Evaluation Of the Protective Effects of Coenzyme Q10, Vitamin E, And Their Combination Against Simvastatin-Induced Myopathy in Male Rats (An Immunohistochemistry Study)

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Abstract:

One of the significant adverse effects of statin medication is simvastatin-induced myopathy, for which there are few effective preventative measures. Simvastatin-induced skeletal muscle myopathy in male rats was assessed in this study utilizing immunohistochemical analysis of BAX (BCL2-associated protein X) expression to determine the preventive effects of Coenzyme Q10, Vitamin E, and their combination.

After being divided into groups, the rats were given simvastatin, Coenzyme Q10, vitamin E, or all three together for 30 days. The treatment groups' levels of BAX expression, a marker of apoptosis, were considerably lower than those of the group taking simvastatin alone. The biggest reduction was seen with the combination treatment, with an average immunohistochemistry (IHC) -intensity score of 1.0 ± 0.577, whereas the induction group's score was 2.857 ± 0.378. These results demonstrated the ability of vitamin E and Coenzyme Q10, particularly when combined, to reduce statin-induced muscle apoptosis. Future suggested treatment approaches to stop myopathy in statin users are based on the findings.

Keywords: simvastatin, CoQ10, vitamin E, BAX, myopathy, immunohistochemistry.

تقييم التأثيرات الوقائية للإنزيم المساعد كيو 10 وفيتامين هـ ومزيجهما ضد الاعتلال العضلي الناجم عن السيمفاستاتين في ذكور الفئران (دراسة مناعية كيميائية)

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الخلاصة:
إحدى التأثيرات الضارة لأدوية الستاتين هو الاعتلال العضلي الناجم عن عقار سيمفاستاتين، والذي لا يوجد سوى عدد قليل من التدابير الوقائية الفعالة له. تم تقييم اعتلال العضلات الهيكلية الناتج عن السيمفاستاتين في ذكور الفئران في هذه الدراسة باستخدام التحليل المناعي الكيميائي لتعبير BAXبروتين X المرتبط بـ BCL2 لتحديد التأثيرات الوقائية للإنزيم المساعد Q10 (CoQ10) وفيتامين E ومزيجهما. بعد تقسيم الفئران إلى مجموعات، تم إعطاء السيمفاستاتين أو CoQ10 أو فيتامين E أو الثلاثة معًا لمدة 30 يومًا. كانت مستويات التعبير عن BAX في مجموعات العلاج، وهو مؤشر على موت الخلايا المبرمج، أقل بشكل ملحوظ من تلك الموجودة في مجموعة السيمفاستاتين فقط. أظهر العلاج المشترك أكبر انخفاض، مع متوسط درجة شدة IHC 1.0 ± 0.577، في حين كانت درجة مجموعة السيمفاستاتين 2.857 ± 0.378. توضح هذه النتائج قدرة فيتامين E وCoQ10، وخاصة عند دمجهما، على تقليل موت الخلايا العضلية الناجم عن الستاتين. وتوفر هذه الدراسة طريقة للاعتماد عليها لمنع او تقليل اعتلال العضلات الناجم عن دواء السيمفاستاتين.

الكلمات المفتاحية: سيمفاستاتين، كو كيو ١٠، فيتامين هـ، BAX، الاعتلال العضلي، الكيمياء المناعية.

Introduction

Myopathy indicates a condition or disease affecting the muscles; typical indications and manifestations of myopathies include debility, rigidity, contractions, and convulsions [1]. Numerous physiological functions heavily rely on skeletal muscle. The primary function of skeletal muscle is to convert chemical energy into mechanical energy, which permits the production of force and power [2]

The drugs most commonly linked to muscular adverse effects are β-Hydroxy β-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, also known as statins [3]. Simvastatin is an oral drug that inhibits the enzyme HMG-CoA reductase. It is combined with diet, exercise, weight loss, and other medications to lower lipid levels and improve cardiovascular health [4]. Simvastatin has been associated with cases of rhabdomyolysis in patients, with the total occurrence of myopathy was found to be 0.025%. The incidence of myopathy was significantly higher among individuals taking a daily dose of 80 mg of simvastatin compared to those on a daily dose of 20 mg in a study involving 12,064 survivors of myocardial infarction [5].

Statins, when they reach a sufficiently high concentration in the body, can hinder the production of cholesterol not only in the liver but also in other tissues like skeletal muscle [6], which impacts the cellular levels of cholesterol and the levels of other substances involved in cholesterol synthesis, including farnesyl-pyrophosphate, geranylgeranyl-pyrophosphate, squalene, ubiquinone, and dolichol [6].

In L6 rat myoblasts, simvastatin inhibited the activity of two cation transporters in the plasma membrane that are necessary for cellular function: Na+/K+ ATPase and Na+/Ca2+ ATPase [7]. Mitochondrial dysfunction reduces the mitochondria's capacity to produce high-energy compounds like adenosine 5′ triphosphate (ATP); this can occur due to either impaired electron transfer along the electron transport chain or the dissipation of the proton gradient across the inner mitochondrial membrane. Multiple investigations have demonstrated that mitochondrial dysfunction may significantly impact the muscle damage caused by statins [8, 9].

Coenzyme Q10 (CoQ10), or ubiquinone, is a lipophilic, vitamin-like compound inherently found in all cellular membranes in our bodies. This enzyme is commonly found in our food but is also produced naturally within our bodies [10, 11]. CoQ10 plays a crucial role in the respiratory chain by facilitating the

transfer of electrons from the NADH dehydrogenase protein complex (protein I) to the succinate dehydrogenase protein complex (protein II) and from protein complex II to protein complex III (bc1 complex). Ubiquinol maintains its reduced state upon acquiring electrons from complexes I and II. After delivering the electrons to complex III, it reverts to its oxidized form as ubiquinone [12, 13]. Several experts hypothesize that statin medications may contribute to the depletion of CoQ10 in the human body. Muscle discomfort and cramping commonly occur as negative side effects of statins, and these symptoms are attributed to reduced levels of CoQ10 [14].

Vitamin E is a lipophilic vitamin extensively researched because of its antioxidant characteristics and non-antioxidant effects [15]. As fat oxidizes and free radical reactions expand, vitamin E, a potent antioxidant, stops the production of reactive oxygen species (ROS) molecules. The polyunsaturated fatty acids in plasma lipoproteins and membrane phospholipids are shielded by its peroxyl radical-scavenging properties [16]. Multiple studies have demonstrated that long-term use of statins has a detrimental impact on the overall levels of the primary type of vitamin E (known as a-tocopherol) and other fat-soluble substances in the bloodstream [17-20]. However, only a few contradictory findings have been made regarding whether these levels increase or remain unchanged after accounting for the primary lipids in the blood [20-22].

There is a lack of studies that examine the protective effect of vitamin E and CoQ10 combination to reduce simvastatin-induced myopathy (SIM). With this in mind, we need to address this knowledge gap. There is sufficient evidence from using both agents alone in ameliorating SIM; we hypothesized that using a combination of vitamin E and CoQ10 would enhance both agents' protective effects on SIM since both agents share similar mechanisms of action.

The presented study aimed to study the ameliorative effect of CoQ10, vitamin E, and their combination to prevent simvastatin-induced skeletal muscle myopathy in rat models; it explored the potential mechanism of CoQ10, vitamin E and their combination protection against simvastatin-induced skeletal muscle myopathy in rat models using immunohistochemistry analysis of BAX (BCL2 associated X) in muscle tissue.

Material and Methods

Chemical

All drugs and specific chemicals utilized in this study were pharmaceutical grade purity (95% and above), Simvastatin pure (98.0% purity, Bidepharm, China), Saline 0.9% NaCl (Pioneer, Iraq), Coenzyme Q10, DL-α-Tocopherol (Meryer, China), Formalin 40% (Scharlau, Spain), Ethanol 99% (Scharlau, Spain), Ketamine 10% vial (Kepro Holland, India), Xylene (Scharlau, Spain), Phosphate buffer system (Pure chemistry, Germany).

Animals housing

Thirty-five previously untreated Sprague-Dawley albino male rats, aged 9 to 13 weeks, weighed 140 to 200 gm were used. They have been obtained from the Ministry of Health's National Center for Drug Control and Research.

These animals were kept at the College of Pharmacy, Mustansiriyah University, and maintained under controlled temperature (25±2℃) and humidity (40-60%) conditions. Fed pellets and tab water were free, with a regular light-dark cycle. Animals were placed in plastic cages; each cage has a dimension of 20x25x35 cm and houses three rats. Animals within similar cages were distinguished by their back hair, marked with a waterproof black marker; the animals were maintained for ten days in these conditions before initiating the study protocol. This

study began on 30th November 2023 and finished on 30th June 2024.

Study design

Thirty-five male Sprague-Dawley albino rats were utilized in this experiment. They were randomly separated into five groups using a complete block design; each group contained seven rats. **Negative control group**: seven rats treated with 0.9% NaCl saline orally by gavage for 30 days. **CMC control group:** seven rats were treated with 0.5% in 0.9% saline solution orally by gavage for 30 days. **Simvastatin induction group**: seven rats were treated with 80 mg/kg/day simvastatin, given orally by gavage equal to daily for 30 days This dose is necessary because rodents exhibit higher hepatic metabolism of statins than humans. Due to differences in pharmacokinetics, rodents require proportionally higher doses to achieve systemic exposures comparable to human doses [23] [24]. **CoQ10 group:** Seven rats received the same induction with simvastatin and CoQ10 (100 mg / kg / day) simultaneously administered orally by gavage once a day for 30 days [24,25]. **Vitamin E group:** seven rats received the same induction with simvastatin and vitamin E (40 mg / kg) simultaneously administered orally by gavage once a day for 30 days [24, 26]. **Combination group:** seven rats received the same induction with simvastatin, CoQ10 (100 mg / kg), and vitamin E (40 mg / kg) simultaneously administered orally by gavage once a day for 30 days.

Drug preparation and dissolution steps

The used preparations are prepared in the following steps

- **carboxymethyl cellulose (CMC)** solution is prepared from powder dissolved in 0.9% NaCl [24].
- **Simvastatin**: Simvastatin was obtained in a powdered form and the drug was **suspended in 0.5% carboxymethyl cellulose (CMC)** before administration [24].
- **Coenzyme Q10**: Suspended in a **0.5% carboxymethyl cellulose (CMC) solution** to enhance solubility [25]
- **Vitamin E**: Dissolved in **cottonseed oil or corn oil**, a commonly used vehicle for lipophilic compounds [24, 26].

Sample collection and preparation

After 30 days, IP injection was used to anesthetize the rats with 50 mg/kg 10% ketamine and then 5 mg kg 2% xylazine (animal euthanasia) [27]. The gastrocnemius muscle was immediately removed and washed with distilled water. After that, it is preserved in 10% formalin for histopathological study [24].

Immunohistochemistry (IHC)

The BCL2-associated protein X (BAX) (Cat#: MBS9503347, MyBioSource, USA) was detected by IHC stain from the skeletal muscle of rats. The processing of slides was done following the Areloegbe *et al.* method [28] and following manufacturer instructions.

IHC protocol description.

- **Fixation and antigen retrieval**: Tissue samples were fixed in **10% formalin**, embedded in paraffin, and sectioned at **4 µm thickness**.
- **Primary antibody incubation**: Anti-BAX antibody (MyBioSource, Cat#: MBS9503347) was applied overnight at 4°C.
- **Detection method**: The DAB (3,3'-diaminobenzidine) system was used to visualize staining.
- **Counterstaining**: Hematoxylin was applied to enhance contrast.

Furthermore, according to intensity, the BAX score was separated into four categories: weak (slightly noticeable faint staining; score: 1), moderate (distinct staining; score: 2), strong (intense, dark staining; score: 3), and none (no apparent staining; score: 0) [29].

Ethical considerations

On November 13, 2023, the Research Ethical Committee of Mustansiriyah University's College of Pharmacy granted clearance for the study (approval number 39, reference number 134).

Sample size calculations

Program G Power was used to calculate the sample size [30] based on Cohen's principles [31]. The table's categories were created by selecting random integers. To reduce the possibility of confusion, the animals were kept in containers with clear labels and were identified by their tail tags [32].

Statistical analysis

The Anderson-Darling test of normality was used to verify that all variables had a normal distribution, and an ANOVA with a post-hoc Tukey test was used for analysis. The average value ± standard deviation is how the data are shown. The data was analyzed using Graph Pad Prism 10.2 to create graphs and figures. A significance level of $P < 0.05$ was considered statistical.

Results

Control Group

Immunohistochemical expression of BAX in the skeletal muscle of rats in the negative control group and also for the CMC control group shows no visible staining with a mean IHC-intensity score of 0.143 ± 0.378, as seen in Figures 1 and 2.

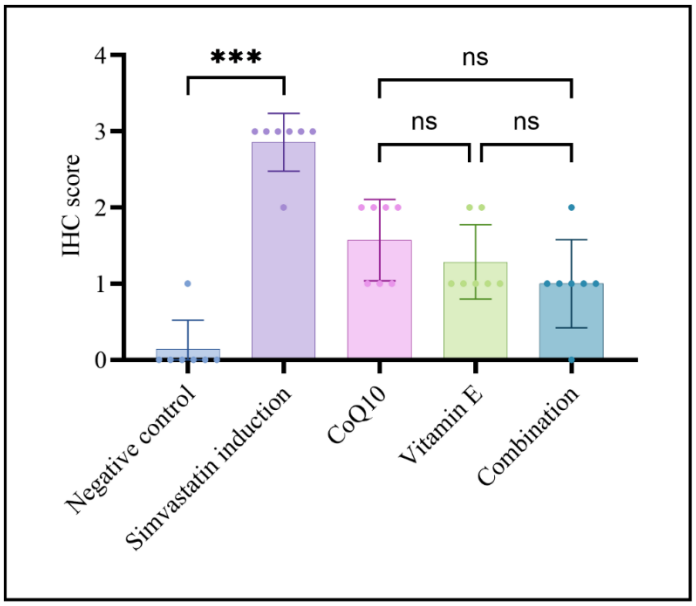

Figure 1: histogram and error bars of BAX expression in rat's gastrocnemius muscle tissue
Significant variations from the control group are indicated by black stars. Disparities between the treatment group members are shown by colored stars. *One-way ANOVA with post hoc Tukey test was used. Data presented as mean ± standard deviation. (^{ns}) denotes p-value >s0.05, (***) denotes p-value <0.0001.*

Induction Group

The immunohistochemical expression of BAX in the skeletal muscle of rats in the simvastatin induction group shows an intense expression, with a mean IHC-intensity score of 2.857 ± 0.378, which was significantly higher compared to the control group, as seen in Figures 1 and 2.

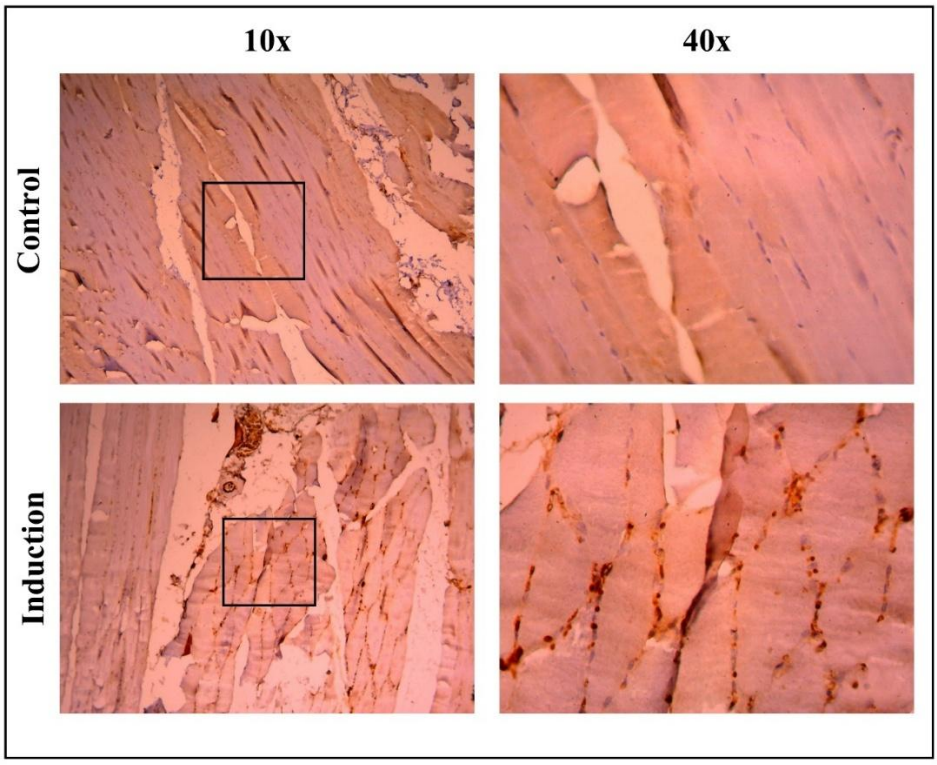

Figure 2: immunohistochemical section of rats gastrocnemius muscle showing the expression of BAX at 4x and 10x magnification in control and induction groups.

CoQ10 treatment group

The immunohistochemical expression of BAX in the skeletal muscle of rats in the COQ10 group shows a distinctive to faint staining, with a mean IHC-intensity score of 1.571 ± 0.535, which was significantly lower compared to the induction group (p-value<0.001), as seen in Figures 1 and 3.

Vitamin E treatment group

The immunohistochemical expression of BAX in the skeletal muscle of rats in the Vitamin E group shows a distinctive to faint staining, with a mean IHC-intensity score of 1.286 ± 0.488, which was significantly lower compared to the induction group (p-value<0.001), as seen in Figures 1 and 3.

Combination treatment group

The immunohistochemical expression of BAX in the skeletal muscle of rats in the combination group shows faint staining, with a mean IHC-intensity score of 1.0 ± 0.577, which was significantly lower compared to the induction group (p-value<0.001), as seen in Figures 1 and 3.

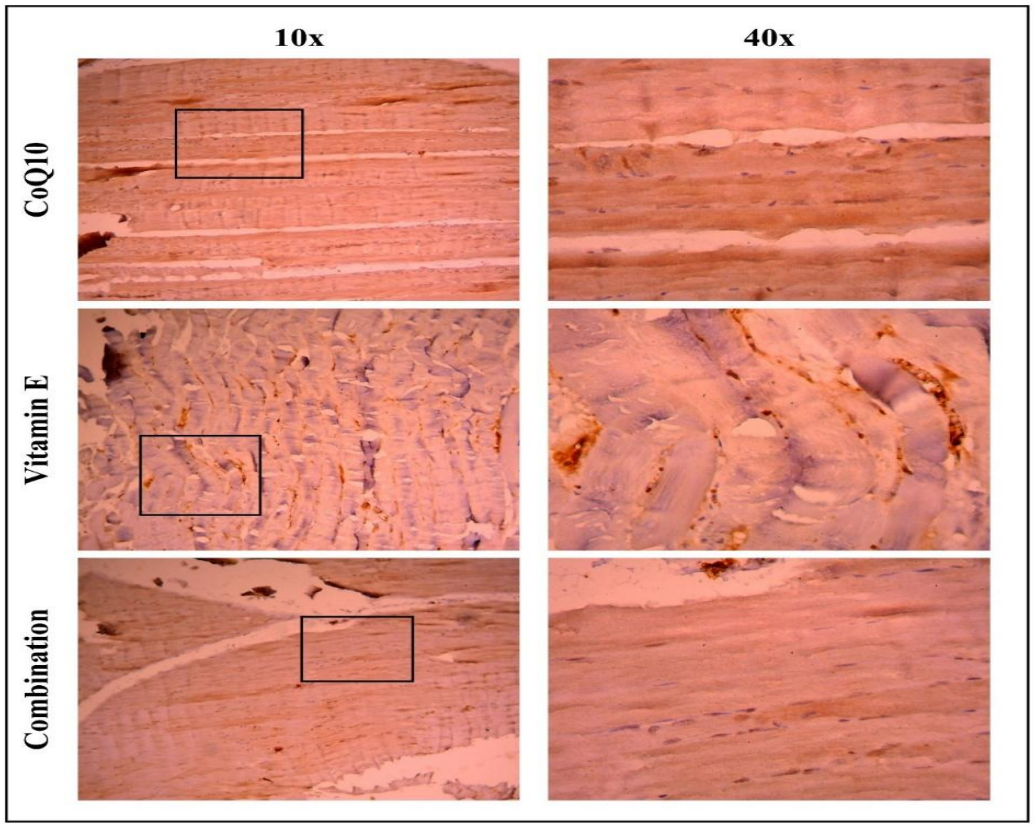

Figure 3: immunohistochemical section of rat's gastrocnemius muscle showing the expression of BAX at 4x and 10x magnification in CoQ10, Vitamin E, and combination groups.

Discussion

Simvastatin, an HMG-CoA reductase inhibitor, is the primary therapy alongside dietary modifications to manage low-density lipoprotein (LDL) cholesterol levels in several clinical situations [(33)]. Reduced circulating lipid levels correlate with diminished risk of cardiovascular disease (CVD) and atherosclerotic cardiovascular disease (ASCVD) [(34)]. Larger dosages of simvastatin and increased plasma concentrations of simvastatin correlate with a greater incidence of side events, particularly statin-associated muscle symptoms (SAMS), a primary reason for treatment cessation [(23)]. The incidence of SAMS was seen to directly correspond with the dosage of simvastatin in clinical investigations, varying from 0.61–0.9% among persons administered an 80 mg dose to 0.02–0.03% among those receiving a 20 mg dose [(23)].

In the presented study, it has been observed that both CoQ10 and vitamin E significantly reduced apoptosis markers, with a more pronounced effect when used in combination. This was accompanied by a notable decrease in Bax protein expression, suggesting modulation of the intrinsic apoptotic pathway [(35)].

CoQ10's role in maintaining mitochondrial membrane potential may prevent cytochrome c release, a key trigger of apoptosis. By scavenging radicals, Vitamin E reduces oxidative damage that can initiate Bax activation. Their combined effect likely offers enhanced protection against mitochondrial dysfunction and subsequent apoptosis [(36)].

The results align with previous studies showing CoQ10's efficacy in reducing apoptosis, and vitamin E's protective effects [36, 37]. Interestingly the combined treatment showed superior effects, supporting the hypothesis of a synergistic interaction [38].

The observed reduction in Bax expression indicates a potential mechanism where CoQ10 and vitamin E modulate the mitochondrial apoptotic pathway. Since Bax promotes cytochrome c release, its downregulation correlates with reduced apoptosis, as evidenced in this study. These findings suggest that CoQ10 and vitamin E, particularly in combination, could serve as potential therapeutic agents in conditions characterized by excessive apoptosis such as myopathy induced by simvastatin.

BAX staining was mostly cytoplasmic, which is in line with its role in pathways leading to mitochondrial apoptosis. None of the treatment groups showed any signs of nuclear localization, which is consistent with other research showing that BAX activation mostly takes place in the cytosol prior to translocation to mitochondria.

These IHC findings showed that vitamin E and CoQ10 protect against simvastatin-induced myopathy. Based on the outcome of this study, CoQ10 potentiates the activity of vitamin E as a protective agent against simvastatin-induced myopathy by antiapoptotic pathways.

Conclusions

In the presented study, vitamin E and Co10 combined showed a protective effect on myopathy induced by simvastatin in rats; both agents reduced the BAX levels in muscle tissues. Thus, vitamin E and CoQ10 treatment will ameliorate muscle death (apoptosis).

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