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Full Length Research Paper

Anti atherosclerotic effects of verjuice on hypocholesterolemic rabbits

Mahbubeh Setorki[1]*, Bahar Nazari[2], Sedighe Asgary[3], Leila Azadbakht[4,5] and Mahmoud Rafieian-Kopaei[6]

[1]Department of Biology, Izeh Branch, Islamic Azad University, Izeh, Iran.
[2]Isfahan Cardiovascular Research Center, Isfahan University of Medical Sciences, Isfahan, Iran.
[3]Isfahan Cardiovascular Research Center, Applied Physiology Research Center, Isfahan University of Medical Sciences, Isfahan, Iran.
[4]Food Security Research Center, Isfahan University of Medical Sciences, Isfahan, Iran.
[5]Department of Nutrition, School of Health, Isfahan University of Medical Sciences, Isfahan Iran.
[6]Medical Plants Research Center, Shahrekord University of Medical Sciences, Sharekord, Iran.

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The aim of the present study was to evaluate the effects of verjuice on atherosclerosis risk factors in rabbits fed a cholesterol-rich diet for how long. Two New Zealand rabbits were used for this study which lasted 8 weeks. The rabbits were divided to four groups and treated as follows normal diet, cholesterol-rich diet, cholesterol-rich diet supplemented with 5 ml verjuice and cholesterol-rich diet supplemented with 10 ml verjuice. The low density lipoprotein-cholesterol (LDL-C), malondialdehyde (MDA), oxidized LDL (ox-LDL), nitrite, nitrate; factor VII, fibrinogen and C-reactive protein (CRP) were measured before and after experimental feeding. In all groups, fatty streak formation in right and left coronary arteries were determined at the end of the study. Administration of low (5 ml) and high (10 ml) dose of verjuice significantly lowered the levels of fibrinogen ($p<0.05$) and atherosclerotic lesion in right and left coronary arteries ($p<0.05$). However, the serum level of nitrite and nitrate increased in both verjuice supplemented groups. Administration of 10 ml verjuice could significantly reduce the amount of MDA, ox-LDL, LDL-C ($p<0.05$). No significant effects were noted for CRP and factor VII after consumption of both doses of verjuice. We conclude that verjuice could reduce some atherosclerotic risk factors in long term treatment.

Key words: Atherosclerosis, fatty streak formation, fibrinogen, verjuice.

INTRODUCTION

Atherosclerosis is a heterogeneous problem and several risk factors might be related to this disease. Increased plasma cholesterol, low-density lipoprotein (LDL), and oxidized LDL (ox-LDL) are important risk factors of this problem (Ross, 1993). Besides the mentioned traditional markers, some components of coagulation system (fibrinogen, factor VII) as well as inflammatory markers are now identified as the new risk factors of athero-sclerosis (Janero, 1990; Gensini et al.,1996; Ross,1999; Libby, 2002). Nowadays, C - reactive protein (CRP) and fibrinogen are considered as two important markers in the pathogenesis of cardiovascular diseases (Gabay and Kushner, 1999; Uhlar and Whitehead, 1999; Libby and Ridker, 2004; Black et al., 2004). The adherence of platelets and leukocytes to the endothelium can be prevented by releasing nitric oxide (NO) from a normal vascular endothelium (Bagchi et al., 2000). Not only NO is an anti atherogenic agent but also it causes relaxation of the vascular smooth muscle and production of vasodilators. Hypercholesterolemia, hypertension and diabetes can damage the endothelium and can decrease the bioavailability of NO (Folts, 2002).

Polyphenol-rich fruit juice such as pomegranate juice, apple and purple grape and their juices has significant antiatherogenic, antioxidant, antihypertensive, and anti-inflammatory effects (Dohadwala and Vita, 2009; Basu and Penugonda, 2009; Rosenblat et al., 2006; Décordé,

*Corresponding author. E-mail: doctor.setorgi@gmail.com. Tel: +989133121589. Fax: +983113373435.

2008; Iriti and Faoro, 2009; Setorki et al., 2009). Evidence is provided that procyanidins, derived from grape seed, may decrease the development of foam cell formation by reducing cholesterol accumulation and modulating the expression of genes interferes in inflammation (Terra et al., 2009). Wines, manufactured from grapes, also contain a large amount of antioxidants, including resveratrol, catechin, epicatechin, and pro-anthocyanidins (Bertelli and Das, 2009). Vinegar, one of grape derives, has some acute effects on biochemical risk factors of atherosclerosis (Setorki et al., 2010a). Verjuice is an acidic juice made from unripe fruit particularly grapes. This ancient product's culinary and medical uses date back to medieval times. It is used extensively in Iranian cuisine as a popular ingredient. Grape juice and verjuice are very similar in structure and both have flavonoid components such as catechin and anthocyanin (Kishi et al., 1999). Some beneficial effects of verjuice have been mentioned in Iranian medicine. Also, in previous study we had shown that verjuice had acute effects on some atherosclerotic factors such as fibrinogen, ox-LDL and Malondialdehyde (MDA) (Setorki et al., 2010b). This study was performed to determine the chronic effects of verjuice on lipid and other risk factors of atherosclerosis including CRP, fibrinogen, factor VII, nitrite, nitrate and development of fatty streak formation.

MATERIALS AND METHODS

METHODS

A botanist identified the species of the grape (*Vitis Sylvestris*, herbarium number 15810) from the Research Center of Isfahan Province Natural Resources. The grapes were collected from the Aminabad region of Isfahan. The anthocyanin, pH, density, vitamin C, acetic acid and flavonoids contents of the verjuice were measured.

Measurement physiochemical factors in verjuice

pH meter was used for measuring pH. Densitometer also was utilized, vitamin C (Mccormick and Greene, 1994), flavonoids (Kumar et al., 2008) total anthocyanin (Schutz et al., 2006) were assayed by spectrophotometry (ShimadzoUV-3100 (Japanese) and acetic acid was quantified by titration (Scharf and Malerich).

Treatment of rabbits

Thirty two male New Zealand white rabbits weighting 1930±287 g were obtained from Razi Institute of Iran. They were acclimatized in an air conditioned room (State the temperature) for two weeks and provided with free access to water and a normal rabbit chow (Dampars Co, Iran) which consisted of 10% protein, 40 to 50% carbohydrates, 2% vegetable fat and 15 to 25% fiber. At the end of this period, rabbits were randomly divided into four groups of diets: no cholesterol, diet containing 1% cholesterol (Merck), diet containing 1% cholesterol with 5 ml verjuice, diet containing 1% cholesterol with 10 ml verjuice. Animals were fasted for 12 to 15 h and venous blood samples were taken pre-experiment and end of study (2 months). Cholesterol-rich diet was prepared by adding 1 g cholesterol (Merck,Germany) in 4 ml olive oil to 0.1 kg of commercial rabbit chow. Verjuice was given with force feeding (Singer et al., 1995; Boger et al., 1997). The study was reviewed and approved by the Research council and the ethics committee of Isfahan University of medical sciences.

Biochemical factors in rabbit measurement

Blood samples were centrifuged at 3500 rpm for 20 min to obtain serum and plasma. The serum that was used to measure the levels of low density lipoprotein-cholesterol (LDL-C) [were determined using standard enzymatic kits (Pars Azmoon Co, Iran) and auto analyzer (Hitachi 902, Japan)], oxidized LDL (ox-LDL) and CRP [(Promokine Co, Germany) and (Kamiya Biomedical Co, USA), were assessed with enzyme-linked immunosorbent assay kit in accord with manufacturer's guidelines], nitrite and nitrate [were measured with a colorimetric assay kit (R&D Systems, USA) that includes the Griess reaction] and plasma was used to determine levels of MDA, fibrinogen and factor VII. MDA was quantified by spectrophotometric way (Kostner et al., 1997), factor VII was quantified using clotting time, in the presence of the STA-Neoplastine reagent of a system in which all the factors are present, constant and in excess except factor VII which is derived from the sample being tested (Diagnostic Stago, French) and fibrinogen was determined using coagulation kit (Mahsayaran Co, Iran).

Assessment of the severity of atherosclerotic lesions

At the end of study, the blood sampling and storage were repeated. All animals in groups euthanized by an overdose of sodium pentobarbital and exsanguinated. The animal's aortas were harvested for pathological investigation. The entire aorta, from the aortic arch to the external iliac arteries, was dissected out and cleaned of excess adventitious tissue. The aortas were fixed in buffered 10% formalin for 24 h and then embedded in paraffin. The paraffin embedded specimens were sectioned at 5 µm (20 sections in succession). Atherosclerotic layer, gained from slicing, was determined in hematoxylin stained sections on an arbitrary scale 1-4.

Trace: Minimal thickness of subintimal with little injury to aorta artery.
Grade 1: Atherosclerotic thickness was less than half as thick as the media with some form of endothelial dysfunction, macrophages and isolated foam cell inside the endothelium.
Grade 2: Atherosclerotic thickness was half as thick as the media with accumulation of intracellular lipid, macrophage and smooth muscle cells.
Grade 3: Atherosclerotic thickness as thick as the media with an abundance of macrophages, smooth muscle cells and connective tissue.
Grade 4: Atherosclerotic thickness more that as thick as the media with a large extracellular intimal lipid core that appears as a large nucleus from the endothelial surface (Chekanov, 2003).

Statistical analysis

Results are given as Mean ± SD. Data were analyzed statistically using One-Way-ANOVA test followed by LSD post test. The differences between the baseline values and the two months values of all measured parameters calculated and were used in statistical analysis. In all instances, p value less than 0.05 was considered significant. For histological data, SPSS software was used to compare mean values between the groups. One-Way ANOVA and

Table 1. Comparison of cardiovascular risk factors among rabbits before (Baseline) and after (End of 2 months) experimental diet.

Biochemical factors		Groups			
		Cholesterolemic diet	5 ml verjuice with 1% chol	10 ml verjuice with 1% chol.	Normal diet
LDL-C (mg/dl)	Baseline	32.50 ±12.24	42.67±15.72	31.83±19.10	49.25± 22.47
	End of 2 months	711.50 ±102.97	498.83±262.70	477.33±235.66*	50.75± 31.64*
MDA (M)	Baseline	1.5 ± 0.24	1.54 ± 0.14	1.5 ± 0.26	1.38 ± 0.20
	End of 2 months	2.85 ± 0.88	2.34±0.59	2.11 ± 0.33*	1.32 ± 0.27*
ox-LDL (ng/ml)	Baseline	32.99 ± 11.2	18.77± 7.25	18.12± 6.16	24.08 ± 6.6
	End of 2 months	73.6 ±6.24	46.61 ± 14.32	37.67 ± 18.91*	20.98± 8.21*
Nitrite (µmol/l)	Baseline	21.3 ± 7.20	22.37±8.13	16.25±4.90	31.9 ± 16.10
	End of 2 months	35.61± 10.64	53.67±9.13.90*	58.18±18.15*	27.17 11.01*
Nitrate (µmol/l)	Baseline	15.49 ± 2.92	12.62±4.34	17.32±8.84	8.11± 0.84
	End of 2 months	22.54 ± 5.88	26.71± 4.63*	31.22±2.7*	8.2 ± 1.63*
Fibrinogen (mg/dl)	Baseline	205.17±20.81	233.33±39.85	207.33±23.69	240.75±16.8
	End of 2 months	293.67±35.10	252.67±41.52*	219.83±44.93*	244.75±13.96*
VII (% activity)	Baseline	200.17±29.54	214.33±50.60	157.83±33.49	230 ±2 2.06
	End of 2 months	338.83±78.62	286.17±72.94	254.5±59.48	231.5 ± 31.46*
CRP(µg/ml)	Baseline	2.53 ± 0.35	2.42±0.27	2.22±0.27	2.45 ± 0.12
	End of 2 months	3.9 ± 0.21	3.62±0.43	3.48±0.09	2.63 ± 0.21*

Mean LDL-C (low density lipoprotein), MDA (malondialdehyde), ox-LDL (oxidized LDL), nitrite, nitrate, fibrinogen, factor VII, and CRP(C-reactive protein) ± SD, (n=8 for each experimental group). *: $p<0.05$, Pairwise comparison of differences between baseline and end of 2 months when compared to cholesterolemic group.

Tukey tests were used for histological data.

RESULTS

Determination of some physiochemical factors in verjuice

Analyzing verjuice showed the following amount of different components vitamin C: 1.8±0.5 (mg/dl), acetic acid percent: 9.81±0.04%, total anthocyanin in: 2.99±1.04 (mg/100 g) and flavonoids: 1.97±0.238 (g /100 ml equivalent catechin). The density and pH: 0.157±0.001 (g/cm^3) and 3.24±0.01respectively.

Cardiovascular risk factors

Comparison of cardiovascular risk factors among rabbits at baseline and after end of 2 months consuming experimental diets are shown in Table 1.

In the high-cholesterol diet group the LDL-C level was increased significantly compared with the normal diet group ($p<0.05$). Following concurrent use 10 ml verjuice with the high-cholesterol diet, the LDL-C level was significantly decreased compared with the high-cholesterol group without verjuice ($p<0.05$). Consumption of 5 ml verjuice with the high-cholesterol diet did not change serum LDL-C level significantly compared with the high-cholesterol diet alone. No significant difference was shown between 5 and 10 ml verjuice

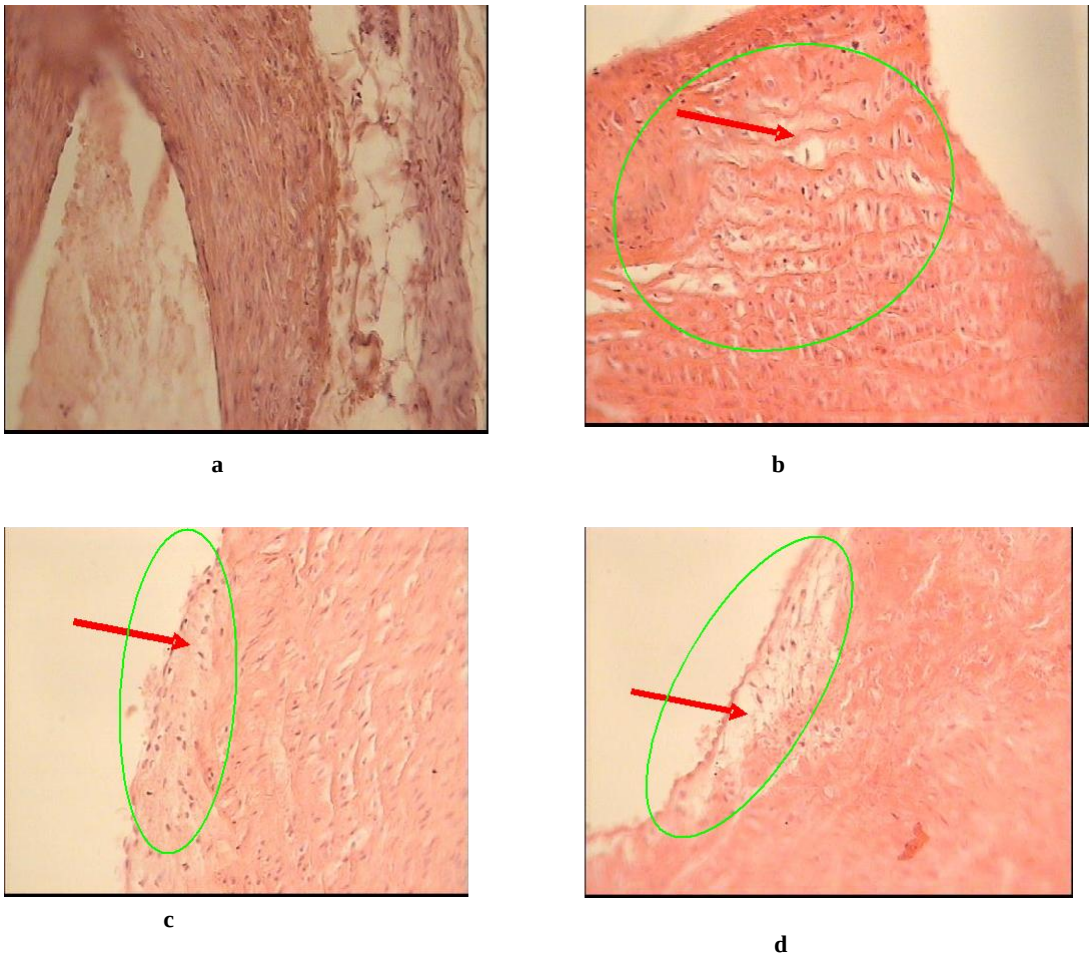

Figure 1. Right Coronary artery intima cross-section in the studied groups at the end of 2 months. (a) Normal diet(X40); (b) hypercholesterolemic diet (X40); (c) 5 ml verjuice +%1hypercholesterolemic diet(X40); (d) 10 ml ver juice +%1 hypercholesterolemic diet(X40).

regarding LDL-C index. Serum nitrite level in the normal-diet group was significantly decreased compared with the high-cholesterol diet one ($p<0.05$). Using 5 and 10 ml of verjuice with the high cholesterol diet induced a significant increase in nitrite and nitrate compared with the high-cholesterol diet alone ($p<0.05$).No significant difference in nitrate and nitrate concentration was found between 5 and 10 ml of verjuice. In the high-cholesterol group, CRP, fibrinogen and factor VII were increased significantly compared with the normal-diet group ($p<0.05$). Significant differences in fibrinogen were observed between both doses of verjuice with the high-cholesterol diet compared with the high-cholesterol diet alone ($p<0.05$). There was no significant difference between the verjuice groups in fibrinogen level. No significant difference in CRP and factor VII were found between 5 and 10 ml verjuice with the high-cholesterol diet compared with the high-cholesterol diet alone. There was also no significant difference in CRP and factor VII between the two doses of verjuice. MDA increased significantly in high-cholesterol diet group compared with the normal-diet group ($p<0.05$). Using of 10 ml verjuice with the high-cholesterol diet induced a significantly reduction in MDA compared with the high-cholesterol diet alone ($p<0.05$). No significant difference was found between the verjuice groups regarding MDA. A significant difference was observed between the high-cholesterol group and the normal-diet group in ox-LDL ($p<0.05$). Consumption of 10 ml of verjuice with the high-cholesterol diet induced a significant decrease in ox-LDL compared with the high-cholesterol diet ($p<0.05$). The difference between the two doses of verjuice was not significant.

Fatty streak formation

Histological sections of right and left coronary arteries stained from the 4 groups were shown in Figures 1 and 2 respectively and the results of atherosclerotic thickness grading in these groups were summarized in Figure 3a and b. Atherosclerotic changes were absent in normal

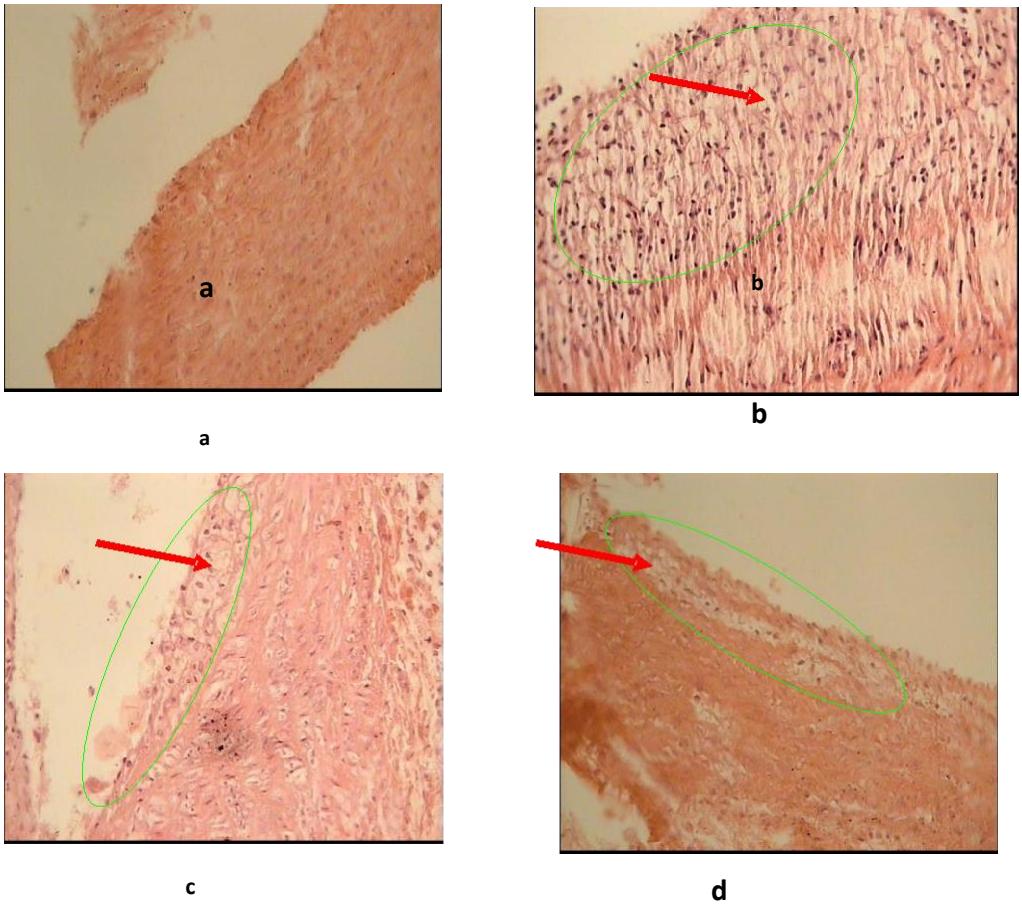

Figure 2. Left Coronary artery intima cross-section in the studied groups at the end of 2 months. (a) Normal diet(X40); (b) hypercholesterolemic diet(X40); (c) 5 ml verjuice +%1 hypercholesterolemic diet(X40); (d) 10 ml verjuice +%1 hypercholesterolemic diet (X40).

diet group (Figures 1a and 2a), whereas in the intimal surface of the coronary arteries from high-cholesterol diet group were seen many fat-laden macrophages. The cytoplasm of the macrophages filled with lipid droplets (foam cell) as the result of lipid digestion by the macrophage. Plaque thickness was also increased to more than half of media thickness, equal to degree 3 of Chekanov scale (Figures 1b and 2b). In the verjuice groups some endothelial dysfunction along a few foam cell and macrophages were seen in the intimal surface of the coronary arteries and plaque degree were 1 (Figures 1c and 2d). Atherosclerotic thickness grade in the verjuice groups decreased significantly compared to the high-cholesterol group (1.8±0.45, 0.75±0.25 in right coronary) (1.98±0.65, 0.82±0.42 in left coronary) ($p<0.05$).

DISCUSSION

The results of this study showed that concomitant consumption of cholesterol enriched diet with verjuice modifies the atherogenic effects of cholesterol and significantly prevents the increase of ox-LDL, MDA, LDL-C, fibrinogen and atherosclerotic lesions in a long term study. In addition verjuice induced a significant increased in nitrite and nitrate.

In our study, nitrite and nitrate level increased in high cholesterol diet compared to normal diet. It seems that increasing of NO synthesis is an effective way to combat with frequently inactivation of NO and defense versus harmful factors (Ferlito et al., 1999).

In the present study, both consumed doses of verjuice induced considerable addition in nitrite and nitrate levels. Different studies have shown that red wine and grape extract cause the formation of NO or increase its activity therefore raise cGMP. Guanylate cyclase account as an intracellular receptor for NO, thus it seems that increased production of NO cause the raising of cGMP levels (Fitzpatrick et al., 1993). Some polyphenols cause endothelium-dependent vasorelaxation through increasing the activiatin of NO synthase.

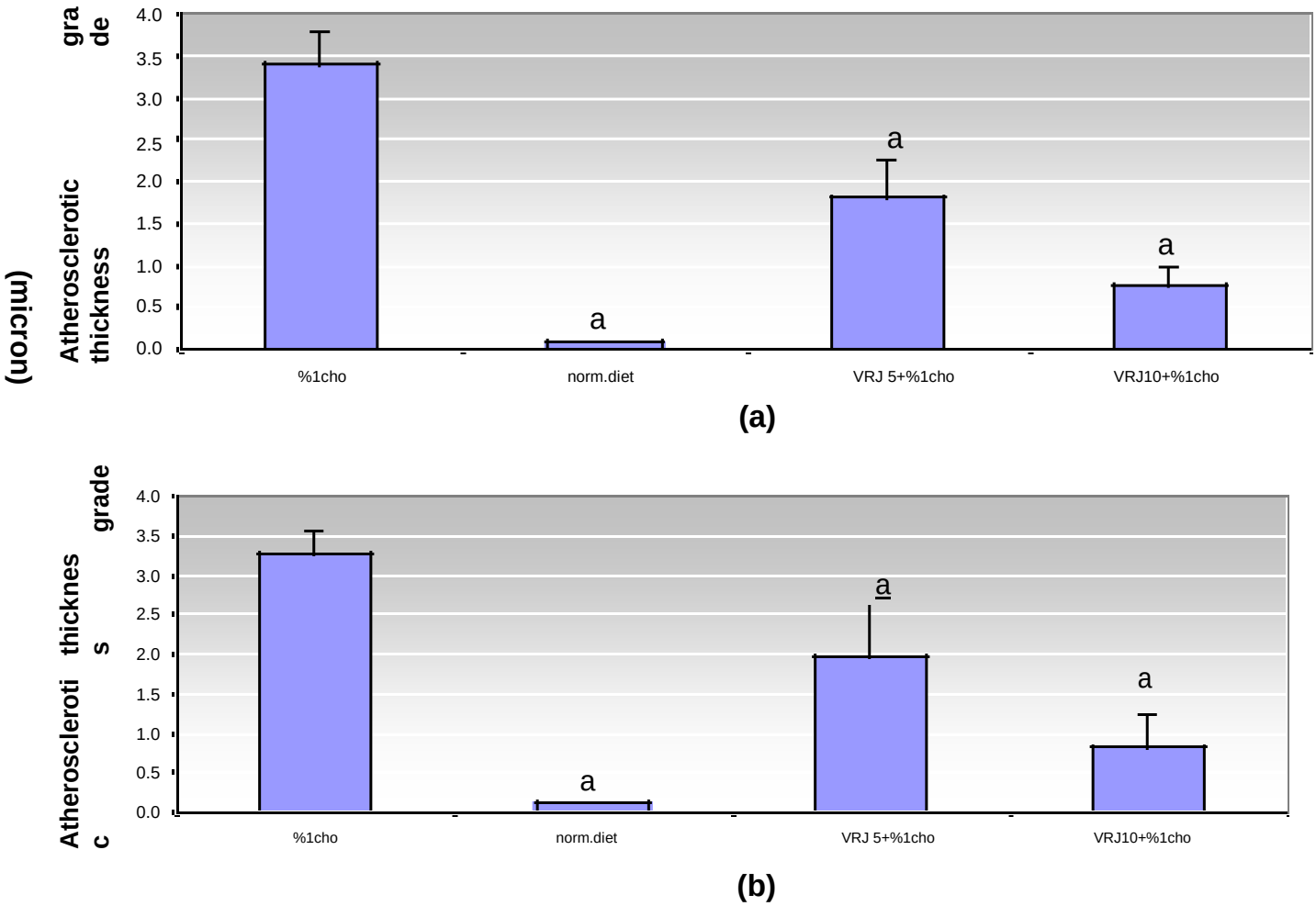

Figure 3. Mean of atherosclerotic thickness grade in the studied groups (right and left coronary arteries) at the end of 2 months. a: $p<0.05$, Comparison between groups with respect to hypercholesterolemic group. 3a: Mean of atherosclerotic thickness grade in the studied groups (right coronary artery), 3b: Mean of atherosclerotic thickness grade in the studied groups (left coronary artery), %1cho: %1hypercholesterolemic diet, norm.diet: normal diet, VRJ5+%1cho: 5ml verjuice+%1hypercholesterolemic diet, VRJ10+%1cho: 10 ml verjuice+%1hypercholesterolemic diet, VRJ10+%1cho: 10 ml verjuice+%1hypercholesterolemic diet.

Increase the intracellular Ca^{2+} concentrations which can induce NO formation and endothelium –dependent vasorelaxation, is the most important phase of NO synthase activity in the endothelial cells (Andriambeloson et al., 1999). Extracellular Ca^{2+} release or release of Ca^{2+} from intracellular sources is responsible for high level of Ca^{2+} concentrations in the endothelial cells. There are two mechanisms in which polyphenolic compounds increase the amount of NO production. First, enhance the activity of NO synthase or stabilize the releasing of NO under basal status. Second, stabilize NO releasing through superoxidase and other different free radicals (Schuldt et al., 2000). Antioxidative activity of tea, red wine and grape could also promote the amount of NO through free radical scavenging. This might be the case also regarding the verjuice.

Several studies have conclude that, dealcoholyzed red wine and flavonoid rich diets contain quercetin or catechin increase endothelium dependent nitric oxide production without changing in O_2 generation, which can lead to relaxation of vessels. Flavonoid compounds raise the generation of NO, the activity of NOS and change the NO-cGMP pathway too (Benito et al., 2002). Therefore, in the present study, the beneficial effects of verjuice might be related to its flavonoid content. It is mentioned that red wine polyphenols could elevate eNOS expression and endothelial NO release (Leikert et al., 2002). This might also be the case for verjuice. However, it needs more experimental studies in this regard.

In this study, both doses of verjuice caused a significant decrease in fibrinogen level, but no significant difference in factor VII level was found between verjuice groups compared to hypercholesterolemic diet group. Studies regarding the red wine intake indicated a significant

reduction in fibrinogen and factor VII (Mukamal et al., 2001). Intake of high and low-dose verjuice did not change serum CRP level significantly in contrast with hypercholesterolemic diet. Following concurrent use of 10 ml verjuice, cause significant reduction in the level of LDL-C, MDA and ox-LDL compared to hypercholesterolemic diet group. Several studied have reported the polyphones activities on inhibiting the enthrohepatic cycling of cholesterol or bile acid (Osada et al., 1997). That is not clear yet that by which mechanism flavonoids inhibit LDL-C exactly. Decreasing the formation of free radicals, or separating the contributing metal ions in oxidation reaction are some possible mechanisms (Yan et al., 1995).

Studies of Decorde et al. (2008) shown that polyphenols components exist in grape and apple extracts can prevent the process of atherosclerosis in rats and the suppression rate of fatty streak formation was 93% in grape extracts, 78% in red grape, 60% in apple extract and 40% in apple. In previous study, we had shown that intake of 10 ml verjuice had significant acute effect on reduction of ox-LDL, MDA and nitrite but it did not have effect on CRP, LDL-C (Setorki et al., 2010b). In this study long term consumption of 10 and 5 ml verjuic intake significantly lowered the levels of fibrinogen and atherosclerotic lesion in right and left coronary arteries. However, the serum level of nitrite and nitrate increased in both verjuice supplemented groups. Consumption of 10 ml verjuice could meaningfully reduce the amount of MDA, ox-LDL, LDL-C ($p<0.05$). No significant effects were observed for CRP and factor VII after consumption of both doses of verjuice. This research showed the beneficial effects of verjuice on the cardiovascular risk factors and atherosclerotic lesions in a long-term study. This may be due to the anti inflammatory effect of verjuice.

Conclusion

The results of this study showed that verjuice consumption with hypercholesterolemic diet decrease destructive effects of a cholesterol rich diet. In future, it is important to confirm the same effect of dietary verjuice on lipid metabolism in human subjects.

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