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# Pomegranate phytoconstituents blunt the inflammatory cascade in a chemically induced rodent model of hepatocellular carcinogenesis

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**Abstract**

Liver cancer, predominantly hepatocellular carcinoma (HCC), represents a complex and fatal malignancy driven primarily by oxidative stress and inflammation. Due to dismal prognosis and limited therapeutic intervention, chemoprevention has emerged as a viable approach to reduce the morbidity and mortality of HCC. Pomegranate fruit is a rich source of phytochemicals endowed with potent antioxidant and anti-inflammatory properties. We previously reported that pomegranate phytochemicals inhibit diethylnitrosamine (DENA)-initiated hepatocarcinogenesis in rats though nuclear factor E2-related factor 2 (Nrf2)-mediated antioxidant mechanisms. Since Nrf2 also acts as a key mediator of the nuclear factor-kappaB (NF-κB)-regulated inflammatory pathway, our present study investigated the anti-inflammatory mechanisms of a pomegranate emulsion (PE) during DENA-induced rat hepatocarcinogenesis. Rats were administered with PE (1 or 10 g/kg) 4 weeks before and 18 weeks following DENA initiation. There was a significant increase in hepatic expressions of inducible nitric oxide synthase, 3-nitrotyrosine, heat shock protein 70 and 90, cyclooxygenase-2 and NF-κB in DENA-exposed rat livers. PE dose-dependently suppressed all aforementioned elevated inflammatory markers. A conspicuous finding of this study involves lack of cardiotoxicity of PE as assessed by monitoring cardiac function using noninvasive echocardiography. Our results provide substantial evidence that suppression of the inflammatory cascade through modulation of NF-κB signaling pathway may represent a novel mechanism of liver tumor inhibitory effects of PE against experimental hepatocarcinogenesis. Data presented here coupled with those of our earlier study underline the importance of simultaneously targeting two interconnected molecular circuits, namely, Nrf2-mediated redox signaling and NF-κB-regulated inflammatory pathway, by pomegranate phytoconstituents to achieve chemoprevention of HCC.
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## 1. Introduction

Liver cancer is one of the most prevalent and life-threatening human malignancies in the world [1]. Hepatocellular carcinomas (HCCs), malignant tumors arising from hepatic parenchymal cells, represent the majority (75%–90%) of the primary liver cancers. The incidence of HCC has dramatically increased in the United States by more than 70% during the past 25 years, with approximately 28,720 new cases and about 20,550 deaths expected to occur in 2012 alone [2]. Chronic oxidative stress and unresolved inflammation represent two major driving forces behind the development and progression of HCC [3,4]. Major risk factors for HCC include chronic infection with hepatitis B virus and hepatitis C virus; however, alcoholic cirrhosis, dietary carcinogens including aflatoxins and nitrosamines, nonalcoholic steatohepatitis and other metabolic liver diseases, obesity, diabetes and smoking also contribute to the development of HCC [5–7].

Lack of effective diagnostic tools for early detection and limited treatment options available to patients with advanced stages of HCC contribute to a dismal prognosis and high mortality rate. Surgical resection represents the treatment of choice for patients with well-preserved hepatic function. Liver transplantation serves a curative option for early tumors, but this option is of limited value due to the inadequate number of qualified donors as well as occurrence of the disease in the transplanted liver. Currently, sorafenib (Nexavar, Bayer) is the only drug approved by the United States Food and Drug Administration for the treatment of advanced HCC. However, only moderate improvement of survival, severe adverse side effects and high costs call for other novel therapeutic as well as preventive approaches [8–10].

The pomegranate (*Punica granatum*, Punicaceae) is a primeval, mystical and distinctive fruit which represents a phytochemical reservoir of heuristic medicinal value [11]. The "superfruit" pomegranate is gaining tremendous importance because of its wide-spectrum health benefits [12–14]. A diverse array of phytochemicals, including polyphenolic constituents (anthocyanins), hydrolyzable tannins (ellagitannins and gallotannins) and condensed tannins

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(proanthocyanidins), has been identified in several parts of the fruit [11]. Some of these compounds have been shown to exhibit potent antioxidant and anti-inflammatory properties implicated in the prevention and intervention of several inflammation-driven diseases, including cancer [11,14]. Although pomegranate-derived products have been shown to prevent chemically induced tumors of skin, breast, lung and colon as well as inhibit the growth and metastasis of xenografted lung and prostate tumors in rodents (reviewed in Ref. 15), the liver cancer chemopreventive potential of this unique fruit has not been explored until very recently.

Using the chemically induced and clinically relevant two-stage (initiation–promotion) model of rat liver carcinogenesis, our laboratory has provided substantial evidence for the first time that pomegranate bioactive constituents afford a striking chemopreventive activity against liver tumorigenesis through potent antioxidant mechanisms achieved by up-regulation of hepatic antioxidant and phase 2 genes regulated by the nuclear factor E2-related factor 2 (Nrf2) signaling pathway [16]. Emerging evidence strongly indicates that Nrf2 may work in concert with other signaling molecules, including nuclear factor-kappaB (NF-κB), a cardinal regulator of inflammation; and there is a possibility of cross talk between Nrf2 and NF-κB (reviewed in Ref. 17). Several studies have shown that, in addition to the activation of antioxidant and phase 2 gene transcription, Nrf2 is also involved in the suppression of proinflammatory signaling mediated by NF-κB (reviewed in Ref. 18). Accordingly, we have hypothesized that pomegranate-mediated activation of Nrf2 during experimental hepatocarcinogenesis may be linked to impairment of inflammatory cascade driven by NF-κB. Hence, the current study was initiated to investigate the possible anti-inflammatory mechanisms involved in the previously observed liver cancer chemopreventive effects of pomegranate phytoconstituents by assessing NF-κB-regulated proinflammatory mediators, including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Since indiscriminate inhibition of COX-2 may comprise the risk of adverse cardiovascular events [19], the safety profile of chemopreventive doses of pomegranate has also been investigated by monitoring cardiac functions *in vivo* using noninvasive transthoracic echocardiography.

## 2. Materials and methods

### 2.1. Materials

Pomegranate emulsion (PE) was purchased from Rimonest Ltd. (Haifa, Israel). The detailed description of the preparation of this formulation has been provided in our previous communication [16]. The chemical analyses revealed a preponderance of mixed octadecatrienoic acids, sterols and steroids, especially 17-alpha-estradiol and the tocol, gamma tocopherol in the lipid phase and gallic acid, 5-hydroxymethylfurfural, ferulic acid, punicalagins A and B, caffeic acid, corilagin, protocatechuic acid, *trans-p*-coumaric acid and ellagic acid in the aqueous phase [16]. Diethylnitrosamine (DENA) and phenobarbital (PB) were procured from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies, such as rabbit polyclonal iNOS, rabbit polyclonal COX-2, rabbit polyclonal NF-κB, rabbit polyclonal inhibitory κB (IκB) and mouse monoclonal β-actin antibody and ABC staining system were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other antibodies, such as rabbit polyclonal 3-nitrotyrosine, were obtained from Fisher Scientific, Inc. (Pittsburgh, PA, USA), and mouse monoclonal heat shock proteins (HSP70 and HSP90) were purchased from Enzo Life Sciences (Farmingdale, NY, USA). Quick RNA mini Prep kit was obtained from Zymo Research (Irvine, CA, USA) and Verso cDNA synthesis kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

### 2.2. Animal treatment and tissue harvesting

Liver tissues used for all assays in this study were harvested from our earlier chemopreventive study in which male Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN, USA) subjected to oral feeding of PE 1 and 10 g/kg body weight exhibited 26% and 50% inhibition of occurrence of hepatic nodules, respectively [16]. The animal study was carried out at the Northeast Ohio Medical University (Rootstown, OH, USA) following an animal protocol approved by the Institutional Animal Care and Use Committee. In short, following an acclimatization period (1 week), the rats were randomly divided into five groups. Group A animals were maintained as untreated normal control, whereas group B animals were fed with a sham emulsion (Rimonest Ltd., Haifa, Israel) through oral gavage at 10 g/kg three times/week. Three remaining groups were similarly given PE at 1 g/kg (groups C) or 10 g/kg (groups D and E). The aforementioned treatment regimen was followed for 4 weeks, and then hepatocarcinogenesis was initiated in all animals belonging to groups B, C and D by a single intraperitoneal injection of DENA (200 mg/kg). Following a period of 2 weeks, PB (a well-known tumor promoter) was added in the drinking water of DENA-initiated animals at a concentration of 0.05% (w/v). Oral feeding of rats with sham or PE was continued till the end of the study. The treatment regimens of various animal groups are as follow: group A (normal control), group B (DENA control), group C (PE at 1 g/kg + DENA), group D (PE at 10 g/kg + DENA) and group E (PE control at 10 mg/kg). All animals were sacrificed 18 weeks following the DENA administration, i.e., 22 weeks after commencement of the study. Liver samples from various rat groups were either preserved in paraformaldehyde or immediately flash-frozen in liquid nitrogen, stored at −70°C and used for various assays as described below.

### 2.3. Immunohistochemical assessment

Serial sections of liver tissue were prepared and processed for immunohistochemical analysis of iNOS, 3-NT, HSP70, HSP90, COX-2, IκB and NF-κB protein expressions following our established techniques [20]. The immunohistochemical slides were visualized under a light microscope, and at least 1000 hepatocytes/animal were analyzed. Results were expressed as percentage of immunopositive cells.

### 2.4. Western blot analysis

Frozen liver tissue samples were first homogenized in ice-cold RIPA lysis buffer to yield a 10% w/v tissue homogenate. The sample was then centrifuged at 4°C at 14,000*g* for 20 min. The supernatant was collected in a separate tube. Protein concentrations in supernatants were quantified using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) following the vendor's instructions. Equal amounts of protein samples were run on a 10% Tris–HCl gel (Bio-Rad Laboratories, Hercules, CA, USA), transferred onto a nitrocellulose and separately reacted with anti-iNOS antibody (1:1000), anti-3-NT antibody (1:200), anti-HSP70 antibody (1:1000), anti-HSP90 antibody (1:1000), anti-COX-2 antibody (1:200), anti-IκB antibody (1:200) or anti-NF-κB antibody (1:200). The immunoreactions were detected by an enhanced chemiluminescence detection kit (Thermo Scientific, Rockford, IL, USA) and analyzed using a Kodak analyzer. Normalization of Western blot was ensured by β-actin.

### 2.5. Gene expression

Total RNA from 20 mg of liver sample was extracted using Quick RNA mini Prep kit following the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the Verso cDNA synthesis kit as per the vendor's protocol. Polymerase chain reaction (PCR) was performed using specific primers for rat NF-κB and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) following the sequence: NF-κB sense: 5'-GAGCCCATGGAGTTCCAGTA-3'; antisense: 5'-ACTTGGTAC-CATGGCTGAGG; GAPDH sense: 5'-AGACAGCCGCATCTTCTTGT-3'; and antisense: 5'-TACTCAGCACCAGCATCACC-3'. The PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

### 2.6. Echocardiography

*In vivo* heart functions were monitored noninvasively by a Vevo 770 system (VisualSonics, Toronto, Canada) equipped with a 710B-075 transducer (20–30 MHz) as per our published technique [21]. In short, animals were anesthetized using 2%–2.5% sevoflurane and placed on a heated platform with electrocardiography electrodes to monitor heart and respiration rates. M-mode and two-dimensional images at the midpapillary level were captured from the parasternal short-axis view. Mitral valve inflow was obtained from apical four-chamber view followed by pulsed-wave Doppler. All measurements were averaged from at least three cardiac cycles. Measurements and calculations were performed using the Vevo 770/3.0 software.

### 2.7. Statistical analysis

Significant differences among various groups were detected by one-way analysis of variance. *Post hoc* analysis was performed by the Student–Neuman–Keuls test. A *P* value less than .05 is considered to be significant. The commercial software SigmaStat 3.1 (Systat software, Inc., San Jose, CA, USA) was used for all statistical analyses.

## 3. Results

### 3.1. PE suppresses elevated levels of hepatic iNOS in DENA-induced hepatocarcinogenesis

Fig. 1A (a–d) represents the immunohistochemical expression of the inflammatory marker iNOS in hepatic sections obtained

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