Impacts of fresh lime juice and peel on atherosclerosis progression in an animal model

Maryam Boshtam[1], Sedigheh Asgary[2], Jamal Moshtaghian[3], Gholamali Naderi[4], Narges Jafari-Dinani[5]

Original Article

Abstract

BACKGROUND: The main protective role of antioxidants in the progression of atherosclerosis has been shown in some studies. Therefore, this project evaluated the effects of Citrus aurantifolia (Christm) juice and peel on antioxidant activity and atherosclerosis progression in rabbits receiving a hypercholesterolemic diet.

METHODS: Forty white New Zealand male rabbits were randomly allocated to four groups. All groups were on hypercholesterolemic diet for two months. While the first group was considered as the hypercholesterolemic control, groups 2 and 3 (intervention groups) received 5 ml/day lime juice and 1 g/day dried lime peel powder, respectively. Group 4 was fed a normal diet (normal control). Before and after the study, weight was measured and a fasting blood specimen was taken from the rabbits. Serum lipids analyses and antioxidant activity evaluations were then performed. The rabbits' aorta and coronary arteries were separated and the presence of fatty streaks was studied.

RESULTS: Comparing to the hypercholesterolemic control group (-25.2 ± 7.0), only the plasma total antioxidant capacity change was significantly more in rabbits supplemented with lime juice (16.3 ± 14.7) and peel (8.6 ± 7.1) ($P = 0.008$). The presence of fatty streaks in coronary arteries and aorta of the intervention groups [juice (0.2 ± 0.01); peel (0.0 ± 0.00)] was significantly decreased compared to the hypercholesterolemic control group (1.2 ± 0.4) ($P < 0.001$).

CONCLUSION: Based on our findings, Citrus aurantifolia peel and juice increase plasma antioxidant capacity in rabbits, and can thus prevent or decelerate the process of atherogenesis. However, lime peel is more effective than lime juice.

Keywords: Animal, Atherosclerosis, Atherogenic Diet, Fatty Streak, Intervention, Lime

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Introduction

Atherosclerosis is a leading cause of mortality and morbidity worldwide.[1] Hypercholesterolemic diet, on the other hand, is a main factor in initiation and progression of atherogenesis.[2] Moreover, the etiology of several chronic diseases, including coronary artery disease and stroke is thought to be associated with oxidative stress.[3] Accordingly, the protective role of antioxidants in progression of atherosclerosis has been shown in some in vitro and in vivo studies.[4]

Scientists have been long seeking effective components to prevent the atherosclerotic process. Although, research on the anti-atherogenic effects of fruits and vegetables has found that their content of various bioactive compounds with high antioxidant capacity seems to protect the body from the harmful effects of oxidative stress.[5-9]

Despite the proven benefits of some fruits and vegetables in this field, evidence for the impact of some others, like citrus fruits, is less consistent.[10]

As rich sources of dietary fiber, vitamin C, phenolic components, and flavonoids, citrus fruits are believed to have potential health-promoting properties.[11] Limonoids, the major cause of bitterness in citrus juice, have been reported to

1- Isfahan Cardiovascular Research Center, Isfahan Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Iran
2- Professor, Apply Physiology Research Center, Isfahan Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Iran
3- Assistant Professor, Department of Sciences, University of Isfahan, Isfahan, Iran
4- Associate Professor, Isfahan Cardiac Rehabilitation Research Center, Isfahan Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Iran
5- Isfahan Hypertension Research Center, Isfahan Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Iran
Correspondence to: Maryam Boshtam, Email: maryamboshtam@gmail.com

possess substantial antioxidant and anticancer activities.[12] In addition to individual actions of citrus antioxidants, a large number of studies have indicated the existence of cooperative/synergistic interactions among antioxidants in plasma.[13,14]

Citrus aurantifolia (Christm) is one of the most popular citrus fruits throughout the world whose anti-atherogenic effect has not yet been ascertained. On the other hand, the antioxidant-rich lime peel is wasted and only lime juice is consumed. Therefore, in this project, using an animal model, the effects of fresh Citrus aurantifolia (Christm) juice and peel on antioxidant activity and atherosclerosis progression were studied.

Materials and Methods

Animals

Forty white male New Zealand rabbits (mean body weight: 2.0 ± 0.3 kg) were purchased from the Pasteur Institute (Karaj, Iran). Before the experiment, the animals were kept in a laboratory for three weeks to allow them to adapt to laboratory conditions. They were then randomly allocated into four equal groups. Animals in group 1 (hypercholesterolemic control group) received only a hypercholesterolemic diet. Groups 2 and 3 were fed a hypercholesterolemic diet supplemented with 5 ml of fresh lime juice and 1 g of dried lime peel powder, respectively. Rabbits in group 4 were normal controls that received a normal diet. The study lasted for 60 days. The study was approved by the ethics committee of Isfahan Cardiovascular Research Center (Isfahan, Iran) which is a member of the Office for Human Research Protections, US Department of Health and Human Services. The animals were handled according to the guidelines of Isfahan University of Medical Sciences for Laboratory Animal Sciences (Isfahan, Iran).

Fruit collection

Fruits of Citrus aurantifolia were collected from Shiraz gardens (Fars Province, Iran) during the fruiting period in 2007. The fruits were authenticated by a botanist at the Department of Biology, School of Sciences, Isfahan University (Isfahan, Iran). A voucher specimen of the fruits is available at the herbarium of Isfahan University (ID: 5527).

Fruit peels were separated and dried at room temperature (in the shade) for four days. The dried peels were ground by an electric blender. The fruit juice was prepared by squeezing fruits exactly before consumption.

Blood analyses

Before and after the interventions, fasting blood samples were obtained from the rabbits' hearts for serum lipid analyses and plasma total antioxidant capacity measurement. Serum lipids were measured by an auto-analyzer (Hitachi 902) using Pars-Azmoon kits (Iran).

In order to determine plasma total antioxidant status, red blood cells (RBCs) were acquired from healthy voluntary donors. After removal of plasma and buffy coat, the RBCs were washed with phosphate buffered saline (PBS) (150.0 mM NaCl + 1.9 mM NaH_2PO_4 + 8.1 mM Na_2HPO_4; pH = 7.4) three times. They were then kept in PBS for subsequent analyses. 20 µl RBC suspension was incubated in a shaking bath for 10 min with 5 µl plasma objected for total antioxidant capacity measurement. After adding 2,2'-azobis (2-amidinopropane) dihydrochloride (APPH) (70 mM in PBS) and two hours of incubation, the suspension was centrifuged at 2500 rounds per minute for 10 minutes. The extent of hemolysis was spectrophotometrically evaluated (Shimadzu UV 3100, Japan) at 540 nm as hemoglobin (Hb) released from cells in the supernatant.[15]

The radical-scavenging activities of the plasmas, represented as inhibition percent of APPH, were calculated according to the following formula:

Inhibition percent = [1-(ODT/OD)] × 100

Where, ODT and OD were the absorbance values of the tested plasma and control after two hours of incubation, respectively.

The rabbits were sacrificed with pentobarbital (60 mg/kg) after two months and their aorta, coronary arteries, liver, and stomach were removed and preserved in formalin solution (10%) until pathologic investigations. Coronary artery and aortic specimens were sectioned and prepared using particular histological methods. After staining with hematoxylin, they were assessed for the presence of fatty streaks by a pathologist. Atherosclerotic thickness was evaluated on a scale of 1-4 as described by Chekanov.[15]

Statistical analysis

All analyses were performed using SPSS for Windows (version 15.5; SPSS Inc., Chicago, IL, USA). Results were expressed as the mean ± SD for fatty streak grade, and plasma antioxidant capacity and serum lipids changes. After the intervention, concentration of serum biomarkers was compared with before the intervention in each group by Wilcoxon test. Moreover, comparison of serum biomarker mean levels of other three study groups with hypercholesterolemic control group was done by the Mann-Whitney U test. Kruskall-Wallis test

was used to compare fatty streak percentage and also serum biomarkers between study groups. P values of less than 0.05 were considered significant.

Results

Table 1 shows comparison of serum lipids and plasma total antioxidant capacity mean level between before and after the intervention in each group.

Except for the normal diet group, for other groups, a significant increase was observed in serum lipids after the interventions (P < 0.02). However, plasma antioxidant capacity significantly decreased after the intervention only in the hypercholesterolemic group (P = 0.017), and in other groups it increased but not significantly.

The comparison of serum lipids and plasma total antioxidant capacity change during the study period between the groups is presented in table 2. There were no significant differences in the change of serum total cholesterol (TC) (P > 0.05), high density lipoprotein cholesterol (HDL-C) (P > 0.55), and triglyceride (TG) (P > 0.14) levels between the hypercholesterolemic group and the lime peel or juice consumers. However, hypercholesterolemic controls and rabbits supplemented with lime juice were significantly different in the mean changes in serum low density lipoprotein cholesterol (LDL-C) levels (P = 0.04). Compared to the hypercholesterolemic group (group 1), the plasma total antioxidant capacity increase was significantly higher in the lime juice and lime peel administrated rabbits (groups 2 and 3) (P < 0.05). Serum LDL-C levels had significantly higher increments in rabbits receiving lime juice than in those receiving peel (P = 0.043). The opposite was true in the case of plasma antioxidant capacity increase (P < 0.05).

The stage of fatty streak in coronary arteries and aorta of groups 2 and 3 was significantly decreased compared to the hypercholesterolemic group (P < 0.001) (Table 3).

Table 1. Comparison of serum lipids and plasma total antioxidant capacity mean level between before and after the intervention in each group

Serum marker	Hypercholesterolemic diet		Hypercholesterolemic diet + lime juice		Hypercholesterolemic diet + lime peel		Normal diet	
	Before intervention	After intervention	Before intervention	After intervention	Before intervention	After intervention	Before intervention	After intervention
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
TC (mg/dl)	45.9 ± 10.2	987.0 ± 227.2*	49.6 ± 5.8	931.6 ± 197.7*	48.5 ± 9.9	999.5 ± 106.1*	68.6 ± 40.9	69.4 ± 38.2
LDL-C (mg/dl)	15.0 ± 5.7	279.9 ± 167.2*	17.6 ± 3.9	454.1 ± 112.3*	16.0 ± 7.4	299.4 ± 121.7*	33.2 ± 35.8	29.2 ± 36.4
HDL-C (mg/dl)	14.2 ± 5.1	110.0 ± 19.7*	15.1 ± 5.2	114.4 ± 28.8*	15.6 ± 5.1	117.6 ± 26.3*	17.6 ± 4.0	23.8 ± 7.4
TG (mg/dl)	59.2 ± 25.4	216.1 ± 267.8*	54.3 ± 13.8	96.7 ± 23.8*	39.6 ± 6.8	138.6 ± 162.7*	67.0 ± 33.7	81.8 ± 18.5
Antioxidant capacity (%)	62.3 ± 19.1	35.0 ± 15.2*	31.7 ± 8.3	40.2 ± 25.0	57.9 ± 10.9	67.1 ± 16.1	48.9 ± 4.2	47.8 ± 5.1

* Significant difference between before and after the intervention (P value of Wilcoxon test < 0.02); TC: Total cholesterol; LDL-C: Low density lipoprotein cholesterol; HDL-C: High density lipoprotein cholesterol; TG: Triglyceride

Table 2. Comparison of the mean change in serum lipids and plasma total antioxidant capacity during the study period between the groups

Parameter	Hypercholesterolemic diet	Hypercholesterolemic diet + lime juice	Hypercholesterolemic diet + lime peel	Normal diet	P∫
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
TG (mg/dl)	156.9 ± 246.6	42.1 ± 28.7	99.0 ± 162.9	14.8 ± 35*	0.110
TC (mg/dl)	941.1 ± 224.6	882.0 ± 194.4	951.0 ± 101.7	0.8 ± 12.6*	0.002
LDL-C (mg/dl)	264.9 ± 165.1	435.1 ± 114.2	283.4 ± 121.2Ł	-4.0 ± 10.8*	0.001
HDL-C (mg/dl)	95.8 ± 18.3	99.3 ± 26.0	102.0 ± 23.0	6.2 ± 7.9*	0.006
Total antioxidant capacity (%)	-25.2 ± 19.8	16.3 ± 32.8*	8.6 ± 21.3*	1.5 ± 1.2*	0.011

∫ P value of Kruskal-Wallis test; * Significant difference with hypercholesterolemic group (P < 0.05) (P value of Mann-Whitney U test); Ł Significant difference with lime juice users (P < 0.05) (P value of Mann-Whitney U test); TG: Triglyceride; TC: Total cholesterol; LDL-C: Low density lipoprotein cholesterol; HDL-C: High density lipoprotein cholesterol

Table 3. Comparison of mean fatty streak grade between the study groups

Group	Fatty streak stage Mean ± SD
Hypercholesterolemic diet	1.2 ± 0.040
Hypercholesterolemic diet + lime juice	0.2 ± 0.001
Hypercholesterolemic diet + lime peel	0.0 ± 0.000
Normal diet	0.0 ± 0.000
P*	< 0.001

* P value of Kruskal-Wallis test

No abnormality was observed in the stomach tissue or liver of rabbits who consumed lime peel or juice.

Discussion

The results of this study showed that consumption of lime (Citrus aurantifolia) peel or fresh juice could inhibit the process of atherogenesis. Meanwhile, the peel had better effects than the juice.

Hypercholesterolemic diet is considered to play a main role in atherosclerosis progression as it acts through increasing lipid profile, especially LDL-C.[16,17] Such an effect was also observed in our study. On the other hand, the etiology of aging and some diseases like cancer, atherosclerosis, coronary artery disease, stroke, ischemic injury, and inflammation has been reported to associate with reactive oxygen species.[18] Therefore, antioxidant compounds should improve them. Antioxidant vitamins, enzymes, and some poly-phenolic compounds comprise the human's total antioxidant defense system.[19] Fruits and vegetables are the main sources of bioactive and antioxidant compounds.

Flavonoids, one of the important bioactive components of vegetables and fruits, may contribute to protection against the diseases through enhancing the human immune system.[20] Several in vivo and in vitro studies have revealed the preventive effects of flavonoid contents of fruits and vegetables on oxidative stress.[21] Based on our findings, changes in the total serum antioxidant capacity were significantly higher in rabbits consuming lime juice or peel than in controls. This increase was significantly more in the juice users than in the peel consumers. Phenolic compounds (i.e. flavonoid content of lime peel and juice) should be responsible for the observed differences. In other words, lime peel and juice might have different types of flavonoids. Research has indicated that hesperidin, naringenin and eriocitrin exist in lime juice.[22] The peel, on the other hand, contains polymethoxylated flavones (PMF), limonoid, and diosmin.[23-25] The high ascorbic acid content of fresh lime juice can justify the significantly higher serum antioxidant capacity among rabbits supplemented with lime juice than those with lime peel.

Diets rich in fruits and vegetables have been suggested to be inversely related with the risk of various diseases.[26] For instance, seven-day consumption of red orange juice by non-diabetic patients with cardiovascular diseases could increase endothelial function and decrease inflammation.[27] We found that consumption of lime peel or fresh lime juice prevented atherosclerosis in rabbits with atherogenic diet. Although lime juice induced greater changes in serum antioxidant capacity than did lime peel, the latter caused significantly more reductions in fatty streak grade. This difference can again be related to the effects and absorption of each type of flavonoid. Unfortunately, we have no information on the type and amount of absorbed flavonoids.

Numerous studies have assessed the effects of separate bioactive components of lime juice and peel. Some have also investigated how the compounds play their atherogenic role. Yen et al. concluded that 5-demethyl-nobiletin (a flavonoid in citrus fruits) has antiatherogenic properties and inhibits monocyte-to-macrophage differentiation and foam cell formation. This flavonoid, and nobiletin may increase the expression of LDL-C receptor gene and simultaneously decrease diacylglycerol acyltransferase 2 (an acyl coenzyme A) expression.[28] A previous study reported a 70%-reduction in atherosclerosis among mice treated with naringenin.[29] Similarly, Lee et al. showed that naringin could prevent atherosclerosis through Phosphatidylinositol 3-kinase (PI3K)/Akt and mammalian target of rapamycin (mTOR)/p70S6K pathways (PI3K/AKT/mTOR/p70S6K pathway) repression, invasion, and migration, and the subsequent suppression of matrix metalloproteinase-9 (MMP-9) expression.[30] Eguchi et al. found nobiletin to suppress the activator protein-1 transcriptional activity and hence regulate atherosclerosis.[31] In another animal study, citrus juice inhibited atherosclerosis and decreased serum

TCho and TG. Ascorbic acid in a dose similar to citrus juice showed the same effect on atherosclerosis.[32] In a research on male New Zealand white rabbits receiving a hypercholesterolemic diet for eight weeks, Choe et al. suggested naringin to cause antiatherogenic effects as it decreased fatty streak formation and intercellular adhesion molecule 1 expression in endothelial cells.[33]

Another component of lime peel is pectin. Grapefruit pectin has been shown to inhibit hypercholesterolemia and atherogenesis. Such antiatherogenic effect may be related to a mechanism independent of cholesterol levels.[34] A research on pigs receiving grapefruit pectin indicated the same results.[35]

A limitation of the present study was not measuring cellular and molecular pathways that could cause such results. In addition, it was better to assess the specific effects of each flavonoid separately.

Conclusion

Although both fresh lime (Citrus aurantifolia) juice and peel could prevent atherogenesis through increasing serum antioxidant capacity, the antiatherogenic effect of the latter was significantly stronger. It is thought that the type and amounts of flavonoids in lime peel and juice are responsible for this difference.

Further research is warranted to clarify the effects of each flavonoid in lime juice and peel on atherosclerosis and cellular and molecular pathways.

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Conflict of Interests

Authors have no conflict of interests.

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