Original Article

Comparative Study of Prophylactic Role of Vitamin D Versus Coenzyme Q10 Against Statin Induced Myopathy in Adult Male Albino Rats: Histological and Immunohistochemical Study

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ABSTRACT

Introduction: Atorvastatin was chosen for this model because its elimination half-life is nearly 14 hours, property that stimulates drug's efficacy for lowering LDL compared with other Statins. Furthermore, it is given in an active form that prolongs its effect on HMG-C0A reductase. Therefore muscle impairment was intensified by Atorvastatin. C0-Q10 acts as free radical scavenger in skeletal muscle mitochondria. Vitamin D is strong antioxidant which maintains stable activities of mitochondria .

Aim of the Work: This work aimed to study changes in rat's skeletal muscle with atorvastatin administration and prophylactic role of coenzyme Q10 and vitamin D.

Material and methods: Fifty adult male albino rats were separated into five equal groups. Control group, Statin group , rats were received Atorvastatin at a dose 50 mg/kg/day , liquefied in distilled water and given by gastric tube for 4 weeks. Statin & Coenzyme Q10 treated group at which rats received Coenzyme Q10 at dosage of 3 mg/kg b.wt. during the period of Statin treatment. Statin &Vit D treated group , rats were treated with statin like that of group II and given Vit D orally at dosage of 0.5µg/kg/day and Withdrawal group at which rats retained for one month without treatment after 4 weeks of statin treatment. Skeletal muscle tissue was examined for histopathological and immunohistochemical changes.

Results: group treated with Atorvastatin revealed degenerated and disorganized muscle fibers. Also numerous collagen fibers were present within the CT septa in the masson & Van Gieson stained muscle sections. Strong KI-67 immunoreactivity and weak Desmin and Myogenin immuno expression. Coenzyme Q10 and vit D decreases the effect of statins on skeletal muscle tissue, but Coenzyme Q10 shown a significant diminution in collagen fibers deposition, KI-67 immunoreactivity and significant increase in Desmin & Myogenin immuno expression compared with that in Statin treated group.

Conclusion: Intake of Vitamin D during the period of Statin treatment have a protecting effect against statin induced myopathy. Meanwhile the consumption of Coenzyme Q10 has a more protection

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INTRODUCTION

Statins are generally used in the avoidance and management of coronary heart disease[1]. They inhibit 3-hydr0xy-3-methylglutaryl c0enzyme A reductase (HMG-C0A reductase) an enzyme involved in the synthesis of cholester0l. Also they are used in treatment of hyperlipidemia in patients with chr0nic liver diseases and are the most effective drugs for decreasing LDL cholester0l[2]. Statins are broken down to reactive metabolites, increase creation of reactive oxygen species (ROS) and induce mitochondrial injury[3]. The precise mechanism of myopathy caused by statin is still unknown. However, numerous mechanisms have been theorized in this concern, statins have been related to inhibition of mitochondria complex III leading to dysfunction of the skeletal muscle mitochondria[4]. Many patients must use higher doses of statin to attain lower level of cholesterol, therefore the risk of myopathy is raised[5].

Coenzyme Q10 (C0-Q10) is a quinone which is naturally occurring and fat-soluble present in the hydrophobic portions of the cell membranes[6]. It is produced in the HMG-C0A reductase pathway. It is an isoprenoid which shows an important role in the chain of electron transport, and decrease in CoQ10 could cause abnormal mitochondrial respiratory function, leading to diminished production of energy and cause myopathy[7]. C0-Q10 protects against statin-induced oxidative damage in skeletal muscle mitochondria because it acts as free radical scavenger[8]. A prior study revealed that treatment with statin could result in decline in circulating C0Q10[9]. C0enzyme Q10 (C0Q10) depletion may interpret for the prospective myotoxicity effect of statins[10].

Vitamin D is a steroid hormone that is regularly acquired from dietary sources. In addition, it is also formed in the skin subsequent to exposure to UVB light which permits the synthesis of vitamin D3[11]. It is also a powerful antioxidant that allows stable mitochondrial activities, inhibiting oxidative stress-related protein oxidation, lipid peroxidation, and DNA damage[12]. Low level of vitamin D is associated with myopathy and weakness of muscle[13].

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A previous study showed that statin-induced myopathy could be revocable with supplementation of vitamin D[14].

MATERIAL AND METHODS

experimental design and animals

This experimental study was done on 50 healthy adult male albino rats (2 months-old) their weight 180-200 gram. The rats were gotten from the unit of Laboratory Animals, Faculty of Veterinary Medicine, Benha University, Egypt. The rats were retained in cages formed of plastic to avoid any metallic contact under environmental laboratory circumstances at 20 ± 2°C. The animals were exposed to a controlled photo period (14 h: 10 h light:dark), were nourished standard diet and allowed water ad-libitum. The experiment was completed in agreement with the "Guide for Care and Usage of Laboratory Animals". The protocol of experiment was permitted by the Committee of Ethics, Benha University. After a period of 1 week for acclimatization, rats were randomly divided into 5 equal groups: I, II, III , IV& V

Group I (control group): Ten rats were divided into 2 subgroups, each 5 rats:

- Group Ia: rats were given only standard diet and tap water .
- Group Ib: each rat was given distilled water by gastric tube.

Group II (statin treated group): Ten rats that were received Atorvastatin at a dose 50 mg/kg/day, which dissolved in distilled water and given through a gastric tube for 4 weeks[15].

Group III: (statin+ coenzyme Q10 treated group): Ten rats that received atorvastatin at a dose 50 mg/kg/day dissolved in distilled water combined with coenzyme Q10 treatment at a dose 3 mg/day/animal liquefied in distilled water, which was corresponding to 200 mg/day for humans and was given using a gastric tube for 4 weeks[16].

Group IV (statin +vit D treated group): Ten rats that were received atorvastatin at a dose 50 mg/kg/day and were treated at the same time with Alfacalcidol (0.5µg/kg/day) liquefied in distilled water using a gastric tube for 4 weeks[17].

Group V (Withdrawal group): Ten rats kept for one month without any treatment after 4 weeks treatment with atorvastatin at a dose 50 mg/kg/day which was liquefied in distilled water.

Reagents

Atorvastatin: Trade name ATOR, produced by Egyptian Int. Pharmaceutical Industries Co., in the form of tablets (each 40 mg).The drug prepared by crushing and dissolving it in distilled water.

Coenzyme-Q10: was produced by Arab Company of Pharmaceutuical and Medicinal plants, MEPACO-Egypt in capsule form of 30 mg using distilled water to dissolve it.

Alfacalcidol (ALF): Trade name BON-ONE, manufactured by Minipharm Egypt, in a tablet form(each 1 µg active ingredient) that were crushed and dissolved in distilled water.

Collection of blood samples

1. At the termination of the experimental study , the animals were retained without eating all over the night and then they were given intraperitoneal (50 mg/kg) of thiopental in the morning. the abdominal cavity was opened longitudinally after fixation of The animals on the dissecting table.
2. collection of blood samples through using a syringe from the heart, and then Centrifuged at 600g for a period of 10 minutes to get the serum, it was kept at −20 °C until assess.

Biochemical analysis

1. The total plasma level CPK was measured through standard spectrophotometric analysis via using the obtainable diagnostic kit of (Sigma Aldrich Co.,Cairo, Egypt) .
2. Malondialdehyde (MDA) measured by using the colorimetric kits (Bi0-Diagnostics, Cair0, Egypt[18]

Histological and immunohistochemical examinations

At the end of experimentation according to timing mentioned in each group, the rats have been anesthetized using an intraperitoneal sodium pentobarbital injection (30 mg/kg body weight of Nembutal) for sacrificing.

Specimens from the middle part of biceps femoris muscle of both limbs were dissected and excised.

Specimens from both limbs were fixed for 24 hours using 10% buffered formalin solution, dehydrated in ethanol by using ascending grades of it and then inserted in paraffin. Sections of 5 µm were subjected to:

- Hematoxylin and Eosin stain (H&E): for studying the general structure[19]
- Masson's trichrome stain: was used to stain the collagen fibers (stains the collagen-rich fibrotic regions in blue)
- Van Gieson's stain was used to detect collagen fibers which stained red.

Immunohistochemical analysis

KI67

The tissues were fixed using 10% formalin, dehydrated, cleared, and then inserted in paraffin. 5 µm Sections were deparaffinized and hydrated in ascending sequences of alcohols. The tissue sections were kept with 3% H2O2 solution at 27°C for 20 minutes. Then kept with standard rabbit serum working solution (Biosharp, Hefei, China) at 27°C for 30 minutes. Next, the sections were kept overnight at 4°C with a rabbit anti-mouse Ki-67 monoclonal

antibody (1:400 dilution; Cell Signaling Technology) from Sigma (St. Louis, MO, USA). rabbit anti-mouse IgG secondary polyclonal antibodies (dilution, 1:1,000; cat. no. ab6789; Abcam, Cambridge, MA, USA) were used next for incubation for 20 minutes at 27°C. Finally, the sections were kept with horse-radish peroxidase-labeled streptavidin working solution for 20 minutes at 27°C. The sections were washed 3 times with phosphate-buffered saline, each time 5 minutes, and addition of DAB solution. The sections were counterstained with hematoxylin to stain nuclei, dehydrated, cleared in xylene and mounted with DPX.[20]

Myogenin immunohistochemistry

Myogenin immunoperoxidase staining was achieved using the myf-4 antibody (L026, 1:10, mouse monoclonal, Novocastra Labs, Burlingame, CA), as stated by the manufacturer's mentioned protocol. Antigen recovery was done in a pressure cooker for 40 minutes in 10 mM citrate buffer with 0.1% Tween 20 at pH 6.0. Slides were kept with the primary antibody overnight, followed by detection using standard protocol on an automated immunostainer (Ventana Medical Systems, Tuscon, AZ), according to the instructions of manufacturer.[21]

Desmin immunohistochemistry

Tissue sections were deparaffinized using xylene, rehydrated using alcohols in descending sequences, and then transported to distilled water. By using 3 % H2O2 in distilled water for 10 minutes at RT, endogenous peroxidase activity was blocked, then sections were washed for 3×5 min duration in phosphate-buffered saline its pH was 7.3. The slides were transported into a humidified place, and then adding blocking serum for 20 minutes at RT. Sections incubated with pre-diluted desmin antibody for 30 minutes at RT, washed with phosphate-buffered saline (pH 7.2) for a period of 5 minutes, kept with secondary antibody for a period of 30 minutes, and followed by washing in several modifications of PBS. Visualization was with diaminobenzidine (DAB) chromogen by addition of 14 µl of hydrogen peroxide to 2 ml of diaminobenzidine stock solution, and sections were kept for 7 minutes in a dark place. Mayer's hematoxylin for 2 minutes to counterstained Nuclei, cleaned with tap water, then dehydrated using ascending sequences of alcohol, cleared by using xylene, and cover slips were fixed using DPX.[22]

Morphometric study

Successive sections were evaluated morphometrically by using image analyzer computer system Ltd "Leica Qwin 500 C". (Cambridge, England). That was done in 5 non overlying fields of 5 dissimilar sections from 5 dissimilar rats in every group at × 400. to measure the following parameters:

1. Area % of collagen fibers in section stained with Masson's trichrome
2. Area % of the immunopositive reaction of sections stained with KI 67, Myogenin and desmin immunostain.

Statistical analysis

The data achieved from the biochemical records and image analyzer were subjected to (SPSS Inc., Chicago, IL,USA) ® version 22 for Windows. From all groups data were stated as (mean ± standard deviation).

One-way ANOVA was used to relate between more than two groups. when $P < 0.05$ was considered significant.

RESULTS

Biochemical results

Mean Serum MDA & CPK levels were increased significantly in Atorvastatin treated group compared to control group ($P \leq 0.05$), while in Atorvastatin & Coenzyme Q10 treated group (group III) these serum levels were decreased significantly in comparing with Atorvastatin treated and recovery groups (groups II & V) ($P \leq 0.05$) and increased insignificantly in comparison to control group ($P > 0.05$), in Atorvastatin & Vitamin D treated group (group IV) these levels were insignificantly increased in comparing with control and Atorvastatin & Coenzyme Q10 treated groups ($P > 0.05$) but they were significantly decreased in comparing with Atorvastatin treated and recovery groups (groups II & V) ($P \leq 0.05$) (Tables 1,2, Histograms 1,2).

Microscopic Results

Histological stains

H &E stain

Group I (Control group): Tissue analysis of control subclasses; Ia and Ib revealed nearly the same configuration. We used figures of the control subgroup Ib to discriminate with other groups. Transverse sections of the skeletal muscle fibers of the control group I showed regular organization of skeletal muscle bundles. Each bundle consisted of a group of muscle fibers surrounded by C.T. perimysium. Muscle fibers appeared polyhedral with peripherally located oval nuclei and acidophilic sarcoplasm separated by narrow C.T. endomysium (Figure 1A). Longitudinal sections of the skeletal muscle of the control group (GI) exposed parallel elongated, cylindrical muscle fibers. They were separated from each other by fine loose connective tissue (endomysium). Muscle fibers have acidophilic sarcoplasm, multiple peripheral oval nuclei and showed well-defined transverse striations (Figure 1B).

Group II (Atorvastatin treated group): Histological inspection of H&E stained T.S. of skeletal muscle of Atorvastatin treated group (G2) shown many fibers that acquired an irregular outline rather than polygonal, Others were rounded. Focal areas of the sarcoplasm of different muscle fibers appeared either deeply acidophilic or lightly stained in others. Loss of striations, centrally placed inflammatory cells, vacuolization and fragmentation were perceived (Figure 2A). In L.S., disorganized, fragmented

and discontinued muscle fibers were observed . Darkly stained nuclei were shown with Loss of striations. Endomysial and sarcoplasmic mononuclear cellular infiltration and degenerated fibres were seen (Figure 2B).

Group III (Atorvastatin + Coenzyme Q10 treated group): Histology of Atorvastatin& Coenzyme Q10 treated group (G III) have appeared nearly similar to control group. Examination of H&E stained T.S. showed apparently normal polygonal muscle fibers with acidophilic sarcoplasm (Figure 3A). L.S. showed regularly arranged parallel muscle fibers. Some nuclei were peripheral and oval; and others were pale and centrally located (Figure3B).

Group IV (Atorvastatin +Vit.D treated group): Histology of Atorvastatin& Vit.D treated group (G IV) have appeared nearly similar to control group .Examination of H&E stained T.S. showed bundles of apparently normal polygonal muscle fibers with acidophilic sarcoplasm, while few muscle fibers appeared rounded with peripherally arranged oval dark nuclei but some nuclei were centrally located. Few fibers appeared with disintegration and hyaline degeneration (Figure 4A). L.S. showed regularly arranged parallel muscle fibers. Nuclei were dark, peripheral and oval. Centrally located inflammatory cell was seen (Figure 4B).

Group V (Withdrawal group): Transverse section of rat skeletal muscle of withdrawal group showing many fibers with irregular outline, Focal areas of sarcoplasm are deeply acidophilic and others are lightly stained, endomysial mononuclear cellular infiltration ,vacuolaization of sarcoplasm and darkly stained nuclei displaced away from the periphery (Figure 5A). Longitudinal section of rat skeletal muscle of withdrawal group showing disorganized, fragmented and degenerated muscle fibers with darkly stained nuclei. Loss of striations and sarcoplasmic mononuclear cellular infiltration were seen (Figure 5B).

Masson trichrome stain

Little amount of collagen fibers was detected in between muscle fibers of Masson stained skeletal muscle sections of control group (Figure 1c)

Increased amount of collagen fibers around and within the muscle fibers were noticed in the Masson stained skeletal muscle sections of (Atorvastatin treated group) (Figure 2c).

Minimal amount of collagen fibers in-between muscle fibers could be seen in Masson stained skeletal muscle sections of (Atorvastatin & Coenzyme Q10 treated group) (Figure 3c).

Minimal amount of collagen fibers were seen in-between muscle fibers of Masson stained skeletal muscle sections of (Atorvastatin + Vitamin D treated group) (Figure 4c).

Moderate amount of collagen fibers around degenerated muscle fibers and also around the nerve fibers were detected in Masson stained skeletal muscle sections of withdrawal group (Figure 5c).

Immuno-stained results

Desmin

The immunohistochemical results revealed increased brown coloration of sarcoplasm that indicates strong positive cytoplasmic reaction for desmin in the skeletal muscle of the control group (G1) (Figure 1D).

Decreased brown coloration of sarcoplasm that indicates negative cytoplasmic reaction for desmin were shown in skeletal muscle sections of the Atorvastatin treated group (G2) (Figure 2D).

Skeletal muscle sections of Atorvastatin & Coenzyme Q10 treated group showing moderate brown coloration of sarcoplasm that indicates positive cytoplasmic reaction for desmin (Figure 3D) .

Skeletal muscle sections of Atorvastatin & Vitamin D treated group revealed moderate brown coloration of sarcoplasm that indicates positive cytoplasmic reaction for desmin (Figure 4D) .

While skeletal muscle sections of Withdrawal group showing decreased brown coloration of sarcoplasm that indicates negative cytoplasmic reaction for desmin. (Figure 5D).

Myogenin

Negative myogenin immunoreactivity was shown in nuclei of skeletal muscle sections of control group (G1) (Figure 1E).

Results revealed negative expression of myogenin in the nuclei of skeletal muscle fibers of Atorvastatin treated group (G2) (Figure 2E).

Nuclei of the skeletal muscle fibers of Group III (Atorvastatin & Coenzyme Q10 treated group) revealed strong expression for myogenin (Figure 3E) .Also nuclei of skeletal muscle fibers of Group IV (Atorvastatin & Vitamin D treated group) revealed moderate myogenin expression (Figure 4E).

While in Withdrawal group (group V), there is very weak positive myogenin immune-expression (Figure 5E).

KI-67

The immunohistochemical results revealed the negative expression of KI-67 in the nuclei of skeletal muscle fibers of the control group (G1) (Figure 1F).

A strong KI-67 immunoreactivity was shown in skeletal muscle sections of Atorvastatin treated group (G2) (Figure 2F).

Nuclei of the skeletal muscle fibers of Group III (Atorvastatin & Coenzyme Q10 treated group) revealed very weak KI-67 expression (Figure 3F) in comparison with both the control group and Atorvastatin treated group.

Also nuclei of skeletal muscle fibers of Group IV (Atorvastatin & Vitamin D treated group) revealed weak KI-67 expression (Figure 4 F) .

While in withdrawal group (group V), there is moderate KI-67 expression (Figure 5F).

Van Geisson Stain

Van Geisson stained skeletal muscle sections of control group(GI) showing decreased reddish coloration which means decreased collagen fiber deposition (Figure 1G).

Van Geisson stained skeletal muscle sections of Atorvastatin treated group (G II) showing increased reddish coloration which means increased collagen fiber deposition (Figure 2G).

Van Geisson stained skeletal muscle sections of Atorvastatin & Coenzyme Q10 treated group (G III) showing decreased reddish coloration which means decreased collagen fiber deposition (Figure 3G).

Van Geisson stained skeletal muscle sections of Atorvastatin & Vitamin D treated group (G IV) showing decreased reddish coloration which means decreased collagen fiber deposition (Figure 4G).

Van Geisson stained skeletal muscle sections of withdrawal group (G V) showing increased reddish coloration which means increased collagen fiber deposition (Figure 5G).

Morphometric results

The mean area % of collagen deposition in masson stained sections for all groups has been represented in (Table 3, Histogram 3). There was significant rise in mean area% of collagen deposition ($P \leq 0.02$) in group II in comparing with groups I,III &IV. But mean area percentage of collagen deposition has increased in groups III & IV without significant difference in comparison to control group ($P>0.05$). Also, mean area % of collagen deposition has increased insignificantly in group IV in comparing with group III ($P>0.05$). There was high significant increase in the mean area % of collagen deposition ($P \leq 0.02$) in group V in comparing with groups I,III & IV.

The mean area percent of Desmin immuno-expression for all groups was represented in (Table 4, Histogram 4). The mean area percent of Desmin immunoreactivity has highly significantly diminished in group II in comparing with control group I ($P< 0.02$). In group III, the mean area percent of Desmin immunoreactivity has decreased with no significant difference in comparing with control group ($P> 0.05$) and it has highly significantly increased in comparing with group II ($P<0.02$). Also, the mean area percent of Desmin immunoreactivity has highly significantly increased in group IV in comparing with group II ($P< 0.02$) but it has decreased with no significant difference when compared with control group ($P> 0.05$) and it has insignificantly decreased in comparing with group III ($P> 0.05$). The mean area percent of Desmin immunoreactivity has highly significantly decreased in withdrawal group in comparing with groups I,III & IV ($p \leq 0.02$), but it has slightly decreased with no significant difference in comparing with group II.

The mean area percent of Ki-67 immuno-expression for all groups was represented in (Table 5, Histogram 5). There was significant rise in mean area% of Ki-67 ($P \leq 0.02$) in group II in comparing with groups I, III &IV. But the mean area percentage of Ki-67 has increased in groups III & IV without significant difference in comparison to control group ($P>0.05$). Also, mean area % of Ki-67 has increased insignificantly in group IV in comparing with group III ($P>0.05$). There was high significant increase in the mean area % of Ki- 67 ($P \leq 0.02$) in group V in comparing with groups I,III & IV.

The mean area percent of myogenin immuno-expression for all groups was represented in (Table 6, Histogram 6). There was significant rise in mean area% of myogenin ($P \leq 0.02$) in group III and group IV in comparing with groups I, II & V. Also, the mean area % of myogenin has increased insignificantly in group III in comparing with group IV ($P>0.05$).

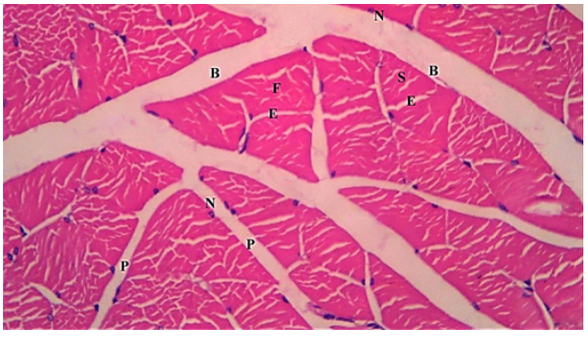


Fig. 1A: A photomicrograph of transverse section of rat skeletal muscle of control group showing normal organization of skeletal muscle bundles (B). Each bundle consisted of a group of muscle fibers surrounded by C.T. perimysium (P). Muscle fibers appeared polyhedral (F) with peripherally located oval nuclei (N) and acidophilic sarcoplasm (S) separated by narrow C.T. endomysium (E). (H&E X400)

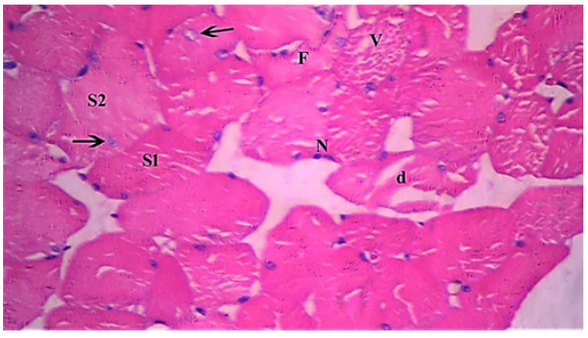


Fig. 2A: A photomicrograph of transverse section of rat skeletal muscle of group treated with Atorvastatin showing many fibers with irregular outline (F), Focal areas of sarcoplasm are deeply acidophilic (S1) and others are lightly stained (S2), Vacuolaization of sarcoplasm (V) , darkly stained nuclei (N) , centrally placed Inflammatory cells (black arrow) and some muscle fibers are disintegrated (d). (H&E x 400)

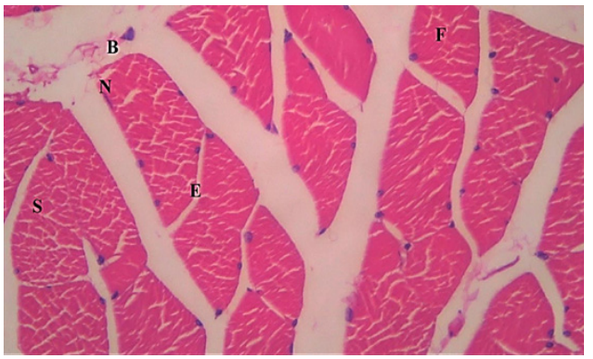


Fig. 3A: A photomicrograph of transverse section of rat skeletal muscle of Atorvastatin & Coenzyme Q10 treated group showing normal organization of skeletal muscle bundles (B), Muscle fibers appeared polyhedral (F) with peripherally located oval dark nuclei (N) and acidophilic sarcoplasm (S) separated by narrow C.T. endomysium (E). (H&E x400)

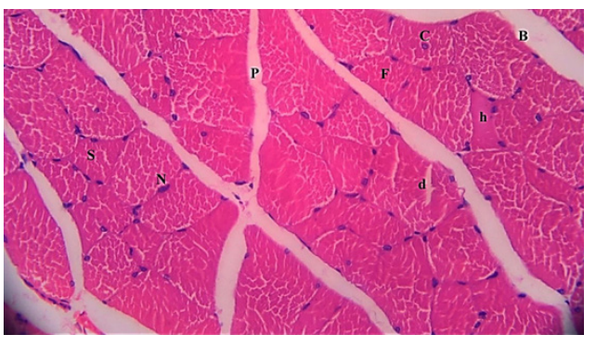


Fig. 4A: A photomicrograph of transverse section of rat skeletal muscle of Atorvastatin & vitamin D treated group showing normal organization of skeletal muscle bundles (B), Muscle fibers appeared polyhedral (F) with peripherally located oval dark nuclei (N) , few centrally located inflammatory cells (C) and acidophilic sarcoplasm (S),some muscle fibers show disintegration (d) and others show hyaline degeneration (h). (H&E x400)

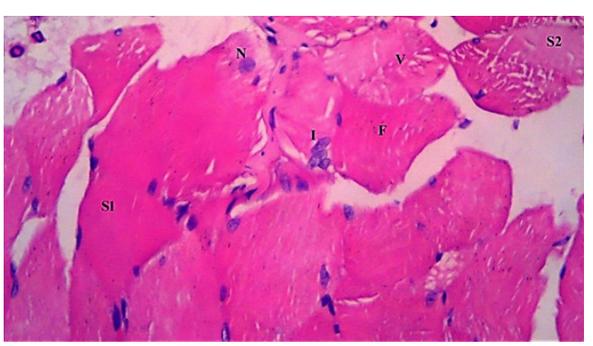


Fig. 5A: A photomicrograph of transverse section of rat skeletal muscle of withdrawal group showing many fibers with irregular outline (F), Focal areas of sarcoplasm are deeply acidophilic (S1) and others are lightly stained (S2), endomysial mononuclear cellular infiltration (I),Vacuolaization of sarcoplasm (V) and darkly stained nuclei displaced away from the periphery(N). (H&E X 400)

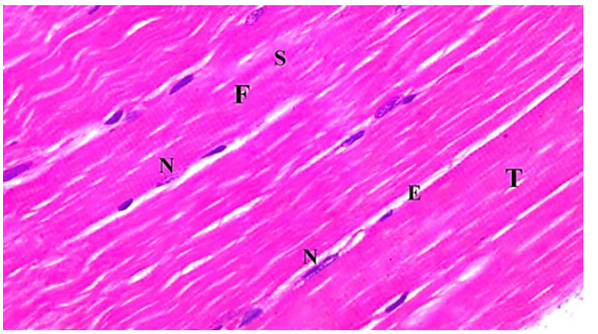


Fig. 1B: A photomicrograph of longitudinal section of rat skeletal muscle of control group showing parallel elongated, cylindrical muscle fibers (F) with well-defined transverse striations (T) . They were separated from each other by fine loose connective tissue i.e endomysium (E). Muscle fibers have acidophilic sarcoplasm(S), multiple peripheral oval nuclei (N). (H&E X400)

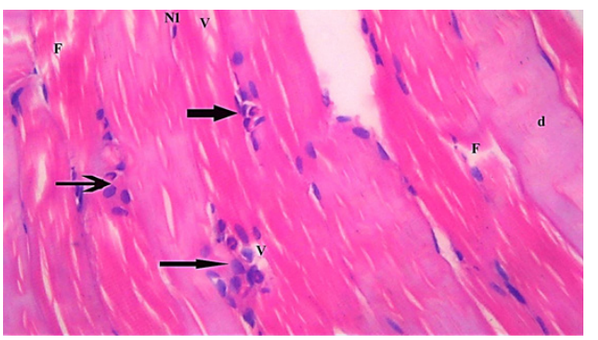


Fig. 2B: A photomicrograph of longitudinal section of rat skeletal muscle of Atorvastatin treated group showing disorganized, fragmented and discontinued muscle fibers (F) with darkly stained nuclei(N1) ,degenerated fibres were seen(d). Loss of striations , vacuolization and fragmentation were seen(V) ,endomysial and sarcoplasmic mononuclear cellular infiltration appeared (black arrow). (H&E X 400)

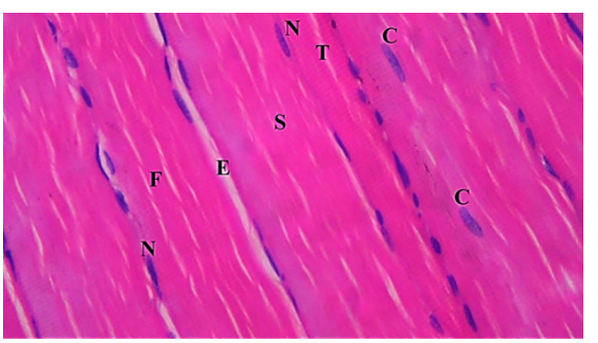


Fig. 3B: A photomicrograph of longitudinal section of rat skeletal muscle of Atorvastatin & Coenzyme Q10 treated group showing parallel elongated, cylindrical muscle fibers (F)with well-defined transverse striations(T) . They were separated from each other by fine loose connective tissue i.e endomysium (E). Muscle fibers have acidophilic sarcoplasm (S), multiple peripheral oval nuclei (N) but there is some centrally placed nuclei (C) . (H&E X400)

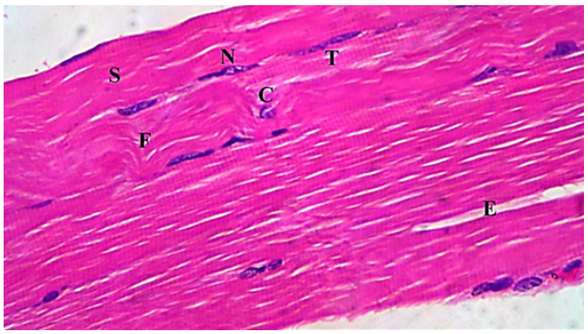


Fig. 4B: A photomicrograph of longitudinal section of rat skeletal muscle of Atorvastatin & Vitamin D treated group showing parallel elongated, cylindrical muscle fibers (F)with well-defined transverse striations(T) . They were separated from each other by fine loose connective tissue i.e endomysium (E). Muscle fibers have acidophilic sarcoplasm (S), multiple peripheral oval nuclei (N) and few centrally placed inflammatory cells (C) . (H&E X400)

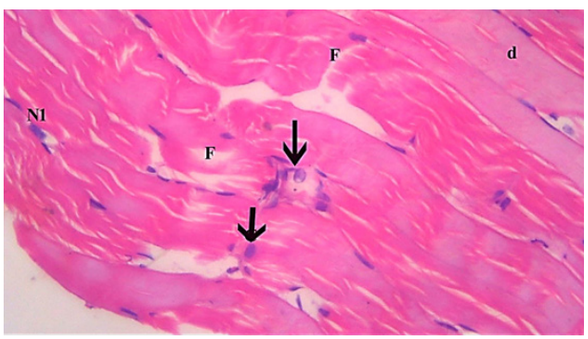


Fig. 5B: A photomicrograph of longitudinal section of rat skeletal muscle of withdrawal group showing disorganized, fragmented (F) and degenerated (d) muscle fibers with darkly stained nuclei (N1). Loss of striations and sarcoplasmic mononuclear cellular infiltration (black arrow) were seen. (H&E X 400)

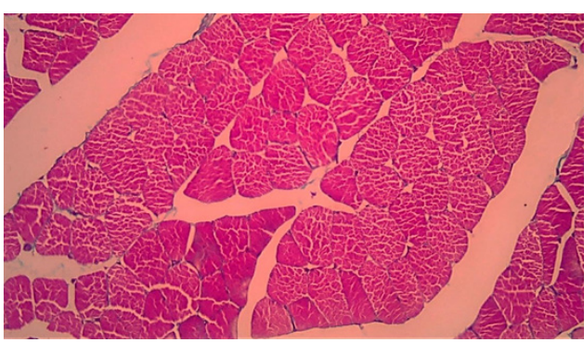


Fig. 1C: A photomicrograph of a section of the skeletal muscle of control adult rat showing minimal amount of collagen fibers in-between the muscle fibers. (Masson ,s trichrom X 200)

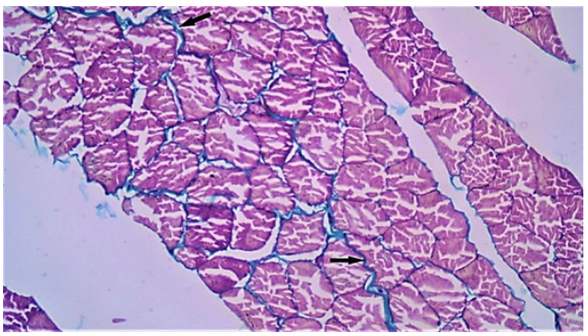


Fig. 2C: A photomicrograph of a section of adult rat Skeletal muscle treated with Atorvastatin showing increased amount of collagen fibers (Black arrow) in between muscle fibers. (Masson ,s trichrom X 200)

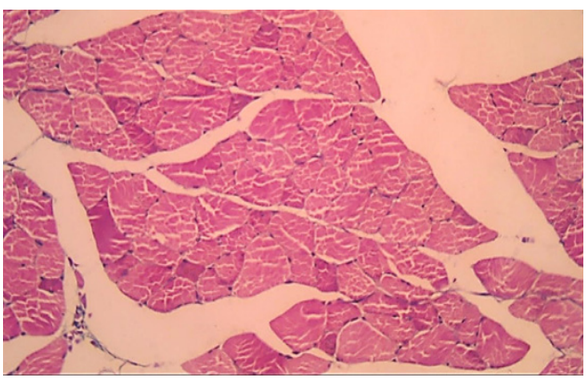


Fig. 3C: A photomicrograph of a section of the skeletal muscle of Atorvastatin & Coenzyme Q10 treated group showing minimal amount of collagen fibers in-between the muscle fibers. (Masson ,s trichrom X 200)

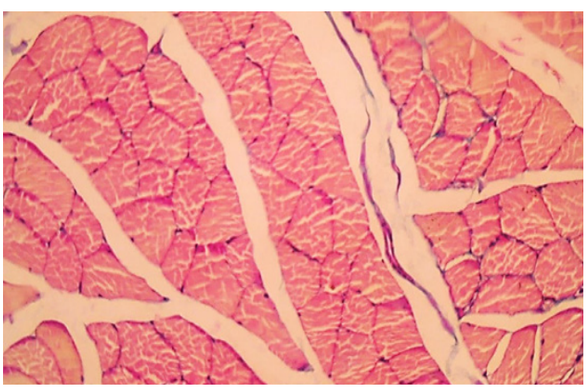


Fig. 4C: A photomicrograph of a section of the skeletal muscle of Atorvastatin & Vitamin D treated group showing minimal amount of collagen fibers in-between the muscle fibers. (Masson ,s trichrom X 200)

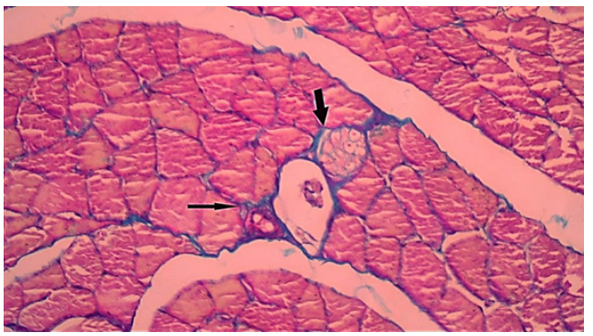


Fig. 5C: A photomicrograph of a section of adult rat skeletal muscle of withdrawal group showing moderate amount of collagen fibers inbetween degenerated muscle fibers and also around the nerve fibers (Black arrow). (Masson ,s trichrom X 200)

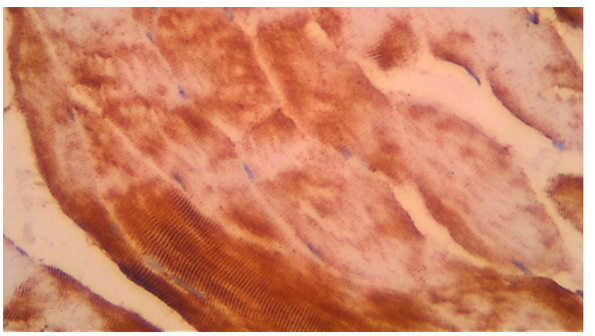


Fig. 3D: An immunostained photomicrograph of skeletal muscle of Atorvastatin & Coenzyme Q10 treated group showing moderate brown coloration of sarcoplasm that indicates positive cytoplasmic reaction (Desmin immune staining with counter stain hematoxylin X 400)

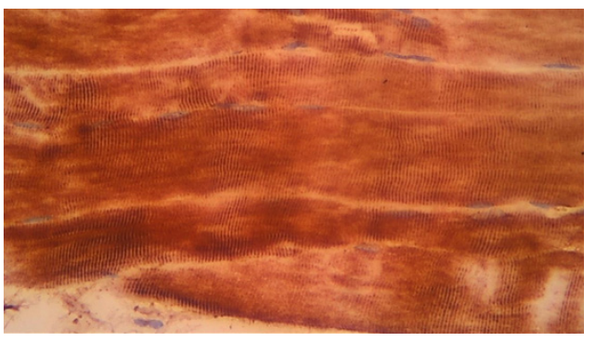


Fig. 1D: An immunostained photomicrograph of skeletal muscle of control group showing increased brown coloration of sarcoplasm that indicates positive cytoplasmic reaction (Desmin immune staining with counter stain hematoxylin X 400)

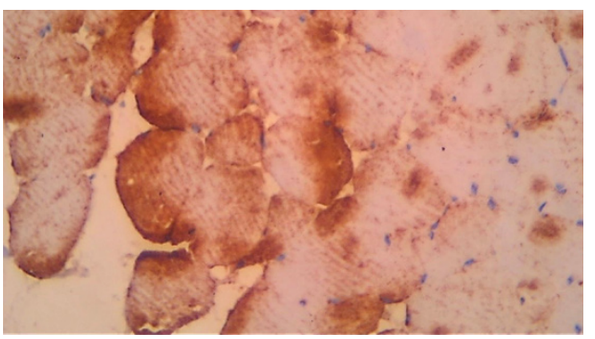


Fig. 4D: An immunostained photomicrograph of skeletal muscle of Atorvastatin & Vitamin D treated group showing moderate brown coloration of sarcoplasm that indicates positive cytoplasmic reaction (Desmin immune staining with counter stain hematoxylin X 400)

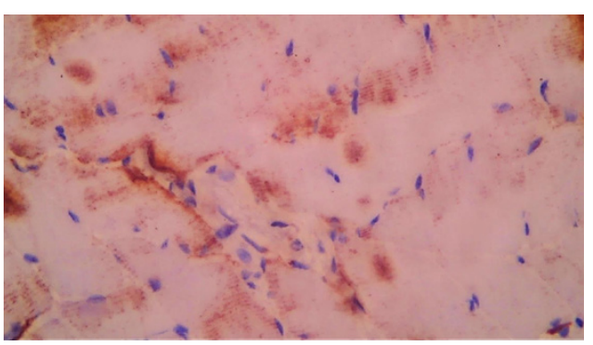


Fig. 2D: An immunostained photomicrograph of skeletal muscle of Atorvastatin treated group showing decreased brown coloration of sarcoplasm that indicates negative cytoplasmic reaction. (Desmin immune staining with counter stain hematoxylin X 400)

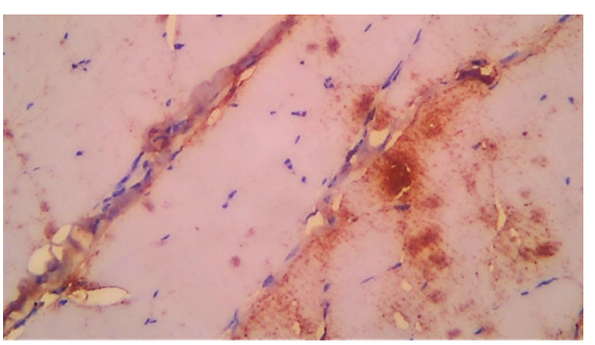


Fig. 5D: An immunostained photomicrograph of skeletal muscle of withdrawal group showing decreased brown coloration of sarcoplasm that indicates negative cytoplasmic reaction. (Desmin immune staining with counter stain hematoxylin X 400)

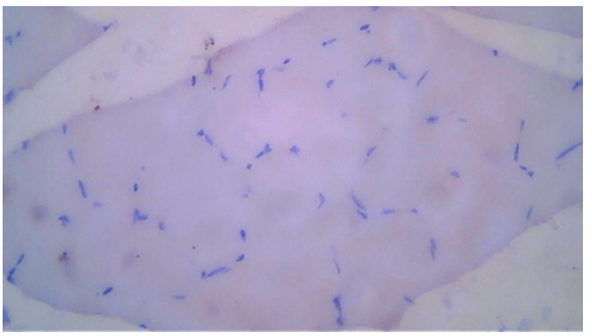


Fig. 1E: An immunostained photomicrograph of transverse section of skeletal muscle of control group showing negative immune reaction. (Myogenin immune staining with counter stain hematoxylin x 400)

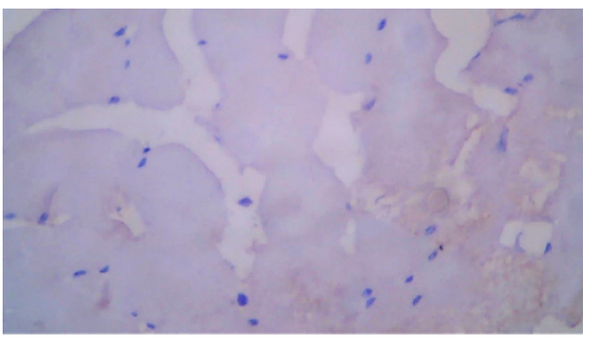


Fig. 2E: An immunostained photomicrograph of transverse section of skeletal muscle of Atorvastatin treated group showing negative immune reaction. (Myogenin immune staining with counter stain hematoxylin x 400)

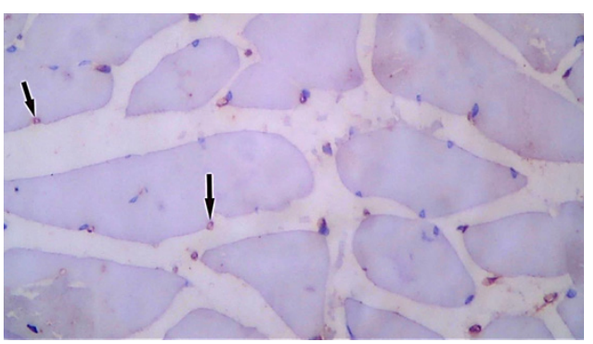


Fig. 3E: An immunostained photomicrograph of transverse section of skeletal muscle of Atorvastatin & Coenzyme Q10 treated group showing multiple immunopositive nuclei close to sarcolemma of muscle fibers (Black arrow). (Myogenin immune staining with counter stain hematoxylin x 400)

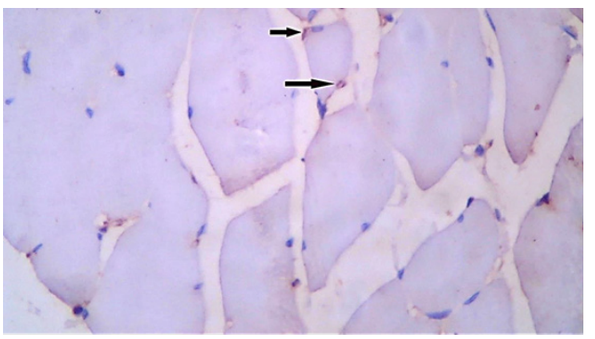


Fig. 4E: An immunostained photomicrograph of transverse section of skeletal muscle of Atorvastatin & Vitamin D treated group showing some immunopositive nuclei close to sarcolemma of muscle fibers (Black arrow). (Myogenin immune staining with counter stain hematoxylin x 400)

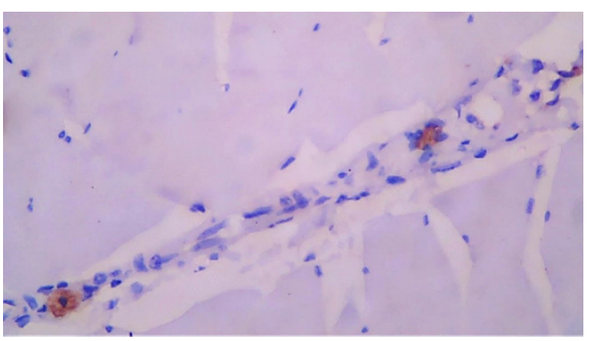


Fig. 5E: An immunostained photomicrograph of transverse section of skeletal muscle of withdrawal group showing weak positive immune reaction. (Myogenin immune staining with counter stain hematoxylin x 400)

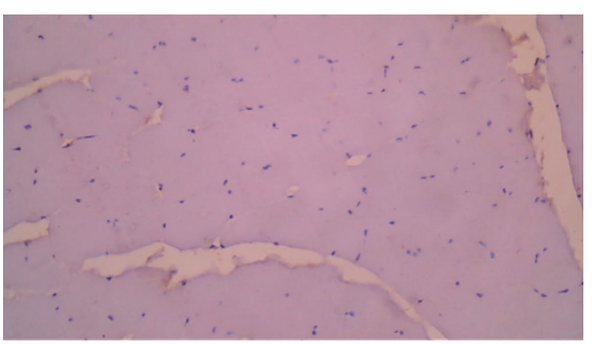


Fig. 1F: An immunostained photomicrograph of skeletal muscle of the control group showing a negative immunostaining for KI67. (KI67 immune staining with counter stain hematoxylin X200)

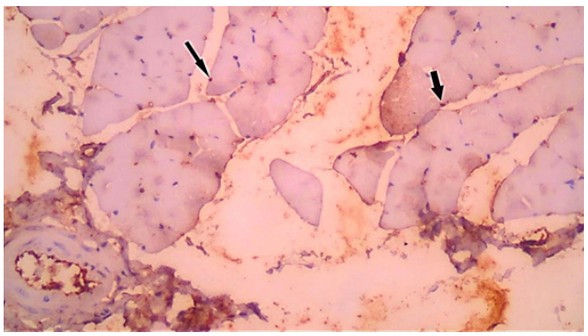


Fig. 2F: An immunostained photomicrograph of skeletal muscle of Atorvastatin treated group showing a strong positive nuclear immunostaining for KI67 (black arrow). (KI67 immune staining with counter stain hematoxylin X200)

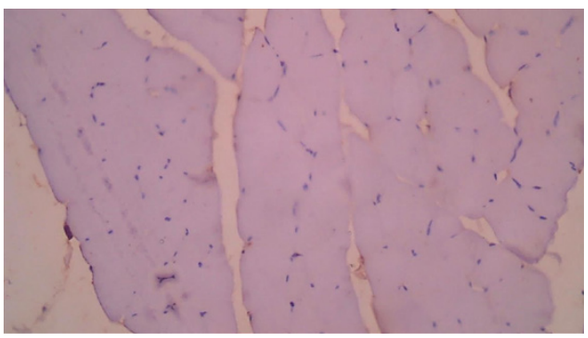


Fig. 3F: An immunostained photomicrograph of skeletal muscle of Atorvastatin & Coenzyme Q10 treated group showing a very weak nuclear immunostaining for KI67. (KI67 immune staining with counter stain hematoxylin X200)

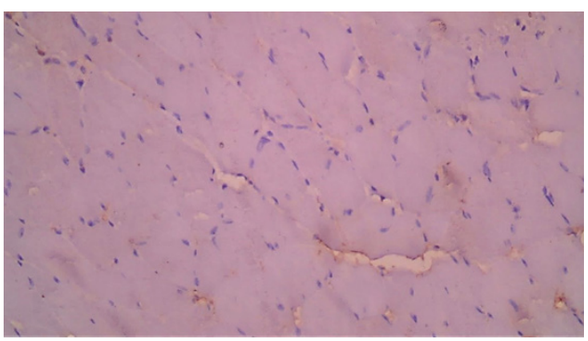


Fig. 4F: An immunostained photomicrograph of skeletal muscle of Atorvastatin & Vitamin D treated group showing a weak positive nuclear immunostaining for KI67. (KI67 immune staining with counter stain hematoxylin X200)

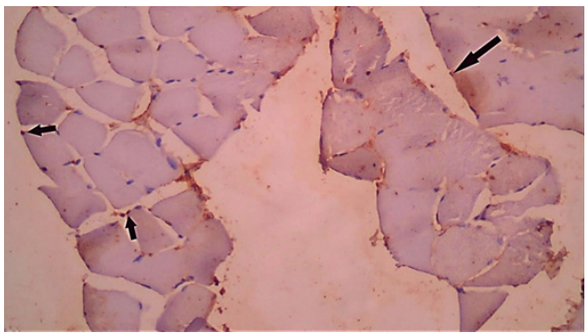


Fig. 5F: An immunostained photomicrograph of skeletal muscle of the withdrawal group showing a positive nuclear immunostaining for KI67 (black arrow). (KI67 immune staining with counter stain hematoxylin X200)

Fig. 1G: A photomicrograph transverse section of rat skeletal muscle of control group showing decreased reddish coloration which means decreased collagen fiber deposition. (Van Geisson X 400)

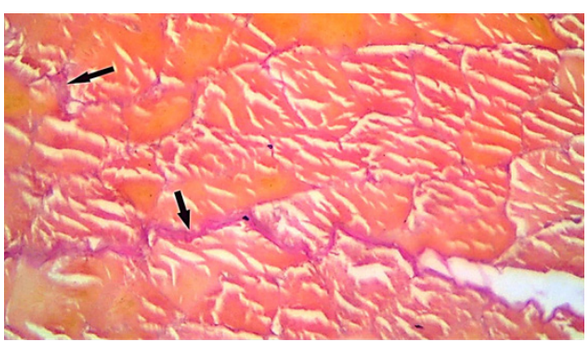


Fig. 2G: A photomicrograph transverse section of rat skeletal muscle of Atorvastatin treated group showing increased reddish coloration (Black arrow) which means increased collagen fiber deposition. (Van Geisson X 400)

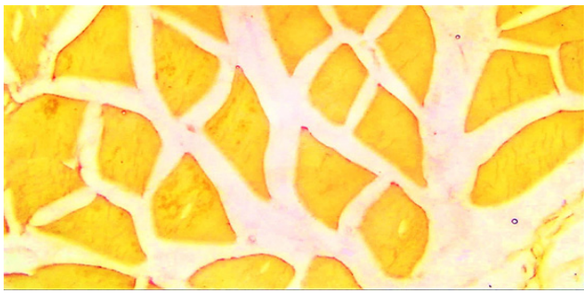


Fig. 3G: A photomicrograph transverse section of rat skeletal muscle of Atorvastatin & Coenzyme Q10 treated group showing decreased reddish coloration which means decreased collagen fiber deposition. (Van Geisson X 400)

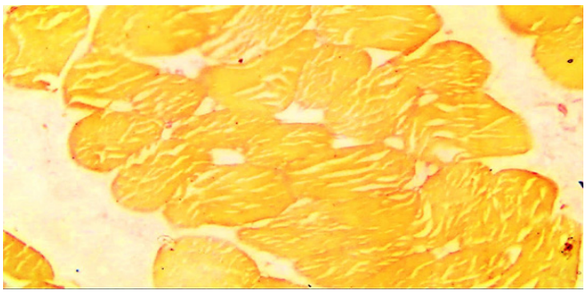


Fig. 4G: A photomicrograph transverse section of rat skeletal muscle of Atorvastatin & Vitamin D treated group showing decreased reddish coloration which means decreased collagen fiber deposition. (Van Geisson X 400)

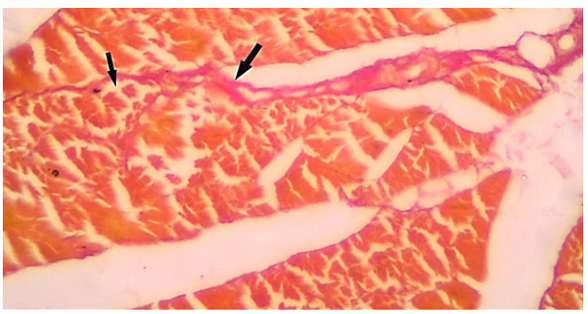


Fig. 5G: A photomicrograph transverse section of rat skeletal muscle of withdrawal group showing increased reddish coloration (Black arrow) which means increased collagen fiber deposition. (Van Geisson X 400)

Table 1: showing mean values of MDA nmol/ml ± SD in the 5 groups

Mean % ± SD	Group I	Group II	Group III	Group IV	Group V
MDA	12.4 ± 0. 6	19 ± 2	13.3 ±1.04	14.4 ± 1.3	19 ± 1
Significance ≤ 0.05	With groups II, &V	With groups I,III & IV	With groups II & V	With groups II &V	With groups I,III & IV

Table 2: showing mean values of CPK IU/L ± SD in the 5 groups

Mean % ± SD	Group I	Group II	Group III	Group IV	Group V
CPK	132 ±19.47	331 ± 18.5	160.3 ± 16.5	186.7 ± 7.6	307± 20.4
Significance ≤ 0.05	With groups II, &V	With groups I,III & IV	With groups II & V	With groups II &V	With groups I,III & IV

Table 3: showing mean values of area percent of collagen fibers deposition ± SD in the 5 groups

Mean % ± SD	Group I	Group II	Group III	Group IV	Group V
Masson%	2.47± 0.25	27.63± 6.9	2.8± 1.5	3.2± 1.2	26.57±7.6
Significance ≤ 0.05	With groups II &V	With groups I,III & IV	With groups II & V	With groups II &V	With groups I, III & IV

Table 4: showing mean values of area percent immunoreactivity of desmin ± SD in the 5 groups

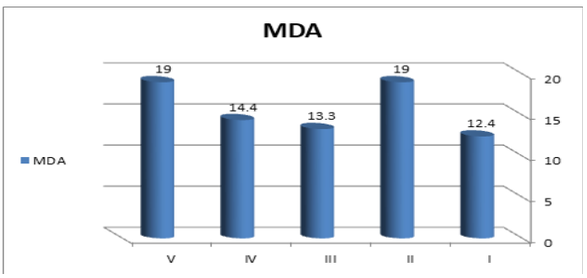
Mean % ± SD	Group I	Group II	Group III	Group IV	Group V
MA	38.9± 9.5	8.07± 2.5	35.5± 3.7	32.5± 5.5	8.05± 3.3
Significance ≤ 0.05	With groupsII &V	With groups I,III & IV	With group II & V	With groups II &V	With groups I,III & IV

Table 5: showing mean values of area percent immunoreactivity of Ki 67 ± SD in the 5 groups

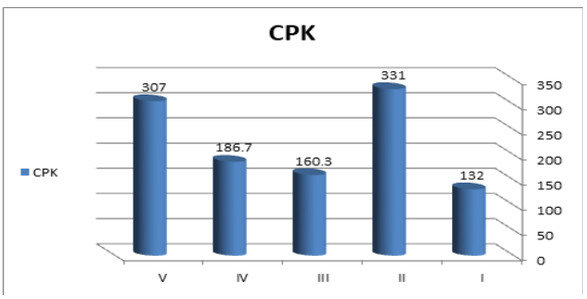
Mean % ± SD	Group I	Group II	Group III	Group IV	Group V
Ki67%	1.77± 0.3	23.5± 5.7	2.77± 1.7	4.4± 2.2	21.2± 7.4
Significance ≤ 0.05	With groupsII &V	With groups I,III & IV	With group II & V	With groups II &V	With groups I,III & IV

Table 6: showing mean values of area percent immunoreactivity of Myogenin ± SD in the 5 groups

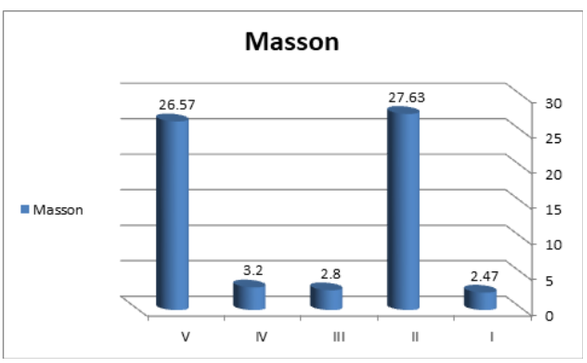
Mean % ± SD	Group I	Group II	Group III	Group IV	Group V
Myogenin	1.3 ± 0.5	2.8 ± 0.4	9.6 ± 1.1	7.6 ± 1.5	3.9 ± 1.2
Significance ≤ 0.05	With groupsII &V	With groups I,III & IV	With group II & V	With groups II &V	With groups I,III & IV



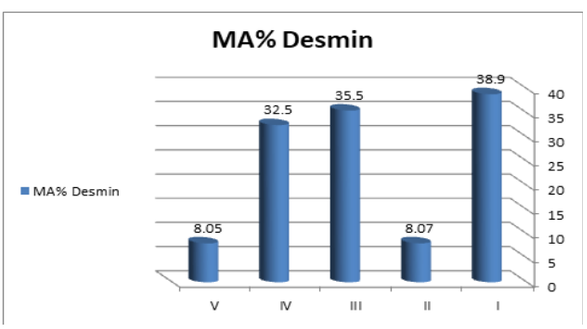
Histogram 1: showing mean values of MDA nmol/ml in the 5 groups



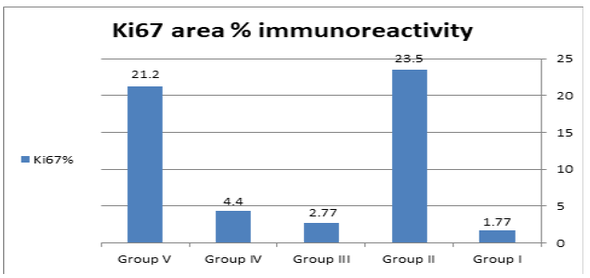
Histogram 2: showing mean values of CPK IU/L protein in the 5 groups



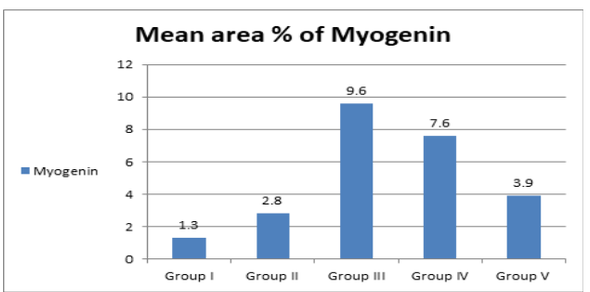
Histogram 3: showing mean values of area percent of collagen fibers deposition in the 5 groups



Histogram 4: showing mean values of area percent immunoreactivity of desmin in the 5 groups



Histogram 5: showing mean values of area percent immunoreactivity of ki 67 in the 5 groups



Histogram 6: showing mean values of area percent immunoreactivity of myogenin in the 5 groups

DISCUSSION

Myopathy is more likely to occur with lipophilic Statins[23]. Statins cause injury to the muscle cell by changing cholesterol content in cell membranes, and myocytes have special separation of lipids and proteins causing it liable to injury by statins[24]. Decreased cholesterol controls fluidity and hinders the function of Na+ K+ pumps which could lead to disintegration of organelles and thus causing cell damage[25]. Deficiency of vitamin D alone can cause myopathy of skeletal muscle as well as decreased muscle strength[13]. Deficiency of coenzyme Q10 is the most probable mechanism of statin-related myopathy[26].

This study was planned to evaluate the protective effect of vitamin D and coenzyme Q10 against statin induced myopathy.

In our study the biochemical assay showed that the mean Serum MDA & CPK levels were increased significantly in group treated with Atorvastatin compared to control group, while in Atorvastatin & Coenzyme Q10 treated group these serum levels were decreased significantly in comparing with Atorvastatin treated. These levels were insignificantly increased in Atorvastatin & Vitamin D treated group comparing with control and Atorvastatin & Coenzyme Q10 treated groups.

This in line with El-Deeb *et al* ,2018 who revealed that serum CPK showed a significant rise in its mean values in Ator group versus control[17]. Also El-Ganainy *et al*,2016 stated that myopathy in rats is characterized by an elevation of CPK level along with muscle necrosis[27].

Elshama *et al*.,2016 stated that CPK is a biochemical indicator for muscle damage and its leakage from the muscle cytosol following cell membrane damage can affect energy metabolism by lowering its ability to generate ATP[28].

Also Khalil *et al* , 2015 revealed that Atorvastatin increase the creatine kinase which was decreased by giving coenzyme Q10[23]. In the same line Choi *et al*, 2016 stated that Co-Q10 supplementation when given with Statin, it reduced creatine kinase significantly in serum[29].

In statin group CK level was significantly increased when compared with the control group, signifying damage of skeletal muscle due to statin treatment. However, in statin plus vit D group the CK level was reduced[30].

In our study, Mean Serum MDA levels were significantly increased in Atorvastatin treated and Withdrawal groups (groups II & V) compared to control group.

Some cases of statin-induced myopathy characterized by high serum level of creatine kinase (CK), moreover persistent and progressive muscular weakness inspite of statin stoppage was detected[31]

Ren *et al*,2020 showed that in statin treated mice, there was increase in MDA compared to the control ones. In contrast, vitamin D supplementation in statin treated group decrease the MDA level[30]. while Ben-Meir *et al*, 2015 Stated that usage of supplements such as Co-Q10 decreased oxidative stress and toxic impacts of injury caused by statins[32].

In our study histological examination of Atorvastatin treated group showed degenerated muscle fibres, Loss of striations and central nuclei. More vacuolization and fragmentation were seen. While histology of Atorvastatin& Coenzyme Q10 treated group (G III) have appeared nearly similar to control group, with cellular proliferation with multiple nuclei were seen. Also histology of statin& Vit.D treated group(G IV) have appeared nearly similar to control group. It showed bundles of apparently normal polygonal muscle fibers with acidophilic sarcoplasm, while few muscle fibers appeared rounded with peripherally arranged oval dark nuclei with centrally located inflammatory cell was seen but some nuclei are centrally located. Few fibers appeared with disintegration and hyaline degeneration.

This is in line with Chogtu *et al*, 2020 who showed that in the group treated with statin-only, swollen degenerated fibres, Loss of striations ,concentrated nuclei in the centre and Inflammatory cellular infiltration were seen[33].

Soliman *et al* ,2017 revealed that in statin plus coenzyme Q-10 treated group there was mild focal histological changes, a transverse section of the muscle showed partial splitting and distortion of some muscle fibers also Areas of loss of transverse striations were observed in a longitudinal section associated with some hemorrhagic spots and mononuclear cellular infiltration[16].

Choi *et al* ,2016 showed that various mechanisms are deliberated to be engaged in pathogenesis of myotoxicity caused by statins. One of them, mevalonate, a precursor of both cholesterol and Co-Q10 was decreased with statin treatment and thus causes Coenzyme Q10 depletion. The exhaustion of CoQ10 in myocyte mitochondria may inhibit ATP production and consequently cause myotoxicity[29].

Serum levels of Co-Q10 diminished during statin treatment, but Co-Q10 is transported in LDL particles and its decrease is consistent with the decrease in blood cholesterol[34]

El-Deeb *et al*, 2018 showed histological improvement of LM findings in group treated with vit D and statin that appeared in the form of well-organized muscle fibers nearly similar to the control with clear regular striations[17]

Furthermore, vitamin D was proved to have anabolic effect on muscular tissue, as it promotes synthesis of muscle cytoskeletal protein[35]

Chogtu *et al* ,2020 stated that combination of vit D with statin showed regeneration in the form of increased cellularity with multiple nuclei. The histology of this group was similar to that of the control group[33].

Also Ahmed *et al*,2019 revealed that vitamin D3 administration with atorvastatin led to improved histological findings in muscle tissues with reduction of necrotic and degenerative changes[15].

Improvements in the group treated with statin plus vit D was based on the possible link between vitamin D and Atorvastatin. Vitamin D being an inducer of Cytochrome P enzyme that stimulates the metabolism of Atorvastatin, thus can result in less toxic metabolites[36]. Therefore, it was suggested that the simultaneous administration of vitamin D with Atorvastatin might decrease the risk of its adverse effects on muscle[37] It has been proposed that vitamin D deficiency may aggravate muscle pain induced by statin[38].

In our study increased amount of collagen fibers around and within the muscle fibers were noticed in the Masson stained skeletal muscle sections of Atorvastatin treated group .minimal amount of collagen fibers in between muscle fibers could be seen in Masson stained skeletal muscle sections of Atorvastatin & Coenzyme Q10 treated group (G III). Also minimal amount of collagen fibers were seen in between muscle fibers of Masson stained skeletal muscle sections of Atorvastatin + Vitamin D treated group (G IV) .moderate amount of collagen fibers around and also around the blood vessel were detected in Masson stained skeletal muscle sections of Withdrawal group.

Soliman *et al* ,2017 revealed that Mallory's trichrome stain in statin treated group showed a marked increase in collagen fibers between muscle fibers and bundles.

Meanwhile animals treated with statin and coenzyme Q10 showed Mild deposition of collagen fibers with Mallory's trichrome stain[16]. Also Khalil *et al* ,2015 showed excess collagen fibers in statin treated and these changes were improved by alongside giving coenzyme Q10[23].

In this study The immunohistochemical results revealed a strong myogenin immunoreactivity in the nuclei of skeletal muscle sections of control group also strong positive cytoplasmic reaction for desmin and negative expression of KI-67 in the nuclei of muscle fibers . In atorvastatin treated group revealed the negative expression of myogenin in the nuclei of skeletal muscle fibers also show negative cytoplasmic reaction for desmin and a strong KI-67 immunoreactivity .In Atorvastatin & Coenzyme Q10 treated group (G III), Nuclei of the skeletal muscle fibers revealed strong expression for myogenin and strong positive cytoplasmic reaction for desmin and very weak KI-67 expression .Also nuclei of skeletal muscle fibers of Atorvastatin & Vitamin D treated group (G IV) revealed moderate myogenin expression and positive cytoplasmic reaction for desmin and weak KI-67 expression. While in the Withdrawal group (group V), there is weak myogenin expression, negative cytoplasmic reaction for desmin and positive reaction for KI67.

In Atorvastatin treated rats immunohistochemical analysis of tissue sections signposted the presence of apoptotic changes which were affirmed by histopathological findings of necrosis and cellular damage[15].

Also El-Deeb *et al* ,2018 revealed that in atorvastatin treated rats there was a significant rise in the mean area percent of immunoreactivity of cytochrome C stain versus control[17].

Khalil *et al* ,2015 showed that atorvastatin increased the strength of the immune-positive reactions of cytochrome C and Bax. These changes were improved by simultaneous coenzyme Q10 intake[23].

Administration of Alfacalcidol ameliorated Ator-induced myopathy and kept muscle fibers structure, based on lower CPK level, decreased immunoexpression of cytochrome C and nearly normal muscle fibers appearance[17].

Also Ahmed *et al*, 2019 revealed that vitamin D3 administration with atorvastatin led to improved histological findings in skeletal muscle tissue with decrease of necrotic and degenerative changes[15].

Rallidis *et al*.,2011 stated that statin induced myotoxicity caused by exhaustion of isoprenoids that regulate the rate of muscle fiber apoptosis[39].

Additional studies discovered that treatment with statin weakened oxidative phosphorylation induced by raised level of ADP in muscle cells, leading to increased production of ROS and apoptosis[40].

Statin myopathy may be due to decreased Co-Q10 levels; so, Co-Q10, acting as radical scavengers and can inhibit oxidative mitochondrial damage and protect the muscles[41]

Other studies have found that statins intolerance due to myalgia, myositis, myopathy, or muscular necrosis associated with vitamin D deficiency can be safely resolved by vitamin D supplementation[42].

Kang *et al*. concluded that replenishment of vitamin D deficiency in patients with statin-induced myopathy appears to be an effective policy in improving adherence to medication[43].

Vitamin D supplementation may inverse statin intemperance in hypercholesterolemic patients with a vitamin D deficiency who did not tolerate statin therapy[44].

CONCLUSION

Intake of Vitamin D during the period of Statin treatment considered to have a protecting effect against statin induced myopathy. Meanwhile the consumption of Coenzyme Q10 has a more protection than Vitamin D.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

دراسة مقارنة للدور الوقائي لفيتامين (د) مقابل الانزيم المساعد Q10 ضد الاعتلال العضلى الناجم عن الستاتين فى ذكور الجرذان البيضاء البالغة: دراسة نسيجية وكيميائية مناعية

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الخلفية: تم اختيار أتورفاستاتين لهذه الدراسة لأن نصف عمر التخلص منه يقارب ١٤ ساعة ، وهي خاصية تحفز فعالية الدواء لخفض البروتين الدهني منخفض الكثافة مقارنة مع الستاتينات الأخرى .علاوة على ذلك ، يتم إعطاؤه في شكل نشط يطيل تأثيره على اختزال HMG-CoA. لذلك ازداد ضعف العضلات بواسطة أتورفاستاتين. يعد الإنزيم المساعد Q١٠ مزيل للجذور الحرة من ميتوكوندريا العضلات الهيكلية. فيتامين د هو أحد مضادات الأكسدة القوية التي تحافظ على الأنشطة المستقرة للميتوكوندريا.

الهدف من العمل: يهدف هذا العمل إلى دراسة التغيرات التي تحدث في العضلات الهيكلية للفئران أثناء إعطاء أتورفاستاتين والدور الوقائي لأنزيم Q١٠ وفيتامين د.

المواد والطرق: تم فصل خمسين من ذكور الجرذان البالغة إلى خمس مجموعات متساوية. المجموعة الضابطة ، مجموعة الستاتين ، تم إعطاء الفئران أتورفاستاتين بجرعة ٥٠ مجم / كجم / يوم ، تمت تسييلها في ماء مقطر وتم إعطاؤها بواسطة أنبوب معدي لمدة ٤ أسابيع. المجموعة المعالجة بالستاتين والإنزيم المساعد Q١٠ والتي تلقت فيها الفئران الإنزيم المساعد Q١٠ بجرعة ٣ مجم / كجم من وزن الجسم. خلال فترة العلاج بالستاتين. المجموعة المعالجة بالستاتين وفيتامين (د)عندها ، عولجت الفئران بالستاتين مثل المجموعة الثانية وأعطيت فيتامين (د) عن طريق الفم بجرعة ٠,٥ ميكروغرام / كغ / يوم ومجموعة الانسحاب التي احتفظت فيها الجرذان لمدة شهر واحد دون علاج بعد ٤ أسابيع من العلاج بالستاتين.تم فحص أنسجة العضلات الهيكلية من أجل التغيرات النسيجية المرضية والكيميائية المناعية.

النتائج: أظهرت المجموعة التي عولجت بـ أتورفاستاتين ألياف عضلية متدهورة وغير منظمة. كما توجد العديد من ألياف الكولاجين داخل حواجز النسيج الضام في أقسام العضلات الهيكلية المصبوغة بماسون وفان جيسون.نشاط مناعي قوي لـ KI-٦٧ وضعف التعبير المناعي للدسمين والميوجينين .يقلل الإنزيم المساعد Q١٠ وفيتامين(د) من تأثير الستاتينات على أنسجة العضلات الهيكلية، لكن الإنزيم المساعد Q١٠ أظهر انخفاضًا كبيرًا في ترسب ألياف الكولاجين و KI-٦٧ المناعية وزيادة في التعبير المناعي للدسمين و الميوجينين مقارنة مع تلك الموجودة في المجموعة المعالجة بالستاتين.

الخلاصة: إن تناول فيتامين (د) خلال فترة العلاج بالستاتين له تأثير وقائي ضد الاعتلال العضلي الناجم عن الستاتين. وفي الوقت نفسه ، يتمتع استخدام الإنزيم المساعد Q١٠ بحماية أكبر.