Original Article

The Possible Therapeutic Effect of Stem Cells-Conditioned Medium on Statin Induced Animal Model of Myopathy: Biochemical, Histological and Immuno-Histochemical Study

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ABSTRACT

Introduction: Myopathies are diseases with high prevalence rate all over the world. This study aimed to investigate the ability of stem cell conditioned medium (SC-CM) administered by 2 different routes (IM\IV) to repair damaged skeletal muscle.

Materials and Methods: This study included 60 adult male albino rats divided equally into five groups: the control group; the myopathy group in which myopathy was induced by administration of ATOR (10 mg/kg/day orally for 4 weeks); IV conditioned medium treated group in which myopathy was induced then the rats were IV injected with 0.5 ml of SC-CM in tail vein, IM conditioned medium treated group the rats were locally injected with 0.5 ml of SC-CM after induction of myopathy, the untreated myopathy group was left without treatment for 4 weeks. Blood samples and muscular specimens from all groups were collected for biochemical and histological studies.

Results: The myopathy group showed marked muscle degeneration, with inflammatory cells infiltration. Compared with control, there was significant decrease in the area% of PAS and significant increase in mean creatinine kinase and Myoglobin levels , in area% of collagen and caspase3 positive reaction and the number of myogenin positive cells. Four weeks after SC-CM injection (IM\IV), there was marked improvement in histological architecture of the skeletal muscle as there was significant decrease in mean creatinine kinase and Myoglobin levels and in area% of collagen and caspase positive cells and significant increase in area% of PAS and number of myogenin positive cells and the improvement was more obvious in IV injected group.

Conclusion: SC-CM injection may be of great help in the management of critical cases of myopathy especially by IV route.

Key Words: Conditioned medium, myopathy, statin.

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INTRODUCTION

Myopathies are a diverse collection of diseases that affect the structure, metabolism, or function of the skeletal muscles and are characterized by segmental necrosis and degeneration of individual muscle fibers (**Squecco *et al*., 2021**). Acquired myopathies have a wide range of etiologies, including endocrine, infectious, inflammatory, paraneoplastic, metabolic, pharmacological, and toxin-induced myopathies (**Saint-Gerons *et al*., 2022**).

One of the most common causes of drug induced myopathy is statins which are widely used to lower blood cholesterol levels and to reduce the risk of cardiovascular disease. Statins can induce myopathy through their deleterious effect on mitochondria and induction of skeletal muscle apoptosis (**Saint-Gerons *et al*., 2022**).

Mesenchymal stem cell culture medium (SC-CM) which known as MSC's secretome is a rich source of many trophic factors (**Fui *et al*., 2019**) which represent growth factors and cytokines, microvesicles and exosomes, that are involved in the transport of proteins and genetic material (e.g., microRNAs) to other cells (**Vilaça-Faria *et al*., 2021**). Therefore, (SC-CM) can be considered as a promising regenerative therapy for many degenerative diseases as myocardial infarction, stroke, bone diseases and degenerative myopathies (**Gunawardena *et al*., 2019**).

Our study aims to assess at the effect of different routes of administration (IM\IV) of SC-CM on the statin induced myopathy in adult male albino rats through biochemical, histological and immune-histochemical methods.

MATERIALS AND METHODS

1) Animals:

The study was carried on 60 adult male albino rats 8-12 weeks old and their weights approximately 150-180 gm and another 10 donor rats 4-6 weeks old with average weight of 70-80 gm for SC-CM preparation. All animal′s procedures were performed according to the local guidelines of the ethical committee of Faculty of Medicine, Minia University (Approval No.230:2022) according to the international guidelines (Act 1986).

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2) Isolation and culture of BMSCs was done according to the protocol described by (**Abdelwahab *et al*., 2021**) in Stem Cell Unit in Ain-Shams University.

3) Conditioned medium preparation (**Ibrahim and Allam, 2022**):

For 48 hours, BMSCs (2x106) were cultivated in 2 ml of FBS-free DMEM. The

conditioned media was removed, collected in an Eppendorf tube, and filtered using a sterile syringe filter with a 0.20 um pore size (Corning, NY14831, Germany).

4) Experimental design:

Sixty rats were served as the experimental group which was randomly divided into five equal groups:

A. Group I (Control group):

Included 20 animals that subdivided into 4 subgroups:

1- Subgroup A: animals were left without any intervention.

2- Subgroup B: animals received 1 ml DMSO per day orally for 4 weeks and then sacrificed.

3- Subgroup C: animals received 1 ml DMSO per day orally for 4 weeks, then received single intravenous injection of 0.5 ml saline in rat's tail vein and were sacrificed after 8 weeks

4- Subgroup D: animals received 1 ml DMSO per day orally for 4 weeks, then received single intramuscular injection of 0.5 ml saline in gastrocnemius muscle and sacrificed after 8 weeks

Group II (Myopathy group):

Included 10 Animals that received 10mg/kg (BW) per day of ATOR , dissolved in DMSO, once a day orally for 4 weeks then were sacrificed (**El-Deeb *et al*., 2018**).

B. Group III (Intravenous conditioned medium (IVCM) group):

After induction of myopathy; animals were intravenously injected by 0.5 ml of SC-CM in rat tail vein then were sacrificed after another 4 weeks.

C. Group IV (Intramuscular conditioned medium (IMCM) group):

After induction of myopathy; animals were intramuscularly injected by 0.5 ml of SC-CM in gastrocnemius muscle then were sacrificed after another 4 weeks.

D. Group V (Recovery group):

After induction of myopathy; animals didn't receive any treatment and were sacrificed after another 4 weeks.

Rats of all groups were decapitated and sacrificed under sedation with halothane. Rat tail veins were venipunctured to obtain blood samples, which were then centrifuged to extract the serum and submitted for biochemical analysis. Specimens of gastrocnemius muscle were rapidly removed and dissected for histological preparation. Muscle samples were quickly fixed in 10% formal saline for 48 hours then processed to make paraffin sections for the histological and immunohistochemical tests.

5) Motor Functional assessment (Forelimb grip strength test) (Seo et al., 2020):

This test was done using automated grip strength meter (Columbus Instruments). The mouse's forelimb was brought into touch with a grid attached to the device while being held by the tail. The mouse spontaneously pulled the grid, and the strength of its grasp was assessed. Each animal was maintained in position for 5seconds and was examined three times, with a 5-minute gap between each measurement. By averaging the results of the three best trials, we determined the maximum forelimb strength. The test was performed for all rats of all groups.

6) Biochemical study

1- Measurement of Rat Creatine Kinase MM Isoenzyme (CK-MM) activity:

Rat Creatine Kinase MB Isoenzyme (CK-MM) ELISA Kit manufactured by: CUSABIO (Catalog Number. CSB-E14403r) and done according to manufacturer's instructions.

2- Measurement of Rat Myoglobin (MYO) activity:

Rat Myoglobin (MYO) ELISA Kit manufactured by: Immunology Consultants Laboratory, Inc. (ICL) (Catalog Number. E-25MY) and done according to manufacturer's instructions.

7) Histological studies

Hematoxylin and Eosin (H&E) stains, Masson's trichrome and periodic Acid Schiff (PAS) (Suvarna *et al*., 2018):

Sections of muscle from all groups were stained with hematoxylin and eosin (H&E), Masson's trichrome for detection of collagen deposition and PAS stain for detection of glycogen content.

Immunohistochemical study (Cemek *et al*., 2008):

Five µm sections were sectioned for immune labelling. The procedure was done according to the manufacture instructions.

Briefly, tissue sections were deparaffinized in xylene, rehydrated, and then submerged in 0.1% hydrogen peroxide for 15 minutes to block endogenous peroxidase. To stop the background nonspecific staining, sections were incubated in the ultra-vision block for 5 minutes at room temperature. Primary anti-caspase 3 (Cat. No. 11319, ABclonal, Egypt) and anti-myogenin (Cat. No. NBP2-44328, Novus Biological, USA) antibodies were diluted at 1:100 in antibody diluent and incubated with the tissue for 1 hour at room temperature. After washing in a buffer, sections were incubated with biotinylated goat anti-rabbit secondary antibodies (1:1000) for 30 minutes before being washed. After that, incubate for a further 30 minutes with Vectastain ABC kits (Avidin Biotinylated horse radish peroxidase Complex) and washing for 10 minutes. Using the Ultra-vision One Detection System, HRP Polymer, and (diaminobenzidine) DAB Plus Chromogen (Thermo Fisher Scientific, USA), the reaction was seen. Counterstaining was carried out using hematoxylin and dehydrated by passing through ascending concentrations of alcohol then cleared by xylene then mount the cover slip using mounting media. The changes in the immunohistochemical reaction of the muscle tissues of the treated animals were detected through a comparative examination of the corresponding tissues of the normal control animals. The identical procedure was used on negative control slides, but no primary antibody was added.

Caspase 3 is a marker for apoptosis. and expressed in the cytoplasm of the degenerated skeletal muscle (Huang *et al*., 2019). Myogenin is a marker for muscle regeneration that expressed in the nuclei of satellite cells in developing muscle tissue (**Ibrahim and shaheen, 2022**).

8) Morphometric study:

The morphometric evaluation was performed for all groups. Ten non overlapping fields were used from 5 animals in every group of the different experimental groups.

1- Measuring endomysium space in transverse sections:

Endomysium space was measured using Image J software in a standard measuring frame using a magnification X400 by light microscopy transferred to the monitored screen. Photomicrographs of transverse sections of all groups were used for evaluation as previously described by (**Seo *et al*., 2020**)

2- Area % of collagen fibers, glycogen and caspase 3 immunopositivity:

Image J software was used to measure area fraction in a standard measuring frame using a magnification X400 by light microscopy transferred to the monitored screen (**Pereira *et al*., 2014**). Regardless of the staining intensity, areas with positively immunostained tissues were used for evaluation.

3- Counting Myogenin positive cells:

The myogenin positive cells were manually counted in myogenin immune stained slide X400.

STATISTICAL ANALYSIS

Quantitative data was analyzed by Graph Pad Prism (version 7.01 for Windows, Graph Pad Software, San Diego, California, USA, www.graphpad.com). For the parameters of each group, the mean and standard error of the mean (SEM) were determined. Mean and SEM were used to express values. The one-way ANOVA followed by the Tukey-Kramar post hoc test was performed for multiple comparisons, and the student t test was utilized to determine whether there were any significant differences between each pair of groups. Statistics are deemed significant for P-values below 0.05.

RESULTS

All the control subgroups showed the same results, so they are represented as one group

1. Motor Functional assessment:

The mean values of forelimb grip strength of rats in different groups (Figure 1c)

The myopathy and recovery groups showed a significant ($P<0.0001$) decrease compared to control group. The IVCM and IMCM groups showed a significant ($P<0.0001$) increase compared to myopathy rats. Comparing the CM treated groups; there was a significant ($P<0.05$) increase in the IVCM group compared to the IMCM group.

2. Biochemical results:

The mean values of Rat Creatine Kinase MM Isoenzyme (CK-MM) and Myoglobin (MYO) in different groups (Figure 1; a, b):

Statistical analysis of CK-MM and MYO level levels among groups showed that, the myopathy and recovery groups showed a significant increase compared to the control group ($P<0.0001$). While IVCM and IMCM groups showed a significant ($P<0.05$) decrease compared to the myopathy and recovery groups but there was no significant ($P>0.05$) difference between 2 groups.

3. Histological Results:

H&E results:

Control group: Longitudinal sections of skeletal muscles revealed normal histological structure in the form of regularly arranged parallel muscle fibers with acidophilic sarcoplasm, regular transverse striations and multiple flat peripheral nuclei. Flat nuclei of fibroblasts were also noticed in the endomysium (Figure 2; a1, a2). while transverse sections revealed bundles of polygonal skeletal muscle fibers that were separated by CT perimysium. The individual muscle fibers exhibited multiple flat peripheral nuclei with acidophilic cytoplasm (Figure 2; b1, b2).

Myopathy group: Longitudinal sections of skeletal muscles showed extensive histological changes including waviness, disruption and degenerated muscle fibers, with tapering to the extent of separation and widening of endomysial space. Some muscle fibers showed areas of strong acidophilic sarcoplasm and deeply stained nuclei. Loss of striations was noticed in most fibers. (Figure 3; a1, a2, a3). Congested blood vessels with inflammatory cells was noticed (Figure 3; a4, a5, a6). Transverse sections showed widely separated fibers with irregular outline. There were degeneration and disruption of fibers with wide endomysium between muscle fibers. Some fibers had pale centrally located nuclei (Figure 3; b1, b2).

IVCM group: Longitudinal sections of skeletal muscles showed restoration of their normal regular parallel arrangement with multiple flat peripheral nuclei and regular transverse striations (Figure 4; a1, a2). Transverse sections showed restoration of normal polygonal muscle fibers with apparent multiple peripheral flat nuclei with C.T. perimysium in between muscle bundles (Figure 4; b1, b2).

IMCM group: Longitudinal sections showed apparently regularly arranged parallel muscle fibers with numerous peripheral flat nuclei. Pale acidophilic sarcoplasm and loss of transverse striations were noticed in some muscle fibers (Figure 5; a1, a2). Transverse sections of skeletal muscles of IMCM group showed restoration of normal appearance of polygonal muscle fibers with peripheral flat nuclei that separated by CT perimysium with disruption of some muscle fibers (Figure 5; b1, b2).

Recovery group: Longitudinal sections of this group still showed some degenerated muscle fibers with loss of striations and faint acidophilic sarcoplasm. Few fibers with centrally located nuclei were also noticed (Figure 6; a1, a2). Transverse sections showed some areas with faint acidophilic sarcoplasm, but other areas showed strong acidophilic sarcoplasm. Wide endomysium in between muscle fibers was noticed (Figure 6; b1, b2).

Masson's trichrome results:

The control group showed minimal collagen deposition in the endomysium of muscle fibers while the myopathy and recovery groups revealed excessive collagen deposition. In IVCM treated group, there was little collagen deposition in endomysium; while in IMCM treated group, the collagen deposition was moderate (Figure 7; a, b, c, d, e).

Statistically, analysis of area % of the collagen fibers among groups showed the myopathy and recovery groups with a significant ($P<0.001$) increase in collagen deposition compared to all groups. The IVCM and IMCM groups revealed a significant ($P<0.05$) decrease compared to the myopathy and recovery groups. Comparing the CM treated groups; there was a significant ($P<0.05$) increase in the IMCM group compared to the IVCM groups. (Figure 7f)

Periodic acid Schiff (PAS) results:

PAS-stained sections of the control group showed strong PAS reaction in the muscle fibers while there was faint PAS reaction in the myopathy group. The IVCM and IMCM treated groups revealed strong PAS reaction with few faint patchy areas. In the recovery group there was moderate PAS reaction in some areas of muscle fibers (Figure 8).

Statistically, analysis of area % of the glycogen content among groups showed that, the myopathy and recovery groups with a significant ($P<0.0001$) decrease in glycogen content compared to other groups. The IVCM and IMCM groups showed a significant ($P<0.05$) increase compared to the myopathy and recovery groups. Comparing the treated groups; there was a significant ($P<0.05$) increase in the IVCM group compared to the IMCM groups (Figure 8f).

Immunohistochemical results for anti-caspase 3 antibody:

Immunohistochemical staining of caspase 3 resulted in that the control group revealed negative immune expression in the sarcoplasm of muscle fibers while there was strong positive expression in the myopathy and recovery groups. In IVCM group, there was faint caspase 3 reaction while the IMCM group showed moderate positive expression (Figure 9; a, b, c, d, e).

Statistically, analysis of area fraction of caspase 3 immunopositivity among groups showed that, the myopathy and recovery groups with a significant ($P<0.05$) increase compared to other groups. The IVCM and IMCM groups showed a significant ($P<0.05$) decrease compared to the myopathy and recovery groups. Comparing the treated groups; there was a significant ($P<0.05$) increase in the IMCM group compared to the IVCM groups (Figure 9f).

Immunohistochemical results for anti-myogenin antibody:

The control group showed negative nuclear expression for myogenin in skeletal muscle cells while the myopathy and recovery groups showed few positive nuclei of satellite cells. The IVCM group showed numerous nuclei of satellite cells with positive expression. The IMCM group showed many immunopositive nuclei of satellite cells (Figure 10; a, b, c, d, e).

Statistically, analysis of number of myogenin positive cells among groups showed that, there was a significant ($P<0.05$) increase in the mean number of myogenin positive cells in the myopathy and recovery groups compared to the control group. The IVCM and IMCM groups showed a significant ($P<0.05$) increase compared to all other groups. Comparing the treated groups; there was a significant ($P<0.05$) increase in the IVCM group compared to the IMCM groups (Figure 10f).

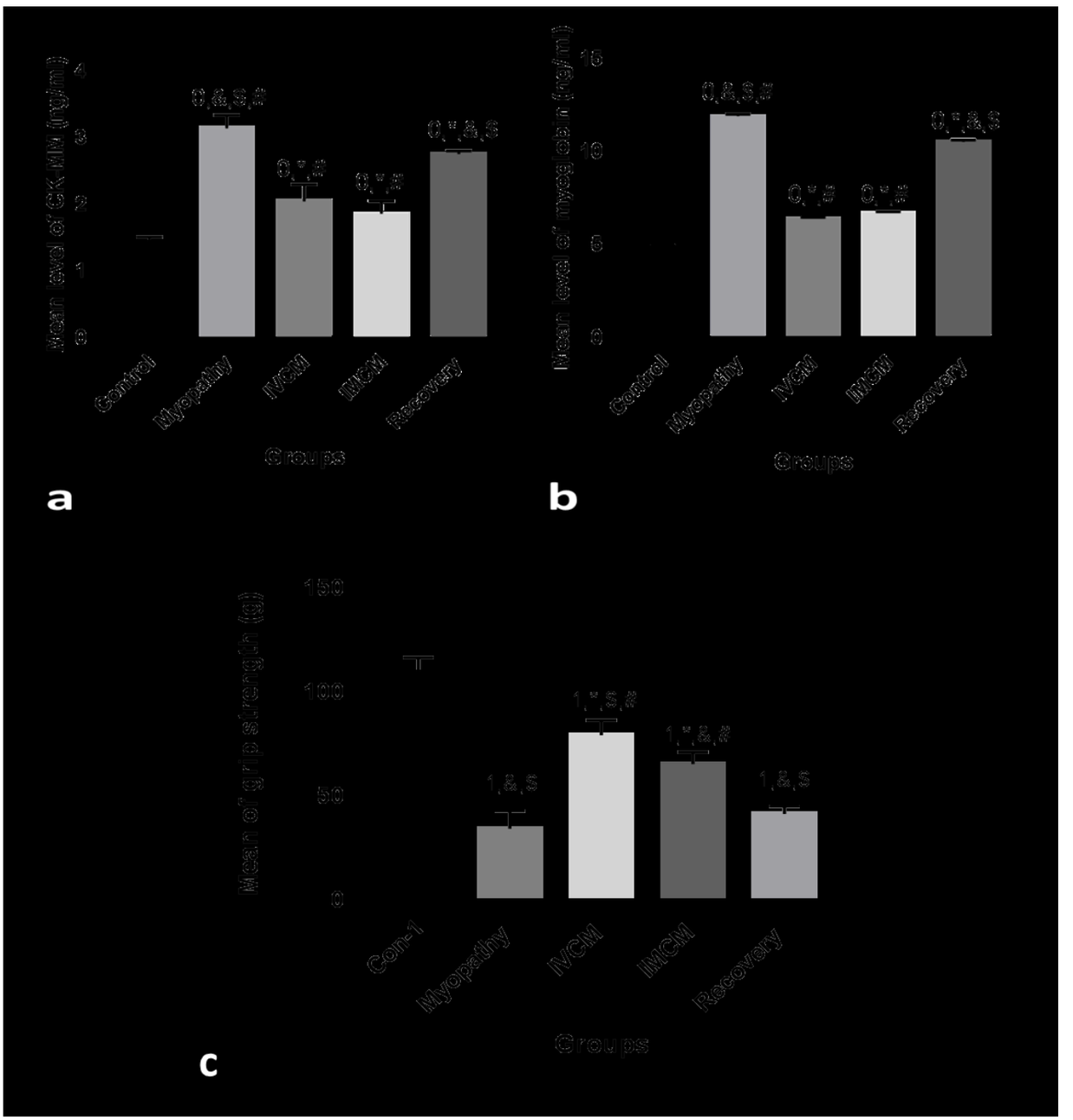

Fig. 1: Bar charts showing a) The mean values of Rat Creatine Kinase MM Isoenzyme (CK-MM) in different groups. b) The mean values of Rat Myoglobin (MYO) in different groups. C) The mean values of forelimb grip strength of rats in different groups. (Significant: 0 vs control group, * vs myopathy group, & vs IVCM group, $ vs IMCM group, # vs recovery group)**.**

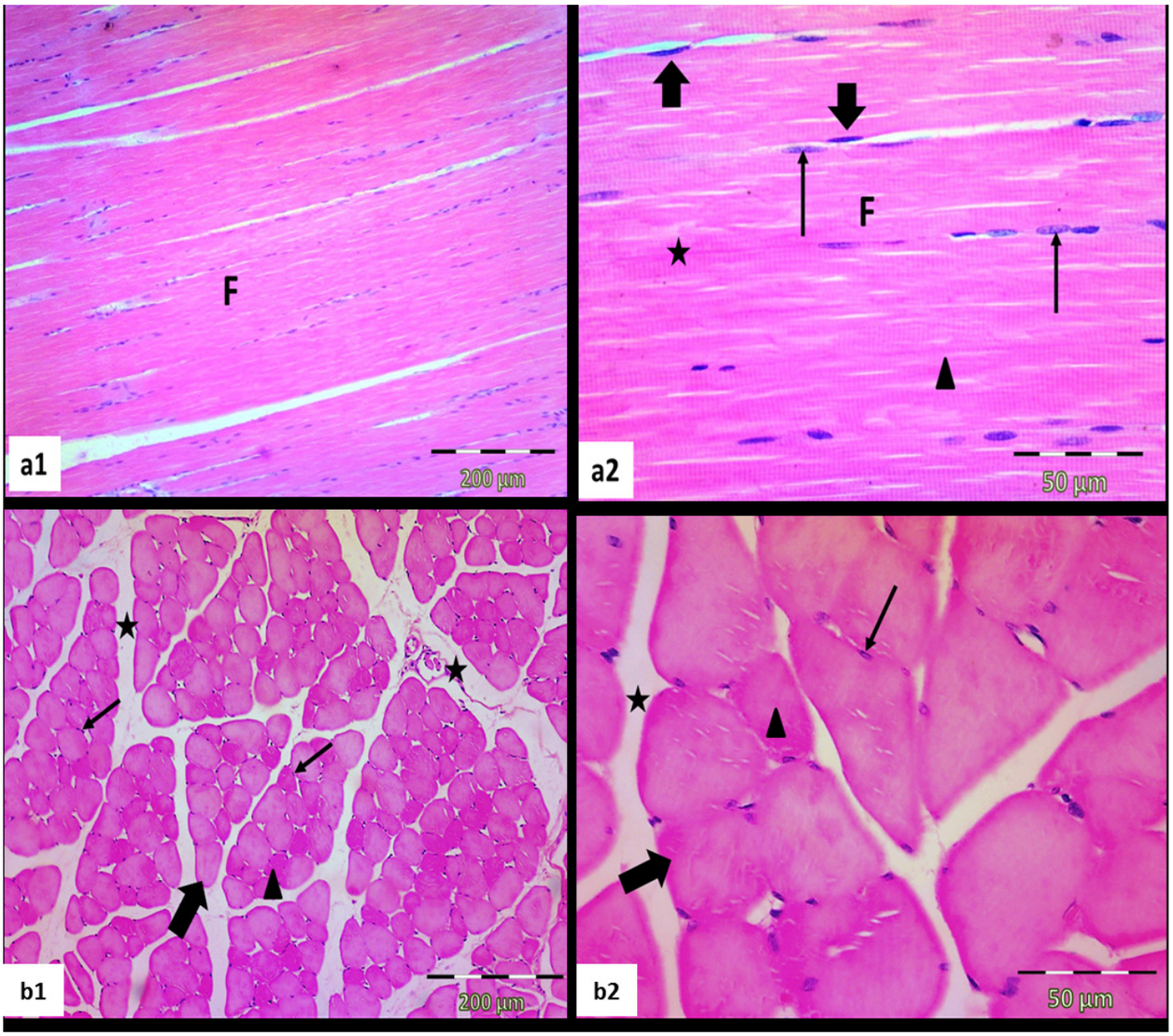

Fig. 2: Representative Photomicrographs of a1, a2)longitudinal section and b1, b2) Transverse section of rat's gastrocnemius muscle of control group showing a1, a2) Regularly arranged parallel muscle fibers (F) with acidophilic sarcoplasm (star), regular transverse striations (arrowhead) and multiple peripheral flat nuclei (thin arrows). Notice flat nuclei of fibroblasts in the endomysium (thick arrows). B1, b2) Bundles of polygonal skeletal muscle fibers (thick arrows) separated by CT perimysium (stars), acidophilic sarcoplasm (arrowhead) of muscle fibers with multiple flat peripheral nuclei (thin arrows). (H&E; a1, b1 x 100 , scale bar=200 µm ; a2, b2 x 400, scale bar=50 µm).

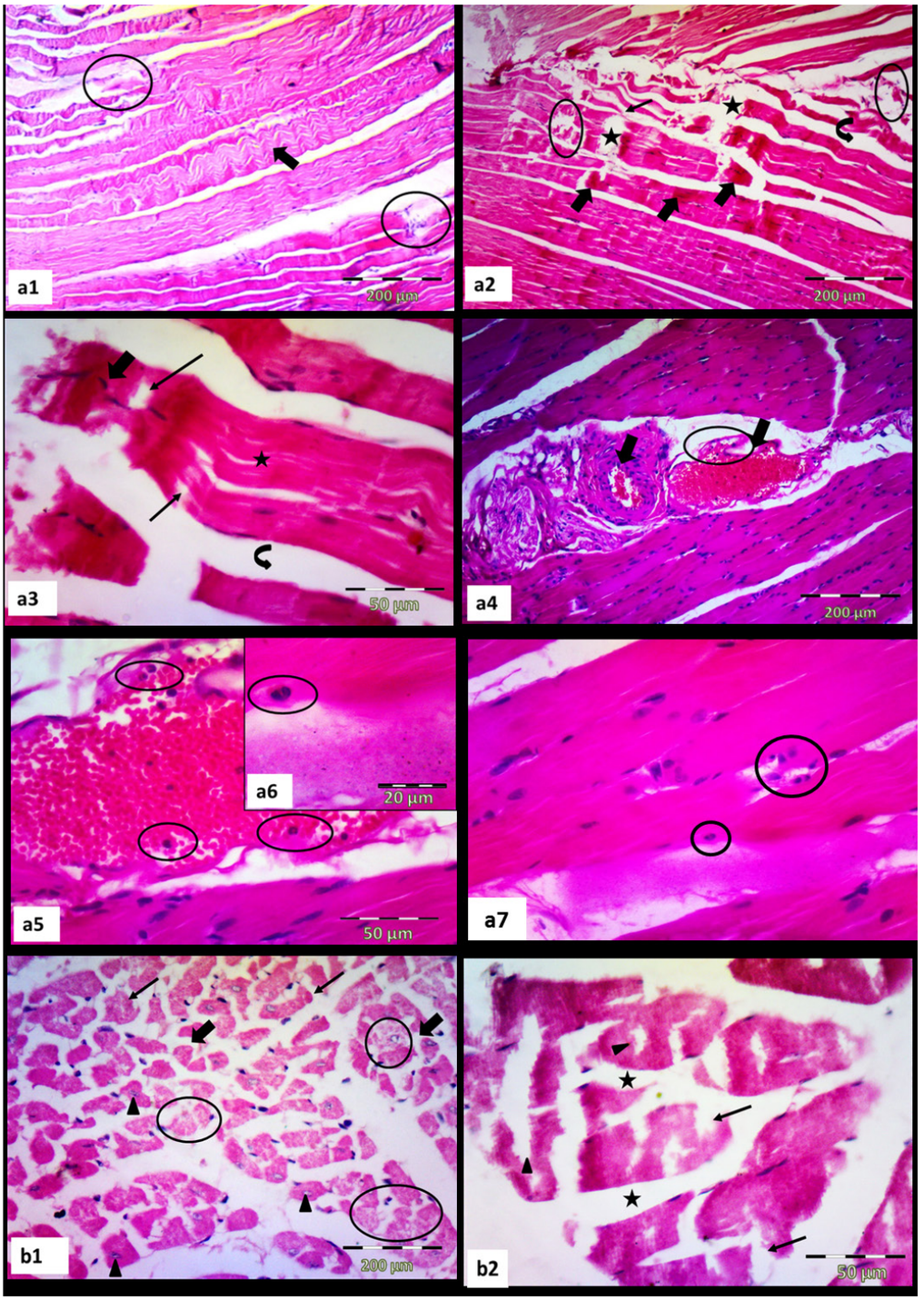

Fig. 3: Representative Photomicrographs of a1, a2, a3, a4, a5, a6, a7) longitudinal section and b1, b2) Transverse section of rat's gastrocnemius muscle of myopathy group showing a1) degenerated (circles) and wavy (thick arrow) skeletal muscle fibers. a2) Disrupted (stars) & degenerated (circles) muscle fibers , tapered fibers to the extent of separation (thin arrow), widening of the endomysium (curved arrow), cells with strong acidophilic sarcoplasm (thick arrows). a3) Higher magnification from (a2) degenerated muscle fibers (thin arrows), wide endomysium (curved arrow), cells with strong acidophilic sarcoplasm and deeply stained nuclei (thick arrows). Notice loss of striations (star). a4, a5, a6) congested blood vessels (thick arrows) with inflammatory cells (circle). a7) inflammatory cells infiltration (circles). b1) fibers with irregular outline (thin arrows), degenerated fibers (circles), wide endomysium (thick arrows). Notice centrally located nuclei(arrow heads). b2) degenerated (thin arrow), widely separated (stars), disrupted (arrows head) fibers. (H&E; a1, a2, a4, b1 x 100 , scale bar=200 µm ; a3, a5, a7, b2 x 400, scale bar=50 µm ; a6 x1000, scale bar=20 µm).

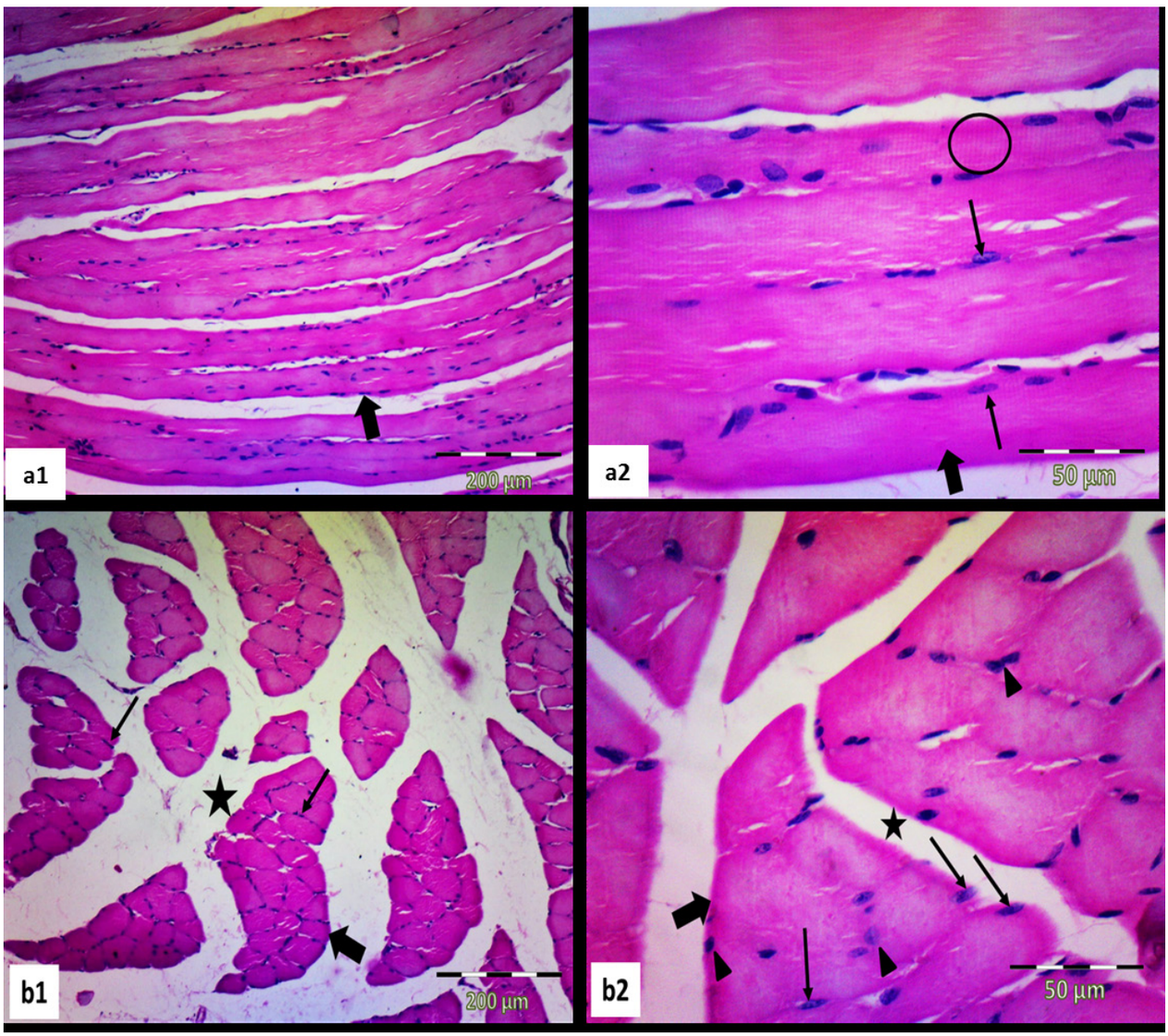

Fig. 4: Representative Photomicrographs of a1, a2) longitudinal section and b1, b2) Transverse section of rat's gastrocnemius muscle of IVCM group. a1, a2) showing apparently regularly arranged parallel muscle fibers (thick arrows) with multiple peripheral flat nuclei (thin arrow). Notice the transverse striations (circle). b1, b2) revealing apparently normal polygonal muscle fibers (thick arrow) with peripheral flat nuclei (thin arrow), separated by C.T. perimysium (star). (H&E; a1, b1 x 100, scale bar=200 µm ; a2, b2 x 400, scale bar=50 µm).

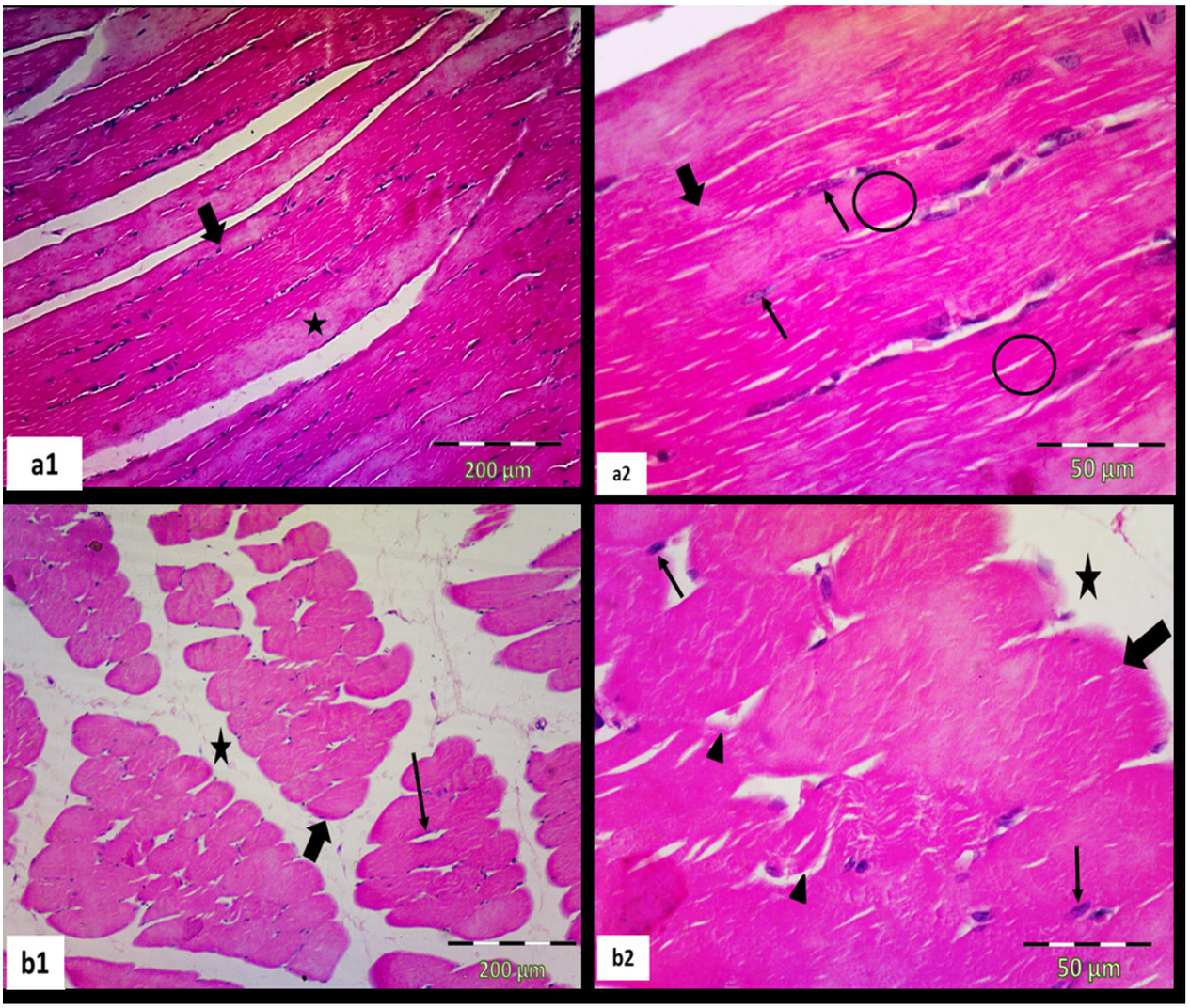

Fig. 5: Representative Photomicrographs of a1, a2) longitudinal section and b1, b2) Transverse section of rat's gastrocnemius muscle of IMCM group showing a1, a2) apparently regularly arranged parallel muscle fibers (thick arrows) with areas of pale acidophilic sarcoplasm (star), and peripheral flat nuclei (thin arrows) of muscle fibers. Notice loss of striation (circles). B1, b2) apparently normal polygonal muscle fibers (thick arrows) with peripheral flat nuclei (thin arrows), separated by C.T. perimysium (stars). Notice disrupted muscle fibers (arrowheads). (H&E; a1, b1 x 100 , scale bar=200 µm ; a2, b2 x 400, scale bar = 50 µm).

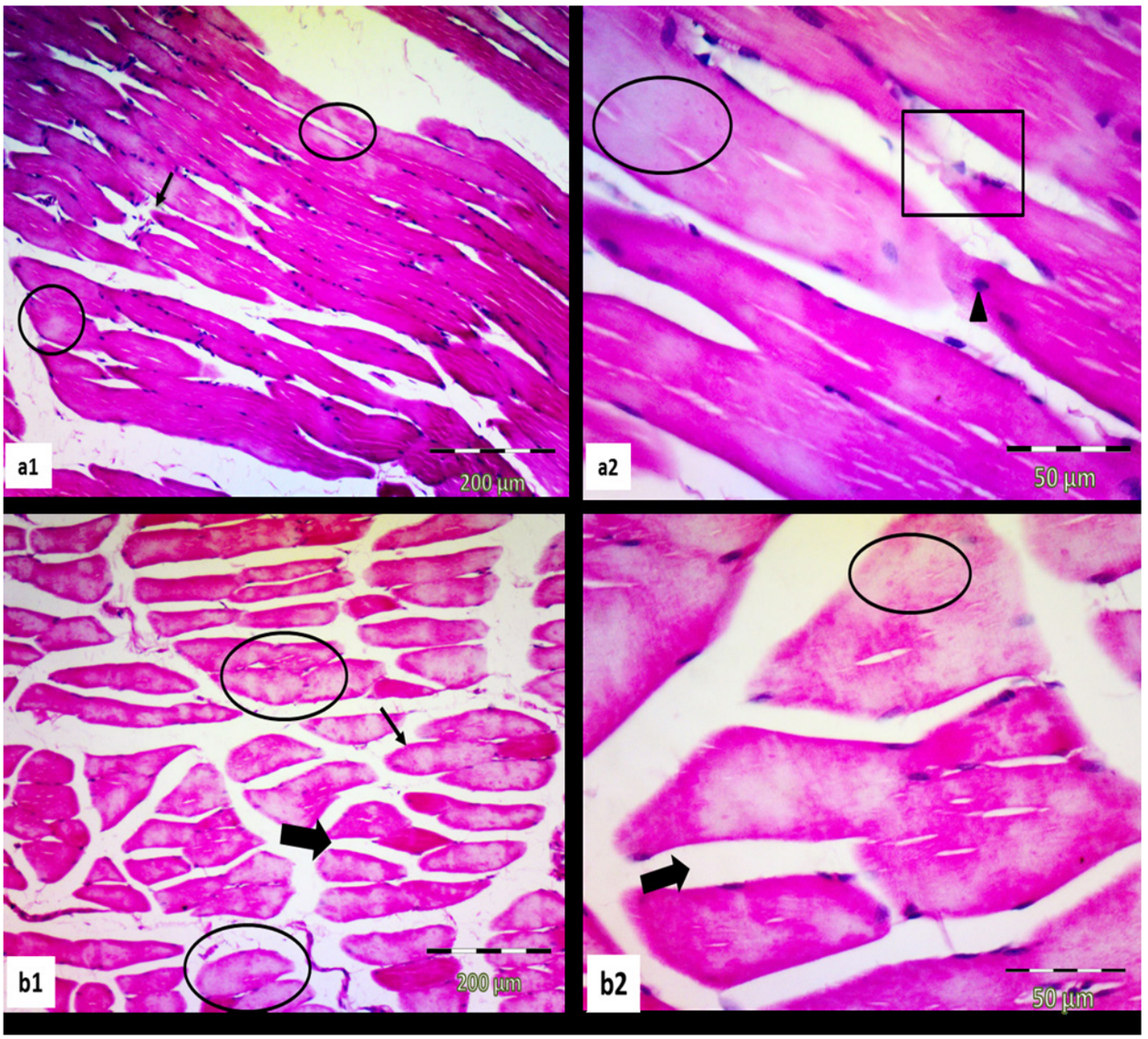

Fig. 6: Representative Photomicrographs of a1, a2) longitudinal section and b1, b2) Transverse section of rat's gastrocnemius muscle of recovery group showing a1, a2) disruption of muscle fibers (thin arrow), loss of striations with faint acidophilic sarcoplasm (circles), and degenerated fibers (square). Notice centrally located nuclei (arrowhead). b1, b2) fibers with faint acidophilic sarcoplasm (circles) and wide endomysium (thick arrows). (H&E; a1, b1 x 100, scale bar=200 µm ; a2, b2 x 400, scale bar=50 µm).

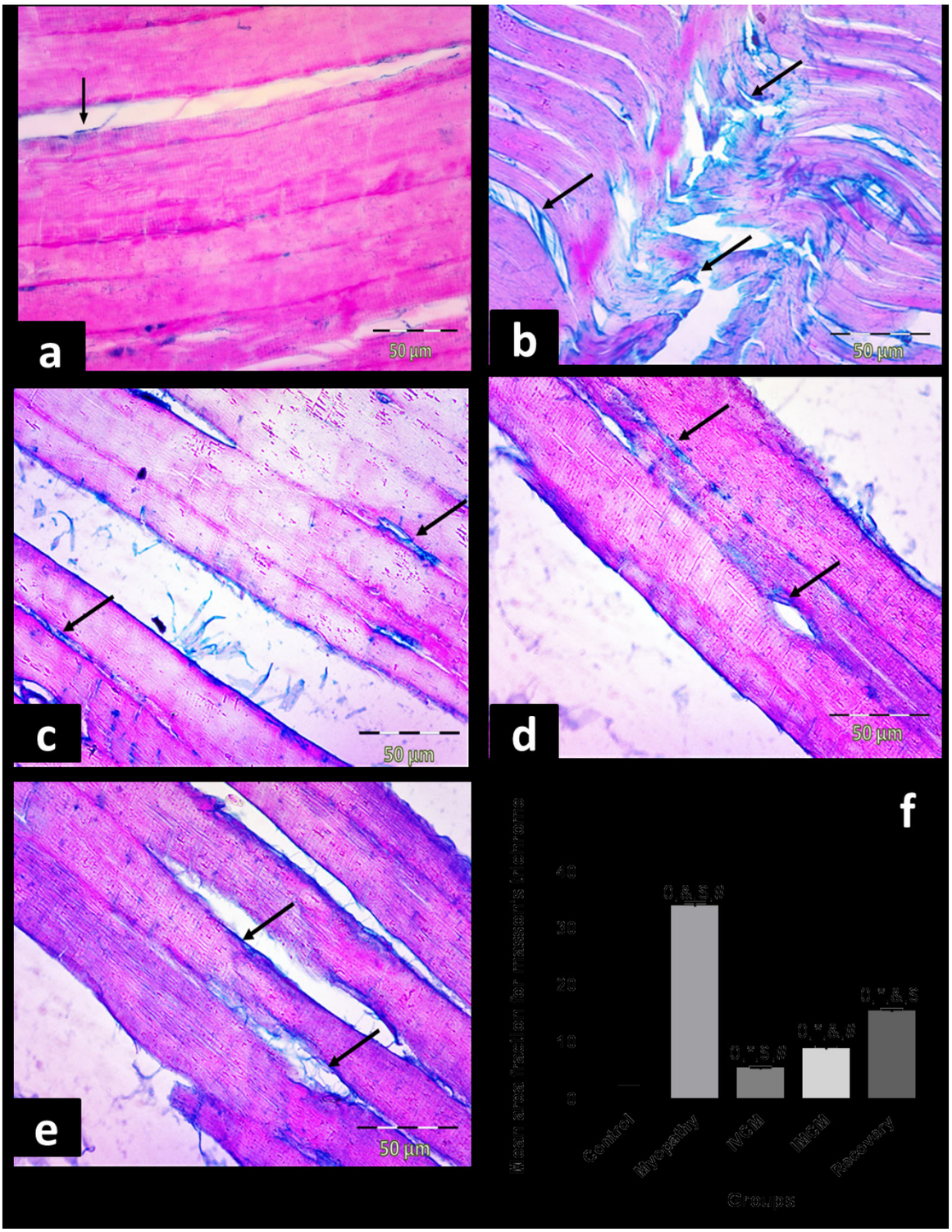

Fig. 7: Representative Photomicrographs of longitudinal section of rat's gastrocnemius muscle of a) control group; showing minimal collagen deposition in the endomysium (thin arrow). b) myopathy group; showing excessive collagen deposition in muscle fibers (thin arrow). C) IVCM group; showing little collagen deposition in the endomysium (thin arrows). d) IMCM group; showing moderate collagen deposition in the endomysium (thin arrows). e) recovery group showing abundant collagen deposition in the endomysium (thin arrows). (Masson's trichrome ; x400, scale bar=50 µm). f) bar chart showing The mean area percentage of the collagen fibers in different groups (Significant: 0 vs control group, * vs myopathy group, & vs IVCM group, $ vs IMCM group, # vs recovery group).

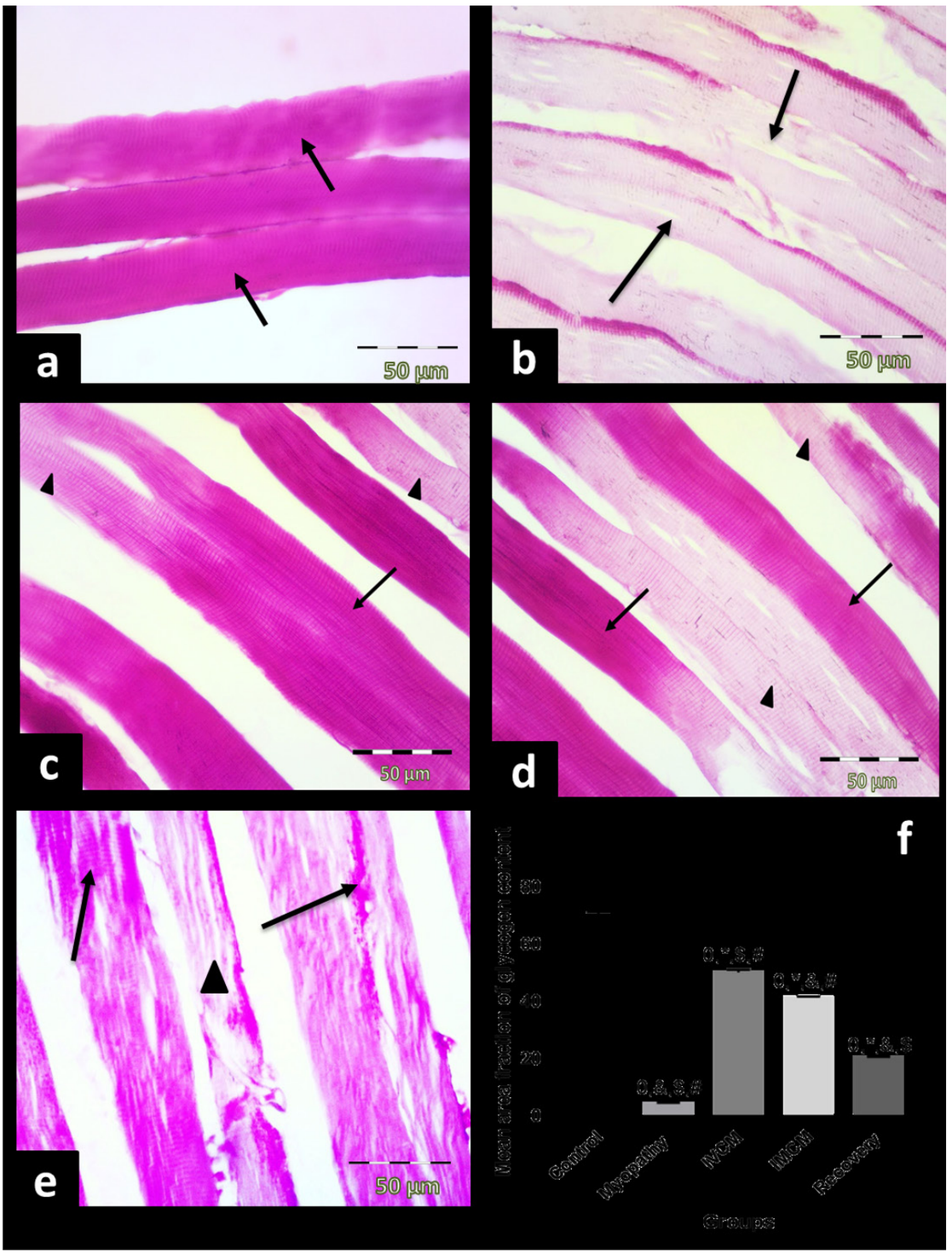

Fig. 8: Representative Photomicrographs of longitudinal section of rat's gastrocnemius muscle of a) Control group; showing strong PAS reaction in the muscle fibers(thin arrows). b) myopathy group; showing marked decrease in PAS reaction in the muscle fibers(thin arrow). C) IVCM group; showing marked PAS reaction in the muscle fibers(thin arrow) with patchy areas of faint reaction (arrowheads). d) IMCM group showing areas of strong PAS reaction (thin arrows) with other areas with faint reaction (arrowheads). e) recovery group showing moderate PAS reaction in some areas of the muscle fibers (thin arrows) with other areas of faint reaction (arrowhead). (PAS ; x400, scale bar=50 µm). f) bar chart showing The mean area percentage of glycogen content in different groups (Significant: 0 vs control group, * vs myopathy group, & vs IVCM group, $ vs IMCM group, # vs recovery group).

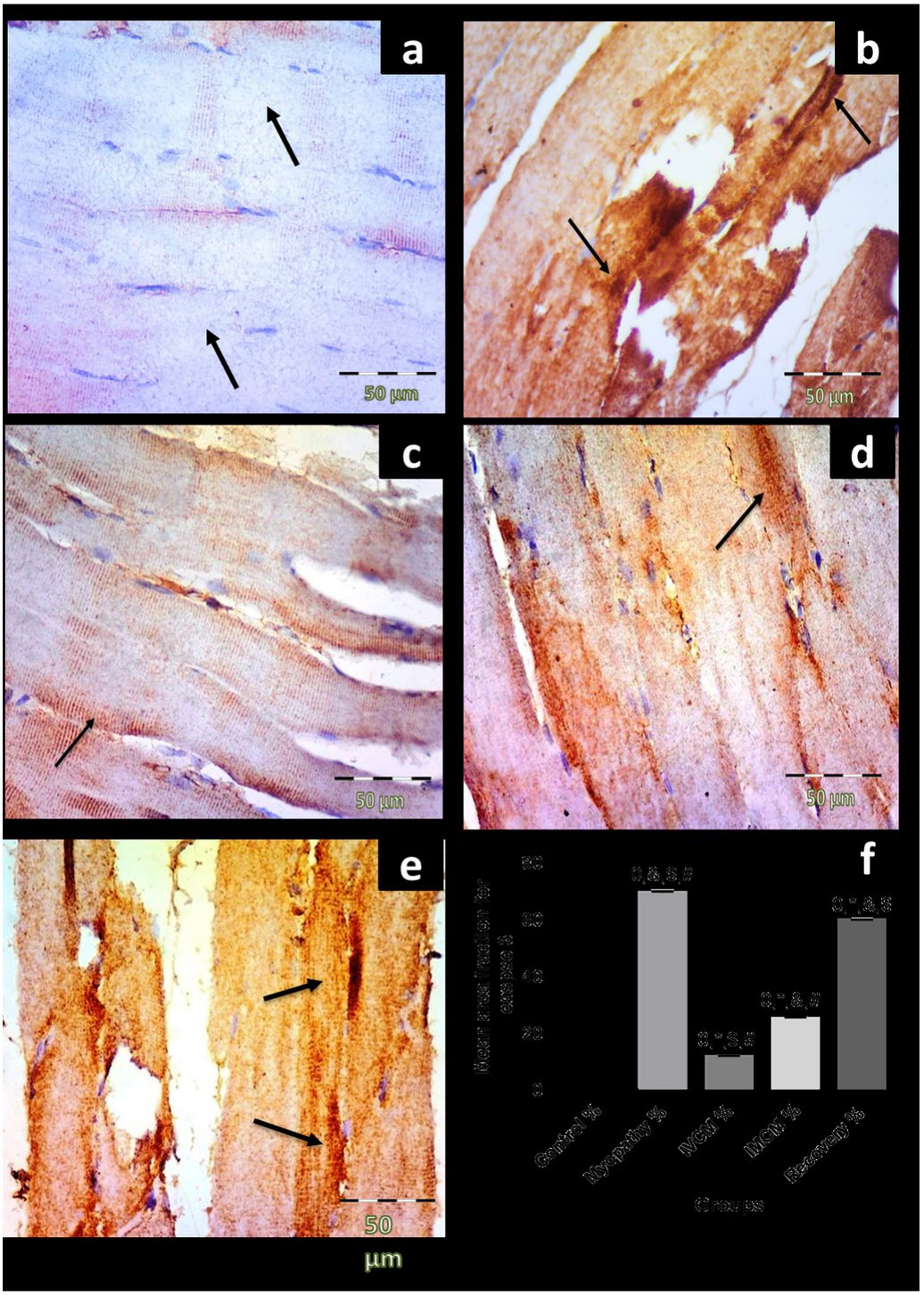

Fig. 9: Representative photomicrographs of longitudinal section of rat's gastrocnemius muscle immunostained for caspase 3 of a) Control group; showing negative immune expression in the sarcoplasm of muscle fibers (arrows). b) myopathy group; showing strong positive expression in the sarcoplasm of muscle fibers (arrows). C) IVCM group showing little positive expression in the sarcoplasm of muscle fibers (arrow). d) IMCM group showing moderate positive expression in the sarcoplasm o muscle fibers (arrow). e) recovery group showing strong positive expression in the sarcoplasm of muscle fibers (arrows). (immunohistochemistry for caspase 3 ; x400, scale bar=50 µm). f) Bar chart revealing the mean area fraction of caspase 3 immunopositivity in different groups (Significant: 0 vs control group, * vs myopathy group, & vs IVCM group, $ vs IMCM group, # vs recovery group).

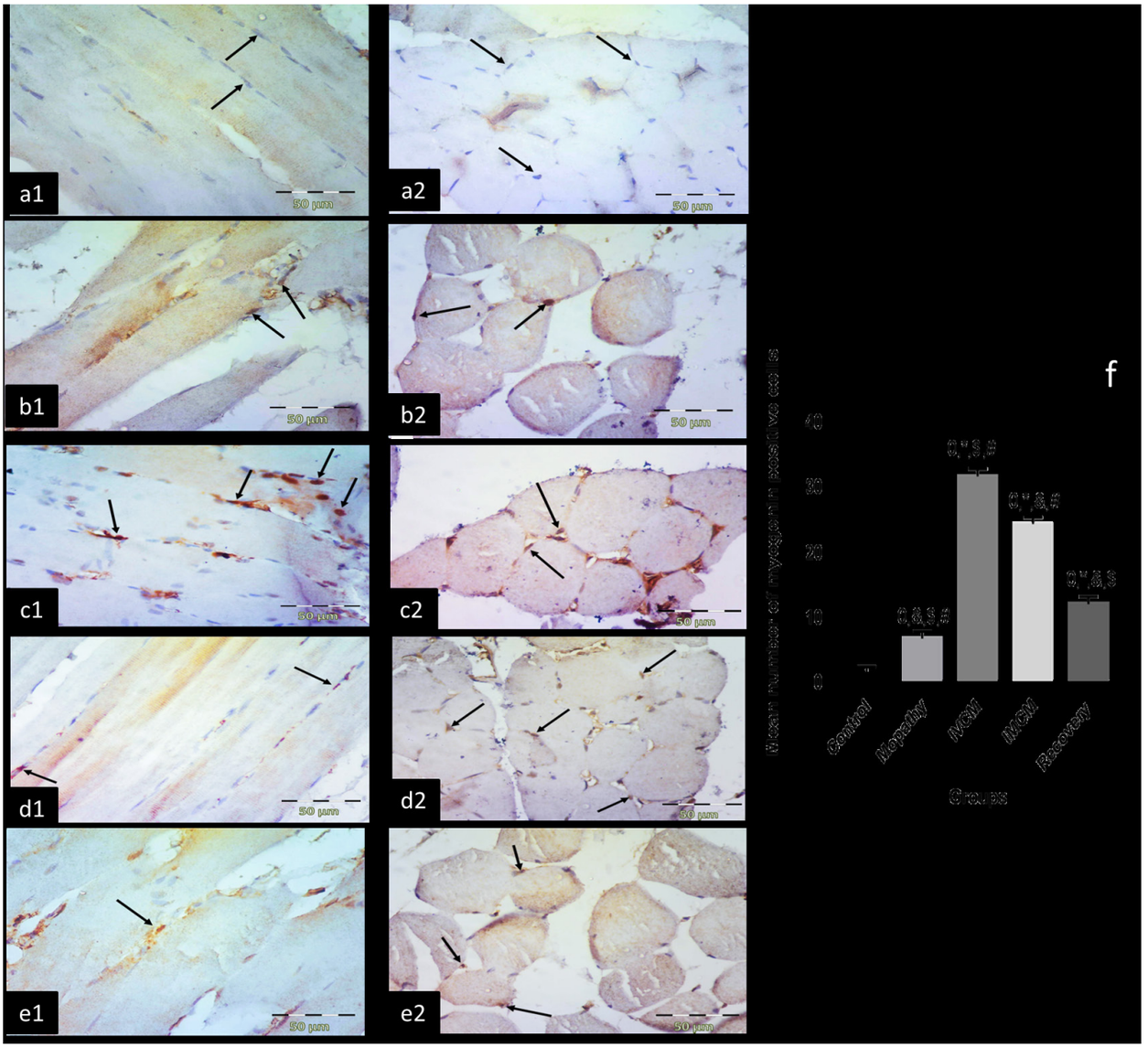

Fig. 10: Representative photomicrographs for myogenin nuclear expression in satellite cells among different experimental groups showing a1, a2) longitudinal and transverse sections in control group respectively showing negative nuclear expression in skeletal muscle cells (arrows). b1, b2) longitudinal and transverse sections in myopathy group respectively showing few nuclei with positive expression in skeletal muscle cells (arrows). c1, c2) longitudinal and transverse sections in IVCM group respectively showing numerous nuclei of satellite cells with positive expression in skeletal muscle cells (arrows). d1, d2) longitudinal and transverse sections in IMCM group respectively showing many nuclei of satellite cells with positive expression in skeletal muscle cells (arrows). e1, e2) longitudinal and transverse sections in recovery group respectively showing scattered nuclei with positive expression (thin arrows). (immunohistochemistry for myogenin; A&B x400, scale bar=50 µm). f) Bar chart showing mean number of myogenin positive cells in different groups (Significant: 0 vs control group, * vs myopathy group, & vs IVCM group, $ vs IMCM group, # vs recovery group).

DISCUSSION

Many recent studies gave an evidence for the beneficial effects of stem cells in regenerative medicine depending on their ability to secrete growth factors and bioactive molecules with multiple functions into their CM (**Kumar *et al*. 2019**).

Anti-hyperlipidemic drugs are widely used nowadays in prevention of great cardiovascular events like ischemic strokes. Statins are the most popular type of those drugs (**Reynolds *et al*., 2021**). There are accumulative evidences that such drugs have a bad impact on skeletal muscle structure and function. Amelioration of such deleterious effect is an urgent medical demand as skeletal muscles

comprise about 40% of total body mass and have an important role in overall metabolism, Therefore, an optimal function of skeletal muscles is extremely needed (**Das *et al*., 2020**).

The present study was done to demonstrate the possible myotoxicity of atorvastatin and the potential histological, biochemical and immunohistochemical changes behind it and to provide experimental evidence of the ability of CM to repair the muscle damage.

The grip strength test is used to assess the neuromuscular function (**Padilla *et al*., 2021**) by measuring forelimb grip strength. We found that atorvastatin treatment and even after its cessation caused reduction in muscular strength that was in agreement with (**Seo *et al*., 2020**). They reported that statin treatment impairs skeletal muscle functions as statins target ion channels particularly ClC-1 chloride channels which are important for skeletal muscle excitability and contractility (**Camerino *et al*., 2021**).

On the contrary, CM treated groups especially IVCM group showed significant improvement that was in line with (**Rudnicka-Drożak *et al*., 2022**). This may be explained that CM growth factors can cause regeneration of damaged muscle and replace the damaged with functional tissue (**Pereira *et al*., 2014**).

In the current study, analysis of serum of creatine kinase (CK),which helps skeletal muscle to produce energy (**Aujla and Patel, 2022**), and myoglobin, which is an oxygen storage protein, are biochemical markers for muscle damage (**Mami *et al*. 2019**).They revealed a significant increase in their mean values in the myopathy and recovery groups compared to the control group. This was in line with (**Kim *et al*., 2020**). This finding was explained by (**Gawish *et al*., 2022**) who reported that statins cause sarcolemma damage so, CK leaks from the muscle cytosol and decreasing its ability to generate ATP and deteriorate the stability of contractile filaments. While myoglobin, which has low molecular weight, is released quickly after muscle damage (**Gondal *et al*., 2022**).

CM treated groups especially IVCM group showed a significant decrease in serum CK and myoglobin compared to the myopathy group that may be explained by the study of (**Squecco *et al*., 2021**). They reported that administration of CM improve muscle recovery and counteract the muscular damage due to the presence growth factors as Insulin like growth factor 1 (IGF-1), fibroblast growth factor 2 (FGF-2) and adrenomedullin that help in muscle regeneration with cytoskeleton organization and cell membrane stability.

The histological study by H&E of the myopathy and recovery groups revealed marked degenerative changes and disruption in the muscle histology that were noticed by (**Abu-Dief *et al*., 2022**). The waviness of myofibrils and sarcolemma may be explained by slippage of myofibrillar alignment due to muscle atrophy and disruption of the connection between the muscle fibrils and the sarcolemma as mentioned by (**Kashef and Elswaidy, 2022**). Some centrally located nuclei were noticed and explained by (**Salem *et al*., 2016**) as the newly regenerated myofibers had centrally located nuclei. We noticed also congested blood vessels with inflammatory cells that are stimulated by the degenerative changes of the muscles (**Dias and Nylandsted, 2021**).

In the CM treated groups especially IVCM group, obvious improvement in the myopathic muscle fibers structure was noticed. This regenerative capacity of CM was reported by (**Sandonà *et al*., 2021; Squecco *et al*., 2021**) and explained by (**Abdelwahab *et al*., 2021**) who reported that the regenerative effect of CM may be due to presence of many growth factors and cytokines as IGF-1, FGF-2 and interleukin 10 (IL-10) that secreted by stem cells in the medium and may stimulate differentiation of muscle stem cells and promote new fibers formation.

Histological results were confirmed with measuring endomysium space in transverse sections of all groups that showed widening in this space in the myopathy and recovery groups .This was in agreement with (**Seo *et al*., 2020**) while in the CM treated groups the endomysium space almost returned to normal indicating regeneration of muscles in line with (**Kim *et al*., 2016**).

Masson's trichrome is special stain for demonstration of collagen fibers to detect fibrosis in the muscle (Van De Vlekkert ***et al***., 2020). In the current study, there were abundant collagen fibers in between the muscle fibers in myopathy and recovery groups in line with (**Mehanna *et al*., 2020**). This was supported by (**Sheets *et al*., 2022**) who reported that this fibrotic process is promoted by release of some growth factors as platelet-derived growth factor and transforming growth factor-β1, that trigger differentiation of myogenic cells to myofibroblasts and stimulate the extracellular matrix cells to produce collagens. In addition, the presence of inflammatory cells may trigger fibrosis as mentioned by (**Mack 2018**).

In CM treated groups, collagen fibers decreased significantly especially in IVCM group compared to the myopathy group due to regeneration of muscle fibers and reducing inflammation and fibrosis development which was in line with (**Świerczek-Lasek *et al*., 2022**) .They reported that CM limit fibrosis by its high concentration of IGF-1 which enhances myofiber formation and promotes macrophage differentiation into the anti-inflammatory M2 phenotype. This was also supported by (**Sandonà *et al*., 2021**) who stated that CM had an anti-fibrotic action due to it paracrine effect on matrix metalloproteinase-1 (MMP-1).

As regard PAS staining, there was marked decrease in PAS reaction in myopathy and recovery groups and this was in agreement with (**Mehanna *et al*., 2020**) and (**Camerino *et al*., 2021**) who reported that administration of statins caused depletion of glycogen stores in the hepatocyte and this could be what happened in its stores in muscle also. This might be due to statins' effect on glucose absorption or on the enzymes of glycogenesis and glycolysis.

The CM treated groups showed marked increase in PAS reaction especially in IVCM group compared to the myopathy group which suggests that CM restored the muscles' ability to store glycogen. As CM can stimulate the phosphoinositol-3-kinase (PI3K)/Akt signaling cascade which promotes metabolism, proliferation, survival and growth of cells (**Kim *et al*. 2016**).

As an attempt to understand the underlying mechanism of the therapeutic effect of CM we studied the expression of Caspase 3, as a marker for apoptosis (**Asadi *et al*., 2022**). In our study there was a significant increase in caspase 3 expression in the myopathy and recovery groups that was in accordance with (**Mollazadeh *et al*., 2021**) who reported that statin induced myopathy occurred through depletion of isoprenoids which control the rate of muscle apoptosis and are linked to some proteins that when reduced, cytosolic calcium increases and activates number of events leading to activation of caspase 3.

In the CM treated groups obviously in IVCM group, there was a significant decrease in caspase 3 expression compared to the myopathy group. This was in agreement with (**Squecco *et al*., 2021**) who reported that treatment with CM protects against apoptosis by inhibiting the activated mitochondria-dependent caspase 3 pathway leading to decreased caspase 3 expression.

Myogenin is a marker for muscle regeneration which is expressed in the nuclei of satellite cells in developing muscle tissue (**Dabbs, 2021**). In the current work, the expression of anti myogenin antibody in the nuclei of skeletal muscles of the control group was negative in agreement with (**Nada *et al*., 2018**). While in the myopathy and recovery groups, there were few nuclei with positive expression that was in agreement with (**Ibrahim and shaheen, 2022**). This may be due to activation of satellite cells in response to muscular injury (**Nada *et al*., 2018**).

CM treated groups especially in IVCM group showed strong nuclear expression for anti myogenin antibody in many skeletal muscle cells compared to the myopathy group. (**Ahmad *et al*., 2022**) stated that CM is important for proliferation and differentiation of satellite cells into myoblasts due to presence of fibroblast growth factor 2 (FGF2).

There was a significant difference between IVCM and IMCM groups in favor of the IVCM group that showed better histological and immunohistochemical results . This may be due to IV injection of CM helped their growth factors to reach tissues faster and in higher concentration, so regeneration of muscle was earlier than in IM injection. Moreover, I.M. injection may act as local injection that affect the injected muscle, but statin induced myopathy is a systemic disorder affecting many skeletal muscles so I.V route may be more effective. This was supported by the results of the strength test that confirm generalized improvement of muscles including forelimb muscles. In agreement, (**Kanazawa *et al*., 2018**) and (**Zhao *et al*., 2012**) reported that systemic administration of MCs is more effective than MCs local injection in different studies.

CONCLUSION

Taken together the previously mentioned data indicated that CM is a promising therapeutic approach in treatment of statins induced myopathy, but the intravenously injected conditioned medium had more rapid and effective role than the intramuscularly injected. This may pave the future for stem cell conditioned medium to be used as a cell-free therapy in case of statin induced myopathy and other muscular diseases, but further investigation is needed for better understanding to the underlying cellular and molecular mechanism before clinical use.

CONFLICT OF INTEREST

There are no conflicts of interest.

REFERENCES

Abdelwahab, S., Elsebay, S.A.G., Fouli Gaber, M., and Abdel-Hafez, S.M.N. (2021). Comparative study between bone marrow mesenchymal stem cell and their conditioned medium in the treatment of rat model of Parkinsonism. Journal of Cellular Physiology 236, 440-457.

Abu-Dief, A.M., Shaaban, E.A., Abdelhaleem, N.G., and Mohamed, D.S. (2022). The Possible Role of Nanocurcumin in Rat Model of Statin Induced Myopathy: Histological and Immune Histochemical Study. Journal of Advances in Medicine and Medical Research, 76-88.

Ahmad, S., Chun, H., Ahmad, K., Shaikh, S., Lim, J., Ali, S., Han, S., Hur, S., Sohn, J., and Lee, E. (2022). The roles of growth factors and hormones in the regulation of muscle satellite cells for cultured meat production. Journal of Animal Science and Technology.

Asadi, M., Taghizadeh, S., Kaviani, E., Vakili, O., Taheri-Anganeh, M., Tahamtan, M., and Savardashtaki,

A. (2022). Caspase-3: structure, function, and biotechnological aspects. Biotechnology and Applied Biochemistry 69, 1633-1645.

Aujla, R., and Patel, R. (2022). Creatine phosphokinase. StatPearls.

Camerino, G.M., Tarantino, N., Canfora, I., De Bellis, M., Musumeci, O., and Pierno, S. (2021). Statin-induced myopathy: translational studies from preclinical to clinical evidence. International Journal of Molecular Sciences 22, 2070.

Cemek, M., Kağa, S., Şimşek, N., Büyükokuroğlu, M.E., and Konuk, M. (2008). Antihyperglycemic and antioxidative potential of Matricaria chamomilla L. in streptozotocin-induced diabetic rats. Journal of natural medicines 62, 284-293.

Dabbs, D.J. (2021). Diagnostic immunohistochemistry e-book: theranostic and genomic applications (Elsevier Health Sciences).

Das, D.K., Graham, Z.A., and Cardozo, C.P. (2020). Myokines in skeletal muscle physiology and metabolism: Recent advances and future perspectives. Acta Physiologica 228, e13367.

Dias, C., and Nylandsted, J. (2021). Plasma membrane integrity in health and disease: significance and therapeutic potential. Cell discovery 7, 4.

El-Deeb, D.F., Youssef, M.F., Yousry, M.M., and Ahmed, A.M. (2018). Can Alfacalcidol ameliorate Atorvastatin-induced myopathy in adult male rats? A histological study. Egyptian Journal of Histology 41, 285-299.

Fui, L.W., Lok, M.P.W., Govindasamy, V., Yong, T.K., Lek, T.K., and Das, A.K. (2019). Understanding the multifaceted mechanisms of diabetic wound healing and therapeutic application of stem cells conditioned medium in the healing process. Journal of tissue engineering and regenerative medicine 13, 2218-2233.

Gawish, M.F., Selim, S.A., Abd El-Star, A.A., and Ahmed, S.M. (2022). Histological and immunohistochemical study of the effect of ozone versus erythropoietin on induced skeletal muscle ischemia-reperfusion injury in adult male rats. Ultrastructural Pathology 46, 96-109.

Gondal, A.Z., Foris, L.A., and Richards, J.R. (2022). Serum myoglobin. In StatPearls [Internet] (StatPearls Publishing).

Gunawardena, T.N.A., Rahman, M.T., Abdullah, B.J.J., and Abu Kasim, N.H. (2019). Conditioned media derived from mesenchymal stem cell cultures: The next generation for regenerative medicine. Journal of tissue engineering and regenerative medicine 13, 569-586.

Huang, J., Ding, Z., Luo, Q., and Xu, W. (2019). Cancer cell-derived exosomes promote cell proliferation and inhibit cell apoptosis of both normal lung fibroblasts and non-small cell lung cancer cell through delivering alpha-smooth muscle actin. American journal of translational research 11, 1711.

Ibrahim, M.F.G., and Allam, F.A.F.A. (2022). Potential stem cell-Conditioned medium and their derived exosomes versus omeprazole in treatment of experimental model of gastric ulcer. Acta Histochemica 124, 151896.

Ibrahim, m.H., and shaheen, N.f. (2022). Comparative study of Prophylactic role of Vitamin D versus Coenzyme Q10 against Statin induced myopathy in adult male albino rats: Histological & Immunohistochemical study. Egyptian Journal of Histology, -.

Kanazawa, M., Atsuta, I., Ayukawa, Y., Yamaza, T., Kondo, R., Matsuura, Y., and Koyano, K. (2018). The influence of systemically or locally administered mesenchymal stem cells on tissue repair in a rat oral implantation model. International journal of implant dentistry 4, 1-11.

Kashef, S.M., and Elswaidy, N.R. (2022). The Possible Role of Allicin in Ameliorating Azithromycin Induced Cardiotoxicity in Adult Male Albino Rat: A Histological and Immunohistochemical Study. Egyptian Journal of Histology 45, 863-874.

Kim, M.C., Ahn, Y., Cho, K.H., Sim, D.S., Hong, Y.J., Kim, J.H., and Jeong, M.H. (2020). Image of Statin-Induced Rhabdomyolysis. Korean Circulation Journal 50, 738.

Kim, M.J., Kim, Z.-H., Kim, S.-M., and Choi, Y.-S. (2016). Conditioned medium derived from umbilical cord mesenchymal stem cells regenerates atrophied muscles. Tissue and Cell 48, 533-543.

Mehanna, M., Abd Allah, E.E.-D.E.-S., Alshahed, F.A.N., and Al-Azab, H.W. (2020). Effect of Simvastatin on the Skeletal Muscles of Senile Male Albino Rats and Possible Protective Role of L-Carnitine. A Histological Study. Egyptian Journal of Histology 43, 286-300.

Mollazadeh, H., Tavana, E., Fanni, G., Bo, S., Banach, M., Pirro, M., Von Haehling, S., Jamialahmadi, T., and Sahebkar, A. (2021). Effects of statins on mitochondrial pathways. Journal of Cachexia, Sarcopenia and Muscle 12, 237-251.

Nada, S.M., El-Deeb, D.F., AboulKhair, A.G., and Hosny, S.A.A. (2018). A histological Study Comparing the Possible Protective Mechanisms of Oxytocin and Vitamin E on ischemia reperfusion injury in Rat skeletal muscle. Egyptian Journal of Histology 41, 570-581.

Padilla, C.J., Harrigan, M.E., Harris, H., Schwab, J.M., Rutkove, S.B., Rich, M.M., Clark, B.C., and Arnold, W.D. (2021). Profiling age-related muscle weakness and wasting: neuromuscular junction transmission as a driver of age-related physical decline. Geroscience 43, 1265-1281.

Pereira, T., Armada-da Silva, P., Amorim, I., Rêma, A., Caseiro, A., Gärtner, A., Rodrigues, M., Lopes, M., Bártolo, P., and Santos, J. (2014). Effects of human mesenchymal stem cells isolated from Wharton's jelly of the umbilical cord and conditioned media on skeletal muscle regeneration using a myectomy model. Stem Cells International 2014.

Reynolds, T.M., Pottle, A., and Quoraishi, S.H. (2021). Current perspectives on the attainment of lipid modification goals relating to the use of statins and ezetimibe for the prevention of cardiovascular disease in the United Kingdom. Vascular Health and Risk Management, 227-237.

Rudnicka-Drożak, E., Drożak, P., Mizerski, G., and Drożak, M. (2022). Endothelial Progenitor Cells in Neurovascular Disorders—A Comprehensive Overview of the Current State of Knowledge. Biomedicines 10, 2616.

Saint-Gerons, M., Rubio, M.A., Aznar, G., and Matheu, A. (2022). Ophthalmological Manifestations of Hereditary Myopathies. Journal of Binocular Vision and Ocular Motility 72, 4-17.

Salem, M.Y., El-Eraky El-Azab, N., and Ibrahem, R. (2016). Effect of the route of stem cell transplantation on its therapeutic potential on induced myopathy in rats: a histological and immunohistochemical study. The Egyptian Journal of Histology 39, 74-86.

Sandonà, M., Di Pietro, L., Esposito, F., Ventura, A., Silini, A.R., Parolini, O., and Saccone, V. (2021). Mesenchymal stromal cells and their secretome: new therapeutic perspectives for skeletal muscle regeneration. Frontiers in Bioengineering and Biotechnology 9, 652970.

Seo, D.Y., Heo, J.-W., No, M.-H., Yoo, S.-Z., Ko, J.R., Park, D.-H., Kang, J.-H., Kim, C.-J., Jung, S.-J., and Han, J. (2020). Exercise training protects against atorvastatin-induced skeletal muscle dysfunction and mitochondrial dysfunction in the skeletal muscle of rats. Journal of Clinical Medicine 9, 2292.

Sheets, K., Overbey, J., Ksajikian, A., Bovid, K., Kenter, K., and Li, Y. (2022). The pathophysiology and treatment of musculoskeletal fibrosis. Journal of Cellular Biochemistry 123, 843-851.

Squecco, R., Tani, A., Chellini, F., Garella, R., Idrizaj, E., Rosa, I., Zecchi-Orlandini, S., Manetti, M., and Sassoli, C. (2021). Bone marrow-mesenchymal stromal cell secretome as conditioned medium relieves experimental skeletal muscle damage induced by ex vivo eccentric contraction. International Journal of Molecular Sciences 22, 3645.

Suvarna, K.S., Layton, C., and Bancroft, J.D. (2018). Bancroft's theory and practice of histological techniques E-Book (Elsevier health sciences).

Świerczek-Lasek, B., Tolak, L., Bijoch, L., Stefaniuk, M., Szpak, P., Kalaszczynska, I., Streminska, W., Ciemerych, M.A., and Archacka, K. (2022). Comparison of Muscle Regeneration after BMSC-Conditioned Medium, Syngeneic, or Allogeneic BMSC Injection. Cells 11, 2843.

Van De Vlekkert, D., Machado, E., and d'Azzo, A. (2020). Analysis of generalized fibrosis in mouse tissue sections with Masson's trichrome staining. Bio-protocol 10, e3629-e3629.

Vilaça-Faria, H., Marote, A., Lages, I., Ribeiro, C., Mendes-Pinheiro, B., Domingues, A.V., Campos, J., Lanceros-Mendez, S., Salgado, A.J., and Teixeira, F.G. (2021). Fractionating stem cells secretome for Parkinson's disease modeling: Is it the whole better than the sum of its parts? Biochimie 189, 87-98.

Zhao, W., Li, J.-J., Cao, D.-Y., Li, X., Zhang, L.-Y., He, Y., Yue, S.-Q., Wang, D.-S., and Dou, K.-F. (2012). Intravenous injection of mesenchymal stem cells is effective in treating liver fibrosis. World journal of gastroenterology: WJG 18, 1048.

الملخص العربى

التأثير العلاجي المحتمل للوسط المكيف للخلايا الجذعية على النموذج الحيواني المستحث بالستاتين للاعتلال العضلي: دراسة كيميائية حيوية وهستولوجية وهستوكيميائية مناعية.

منار فولي جابر إبراهيم، سها عبدالقوي عبدالوهاب، هاجر علي حسن محمد، أميرة فتحي أحمد

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مقدمة: الاعتلالات العضلية هي أمراض ذات معدل انتشار مرتفع في جميع أنحاء العالم. هدفت هذه الدراسة إلى التحقق من قدرة الوسط المكيف للخلايا الجذعية (SC-CM) الذي يتم إعطاءه بواسطة طريقين مختلفين (عضليا/وريديا) لإصلاح العضلات الهيكلية التالفة.

المواد والطرق: اشتملت هذه الدراسة على ٦٠ من ذكور الجرذان البيضاء البالغة مقسمة بالتساوي على خمس مجموعات: المجموعة الضابطة؛ مجموعة الاعتلال العضلي حيث تم إحداث الاعتلال العضلي عن طريق إعطاء عقار أتورفاستاتين (١٠ مغ / كغ / يوم عن طريق الفم لمدة ٤ أسابيع) ؛ المجموعة المعالجة بالوسط المكيف وريديا حيث تم إحداث الاعتلال العضلي ثم حقنت الجرذان في الوريد بـ ٠,٥ مل من الوسط المكيف للخلايا الجذعية في وريد الذيل ؛ المجموعة المعالجة بالوسط المكيف عضليا تم حقنها موضعيا بـ ٠,٥ مل من الوسط المكيف للخلايا الجذعية بعد إحداث الاعتلال العضلي ؛ تُرِكت مجموعة الاعتلال العضلي غير المعالجة دون علاج لمدة ٤ أسابيع. تم جمع عينات الدم وعينات العضلات من كل المجموعات للدراسات الكيميائية الحيوية والهستولوجية.

النتائج: أظهرت مجموعة الاعتلال العضلي تنكسا ملحوظا في العضلات، مع ارتشاح خلايا الالتهاب. بالمقارنة مع المجموعة الضابطة، كان هناك انخفاضا ذا دلالة إحصائية في المساحة المصبوغة بصبغة شيف للحمض البريودي وارتفاعا ذا دلالة إحصائية في متوسط مستويات الكرياتين كيناز والميوجلوبين، ومساحة التفاعل الإيجابي للكولاجين والكاسباس٣ وعدد الخلايا ذات التفاعل الإيجابي للميوجينين. بعد أربعة أسابيع من حقن الوسط المكيف للخلايا الجذعية (عضليا/وريديا)، كان هناك تحسنا ملحوظا في البنية النسيجية للعضلات الهيكلية حيث كان هناك انخفاضا ذا دلالة إحصائية متوسط مستويات الكرياتين كيناز والميوجلوبين وفي مساحة التفاعل الإيجابي للكولاجين والكاسباس٣ وارتفاعا ذا دلالة إحصائية في المساحة المصبوغة بصبغة شيف للحمض البريودي وفي عدد الخلايا ذات التفاعل الإيجابي للميوجينين وكان التحسن أكثر وضوحا في المجموعة المعالجة وريديا.

الاستنتاج: قد يكون حقن الوسط المكيف للخلايا الجذعية مفيدا بشكل كبير في علاج الحالات الحرجة للاعتلال العضلي خاصة عن طريق الحقن الوريدي.