Effects of pomegranate juice consumption on inflammatory markers in patients with type 2 diabetes: A randomized, placebo-controlled trial

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Background: Diabetes causes the increased concentration of circulatory cytokines as a result of inflammation. Considering that pomegranate juice (PJ) is known to have antioxidant and anti-inflammatory properties, the purpose of this study was to determine the effects of PJ consumption on markers of inflammation in patients with type 2 diabetes (T2D). **Materials and Methods:** In a randomized, double-blind clinical trial study, 50 patients with T2D (40-65 years old) were randomly assigned to one of two groups. Participants in each group received either 250 mL/day PJ or a control beverage for 12 weeks. Biochemical markers including fasting plasma glucose (FPG), insulin and inflammatory markers were assayed on the baseline and follow-up blood samples. **Results:** In all, 44 patients in two groups were included in the analysis: PJ ($n = 22$) and placebo ($n = 22$). After 12 weeks of intervention, in the PJ group, there were 32% and 30% significant decreases in plasma C-reactive protein (hs-CRP) and Interlukin-6, respectively ($P < 0.05$). The mean ± SD plasma interlukin-6 (7.1 ± 5.6 vs. 11.9 ± 14.4 mg/L) and hs-CRP (1791 ± 1657 and 1953 ± 1561 ng/mL) concentrations in the PJ group were significantly lower than the placebo group after intervention ($P < 0.05$). **Conclusion:** PJ consumption by patients with T2D does not affect FPG or the insulin resistance index (HOMA-IR), whereas it does reduce Interlukin-6 and hs-CRP concentrations in plasma. Therefore, PJ consumption may have an anti-inflammatory effect in patients with T2D.

Key words: Diabetes mellitus, inflammation, pomegranate

INTRODUCTION

Type 2 diabetes (T2D) is one of the main noncommunicable chronic diseases, and its complications have become a major cause of morbidity and mortality worldwide. The increase in adipose tissue mass observed in obesity can lead to chronic activation of the innate immune system, which can lead to insulin resistance and T2D.[1] All of the following, heart disease, metabolic syndrome and T2D, have in common the increased concentration of circulatory cytokines as a result of inflammation.[2] Low-grade systemic inflammation is characterized by a two- to threefold increase in systemic plasma concentrations of cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6 and C-reactive protein (CRP).[1] The cytokines produced by adipose tissue have a key role in promoting atherosclerosis and, therefore, cardiovascular disease (CVD).[2]

Pomegranate juice (PJ) contains diverse groups of polyphenols, including ellagitanins, gallotannins, and ellegic acid, as well as flavonoids, such as anthocyanins.[3] However, its antioxidant activity is mainly due to hydrolysable tannin, including punicalagins, anthocyanins and ellagic acid.[4] PJ antioxidant activity was found to be three times higher than that of red wines and green teas, and two- to six-folds stronger than the other natural beverages.[5]

Evidence for the clinical benefits of PJ has been reported in some studies,[6-8] but few studies that have assessed its impact on inflammation have been conducted *in vitro* or used pomegranate extract.[9,10] Although patients with T2D are exposed to severe systemic oxidative stress and inflammation, data on the effect of PJ intake among these patients is limited. In this study, the effects of PJ consumption on markers of inflammation in patients with T2D were investigated.

MATERIALS AND METHODS

This study was a randomized, double-blind, placebo-controlled trial.

Subjects and ethical aspects

T2D patients, aged 40-65 years, with a diagnosis of at least 1 year, were recruited from the Charity Foundation for Special Diseases and Health Center of District 2 of Tehran, Iran. All the patients controlled their diabetes with oral hypoglycemic agents. Patients were excluded from the trial if they were smoking, suffering from any other chronic diseases and taking estrogen or progesterone (if female) or antioxidant supplements or used insulin as diabetes medication.

At baseline, patients were stratified by sex and randomly assigned to one of two groups: Group A (PJ, *n* = 25) and group B (placebo, *n* = 25). Random allocation of patients to treatment groups was performed by sequentially numbered containers. Randomization was performed by an assistant and the group allocation was blinded for the investigator and participants.

Written informed consent was obtained from all patients. Ethic approval for the trial was obtained from the ethical committee of the National Nutrition and Food Technology Research Institute (Tehran, Iran). This clinical trial has been registered in the Iranian Registry of Clinical Trials at htpp://www.irct.ir with the following identification: IRCT201206144010N8, and was conducted between November 2012 and March 2013.

Protocol

At baseline, patients were stratified by sex and randomly assigned to consume 250 mL/day PJ or a control beverage of similar color and energy content for 12 weeks. The study product was packaged in single-serving bottles labeled such that neither subjects nor staff members were aware of the treatment assignment.

Subjects were advised not to change their dietary habits, physical activities or drug medication. The dietary intakes of subjects were assessed using a 3-day dietary recall (two weekdays and one weekend day) at baseline and at the end of 12 weeks. Patients' diets were analyzed by the Nutritionist IV software (N Squared Computing, San Bruno, CA, USA).

Measurements

At baseline and after 12 weeks of intervention, 10 mL blood was collected from each patient after a 12-14-h overnight fasting. Blood samples to which an anti-coagulating agent was added were centrifuged at 4000 rpm for 10 min. The plasma samples were separated into aliquots and were frozen at -70ºC until they were assayed. Fasting plasma glucose (FPG) concentration was assessed using the enzymatic colorimetric method using glucose oxidase by commercial kits (Pars Azemoon, Tehran, Iran) and a Selectra 2 auto-analyzer (Vital Scientific, Spankeren, the Netherlands). The coefficient of variation (CV) for FPG

was 1.3%. Plasma insulin and inflammatory markers were assessed using the enzyme-linked immunosorbent assay method (ELISA). Insulin was measured using a commercial kit (Mercodia, Uppsala, Sweden), with a CV of 5.0%. The plasma concentration of TNF-α and high-sensitive CRP (hs-CRP) were assayed by related kits (Komobiotech Inc., Gangseo-gu Seoul, Korea) and Diagnostics (Biochem, Canada Inc., Ontario, Canada), respectively. Interlukin-6 was measured using commercial kits (Biolegend, CA, USA). All immunoassays were performed by an ELISA reader Sunrise, Tecan Co. Salzburg, Austria. CVs for TNF-α, hs-CRP and IL-6 were 6.7, 6.3 and 7.1, respectively.

Weight and height were measured using a balance Seca scale with a stadiometer at the baseline and at the end of the intervention. Weight was measured while the subjects were minimally clothed and not wearing shoes, and was recorded to the nearest 100 g. Height was measured while subjects were standing without shoes, with their shoulders in a normal position, and was recorded to the nearest 0.5 cm. Body mass index (BMI) was calculated as weight (kg) divided by square of height (m).

In addition, HOMA-IR was calculated as: [Glucose (mmol/L) × Insulin (µU/mL)]/22.5.

Compliance

To ascertain patient compliance, we provided each patient with a fixed number of PJ bottles and instructions to return the unused bottles at the end of the study. Based on the number of returned bottles by each patient, their compliance was determined, which was 90% for our participants. No adverse events were reported.

Pomegranate and placebo juice

In order to choose the commercial PJ with the highest polyphenol levels, several hand-squeezed and various commercially available juices were analyzed using the colorimetric assay. The phenols were determined by the Folin-Ciocalteu reagent, using Gallic acid as a standard.[11] Total flavonoid content was measured by the aluminum chloride colorimetric assay using Catechin as a standard.[12] PJ was diluted 1:10 (v:v) to measure its total antioxidant capacity (TAC), which was based on the inhibition percent of ABTS and comparison with bovine serum albumin (BSA) standard curve.[13] PJ composition is shown in Table 1.

PJ and placebo was prepared by Alifard Inc., Tehran, Iran. We used special essence and formula of Wonderful Variety, Pom, supplied by Roll International Corporation, Los Angeles, CA, USA, for preparing the placebo. The sugar content of the placebo was 50% of glucose and 50% of fructose. Analyzing the placebo juice using the colorimetric assay verified that it had no polyphenols.

The juice and the placebo were kept at room temperature (<25°C) until opened, as recommended by the manufacturer.

Sample size estimation and statistical analysis

The results are expressed as mean ± SD for quantitative variables and n (%) for qualitative variables, and differences were considered significant at $P \leq 0.05$.

Statistical analysis of data was performed using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) for Windows version 16.0. A χ^2 test was used to compare the qualitative variables between the two groups.

Because all quantitative parameters according to the Kolmogorov–Smirnov test had normal distributions, a paired *t*-test was used to compare the pre- and postintervention variables within groups, and the mean changes within groups were tested using the Student's *t*-test. Adjustment for differences in baselines covariates and changes in variables during the study were performed by analysis of covariance using general linear models.

RESULTS

The sample size was designed to detect a 2 ng/L difference of plasma IL-6 between the groups with 95% confidence interval and 90% power.[14] In all, 44 patients [PJ (*n* = 22), placebo (*n* = 22)] were included in the analysis as presented in the flow chart [Figure 1].

The baseline characteristics of patients did not differ significantly between the PJ-treated and the placebo groups [Table 2]. Anthropometric factors had no significant differences between the two groups at the baseline or at the end of Week 12; in addition, these factors did not change significantly within the groups during the study [Table 3].

Dietary energy and carbohydrate intake showed significant differences between the two groups at the baseline and at the end of the study ($P < 0.05$); however, none of the dietary factors changed significantly within each group during the study [Table 3].

No serious adverse events or side-effects were reported. All analyses were adjusted for energy and carbohydrate intake by analysis of covariance.

Plasma interlukin-6 and hs-CRP concentration reduced significantly in the PJ group at the end of the 12th week compared with baseline ($P < 0.05$), whereas no significant change was observed in the placebo group. Decreases in plasma interlukin-6 and hs-CRP concentrations in the PJ
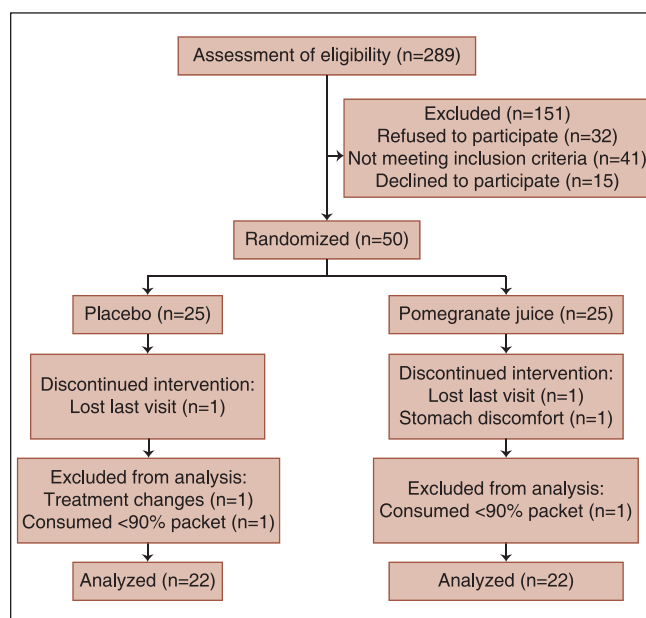

Figure 1: Flow chart of the study

Table 1: Composition of pomegranate juice

	Pomegranate juice	Placebo
Total polyphenols (mg/L)	1946	0
Total flavonoid (μg/mL)	345.87	0
TAC (mmol/L) BSA	7	0
Energy (kcal)*	126	126
Sugar (g)*	24	24
Protein (g)*	1.4	0
Fiber (g)*	0	0

*Values are per 250 mL; TAC = Total antioxidant capacity; BSA = Bovine serum albumin

Table 2: Baseline characteristics of patients in the pomegranate juice-treated and placebo groups

Characteristics	Pomegranate (*n* = 22)	Placebo (*n* = 22)
Age (years)[1]	55±6.7	56.9±6.8
Duration of diabetes (years)[1]	5.8±3.2	6.5±4.3
Sex		
Men	11 (50%)	12 (54.5%)
Women	11 (50%)	10 (45.5%)
Anti-hypertensive drugs (%)	4 (18.2%)	6 (27.3%)
(ACE-I or ARB)	3 (13.6%)	4 (18.2%)
Anti-hyperlipidemic drugs (%)		

[1]Age and duration of diabetes are presented as mean ± SD; ACEI = Angiotensin converting enzyme inhibitors; ARB = Angiotensin receptor blockers; *P* - value > 0.05

group were significant in comparison with the placebo group ($P < 0.05$; Table 4), plasma TNF-α concentration did not change significantly within each group or between groups during the study [Table 4].

Mean differences of inflammatory variables compared with baseline values are presented in Table 4. There were 30% and 32% significant decreases in plasma IL-6 and hs-CRP in the PJ and placebo groups ($P < 0.05$), respectively.

Table 3: Anthropometric and dietary factors in the pomegranate juice-treated and placebo groups[1]

Factors	Groups	Baseline	Week 12
Weight (kg)	Pomegranate	76.9±15	77.4±15.4
	Placebo	75.2±15	74.9±9.7
BMI (kg/m²)	Pomegranate	29.4±3.9	29.4±3.9
	Placebo	28.6±4.2	28.4±4.4
Energy (kcal/d)	Pomegranate	1784±238[b]	1787±233[b]
	Placebo	2028±241	2011±221
Protein (g/d)	Pomegranate	54±12	58±12
	Placebo	59±10	65±9
Carbohydrate (g/d)	Pomegranate	234±35[b]	266±41[b]
	Placebo	232±27	257±37
Fiber (g/d)	Pomegranate	15±4	15±3
	Placebo	16±3	15±4
Fat (g/d)	Pomegranate	73±20	72±19
	Placebo	84±15	83±19
SAFA (g/d)	Pomegranate	16.2±5.5	17.2±6.4
	Placebo	23.7±8.8	21.3±8.2
MUFA (g/d)	Pomegranate	30.7±11	29.8±8.8
	Placebo	32.5±6.1	33.6±8.7
PUFA (g/d)	Pomegranate	21.4±6.7	19.4±4.4
	Placebo	20.5±6.1	21.7±5.9
Cholesterol (mg/d)	Pomegranate	134.3±60.7	136.1±48.6
	Placebo	169.6±78	189.9±58.2
Vitamin E (mg/d)	Pomegranate	22.7±5.9	20.7±4.7
	Placebo	20.5±6.6	22.1±6.6
Vitamin C (mg/d)	Pomegranate	78.7±54.3	77.3±32.9
	Placebo	60.1±32.8	66.6±32.1
Vitamin A (UI/d)	Pomegranate	559.7±478.3	719.9±609
	Placebo	440.8±349.4	359.7±170.2

All values are presented as mean ± SD; [1]*n* = 22 for all values; BMI = Body mass index; MUFA = Monounsaturated fatty acids; SAFA = Saturated fatty acids; PUFA = Polyunsaturated fatty acids; a) *P* < 0.05 vs baseline, b) *P* < 0.05 vs the placebo group

PJ consumption by the patients did not significantly affect the levels of parameters such as FPG, insulin and HOMA-IR [Table 4].

DISCUSSION

We found that consumption of PJ had beneficial effects on some inflammatory markers in patients with T2D through a significant decrease in IL-6 and hs-CRP in plasma. Adjustment for confounding factors including energy and carbohydrate intake provide the same results.

Although there are some clinical trials that have described the effects of PJ consumption on the oxidative state and blood pressure,[6,15-17] only a limited number of *in vitro* studies have assessed the impact of pomegranate on inflammation using the pomegranate extract.[9,10] To the best of our knowledge, this is the first study that examines the effects of PJ consumption on markers of inflammation in individuals with T2D.

A 1-year intake of PJ by hemodialysis patients showed a significant reduction in the level of all oxidative stress and inflammatory biomarkers compared with placebo, with a significant reduction in IL6 and TNF-α.[3] Decreased circulating levels of vascular cell adhesion molecule 1 (VCAM-1) were reported with the consumption of PJ for a period of 2 weeks in hypertensive subjects, but no significant effect was observed from PJ on the serum levels of intercellular adhesion molecule 1 (ICAM-1), hs-CRP, IL-6 and lipid profile.[18]

Table 4: Plasma concentrations of fasting glucose, insulin, HOMA-IR, inflammatory markers in the pomegranate juice-treated and placebo groups[1]

Parameters	Groups	Baseline	Week 12	*P*-value (within groups)	Mean changes[2]	Mean changes[3] (%)
Fasting plasma glucose (mg/dL)	Pomegranate	160.3±47.8	162.3±35.2	NS	2±45.3	6±27
	Placebo	148.7±42.1	158.6±49.1	NS	9.8±43.3	10±35
P-value (between groups)		NS	NS		NS	NS
Insulin (mU/L)	Pomegranate	7.6±3.8	7.1±2.6	NS	−0.52±3.2	−0.45±28
	Placebo	7.6±3.8	7.1±3.1	NS	−0.48±2.4	−3±27
P-value (between groups)		NS	NS		NS	NS
HOMA-IR	Pomegranate	54.3±29.9	51.4±21.8	NS	−2.9±29.7	6±40
	Placebo	48.4±20.3	47.0±16.7	NS	−1.3±15.9	4±34
P-value (between groups)		NS	NS		NS	NS
hs-CRP (ng/mL)	Pomegranate	3243±2935	1791±1657	*P*<0.01	−1441±2251	−32±31.8
	Placebo	2667±1577	1953±1561	*P*<0.05	−271±1322	−2±41.7
P-value (between groups)			NS	*P*<0.05	*P*<0.05	*P*<0.05
Interlukin-6 (ng/L)	Pomegranate	10.9±4.4	7.1±5.6	*P*<0.01	−3.8±6.1	−30±46
	Placebo	12.5±14.3	11.9±14.4	NS	−0.6±2.6	−5±25
P-value (between groups)		NS	*P*<0.05		*P*<0.05	*P*<0.05
TNF-α (ng/L)	Pomegranate	37±19.3	30.4±17.5	*P*<0.01	−6.6±9.2	−16±17
	Placebo	36±20.4	30.8±18.0	*P*<0.01	−5.2±7.1	−11±22
P-value (between groups)		NS	NS		NS	NS

All values are presented as mean ± SD; [1]*n* = 22 for all values; [2]Mean changes reflect Week 12 − baseline values; [3]Mean changes% reflect (mean changes/baseline)*100; NS = None significant; HOMA-IR = Homeostatic model assessment for Insulin Resistance; Hs-CRP = High sensitive C-reactive protein

In vitro studies have shown the positive inhibitory effect of pomegranate punicic acid on the production of TNF-α, induced by priming of reactive oxygen species,[19] on the modulation of inflammatory cell signaling in colon cancer cells via pomegranate ellagitannins[9] and reducing pro-inflammatory cytokine production via inhibiting the gene expression (pomegranate fruit extract).[10] However, findings from *in vitro* studies often conflict with findings obtained from *in vivo* ones.

Most studies investigating the antioxidant and anti-inflammatory effects of polyphenols and flavonoids have been conducted *in vitro*. It has been indicated that flavonoid metabolites have different biological and antioxidant properties than their parent compounds, which suggested that data from *in vitro* studies using nonmetabolites of flavonoids are of limited relevance to *in vivo* studies.[20]

Low-grade systemic inflammation such as diabetes is characterized by an increase in systemic plasma concentrations of cytokines such as TNF-α, IL-6 and CRP.[1] In our study, IL-6 and hs-CRP decreased significantly but TNF-α did not show any significant reduction.

Zhao *et al.* have suggested that plasma TNF-α level may not be detectable in a single blood sampling as this may not reflect the host production of this inflammatory mediator.[21] Human blood mononuclear cells are known to synthesize and secrete TNF-α. It has been suggested that activated monocytes and macrophages are important cellular sources for circulating pro-inflammatory cytokines.[22]

The main antioxidant compounds in PJ are hydrolysable tannins, anthocyanins and ellagic acid derivatives, which contribute to the total antioxidant capacity of the juice.[4] Some mechanisms have been also proposed for the anti-inflammatory properties of PJ, including inhibition of enzymes related to inflammation, such as peroxisome proliferators active receptors (PPARs), nuclear transcription factor kappa B (NF-kB), and NSAID activated gene-1 (NAG-1),[23] scavenging-free radicals[4] or production of urate, which is stimulated by fructose and other compositions of flavonoid-rich foods.[24] In addition, pomegranate enhances endothelial nitric oxide production and bioavailability[25] and protects nitric oxide against oxidative destruction.[26]

Strengths of the current study are a randomized, double-blind, placebo-controlled trial design, acceptable response rate and detailed data collection through face-to-face meetings. Moreover, PJ and placebo were provided for all patients. This study had limitations because we could not measure plasma polyphenol derivatives' levels nor did we evaluate the level of TNF-α in the blood mononuclear cells. Hence, it is suggested these two be measured in future studies.

In conclusion, our findings suggest that PJ, which is a source of natural sugars, does not affect FPG and insulin resistance index (HOMA-IR) in patients with T2D, and it also acts as an anti-inflammatory agent, lowering some inflammatory factors including IL-6 and hs-CRP. Further studies with a longer intervention period as well as a bigger sample size or using PJ with different polyphenol contents are needed in order to evaluate its protective role against diabetes and its complications and the anti-inflammatory effects of PJ.

ACKNOWLEDGMENTS

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