Promising Therapeutic Efficacy of *Trigonella-foenum graecum* and Bone Marrow-Derived Mesenchymal Stem Cells on Skeletal Muscle Atrophy in Experimental Rat Model

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Abstract: Skeletal muscle plasticity is maintained by various ways to regulate cell and protein turnover. When muscle atrophy occurs, proteolytic cascades are activated, resulting in the removal of organelles and contractile proteins, leading to myofibrillar shrinkage and great loss of the total muscle mass. This is linked to improper diagnosis in several diseases such as myopathies and muscular dystrophies, systemic disorders, and many catabolic diseases. The present study was carried out to evaluate the powerful mitigatory ability of Bone marrow mesenchymal stem cells (BM-MSCs) and/or *Trigonella foenum-graecum* Linn. (Tfg) against simvastatin induced muscular atrophy model in experimental rats. A total number of 50 rats were divided mainly into 2 groups; the first group served as the normal control group (con.) (n=10) and was allowed to take normal saline via oral gavage tube. While the remaining 40 rats were subjected to simvastatin to induce muscular atrophy at a dose of 80 mg/kg b.wt., after 46 days, the 40 rats were divided into 4 groups (n=10): (1): Muscular atrophy group (MA) served as a positive control group and sacrificed by the end of the 46 days, (2): (MA+Tfg) which were treated with Tfg only (500mg/day.), (3): (MA + BM-MSCs) each rat received a single dose of 0.5 ml phosphate-buffered saline as a vehicle of stem cells injected into the tail vein and (4): received co-treatment of (Tfg+ BM-MSCs). All the groups' compromised rats were sacrificed after 30 days of the different treatments. In order to figure out muscle atrophy and the treatment efficacy serum biomarkers CK and Troponin, oxidative stress, apoptosis, and inflammatory markers were also determined, molecular investigations of atrogin-1, FoxO-3, mTOR, C-miR-486, and PAX3 were carried out accompanied to histopathological analysis using hematoxylin and eosin (H & E) stain. Tfg and/or BM-MSCs showed gradual improvement in most of the parameters confirmed by the histopathological determinations, confirming the present scientific team's assumption that Tfg and BM-MSCs have a promising therapeutic role in the treatment of muscular atrophy, which needs more studies and investigations.

Keywords: muscular atrophy; BM-MSCs; *Trigonella foenum-graecum*; oxidative stress; apoptosis; inflammation; molecular investigations.

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1. Introduction

Skeletal muscles are one of the main and important soft tissues in the body as they represent about 35-45% of the body weight [1]. Differently from smooth and cardiac muscles, skeletal
Muscles are totally adjusted (contracted and relaxed) under self-control in the healthy person, where it is divided into three main types: 1) type (1) slow fibers with anti-fatigue effect; 2) type (2A) fast fibers with moderate anti-fatigue effect; 3) type (2B) fast fibers with the worst anti-fatigue effect [2].

Muscular atrophy (MA) is a systemic response that happens under different circumstances; inactivity comes on its top such as in bedridden patients [3], prolonged fasting and skeletal muscle denervation due to trauma, nephropathy, viral infections such as polio, spinal muscular atrophy (SMA) and amyotrophic lateral sclerosis (ALS) [4]. Also, various catabolic diseases lead to MA as AIDS, Cachexia, trauma, excessive glucocorticoids, as in Cushing syndrome, and cardiac disorders [5]. Animal models have shown that skeletal muscles suffer from atrophy in response to different catabolic stimuli, which are activated by the ubiquitin-proteasome system (UPS) [6] and autophagy [7]. UPS breaks down muscle proteins as the myofibrillar components cause a reduction in muscular strength accompanied by loss of contractile machinery. At the same time, degradation of the organelles, especially the mitochondria, takes place by autophagy leading to decreased endurance capacity within the atrophied muscle. All these cascades are controlled by a set of transcriptional factors activating protein degradation over protein synthesis and thus leading to muscle wasting [8, 9].

Microarray studies demonstrated a set of about 120 genes called atrophy-related genes (atrogenes); among these atrogenes are those encoding Ubiquitin (Ub), proteasome subunits, and Ub ligases, and also genes encoding different proteins mediating autophagy [10]. The present study focused on estimating one of the main Ub-liageses, Atrogeine-1 (also known as MAFBX), which is markedly elevated in almost all types of atrophy and used as a marker of accelerated proteolysis and consequently the atrophy process.

The main pathway which controls the overall protein synthesis and inhibits protein degradation in non-dividing muscles is the phosphoinositol 3-kinase (PI3K)–AKT–mammalian target of rapamycin (mTOR) signaling pathway [11]. As it stimulates protein accumulation by deactivation of forkhead box protein O (FoxO) transcription factors [12] which stimulate or suppress the atrogene program [13]. However, during catabolic diseases or starvation PI3K–AKT–mTOR signaling is suppressed, and in turn, protein degradation is up-regulated via activation of FoxO-mediated expression of the atrogene program as stimulation of FoxO3 only is sufficient to enhance the atrophy process through UPS and autophagy, and it is present in all types of atrophy [13, 14]. So, one of the main targets in this study is to demonstrate the levels of FoxO3 and mTOR to evaluate the balance between protein degradation and synthesis during atrophy.

In chronic diseases and catabolic disorders, oxidative stress as reactive oxygen species (ROS) promotes inflammation by increasing the pro-inflammatory transcription factors as NFκB, which induces and activates other pro-inflammatory cytokines like IL-6 and TNF-α [15]. ROS not only activates inflammatory cytokines but also has a great impact on apoptosis. As elevated levels of ROS can directly or indirectly activate the mitochondrial apoptotic pathway either by the extrinsic pathway due to elevation of TNF family or by the intrinsic pathway due to imbalance between anti-apoptotic factors as Bel-2 and apoptotic factors as Bax inducing cytochrome C, which in turn binds to protease-activating factor-1 (Apaf-1) activating the initiator caspase 9. Both pathways ultimately stimulate the effector caspase 3, which triggers DNA fragmentation degradation of genetic material by proteases [16]. Oxidative stress, inflammation, and apoptosis can promote MA by enhancing ROS production and stimulating UPS, forming a positive feedback mechanism [17, 18].
Skeletal muscle injuries caused great burdens to all the medical systems worldwide as there has been no efficient treatment until now [19]. However, recently various studies on stem cells postulated novel strategies to deal with skeletal muscle problems [20]. Skeletal muscles contain stem cells known as satellite stem cells which are able to differentiate into muscle cells but only in theory. In vitro, they have limited regenerative capacity on skeletal muscles because of high heterogeneity, small quantity, and loss of myogenic differentiation potential [21]. Also, inflammation has a negative effect on the myogenic differentiation potential of satellite stem cells [22]. So, whether satellite cells have sufficient regenerative capacity, scientists have demonstrated that BM-MSCs may contribute to tissue repair with their myogenic, neurogenic, and angiogenic differentiation ability in tissue repair.

BM-MSCs regulate the regeneration of skeletal muscles via complex mechanisms as they are able to secrete growth factors, trophic factors, and extracellular matrix (ECM) into the microenvironment [23]. Konala et al. [24] mentioned that BM-MSCs also help nourish the damaged skeletal muscles to enhance the regeneration process in vivo. Also, BM-MSCs were proved to secrete anti-inflammatory cytokines IL-10 and inhibit inflammatory cytokines IL-6 [25]. Regarding protein synthesis BM-MSCs participate in promoting protein synthesis by secreting insulin-like growth factor-1 (IGF-1) [26], which directly activates PI3K–AKT–mTOR signaling [27], which inhibits activation of FoxO3 and consequently blocks the atrogeneprogramme as mentioned above. Therefore, ameliorating inflammation and enhancing protein synthesis renewing muscle fibers, nerves, and blood vessels.

microRNAs (miRNAs) are a class of small non-coding RNAs that play key roles in various physiological processes, mainly in modulating intracellular signaling pathways of development and disease [28]. miRNAs play key roles in muscle cells specification, proliferation, differentiation, and regeneration [29, 30]. C-miRNA-486 is a muscle-enriched miRNA that regulates muscle growth by stimulating the PIK3/AKT signaling pathway by down-regulation of PTEN (phosphatase and tensin homolog) and FoxO1 genes [31]. It was found that c-miRNA-486 is significantly depressed during atrophies, while its over-expression improves muscle strength and endurance [32].

Due to the multifactorial pathogenesis of muscle atrophy, combining drugs and transport interventions such as exercising, nutritional therapy, or electrical stimulation are the most promising treatments up to date. However, plant-derived natural products have emerged strongly towards treating or at least helping in the treatment of many diseases Trigonella foenum graecum Linn (Tfg). (Fenugreek) is a short-living annual plant, a member of the Fabaceae family. Found in many parts of Asia, Africa, and Europe also, strongly participate in the folk, Chinese and Indian medicine [33]. Nowadays, a large number of studies and recent researches have spotlighted the light on the medicinal importance of Fenugreek as an antioxidant [34], anti-inflammatory [35], anti-diabetic [36], anti-obesity [37], and anti-cancer [38]. All these benefits that promote health can be attributed to the presence of a different array of phytochemicals, especially flavonoids with a variety of pharmacological and biological activities. So, the present work postulated that the high percentage of the protein content in Tfg [39] might help maintain protein synthesis while treating atrophy.

Therefore, the present study was undertaken to explore the mechanisms by which Tfg and BM-MSCs could activate the antioxidant system, repress inflammation, apoptosis, molecular and genetic expression and consequently, ameliorate MA disease in the experimental model.
2. Materials and Methods

2.1. Animals.

Adult male albino rats (n=50) of about 90 days and weight (130±10 g) were purchased from the National Research Centre, Giza, Egypt. Animals were kept acclimatizing before the experimental onset for 1 week in polypropylene cages under controlled conditions of illumination (12/12 h light-dark cycles), temperature (25-30°C), and humidity about 60% with free access to food and water.

All the experiments involving animals and tissue samples were conducted according to the principles and guidelines of the care and use of laboratory animals of the national institute of health (NIH), (USA), and approved by the ethical committee for animal experiment national research Centre, Egypt.

2.2. Chemicals and drugs.

Simvastatin: SIMVACOR® drug by SIGMA Pharmaceutical Company was obtained from Commercial Market, Cairo, Egypt. Trigonella foenum-graecum (Tfg) was supplied from Nature'sway brand, LLC Green Bay, WI (USA). All the other reagents, solvents, and chemicals used for analysis met the quality criteria in agreement with the International Standards.

2.3. Induction of muscular atrophy (MA).

MA was induced by simvastatin at a dose of 80mg/kg b.wt, according to Farouk et al. [40]. Each SIMVACOR® box contains 7 coated tablets: each with a concentration of 80 mg simvastatin as the active ingredient.

2.3.1. Dose preparation

Each simvastatin tablet was mashed and dissolved in 2mL distilled water to form a suspension which is introduced orally by the gavage tube daily for 46 days; by the beginning of each new week, rats were weighed, and the dose was modified according to the weight gain.

2.4. Preparation and isolation of BM-derived BM-MSCs.

White albino rats with 6-week-old were used for preparation and isolation of BM-MSCs, bone marrow was harvested by flushing of tibiae and femurs with Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL), augmented with 10% fetal bovine serum (GIBCO/BRL). Separation of nucleated cells was considered with a density gradient; then, it is suspended in a complete culture medium supplemented with 1% penicillin-streptomycin (GIBCO/BRL). Cells are incubated in 5% humidified CO2 for 12–14 days at 37°C for 12-14 days. After 80-90% confluence and formation of large colonies, culture was washed with phosphate-buffered saline (PBS) and allowing it to be trypsinized using 0.25% trypsin in 1 mL EDTA (5 min at 37°C) (GIBCO/BRL). Following cells centrifugation, they were resuspended with a serum-supplemented medium and incubated in a 50 cm² culture flask.

MSCs of 2nd passage had been used in the experiment and characterized by their adhesiveness and fusiform shape detected by microscopic investigations [41]. Moreover, flow-cytometric analysis of cell surface markers was performed in Giza Egypt's National Research Centre. The expression of surface antigens markers CD34 (-ve), CD90 (+ve), and CD29 (+ve)
were examined. The expression of surface antigens of cells was detected by a fluorescence-activated cell sorter (FACS) flow cytometer (Coulter Epics Elite, Miami, FL) (Figure 1).

Injection of BM-MSCs into the rats: MSCs of the 2nd generation was washed twice using PBS, and cells were released with 0.25% trypsin in 1 mM EDTA for 5 min at 37 °C. Allowing it to centrifuge for 20 min. at 2400 rounds per minute (RPM), cells were counted under a light microscope using a hemocytometer. MSCs (1 x 106 cells/0.5 ml), suspended in PBS, were injected into the tail vein [42].

Labeling of stem cells: with Paul Karl Horan 26 (PKH 26) (red fluorescence cell linker): MSCs were collected during the 2nd passage, labeled with PKH 26 dye [43]. Cells were centrifuged and washed twice in a serum-free medium. Cells were pelleted and suspended in the dye solution.

Detection of stem cells homing: Sections of skeletal muscle of BM-MSCs and (Tfg + BM-MSCs) treated groups showed PKH26 labeled stem cells appearing as bright dots along the course of the muscle fibers (Figure 2). They were examined by fluorescent microscope in Giza Egypt's National Research Centre.

2.5. *Trigonella foenum-graecum*.

(Tfg) was supplied from Nature's way brand, LLC Green Bay, WI (USA), used in a dose 500mg/day [44].

2.6. Experimental design.

The experiment was conducted totally on 50 rats divided mainly into 2 groups; the first group served as the normal control group (con.) (n=10) and was allowed to take normal saline via oral gavage tube. While the remaining 40 rats were subjected to simvastatin to induce muscular atrophy at a dose of 80 mg/kg b.wt., after 46 days, the 40 rats were divided into 4 groups, each containing 10 rats: (1): Muscular atrophy group (MA) and served as a positive control group and immediately sacrificed by the end of the 46 days, (2): (MA+Tfg) which were treated with Tfg only (500mg/day.), (3): Muscular atrophy + BM-MSCs (MA + BM-MSCs) this group was treated with BM-MSCs only where Each rat received a single dose of 0.5 ml phosphate-buffered saline as a vehicle of stem cells injected into the tail vein and (4): received co-treatment of (Tfg+ BM-MSCs), all groups compromised rats were sacrificed after 30 days of the different treatments.

2.7. Biochemical determinations.

Determination of serum creatine kinase (CK) was tested using a kit bought from DiagnosticumZrt., referred to [45]. Serum Troponin was assessed by enzyme-linked immunosorbent assay (ELISA) method using Gscience kits obtained from Glory Science Co., Ltd, USA, according to the manufacturer's instructions. Serum C-reactive protein (CRP) was determined by ELISA method using rat c-reactive protein assay kit purchased from GenWay, Inc. Co., San Diego, CA, USA, according to the assay of [46]. Serum Interleukin-6 (IL-6) and Interleukin-10 (IL-10) were estimated by ELISA technique, a kit purchased from Uscn life science Inc. USA. While Nuclear factor-kappa B (NFkB) was evaluated via ELISA technique using rat nuclear factor kappa B ELISA kit purchased from Glory Science Co., Ltd, USA according to the manufacturer's instructions.
Caspase-3 was evaluated applying ELISA technique in accordance with the kit purchased from Usclife science Inc. USA. And B-cell lymphoma 2 (Bcl2) was assessed by ELISA method using a kit bought from Glory Science Co., Ltd, USA, according to the manufacturer's instructions. Superoxide dismutase (SOD) was determined according to [47]. Malondialdehyde (MDA) content was determined by colorimetric methods using Bio diagnostic kit (Egypt) following the methods of Satoh [48].

2.8. Molecular study for determination of Atrogin-1, FoxO-3, mTOR, PAX3, and miRNA-486 expression levels.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis with a quantitative approach Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the kit's instructions, and NanoDrop was used to measure it (Thermo Fisher Scientific, USA). MicroRNA First Strand cDNA Synthesis (Poly A Tailing, ShengGong, China) was used to reverse transcribe miR-486, and PrimeScript RT Reagent Kit was used to reverse transcribe mRNA (Takara RR047A, Japan). According to the manufacturer's instructions, the samples were then subjected to PCR with an SYBR Premix Ex TaqII kit (Takara RR820A, Japan). MiR-486, atrogen, foxo, mtor, and pax3 were detected using RT-PCR, which was performed by using an ABI StepOne system (Applied BioSystems, USA), and their levels were relatively measured using the 2–ΔΔCT method. U6 and β-actin were used as internal references for miR-486 and mRNA, respectively.

### Table 1. Primer sequences used for RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primers</th>
<th>Reverse primers</th>
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</thead>
<tbody>
<tr>
<td>Atrogin-1</td>
<td>5′-GGGAGGAAGCAGAACAGAAGAGGA-3′</td>
<td>5′-TCTCCATAGCAATTCCGCTCC-3′</td>
</tr>
<tr>
<td>FoxO-3</td>
<td>5′-GAGGTTGCAAATGTGGGAAGAAT-3′</td>
<td>5′-TTGAATGAAATGGCAAAAGCA-3′</td>
</tr>
<tr>
<td>mTOR</td>
<td>5′-GGTGGAGCGACCTTTTGCTCA3′</td>
<td>5′-AGGAGCCCTAAACCTCGGAT-3′</td>
</tr>
<tr>
<td>PAX3</td>
<td>5′-CAGCCACCAGTCTATCCCCAC-3′</td>
<td>5′-CACGAAGCTGTCGGGTAGC-3′</td>
</tr>
<tr>
<td>miRNA-486</td>
<td>5′-ATCCCTGTACTGAGCTGCC-3′</td>
<td>Matching sequence provided in MicroRNA First Strand cDNA Synthesis kit (reverse) (Poly A Tailing, ShengGong, China).</td>
</tr>
<tr>
<td>β-actin</td>
<td>5′-GGAGATTACTGGCGCTCCCTA-3′</td>
<td>5′-GACTCATGTAATCTGGTGAC-3′</td>
</tr>
<tr>
<td>U6</td>
<td>5′-GCTTCGCGAGCAGCATATAACTAAAAT3′</td>
<td>5′-CGCTTCAGGAATTGCGGTCTCAT-3′</td>
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</table>

2.9. Histopathological examination of skeletal muscles.

Muscle specimens were collected and fixed in neutral buffered formalin 10%, routinely processed, and embedded in paraffin wax. Sections of 4-5 µm thickness were prepared and stained with Hematoxylin and Eosin for histopathological examination by light microscope (Olympus CX 41, Japan). Histopathological alterations were graded as (0) indicated no changes, (1), (2), and (3) indicated mild, moderate, and severe alterations, respectively [49].

2.10. Statistical analysis.

All the data present in the current study were expressed as mean±SE of the mean. Using the statistical Package for the Social Sciences (SPSS) program, version 14.0 was used to compare the significant difference between every two groups. The difference was considered significant when P <0.05. Percentage difference representing the percent of variation with respect to the corresponding control group was calculated according to the following formula:

\[
\text{% Difference} = \left(\frac{\text{Treated value} - \text{control value}}{\text{control value}}\right) \times 100
\]
3. Results and Discussion


An Immunophenotype of BM-MSCs cell was examined by flow cytometry. BM-MSCs cells were negative for the hematopoietic marker CD34 while strongly positive for mesenchymal stem cell-specific markers, including CD29 and CD90. The blue histograms represent antibody-labeled cells, while the grey histogram shows the profile of the isotype control.

![Figure 1](https://biointerfaceresearch.com/)

**Figure 1.** The immunophenotype of BM-MSCs was examined by flow cytometry. BM-MSCs cells were negative for the hematopoietic marker CD34 while strongly positive for mesenchymal stem cell-specific markers, including CD90 and CD29. The blue histograms represent antibody-labeled cells, while the grey histogram shows the profile of the isotype control.

3.2. Effect of treatment with Tgf and BM-MSCs on biochemical analysis in MA model.

Data in Table 2 illustrated the effect of treatment with Tgf and BM-MSCs on CK and Troponin MA-induced rats. In comparison with the normal control group, there was a significant increase (P<0.05) in the levels of CK and Troponin. On the other hand, the treatment of MA group with Tgf and BM-MSCs or both of them produced a significant reduction (P<0.05) in the levels of CK and Troponin as compared to MA-group.

**Table 2.** The effect of treatment with Tgf and BM-MSCs on CK and Troponin in MA model. (Means±SE, n=10).

<table>
<thead>
<tr>
<th></th>
<th>Con.</th>
<th>MA</th>
<th>MA+Tgf</th>
<th>MA+BM-MSCs</th>
<th>BM-MSCs + Tgf</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK (IU/L)</td>
<td>111.70±0.42</td>
<td>220.34±1.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>172.66±1.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>177.18±0.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>163.76±1.97&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Troponin</td>
<td>0.4490±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.3730±0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.2260±0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.0590±0.008&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.9360±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>MA+BM-MSCs</th>
<th>MA+BM-MSCs</th>
<th>MA+BM-MSCs</th>
<th>MA+BM-MSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK (IU/L)</td>
<td>177.18±0.64</td>
<td>177.18±0.64</td>
<td>177.18±0.64</td>
<td>177.18±0.64</td>
</tr>
<tr>
<td>Troponin</td>
<td>1.2260±0.09</td>
<td>1.2260±0.09</td>
<td>1.2260±0.09</td>
<td>1.2260±0.09</td>
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</table>

a: Significant change at P< 0.05 compared to the normal control group; b: Significant change at P< 0.05 against MA group.
The data in Table 3 showed the effect of treatment with Tfg and BM-MSCs on apoptotic markers, SOD, and MDA of MA-induced rats. It was revealed that simvastatin administration led to an increase (P<0.05) in the levels of apoptotic marker caspase-3 and MDA accompanied with the reduction (P<0.05) in the levels of anti-apoptotic marker Bcl2 and SOD as compared to the normal control group. In contrast, treatment of MA group with Tfg and BM-MSCs led to a significant reduction (P<0.05) in the levels of apoptotic marker caspase-3 and MDA and significant elevation in the levels of the Bcl2 and SOD as compared to the untreated MA group.

Table 3. The effect of treatment with Tfg and BM-MSCs on apoptotic markers, SOD, and MDA levels in MA model. (Means±SE, n=10).

<table>
<thead>
<tr>
<th></th>
<th>Con.</th>
<th>MA</th>
<th>MA+Tfg</th>
<th>MA+MSCs</th>
<th>BM-MSCs</th>
<th>BM-MSCs + Tfg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl2 (ng/mg protein)</td>
<td>233.78±1.99</td>
<td>107.88±1.50a (a=-53.85%)</td>
<td>174.96±1.17b (b=62.18%)</td>
<td>187.59±0.92b (b=73.38%)</td>
<td>202.18±1.25b (b=87.41%)</td>
<td></td>
</tr>
<tr>
<td>Caspase-3 (ng/mg protein)</td>
<td>2.36±0.05</td>
<td>11.74±0.28a (a=397.45%)</td>
<td>6.87±0.27b (b=41.48%)</td>
<td>5.80±0.19b (b=-50.59%)</td>
<td>4.43±0.74b (b=-62.26%)</td>
<td></td>
</tr>
<tr>
<td>SOD (U/mg tissue)</td>
<td>8.68±0.11</td>
<td>1.73±0.11a (a=-80.06%)</td>
<td>4.28±0.55b (b=143%)</td>
<td>4.17±0.06b (b=141%)</td>
<td>3.22±0.08b (b=86.12%)</td>
<td></td>
</tr>
<tr>
<td>MDA (nmol/mg protein)</td>
<td>28.80±0.64</td>
<td>140.70±1.92a (a=388.54%)</td>
<td>73.37±1.44b (b=47.85%)</td>
<td>59.42±1.08b (b=-57.76%)</td>
<td>57.64±1.19b (b=-59.03%)</td>
<td></td>
</tr>
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</table>

a: Significant change at P< 0.05 compared to the normal control group; b: Significant change at P< 0.05 against MA group.

The results in Table 4 showed the effect of treatment with Tfg and BM-MSCs on cytokines and CRP levels in MA model. In comparison with the negative control group, there was a significant increase (P<0.05) in the values of NFkB, pro-inflammatory cytokine (IL-6), and CRP associated with a significant reduction (P<0.05) anti-inflammatory cytokine (IL-10) in MA group. On the other hand, the treatment of MA group with Tfg, BM-MSCs, or both of them resulted in a significant decrease (P<0.05) in the levels of NFkB, pro-inflammatory cytokine (IL-6), and CRP. Moreover, significant elevation (P<0.05) in the anti-inflammatory cytokine (IL-10) level in MA group treated with Tfg, BM-MSCs, or both of them was recorded compared with the untreated MA group.

Table 4. The effect of treatment with Tfg and BM-MSCs on cytokines and CRP levels in MA model. (Means±SE, n=10).

<table>
<thead>
<tr>
<th></th>
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<th>MA+BM-MSCs</th>
<th>BM-MSCs</th>
<th>BM-MSCs + Tfg</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFkB (ng/mg protein)</td>
<td>83.45±2.44</td>
<td>160.43±12.5a (a=92.24%)</td>
<td>150.05±2.27b (b=-6.47%)</td>
<td>143.48±1.17b (b=10.56%)</td>
<td>109.81±2.56b (b=-31.55%)</td>
<td></td>
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<tr>
<td>IL-6 (pg/mg total protein)</td>
<td>14.27±0.28</td>
<td>89.03±1.70a (a=523.90%)</td>
<td>66.59±0.54b (b=25.20%)</td>
<td>69.43±0.49b (b=-22.01%)</td>
<td>37.21±0.62b (b=-58.20%)</td>
<td></td>
</tr>
<tr>
<td>IL-10 (pg/mg total protein)</td>
<td>134.92±2.13</td>
<td>62.39±1.31a (a=-51.83%)</td>
<td>92.57±0.51b (b=48.37%)</td>
<td>99.19±0.79b (b=58.98%)</td>
<td>107.00±1.92b (b=71.50%)</td>
<td></td>
</tr>
<tr>
<td>CRP (ng/mL)</td>
<td>1.22±0.04</td>
<td>9.17±0.26a (a=651.63%)</td>
<td>4.79±0.08b (b=47.76%)</td>
<td>3.76±0.47b (b=-58.99%)</td>
<td>2.85±0.29b (b=-68.92%)</td>
<td></td>
</tr>
</tbody>
</table>

a: Significant change at P< 0.05 compared to the normal control group; b: Significant change at P< 0.05 against MA group.

3.3. Effect of treatment with Tfg and BM-MSCs on molecular determinations in MA model.

Table 5 and Figure 3 (a-e) represent the molecular genetic expression of catabolic, anabolic genes, and miR-486 as shown in MA group a remarkable increase (P<0.05) in the expression of catabolic genes (Atg-1 and FoxO-3) and a significant decrease (P<0.05) in the expression of anabolic genes (mTOR and PAX-3) and miR-486 expression in comparison to the negative
control. In contrast, results of the treated groups show gradient improvement as Tfg and BM-MSCs treated groups induce average improvement contra the double treatment of (Tfg + BM-MSCs), which in general induces a significant amelioration in comparison to the MA group.

Table 5. The effect of treatment with Tfg and BM-MSCs on Atg-1, FoxO3, mTOR, PAX3 A, and miR-486 in MA model. (Means±SE, n=10).

<table>
<thead>
<tr>
<th></th>
<th>Con.</th>
<th>MA</th>
<th>MA+Tfg</th>
<th>MA+ BM-MSCs</th>
<th>BM-MSCs + Tfg</th>
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</thead>
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<tr>
<td>Atg-1 expression</td>
<td>1.27±0.07</td>
<td>5.88±0.34 a</td>
<td>4.12±0.23 b</td>
<td>3.55±0.12 b</td>
<td>2.91±0.25 b</td>
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<td>FoxO3 expression</td>
<td>1.56±0.17</td>
<td>5.93±0.18 a</td>
<td>3.85±0.17 b</td>
<td>3.27±0.20 b</td>
<td>2.04±0.12 b</td>
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<td>mTOR expression</td>
<td>1.45±0.15</td>
<td>0.25±0.21 a</td>
<td>0.48±0.27 b</td>
<td>0.67±0.22 b</td>
<td>0.84±0.24 b</td>
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<tr>
<td>PAX3 expression</td>
<td>1.12±0.03</td>
<td>0.27±0.27 a</td>
<td>0.44±0.02 b</td>
<td>0.68±0.33 b</td>
<td>0.83±0.18 b</td>
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<tr>
<td>miR-486 expression</td>
<td>1.13±0.03</td>
<td>0.27±0.13 a</td>
<td>0.60±0.03 b</td>
<td>0.71±0.02 b</td>
<td>0.86±0.01 b</td>
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a: Significant change at P < 0.05 compared to the normal control group; b: Significant change at P< 0.05 against MA group.

Figure 3. Agarose gel electrophoresis showing (a) Atrogin-1; (b) FoxO3; (c) mTOR; (d) PAX3; (e) Beta-actin expression in the skeletal muscle tissue by RT-PCR analysis. Lane 1: Control group, Lane 2: MA group, Lane 3: MA+Tfg group, Lane 4: MA+ BM-MSCs group, Lane 5: Tfg+ BM-MSCs.

3.4. Histological examination.

Transverse sections of skeletal muscles stained with hematoxylin and eosin represented in Figure 4 (a-e) showed normal muscle architecture within the control group. In contrast, the MA group revealed severe alterations in the muscular organization by treating the atrophied muscles with Tfg only we noticed no recovery. In contrast, the treatment with BM-MSCs showed moderate mitigation. On the other hand, the double treatment of Tfg with BM-MSCs showed an obvious recovery in muscle fibers’ size, nuclei, and sarcoplasm.
Figure 4. Histopathological examination of transverse sections in the control group showed the normal organization of skeletal muscle bundles, where each bundle consisted of a group of polyhedral muscle fibers with acidophilic sarcoplasm and multiple peripheral oval nuclei separated by narrow C.T. endomysium (a). The muscular atrophy (MA) group receiving simvastatin showed severe muscular histopathological alterations, where muscle fibers are atrophied and angulated with various shapes and sizes. Sarcoplasm is fragmented hypereosinophilic, separated by wide C.T. endomysium and severe widening of perimysium (b). The third group receiving only Tfg didn't show any recovery. The muscle fibers were atrophied of various shapes and sizes with fragmented sarcoplasm separated by wide C.T. endomysium and severe widening of perimysium (c). While group 4, which received BM-MSCs showed mild recovery, where muscle fibers are still atrophied with acidophilic fragmented sarcoplasm and multiple peripheral oval nuclei separated by wide C.T. endomysium (d). On the other hand, the double treatment of Tfg and BM-MSCs declared a marked recovery. Muscle fibers are polyhedral and larger with acidophilic sarcoplasm and multiple peripheral oval nuclei separated by narrow C.T. endomysium (e).

3.5. Discussion.

MA is the result of myofibrils shrinkage [50], reduction in muscle mass, muscle strength, protein content, organelles, cytoplasm, and cross-section area (CSA), as well as the alteration in the type of muscle fiber [51].

Novel studies stated a complicated scenario by which catabolic mechanisms support one another at various levels leading to the balance between protein degradation and synthesis, which determines the physiological state of muscle fiber. And the general protein homeostasis of the whole body as muscles are the largest protein reservoir in the body [50].

The present study demonstrated an increase in serum CK and serum Troponin levels in MA group. Several studies postulated that under several catabolic conditions such as aging, cancer cachexia, muscle dystrophy, immobilization, denervation, prolonged fasting, excessive glucocorticoids as in cushing syndrome patients, and many other conditions that develop muscle atrophy. A significant elevation in serum CK and serum Troponin levels is resulted, which is an alert of necrosis and tissue damage post-acute or chronic muscular disorders [52]. However, values of CK and Troponin showed improvement in the treated groups, which was mild in Tfg and BM-MSCs treated groups and ultimate in the double treated group (Tfg + BM-MSCs). These results may be attributed to the anti-apoptotic effect of Tfg, which aids and support the anti-apoptotic effect of BM-MSCs [53, 54].
Current data has shown significant depression in SOD coupled with an elevation of MDA levels within the group of MA. Muscle atrophy affects mitochondrial content, morphology, and function; changes that occur in the mitochondria cause ROS production, which impacts muscle function and triggers the catabolic signaling pathway, which promotes the activation of muscular atrophy [55]. Mitochondrial ROS (mtROS) is normally removed by a cellular antioxidant defense system comprised of superoxide dismutase (SOD), catalase, and glutathione peroxidase. However, ROS are accumulated and overproduced under pathological conditions, leading to mitochondrial damage overwhelming the antioxidant capacity and impairing the mitochondria [56]. So, SOD activity may be reduced due to mitochondrial damage, which induces ROS. In addition, increased MDA levels in the MA group are evidence of the increment of ROS levels inducted due to mitochondrial damage-causing lipid peroxidation (LPO) and up-regulation of MDA [57]. On the other hand, improvement in SOD and MDA levels was shown on the treatment with Tfg and BM-MSCs either alone or combined, which indicates the amelioration of oxidative stress accompanied by reduction of ROS levels.

In agreement with the present results, Bhatt et al. [58] found that Tfg has a powerful antioxidative capacity due to containing total phenols and flavonoids which scavenge ROS, control the formation of ROS and stop their reaction with biological structures, which in turn limit lipid peroxidation associated with decreased levels of MDA. All these antioxidative advantages of the natural antioxidant Tfg boost the tremendous antioxidative role of BM-MSCs as they can manage the redox context through several mechanisms by reducing mitochondrial induced-ROS specially superoxides through mitigating the redox environment [59], releasing various antioxidant factors, and enhancing the uncoupling respiration of mitochondria suppressing oxidative stress [60]. In agreement with Jeziorska-Wozniak et al. [61]. BM-MSCs can modulate oxidative stress by a dual action of scavenging excessive ROS and increasing the activity and concentration of antioxidative enzymes leading to total neutralization of oxidative stress.

Dirks and Leeuwenburgh [62] evidenced that apoptosis may be considered as a key role in skeletal muscle atrophy; the current data showed a remarkable depression in Bcl2 levels and elevation in Caspase-3 levels within the MA group, and this may be attributed to mitochondrial damage illustrated previously as according to Vringer and Tait [63]. Mitochondria is the major player of apoptosis. When it is severely damaged by any means, it could release cytochrome C in the cytosol, which is the initiation step of the mitochondrial apoptotic pathway (intrinsic pathway). Elena-Real et al. [64] stated that following the release of cytochrome C, formation of an-apoptotic initiating complex and apoptotic protease activating factor-1 (Apaf-1) and caspase-9, which activates in turn caspase-3 and a group of other caspases. Caspase-3 activation plays an important role in cytoskeleton reorganization, stops DNA replication and repair system, destroys DNA, disrupts the nuclear structure finally leads to cell destruction.

Du et al. [65] mentioned that proteins of the Bcl-2 family are one of the main regulators of the mitochondria-initiated caspase activation pathway as the anti-apoptotic Bcl-2 preserve the mitochondrial integrity and prevent the release of cytochrome C in the presence of apoptotic stimuli. So, when the levels of Bcl-2 are decreased due to increased ROS levels, caspase-3 is elevated to the interruption in the mitochondrial integrity, and apoptosis is initiated.

On the other hand, the Tfg group showed a considerable effect on both levels of Bcl-2 and caspase-3, and this may be due to the fact that Tfg is rich in flavonoids [53], as Flavonoids have vast anti-apoptotic effect by up-regulation of anti-apoptotic Bcl-2 and inhibition of Caspase-3 [66]. At the same time, BM-MSCs only treated group showed a mild increment in Bcl-2 and decrement
in caspase-3. At the same time, the treatment with BM-MSCs associated with Tfg induced the ultimate mitigatory effect, and this could be due to the extraordinary anti-apoptotic effect of BM-MSCs as they can directly synthesize Bcl-2, therefore, inhibiting apoptosis and restoring tissue homeostasis [54]. Moreover, BM-MSCs help in the down-regulation of caspase-3 and Bcl-2 associated protein (BAX) expression, and due to this, it prevents the cells from responding to apoptotic signals [67].

Inflammation is a complex process of innate immunity in response to physiological, physical, and oxidative stress; it is mediated by pro-inflammatory cytokines and free radicals [68]. At the onset of muscular atrophy, our data showed that IL-6, NF-κB, and CRP levels were significantly associated with a decrement of the anti-inflammatory IL-10 compared to the normal control group. In agreement with A.Ganguly [69], CRP levels are increased due to inflammation and muscle loss. Regarding NF-κB, it is a protein complex involved in the cellular response to various stimuli such as oxidative stress, cytokines, and ROS [70]. It is considered the first responder to deleterious cellular stimuli [71] as it is already expressed in all types of cells in an inactive form [72]. Elevated ROS and oxidative stress promote the activation of NF-κB via, the canonical pathway [73]. When the muscular tissue is stimulated by any means leading to its injury, the balance between regeneration and apoptosis is broken, leading to atrophy [74], where inflammatory factors enter the damaged sites and stimulate the early inflammatory response, releasing various cytokines chemokines. TNF-α (Cachectin) is the main player upon inflammation, apoptosis, and the immune response towards atrophy [75]. Also, studies have associated the TNF-α expression with inadequate muscular production via acting on NF-κB, which regulates the expression of various pro-inflammatory cytokines, including IL-6. In a positive feedback loop, overexpression of these pro-inflammatory cytokines leads to activation of NF-κB, causing more atrophy [75].

Meanwhile, the effect of Tfg on ameliorating inflammation was obvious in the present data by decreasing NF-κB, IL-6, and CRP levels as well as increasing the anti-inflammatory cytokine IL-10 as Al-Khawaga and M. Abdelalim [76] and Salvadori et al. [77] were in agreement with our data, and since it is a powerful antioxidant, so it helps in mitigating inflammation by quenching excessive ROS. Moreover, BM-MSCs greatly improved the inflammatory environment by decreasing levels of NF-κb, IL-6, and CRP and upregulation of IL-10 due to inducing dendritic cells (DCs). As IL-10 has an essential role in keeping the balance between pro-inflammatory and anti-inflammatory responses to maintain homeostasis, also it inhibits the activity and synthesis of pro-inflammatory cytokines [78]. As well as it suppresses inflammation by counteracting the Toll-like receptor-mediated inflammation [79], and it selectively inhibits the transcription and translation of inflammatory genes [80]. Furthermore, IL-10 decreases the expression of various chemokines that recruit macrophages as mice with overexpressed IL-10 were shown to manufacture less pro-inflammatory cytokines [81]. Our data revealed that in treated groups, along with decreasing ROS as mentioned above, the elevation of IL-10 levels causes depression in IL-6 and also TNF-α, which in turn caused the depression of NF-κB and finally causes improvement in the whole inflammatory conditions.

Under catabolic conditions that boost muscular atrophy, the present data conveyed a notable elevation in FoxO3 and Atrogin-1 in the MA group correlated with a clear depression in the expression of both anabolic transcriptional factors mTOR and PAX3.

Consistent with the present findings, Kang et al. [82] explained that Forkhead box (FOX) O proteins, including FoxO1, 3, 4, and 6 are expressed in skeletal muscles and are known for their
major role in the atrophy cascade by activating E3 ubiquitin ligases as MuRF-1 and Atrogin-1 to enhance both catabolic pathways UPS and autophagy triggering muscular atrophy.

Deficiency in insulin/IGF-1 signaling under catabolic conditions is accompanied by decreased activity of PI3K-Akt pathway [83], which suppresses mTOR and phosphorylates FoxO3, thus accelerating protein degradation represses protein synthesis [84]. Moreover, the up-regulation of the catabolic transcriptional factor FoxO3 leads to activation of Atrogin-1 which is considered an E3 ubiquitin ligase, escalating UPS and promoting protein degradation. Atrogin-1 not only promotes proteolysis but also was found to suppress protein synthesis by stimulating eIF-3f (an important activator of protein synthesis) degradation and diminishing its level in muscular tissue [85].

Regarding Tfg's positive role in alleviating the catabolic state and degradation of myoproteins, as shown in the above results, its strong antioxidative, anti-apoptotic, and anti-inflammatory effects all stimulate the PI3K-Akt pathway and up-regulate protein synthesis via mTOR and PAX 3. In addition, Kyoung et al. [86] stated that flavonoid, a major constituent of Tfg, modulates cell survival signaling. BM-MSCs have the ability to modulate skeletal muscle regeneration by complex mechanisms, which primarily contain paracrine effect and immunomodulation by releasing growth factors, trophic factors, and extracellular matrix within the injured microenvironment [19]. Konala et al. [24], in their study, have thought that BM-MSCs have the total ability to nourish the damaged skeletal muscles and enhance regeneration of tissues in vivo. In addition, exosomes induced by BM-MSCs have different kinds of cytokines as granulocyte colony-stimulating factor (GCSF), fibroblast growth factor-2 (FGF-2), and platelet-derived growth factor-BB (PDGF-BB), which are mainly involved in the regeneration of skeletal muscles [19]. Furthermore, BM-MSCs can synthesize IGF-1 to boost their myogenic differentiation processes [26], which is the main controller of the PI3K-Akt mechanism.

Recent studies as Chang et al. [87] and Liu et al. [88] pointed out the importance of Circulating MicroRNA-486 (C-miR-486) as an essential potential biomarker regarding the issue of protein turnover in skeletal muscles under catabolic conditions such as sarcopenia and Cancer-associated cachexia, which led eventually to decreasing the muscular mass causing atrophy.

In parallel with the previous studies, the present results recorded a reduction in the expression of C-miR-486 and PAX3 in MA group in comparison with the normal control group. However, a clear alleviation in their levels is shown within the MA treatment groups compared with the MA group. Reduced C-miR-486 and PAX3 levels occurred due to continuous loss of muscle mass [88]. Moreover, C-miR-486 downregulation enhances AKT/FoxO mediated muscle loss [87]. Also, decreased C-miR-486 in the aging process, which is one of the main catabolic conditions, may impair the protein homeostasis of skeletal muscles, leading to loss of contractile proteins and accumulation of damaged proteins [89]. C-miR-486 is highly expressed in skeletal muscles and was significantly up-regulated during muscular differentiation by targeting PAX3 and PAX7 consequently boosting myoblast differentiation [90]. Furthermore, C-miR-486 suppresses FoxO-1 genes, which negatively activates PI3K/Akt signaling associated with increased transcription of atrogenes (MuRF-1 and Atrogin-1) [31]. As discussed above and consistent with [54, 61, 77] this study recorded that the antioxidative, anti-inflammatory, and proliferative effect of both Tfg and BM-MSCs either each alone or combined have an influence on muscle regeneration and enhance strength as well, leading to increment of C-miR-486 and PAX3 expression.
Histopathological findings showed severe development of degeneration, atrophy, and necrosis in rat muscles following the administration of simvastatin, where muscle fibers are angulated with various shapes and sizes, and sarcoplasm is fragmented hypereosinophilic separated by wide C.T. endomysium and severe widening of perimysium. These findings were in agreement with Westwood et al. [91] and Mehanna et al. [92], who reported that simvastatin induced multiple changes of muscles as loss of transverse striations, splitting of myofibrils, and presence of central nuclei. H&E-stained sections also showed myofibers degeneration, sarcoplasmic fragmentation, and myofiber splitting, as mentioned by Meregalli et al. [93], who concluded that simvastatin brought out significantly morphological and structural skeletal muscular damage-causing hyalinization as a result of over contraction precipitated by excessive intracellular $Ca^{2+}$, allowing muscle fibers to become necrotic or apoptotic and initiate inflammatory reaction via releasing of mitogenic chemoattractants. Splitting of the myofibrils might be due to the insufficient oxygen supply and metabolites exchange to the enlarged and hypertrophied fibers [94]. Marked recovery was observed in the Tfg treated group as seeds of Fenugreek possess estrogen-like action and protective effect along with the stem cells administration [95], improve glucose and lipid metabolism, enhance insulin sensitivity, increase antioxidant defense, and downregulation of lipogenic enzymes [96].

4. Conclusions

The present results could conclude that *Trigonella foenum-graecum* and bone marrow mesenchymal stem cells have a promising therapeutic role against Muscle atrophy induced in male rats by simvastatin, as indicated by the observed improvement in biochemical and molecular genetic markers, which were confirmed by histological examination. This role was achieved through powerful antioxidants activity, anti-inflammatory properties, and anti-apoptotic effects of the active constituents of Tfg and BM-MSCS.

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Conflicts of Interest

The authors declare no conflict of interest.

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