Original Article

Histological Study on Possible Regenerative Effect of Bone Marrow Derived Mesenchymal Stem Cells versus Platelet Rich Plasma in Skeletal Muscle Injury Model in Adult Male Albino Rat

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ABSTRACT

Introduction and Aim of Work: Skeletal muscle injuries are one of the most common injuries.As the complete recovery is compromised due to fibrosis. This study was designed to evaluate and compare the possible regenerative effects of bone marrow-mesenchymal stem cells (BM-MSCs) versus platelet rich plasma (PRP) in skeletal muscle laceration injury in adult male albino rat model.

Materials and Methods: Forty four adult rats were divided into 4 groups: Group I (Control)=12 rats, Group II=12 rats subjected to right gastrocnemius muscle laceration and subdivided into subgroup IIA=4 rats sacrificed at day0, subgroup IIB (recovery)=4 rats sacrificed at day 7, subgroup IIC (recovery)=4 rats sacrificed at day 14. Group III=10 rats (local IM injection of 1ml PBS containing 1 million BM-MSCs) subdivided into subgroup IIIA=4 rats sacrificed at day 7, subgroup IIIB=4 rats sacrificed at day14. Group IV=8 rats (local IM injection of 1 ml of PRP) subdivided into Subgroup IVA =4 rats sacrificed at day 7, Subgroup IVB =4 rats sacrificed at day 14. Samples were obtained and processed for biochemical, histological, and immunohistochemical techniques.

Results: H&E, subgroup IIA confirmed the injury. Subgroup IIB showed irregular muscle fibers, inflammatory, fat cells infiltration and few fibers showed myotubes. Subgroup IIC showed some regular and many irregular muscle fibers with extensive apparent fibrosis. Groups III and IV showed improvement of muscular structure. Mallory stain, group IIA showed disturbed endomysium. Subgroups IIB&IIC showed significant increase in Collagen fibers deposition which significantly decreased in groups III&IV.As regard myogenin and VEGF reaction, treated groups were significantly higher than recovery groups, while group III was the highest .CPK value in subgroup IIA was significantly the highest with significant drop in recovery groups with more drop in treated groups.

Conclusion: MSCs and PRP provided evidence for their therapeutic effects in muscle injury. Meanwhile, MSCs have the upper hand in restoring muscle structure.

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Key Words: BM-MSCs, laceration, PRP, skeletal muscle.

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INTRODUCTION

Skeletal muscle is a vital tissue that is in charge of voluntary motion and maintaining posture. It accounts for about 40% of the body's mass and is the most prevalent tissue in the human body. Skeletal muscle injuries may manifest clinically as laceration, strain, contusion, or as a mix of these signs[1].

Skeletal muscle consists of hierarchically arranged myofibers, blood vessels, nerves, and connective tissue. Skeletal muscle's functional unit, myofibers are multi-nucleated fibers created through the fusion of myoblasts. Numerous nuclei allow for the generation of an enormous amount of proteins and enzymes, which are essential for these cells to function effectively[2].

Local stem cells diverse populations are necessary for regeneration of skeletal muscle; they are called satellite cells and are mainly regulated by extracellular matrix (ECM) proteins, and secreted factors[3].

In order to repair injured muscle, satellite cells—which are normally quiescent—become activated, multiply, develop into myocytes, and merge with damaged myofibers or with each other. However, the skeletal muscle fibres' limited ability to regenerate is due to paucity of satellite cells. Fibrous tissue forms and causes mechanical dysfunction when muscles fail to mend properly after severe injuries[1].

Researchers are experimenting with different approaches to develop novel treatments for dystrophic or traumatic skeletal muscle injuries and degeneration. It is commonly recognized that in the bone marrow milieu, mesenchymal stem cells (MSCs) form distinct niche that promotes regeneration[4].

Satellite cell myogenic differentiation and musculoskeletal tissue regeneration can be triggered by bone marrow mesenchymal stem cells (BM-MSCs) which are controlled mainly by paracrine effect and immunomodulation[5].

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Also, one of the potentially effective medicinal substances that can promote the healing of many tissues, including striated muscles, is platelet-rich plasma (PRP). Studies showed that the infusion of autologous conditioned serum stimulates regeneration. It has a high concentration of autologous growth factors which accelerate regeneration[6].

The transfer of important growth factors and cytokines from platelets' granules to the designated area is the basis for the justification for using PRP, which enhances the healing process in different tissues and acts as cell cycle regulators[7].

The purpose of this work was to make comparison between the regenerative outcomes of BM-MSCs versus PRP in skeletal muscle laceration injury in model of adult albino rat at different time intervals. This was done using histological, immunohistochemical, biochemical and morphometric studies.

MATERIALS AND METHODS

Anesthesia

A cocktail of ketamine chlorhydrate 60 mg/kg (Pfizer pharmaceuticals, Egypt) associated with xylazine 15mg/kg (Adwia pharmaceuticals, Egypt) was administered intra-peritoneal[8] just before trauma induction.

Bone marrow derived mesenchymal stem cells (BM-MSCs)

BM-MSCs were labeled with Paul Karel Horan-26 (PKH-26) and prepared at Histology Department, Tissue Culture Unit, Faculty of Medicine, Cairo University.

Platelet rich plasma (PRP)

PRP was prepared in Biochemistry department, Faculty of Medicine, Cairo University.

Animals

Fourty-four adult male albino rats (weight 180-200 grams) were used. The animals were bred at the Animal House, Faculty of Medicine, Cairo University. They were housed in stain-steel cages with hygiene standers, in clean properly ventilated rooms and authorized unrestricted access to standard diet and water. The rats were kept for 48 hours in these conditions before the start of the experiment to allow them to adapt to their new environment. Every procedure was done in compliance with the standers of Animal Ethics Committee of Cairo University (approval no: CU III F 21 23).

Animals had sorted into the subsequent groups

I. Donor Group, 2 rats:They were used for BM-MSCs isolation, culture, phenotyping and labeling.

II. Group I (Control group), 12 rats:

These rats were randomly subdivided into 3 subgroups (4 rats each):

- Subgroup IA: The rats received no treatment throughout the whole experimental duration. Two rats were sacrificed at day 7, and then the remaining two rats were sacrificed at day 14.
- Subgroup IB (Sham group): The rats were given anesthesia, and skin was incised over the right gastrocnemius muscle, then skin suturing was done. Two rats were sacrificed at day 7, and then the remaining two rats were sacrificed at day 14.
- Subgroup IC: Each rat treated as in subgroup IB, and then local injection of one milliliter of phosphate buffer saline (PBS) into the muscle was given. Two rats were sacrificed at day 7, and then the remaining two rats were sacrificed at day 14.

III. Group II (Injury group),12 rats:

Laceration injury of right gastrocnemius muscle was done[9]. Suturing was done, and then they were randomly subdivided into 3 subgroups:

- Subgroup IIA, 4 rats: They were sacrificed at day 1 (4 hours after injury).
- Subgroup IIB, 4 rats: They were sacrificed at day 7.
- Subgroup IIC, 4 rats: They were sacrificed at day 14.

IV. Group III (BM-MSC treated group), 10 rats:

Laceration injury of right gastrocnemius muscle was done. In addition, rats were given a 1ml of PBS IM containing 1 million BM-MSCs at the injury site before suturing[1].

In order to identify the homing of stem cells in muscle tissue, two rats were randomly selected and scarified on day 5 of the experiment[10].

The remaining 8 rats were randomly subdivided into:

- Subgroup IIIA, 4 rats: They were sacrificed at day 7.
- Subgroup IIIB, 4 rats: They were sacrificed at day 14.

V. Group IV (PRP treated group), 8 rats:

Rats were injected IM by 1 ml of PRP before suturing[11]. Rats were randomly subdivided into:

- Subgroup IVA, 4 rats: They were sacrificed at day 7.
- Subgroup IVB, 4 rats: They were sacrificed at day 14.

Methods

Preparation of Bone Marrow Derived Mesenchymal Stem Cells (BM-MSCs)

Tibia and femur of male Westar 8-week-old rats used to get bone marrow. Bone marrow was flushed with fetal bovine serum [FBS, GIBCO/BRL] and glucose low

Dulbecco's Modified Eagle's Medium (DMEM) [Gibco, Gainesville, MD, USA].The cells growing was done in 1% penicillin/streptomycin (GIBCO) containing medium. They kept as primary culture for 2 weeks at 37°C. The cells were PBS washed at 80% confluence. During five minutes at 37°C, they were handled with trypsin (0.25%) in ethylene diamine tetra acetic acid. Then they were centrifuged, immersed in serum-containing media (10% FBS) then cultured on Falcon flasks measuring 50 cm2. To increase the cell population, the subculture passage was continued until third passage .BM-MSCs were distinguished by their ability to adhere, fusiform shape, and surface marker identification by flow cytometer (CYTOMICS FC 500, Beckman Coulter, Champaign, IL, USA). They were positive for CD105&CD90 and negative for CD34&CD45)[12].

Labeling of BM-MSCs by PKH26

PKH dyes stain biological and artificial membranes. These dyes rapidly establish powerful noncovalent bonds with lipid bilayer, so enhance durability of dye and steady fluorescence[13]. For labeling with PKH26, BM-MSCs were grown in a staining mixture that contained DMEM medium with PKH26 solution (Sigma, USA, Catalog Number MIN126) and incubated for one hour in a humidified Incubator, 37°C, 5% carbon dioxide[14].

Preparation of Platelet-Rich Plasma(PRP)

Rat tail venous blood (24 mL) was drawn into blood collecting tubes with sodium citrate (3.8%). Samples of blood underwent centrifugation at 800× g for ten minutes. After aspirating the plasma, it was gathered into 15-mL containers with centrifugation at 1,000×g for an additional 5 minutes. After discarding the upper layer of plasma, 5 mL of the remaining plasma was left at the bottom. This plasma will be collected as platelets, stored at -20 °C, and thawed before being used[15].

Steps of muscle injury

Anesthesia was given to rats; then following hair removal from the right limb, povidone iodine (PVPI) was used as a local antiseptic treatment. To expose the gastrocnemius muscle, the skin and subcutaneous tissue were dissected and retracted. Laceration of the gastrocnemius muscle at 75% of its width (about 1.0 cm), 60% of its entire length (approximately 2.5 cm up from the calcaneus flexed at 90 degrees), and 50% of its overall thickness; was performed using blade (15). For every measurement, a digital caliper ruler was used. Applying nylon thread sutures to the skin. Local antisepsis was performed following surgery[16].

Sample collection

According to time intervals, rats were sacrificed after anesthesia by phenobarbital (80mg/kg) (Intra-peritoneal)[17]. Samples obtained during dissection of the right gastrocnemius muscle, encompassing the area of injury in the middle[1]. Specimens were preserved in 10% formalin saline solution for 24 hours, and embedded in paraffin 5 micrometer thickness sections were cut serially. These sections were used in the next studies:

Histological study

a. Hematoxylin and Eosin stain (H&E)[18].

b. Mallory trichrome stain[19] to demonstrate collagen fibers.

c. Immunohistochemical staining[20]:

VEGF: For detection of new vessels formation during muscle regeneration[21].

Myogenin: Myogenin is a specific gene for skeletal muscle which encodes a transcription factor. It controls how myoblasts fuse together during development. Furthermore, it expresses itself early in the process by which satellite stem cells differentiate into specialized myogenic cells[22].

Primary antibodies were used for one hour. It is ready to use mouse monoclonal human reactive VEGF Ab [product number V7259, Sigma_Aldrich, Saint Louis, Missouri, USA] and rabbit polyclonal anti-myogenin antibody [product number YPA2269A, Chongqing Biospes Co, Ltd, china]. It was used as 7.0ml of ready to use antibody (was diluted before in 0.05mol/L Tris-HCL, PH 7.6 containing stabilizing protein and 0.015mol/L sodium azide).To complete immunostaining, Ultra-vision detection system (TP-015-HD) was used and for counter staining Mayer's Hematoxylin (TA-060-MH) was used. Citrate buffer, Ultra vision detection system and Mayer's hematoxylin were acquired from Lab vision Thermo Scientific, Fremont, California, USA. Same steps were done for negative controls where adding primary antibodies step was skipped.

Fluorescent Microscopic Study

Examination of PKH26 labeled BM-MSCs in unstained sections to detect homing of BM-MSCs in the muscle tissue.

Biochemical study

Blood samples were collected at the last day of each time interval just before sacrifaction. Samples were analyzed for creatine phosphokinase (CPK). Analysis was done in Biochemistry Unit, Faculty of Medicine, Cairo University.

Morphometric Study

Ten non-overlapping fields for each animal were analyzed under the light microscope and the data will be obtained by "Leica Qwin 500C" image computer analysis system (Leica Imaging System Ltd, Cambridge, UK) for detection of:

a. The mean number of central nuclei (myotubes) per field in longitudinal sections.

b. Area percent of collagen fibers in sections stained by Mallory trichrome.

c. Area percent of VEGF positive immunoreaction

d. Number of cells stained with myogenin antibody.

Statistical study

The measurements obtained were analyzed utilizing version 16 of the Statistical Package for Social Science (SPSS) program. (SPSS, Chicago, USA). To compare various groups, one-way analysis-of-variance was used (ANOVA) followed by post Hoc-Tukey test. The results were stated as mean ± standard deviation (SD). The variations were regarded statistically with significance when probability (p) value was < 0.05[23].

RESULTS

Histological Results

Hematoxylin and Eosin Results: (Figure 1)

Samples obtained from gastrocnemius muscle of subgroup IA, subgroup IB and subgroup IC showed similar histological findings. They are showing cylindrical parallel muscle fibers with peripheral flat nuclei separated by endomysium (Figure a). Sections from subgroup IIA are showing disorganized muscle fibers with focal areas of homogenous deep acidophilic cytoplasm , peripheral nuclei and cellular infiltrate (Figure b).Subgroup IIB is showing less disorganized muscle fibers with cellular infiltrate, fat cells (y and few fibers show central located nuclei (Figure c). Subgroup IIC is showing regular muscle fibers , many wavy fibers , few fibers with central nuclei and widening of CT (Figure d).Subgroup IIIA is showing localized cellular infiltrate , regularly arranged muscle fibers and some with many central nuclei (Figure e). Subgroup IIIB is showing regular muscle fibers with peripheral flat nuclei, few fibers are slight wavy exhibiting centrally located nuclei (Figure f). Subgroup IVA is showing regular muscle fibers, others are wavy, some fibers showed central nuclei, homogenous deep acidophilic cytoplasm, cellular infiltrate and congested blood vessel (Figure g). Subgroup IVB is showing parallel regular muscle fibers, few fibers wavy and some fibers with central nuclei. Cellular infiltrate is seen between muscle fibers (Figure h).

Mallory Trichrome Results: (Figure 2)

LS sections are showing ;in group I ,cylindrical parallel muscle fibers separated by minimal amount of endomysium (Figure a).Subgroup IIA is showing increased collagen fibers around blood vessels between disorganized muscle fibers (Figure b). Subgroup IIB is showing wide areas of collagen between muscle fibers with congested dilated blood vessel (Figure c).Subgroup IIC is showing large amount of collagen between muscle fibers (Figure d). Subgroup IIIA is showing minimal collagen between muscle fibers (Figure e). Subgroup IIIB is showing minimal collagen between well-organized muscle fibers (Figure f). Subgroup IVA is showing collagen in between muscle fibers and around congested blood vessel (Figure g). Subgroup IVB showed considerable amount of collagen between muscle fibers (Figure h).

Immunohistochemical Results for Myogenin: (Figure 3)

Group I (LS) is showing negative nuclear immunostaining in the muscle fibers (Figure a). Subgroup IIA (TS) is showing occasional +ve nuclei among muscle fibers (Figure b). Subgroup IIB (TS) is showing few +ve nuclei among muscle fibers (Figure c). Subgroup IIC (TS) is showing some +ve nuclei among muscle fibers (Figure d). Subgroup IIIA (TS) is showing multiple +ve nuclei among muscle fibers (Figure e). Subgroup IIIB (TS) is showing numerous +ve nuclei among muscle fibers (Figure f). Subgroup IVA (TS) is showing many +ve nuclei among muscle fibers (Figure g). Subgroup IVB (TS) is showing multiple +ve nuclei among muscle fibers (Figure h).

Immunohistochemical Results for VEGF: (Figure 4)

Examination of sections obtained from gastrocnemius muscle of group I revealed +ve cytoplasmic immunostaining in endothelial lining of blood vessel between muscle fibers (Figure a). Subgroup IIA is showing +ve endothelial lining of few blood vessels (Figure b). Subgroup IIB (TS) is showing few blood vessels with positive endothelial cytoplasmic reaction (Figure c). Subgroup IIC (TS) is showing few blood vessels with +ve endothelial lining (Figure d). Subgroup IIIA (TS) is showing +ve endothelial lining (red arrows) in multiple blood vessels (Figure e). Subgroup IIIB (TS) is showing +ve endothelial lining in numerous blood vessels (Figure f). Subgroup IVA (TS) is showing +ve endothelial lining in some blood vessels (Figure g). Subgroup IVB (TS) is showing +ve endothelial lining in multiple blood vessels (Figure h).

Immunofluorescent Results: (Figure 5)

The sections of skeletal muscle in group III (stem cells-treated group, Sacrificed at (day 5) showed MSCs labeled with PKH26 fluorescence dye in muscle fibers.

Biochemical Results: (Table1, Histogram 1)

The mean value of CPK at day 1 after injury in subgroup IIA was significantly the most between all other groups (P<0.05). However this value was significantly decreased in the recovery group after 7 days (Subgroup IIB) with further significant decrease at 14 days (subgroup IIC). Even though CPK level in all recovery groups was still significant more than that of the control group (P<0.05). CPK level in treated groups; stem cell (group III) and PRP (group IV) was significantly lower than recovery groups in corresponding days but still significantly more than the control group(P<0.05). In all treated groups (group III, IV) value at day 14 was significantly less than same group at day 7 (P<0.05). Both treated groups (III, IV) showed no significant difference in mean CPK level values in corresponding days (P<0.05).

Morphometric& statistical results: (Table 2, Histograms 2-5)

As regard the mean number of both myotubes and myogenin immunoreactive nuclei in recovery groups; the

values were significantly increased in subgroup IIB with further significant rise in subgroup IIC as compared to control group (P<0.05).The mean values in treated groups, stem cell (group III) and PRP (group IV) were significantly more than that of recovery groups in the corresponding days (P<0.05).In all treated groups (group III, IV) value at 14 days was significantly more than same group at 7 days (P<0.05). Stem cell treated groups (group III) showed significant higher values than PRP treated group (group IV) in corresponding days (P<0.05).

The mean value of Mallory trichrome area percent in recovery groups was significantly high in subgroup IIB with further significant increase in subgroup IIC (P<0.05). The value in treated groups, was significantly lower than recovery groups in the corresponding days (P<0.05). Stem cell treated groups (group III) showed significant lower values than PRP treated group (group IV) in the corresponding days (P<0.05).

As regard laceration group (group II), the mean value of VEGF area percent raised significantly at day 1 after injury (subgroup IIA).However this value revealed significant rise in the recovery group after 7 days (Subgroup IIB) with further significant increase at 14 days (subgroup IIC) as compared to control group (P<0.05).The mean value of VEGF area percent in treated groups; stem cell (group III) and PRP(group) was significantly greater than recovery groups in corresponding days (P<0.05). In all treated groups (group III, IV) value at 14 days was significantly more than same group at 7 days (P<0.05). Stem cell treated groups (group III) showed significant higher values than PRP treated group (group IV) in corresponding days (P<0.05).

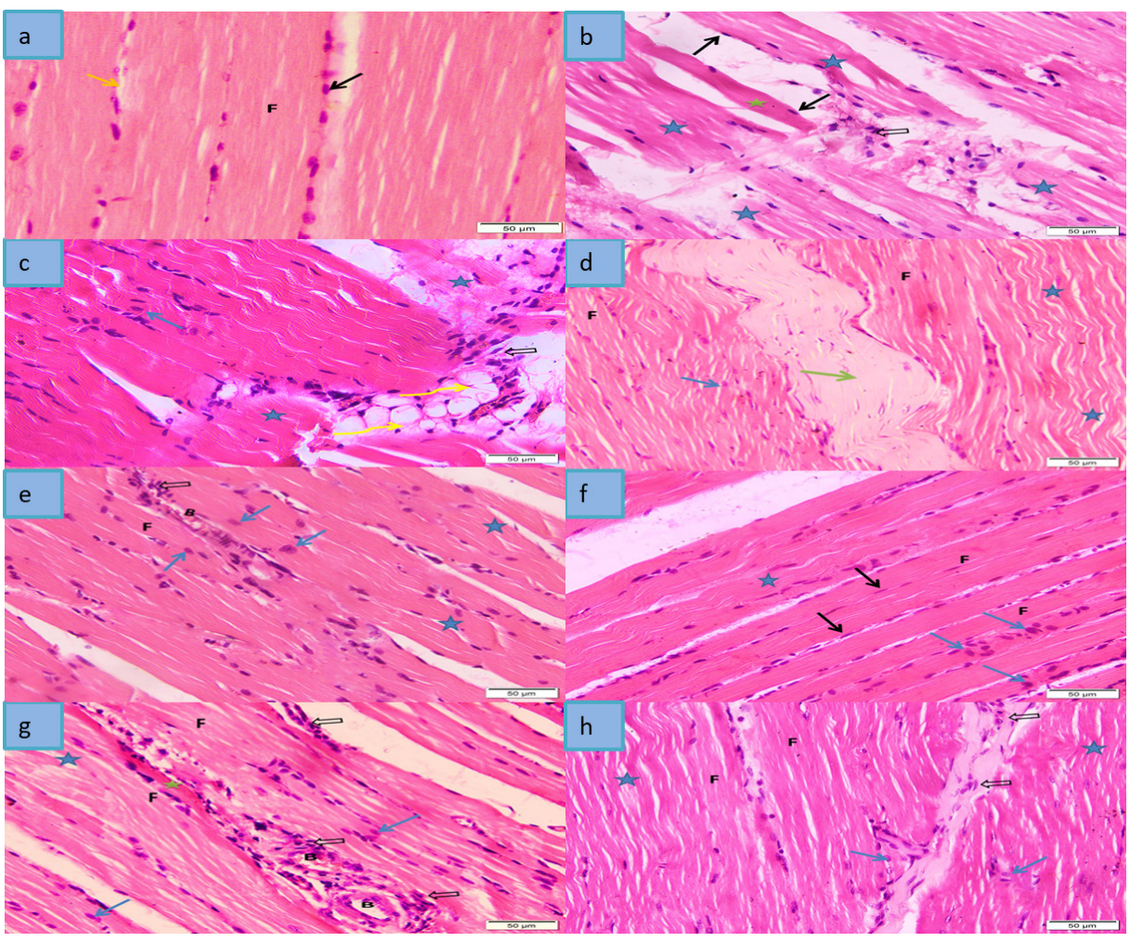


Fig. 1: LS sections in the skeletal muscle of rats stained with H&E (x200) (a): group I showing cylindrical parallel muscle fibers (F) with peripheral flat nuclei (black arrows) separated by endomysium (yellow arrow). (b): subgroup IIA showing disorganized muscle fibers (blue stars) with focal areas of homogenous deep acidophilic cytoplasm (green star), peripheral nuclei (black arrows) and cellular infiltrate (hollow arrow). (c): subgroup IIB showing less disorganized muscle fibers (blue stars) with cellular infiltrate (hollow arrow), fat cells (yellow arrows) and few fibers show central located nuclei (blue arrow). (d): subgroup IIC showing regular muscle fibers (F), many wavy fibers (blue stars), few fibers with central nuclei (blue arrow) and widening of CT (green arrow). (e): subgroup IIIA showing localized cellular infiltrate (hollow arrow), regularly arranged muscle fibers (F) and some with many central nuclei (blue arrows).(f): subgroup IIIB showing regular muscle fibers (F) with peripheral flat nuclei (black arrows), few fibers are slight wavy (blue star) exhibiting centrally located nuclei (blue arrows). (g): subgroup IVA showing regular muscle fibers (F), others are wavy (blue star), some fibers showed central nuclei (blue arrows), homogenous deep acidophilic cytoplasm (green stars), cellular infiltrate (hollow arrows) and congested blood vessel (B). (h): subgroup IVB showing parallel regular muscle fibers (F), few fibers wavy (blue stars) and some fibers with central nuclei (blue arrows). Cellular infiltrate is seen between muscle fibers (hollow arrows).

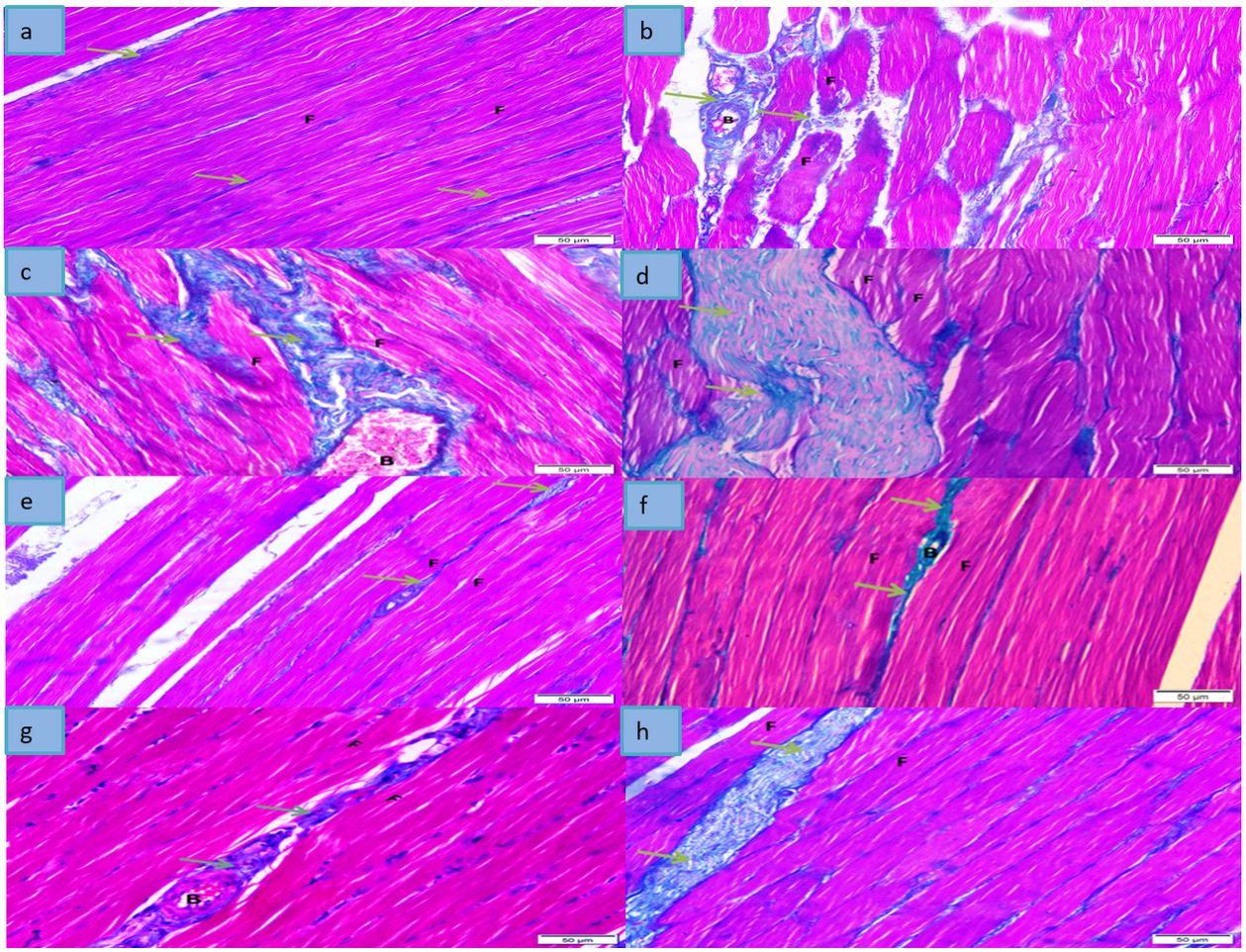


Fig. 2: LS skeletal muscle sections of rats stained with Mallory trichrome (x200). (a): group I showing cylindrical parallel muscle fibers (F) separated by minimal amount of endomysium (green arrows). (b): subgroup IIA showing increased collagen fibers (green arrows) around blood vessels (B) between disorganized muscle fibers (F). (c): subgroup IIB showing wide areas of collagen (green arrows) between muscle fibers (F) with congested dilated blood vessel (B). (d): subgroup IIC showing large amount of collagen (green arrows) between muscle fibers (F). (e): subgroup IIIA showing minimal collagen (green arrows) between muscle fibers (F). (f): subgroup IIIB showing minimal collagen (green arrows) between well-organized muscle fibers (F). (g): subgroup IVA showing collagen (green arrows) in between muscle fibers (F) and around congested blood vessel (B). (h): subgroup IVB showed considerable amount of collagen (green arrows) between muscle fibers (F).

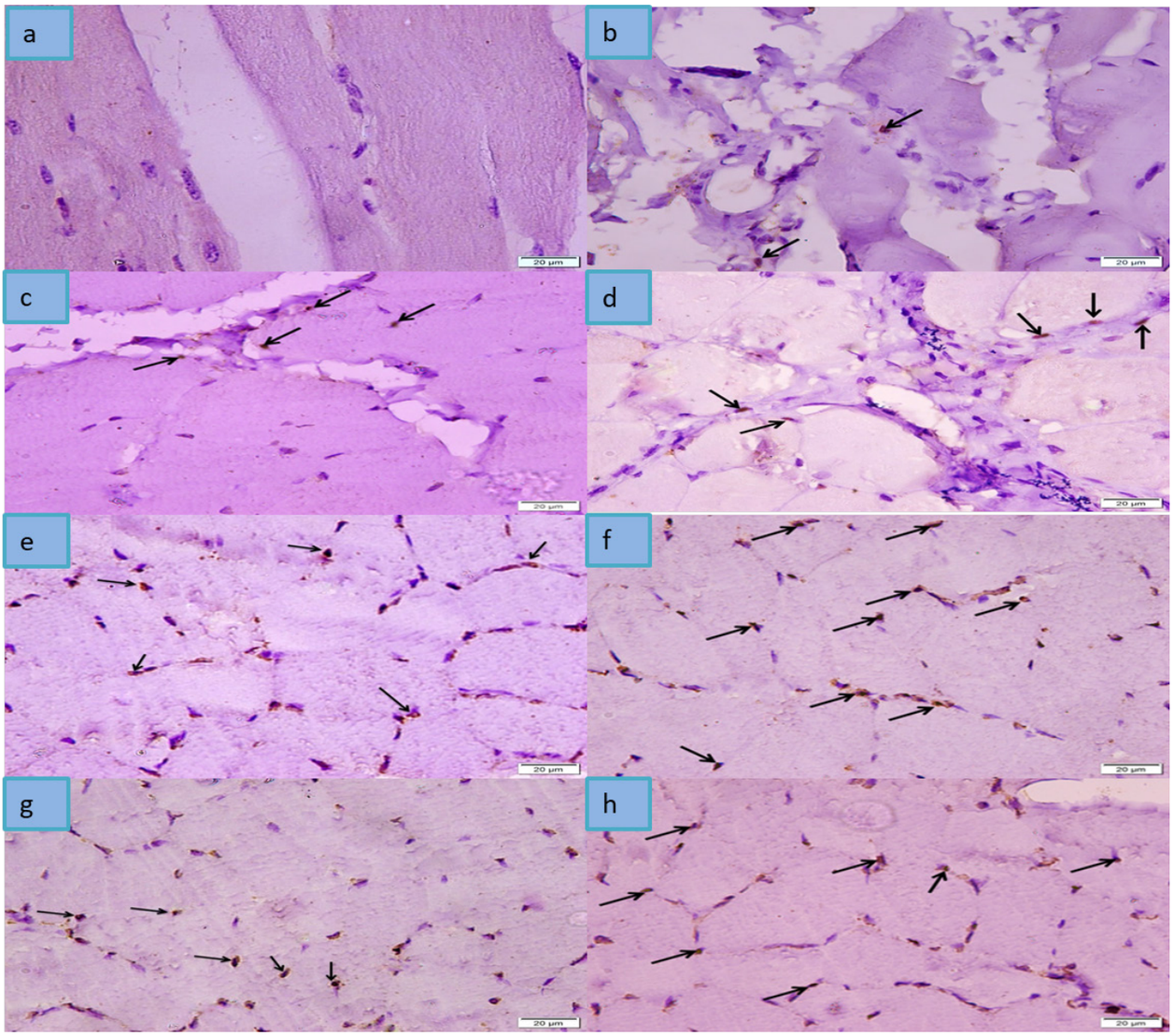


Fig. 3: sections in the skeletal muscle of rats stained with anti myogenin immunostaining (x400). (a): group I (LS) showing negative nuclear immuno-staining in the muscle fibers. (b): subgroup IIA (TS) showing occasional +ve nuclei (arrows) among muscle fibers. (c): subgroup IIB (TS) showing few +ve nuclei (arrows) among muscle fibers. (d) subgroup IIC (TS) showing some +ve nuclei (arrows) among muscle fibers. (e): subgroup IIIA (TS) showing multiple +ve nuclei (arrows) among muscle fibers. (f): subgroup IIIB (TS) showing numerous +ve nuclei (arrows) among muscle fibers. (g): subgroup IVA (TS) showing many +ve nuclei (arrows) among muscle fibers. (h): subgroup IVB (TS) showing multiple +ve nuclei (arrows) among muscle fibers.

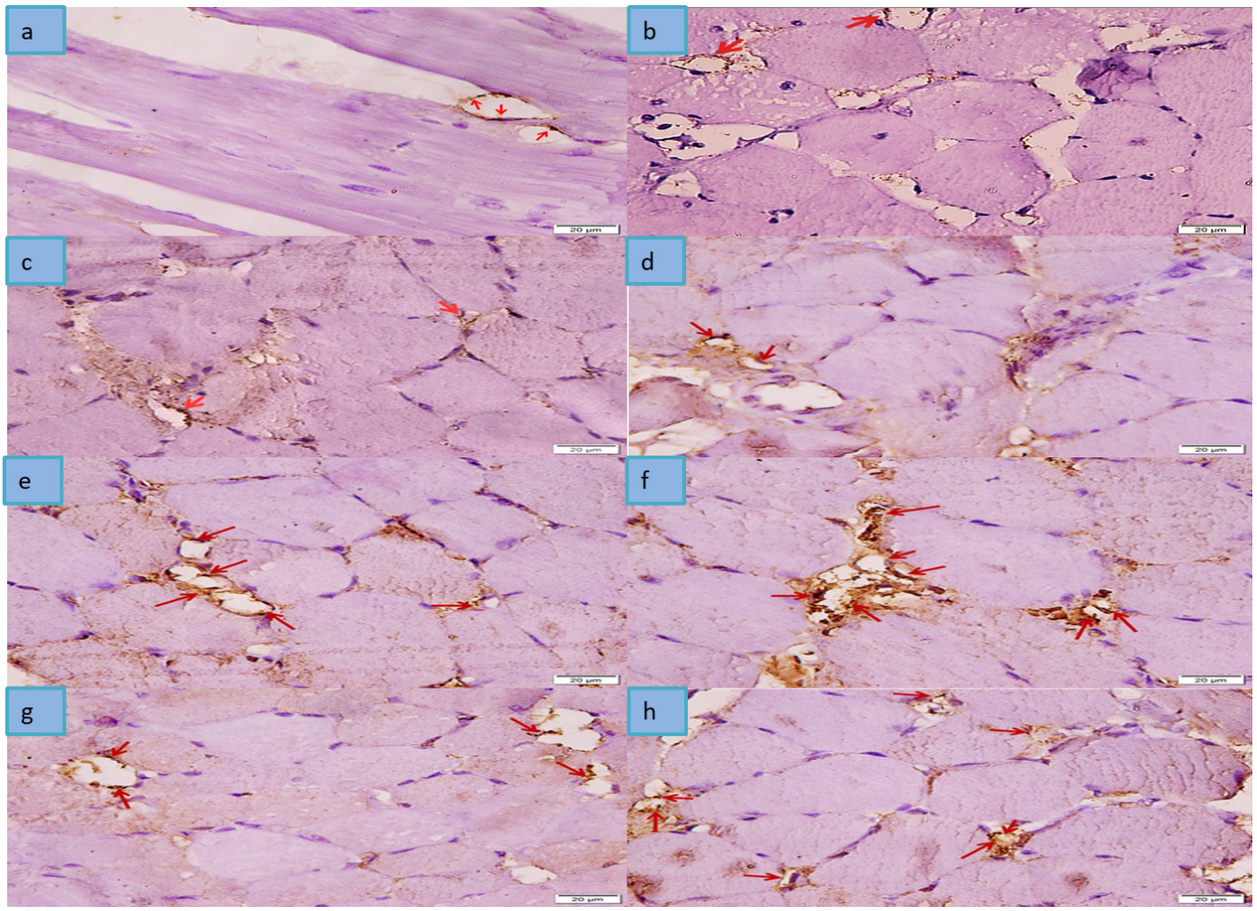


Fig. 4: sections in the skeletal muscle of rats stained with anti VEGF immunostaining (x400). (a): group I (Ls) showing +ve cytoplasmic immunostaining (red arrows) in endothelial lining of blood vessel between muscle fibers. (b): subgroup IIA (TS) showing +ve endothelial lining of few blood vessels (red arrows). (c): subgroup IIB (TS) showing few blood vessels with positive endothelial cytoplasmic reaction (red arrows). (d): subgroup IIC (TS) showing few blood vessels with +ve lining (red arrows). (e): subgroup IIIA (TS) showing +ve endothelial lining (red arrows) in multiple blood vessels. (f): subgroup IIIB (TS) is showing +ve endothelial lining (red arrows) in numerous blood vessels. (g): subgroup IVA (TS) showing +ve endothelial lining (red arrows) in some blood vessels. (h): subgroup IVB (TS) showing +ve endothelial lining (red arrows) in multiple blood vessels.

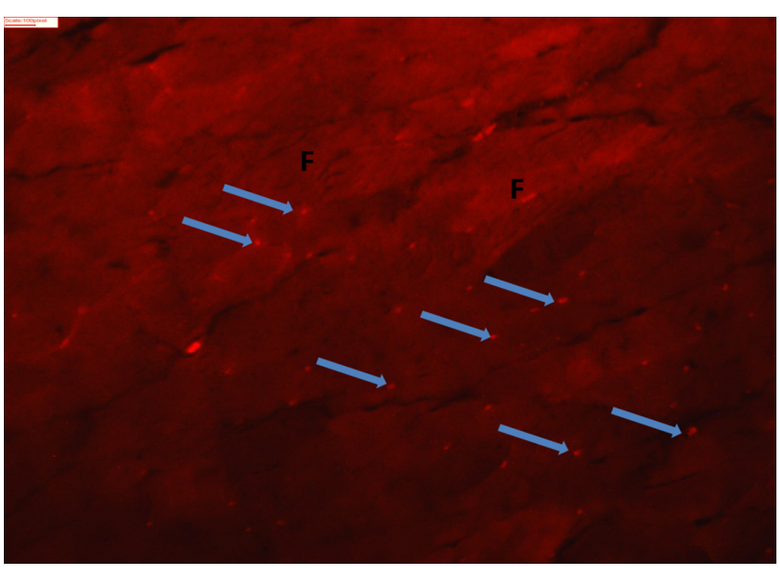


Fig. 5: LS section in the skeletal muscle of rat (immunofluorescent): group III (stem cells-treated group, Sacrificed at (day 5) showed MSCs labeled with PKH26 fluorescence dye in muscle fibers (arrows).

Table 1: Mean CPK level ± SD among the studied Groups

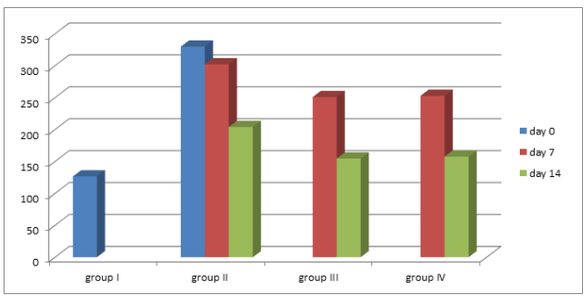
Groups	Mean CPK level ± SD
group I	126.7±0.99
group IIA	330±9.6*
group IIB	302.3±3.77*^
group IIC	204±4.1*^#
group IIIA	251±7.69*^#+
group IIIB	154.7±3.65* ^+$
group IVA	253±6.6*^#
group IVB	157.7±7.99*^%

*: significant P as compared to control group (group I) (P<0.05).
^: significant P as compared to trauma group (IIA) (P<0.05).
#: significant P as compared to recovery group at 7 days (IIB) (P<0.05).
+: significant P as compared to recovery group (IIC) (P<0.05).
$: significant P as compared to treated subgroup IIIA.
%: significant P as compared to treated subgroups IVA.

Table 2: Mean number of myotubes (± SD), mean area percent of Mallory Trichrome (± SD), mean number of myogenin immunoreactive nuclei (±SD) and mean area percent of VEGF positive immunoreaction (± SD) among the studied groups

Parameters and groups	Mean number of myotubes	mean area % of Mallory Trichrome	mean number of myogenin immunoreactive nuclei	mean area % of VEGF positive immunoreaction
Group I	0	2.5±0	0.1±0.214	1.8±.11
Group IIA	0	2.6±0.51	0.1±0.54	2.99±0.54*
Group II B	2.34 ± 0.54*^	6.5±0.52*^	2±0.23* ^	3.7±0.62*^
Group IIC	3.66 ± 0.58*^#	10.5±0.52*^#	3.5±0.56 *^#	4.9±0.82*^#
Group IIIA	10.50 ± 2.5 *#0	3.5±0.55*#0	4±0.97*# 0	8.8±0.72*#0
Group III B	13.00 ± 5.3 *+$0	2.8±0.52*+0$	5.9±0.89*+0$	10.5±0.46*+0$
Group IVA	6.39 ± 2.24 *#	4.1±0.54*#	2.9±0.81 * #	6.3±0.44*#
Group IV B	9.50 ± 2.03*+%	5.8±0.54*+%	4.3±0.32*+%	7.9±0.67*+%

*: significant *P* as compared to control group (group I) (P<0.05).
^: significant *P* as compared to trauma group (IIA) (P<0.05).
#: significant *P* as compared to recovery group at 7 days (IIB) (P<0.05).
+: significant *P* as compared to recovery group (IIC) (P<0.05).
$: significant *P* as compared to treated subgroup IIIA.
%: significant *P* as compared to treated subgroups IVA
0: significant *P* as compared to (PRP treated groups) subgroup IVA, IVB in corresponding days (P<0.05).



Histogram 1: Mean CPK level ± SD among the studied Groups

*: significant *P* as compared to control group (group I) (P<0.05).

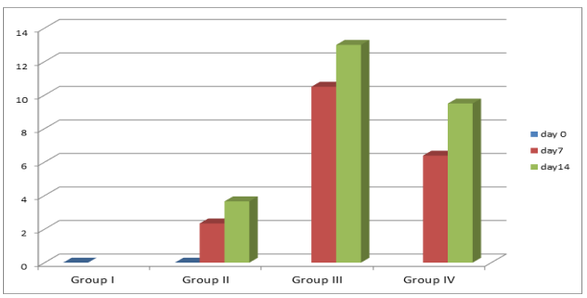
^: significant *P* as compared to trauma group (IIA) (P<0.05).

#: significant *P* as compared to recovery group at 7 days (IIB) (P<0.05).

+: significant *P* as compared to recovery group (IIC) (P<0.05).

$: significant *P* as compared to treated subgroup IIIA.

%: significant *P* as compared to treated subgroups IVA.



Histogram 2: Mean number of myotubes (± SD) among the studied groups

*: significant *P* as compared to control group (group I) (P<0.05).

^: significant *P* as compared to trauma group (IIA) (P<0.05).

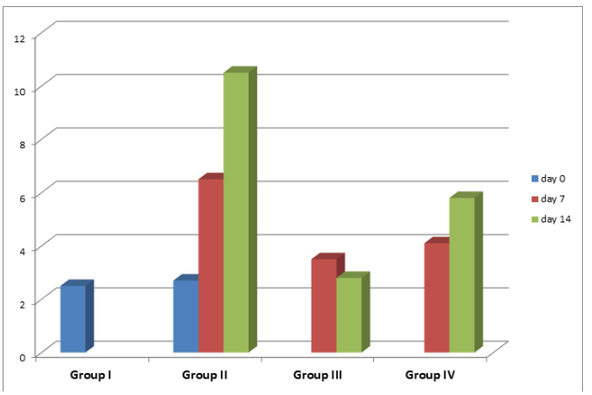
#: significant *P* as compared to recovery group at 7 days (IIB) (P<0.05).

+: significant *P* as compared to recovery group (IIC) (P<0.05).

$: significant *P* as compared to treated subgroup IIIA.

%: significant *P* as compared to treated subgroups IVA

0: significant *P* as compared to (PRP treated groups) subgroup IVA, IVB in corresponding days (P<0.05).



Histogram 3: Mean area% of Mallory trichrome (± SD) among the studied groups

*: significant *P* as compared to control group (group I) (*P*<0.05).

^: significant *P* as compared to trauma group (IIA) (*P*<0.05).

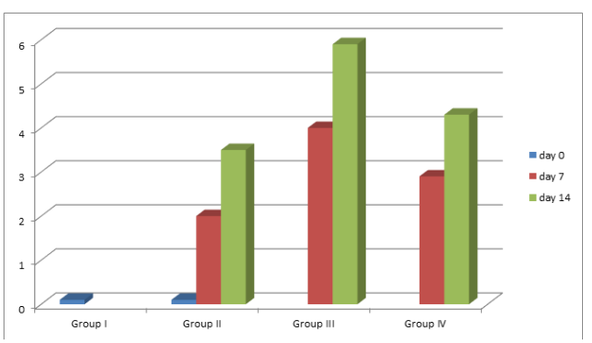
#: significant *P* as compared to recovery group at 7 days (IIB) (*P*<0.05).

+: significant *P* as compared to recovery group (IIC) (*P*<0.05).

$: significant P as compared to treated subgroup IIIA.

%: significant P as compared to treated subgroups IVA

0: significant *P* as compared to (PRP treated groups) subgroup IVA, IVB in corresponding days (*P*<0.05).



Histogram 4: Mean number ± SD of myogenin immunoreactive nuclei among the studied groups

*: significant *P* as compared to control group (group I) (*P*<0.05).

^: significant *P* as compared to trauma group (IIA) (*P*<0.05).

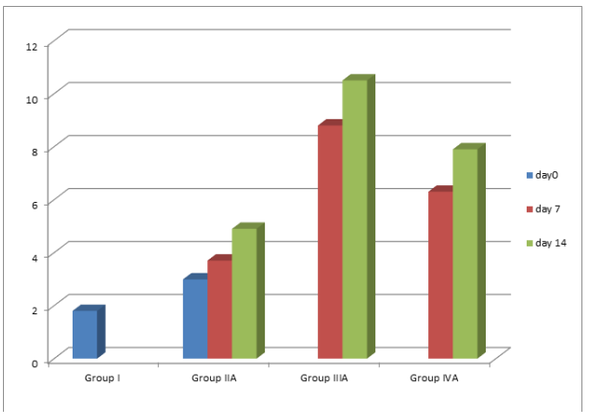
#: significant *P* as compared to recovery group at 7 days (IIB) (*P*<0.05).

+: significant *P* as compared to recovery group (IIC) (*P*<0.05).

$: significant *P* as compared to treated subgroup IIIA.

%: significant *P* as compared to treated subgroups IVA

0: significant *P* as compared to (PRP treated groups) subgroup IVA, IVB in corresponding days (*P*<0.05).



Histogram 5: Mean area percent of VEGF immune reaction ± SD among the studied groups

*: significant *P* as compared to control group (group I) (*P*<0.05).

^: significant *P* as compared to trauma group (IIA) (*P*<0.05).

#: significant *P* as compared to recovery group at 7 days (IIB) (*P*<0.05).

+: significant *P* as compared to recovery group (IIC) (*P*<0.05).

$: significant *P* as compared to treated subgroup IIIA.

%: significant *P* as compared to treated subgroups IVA

0: significant *P* as compared to (PRP treated groups) subgroup IVA, IVB in corresponding days (*P*<0.05).

DISCUSSION

Muscle injuries are one of the most relevant injuries which can be described clinically as strains, contusions, lacerations or mixture of these ailments[1]. Skeletal muscle can heal itself; the process may not be fully completed, which could result in a reduction in strength and function[24]. It was reported that BM-MSCs convey a unique multipotent, non-hemopoietic adult stem cell, which can be utilized for repair of skeletal muscle due to their capacity for myogenic growth[2]. In regenerative medicine, platelet rich plasma (PRP) is frequently utilised, particularly in orthopaedic sports medicine. Studies demonstrate that PRP has beneficial impacts on musculoskeletal system[25].

Therefore, the current study was created to make comparison between the regenerative outcomes of BM-MSCs versus PRP in skeletal muscle laceration injury in adult albino rat model at different time intervals.

The experiment duration was classified into two phases; 7 days and 14 days[1] to compare the results of muscle tissue damage and the possible role of BM-MSCs and PRP in muscle damage repair over different durations. The present study focused on the gastrocnemius muscle

since it is frequently the site of acute injuries sustained while practicing various sports[26].

In the present study, subgroup IIA at day of injury revealed disorganized, discontinued muscle fibers, homogenous acidophilic cytoplasm and apparently numerous inflammatory cells which indicate that the inflammatory response could occur few hours after injury. It was reported that inflammatory response can be detected immediately after injury with leukocytosis, mostly seen as a brief rise in neutrophil counts, mostly noticeable in the first 24 hours. This increase in neutrophils is followed by the mobilization of other inflammatory cells as well as a transport of fluid and plasma proteins into the injured muscle[27].

In current work, inflammatory response in group IIA was accompanied with rise in the mean area percent of VEGF immunostained sections when compared with the control group. One such crucial modulator of the cascade of tissue healing and angiogenesis is VEGF. Growth factors such as vascular endothelial growth factor and platelet-derived growth factor are released by the endothelial cells in damaged vessels[1].

In the current work, the destructive changes in the injured muscle were accompanied with significant rise in CPK value in group IIA as compared with the group I. Group IIA showed the highest value of CPK between all groups. Serum CPK starts to elevate within 2 to 12 hours as soon as a muscle injury occurs[28]. CPK presents in cardiac, skeletal muscles, etc. However, upon muscular injury, there is leak of CPK into the bloodstream[29]. Thus, CPK is an indicator of muscular degeneration and damage. In a trial to explain this finding, it was found that injury to myofibrils damages membranes and has an impact on the sarcolemma's channels. After an injury, there is a sustained rise in the membrane permeability of many muscle proteins, the most notable being creatine kinase and myoglobin, which can then be detected in plasma and utilized as injury biomarkers[27].

In the present study the mean numbers of both myotubes and myogenin immunostained nuclei in subgroup IIA were not significantlyvariant as compared to group I (control). It seems that the myogenin expression is correlated with myotubes formation and regeneration process.The previous finding is in accordance with Pizza & Buckley., (2023)[30] who explained this by the process of muscle regeneration starts a few days after injury, when satellite cells begin to multiply and give rise to myoblasts.

In the current study, subgroup IIB (recovery group) showed less disorganized muscle fibers and mononuclear inflammatory and fat cells infiltration between muscle fibers. These features improved in subgroup IIC which revealed some fibers became regular with restoration of normal structure, which could be a mark of caseation of inflammation.

At day 7, number of infiltrating inflammatory cells starts to decline with further drop by time, and damaged fibers are replaced by regenerating fibers. Deposition of extracellular matrix is exaggerated (fibrous tissue formation) at this stage to assist the structure[31]. The presence of fat cells at 7 days can be explained as fibroadipogenic progenitors (FAPs), a subset of activated stem cells, are crucial for the regeneration of skeletal muscle. These tissue-resident Stem cells possess the capacity to develop into either adipocytes or fibroblasts. FAPs promote satellite cell activation and differentiation in acute skeletal muscle damage, and satellite cells subsequently prevent FAP conversion into adipocytes[31]. Disappearance of inflammatory features after 14 days can be explained as the immune system switches its attention from inflammation to tissue repair during regeneration. ECM synthesis and hematoma replacement with ECM are specifically facilitated by M2 macrophages and T helper cells (TH2)[32].

Also there was increase in mean values of both myotubes and myogenin immunostained nuclei in subgroup IIB with further progressive increase in subgroup IIC as compared to the control and subgroup IIA. These findings indicate start of regeneration and myogenesis in subgroup IIB with continuation of regeneration process in subgroup IIC. Satellite cell/myoblast proliferation occurs 3–7 days following injury. To create myotubes, myoblasts differentiate and fuse together. Myofibers were formed by the fusion of myotubes. The center location of nuclei defines myotubes and regenerating myofibers[30]. According to previous studies; pro-inflammatory macrophages (M1) stimulate myoblast multiplication but impede myoblast fusion and differentiation, whereas anti-inflammatory macrophages (M2), which are primarily involved in regeneration, encourage the development and differentiation of myotubes[31].

In addition to the previous finding, subgroup IIB and IIC showed evident fibrosis which was measured by area percent of Mallory stained collagen fibers which showed significant increase in subgroup IIB with further more increase in subgroup IIC as compared to control and group IIA. Subgroup IIC showed the highest value of area percent of collagen fibers in all groups which indicates incomplete regeneration and healing by fibrosis. It was found that the anti-inflammatory macrophages (M2) release transforming growth factor-β (TGF-β) that enhances fibroblast proliferation and collagen synthesis[33].

In the current work, as regard VEGF expression in subgroup IIB showed significant increase in expression of VEGF with further t increase in subgroup IIC as compared with the group I and group IIA, but the values were less than that of treated groups in the corresponding days. This can be explained as VEGF is active during the wound repair process and its levels can affect the speed and efficacy of the repair, low VEGF levels lead to impaired wound healing[34].

In the present work the regeneration process in subgroups IIB and IIC was confirmed by the progressive significant drop in the CPK levels in both subgroups IIB and IIC concomitantly as compared to group IIA, but values still significantly greater than the group I.It was reported that the progression of muscle injury and recovery can be tracked using CPK as a marker. Recovering skeletal muscle or losing mass in functional muscles due to fibrous tissue formation is a distinctive cause of a decline in serum CPK level[35]. Our findings in recovery groups confirmed the start of regeneration. However; the regeneration process was incomplete as it resulted in fibrous tissue formation instead of formation of new muscle fibers.

In present work as regard stem cell treated groups; in subgroup IIIA revealed few cellular infiltrations in the adjacent connective tissue with many regular fibers. After 14 days there was more regression of the muscle damage in subgroup IIIB as it showed parallel regularly arranged muscle fibers with peripherally arranged flat nuclei. This is consistent with Zhang *et al*., (2020)[36] and Moussa *et al*., (2020)[1] who reported that BM-MSC can suppress the release of pro-inflammatory cytokines and increases the production of anti-inflammatory IL-10 cytokines.

Meanwhile, in our work, subgroup IIIA showed significant rise in mean values of both myotubes and myogenin immunoreactive nuclei with further more increase in subgroup IIIB. The mean numbers of myotubes and myogenin stained nuclei in stem cell treated groups were the highest among all other groups in corresponding days which reflects the high rate of myogenesis and regeneration.It is also in agreement with Moussa *et al*., (2020)[1], who stated that BM-MSCs induced the regeneration of muscle fibers by myogenic proliferation, and myoblasts differentiation. The primary element influencing BM-MSCs' curative efficacy was their capacity to generate paracrine substances, such as a range of growth factors and cytokines. This is also in accordance with Alarcin *et al*., (2021)[2] who documented that When BM-MSCs were injected directly into the injured rat muscle, the number of mature muscular fibers and skeletal muscle regeneration were higher than the non-treated group. In addition in our work, the regression of damage of muscle fibers was confirmed by significant drop in CPK level in subgroup IIIA which with further more drop in subgroup IIIB .The values were significantly lower than recovery groups in corresponding days.

In our study, VEGF-stained sections from subgroup IIIA confirmed the previous finding as they showed significant increase in VEGF expression with further significant increase in subgroup IIIB. The values were significantly more than all study groups in the corresponding days, indicating higher rate of angiogenesis and regeneration. Bone marrow-derived mesenchymal stem cells have the capacity to develop into a various cell types, such as pericytes and endothelial cells, as well as monocytes and macrophages, which are involved in tissue repair and revascularization[37].

In the current study subgroup IIIA showed significant decrease in fibrosis by Mallory trichrome with more decrease after 14 days in subgroup IIIB. Stem cell treated groups showed the least values of area percent of collagen fibers among all other groups in corresponding days indicating very minimal fibrosis. Studies have reported that MSCs target TGFβ receptors playing anti-fibrotic effect in skeletal muscle healing. Along with inflammation and fibrosis, up-regulation of TGFβ activity disrupts the balance necessary for healthy and effective muscle regeneration and alters myoblast proliferation[38].

In current study, sections from subgroup IVA (PRP treated Group) showed many regularly arranged muscle fibers, others irregular with some inflammatory cellular infiltrate. These finding showed more improvement of muscular tissue in subgroup IVB as it showed parallel regularly arranged muscle fibers with peripheral arranged flat nuclei, few fibers were irregular. It has been demonstrated that Platelets can control regulation of inflammatory cells and macrophage polarization. Furthermore, platelet mediators can convert monocytes into M2 macrophages, which can lessen inflammation and carry out tissue healing[39].

In the current work, in addition to previous findings of subgroup IVA, there was significant increase in the mean value of myotubes and the mean value of myogenin immunoreactive nuclei with further increase in subgroup IIIB. The values were higher than recovery groups but lower than SCs treated groups in the corresponding days. These findings were in agreement with García *et al*., (2022)[40] who found greater expression of myogenin and other myogenic factors during the PRP-induced muscle regeneration. This finding, suggests that PRP therapy accelerating the regeneration and the repair process of muscle injury.

In the current work, the features of repair and restoration of structure in subgroup IVA were confirmed by mean CPK level which showed significant drop in subgroup IVA with further decrease after in subgroup IVB. The values were lower than recovery groups but not significantly differ from stem cell treated groups in corresponding days.

VEGF-stained sections also confirmed repair as they showed increased expression of VEGF in subgroup IVA with more expression in subgroup IV , the values were significant higher than recovery groups, but they were significantly lower than stem cell treated groups at corresponding days.It was reported that after injection of PRP, an extensive tissue repair process is started. Pro-angiogenic and growth factors and other granular components are released by activated platelets. One of the most powerful platelet growth factors, VEGF, is crucial for angiogenesis[41].

In currant study, the area percent of collagen fibers in subgroup IVA showed significant decrease in fibrosis with more improvement in subgroup IVB as compared by recovery group at corresponding days but fibrous

tissue was still higher than that in stem cell treated groups in corresponding days. This could be explained as PRP hinders TGF-β1-induced conversion of fibroblasts to myofibroblasts, regulates fibrogenic markers expression possibly by blockage the TGF-β1 pathway of transduction[42]. However, Mariani and Pulsatelli (2020)[43] and Tonogai *et al* (2018)[44] reported in their work that PRP has a limited ability in hindering of muscle fibrosis. Also Kuffler, (2019)[45] and Zhang *et al*., (2023)[46] stated that PRP could reduce pain sensation by its anti-inflammatory effect. However, PRP's potential to prevent fibrosis is still debatable.

CONCLUSION

According to our results, both stem cell and PRP injection showed improvement of muscle healing and regeneration in compare with non-treated recovery group. Stem cells had the upper hand in improvement of muscular structure, decrease fibrosis and induction of angiogenesis.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

دراسة هستولوجية للتأثير التجددي المحتمل للخلايا الجذعية الوسيطية المشتقة من نخاع العظام مقابل البلازما الغنية بالصفائح الدموية علي نموذج اصابة العضلات الهيكلية في ذكر الجرذ الأبيض البالغ

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قسم علم الأنسجة، كلية الطب، جامعة القاهرة، مصر

الخلفية والهدف من العمل: تعتبر إصابات العضلات الهيكلية واحدة من أكثر الإصابات شيوعًا. نظرًا لأن التعافي الكامل يكون معرضًا للخطر بسبب التليف. صُممت هذه الدراسة لتقييم ومقارنة التأثيرات التجددية المحتملة للخلايا الجذعية الوسيطة للنخاع العظمي مقابل البلازما الغنية بالصفائح الدموية في إصابة تمزق العضلات الهيكلية في نموذج الجرذ الأبيض الذكر البالغ.

المواد والطرق: تم تقسيم أربعة وأربعين جرذا بالغًا إلى ٤ مجموعات: المجموعة ١ (الضابطة)(١٢ جرذ), المجموعة٢ (١٢ جرذ) تعرضت لتمزق عضلة الساق اليمنى وتم تقسيمها إلى مجموعات فرعية؛مجموعة فرعيى ٢- أ (٤ جرذان) تم التضحية بهم في يوم التجربة, المجموعة الفرعية (٢- ب)(٤ جرذان) تمت التضحية بهم في اليوم ٧ المجموعة الفرعية (٣- ج) (٤ جرذان) تمت التضحية بهم في اليوم ١٤ .المجموعة ٣ (١٠ جرذان))حقنة عضلية تحتوي علي ١ مل محلول ملح الفوسفات تحتوي على مليون خلية جذعية (ثم تم تقسيمها الي المجموعة الفرعية (٣- أ)(٤ جرذان) تمت التضحية بهم في اليوم ٧ ,المجموعة الفرعية (٣- ب)(٤ جرذان) تم التضحية بهم في اليوم ١٤, المجموعة ٤(٨ جرذان) (حقنة عضلية بمقدار ١ مل من البلازما). ثم تقسيمهم إلى المجموعة الفرعية (٤- أ): ٤ جرذان تم التضحية بهم في اليوم ٧ ,المجموعة الفرعية (٤- ب):، ٤ جرذان تم التضحية بهم في اليوم ١٤. تم الحصول على العينات ومعالجتها باستخدام التقنيات الكيميائية الحيوية والنسيجية والمناعية.

النتائج: (الهيماتوكسيلين والإيوسين) المجموعة الفرعية ٢- أ أكدت الاصابة.المجموعة الفرعية٢-ب كشفت أليافًا عضلية غير منتظمة مع تغلل لخلايا الالتهاب و الخلايا الدهنيةو ألياف قليلة أظهرت أنابيب عضلية. المجموعة الفرعية(٢- ج) بعض الألياف العضلية المنتظمة والعديد من الألياف غير المنتظمة مع تليفًا ظاهري واسع النطاق. المجموعات الفرعية ٣- أ, ٣-ب,٤-أ,٤-ب أظهروا تحسن في البنية العضلية. صبغة مالوري,المجموعة الفرعية ٢-أ أظهرت اضطراب في الغشاء العضلي,المجموعات الفرعية ٢-ب ,٢-ج أظهروا زيادة ذات دلالة احصائية في ترسيت الكولاجين و التي انخفضت في كلتا المجموعتين الثالثة و الرابعة.فيما يخص التفاعل لكل من الميوجينين و عامل نمو البطانة المجموعات المعالجة أظهرت ارتفاعا ذا دلالة احصائية اكثر من مجموعات التعافي ولكن المجموعة ٣ كانت الأعلي. كان متوسط قيمة فوسفوكيناز الكرياتين في المجموعة الفرعية (٢- أ) هو الأعلى احصائيا مع انخفاض ملحوظ حدث في لمجموعات التعافي مع انخفاض أكبر في المجموعات المعالجة. الاستنتاج:كل من الخلايا الجزعية الوسيطة لنحاع العظام و البلازما الغنية بالصفائح الدموية قدمت دليلا على آثارها العلاجية في إصابة العضلات و لكن الخلايا الجذعية لها اليد العليا في استعادة بنية العضلات.