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Full Length Research Paper

Combined treatment with resveratrol prevents the atorvastatin-induced myopathy in rat skeletal muscle

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Statins are widely used besides their myopathic side effects, ranging from mild myalgia to fatal rhabdomyolysis. Resveratrol is one of the most popular over the counter products used for similar purposes with statins. The aim of this study was to elucidate the myopathic effects of atorvastatin and coadministered resveratrol in male rat skeletal muscle via morphological analyses and immunohistochemistry studies. Control group received 1.5 ml of drinking water by oral gavage and 1 ml 15% ethanol (vehicle of resveratrol) i.p. for 14 days daily; atorvastatin group was treated with 40 mg/kg atorvastatin by oral gavage and 1 ml 15% ethanol i.p. for 14 days daily. Resveratrol + atorvastatin group was treated with 40 mg/kg atorvastatin by oral gavage and 20 mg/kg i.p resveratrol for 14 days daily. Atorvastatin treatment resulted with a moderate inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) immunoreactivity in nucleus and strong immunoreactivity in fibers. Control group and resveratrol + atorvastatin group showed weak iNOS and eNOS immunoreactivity in nucleus and moderate immunoreactivity muscular fibers. Treatment with atorvastatin resulted in a significantly shortened fibrils, and resveratrol co-treatment reversed this effect. Resveratrol and atorvastatin co-treatment could be an alternative treatment to prevent the myositis adverse effects of atorvastatin on skeletal muscle.

Key words: Atorvastatin, myositis, resveratrol.

INTRODUCTION

5-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase reaction is the rate limiting step of cholesterol biosynthesis and thus the primary mechanism of action of HMG-CoA reductase inhibitors (statins) is to lower cholesterol levels (Evans and Rees, 2002). Statins are widely used in the prevention of cardiovascular events. Although they are generally well tolerated, different grades of myopathy, ranging from mild myalgia to fatal rhabdomyolysis has been reported (Abourjaily et al., 2003; Omar et al., 2002; Graham et al., 2004). The most serious risk associated with statins is myositis with rhabdomyolysis. The incidence of rhabdomyolysis has been estimated to be 0.44 to 0.54 cases per 10,000 person/years (Shek and Ferrill, 2001; Graham et al., 2004; Arora et al., 2006). The prevalence of milder muscle complaints like myalgia has been reported by statin

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users range as high as 20% (Foley et al., 2004; O'Meara et al., 2004; Buettner et al., 2008). Some authors have also reported the incidence of myalgia approximately in 5 to 7% of all patients on statins (Arora et al., 2006). Although the risk of rhabdomyolysis with currently marketed statins is very low, symptomatic muscle weakness and pain are much more frequent. Several possible mechanisms such as depletion of secondary metabolic intermediates, induction of apoptosis and alterations of chloride channel conductance within myositis have been proposed for statin-associated myopathy (Pierno et al., 2009). But the reason why myopathy develops in some patients as a result of statin treatment is still not well understood and this side effect prevents patients and their physicians from complying with statin therapy guidelines.

Hypercholesterolemic patients may sometimes direct to various dietary components and natural compounds to regulate serum lipid concentrations because of these doubts on the safety of statins. Resveratrol is one of the most popular over the counter (OTC) products used for similar purposes. Resveratrol (trans-3,5,4'-trihydroxystilben) is a polyphenol (phytoalexin) naturally found mostly in red wine and different therapeutic plants. By *in vitro* experiments, it has been shown that the cardiovascular protective effects of resveratrol might be through a variety of mechanisms such as inhibition of smooth muscle cells proliferation, platelet aggregation, and the oxidation of low-density lipoprotein (LDL) cholesterol. Resveratrol also reduces the synthesis of lipids and eicosanoids, which promote inflammation and atherosclerosis (Soner et al., 2010). Such multiple protective effects of resveratrol increase its demand as an OTC product even for statin users.

The present study was designed to elucidate the effects of combined treatment of resveratrol on atorvastatin-induced myopathy in male rat skeletal muscle via morphological analyses and immunohistochemistry studies.

MATERIAL AND METHODS

Animals and experimental protocol

Three groups of male Wistar-albino rats of 8 weeks, weighing 260 to 280 g were used in the experiments. Animals were housed identically in cages in an air conditioned room under a 12 h light dark cycle. Temperature and humidity were controlled within the limits 21 ± 2°C and 55 ± 15% relative humidity (RH). All animals became acclimatized for at least 7 days before the outset of the study. A standard diet and tap water were provided *ad libitum*. The experimental protocols were approved by the Animal Ethics Committee of Selcuk University, Meram Medical School. Control group received 1.5 ml of drinking water by oral gavage and 1 ml 15% ethanol (vehicle of resveratrol) i.p. for 14 days daily (n = 8); atorvastatin group was treated with 40 mg/kg atorvastatin (Lipitor®, prepared daily and dissolved in drinking water) by oral gavage and 1 ml 15% ethanol i.p. for 14 days daily (n = 6). Resveratrol + atorvastatin group was treated with 40 mg/kg atorvastatin by oral gavage and 20 mg/kg i.p resveratrol for 14 days daily (n = 8). Rats were weighted every 5 days to rearrange dosing schedule and observed every day or as necessary. When the body weight loss exceed 15% of day 1, rats were excluded from the study group. On the 15th day, the following muscle tissues were sampled: trapezius, gastrocnemius, semitendinosus and biceps femoris.

Tissue processing and immunohistochemistry

Paraformaldehyde fixation of tissues continued for 24 h, at 4°C and processed for embedding in paraffin wax using routine protocols. 5 µm-thick coronal sections were cut using a microtome (Leica MR 2145, Heerbrugg, Switzerland); they were then dewaxed and rehydrated through a graded ethanol series using routine protocols. Sections were then washed with distilled water and phosphate buffered saline (PBS) for 10 min, then treated with 2% trypsin (Sigma Chemical Co., St. Louis, Missouri, USA) in 50 mM Tris buffer (pH 7.5), at 37°C, for 15 min. Sections were delineated with a marker pen (Dakopen, Glostrup, Denmark) and incubated in a solution of 3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity. To reduce non-specific background staining, slides were incubated at room temperature for 30 min in 0.3% bovine serum albumin/1 × Tris-buffered saline. Then, sections were incubated with primary antibodies directed against inducible nitric oxide synthase (iNOS) (1:100 dilution; Abcam, Cambridge, UK), endothelial nitric oxide synthase (eNOS) (1:200 dilution; Abcam, Cambridge, UK) for 18 h at 4°C in a humid chamber. Sections were then incubated with biotinylated secondary antibody and then with streptavidin conjugated to horseradish peroxidase (Histostain plus peroxidase kit, Zymed Laboratories Inc., South San Francisco, CA, USA) for 30 min in accordance with the manufacturer's instructions. Finally, sections were incubated with diaminobenzidine (DAB) for 5 min to reveal immunolabelling. All dilutions and thorough washes between stages were performed using PBS. Sections were counterstained with Mayer's hematoxylin (Sigma Chemical Co., St. Louis, Missouri, USA).

After washing with tap water, sections were dehydrated through a graded ethanol series, cleared in xylene and mounted with entellan. Negative control samples were processed as described except that primary antibodies were omitted and replaced with PBS alone. Positive controls were represented by sections of a neuroblastoma specimen known to be positive for the markers of interest. The intensity of iNOS and eNOS immunohistochemical stainings was graded semiquantitatively according to the nuclear and cytoplasmic immunoreaction in sections as follows: (-) no immunostaining, (+) weak staining, (++) moderate staining, (+++) strong staining. Light microscope, equipped with a camera (Olympus BX-51 and Olympus C-5050 digital camera, Olympus Co., Tokyo, Japan) was used. The slides were examined by two investigators.

Morphometrical analysis

Longitudinal sections were cut and stained with Gomori's trichrome. The first and second of the three consecutive serial sections were omitted and 12 sections from each subject were taken for quantification from the third. Preparations were screened systematically using a random start. Visualization of specimens at ×40 magnification was started from the top right corner of the preparation. Sections were examined under a light microscope

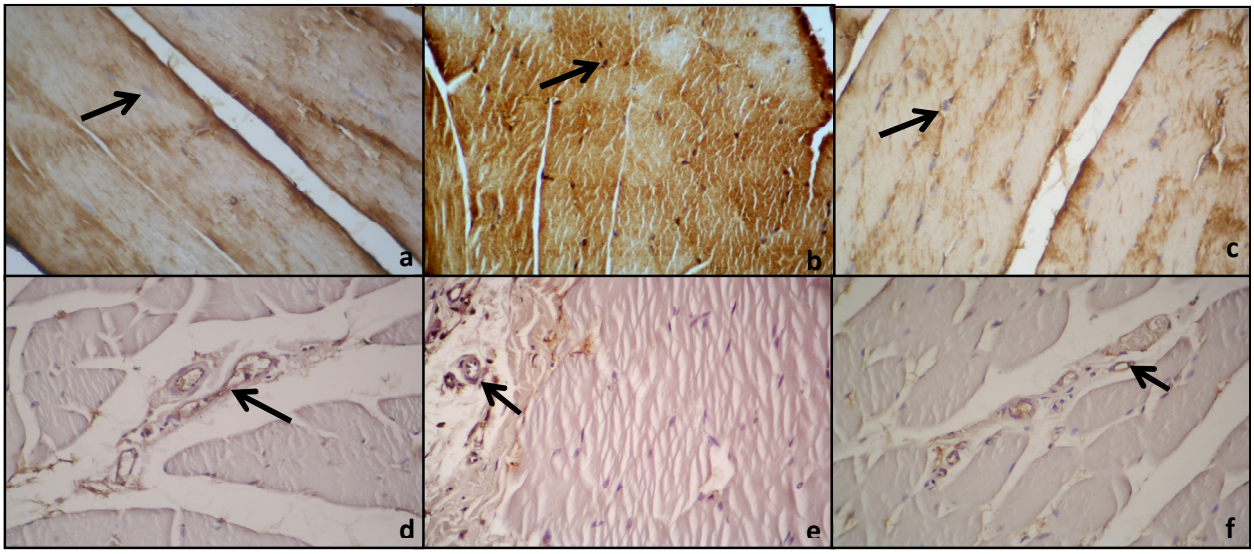

Figure 1. iNOS immunoreactivity in control group (a) with weak nuclear immunoreactivity (arrow indicates negative immunostaining) and moderate in muscular fibers were demonstrated. Statin group (b) showed that moderate immunoreactivity in nucleus and strong immunoreactivity in fibers where in the resveratrol+atorvastatin group (c) negative immunostaining in nucleus, weak immunoreactivity in fibers were observed. eNOS showed similar results in control group (d) and in resveratrol+atorvastatin group (f) with weak immunoreactivity in nucleus and weak in fibers. On the other hand, statin group (e) with moderate nuclear immunostaining and weak muscle fiber staining were observed. Arrows showed positive immunoreactivity (×400).

(Olympus BX-51, Olympus, Tokyo, Japan) equipped with a camera (Olympus C-5050 digital camera, Olympus). Image was transferred to a desktop computer system and length of muscle fibrils determined using image analysis software program (Image-Pro Express, Media Cybernetics, Bethesda, MD) for morphometric analysis. Evaluation of the specimen was performed by an experienced histologist blinded to the surgical groups.

Statistical analysis

The statistical significance of differences of groups was analyzed by one-way analysis of variance (ANOVA) or Student's t-test. p-Values of < 0.05 were considered significant.

RESULTS

Observations

After the 10th day of atorvastatin treatment, rats showed piloerection, hunched posture, thin appearance with weight loss, pale appearance and decreased activity. Control group and resveratrol + atorvastatin group showed no significant alterations.

Immunoreactivity

Control group showed weak iNOS immunoreactivity in nucleus and moderate immunoreactivity in muscular fibers. 40 mg/kg atorvastatin for 14 days has resulted with a moderate immunoreactivity in nucleus and strong immunoreactivity in fibers. Co-administration of 20 mg/kg i.p resveratrol for 14 days with atorvastatin elicited a negative immunostaining in nucleus and weak immunoreactivity in fibers (Figure 1a, b and c). eNOS immunoreactivity results were similar in control group and in resveratrol + atorvastatin group, with weak immunoreactivity in nucleus and weak in fibers. On the other hand, in atorvastatin group, moderate nuclear immunostaining and weak muscle fiber staining were observed (Figure 1d, e and f).

Structural modification of rat skeletal muscles

Our results showed that the treatment of rats with atorvastatin have resulted with a significantly shortened muscle fibrils in trapezius, gastrocnemius, semitendinosus and biceps femoris muscles (20.48 ± 0.91, 18.07 ± 1.6, 19.53 ± 1.1, 20.52±1.0 µm, respectively) when compared with control group (34.22 ± 1.07, 32.01 ± 1.52, 33.0 ± 2.10, 31.65 ± 1.0 µm, respectively) ($p < 0.05$ for trapezius, gastrocnemius, semitendinosus and $p < 0.01$ for biceps femoris). Co-treatment with resveratrol prevented the shortening of muscle fibrils caused by statin treatment alone. Fibril lengths were 29.98 ± 2.4, 28.32±1.6, 27.84±1.9, 40.10±0.8 µm in resveratrol + atorvastatin

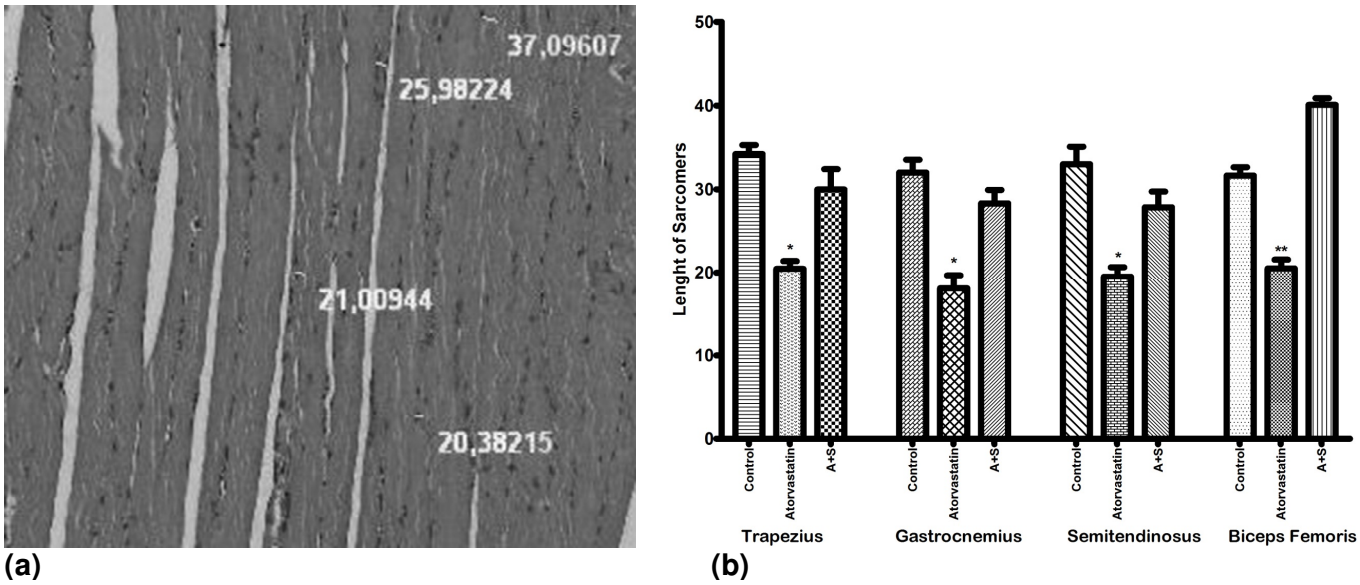

Figure 2. (a) Displays representative skeletal muscle fibril images of rats treated with atorvastatin measured from different fibers. Comprehensive analysis of the length of fibers are shown in (b). As shown in (b), treatment of rats with atorvastatin has significantly shortened fibril length in trapezius, gastrocnemius, semitendinosus and biceps femoris muscles compared with control group. Resveratrol co-treatment prevented the shortening of fibrils caused by atorvastatin treatment alone. Strong correlation between the atorvastatin dose and shortening of fibrils has been evaluated and resveratrol treatment has prevented this effect of statins (*$p < 0.01$, **$p < 0.05$). A+S: resveratrol+atorvastatin group

treated groups for trapezius, gastrocnemius, semitendinosus and biceps femoris muscles, respectively (Figure 2a and b). A strong correlation between the atorvastatin and shortening of muscle fibrils has been found, and treatment with resveratrol has prevented the effect of atorvastatin.

DISCUSSION

Indications for statin therapy as recommended by The National Institute for Health and Clinical Excellence were defined as: ischemic heart disease (angina, myocardial infarction, chronic heart disease), cerebrovascular accidents (transient ischemic attack), hypercholesterolemia, peripheral arterial disease and combination of risk factors (hypertension, type 2 diabetes mellitus, age > 70 years). They also recommend that adults with a history of cardiovascular disease (CVD) and adults with a 10-year risk of developing CVD equal to or greater than 20% should start statin therapy as primary prevention (NICE, 2008). These indications make statins one of the world's most prescribed drugs.

Effects of statins on early cellular changes, inflammation markers, have been shown in rats. Our study has also evaluated muscle fiber lengths in early myositis in rats. Atorvastatin treatment has significantly shortened the fibrils of skeletal muscles. Resveratrol has completely reversed the shortening of fibrils, showing its protective effect on atorvastatin induced myositis.

Similar effect of statins has been shown in zebra fish and this effect has been attributed to an inhibition on biosynthetic pathway or an impaired production of mevalonate caused by statins. Such a destructive effect on myosin filament might result with impaired muscular functions which can be projected into clinical symptoms of myopathy (Huang et al., 2011). To our knowledge, this is the first study showing the shortening of myosin fibril in a rat toxicity model for skeletal muscles with deleterious muscle manifestations induced by atorvastatin. Since some muscle biopsies have documented statin-associated myopathy with normal creatine kinase (CK) levels (Phillips et al., 2002); the use of creatine phosphokinase (CPK) as a reliable biomarker for muscle diseases has consequently been questioned (Gunst et al., 1998; Phillips et al., 2002).

CPK levels do not invariably correlate with clinical symptoms of myopathy because elevated CPK values can be associated with various diseases. Myosin fibril length could also be a marker for myositis in patients with normal CK levels. A change of the myosin fibril length associated with myopathy might allow a diagnosis of myopathy before the occurrence of clinical and laboratory symptoms such as elevated CPK levels. All isoforms of

nitric oxide synthase are expressed in skeletal muscle of all mammals (Stamler and Meissner, 2001) and it has been shown that inflammatory cytokines upregulates iNOS in myositis (Williamset al., 1994; Park et al., 1996).

Our results showed that atorvastatin caused an increase in iNOS and eNOS and when resveratrol was co-administered, iNOS and eNOS upregulation has been recovered to control group. This possible down regulation of iNOS in resveratrol + atorvastatin group can be attributed to the effect of resveratrol which has been shown to inhibit iNOS induction in skeletal muscle (Centeno-Baez et al., 2011). Anti-inflammatory effects of resveratrol have been shown in several studies but for the first time, we have shown the effect of resveratrol on iNOS regulation when co-administered with atorvastatin.

Myotoxic effects of atorvastatin are known to be dose-dependent. As pointed out, impaired metabolism of statins, pharmacokinetic interactions and/or genetic effects are all probable causes of the myotoxicity (Laaksonen, 2006). Co-administered agents effecting on similar metabolic pathways (eg fibrates) increases their myotoxic side effects besides their lipid-lowering action (Ballantyne et al., 2003). An alternative molecule that has the same protecting effect by a different mechanism is needed to decrease the adverse effects of atorvastatin without affecting its effects. With further studies, resveratrol could be an alternative to decrease the effects of statins on skeletal muscle.

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