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Formoterol induces muscle regeneration through myogenic regulatory factor MyoD in rat model of statin-induced myopathy

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ABSTRACT

Background and Objective: Statins can induce myopathy; however, the impact on muscle regeneration remains unclear. β 2-agonists may mitigate statin myotoxicity. This study investigated the effects of simvastatin and formoterol on skeletal muscle regeneration and injury using a rat model.

Methods: Adult Sprague-Dawley rats ($n = 90$) were divided into three groups: control (A), simvastatin treatment (B), and simvastatin plus formoterol treatment (C). The rats were kept at room temperature for 3 months where an animal house diet was available *ad libitum*. Simvastatin was administered orally once a day, by oral gavage for 12 weeks; formoterol was concurrently administered to one group. Extensor digitorum longus muscles were analyzed for the myogenic differentiation factor (MyoD) by immunohistochemistry. Serum lactate dehydrogenase (LDH) levels were quantified as a marker of muscle damage.

Results: MyoD-positive myonuclei were significantly increased in the C group (simvastatin plus formoterol group) compared to the A and B Groups (control and simvastatin-only groups) ($p < 0.05$). Serum LDH levels were significantly lower in group C than in groups A and B ($p < 0.05$).

Conclusion: Formoterol alleviates statin-associated muscle injury in a rat model as evidenced by increased MyoD expression and decreased LDH levels. The activation of dormant satellite cells and increased expression of MyoD indicate the potential of formoterol to promote muscle regeneration.

Keywords: Statin myotoxicity, β 2-agonists, formoterol, MyoD, lactate dehydrogenase.

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Introduction

Statins are cholesterol-lowering agents extensively prescribed as first-line agents for the treatment of hypercholesterolemia and prevention of coronary heart disease¹. Statin-induced myopathy is the leading cause of statin intolerance and withdrawal². Statin-induced myopathies deserve special importance because they are often overlooked, resulting in misdiagnosis and improper care and treatment. Patients report to clinicians with symptoms of fatigue, muscle weakness, pain and tenderness, night time cramping, and worsening of these complaints with enhanced physical activities³. Many theories have been suggested to elucidate statin-induced myotoxicity. These include the reduction of mevalonate pathway products, including metabolites of

isoprenoids, mitochondrial dysfunction, and the induction of apoptosis. Gaining insights into the molecular, cellular, and environmental factors governing skeletal muscle repair may help in the development of effective therapies for a variety of skeletal muscle diseases and injuries. Skeletal muscle satellite cells (SCs) are dormant mononucleated myogenic cells responsible for postnatal muscle growth and regeneration in response to injury^{4,5}. They are activated from this dormant state to accomplish their roles in the maintenance and repair of adult skeletal muscle in postnatal development and repair of skeletal muscle^{4,6}. In response to muscle fiber injury, quiescent SCs become active and express myogenic regulatory factors (MRFs). MRFs regulate gene expression and myoblast proliferation and differentiation⁷.

Myogenic differentiation factor (MyoD) is an important MRFs in myogenic determination⁸. It is expressed in proliferating myoblasts and continues to be expressed even after muscle determination has occurred. MyoD stimulates skeletal muscle cell division and replication and enhances the incorporation of SCs into existing myofibers. It has been seen that the structural and functional recovery of myotoxic injury can be accelerated by the stimulation of β -adrenoceptors in skeletal muscles⁹. Thus, the basis of the effectiveness of β -agonists in skeletal myopathy is their ability to stimulate muscle protein synthesis and diminish muscle protein degradation¹⁰. Newly established β 2 agonists, such as formoterol, have more significant and potent protective effects on skeletal muscle histology than older generations of β 2¹¹.

Serum lactate dehydrogenase (LDH) level is an indicator of muscle tissue damage. LDH levels help to ascertain the progress of muscle repair and regeneration. This biochemical marker is also used to assess the effectiveness of various therapeutic modalities in muscular diseases^{12,13}. The extensor digitorum longus (EDL) muscle is an optimal model for analyzing skeletal muscle histology and regeneration due to its mixed fiber-type composition, superficial anatomy that allows direct access without disturbing other structures, and established utility in prior muscle studies.

This study aimed to investigate whether the β 2-agonist formoterol could mitigate statin-induced muscle injury in a rat model by evaluating its effects on MyoD expression and skeletal muscle regeneration, as assessed by immunohistochemical analysis of MyoD+ myonuclei and serum LDH levels. Gaining insights into the comprehensive understanding of how β 2-agonists affect MRFs within myofibers will help us establish their specific role in muscle development, growth, and regeneration. This in turn may help in the development of effective treatment modalities for a variety of skeletal muscle diseases in humans.

Methods

This study was a laboratory-based experimental randomized controlled trial. This research was carried out at the Anatomy Department of the Army Medical College, Rawalpindi, in collaboration with the National Institute of Health (NIH) Islamabad and the Armed Forces Institute of Pathology (AFIP) Rawalpindi. Ninety adult male Sprague-Dawley rats with an average age of approximately 70-80 days and weighing 250 ± 50 g were procured from NIH Islamabad. The rats were kept at a room temperature of 18°C - 26°C in separate cages for 3 months, where a normal animal house diet was available *ad libitum*. The animals were randomly divided into 3 groups, each with 30 rats, numbered separately from 1 to 30. Control Group A (Group A) did not receive any medication. The rats in Group B were administered simvastatin dissolved in

distilled water once daily by oral gavage (60 mg/kg/day)^{14,15} for 12 weeks. Group C received formoterol by oral gavage ($3 \mu\text{g/kg/day}$)¹⁵ dissolved in distilled water once a day for 12 weeks in addition to simvastatin. A 5 ml blood sample was collected from the heart of each animal directly into a plain tube to quantitatively measure the levels of serum LDH before animal sacrifice. The collected samples were labeled appropriately based on the respective experimental groups.

After the experimental period, the animals were sacrificed and the EDL muscle was dissected along with its tendon. A 0.5 cm thick transverse section was obtained from the mid-belly region of the muscle for processing. The tissues were processed to form paraffin-embedded blocks. Standard tissue processing protocols were followed to fix, dehydrate, clear, infiltrate, embed, section, and mount the EDL muscle pieces to create paraffin-embedded blocks for further analysis. Immunohistochemical staining was performed on the slides using a mouse anti-human MyoD antibody for MyoD expression (Santa Cruz Biotechnology, Catalogue number:SC-32758). One cross section was selected from each specimen for immunohistochemical analysis. An indirect immunohistochemical technique through avidin-biotin complex (ABC) method was used for MyoD staining. Briefly, antigen retrieval was performed by heating the tissue sections in citrate buffer at pH 6.0 for 10 minutes. Endogenous peroxidase activity was quenched using 3% H_2O_2 in methanol for 30 minutes. Sections were blocked with 5% normal goat serum for 60 minutes and then incubated with the primary antibody overnight at 4°C . Biotinylated goat anti-mouse IgG secondary antibody (Vector Labs, catalog #BA-9200) was applied for 30 minutes, followed by incubation with ABC reagent (Vector Labs) for 30 minutes. Color development was performed using a diaminobenzidine substrate kit (Vector Laboratories, Burlingame, CA). The sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted. The interpretation of MyoD positivity was based on the study by Cornelison and Wold¹⁶ where MyoD positive myonuclei stained dark brown, while MyoD negative myonuclei lacked staining and appeared blue. The total number of MyoD positive (brown) myonuclei was counted in eight microscopic fields under $40\times$ objective (by ImageJ software which is a Java-based image processing program) and the average number was recorded.

Statistical analysis

Data were analyzed using the IBM SPSS 27. Quantitative variables are expressed as the mean \pm SEM. Analysis of variance (ANOVA) followed by *post-hoc* Tukey's test was used to determine the differences among the various groups. Statistical significance was set at $p < 0.05$.

Table 1. Comparison of immunohistochemical expression of MyoD+ nuclei among three groups A, B, and C.

Parameters	Groups	Mean \pm SEM	Statistical significance (ANOVA) followed by post-hoc Tukey's test		
			Group A/B	Group A/C	Group B/C
Number of MyoD+ myonuclei	A	17.23 \pm 0.259	$p < 0.001$	$p < 0.001$	$p < 0.001$
	B	22.60 \pm 0.334			
	C	31.63 \pm 0.344			

Results

Microscopic examination of the EDL muscle of the control group revealed a normal histological structure of the skeletal muscle, evident by uniformly distributed muscle fibers within fascicles and peripherally positioned myonuclei. In the control group A, MyoD+ myonuclei appeared dark brown upon immunohistochemical staining. The mean number of MyoD+ myonuclei in the control group was 17.23 ± 0.259 (Table 1).

The cross-section of the skeletal muscle of group B showed polygonal bundles of skeletal muscle fibers with peripherally located nuclei. The mean number of MyoD+ myonuclei in the skeletal muscle sections of group B (Figure 1) was 22.60 ± 0.334 . This difference was statistically significant in control group A ($p < 0.001$) (Table 1).

Cross section of EDL of group C showed polygonal-shaped bundles of skeletal muscle fibers having peripherally located nuclei. The number of MyoD+ myonuclei was counted in sections of skeletal muscle (Figure 2) and a mean count of 31.63 ± 0.344 was observed. It was significantly higher when compared to groups A and B ($p < 0.001$) (Table 1).

Serum LDH levels were measured at the conclusion of the 3-month study period. In group A, the mean LDH levels were 158.18 ± 1.457 IU/l while it was 195.84 ± 2.185 IU/l for group B with a significant difference among both groups ($p < 0.001$). However, in group C, the mean LDH was 171.54 ± 3.350 IU/l which was significantly lower than that of group B but significantly higher than that of control group A ($p < 0.001$) (Table 2).

Discussion

Skeletal muscle has a remarkable capacity for regeneration, which relies on muscle stem cells known as SCs. Upon muscle injury, SCs are activated to proliferate and fuse to form new muscle fibers for repair¹⁷. This process requires coordinated expression of MRFs such as MyoD¹⁸.

A key finding of the current study was that treatment with the β_2 -adrenergic agonist formoterol significantly increased MyoD+ myonuclei in the regenerating muscle, indicating enhanced SC activation and myogenic potential to stimulate muscle repair. Specifically, formoterol administration resulted

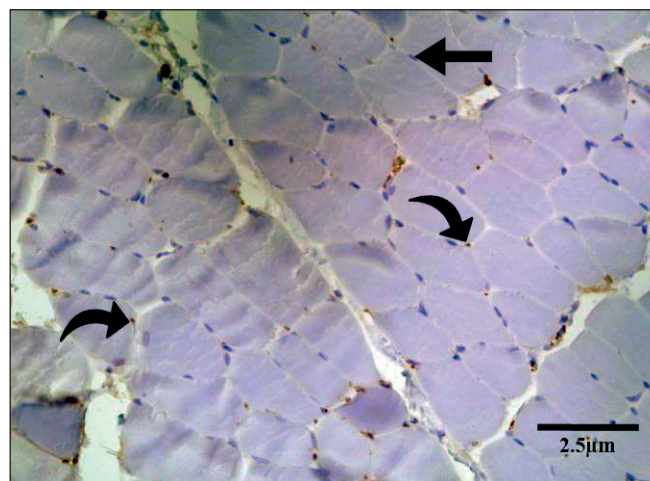


Figure 1. Transverse section of the EDL of a rat from group B. There are scattered MyoD positive satellite nuclei (curved arrow). MyoD-negative myonuclei lacked staining and appeared blue (straight arrows).

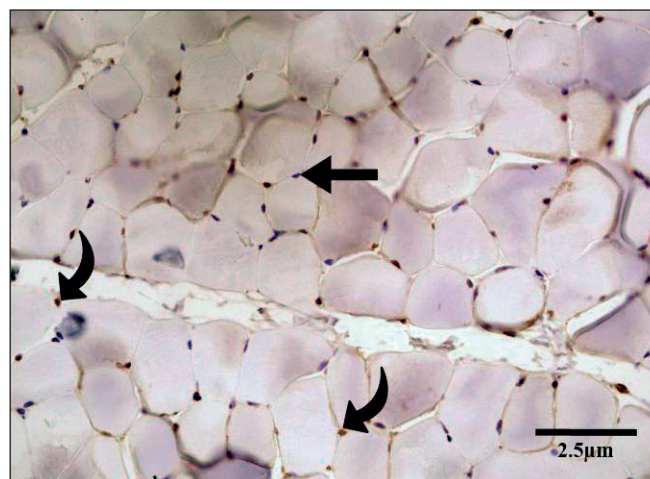


Figure 2. Transverse section of the EDL of a rat in Group C. There were numerous MyoD-positive satellite nuclei (curved arrows). (IHC, 400 \times). MyoD-negative myonuclei lacked staining and appeared blue (straight arrows).

in greater restoration of muscle architecture by day 90 post-injury compared to untreated injured muscle.

Previous studies of formoterol have provided insights into the mechanisms underlying its beneficial effects on muscle regeneration. Formoterol acts via β_2 -adrenergic receptors

Table 2. Comparison of LDH levels in three groups.

Parameters	Groups	Mean \pm SEM	Statistical significance (ANOVA) followed by <i>post-hoc</i> Tukey's test		
			Group A/B	Group A/C	Group B/C
LDH (IU/l)	A	158.18 \pm 1.457	$p < 0.001$	$p < 0.001$	$p < 0.001$
	B	195.84 \pm 2.185			
	C	171.54 \pm 3.350			

on muscles, triggering intracellular cyclic adenosine monophosphate-dependent signaling involved in muscle growth¹⁹. In rats, formoterol treatment activates anabolic pathways, leading to muscle hypertrophy and strength gain²⁰. Formoterol also inhibits proteolytic pathways and prevents muscle wasting in immobilized rats²¹.

In our study, formoterol-treated muscles showed increased MyoD+ nuclei and new myofiber formation, indicative of muscle hyperplasia. While we did not quantify the fiber number, prior work showed that formoterol increased myofiber formation in rats following botox-induced muscle paralysis²². MyoD drives myogenic differentiation, implying that formoterol improves regeneration by enhancing MyoD-mediated SC differentiation into mature muscles²³.

The analysis of serum LDH levels in the different experimental groups revealed significant variations. In group C, the LDH levels were significantly lower than those in group B, but significantly higher than those in group A (Table 2). These findings suggest that formoterol administration contributes to a reduction in LDH levels by promoting the restoration of skeletal muscle structure in degenerated muscles. Notably, animals exhibiting more severe histomorphological changes displayed higher LDH levels, whereas those showing better restoration of muscle architecture exhibited reduced LDH levels.

Our findings align with the already published literature, indicating that formoterol can stimulate muscle growth and regeneration²⁴. These results shed light on the potential of formoterol as a treatment for muscle diseases. Additional preclinical studies should be conducted to further explore the efficacy of formoterol in treating myopathies. Examining markers such as MyoD and LDH gives us clues into how formoterol may improve muscle repair at the molecular level. Overall, this study adds to the evidence that formoterol could prove useful as a therapy for certain muscle disorders. Further animal model testing is warranted to better understand whether formoterol may benefit patients with different types of muscle pathology.

Conclusion

This study concludes that formoterol improved the regenerative abilities of skeletal muscle cells in a rat model

of statin-induced myopathy. The activation of SCs increased MyoD expression and decreased LDH levels suggesting that formoterol may promote muscle regeneration. These results add to our understanding of how muscles can be restored after injury and point to potential therapies for muscle damage and disease in the future.

Limitations of the study

Despite providing valuable insights, this study has several limitations. It relies on animal models and lacks clinical data from human subjects. Although animal studies are informative, the lack of human data makes it difficult to directly apply these findings to real-world medical situations. In addition, the 12-week duration, although reasonable, may not fully capture the long-term impacts of the treatments that could emerge over longer periods. These results support the use of formoterol as a possible therapy for patients with high cholesterol and pulmonary diseases who also have muscle-related morbidities subsequent to the use of statins. Combining formoterol with statins could potentially control asthmatic symptoms and alleviate the myopathic side effects. Further clinical studies in humans are recommended to directly test the effects and safety of formoterol over extended periods of time.

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List of Abbreviations

EDL	Extensor digitorum longus
LDH	Lactate dehydrogenase
MRF	Myogenic regulatory factor
MyoD	Myogenic differentiation factor
SC	Satellite cell

Conflict of interest

None to declare.

Grant support and financial disclosure

None to disclose.

Ethical approval

This study was approved by the Ethical Review Committee of Army Medical College, Rawalpindi, Pakistan, vide Letter No. ERC ID/375 dated 22 Oct 2021.

Authors' contributions

AQ: Conception and design of study, acquisition of data, drafting of the manuscript with critical intellectual input.

SH: Design of study, acquisition, and analysis of data, drafting of the manuscript

FM: Acquisition of data, critically intellectual input

MSA: Acquisition of data, drafting of the manuscript

FU: Critical intellectual input, drafting of the manuscript

RK: Analysis of data.

ALL AUTHORS: Approval of the final version of the manuscript to be published

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