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EXPERIMENTAL STUDY

Protective effect of betaine against skeleton muscle apoptosis in rats induced by chronic alcohol and statin consumption

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ABSTRACT
AIM: The aim of the present study was to investigate the effect of apoptosis on rat skeletal muscle caused by chronic alcohol and statin consumption with modified liquid diet and to elucidate protective effects of betaine supplementation.
METHODS: TNF-α (tumor necrosis factor), NF-kB (Nuclear Factor kappa B), cytochrome c and caspase-3 levels with or without betaine treatment in alcohol and/or statin-induced skeleton muscle apoptosis rats as well as in controls were measured in serum and tissue. Histologic examinations of the muscle tissues were also performed.
RESULTS: In our study, betaine treated treatment groups we found that calpain and caspase activities and cytokine c release were decreased caused by alcohol, statin and more importantly alcohol+statin group and TNF and NF-kB levels were also close to the levels of control group. Similarly, significant improvements have been observed in our morphological and histological examination results also supporting our biochemical data.
CONCLUSION: We found that combined consumption of ethanol and statin is capable of triggering apoptotic cell death in rat muscles more than the consumption of only alcohol or only statin. Betaine was able to reduced this muscle cell death induced by alcohol and/or statin consumption *(Tab. 4, Fig. 4, Ref. 43)*. Text in PDF *www.elis.sk*
KEY WORDS: alcohol, statin, rat, muscle, apoptosis.

Abbreviations: ADP: Adenosine diphosphate, AMC: 7-amino-4-methyl coumarin, ATP: Adenosine triphosphate, CK: Keratin Kinase, JNK: Jun-N-terminal kinases, MLD: Modified Liquid Diet, NF: Nuclear Factor, NF-kB: Nuclear Factor kappa B, pNA: p-Nitroaniline, SAH: S-adenosylhomocysteine, SAM: S-adenosylmethionine, TNF-α: Tumor Necrosis Factor

Introduction

Primary disease of skeletal muscle is expressed in terms of myopathy. Myopathies may have genetic, infectious, autoimmune and toxic reason. Toxic myopathies can develop depending on using drugs and alcohol. Alcohol can lead to acute and chronic myopathy depending on the period of use (1, 2). There are many medicines that are held responsible for the development of toxic myopathy. Statin derivative drugs that are used in the treatment of hypercholesterolaemia cause the development of myopathy (3). Although the mechanisms of statin induced myopathy is not fully understood, several theories have been proposed by researchers. These include alteration of the level of ubiquinone or alteration of the sarcolemic cholesterol content or proteolysis and induction of apoptosis. The most important group of membrane receptors involved in apoptosis is actively induced by several structurally related receptors, called death receptors. The best known are TNF-alpha and Fas receptors (4). TNF-α is caused by apoptosis with 3 important signaling pathways. The first pathway triggers apoptosis by triggering caspases through the FADD-associated death zone (FADD). The second pathway activates Jun-N-terminal kinases (JNK) and transcription factor AP-1, again leading to apoptosis. In the third pathway, it activates NF-κB, the main mediator in the control of TNF-α transcription. TNF-α is important in the activation and translocation of NF-κB in the skeleton (5). NF-kB increases the expression of several members of the Bcl 2 family that inhibits apoptosis by inhibiting cytochrome c release and mitochondrial permeability (6). The Bcl-2 (antiapoptotic protein) family is the most important group in the control of the apoptotic cascade and has a large number of squamous cells. Bcl-2 protect mitochondria membrane permeability. It inhibits apoptosis by in-

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hibiting proapoptotic proteins (Bax and Bad) (7). Bax protein is a proapoptotic protein and has an important role in the induction of apoptosis (8). In healthy cells Bax is found in cytosol. Apoptotic stimulation leads to cytosolic bax to mitochondria and, as a result of various changes, hydrophobic C-terminus of Bax is released and causes cytochrome c release (7). Released cytochrome c plays an important role for caspase-3 activation in apoptotic cell death. Activation of the cofactor nucleotide triphosphate (d-ATP and ATP) activates cytochrome c and apaf-1 to activate procaspase-9. Activated caspase-9 also activates caspase-3 and triggers the other caspase cascade (9). The release of Bax is stimulated by the calpain, resulting in the release of cytochrome c (7). Calpains are calcium-dependent thiol-proteases (10). Calpains are proteases involved in cell skeleton and signal transduction. They are also involved in other physiological and pathophysiological processes such as cell cycle regulation, apoptosis, inflammation, ischemia, muscular dystrophy, cataractogenesis, Alzheimer's and Parkinson's diseases (11). Activated calpains promote apoptosis by causing caspase 9 and 3 activation (7).

Skeletal myopathy is observed in 30 % to 50 % of high-dose chronic alcohol consumers and it has been related to a direct dose-dependent toxic effect of ethanol. However, its pathogenesis has not been clearly established (12). Apoptosis may be responsible for the progressive intrinsic contractile dysfunction of skeletal myocytes and progressive myocyte loss in alcoholic myopathy (13). Ethanol may activate apoptosis through diverse mechanisms, with the increase in TNF, activation of nuclear-factor-(NF), activation of mitochondrial caspases and disruption of intracellular (Ca^{2+}) transients being the most frequently implicated (12). The change in intracellular calcium level triggers pathways that cause apoptosis. It is thought that calcium plays a role in dephosphorylation of Bad which affects cytochrome c release. Similarly, (Ca^{2+}) -dependent cysteine protease calpain, providing Bax cleavage and it causes Bax to perform its proapoptotic activity. Furthermore, (Ca^{2+}) has been implicated in the release of cytochrome c by affecting the mitochondrial membrane transition pores during apoptosis (14). Cytochrome c can initiate the activation cascade of caspases once it is released into the cytosol and lead to apoptosis (15).

Natural products have been the starting point for the discovery of many modern medicines. Betaine was first discovered in the sugar beet (Beta vulgaris) juice in the 19th century and is known to be present in some other organisms (16). Betaine, a molecular chaperone task, is an inhibitor of oxidative stress and mitochondrial damage (17). It is the methyl source of the transmethylation reactions used in many biochemical pathways, thus providing single carbon units to be used in the formation of methionine and cholines necessary for optimal nutrition (16). The conversion of homocysteine to methionine is necessary for the maintenance of the level of methionine, the detoxification of homocysteine and the production of S-adenosylmethionine (SAM). Ethanol consumption increases the rate of S-adenosylhomocysteine (SAH) in liver cells (19). Betaine reduces the rate of increasing SAH (17). In recent years, an increase in the number of intracellular SAHs has been reported with increasing apoptosis in many different cell types (18, 19).

In our study, supported by Tübitak 1002 support program, the role of statin use on skeletal muscle apoptotic cell death and the protective role of betaine in chronic alcohol consumption were investigated biochemically and histologically. Orally, betaine treatment has been shown to reduce liver necrosis, liver fat accumulation, apoptosis induced in various forms. A similar result was aimed at seeing when chronic alcohol use and statin use coexist. In this context; as a result of chronic alcohol use TNF-α, NF-kB, cytochrome c and caspase-3 levels as markers of skeletal apoptosis and statin consumption in rat muscle tissues were determined from cysteine proteases known to be involved in triggering apoptosis. CK enzyme levels, which are muscle injury indicators in serum of rats, were measured. Bcl-2 and Bax levels were determined by immunohistochemical analysis, TUNEL method and light microscopy. The results obtained here provide us with the first information in the literature about whether there is a protective role for betaine on the use of statin along with chronic alcohol consumption and will shed light on the development of different treatment strategies for the use of stain during chronic alcohol use.

Materials and methods

Materials

56 adult male Sprague-Dawley rats weighting 160–195 g were obtained from TICAM (Medical and Surgical Experimental Research Center, Eskisehir-TURKEY) and housed in polycarbonate cages in a room with controlled temperature (22 ± 2 DC), humidity (50 ± 5 %), and a 12 hour cycle of light and dark (07:00 AM-07:00 PM light). The rats were placed in separate cages and acclimated to laboratory conditions for one week before starting the experiments. The rats were randomized into 7 groups, each consisting of 8 animals.

Control group (n = 8): Received isocaloric modified liquid diet (MLD)

Alcohol group (n = 8): Received MLD solution with ethanol

Statin group (n = 8): Received isocaloric MLD and 10 mg/kg atorvastatin

Alcohol+statin group (n = 8): Received MLD solution with ethanol and 10 mg /kg atorvastatin

Alcohol+betaine group (n = 8): Received MLD solution with ethanol and betaine (1 % w/v).

Statin+betaine group (n = 8): Received isocaloric MLD, 10 mg/kg atorvastatin and betaine (1 % w/v)

Alcohol+statin+betaine group (n = 8): Received MLD solution with ethanol, 10 mg/kg atorvastatin and betaine (%1 w/v)

The MLD comprised 925 ml low fat cows' milk (Sütaş, Eskisehir, Turkey), from 25 to 75 ml alcohol (96.5 % v/v ethanol; Merck Millipore, Darmstadt, Germany) and 17 g sucrose (Merck Millipore). The caloric content of the diet was 1,007 kcal/l. The weight of the rats was recorded every day and the daily alcohol intake was also measured. The MLD was prepared daily and given to the animals while fresh. Extra chow or water were not available during the experimental period. At the beginning of the study, the rats were given MLD without alcohol for 2 days. Then, a liquid diet with 2.4 % alcohol was administered for 3 days. The alco-

Tab. 1. Biochemical results of groups.

Group	CK-MB (ng/ml)	Cytochrome C (C/M)	TNFα (pg/mg protein)	NF kB (ng/mg protein)	Caspase 3 (μmol pNA/minute/ /mg protein)	Calpain (pmol/minute/ /mg protein)
Control	24.63±4.32	1.40±0.46	10.50±2.66	11.52±2.86	0.25± 0.14	1.54±0.32
Alcohol	32.97±6.75[a]	3.05±0.78[a]	16.71±1.65[a]	17.78±2.06[a]	0.35±0.15	4.93±1.79[a]
Statin	36.92± 7.72[a]	1.78±0.57	16.06±3.94[a]	14.66±3.57	0.31± 0.03	3.15±0.75[a,b]
Alcohol+Betaine	22.43±2.38[b,c,d]	1.48±0.41[b]	11.17±2.16[b,c,d]	12.25±2.29[b]	0.30±0.21[d]	3.23±1.26[a,b]
Alcohol+Statin	39.06±5.12[a]	3.20±1.98[a,c]	16.39±1.93[a]	16.54±3.06[a,e]	0.90± 0.39[a,b,c]	3.96±0.97[a]
Statin+Betaine	26.17±5.86[c,d]	1.46±0.85[b,d]	12.92±2.52[b]	13.10±2.25[b]	0.21±0.23	2.54±0.86[b]
Alcohol+Statin+Betaine	26.51±6.38[c,d]	1.58±0.63[b,d]	11.97±1.69[b,c,d]	13.08±1.77[b]	0.39±0.15[d]	3.14±0.81[a,b]

Distribution of creatine kinase, cytochrome c, TNF-α, NF Kb, caspase-3 and calpain levels in rat groups. The results are expressed as mean ± standard deviation. a: Significant difference from control group ($p < 0.05$), b: Significant difference from alcohol group ($p < 0.05$), c: Significant difference from statin group ($p < 0.05$), d: Significant difference from alcohol+statin group ($p < 0.05$), d: Significant difference from alcohol+betaine group ($p < 0.05$)

Tab. 2. Histological results of groups.

	Groups (median 25–75%)				
	Necrosis	Myofibrillar degeneration	Nuclear proliferation	Nuclear hypertrophy	Edema
Control	0.00 (0.00–0.00)	0.20 (0.00–1.00)	0.00 (0.00–0.00)	0.10 (0.00–1.00)	0.00 (0.00–0.00)
Alcohol	1.25 (1.00–2.00)[a]	1.30 (1.00–2.00)[a]	1.40 (1.00–2.00)[a]	1.30 (1.00–2.00)[a]	1.30 (1.00–2.00)[a]
Statin	1.25 (1.00–2.00)[a]	1.40 (1.00–2.00)[a]	1.30 (1.00–2.00)[a]	1.20 (1.00–2.00)[a]	1.40 (1.00–2.00)[a]
Alcohol+Statin	2.80 (2.00–3.00)[a, b]	2.70 (2.00–3.00)[a, b]	2.90 (2.00–3.00)[a, b]	2.70 (2.00–3.00)[a, b]	2.80 (2.00–3.00)[a, b]
Alcohol+Betaine	0.75 (0.00–1.00)[a, c]	0.60 (0.00–1.00)[b]	0.80 (0.00–1.00)[a, b, c]	1.00 (0.00–2.00)[a, c]	0.80 (0.00–2.00)[a, c]
Statin+Betaine	0.50 (0.00–1.00)[a, b]	0.80 (0.00–1.00)	0.80 (0.00–1.00)[a]	0.80 (0.00–1.00)[a]	0.80 (0.00–2.00)[a]
Alcohol+Statin+Betaine	0.25 (0.00–1.00)[b, c, d]	0.20 (80.00–1.00)[b, c, d]	0.10 (0.00–1.00)[b, c, d]	0.30 (0.00–1.00)[b, c, d]	0.20 (0.00–1.00)[b, c, d]

Light microscopic evaluations: (0) no injury. (1) mild. (2) moderate and (3) severe. a: Statistical difference compared with control group $p < 0.05$. b: Statistical difference compared with alcohol group $p < 0.05$. c: Statistical difference compared with alcohol+statin groups $p < 0.05$. d: Statistical difference compared with alcohol+betaine groups $p < 0.05$

hol concentration was increased to 4.8 % during the following 4 days and finally reached 7.2 % over the next 21 days. Isocaloric modified liquid diet containing sucrose as a caloric substitute for alcohol (96 g sucrose and 75 ml cows' milk replaced 60.75 g or 75 ml ethanol) (20, 21).

10 mg / kg atorvastatin was dissolved in 0.9 % NaCl by a magnetic stirrer and given oral gavage for 30 days at the same time.

The rats were anesthetized (Ketamine / Xylazine 3: 1, 1.32 mg / kg intraperitoneal) and blood was taken from their hearts. Skeletal muscle was surgically removed. Tissues used for biochemical studies were frozen and kept at –80 °C until they were tested. For histological studies, the tissues were fixed in neutral form for 24–48 hours. Routine histologic follow-up of tissues was done after fixation. All experiments were carried out in accordance with institutional guidelines for animal welfare (Eskisehir Osmangazi University Animal Care and Use Committee, Eskisehir, Turkey) and were approved by the Ethics Committee of the Medical and Surgical Experimental Research Center of Osmangazi University

Methods

Measurement of CK Activity: Serum was measured using the CK Elisa kit (MAK116, Sigma-Aldrich).

Measurement of TNF-α Levels: Tissue TNF-a levels were measured using a quantitative ELISA test kit (RAB0479, Sigma-Aldrich).

Measurement of NF kB Levels: Tissue NF kB levels were measured using a quantitative ELISA test kit (CSB-E13148r, Cusabio).

Measurement of Caspase-3 Activities: Tissue samples were homogenized (1/10 w/v) in lysis buffer (250 mM HEPES, pH 7.4, 1 % CHAPS, 50 mM DTT, 10 mM EDTA) and then centrifuged for 20 min at 3,000 rpm and 4 °C. Supernatants were collected and centrifuged twice at 14,000 rpm for 15 min (22). Commercial assay kit (Sigma CASP-3-C) was used to measure caspase-3 activities in supernatant fractions. Results were expressed in μmol pNA/minute.

Measurement of Cytochrome-c Release: Cytochrome c levels were measured in both cytosolic and mitochondrial fractions using a commercial assay kit (R&D Systems MCTC0) and cytosolic fraction/mitochondrial fraction ratios were used as indicators of mitochondrial damage (23, 24).

Calpain Activity Assay: The difference between measurement rates in calcium free and calcium containing assay buffer was used to determine calpain activity. Calpain activity was determined as the difference between the calcium-dependent fluorescence and the

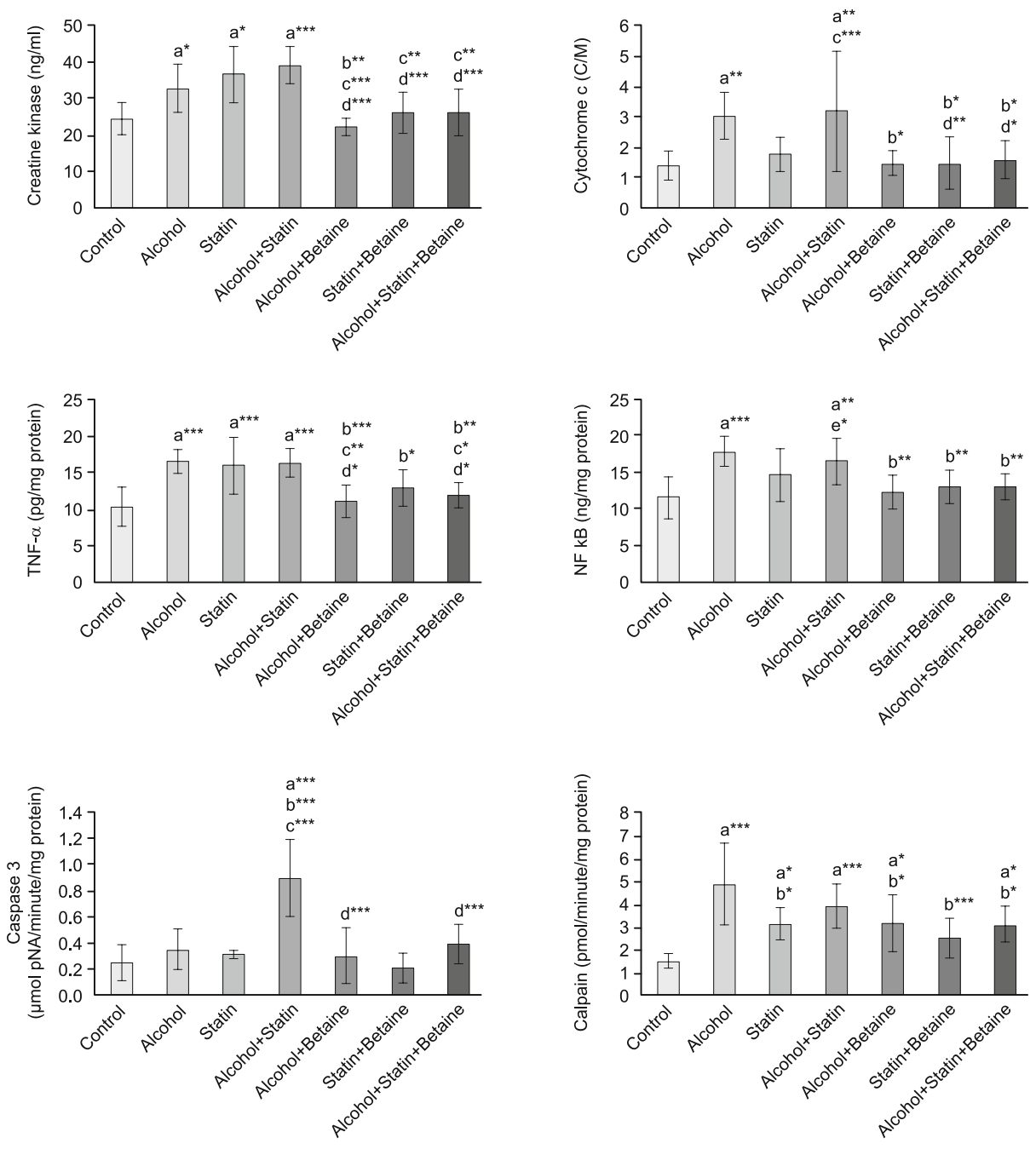

Fig. 1. Distribution of creatine kinase, cytochrome c, TNF-α, NF Kb, caspase-3 and calpain levels in rat groups. The results are expressed as mean ± standard deviation in eight rat of each groups. a*: Compared with control group p<0.047, a: Compared with control group p<0.010, a***: Compared with control group p<0.001, b*: Compared with alcohol group p<0.047, b**: Compared with alcohol group p<0.010, b***: Compared with alcohol group p<0.001, c*: Compared with statin group p<0.047, c**: Compared with statin group p<0.010, c***: Compared with statin group p<0.001, d*: Compared with alcohol+statin group p<0.047, d**: Compared with alcohol+statin group p<0.010, d***: Compared with alcohol+statin group p<0.001, e*: Compared with alcohol+betain group p<0.047**

noncalcium-dependent fluorescence. A 7-amino-4-methyl coumarin (AMC) standard curve was constructed. Calpain activity was expressed as pm of AMC released per minute of incubation time per minute of total protein (25).

Histological analysis

At the end of the experimental period all the groups were sacrificed with anesthesia and muscle specimens of rats were fixed in 10 % formalin fixative for 48 hours in order to be able to perform his-

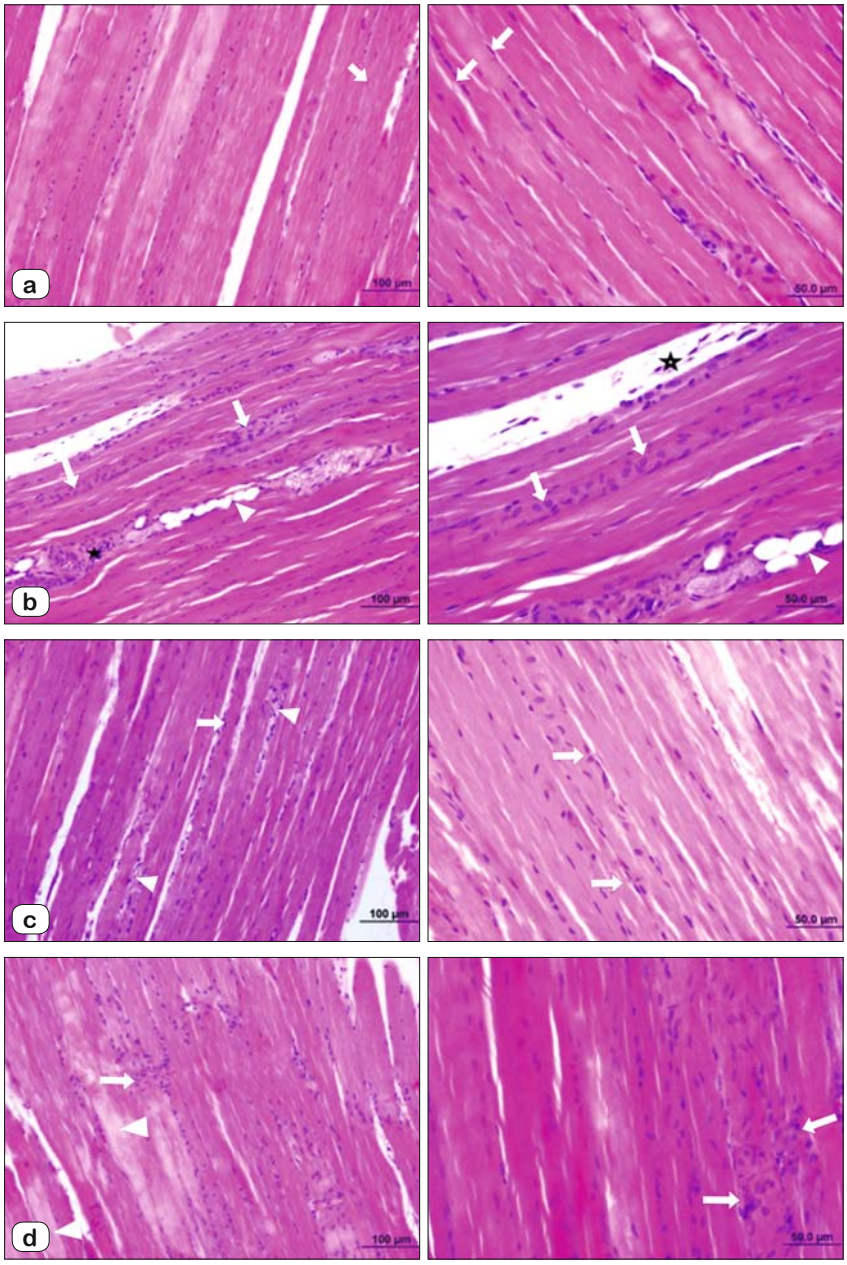

Fig. 2. Light microscope results in rat groups (a-g). a) Control group, the overall appearance. Located peripheral nuclei →), (HE, scale bar: 100 µm, X20, scale bar: 50.0 µm, X40). b) Alcohol group: (*) partial edema, (►) vacuolization, (→) nuclear hypertrophy (HE, scale bar: 100 µm, X20, scale bar: 50.0 µm, X40). c) Statin group: (►) partial edema, (→) nuclear hypertrophy (HE, scale bar: 100 µm, X20, scale bar: 50.0 µm, X40). d) Alcohol+statin group: ►) necrotic myofibrils, (→) nuclear proliferation (HE, scale bar: 100 µm, X20, scale bar: 50.0 µm, X40).

tological examinations at light microscopic level. The samples were subjected to routine procedures, were processed into paraffin-embedded blocks. 5 µm-sections were obtained from each paraffin-embedded block, and these sections were stained with Hematoxylin Eosin and examined under Olympus BH-2 microscope and photographed. In addition, sections were taken for TUNEL staining to determine apoptotic cells from all muscle tissue specimens. The paraffins of the preparations were melted at 60 degrees for 1 hour. Then TUNEL staining technique was applied to muscle tissue specimens. Immunohistochemically, sections were taken from the same muscle tissue samples and Bcl-2 and Bax levels were measured.

Statistical analysis

SPSS software, version 15.0 for Windows (SPSS, Inc., Chicago, IL, USA) was used for the statistical analysis of biochemical data. In order to assess differences between groups, one-way analysis of variance (ANOVA) and Tukey's multiple comparison test were used. Results are presented as mean ± standard deviation and $p<0.05$ was considered to indicate a statistically significant result.

Results

Biochemistry results

CK levels: Serum CK levels in the alcohol ($p < 0.047$), statin ($p < 0.010$) and alcohol+statin ($p < 0.001$) groups were significantly increased compared with the control group. The CK levels in the alcohol+betaine group exhibited a significant reduction compared with the alcohol, statin and alcohol+statin group (respectively $p < 0.010$, $p < 0.001$, $p < 0.001$). The CK levels in the statin+betaine and alcohol+statin+betaine group were decreased compared with the statin group ($p < 0.010$).The CK levels of the statin+betaine and alcohol+statin+betaine were found to be significantly lower compared with alcohol+statin group ($p < 0.001$) (Tab. 1).

Cytochrome-c release: Cytochrome-c release in the alcohol, statin and alcohol+statin groups was increased compared with the control group and this increase was statistically significant only for the alcohol+statin group ($p < 0.001$). In the alcohol+statin group it was significantly increased compared with the statin group ($p < 0.001$). The release cytochrome c levels of the statin+betaine group were found to be lower compared with alcohol and alcohol+statin group (respectively $p < 0.047$, $p < 0.010$). The release cytochrome c levels in the alcohol+statin+betaine group exhibited a significant reduction compared with the statin and alcohol+statin group (respectively $p < 0.010$, $p < 0.001$) (Tab. 1).

TNF-α Levels: TNF- α levels in the alcohol, statin and alcohol+statin groups were significantly increased compared

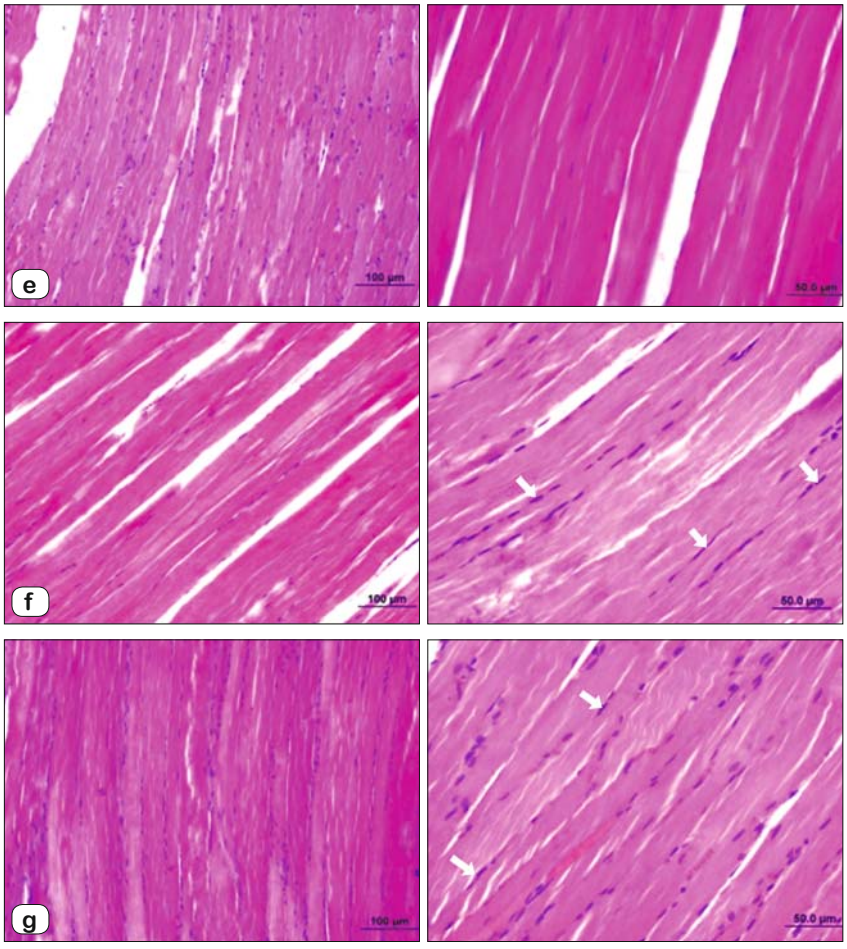

Fig. 2. Light microscope results in rat groups (a-g). e) Alcohol+betaine group: (→) Located peripheral nuclei (HE, scale bar: 100 µm, X20, scale bar: 50.0 µm, X40). f) Statin+betaine group: (→) Located peripheral nuclei (HE, scale bar: 100 µm, X20, scale bar: 50.0 µm, X40). g) Alcohol+statin+betaine group: (→) Located peripheral nuclei (HE, scale bar: 100 µm, X20, scale bar: 50.0 µm, X40).

with the control group ($p < 0.001$). The TNF-α levels of the alcohol+betaine group were found to be lower compared with alcohol, statin and alcohol+statin group (respectively $p < 0.001$, $p < 0.010$, $p < 0.047$). In the statin+betaine group the levels were decreased compared with the alcohol group ($p < 0.047$). The TNF-α levels in the alcohol+statin+betaine group exhibited a significant reduction compared with the alcohol, statin and alcohol+statin group (respectively $p < 0.010$, $p < 0.047$, $p < 0.048$) (Tab. 1).

NF-kB Levels: Compared with the control group, NF kB levels in the alcohol and alcohol+statin group were found to be statistically significantly higher (respectively $p < 0.001$, $p < 0.010$). In the alcohol+betaine, statin+betaine, alcohol+statin+betaine groups the levels were found to be decreased compared with the alcohol group and this decreased was statistically significant ($p < 0.010$). The NF kB levels in the alcohol+statin group exhibited a significant increment compared with the alcohol+betaine group ($p < 0.047$) (Tab. 1).

Caspase 3 enzyme activity: When the caspase 3 activities of the groups were assessed significantly, it was seen that the caspase 3 activity of alcohol+statin group statistically showed a significant increase compared to the control group ($p < 0.001$). Caspase 3 activities of alcohol+statin group were statistically significantly increased compared with the alcohol and statin groups ($p < 0.001$). The caspase 3 enzyme activities of the alcohol+betaine and alcohol+statin+betaine groups were found to be statistically lower compared with alcohol+statin group ($p < 0.001$) (Tab. 1).

Calpain activity: Compared with the control group, calpain activities in the alcohol, alcohol+statin, statin and alcohol+betaine group were found to be statistically significantly higher (respectively $p < 0.001$, $p < 0.001$, $p < 0.047$, $p < 0.047$). In the statin, alcohol+betaine and alcohol+statin+betaine groups the levels were found to be statistically decreased compared with the alcohol group ($p < 0.047$). The calpain activity of the statin+betaine group was found to be statistically lower compared with alcohol group ($p < 0.001$) (Tab. 1).

Histological results

Light microscope evaluation: Light microscopy results are shown in Figure 1. In light microscope evaluation, samples were scored according to the presence of necrosis, myofibrillar degeneration, nuclear proliferation, nuclear hypertrophy and edema. Obtained data was compared statistically. According to the data, necrosis rate was quite high in alcohol, statin and alcohol+statin groups compared to control group and this increase was significant ($p < 0.001$). Necrosis rate was moderately high in statin+betaine and alcohol+betaine group compared to control group ($p < 0.046$). Necrosis in the alcohol + statin group was significantly increased compared to alcohol and statin groups. Necrosis rate was quite significantly decreased in alcohol+statin+betain group compared to alcohol and statin groups. Necrosis in the statin+betaine group was significantly reduced compared to statin groups ($p < 0.046$). Necrosis rate was quite high in alcohol+statin group compared to statin+betaine and alcohol+betaine group and this increase was significant ($p < 0.001$) (Tab. 2).

Myofibrillar degeneration in the alcohol, statin and alcohol+statin groups were increased quite significantly compared to control group ($p < 0.001$). Myofibrillar degeneration in the alcohol+statin group was increased quite significantly ($p < 0.001$), alcohol+betaine and alcohol+statin+betaine groups were quite significantly decreased compared with alcohol group ($p < 0.001$). Myofibrillar degeneration in the alcohol+statin group was quite

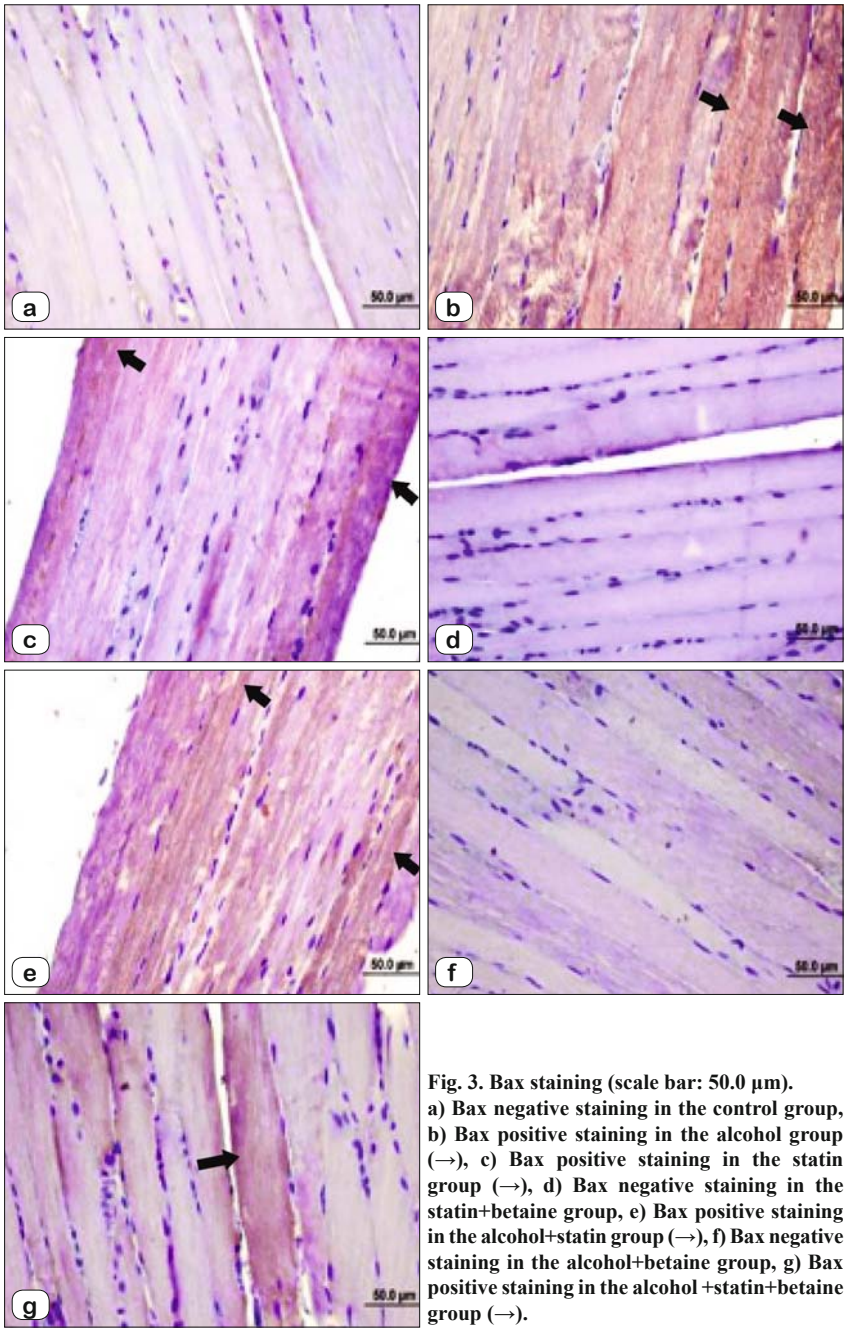

Fig. 3. Bax staining (scale bar: 50.0 µm).
a) Bax negative staining in the control group, b) Bax positive staining in the alcohol group (→), c) Bax positive staining in the statin group (→), d) Bax negative staining in the statin+betaine group, e) Bax positive staining in the alcohol+statin group (→), f) Bax negative staining in the alcohol+betaine group, g) Bax positive staining in the alcohol +statin+betaine group (→).

significantly increased ($p < 0.001$), whereas in the alcohol+betaine and alcohol+statin+betaine significantly decreased compared to statin group (respectively $p < 0.010$ and $p < 0.001$). Myofibrillar degeneration in the alcohol+statin+betaine group was decreased quite significantly compared to alcohol+statin group ($p < 0.001$) (Tab. 2).

Nuclear proliferation in myofibrills in the alcohol, statin, alcohol+statin and statin+betaine groups were increased quite significantly compared to control group ($p < 0.001$). Compared with alcohol group, nuclear proliferation was increased statistically significantly in alcohol+statin group ($p < 0.001$). A significant decrease was seen in statin+betaine, alcohol+betaine and alcohol+statin+betaine groups (respectively $p < 0.05$, $p < 0.05$, $p < 0.001$). There was a significant increase in the statin group compared to the alcohol+statin group ($p < 0.001$). This increase in proliferation was decreased significantly in the alcohol+statin+ betaine group ($p < 0.001$). A significant decrease was observed in the alcohol+statin+ betaine group compared to the statin+betaine group ($p < 0.001$). Compared with alcohol+statin group, a significant decrease was found in alcohol+betaine and alcohol+statin+betaine groups ($p < 0.001$) (Tab. 2).

Hypertrophy in alcohol, statin, statin+betaine, alcohol+statin and alcohol + betaine groups was increased quite significantly compared to control group (respectively $p < 0.001$, $p < 0.001$, $p < 0.046$, $p < 0.001$, $p < 0.001$). A significant decrease was found in the alcohol+statin+betaine group ($p < 0.001$), whereas a significant increase was observed in the alcohol+statin group compared to alcohol and statin groups ($p < 0.001$). Hypertrophy in the alcohol + statin group was significantly higher than in the alcohol + betaine group ($p < 0.001$). Compared to alcohol + statin group, hypertrophy in the alcohol+statin+betaine groups was decreased statistically significantly ($p < 0.046$) (Tab. 2).

Edema in connective tissue surrounding myofibrils of statin, alcohol and alcohol + statin groups was quite statistically higher than in the control group ($p < 0.001$). Statin + betaine and alcohol + betaine groups showed a significant increase in edema compared to the control group ($p < 0.010$) (Tab. 2).

Edema in alcohol + statin group was statistically significantly higher than in the alcohol and statin groups ($p < 0.001$), but

Tab. 3. Bax levels.

	Bax
Control	0.20 (0.00–1.00)
Alcohol	1.30 (1.00–2.00) [a]
Statin	1.30 (1.00–2.00) [a]
Alcohol+Statin	2.90(2.00–3.00) [a.c.d]
Alcohol+Betaine	0.10 (0.00–1.00) [c]
Statin+Betaine	0.80 (0.00–1.00) [b]
Alcohol+Statin+Betaine	0.80 (0.00–1.00) [b.e]

a: Compared with control group $p < 0.05$, b: Compared with control group $p < 0.05$, c: Compared with alcohol group $p < 0.05$, d: Compared with statin+betaine group $p < 0.05$, e:Compared with alcohol+statin group $p < 0.05$

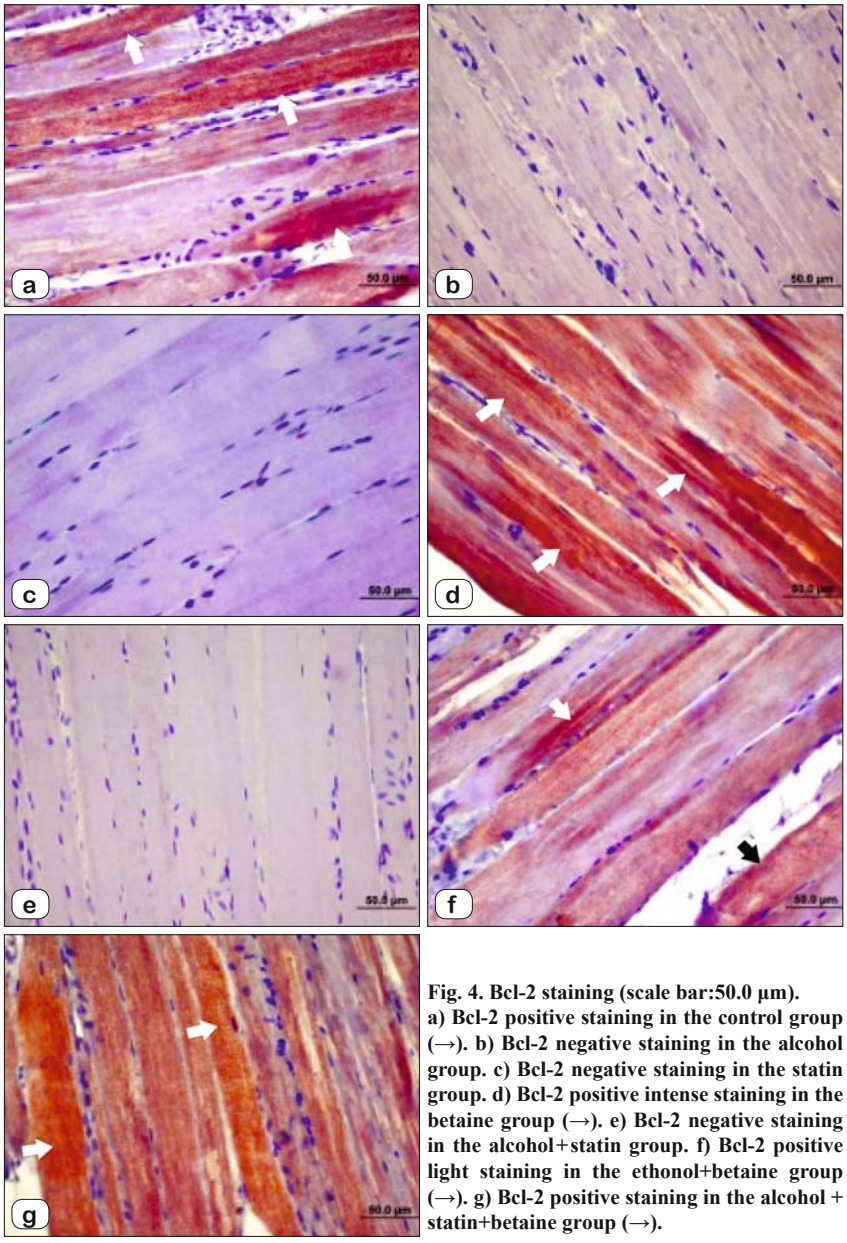

Fig. 4. Bcl-2 staining (scale bar:50.0 µm). a) Bcl-2 positive staining in the control group (→). b) Bcl-2 negative staining in the alcohol group. c) Bcl-2 negative staining in the statin group. d) Bcl-2 positive intense staining in the betaine group (→). e) Bcl-2 negative staining in the alcohol+statin group. f) Bcl-2 positive light staining in the ethonol+betaine group (→). g) Bcl-2 positive staining in the alcohol + statin+betaine group (→).

this increase was significantly reduced in the alcohol+statin+ betaine group ($p < 0.001$). The edema in the alcohol+statin group was significantly higher than in the alcohol +betaine and alcohol+statin+betain groups ($p < 0.001$) (Tab. 2).

Bax Levels: When bax levels were examined in skeletal muscle specimens constituting all groups, there was a progressive increase in alcohol, statin, alcohol+statin groups ($p < 0.001$), a statistically significant increase in statin+betaine and alcohol+statin+betaine groups ($p < 0.046$). There was a significant increase in alcohol+statin group compared to alcohol group ($p < 0.001$), this increase was significantly reduced in the alcohol+betaine group ($p < 0.001$). Bax levels in alcohol + statin group were higher than in the statin+ betaine group ($p < 0.001$). Statistically significant reduction in the alcohol+statin+betaine group was observed when compared with the alcohol + statin group ($p < 0.001$) (Tab. 3, Fig. 2).

Bcl-2 Levels: Bcl-2 levels were examined in skeletal muscle samples constituting all groups and it was found low in alcohol and statin groups and high in alcohol+ statin+betaine group when compared with control group ($p < 0.001$). Bcl levels were high whereas it was low in alcohol+betaine group compared to alcohol group ($p < 0.001$). Bcl-2 levels in alcohol+statin and statin+betaine groups were lower than in the statin group ($p < 0.047$). A significant increase was seen in the alcohol+statin+betain group compared with the statin group ($p < 0.001$) (Tab. 4, Fig. 3).

TUNEL results: Skeletal muscle samples were stained with TUNEL method and brown stained samples were evaluated as TUNEL (+). TUNEL values were increased in skeletal muscle myopathy caused by alcohol, statin and combine using of alcohol+statin. Also this increased TUNEL values were decreased in the alcohol+statin+betaine group (Fig. 4).

Discussion

Excessive alcohol consumption and alcohol-related problems are also a major public health problem and result in large proportions of mortality and morbidity. Many factors influence the development of alcohol-related problems (26, 27). One of the most common causes of myopathy is the use of drugs from the statin group (7). The most commonly used antihyperlipidemic drugs are statins, HMG CoA reductase inhibitors, due to theit high activity and low side effect profile (28). Tiredness, weakness symptoms and myopathy are the most common side effect of statin drugs (7). It is estimated that at least 1.5 million

Tab. 4. Bcl-2 levels.

	Bcl–2
Control	1.40 (1.00–2.00)
Alcohol	0.20 (0.00–1.00) [a]
Statin	0.10 (0.00–1.00) [a]
Alcohol+Statin	0.80 (0.00–1.00) [c]
Alcohol+Betaine	1.00 (0.00–2.00) [b]
Statin+Betaine	0.80 (0.00–1.00) [c]
Alcohol+Statin+Betaine	2.70 (2.00–3.00) [a,b,c,d]

a: Compared with control group $p < 0.05$, b: Compared with alcohol group $p < 0.05$, c: Compared with statin group $p < 0.05$, d: Compared with alcohol+statin+betaine group $p < 0.05$

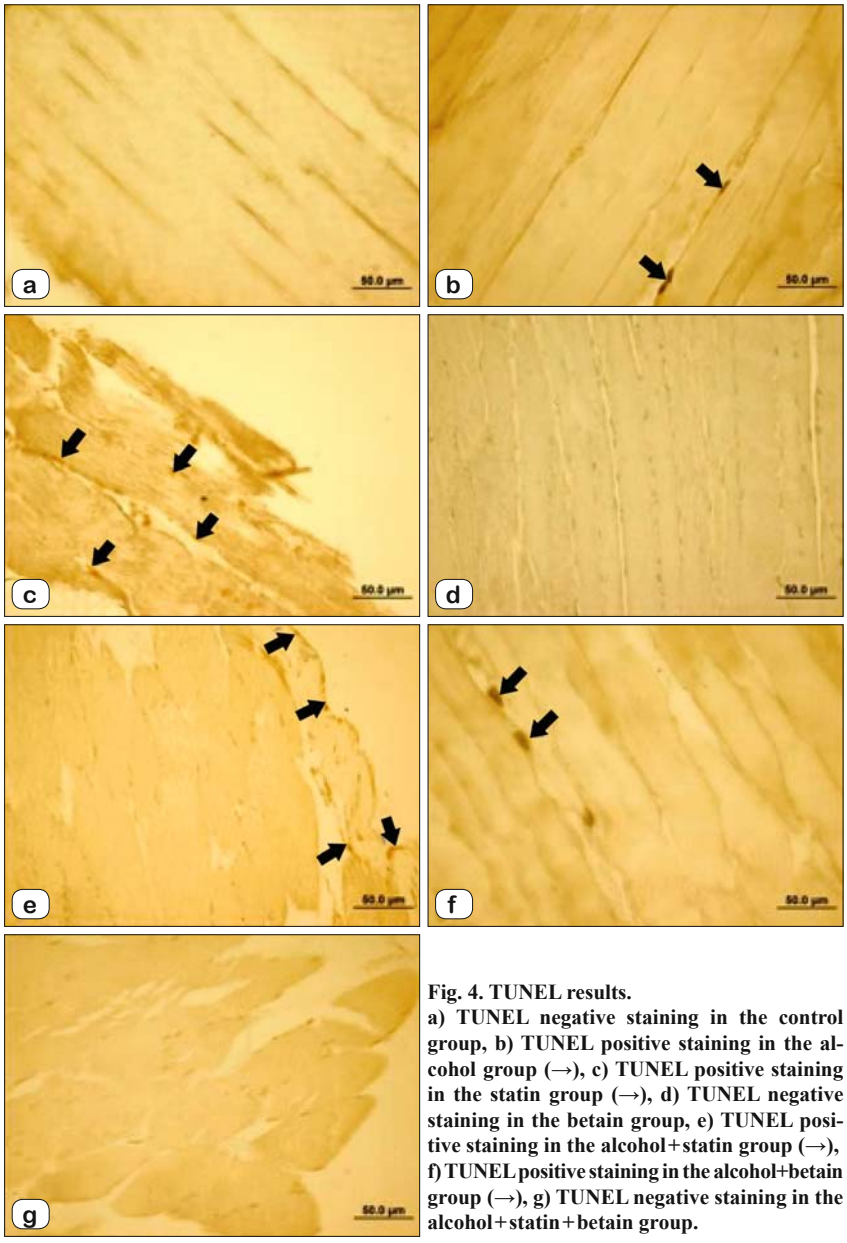

Fig. 4. TUNEL results.
a) TUNEL negative staining in the control group, b) TUNEL positive staining in the alcohol group (→), c) TUNEL positive staining in the statin group (→), d) TUNEL negative staining in the betain group, e) TUNEL positive staining in the alcohol+statin group (→), f) TUNEL positive staining in the alcohol+betain group (→), g) TUNEL negative staining in the alcohol+statin+betain group.

people have muscle complaints related to statin use annually (29). An increase in creatine kinase levels biochemically is observed in statin-bound myotoxicity (30). In our study, creatinine kinase levels were increased in alcohol and statin treated groups. Pasternak and colleagues concluded that the combined use of ethanol and statin in the study was an increasing risk factor for myopathy (31). In parallel with this study, in our study the greatest increase in creatine kinase levels was also noted in the group in which alcohol and statin were given together. However, Studies have also shown normal creatine kinase levels in statin-dependent myotoxicity (32, 33). In our study creatine kinase levels were increased in the alcohol and statin given groups. In previous study, Pasternak and colleagues have argued that the combined use of ethanol and statin represents an increased risk for the formation of myopathy (31). Paralleling this study, increase in creatine kinase levels was observed in combining of alcohol and statin group.

It is known that long term alcohol use has an apoptosis-initiation effect by impairing mitochondrial permeability. In this way, cytochrome c is released into the cytosol and binds with Apaf-1 and activated caspase-9. The proteolytic activity of caspase-9 activates caspase-3 and apoptosis is initiated (34). In our study to determine the effect of statin and alcohol use on muscle, we measured the release of mitochondrial cytochrome c into the cytosol. We also measured the amount of cytochrome c remaining in the mitochondria and estimated cytosol to mitochondria ratio. Because of this ratio, as the cytochrome c content decreases in the mitochondrial fraction when the cytosol penetration increases, the ratio between them also increases remarkably. As in earlier studies, this rate represents mitochondrial damage (35). In our study this ratio increased in alcohol, statin, and alcohol + statin groups, but the highest increase was seen in alcohol + statin group. These results have also affected the results of caspase-3, since cytosol-releasing cytochrome c is involved in the caspase-9 activation necessary for the activation of caspase-3 (36). In parallel with our cytochrome c results, the highest increase was in our alcohol + statin group. We attributed the highest cytrochrome c and caspase 3 results in the alcohol + statin group to the effect of apoptosis both using alcohol and statin separately. Human and rodent studies have shown that statin administration induced apoptosis in the skeleton (37, 38, 39).

The use of statin causes an increase in cytosolic calcium resulting in a change in synthesis of isoprenoid. Increased levels of calcium also cause activation of the calpains. Calpains generate apoptosis by initiating caspase cascade (7). In our study, there was a statistically significant increase in the statin group compared to the control group. Rajgopal et al. demonstrated that calpains are active in cell death caused by ethanol (40). In our study, calpain levels of alcohol and alcohol + statin group had a significant increase compared to control group.

Studies have shown that there is a decrease in the ratio of Bcl2/Bax that causes cytochrome c release in statin-induced apoptosis in skeletal muscle (7). Our results of our Bcl-2/Bax in our histology findings support this decrease. In another study; an increase in TUNEL values was observed in skeletal muscle myopathy caused by alcohol (41).

Histological examinations of our study showed that, necrotic myofibrils and intense nuclear proliferation are evident in alcohol and statin groups, while partial edema, vacuolization, centrally located nuclei, nuclear proliferation in the myofibrils and necrotic myofibrils in the alcohol+statin group in association with these were shown previous studies. Alcohol +betaine+statin group showed decreased myofibril damage and muscular cells in histological structure near normal cells.

Betaine is lipotropic and passes through the lipid layer quickly when given externally and inhibits lipid peroxidation in cellular membranes. The addition of betaine to protect against necrotic damage is shown in a study by Kim et al (42). As in our previous study with betaine (43), in this study, betaine-treated treatment groups showed that calpain and caspase activities and cytokine c release were decreased caused by alcohol, statin and more importantly alcohol + statin, and TNF and NF kB levels were also close to the control group.

Similarly, significant improvements have been observed in our morphological and histological examination results that are also supported by our biochemical data.

In conclusion, our findings in this experimental study suggested that alcohol and statin combined consumption increase apoptotic cellular degeneration in the skeletal muscles and following betaine treatments can cause a decrease in this apoptotic damage. We have found protective efficacy of betaine and believe that these findings will be a stepping stone for future studies. Further studies are also needed about the possible effects of application of this supplement.

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