

THE EFFECT OF ORALLY INGESTED ATORVASTATIN ON THE MASSETER MUSCLE OF WHITE ALBINO RATS (HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDIES)

Mohammed Shredah* and Saher Sayed**

ABSTRACT

Statins are important class of cholesterol lowering drugs that provide cardio-protective effect. They act by inhibiting 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMG Co reductase), the rate limiting enzyme involved in cholesterol biosynthesis pathway. A lot of researches are in progress to reveal the other effects of statins including anti-inflammatory activity and antioxidant activity properties. However, statin-induced myopathy, which is considered a side effect of statins' use, limits their utilize. Statins induced myopathy may result from reduced muscular coenzyme Q10 levels.

This study was carried to evaluate the effects of ingestion of two different doses of atorvastatin for six weeks on the masseter muscle of rats. Eighteen males Sprague Dawley albino rats weighted 200-250 grams at the age of 6-8 weeks were used in this study. They were divided into 3 groups, group I (control group), group II (20 mg/kg b.w atorvastatin received rats) and group III (40 mg/kg atorvastatin received rats). Masseter muscle specimens were fixed in formal saline solution, dehydrated using ascending grades of alcohol, cleared using xylene then infiltrated with paraffin and finally embedded in it. Histological results revealed fragmented and disorganized muscle fibers, centrally located nuclei and clear external rims around some fibers. Immunohistochemical results using Bax showed increased in the cytoplasmic immunopositivity in the muscle fibers of both experimental groups. These results suggested that atorvastatin exhibits significant myotoxicity on rats' masseter muscle. Atorvastatin side effects were found to be dose dependent.

Key words: Atorvastatin, masseter muscle, Bax, myotoxicity

INTRODUCTION

Statins are among the most widely taken prescription medications in the world. They are used to reduce the risk of cardiovascular diseases

by reducing hypercholesterolemia. Statins include cerivastatin drug which is the most potent, followed by rosuvastatin, atorvastatin, simvastatin, lovastatin, pravastatin and fluvastatin (Shepherd et al., 2003)⁽¹⁾.

* Assistant Professor, Oral Biology Department, Faculty of Dentistry, Damanhour University.

** Lecturer of Oral Biology, Oral Biology Department, Faculty of Dentistry, Minia University.

Statins mode of action depends on the inhibition of 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMG-CoA reductase), a key enzyme in the cholesterol biosynthetic pathway (**Egan & Colman, 2011**)⁽²⁾. The most common factor that limits their use is the occurrence of myopathy. It is more likely to occur with lipophilic statins such as atorvastatin, simvastatin and lovastatin compared to the hydrophilic statins like pravastatin and fluvastatin (**Kiener et al., 2001**)⁽³⁾. The myotoxic effect of lipophilic statins is attributed to their penetration to the muscle tissue. Statins mainly affect the mitochondria of the skeletal muscle by decreasing the synthesis of melanovate, which is a critical intermediary in the cholesterol synthesis pathway, via the competitive inhibition of the HMG-COA reductase. The inhibitory effect of statins on HMG-COA reductase leads to a reduction of farnesyl pyrophosphate synthesis which is an intermediate in the synthesis of CoQ 10 (ubiquinone) (**Macroff & Thompson, 2007**)⁽⁴⁾.

Atorvastatin is rapidly absorbed following administration (**Gaw et al., 2000**)⁽⁵⁾. Its half-life is approximately 14 hours (**Cilla et al., 1996**)⁽⁶⁾. It is metabolized by hepatic cytochrome P450 enzymes (**Bottorff & Hansten, 2000**)⁽⁷⁾.

Graham et al., 2004⁽⁸⁾ stated that statin-induced rhabdomyolysis is characterized by skeletal muscle necrosis that leads to the release of muscle proteins and compounds into the blood. The release of potassium can lead to disruption in heart rhythm, and phosphates can cause hypocalcemia by precipitating with calcium in the blood (**Vanholder et al., 2000**)⁽⁹⁾.

On the other hand, **Mendes et al, 2014**⁽¹⁰⁾ proved that statin-induced rhabdomyolysis was more commonly reported when statins were used in conjunction with other drugs, which potentiated its effect.

The most severe consequence of rhabdomyolysis is the accumulation of myoglobin in kidney tubules, which can severely damage the kidney via acute tubular necrosis and eventually lead to kidney failure if left untreated (**Charatan, 2001**)⁽¹¹⁾.

Pannonnummal & Varkey 2014⁽¹²⁾ found that rats received 20mg/kg atorvastatin showed characteristic pathological changes in kidney such as glomerular atrophy, glomerular nephritis and dilated tubules with atrophied tubular epithelium.

Apoptosis is a cell suicide program that occurs via activation of specific signalling pathways. In apoptotic cells, DNA fragmentation, nuclear condensation, and formation of apoptotic bodies can be clearly detected. The cells are then engulfed by macrophages or neighbouring cells without initiating an inflammatory response (**Pollack et al., 2002**)⁽¹³⁾.

The mitochondrion plays an important role in regulating apoptosis. It can release cytochrome c into the cytosol that forms an apoptosome with procaspase-9. Once the apoptosome is formed, procaspase-9 can cleave and activate itself into caspase-9. Caspase-9 then activates procaspase-3, leading to apoptosis. This process is highly regulated. The Bcl-2 family of proteins is among the most effective proteins that regulate the release of cytochrome c. This family consists of several proteins, which are antiapoptotic or proapoptotic. Bax favours cytochrome c release and is therefore considered proapoptotic (**Pollack & Leeuwenburgh, 2001**)⁽¹⁴⁾.

It was proved that statins can induce apoptosis in many cell types, such as rheumatoid synovial cells, pericytes, smooth muscle cells, cardiac myocytes, and several types of cancer cells (**Dirks & Jones, 2006**)⁽¹⁵⁾.

Several studies were concerned with the clinical and histopathological effects of statins on various skeletal muscles in the body. However, only a few

clinical studies are available regarding its influence on the masticatory muscles. So, this study aimed to evaluate the effect of orally ingested atorvastatin on the masseter muscle of white albino rats through immunohistochemical staining using Bax.

MATERIALS AND METHODS

Eighteen males Sprague Dawley albino rats at the age 6-8 weeks, weighting 200-250 grams, specific pathogens free were used in this study. Each animal group was housed in separate clean plastic cage at faculty of dentistry, Minia University.

All animals were given food and water ad-libitum and were kept at constant humidity and temperature. The experiment was approved by the ethical committee for animal handling for research work in Minia University.

Reagents:

Atorvastatin (Lipitor) 40 mg tablets were obtained from Pfizer. The tablets were ground and then the different doses 20 and 40 mg/kg were calculated. Each dose was freshly dissolved in distilled water before the oral intake.

Experimental design

Animals were randomly divided into three groups as following:

Group I (control group):

The control group included 6 rats received distilled water for six weeks.

Group II:

This group included 6 rats received 20 mg/kg/day of atorvastatin dissolved in distilled water by a gastric tube for six weeks.

Group III:

This group included 6 rats. Rats received 40 mg/kg/day of atorvastatin dissolved in distilled water by a gastric tube for six weeks.

Histological procedures:

Rats from all groups were sacrificed after six weeks by decapitation under light halothane anaesthesia. Masseter muscle tissue samples were obtained for tissue preparation. The specimens were rapidly put and fixed in 10% formal saline for 48 hours, then they were washed with tap water and processed to prepare tissue sections for histological and immunohistochemical studies. Specimens were dehydrated using ascending grades of alcohol (50%, 70%, and 90% then in absolute alcohol). They were then cleared from alcohol with xylene (clearing agent) followed by infiltration with paraffin in constant temperature oven (60° C) for 2-3 hours. As the specimens were completely infiltrated, they were removed and placed in the center of the box of melted paraffin, the bottom of which was the surface of cutting. The box containing the paraffin embedded specimen was then immersed in cool water to harden. By the use of microtome, serial sections were done from the paraffin blocks. The cutting was done to obtain sections with 4-6 microns in thickness. Suitable lengths of the paraffin ribbon were mounted on the prepared microscope slides. The slides were placed on a constant temperature drying table at about 37-42° C (Luna, 1968⁽¹⁶⁾).

Immuno-reactivity for Bax was recognized using a streptavidin-biotin-peroxidase detection system (DAKO Corp. catalyzed signal amplification system, DAKO Corp.). The paraffin-embedded tissue sections were deparaffinized and put in Target Retrieval Solution (DAKO Corp.) to amplify the signal, then placed in a water bath (95-97° C) for 20-40 minutes. Tissue sections were cooled for 20 minutes at room temperature. After blocking endogenous peroxidase activity with a 3% hydrogen peroxidase solution for 10 minutes, intrinsic biotin with an endogenous avidin/ biotin blocking kit (Nichirei Corp., Tokyo Japan), and nonspecific binding by serum-free protein, the tissue

was incubated with pre-diluted rabbit polyclonal antibodies (5 mg/mL) for 15 minutes, followed by 15-minutes incubation with a biotinylated rabbit antimouse IgG1M1A antibody and 15-minutes incubation with streptavidin-biotin-peroxidase complex, biotinyl tyramide, hydrogen peroxide, and streptavidin conjugated with peroxidase. Staining was completed with diaminobenzidine for 5 minutes, and the specimens were counterstained with Mayer's hematoxylin for 2–5 minutes (Krajewska et al., 1996⁽¹⁷⁾).

Measuring area fraction of Bax immunopositivity

Image J 22 soft ware was used for area fraction measurement of the Bax immunopositivity. Area fraction was measured in a standard measuring frame per 5 photomicrographs in each group using a magnification x 400 by light microscopy transferred to the monitored screen. Areas containing positively immunostained tissues were used for evaluation regardless the intensity of staining. These areas were masked by a red binary color that could be measured by the computer system as follows:

- 1- Soft ware converted the image type to 8-bit grey scale.
- 2- The image was then colour threshold to select only the colour of interest which is the brown colour of the immunopositivity.
- 3- The colour was then masked by a red binary colour to measure area fraction which is the percentage of the pixels in the brown colour that have been highlighted in red.

Statistical analysis

The results of the area fraction immuno-reactivity of Bax were summarized as mean and standard deviation. Results' significance was assessed by determining the probability factor (P value), were $P \leq 0.05$ is considered statistically significant.

Calculations were made using SPSS (Statistical Package for the Social Sciences) version 15.

RESULTS

1) The histological results using hematoxylin and eosin

a) Group I (control group)

Longitudinal sections (LS) of masseter muscle in control group revealed long cylindrical, non-branching, striated muscle fiber cells with acidophilic sarcoplasm. Their nuclei were multiple, elongated, vesicular and peripherally located just beneath the sarcolemma (Fig.1).

In transverse section (TS), the masseter muscle fibers appeared polygonal with acidophilic sarcoplasm and peripherally located nuclei. The muscle fibers were grouped in to bundles. Each bundle was surrounded by a connective tissue sheath (perimysium) (Fig.2).

b) Group II (20 mg/kg atorvastatin treated group):

Longitudinal sections in this group showed connective tissue ingrowths through the muscle fibers leading to their splitting. Some of the muscle fibers showed central nuclei instead of the flat peripheral nuclei. Other fibers were fragmented (Fig.3).

The TS of masseter muscle fibers showed rounding or loss of the polygonal appearance of some muscle fibers. Some other muscle fibers revealed rounded internal nuclei and there were clear external halos around them (Fig. 4).

c) Group III (40 mg/kg atorvastatin treated group)

Longitudinal sections in masseter muscle fibers of this group showed a wavy appearance of the fibers. Some nuclei situated in a central instead of the peripheral position. Fragmented muscle fibers were also clearly identified (Fig. 5).

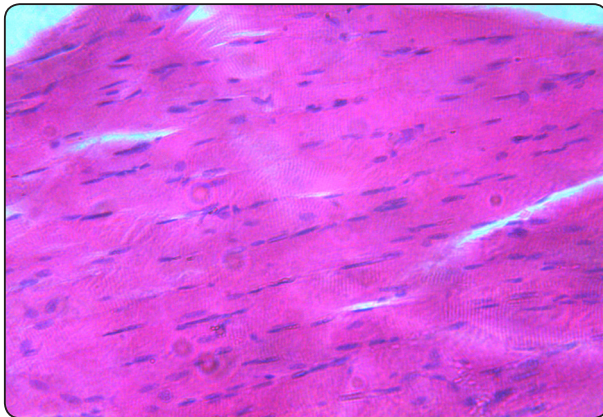


Fig. (1): A Photomicrograph of longitudinal section in the control group of masseter muscle showing long cylindrical, non-branching, striated muscle fiber cells with acidophilic sarcoplasm and peripherally located nuclei (H & E, X 400).

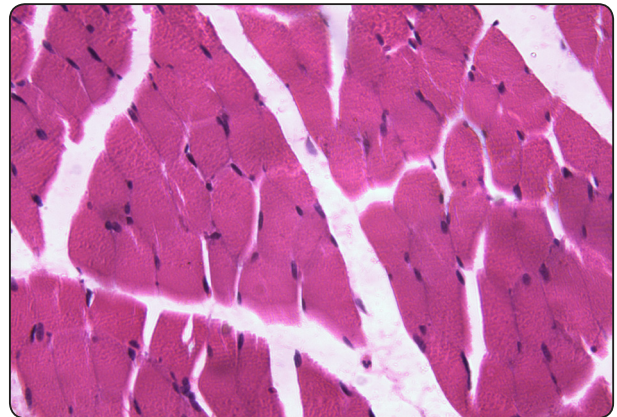


Fig. (2): A Photomicrograph of transverse section in the control group of masseter muscle showing polygonal muscle fibers with acidophilic sarcoplasm and peripherally located nuclei (H & E, X 400).

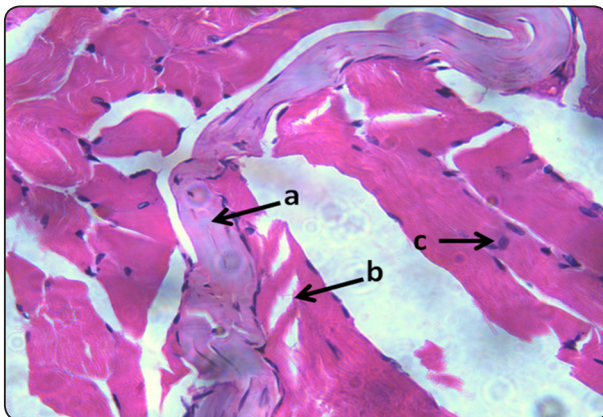


Fig.(3): Photomicrograph of longitudinal section in group II showing: a- connective tissue ingrowths b- fragmented muscle fiber c- centrally located nucleus (H & E, X 400).



Fig. (4): Photomicrograph of transverse section in group II showing: a- rounded muscle fiber b- centrally located nucleus c- clear external halo (H & E, X 400).

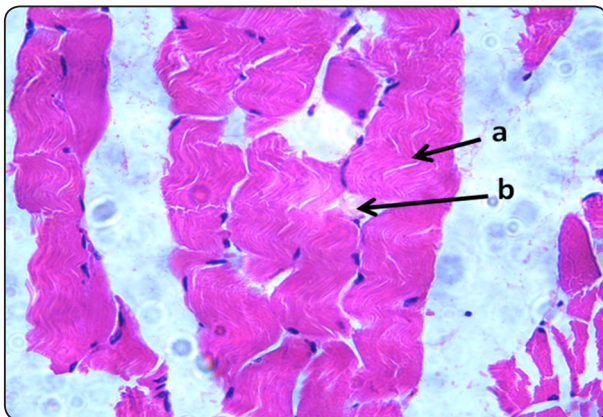


Fig. (5): Photomicrograph of longitudinal section in group III showing: a- wavy muscle fiber b- fragmented muscle fiber (H & E, X 400).

2) The immunohistochemical results using Bax:

The positive immunoreactivity for Bax appeared in the form of brown coloration of the sarcoplasm of the immunoreactive cells.

a) Group I (control group):

Longitudinal sections of rats' masseter muscle fibers in the control group showed negative immunolabeled sarcoplasm and nuclei for Bax in the masseter muscle fiber cells (Fig .6).

b) Group II (20 mg/kg atorvastatin treated group):

The longitudinal sections of the masseter muscle fibers in the 20 mg/kg atorvastatin treated rats revealed moderate sarcoplasmic immunopositive reaction for Bax together with negative immunoreaction in the nuclei of the masseter muscle fibers (fig. 7)

c) Group III (40 mg/kg atorvastatin treated group):

Most of the skeletal muscle fibers of this group showed intense immunopositive sarcoplasmic reaction for Bax while the nuclei negatively reacted for Bax (Fig. 8).

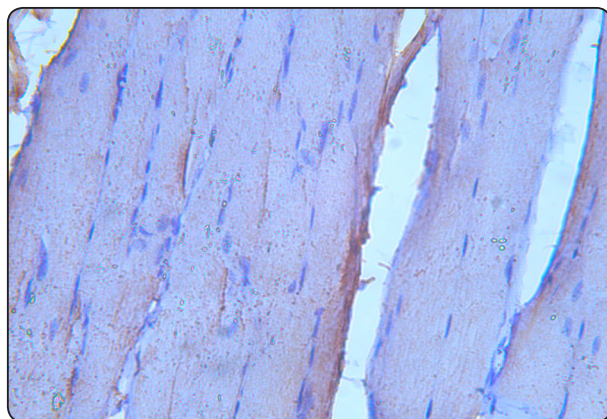


Fig. (6): A photomicrograph of longitudinal section in masseter muscle fibers of the control group showing negative sarcoplasmic and nucleic immunoreactions for Bax (Bax, X 400).

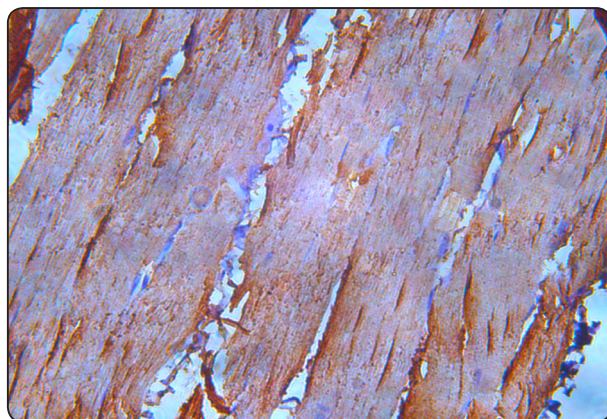


Fig. (7): A photomicrograph of longitudinal section in masseter muscle fibers of group II showing moderate sarcoplasmic Bax immunostained muscle fiber cells (Bax, X 400).

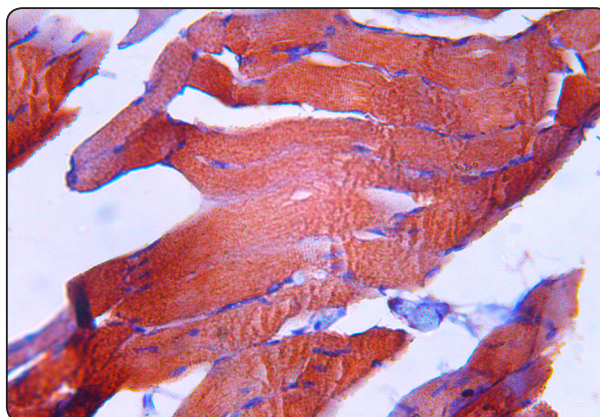


Fig. (8): A photomicrograph of longitudinal section in masseter muscle fibers of group III showing intense sarcoplasmic Bax immunostained fiber cells with negatively immunoreacted nuclei (Bax, X 400).

3) Image analysis results of area fraction:

On measuring the area fraction of Bax immunopositivity, disappearance of the red binary colour was observed in the control group while an excessive increase in the binary colour could be easily detected in the both experimental groups.

P-value and mean ± SD results revealed a significant increase in Bax immunopositivity from control to both experimental groups. Moreover, statistical analysis results revealed a significant increase in Bax immunopositivity from group II to group III [table 1; fig (9)].

TABLE (1): Comparison between the studied groups regarding area fraction results

	Range	Mean ±SD	P 1	P2	P3
Control	10.9-15.8	13.7±1.9	0.001*	0.001*	0.001*
20 mg dose	43.5-46.2	44.5±1.02			
40 mg dose	64.8-72.06	66.9±3.01			

*= significant, P1= control vs 20 mg, P2= control vs 40 mg, P3= 20mg vs 40 mg.

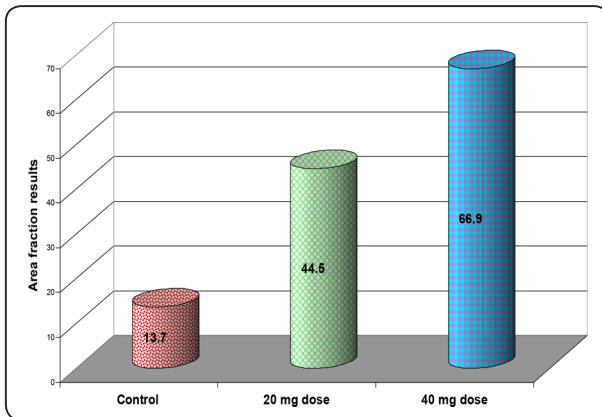


Fig. (9): A histogram showing comparison between the control and both experimental groups regarding area fraction

DISCUSSION

Statins are one of the most commonly used drugs for reducing hypercholesterolemia. They are also used to treat and prevent various other diseases such as cancer, osteoporosis, multiple sclerosis, rheumatoid arthritis, type 2 diabetes, and Alzheimer's disease. Consequently, the number of patients taking statins is expected to increase. Statin-induced myopathy which is a side effect of statins may result from reduced muscular coenzyme Q10 levels and limit their use (Khalil et al., 2015)⁽¹⁸⁾.

Statins are generally well tolerated. However, different grades of myopathy ranging from mild myalgia to fatal rhabdomyolysis have been reported (Soner et al., 2013)⁽¹⁹⁾.

Several studies have investigated the effects of statins administration on various skeletal muscles of the body. In particular, there is no histopathological information about the effect of statins on masticatory muscles.

The present work was done to show the potential myotoxicity of atorvastatin on masseter muscle and whether these effects are dose dependent or not using histological and immunocytochemical techniques.

Masseter muscle was chosen for this study because it is mainly formed of type II muscle fibers (Sano et al., 2007)⁽²⁰⁾ which are selectively subjected to statin induced myotoxicity (Hanai et al., 2007)⁽²¹⁾.

In the current study, different structural changes were detected in the masseter muscle tissue by light microscope in rats receiving 20 and 40 mg/kg body weight of atorvastatin. Using hematoxylin and eosin, rounding and splitting of the muscle fibers and dense centrally located nuclei were detected in the two groups. This study also reported that the dose of 40 mg/kg produced a wavy appearance of the myofibril. Muscle fibers appeared disorganized, fragmented and discontinued and some of them were surrounded by clear external halo rims.

The centrally located nuclei that have been observed in this work were used by Mazroa & Asker, 2010⁽²²⁾ as marker for muscle fibers damage. In addition, Sugarman, 2002⁽²³⁾ agreed with our finding by demonstrating central nuclei in skeletal muscle degenerative myopathies.

Sirvent et al., 2005⁽²⁴⁾ explained our findings regarding disorganized and fragmented muscle fibers. They reported data showing that simvastatin induces an efflux of Ca²⁺ from the mitochondria of isolated human muscle fibers. They suggested that the efflux is caused by a disruption in mitochondrial function, as a result of a direct inhibition of one or more of the complexes of the mitochondrial electron transport chain. Altered Ca²⁺ homeostasis in the muscles could lead to muscle dysfunction and dysregulation.

Moreover, it was reported that the inhibition of HMG-CoA pathway deprives the cell from coenzyme Q10 which is needed for mitochondrial electron transport. This leads to mitochondrial dysfunction, decrease of energy production and cell degeneration (Westwood et al., 2005)⁽²⁵⁾.

On the other hand, many studies proved that the serum coenzyme Q10 levels were decreased during statin treatment but its myocyte levels

were not consistently decreased (**Rallidis et al., 2012**)⁽²⁶⁾. In addition, CoQ10 supplementation studies failed to prove an etiologic role of coenzyme Q10 deficiency in statin-associated myopathy (**Schaars & Stalenoef, 2008**)⁽²⁷⁾.

The clear external halos surrounding some muscle fibers detected in our study might be attributed to the increase in lipid deposition in and around these fibers. This finding came with **Lammens & Laak, 2004**⁽²⁸⁾ who found that intracellular lipid deposition is characteristic of mitochondrial disorder.

Meanwhile, **Khalil et al., 2015**⁽⁴⁸⁾ agreed with our results as they found that 10 mg/kg body weight received rats demonstrated halos of clear external rims around the extensor digitorum longus muscle fibers of rats.

Moreover, in this study, atorvastatin also induced excess collagen deposition between the muscle fibers. **Lańcut et al., 2004**⁽²⁹⁾ explained our finding as they stated that the increase of the collagen fibers represents a response to muscle fiber damage where fibroblasts replace the damaged area with subsequent formation of collagen fibers.

Our results also came in line with **Mazroa & Asker, 2010**⁽²²⁾ who recorded fibrosis in the intercellular spaces after administration of 10 mg/kg/day of atorvastatin.

Clinically, **González et al., 2016**⁽³⁰⁾ supported our histopathological findings detected in masseter muscle. They found that the risk of suffering muscular alterations during oral device treatment in a group of patients with a diagnosis of obstructive sleep apnea is significantly higher in statin patients compared to non-statin patients. These muscular side effects were in the form of mandibular rigidity and fatigue, tension and sensitivity of the masticatory muscles.

In contrast, **Nasri et al., 2016**⁽³¹⁾ found that intraperitoneally administered atorvastatin for 7 days was nephrotoxic only at high dose of 150 mg/kg body weight. Lower doses of 10 and 50 mg/kg

body weight were found to be not accompanied by cellular renal injury.

Atorvastatin induced myopathy could be attributed to apoptosis which involves the mitochondrial pathway. This involves the release of cytochrome c into the cytosol as a sequence of the increased permeability of mitochondrial transition pore for Bax (**Bouitbir et al., 2012**)⁽³²⁾.

The Bcl-2 family of proteins regulates apoptosis by controlling mitochondrial permeability. The pro-apoptotic Bcl-2 proteins including Bax reside in the cytoplasm but translocate to mitochondria following death signalling, where they enhance the release of cytochrome c. Bax influence the voltage-dependent anion channel (VDAC), which plays a role in regulating cytochrome c release. After release from mitochondria, cytochrome c binds to Apaf-1 and forms an activation complex with caspase-9. (**Lindsay et al., 2011**)⁽³³⁾

Our immunohistochemical results showed increased immunopositive staining of Bax in the atorvastatin treated groups. This finding was in agreement with **Khalil et al., 2015**⁽⁴⁸⁾ who found marked immunopositive Bax staining in the majority the extensor digitorum longus muscle fibers of rats treated with 10 mg/kg body weight of atorvastatin for four weeks.

These results could be explained by **Mazroa & Asker, 2010**⁽²²⁾ who observed the expression of active caspase 9 in rat skeletal muscle after 10 mg/kg of atorvastatin treatment. In addition, **Xu et al., 2007**⁽³⁴⁾ proved that atorvastatin treatment caused apoptosis in vascular smooth muscle and **Yu et al., 2009**⁽³⁵⁾ reported that simvastatin induced apoptosis in skeletal muscles.

However, the studies investigating the apoptotic effect of atorvastatin on the skeletal muscle are relatively scarce. It has been reported in the study of **Urso et al., 2005**⁽³⁶⁾ that Atorvastatin induces apoptosis in the skeletal muscle at a genetic level after muscle exercise.

Our statistical analysis of the area fraction of Bax immunopositivity revealed a significant increase in Bax immunoreactivity from group II to group III, indicating that apoptosis produced by atorvastatin was dose dependent.

Our image analysis results came in line with **Aprigliano et al., 2008**⁽³⁷⁾. They investigated the effect of atorvastatin on the cell cycle and on the fate of activated hepatic stellate rat cells (HSCs) in primary culture. They recorded that atorvastatin dose dependently induced apoptosis in a percentage of 68% and resulted in G2 phase arrest at therapeutic drug concentrations.

Moreover, **Liu et al., 2015**⁽³⁸⁾ supported our findings as they found that atorvastatin induced apoptosis of the leukemic cell lines (Jurkat, K562 and HL-60). They reported that apoptosis levels of the three types of cells treated with atorvastatin with different concentration for 24 hours using the flow cytometry were increased with the increase of drug concentration.

CONCLUSION:

It has been concluded that atorvastatin has toxic effect on the skeletal muscles including the masseter one. This effect was proved to be dose dependent in our study. Atorvastatin toxic effect involves type II muscle fibers, the effect that has been clearly shown in the masseter muscle fibers.

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