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ARAŞTIRMA MAKALESİ /**RESEARCH ARTICLE**

GPSE (GRAPE SEED PROANTHOCYANIDIN EXTRACT) SIMVASTATIN AND SILYMARIN EFFECT ON HIGH CHOLESTEROL-FED ANIMALS

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ABSTRACT

The present study was designed to test the hypothesis that cholesterol rich diet-induced hypercholesterolemia in rabbits can be reversed by long-term treatment of antioxidants (proanthocyanidin and silymarin) and antihyperlipidemic drug(simvastatin).

In this study 35 adult New Zealand White (NZW) male rabbits were used and divided into five groups. The rabbits in Group 1(control) was fed with rabbit chow diet. Group 2 was fed with only cholesterol (2% w/w) rich diet to induce hypercholesterolemia. The other groups received GPSE 100mg/kg (Group 3), silymarin 10mg/kg (Group 4) and simvastatin10mg/kg (Group 5) with cholesterol in addition to rabbit chow diet. At the end of the experiment, the animals were sacrified and their aorta were excised for intimal lesion analysis. Vessells were dissected, trimmed, and then placed fixed in neutral formalin. All tissue sections were stained with hematoxylin and eosin(H&E). Apoptotic process was detected by using TUNEL.

Cholesterol treatment produced a sustainable state of atheromatous plaque formation In addition, only cholesterol rich diet-induced animals have shown a considerably high number apoptotic cells by using TUNEL and plasma total cholesterol (TC) and low density lipoprotein (LDL) levels were found

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to be significantly high in this group ($p<0.05$). But simvastatin was significantly effective on the decreased plasma lipid levels. Histomorphometric intimal lesion analysis of the aorta showed less atheromatous plaque formation in simvastatin, GPSE and silymarin groups, respectively. Relaxation responses were significantly decreased in cholesterol group, but simvastatin treatment and also antioxidants significantly increased ach responses to the control level.

Cholesterol rich diet-induced hypercholesterolemia in rabbits partially was reversed by adding antioxidants prolonging its use. In addition simvastatin modulated lipid profiles.

Keywords: Atherosclerosis, Grape seed extract, Proanthocyanidin, Simvastatin, Silymarin.

YÜKSEK KOLESTEROL DİYETİ İLE BESLENEN HAYVANLARDA ÜZÜM ÇEKİRDEĞİ EKSTRESİ (PROANTHOSIYANIDIN), SIMVASTATIN VE SILYMARININ ETKİSİ

ÖZ

Araştırmamızda kolesterolden zengin diyet ile beslenen tavşanlarda hiperkolesterolemi üzerine uzun süreli antioksidanların (üzüm çekirdeği ekstresi ve silymarin) ve antihiperlipidemik ilaçların (simvastatin) kullanımının etkisi incelenmiştir.

Araştırmamızda 35 adet yetişkin Yeni Zelanda türü erkek tavşan kullanılarak 5 grup oluşturuldu. Grup 1 kontrol grubu olup hayvanlar sadece tavşan yemi ile beslendi. Grup 2 kolesterolden zengin diyet ile beslendi. Diğer üç grup ise kolesterolden zengin diyetin yanısıra üzüm çekirdeği ekstresi/proanthosiyanidin (100mg/kg-grup 3), silymarin (10mg/kg-grup 4) ve simvastatin (10mg/kg-grup 5) ile beslendi. Deney sonunda tüm hayvanlar anestezi altında sakrifiye edildi ve aortaları alınarak intiba lezyonları incelendi. Damarlar daha sonra çevresel dokulardan temizlenerek nötral formalinde fikse edildi. Kesitler hematoksilen ve eosin ile boyandı. Apoptoz ise TUNEL metodu ile belirlendi.

Kolesterol diyeti ile beslenen hayvanlarda, ateromatöz plak oluşumu çok sayıda TÜNEL pozitif hücrelerin yanısıra plazma total kolesterol miktarı ile LDL diğer gruplara oranla anlamlı olarak (p 0.05) yüksek bulundu. Simvastatin ise plazma lipid seviyelerini belirgin olarak azalttı. Aortaların intima lezyonlarının histomorfometrik analizlerinde ise ateramatöz plaklar etki sırasına göre simvastatin, üzüm çekirdeği ekstresi, silymarin gruplarında azalmıştı Gevşeme yanıtları ise kolesterol grubunda en az, simvastatin ve antioksidan gruplarında artmış olup kontrol grubu değerlerine yakındı.

Sonuç olarak kolesterolden zengin diyetle beslenen tavşanlardaki hiperkolesterolemi uzun süre antioksidan kullanımı ile kısmen önlenmiştir. Simvastatin ise lipid profillerini düzenlemiştir.

Anahtar Kelimeler: Ateroskleroz, Üzüm çekirdeği ekstresi, Simvastatin, Silymarin.

1. INTRODUCTION

Atherosclerosis is a complex and relatively slow-progressing disease. It is clearly demonstrated that hypercholesterolemia, due to high level LDL in plasma and the subsequent oxidation of these lipoproteins giving ox-LDL, is one of the main causes of atherosclerosis in human and different animal models (Ballatyne et al. 2001; Choy et al. 2003; Heinecke, 2003; Noguchi, 2002; Upston, 2003). The oxidative hypothesis of atherosclerosis indicates that inhibiting the oxidation of LDL may help to prevent the development of atherosclerotic disease. In addition study indicated that antioxidants are new preventative and therapeutic agents which may take their place alongside cholesterol lowering drugs (Amom et al. 2008). Some investigators demostrated that proanthocyanidin provides excellent protection against free radicals in both in vitro and in vivo human and animal models (Ariga, 2004; Bagchi et al. 2000, 2002). Proanthocyanidins have been demostrated to exhibit a wide range of biological effects including antibacterial, antiapoptotic antiatherosclerotic, antiviral, antiinflammatory, antiallergic and vasorelaxing activity, modulation of lipid metabolism and inhibition of LDL oxidation (Bagchi et al. 1998; 2003, Fine 2000; Hecth and Harman 2003; Joshi et al., 2000; Waters 2005;Yamakoshi et al. 1999). There are some data indicating capability of silymarin to modulate and positively affect lipoprotein metabolism (Skottava et al., 1997, 1999). In addition, statins are the most potent drugs available for reducing plasma ox-LDL cholesterol concentrations (Rosenson, 2004).Treatment of hypercholesterolemic patients with simvastatin has been shown to reduce monocyte adhesion to endothelial cells and inflammation in atherosclerotic plaques (Hernandez-Presa et al. 2003) and in vitro studies have demostrated that lipophilic inhibitors (statins) inhibit proliferation of SMCs (Bellosta et al. 1999; Shiomi et al. 2005).

The aim of this study is to compare the effect of proanthocyanidin rich extract from GPSE, simvastatin and silymarin on some biochemical parameters and vascular reactivity in high cholesterol-fed rabbits.

MATERIALS AND METHODS

Animals

Young, 7-8 months old, thirty-five male New Zealand White rabbits weighing between 2.2-2.6 kg were divided into five groups (Table 1) after 2 weeks of adaptation. They were housed in metal cages in an air-conditioned room (23±1 ºC, 55±5 % humidity)under a 12-h light and 12-h dark cycle. The experiments were approved by the Ethics Committee of the Eskisehir Osmangazi University, and the animal care was taken according to approved standards of Laboratory Animal Care. The animals were given a standard laboratory diet and vegetables. The rabbits were on their respective diets for 10-12 weeks. The diets were prepared freshly every one week period and stored at 4º C until their use in order to prevent oxidation and loss of antioxidants (Amom et al. 2008; Prasadi 2005). Throughout the experimental period, they were given in restricted amounts (90g/head per day of each diet). The rabbits in Group 1 were fed by rabbit chow diet. The other groups received simvastatin, silymarin and GPSE with cholesterol only, in addition to rabbit chow diet. The diet was specially prepared and did not contain any antioxidants. Water and food supplied ad libitum.

Groups
I (n=7) Control (rabbit chow diet bought from Oguzlar Yem - Turkiye)
II (n=7) Cholesterol diet 2%(w/w)for 12 weeks (Cholesterol, Sigma, U.S.A.) (Chol group)
III (n=7) GPSE (Proanthocyanidin 100mg/kg) + Cholesterol diet (Pure Grape Seed Extract, GNC Products, 736112, U.S.A.) (GPSE+Chol group)
IV (n=7) Silymarin (10mg/kg) +Cholesterol diet (Silymarin Sigma S-292 USA) (Sily+Chol group)
V (n=7) Simvastatin (10mg/kg) +Cholesterol diet (Simvastatin, Zocor, Merck Sharp and Dohme USA) (Sim+Chol group)
Blood samples (from ear marginal artery) were collected before (time 0) and after 1,5 months on their respective diets for measurements of lipid profiles. At the end of the protocol rabbits were

Table 1. Vessel wall thickness

Groups	n	$\overline{X} \pm S_{\bar{x}}$	
Chol	7	21.44 ± 0.65	$F_{3;16} = 2.99$ $P<0.05$
GPSE +Chol	7	15.76 ± 2.20	
Sily+Chol	7	16.46 ± 2.20	
Sim+Chol	7	13.25 ± 2.37	

Chol group is significantly different in all groups ($p<0.05$). There are no difference between GPSE+Chol, Sily+Chol and Sim+Chol ($p>0.05$).

anesthetized with xylasine (5mg/kg intraperitoneal) and ketamine (35mg/kg intraperitoneal) and bloods were collected by intracardiac injection. Then, aortas were removed for assessment of atherosclerotic changes. Firstly, one descending aorta segment was carefully excised and placed in Krep's solution for organ bath. Then, other aortas were divided into six transverse sections taken from whole regions for histologic analysis and about randomly 30 slices selected were taken from each section. Atherosclerotic lesions were measured in these slices. Serial sections (5μm) were prepared for each segment. On average 180-200 slices were collected per rabbit. Sections were stained with hematoxylin & eosin, Massons' trichrome and for elastin with Verhoff's and for apoptosis with TUNEL staining. Digital images were obtained by Olympus BX-61 microscope with DP70 digital camera.

Transmission Electron Microscopy

To provide morphological evidence of intimal variations and foam cells, we performed transmission electron microscopy of aortas (Hasdai et al.,1999). Tissue of aortas from Groups 1,2,3,4 and 5 were fixed in 2.5 % gluteraldehyde (pH:7.3). Semithin sections were stained with toluidine blue and ultrathin section of the areas of interest stained with uranyl acetate and lead citrate and examined with JEOL TEM 1220 (Tokyo, JAPAN) electron microscope.

Quantitative Analysis of Atherosclerotic Lesions

Quantitative histomorphometric measurements were obtained by using calibrated microscope. The morphology of atherosclerotic lesions in aortas were assessed using an ocular micrometer standardized against a stage micrometer. Arterial sections stained with elastic Verhoff were manually traced with the following parameters measured (Hasdai et al.1999; Singh et al. 2000).

1- vessel wall thickness between the internal elastic lamina and external elastic lamina
2- surface area of atherom plaques

The degree of aortic atherosclerosis was evaluated by the lesion area on the surface of whole intima (surface area of lesion/ surface area of the whole intima) of six segments of each rabbit.

In Situ Apoptosis Detection

Cleavage of genomic DNA during apoptosis may yield double stranded, low molecular-weight DNA strand breaks that can be labeled by terminal deoxynucleotidyl transferase (TdT) which catalyzes polimerization of labeled nucleotides to free 3'-OH in a template-independent manner (TUNEL reaction). The quantity of apoptotic cell death was evaluated using an in situ cell death detection kit (Cat. No. 1684795; Roche Molecular Biochemicals, Mannheim Germany). First, the parafin sections were placed onto glass slides pretreated with poly-L-lysine, then tissue sections were deparafinized by incubation of slides at 60°C for 30 min and washed twice in xylene for 10 min and rehydrated through a series of decreasing concentrations of ethanol. Next, the sections were partially digested with proteinase K(20 μg/ml; Roche Molecular

Biochemicals) in Tris-HCl buffer (10mM, pH 8.1) at 37°C for 30 min and the slides were washed 4 times in phosphate- buffer solution. Thereafter,the tissue sections were incubated at 37°C for 60 min with TUNEL reaction mixture (1:10) enzyme TdT/label solution) in a humidified atmosphere in the dark. After this incubation, the slides were washed 3 times with phosphate buffer saline and surface dye. DABCO[1,4-diazobiocyclo (2.2.2)octane] was added onto the slides so as to clearly depict the cells with apoptosis. For analysis of apoptotic cells, 25 randomly selected areas for each aorta segment were evaluated under an interface-contact epiflourecence microscope (Zeiss Axiopot, Carl Zeiss AG, Germany).

Biochemical Analysis

The blood samples were centrifugated at 1000Xg for 15 min at 4°C. The serum was then separated. Total cholesterol, triglycerides and HDL-Cholesterol levels of serum were measured by means of Roche Hitachi 747 autoanalyzer using Roche Diagnostic GmbH, D- 68298 Mannheim kits, U.S.A.).

LDL-Cholesterol was calculated according to the Formula; LDL-Cholesterol = Total Cholesterol – HDL- Cholesterol–Triglyceride/5.

Pharmacological Analysis

In order to evaluate the contraction response of aortas in isolated organ bath; two centimeter part of descending aorta at abdominal start was carefully separated from peripheral tissues and placed in Krebs solution (mM/L: 118 NaCl, 4.75 KCl, 2.5 CaCl2, 1.2 MgS02, 1.2 NaH2P04, 2.4 NaHC03, 11 Glycose).

Afterwards, two samples of 0,5 cm each were prepared and oxidized with a gaseou solution of % 95 O2 and %5 CO2. These two samples in Krebs solution were hanged at isolated organ bath at 37°C. They were washed for 15 minutes at a tension of 2 grams. After one and a half hour, the process below was applied on the aorta at isolated organ bath (IOB).

Groups (1,2,3,4 and 5) treatments are:
1- NA 10-3+Ach 10-7 M
2- NA 10-3+Ach 10-6 M
3- NA 10-3+Ach 10-5 M
4- NA 10-3+Ach 10-4 M
5- NA 10-3+Ach 10-3 M
% relaxation values were obtained for these results. Arc.Sin transformation was applied.

Statistical Analysis

Results were expressed in means of ± S.E. One way analysis of variance (ANOVA) was used for the analysis and comparison of data within and between groups were conducted. Two way analysis of variance was used for the analysis and comparison of data within and between experimental periods were conducted.

RESULTS

Histology and morphometry

At the end of experimental period, rabbits were sacrified and their aortas were resected. Atherom plaques were observed in animals exposed to Chol (Figure 1), GPSE+Chol, Sily+Chol and Sim+Chol groups by light microscopy. The vessel wall thickness were greater in hypercholesterolemic rabbits compared with other groups. Vessel wall thickness is significantly different between Sim+Chol ($p<0.05$) and Chol groups. Vessel wall thickness is also decreased in GPSE+Chol, Sily+Chol groups but any difference was not observed in other groups. The details of statistical analysis are shown in Table 1.

Atherom plaques decreased in Sim+Chol group compared with Chol groups (Fig 2). In many animals lipid-loaded foam cell were observed by electron microscope in cholesterol and experiment groups (Fig 3b). Also foam cells were observed in semithin sections (Fig 3a) Surface area of atherom plaques were greatest in Chol group. However, treatment of the rabbits with simvastatin and proanthocyanidin and silymarin plaques were decreased, but there were no statistical differences between these groups. The details of statistical analysis are shown in Table 2.

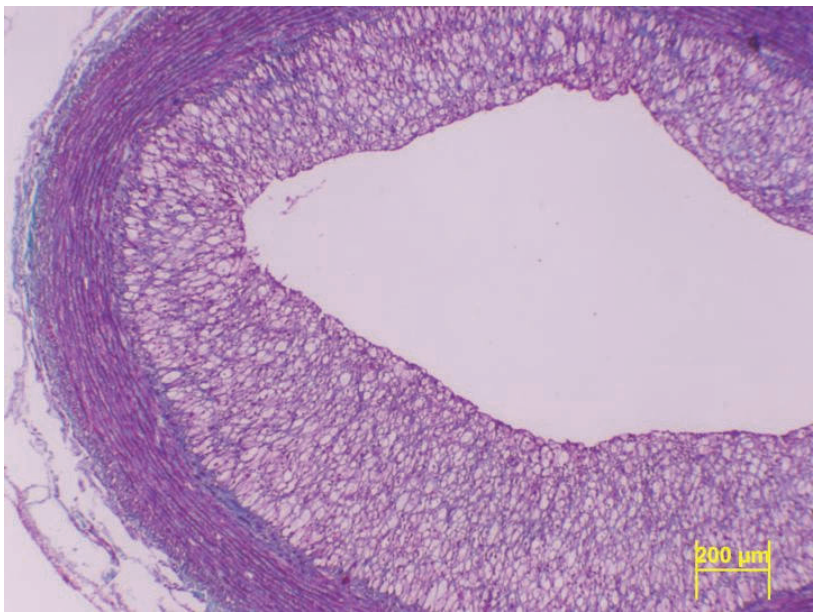

Figure 1. Atherosclerotic lesion of rabbit fed cholesterol was seen in aorta. The rabbits from the Chol group showed more developed lesions and a more enlarged intimal and total area than those from the control group. Masson trichrome, orig mag.X10.

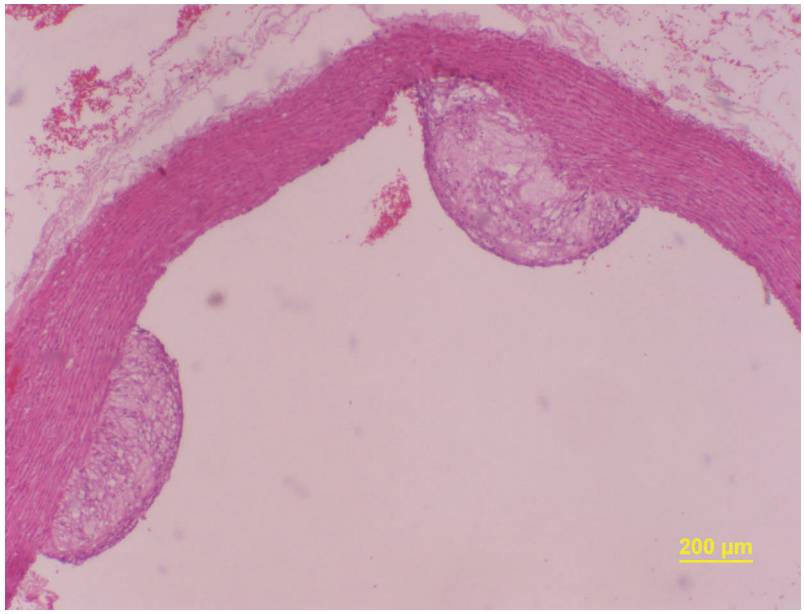

Figure 2. Decreased atherosclerotic lesions of rabbit fed simvastatin and cholesterol were seen in aorta. The lesions was reduced in the Sim+Chol group. HXE staining, orig mag.X10.

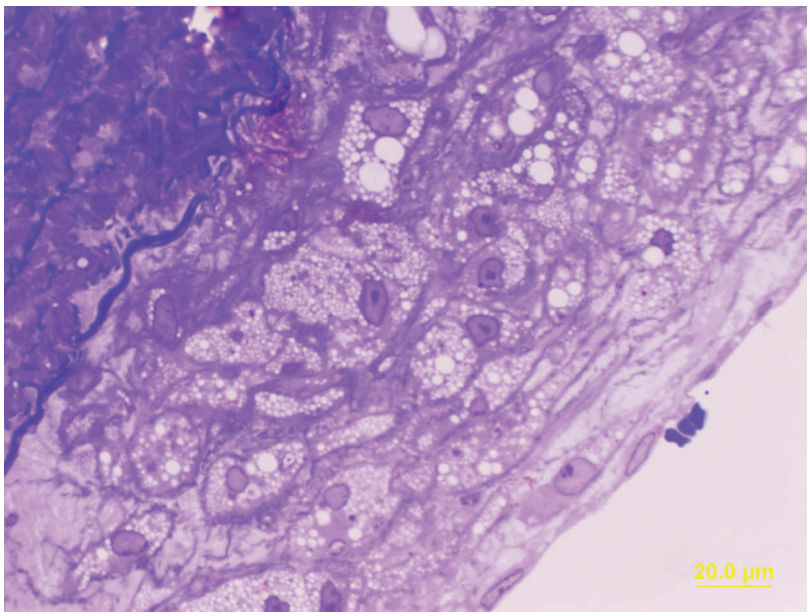

Figure 3a

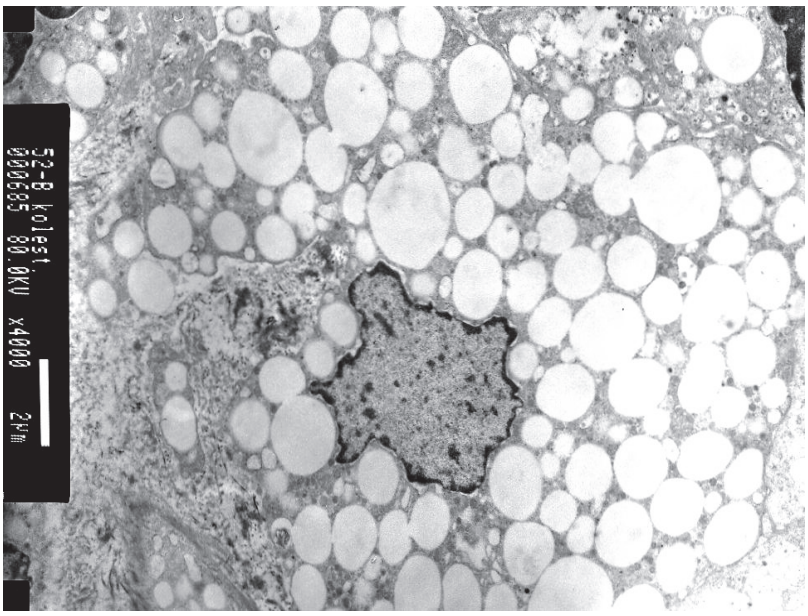

Figure 3b

Figure 3a. Semithin section of an area of the aortic intima from a Chol group. Lipid-loaded foam cell was seen in cholesterol group. b. Electron micrographs of an area of the aortic intima from a Chol group. Lipid-loaded foam cell was seen in cholesterol group. TEM. Orj.MagX4000.

Table 2. Surface area of atherom plaques.

Groups	N	$\overline{X} \pm S_{\bar{x}}$	
Chol	7	1671.81 ± 456.58	$F_{3;16} = 0.77$ P>0.05
GPSE +Chol	7	1095.41 ± 304.49	
Sily+Chol	7	1143.72 ± 67.10	
Sim+Chol	7	1029.08 ± 385.02	

Surface area of atherom plaques were greatest in chol group but there were no statistical differences between groups (p>0.05).

In situ detection of apoptotic cells

TUNEL staining for apoptosis was positive in Chol, GPSE+Chol, Sily+Chol and Sim+Chol groups with variability in the number of cells stained for apoptosis between different specimens. TUNEL positive cells numbers are significantly different in Chol and other groups (P<0.001), but there were no difference in between GPSE+Chol, Sily+Chol and Sim+Chol groups. TUNEL positive cells numbers are also significantly different in between Sily+Chol and Sim+Chol groups(P<0.05). The details of statistical analysis are shown in Table 3. In contrast, TUNEL stain was not detected in any of the control aortas, suggesting that this process occurs at a very limited rate in normal aortas. In the Chol group positively stained by TUNEL. TUNELl positive cells were primarily detected in the intima and media. Cells showing high-intensity fluorescence (high nuclear fragmentation) which are characteristic morphological features of apoptosis are shown in figures. TUNEL positive cells were also detected in Chol (fig 4) and GPSE+Chol (fig 5), Sim+Chol and Sily+Chol groups, but they were slightly decreased compared with the Chol group.

Biochemical results

The atherogenic diet induced a marked increase in all lipid parameters, except in HDL levels. The details of statistical analysis are shown in Table 4. In 45th day total cholesterol (TC) levels are significantly different in control and Sim+Chol groups, but no differences between other groups. Total triglyceride (TG) level is significantly different in all groups. TG levels are highest in Chol group. HDL levels are higher in control group and lower in the other groups, but there were no difference in between Chol, GPSE+Chol and Sily+Chol groups. LDL levels are lower in control group and higher in Chol group. There is a significant difference in between control and other groups, and Sim+Chol and other groups. The simvastatin group showed a decrease in blood LDL lipid levels, especially in the 90th day. In 90th day total cholesterol (TC) levels are significantly different in control and other groups. TC levels decreased in Sim+Chol group. It is observed that TG levels are significantly different in all groups. TG levels were very high in Chol group, but decreased in Sily+Chol and Sim+Chol groups. HDL levels are significantly different in Chol and other groups. HDL levels are high in control group, but there was no statistical difference in between groups. LDL levels are significantly different in between control and other groups. In Sim+Chol group, LDL level is significantly decreased.

Pharmacological results

At presence of Noradrenalin (10-3) belonging to experimental and control groups; relaxation responses related to Acetylcholine (Ach 10-3–10-7) are given as percentage relaxation of maximum contraction response (Figure 6). When rabbit aorta responses at IOB are investigated and pharmacologically evaluated, concentration dependent relaxation response is received at control group during treatment (p>0.05). At Chol group, a significant reduction in relaxation response is observed compared to control group (p<0.05). At Pro+Chol and Sily+Chol groups, less relaxation response is observed compared to Sim+Chol group. However, GPSE +Chol and Sily+Chol groups give more relaxation response compared to cholesterol group which is found to be statistically different (p<0.05). Meanwhile; there is not a statistical difference in between GPSE + Chol and Sily+Chol groups (p>0.05). A dose-concentration dependent relaxation response wasreceived at all groups during treatment (p>0.05). It is observed that they become more effective when their concentration increased at IOB.

Table 3. Apoptotic cell number in atherom plaques.

Groups	N	$\overline{X} \pm S_{\bar{x}}$	
Chol	7	155,14± 4,57	$F_{3;24} = 43.5$ $P<0.001$
GPSE +Chol	7	91,57± 6,30	
Sily+Chol	7	105,14±3,66	
Sim+Chol	7	82,42±4,75	

Chol group is significantly different in all groups ($p<0.001$). There are no difference between GPSE+Chol, Sily+Chol and Sim+Chol ($p>0.05$).

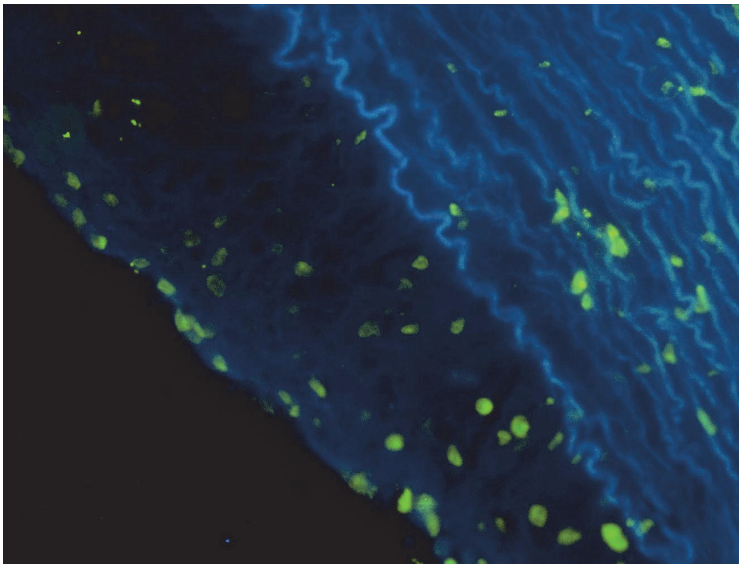

Figure 4. A lot of TUNEL positive cells in the atherosclerotic lesion and vessel wall of a rabbit fed cholesterol. Nuclei positive for active TUNEL stain in green. TUNEL positive cells are located in media and intima. Orj. mag. X40

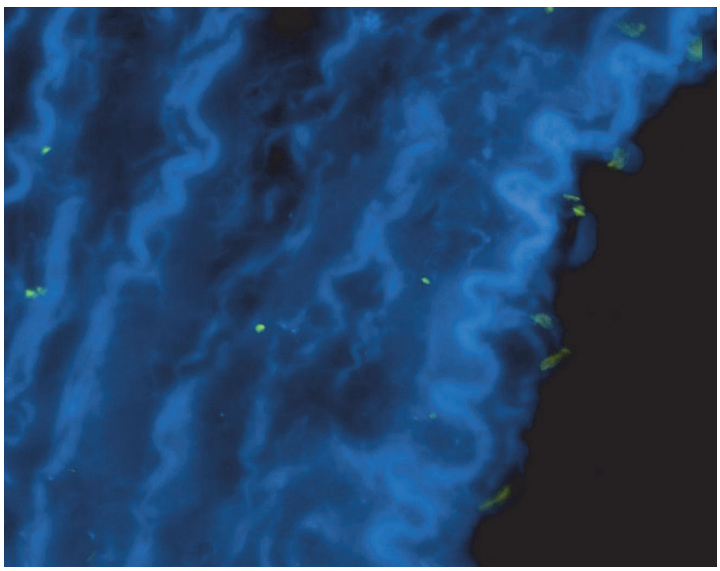

Figure 5. Limited number of TUNEL positive cells are seen in the intima and media of a rabbit aorta in GPSE group. Orj. mag. X100

Tablo 4. In 45th day and 90th day serum TC, TG, HDL, LDL levels from rabbits

Day	Groups	TC	TG	HDL	LDL
45th	Control	123.43±3.22 [a]	95.00±4.44[a]	42.86±2.54[a]	61.56±1.65[a]
45 th	Chol	1767.71±45.94	200.00±4.86[c]	25.43±1.70	1702.71±1.17
45 th	GPSE +Chol	1584.29±12.54	182.86±2.39[d]	30.43±1.17	1516.57±12.90
45 th	Sily+Chol	1661.86±25.08	147.14±3.23	31.71±2.11	1599.57±25.34
45 th	Sim+Chol	1315.57±41.79 [b]	161.86±3.60	31.71±2.11	1251.29±41.72 [b]
	p<0.001	$F_{4;30}$=487.42	$F_{4;30}$=111.92	$F_{4;30}$=11.24	$F_{4;30}$=190.73
90 th	Control	123.43±3.22 [a]	95.00±4.44[g]	42.86±2.54	61.56±1.65[a]
90 th	Chol	1934.29±25.53[c]	240.57±5.59[c]	18.16±1.06[c]	1867.29±25.80[c]
90 th	GPSE +Chol	1380.43±21.40 [e]	176.71±3.62[e]	35.57±2.21	1309.57±20.63[e]
90 th	Sily+Chol	1611.86±25.08 [f]	109.57±4.64	40.57±1.81	1549.43±23.43[f]
90 th	Sim+Chol	1011.14±35.62 [b]	122.71±2.25[h]	38.57±1.31	948.14±36.12[b]
	p<0.001	$F_{4;30}$=801.57	$F_{4;30}$=197.24	$F_{4;30}$=26.22	$F_{4;30}$=816.28

a: Control group is significantly different other groups
b: Sim+Chol group is significantly different other groups
c: Chol group is significantly different other groups
d: GPSE +Chol group is significantly different between Sim+Chol and Sily+Chol groups
e: GPSE+Chol group is significantly different other groups
f: Sily+Chol groups is significantly different other groups
g: Control group is significantly different between Chol, Sim+Chol, GPSE+Chol and Sily+Chol groups.
h: Sim+Chol group is significantly different GPSE+Chol group.

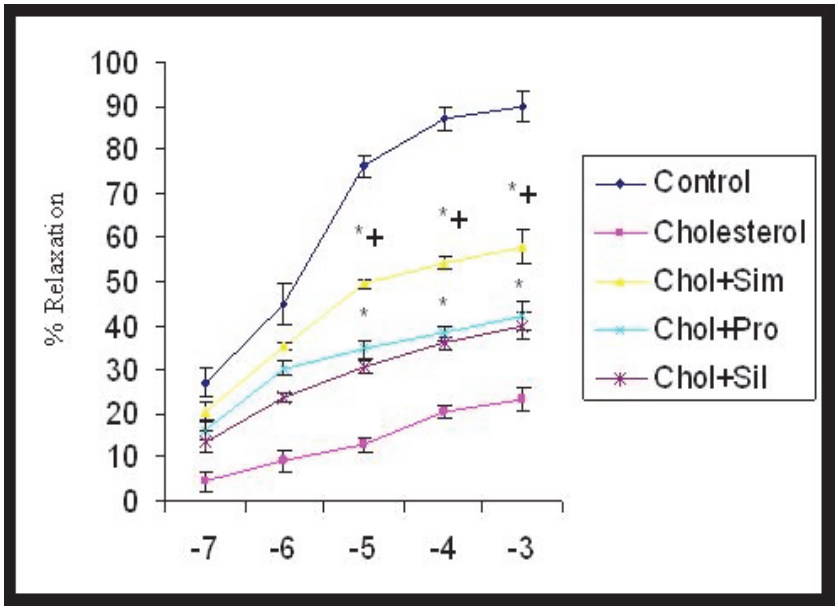

Figure 6. Ach (10-7, -3) concentration-dose dependent invitro responses at presence of NA 10-3 in experimental groups. *For the control group p<0,05 is accepted to be statistically difference. For the cholesterol group p<0,05 is accepted to be statistically difference.

DISCUSSION

The present study has definitely established a rabbit model of atherosclerosis, by showing an atherosclerotic effect of cholesterol following 10-12 weeks of treatment.

The results of many researches performed by using various animal species indicated that proanthocyanidin reduces atherosclerotic lesion development by inhibiting LDL oxidation (Agli et al. 2004; Ariga 2004; El-Afy et al. 2005; Pataki et al. 2002; Shao et al. 2003; Yamakoshi et al. 2002). In our research, rabbits were administered 100 mg/kg high-dose GPSE for 12 weeks. Following histological examination of aortas, we observed that this amount of dose, which we preferred in the test protocol, plays a significantly higher role in inhibition of plaque. Some researchers also demostrated that GPSE affects on the cholesterol and trigliserid levels (Vinson et al. 2002). According to our biochemical findings, proanthocyanidin was not effective in reducing total cholesterol, HDL and LDL levels in rabbits at the end of the 45th day, but was only able to reduce these parameters at the end of the 90th day. As mentioned in researches, we are in the opinion that proanthocyanidin may be provides antiatherosclerotic activity reducing lipid parameters. As mentioned in all research studies, proanthocyanidins are natural compounds having strong antioxidant features. Although histological examination of aortas taken at the end of our test indicated no statistically significant difference in terms of the areas covered by atherosclerotic plaques, as may be seen in Table 1, plaques respectively in Sim+Chol and GPSE+Chol groups were reduced in size compared to Chol group. The examination of vessel wall thicknesses indicated that there is a protection for Sim+Chol, GPSE+Chol and Sily+Chol groups in comparison with Chol group, as may be observed in Table 2. According to our research findings, silymarin has significant antiatherogenic features which are similar to proanthocyanidin. It was observed in a research performed with high cholesterol-fed rats that silymarin reduces LDL and increases HDL in blood thanks to its polyphenol content, but it does not have any effect on total cholesterol or LDL (Skottova et al. 1997; 1998; 2003; 2004). Silymarin can be very effective on antioxidant capacity mainly due to its phenolic compounds and inhibits lipid peroxidation in plasma (Ashar and Masood, 2008). While silymarin inhibits accumulation of cholesterol in the liver, it also increases LDL intake of the liver. The effect of silymarin on cholesterol metabolism is thought to be the inhibition of cholesterol absorption by intestines (Sobolova et al. 2006). Our research findings show that the response of silymarin is quicker than that of proanthocyanidin in terms of reducing TG levels at the end of the 90th day. In line with the researches, we also think that the effects of silymarin on reducing the formation of atherosclerotic lesions may have inhibited cholesterol absorption by intestines, and that silymarin reduces oxidative stress in the medium by strengthening endogenous antioxidant defense, thus inhibiting LDL oxidation. Some investigators suggest that HMG-CoA reductase inhibitors exert a direct antiatherosclerotic effect on the arterial wall dependence of their lipid-lowering properties (Al-Zuhair et al. 1997; Gotto 2005). This activity, which affects move process involved in the formation of atherosclerotic lesions, is linked to the local modulation of the mevalonic acid (Bellosta et al. 1998). HMG-CoA reductase inhibitor of simvastatin has an antiinflammatory effect on experimental atherosclerosis beyond its ability to reduce cholesterol levels. This effect is also evident in circulating blood mononuclear cells whose inflammatory activity correlates with that of atherosclerotic plaques (Hernandez-Presa et al. 2003). Recent data suggest that statins in addition to their lipid –lowering ability, can also reduce the production of reactive oxygen species and increase the resistance of LDL to oxidation (Rosenson 2003). According to our findings, atherosclerotic lesions reduced in size and vessel wall thicknesses decreased in simvastatin group more than cholesterol group, and biochemical analysis of blood samples indicated a decrease in T-kol, TG, LDL cholesterol levels on the 45th and 90th days. HDL cholesterol increased on the 90th day. It is clear that protective effect of simvastatin against atherosclerosis is multidimensional due to its related features mentioned in previous researches. We believe that variations caused by simvastatin on LDL particles and decrease again caused by simvastatin on atherogenic features of LDL are crucial besides its well-known effect on lipid profile

decrease. Reduction in sizes of atherosclerotic plaques observed in Sim+Chol group was interpreted as a potential decrease in macrophage infiltration to the lesion. Simvastatin is an antihyperlipidemic drug and also an antioxidant. Researches indicate that due to its antioxidant feature, simvastatin inhibits endothelial damage by capturing free oxygen radicals in atherosclerosis and provides an endothelium-dependent relaxation response (Jiang et al. 2004; Rosenson 2003) and that treatment with NO donor L-Arginin inhibits lipid peroxidation and oxidative stress in diabetic rats (Özcelikay et al. 1999). It was also observed that simvastatin plays an important role in protection of vascular endothelial structure by decreasing TNF alpha level, which is a inflammatory cytokine (Jiang et al. 2004). Simvastatin administration increased the antioxidant potential of the serum, diminished transtosis of lipoproteins and restored the endothelium dependent relaxation. Accordingly, they have been demostrated to improve endothelial function and to reduce inflammation and blood thrombogenicity. Treatment of hypercholesterolemic patients with simvastatin and lovastatin has been shown to reduce monocyte adhesion to endothelial cells. Macrophage growth stimulated by ox-LDL can be also inhibited by statins (Simionescu et al. 2002). According to our results, we especially think that simvastatin inhibits tissue damage due to its antioxidant feature, and thus, it provides an endothelium-dependent relaxation response. Statins decrease the incidence of acute coronary events (Shabanzadeh et al. 2005) which are due to plaque thrombosis. Furthermore, simvastatin suppressed the macrophage accumulation or extracellular lipid deposition and supressed ox-LDL induced macrophage growth (Lefer and Granger 1999; Sakai et al. 1997; Shiomi et al. 2005). In vitro studies demostrated that all lipophilic statins including simvastatin showed inhibitory effects on SMCs proliferation and an increase in apoptosis in cultured SMCs. Simvastatin blocks the formation of cholesterol at various stages in its biosynthetic pathway resulting in decreasing total cholesterol and LDL level in hypercholesterolemia (Alegret et al. 1998; Al-Zuhair et al. 1997). In this study, simvastatin significantly lowered the concentrations of LDL and markedly elevated HDL at the end of the protocol. These changes could be attributed to the fact that simvastatin is one of the HMGCoA reductase inhibitors that can inhibit cholesterol synthesis. In recent studies, a potential role of oxidative mechanism has been suggested in the apoptosis of vascular cells (Haunstetter and Izumo 1998). In the present study, TUNEL positive cells are mainly detected in the intima and media of aorta from hypercholesterolemic animals. TUNEL positive cells marked an increase in the aortas of cholesterol treated animals compared to controls. In addition we observed that TUNEL positive cells were slightly decreased in GPSE and silymarin groups. Some researchers demostrated that GPSE protects cardiomyocytes from apoptotic cell death by reducing the expression of proapoptotic genes during ischemia/reperfusion (Sato et al. 1999) and apoptosis is an integral process of early coronary atherosclerosis (Kockx et al. 1996). Exposure to GPSE resulted in a significant reduction in apoptosis in response to chemotherapeutic agents. Investigators suggest that some of the chemoprotective effects of GPSE are mediated by upregulating Bcl-2 and downregulating c-myc and p53 genes (Joshi et al. 2001). Silymarin strongly reduced UV induced cell apoptosis and that the inhibitory mechanism has a relationship with caspase(s) and Bcl-2 family members as well as with extracellular signal-regulated protein kinase (ERK)/MAPK (Krecman et al. 1998; Li et al. 2004). Silymarin has been shown to inhibit skin carcinogenesis in mice. The results of this study induced apoptosis in cells primarily mediated through a p53 dependent pathway which involves the proteins of Bcl-2 family, cytocrome c, and activation of caspase (Katıyar 2002). These results are correlated with previous studies.Statins have been shown in numerous clinical trials to reduce death and myocardial infarction in both primary and secondary prevention studies (Feng-Xia et al. 2003; Rosenson 2003). The fact that these effects occur with little reduction in overall plaque burden has emphasized that alterations in plaque stability, including plaque cell apoptosis, underlie their efficacy. In addition, Some investigators detected that statins suppressed the apoptosis in atherosclerotic lesions and vessel wall (Bonomini et al. 2008; Chien 2003; Mayr and Xu 2001; Stoneman and Bennett 2004). Simvastatin treatment reduced proliferation and increased apoptosis of pathologic smooth muscle cell in the neointima and medial walls of pulmonary arteries (Nishimura and et al. 2003). Apart from many effects of

simvastatin, it reduces factor nuclear kappa ß (NF-κß) activity in peripheral mononuclear cells. Therefore, it inhibits macrophage infiltration to lesions and smooth muscle proliferation, migration and apoptosis in tunica media. As is known, NF-κß is activated in atherosclerosis. Silymarin reduces NF-κß activity just as similar to simvastatin (Skottova et al. 2003). Our results show that simvastatin reduced TUNEL positive cells in the neointima compared with cholesterol group. In this study, we observed that vessel wall thickness of normal rabbit aorta has not changed and that endothelial layer remained constant without any interruption. The examination of IOP responses of rabbits in control group indicated the formation of the expected Ach-dependent relaxation response at a level supporting histological findings (90%-27%). It is known that the existence of endothelium is crucial during the noradrenalin (NA) response and that the activity decreasing contraction during the response is mediated by the endothelial cell (Simionescu et al. 2002). These Ach-dependent responses are endothelium dependent (NO) responses. NOoscillation dependent vasodilatation was observed in endothelial aortic rings which were not disturbed. In our research, we determined that vessel wall thicknesses of cholesterol-fed rabbits were variable and that there were many developed atherosclerotic lesions in tunica intima. Examination of rabbit-isolated organ bath responses indicated a significantly high level of decrease (25%-4,6%) in relaxation responses of aortic rings compared to the control group, which was consistent with histological findings. These results indicated a decrease in Ach and endothelium dependent (NO) relaxation response due to disturbance of integrity of NO oscillation and endothelial layer. In Pro+Chol and Sily+Chol groups, less relaxation response is observed compared to Sim+Chol group. However, Pro+Chol and Sily+Chol groups give more relaxation response compared to cholesterol group. Regarding this issue, we suggest that statins are more effective compared to antioxidants while silymarin and proantocyanidin are thought to have less effects. Besides, it is believed that they have contribution in restriction of progress of atherosclerosis. In general, antioxidants can not heal the developed atherosclerosis, however, they help to keep them in their existing level and prevent their progress. Meanwhile, they have a regulating role on blood lipid profiles. Statin tests, especially with lovastatin (Feng-Xia et al. 2003) were carried out in previous researches and our results are consistent with general statin results. However, researches including isolated organ bath responses related to silymarin and proanthocyanidin could not be obtained. It was concluded that proanthocyanidin and silymarin reduce endothelial damage due to their antioxidant features, but upon examination of endothelium dependent relaxation responses, it was determined that they are not as effective as statins. The effects of silymarin and proanthocyanidin used for inhibiting formation of atherosclerosis in hypercholesterolemic rabbits were first evaluated in our isolated organ bath research, and we are in the opinion that the results of this research will shed light to future experimental studies about atherosclerosis. In summary, the results of the current study provide evidence that based on the oxidative stress hypothesis of cholesterol action, hypercholesterolemia is a well documented model for investigating the pathology of atherosclerosis. Antihyperlipidemic drug of simvastatin affords a protection against atherosclerosis by decreasing the numbers of TUNEL positive cells and decreasing LDL cholesterol levels. In addition GPSE (Grape Seed Extract) and silymarin are natural, safe and protective antioxidants may be used for support in the atherosclerosis. Extrapolating these results to human settings needs further investigations.

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