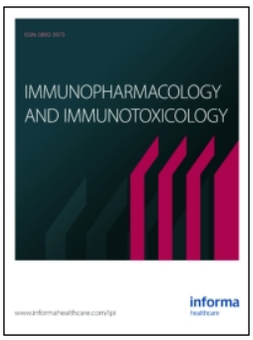

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RESEARCH ARTICLE

Immunomodulatory capacity of the local mesenchymal stem cells transplantation after severe skeletal muscle injury in female rats

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ABSTRACT

Context: Cell therapy technique with stem cells is a very attractive strategy for the treatment of muscle disorders.

Objective: The objective of this study was to investigate the mechanism of local transplantation of mesenchymal stem cells (MSCs) which could contribute to skeletal muscle healing.

Materials and methods: Female rats were divided into three equal groups as the following: group 1, the negative control group (untreated group), group 2, sham-treated group, rats with muscle injuries involving volumetric muscle loss (VML) of adductor brevis muscle and injected locally with phosphate-buffered saline (PBS) 0.5 ml without stem cells after 7 d of muscle injury, group 3, treated group, rats with VML and injected locally (intramuscular) with 1.5×10^6 bone marrow MSCs suspended in PBS 0.5 ml (1) after 7 d of muscle tissue injury. All animals were sacrificed after 4 weeks of stem cell transplantation.

Results: *In vitro* culture the morphology of MSCs reached confluence and appeared as long spindle in shape on 9–14 d. Most of the cells did not express the hematopoietic cell marker, CD34 and CD45 but expressed MSCs marker CD44, CD90 and CD105. The remarkable increase of proliferating cell nuclear antigen positive nucleus was recorded in MSCs group as compared to PBS group. After 28 d of injection, administration of only PBS into the site of muscle injury caused up-regulation in the levels of interleukins IL-1β, IL-6, tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β1), interferon alpha (IFN-α) and down-regulate the level of IL-10 in muscular tissue comparing to the untreated control. Bone marrow MSCs + PBS injected at the site of muscle injury significantly down-regulate the inflammatory cytokines levels IL-1β and IL-6 and TNF-α, TGF-β1 and IFN-α and up-regulate the level of IL-10. Collagen concentrations in the injured skeletal muscle estimated by enzyme-linked immuno sorbent assay and stained with Masson trichrome stain were increased with PBS group and decreased after transplantation of bone marrow MSCs in the site of injury. Muscle sections stained with H&E showed a higher number of centronucleated regenerating myofibers in the stem-cell-treated group than in the (PBS) and untreated control group. Microvasculature of skeletal muscle was decreased as demonstrated by immunostaining technique for CD34 in PBS group from untreated control. The MSCs group showed angiogenesis and marked increase of skeletal muscle microvasculature than PBS group.

Conclusion: MSCs can modify the local immunological responses and improve muscle regeneration by suppressing of inflammatory cytokines, activating of the anti-inflammatory cytokine, restoration of muscle fibers and angiogenesis. By means of increase in TGF-β production in response to muscle injury prevent the repair of injured fibers and increase connective tissue production (collagen fibers), thus propagating skeletal muscle weakness and fibrosis whereas MSCs + PBS injected at the site of muscle injury significantly down-regulate (TGF-β1) and hence the level of collagen (fibrosis or scar areas). MSCs are able to block the fibrotic signaling cascade by declining TGF-β1 and scar areas in the injured muscle.

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Mesenchymal stem cells; interleukins; tumor necrosis factor alpha; transforming growth factor; interferon alpha; fibrosis; angiogenesis

Introduction

Skeletal muscle injury resulting in tissue loss poses unique challenges for surgical repair[1]. Injuries involving volumetric muscle loss (VML) are often seen in military personnel wounded in action by gunshots and blasts[2]. Complete repair of VML is dependent on the ability of an implant to fill the void in the tissue while allowing for the growth and development of functional myofibers, blood vessels and nerves[1]. In response to damage, the physiological regeneration of skeletal muscle follows three sequential phases: i) inflammation with the presence of macrophages, ii) activation and fusion of satellite cells and iii) the maturation of newly formed myofibers and the development of fibrosis[3].

Satellite cells are considered high-quality cells because of their intrinsic myogenic potential[4]. Nevertheless, they are poorly expandable *in vitro*, rapidly undergo senescence and show a poor survival rate of transplanted cells *in vivo*[5]. Bone-marrow-derived mesenchymal stem cells (MSCs) have the potential to differentiate and fuze with myoblasts *in vitro*[6] and to contribute to muscle healing and treatment of muscle

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disorders[7]. Stem cells are unspecialized cells with the ability to renew themselves for long periods without significant changes in their general properties. They can differentiate into various specialized cell types under certain physiological or experimental conditions[8] and can be successfully expanded *in vitro*[9]; they can also exert strong therapeutic effects in the musculoskeletal system. The full repair of a large defect in a skeletal muscle will require the growth of myofibers, but it also requires blood vessel integration and nerve innervation of the myofibers. The implantation of myoblasts or other myogenically committed cells might not be capable of regenerating vessels and nerve. Cells from the bone marrow are known to participate in skeletal muscle regeneration naturally[10]. Moreover, Roth et al.[3] found that muscles that underwent MSCs transplantation developed a higher amount of fast myosin heavy chain (MHC) fibers compared to untreated animals. MSCs have been shown to be effective in the regeneration of periodontal tissue defects, diabetic critical limb ischemia, bone damage caused by osteonecrosis and burn-induced skin defects[11]. In addition to their ability to treat tissue injury, MSCs are also applied to alleviate immune disorders because MSCs have a powerful capacity of regulating immune responses[8]. Gnecchi et al.[12] postulated a paracrine mode of action, which they concluded from decreased fibrosis and apoptosis after local injection of the MSCs-conditioned medium in myocardial infarction. The effect of a local MSCs transfer on skeletal muscle regeneration has been examined in a few experimental studies[1]. Earlier studies have shown that MSCs treatment in muscle crush trauma apparently allows enhancing muscle regeneration, but the underlying mode of action or structural change have remained unclear so far[3]. The aim of this study was to investigate the mechanism of local transplantation MSCs that could contribute to skeletal muscle healing after an injured skeletal muscle with VML in the rat model through the study of MSCs immunological properties and the level of cytokines.

Materials and methods

MSCs isolation and cultivation

Bone marrow MSCs were isolated according to the method described by McFarlin et al.[13] The bone marrows of 10 young male rats, *Wistar* rats (6-week-old) were harvested by flushing the tibiae and femurs with Dulbecco's modified Eagle's medium (DMEM) (Lonza Group Ltd, Basel, Switzerland). The harvested cell suspension was divided into seven flasks 25 cm with DMEM containing 12% fetal bovine serum (FBS) (Lonza Group Ltd, Basel, Switzerland), 1% penicillin- streptomycin (Lonza Group Ltd, Basel, Switzerland) and 0.05% Amphotericin B (Lonza Group Ltd, Basel, Switzerland). Cells were incubated at 37 °C in 5% humidified CO_2; the cultured cells were examined daily by using the inverted microscope to follow up the growth of the cells and to detect the appearance of any bacterial or fungal infection of the cultured cells. After 5 d, the supernatant that contained the non-adherent cells was removed by aspiration using a sterile pipette. The adherent cells were then washed twice with a sterile phosphate buffer saline (PBS). Finally, 10 ml of fresh complete media was added to the dish. MSCs were distinguished from other bone marrow cells by their tendency to adhere to tissue culture plastic[14]. The second exchange of media was done after 3 d[15]. On day 9, the cultured cells showed confluent appearance. The medium was aspirated by using a sterile pipette. Then the adherent cells were washed twice, using sterile PBS. The adherent MSCs were fixed, on day 9 of culture, by using freshly prepared pre-cooled (−20 °C) mixture of acetone/methanol (A:M at 1:1 V/V). The mixture of pre-cold A/M was added to the culture dish for 10 min at room temperature[16]. The fixed adherent MSCs were stained by Giemsa stain. The staining solution was added to the culture dishes for 30 min and then the dishes were washed by tap water. Examination of dishes and taking photographs were done by using the inverted microscopy. On day 12, the culture was washed with PBS and released with 0.25% trypsin in 1 mmol/l ethylenediamine tetraacetic acid (EDTA) (Lonza Group Ltd, Basel, Switzerland) (4 min at 37 °C). After centrifugation, the cells were resuspended and counted with trypan blue stain 0.4% using Neubauer hemocytometer[17]. MSCs were distinguished from other bone marrow cells by negative expressions of CD34 and CD45 and positive expression of CD44, CD105 and CD90 (Figure 1(e,f)) in immune staining, described by Li et al.[18] Briefly, the cultured cells were fixed in Petri dishes on day 12 of culture by A:M (1:1) then covered by H_2O_2 (10%). Dishes then immersed in a preheated citrate buffer solution (pH 6) and maintaining heat in a microwave, then left to cool, washed in distilled water and incubated in normal blocking goat serum. The primary antibodies for all (CD44, CD105, CD90, CD34 and CD45) were monoclonal mouse antihuman, (CD44v6 Ab-1, Clone VFF-7 and CD34, clone 8G12, (Lab-Vision, Biocompare com, CA)), (CD105, Clone 1G2; CD90, clone 5E10; CD45 clone 30-F11, Beckman Coulter, Germany). The primary antibodies were added and left overnight in the humidified chamber, then washed. The secondary biotinylated anti-mouse antibody was added and incubated. Then, dishes were washed and covered by streptavidin-horseradish peroxidase conjugate. The color was developed using diaminobenzidine.

Animals

The experiment was performed on female *Wistar* rats (156–158 g; 9-week-old). Animals were housed with free access to food and water and were maintained at standard laboratory conditions (28 ± 2 °C and 12-h light/dark cycle). Animal housing and experimental procedures including stem cell transplantation were approved by Medical Research Center, Stem Cell Unit, Ain Shams University. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Medical Research Center, Ain Shams University.

Experimental groups

A total number of 18 female rats were divided into three groups of six rats each as the following: group 1, the

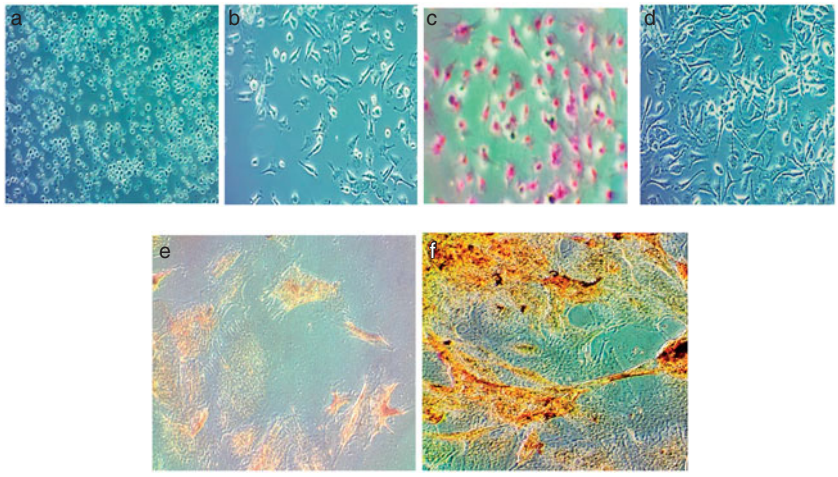

Figure 1. Morphology of MSCs *in vitro* culture following (a 400×) day one and (b 400×) day 7 of primary culture. (c 400×) On day 9, adherent MSCs stained with Giemsa appeared as fibroblastic in shape. (d 400×) On day 12, MSCs were appeared as long spindle-shaped cells. (e) MSCs did not express the hematopoietic marker, $CD45^{-}$. (f 400×) MSCs expressed marker $CD90^{+}$.

negative control group constituted of an untreated group. Group 2, sham-treated group, rats with muscle injuries involving VML and injected locally with PBS 0.5 ml without stem cells, group 3, treated group, rats with VML and injected locally (intramuscular) with 1.5×10^6 bone marrow MSC suspended in PBS 0.5 ml[1].

Skeletal muscle injury

One week before stem cells transplantation, rats were anesthetized with an intraperitoneal injection of ketamine and xylazine (80 and 5 mg/kg), and an adequate depth of anesthesia was maintained such that the rats were unresponsive to tactile stimulation. Left legs were shaved and disinfected with povidone-iodine. The adductor brevis muscles were used in all rats. The rat muscle injury model was based on a previous report[1,19]. A lateral skin incision (2 cm) was made in the left leg on the side of the thigh of 12 female rats, and the adductor brevis muscle was exposed. The fascia was incised transversely and carefully released from the muscle. The muscle was then lacerated transversely, using a scalpel. The defect's size was approximately 6 mm long, 4 mm wide and 5 mm deep. The wounds were closed in one layer with simple interrupted stitches of silk suture (4–0; Ethicon).

Cell transplantation

After 7 d of muscle tissue injury, female rats were anesthetized as described earlier and prepared for the injection of cells. The original skin incision was opened at the defect of the adductor brevis muscle. MSCs were injected in 4–6 locations throughout the adductor brevis defect area. All animals were evaluated macroscopically during healing and regeneration at 1 and 4 weeks after injury. The bridging tissue occupying the lacerated sites was observed. All animals were sacrificed after 4 weeks of cell transplantation by decapitation. For each animal, the adductor brevis muscle of the left leg was cut transversely into two pieces. One piece was stored at −70 °C until used for enzyme-linked immuno sorbent assay (ELISA) or biochemical studies and the other one was fixed in 10% neutral buffered formalin. After fixation, muscle samples were routinely processed for paraffin embedding, with the fibers oriented perpendicularly to the plane of sectioning in order to permit a cross-sectional view. Muscle samples were sectioned (5 μm). Tissue sections were analyzed under a light microscope.

Identification of PCNA labeled MSCs

To confirm the proliferation of transplanted cells at the site of injection, the muscle tissues were labeled using mouse-derived monoclonal antibodies to detect proliferating cell nuclear antigen (PCNA) after 28 d of MSCs transplantation which was carried out by using the avidin–biotin peroxidase complex ABC technique[20]. Paraffin sections were deparaffinized and hydrated. After blocking the endogenous activity of peroxidase using 10% hydrogen peroxide, the sections were incubated with mouse monoclonal anti-PCNA antibody (PC 10, Novocastra, Milton and Keynes, USA). After washing with PBS, the secondary antibody was applied (biotinylated goat antirabbit). The slides were incubated with labeled avidin–biotin peroxidase, which binds to the biotin on the secondary antibody. The site of antibody binding was visualized after adding (diaminobenzidine) chromogen, which is converted into a brown precipitate by peroxidase. PCNA-positive cells showed brown nuclear deposits.

Determination of cytokine concentrations in the muscular tissue

The muscle tissues were homogenized in proteases inhibitors centrifuged to remove debris and concentrated five-fold using a 3000 molecular-weight cutoff (MWCO) filters (Centricon 3, Millipore, Billerica, MA). Cells were collected by scraping into cold PBS with 2% FBS and protease inhibitors, and lysed by sonication, followed by centrifugation[21].

The evaluation of interleukins (IL-1β, IL-6, IL-10), transforming growth factor beta (TGF-β1), interferon alpha (IFN-α),

tumor necrosis factor alpha (TNF-α) concentrations were estimated in the rat muscular tissue in all groups by using sandwich ELISA using capture/biotinylated detection monoclonal antibodies (Sigma, UK). The results were presented as an absolute ratio: cytokine concentration/protein concentration.

Determination of collagen fibers concentrations in the muscular tissue

Rat ELISA of type 1 collagen levels in tissue were performed using sandwich ELISA kits (Sigma, St. Louis, MO), according to the manufacturer's instructions.

Evaluation of fibrosis by Masson trichrome stain, muscle regeneration and capillary density in the skeletal muscle sections

For collagen analysis, the transverse muscle sections stained with Masson trichrome reagents[22] were examined by using the objective lens at 400× magnification. Fifteen random fields were counted for each sample. The percentage of the collagen fiber area that was expressed was calculated by dividing the collagen fiber area by the sum of muscle fiber area and collagen fiber area[23].

For evaluation of muscle regeneration, Hematoxylin and Eosin staining were performed. Muscle regeneration was assessed by counting the number of centronucleated regenerating myofibers 4 weeks after treatment. Ten random fields from a single cross section area were examined at 400× magnification for each muscle. The average number of regenerating myofibers was compared between groups[24,25].

Immunohistochemical staining with CD34, a marker for rat endothelial cell of blood capillaries within the skeletal muscle, was performed and the capillary density was evaluated quantitatively. The immunopositive area for CD34 in 10 fields of view from each animal was calculated at 400× magnification. The results underwent statistical analysis. Immunohistochemical stainings were performed by using primary antibodies against antigens: CD34 (Lab-Vision, Biocompare com, CA). Paraffin was removed from muscle tissue sections by rinsing in xylene and ethanol. Sections were incubated in a solution of 10% H_2O_2 to block endogenous peroxidase. Tissue sections were incubated with primary antibodies for 60 min and then rinsed with PBS. Then, the slices were subjected to Diaminobenzidine (DAB) chromogen staining, which reacts with the enzyme indicator, reveals sought in the form of the brown-antigen reaction product. Tissue sections were analyzed under light microscope[26].

Statistical analysis

Data are expressed as mean values ± SD of replicate determinations. Statistical analysis was performed using one-way analysis of variance to assess significant differences among treatment groups. For each significant effect of treatment, the *post hoc* Least Significant Difference (LSD) test was used for comparisons. All statistical analyses were performed by using SPSS statistical version 17 software package (SPSS Inc. Chicago, IL).

Results

Morphology of MSCs in vitro culture

Day one in culture, MSCs appeared as a monolayer of broad flat cells (Figure 1(a)), the cells were differentiated into a more spindle and fibroblastic shaped cells on day 7 (Figure 1(b)). On day 9 of culture and after fixation with A:M, adherent MSCs stained with Giemsa appeared fibroblastic in shape (Figure 1(c)). The cells reached confluence and appeared as long spindle in shape on 9–14 d, (Figure 1(d)), and were attached to the culture flasks. Most of the cells did not express the hematopoietic cell marker, CD34 and CD45 but expressed MSCs marker CD44, CD90 and CD105 (Figure 1(e,f)).

Immunohistochemical staining of proliferating cell nuclear antigen (PCNA) in skeletal muscles

Proliferated cells were examined by PCNA in skeletal muscle of untreated control, injured skeletal muscle (PBS) group and injured skeletal muscle after 28 d of MSCs transplantation (Figure 2). The numbers of PCNA positive nuclei increased significantly in skeletal muscle sections in both PBS and MSCs groups as compared to untreated control. Remarkable increase (+151.39%) of PCNA positive nucleus was recorded in MSCs group as compared to PBS group as shown in Figure 2(d). The level of increase in the proliferated cells

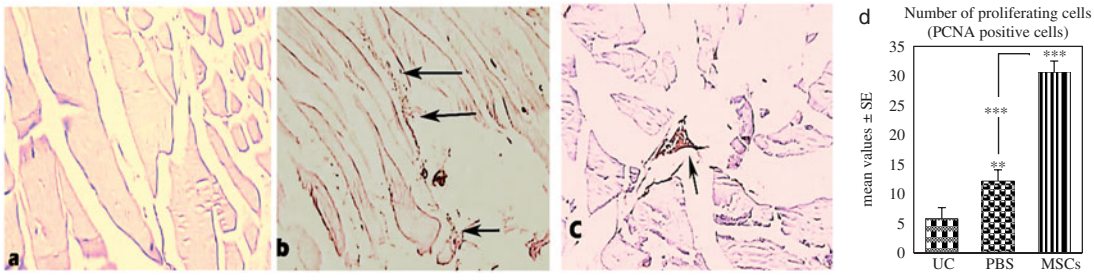

Figure 2. PCNA immunohistochemistry was used to evaluate cell replication in skeletal muscles of (a 100×) untreated control, (b 100×) injured muscle treated with PBS and (c 100×) injured muscle treated with stem cells to show few number of PCNA positive nuclei (arrow) in PBS group (b) and many number of PCNA positive nuclei (arrow) in injured skeletal muscle after MSCs transplantation (c). (d 400×) Quantification number of PCNA positive nuclei in 10 randomly selected fields. Results are expressed as mean values ± SE, different superscripts designate significant differences $^{**}p < .01$, $^{***}p < .001$. Untreated control (UC) injured muscle group treated with PBS (PBS) and injured muscle group treated with stem cells (MSCs).

were statistically recorded ($p = .01$) in PBS group and ($p = .001$) in MSCs group as compared to negative control group. There was a statistically significant difference ($p = .001$) was detected in terms of increase in a number of proliferated cells between MSCs group and PBS group.

Cytokines levels in the muscular tissue

Analysis of IL-1β and IL-6 in muscular tissue revealed that administration of only PBS into the site of muscle injury caused highly significant ($p < .001$) up-regulation of both IL-1β and IL-6 comparing to the untreated control after 28 d of injection. The levels of both IL-1β and IL-6 were increased (+120.4% and+ 105.9%, respectively) from the negative control. Bone marrow MSCs + PBS injected at the site of muscle injury significantly ($p < .001$) down-regulate the inflammatory cytokines levels IL-1β and IL-6 after 28 of injection to become (−31.02% and −32.27%, respectively) from PBS group (Figure 3). There were statistically significant difference were detected ($p = .001$) in terms of down regulation in the inflammatory cytokines levels IL-1β and IL-6 between MSCs group and PBS group.

On contrast, down-regulation in the level of IL-10 was recorded in the group of rats treated with PBS only without stem cells and reached to (−48.5%) of negative controls. While transplantation of MSCs into the skeletal muscle induced a statistically significant ($p < .001$) up-regulation of IL-10 to become (+52.96%) of PBS group (Figure 3).

Statistically significant difference was noticed ($p = .001$) in terms of up-regulation in the IL-10 between MSCs group and PBS group.

The level of TNF-α increased significantly ($p < .001$) in the PBS-treated group without stem cells to reach 120.5% from negative untreated control. While injection of MSCs + PBS decreased significantly ($p < .001$) the level of TNF-α to (−18.8%) from PBS group (Figure 4). On the same line, the levels of TGF-β1 in muscular tissue were elevated 80% ($p < .001$) from untreated control after 28 of injection whereas treatment muscle injury by bone marrow MSCs reduced significantly ($p < .001$) the level of TGF-β to reach only −28.6% from PBS group (Figure 4).

The group of rat that exposed to the muscle injury and treated with PBS only for 4 weeks showed statistically ($p < .001$) higher levels of IFN-α to reach (+ 143.9%) with respect to untreated control. However, transplantation of bone marrow MSCs into the site of injury was statistically ($p < .001$) lower the level of IFN-α to (−30.73%) as compared to PBS group (Figure 4). There were statistically significant difference were detected ($p = .001$) in terms of reduction in the levels of TNF-α, TGF-β1 and IFN-α between MSCs group and PBS group.

Estimation of fibrosis by ELISA and Masson trichrome stain

The levels of collagen in the injured skeletal muscle estimated by ELISA were increased significantly ($p < .001$) about +55.6% of negative control after 28 d of injury and decreased significantly ($p < .001$) to −27.7% of PBS after transplantation of bone marrow MSCs in the site of injury (Figure 5(d)). In addition, muscle sections stained with Masson trichrome stain (Figure 5) showed an increase in the percentage of green positive areas with massive fibrosis in PBS group as compared to the untreated control group, which was decreased significantly ($p < .001$) after treatment of muscle injury with MSCs. The mean percentage of collagen fiber area recorded (44.66 ± 4.04) in PBS group and decreased to (10.52 ± 1.79) in MSCs group (Figure 5(e)).The increase in the collagen fibers were statistically recorded ($p = .001$) in PBS group and ($p = .05$) in MSCs group as compared to negative control group. There was a statistically significant difference ($p = .001$) was detected in terms of decrease in a number of collagen fibers between MSCs group and PBS group.

Skeletal muscle fibers regeneration

From the examination of muscle sections stained with H&E (Figure 6) within injured site, we observed that a higher number of centronucleated regenerating myofibers in the stem-cell-treated group (12.11 ± 0.67) than in the (PBS) group

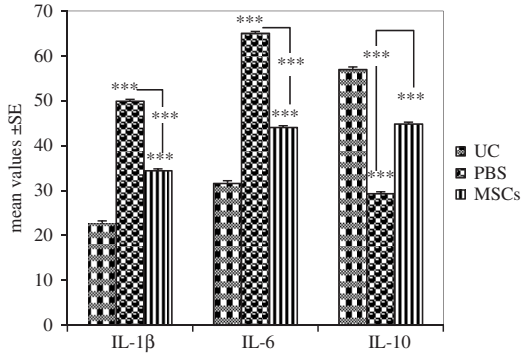

Figure 3. The concentrations of cytokines: IL-1β, IL-6 and IL-10 in the muscle tissue: untreated control (UC; $n = 6$), after PBS injection (PBS; $n = 6$) and after MSCs transplantation (MSC; $n = 6$). Results are expressed as mean values ± SE. Significantly $^{***}p < .001$ compared with untreated control group (UC).

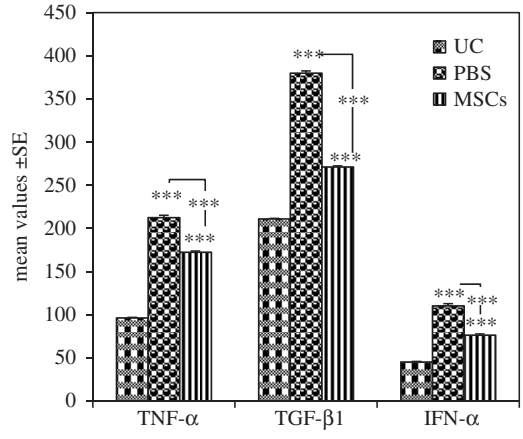

Figure 4. The concentrations of cytokines: TNF-α, TGF-β1 and IFN-α in the muscle tissue: untreated control (UC; $n = 6$), after PBS injection (PBS; $n = 6$) and after MSCs transplantation (MSCs; $n = 6$). Results are expressed as mean values ± SE. Significantly $^{***}p < .001$ compared with untreated control group (UC). Untreated control (UC) injured muscle group treated with PBS (PBS) and injured muscle group treated with stem cells (MSCs).

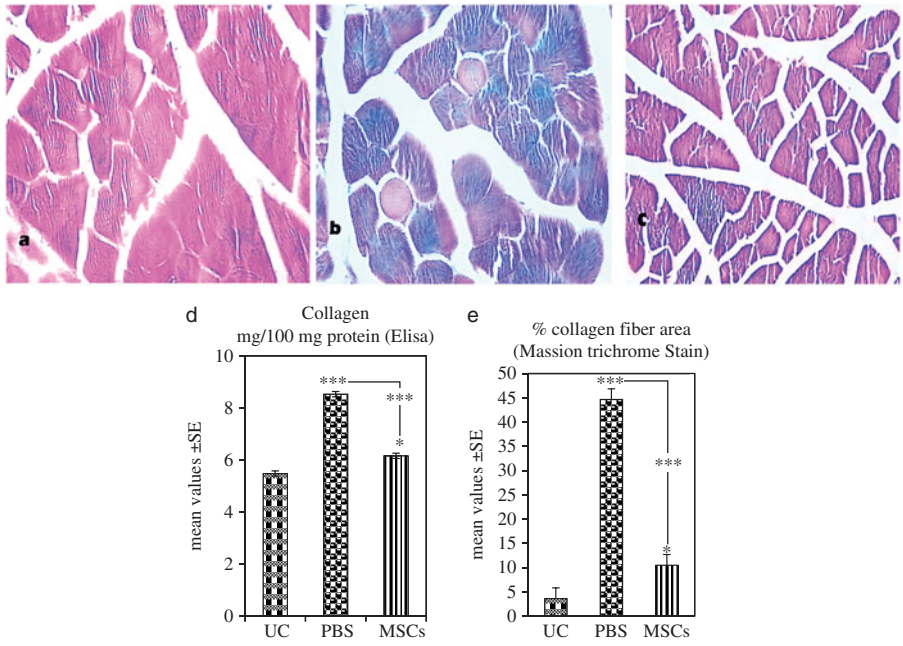

Figure 5. Transverse sections of skeletal muscles stained with Masson trichrome to evaluate the areas of fibrosis in (a 200×) untreated control, (b 200×) injured muscle group treated with PBS and (c 100×) injured muscle group treated with stem cells. Multiple scattered areas of fibrosis as positive green areas were seen in (b) PBS group whereas injured skeletal muscle treated with MSCs stem cells (c) exhibited fewer number of skeletal muscle fibrosis as compared to (b). (d, ELISA) Quantification analysis of the level of collagen in skeletal muscle tissue and (e, Masson trichrome stain ×200) of untreated control (UC), injured muscle group treated with PBS (saline) and injured muscle group treated with stem cells (MSCs). Results are expressed as mean values ± SE ($n = 6$), different superscripts designate significant differences $^{*}p < .05$, $^{***}p < .001$ compared with normal group (UC).

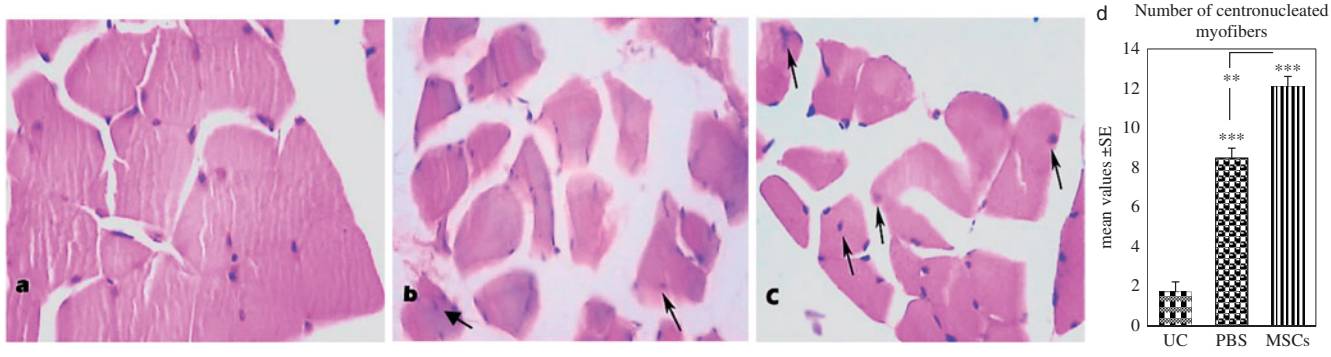

Figure 6. Transverse sections of skeletal muscles stained with H&E to evaluate the centronucleated regenerating myofibers in untreated control (a 200×), injured muscle group treated with PBS (b 200×) and injured muscle group treated with stem cells (c 200×) to show few centronucleated regenerating myofibers in (b) PBS group and (c) several number of centronucleated regenerating myofibers in injured skeletal muscle after MSCs transplantation. (d) Quantification analysis of centronucleated regenerating myofibers in 10 randomly selected fields (at 400× magnification). Data are expressed as mean values ± SE, different superscripts designate significant differences $^{**}p < .01$, $^{***}p < .001$ compared with normal Group (UC). Untreated control (UC), injured muscle group treated with PBS (PBS) and injured muscle group treated with stem cells (MSCs).

(8.49 ± 0.97) and untreated control group (1.73 ± 0.28). This means that MSCs injection in the site of injury led to an increase of centronucleated regenerating myofibers to reach (+42.63%) of PBS group (Figure 6(d)). Statistically significant difference was estimated ($p = .01$) in terms of augmentation in the number of centronucleated regenerating myofibers between MSCs group and PBS group.

Capillary density of skeletal muscle

Microvasculature of skeletal muscle was decreased as demonstrated by immunostaining technique for CD34 in PBS group (−30.46%) from untreated control. MSCs group showed angiogenesis and marked increase of skeletal muscle microvasculature to reach (+111.19%) of PBS group (Figure 7(d)). Histologically, few CD34 + ve spindle cells were observed at the periphery of the fibers in the untreated control (Figure 7(a)) and PBS groups (Figure 7(b)). In contrast, a noticeable increase of CD34 positive cells were observed in the lumen of blood vessels and occasionally between muscle fiber or within muscle fibers in injured skeletal muscle after MSCs transplantation (Figure 7(c)). The increase in the muscle microvasculature were statistically recorded ($p = .01$) in MSCs group as compared to negative control group. There was a statistically significant difference was recorded ($p = .001$) in terms of increase in the number of skeletal muscle microvasculature between MSCs group and PBS group.

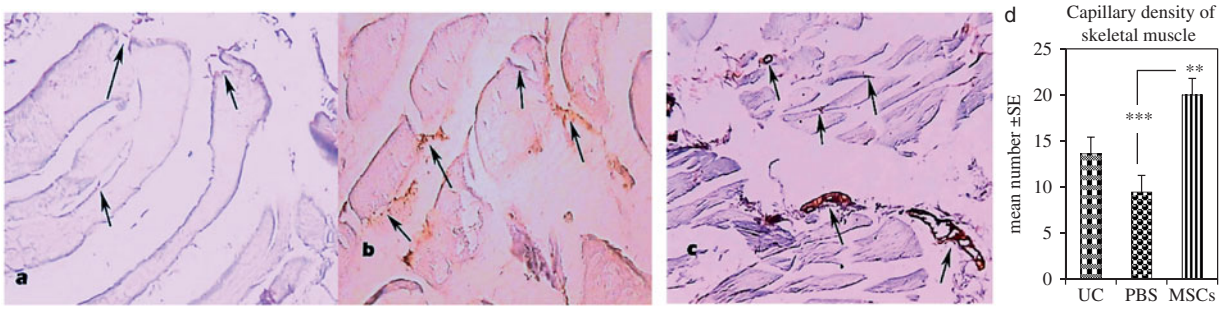

Figure 7. Skeletal muscles sections stained with immunohistochemical antibody (CD34), a marker for rat endothelial cell of blood capillaries within the skeletal muscle to show few CD34 + ve spindle cells (arrows) at the periphery of the fibers in untreated control group (a 200×), few CD34 + ve spindle cells in PBS group (b 200×) and marked CD43 positive cells were observed in the lumen of blood vessels and between muscle fiber or within muscle fibers (arrows) in the injured skeletal muscle after MSCs transplantation (c 100×). (d) Quantification number of capillaries in 10 randomly selected fields (CD34 immunostaining, 400×). Results are expressed as mean values ± SE, different superscripts designate significant differences $^{**}p < .01$, $^{***}p < .001$. Untreated control (UC), injured muscle group treated with PBS (PBS) and injured muscle group treated with stem cells (MSCs) groups.

Discussion

MSCs are an attractive cell therapy candidate is that they are easily obtained from the bone marrow and can be expanded in culture to provide clinically relevant quantities of cells[27]. Limitations of the present study directly linked to the interaction between the transplanted bone marrow MSCs and the immune response in the rat model. Among these limitations inflammatory and immune responses after cell transplantation including cell migration and differentiation. Because of gender that is becoming increasingly important in musculoskeletal research and therapy, we used female rats instead of males as female cells have higher skeletal muscle regeneration efficiently than males. Deasy et al.[28] found that dystrophic female cells have higher skeletal muscle regeneration efficiently than dystrophic male mice after muscle-derived stem cells transplantation. The increased number of PCNA positive cells that were seen on the site of injured muscle after MSCs transplantation as compared to PBS group indicated activation and proliferation of satellite cells that enter the S phase of the cell cycle[29]. It was demonstrated that satellite cells located on the damaged fibers undergo a series of processes including activation of mitotic quiescence, proliferation, differentiation and self-renewal. Ultimately, some of these daughter cells differentiate, and fuze into existing myofibers or form new fibers[30]. We and other previous study were able to identify MSCs in the interstitium of the injured muscles up to 4 weeks after transplantation[31]. The transplantation of stem cells to the injured skeletal muscle was performed 1 week after muscle injury as MSCs administration after disease onset may be better than at the same time of disease induction[32] owing to MSCs transplanted directly after trauma would not be able to exert their effect on regeneration through paracrine pathways, due to the hostile microenvironment[33]. The results of the present study showed highly significant up-regulation of cytokines IL-1β, IL-6, TNF-α, TGF-β1, IFN-α and collagen fibers accompanied with down-regulation in the level of IL-10 after 28 d of the muscle injury. Cytokine priming should be attempted to improve the therapeutic effect of MSCs. Skeletal muscle injury is associated with chronic elevations in circulating proinflammatory cytokines, soon after damage, a wave of neutrophils invades the area with a peak in their concentration at 24 h[34]. Neutrophils release proinflammatory molecules (such as CXCL8 and another IL-6) which recruit macrophages into the tissue[35]. Macrophages are rapidly activated and polarized towards an M1 inflammatory phenotype. Neutrophils and M1 macrophages produce an array of molecules, including cytokines, chemokines, nitric oxide and prostaglandins that sustain and amplify local inflammation[36]. TNF-α is the main proinflammatory cytokine present upon skeletal muscle damage[37]. However, TNF-α level remain raised for 2 weeks after acute injury, and this rise is paralleled by an increased expression of type I TNF α receptor in injured muscle fibers[38]. It has been demonstrated that TNF-α activates satellite cells to enter the cell cycle and accelerates G1-to-S phase transition[39]. TNF- α together with IL1, synthetized by invading macrophages as well, can promote muscle differentiation[40]. In both skeletal muscle disease and injury, TGF-β appears to be a major determinant for connective tissue proliferation and fibrosis[41]. Although TGF-β is typically regarded as a profibrotic cytokine, *in vitro* studies have shown that TGF-β plays a role in delaying myogenesis[42]. Therefore, an increase in TGF-β production in response to chronic muscle injury may inhibit the repair of injured fibers and increase connective tissue production, thus propagating skeletal muscle weakness and fibrosis[43]. An earlier study provided some evidence that the increase in plasma IL-6 was a consequence of an immune response due to local damage in the working muscles[44]. The marked and immediate increase in plasma IL-6 in response to muscle damage is followed by repair mechanisms including invasion of macrophages into the muscle leading to IL-6 production[45]. IL-6 induces the production of C-reactive protein, which has a role in the induction of anti-inflammatory cytokines in circulating monocytes and in the suppression of the synthesis of proinflammatory cytokines in tissue macrophages[46]. The skeletal muscle injury was significantly ($p < .001$) improved following injection of bone marrow-MSCs locally at the wound site by down-regulate the inflammatory cytokines levels IL-1β, IL-6, TNF-α, TGF-β1, IFN-α, collagen fiber and up-regulate the level of IL-10 after 28 of injection as compared to PBS group. These results are consistent with previous findings by others[47,48]. *In vitro* and *in vivo* studies, MSCs have been shown to suppress the excessive immune

responses of T cells, B cells, dendritic cells, macrophages and natural killer cells[49]. Moreover, after MSCs transplantation to the injured muscle it will recruit as the following; CX3CR1lo/Ly-6C + blood monocytes[50] within the muscle they release proinflammatory cytokines (TNF-α, INF-γ and IL-1β) that amplify tissue damage[35]. These cells were named CD68 + inflammatory (M1) cells and they are activated by INF-α, they will suffer a phenotypic and functional switch by the interference of a set of cytokines IL-4, IL-10 and IL-13, to the activated (M2) cells, alternatively activated macrophages that are involved in wound repair and tissue remodeling. Such cells were found near the regenerating muscle fibers. In the present work, the capillary density of skeletal muscle was decreased significantly after 28 d of injury while the noticeable increase of CD34 positive cells and capillary density was observed in between muscle fiber or within muscle fibers of the injured skeletal muscle after MSCs transplantation. Also, a higher number of centronucleated regenerating myofibers was detected in the stem-cell-treated group than in the (PBS) group. Implanting stem cells into skeletal muscle, promoting angiogenesis to activate resident satellite cells to aid in skeletal muscle healing[1]. Regeneration/repair phase initiates in the first week after injury and peaks at 2 weeks, and consists of three major stages starting with the activation and differentiation of muscle stem cells followed by maturation of the myofibers and paralleled by the formation of new vessels by angiogenesis to revascularize the newly formed myofibers[51]. After MSCs home to damaged tissue sites for repair, they interact closely with local stimuli, such as inflammatory cytokines and ligands of Toll-like receptors, which can stimulate MSCs to produce a large amount of growth factors that perform multiple functions for tissue regeneration[52]. Many of these factors are critical mediators in angiogenesis and the prevention of cell apoptosis, such as vascular endothelial growth factor, insulin-like growth factor 1, basic fibroblast growth factors, hepatocyte growth factor, IL-6 and CCL-2[53].

In conclusion, data from our study lead us to suggest that, local injection of MSCs has the potential to enhance muscular regeneration in rats. MSCs modify local immunological responses and can improve muscle regeneration by suppressing of inflammatory cytokines, activating of anti-inflammatory cytokine (IL-10), restoration of muscle fibers and angiogenesis. In addition to this, MSCs have proven to be remarkably antifibrotic able to block the fibrotic signaling cascade induced by skeletal muscle injury by declining TGF-β1 and scar areas in injured skeletal muscle. The present results suggested that MSCs could provide an appropriate environment for the injured skeletal tissue to recruitment and restore its integrated functions. Thus, cell therapy using MSCs derived from multipotent stem cells is one of the effective and safe ways to improve functional recovery of damaged skeletal muscles.

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Disclosure statement

The authors declare that they have no conflicts of interest in the research.

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