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POMEGRANATE JUICE ATTENUATES ELEVATION OF ASYMMETRIC DIMETHYLARGININE IN INDOMETHACIN INDUCED PEPTIC ULCER

Jihan Hussein[1*], Yasmin Abdel Latif[1], Dalia Medhat[1], Eman Refaat[1], Islam El Nahrery[2], and Abdel Razik Farrag[3]

[1]Medical Biochemistry Department, National Research Center. Doki, Giza, Egypt.
[2]Chemistry Department, Biochemistry Branch, Faculty of science. Suez University Egypt.
[3]Pathology Department, National Research Center, Doki, Giza, Egypt.

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***Correspondence for Author**
Jihan Hussein
Medical Biochemistry Department, National Research Cente. Doki, Giza, Egypt.

ABSTRACT

Pomegranate (*Punica granatum L.*) is a well-known table fruit of tropical and subtropical regions of the world. In this study, we aimed to identify the effect of pomegranate juice (PJ) on gastric ulcers induced by Indomethacin in experimental rats and exhibit its powerful antioxidant properties as well as its role in reducing asymmetric dimethylarginine (ADMA) that may affect the production of nitric oxide (NO). Forty male albino rats were used in this study and divided into four groups (ten in each group), classified into control, pomegranate, indomethacin and treated groups. Stomach paraoxonase activity and NO were determined, plasma asymmetric dimethylarginine was estimated by HPLC; separation was achieved on reversed phase column (250 X 4.6 mm C18) with 5 µm particle size. The mobile phase consists of sodium acetate buffer, methanol and tetrahydrofuran (THF), the wavelength of UV detector was set at 254 nm. Stomach paraoxonase activity was significantly increased in treated group compared to indomethacin group, in addition to the elevation of NO concomitant with a reduction of ADMA in treated group compared to indomethacin. Pomegranate juice offered significant protection against indomethacin induced gastric ulcer in the experimental rats. It also reduced ulcer index to 2.16 ± 0.12, showing 69.4 % prevention. The biochemical and pathological results in this study appeared the potential effect of pomegranate juice in attenuating the ulceration induced by indomethacin in rat model.

KEYWORDS: Pomegranate juice, indomethacin, peptic ulcer, ADMA, paraoxonase activity.

INTRODUCTION

Peptic ulcer is erosion in the lining of the stomach or duodenum occurring at a site where the mucosal epithelium is exposed to acid and pepsin. Smoking, stress, nutritional deficiencies and ingestion of nonsteroidal-anti-inflammatory drugs increase gastric ulcer incidence. [1] Non-steroidal anti-inflammatory drugs (NSAIDs) cause marked reduction in mucosal blood flow, mucus bicarbonate secretions, impaired platelet aggregation, reduced epithelial cell renewal and increased leukocyte adherence that are responsible for pathogenesis of ulceration. [2] Indomethacin is an indol derivative, non-steroidal, anti-inflammatory drug with anti-inflammatory, analgesic, and antipyretic effects. Indomethacin became the first choice drug to produce an experimental ulcer model as a result of having a higher ulcerogenic potential than other NSAIDs. [3]

The current medical treatment of peptic ulcer is generally based on the inhibition of gastric acid secretion by H2-antagonists, such as omeprazole and antimuscarinics, as well as the acid-independent therapy like that provided by sucralfate and bismuth. [4] The most important problem in ulceration treatment is the recurrent of ulcer within one year after stopping treatment is between 40 and 80%. [5] In addition to various side effects of these products such as hepatotoxicity and anaphylaxis. [6]

From this point of view, several natural products have been reported to to pose anti-ulcerogenic activity by virtue of their predominant effects on mucosal defensive factors including apple bananas, papeeta, and brindle berry. [7] Pomegranate (Punica granatum L.) is a well-known table fruit of tropical and subtropical regions of the world. Some botanists place it in the family Lythraceae, of the peculiar type of fruit, called as balausta, most authorities make it the only genus in the family Punicaceae. [8]

The biological activity of Pomegranate (PG) has been widely investigated, including in vitro, in vivo, and clinical studies. The beneficial effects are mostly the cardiovascular protective role, neuroprotective activity, hypoglycemic effect, and anticancer properties, in particular against prostate, colon, and breast cancer; the anticancer effect is limited only to in vitro and animal studies. [9] Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthase (NOS) enzyme. Nitric oxide (NO), synthesized from L-arginine by NOS

enzyme, contributes to the regulation of blood pressure and to host defense. Nitric oxide is a vasodilator and inhibitor of platelet aggregation, leucocytes migration, cellular adhesion and vascular smooth muscle proliferation. [10, 11] The main function of NO is to provide the vascular homeostasis. When the NO levels is decreased, endothelial homeostasis is impaired in the direction of vasoconstriction and endothelial dysfunction begins. [12]

Paraoxonases (PONs) are a family of three enzymes termed PON1, PON2 and PON3. [13] The best known among the paraoxonases is PON1 which degrades oxidized phospholipids in low-density lipoproteins (LDL) and HDL and, as such, plays a role in the organism's antioxidant system [14]. Alterations in circulating PON1 levels are associated with a variety of diseases involving oxidative stress. [15, 16] The liver plays a key role in the synthesis of PON1, [17] and chronic liver diseases are associated with increased oxidative stress and lipid peroxidation, also a reduction of serum PON1. [18] The aim of this study was to identify the effect of pomegranate on gastric ulcers induced by indomethacine in experimental rats and exhibits its powerful antioxidant properties.

MATERIALS AND METHODS

Materials

Chemicals

1. Asymmetric dimethylarginine (ADMA) (HPLC standard), O-Phthaldialdehyde (OPA), mercaptoethanol, acetic acid, phenylacetate and boric acid were purchased from Sigma Aldrich Medical Company St.Louis USA. Indomethacine as Liometacen ampoules was purchased from The Nile Company for Pharmaceuticals and Chemical Industries.
2. Methanol (HPLC grade), Tetrahydrofuran (THF), sodium acetate and 5-sulfosalicylic acid (5-SSA) were purchased from Merck (Merck, Germany).
3. Pomegranate was purchased from local market.

Experimental animals

Forty adult male albino rats weighing 180-200 g were obtained from the animal house colony of the National Research Center, Cairo, Egypt. The rats were maintained under standard conditions with free access to tap water and a standard pellet diet. Rats were acclimated to these conditions for two weeks before beginning the experiment. The animal experimental protocol was approved by the Ethical Committee for Medical Research, National Research Center, Egypt. After the acclimatization period, the animals were randomly assigned into four groups; each group was comprised of ten rats.

METHODS

Induction of peptic ulcer

Acute gastric ulcers were induced by oral administration of indomethacin at a dose of 48 mg/kg body weight once five hours before rats were sacrificed. [19]

Preparation of plant extract

Pomegranate fruit was crushed, squeezed, and treated enzymatically with pectinase to yield the PJ and byproducts, which included the inner and outer peels and the seeds. Pectinase hydrolyzes α-1, 4-galacturonide bonds in pectin and thus improves extraction and filtration and prevents the formation of pectin gels. The juice was filtered and stored at -18 ˚C. [20]

Experimental design

Forty male albino rats were classified into four groups (10 rats in each group) as follows:

Group I: healthy rats, received a vehicle (0.5 ml distilled water / rat/ day orally) for four weeks.

Group II: healthy rats, received 0.5 ml pomegranate juice (100mg/kg body weight / day orally) for four weeks.

Group III: healthy rats, received a vehicle for four weeks, then injected with indomethacin.

Group IV: healthy rats, received 0.5 ml pomegranate juice (100mg/kg body weight / day orally) for four weeks, then injected with indomethacin.

After the experimental period (4 weeks), animals were kept fasting for 12 hours before blood sampling, blood was withdrawn from the retro-orbital venous plexus of the eye using capillary tubes and collected in clean centrifuge tubes for separation of serum samples for biochemical analysis. Stomach was removed quickly; part of it was homogenized and prepared for estimation of other biochemical parameters. Other part of the stomach was kept immersed in 10 % formalin for histopathological and histochemical examinations.

Preparation of tissue homogenate

The frozen tissues were cut into small pieces and homogenized in 5 ml cold buffer (0.5 g of Na_2HPO_4 and 0.7 g of NaH_2PO_4 per 500 ml deionized water (pH 7.4) per gram tissue, then centrifuged at 4000 rpm for 15 minutes at 4°C and the supernatant was removed and used in estimation of chemical parameters. [21]

Determination of stomach paraoxonase activity

The arylesterase activity of paraoxonase was measured spectrophotometrically in supernatants using phenylacetate as a substrate. [22, 23] In this assay, arylesterase/paraoxonase catalyzes the cleavage of phenyl acetate, resulting in phenol formation. The rate of phenol formation is measured by monitoring the increase in absorbance at 270 nm at 25 º C. The working reagent consisted of 20 mM Tris/HCl buffer, pH 8.0, containing 1 mM $CaCl_2$ and 4 mM phenyl acetate, as the substrate. Samples diluted 1:3 in buffer were added and the change in absorbance was recorded following a 20-s lag time. Absorbance at 270 nm was taken every 15 s for 120 s using a UV I8 Recording Spectrophotometer.

Nitric oxide

Nitric oxide was measured according to the method described by [24] as nitrite was determined by using Griess reagent, where nitrite, the stable end product of nitric oxide radical is mostly used as indicator for the production of nitric oxide.

Determination of serum Asymmetric dimethylarginine by HPLC

ADMA was determined by HPLC after modification of the method described previously. [25]

Chemical preparation

Borate buffer was prepared by dissolving 2.473g of boric acid in 100 ml distilled water (pH was adjusted to 10.0 using potassium hydroxide). OPA solution was prepared by 500 ul methanol + 2 ml borate buffer +30 ul mercabtoethanol and 10 mg OPA (this solution is stable only 2 days at 2-8 ˚C). Sodium acetate buffer was prepared by dissolving 6.804 Sodium acetate in 1 L distilled water; pH was adjusted at 6.8 by acetic acid and filtered two times through a 0.2µm filter before use.

Standard preparation

0.5 mM ADMA was prepared by dissolving 1.375 mg of ADMA standard in 10 ml HCL (0.1 M) as stock solution , then several dilutions were prepared by diluting with 0.1 M HCL.

Sample preparation

25 mg 5-sulfosalicylic acid (5-SSA) were added to 1 mL serum and mix well then left in an ice-bath for 10 min. The precipitated protein was removed by centrifugation at 2000 g for 10 min. The supernatant was filtered through 0.2 µm filter, then mix 60 µL of sample and 600 µL of OPA solution and left for 3 min. before injecting onto HPLC.

HPLC condition

20 µl of sample-OPA were injected in HPLC; separation was achieved on reversed phase column (250 X 4.6 mm C18) with 5 µm particle size. The mobile phase consists of sodium acetate buffer, methanol and THF (82:17:1) v/v respectively for solution A and (22:77:1) v/v respectively for solution B and eluted by gradient method as shown in table 1. Column temperature was adjusted at 37^{o}c and flow-rate was 1.0 ml/min. The wavelength of UV detector was set at 254 nm. A linear standard curve was constructed by plotting peak areas versus the corresponding concentrations. The concentrations in samples were obtained from the curve.

Table 1: Gradient program for ADMA separation by HPLC.

Time (min.)	Mobile phase A	Mobile phase B
0	95	5
5	85	15
15	60	40
30	20	80
32	0	100
35	95	5

Macroscopic of stomach tissues and lesions analysis

Each stomach was incised along the greater curvature and examined for linear haemorrhagic lesions in the glandular region. The degree of gastric mucosal damage was evaluated in terms of the ulcer index (UI) [26, 27] which depends on the calculation of a lesion index by using of a 0–5 scoring system based on the severity of each lesion. The severity factor was defined according to the length of the lesions. Severity factor 0 = no lesions; 1 = petechiae; 2 = erosions < 1mm; 3 = erosions of 1-2 mm; 4 = erosions of 2-4 mm and 5 = erosions > 4 mm. The partial scores were then summed to obtain the ulcer index of the animal examined. The percent protection with each test drug dose was also calculated by the following formula [28]:

% Protection = $[(UI_{indomethacin} - UI_{pomegranate\ and\ indomethacin}) / UI_{indomethacin}] \times 100$, where UI stands for ulcer index.

Statistical analysis

All data were expressed as mean ± standard error. Data were analyzed using one-way ANOVA using SPSS (Version 16). Duncan's new multiple-range test was used to assess differences between means. Pearson's correlation test was used to assess correlations between means. A significant difference was considered at the level of $P < 0.05$.

RESULTS AND DISCUSSION

In this study, stomach paraoxonase activity was significantly decreased in peptic ulcer group compared to control indicating the elevation of oxidative stress induced by indomethacin (table 2) thus, indomethacin is known to induce the reactive oxygen metabolites in animal models, which may contribute to mucosal injury, these free radicals also damage the cellular antioxidant enzymes which acting as the first line of cellular defense against oxidative stress. This might lead to aggravated tissue damage during stomach ulceration. [26] Indomethacin interferes with gastric protective mechanisms, such as mucus and bicarbonate secretion, surface epithelial hydrophobicity, and mucosal blood flow. Acetylsalicylic acid mainly interferes with the biosynthesis of cytoprotective prostaglandins through the inhibition of cyclooxygenase (COX); this effect results in an over production of leukotrienes and other products of the 5-lipoxygenase pathway, in addition to the reduction of NO and elevation of ADMA as was found in our study (table 3).

Table 2: Paraoxonase 1 activity in different studied groups.

Parameters / Groups	Paraoxonase activity µmol/g. tissue
Control group mean ± SE	27± 0.3 [b]
Pomegranate juice group mean ± SE%a	29± 0.1 [b] 7.4 %
Indomthacin group mean ± SE%a	11.1 ± 0.2 [a] -58.8 %
Treated group mean ± SE%a%b	23.2± 0.1 [a, b] -14%109.0%

Significant p value < 0.05

a = significant difference compared to control group

b = significant difference compared to indomethacine group

%a = % of change from control group

%b= % of change from indomethacine group

Table 3: Serum ADMA and nitric oxide levels in different studied groups.

Parameters / Groups	ADMA µmol/L	NO µmol/L
Control group mean ± SE	0.5 ± 0.06	20 ± 0.21
Pomegranate juice group mean ± SE %a	0.4±0.11-20%	20.7±0.113.5 %
Indomthacin group mean ± SE %a	1.2±0.09 140%	13.03±0.28 -34.85 %
Treated group mean ± SE %a %b	0.9 ±0.87 80% - 25%	17.87±0.25 -10.65 % 37.15 %

Significant p value < 0.05

a = significant difference compared to Control group

b = significant difference compared to Indomethacine group

%a = % of change from control group

%b= % of change from indomethacine group

Table 4: Scoring shows the severity of lesions of indomethacin induced gastric ulcer in different groups of rats.

Treatment and dose	Histological lesions					
	no lesions	Petechiae	Erosions (<1 mm)	Erosions (1-2mm)	Erosions (2-4mm)	Erosions (>4mm)
Control group	0	0	0	0	0	0
Pomegranate juice group	0	0	0	0	0	0
Indomethacin group	-	1	2	3	4	5
Treated group	-	-	2	3	-	-

Table 5: Effect of pomegranate juice against indomethacin induced gastric ulcer in rats.

Treatment	Ulcer Index	% of protection
Control group	-	-
Pomegranate juice group	-	-
Indomethacin group mean ± SE	9.1 ± 0.21	-
Treated group mean ± SE	2.16 ± 0.12	69.4*

Under physiological conditions, NO is produced by NOS from its substrate L-arginine, which is metabolized to amino acid and L-citrulline. Under pathological conditions, L-arginine may be involved in another metabolic pathway catalyzed by protein arginine methyltransferase (PRMT). The activity of PRMT, in the presence of proteins containing methylated arginine residues, leads to formation of asymmetric dimethylarginine (ADMA) and symmetric methyl arginine (MMA) [29]. Previous studies documented that ADMA acts as the endogenous NOS inhibitor and the inhibition of this enzyme results in a decrease of NO production. The excessive accumulation of ADMA can decrease NO bioavailability in many cells causing an impairment of multiple systems including gastrointestinal tract. [30] However, administration of pomegranate juice in this study significantly increased paraoxonase activity in treated group compared to endomethacin group (table 2), in addition to the reduction of ADMA level which attenuated the reduction of NO (table 3). The health benefits of pomegranate have been attributed to its wide range of phytochemicals. The phytochemicals found in pomegranate are predominantly polyphenols, including primarily hydrolysable ellagitannins,

anthocyanins and other polyphenols. Ellagitannins found in the outer part of the fruit are largely responsible for the antioxidant activity of the pomegranate juice. [31] The antioxidant protection of pomegranate juice elicited decreased cellular production and release of oxygen radicals in the vascular wall, inhibits endothelial activation of oxidation-sensitive genes, and improves the biologic activity of NO through a cell- or tissue-specific antioxidant action. [32]

In addition, high PON1 activity may reduce the formation of oxidized- LDL (ox- LDL). [33] Which has an important role in NOS downregulation and injury to endothelial cells via activation of different signal transduction pathways such as those involving protein kinase-C (PKC) and mitogen-activated protein kinases (MAPKs). [34] Besides, PON1 is directly involved in the pathogenesis of atherosclerosis due to modulation of NO bioavailability. [35] The possibility that flavonoids could support NO synthase activity by aiding efficient catabolism of ADMA remains open, but there is no direct evidence supporting this possibility.

The biochemical results in this study were confirmed by pathological results , thus, On gross examination of stomach, no lesions were seen in any of control or pomegranate juice administered animals, while almost all animals that given indomethacin showed lesions including petechiae and different areas of erosions in the gastric mucosa. On the other hand, pomegranate juice pretreatment with indomethacin showed very mild lesions (Table 4). Indomethacin administration caused a remarkably high ulcer index (9.1 ± 0.21) when compared to control group. Pretreatment with pomegranate juice offered significant protection against indomethacin induced gastric ulcer in the experimental rats. Pomegranate juice reduced ulcer index to 2.16 ± 0.12, showing 69.4 % prevention (Table 5).

CONCLUSION

Treatment with antioxidant polyphenols contained in pomegranate juice may promote a sustained correction of the NOS dowregulation induced by ox - LDL and ADMA in peptic ulcer. These findings may have important implications for the prevention of peptic ulcer by pomegranate juice.

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