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Effect of pomegranate juice supplementation on serum total cholesterol level of hypercholesterolemic rats

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To investigate the effect of pomegranate (*Punica granatum)* juice on serum total cholesterol level in an animal model, an experimental study was carried out in twenty Wistar strain rats (10 males and 10 females, 200-250 gm body weight,). To induce hypercholesterolemia, all rats were supplemented with a high cholesterol diet which was enriched with coconut oil, cholesterol and sugar (25%, 1%, and 16% by weight, respectively) to the ordinary feed for thirty days. After dietary inducement, ten of twenty hypercholesterolemic rats whose serum total cholesterol levels increased by 75% and above 75% than their basal levels were administered orally, an alcohol extract of pomegranate seeds (0.056 gm) and 3 ml of fresh pomegranate juice (PJ) daily for ninety days. Another ten hypercholesterolemic rats (control group) were administered orally, 3 ml of distilled water daily for ninety days. Serum total cholesterol levels were measured on Day 30, Day 60, and Day 90 of pomegranate juice and distilled water supplementation. Serum total cholesterol levels of the test group were lower than those of the control group on Day 30 (91.44 ± 22.9 mg/dl vs 109.4± 27.19 mg/dl), and on Day 60 (82.6±17.3 mg/dl vs 89.7±13.96 mg/dl) of supplementation, although they were not statistically significant ($p>0.05$ for both). On Day 90 of supplementation, serum total cholesterol level of test group was significantly lower than that of control group (74.4 ±10.11 mg/dl vs 86.8± 10.52mg/dl), ($p=0.043$). Therefore, a 90-day supplementation of PJ reduces serum total cholesterol level in diet-induced hypercholesterolemic rats.

INTRODUCTION

Hypercholesterolemia in humans is considered to be elevated blood cholesterol level (ie. 240 mg% and above). Hypercholesterolemia is usually discovered during routine screening and is an asymptomatic condition. It is more common in individuals with family history of it, but life style factors (eg. diet in saturated fat) clearly play major role. Having too much cholesterol in blood is not a disease itself but can lead to the hardening and narrowing of the arteries (atherosclerosis) in the major vascular systems. Therefore, it is associated with myocardia infarction, cerebrovascular accident and intermittent claudication.

At early stage of atherosclerosis, macrophage cholesterol (and oxidized lipid) accumulation and foam cell formation take place, leading to the development of the complicated atherosclerotic lesion [1]. Major contributors to cholesterol accumulation in arterial cells during atherogenesis include high plasma cholesterol concentration [2], increased oxidative stress [3], reduced serum paraoxonase activity [4], increased uptake of atherogenic lipoproteins by arterial cells [5], enhanced macrophage cholesterol esterification rate [6], and decreased cholesterol efflux from arterial cells [7]. Since oxidative stress is believed to play an important role in early atherogenesis, antioxidants (polyphenolic flavonoids) which are rich in red wine, grapes, licorice, ginger and pomegranate, significantly reduce oxidative stress by inhibiting the formation of OX-LDL and macrophage lipid peroxidation.

Pomegranate (*Punica granatum L, Punicaceae*) or *Thalathee* (ovJoD:), likely originated in Iran and Afghanistan, is currently grown mainly in Iran, India, and the United States, but also in most near and far east countries [8]. Historically, the fruit is mentioned by various cultures and religions. Greek and Persian mythologies mention the fruit as representing life, regeneration, and marriage. The ancient Chinese believed that the seeds symbolized longevity and immortality. The fruit is also a symbol of resurrection and life in Christianity, and it is one of the three "blessed fruits" in Buddhism [9].

Aviram *et. al.* [10] showed that PJ supplementation of apolipoprotein E deficient (E^0) mice reduced the size of their atherosclerotic lesions by 44%. It was also found that PJ consumption for 3 years by patients with carotid artery stenosis reduced common carotid intema-media thickness, blood pressure and LDL oxidation [11]. Therefore, the effect of pomegranate grown in Myanmar on plasma lipids profile has to be investigated. The objective of this study was to investigate the effect of pomegranate (*Punica granatum)* juice on serum total cholesterol level of rats with a high cholesterol diet prior to pomegranate juice supplementation.

MATERIALS AND METHODS

An experimental study design with controlled trial was used in twenty Wistar strain rats (200-250 gm body weight, 10 males and 10 females). To induce hypercholesterolemia, all rats were fed with a high cholesterol diet for thirty days before pomegranate juice (PJ) supplementation. After dietary inducement, the rats whose serum total cholesterol levels were increased by 75% and above 75% than their basal levels were defined as hypercholesterolemic rats and they were allocated into two groups (Test and Control Group). Then, the high cholesterol diet was changed into the routine diet on the first day of PJ supplementation and continued throughout the study. All rats were housed individually in metabolic cages for three days to measure food intake during dietary inducement, first, second and third month of PJ supplementation. Body weights and serum total cholesterol levels were determined before and after dietary inducement and Day 30, Day 60, and Day 90 of PJ supplementation.

Dietary inducement for hypercholesterolemia

All rats were maintained at 24 C° with a 12-hour light and dark cycle. Initial body weights were measured with an animal balance and basal serum total cholesterol levels of them were determined. To make a high cholesterol diet, a routine diet was enriched with coconut oil, sugar, and cholesterol in the ratio of 25%, 16%, and 1% by weight of feed respectively. Then, all rats were given freely access to high cholesterol diet for thirty days. After that, serum total cholesterol levels were measured and by an operational definition, the hypercholesterolemic rats were undergone the intervention study. If the rats whose serum total cholesterol levels increased by less than 75% than their basal levels, they were continued to feed the high cholesterol diet for next thirty days.

Pomegranate Juice (PJ) Preparation

Pomegranates were provided by Ministry of Agriculture and Irrigation, in February, 2005. After peeling off the covers, the red juicy edible sacs together with seeds were crushed with a blender. The pink fluid and crushed seeds were separated by filtration and pink fluid was kept as 15 ml aliquots and was stored at –80C°. The crushed seeds were kept to be dry under shadow and extracted with 50% ethyl alcohol. The dried alcohol extract of the crushed seeds was stored separately and immediately before administration to rats, pink fluid and alcohol extract of crushed seeds were mixed to make pomegranate juice for rats.

Pomegranate Juice (PJ) Supplementation

After dietary inducement, the high cholesterol diet was changed to the routine diet and hypercholesterolemic rats were allocated into two groups: 10 in test group and 10 in sex-matched control group. If a person ate a pomegranate a day, a rat would have 0.5ml fresh juice containing 0.00028g of alcohol extract of seeds because of the differences between their body weights. To evaluate pharmacological effects of herbs, the dose of PJ for administration to rats was increased up to six times of human dose. Therefore, it became 3 ml of pomegranate pink fluid containing 0.056 gm of alcohol extract of pomegranate seeds. Test and control groups were administered orally pomegranate juice and three ml of distilled water, respectively, for ninety days. Either a high cholesterol diet or routine diet, all rats were fed without restriction till the end of the study. Before PJ supplementation, qualitative phyto-chemical analysis of seeds and fluid was carried out in Laboratory of Pharmacology Research Division [12].

Blood samples were collected from tails to determine serum total cholesterol levels on Day 30, Day 60, and Day 90. Serum total cholesterol levels were determined spectophotometrically with commercially available reagent kits (Hospitex Diagnostics s.r.l., ITALY).

Student's t tests were used for the differences between (food intakes, body weights and serum total cholesterol levels) of test and control groups. Statistical significance was accepted at $p<0.05$.

RESULTS

Table 1 shows constituents of pomegranate seeds and juice, which were analyzed separately. Reducing sugar and Vitamin C were found in squeezed fluid but not in crushed seeds. However, there were flavonoids and tannin (Antioxidants) in both fluid and seeds.

Table 1. Chemical constituents of pomegranate seeds and fluid

Chemical Constituents	Pomegranate Seeds	Pomegranate Fluid
Alkaloid	(-)	(-)
Terpene	(-)	(+)
Reducing sugar	(-)	(+)
Flavonoid	(+)	(+)
Steroid	(-)	(-)
Glycoside	(+)	(+)
Tannin	(+)	(+)
Saponin	(+)	(-)
Amino acid	(+)	(+)
Vitamin C	(-)	(+)

(+) Present (-) Absent

Figure 1 shows three-day food intake of both groups during the period of PJ supplementation. Food intake of all rats was lower during the period of high cholesterol diet feeding than that of usual food feeding (21.9 ± 5.6g/day vs 25.1 ± 3.4g/day, $p>0.05$), but not shown in the figure. Food intakes of test and control groups were not different during first month of PJ supplementation (18.3 ± 4.1g/day vs 24.1 ± 2.6g/day, $p>0.05$) but during second and third month of PJ supplementation, food intake of test group were significantly lower than those of test group (16.6 ± 2.6g/day vs 25.3 ± 5.6g/day, 17.8 ± 3.4g/day vs 26.8 ± 4.8 g/day, $p<0.005$ for each).

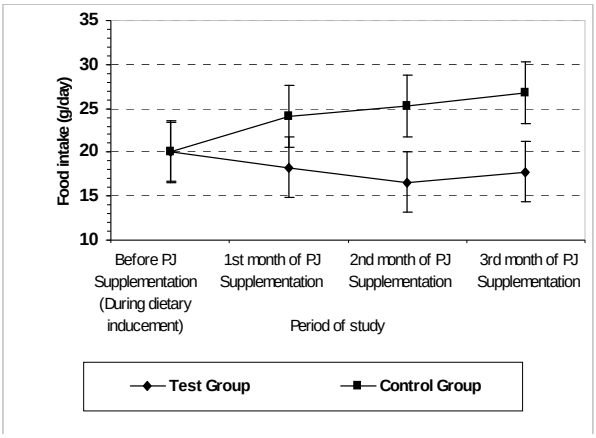

Fig. 1. Food intake (g/day) of test and control groups before and after PJ supplementation

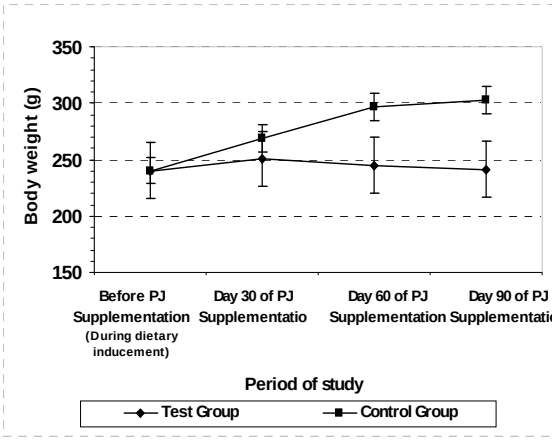

Fig.2. Body weights of test and control groups before and after PJ supplementation

Figure 2 shows body weights of test and control groups on Day 30, Day 60 and Day 90 of PJ supplementation. Body weights of test group were lower than those of control group (250.6±34.3 vs 268.8±11.8 g) although it was not statistically different ($p>0.05$) but significantly lower body weights of test group were found in Day 60 and Day 90 of PJ supplementation (245.1±22.9 vs 296.3±11.4 g) and (241.3±37.3 vs 302.8±12.6 g) , $p<0.05$ for each).

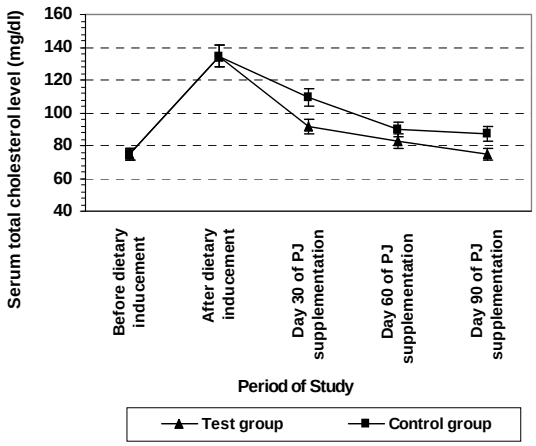

Fig. 3. Serum total cholesterol levels of test and control groups before and after PJ supplementation

Figure 3 shows serum total cholesterol levels of both groups. Serum total cholesterol levels of the test group were lower than those of the control group on Day 30 (91.44 ± 22.9 mg/dl vs 109.4± 27.19 mg/dl), and on Day 60 (82.6± 17.3 mg/dl vs 89.7± 13.96 mg / dl) of PJ supplementation, although they were not statistically significant ($p>0.05$ for both). On Day 90 of supplementation, serum total cholesterol levels of test group was significantly lower than those of control group (74.4 ±10.11 mg/dl vs 86.8± 10.52mg/dl), ($p=0.043$).

DISCUSSION

The pomegranates have been shown to contain powerful antioxidant compounds as well as macro and micronutrients. Edible parts of pomegranate fruit (about 50% of total fruit weight) comprise 80% juice and 20% seeds. Pomegranate juice (PJ) was shown recently its impressive antioxidative properties. Fresh juice contains 85% water, 10% total sugars, and 1.5% pectin, ascorbic acid, and polyphenolic flavonoids (tannins, anthocyains, ellagic acid derivatives). Pomegranate seeds are rich in sources of crude fibers, pectin, and sugars. Pressing the whole fruit results in much higher contents of pericarp polyphenols in juice. The seed oil consists of 63.5% punicic acid- a rare trans 18-carbon fatty acid (structurally related to conjugated linolenic acid) [13, 14].

According to USDA National Nutrient Database, nutritive values of 100g edible portion of pomegranate contain 15% carbohydrate, 1.6% protein, 0.7% minerals, 5% fibers, 10 mg calcium, 0.3 mg iron, 70mg phosphous and 16mg vitamin C. The principal amino acids are glutamic and aspartic acids.

In the study, in comparison of food intake of the controls, that the test group's food intake was significantly lower in second and third month of PJ supplementation might be due to less energy getting from PJ than from food despite the presence of macronutrients in PJ. This finding was not found in a study where long - term oral administration of pomegranate flower extract could not reduce body weight in Zucker diabetes fatty rats and appetite suppressing activity of PJ has not been proved yet [15].

Cholesterol, the characteristic of alcohol of animal tissues, performs a number of essential functions in the body. Thus, a complex series of transport, biosynthetic, and regulatory mechanisms has evolved. Cholesterol enters the liver's cholesterol pool from a number of sources including dietary cholesterol as well as de novo synthesis by extrahepatic tissues as well as by the liver itself. Cholesterol is eliminated from the liver as unmodified cholesterol in the bile or it can be converted to bile salts that are secreted into the intestinal lumen. It can also serve as a component of plasma proteins sent to the peripheral tissues. In humans, the balance between cholesterol influx and efflux is not precise, resulting in gradual deposition of cholesterol in the tissue, particularly in the endothelial linings of blood vessels.

There are two forms of cholesterol: circulating and cellular cholesterol. Serum total cholesterol, measured in this study is a kind of circulating cholesterol. HMG CoA (3-hydroxy-3-methylglutaryl CoA) reductase is the major rate-limiting enzyme for cholesterol biosynthesis and its gene expression is controlled by a transcription factor (Sterol regulatory element binding protein or SREBP). Other two enzymes: acyl CoA cholesterol acyltranferase (ACAT) and lecithin cholesterol acyltransferase (LCAT) are to change the free cholesterol to the storage forms. The activity of ACAT is enhanced in the presence of increased intracellular cholesterol.

A group of researchers from Iran showed that after 8-week consumption of concentrated pomegranate juice, plasma total cholesterol and LDL-cholesterol levels were lower significantly ($p<0.006$ for each) in diabetic patients with hyperlipidemia than those of the controls but mechanism of hypolipidemia could not be explained [16]. Fuhrman found that macrophage cholesterol biosynthesis was inhibited by 50% ($p<0.01$) after cell incubation with pomegranate juice [17]. This inhibition, however, was not mediated at the HMG CoA reductase level along the biosynthetic pathway. In a study of *in vivo* and *vitro*, long term oral administration of pomegranate flower extract (500mg/Kg) reduced cardiac triglyceride (TG) content, accompanied by a decrease in plasma levels of TG and total cholesterol in Zucker diabetic fatty rats. They suggested that improvement of cardiac lipid metabolism in these rats was due to activation of PPAR-α (peroxisome proliferation-activated receptors- α) and thereby lowering circulating lipid and inhibiting its cardiac uptake [15].

Hypolipidemic effect of PJ may be due to polyphenolic flavonoids which are abundantly present in it. *In vitro* studies of Chen et al and Chang *et al.* [18, 19], inhibitory effects of polyphenolic flavonoids and tannin derivatives on HMG CoA reductase of vero cells, a cell line obtained from kidneys of African green monkeys, were found. Mullen *et. al.* [20] hypothesized that maturation of sterol regulatory element binding proteins (SREBPs) and SREBP-regulated genes in HepG2 cells by isoflavones produce an increase in surface LDL receptor expression that increases the clearance of plasma cholesterol, thus decreasing plasma cholesterol levels.

In vivo experimental study of Bok *et al.* [21] showed lipid lowering efficacy of flavonoids in high cholesterol fed rats. They found that flavonoids supplementation on male rats with a high cholesterol diet significantly lowered the levels of plasma cholesterol (2.44 ± 0.59 vs 3.8 ± 0.28 mmol/L, $p<0.05$), hepatic cholesterol (0.143 ± 0.017 vs 0.181 ± 0.003 mmol/g, $p<0.05$ and hepatic triglycerides (0.069 ± 0.007 vs 0.095 ± 0.002 mmol/g, $p<0.05$) compared to those of the controls. They also showed that significant reduction activities of HMG CoA and ACAT.

Vitamin C also favors cholesterol excretion (bile acids formation at the 7α- hydroxy-lation step). According to USDA National Nutrient Database, relatively high content of

polyunsaturated fatty acids (PUFA) in PJ stimulates cholesterol excretion into the intestine and oxidation of cholesterol to bile acids and up-regulation of LDL receptors leading to distribution of cholesterol from the plasma into the tissues. Therefore, in the present study, significant reduction of serum total cholesterol levels in hyper-cholesterolemic rats after 90-day PJ supplementation may be due to contents of antioxidants (mainly flavonoids and tannin) and vitamin C in PJ. Furthermore, low food intake during PJ supplementation needs to be considered the fact of lowering serum total cholesterol level.

In conclusion, 90-day supplementation of PJ significantly reduces serum total cholesterol levels in hypercholesterolemic rats compared to their controls. Thus, daily consumption of PJ may prevent to increase serum total cholesterol level in human. However, as this study was carried out only on a type of laboratory animal, effect on human needs to be studied.

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