Original Article

Therapeutic Influence of Exosomes Derived from Non-heat versus Heat Shocked Stem Cells on Experimentally Induced Myocardial Infarction in Adult Male Albino Rats

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ABSTRACT

Background: Worldwide, myocardial infarction (MI) is considered as a principal cause of mortality. It results in death of the cardiac myocytes and declining of the cardiac functions. Exosomes of the bone marrow mesenchymal stem cells (BMMSCs) are cell-free, nano-sized extracellular vesicles that have the same reparative potentiality of the stem cells. Heat shock is one of the stresses that increase the production of heat shock proteins in the stem cells augmenting their survival capabilities.

Aim of the Work: The current study aimed at assessing and comparing the therapeutic potentiality of exosomes derived from the non-heat and the heat shocked BMMSCs ($^{non\text{-}HS}$BMMSCs-EXOs and ^{HS}BMMSCs-EXOs) on experimentally induced MI (acute & chronic stages) with underlining the probable explanation for that difference.

Materials and Methods: 56 adult male albino rats were divided into the donor group (for $^{non\text{-}HS}$BMMSCs-EXOs & ^{HS}BMMSCs-EXOs preparation) and the experimental groups (control, MI, MI-$^{non\text{-}HS}$BMMSCs-EXOs & MI-^{HS}BMMSCs-EXOs). Four control rats with two rats from each MI-$^{non\text{-}HS}$BMMSCs-EXOs & MI-^{HS}BMMSCs-EXOs groups were sacrificed 1 day following MI to confirm BMMSCs-EXOs homing while other rats were sacrificed after 3 &28 days. Serological, biochemical, histological and morphometric studies were done.

Results: The acute stage of MI revealed degenerative and inflammatory features whereas the chronic stage demonstrated marked fibrotic changes. Such changes were regressed in MI-$^{non\text{-}HS}$BMMSCs-EXOs & MI-^{HS}BMMSCs-EXOs groups except that of MI-$^{non\text{-}HS}$BMMSCs-EXOs sacrificed after 28 days.

Conclusion: Both $^{non\text{-}HS}$BMMSCs-EXOs and ^{HS}BMMSCs-EXOs had therapeutic capabilities in the acute stage of MI which was more evident with ^{HS}BMMSCs-EXOs. In the chronic stage, $^{non\text{-}HS}$BMMSCs-EXOs exhibited non-curative effect despite the perfect effect of ^{HS}BMMSCs-EXOs.

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INTRODUCTION

Myocardial infarction, also called heart attack, is a focus of acute cardiac muscle cell death occurred as a sequel of reduced cardiac blood supply. It may result in cardiogenic shock and cardiac arrest[1]. Worldwide, its incidence is increased progressively, about 15×10^6 cases are recorded every year[2]. Epidemiologically, it was reported that most of the cases of MI ensued from coronary artery obstruction that might occur as a result of high blood pressure, high cholesterol, diabetes or smoking[3]. Myocardial infarction is associated with consequent great morbidity and mortality rates[1]. This is not only due to the lesion itself but also due to the aggressive management interferences used in its treatment such as percutaneous coronary intervention (PCI) and coronary artery bypass graft (CABG)[4]. So, there is a crucial necessity to achieve long term therapy that is safer and more appropriate.

Mesenchymal stem cells (MSCs) have been found to retain obvious therapeutic potentiality in many different pathologies[5] which has been extended to cure cerebral ischemia[6]. Such therapeutic capabilities of MSCs are based on their unique features as self-renewing, proliferating, multipotent differentiating and immunomodulatory properties[5].

In the case of MI, the use of stem cells showed limited improvement of the cardiac functions and infarct size where they undergo enormous cell death due to tissue hypoxia. It was stated that 2 and 18 h after stem cells transplantation, only 5% and 1 % of the stem cells could be spotted in the myocardium, respectively[7]. Moreover, the stem cells recruitment to the ischemic myocardium aroused by the interaction between injured myocytes′ stromal cell-derived factor-1 (SDF-1) and its receptor CXC chemokine receptor 4 (CXCR4) was dampened after 3 days of MI. This occurs since SDF-1 release is peaked 1-3 days then dropped to its basal level[8]. These hindrances in addition to the other potential threats of immune rejection and tumour development restrict the clinical use of stem cells in MI.

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Recently, MSCs have been documented to function through secretion of trophic factors (cytokines, chemokines, mRNAs, microRNAs and growth factors) that act on adjacent cells via a paracrine mechanism. These trophic factors are produced by MSCs as extracellular vesicles (EVs), namely MSCs secretomes. Accordingly, MSCs secretomes, rather than MSCs differentiation, are considered the primary way through which MSCs exert their therapeutic effects[9] where the differentiated number of stem cells was very few to explain their magical reparative effects[10].

These secretomes are partitioned based on their sizes and cellular origin into exosomes (EXOs), microvesicles (MVs) and apoptotic bodies; EXOs (30-150 nm) are produced in the endosomal system, MVs (100-1000 nm) and apoptotic bodies (500-2000 nm) both originate from the cell membrane. Both EXOs and MVs are released by the cells for intercellular communication and control of the recipient cells functions, via their contents (RNA, microRNA, proteins and others). However, apoptotic bodies are liberated from cells undergoing apoptosis to facilitate their phagocytosis[11]. Additionally, EXOs derived from MSCs (MSCs-EXOs) are of attracting interest as they are produced in large numbers and characterized by being stable, of low immunogenicity and well-tolerated in different body fluids[12]. Moreover, multiple recent studies proposed the potentiality of MSCs-EXOs as cell-free treatment in cases of MI[13].

Heat shock proteins (Hsps), a family of endogenous proteins, produced in response to different cellular stresses and diseases such as hyperthermia[14] and hypoxia[15]. They are considered as molecular chaperones i.e. they stabilize the cellular proteins through controlling folding and translocation of endogenous proteins and disposal of the incorrectly folded and the irretrievably denatured ones. So, they could protect the cells against these stresses and increase their survival[14].

Heat shock protein70 (Hsp70) is the main Hsp involved in cardiac protection following MI where its induction decreases the infarct size and apoptosis of cardiac myocytes[16]. Furthermore, it was described that MSCs exposed to various forms of stresses (hypoxia and hyperthermia) during their culture, overexpressed Hsps (especially Hsp70) to augment their survival[17].

This study was designed to evaluate and compare the potential therapeutic effect of $^{non\text{-}HS}$BMMSCs-EXOs versus ^{HS}BMMSCs-EXOs on experimentally induced MI in adult male albino rats (acute and chronic stages), with highlighting the possible explanation for that difference.

MATERIALS AND METHODS

Experimental Design

Fifty-six adult male albino rats (~250 g) were treated according to the guidelines granted by the Animal Use Committee of Cairo University(CU-III-F-12-20). The rats were housed in the Laboratory Animal House Unit of Kasr Al-Aini, Faculty of Medicine, Cairo University and divided as follows:

Donor Group

Two rats were used for BMMSCs isolation, culture, phenotyping, EXOs isolation and labelling.

Experimental Groups

The remaining animals were divided randomly into 4 main groups (control, MI, MI-$^{non\text{-}HS}$BMMSCs-EXOs &MI-^{HS}BMMSCs-EXOs groups). Then the rats in each group were furtherly divided into 2 equal subgroups according to the time of sacrifice (3 and 28 days).

Control Group (20 rats)

The animals of each subgroup (10 rats) were subdivided into: Two rats, not subjected to any procedure. Two rats (sham-operated), subjected to the same procedure as those of MI group but without left anterior descending coronary artery occlusion (LCAO). Three rats, prepared as sham-operated rats then each rat was given intravenous (IV) injection of 400µg of PKH26 labelled $^{non\text{-}HS}$BMMSCs-EXOs suspended in 200µl PBS via the tail vein. Three rats, prepared as the previous three rats but the animals were given PKH26 labelled ^{HS}BMMSCs-EXOs instead of $^{non\text{-}HS}$BMMSCs-EXOs.

MI Group (MI-3 and MI-28, 5 rats each)

Animals of this group were subjected to permanent LCAO as previously described[18] at the Laboratory Animal House Unit of Kasr Al-Aini, Faculty of Medicine, Cairo University. Briefly, the animals were anaesthetized with ketamine (90 mg/ kg)/xylazine (15 mg/kg) intraperitoneal (IP) injection and immobilized face-up on a warm pad. They were ventilated using a rodent ventilator through tracheal intubation. A left thoracotomy was done, between the 4th and the 5th intercostal spaces. Then lateral compression of the chest was applied to exteriorize the heart. Ligation of the left anterior descending coronary artery (2 mm from its origin, between the left atrium border and the pulmonary artery sulcus) was achieved using 5-0-size silk suture thread. The heart was quickly returned to the thoracic cage, the lungs were inflated with 100% oxygen, positive ventilation and the wound was closed. Myocardial infarction was confirmed by colour loss in the area below the ligation point.

MI-$^{non\text{-}HS}$BMMSCs-EXOs Group (MI-^{nHS}E-3 and MI-^{nHS}E-28, 6 rats each)

The rats in this group were subjected to permanent LCAO as in MI group. Immediately after LCAO, each rat was given IV injection of 400µg of PKH26 labelled $^{non\text{-}HS}$BMMSCs-EXOs suspended in 200µl PBS through the tail vein[1].

MI-^{HS}BMMSCs-EXOs Group (MI-^{HS}E-3 and MI-^{HS}E-28, 6 rats each)

The rats in this group were treated as in MI-^{nHS}E group but they received PKH26 labelled ^{HS}BMMSCs-EXOs instead of $^{non\text{-}HS}$BMMSCs-EXOs.

In Vitro Studies

PKH26 labelled $^{non\text{-}HS}$BMMSCs-EXOs & ^{HS}BMMSCs-EXOs

They were purchased [as suspension of 400µg EXOs/200µl of phosphate-buffered saline (PBS)] from Stem Cell Research Unit, Biochemistry Department, Faculty of Medicine, Cairo University after being freshly prepared as follows:

Isolation and Culture of BMMSCs

This was done according to previously described methodology[19] till the end of the 4th passage which was used in this study.

Preparation of ^{HS}BMMSCs[14]

Part of BMMSCs was cultured in culture flasks (3×10^4 cells/cm^2), heat-sealed and immersed in a 42°C water bath for 60 min. Then, they were incubated at 37°C for 24 h to recover before being used.

$^{non\text{-}HS}$BMMSCs-EXOs and ^{HS}BMMSCs-EXOs Extraction[20]

Their extraction was done under complete aseptic conditions using differential centrifugation. Then, the exosomal pellets were re-suspended in PBS at - 80 °C to be used in this work.

PKH26 Labelling of $^{non\text{-}HS}$BMMSCs-EXOs and ^{HS}BMMSCs-EXOs[21]

Using Red PKH26 Fluorescent Cell Linker Kit (Sigma, USA, MINI26) according to the manufacturer's protocol.

Measuring Value of heat shock factor 1 (HSF1) in $^{non\text{-}HS}$BMMSCs-EXOs and ^{HS}BMMSCs-EXOs

This was accomplished at Biochemistry Department, Faculty of Medicine, Cairo University, using Enzyme-Linked ImmunoSorbent Assay (ELISA) according to manufacturer's instructions after adding 100 ml of each exosomal samples to the primary HSF1 antibody (rat monoclonal antibody, MA5-27688, Invitrogen, ThermoFisher Scientific, USA) in the wells of a 96-well plate of an ELISA kit (R&D system, USA).

Animal Studies

Serological Study

Just before the sacrifice of each experimental group, blood samples from the tail vein were obtained to measure the serum level of the early MI markers [creatine phosphokinase (CPK) and lactate dehydrogenase (LDH)[22] using spectrophotometer (UV-1601PC, Shimadzu, Japan). This was done at Biochemistry Department, Faculty of Medicine, Cairo University.

Animals sacrifice

At the Laboratory Animal House Unit of Kasr Al-Aini, Faculty of Medicine, Cairo University, the rats were sacrificed by cervical dislocation after being anaesthetized with IP injection of ketamine (90 mg/kg)/xylazine (15 mg/kg)[23]. Two of the control animals received $^{non\text{-}HS}$BMMSCs-EXOs and another two received ^{HS}BMMSCs-EXOs together with one animal from subgroups MI-^{nHS}E-3, MI-^{nHS}E-28, MI-^{HS}E-3 and MI-^{HS}E-28 were sacrificed 1 day after EXOs administration while the remaining animals were sacrificed after 3 and 28 days. The chests were opened and the hearts were dissected. Three slices (2-2.5 mm in thickness) were longitudinally sliced from the apex to the base of the left ventricles (one for quantitative real-time polymerase chain reaction [qRT-PCR], one for preparation of cardiac homogenates and one for histological examination).

Quantitative real-time polymerase chain reaction (qRT-PCR)[24]

It was done at Biochemistry Department, Faculty of Medicine, Cairo University to detect relative mRNA expression of Bax (pro-apoptotic gene) and microRNA-34a (miR-34a): After total RNA extraction and complementary DNA (cDNA) synthesis, qRT-PCR of the studied genes was done using Rotor Gene 6000 series software version 1.7 (Corbett Life Science, USA) and the primers. Then the results were expressed as a normalized ratio. The PCR primer sequences used were Bax (forward: 5′- GTTGCCCTCTTCTACTTTG-3′; reverse: 5′- AGCCACCCTGGTCTTG -3′), miR-34a (forward: 5′- TTGAATTCTAACACCTTCGTGGCTACAGAG-3′; reverse:5-TTAGATCTCATTTATCGAGGGAAGG ATTG-3′) and GAPDH [internal control] (Forward: 5′- CTCCCATTCTTCCACCTTTG-3′; Reverse: 5′- CTTGCTCTCAGTATCCTTGC-3′).

Cardiac Homogenates and ELISA

Cardiac homogenates were done at Biochemistry Department, Faculty of Medicine, Cairo University, based on previous methodology[24]. Then ELISA was done according to the manufacturer's instructions to measure values of HSF1 and Hsp70 using a 96-well plate of a rat ELISA kit (R&D system, USA) and primary antibodies for HSF1 (rat monoclonal antibody, MA5-27688, Invitrogen, ThermoFisher Scientific, USA) and for Hsp70 (mouse monoclonal antibody, 33-3800, Invitrogen, ThermoFisher Scientific, USA).

Histological Study

At Histology Department, Faculty of Medicine, Cairo University, the slices for the histological examination were fixed in 10% formol saline for 24 h. Paraffin blocks were processed and 5µm thick sections were cut:

Unstained sections of the control group and subgroups MI-^{nHS}E-3, MI-^{nHS}E-28, MI-^{HS}E-3 & MI-^{HS}E-28 sacrificed after 1 day were examined by the fluorescent microscope. However, sections of different groups sacrificed after 3 and 28 days were subjected to:

Hematoxylin and Eosin stain (H&E)[25].

Masson's trichrome stain[26].

Immunohistochemical staining for:

a. Caspase 3 (rabbit polyclonal antibody, ab4051, abcam, USA): it appears as a cytoplasmic reaction in the apoptotic cells.

b. Connexin 43 (rabbit polyclonal antibody, ab11370, abcam, USA): it is a marker for gap junction of the intercalated discs. It appears as a membranous reaction (at the sites of connection between the adjacent cardiomyocytes).

c. HSF1 (rabbit polyclonal antibody, ab131081, abcam, USA): it appears as a cytoplasmic and/or nuclear reactions localizing HSF1.

d. Hsp70 (mouse monoclonal antibody, MA3-008, Invitrogen, ThermoFisher Scientific, USA): it is a marker for Hsp70 that appears as a cytoplasmic reaction.

Immunostaining using avidin-biotin technique required pretreatment[26], this was carried out by 10 min boiling in 10 mM citrate buffer (cat no 005000) pH 6 for antigen retrieval. Sections were left to cool for 20 min in room temperature. Then, incubation of the sections for one hour with the primary antibodies was done. Immunostaining was completed by the use of Ultravision One Detection System (cat no TL - 060- HLJ). Counterstaining was carried out using Lab Vision Mayer's hematoxylin (cat no TA- 060-MH). Negative control sections were prepared by the same process after excluding the primary antibodies. Citrate buffer, Ultravision One Detection System and Ultravision Mayer's hematoxylin were purchased from Labvision, ThermoFisher Scientific, USA.

Morphometric study

Image analysis by Leica Qwin-500 LTD-software image analysis computer system (Cambridge, England) was done at Histology Department, Faculty of Medicine, Cairo University to measure mean area percent of collagen fibres in Masson's trichrome-stained sections and of caspase 3, connexin 43, HSF1 and Hsp70 immuno-expression in the corresponding immunostained sections. Each of these measurements was done in ten non-overlapping fields (×100).

Statistical analysis[27]

All morphometric and biochemical measurements were expressed as mean ± standard deviation (SD). They were statistically analyzed using one-way analysis of variance (ANOVA) followed by "tukey" post hoc test for all measurements except exosomal HSF1 level where independent samples T-test was used. All calculations were done using the IBM Statistical Package for the Social Sciences (SPSS) version 21 the results were considered statistically significant when *P-value* was < 0.05.

RESULTS

General observations

Lethargy was observed in MI subgroups throughout the whole experimental duration and in subgroup MI-^{nHS}E-28, 7 days after induction of MI. No deaths nor abnormal behaviour was observed in any of the experimental animals.

Similar serological, biochemical and histological results were found in the control subgroups so, they were collectively called the control group.

ELISA Results for Exosomal HSF1

Mean level of HSF1 was 8.30±0.50 and 17.56±1.34 ng/ml in $^{non\text{-}HS}$BMMSCs-EXOs and ^{HS}BMMSCs-EXOs, respectively. Statistically, this indicated a significant increase in ^{HS}BMMSCs-EXOs when compared to $^{non\text{-}HS}$BMMSCs-EXOs.

Animal Data

Serological Results (Table 1)

Levels of cardiac enzymes (CPK & LDH) showed a significant increase in all experimental subgroups versus the control group except subgroups MI-^{HS}E-3 & MI-^{HS}E-28. Additionally, there was significant decrease in subgroups MI-28 & MI-^{nHS}E-28 when compared to subgroups MI-3 & MI-^{nHS}E-3, respectively and in subgroup MI-^{nHS}E-3 versus subgroup MI-3. Moreover, there was significant decrease in subgroup MI-^{HS}E-3 versus subgroups MI-3 & MI-^{nHS}E-3 and in subgroup MI-^{HS}E-28 versus subgroups MI-28 & MI-^{nHS}E-28.

QRT-PCR Results for cardiac Bax and miR-34a (Table 1)

Levels of both Bax and miR-34a expressions revealed significant increase in all experimental subgroups except subgroups MI-^{HS}E-3 & MI- ^{HS}E-28 versus the control group. Also, there was a significant decrease in subgroups MI-28 & MI-^{nHS}E-3 versus subgroup MI-3 and a significant increase in subgroup MI-^{nHS}E-28 versus subgroup MI-^{nHS}E-3. Subgroup MI-^{HS}E-3 showed a significant decrease versus subgroups MI-3 & MI-^{nHS}E-3 and subgroup MI-^{HS}E-28 demonstrated a significant decrease versus subgroups MI-28 & MI-^{nHS}E-28.

ELISA Results for HSF1 and Hsp70 in Cardiac Homogenates (Table 1)

Levels of HSF1 & Hsp70 demonstrated a significant increase in subgroups MI-3, MI-^{nHS}E-3 & MI-^{HS}E-3 than control group, subgroups MI-3 and MI-^{nHS}E-3, correspondingly. In addition, there was a significant decrease in subgroups MI-28 & MI-^{nHS}E-28 versus subgroups MI-3 & MI-^{nHS}E-3, respectively. Furthermore, subgroup MI-^{HS}E-28 demonstrated a significant increase than subgroups MI-28, MI-^{nHS}E-28 & control group.

Histological Results

Fluorescent labelled sections

PKH26 labelled BMMSCs-EXOs were absent in control rats received $^{non\text{-}HS}$BMMSCs-EXOs (Figure 1a) and ^{HS}BMMSCs-EXOs (Figure 1b) and present in subgroups MI-^{nHS}E (Figure 1c) & MI-^{HS}E (Figure 1d).

H&E stained sections

In the control group (Figure 2a), the myocardium was formed of longitudinally cut transversely striated cardiac myocytes with central oval pale nuclei. They were joined together by intercalated discs and appeared branching and anastomosing forming muscle sheets. In-between the cardiac myocytes, there was a delicate layer of CT with well-demonstrated blood vessels. In subgroups MI-3 and MI-28 (Figures 2b, 2c), the myocardium demonstrated structural disorganization with features of inflammation and cellular damage that were more obvious in subgroup MI-3. However, widening of the intercellular spaces with active fibroblasts (pale nuclei) and thickened CT was more evident in subgroup MI-28. Sections of subgroup MI-^{nHS}E-3 (Figure 2d) presented minimal features of myocardial lesion. Whereas, sections of subgroup MI-^{nHS}E-28 (Figure 2e) showed marked deterioration of the myocardial histological structure to be similar to that of subgroup MI-28. Nearly normal histological architecture was shown in subgroups MI-^{HS}E-3 and MI-^{HS}E-28 (Figures 2f, 2g), except for the presence of very few shrunken darkly stained nuclei.

Masson's trichrome stained sections

Occasional collagen fibers were shown in the intercellular CT of the control group, subgroups MI-^{nHS}E-3, MI-^{HS}E-3 and MI-^{HS}E-28 (Figures 3a, 3d, 3f, 3g). However, this amount was increased in subgroup MI-3 (Figure 3b) to become abundant in subgroups MI-28 and MI-^{nHS}E-28 (Figures 3c, 3e).

Caspase 3 immunostained sections

The positive immunoreaction was sporadic in the control group (Figure 4a), dramatically increased in subgroup MI-3 (Figure 4b), moderate in subgroups MI-28, MI-^{nHS}E-3 and MI-^{nHS}E-28 (Figures 4c, 4d, 4e) and infrequent in subgroups MI-^{HS}E-3 and MI-^{HS}E-28 (Figures 4f, 4g).

Connexin 43 immunostained sections

Abundant positive immunoreaction was visualized in the control group (Figure 5a), then the reaction was radically reduced in subgroups MI-3, MI-28 and MI-^{nHS}E-28 (Figures 5b, 5c, 5e). In subgroups MI-^{nHS}E-3, MI-^{HS}E-3 and MI-^{HS}E-28, the positive immunoreaction regained its abundance (Figures 5d, 5f, 5g).

HSF1 immunostained sections

Widely spread positive immunoreaction was detected in subgroups MI-3, MI-^{nHS}E-3, MI-^{HS}E-3 and MI-^{HS}E-28 (Figures 6b, 6d, 6f, 6g). This was in contrast to the reduced reaction recognized in the control group, subgroups MI-28 and MI-^{nHS}E-28 (Figures 6a, 6c, 6e).

Hsp70 immunostained sections

The demonstrated positive immunoreaction increased gradually from control group and subgroups MI-28 and MI-^{nHS}E-28 (Figures 7a, 7c, 7e) passing through subgroups MI-3 and MI-^{nHS}E-3 (Figures 7b, 7d) to subgroups MI-^{HS}E-3 and MI-^{HS}E-28 where it appeared in most of the myocytes (Figures 7f, 7g).

Morphometric Results

Mean area percent of collagen fibres (Figure 3h) showed a significant increase in subgroups MI-3, MI-28 and MI-^{nHS}E-28 when compared with the control group and subgroups MI-3 and MI-^{nHS}E-3, respectively. Also, there was a significant decrease in subgroup MI-^{nHS}E-3 versus MI-3 and its significant increase versus the control group. Moreover, a significant decrease in subgroups MI-^{HS}E-3 and MI-^{HS}E-28 compared to subgroups MI-^{nHS}E-3 and MI-^{nHS}E-28, correspondingly was detected. Besides, significant decrease in subgroup MI-^{HS}E-28 versus subgroup MI-28 was reported.

Caspase 3 mean area percent (Figure 4h) revealed similar result to that of the statistical analysis of Bax level.

Regarding the mean area percent of connexin 43 (Figure 5h), it revealed a significant decrease in subgroups MI-3, MI-28 and MI-^{nHS}E-28 when compared with the control group and subgroups MI-3 and MI-^{nHS}E-3, correspondingly. Additionally, subgroup MI-^{nHS}E-3 showed a significant increase than MI-3 and a significant decrease than the control group. Moreover, there was a significant increase in subgroups MI-^{HS}E-3 and MI-^{HS}E-28 versus subgroups MI-^{nHS}E-3 and MI-^{nHS}E-28, respectively. Furthermore, subgroup MI-^{HS}E-28 exposed significant increase versus subgroup MI-28.

Mean area percent of HSF1 and Hsp70 (Figures 6h,7h) revealed results parallel to the statistical results of their biochemical levels.

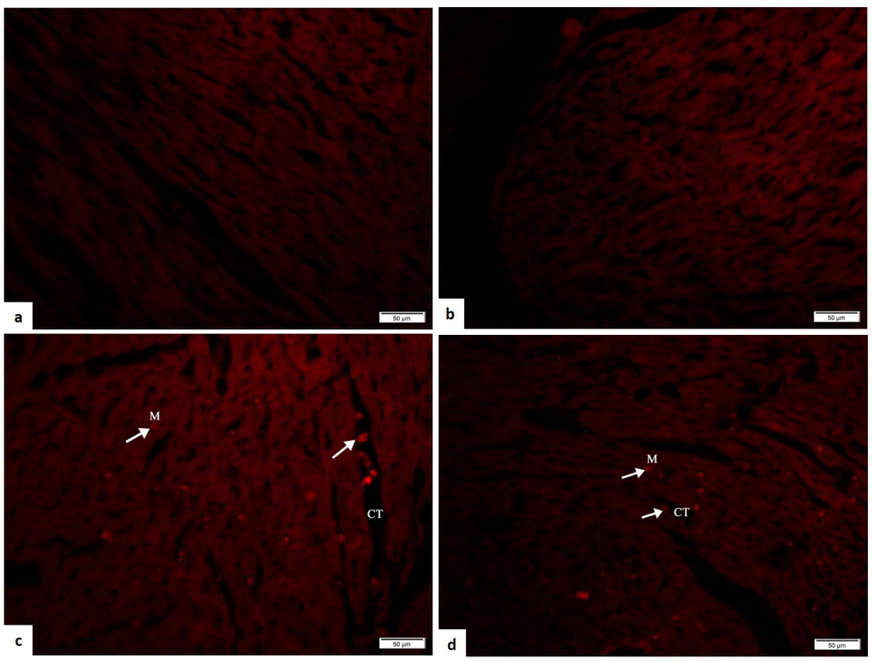

Fig. 1: showing (a & b) absence of PKH26 labeled BMMSCs-EXOs in both control subgroups received [non-HS]BMMSCs-EXOs & [HS]BMMSCs-EXOs. (c & d) presence of PKH26 labeled [non-HS]BMMSCs-EXOs and [HS]BMMSCs-EXOs (arrows) in the connective tissue (CT) and cardiac myocytes (M) of subgroups MI-[nHS]E & MI-[HS]E, respectively. (PKH26, x 200)

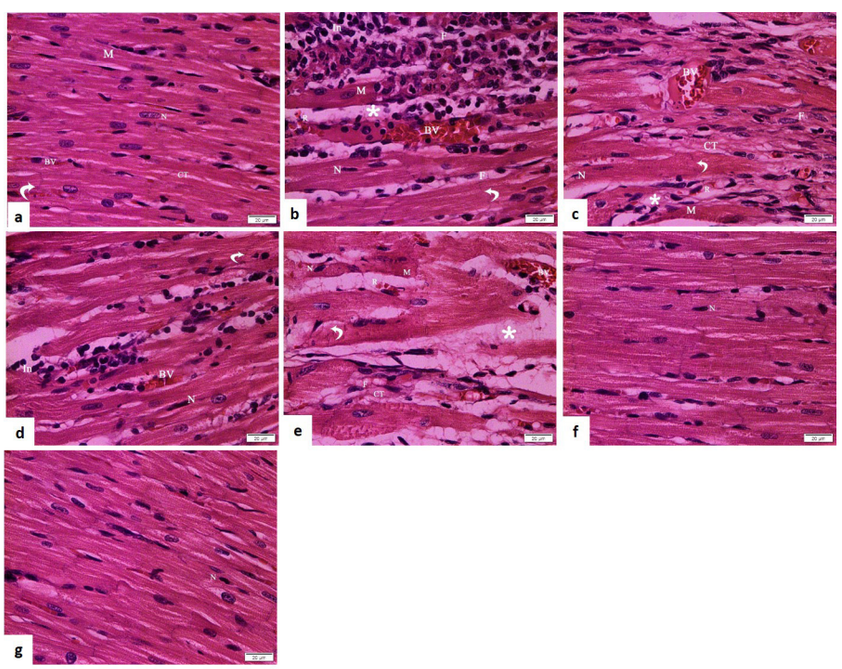

Fig. 2: showing (a) cylindrical branching and anastomosing longitudinally arranged cardiac muscle fibers (M) joined by intercalated discs (curved arrow), exhibiting transversely striated acidophilic sarcoplasm and central oval pale nuclei (N) and separated by delicate CT (CT) with blood vessels (BV) in the control group. (b) widening of the intercellular spaces (star), a dilated congested blood vessel (BV), extra-vasated RBCs (R), mononuclear cellular infiltration (In), active fibroblasts (F), disruption of the intercalated discs (curved arrow), myocytes (M) with deeply acidophilic sarcoplasm and lost striations and others with shrunken darkly stained nuclei (N) in subgroup MI-3. (c) enormous widening of the intercellular spaces (star), obvious CT (CT) thickening, numerous active fibroblasts (F), blood vessels (BV) dilatation and congestion, extra-vasated RBCs (R), disrupted intercalated disc (curved arrow) and few myocytes (M) with deeply acidophilic sarcoplasm and lost striations or shrunken darkly stained nuclei (N) in subgroup MI-28. (d) few shrunken condensed nuclei (N), intact intercalated discs (curved arrow), minimal inflammatory cell infiltration (In) and a dilated congested blood vessel (BV) in subgroup MI-[nHS]E-3. (e) wide intercellular spaces (star), thickened CT (CT), active fibroblasts (F), a dilated congested blood vessel (BV), extra-vasated RBCs (R), disrupted intercalated discs (curved arrow), myocytes (M) with deeply acidophilic sarcoplasm and lost striations, shrunken darkly stained nuclei (N) in subgroup MI-[nHS]E-28. (f & g) very few shrunken darkly stained nuclei (N) in subgroups MI-[HS]E-3 &MI-[HS]E-28. (H&E, x400)

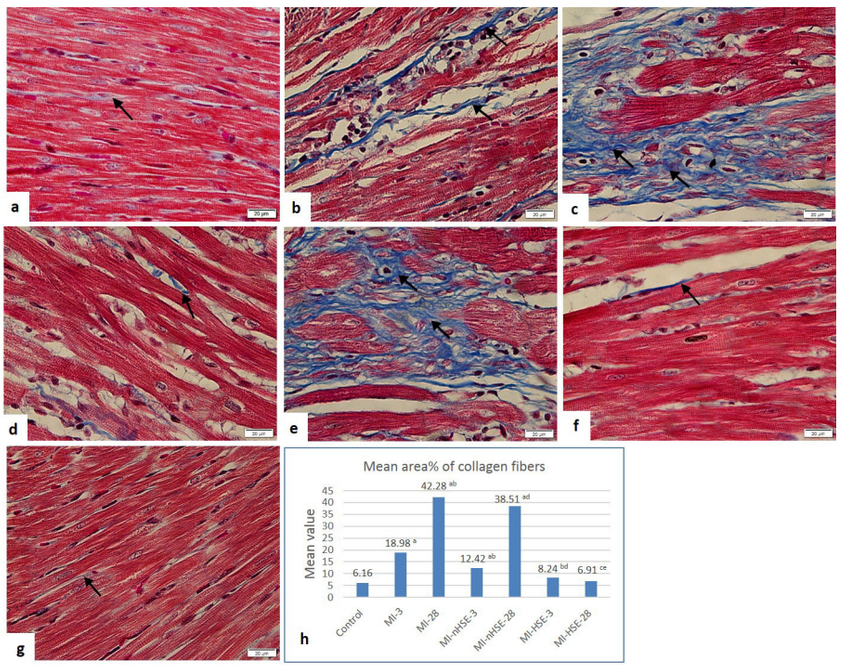

Fig. 3: demonstrating: (a, d, f & g) occasional fine collagen fibers (arrow) in control group, subgroups MI-^{nHS}E-3,MI-^{HS}E-3 & MI-^{HS}E-28. (b) increased collagen fibers content (arrows) in subgroup MI-3. (c & e) copious collagen fibers (arrows) in subgroups MI-28 & MI-^{nHS}E-28. (Masson's trichrome, ×400) (h) showing mean area % of collagen fibers: a, b, c, d &e as compared to control group & subgroups MI-3, MI-28, MI-^{nHS}E-3 &MI-^{nHS}E-28, respectively (significant difference at $P < 0.05$)

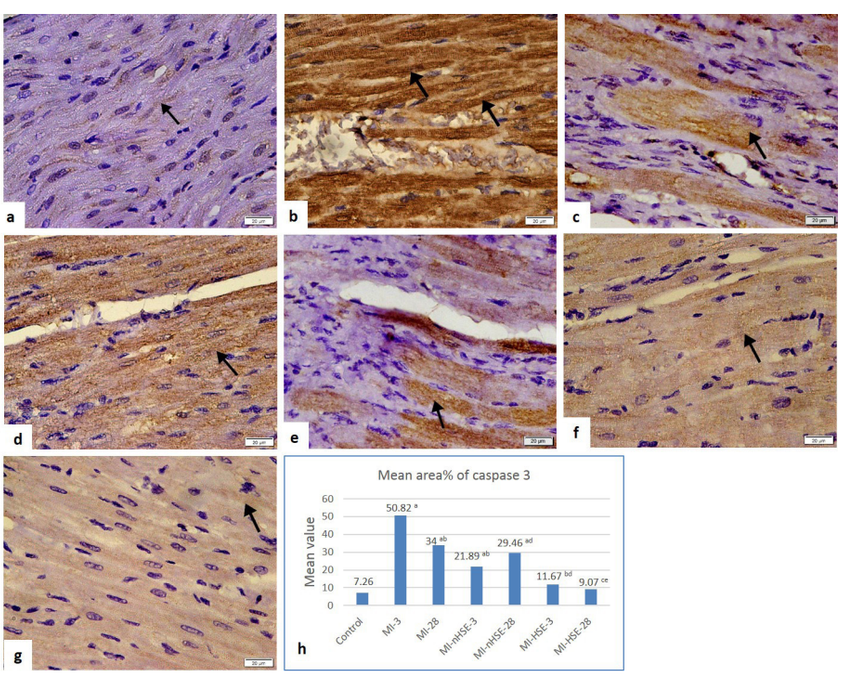

Fig. 4: showing positive cytoplasmic immunoreaction (arrow) in: (a) very few myocytes in control group. (b) almost all myocytes in subgroup MI-3. (c, d & e) some myocytes in subgroups MI-28, MI-^{nHS}E-3 & MI-^{nHS}E-28. (f & g) few myocytes in subgroups MI-^{HS}E-3 &MI-^{HS}E-28. (Immunohistochemical stain for caspase 3, x400) (h) showing mean area % caspase 3: a, b, c, d &e as compared to control group & subgroups MI-3, MI-28, MI-^{nHS}E-3 &MI-^{nHS}E-28, respectively (significant difference at $P < 0.05$)

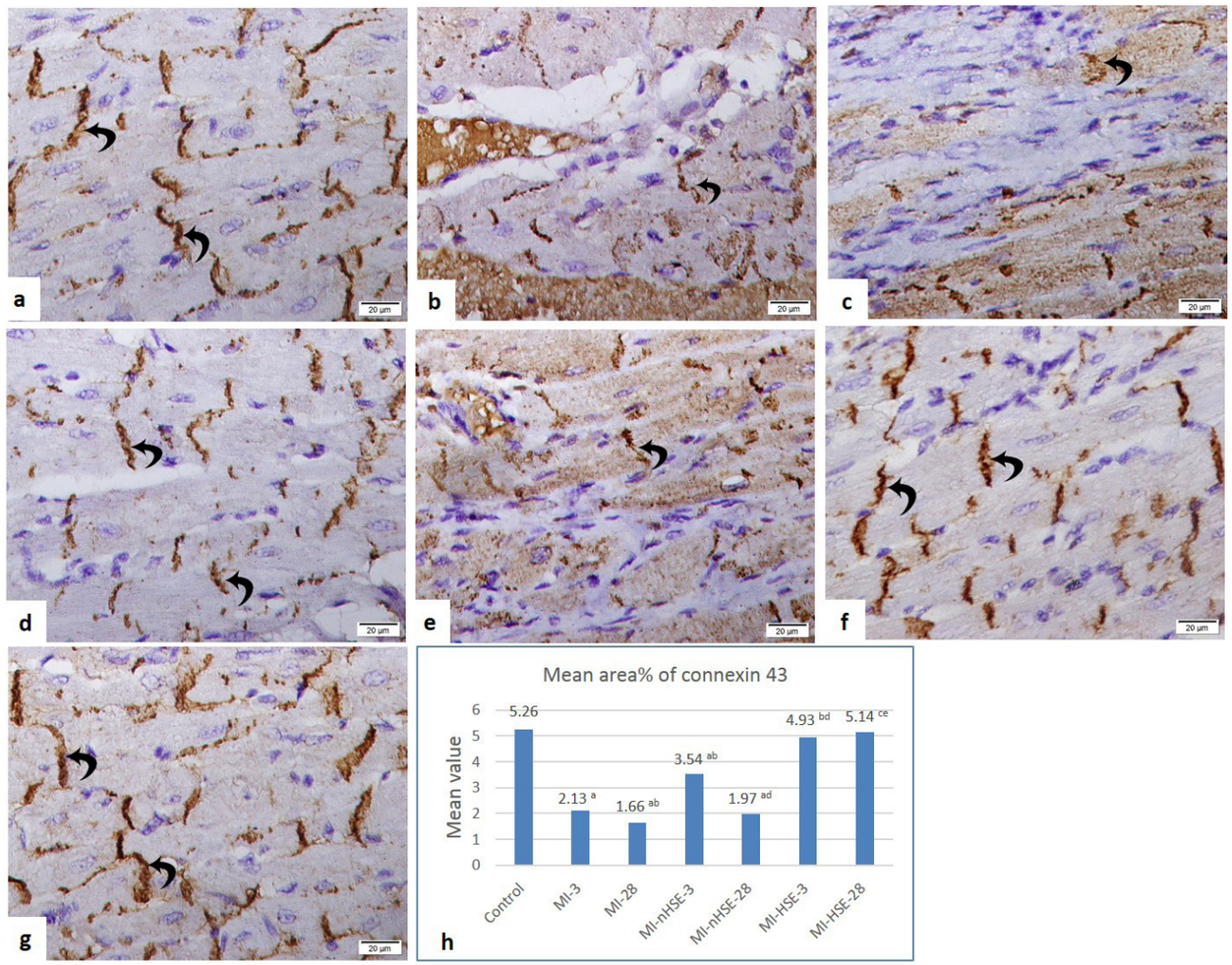

Fig. 5: Showing positive membranous immunoreaction (curved arrow) which is: (a, d, f & g) abundant in control group and subgroups MI-[nHS]E-3, MI-[HS]E-3 and MI-[HS]E-28. (b, c & e) minimal in subgroups MI-3, MI-28 and MI-[nHS]E-28. (Immunohistochemical stain for connexin 43, x400) (h) showing mean area % of connexin 43: a, b, c, d &e as compared to control group & subgroups MI-3, MI-28, MI-[nHS]E-3 &MI-[nHS]E-28, respectively (significant difference at $P < 0.05$)

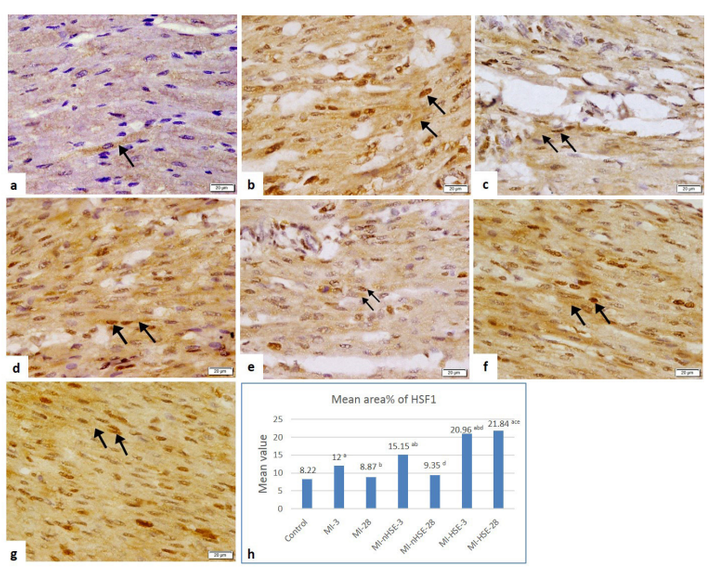

Fig. 6: Revealing positive immunoreactivity (arrow) in: (a) cytoplasm of some myocytes in control group. (b, d, f, & g) nuclei and cytoplasm of most of the muscle fibers in subgroups MI-3, MI-[nHS]E-3, MI-[HS]E-3 and MI-[HS]E-28. (c & e) nuclei and cytoplasm of some of the cardiac myocytes in subgroups MI-28 and MI-[nHS]E-28. (Immunohistochemical stain for HSF1, x400)
(h) showing mean area % HSF1: a, b, c, d &e as compared to control group & subgroups MI-3, MI-28, MI-[nHS]E-3 &MI-[nHS]E-28, respectively (significant difference at $P < 0.05$)

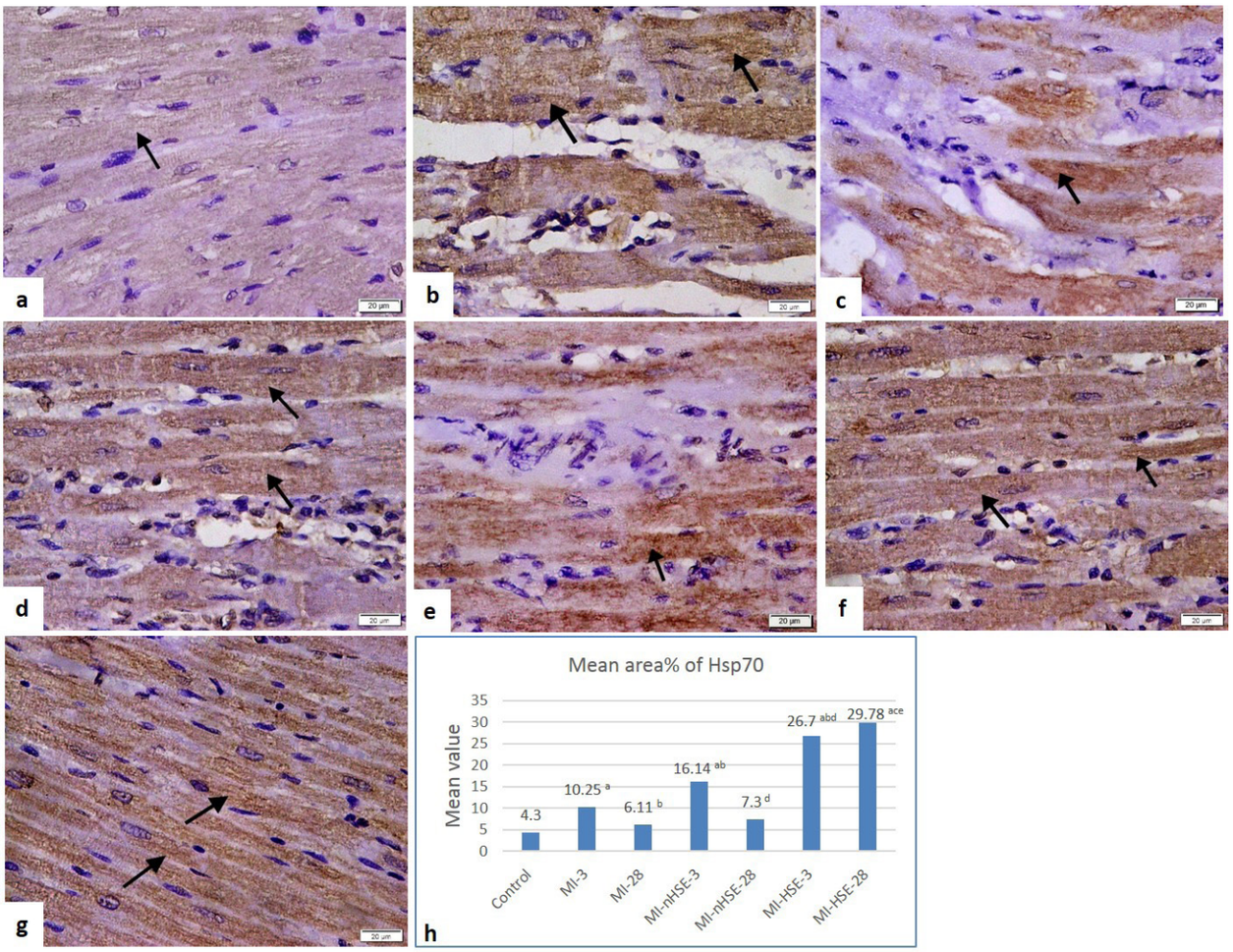

Fig. 7: Illustrating positive cytoplasmic immunoreaction (arrow) in: (a) some myocytes of control group. (b, c, d & e) more muscle fibers in subgroups MI-3, MI-28, MI-^{nHS}E-3 &MI-^{nHS}E-28. (f & g) most of the fibers in subgroups MI-^{HS}E-3 and MI-^{HS}E-28. (Immunohistochemical stain for Hsp70, x400) (h) showing mean area % of Hsp70: a, b, c, d &e as compared to control group & subgroups MI-3, MI-28, MI-^{nHS}E-3 &MI-^{nHS}E-28, respectively (significant difference at $P < 0.05$)

Table 1: Mean value ± SD of biochemical parameters in all groups

	Control group	Subgroup MI-3	Subgroup MI-28	Subgroup MI-^{nHS}E-3	Subgroup MI-^{nHS}E-28	Subgroup MI-^{HS}E-3	Subgroup MI-^{HS}E-28
Serum CPK (U/L)	117.60±10.52	234.10±9.09	184.50±21.56^{ab}	199.9±16.31^{ab}	171.60±14.22^{ad}	131.90±7.74^{bd}	124.30±11.35^{ce}
Serum LDH (U/L)	143.10±13.04	330.00±18.37	211.50±14.75^{ab}	243.70±11.32^{ab}	197.50±15.54^{ad}	157.40±6.87^{bd}	149.80±10.03^{ce}
Cardiac Bax expression	0.16±0.01	1.56±0.15^{a}	1.12±0.10^{ab}	0.74±0.15^{ab}	1.05±0.12^{ad}	0.30±0.03^{bd}	0.20±0.04^{ce}
Cardiac miR-34a expression	1.04±0.14	3.87±0.43^{a}	2.48±0.41^{ab}	1.66±0.05^{ab}	2.17±0.22^{ad}	1.31±0.05^{bd}	1.26±0.07^{ce}
Cardiac HSF1 (ng/mg ptn)	4.96±0.28	7.86±0.52^{a}	5.11±0.47^{b}	9.09±0.61^{ab}	5.66±0.42^{d}	12.89±0.63^{abd}	13.65±0.91^{ace}
Cardiac Hsp70 (pg/mg ptn)	3.20±0.31	8.58±1.11^{a}	3.61±0.40^{b}	11.37±0.94^{ab}	4.00±0.40^{d}	13.84±1.61^{abd}	15.02±1.19^{ace}

[a] $P < 0.05$ as compared to control group
[b] $P < 0.05$ as compared to subgroup MI-3
[c] $P < 0.05$ as compared to subgroup MI-28
[d] $P < 0.05$ as compared to subgroup MI-^{nHS}E-3
[e] $P < 0.05$ as compared to subgroup MI-^{nHS}E-28

DISCUSSION

This work aimed at assessing and comparing the potential therapeutic influence of [non-HS]BMMSCs-EXOs versus [HS]BMMSCs-EXOs on experimentally induced MI (acute and chronic stages) in adult albino rats and underlining the possible explanation for that difference. Male rats were chosen for this study to avoid the protective effect of the female hormone (estrogen) on the cardiac muscle[28].

Heat shock factor 1 (HSF1) is a naturally present inactive cytoplasmic protein that becomes activated by phosphorylation after any cellular stress. The phosphorylated HSF1 (p-HSF1) is translocated to the nucleus where it activates the transcription of Hsp70 gene with a consequent increase in its production[15]. The link between p-HSF1 and Hsp70 was found to be through pro-apoptotic miR-34a where p-HSF1 binds to the promotor region of miR-34a gene preventing its transcription. Recently, miR-34a was known to be a suppressor for Hsp70 gene through blocking of its promotor region. So, suppression of miR-34a was documented to increase the production of Hsp70[17].

Based on that, the hypoxia occurred during the acute stage of MI in the current study with its subsequent release of reactive oxygen species (ROS) and oxidative stress OS[29] demonstrated a significant increase of HSF1 and Hsp70, both biochemically and immunohistochemically, than the control group which was similarly reported for Hsp70[16]. However, this increase in HSF1 was suggested not to be sufficient to suppress the increased miR-34a production occurred secondary to increased P53 protein induced by severe hypoxia[30,31]. This suggestion was enforced by the significant increase in miR-34a level in subgroup MI-3 when compared to control.

This increase in miR-34a together with the persistent lesion led to inevitable myocytes apoptosis documented by deeply eosinophilic sarcoplasm, pyknotic nuclei and a significant increase in caspase 3 area percent and Bax level in subgroup MI-3 versus the control group. Such apoptotic cell death was similarly stated in a previous study[29]. Additionally, there was a significant increase in the serum levels of the cardiac enzymes (CPK and LDH) in this subgroup. This finding could be explained by the cardiac myocytes' membrane damage with subsequent prompt release of the cardiac enzymes to blood resulted from hypoxia-induced OS[32].

Cardiac myocytes death was found to disrupt the cardiac contractility and functionality through disruption of the intercalated discs and their gap junction. This suggestion was enforced by the significant decrease in the area percent of connexin 43 in this subgroup compared to the control group. Further support came from the former study[1] in which left coronary artery ligation was followed by cardiac myocytes apoptosis and cardiac functions deterioration.

Moreover, hypoxia-induced OS is followed by the production of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor (TNF)-α, and initiation of the inflammatory response[33]. This was supported, in subgroup MI-3, by dilatation and congestion of the blood vessels, extravasation of blood, inflammatory cell infiltration and edematous widening of the intercellular spaces with fibroblasts activation. More support was achieved by the significant increase in the area percent of collagen fibres in this subgroup compared to the control group.

In the chronic stage of MI (subgroup MI-28), histological signs of cell death and inflammatory reaction were diminished with the appearance of well-demarcated areas of fibrosis. These findings were furtherly backed by the significant decrease in the area percent of caspase 3 and the levels of Bax and cardiac enzymes and a significant increase in the area percent of collagen fibres versus subgroup MI-3. Such results are concomitant to those previously reported[34] where they were explained by death and phagocytosis of most of the cells and their replacement by fibrous tissue.

The obvious loss of myocardial cells in subgroup MI-28 unsurprisingly resulted into a significant decrease in HSF1, Hsp70 and miR-34a than subgroup MI-3. Additionally, it was demonstrated that HSF1 and Hsp70 were non-significantly increased than the control with a consequent significant increase in miR-34a. Such increase was certainly accompanied by a significant increase in caspase 3 area percent and levels of Bax and cardiac enzymes in this subgroup compared to the control group.

Chronic stage of MI is associated with marked impairment of the myocardial function[35] which could be explained in this work by the evident loss of cardiac myocytes and the widely spread fibrous tissue disrupting the intercalated discs. Such explanation was backed by the significant decrease in the area percent of connexin 43 in subgroup MI-28 versus MI-3.

In the present work, injection of EXOs, produced by either [non-HS]BMMSCs or [HS]BMMSCs was followed by their homing at the site of the lesion, 1 day after MI induction, and their absence in the control rats. This could be explained by their attraction to the site of the lesion via interaction between cardiac myocytes' SDF-1 released during lesion and EXOs' CXCR4[36]. This attraction is followed by EXOs internalization inside the lesioned cells through endocytosis, direct fusion or phagocytosis in certain cells[37] transferring their contents to the recipient cells. These contents are variable according to the EXOs' cellular origin[38].

Subgroup MI-[nHS]E-3 revealed nearly normal histological architecture as EXOs transported their contents derived from [non-HS]BMMSCs to the cardiac myocytes. These contents were shown formerly[1,5,17,21,38] to be anti-apoptotic [B-cell lymphoma 2 (Bcl-2)], anti-inflammatory [interleukin-10 (IL-10)], anti-oxidant [catalase], angiogenic [vascular endothelial growth factor (VEGF)], growth factors [basic fibroblast growth factor and transforming growth factor

(TGF)-α], mRNA, microRNA and minimal amount of HSF1. Exosomal HSF1 was assumed to be added to the endogenous one produced by the stressed myocytes resulting in its significant increase versus subgroup MI-3. This exosomal HSF1, after being activated, causes more blockage of miR-34a gene expression than in subgroup MI-3 and consequently, overexpressed Hsp70. Such genetic modification was supported by the significant decrease in miR-34a level and the significant increase in Hsp70 in this subgroup versus subgroup MI-3.

This exosomal preservation was reinforced by the significant decrease in area percent of caspase 3 and collagen fibres in addition to the levels of Bax and cardiac enzymes in this subgroup compared to subgroup MI-3. However, these parameters were still significantly increased than the control group. This finding could be enlightened by that the HSF1 increase was still not adequate to sufficiently block the marked augmentation of miR-34a production. This explanation was defended, in the current study, by the significant increase in miR-34a level in this subgroup compared to the control group.

Such myocardial histological conservation was predictably followed by cardiac functions preservation. This was backed by the significantly increased area percent of connexin 43 in subgroup MI-^{nHS}E-3 versus subgroup MI-3. Nevertheless, the presence of some cardiac myocytes deaths led to disruption of some intercalated discs and consequently the significant decrease in connexin 43 area percent in this subgroup compared to the control group.

Surprisingly 28 days following MI and $^{non\text{-}HS}$BMMSCs-EXOs (subgroup MI-^{nHS}E-28), there was a marked deterioration of the histological and functional aspects of the myocardium where it appeared similar to those of the chronic fibrotic stage of MI (subgroup MI-28). This was reinforced by the non-significant differences between the two subgroups regarding different biochemical and morphometric parameters. Further support came from a previous study[39] where there was no difference in the cardiac functions (left ventricular ejection fraction, left ventricular volume and infarct size) between placebo and BMMSCs, in chronic cases of MI.

Such deterioration could be illuminated as SDF-1 derived from the stressed myocytes to attract EXOs is declined to its normal level after 3 days[8]. Accordingly, there was no more EXOs at the lesion site and no more trophic factors internalization inside the stressed fibres[21]. Successively, there was no more exosomal support to the lesioned cells and the only support was suggested to come from the endogenously released HSF1 which was documented in subgroup MI-3 to be not sufficient to relieve the hypoxic and oxidative stresses. Additionally, the genetic modification produced by the minimal amount of exosomal HSF1 transferred to the stressed cells during the first 3 days was also proved in subgroup MI-^{nHS}E-3 to be not enough to ameliorate the stresses. Thus, cardiac myocytes started to express the signs of these lesions passing through the acute stage until they reached the chronic fibrotic stage.

Subgroup MI-^{HS}E-3 revealed apparently normal histological and functional pictures of the myocardium more than in subgroup MI-^{nHS}E-3. This was supported by the significant decrease in the area percent of caspase 3 and collagen fibres and in the levels of Bax and cardiac enzymes and the significant increase in the area percent of connexin 43 in this subgroup versus subgroup MI-^{nHS}E-3. Moreover, there were non-significant differences in these statistical results between this subgroup and the control group. More support was obtained from the results of the prior study[17] that stated impressive improvement of the cardiac functions (improved ejection fraction and decreased left ventricular systolic and diastolic dimensions) 1 to 4 weeks following ^{HS}BMMSCs injection in cases of MI.

Such better preservation compared to subgroup MI-^{nHS}E-3 could be explained by the large amount of HSF1 translocated from ^{HS}BMMSCs to the recipient cardiac cells via EXOs. As EXOs' contents varied according to the environmental stimuli to which parent cells are subjected[40]. This explanation was supported by an earlier in vitro study[17] where ^{HS}BMMSCs was proved to produce much more HSF1 in their cytoplasm than $^{non\text{-}HS}$BMMSCs. This clarification was furtherly supported in the current work by the significant increase in ^{HS}F1 level in ^{HS}BMMSCs-EXOs than $^{non\text{-}HS}$BMMSCs-EXOs and in this subgroup compared with subgroup MI-^{nHS}E-3. High level of EXOs' HSF1 was suggested to trigger much more genetic modification in myocardial cells with consequent sufficient decrease in miR-34a level (significantly decreased than MI-^{nH}SE-3 and not significantly increased than the control) and appropriate increase in Hsp70 level (significantly increased than both subgroup MI-^{nH}SE-3 and the control group).

Although ^{HS}BMMSCs-EXOs were no more attracted to the lesion site after 3 days, they efficiently continued to support the myocardium 28 days after MI (subgroup MI-^{HS}E-28). This finding was reinforced by the non-significant difference in the area percent of caspase 3, collagen fibres and connexin 43 and in the levels of Bax and cardiac enzymes in this subgroup versus both subgroup MI-^{HS}E-3 and control group. Similar results were documented previously[17] following ^{HS}BMMSCs transplantation to the hearts with MI for 28 days.

This sustained support could be elucidated by the persistence of the genetic modification induced by the prominent exosomal HSF1 transported to the myocardial cells in the first 3 days. This, in turn, resulted into the significant decrease in miR-34a level and the significant increase in HSF1 and Hsp70 levels in this subgroup versus subgroup MI-^{nH}SE-28 in addition to the non-significant increase in miR-34a level and the significant increase in HSF1 and Hsp70 levels versus the control group.

It could be concluded that both $^{non\text{-}HS}$BMMSCs-EXOs and ^{HS}BMMSCs-EXOs were effective in the conservation of the biochemical, histological and functional aspects

of the left ventricular myocardium in the early stage of MI, which was more obvious with [HS]BMMSCs-EXOs. However, in the chronic stage of MI [non-HS]BMMSCs-EXOs had a non-therapeutic effect on the myocardium, in contrast to the marked curative and conservative effects of [HS]BMMSCs-EXOs. Such flawless preservation was shown to be due to the ability of [HS]BMMSCs-EXOs to manipulate and change the gene expression and the subsequent protein production by the myocardial cells via their high level of HSF1.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

التأثير العلاجي للإكسوسومات المستمدة من الخلايا الجذعية الغير معرضة للصدمة الحرارية مقابل المعرضة للصدمة الحرارية على احتشاء عضلة القلب المحدث تجريبيا في ذكور الجرذان البيضاء البالغين

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المقدمة: يعتبر احتشاء عضلة القلب (MI) في جميع أنحاء العالم سببا رئيسيا للوفيات، حيث يؤدي إلى موت عضلة القلب وضعف وظائف القلب. إن إكسوسومات الخلايا الجذعية الوسيطة للنخاع العظمي (BMMSCs)هي عبارة عن حويصلات خارجة من الخلايا، نانوية الحجم ولها نفس الإمكانيات التعويضية للخلايا الجذعية. وتعتبر الصدمة الحرارية واحدة من الضغوط التي تزيد من إنتاج بروتينات الصدمة الحرارية في الخلايا الجذعية مما يزيد من قدراتها على البقاء.
الهدف من العمل: تهدف الدراسة الحالية إلى تقييم ومقارنة الإمكانات العلاجية للإكسوسومات المستمدة من BMMSCs الغير معرضة للصدمة الحرارية و المعرضة للصدمة الحرارية (^{HS}BMMSCs-EXOs &$^{non-HS}$BMMSCs-EXOs) على احتشاء عضلة القلب المحدثة تجريبيا (المراحل الحادة والمزمنة) مع توضيح التفسير المحتمل لهذا الاختلاف.
المواد وطرق البحث: تم تقسيم سته وخمسون من ذكور الجرذان البيضاء البالغين إلى المجموعة الواهبة (لإعداد ^{HS}BMMSCs-EXOs &$^{non-HS}$BMMSCs-EXOs) والمجموعات التجريبية (الضابطة، &MI-$^{non-}$، MI &MI-^{HS}BMMSCs-EXOs، ^{HS}BMMSCs-EXOs). وقد تم التضحية بأربعة جرذان من المجموعة الضابطة وجرذين من كلا من المجموعتين ^{nonHS}BMMSCs-EXOs& MI-^{HS}BMMSCs-EXOs بعد يوم واحد من MI لتأكيد وصول BMMSCs-EXOs بينما تم التضحية بالجرذان الاخرى بعد ٣ و ٢٨ يومًا. وتم إجراء الدراسات المصلية والكيميائية والنسيجية والقياسات المترية الشكلية.
النتائج: اظهرت المرحلة الحادة من احتشاء عضلة القلب تحلل الخلايا وملامح التهابية بينما أظهرت المرحلة المزمنة تغيرات ليفية ملحوظة. وقد تراجعت هذه التغييرات في مجموعات MI-^{nonHS}BMMSCs-EXOs و-^{HS}BMMSCs EXOs باستثناء تلك التي تنتمى لمجموعة MI-^{nonHS}BMMSCs-EXOs وتم التضحية بجرذانها بعد 28 يومًا.
الاستنتاج: كان لكلا من ^{nonHS}BMMSCs-EXOs و ^{HS}BMMSCs-EXOs قدرات علاجية في المرحلة الحادة من MI والتي كانت أكثر وضوحًا مع ^{HS}BMMSCs-EXOs.و في المرحلة المزمنة، لم يظهر أي تأثير علاجي لل ^{nonHS}BMMSCs-EXOs على الرغم من التأثير المثالي لل ^{HS}BMMSCs-EXOs.