The Effect of Bone Marrow Mesenchymal Stem Cells on Experimentally-Induced Gastrocnemius Muscle Injury in Female Albino Rats

Original Article

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ABSTRACT

Objective: Skeletal muscle injury has become an important condition to treat. With traumas increasing and poor health outcome it has become important to find a suitable treatment to enhance the regeneration of this tissue. Many treatment options are available but there is still no best technique to treat the skeletal muscles in a rapid and effective way. Bone marrow-derived mesenchymal stem cells (BM-MSCs) need to be demonstrated to be able to do this in a very well way.

Aim: To evaluate the therapeutic potential of BM-MSCs in treatment of gastrocnemius muscle injury after 14 days.

Material and Methods: Fifteen female rats were randomly divided into three groups:

1. Group I (Control Group)
2. Group II (Gastrocnemius muscle injury without BM-MSCs local injection)
3. Group III (Gastrocnemius muscle injury with BM-MSCs local injection)

The muscle specimens were processed for the light microscope, transmission electron microscope examination, and real time-quantitative polymerase chain reaction (RQ-PCR) assay for Y-chromosome, and CM-Dil.

Results: Group II showed destruction of the muscle fibres and replacement with fibrous tissue. There was depletion of the cytoplasmic content. Group III showed regeneration of the muscle fibres with minimal amount of fibrous tissue. The nuclei of the muscle fibres regained their normal architecture and bridging between the muscle fibres remained. The BM-MSCs become part of the muscle fibres as proved by CM-Dil.

Conclusion: The results confirmed that BM-MSCs are a great option for the use in skeletal muscle regeneration. They were capable of both accelerating the healing process and allowing the skeletal muscle to repair with health new muscular tissue.

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Key Words: BM-MSCs, CM-Dil, skeletal muscle injury.

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INTRODUCTION

Most injuries of the muscle are either bruises or sprains making up-to 90% of all injuries[1]. A quick hit to the muscle induces a bruise[2], in the other hand a sprain is formed by pulling out the muscle more than it can cope. In every condition of injury, pain is always an outcome. The pain can interrupt daily activities and sports related work[3] Because of the high variation between the injuries and each other, many studies need to be performed[4], so as to identify the most appropriate for each injury[5].

The variation in the mechanism of injury is vast, and so is the different therapy options[6]. The therapy options generally are directed towards fixing muscular tissue as quick as possible. These include:

1. Stopping of movement followed by rehabilitation[7],
2. Non-steroidal anti-inflammatory drugs[8],
3. Corticoids[9],
4. Therapeutic ultrasound[10],
5. Laser therapy[11], and
6. Growth factors[12].

Although a lot of research is done the optimum therapeutic option is still not known.

The mesenchymal cells (MSCs) have the ability to regenerate damaged tissue via paracrine molecules that allow cell to cell cross talk and thus balance out the inflammatory process and help the progenitor cells inside the tissue to develop and transform. These molecules include growth factors, morphogens, and chemokines)[13,14]

This ability of these cells allows them to reduce inflammatory reactions and also fibrosis whilst enhancing the development of blood vessels and new tissue formation.

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They provide a favorable region for regeneration of the tissue by its own self[15].

In this work, we studied the effect of bone marrow-derived mesenchymal stem cells (BM-MSCs) on reducing the damage of experimentally-induced gastrocnemius muscle injury in female albino rats and its regenerative potential on the injury.

MATERIAL AND METHODS

The study was carried on twenty healthy adult albino rats (fifteen females and five males). Female albino rats had an average age between twelve to fifteen weeks and weighed from 180 to 200 grams. Male albino rats had an average age between two to three weeks and weighed from 30-50 grams. The male albino rats were only a source of stem cells.

The animals were housed at the Animal House, Faculty of Medicine, University of Alexandria. They were allowed to acclimatize, and were maintained under standard laboratory conditions of temperature, humidity, food, and water. Guidelines for care and use of animals, approved by the Research Ethics Committee, Faculty of Medicine, University of Alexandria was followed.

The animals were randomly divided into three group:[16–20]

1. **Group I:** Control Group: (n=5 adult female albino rats) serving as control without gastrocnemius muscle injury.
2. **Group II:** Experimental Control Group: (Gastrocnemius muscle injury without BM-MSCs local injection) (n=5 adult female albino rats). The purpose of this group is to assess the possibility for spontaneous regeneration. They were sacrificed fourteen days after gastrocnemius muscle injury.
3. **Group III:** Experimental Group: (Gastrocnemius muscle injury with BM-MSCs local injection) (n=5 adult female albino rats). They were sacrificed fourteen days after gastrocnemius muscle injury and stem cell injection.

Isolation of bone marrow derived mesenchymal stem cells[21–23]

The five male rats were sacrificed by overdose anesthesia (100 milligram per kilogram phenobarbital), sprayed with 70% ethanol, and under a Class II biosafety cabinet, for the collection of bone marrow.

The process included the following:

a. A rat was positioned on its dorsal back on a clean blotting sheet and thoroughly sprayed with 70% ethanol for disinfection.

b. An incision was made in each hind leg using blunt-end sterile scissors. Skin was firmly grasped and gently pulled downwards to expose the muscles.

c. The hind limbs were cut just above the hip joint using sharp and sterile dissecting scissors, ensuring that the epiphysis remains intact without exposing off its contents to outside.

d. The hind leg was cut just below the knee-joint through ligaments to remove off the tibia, ensuring that the epiphysis remains intact.

e. The femur and tibia were dissected from the surrounding muscles and all excess tissue was removed using sterile forceps and scissors, keeping the ends of the bone intact.

f. The four dissected intact bones were soaked in 70% ethanol in a sterile petridish for 2 minutes for disinfection, then transferred to phosphate buffer saline (PBS) (PBS, Lonza, Belgium) in a sterile petridish for rinsing off ethanol, and finally placed in culture medium in a sterile petridish for flushing the bone marrow.

g. Bone marrow flushing: the proximal and the distal ends of both tibiae and femurs were clipped and removed. The exposed bone marrow was flushed by inserting a 23-gauge needle attached to a 5 millilitre syringe with complete culture medium (CCM). CCM consisted of low glucose Dulbecco's modified Eagle's medium (LG-DMEM) (1.0 gram per litre glucose, Lonza) supplemented with 20% fetal bovine serum (FBS, HyClone), 2 mM L-glutamine, 1% penicillin, streptomycin (P/S, 10000 IU/ml penicillin per 10000 µg/ml streptomycin, Lonza).

h. The cells were re-suspended and aspirated. The cell suspension was filtered through a 70 millimetre filter mesh to remove any bone spicules or muscle and cell clumps then was centrifuged at 1200 rotations per minute for 5 minutes.

i. Bone marrow cells were cultured in CCM in 25 centimetre squared tissue culture flask (T25) and incubated in the carbon dioxide incubator at 37oC in a humidified atmosphere with 5% carbon dioxide and 95% oxygen (cells cultured were labeled as primary culture or Passage 0; P0).

j. Bone marrow cells were cultured in growth media and incubated in a carbon dioxide incubator at 37oC in a humidified atmosphere with 5% carbon dioxide and 95% oxygen.

k. Two days after culture initiation, the medium was discarded, and the adherent cells were washed two to three times with PBS and fed with a fresh complete medium. During the expansion period, the medium is replaced two times per week in BM -MSCs till reaching confluency.

Induction of skeletal muscle injury

Female albino rats of Groups II and III were subjected

to experimentally-induced gastrocnemius muscle injury of the left hind limb[16]. The location of the injury was determined in all animals at the largest diameter of the muscle[16,24]. The injury was induced by a curved artery forceps repeated three times across the muscle for 20 seconds each at a constant pressure gauge[16,24,25].

Injection of bone marrow derived mesenchymal stem cells

Immediately after injury, female albino rats of Group III were injected BM-MSCs locally into the injured skeletal muscles. The dose of injected cells was approximately 2.5x106 cells[16,24].

Assessment of the effect of bone marrow derived mesenchymal stem cells on gastrocnemius muscle regeneration

The gastrocnemius muscle of each rat was crossly divided into four pieces:

a. The first piece was fixed in 10% formol saline and processed to get 6 µm thick paraffin sections. These sections were stained with Haematoxylin and Eosin (H&E) stain[26] and Gomori's trichrome stain[27] for light microscopic examination.

b. The second piece was cut into small cubes of 0.5 to 1 millimtre cubed, fixed in 3% glutaraldehyde solution and processed for transmission electron microscopic examination[28].

c. The third piece was immediately cut and put at -80oC for assessing the expression of Y-chromosome using the Real-time PCR.

d. The fourth piece was placed in 4% formol saline and processed to get 6 µm thick paraffin sections. These sections were stained with Hoechst 33342 fluorescent nuclear stain (Thermo Fisher Scientific, Cat # 62249; Excitation / emission wave lengths 361/ 497 nm). The sections were examined by confocal laser scanning microscopy (Leica TSC SPE II/ DMi 8).

RESULTS

In In vitro part of the study

1. Morphological characterization of BM-MSCs

The BM-MSCs were identified after two weeks in culture with an inverted microscope as spindle shaped cells (Figure 1).

2. Immunophenotyping of BM-MSCs by flow cytometry

The flow cytometric analysis of cell-surface markers of BM-MSCs at passage three, using antibody against BM-MSCs marker (CD90) and another one against hematopoietic antigen (CD45) showed that, 94.5% of cells were positive for anti-CD90, while 0.5% only of cells were positive for anti-CD45 (Figure 2).

II. In vivo part of the study

1. Light microscopic results

a. Hematoxylin & Eosin stain

i. Group I (Control Group)

Light microscopic examination of sections of the rat gastrocnemius muscles of the control group revealed the normal appearance of skeletal muscle fibres. In the longitudinal sections, the muscle fibres were cylindrical in shape and parallel to each other with eosinophilic sarcoplasm (Figures 3a, b). The bundles were separated by the connective tissue of endomysium (Figure 3b).

The control group showed multiple flattened pale nuclei which were peripherally situated beneath the sarcolemma (Figure 3b). The nuclei of the fibroblast appeared as slit like (Figure 3a, b).

ii. Group II (Experimental Control Group)

On examination of sections of the gastrocnemius muscles of this subgroup, some muscle fibres appeared shrunken (Figures 4a, b, c). Collagen fibres deposition was extensive (Figure 4b). Haemorrhage remained persistent. Cellular infiltration was also evident (Figure 4b).

iii. Group III (Experimental Group)

On examination of sections of the gastrocnemius muscles of this group, muscle fibres seemed to be more or less regular. The newly formed fibres seemed to have regained most of their striations. The fibres were more or less straight, parallel and regular trying to regain their full normal architecture similar to control pattern (Figures 5a, b, c).

Some areas still showed some bridging points between the muscle fibres (Figures 5a, b, c). Some nuclei of the muscle fibres appeared to be internalized and pale while other nuclei remained peripheral and dark (Figure 5c). Residual cell infiltrates were also still present (Figure 5a).

b. Gomori's Trichrome stain

i. Group I (Control Group)

Examination of the trichrome stained sections of the gastrocnemius muscles of the control group revealed scanty amount of collagen fibres in the endomysium with longutidinal arranged muscle fibres (Figure 6).

ii. Group II (Experimental Control Group)

On examination of the gastrocnemius muscles of this group, evident increase of collagen distribution was observed, where a large amount of collagen fibres was seen in between the injured muscle fibres and in the endomysium (Figures 7a, b).

iii. Group III (Experimental Group)

On examination of the gastrocnemius muscles of this group, almost control pattern of collagen distribution was

observed, where a scanty amount of collagen fibres were seen in between the regenerated muscle fibres. There were bridges between the muscle fibres (Figure 8).

2. Ultrastructural results

i. Group I (Control Group)

Electron microscopic examination of the ultra-thin sections of the gastrocnemius muscle of the control group showed skeletal muscle fibres formed mainly by myofibrils. These myofibrils were composed of myofilaments that were arranged in a specific ordered organization responsible for the striated pattern of the muscle fibres. These striations were composed of alternating dark and light bands that occurred in register across the whole muscle fibres (Figures 9a, b, c).

The dark band (A band) was bisected by a paler H band, which itself was bisected by a dense M line (Figures 9a, b). The light band (I band) was bisected by a dense line called Z line (Figure 9a).

The muscle fibre appeared invested by the sarcolemma with sub-sarcolemmal elongated nuclei (Figures 9a,b,c). The sarcoplasm at the poles of the nuclei contained numerous mitochondria (Figures 9a, b). In addition, the mitochondria were also seen in the spaces between the myofibrils (Figures 9a, b).

The transverse tubules (T-tubules) were depicted at the A-I junctions. Each T-tubule was surrounded by two terminal cisternae from the sarcoplasmic reticulum forming a triad (Figure 9b).

ii. Group II (Experimental Control Group)

Examination of the gastrocnemius muscle of rats of this group showed fragmentation, thinning out of the myofilaments (Figures 10a, b). There was widening of the spaces between the myofilaments with loss of the cytoplasmic content (Figure 10a). Cytoplasmic depletion was also apparent under the sarcolemma (Figure 10b).

The sarcolemma appeared corrugated (festooning) in some parts, indicating loss of its normally smooth nature (Figure 10b).

iii. Group III (Experimental Group)

Examination of the gastrocnemius muscle of rats of this subgroup showed more or less normal myofibrils (Figures 11a, b). Their arrangement was regular. The nucleus appeared return to normal state with prominent nucleolus (Figure 11a).

The terminal cisternae were seen to return to normal state although some were still dilated (Figure 11b). Most of all the sarcolemma was normal in structure but a small part was scalloped (Figure 11b).

These findings were similar to the control group.

3. Real-time quantitative polymerase chain reaction (RQ-PCR): for detection of Y-chromosome:

RQ-PCR results showed that Y-chromosome was expressed in female rats injected by male BM-MSCs (Group III) and not detected in Group I. The cycle threshold (cT) at which Y-chromosome amplification was detected, was at 34.69 in Group III.

4. Fluorescence micrograph for detection of CM-DiI labelled mesenchymal cells

Fluorescence micrograph results showed that CM-DiI labelled MSCs were present in female rats injected with CM-DiI labeled MSCs of Group III.

Fluorescence micrograph of the rat skeletal muscle Group III showed a low number of CM-DiI labeled MSCs in the connective tissue stroma (6 Mean ± SD for N° of Dil signals / microscopic field x 200 in connective tissue stroma) and a high number in muscle fibres (15 Mean ± SD for N° of Dil signals / microscopic field x 200 in muscle fibres) (Figures 12a, b).

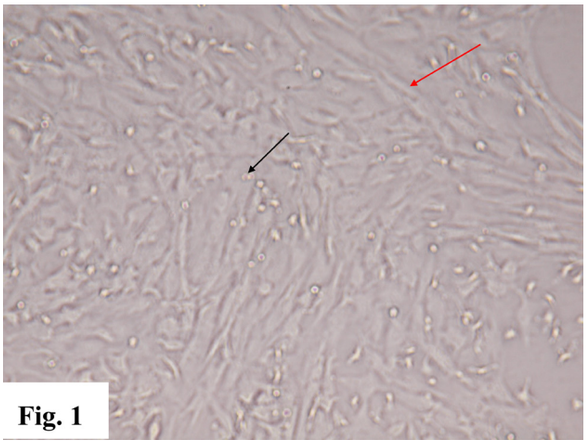


Fig. 1

Fig. 1: Primary culture, with 80% confluence. Mitotic cell is also noticed (black arrow). Spindle shaped cells (red arrow). (Inverted phase contrast microscope, Mic. Mag x200).

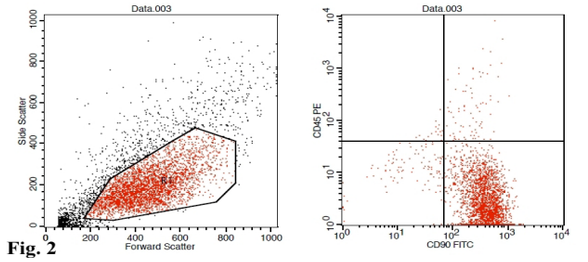


Fig. 2

Fig. 2: Scattered plot chart for FACS analysis for BM-MSCs at P3 showing, anti CD90 cells (BM-MSCs) represent 94.5% (lower right quadrant) while cells positive for anti CD45 only represent 0.5% of population (upper left quadrant).

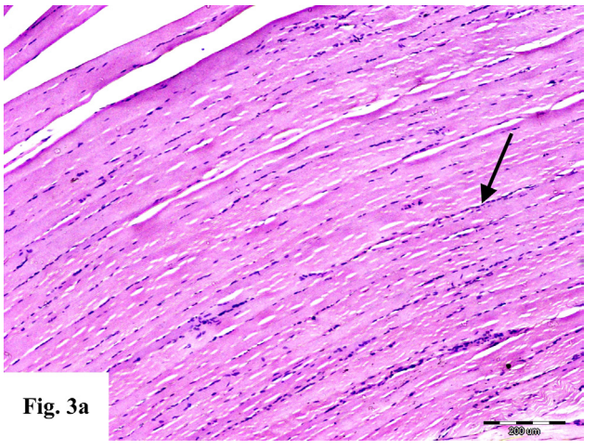


Fig. 3a

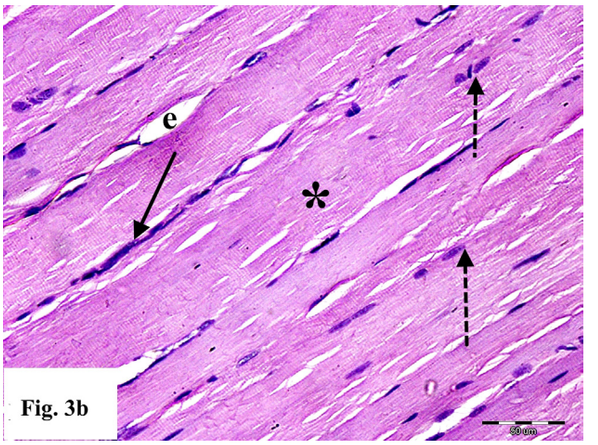


Fig. 3b

Fig. 3a, b: Light photomicrograph of normal control rat skeletal muscle fibres demonstrating: (a) Closely arranged longitudinal muscle fibres with narrow interstitium showing fibroblasts with slit- like nuclei (black arrow). (b) The skeletal muscle fibers demonstrate fine cross striations (*) with peripherally located ovoid pale nuclei (dashed arrows). the narrow interstitium is visible(endomysium, e). (H&E stain. Mic. Mag. (a)x100,(b)x200).

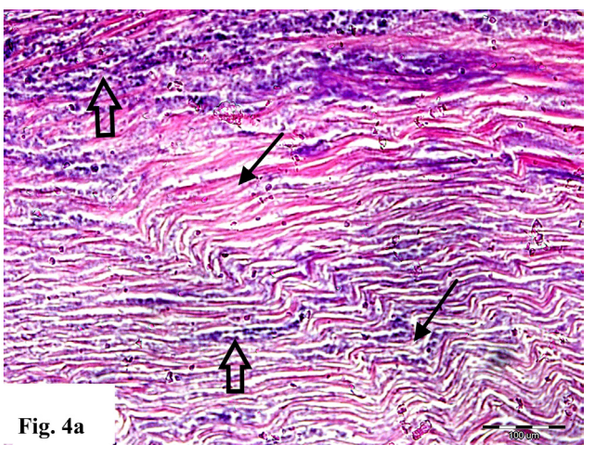


Fig. 4a

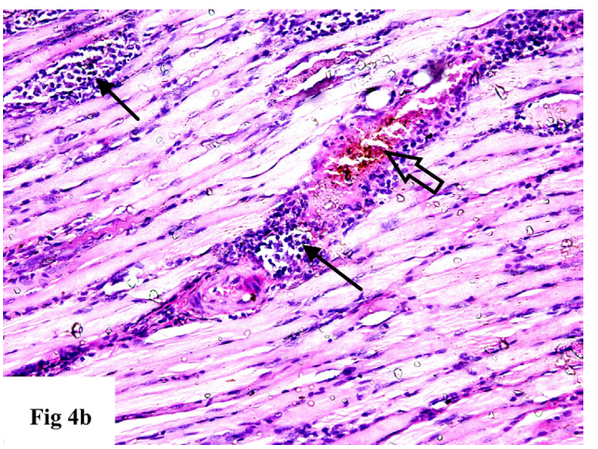


Fig 4b

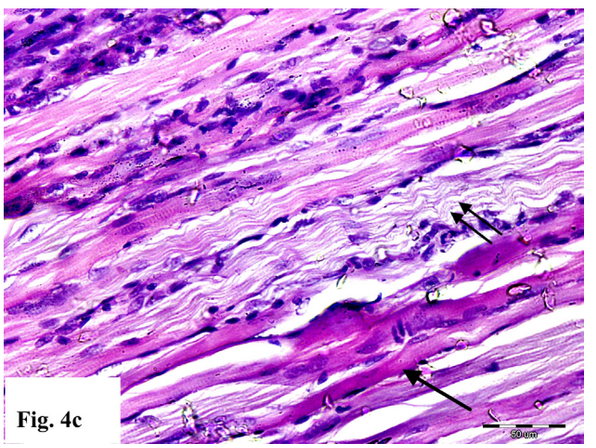


Fig. 4c

Fig. 4 a, b, c: Light photomicrograph of rat gastrocnemius muscle 14 days after experimentally induced crush injury showing: (a) closely arranged corrugated thin muscle fibres (black arrow) with cellular infiltrates at some foci (hollow arrow). (b) Another section from the same group demonstrating the connective tissue in the perimysium between bundles of muscle fibres infiltrated by hemorrhages (hollow arrow). The muscle fibres are closely arranged with persistent cellular infiltration of variable degrees (black arrow). (c) High power view of the muscle fibres depicting individual shrunken eosinophilic muscle fibres (single arrow) alternating with paler less compact muscle fibres with failure to recognize the characteristic cross striations (double arrows). (H&E stain. Mic. Mag. (a) x100, (b)x200, (c)x400).

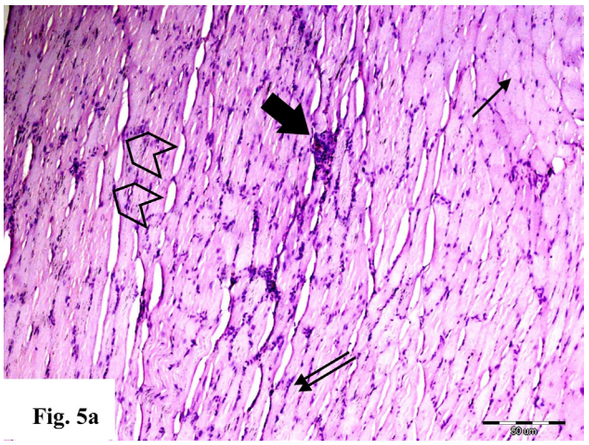


Fig. 5a

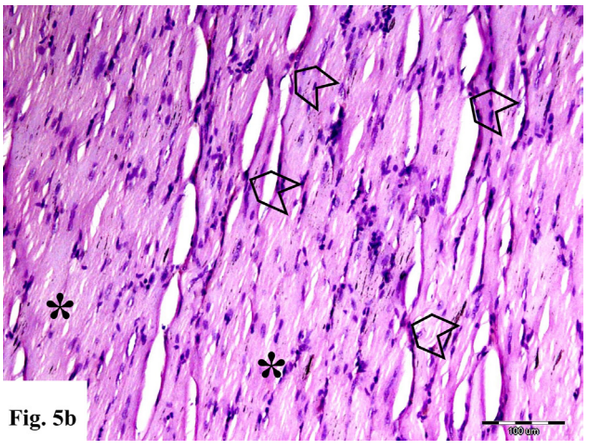


Fig. 5b

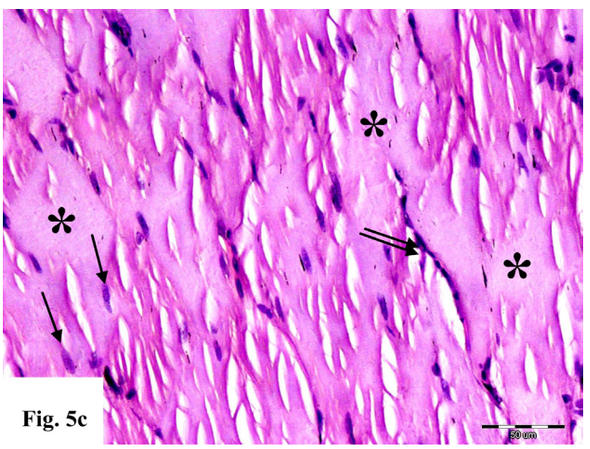


Fig. 5c

Fig. 5 a, b, c: Light photomicrograph of rat gastrocnemius muscle 14 days after experimental induction of crush injury followed by local injection of BM-MSCs. (a) The muscle fibres appear closely organized and in register where some fibers are longitudinally arranged (double arrows) while others show polyhedral transverse cross sections (black arrow). Nuclei of residual cell infiltrate is depicted at one focus (thick black arrow). Note the arrow heads pointing to bridging between muscle fibres. (b): Another section from the same group demonstrating the points of bridging between individual muscle fibres (arrow head). Fusion between fibres is also observed forming large bulky muscle fibres (*). (c): Higher magnifications of the muscle fibers illustrating fusion between fibres forming large bulky muscle fibres (*). Note the internalized oval pale nuclei of the muscle fibres (black arrows) and the chain of dark nuclei at the peripheral boundary of individual muscle fibres (double arrows). (H&E stain. Mic. Mag. (a)x100, (b)x200, (c)x400).

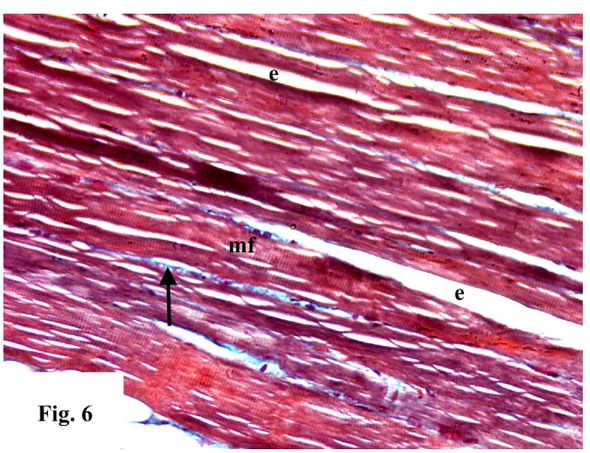
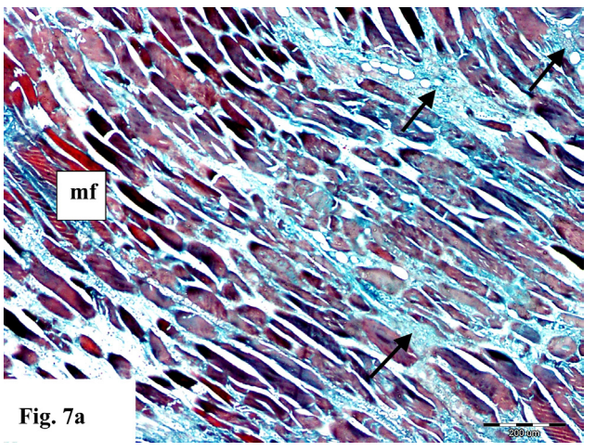


Fig. 6

Fig. 6: Light photomicrograph of rat normal control gastrocnemius muscle showing closely arranged longitudinal muscle fibres with cross striations separated by narrow endomysium composed of minimal greenish collagen fibres (arrow). (Gomori's trichrome stain. Mic. Mag. X100).



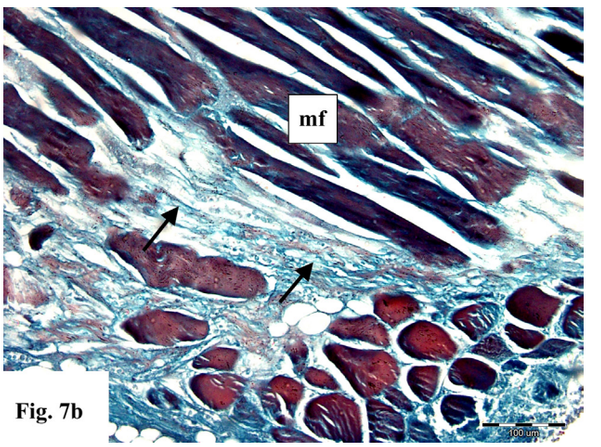


Fig. 7 a, b: Light photomicrograph of rat gastrocnemius muscle 14 days after experimentally induced crush injury. (a) Widespread collagen fibres deposition (arrows) forming bundles between the disrupted muscle fibres (mf). (b) Bundles of collagen fibres (arrow) replace the lost muscle fibers (mf). (Gomori's trichrome stain. Mic. Mag. (a)x100, (b)X200)

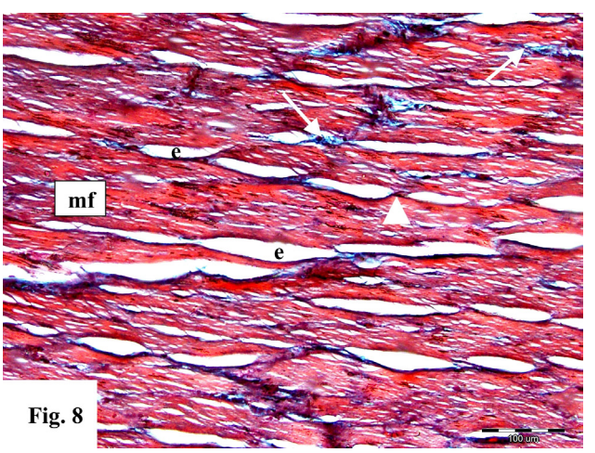


Fig. 8: *Light photomicrograph of rat gastrocnemius muscle 14 days after experimentally induced crush injury followed by local injection of BM-MSCs. Minimal collagen fibres (white arrows) are depicted in the narrow endomysium (e). Note the fused muscle fibres (mf) and inter fiber bridges (white arrow head). (Gomori's trichrome stain. Mic. Mag. X200).*

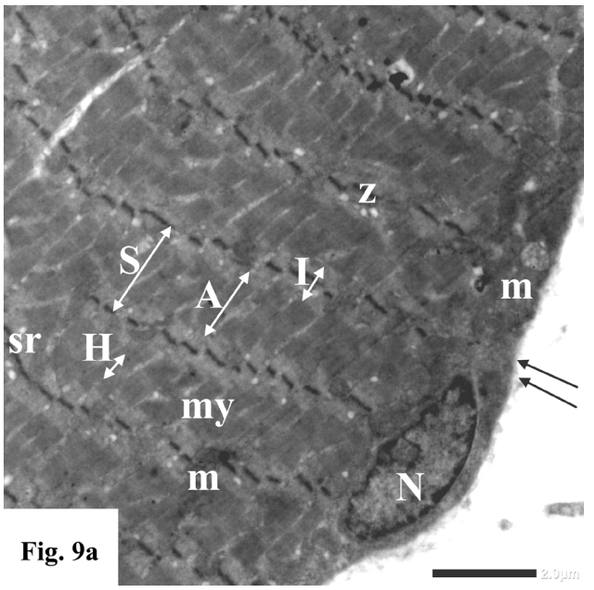


Fig. 9a

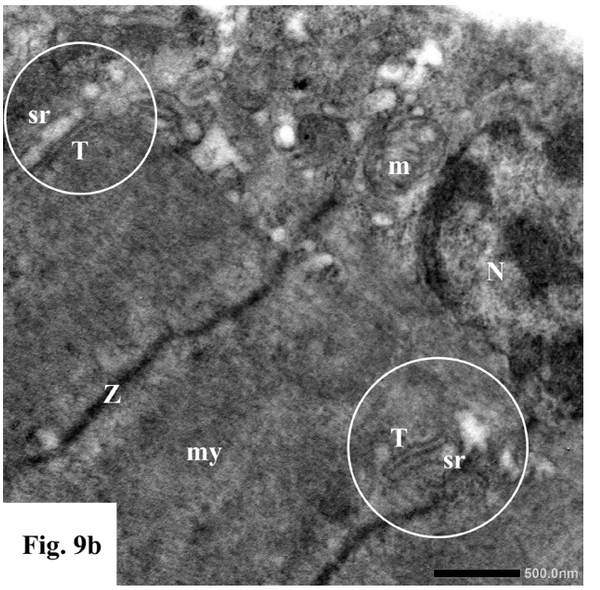


Fig. 9b

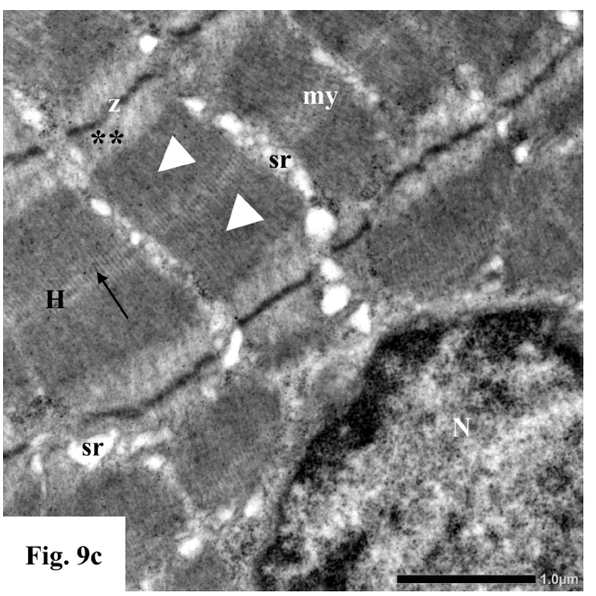
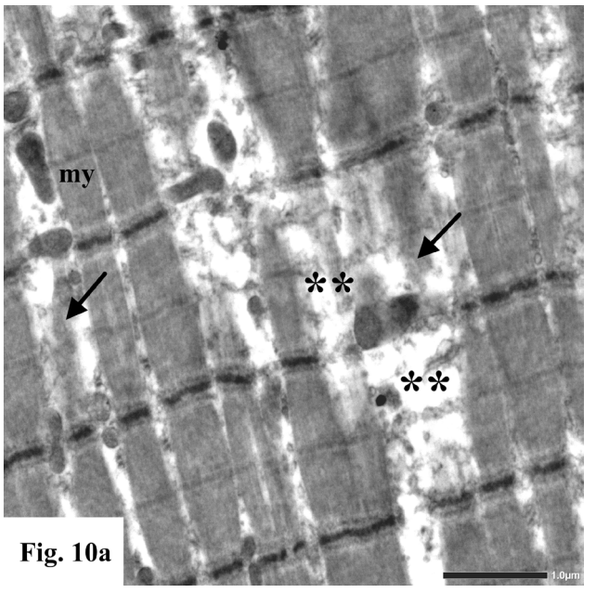


Fig. 9c

Fig. 9 a, b, c: Transmission electron micrograph (TEM) of normal control rat gastrocnemius skeletal muscle fibre. (a) Regularly arranged myofilament (my) and the intervening vesicles of sarcoplasmic reticulum (sr) are seen. The sarcomere is formed of isotropic light bands (I) crossed Z lines (Z) in register with darker anisotropic bands (A) that include a paler H zone (H). A vesicular nucleus (N) is located at the periphery of the muscle fibre just underneath the sarcolemma (double arrows). m= mitochondria. (b) High power of Fig. (9a) demonstrating the triad of T-tubules (T) and sarcoplasmic reticulum (sr) (white rounded circle). (c) Higher magnification of normal sarcomeres illustrating the alternation between light bands (**) and dark bands (white arrow heads), the H zone intersected by the M line (arrow). N= nucleus, sr= sarcoplasmic reticulum. (Lead citrate / uranyl acetate stain. Mic. Mag. (a)x3000, (b)x10000, (c)x8000).



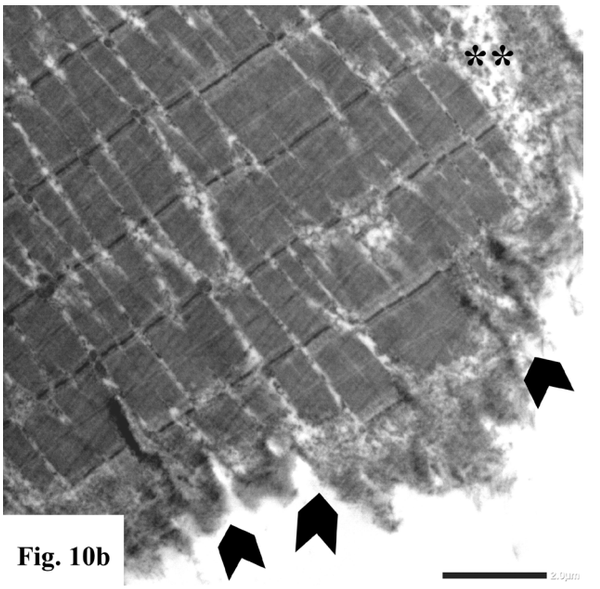
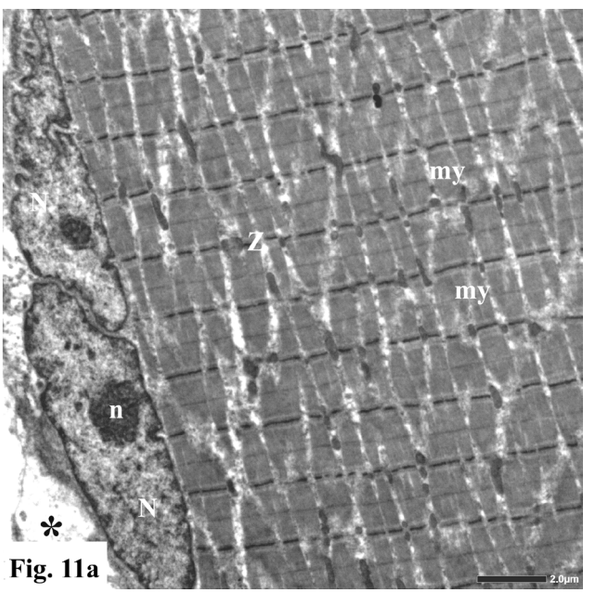


Fig. 10 a, b: TEM of rat gastrocnemius muscle fiber 14 days after experimental induction of crush injury. (a) Myofilament appear fragmented (my) with Z lines in registered order. Other myofilaments are discontinuous and thinned out (arrows). Wide spaces are seen between the myofilaments with depletion of cytoplasmic content (**). (b) Evident festooning of the sarcolemma (black arrow heads). Note foci of cytoplasmic depletion under the sarcolemma (**). (Lead citrate/ uranyl acetate stain. Mic. Mag. (a)x6000, (b)x3000).



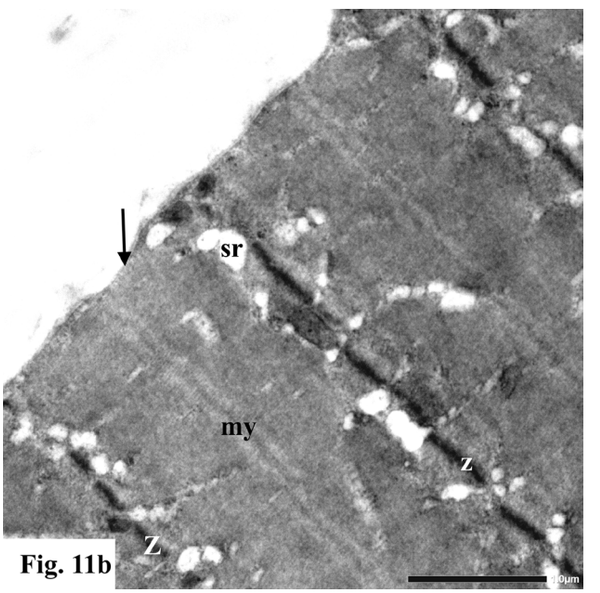


Fig. 11 a, b: TEM of rat gastrocnemius muscle fibers 14 days after experimental induction of crush injury followed by local injection of BM-MSCs. (a) A muscle fibre is showing a chain of peripheral intact vesicular nuclei (N) with prominent nucleoli (n). Note the persistent scalloped sarcolemma (*). (Z)= z lines, (my)= myofilaments. (b) Another muscle fibre from the same experimented group showing restoration of myofilaments (my), Z lines (Z) and sarcolemma (arrow). Note the sarcoplasmic cisternae (sr) trying to return to normal size (Lead citrate/ uranyl acetate stain. Mic. Mag. (a)x2000, (b)x8000).

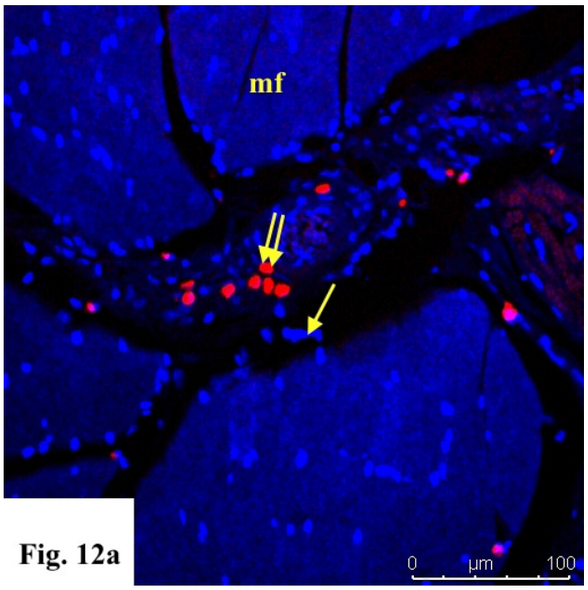


Fig. 12a

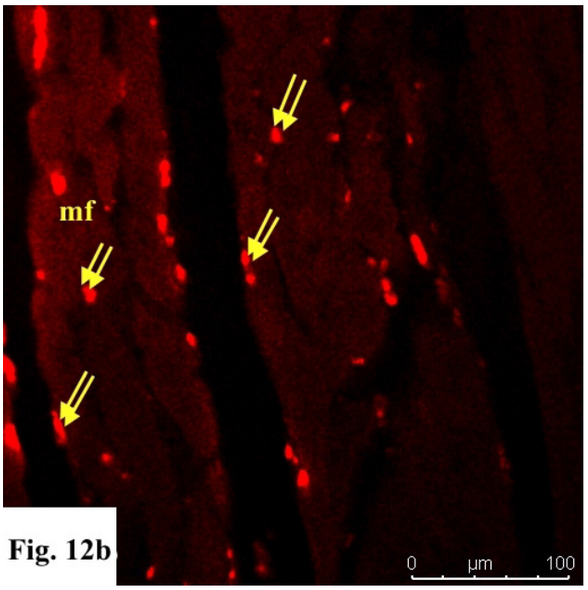


Fig. 12b

Fig. 12 a, b: Confocal Laser Scanning photomicrograph of rat gastrocnemius muscle 14 days after experimental induction of crush injury followed by injection of Dil labeled BM-MSCs. (a) The interstitium between the transverse sectioned muscle fibers (mf) shows few nuclei with red signals (double yellow arrow) among nuclei with blue signals (single yellow arrow). (b) Strong red signals (double yellow arrows) are depicted in the nuclei of cells demarcating the outer border of longitudinal muscle fibers (mf) as well as within the fibres. (a) Overlayed fluorescent mode; (b) Non overlayed fluorescent mode. (a, b) Hoechst nuclear stain with emission at 488 nm wavelength, Dil tracker with emission at 500 nm wavelength. Mic. Mag. X200).

DISCUSSION

The incredibly fast research which is performed by stem cell has become the new path to solve the problems of treating diseases which have no current therapy option.The ability of these cells to transform into other cells has made them the number one option for regenerative medicine[29]. This has caused many research work to be done towards the treatment of a wide array of diseases. At the time being, MSCs are the most used as they can be obtained from different tissues and have the potential to multiply well outside the body[30].

Damage to skeletal muscles occurs mainly in sports and needs to be well treated. This damage can lead to loss of function and many diseases. Reaching as high as half of all sport related injuries skeletal muscle damage affects professional athletes and also non[31].

Deasy *et al*[32] found that sex differences is important in skeletal muscle diseases and that women have a better regenerating power as compared to men. That is why the present study used female rats as the injury model. Other studies used male rats.

In this study, the BM-MSCs were transplanted directly into the injured gastrocnemius muscle. Hocking *et al*[33] also used this technique, and we both found that this allowed both cell to cell interaction and some cells can differentiate to adult cells[33]. Thus BM-MSCs can act also by bringing other cells to this site[34]. The concentration of the accumulated cells is thus high[35].

The number of cells injected into the tissue was chosen on the basis of other research work which showed that the smallest amount of cells needed to preform regeneration inside skeletal muscles is 1 million. In our present study we used 2.5 million cells which is higher so we can ensure optimum results.

Kaariainen *et al*[36] and Li and Huard[37] mention that the damage of skeletal muscle is followed by an inflammatory reaction. In this reaction neutrophils and macrophages are the primary cells. Tidball[38] then continues to state that this response can last a few weeks. Jarvinen and Sorvari[39] explain that the dead tissue is scavenged and then new tissue is formed after 3 days of the damage, beginning with satellite cell activation. The MSCs in the skeletal muscle still are being investigated (Jackson *et al*[39]). MSCs transplanted directly after the trauma are thought not to be able to help new tissue formation due to the microenvironment being highly stressful. Winkler *et al*[40] and this present study had other findings to portray.

After the induction of the muscular damage, a lot of active molecules are released inside the tissue and neutrophils start entering the muscles reaching their highest levels at around 1 day post injury[41]. Then the neutrophils have the ability to call macrophages by releasing another set of bioactive molecules as interleukin-6 (IL-6)[42]. The macrophages transform into their M1 type. Both these calls then start to release other molecules as nitric oxide and also prostaglandins. The main one of these molecules is tumor necrosis factor-α (TNF-α). This molecule can remain for up-to two weeks inside the tissue and its presence up regulates the expression of its receptor. TNF-α has a vital function of stimulating satellite cells to start to proliferate.

TNF-α can also help transforming cells to their adult forms[43].

On the other hand transforming growth factor-β (TGF-β) plays a role on the matrix of the skeletal muscle. TGF-β can accelerate the process of fibrosis and also maturation of skeletal muscles. So in case of increased level of TGF-β is a disadvantage as the end result will be extensive fibrosis which will lead to muscle weakness and loss of function[44]. In Group II the inflammatory process continues down the path to form fibrous tissue.

Just like TGF has an important role, IL-6 also has one. It increases post muscle damage. It helps invite the macrophages to the site of the skeletal muscle damage which further increase the level of IL-6. After helping propagate the inflammatory reaction a feedback mechanism is initiated with the increase of level of C-reactive protein (CRP) which then helps to stop the inflammatory process by inhibiting circulating monocyte transformation and also inhibiting macrophages bioactive material production[45].

The case of injecting BM-MSCs into the damaged skeletal muscle these molecules including TGF-α and -β, IL-6, and other are depreseed while some others as IL-10 can be increased[46].

Most of the immune cells including B and T cells and macrophages can be inhibited post MSC injection. This is proved in both animal and culture studies. Some studies mention that there is activation of some cells as CX3CR1lo/Ly-6C blood monocytes which can release chemical mediators to enhance the inflammation as an intermediate process at first[42] CD68 inflammatory (M1) cells as they are called appear as an intermediate stage and they are switched on by TNF-α, they undergo a swap in nature by these molecules IL-4, IL-10 and IL-13, to become the new (M2) cells. These cells are known for their ability to improve regeneration of tissue. We can see this in Group III where the regenerative process is evident. In the process of regeneration these macrophages appear to help regeneration then disappear again.

Regeneration and fibrosis are thought to happen at the same time and battle with each other[47]. According to one study MSCs transplanted after 7 days from the injury reduced fibrosis the most. MSSC transplanted after 4 days had a better effect as compared to 1 day after the injury. This study was trying to prove the later dates had better effects[48]. This was not the case in our study were an immediate injection had a promising effect on Group III.

TGF-β as mentioned before has the ability to enhance fibrosis of the tissue. Wagatsuma[49] has shown together with other studies that this molecule reaches its high most 2-3 days after the injury to the skeletal muscle. In case of some studies mentioned if BM-MSCs are transplanted before the injury most of the cell turn to fibroblasts and enhance fibrosis of the muscle and loss of its function[37].LI *et al* explains this by exposure of the stem, cells to the high levels of TGF-β which is a hostile environment for these cells[50]. In Ota *et al*,[48] he explains the fact by comparing day 1, 4, and 7 injection of the cells and saying that at day 1 most cells became fibroblasts. Ota *et al*[48] was trying to prove this point. In the case of this study these findings were not corresponding to the results where it was found that even an immediate injection of BM-MSCs after the injury had an excellent outcome in reducing fibrosis and enhancing the regenerative process. It can be seen at ease in both are LM and EM photomicrographs.

In some studies as Andrade *et al*[51] BM-MSCs also did not help in reducing fibrosis. It was stated that the end result was always fibrosis and the connective tissue matrix was not reduced. Carvalho *et al*[52] and Quintanilha *et al*[53] tested this on liver tissue and found that fibrosis was not reduced. Pecanha *et al*[54] whom used skeletal muscle tissue also had an extensive scar . Lee *et al*,[55] Terada *et al*,[56] and also Park *et al*[57] then came up with the solution to add antifibrotic agents which was successful in their case. The amount of fibrous tissue in Group III as compared to Group II decreased proving that BM-MSC had the ability to reduce fibrosis in the absence of an antifibrotic agent. This is seen clearly in both the H&E and the trichrome photomicrographs.

These results may be different from the results of other studies, that found that BM-MSCs (von Roth *et al*[58] and Winkler *et al*[40]), adipose MSCs, embryonic stem cells (Ninagawa *et al*[59]), umbilical cord MSCs (Grabowska *et al*[60]) and skeletal muscle MSCs (Meligy *et al*[61]) contribute to muscle regeneration and improve muscle force after injury. These findings agreed with this study.

Regeneration of skeletal muscle is accompanied with the nucleus being first in the center then taking an external position[62]. This is found in the LM photomicrographs of Group III as a clear finding. Thus, the number of fibres with centrally located nuclei represented early myoblastic cells. Then the fibres starting regaining their mature forms[63] Addition of BM-MSCs increases the number of cells with central nucleus and thus activates regeneration. This technique helps to enhance the number of building blocks needed to build up the tissue again. This shows these cells either enhance stem cells already present in the tissue or they themselves can become integrated within the tissue. Also vascular new formation can be one factor which increases the blood supply to the tissue and thus accelerates regeneration[64]. In some studies as Andrade *et al*,[51] the number of cells having a nucleus in the middle was found to be less. This could be confusing but the fact that the test observed the changes after 28 days could be explained by the fact that the muscle fibres had already differentiated.

Satellite cells have the ability to proliferate[65]. At the time of muscle damage these satellite cells transform from their quiet sleeping phase to highly active cells. Some of them stick to the damaged fibers[66]. In the case of Winkler *et al*,[67] and also in this study, MSCs were identified in the interstitium of the injured muscles up to 2 weeks

after transplantation which is in an indicator that some of the stem cells can fuse directly to the cells while others can enhance the effect of locally present satellite cells to accelerate the regeneration.

T-tubules can be affected by glycerol efflux and influx. This can end the network to become vacuolated and separated from each other[68].

This affection can sometimes return to normal alone[69] No other organelle is thought to be affected. There are other diseases where a similar condition may be seen[70] As a result of these changes a lot of process that are performed by the T-tubules are affected[70]. These steps include returning to normal state after excessive exercise, cell volume balance and transportation of substances to and from the cell[71]. In the present study similar pathology was seen in the T-tubule network which dilated as part of the skeletal muscle injury and was seen in Group II. In Group III they tried to return to normal size.

In contrast to Natsu *et al*,[72] we did find transplanted cells in host muscle tissue, as described by Corbel *et al*[73] and Dezawa *et al*[74]. In the present results this is proved by both the positive reaction seen in the CM-Dil slides were the DAPI/Dil photomicrographs continued to show a positive reaction at Group III. This indicated the presence of the BM-MSCs till 14 days after injection inside the tissue. Also the RQ-PCR insured these findings.

Over viewing the results of the present study, it was found that 2.5x106 bone marrow mesenchymal stem cells produces a regenerating effect on the damaged skeletal muscle up to 14 days after injury.

The reversibility of the damage is proved by the presence of the stem cells. Although the presence of many treatment options for skeletal muscle injury, stem cells showed clearly their beneficial effects in all aspects of recovery enabling the muscle to regain its structure in a very short time and to a very high degree. This will make bone marrow mesenchymal stem cells one of the best choices for regeneration of tissues in the future and a first line therapy especially in severe skeletal muscle injuries.

CONCLUSION

After the accomplishment of the current work, it could be concluded that:

1. Skeletal muscle injury induces an inflammatory reaction which continues till 14 days.
2. This inflammatory reaction leads to the development of fibrous tissue and the damage of many cells.
3. Intramuscular injection of BM-MSCs in the injured tissue induces regeneration of the muscle which is visible after 14 days.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

الفعل المؤثر للخلايا الجذعية المستمدة من نخاع العظم على الإصابة المحدثة تجريبيًا للعضلة التوأمية عند إناث الفئران البيضاء

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المقدمة: تعتبر إصابات العضلات من المشكلات الشائعة التي يواجهها الأطباء. وتزداد مخاطر إعادة الإصابة بعد إجهاد العضلات، في حالة عدم الشفاء الكامل. وغالبًا ما تكون الإصابات المتكررة أشد من الإصابة الأولية، وتؤدي إلي فترات طويلة بعيدة عن النشاط. وبالتالي، ركزت العديد من الدراسات جهودها علي التدخلات العلاجية لتعزيز شفاء العضلات بعد التعرض للإصابة.

وتشكل الخلايا الجذعية نهجًا واعدًا في علاج إصابة العضلات الهيكلية. أما الخلايا الجذعية الميزنكيمية المستمدة من نخاع العظام، فهي أكثر جدوي وجاذبية للتجارب القبل سريرية والسريرية.

ويجري حاليًا استخدام الحقن الموضعي للخلايا الجذعية الميزنكيمية المستمدة من نخاع العظام، ويتم تجربته في العديد من الأمراض، ويؤدي استخدامه إلي نتائج واعدة. وأيضاً، هناك حاجة إلى إجراء المزيد من الدراسات والأبحاث للتأكد من تلك التأثيرات.

الهدف من البحث: هدف العمل الحالي في دراسة التغيرات المحدثة تجريبيًا على إصابة العضلة التوأمية عند إناث الفئران البيضاء، ووضع نهج في مجال الزراعة التجريبية للخلايا الجذعية الميزنكيمية المستمدة من نخاع العظام في العضلة التوأمية لإناث الفئران البيضاء، وأيضًا هدف في استكشاف الإمكانية التجديدية الممكنة للخلايا الجذعية الميزنكيمية المستمدة من نخاع العظام المزروعة، وأثرها علي علاج إصابات العضلة التوأمية.

مواد وطرق البحث: وتضمنت مواد وأساليب العمل الحالي عشرون من الفئران البيضاء البالغة ذات الصحة الجيدة. ومنها، خمسة عشر إناث بمتوسط عمر بين 15-12 أسبوع وتزن من مائة وثمانون إلي مائتين جرام (من 180 إلي 200 جرام) وخمسة ذكور يبلغ أعمارهم من 3-2 أسابيع ويبلغ وزنهم من ثلاثون الى خمسون جرام (من 30 إلى 50 جرام). ولقد تم الإستعانة بذكور الفئران في هذه الدراسة لجمع نخاع العظام حيث، يمثل مصدر مختلف للخلايا الجذعية الميزنكيمية، وبالتالي اقتفاء أثر تمايز تلك الخلايا بتفاعل البوليميراز المتسلسل بالزمن الحقيقي.

وتم تعيين 3 مجموعات من إناث الفئران؛

المجموعة I: مجموعة المراقبة: (العدد = 5 فئران بيضاء إناث بالغة) تعمل بمثابة مجموعة مراقبة وهي خالية من إصابة العضلة التوأمية.

المجموعة II: مجموعة المراقبة التجريبية: (تعاني من إصابة في العضلة التوأمية من دون الحقن الموضعي للخلايا الجذعية الميزنكيمية المستمدة من نخاع العظام) (العدد = 5 فئران بيضاء إناث بالغة). إن الهدف من تلك المجموعة هو تقييم إمكانية التجديد التلقائي. تم قتل فئران التجارب بعد أربعة عشر يوم من إصابة العضلة التوأمية.

المجموعة III: المجموعة التجريبية: (تعاني من إصابة في العضلة التوأمية مع الحقن الموضعي للخلايا الجذعية الميزنكيمية المستمدة من نخاع العظام) (العدد = 5 فئران بيضاء إناث بالغة). تم قتل فئران التجارب بعد أربعة عشر يوم من إصابة العضلة التوأمية وحقن الخلايا الجذعية.

تم عزل الخلايا الجذعية الميزنكيمية وتمييزها وزراعتها وتمريرها في المعمل. ولقد تم القيام بجميع تلك العمليات في معمل الخلايا الجذعية في مركز التميز في أبحاث الطب التجديدى وتطبيقاته، بكلية الطب، جامعة الإسكندرية. بعد التمرير الثالث وبمجرد انتشارها في الوسط بنسبة 80%-70، تم حقن الخلايا الجذعية الميزنكيمية في العضلة التوأمية عند إناث الفئران البيضاء بمجموعة III.

تم القيام بتمييز الخلايا الجذعية الميزنكيمية كالآتي:

1. الدراسة اليومية للشكل الخارجي للخلايا المنزرعة باستخدام مجهر ضوئي ذو أطوار متباينة مقلوب.
2. تحليل قياس التدفق الخلوي لعلامات سطح الخلايا المنزرعة.

تم قتل فئران التجارب بعد مرور فترة زمنية معينة أثناء الدراسة بعد التخدير بالإثير. تم أخذ عينات حديثة من العضلة التوأمية في جميع المجموعات التي تم دراستها. وتم تحضيرها للدراسات التالية:

1. دراسة مجهرية:

أ. صبغة هيماتوكسيلين ايوسين.

ب. صبغة جوموري (بروتكول صبغ ثلاثي الألوان).

2. الدراسة بالمجهر الإليكتروني: تم دراسة مقاطع رفيعة جدًا باستخدام المجهر الإلكتروني النافذ.
3. تفاعل البوليميراز المتسلسل بالزمن الحقيقي: لاكتشاف كروموسوم واي.
4. صبغة CM-DiI (صبغة الفلورسنت الحمراء).

النتائج: اتضح من الفحص النسيجي لمجموعة II (التي تعاني من إصابة في العضلة التوأمية من دون الحقن الموضعي للخلايا الجذعية الميزنكيمية المستمدة من نخاع العظام) وجود نزيف، وخلايا فجوية، وارتشاح التهابي خلوي، وتليف.

اتضح من صبغة جوموري (بروتكول صبغ ثلاثي الألوان) زيادة ألياف الكولاجين.

أكدت دراسة البنية المستدقة علي النتائج المذكورة أعلاه وأيضًا اتضح منها بلاعم الألياف العضلية الموزعة ذات الفجوات السيتوبلازمية، وتطاريف غشاء الليف العضلي، ونضوب السيتوبلازم.

أوضحت دراسة مجموعة III للعضلة التوأمية للفئران (التي تعاني من إصابة في العضلة التوأمية مع الحقن الموضعي للخلايا الجذعية الميزنكيمية المستمدة من نخاع العظام) تنكس أقل، ومزيد من الألياف التجددية، ووصل بين الألياف، ونواة أكثر في منتصف ألياف العضلة.

واتضح بعد 14 يوم، وجود ألياف الكولاجين ذات المستويات الدنيا عند استخدام صبغة جوموري (بروتكول صبغ ثلاثي الألوان).

أوضحت الصورة المجهرية الكترونية للعضلة التوأمية للفئران في مجموعة III، وجود ألالياف العضلية التجددية، وعودة الميتوكوندريا تقريبًا لتشكيلتها العادية، والزيادة النووية.

أيضا تم استخدام صبغة CM-DiI (صبغة الفلورسنت الحمراء) في مجموعة III واتضح أن هناك انتقال للخلايا الجذعية من سدى الأنسجة الضامة، لتصبح جزء من الألياف العضلية، وبالتالي يتم توجيه الخلايا بنجاح.

هناك المزيد من الدلائل فيما يتعلق بتوجيه الخلايا الجذعية الميزنكيمية في العضلة التوأمية المصابة. وقد تم البرهنة علي ذلك من خلال التعبير الجيني للكروموسوم واي لنماذج الفئران الإناث التي تم حقنها. ومن الممكن أن يؤدي تمايز تلك الخلايا الى تجديد العضلات الهيكلية، وذلك من خلال تمييزها أو من خلال تأثيرات باراكرانية لها.

الاستنتاج: ولقد أوضحت النتائج الحالية فعالية الخلايا الجذعية الميزنكيمية في معالجة إصابة العضلات الهيكلية وفعالية الخلايا الجذعية الميزنكيمية المستمدة من نخاع العظام في تجديد العضلات الهيكلية وتقليل التليف.