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Mesenchymal stem cells versus intermittent fasting on statin induced myopathy in rats: the possible involvement of autophagy

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Abstract

Statins, an anti-hyperlipidemic drug, is associated with skeletal muscle myopathy, which may be severe enough to discontinue statin therapy. This study assessed the effect of mesenchymal stem cells (MSCs) versus intermittent fasting on simvastatin induced myopathy in rats and studied the possible autophagy role. This study included forty adult female and four male Sprague Dawely rats. Male rats were used for MSCs isolation. The female rats were divided as follow: Group I served as the control group. Group II (the myopathy group) rats were given a daily oral dose of simvastatin for 30 days. Group III (discontinuation group) rats were given a daily oral dose of simvastatin for 30 days and and were left without treatment for 15 days. Group IV (the intravenous stem cell group) rats were given a daily oral dose of simvastatin for 30 days and then a single stem cell injection was administered intravenously. Group V (the intermittent fasting group) rats were given a daily oral dose of simvastatin for 30 days and then an intermittent fasting protocol was started. Gastrocnemius muscle contractility, histopathological examination plus serum creatine kinase (CK) level, autophagy markers (LC3 and P62) expression and MSCs homing by PCR were carried out.Simvastatin significantly altered contractile properties, induced atrophy of skeletal muscle, increased CK, LC3 and P62 levels. Meanwhile, MSCs injection and intermittent fasting significantly restored all these alterations which were confirmed by histopathological improvement. They reduced skeletal muscle atrophy and CK level, and improve the skeletal muscle contractility and autophagy flux. In conclusion:Statin myopathy is revealed to be due to autophagy flux inhibition. MSCs injection and intermittent fasting induced pronounced skeletal muscle regeneration with improvement of functions. The improvement recorded in intermittent fasting group was significantly better than that observed withMSC injected group.

Key words: statins, myopathy, mesenchymal stem cells, intermittent fasting, autophagy.

1. Introduction

Statins are the cornerstone drugs in reducing cardiovascular diseases incidence and improving rate of survival (1, 2). They act by 3 hydroxyl, 3-methyl glutaryl CoA (HMG co-A) reductase enzyme inhibition, which is in charge of cholesterol synthesis (2). Regardless of its clinical benefits, the most well documented adverse effect of its use is statin-associated muscle symptoms (SAMSs)(3). The main manifestations of skeletal muscle myopathy are decreased muscle mass and muscle force as well as contractile properties alteration. Reduced compliance of the patients due to statin-associated myopathy, exposes them to the high morbidity and mortality risks(4). In some statin-induced myopathy cases, despite drug removal, high level of serum creatine kinase (CK), plus persistent weakness of skeletal muscules were reported (5).

As regard the exact underlying mechanism(s) of statin-induced myopathy, a variety of hypotheses have been proposed. These include the depletion of cholesterol in the cell membranes of skeletal muscle, deficiency of ubiquinone, oxidative stress, mitochondrial dysfunction, altered ca homeostasis and induction of apoptosis (6). In addition to the inhibition of protein geranylgeranylation of RAP1A, RAP1A regulate the structural organization of late endosomes and lysosomes and therefore influence intracellular degradative pathways. Also, statin treatment reduced PKD activation, impaired activation of PKD–Vps34, preventing normal maturation of autolysosomes and reduces the capacity for autophagy flux. Additionally, statin treatment led to reduced mTOR activity, which is a trigger for autophagy induction. The combination of enhanced autophagy initiation and impaired capacity for flux induced statin-related myopathy (7).

Mesenchymal stem cells (MSCs) are multipotent stromal cells that can differentiate into a variety of lineages(8). From bone marrow and other

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mesenchymal tissues, including dental pulp, adipose tissue, the umbilical cord and the placenta, they can be isolated and subsequently rapidly expanded *ex vivo*(9).There is growing evidence that suggests that MSCs augment of autophagy may be an effective approach to help muscle cells repairing against various extra-/intracellular noxious stimuli (10). The differentiation capacity, in addition to the documented immunomodulatory and trophic effects of MSCs that protect parenchymal cells from apoptotic death, and promote endogenous precursors differentiation and proliferation, hold great promise for cell therapies and tissue engineering (11).

Intermittent fasting **(IF)** is a broad term describing patterns of eating alternating between eating and extended fasting, occurring on a recurring basis (12). It includes Ramadan fasting (RF) which is a time restricted eating pattern, intermittent restriction of energy (the 5:2 diet), and alternate-day fasting (consuming no calories for one day and eating the next day without restriction)(13).Popularity of IF was attributed to its potential valuable effects on health(13). One way that intermittent fasting has proven beneficial is through the increased amount of autophagy occurring in the body. IF has been found to promote organs regeneration by increasing production of stem cell, and increase lifespan (14).

Autophagy is necessary to the human body, as it breaks down and recycles proteins and organelles. The purpose of this degradation is either to rid the cells of misfolded proteins and damaged organelles, or to use the components at another more vital location when there is low nutrient availability (15). Regulated proteins and organelles removal by autophagy-lysosome system is in fact critical for homeostasis of the muscle. Excessive activation of autophagy-dependent degradation contributes to atrophy of the muscle and cachexia. Conversely, autophagy inhibition causes protein aggregates and abnormal organelles accumulation, leading to myofibers degeneration and myopathy(16, 17)**.**

The purpose of the current study is to assess the influence of stem cell therapy and fasting on skeletal muscle in an animal model of simvastatin-induced myopathy, identify the possible role of autophagy and compare the potential effect of bone marrow-derived mesenchymal stem cells and fasting in modulation of statin induced myopathy

Materials and methods:

Experimental animals

The study was performed using forty adult female Sprague Dawely rats, aged 6-8 weeks, weighing 150-200 grams. Females were used as they are reported to have a higher risk for statin induced myopathy than males. Animals were housed and maintained at the Medical Experimental Research Center (MERC) animal house, Faculty Of Medicine, Mansoura University at controlled environmental conditions (12 hours light/dark cycles and temperature of (24 °C) fed a standard laboratory chow and had a free access to tap water. All experimental protocols were approved by our local committee of animal care and ethics. The research protocol was approved by the Mansoura Institutional Research Board (Code number MDP.19.03.20).

Drug used

Simvastatin is a drug available commercially and manufactured by Egypharma for pharmaceutical industries, Egypt.

Experimental design

The animals were randomly divided into five groups each contain eight rats as follows: Group I (C): served as a control group receiving distilled water by gastric gavage. Group II (S): was administered simvastatin at a dose of 50 mg/kg body weight /day for 30 days by gastric gavage tube. By the end of 30 days rats were sacrificed**.** Group III (D):Simvastatin was administered for 30 days by gastric gavage tube at a dose of 50 mg/kg body weight /day, and then after 15 days of simvastatin discontinuation rats were sacrificed.Group IV (M): Simvastatin was administered for 30 days at a dose of 50 mg/kg body weight/day by gastric gavage tube, with mesenchymal stem cells intravenous injection via rat tail vein once on day 31, each rat received $1x10^6$ cells suspended in 0.5 mL of phosphate-buffered saline then rats were sacrificed after 15 days(18, 19). Group V (F): Simvastatin was administered for 30 days by gastric gavage tube at a dose of 50 mg/kg body weight /day, then rats were submitted to a program of intermittent fasting for another 15 days. The intermittent fasting program includes alternate periods of 24hrs of fasting and feeding and then rats were sacrificed after 15 days.

Blood samples collection

At the day of sacrifaction, overnight fasting animals were anesthetized with a piece of cotton soaked with 10 ml halothane and then rats were left for 30 seconds. On the dissecting table, the animals were fixed and the abdominal cavity was opened longitudinally. After that, the samples of blood were collected directly from the heart by cardiac puncture and centrifuged at 2000 rate per minute (rpm) for 10 minutes to obtain serum that stored at −20 °C for subsequent biochemical assay.

Preparation of (MSCs) from male rats:

By flushing the tibiae and femurs of male Sprague Dawely rats, bone marrow was harvested. Nucleated cells were isolated and cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine medium and 1% penicillin-streptomycin. Cultures were incubated at 37°C in 5% humidified CO2. After 24 or 48 h, non-adherent cells were discarded and the adherent cells were washed thoroughly with phosphate buffered saline (PBS). The fresh complete medium was added and replaced every 2 or 3 days. Wash of the primary cultures when it became almost confluent (80-90%) was done for 2 times with PBS, and digested for 2 minutes at 37°C With 0.25% trypsin and 0.01% EDTA. The confluent cells were dissociated, and sub-cultured. These procedures were repeated several times(18, 20). In culture, MSCs were characterized by their adhesiveness, fusiform shape, and by flow cytometry analysis of rat MSCs surface markers.

Biochemical investigation:

Using the kit obtained from Sigma Aldrich Company, Cairo, Egypt, plasma level of total CK was determined through standard enzymatic spectrophotometric analysis following the manufacturer's instructions (4).

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Harvesting muscle specimens

In order to detach the tendon of the right gastrocnemius muscle from the underlying bone, careful dissection was done. And then with a thread, the tendon was tied maintaining the muscle's nerve supply and the knee joint attachment. This specimen was immersed in Krebs solution at $30^{\circ C}$ and used for skeletal muscle contractility recording. For further histopathological and immunostaining tests, the left gastrocnemius muscle was taken and left in formalin (10%). In MSCS injected group fresh specimens were preserved and stored in liquid nitrogen for PCR.

Detection of muscle homing of the male-derived mesenchymal stem cells by Polymerase Chain Reaction: By real time PCR, the presence or absence of sex determination region on the Y chromosome male (sry) gene in muscle of recipient female rats was assessed(18, 20). From homogenate of the muscle tissue of rats injected with stem cells, genomic DNA was extracted. The sequences of the primer pairs are: sex determining region Y (Sry): Reverse 5'-TGCCTTCCTCATCAGATGGG- 3'/ Forward 5'-TTGGCTCAACAGAATCCCAG- 3'; glyceraldehyde-3-phosphate dehydrogenase (Gapdh): Forward 5'-TTGTGCAGTGCCAGCCTCGT-3'/ Reverse 5' TGCCGTTGAACTTGCCGTGG - 3'.

Muscle contractility recordings:

By using a Biopac Lab system (BSL 3.7.5 software), (MP36) data analysis unit, isometric force transducer assembly (SS12LA), BIOPAC BSLSTM stimulator and needle electrodes (ELSTM2), gastrocnemius muscle contractility was recorded. The stimuli intensity was adjusted up to 80V and directly delivered to the gastrocnemius preparation of rats (21). The following parameters of contractilty were recorded; Twitch Kinetics (maximal twitch force, time to peak (time taken to reach maximum force) and half-relaxation time (time taken for force to fall to half of the maximum), Tetanus Kinetics (maximum tetanic force). Fatigue was measured by repetitive tetanic stimuli until the force decreased to about 50% of the original force.

Histopathological study:

Collected muscle tissues were washed with saline and immediately stored in 10% buffered neutral formalin solution. Then, by standard procedure processed for paraffin embedding and serial sections were cut (5 µ). The sections were stained with hematoxylin and eosin. Under light microscope X: 400, bar = 50 µm, histopathological evaluation was performed. Cross sectional areas were measured by scaling histopathological specimens.

Immunohistochemical examination for LC3 and P62:

The tissue sections were deparaffinized, rehydrated, washed, immersed in 3% H2O2, and then with pepsin were digested for retrieval of antigen. After the unspecific binding blocking by serum, the sections were incubated with polyclonal LC3B (cat# A5601, 1:150) and P62 (cat# A7758, 1:75) antibodies, purchased from Abclonal, USA, at 4°C overnight. To produce a brown-colored signal, Diaminobenzidine / peroxidase substrate was used. The section was counterstained, dehydrated, cleared, and cover slipped. To replace primary antibody, phosphate buffered solution (PBS) was used and adjacent sections were used as negative control. For the semi-quantitative morphometric analysis, the numbers of LC3 and p62 positive cells was quantified as the percent of muscle area occupied by positive staining (calculated by averaging five fields per slide at a 100X) for each muscle area by using image J software.

Statistics analysis

Via using SPSS Package version 23, statistical analysis was performed. As the mean ± standard deviation, data were represented. For detection of variation between different groups, the ANOVA test was used. *p*- value ≤ 0.05 was considered to be statistically significant.

Table (1): Contractility parameters in different experimental groups

	C	S	D	M	F
Isometric twitch tension (SMTMax)(gm)	80.09±3.74	41.44±3.72*	60.68±4.92$^{*\#}$	72.74±4.38$^{*\#\$}$	77.43±3.35$^{\#\$}$
Percent of change				75.5309% increase	86.8485% increase
Time to peak(sec)	0.035±0.003	0.091±0.004*	0.0655±0.004$^{*\#}$	0.0376±0.004$^{\#\$}$	0.0361±0.003$^{\#\$}$
Percent of change				58.6813% decrease	60.3297% decrease
Half relaxation time(HRT)(sec)	0.0525±0.004	0.1029±0.004*	0.0877±0.003$^{*\#}$	0.0718±0.002$^{*\#\$}$	0.0575±0.004$^{\#\$@}$
Percent of change				30.2235% decrease	44.1205% decrease
Maximum tetanic tension(gm)	115.74±5.75	68.19±5.41*	89.51±3.14$^{*\#}$	98.49±4.34$^{*\#\$}$	111.71±6.59$^{\#\$@}$
Percent of change				44.4347% increase	63.8217% increase
Time to fatigue(sec)	134.52±4.33	89.40±4.85*	99.09±3.98$^{*\#}$	114.38±5.89$^{*\#\$}$	125.60±5.68$^{*\#\$@}$
Percent of change				27.9418% increase	40.4922% increase

C: control group, S: simvastatin treated group, D: discontinuation group, M: mesenchymal stem cells injected group F: intermittent fasting treated group. Parameters described as mean ±SD, P: Probability, *: significance <0.05, Test used: One way ANOVA followed by Tukeyposthoc test. *: significance with control group, #: significance with S group, $: Significance with D group, @: Significance with M group

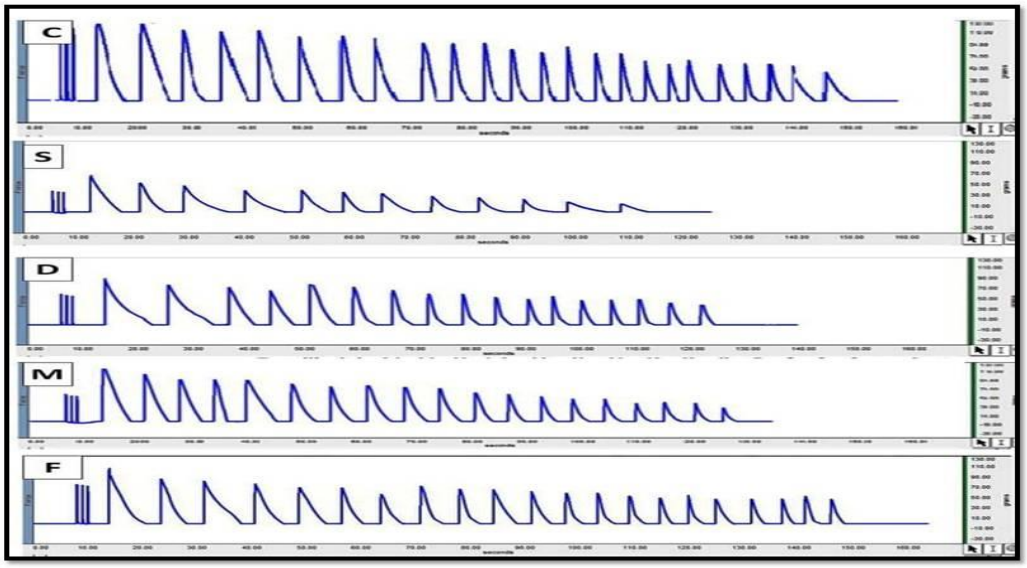

Fig (1): Record of Contractile properties of rat gastrocnemius muscle in different experimental groups. (C) Control group. (S) simvastatin treated group. (D) discontinuation group. (M) mesenchymal stem cells injected group. (F) intermittent fasting treated group.

Results

Contractility recording

Regarding the isometric contractile properties, simvastatin show a significant decrease in forces of contraction (maximum twitch force, maximum Tetanic force) and decreased fatigue resistance together with a significant increase in twitch times (time to peak twitch, half relaxation time twitch) when compared to the control group (Table 1, Figure 1). These parameters were significantly enhanced in discontinuation group, mesenchymal stem cells injected group, and intermittent fasting treated group when compared to the simvastatin group. Intermittent fasting treated group revealed the best significant improvement.

Biochemical analysis

As regard the biochemical analysis, the present study revealed that administration of simvastatin significantly results in serum CK level increase, exceeding the control group by several folds. This elevated level of creatine kinase was significantly decreased in discontinuation group compared to simvastatin treated group, but still significantly higher than normal control group. It was apparent that creatine kinase level was significantly decreased in mesenchymal stem cells injected group in comparison with both simvastatin and discontinuation group. Also, intermittent fasting treated group showed significant decrease in creatine kinase level when compared to simvastatin and discontinuation groups. (Figure2).

Cross sectional area of gastrocnemius muscle:

It was significantly decreased in simvastatin treated group when compared to normal control group. A significant increase in its value was shown in discontinuation group compared to simvastatin treated group, but still significantly lowers than the value of normal control group. Also a significant increase was observed in mesenchymal stem cells injected group and intermittent fasting treated group when compared with simvastatin treated and discontinuation groups (figure 3).

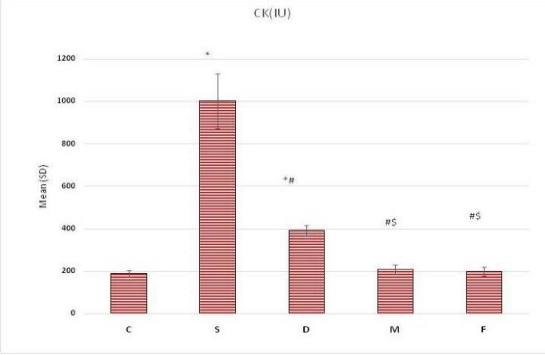

Fig (2): Serum levels of creatine kinase in different studied groups. C: control group, S: simvastatin treated group, D: discontinuation group, M: mesenchymal stem cells injected group F: intermittent fasting treated group. All parameters described as mean ± SD. *: significance with control group, #: significance with S group, Significance with D group, @:
Significance with M group. One-way ANOVA with post hoc Tukey test.

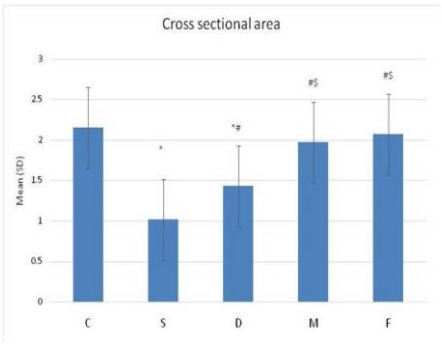

Fig (3): Mean cross sectional area among studied groups. C: control group, S: simvastatin treated group, D: discontinuation group, M: mesenchymal stem cells injected group F: intermittent fasting treated group. All parameters described as mean ± SD. *: significance with control group, #: significance with S group, $: Significance with D group, @:Significance with M group. One-way ANOVA with post hoc Tukey test

Histopathological examination

Figure (4) shows normal architecture of muscle fibers with regular parallel arrangement and peripherally placed nuclei in the gastrocnemius specimen obtained from the control group (C). Simvastatin treated muscles (S) showed loss of striations, splitting of muscle fibers, hyper-cellularity with numerous enlarged vesicular nuclei, inflammatory cells infiltration, congestion, fibrosis and interstitial edema. Discontinuation group (D) showed loss of striations, focal area of fibrosis and few inflammatory cells infiltration. While, muscle specimens obtained from the stem cells injected group (M) still show loss of striations, minimal degenerative changes and very few inflammatory cells infiltration. Meanwhile, skeletal muscles sections from intermittent fasting group showing retained normal histological appearance.

Immunohistochemical examination for autophagy (LC3 markers (LC3 and P62)

In the simvastatin treated rats LC3II expression in gastrocnemius muscle was significantly increased compared to normal control group. In discontinuation group this value was significantly increased when compared with simvastatin treated rats and normal

control group. Further increase was detected in mesenchymal stem cells injected group when compared with the simvastatin treated rats, discontinuation and normal control group. Intermittent fasting treated group showed significant increase when compared with normal control, discontinuation, simvastatin treated and mesenchymal stem cells injected group (figure 5, 6).

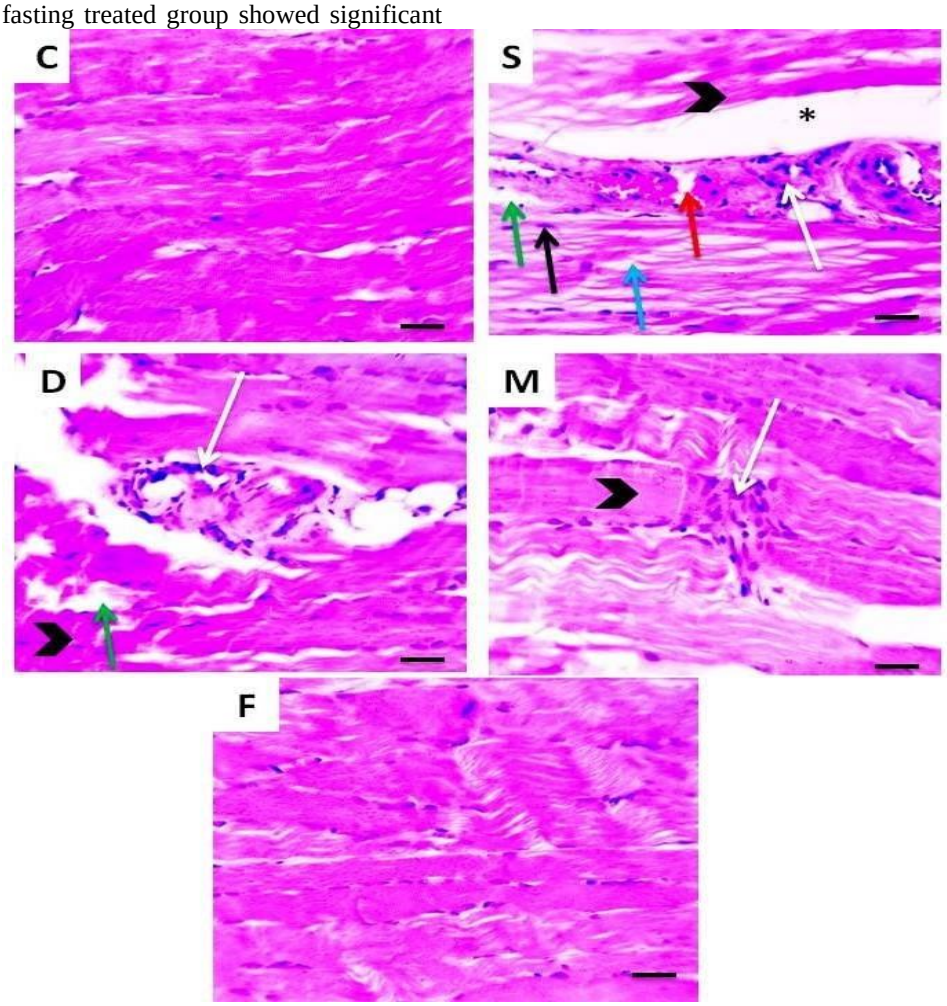

Fig (4): Microscopic pictures of H&E stained sections from gastrocnemius muscles of control group (**C**) showing normal elongated muscle fibers with numerous flattened peripheral nuclei. Muscles sections from simvastatin treated group (**S**) group showing loss of striations (black arrowheads), splitting of muscle fibers (blue arrows), hyper-cellularity with numerous enlarged vesicular nuclei (black arrows), inflammatory cells infiltration (white arrows), congestion (red arrow), fibrosis (green arrows) and interstitial edema (asterisks). Microscopic pictures of sections muscles from discontinuation group (**D**) showing loss of striations (black arrowheads), focal area of fibrosis (green arrows) and few inflammatory cells infiltration (white arrows). Muscles sections from mesenchymal stem cells injected group (**M**) showing loss of striations (black arrowheads) and very few inflammatory cells infiltration (white arrow). Meanwhile, skeletal muscles sections from intermittent fasting treated group (**F**) showing retained normal histological appearance. X: 400, bar = 50 µm.

It was noticed that P62 expression was significantly increased in simvastatin treated rats in comparison with normal control group. With discontinuation group the value was decreased significantly when compared to simvastatin treated rats, but still significantly higher than normal control group. In mesenchymal stem cells injected group its expression was significantly decreased as compared to simvastatin treated rats and discontinuation group. The most obvious decrease in p62 value was detected in intermittent fasting treated group when compared with simvastatin treated rats, discontinuation group and mesenchymal stem cells injected group, but insignificant change with normal control group (figure 7,8).

Morphological features of cultured rat BM-MSCs:

On Day 1, most of the cells were still mononuclear cells and fat droplets were frequently seen. On Day 2, some spindle shaped cells appeared among the mononuclear cells and fat droplets. On Day 3, the number of spindle-shaped cells continued

increasing. On Day 4, the spindle-shaped cells reached about 60-80% confluence. On Day 5, the spindle-shaped cells already formed cell layers (Fig.9)

Confirmation of phenotype of rat BM-MSCs:

Flow cytometry analysis results showed that the established cells were strongly positive for MSC markers CD90, CD105, and negative for the haematopoietic cell marker CD45 and cell marker CD34 (Fig.10).

Detection of male derived (MSCs) homing in female rats muscles:

In myopathic rats that received simvastatin for 30 days (50 mg/kg body weight/day) with injection of MSC on day 31, a marker of Y chromosome, Sry gene was expressed. The sry gene expression by the female rat's injured muscle tissue after intravenous injection of male derived MSCs, proved homing of injected cells in the affected muscles (Figure 11).

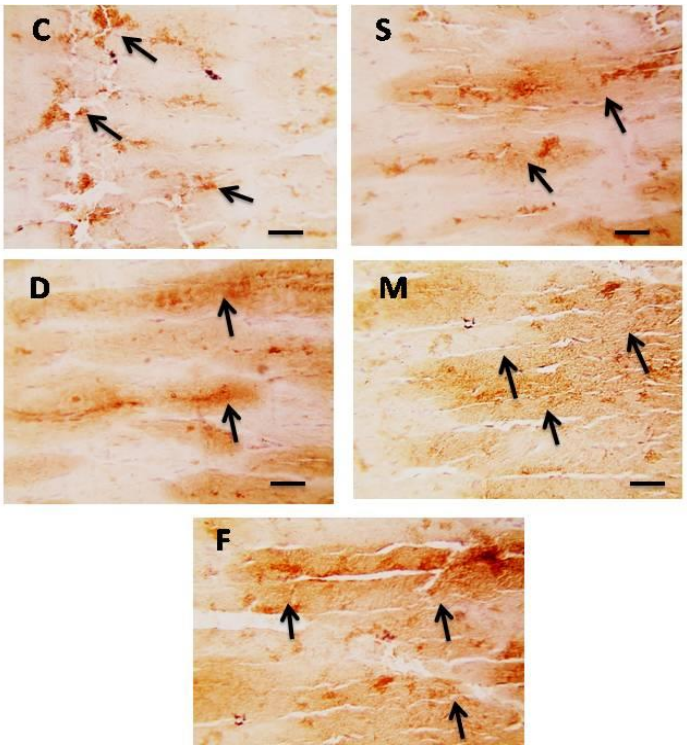

Fig (5): Microscopic pictures of immunostained sections from gastrocnemius muscles against LC3 showing higher expressions in muscle fibers from simvastatin treated (S) group (black arrows) when compared to control (**C**) group. The positive immune reaction slightly increased in discontinuation (**D**) group, moderately increased in mesenchymal stem cells injected group (**M**) and markedly increased in intermittent fasting (**F**) group. IHC counterstained with Mayer's hematoxylin.X:400, bar = 50 µm.

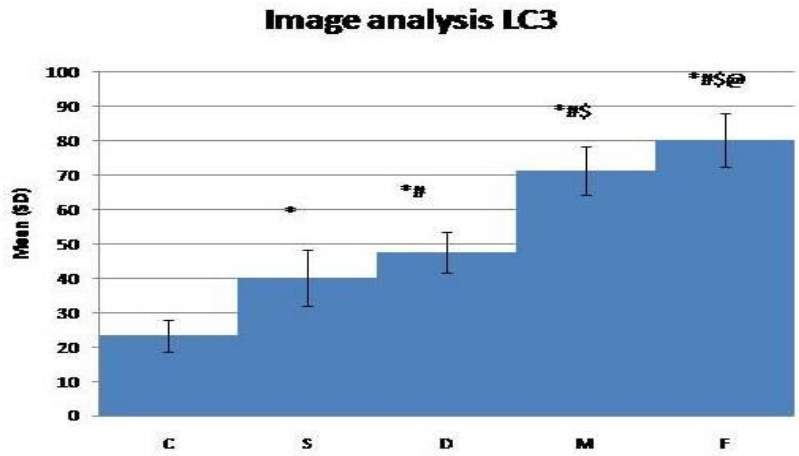

Fig (6): Mean image analysis LC3 between studied groups*: significance with control group, #: significance with S group, $: Significance with D group, @:Significance with M group

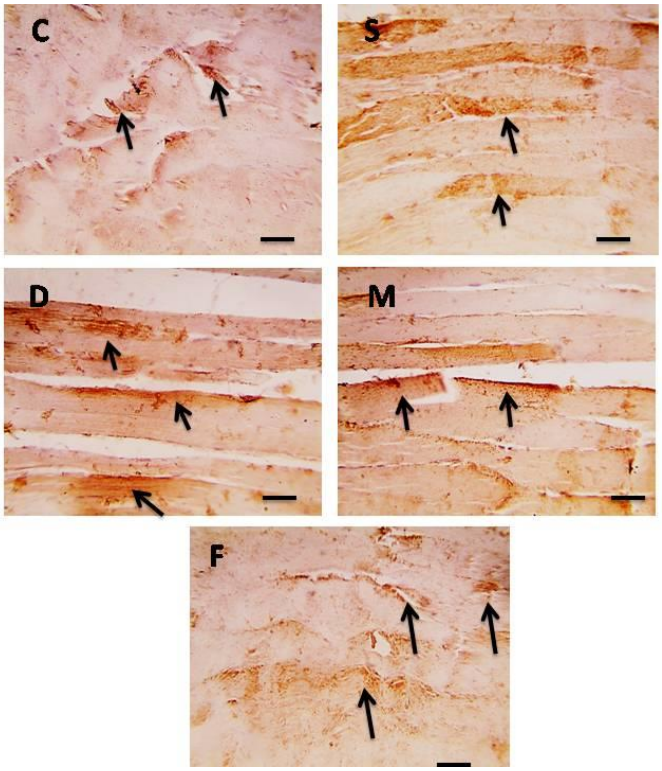

Fig (7): Microscopic pictures of immunostained sections from skeletal muscles against P62 showing higher expressions in muscle fibers from simvastatin treated (**S**) group (black arrows) when compared control (**C**) group. The positive immune reaction slightly decreased in discontinuation (**D**) group, moderately decreased in mesenchymal stem cells injected group (**M**) and markedly decreased in intermittent fasting treated (**F**) group. IHC counterstained with Mayer's hematoxylin.X:400, bar = 50 µm.

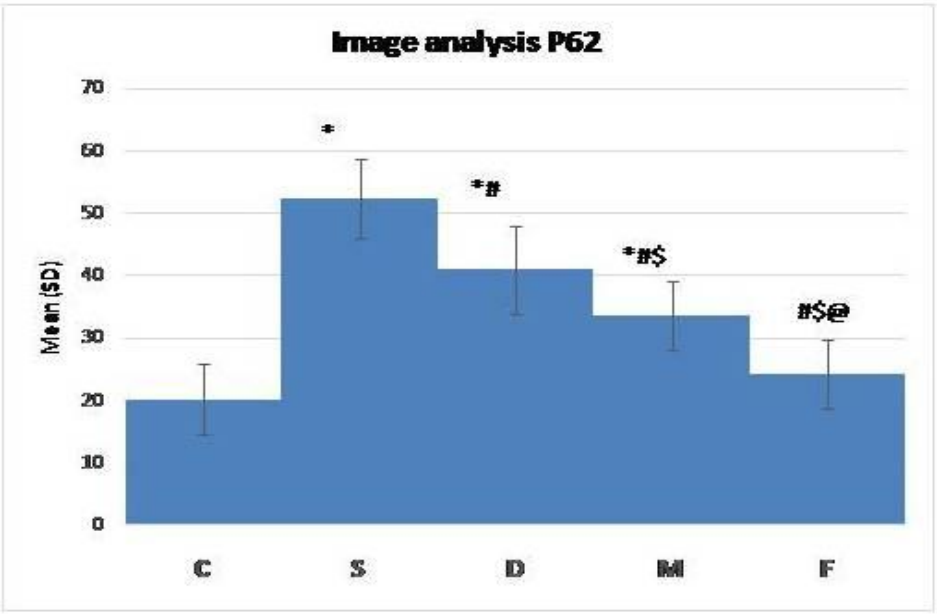

Fig (8): Mean image analysis P62 between studied groups. *: significance with control group, #: significance with S group, $: Significance with D group, @:Significance with M group.

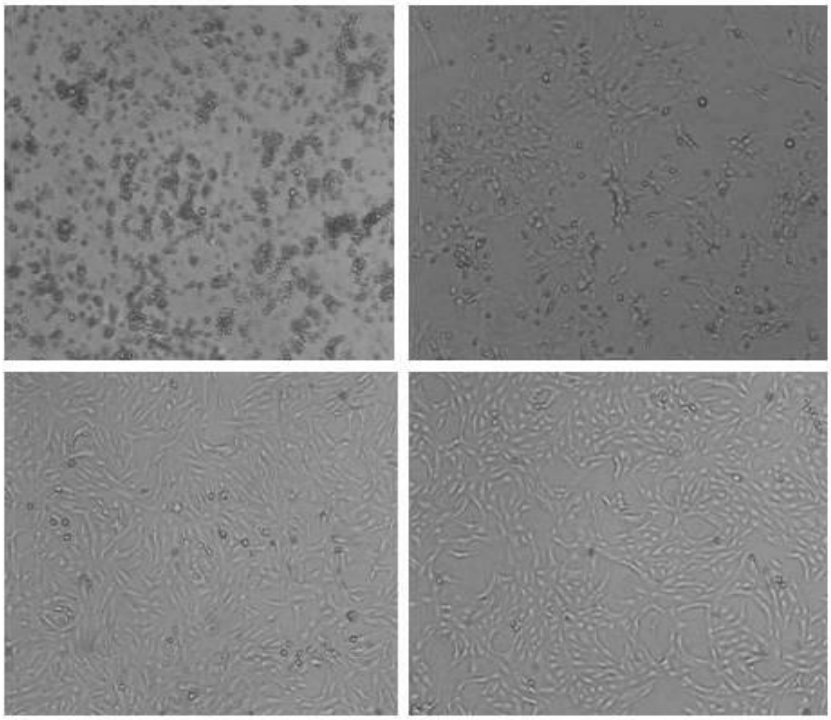

Fig (9): Morphological features of the cultured rat bone marrow mesenchymal stem cells

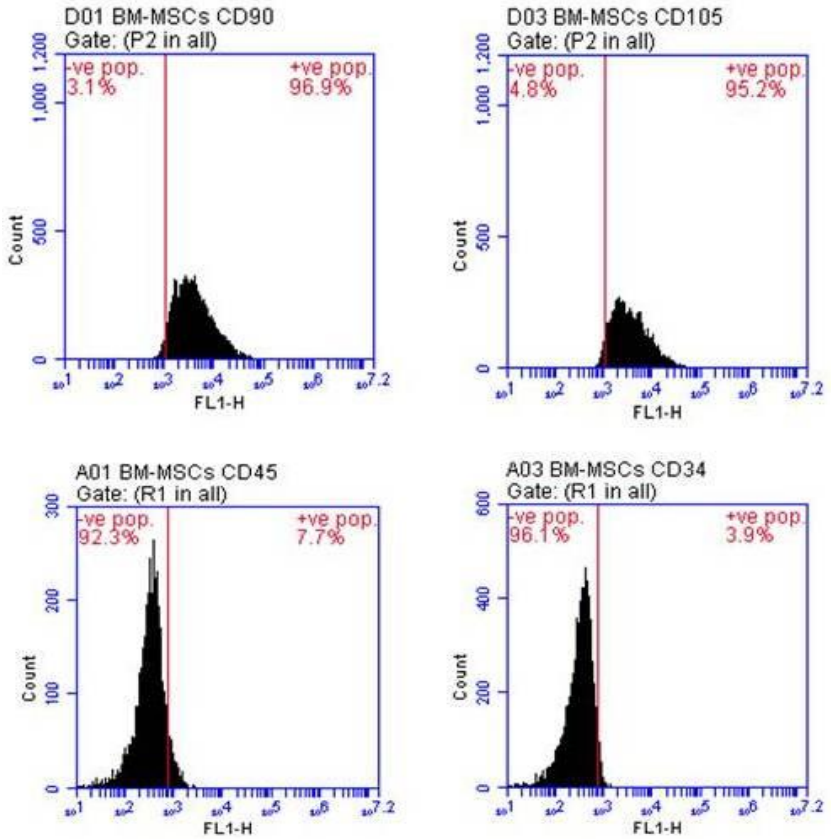

Fig (10): Flow cytometry analysis of rat bone marrow mesenchymal stem cell (MSC) surface markers, showed that the cells were positive for MSC markers CD90 and CD105. Cells were negative for CD45 and CD34

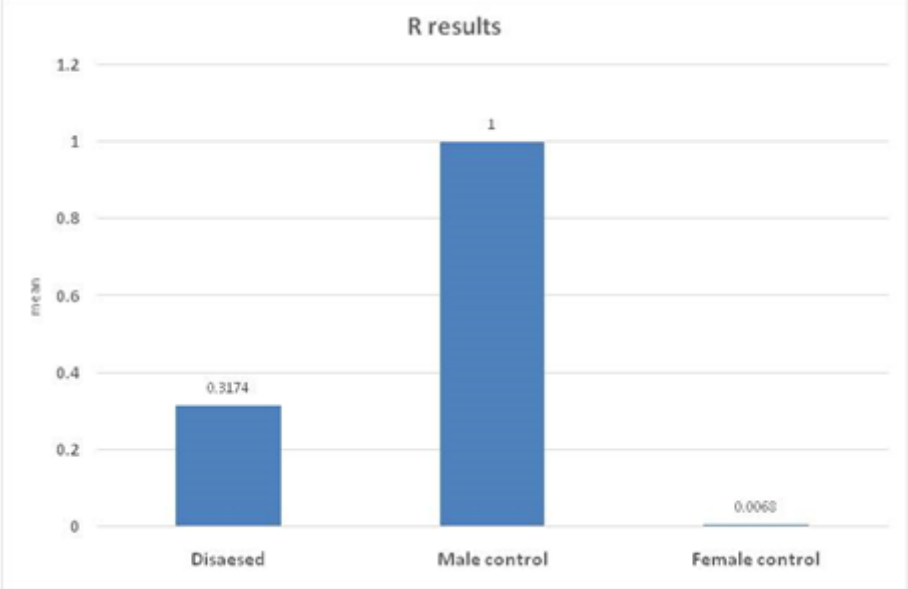

Fig (11): Real time PCR expression of Sry gene for detection of MSCs homing.

Discussion

Statins are statins are a widely prescribed anti-hyperlipidemic drugs. Despite the widespread use of statins to reduce cardiovascular morbidity and mortality, their therapeutic application can be rigorously restricted due to statin-associated muscle symptoms (SAMSs)(22)**.** These can present as myalgia, nocturnal muscle cramping, myopathy and myositis, up to its most severe form, rhabdomyolysis(23)**.**Simvastatin was chosen because it has been hypothesized that lipophilic statins (e.g., simvastatin or atorvastatin) are more likely to cause muscular side effects than hydrophilic statins due to increased passive diffusion into muscle cells (24)**.** In this study, the gastrocnemius muscle was selected because most of its fibers are formed of type II white muscle fibers. Type II muscle fibers were reported by previous studies to be selectively vulnerable to statin induced myotoxicity(25)**.**

In this study, the injurious effect of simvastatin administration on skeletal muscles was detected by significant increase in serum level of creatine kinase(CK) by several folds, and dramatic decrease in muscle cross sectional area, as compared to the control group. This result is contradictory to **Piette et al.(26)**who reported that short-term statin treatment for 28 days did not induce muscle mass loss, muscle fiber atrophy, or creatine kinase (CK) release. Yet it is coherent with several other reports (27-29).These findings could be explained by the activation of muscle atrophy genes(30)**.** It has been postulated thatstatins stimulate the induction of muscle–specific lysosomal proteolysis and ubiquitin proteasome system by the FOXO downstream target genes upregulation, Muscle Ring Finger 1 (MuRF1), cathepsin-L mRNA and F-box (MAFbx), as well as apoptotic caspases activation resulting in accelerated myofibrillar degradation, protein synthesis reduction, and atrophy of myotubes**(**31, 32).

Our results revealed significant prolongation in time of contraction and relaxation together with decrease in forces of contraction and time to fatigue in gastrocnemius of rats supplemented with simvastatin as compared with control ones suggesting muscular dysfunction which is in line with previous studies (4, 33, 34)**.**These contractile effects could be understood by statin induced skeletal muscle functional and structural alterations .These include cholesterol depletion in the skeletal muscle cell membranes which destabilizes the muscle membrane, mitochondrial dysfunction, deficiency of ubiquinone (coenzyme Q10), cellular oxidative stress, disturbed Ca homeostasis and induction of apoptosis(31, 35). It affected also the function and kinetics of ion channels and carriers and results in hyperpolarizing potentials of the contraction mechanical threshold, an excitation-contraction coupling index(4)**.**Statin mediated inhibition of pyruvate dehydrogenase complex and oxidation of CHO may contribute to these side-effects (36)**.**

All of the previous findings were confirmed by the histopathological examinations. In the present study there was a structural change in gastrocnemius muscle that confirm myopathic changes of simvastatin, which is consistent with previous studies as (18, 19, 25)**.**

Moreover, this study try to explain the role of autophagy in statin induced myopathy, as regulated

proteins and organelles removal by autophagy-lysosome system is in fact vital for muscle homeostasis. Autophagy deficient muscles also show abnormal mitochondria accumulation, disorganization of sarcomeres, sarcoplasmic reticulum distension, and activation of apoptosis, contributing to a dramatic myofiber size and force generationdecrease (37, 38). It has been shown that constitutive autophagy is required for satellite cells to maintain their stem cell fitness (39**).**

The process of autophagy starts with formation of isolation membrane, phagophore, which engulf certain cargo (protein aggregates or damaged organelles) forming autophagosome (AP). The process is completed by fusion of AP with lysosomes to form autolysosomes where cargo is degraded by lysosomal enzymes(40). LC3II is considered the only reliable protein marker associated with autophagosomes formation. Meanwhile, p62 is responsible for attraction of ubiquitinated cargo proteins to the autophagosomes facilitating their degradation by fusion with the lysosome and completion of autophagic process(41). Therefore, accumulation of p62 denotes inability to complete autophagic process (42).
In order to assess autophagy, LC3 and P62 expression in gastrocnemius muscle were detected in our study through immunohistochemical examination and it has been shown that LC3 expression was significantly increased in simvastatin treated rats when compared to normal control group. This finding came in accordance with Ghavami et al. (43) who stated that statins can induce autophagy in different types of cells. It can be attributed to mTOR (mammalian target of rapamycin) inhibition and that its inhibition stimulates autophagy (44**).** Also, simvastatin inhibits autophagy flux as, P62 expression was significantly increased, and this is in line with previous study(45).

Our study results revealed that discontinuation of simvastatin results in partial recovery as there were still significant myopathic changes as compared to the control, which is in agreement with several studies (18, 46**).** This could be due to the adult skeletal muscle normal regenerative capacity. This regenerative response is due to the effect of resident stem cells, including satellite cells chiefly, and other progenitor cells (47**).**

At the level of skeletal muscle, the satellite cells are low in intact skeletal muscle (48). So, it is suggested that derived stem cells from other sources, such as bone marrow stem cells, would participate in injured skeletal muscle regenerative process. Consequently, by their myogenic potential, bone marrow mesenchymal stem cells constitute an attractive candidate for regeneration of muscle tissue in cases of diseases associated with muscle dysfunction(49).

In the current work Mesenchymal stem cells administered intravenously to simvastatin treated rats improved the isometric twitch tension by 75.5309% increase, and tetanic tension by 44.4347% increase. It shortened time taken to peak twitch by 58.6813% decrease and half relaxation time by 30.2235% decrease, while time taken to fatigue was prolonged by 27.9418% increase. Also, resulted in Improvement of histopathological findings, decreased CK level by 79.201%, increased cross sectional area by 93.8976%, increased LC3 by 77.8719% and decreased P62 expression by 35.9429%, all as compared to the simvastatin group. In accordance, **Farouk et al (18)**and **Salem et al (19)**reported that mesenchymal stem cells administration has significant effects on improving simvastatin induced myopathy**.**

These findings may be due to improved autophagy flux, and secretion of a wide range of paracrine bioactive growth factors that finally improve muscle strength, such as IGF, bFGF, VEGF, HGF, EGF and Wnt1. Therefore, MSCs could provide trophic support for injured tissue by induction of differentiation and proliferation of local precursor to improve irrigation of damaged tissue and prevent cell apoptosis (50). MSC treatment also produces an anti-inflammatory response, prevented oxidative stress, and Ubiquitin proteasome pathwayoveractivation(11).MSCs mediated repair can be also through release of microvesicles, exosomes microRNAs, and mitochondrial transfer. Mitochondrial dysfunction is associated with a majority of degenerative diseases. Thus, mitochondrial transfer has appeared as a great therapeutic strategy as it can restore the bioenergetic needs of damaged cells (51**).**

In contrast to the results of the present study, one study stated that MSCs intravenous injection had a non-beneficial effect in myopathy treatment. This study attributed the failure to the possibility that the degenerated muscle background fails to stimulate stem cells to trigger their expansion and myogenic differentiation and to the poor survival of stem cells after transplantation and their limited migratory ability(52).

Intermittent fasting protocol applied to simvastatin treated rats in form of alternate-day fasting revealed marked improvement in simvastatin myopathic changes. It resulted in Improved

histopathological findings showing almost normal muscle fibers, decreased CK level by 80.1119%, increased cross sectional area by 103.74%, improved contractile forces as the isometric twitch tension and tetanic tension increased by 86.8485% and 63.8217% respectively. It also shortened time taken to peak twitch by 60.3297% decrease and half relaxation time by 44.1205% decrease, while time taken to fatigue was prolonged by 40.4922% increase, increased LC3 by 99.9751% and decreased P62 expression by 53.5619%, all when compared to the simvastatin group.

Studies have demonstrated that the effect of IF is related to the adaptive energy metabolic response of organs, tissues, and cells triggered by IF (mainly the metabolic conversion from glucose to ketone bodies as an energy source), which manifested as increased ketone body production, autophagy induction, DNA repair, and anti-stress abilities, and antioxidant defense. During periods of recovery (including eating and sleeping), the conversion from ketones to glucose as the main energy source of cells results in an enhanced ability to produce glucose and synthesize intracellular proteins and increased mTOR expression and mitochondrial biogenesis (53). Also, metabolic switch flipping in muscle cells enhanced AMPK activation (54). Consequently, programs of gene expression that promote cellular stress resistance and mitochondrial biogenesis are activated. PGC-1α, a master regulator that mediates the growth and division of mitochondria, expression is up-regulated by AMPK. Fasting induces SIRT3 expression in skeletal muscle cells that results in protection of muscle cells against oxidative stress by deacetylating and activating SOD2 (12).

Data also revealed that in muscle cells fasting stimulates autophagy through mechanisms including AMPK-mediated mTOR signaling inhibition and autophagy-promoting proteins up-regulation (55). AMPK also contributes to anti-inflammatory effect of fasting and increased muscle cells sensitivity to insulin. Moreover, muscle-specific AMPK knockout mice show hyperglycemia and impaired gluconeogenesis, which may be a result of autophagy impairment in muscle cells (56). AMPK is involved also in muscle stem cells (MuSCs) themselves regulation. *Via* the Notch signaling activation, an essential pathway for the maintenance of MuSC, the AMPK-regulated transcription factor FOXO3 enhances MuSC self-renewal (57).

In conclusion, we found that simvastatin inhibits autophagy flux, which was reported to increases the simvastatin myotoxicity. Simvastatin-induced myopathy is proved by the altered parameters of contractility as regard to the force, time to peak, half relaxation time and time to fatigue, elevation of plasma CK levels, muscle atrophy and decrease muscle mass and histopathological degenerative changes. Mesenchymal stem cells and intermittent fasting improve autophagy flux and play a role in ameliorating these myopathic changes. The improvement recorded in intermittent fasting group is significantly better than that observed withMSC injected groups. Marked improvement in the skeletal muscles regenerative capacity after intermittent fasting provides a new hope for patients suffering from myopathies as a natural health protocol that can avoid potential drawbacks of stem cell therapy as immunogenicity, short survival after administration, differentiation into undesirable tissue plus high maintenance cost.

Abbreviations:
bFGF: basic fibroblast growth factor
EGF: epidermal growth factor
HGF: hepatocyte Growth Factor

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