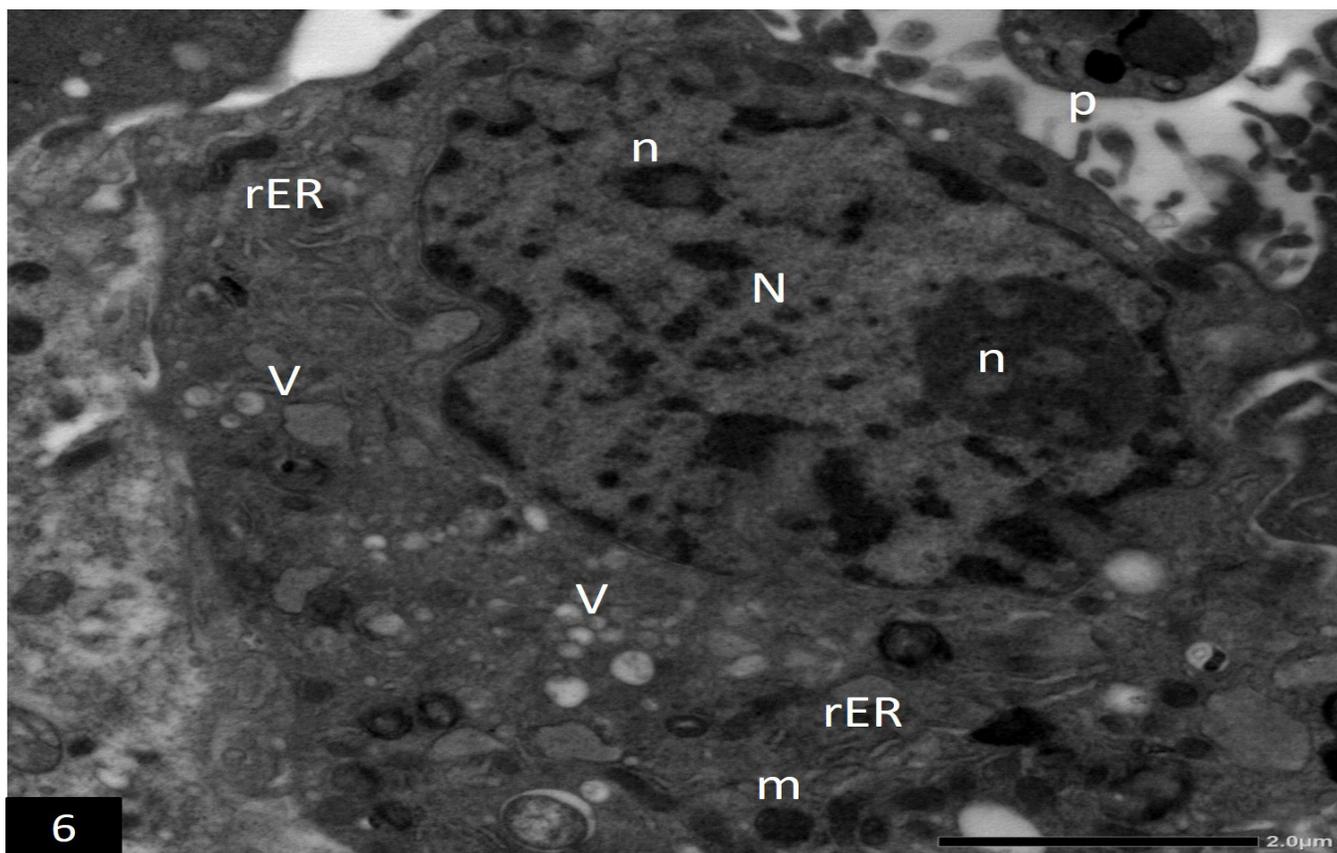

Fig. 6: An electron micrograph of undifferentiated BM-MSCs at passage3 showing a rounded cell with multiple cell processes (p), the nucleus (N) is large, eccentric in position and euchromatic with 1-2 prominent nucleoli (n). The cytoplasm contains profiles of rough endoplasmic reticulum (rER), mitochondria (m) and vacuoles (V). (Uranyl Acetate/ lead citrate stain. Mic.Mag. x 4000)
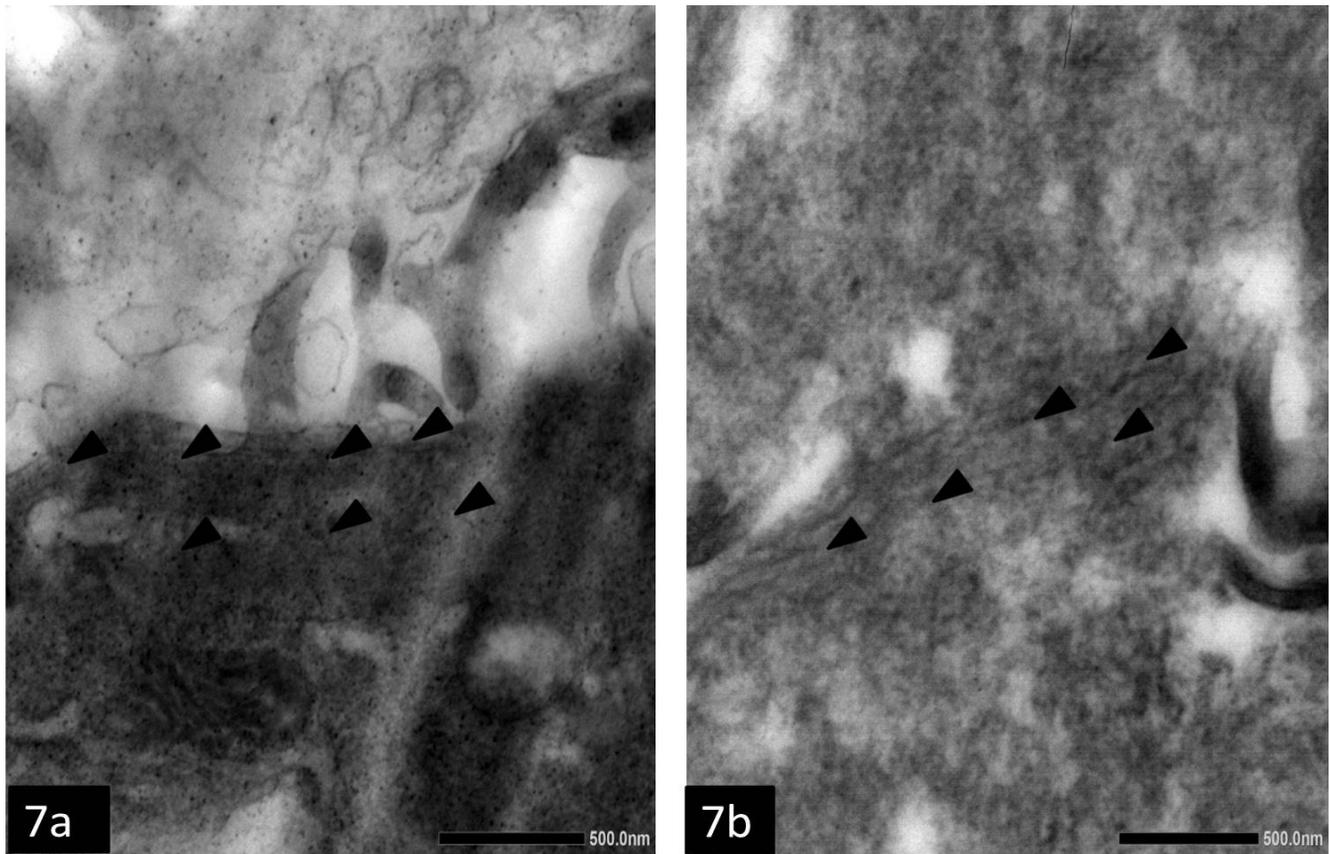
Fig. 7: Electron micrographs of differentiating MSCs after 3 weeks of induction of group II (a) and group III (b), showing primitive myofilaments in the cytoplasm (arrow heads). (Uranyl Acetate/lead citrate stain. Mic.Mag. X 15000)
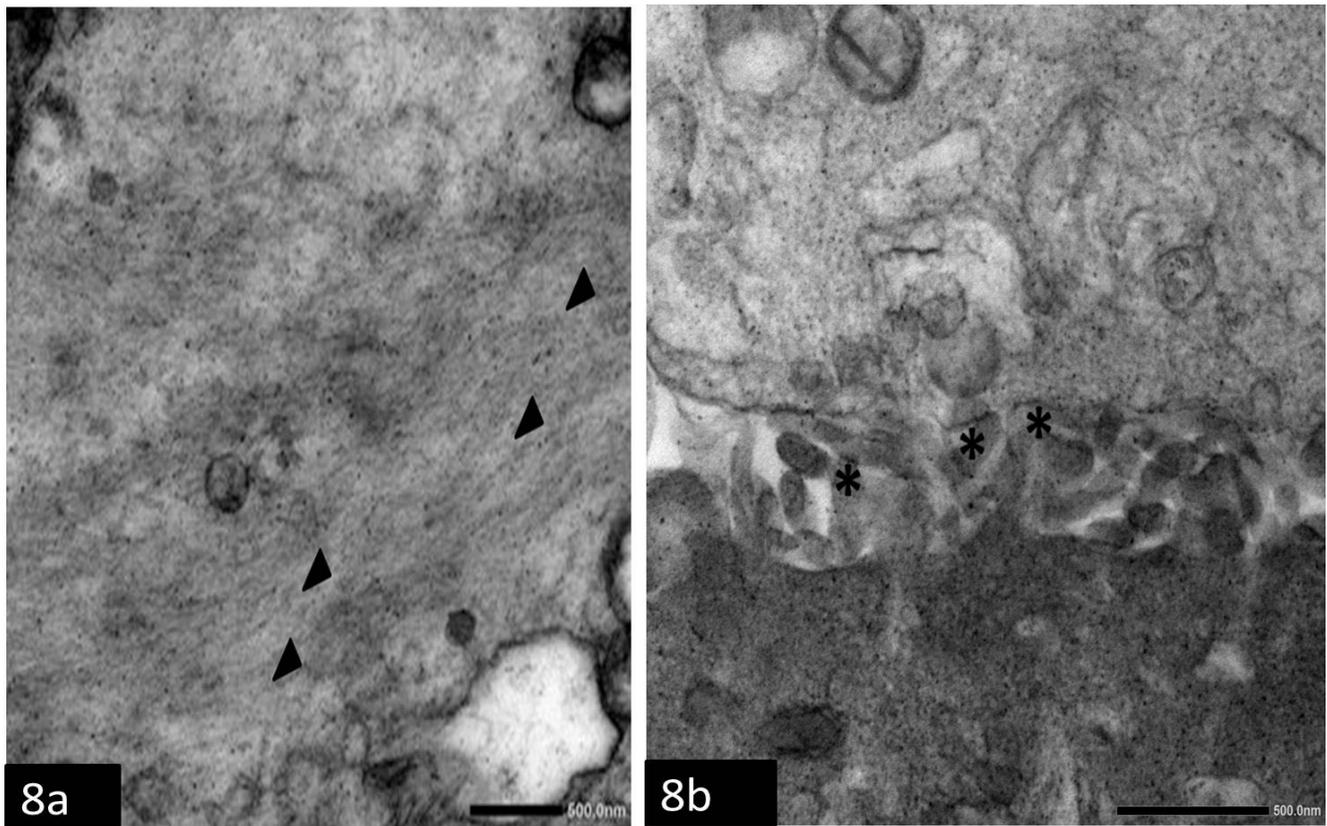

Fig. 8: Electron micrographs of differentiating MSCs in group IV after 3 weeks of induction showing: a- evident primitive myofilaments in the cytoplasm (arrow head). b- Electron dense lines are seen between the adjacent cells (asterisks). (Uranyl Acetate/lead citrate stain. a- Mic.Mag. X10000, b- Mic.Mag. X 2000)
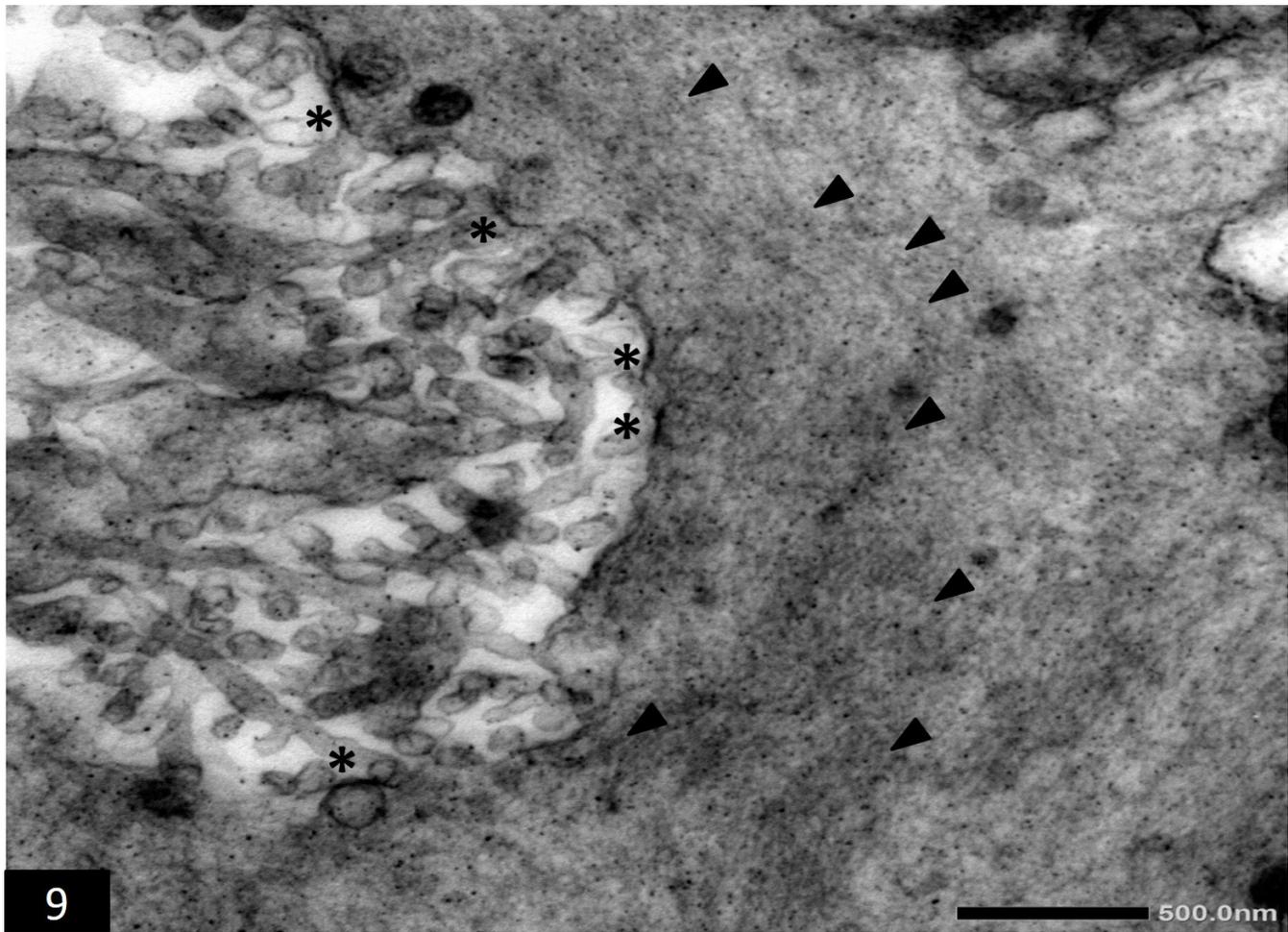
Fig. 9: An electron micrograph of differentiating MSCs in group V after 3 weeks of induction showing prominent primitive myofilaments in the cytoplasm (arrow head) with many electron dense lines (asterisks). (Uranyl Acetate/ lead citrate stain. Mic.Mag. X 10000)
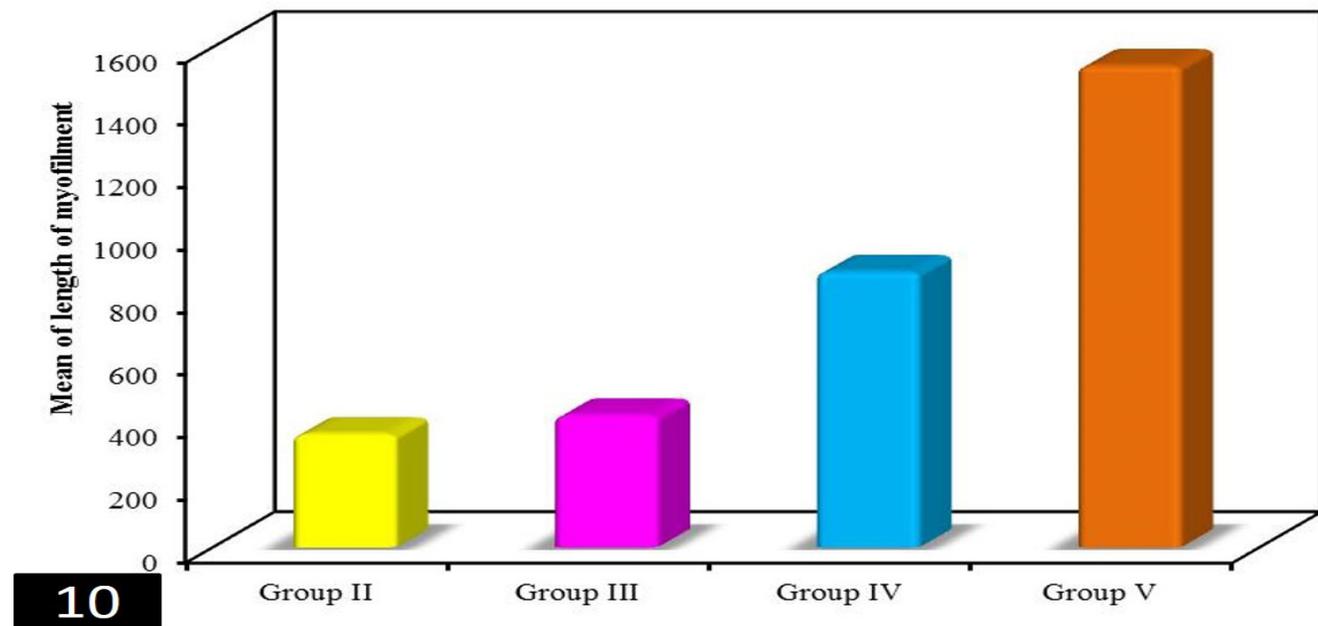

Fig. 10: A bar chart showing comparison between the different studied groups according to the length of the myofilaments
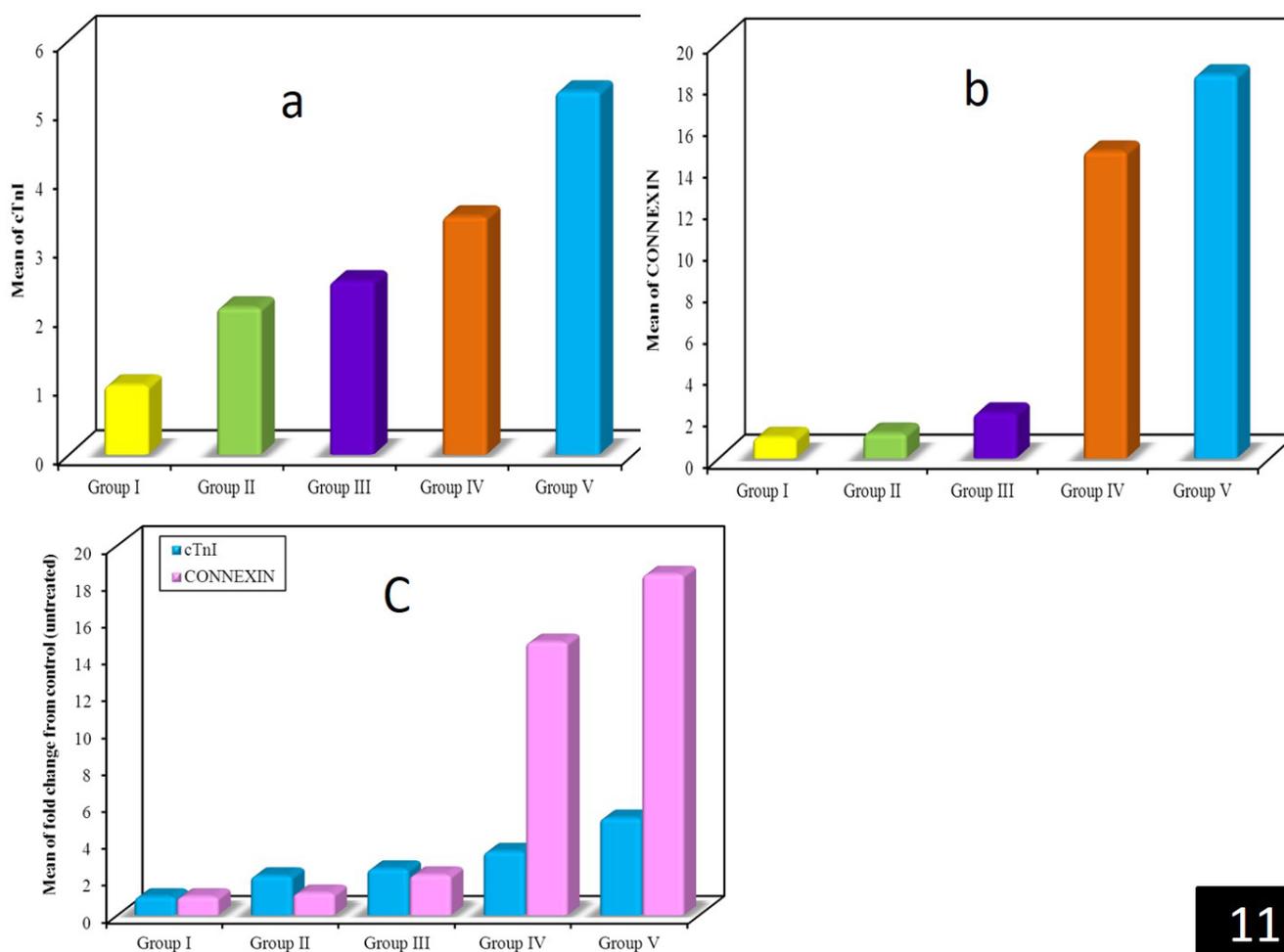
Fig. 11: Bar charts showing comparison between the five studied groups according to a- cTnI expression, b- Cx43 expression, c- cTnI and Cx43 fold change from the control

Table 1: Comparison between the studied groups according to viability of the cells after applying the 10μmol/ L of 5-aza for different durations

	Group I (Control)	Group II (applying 5-aza for 24 h)	Group III (applying 5-aza for 72h)	Group IV (applying 5-aza with every medium change)	Group V (applying 5-aza daily)	F(p)
Min. – Max.	3.29 – 3.29	3.29 – 3.35	2.02 – 3.29	1.95 – 2.10	0.82 – 0.97	131.407* (<0.001*)
Mean ± SD.	3.29 ± 0.0	3.29 ± 0.02	2.84 ± 0.43	2.03 ± 0.07	0.89 ± 0.06	
Median	3.29	3.29	2.98	2.06	0.91	
p		1.000	0.003*	<0.001*	<0.001*	
Sig. between groups			p1=0.002*, p2<0.001*, p3<0.001* p4<0.001*, p5<0.001*, p6<0.001*			
% viability	100.0	100.0	86.3	61.7	27.1	
% cytotoxicity		zero	13.7	38.3	72.9	

F: F for ANOVA test, Pairwise comparison between. Each 2 groups was done using Post Hoc Test (Tukey)

P: *p value* for comparing between the studied groups

P Control: *p value* for comparing between control and each other groups

P1: *p value* for comparing between group 2 and group 3

p2: *p value* for comparing between group 2 and group4

p3: *p value* for comparing between group 2 and 5

p4: *p value* for comparing between group 3 and 4

p5: *p value* for comparing between group 3 and 5

p6: *p value* for comparing between group 4 and 5

*: Statistically significant at $p \leq 0.05$

Table 2: Comparison between the studied groups according to length of myofilament

Length	Group II (n= 5)	Group III (n= 5)	Group IV (n= 6)	Group V (n= 5)	F(p)
Min. – Max.	286.28 – 439.13	408.41 – 433.84	470.75 – 1114.37	1236.24 – 2074.19	
Mean ± SD.	361.90 ± 55.56	421.99 ± 10.27	881.18 ± 234.43	1540.18 ± 338.93	33.774* (<0.001*)
Median	350.85	420.88	980.80	1492.59	
p$_1$		0.968	0.004*	<0.001*	
Sig. bet. grps			p2=0.010*, p3<0.001*, p4<0.001*		

F: F for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)
p: *p value* for comparing between the studied groups
p1: *p value* for comparing between Group 2 and other groups
p2: *p value* for comparing between Group 3 and Group 4
p3: *p value* for comparing between group 3 and Group 5
p4: *p value* for comparing between Group 4 and group 5
*: Statistically significant at $p \leq 0.05$

Table 3: Comparison between the three studied samples according to the expression of cTnI and Cx43

Fold change from control (untreated)	Group I (n= 3)	Group II (n= 3)	Group III (n= 3)	Group IV (n= 3)	Group V (n= 3)	F	P
CTnI							
Min. – Max.	1.0 – 1.0	2.0 – 2.30	2.43 – 2.60	3.30 – 3.60	4.98 – 5.60		
Mean ± SD.	1.0 ± 0.0	2.13 ± 0.15	2.51 ± 0.09	3.45 ± 0.15	5.26 ± 0.31	252.191*	<0.001*
Median	1.0	2.10	2.50	3.45	5.20		
p$_1$		<0.001*	<0.001*	<0.001*	<0.001*		
Sig. bet. grps.		p2=0.134, p4<0.001*, p5<0.001*, p6<0.001*, p7<0.001*, p8=<0.001*					
Cx43							
Min. – Max.	1.0 – 1.0	1.10 – 1.30	2.04 – 2.30	14.21 – 15.20	17.92 – 19.30		
Mean ± SD.	1.0 ± 0.0	1.20 ± 0.10	2.18 ± 0.13	14.77 ± 0.51	18.44 ± 0.75	1250.932*	<0.001*
Median	1.0	1.20	2.20	14.90	18.10		
p$_1$		0.973	0.036*	<0.001*	<0.001*		
Sig. between groups.		p2=0.089, p3<0.001*, p4<0.001*, p5<0.001*, p6<0.001*, p7=<0.001*					

F: F value for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)
p: *p value* for comparing between the three samples
p1: *p value* for comparing between group I and each other group
p2: *p value* for comparing between group II and group III
p3: *p value* for comparing between group II and group IV
p4: *p value* for comparing between group II and group V
p5: *p value* for comparing between group III and group IV
p6: *p value* for comparing between group III and group V
p7: *p value* for comparing between group IV and group V
*: Statistically significant at $p \leq 0.05$

DISCUSSION

Regenerative medicine is considered the most emerging and recent field of medicine, dealing with the restoration of certain tissues or organ functions in patients suffering from chronic diseases or severe injuries, in case of bodies, own regenerative responses do not suffice[24].

The presence of adequate cell sources to populate degenerated or injured tissues is the main issue in regenerative medicine. Stem cells are suitable candidates due to their capacity to self-renew and differentiate into multiple cell types[25].

In order to improve the outcome of the therapeutic use of stem cells regarding their survival, engraftment and functional integration, several strategies, such as genetic manipulation of the cells prior to their injection,

application of extracellular vesicles, immunologic regulation and pharmacological preconditioning have been tried with promising outcomes in improving the efficacy of the treatment after cell transplantation[26,27].

Among these strategies is to increase the MSCs potential towards cardiomyogenic differentiation through the use of 5-aza, a DNA-methyl transferase inhibitor-1 (DNMT-1)[28].

The activity of 5-aza is attributed to the ability of azanucleoside drugs to be incorporated into a replicating DNA. At high doses, 5- aza is incorporated into the DNA leading to formation of adducts between the DNA and DNMT-1. Therefore, irreversible changes in the DNA occur with inevitable cell apoptosis. Lower doses of 5-aza, on the other hand, give a chance for the formed adducts

to be degraded by mean of proteasome, hence, the DNA is recovered in the absence of DNMT-1 resulting in a powerful inhibition of DNMT-1. In this context, previously silenced genes could be re-expressed in the presence of a low dose of 5- aza[29].

Consequently, 5- aza induces BM-MSCs to differentiate into cardiomyocytes by random demethylation of some inactivated myogenic and other determination loci that were initially silenced due to hyper methylation , allowing induction of cardiomyogenic differentiation[30].

It is well established that azanucleoside drugs are known to be unstable and have a short half- life both in vivo and in vitro. The drug undergoes spontaneous hydrolysis in aqueous solutions, as well as rapid deamination by cytidine deaminase with subsequent degradation. Furthermore, chemical stability of 5- aza is temperature dependent, as the drug decomposition is faster at 37Cº than at 4Cº and 20 Cº indicating more rapid decomposition inside the incubator[31,32].

Based on the unique pharmaceutics of the drug, different study groups were designed in a trial to standardize a protocol ensuring the best differentiation with minimal toxicity. Because of the cytotoxic effect of the drug[33], it was important to seek for the minimum effective duration after which the cells will show signs of differentiation.

Examination of the control group during the whole experimental period indicated the absence of any signs of spontaneous differentiation towards any specific lineage, as control cells cultured and subjected to real time PCR showed baseline expression of cTnI and cx43 confirming that no specific differentiation had occurred without adding the differentiation factor used in this study.

In the present study, cTnI was selected because it is specific to cardiac muscle not skeletal muscles. Meanwhile, cTnI is neither detected in skeletal muscles, nor expressed at any time during their development[34].

In the present research, the dose of 5- aza that was used for induction of cardiac lineage was 10µmol/L. The same dose was used in the work of Ramesh et al[35], Xinyun et al[36] and Mohanty et al[37]. Surprisingly, they reported that 10 µmol/L of 5- aza applied only for 24 h is the ideal concentration of 5-aza for differentiation of MSCs into cardiomyocytes. They observed myotube like structures under the phase contrast inverted microscope together with elevated expression of cardiac specific proteins such as sarcomeric Troponin I and alpha myosin heavy chain.

On the contrary, few researchers succeeded in induction of differentiation using different lower doses of 5- aza as 3 µmol/L, however the expression of cardiac-specific proteins was lower than those obtained by the recommended dose of 10µmol/L[38].

Although the use of 5-aza is a known approach for in vitro initiative of induction of cardiomyocyte differentiation, conflicting data exist regarding the effect of 5-aza on MSCs differentiation into mature cardiomyocytes.

In the present work, a single exposure to 5- aza (group II) had a weaker differentiating effect on MSCs towards cardiomyocytes as confirmed by the electron microscopic examination, the morphometric study and the expression of the cardiac specific proteins. Yet, the obtained results were considered as the initial sign of differentiation towards cardiac lineage.

In accordance with the present study, many previous studies reported that the potency of 5- aza to induce differentiation towards cardiac lineage after applying the recommended dose for 24 h is limited and weak as only a low percentage of MSCs can differentiate under a single induction by 5-aza and the differentiating cells were immature[37-39]. This was confirmed in these studies by the absence of typical sarcomeres.

On the other hand, in the work done by Liu et al[40], immunocytochemistry and western blot levels failed to detect the expressions of cTnI, cardiac myosin heavy chain and Cx43 after a single exposure of BM-MSCs at day 3 of primary culture and the first subculture to the recommended dose of 5- aza. This might be attributed to the differentiation protocol that was applied to a heterogeneous population of the primary and first passage cultures.

In this study, there was no significant difference between single exposure to 5- aza (group II) and repeating the dose for three consecutive days (group III) regarding the expression of both cTnI, CX43 and the morphological appearance of the cells. This was in agreement with Martin et al[41], who found that varying exposure intervals to5-Aza (24h, 3 days) did not enhance any increase in the number of differentiated cells or any more expression of cardiac specific proteins.

In the present study, in a trial to augment the differentiation capability of the drug, 5 aza was applied on BM-MSCs for longer duration to investigate whether increasing the time of exposure will enhance the differentiation or not. Examining group IV in which the cells received CCM supplemented with 5-aza (10 µmol/L) with every medium change, twice weekly, till the end of the experiment, showed enhanced differentiation at both the molecular and the ultrastructural levels.

In the work done by Zhang et al,[42] they reported that using 5-aza every 3 days was optimal in differentiating the cells towards cardiomyocytes than using a single dose of 5- aza. They confirmed this finding through detection of cardiac specific proteins by western blot and immunofluorescence, indeed, the positive percentage of cells expressing cardiac specific proteins raised from 37% in the group exposed to a single dose of 5- aza to 44% in the group continuously exposed to the inducing drug.

In this study, despite the enhancement of the results and more organization and development of the primitive myofilaments seen in group IV and V, no typical sarcomeres were detected in these groups, a finding that is coinciding with Zangh et al work[42], that followed continuous

exposure of 5- aza, found primitive myofilaments and intercalated disc-like structure between adjoining cells but no typical sarcomeres were found.

On the other hand, Fukuda *et al*[43], succeeded to establish a cardiomyogenic cell line from murine bone marrow stroma and found typical striation and sarcomeres formation eight weeks after treatment with 3 μmol /L 5-aza for 24h.

In the current study, the disappearance of intercellular spaces with the presence of evident electron dense lines in between adjacent cells suggested the formation of gap junctions in between the cells, a finding that was supported by the elevated expression of Cx43.

In the present work, daily exposure to 5- aza (group V) presented the maximum differentiation characteristics of the cardiac cells in comparison to the other groups as confirmed by RT-PCR expression of cTnI and Cx43 and the presence of more prominent myofilaments. This was apparently at the expense of the viability of the induced cultured cells which had dropped from 61.7% in group IV to 27.1% in daily exposed cells to 5- aza (group V).

There are few published data about the continuous usage of 5- aza as an inducer for cardiac lineage differentiation. Meanwhile, Zangh *et al* work[42] applied 5-aza continuously to the cultured human first-trimester fetal MSCs, they found that the cell population doubling time (PDT) of this group was 9.09 days, that was significantly longer than the PDT of the control group (6.62 days) indicating that increasing the time of 5-aza exposure slowed down the cell proliferation speed and directed the cells towards the differentiation. However, in this study, cytotoxicity assessment for this prolonged use of the drug was not performed.

Taken together, the use of 5-aza as a differentiation factor showed a valuable differentiating effect on MSCs towards cardiomyocytes. Prolonged use resulted in promotion of the differentiation. Yet, it was at the expense of the viability of these cells. Therefore, the differentiation protocol for obtaining cardiomyocytes using 5-aza must be meticulously monitored to reach the optimum beneficial effect with minimum cytotoxicity.

ABBREVIATIONS

MSCs: Mesenchymal stem cells, **BM-MSCs:** bone marrow- mesenchymal stem cells, 5- aza: 5-azacytidine, **PBS:** phosphate buffer saline, **FACS:** fluorescense activated cell sorting, **APC:** allophycocyanin, **CCK-8:** cell counting Kit, **CCM:** complete culture medium, **CERRMA:** center of excellence for research in regenerative medicine and its applications, **cTnI:** cardiac troponin I, **Cx43:** connexin 43, **DNMT-1:** DNA methyltransferase-1 enzyme, **HF:** heart failure, **L-DMEM:** Low Glucose Dulbecco's Modified Eagle's Medium, **PE:** phycoerythrin, **PDT:** population doubling time, **RT-qPCR:** Quantitative reverse transcription polymerase chain reaction, **TEM:** Transmission electron microscope.

CONFLICTS OF INTEREST

There are no conflicts of interest.

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الملخص العربى

تمايز الخلايا الجذعيه الوسطيه المستمده من نخاع عظام الجرذان الي خلايا القلب العضليه باستخدام نظم مختلفة من الاذاسيتيدين

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المقدمة: المناهج العلاجية التقليدية لأمراض القلب الإقفارية لها فائدة محدودة في منع إعادة تشكيل ما بعد الافتقار وفشل القلب البطيني الأيسر. لذلك ، فإن العلاج المستند إلى الخلايا الجذعية هو أداة واعدة لعلاج أمراض القلب الإقفارية بعد احتشاء عضلة القلب.

الهدف من البحث: تهدف هذه الدراسة الى توحيد بروتوكول يضمن أفضل تمايزلخلايا نخاع العظم الجذعية الوسطية الي خلايا القلب العضلية عن طريق تعرض الخلايا الجذعية إلى اذاسيتيدين مع الحد الأدنى من السمية.

مواد وطرق البحث: تم عزل خلايا نخاع العظم الجذعيه الوسطيه من الفئران البيضاء واللتي بلغ عمرها اسبوعين. وعند الممر الثالث تم توصيف الخلايا عن طريق جهاز عد الخلايا(بقياس التدفق الخلوي) وتم تقسيم الخلايا الي خمس مجموعات:المجموعه الاولي (المجموعه المرجعيه) وتم اعطائها وسط زراعى كامل خالى من اذاسيتيدين، المجموعه الثانيه تعرضت الي الوسط المتمايز المحتوى على الاذاسيتيدين لمده اربع وعشرون ساعه فقط ، المجموعه الثالثه تعرضت الي الوسط المتمايز المحتوى على الاذاسيتيدين لمده اثنان وسبعون ساعه علي التوالي، المجموعه الرابعه تم تجديد الوسط المحتوى على الاذاسيتيدين لها مرتين اسبوعيا و المجموعه الخامسه تم تجديد الوسط الذي يحتوي علي الاذاسيتيدين يوميا . وقد تعرضت كل من المجموعه الثانيه والثالثه والرابعه والخامسه الي نفس التركيزمن الاذاسيتيدين والذي كان عشره ميكرمول لكل لتر وقد تم انهاء التجربه بعد ثلاث اسابيع من بداية البحث. وقد تم ملاحظه التغييرات الشكلية باستخدام المجهر المقلوب الطوري.و تم تقييم تأثيرالفترات المختلفة من التعرض الي الاذاسيتيدين علي قدره خلايا نخاع العظم الجذعيه الوسطيه علي البقاء بواسطه عد الخلايا الحية. وتم استخدام اختبار تفاعل البوليميرازالتنسخي العكسي المتسلسل للكشف عن بروتينات القلب المخصصه مثل تروبينين ا وكونيكسين ٤٣ وتم استخدام المجهر الالكتروني لفحص البنيه التحتيه للخلايا المستحثه و تم إجراء دراسة مورفومترية لتقييم ومقارنة طول الألياف العضلية المشكلة في المجموعات الأربع المعالجة.

النتائج:اعتبرت النتائج التي تم الحصول عليها من المجموعة الثانية و الثالثة الى ان هناك علامه محدوده اوليه نحو التمايز الي خلايا القلب العضليه فى حين اظهرت نتائج المجموعة الرابعة و الخامسة الى علامات امثل للتمايز و ظهرت افضل نتائج فى المجموعة الخامسة و لكن على حساب قابلية الخلايا المستزرعة للبقاء.

الاستنتاج: اظهر استخدلم الاذاسيتيدين كعامل تمايزفعال نحو خلايا القلب العضليه وقد ادي الاستخدام المطول الي تعزيز التمايز ولكن علي حساب قابلية الخلايا علي البقاء