The possible promotive effect of Alfacalcidol on bone marrow derived mesenchymal stem cells in the treatment of Simvastatin induced myopathy in adult albino rats:Histological and immunohistochemical study

Abstract

Background: Statins are the most popular type of anti-hyperlipidemic drugs**.**Benefits of statins in hypercholesterolemia management might be restricted by its myopathic side effects. **Aim:** This study set out to assess the myopathy caused by simvastatin in rats and to find out whether there was any way to treat it by combining alfacalcidol with bone marrow mesenchymal stem cells. **Methods:** 45 adult male rats were divided into five groups each included 9 rats,Group I(control),groupII (Simvastatin induced Myopathy),group III(Alfacalcidol),rats of these three groups were sacrificed at the 6th week,group IV(BM-MSCs),group V(Alfacalcidol+BM-MSCs),rats of these two groups were sacrificed at 8th week.At the end of each experimental period before sacrifice, blood samples collected and analyzed for serum creatine kinase.Gastrocnemius muscle samples were prepared for histological and immunohistochemical examination.**Results:**Simvastatin-treated rats exhibited severe muscle degeneration, inflammation, and abnormal fiber structure.ALF treatment led to partial improvement, with some muscle fibers appearing normal.BM-MSCs treatment showed significant histological recovery, most fibers appearing normal with only limited damage.Combined ALF and BM-MSCs treatment closely restored normal muscle structure resembling control group.Results of Masson's trichrome staining revealed excessive collagen in myopathy group,treatment groups showed reduced collagen deposition,especially the combination group.Immunohistochemistry showed strong myofibroblast reaction in myopathy group, reduced reaction in ALF and BM-MSCs groups, and minimal reaction in combination group.Serum CK levels, were highest in myopathy group;then in treatment groups,especially combined group, showed significant reduction.**Conclusion:**combined ALF and BM-MSCs therapy had better results than the use of stem cell therapy alone.

Key words: Statin Myopathy, Alfacalcidol, Mesenchymal stem cells.

Introduction

Hyperlipidemia is a systemic risk factor that can lead to atherosclerosis, renal failure, myocardial infarction, coronary heart disease, , and cerebral stroke[(1)].

Statins are the most frequently prescribed medications for the treatment of hypercholesterolaemia. Simvastatin is a drug that is frequently prescribed worldwide due to its exceptional hypolipidemic performance and relatively low cost [(2)]. However, there are some side effects associated with statins, and the most common of these are concerns with the muscles[(3)].

Among the most prevalent causes of myopathy are drug-induced myopathies. From moderate myalgia to chronic illness with myasthenia gravis and rhabdomyolysis. Severe cases may result in acute renal failure and mortality[4].

Hypercholesterolemic patients who are intolerant to Statins may re-administer Statins without experiencing recurrent myositis–myalgia due to vitamin D supplementation[5].

The treatment of myopathies may benefit from the application of stem cell therapies as regenerative agents[6]. MSC-mediated tissue repair still lacks a comprehensive understanding of its precise mechanisms[7]. A multitude of cytokines and growth factors secreted by MSCs encourage cell recruitment ,migration, proliferation, angiogenesis and differentiation. MSCs are also recognised for their immunomodulatory properties, which may enable them to have a beneficial impact on the local immune cell population at the site of muscle injury[8].

Materials and Methods

I-Materials

This study is experimental research approved by the Research Ethics Committee of faculty of Medicine, Benha University (MD-13-1-220).

1. Animals

This study was conducted on 45 adult male albino rats that were housed in the experimental animal unit of the anatomy department at the Benha Faculty of Medicine from January 2020 to January 2022. All of the animals came from the Moshtohor Faculty of Veterinary Medicine's animal shelter at Benha University. Rodents were kept in a controlled environment with food and water available to them. At the time of expiration, their average weight was between 150 and 200 grammes. The experimental animal committee of Benha Medical University approved the animal handling in accordance with the national research council guidelines.

2. Chemicals

Simvastatin powder: Purchased from NAMAA Pharmaceuticals in El-Monofia, Egypt. Purity: 98.4%, in the form of granules.the dose was formulated as suspension of water containing 0.5% carboxymethylcellulose (CMC).It was given at adose of 80 mg/kg once daily suspended in 2.5 ml /kg of 0.5% carboxymethyl cellulose for six weeks[9]..

Alfacalcidol (ALF): The BON-ONE brand, made by Minipharm Egypt, consists of crushed tablets containing **1 µg** of active ingredient that is dissolved in distilled water. For six weeks, it was given at a dosage of half a **microgram per kilogram**

per day, dissolved in 2.5 milliliters of distilled water [10].

3. Bone Marrow Mesenchymal Stem cells (BM-MSCs)

The stem cell lab at Cairo University's Kasr Al-Ainy Faculty of Medicine generated the BM-MSCs at a concentration of 3×10^6 cells in 0.5 ml of phosphate buffer saline (PBS). As an intramuscular injection of a single stem cell in the right gastrocnemius [11].

II-Methods

1. Experimental design

45 male albino rats were divided into five groups each included 9 rats as following:

Group I (control):rats were chosen randomly as healthy rats.

Group II (Simvastatin-induced myopathy):rats received 0.5 ml of 0.5% CMC containing 16mg Simvastatin orally once daily via orogastric tube for 6 weeks.Then rats were sacrificed.

Group III (Simvastatin and ALF):rats received the mentioned dose of Simvastatin as group II concomitantly rats received 0.5 ml distilled water containing 0.1 μg ALF orally once daily via orogastric tube for 6 weeks. Then rats were sacrificed.

Group IV (Simvastatin and MSCs): rats were given Simvastatin for a duration of six weeks. Afterwards, the right gastrocnemius muscle was injected with a single stem cell dose. Two weeks after the stem cell injection, at the conclusion of the eighth week, the rats were sacrificed.

Group V(Simvastatin and ALF + MSCs):rats received Simvastatin as in group II concomitantly with Alfacalcidol as group III for 6 weeks.Then stem cell injection and scarification were done as group IV.

Before sacrifice, blood samples were collected from the tail veins of all groups at the conclusion of each experimental period in heparinised capillary tubes and analysed for creatine kinase (CK). The rats were anesthetized by injecting them intraperitoneally with sodium pentobarbital (Nembutal, 30 mg/kg of body weight). After that, they were killed by cervical dislocation. The next step was to separate and slice the gastrocnemius muscles from each rat's right leg. Prior to being embedded in pure molten paraffin wax, specimens underwent fixation in 10%

formalin, dehydration in ethyl alcohol at ascending concentrations, xylene clearing, impregnation, and then embedding.

Labeling of MSCs with PKH26

Mesenchymal stem cells were extracted during the fourth passage and labelled with PKH26, a red fluorochrome supplied by Sigma Aldrich, USA. The cells were washed twice with serum-free media following centrifugation. A dye solution was used to suspend the cells after they were pelletized. The cells were detected and traced by examining muscle tissues with a fluorescence microscope after two weeks[11].

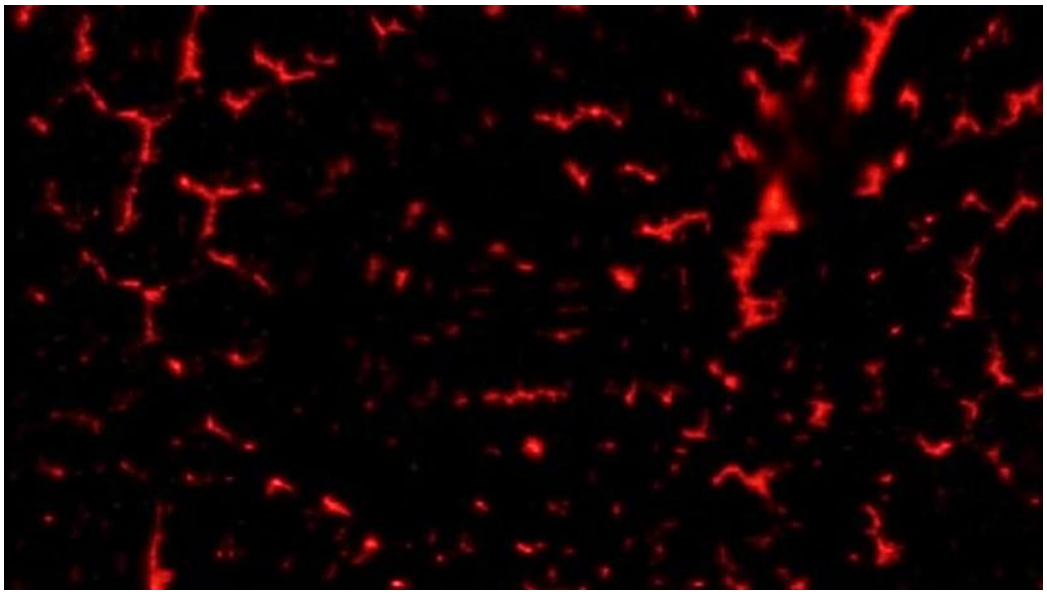

Fig (1) :A photomicrograph showing several immunostained mesenchymal stem cells (PKH 26, red fluorescent) housed in the gastrocnemius muscle .

(Immunofluorescence × 200)

2. Histopathological study:

H&E, Masson trichrome, and Prussian blue histochemical staining were performed on the sectioned Gastrocnemius muscle parrafin blocks, They were sectioned on a rotary microtome to a thickness of 4- 5 µm and mounted on albumenized glass slides. For immunohistochemistry, additional sections were affixed on positively charged slides.

The slide visualization and picture photography were carried out by the Anatomy Department of the Faculty of Medicine at Benha University in Egypt. For this task, we utilized a Nikon Eclipse 80i upright microscope (Japanese manufacturer)

equipped with a Toup CamTM Xcam full HD camera (European manufacturer, Ultramacro Ltd., UK) from ToupTek.

3. Biochemical study[12]:

At the conclusion of each experimental period, blood samples were obtained from the tail veins before sacrifice. A Revco refrigerator was used to segregate the serums and keep them at -20˚C. This was followed by the use of the RayBio® 68CL-CK-S100 creatine kinase (CK) activity colorimetric assay kit. which is manufactured in the United States, to analyse for creatine kinase (CK), a marker of muscle injury.

4. Morphometric Study:

A-Percentage of collagenous fibers area in Masson trichrome stained sections.

B- Number of myofibroblasts (α smooth muscle actin-positive cells) per high-power field.

In ten separate randomly chosen fields, they were evaluated, using a computer system developed by Leica Microsystems Ltd (Cambridge, UK) with the name Leica Qwin 500 image analyzer.

Statistical analysis:
A program called SPSS, developed in Chicago, Illinois, USA, version 19, was used to analyze the collected measurements. We utilized the post hoc Tukey test after running an ANOVA(analysis of variance) to compare the groups.Results were expressed as mean ± standard deviation. Statistical significance was determined by P values lower than 0.05[9].

Results

- **Histological results**
  - **Hematoxylin and Eosin results:**

Control group I

By analyzing longitudinal sections, we can observe that skeletal muscle typically consists of bundles of fibers that are separated by a connective tissue perimysium. Endomysium, a connective tissue, linked the fibers together. Minimal variation in fiber size was observed among the long, parallel, cylindrical, and non-branching fibers. Acidophilic and cross-striated sarcoplasm. Located peripherally beneath the sarcolemma, the nuclei were numerous and elongated **(Fig.2 a)**

Simvastatin-induced myopathy groupII

Showed areas of muscle fiber degeneration with marked cellular infiltration and some hemorrhagic spots,splitting of surrounding fibers,homogenous acidophilic cytoplasm with loss of striations and some fibers showed centrally placed nuclei.**(Fig.2 b)**

Simvastatin and ALF group III

Showed some fibers were apparently normal, massive hemorrhage within one of muscle fibers. also splitting and Cellular infilteration within surrounding fibers and centerally located nuclei were lined up forming chain.**(Fig.2 c)**

Simvastatin and MSCs group IV

Showed many apparently normal parallel muscle fibers with peripherally located nuclei and distinct transverse striations. splitting of some fibers and areas of mild cellular infilteration,wide perimysium and slightly wide endomysium.**(Fig.2 d)**.

Simvastatin and ALF + MSCs group V

Showed that muscle fibers regained its normal structure with intact parallel fibers with peripherally located nuclei,distinct transverse striations with endomysium separating them and nearly normal perimysium separating bundles with occasional cellular infilteration **(Fig.2 e)**.

- **Masson Trichrome results**

Control I and *ALF + MSCsV groups* revealed minimal amounts of blue collagen fibers deposition in between muscle fibers **(Fig.3 a&e)**.*Myopathy group II* revealed marked deposition of blue collagen fibers in between degenerated widely separated muscle fibers **(Fig .3 b).** *ALF group III* showed moderate amounts of collagen fibers deposition in between muscle fibers and around blood vessels and in perimysium surrounding muscle bundles **(Fig.3 c).** Mild quantities of collagen fibers were observed in the perimysium surrounding muscle bundles and in the spaces between muscle fibers in the MCSs group IV **(Fig.3 d)**.

- **Prussian blue results**

Demonstrated iron oxide-labeled stem cells. Positive reaction was present only in sections obtained from *MSCs IV*, and *ALF+MSCs V groups.*Positive cells showed bright Prussian blue stain.*Control group I* revealed absence of iron oxide-labeled stem cells as negative control **(Fig.4 a).** *MSCs IV* and *ALF+MSCs V groups* revealed bright blue iron oxide-labeled stem cells in close relation to damaged muscle fibers **(Fig.4 b&c)**.

- **Immunohistochemical staining results**

Myofibroblasts in the endomysium close to muscle fibers were detected using immunohistochemical labeling of α smooth muscle actin. Immunostaining was seen exclusively in the blood vessel media in the control group, which served as a positive control (**Fig.4 a)**. *Myopathy group* showed strong immunopositive reaction with many myofibroblasts (**Fig .4 b)**. *ALF group III* showed moderate immunopositive reaction (**Fig.4 c)**. *MSCs group IV* showed mild immunopositive reaction (**Fig.4)**. *ALF + MSCs groupV* showed weak immunopositive reaction with few myofibroblasts (**Fig.4 e)**

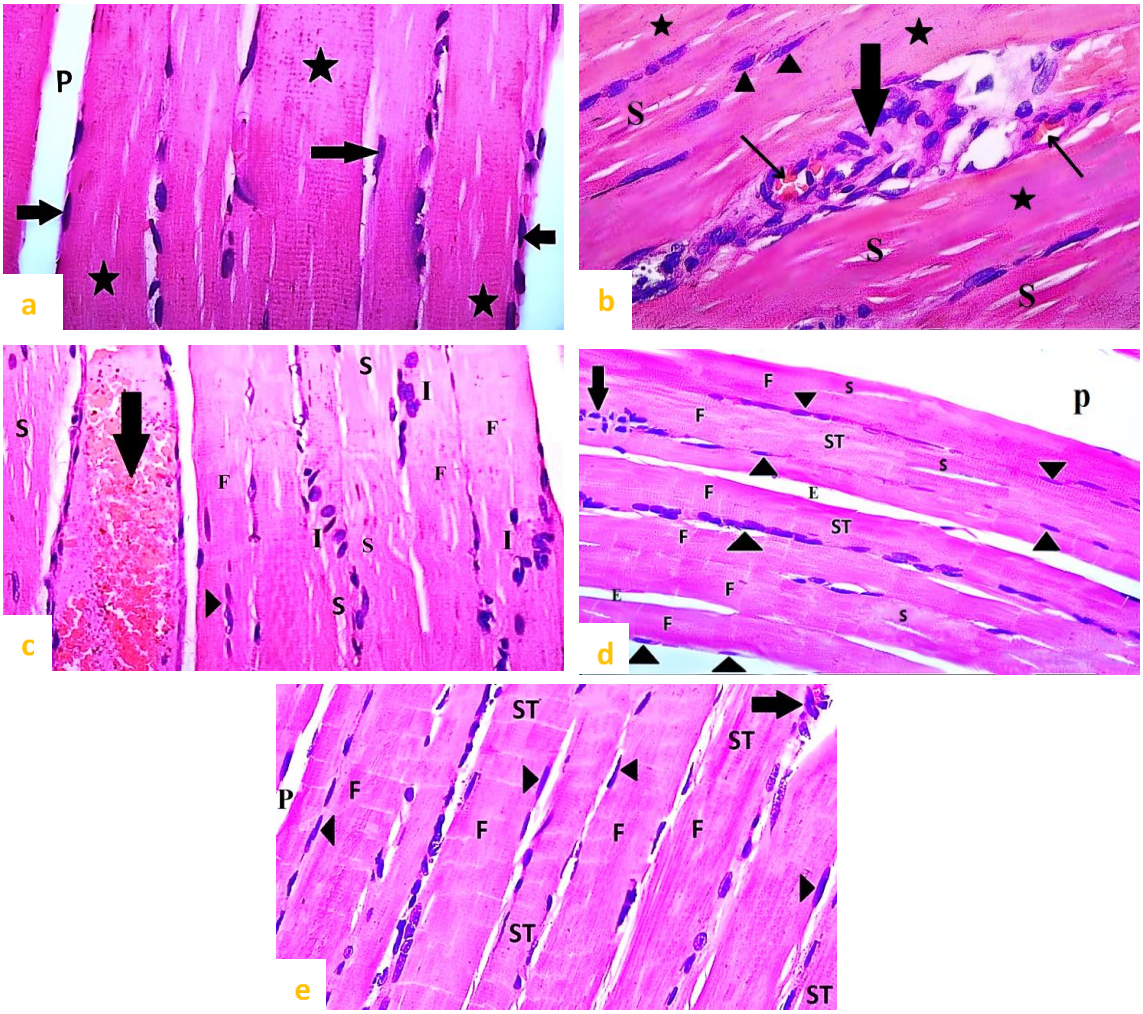

Fig.(2) (a): Parallel muscle fibers with acidophilic sarcoplasm and prominent , uniform, transverse striations (star) and peripherally located multiple nuclei (arrows) are depicted in a photomicrograph of a longitudinal section of the control group **I.(H&E x400). Fig.(2)** (b):A photomicrograph of a longitudinal section of simvastatin - induced myopathy group II showing area of degeneration with marked cellular infiltration (thick arrow) and some hemorrhagic spots (thin arrows) with splitting of surrounding muscle fibers (S) and areas of homogenous sarcoplasm(stars),and centrally located nuclei (triangles).**(H&E x400). Fig.(2)** (c):A photomicrograph of a longitudinal section of Simvastatin and ALF group III showing apperantly normal muscle fibers (F) , massive hemorrhage within one of muscle fibers (arrow). Also splitting of other fibers (S) is seen , There is Cellular infilteration (I), centerally located nuclei lined up forming chain (triangle). **(H&E x400). Fig.(2)** (d) A photomicrograph of a longitudinal section of the Simvastatin and MSCs group IV demonstrates the presence of parallel muscle fibers (F) that appear to be normal, with peripherally located nuclei (triangles) and distinct transverse striation (ST). Additionally, the photomicrograph demonstrates mild cellular infiltration (thick arrow), the splitting of some fibers (S), a wide perimysium (P), and a slightly wide endomysium (E).**(H&E x400). Fig.(2) (**e**):**A photomicrograph of a longitudinal section of Simvastatin and ALF +MSCs group V showing parallel muscle fibers (F) with peripherally located nuclei (triangles) and distinct transverse striations (ST),nearly normal perimysium is seen separating muscle bundles (P),and minimal cellular infilteration (thick arrow).**(H&E x400).**

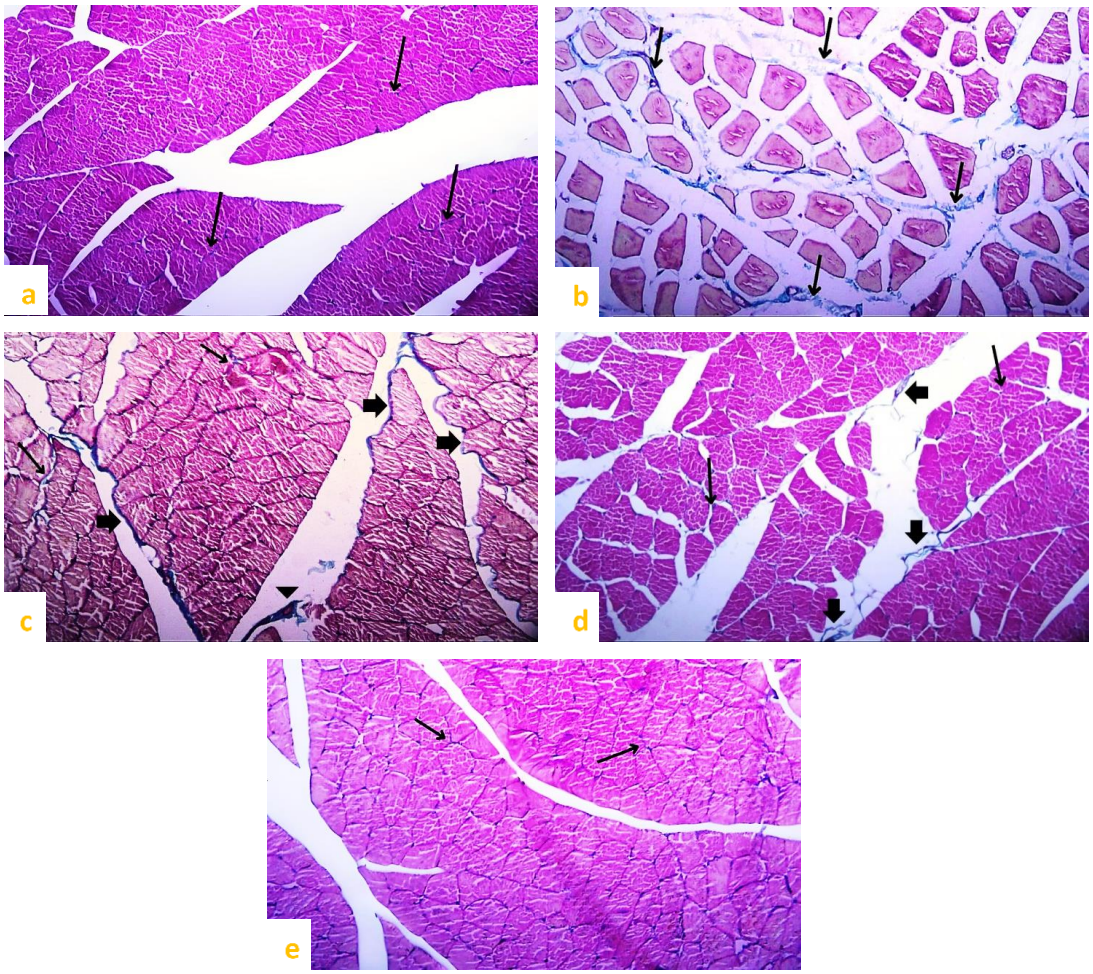

Fig.3(a): A transverse section of the control group I is depicted in a photomicrograph, demonstrating a negligible quantity of collagen fibers between the muscle fibers (thin arrows)**(Masson trichrome x200)**

Fig.3(b): A transverse section of the Simvastatin-induced myopathy group II is depicted in a photomicrograph, demonstrating the significant deposition of collagen fibers (arrows) between degenerated, widely separated muscle fibers **(Masson trichrome x200)**

Fig.3(c): Between muscle fibers (thin arrows) and around muscle bundles (thick arrows), a moderate amount of collagen fibers can be seen in a photomicrograph of a transverse section of the Simvastatin and ALF groups III. Notice ,collagen fibers that encircle the blood vessels (the triangle). **(Masson trichrome x200)**

Fig.3(d): In between muscle fibers (thin arrows) and around muscle bundles (thick arrows), a photomicrograph of a transverse section of the Simvastatin and MSCs group IV illustrates the presence of mild quantities of collagen fibers **(Masson trichrome x200)**

Fig.3(e): A photomicrograph of a transverse section of Simvastatin and ALF + MSCs group V showing muscle fibers fitted together in a mosaic appearance with very few collagen fibers in between (thin arrows).**(Masson trichrome x200)**

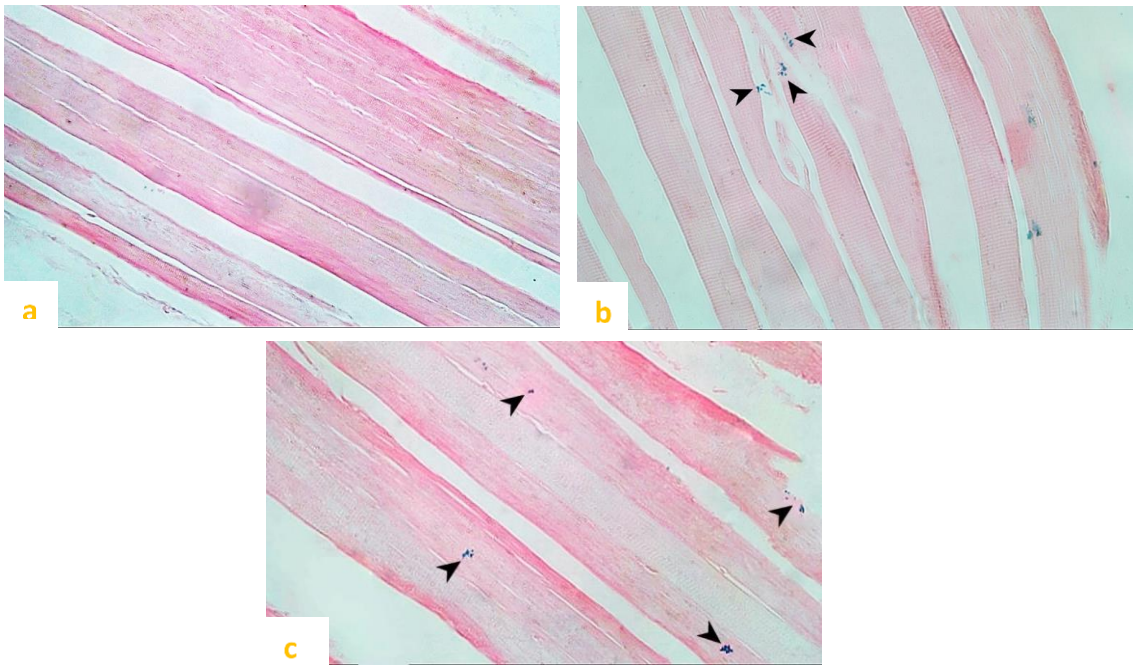

Fig.4(a): A photomicrograph of a longitudinal section of control group I showing no iron oxide-labeled stem cells (negative control).**(Prussian blue ×400)**

Fig.4(b): The photomicrograph shows a longitudinal section of Simvastatin and MSCs group, with iron oxide-labeled stem cells near a damaged muscle fiber (arrow heads).**(Prussian blue ×400)**

Fig.4(c): A photomicrograph of a longitudinal section of the Simvastatin and ALF +MSCs group shows iron oxide-labeled stem cells near a damaged muscle fiber and inside the muscle fibers (arrow heads). **(Prussian blue ×400)**

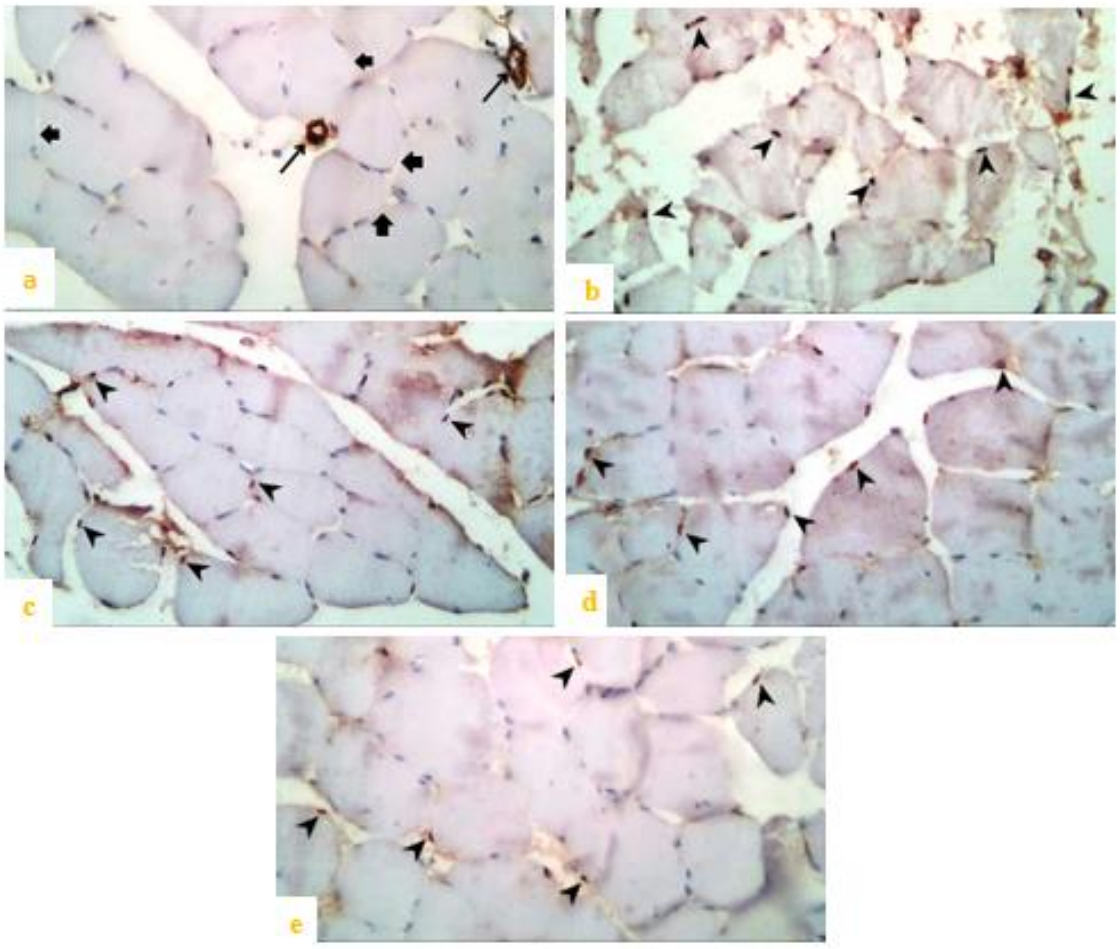

Fig. 5 (a): The endomysium in the control group I does not contain any immunopositive myofibroblasts, as shown by a photomicrograph of a cross-sectional section (thick arrows). The media of blood vessels is the only place where immunostaining is visible (thin arrows) **(Anti-α smooth muscle actin ×400)**

Fig. 5 (b): A transverse section of the Simvastatin-induced myopathy group is pictured in a photomicrograph, revealing numerous immunopositive myofibroblasts (arrow heads). Mainly situated in the endomysium, they are in close proximity to the muscle fibers. **(Anti-α smooth muscle actin ×400)**

Fig. 5 (c): The photomicrograph shows a transverse section of the Simvastatin and ALF group III ,With myofibroblasts, represented by the arrow heads, show a moderate immunopositive reaction **(Anti-α smooth muscle actin ×400)**

Fig.5 (d): A transverse section of the Simvastatin and MSCs group IV is visualized in a photomicrograph, demonstrating a mild immunopositive reaction of myofibroblasts (arrowheads) **(Anti-α smooth muscle actin ×400)**

Fig.5 (e): a photomicrograph of a transverse section of the Simvastatin and ALF+MSCs group V illustrating the presence of few of immunopositive myofibroblasts in close proximity to the muscle fibers (arrowheads). **(Anti-α smooth muscle actin ×400)**

Biochemical results:

Mean values of serum CK level (ng/ml) measured in all five groups represented in (table 1)

Group II (Myopathy group) when compared with group I and other therapeutic groups (III ,IV,V) showed a highly significant increase of serum CK mean levels ($p < 0.001$ for each).

Group III (Alfacalcidol group) showed significant increase ($P<0.001$) when compared with group I and ($p < 0.01$) with group IV and group V. While when compared with group II it showed significant decrease ($P < 0.001$).

Group IV (Mesenchymal stem cell group) showed significant increase($P<0.05$),($P<0.02$) when compared to group I and group V respectively.And there were a significant decrease when compared with group II ($p < 0.001$) and group III ($p <0. 01$).

Group V (Alfacalcidol +Mesenchymal stem cells) when compared to group I showed non significant difference in serum CK mean levels with nearly same control levels ($P > 0.05$),and compared with group II , group III it showed significant decrease in serum CK mean values ($P < 0.001$), ($P < 0.01$) respectively .While there was a slight significant reduction compared to group IV ($P< 0.02$) .

Morphometric results:

Mean area percentage of collagen fiber deposition by Masson's trichrome staining (Table 2)

Group II (Myopathy) when compared with group I and other therapeutic groups (III,IV,V) showed significant increase in the mean area % of collagen deposition ($P \leq 0.001$).

Group III (**Alfacalcidol group)** showed significant increase ($P<0.001$),($P<0.01$),($P<0.001$) when compared with group I, group IV, group V respectively. And showed significant decrease ($P<0.01$) with group II.

Group IV (Mesenchymal stem cell group) showed significant increase (P<0.01),(P<0.02) when compared with group I and group V.And showed significant decrease (P<0.001),(P<0.01) with groups II and III.

Group V (**Alfacalcidol+Mesenchymal stem cells group**) showed non significant difference (P>0.05) when compared with group I.And showed significant decrease (p<0.001) with groups II, III ,and a lesser significant decrease (P<0.02) with group IV.

Mean values of number of immunopositive myofibroblasts/high power- field in α-smooth muscle actin stained sections (Table 3)

Group II (myopathy) showed significant increase (P< 0.001)in number of myofibroblasts/high-power field when compared with group I, group III, group IV and group V.

Group III (Alfacalcidol group) showed significant increase (P<0.001) when compared with group I, group IV, group V and showed significant decrease (P<0.001) when compared with group II.

Group IV (Mesenchymal stem cell group) showed significant increase (P<0.01),(P<0.02) when compared with group I and group V respectively and showed significant decrease (P<0.001) when compared with group II and group III.

Group V (Alfacalcidol + Mesenchymal stem cells) showed non significant difference (P>0.05) when compared with group I ,and showed significant decrease when compared wih group II and group III (P<0.001) and group IV with (P<0.02).

Table (1) Mean serum Ck level (ng/ml) measured in 5 groups

	Group I	Group II	Group III	Group IV	Group V
CK ng/ml	0.75± 0.13	3.9 ± 0.69 $^{a,c,d\ \&\ e}$	2.4 ± 0.16 $^{a,b,d\&\ e}$	0.93 ± 0.1 $^{a,b,c\&e}$	0.76 ± 0.13 $^{a,b,c\&d}$

Data expressed as mean ±SD, *: significance ≤ 0.05 SD = Standard deviation

a: Significance vs Control, b: Significance vs group II, c: Significance vs group III, d: Significance vs group IV, e: Significance vs group V.

Table (2) Mean values of area percent of collagen fibers deposition in Masson stained sections

Control Group	Group I	Group II	Group III	Group IV	Group V
Collagen fiber deposition area%	2.8 ± 0.7	36.6 ± 7.6 a,c,d & e	23.4 ± 2.5 a,b , d & e	10.3 ± 2 a & b & c & e	3.5 ± 0.9 b & c & d

Data expressed as mean ±SD, *: significance ≤ 0.05 SD = Standard deviation

a: Significance vs Control, b: Significance vs group II, c: Significance vs group III, d: Significance vs group IV, e: Significance vs group V.

Table (3) Mean values of number of immunopositive myofibroblasts/ high-power field in α-smooth muscle actin stained sections

	Control Group	Group II	Group III	Group IV	Group V
αSMA area%	4.5 ± 1.4	37.1 ± 2.2 a, c ,d & e	21.5 ± 2.6 a, b ,d & e	10.8 ± 1 a,b & c & e	5.7 ± 1.6 b & c & d

Data expressed as mean ±SD, *: significance ≤ 0.05 SD = Standard deviation

a: Significance vs Control, b: Significance vs group II, c: Significance vs group III, d: Significance vs group IV, e: Significance vs group V.

Discussion

In the present study, myopathy was induced by administering simvastatin, It was reported that oral administration of simvastatin at adose (80mg/kg/day) for 6 weeks. A relvant

myopathy model in rats is provided by the maximal tolerated dose that induces widespread muscle degeneration [9]and[11] .

Myopathic rats revealed marked histological changes on light microscopy (LM), including segmental degeneration, inflammatory and fatty infiltrations, wavy fibers, centrally located nuclei, and fiber splitting. These pathological changes were consistent with previous studies [13,14]. According to [15], these alterations can be attributed to overcontraction caused by sustained elevations in intracellular Ca^{2+}, which leads to hyalinization of injured muscle fibers, similar to what occurs following acute muscle injury. As the affected fibers undergo necrosis or apoptosis, they initiate inflammatory cascades by releasing mitogenic chemoattractants. Furthermore, [16] suggested that statin-induced myotoxicity may be due to the depletion of isoprenoids, which regulate apoptosis. In addition, [17] reported that statin administration impaired oxidative phosphorylation in muscle cells due to elevated ADP levels, which increased production of reactive oxygen species (ROS) and triggered apoptosis.

Myopathic rats had a significantly larger area percentage of collagen deposition than the control group, which is consistent with earlier data [9]and[18].According to[19] Increase in connective tissue is typically a response to the loss of myofibers. The presence of inflammatory cells may also initiate fibrosis, as fibroblasts replace the damaged area and subsequently form collagen fibres.

Immunohistochemical results of myopathic rats in this study showed significant increase in myofibroblasts numbers as compared with control in agreement with [9]and [20] Transforming growth factor-β1 and platelet-derived growth factor are two instances of growth factors that are released locally and stimulate cells of extracellular matrix to produce local collagens and trigger differentiation of myogenic cells into myofibroblasts which deposit collagen as stated by[21] and[22].

This finding was consistent with previous research showing that myopathy is associated with elevated serum CK levels (myopathy group vs. control) [23]and[24] who reported that all animals administered simvastatin for 12 days presented muscle necrosis and increased serum CK levels 3.1 folds above control.[25] reported that statins cause sarcolemma damage so, CK leaks from muscle cytosol and decreases its ability to generate ATP.

In the current study, rats which received ALF for 6 weeks showed partial histological improvement of LM findings in form of some apparently normal fibers,while myopathic changes still present in other fibers in form of splitting,areas of degeneration,cellular and fatty infilterations, and centerally located nuclei in agreement with [26]and[27]. The potential correlation between statins and vitamin D was the basis for the improvements observed in this group. As stated, vitamin D induces the Cytochrome P enzyme, which stimulates the metabolism of statins, resulting in less toxic metabolites [28].

Consistent with earlier studies, the data demonstrated that the Alfacalcidol group had a significantly lower area percentage of collagen deposition than the myopathy group [27].

Immunohistochemical results of Alfacalcidol treated group in the present study showed significant decrease in immunoexpression of myofibroblasts compared to myopathy group indicating improvement.

This study found that blood CK levels were significantly higher in the Alfacalcidol group compared to the control group and significantly lower compared tothe myopathy group; the reduction of mean CK values were consistent with [26]and[29].

In the current study, rats received treatment with BM-MSCs intramascularly showed significant histological improvement of myopathic changes compared to both myopathy and ALF groups in form of well organized muscle fibers as many fibers were apparently normal, meanwhile there were limited myopathic changes in form of splitting ,mild cellular infilteration and centrally located nuclei .These results in agreement with[30],[31]and [32].

Selection of intramuscular route in this study was supported by[33]and[11]who reported that MSCs administered intramuscularly were more effective than intravenously administered and had more therapeutic potentials for statin- myopathy.In accordance to[34] who believed in injecting MSCs directly into muscle avoids clearance of MSCs by liver or kidneys and so ensuring a sufficient amount of stem cells reach the muscle tissue .In contrast,[35]and[36] reported that administration of MSCs intravenously was more effective.

In The current study, intramuscularly injected BM-MSCs cells homed to gastrocnemius muscle were evidenced by iron oxide-labeled stem cells detection using Prussian blue stain in accordance to [9] .Also detection of stem cells labelled with PKH26 dye using fluorescent microscope was done in accordance to[37].

BM-MSCs treated group in this study, showed significant decrease in area percentage of collagen deposition when compared to myopathy group, in agreement with[9]and[37]who reported that this result was due to regeneration of muscle fibers and reduction of inflammation and fibrosis, and when compared to ALF group, The area % of collagen deposition decreased less significantly in this group, indicating more improvement.[38] explained that MSCs limit fibrosis by high concentration of IGF-1 which enhances myofiber formation and promotes macrophage differentiation into anti-inflammatory M2 phenotype.This was supported by[39]who stated that MSCs had anti-fibrotic action due to its paracrine effect on matrix metalloproteinase-1.

Immunohistochemical results of BM-MSCs group in this study showed ahighly significant decrease in immunoexpression of myofibroblasts compared to both myopathy and ALF groups indicating an obvious improvement in agreement with[9].

In the current study, BM-MSCs treated group showed significant increase in serum CK mean levels when compared to control group and showed significant decrease when compared to both myopathy group and ALF treated group this result came in line with [40]and [36].

Consistent with previous research, the BM-MSCs group's findings here supported MSCs' potential as a treatment and regenerative tool for statin myopathy [39],[41]and[42].In separate studies[43]and[42] hypothesized that transplanted MSCs has a novel paracrine effect on muscle repair in addition to secretion of growth factors factors, chemokines and cytokines e.g(IL-10, FGF-2, and IGF-1) in the medium. These cells may stimulate the differentiation of muscle stem cells and promote the formation of new fibres. [31] suggested that the combined effect of BM-MSCs' paracrine support and their function in differentiation into myogenic progenitors was responsible for the influence of BM-MSCs on skeletal muscle, whether systemically or locally injected.

In the current study, H&E sections of rats that received combined treatment with ALF + BM-MSCs showed that sections were more or less similar to control with Occasional cellular infilteration.

The ALF+BM-MSCs group and the control group did not differ significantly with respect to the mean percentage area of collagen deposition.

Immunohistochemical results of ALF+BM-MSCs group showed weak immunopositive expression of myofibroblasts with no significant difference when compared to control.

In the current study, ALF+BM-MSCs group showed no significant difference in serum CK mean levels with nearly same control levels.

Fortunately ALF+BM-MSCs treated group showed marked significant improvement of myopathic picture histologically, immunohistochemically and biochemically. Results were almost similar to control.

Conclusion

From this study, we concluded that combined ALF and BM-MSCs therapy has better results than the use of stem cell therapy alone.Proving to be a promising therapeutic option for statin-induced myopathy.

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