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The Effect of Low Dose Statin Combined with Grapefruit on Skeletal Muscle Structure and the Possible Protective Role of Mesenchymal Stem Cells

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Abstract

Background: Statins are the cornerstone of therapy for dyslipidemia. They are generally well tolerated but can produce skeletal muscle adverse reactions, ranging from myalgia to rhabdomyolysis. Grapefruit juice increases the plasma concentrations of simvastatin thus increasing the risk of adverse effects. Stem cells have been recognized as a potential tool for the development of innovative therapeutic strategies.

Objectives: To evaluate the effect of low dose statin combined with grapefruit on muscle structure and the possible protective role of Bone Marrow Mesenchymal Stem Cells (BM-MSCs) in female rats.

Materials and Methods: Twenty five adult female rats were randomly divided into: Group I (control group, n=10) equally subdivided into: Subgroup IA (grapefruit control); 5 ml/kg oral grapefruit juice was given daily, Subgroup IB (statin control); 20 mg/kg oral Zocor® in tap water was given daily. Group II (statin + grapefruit, n=5); 20 mg/kg Zocor® in 5 ml/kg grapefruit juice was given daily for 45 days, Group III (IM- MSCs, n=5); statin + grapefruit were given as in Group II, in addition to two intramuscular (IM) injections of BM-MSCs. Group IV (IV- MSCs, n=5); statin + grapefruit were given as in Group II, in addition to two intravenous (IV) injections of BM-MSCs. Plasma level of Creatine Kinase (CK) was obtained at various intervals. All animals were sacrificed 12 days after the last dose, gastrocnemius muscles were collected and processed for light and electron microscopic study.

Results: Both control subgroups showed unremarkable muscle changes. However, in Group II, dilated T-tubules, subsarcolemmal space widening, mitochondrial degeneration, disintegrated myofibrils, loss of Z lines and accumulation of large electron dense particles were detected. In Group III and IV these muscle changes were improved.

Conclusion: Statin combined with grapefruit resulted in muscle ultrastructural changes. These changes were ameliorated with either IM and IV injection of MSCs, however IM injection showed better results.

Introduction

Statin (3-hydroxy-3-methylglutaryl coenzyme, HMG coA, reductase inhibitors) is a family of drugs that is best known for its hypolipidimic activity. This activity is attributed to the ability to inhibit the endogenous cholesterol synthesis through inhibition of the HMG CoA reductase enzyme, the rate limiting step in the pathway of cholesterol synthesis [1]. Statins have proved a high efficacy in decreasing risk of both primary and secondary coronary vascular disease and subsequently decreased mortality

rate of cardiovascular incidents [2]. Despite the significant benefit of statins, common side effects have been reported. The most severe and the most common adverse effect of statins is myotoxicity, which varies from myopathy, myalgia and myositis to rhabdomyolysis [3]. This group of adverse effect was the cause of withdrawal of Cerivastatin from the US market by Food and Drug Administration (FDA) in 2001 [3]. Muscle damage is mediated by the deficiency of Fernsyl Pyrophosphate (FPP), one of the intermediates essential for the synthesis Coenzyme Q10 which is an essential transporter in the respiratory chain [4]. Currently existing statins are considered safe from the side effect point of view [5]. However, toxicity might be manifested if it is combined with other myotoxic drugs or if other drugs or natural products have deregulated its own metabolism [6].

Grapefruit is one of the natural agents that is taken to decrease weight or decrease the cholesterol level [7]; however, precaution should be taken if it is combined with other drugs [8]. Combination of grapefruit with statin results in increased risk of myotoxicity in statins groups that are usually have very low risk [9]. This is attributed to the inhibitory effect of grapefruit to the Cytochrome P450 (CYP3A4) responsible for metabolism of statin and hence its accumulation [9].

Bone Marrow Derived Mesenchymal Stem Cells (BM-MSCs) have potential activity in regenerating many mesenchymal tissues including skeletal muscle [10]. Therefore, aiding the process of regeneration of skeletal muscle after muscle injury can carry an extra beneficial effect. In this work, we investigated the effect of intravenous and intramuscular injection routes of BM-MSCs on skeletal muscle after potential skeletal muscle injury induced by combined effect of low dose statin and grapefruit.

Materials and Methods

Drug preparation

Simvastatin used in this experiment was the commercial formula Zocor®, 40 mg (Merck co.). The dose used was adjusted to 20 mg/kg/day dissolved in 5 ml/kg freshly prepared grapefruit [11].

Animals and experimental design

Twenty-five female rats of Wistar strain of average weight 150-200 grams were used in this study. Animals were housed under appropriate conditions of temperature and illumination according to the guidelines for the care and use of laboratory animals approved by the experimental animal ethical committee of Faculty of Medicine, Ain Shams University. They were freely allowed to water and laboratory chow.

Female rats were randomly divided into four groups.

- **Group I** (control group, n=10): was equally subdivided into two subgroups
  - **Subgroup IA** (grapefruit control, n=5); 5 ml/kg grapefruit juice was given daily by gastric gavage tube for 45 days.
  - **Subgroup IB** (statin control, n=5); Zocor® was given daily in a dose of 20 mg/kg suspended in 5 ml/kg tap water administrated by gastric gavage tube for 45 days.
- **Group II** (statin + grapefruit, n=5); 20 mg/kg Zocor® suspended in 5 ml/kg grapefruit juice was given daily for 45 days.
- **Group III** (IM-MSCs, n=5); 20 mg/kg Zocor® suspended in 5 ml/kg grapefruit juice was given daily for 45 days. On days 3 and 33 of the experiment, two Intramuscular (IM) injections of BM-MSCs (1×10^6 cells in 0.5 ml PBS) were given in left gastrocnemius muscle.
- **Group IV** (IV-MSCs, n=5); 20 mg/kg Zocor® suspended in 5 ml/kg grapefruit juice was given daily for 45 days. On days 3 and 33 of the experiment, two Intravenous (IV) injections of BM-MSCs (1×10^6 cells in 0.5 ml PBS) were given via tail vein.

Animals of all groups were sacrificed 12 days after the last dose of statin i.e. 57 days from the beginning of the experiment.

BM-MSCs isolation and culture

Bone marrow was harvested from five male Wistar rats (weight 100-150 grams) by flushing of their femurs and tibiae with Dulbecco's modified Eagle's medium (DMEM, Lonza, Belgium). The marrow plugs dissociated by pipetting and centrifuged at 1800 rpm for 10 min. The cell pellets were then suspended, seeded at a density of 5×10^5 cells/cm2 and incubated at 37 °C in a humidified incubator containing 5% CO_2. When primary cultures became nearly confluent (80-90%), the cells were sub-cultured. Cells of the third passage were used in this experiment [12].

Flow cytometry analysis [13]

The phenotype of the third passaged BM-MSCs was analyzed by flow cytometry. The cells were harvested with trypsin/EDTA and suspended at a concentration of 1×10^5 cells/100 µl of PBS. The cells were stained and incubated with Fluorescein Isothiocyanate (FITC)-conjugated antibodies against CD 29, CD 73, CD105 and CD 34 monoclonal antibodies (Thermo Fisher Scientific, USA). The cells were then, washed in PBS and fixed with 1% paraformaldehyde for 15 min. FAC Scan Flow cytometer (BD Biosciences) was used to analyse the specific surface antigen profile of the cells (Histogram 1).
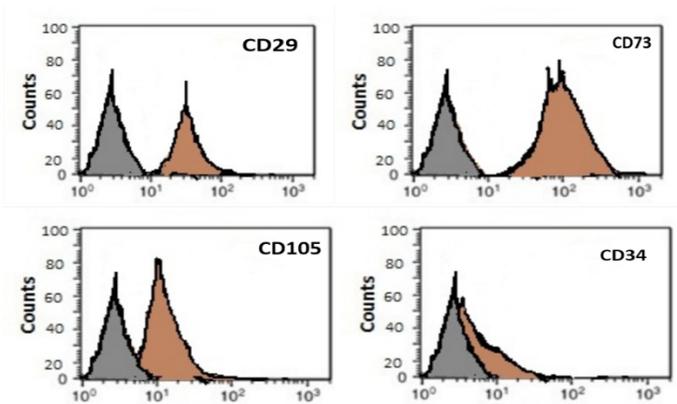
Histogram 1: The expression of cell surface markers of BM-MSCs at P3 analyzed by flow cytometry. They express the mesenchymal markers CD29, CD73 and CD105, but not the hematopoietic marker CD34. Grey curves serve as a negative control.

In situ hybridization technique for sex-determining region Y (SRY) gene

Specimens of gastrocnemius muscle from the female rats (Group IV) were processed for detection of rat Y-chromosome specific SRY-3 gene labelled with digoxigenin by *in situ* hybridization technique. Briefly, paraffin sections were dewaxed by xylene and rehydrated in descending grades of alcohol. Endogenous peroxidase activity was blocked by incubation in 3 ml/L H_2O_2. The sections were then hybridized with rats/mice SRY probe, heated for 5 minutes at 65 °C, incubated overnight at 40 °C and rinsed to wash off the excess unbound probes. The localization of the hybridized probe was revealed by monoclonal antibody mouse anti-digoxigenin (Abcam Biochemical, USA) and visualized with Diaminobenzidine (DAB). Finally, the sections were counterstained with haematoxylin (Sigma Chemical, St. Louis, MO). Nuclei with significantly brown granules were positive [14]. To ensure the specificity of the technique, the same procedure was tested using gastrocnemius muscle sections from male rats which served as positive controls. While, gastrocnemius muscle sections from female rats in the control group were served as negative controls.

Serum creatine kinase analysis

Blood samples were taken from experimental female rats via orbital vein to measure the level of serum Creatine Kinase (CK) on days 4,16,32 and 45 of the experiment. The sera were stored at -20 °C till time of need. All data were subjected to statistical analysis using one-way analysis of variance performed with SPSS, version 21 (IBM Inc., Chicago, Illinois, USA). The data were expressed as mean ± Standard Deviation (SD). P values < 0.05 were considered significant.

Histological study

At the end of the experiment, all female rats were sacrificed, and the left gastrocnemius muscles were obtained. The specimens were fixed in 10% buffered formalin, paraffin blocks were prepared, sectioned at 5 μm thick and stained with hematoxylin and eosin for light microscope examination.

Electron microscopy study

The specimens from left gastrocnemius muscles of all female rat groups were fixed in 2.5% formol buffered glutaraldehyde solution overnight, post fixed for 2 h in 2% osmium tetroxide and processed to epoxy resin blocks. Semithin sections of 1 μm thick were stained with toluidine blue. Ultrathin sections of 50-60 nm were prepared and stained with uranyl acetate and lead citrate [15].

Results

Morphology and immune phenotype of BM-MSCs

Isolated and cultured BM-MSCs reached 80% confluence on day 12. They were of heterogeneous morphology at primary culture (spindle-, star-, triangular-shaped) with numerous non-adherent hematopoietic stem cells. With frequent subcultures beginning from passage 3 (P3), the adherent MSCs were of homogenous fibroblast-like morphology without hematopoietic cell contamination (Figure 1). MSCs at P3 were identified for their surface receptors by flow cytometry, they were positive for CD29, CD73, CD105 and negative for the hematopoietic marker CD34 (Histogram 1).
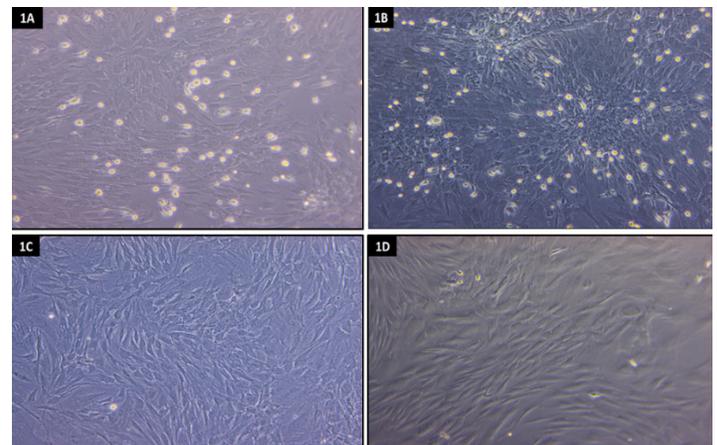

Figure 1: BM-MSCs in culture representing their morphology at different passages. (A) P0; the cells are of heterogeneous morphology. (B and C) P1 and P2 respectively; cell morphology is more or less homogenous. (D) P3; all the cells are of homogenous fibroblastic-like morphology (Phase contrast microscope ×100).

In situ hybridization on SRY gene

Male rat SRY gene was detected with in situ hybridization in the gastrocnemius muscle sections of the female rats that received BM-MSCs transplantation via intravenous route (Figure. 2). These findings proved homing of BM-MSCs into the injured muscle in Group IV. In positive controls, the SRY gene was detected in the gastrocnemius muscle of male rats. However, it could not be detected in gastrocnemius muscles of control female rats (negative controls) (data not shown).
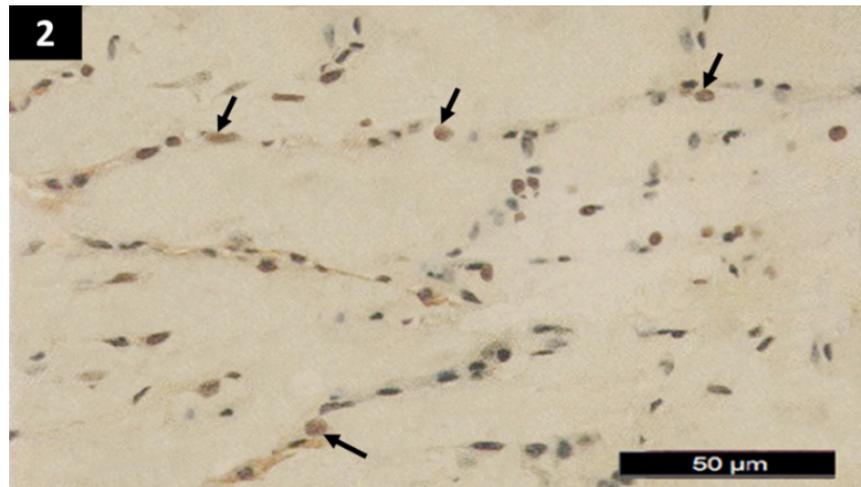

Figure 2: A muscle section of female rats from Group IV (IV-MSCs) showing positive nuclear reaction (↑) to rat SRY gene in fibres that accepted BM-MSCs intravenous transplantation from male rats (in situ hybridization, ×400).

Serum creatine kinase level (Table 1):

Serum CK showed high fluctuations of results between the animals of the same group. Subgroup IA (grapefruit control) showed the significant decrease in the level of CK among all other groups through different durations of sample taking. On day 4 of the experiment, no significant difference was estimated between Group II (statin + grapefruit), Group III (IM-MSCs) and Group IV (IV-MSCs). On the contrary, on days 16, 32 and 45 of the experiment Group II (statin + grapefruit) showed a significant increase with respect to other groups. This reflected the role of BM-MSCs whether injected locally or systemically succeeded in ameliorating muscle injury induced by the combined effect of statin and grapefruit. At the end of the experiment, on day 45, low dose simvastatin alone (Group II) was not able to significantly increase CK level above the BM-MSCs treated groups.

Timing	Subgroup IA (grapefruit control)	Subgroup IB (statin control)	Group II (Statin +grapefruit)	Group III (IM+MSCs)	Group IV (IV+MSCs)
Day 4	1031±130*	1339.3±240*	1973.6±129Δ°	1830±175Δ°	1721±105Δ°
Day 16	1105.6±167*	2287.6±234Δ	3606±120*	2527.3±143Δ	2441±218Δ
Day 32	1052±152*	2179.2±279Δ	3754±137*	2062±214Δ	1986.6±179Δ
Day 45	1101.3±172*	5298.6±200Δ	7957±166*	4193±124Δ	4908.6±132Δ
(*) significant difference with all groups; (Δ)significant difference with Group III, (°) significant difference from subgroup IB					

Table 1: Showing mean ± SD of CK levels in different groups

Light microscopic changes

Examination of gastrocnemius muscle under light microscope showed minimal findings in all groups. Both subgroups of the control group (Group I) revealed the normal structure of skeletal muscle. Muscle fibres appeared as longitudinal parallel multinucleated fibres with acidophilic sarcoplasm. The nuclei appeared oval and located peripherally along the muscle fibres. Cross striations of the fibres were also clearly seen (Figure 3A). Simvastatin administration combined with grapefruit for 45 days (Group II) illustrated less distinct cross striations in some fibres. Other fibres showed disorganized myofibrils and numerous haphazardly arranged nuclei (Figure 3B). In Group III (IM-MSCs) and Group IV (IV-MSCs), parallel muscle fibres having distinct cross striations and subsarcolemmal oval nuclei were demonstrated, while other fibres showed linear centrally situated oval nuclei (Figures 3C, 3D).
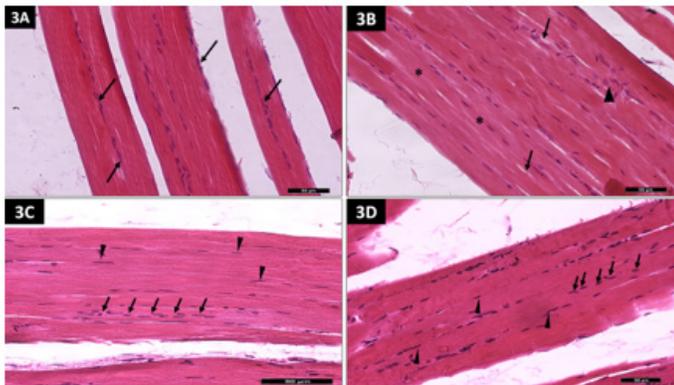

Figure 3: Longitudinal sections in the gastrocnemius muscles showing (A) Group I (control); parallel muscle fibres, having multinucleated subsarcolemmal oval nuclei (↑). Note the well-defined cross striations of the muscle fibres. (B) Group II (statin + grapefruit); parallel muscle fibres. Some fibres show less distinct cross striations (∗). Other fibres show disorganized myofibrils (↑) and numerous haphazardly arranged nuclei (▲). (C) Group III (IM - MSCs); parallel muscle fibres having distinct cross striations. Some fibres have multiple subsarcolemmal oval nuclei (▲), while others have linear centrally situated oval nuclei (↑). (D) Group IV (IV - MSCs); muscle fibres appear parallel well organized with distinct cross striations and subsarcolemmal nuclei (▲). However, linear central nuclei are seen in a muscle fibre (↑), (Hx&E ×400).

Ultrastructural changes

Electron microscopic examination of the two control subgroups (grapefruit subgroup and statin subgroup) detected unremarkable ultrastructural changes in the gastrocnemius muscle (Figure 4A). Myofibers exhibited oval euchromatic nuclei beneath the sarcolemma. The sarcoplasm demonstrated regular profiles of myofibrils with few mitochondria and glycogen granules in between the myofibrils. Each myofibril was formed of uniformly arranged myofilaments into sarcomeres. The unique arrangement of myofilaments formed dark bands (A band) and light bands (I band). The centre of each A band was occupied by a pale H band bisected by a dark M line, while, each I band was bisected by a dark Z line. Between myofibers, few amounts of collagenous fibres representing the endomysium was seen (Figure 4B).
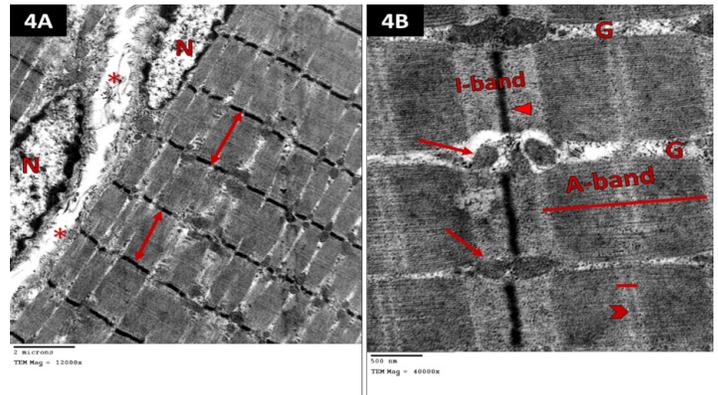

Figure 4: Electron micrograph of longitudinal sections in the gastrocnemius muscle of Group 1 (control). **(A)** Two parallel myofibers are separated by the delicate connective tissue endomysium (*). Each myofiber has oval euchromatic nucleus beneath the sarcolemma (N). The myofiber is formed of parallel myofibrils that extended through the entire length of the muscle fibre. Each myofibril is formed of uniformly arranged myofilaments into sarcomeres (↔), (TEM × 12000). **(B)** A higher magnification of the myofibrils showing sarcomeres. Each sarcomere is formed of a dark band (A-band), having pale H-band (-) at its centre, bisected by a thin M-line (^) and a light band (I-band), bisected by the Z line (▲). Few mitochondria (↑) and electron dense glycogen granules (G) appear in between myofibrils, (TEM ×40000).

In contrast, Group II showed that myofibrils were having a non-uniform diameter and were discontinuous, with wide inter myofibril spaces occupied by remnants of contractile materials and large dense particles. Also, one or multiple consequent sarcomeres show disrupted structure (Figures 5A,5D). T-tubules were distended at A-I junctions with adjacent Z-line degeneration as well as, disarrangement of the myofilaments in the affected sarcomeres (Figure 5B). Moreover, the subsarcolemmal space of a muscle fibres was were widened in some fibres and contained degenerated mitochondria and a large autophagy vacuole. However, the sarcolemma remains intact (Figure 5C). Also, disorganization of myofilaments, swollen mitochondria and dilated sarcoplasmic reticulum were detected (Figure 5D).

The intramuscular injection of BM-MSCs in Group III, restored the normal ultrastructure of the myofibers of the gastrocnemius muscle. The myofibrils were having uniform diameters and were aligned in a precise pattern. Myofilaments were regularly arranged into sarcomeres (Figure 6A). Delicate connective tissue, the endomysium was observed in between myofibers. Few mitochondria were detected deeply in between

myofibrils and Z-lines appeared intact bisecting the I-band with preserved T-tubular system (Figure 6B). However, intravenous injection of BM-MSCs in Group IV, some ultrastructural abnormalities were still detected in the gastrocnemius muscle. Some fibres showed disorganized sarcomeres with myofilament degeneration and T-tubule dilation. Mitochondria were either small located in between the myofibrils, or large located at sites of degenerated Z-lines (Figures 7A, 7B).
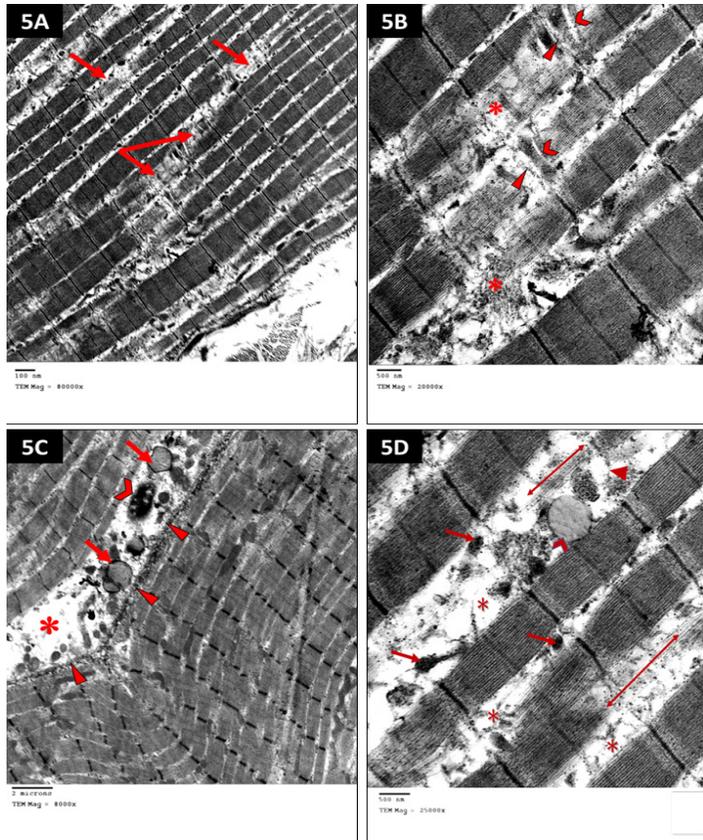

Figure 5: Electron micrographs of longitudinal sections in the gastrocnemius muscle of Group II (statin + grapefruit) showing: (A) myofibrils are having non uniform diameters along the muscle fiber, one or multiple consequent sarcomeres are disrupted (↑). (TEM ×8000). (B) By higher magnification, T-tubules are distended at A-I junctions (^) with adjacent Z-line degeneration (▲). Notice the disarrangement of the myofilaments in the affected sarcomeres (*) (TEM × 20000). (C) The subsarcolemmal space of a muscle fiber is widened (*) and contains degenerated mitochondria (↑) and a large autophagic vacuole (^), while the sarcolemma remains intact (▲). (TEM × 8000). (D) The inter myofibril spaces are widened (*) and are occupied by remnants of contractile materials and large dense particles (↑). Disorganization of myofilaments is obvious (↔). Note the presence of a swollen mitochondrion (^) and dilated sarcoplasmic reticulum (▲), (TEM × 25000).
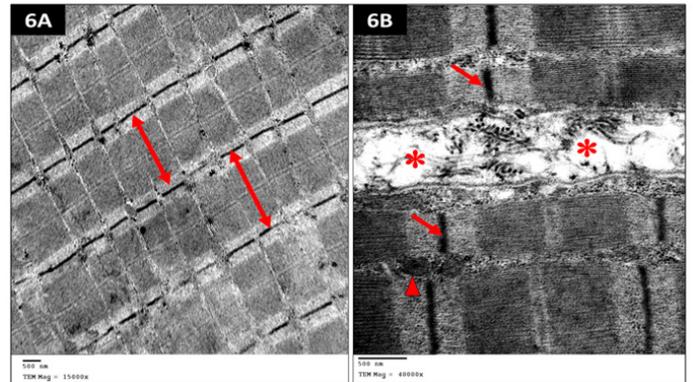

Figure 6: Electron micrographs of longitudinal sections in the gastrocnemius muscle of Group III (IM-MSCs) showing: (A) Myofibrils are having uniform diameters and are arranged parallel to each other. Myofilaments are regularly arranged into sarcomeres (↔) comparable to the control, (TEM × 15000). (B) Two adjacent myofibers are separated by the endomysium (*). Few mitochondria are present deep in between the myofibrils (▲). Z-lines appear intact bisecting the I-band (↑), (TEM × 40000).
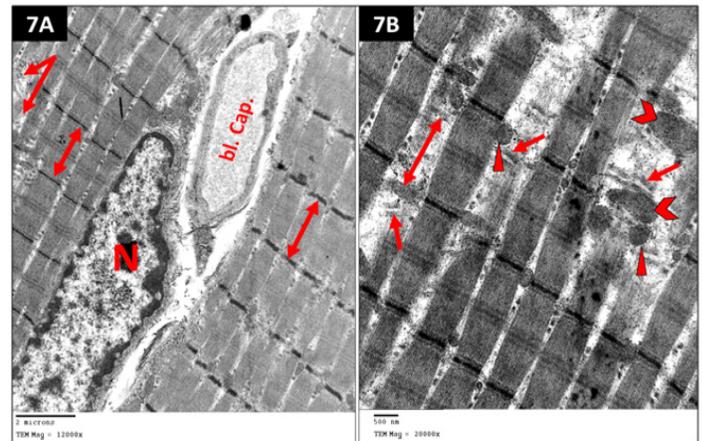

Figure 7: Electron micrographs of longitudinal sections in the gastrocnemius muscle of Group IV. (A) Two adjacent myofibers contain parallelly arranged myofibrils. Each myofibril is formed of uniformly arranged myofilaments into sarcomeres (↔). Few myofibrils still have disorganized sarcomeres (↑). The nucleus of one myofiber is present just beneath the sarcolemma (N) and a blood capillary (bl. cap.) appears in between the myofibers (TEM × 12000). (B) Some sarcomeres show myofilament degeneration (↔) with T-tubule dilation (↑). Small mitochondria are present in between the myofibrils (▲), but larger mitochondria can be noticed at sites of degenerated Z-lines (^), (TEM × 20000).

Discussion

It is well known that, statins (HMG-CoA reductase inhibitor) are widely used as an antihyperlipidemic agents to decrease the concentrations of low-density lipoprotein cholesterol levels in blood through blocking the rate-limiting step in the mevalonate pathway [1]. However, statin-induced myopathy appears to be the most common adverse effect which is dose- dependent as well as concentration-dependent [16].

Many literatures illustrated that muscle necrosis in rats occurred at a simvastatin dose of 80 mg/kg/day by day 12. However, administration of 60 mg/kg/day did not cause any necrotic changes in the muscles [17-19]. Joy and Hegele, (2009) [20], reported that the incidence of myopathy in humans increased with increased simvastatin doses; being 0.02, 0.08 and 0.53% for 20, 40 and 80 mg/kg, respectively.

Fresh grapefruit contains furanocoumarins, which are the main agents responsible for the interference with statins metabolism. They inhibit the activity of the enzyme CYP3A4 in the intestinal wall, consequently inhibiting the first-pass metabolism of statins and increasing their systemic bioavailability. As a result, both the cholesterol lowering effect as well as the risk of concentration-dependent adverse effects of simvastatin are augmented [8].

The present study showed that administration of low dose simvastatin (20 mg/kg/day) alone (subgroup IA) or grapefruit juice alone (subgroup IB) resulted in unremarkable light microscopic and ultrastructural changes in gastrocnemius muscle structure. In contrast to our results, Ozek, et al. (2010) [21] found that at the molecular level low dose simvastatin induced significant structural, functional and compositional alterations as well as protein denaturation in rat extensor digitorum longus muscle**.**

In the present study, light microscopic examination of muscle fibres in Groups III (IM- MSCs) and IV (IV-MSCs) revealed linear arrangement of nuclei into the centre of the muscle fibres. Accordingly, we hypothesized that, centrally located nuclei in the current experiment were a sign of regeneration induced by the influence of injected BM-MSCs either locally or systemically. Centrally located nuclei are routinely found in muscle regeneration. Nuclear movement during myofiber maturation is a step by step process driven by different mechanisms [22]. The first movement is termed centration and involves the migration of myonuclei from fused myoblasts to the centre of the newly formed cell and align on a central axis. Nuclei then migrate from a central position to the cell periphery right below the plasma membrane where they become anchored [22].

In the current study, the most striking results were the ultrastructural muscle changes in Group II in which simvastatin and grapefruit were concomitantly ingested. The myocyte ultrastructural damage was observed as T-tubular system dilation, Z-line loss in adjacent sarcomeres and myofilamentous disorganization in many sarcomeres. Moreover, widened intermyofibrillar and subsarcolemmal spaces, dilated sarcoplasmic reticulum, swollen mitochondria and large electron dense particles. Despite all these changes, the sarcolemma remained intact. These results were in accordance with Draeger, et al**.** (2006) [23] who observed in statin-treated patients, the same changes in T-tubular system and subsarcolemma, while the plasma lemma was intact.

The finding concerning affection of T-tubules but not sarcolemma membrane, could be explained by the different composition of both membranes. Although, sarcolemma and T-tubular membranes are equally rich in cholesterol, yet different in the contents and nature of their cholesterol-stabilizing proteins. The sarcolemma is supported by and persistently adherent to cholesterol-binding caveolin 3, which disappears abruptly at the entrance to the T-tubular system [24].

On the other hand, T-tubules contain members of the annexin protein family, which are calcium dependent and sensitive to fluctuations in intracellular calcium. During muscle relaxation (low calcium), annexin dissociates from the T-tubules membrane, leading to cholesterol extraction associated with statin therapy [25].

The mitochondrial changes observed in this work were in consistent with the study of Kaufmann, et al., (2006) [26] who demonstrated that lipophilic statins as simvastatin are considered as mitochondrial toxins. They inhibited the function of the electron transport chain, impaired mitochondrial β-oxidation and uncoupled oxidative phosphorylation. Thus, decrease the mitochondrial membrane potential with subsequent decrease in ATP synthesis, leading to mitochondrial swelling and apoptosis.

Furthermore, in the present study, the observation of subsarcolemmal autophagic vacuoles were also reported by Rehman, et al. (2016) [27]. They observed a late single membrane autophagic vacuole surrounding electron dense material in 25% of muscle biopsies from patients on statin therapy. These vacuoles resulted from fusion of early autophagic vacuoles with lysosomes. They further added that, muscle fibre necrosis, regeneration and inflammation associated with statins, stimulate a multistep process of autophagy encoded by a family of autophagy related genes. Moreover, Phillips, et al. (2010) [28] reported that, in statin induced rhabdomyolytic muscles, a significant reduction in small G-proteins, which play a crucial role in cell growth and differentiation. While, an upregulation of atrogin-1, a mediator of muscle atrophy was detected.

In the present research, the light and electron microscopic changes were markedly improved in Group III which received two intramuscular injections of BM-MSCs during simvastatin treatment. The muscle fibres of the gastrocnemius muscle were comparable to the control group. On the other hand, Group IV that

received two intravenous BM-MSCs injection, showed almost normal light microscopic structure comparable to the control. Conversely, at the ultrastructural level, few myofibrils showed profiles of disorganized sarcomeres with myofilament degeneration and T-tubule dilation. Moreover, Y chromosome was detected in the muscles of female rats at sacrifice. This proved that male derived BM-MSCs were homed to the injured skeletal muscle.

In this view, LaBarge and Blau, (2002) [29] demonstrated that depletion of endogenous satellite cells after irradiation, could be compensated by endogenous BM-MSCs. They concluded that BM-MSCs could regenerate muscle tissue in a biological stepwise progression from bone marrow stem cells to muscle satellite cell to differentiated multi-nucleate muscle fibre. This potential effect of the endogenous MSC pool could be augmented by administrated of exogenous MSCs.

The ameliorative effect of intramuscular injection of BM-MSCs on muscle injury was reported in many studies. Winkler, et al. (2009) [30] identified that, transplantation of BM-MSCs intramuscularly had improved muscle function in a dose dependent manner in a rat model of skeletal muscle crush injury. Moreover, Shi, et al. (2004) [31] proved the regenerative capacity of injected BM-MSCs injected directly into injured muscle in mice.

On the contrary, Corona and Rathbone (2014) [32] found that when BM-MSCs were injected intramuscularly, they survived up to one-month post-transplantation, but did not improve muscle function. Yet, when injected intravenously, the cells recruited to the site of injury and enhanced muscle regeneration and function. The exact mechanisms of MSC- mediated tissue repair are not fully understood, and many controversies still exist regarding their capacity for myogenic differentiation.

Lee, et al. (2005) [9] identified that intramuscular injection of human BM-MSCs in dystrophin-deficient mice restored dystrophin expression but did not recover the contractile function of the skeletal muscles. The authors suggested that the presence of dystrophin positive myofibers might result from the fusion of the engrafted cells with resident myofibers rather than from the differentiation of BM-MSCs into myogenic progenitors. In other view, Von Roth, et al. (2012) [33] reported that intra-arterial injection of BM-MSCs in a rodent skeletal muscle crush model, resulted in restoration of the muscle function. However, this systemically delivered cells were not detected in the injured muscle. Thus, the authors suggested that the improvements in function were likely due to the paracrine action of the injected stem cells.

Similar to our study, Bittner, et al. (1999) [34] transplanted bone marrow cells from male donor mice into female mice with dystrophic muscles, using the Y chromosome as a cellular marker. It was detected that bone marrow-derived cells were homed to the dystrophic skeletal muscle and underwent muscle-specific differentiation and regeneration.

Accordingly, we suggested that the influence of BM- MSCs on skeletal muscle, either systemically or locally injected, was through the combined effect of the paracrine support they provided through the secretion of many soluble mediators including growth factors and cytokines. In addition, to their role in differentiation into myogenic progenitors.

In the present study, serum CK level was significantly increased when low dose statin was combined with grapefruit. However, low dose statin alone showed a non-significant difference with respect to BM-MSCs treated groups. Therefore, grapefruit greatly augmented the effect of statin even in low doses.

Our results concerning combining low dose statin with grapefruit, was similar to high dose statins in other study. Silva, et al. (2007) [35] found that, high dose statin therapy increased the risk of CK elevations (> 10 times the upper limit of normal range) ten times more than that of low dose statin. On the contrary, other studies claimed that CK would persist at the normal levels despite the presence of significant myopathy because the sarcolemma remained intact in statin myopathy which prevented CK from leakage into the serum [28,36].

Conclusions

It was concluded that low dose simvastatin therapy did not result in skeletal muscle damage on both the microscopic and ultra-structure levels. However, co-administration of low dose simvastatin with grapefruit resulted in myopathic changes especially at the ultrastructural level. These changes were ameliorated with BM-MSCs transplantation either locally or systemically. Despite local IM injection showed better results on the microscopic level, both routes were nearly similar on the biochemical level (CK). This could be attributed to the generalized systemic effect on several muscles through the intravenous route.

Recommendations

We recommended patients on statins therapy, even with low dose statins, not to consume fresh grapefruit juice in a regular fashion. Despite, it would increase the cholesterol lowering effect, it would also increase the adverse effects of simvastatin.

Conflict of interest

The authors have no financial conflict of interest.

Acknowledgment

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