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Effects of Orange Juice and Hesperetin on Serum Paraoxonase Activity and Lipid Profile in Hyperuricemic Rats

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ABSTRACT

Introduction: Hypouricemic, antioxidant and xanthine oxidase inhibitory effects of orange juice and hesperetin have been already indicated. The objective of this study was to investigate the effects of orange juice and hesperetin on paraoxonase and arylesterase activity and lipid profile of hyperuricemic rats. ***Methods:*** Forty eight male Wistar rats were divided into 8 equal groups of healthy control, healthy+orange juice, healthy+hesperetin, healthy+allopurinol, hyperuricemic control, hyperuricemic+orange juice, hyperuricemic+hesperetin and hyperuricemic+allopurinol. Hyperuricemia was induced using potassium oxonate (250 mg/kg ip). The treatments were carried out by daily gavage of 5 ml/kg orange juice, 5 mg/kg hesperetin and 5 mg/kg allopurinol for 2 weeks. Paraoxonase activity in serum was measured spectrophotometrically using paraoxon and phenylacetate as substrates. Serum lipids levels were determined using enzymatic colorimetric methods. ***Results:*** Hyperuricemia-induced reduction of paraoxonase and arylesterase activity was restored after treatment with orange juice and hesperetin ($p<0.05$). The effect of both treatments on lipid profile was marginal and only orange juice could significantly increase the levels of HDL-C. ***Conclusion:*** Supplementation of orange juice and hesperetin could restore paraoxonase and arylesterase activity in hyperuricemic rats. Orange juice could also partially improve the lipid profile. These effects could have major implications with respect to the prevention of cardiovascular disease in hyperuricemic patients. However, more studies are needed in future investigations.

Introduction

Hyperuricemia, characterized by abnormal high levels of uric acid, is a common metabolic disorder with a worldwide distribution (Mo *et al* 2007). It has been considered as an important risk factor for gout and may be associated with oxidative stress conditions such as cardiovascular diseases (Strazzullo and Puig 2007). Allopurinol, an inhibitor of xanthine oxidoreductase (XOR), is the only drug with clinical application to lower uric acid production (Fels and Sundy 2008), but severe side effects such as hepatitis, nephropathy and allergic reactions limit the clinical use of allopurinol and it would be highly desired to search for new XOR inhibitors, in particular from natural sources, as alternatives for allopurinol (Strazzullo and Puig 2007, Nguyen *et al* 2004).

The hypouricemic, antioxidant and XOR inhibitory effects of orange juice and its predominant flavanone, hesperetin, in comparison with allopurinol on potassium oxonate hyperuricemic rats have been already shown. Orange juice and hesperetin were demonstrated to reduce XOR activity, the key enzyme in the catabolism of purines (Haidari *et al* 2009). Decreasing endogenous production of uric acid, serum concentration of malondialdehyde (MDA) and enhancing plasma total antioxidant capacity (TAC) was also found following orange juice and hesperetin administration in Haidari *et al* study (2009). Hesperetin (3′, 5, 7-trihydroxy-4′-methoxyflavanone), which occurs as hesperidin (its glycoside form) in nature, belongs to flavanone subclass of flavonoids and is mainly found in citrus fruits such as orange (Choi *et al* 2006). The predominant mechanism of biological actions of hesperetin is thought to result from antioxidant activity, enzyme inhibition, and the capacity to scavenge free radicals (Kaur *et al* 2006). Concerning the antioxidant effects of orange juice and hesperetin, there is a possibility that orange juice supplementation reverses the oxidative damage in hyperuri-

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cemia (Haidari *et al* 2009). Beside uric acid levels and XOR activity, reactive oxygen species (ROS) and paraoxonase activity are also conceived to play key roles in the pathogenesis of hyperuricemia (Meotti *et al* 2011, Haidari *et al* 2011). Paraoxonase is a Ca-dependent esterase distributed in liver, kidney, intestine and the serum which prevents from peroxidation of lipids in LDLs (Aviram *et al* 1998). Paraoxonase is suggested to possess peroxidase, arylesterase and paraoxonase activities and has been associated with a protective role in oxidative stress and atherosclerosis pathogenesis, which return to paraoxonase's ability in hydrolysis of lipid peroxides (Rodrigo *et al* 1997, Shimoni *et al* 2003). Kirschbaum (2004) indicated the reciprocal relationship between uric acid and paraoxonase activity. Several recent studies also reported that paraoxonase concentration in oxidative stress induced-disease being low and was associated with the lower level of HDL-C and the higher level of lipid peroxidation (Balbir-Gurman *et al* 2011, Suh *et al* 2011). Mechanism of paraoxonase reduction in oxidative stress status is not clearly known; however, it is suspected that ROS overproduction leads to increased deactivation of paraoxonase (Isik *et al* 2007). Due to the correlation of paraoxonase activity with lipid profile (Balbir-Gurman *et al* 2011), evaluating lipid profile levels such as HDL-C seems unavoidable. One recent study has reported that hesperetin and its metabolite, ferulic acid, in hypercholesterolemic hamsters for 12 weeks increases HDL-C/total cholesterol ratio and paraoxonase levels, but decreases the concentration of other lipids (Kim *et al* 2010). Paraoxonase is an important antioxidant enzyme and is responsible for antioxidant effects of HDL-C (Mahrooz *et al* 2011), thus it may have a protective role against oxidative stress in hyperuricemia.

The hypouricemic and antioxidant effects of orange juice and hesperetin have been shown before (Haidari *et al* 2009). However, their effect on paraoxonase activity and lipid profile in hyperuricemia is yet unclear. This investigation is based on the hypothesis that bioactive compounds found in orange juice have paraoxonase-enhancing activity and lipid lowering effect in hyperuricemia. To verify our hypothesis, the present *in vivo* study was aimed to investigate the effects of orange juice and its main flavanone, hesperetin, on paraoxonase activity and lipid profile in potassium oxonate-induced hyperuricemic rats. The results obtained have been compared with those of allopurinol, a potent XOR inhibitor.

Materials and methods

Materials

Hesperetin, potassium oxonate, allopurinol were purchased from Sigma-Aldrich Chemical Co. (Steinheim, Germany). All other reagents used were from of analytical grades. Orange (*Citrus sinensis* L.) was purchased from North region of Iran.

Test compound preparation

Orange is commonly peeled and squeezed for its juice. Hesperetin was first dissolved in propylene glycol and then was added to 0.9% saline (1:20 V: V). Allopurinol was used as a positive control and was prepared in 0.9 % saline (Haidari *et al* 2009).

Animals

Forty eight male Wistar rats (8-10 weeks of age**,** weighing 180-200 grams) were obtained from Laboratory-Animal House of Tabriz University of Medical Sciences, Iran. They were fed with a commercial laboratory diet and allowed food and water *ad libitum* for an acclimatization period of 1 week prior to the experiment. Housing conditions and experimental procedures were set to be in accordance with international standards. All animals were maintained on a 12 h day-night cycle and the temperature and humidity were kept at 22 - 24°C and 50%, respectively. They were handled according to the recommendation of the local and national ethic committees. After accommodation period, rats were randomly divided into eight equal groups of six rats per group as described in Table 1. Treatments were carried out for two weeks after hyperuricemia induction. The freshly prepared test compounds were administrated to respective groups by oral gavage.

Induction of hyperuricemia

Experimentally-induced hyperuricemia in rats due to inhibition of uricase with potassium oxonate was used to study anti-hyperlipidemic and antioxidant effects of test compounds. Briefly, 250 mg/kg potassium oxonate (PO) dissolved in 0.9% saline solution was administrated intraperitoneally to each animal 1 h before oral administration of test compounds (Hall *et al* 1990).

Table 1. Experimental groups

Group	Treatment
Healthy control	Saline 0.9% (vehicle)
Healthy -orange juice-treated	5 ml/kg orange juice
Healthy -hesperetin-treated	5 mg/kg hesperetin
Healthy -allopurinol-treated	5 mg/kg allopurinol
Hyperuricemic control	250 mg/kg oxonate (ip)+saline 0.9% (vehicle)
Hyperuricemic-orange juice-treated	250 mg/kg oxonate (ip)+ 5 ml/kg orange juice
Hyperuricemic-hesperetin-treated	250 mg/kg oxonate (ip)+ 5 mg/kg hesperetin
Hyperuricemic-allopurinol-treated	250 mg/kg oxonate (ip)+ 5 mg/kg allopurinol

Determination of paraoxonase and arylesterase activity

Paraoxonase activity was determined by spectrophotometric analysis using paraoxon (O, O-diethyl-o-p-nitrophenylphosphate) as substrate and measuring the increase in the absorbance of 4-nitrophenol formation at 412 nm. Briefly, the activity was determined by adding 20 µl of serum to Tris-HCl buffer (100 mM, pH 8.0) containing 2 mM $CaCl_2$ and 2mM of paraoxon at 25º C and the rate of 4-nitrophenol formation was determined at 412 nm with a spectrophotometer (Shimadzu 2550, UV/Vis with a temperature control unit). Paraoxonase activity was expressed in nM/min/ml serum (Kuo and La Du 1995).

Arylesterase activity of serum was also determined spectrophotometrically using phenylacetate as the substrate. The generated phenol was measured by spectrophotometer (Shimadzu 2550, UV/Vis with a temperature control unit) at 270 nm. Reaction contained Tris-HCL (100 mM, pH 8.0), phenyl acetate (2 mM), $CaCl_2$ (2 mM) and 10 µl of serum. The activity of arylesterase was expressed in µM/min/ml serum. Both assays were repeated two times (Mahrooz *et al* 2011).

Determination of Lipid profile

Total cholesterol level was determined by an enzymatic colorimetric assay described by Moghadasian *et al* (2002). HDL-C level was also determined by an enzymatic method (Fard *et al* 2004). In this assay, HDL-C concentration is measured after precipitating VLDL-C, LDL-C, IDL-C, chylomicrons and α-lipoprotein. Triglyceride enzymatic determination was carried out according to Zhao *et al* method (1995). LDL-C level was calculated according to Friedewald formula:

LDL (mg/dl) = TC – (HDL-C + TG/5)

Statistical analysis

The results were expressed as the mean ± SD (*n*=6). The statistical comparison of each experimental group with control group was performed by Independent-sample t-test using SPSS computer program. The probabilities of 5% or less ($p<0.05$) were considered significant.

Results

Paraoxonase and arylesterase activity

Serum paraoxonase activity after treatment with orange juice and hesperetin in comparison with healthy control, hyperuricemic control and allopurinol treated groups have been depicted in Table 2.

As it is shown, statistically significant differences were observed in the average level of serum paraoxonase activity in rats between healthy control and hyperuricemic control groups (p=0.008). At the end of study, orange juice and hesperetin could produce a significant increase in the enzyme activity in the treated hyperuricemic groups rather than hyperuricemic control groups (p=0.029 and p=0.042, respectively). Orange juice and hesperetin were also successful in restoring the serum paraoxonase activity in hyperuricemic treated groups; since there was no significant difference in paraoxonase activity between these groups and healthy control group (p>0.05). Administration of orange juice to healthy rats also increased serum paraoxonase activity significantly (p=0.047).

Results from arylesterase activity after treatment with orange juice and hesperetin have been shown in Table 3.

Serum arylesterase activity also decreased in hyperuricemic control rats relatively to healthy control rats (p=0.042). Orange juice and hesperetin caused to a significant increase in serum arylesterase activity compared to hyperuricemic control group (p=0.031 and p=0.045, respectively). Both orange juice and hesperetin could restore the diminished enzyme activity in hyperuricemic rats. The enzyme activity was shown to increase after treatment with orange juice in healthy rats (p=0.035). Both orange juice and hesperetin were found statistically more effective than allopurinol in restoring paraoxonase and arylesterase activity (p=0.048 and p=0.050, respectively).

Table 2. The mean serum paraoxonase activity in normal and hyperuricemic rats after 2 weeks of treatment with orange juice, hesperetin and allopurinol*

Group	Paraoxonase activity (nM/min/ml)	P1	P2	P3
Healthy control	126.33±06.43	-	0.008	0.043
Healthy -orange juice-treated	139.33±09.54	**0.047**	0.000	0.000
Healthy -hesperetin-treated	128.00±12.26	0.156	0.003	0.019
Healthy -allopurinol-treated	125.83±07.67	0.657	0.010	0.024
Hyperuricemic control	105.50±07.73	**0.008**	-	0.998
Hyperuricemic-orange juice-treated	118.66±12.12	0.125	**0.029**	**0.022**
Hyperuricemic-hesperetin-treated	116.83±09.26	0.159	**0.042**	**0.031**
Hyperuricemic-allopurinol-treated	101.00±13.97	0.003	0.998	-

* All values are expressed as mean ± SD (n=6). Independent-sample t-test was used for statistical significance assessment. P1: Comparison with healthy control group; P2: Comparison with hyperuricemic control; P3: Comparison with allopurinol group.

Table 3. The mean serum arylesterase activity in normal and hyperuricemic rats after 2 weeks of treatment with orange juice, hesperetin and allopurinol*

Group	Arylesterase activity (μM/min/ml)	P1	P2	P3
Healthy control	167.83±18.94	-	0.042	0.275
Healthy -orange juice-treated	188.33±15.06	**0.035**	0.007	0.018
Healthy -hesperetin-treated	169.66±13.03	0.680	0.630	0.830
Healthy -allopurinol-treated	166.83±11.58	1.000	0.179	0.334
Hyperuricemic control	144.16±17.47	**0.042**	-	1.000
Hyperuricemic-orange juice-treated	161.83±15.99	0.192	**0.031**	**0.048**
Hyperuricemic-hesperetin-treated	156.66±10.65	0.105	**0.045**	**0.050**
Hyperuricemic-allopurinol-treated	147.16±15.28	0.025	1.000	-

* All values are expressed as mean ± SD (n=6). Independent-sample t-test was used for statistical significance assessment. P1: Comparison with healthy control group; P2: Comparison with hyperuricemic control; P3: Comparison with allopurinol group.

Table 4. The mean serum total cholesterol, triglyceride, HDL-C and LDL-C (mg/dl) in normal and hyperuricemic rats after 2 weeks of treatment with orange juice, hesperetin and allopurinol*

Group	Total cholesterol	Triglyceride	HDL-C	LDL-C
Healthy control	79.93±22.04	69.30±14.83	19.50±2.73	46.57±20.97
Healthy -orange juice-treated	72.60±17.32	69.20±14.39	**24.00±7.15**[a]	33.96±16.73
Healthy -hesperetin-treated	79.16±09.70	68.83±15.23	20.00±2.60	44.66±11.60
Healthy -allopurinol-treated	79.10±19.76	65.03±15.76	19.83±5.52	46.26±13.51
Hyperuricemic control	94.10±23.84	73.33±20.48	18.66±9.68	60.76±17.65
Hyperuricemic-orange juice-treated	67.46±18.54	63.06±09.60	**23.96±6.69**[b,c]	34.85±13.46
Hyperuricemic-hesperetin-treated	78.30±38.67	63.06±13.10	21.00±4.14	54.68±35.26
Hyperuricemic-allopurinol-treated	96.08±23.52	86.68±16.36	16.00±5.05	62.74±18.12

* All values are expressed as mean ± SD (n=6). Independent-sample t-test was used for statistical significance assessment.
a: Compared to healthy control group (p=0.043).
b: Compared to hyperuricemic control group (p=0.032).
c: Compared to hyperuricemic-allopurinol-treated group (p=0.011).

Lipid profile results

Table 4 demonstrates the mean serum concentration of lipid profile (total cholesterol, triglyceride, HDL-C and LDL-C) in groups after treatment with orange juice, hesperetin and allopurinol.

Induction of hyperuricemia in rats could not exert a significant change on lipid profile compared to healthy control group ($p>0.05$). Treatment with orange juice and hesperetin induced a marginally significant reduction in serum triglyceride and total cholesterol level compared to hyperuricemic control rats. Orange juice could also increase serum HDL-C compared to the hyperuricemic control group ($p=0.032$) and allopurinol group ($p=0.011$). After treatment with orange juice in healthy rats, a significant increase in HDL-C concentration was also found compared to the healthy control rats ($p=0.043$).

Discussion

In the present study, we showed that orange juice and hesperetin could increase paraoxonase activity and partially improve lipid profile in hyperuricemic rats. The altered paraoxonase activity and its relation to lipid profile in some oxidative stress- induced diseases were investigated previously (Alvarez-Parrilla *et al* 2010, Kim *et al* 2010, Balbir-Gurman *et al* 2011). Tanimoto *et al* indicated that structurally and functionally changed HDL-C in rheumatoid arthritis patients has lower antiatherogenic properties and suggested that reduced activity of serum paraoxonase activity and changed HDL-C levels are responsible for higher cardiovascular mortality in these patients (2003). Another study reported significantly higher malondialdehyde concentration and lower paraoxonase activity in patients with type 2 diabetes, illustrating a negative correlation between paraoxonase activity and lipid peroxidation (Rasic-Milutinovic *et al* 2012). Baskol *et al* (2011) determined reduced antioxidant paraoxonase activity and increased oxidant XOR activity in women with polycystic ovary syndrome compared to healthy volunteers. The reduction in paraoxonase activity in serum will be related to increased atherosclerosis seen in later life of such patients (Baskol *et al* 2011). There are a few evidences on paraoxonase activity in hyperuricemia until now. We have recently demonstrated the protective effects of onion intake in hyperuricemia. Onion administration (5 g/kg) was shown to increase paraoxonase and arylesterase activity significantly in hyperuricemic rats (Haidari *et al* 2011). Moreover, a

number of epidemiological reports have increasingly linked hyperuricemia with cardiovascular diseases, which may reflect the reduction of antioxidant systems such as paraoxonase (Gaffo and Saaq 2011, Jin *et al* 2012). It seems that one reason for lower paraoxonase activity in hyperuricemia is higher production of free radicals and higher lipid peroxidation (Krishnan 2009), that increases the deactivation of paraoxonase, since paraoxonase is involved in detoxification of lipid peroxides (Isik *et al* 2007). Increased oxidative stress in hyperuricemic patients resulting in the reduction of endogenous antioxidant stores has important roles in the pathogenesis of atherosclerosis (Krishnan 2009, Jin *et al* 2012). Paraoxonase is suspected to be an inducible enzyme and can be affected by diet (Mackness *et al* 2002). The increased activity of paraoxonase and arylesterase following treatment with orange juice and its constitutive flavanone aglycone, hesperetin, in this study can partly be attributed to their antioxidant properties. Dalgard *et al* (2007) in a cross- over study also showed the increased paraoxonase activity after 250 ml orange juice intake in patients with peripheral arterial disease carrying the PON1 L55-allele. Furthermore, hesperetin and its metabolite, ferulic acid, have been shown to increase HDL-C/total cholesterol ratio and paraoxonase levels in hypercholesterolemic hamsters (Kim *et al* 2010). We have recently demonstrated that orange juice and hesperetin administration in hyperuricemic rats increases TAC and decreases MDA concentration, as a biomarker of lipid peroxidation (Haidari *et al* 2009).

In the present study, induction of hyperuricemia in rats did not have a significant effect on serum levels of triglyceride, total cholesterol, LDL-C and HDL-C ($p>0.05$). No treatment neither orange juice nor hesperetin could induce markedly significant change in triglyceride, total cholesterol nor LDL-C in hyperuricemic treated groups. But at the end of the study, orange juice could significantly increase serum HDL-C levels in healthy and hyperuricemic treated groups compared to respective control group ($p= 0.043$ and $p= 0.032$, respectively). Several different studies have been reported controversial results on the effects of orange and hesperetin on serum lipid profile. While some studies indicated no significant effect of orange and/or hesperetin on serum lipid profiles (Franke *et al* 2005, Deyhim *et al* 2007), other studies reported positive and effectual effects (Devaraj *et al* 2006, Cesar *et al* 2010, Kim *et al* 2010). These discrepant results are probably due to the different study subjects. Orange juice might be more effective in improving lipid profile in hyperlipidemic but not in normolipidemic subjects. This is similar to the effect seen with other flavonoid-rich foods exposure (Franke *et al* 2005) and seems to be a favorite effect because lowering normal levels could lead to critical ranges of potentially adverse consequence. Though hyperuricemia induction led to increase of triglyceride, total cholesterol and LDL-C concentrations in this study, no statistical significance was reached for any of these changes. Although it is hard to speculate on the exact mechanism by which the orange juice improves lipid profile, it seems that orange juice components such as flavonoids (hesperetin and naringenin predominantly as glycosides), carotenoids (xanthophylls, cryptoxanthins, carotenes), and vitamin C in addition to other beneficial phytochemicals are involved in lipid lowering effect (Franke *et al* 2005). Flavonoids may also decrease cholesterol absorption by increasing the excretion of bile acids (Devaraj *et al* 2006). Increased HDL-C level can also facilitate the transport of cholesterol from tissues to liver (Cesar *et al* 2010).

Conclusion

In conclusion, supplementation of orange juice and its main constituent flavanone aglycone, hesperetin, could compensate the reduced serum paraoxonase and arylesterase activity in hyperuricemic rats. Orange juice and hesperetin could also partially improve the lipid profile. Both treatments were discovered to be more effective than allopurinol in improving the enzyme activity and lipid profile. Although more studies are needed to confirm paraoxonase-enhancing activity of orange juice in human subjects and investigate the underlying mechanisms, this could have major implications with respect to the prevention of cardiovascular disease in hyperuricemic patients.

Ethical issues

None to be declared.

Conflict of interests

The authors declare no conflict of interests.

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