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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.

Keywords: Chemoprevention; Cyclooxygenase-2; Hepatocarcinogenesis; Inflammation; Nuclear factor-kappaB; Pomegranate

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 non-invasive 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'-ACTTGGTACCATGGCTGAGG; 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

from the various experimental rat groups. Almost no expression to a near absence of iNOS-positive cells is clearly observed in both normal control (Fig. 1A-a) and PE-treated (10 g/kg) (figure not shown) groups, respectively. In contrast, liver sections obtained from the DENA-control animals shows elevated numbers of iNOS-positive cells (Fig. 1A-b), whilst only a slight reduction in iNOS-positive cells is observed in animals treated with PE at 1 g/kg (Fig. 1A-c). Results from sections belonging to animals treated with PE at 10 g/kg alone show very limited numbers of iNOS-positive cells (Fig. 1A-d). Quantitative analysis of the immunohistochemical data shows significant ($P<.001$) increases in iNOS-positive hepatocytes in the DENA-control group compared to the normal control (Fig. 1B). Treatment with PE at both doses is shown to decrease these elevated numbers of iNOS-positive cells; however, a significant ($P<.01$) reduction in the percentage of iNOS-positive cells is demonstrated with the higher dose of PE (10 g/kg) treatment when compared to the DENA-treated control animals. Liver samples, procured from various groups, were subjected to Western blot analysis (Fig. 1C and D), and the subsequent results are in concurrence with our above-mentioned immunohistochemical data. A significant ($P<.001$) value, corresponding to the dramatic increase in iNOS protein expression, is clearly identified in the animals subjected to DENA alone in comparison to the normal animals. On the other hand, a significant ($P<.001$) down-regulation of iNOS expression is noticed for animals subjected to DENA and PE treatment (10 g/kg) compared to the DENA control group.

3.2. PE eliminates increased expression of 3-NT in DENA-mediated liver cancer

The predominant marker of peroxynitrite formation 3-NT was studied in liver sections prepared from different experimental groups using immunohistochemical staining techniques. Sections from the normal control group (Fig. 2A-a) and PE (10 g/kg) group alone (figure not shown) show absence of 3-NT-positive cells. In stark contrast, expression of 3-NT-positive cells is seen to be highly up-regulated in the DENA-challenged group (Fig. 2A-b), while there was slightly diminished immunoreactivity in the groups treated with PE at 1 g/kg (Fig. 2A-c). However, treatment with PE at 10 g/kg (Fig. 2A-d) clearly abrogates the numbers of 3-NT-positive cells. Quantitative analysis performed on the immunohistochemistry data acutely describes a significant ($P<.001$) elevation in the percentage of 3-NT-positive cells in the animals challenged with DENA in comparison to the normal control group (Fig. 2B). Though reduction in the percentage of immunopositive cells is predominantly observed in all PE-administered groups, a significant ($P<.01$) decrease is only noted in the DENA-induced rats treated with PE at a dose of 10 g/kg. Subsequent protein expression analysis using Western blotting technique (Fig. 2C and D) coincides well with our immunohistochemical data, indicating a significant ($P<.01$) increase in protein expression level of 3-NT in the DENA-control group compared to the normal control group. Also observed is a significant ($P<.05$) decrease in 3-NT expression in liver samples obtained from the PE-treated (10 g/kg) group in comparison to the samples obtained from the DENA control rats.

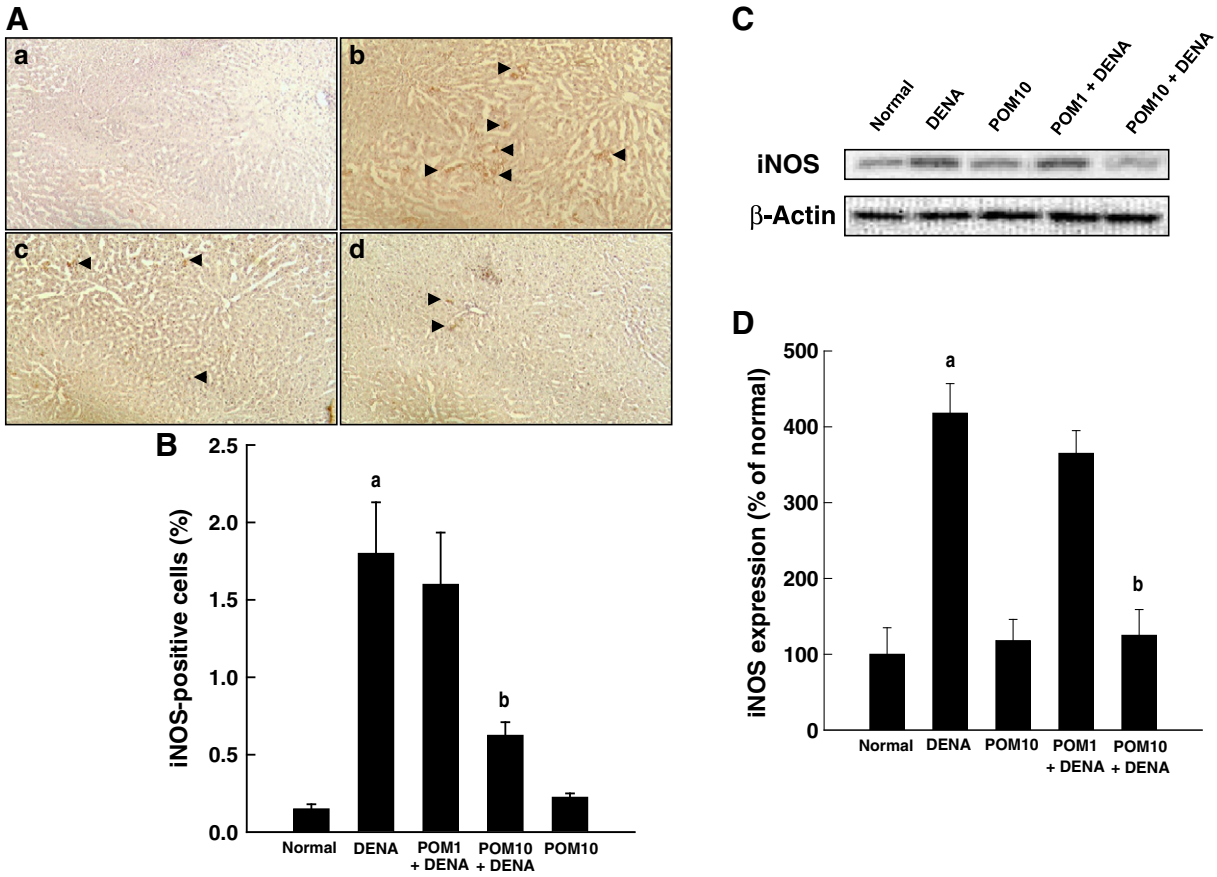

Fig. 1. Effects of PE on hepatic iNOS expression during DENA-initiated hepatocarcinogenesis in rats. (A) Representative immunohistochemical localization of iNOS (magnification: 100×) in various groups: (a) normal, (b) DENA control, (c) PE at 1 g/kg + DENA and (d) PE at 10 g/kg. Arrows indicate immunohistochemical staining of iNOS. (B) Quantification of iNOS-positive cells based on 1000 hepatocytes per animal and 4 animals per group. Each bar represents the mean±S.E.M. ($n=4$). [a]$P<.001$ as compared with normal group; [b]$P<.01$ as compared with DENA control. (C) Representative Western blot and (D) densitometric analysis of hepatic iNOS expression. Each bar represents the mean±S.E.M. ($n=4$–6). [a]$P<.001$ as compared with normal group; [b]$P<.001$ as compared with DENA control.

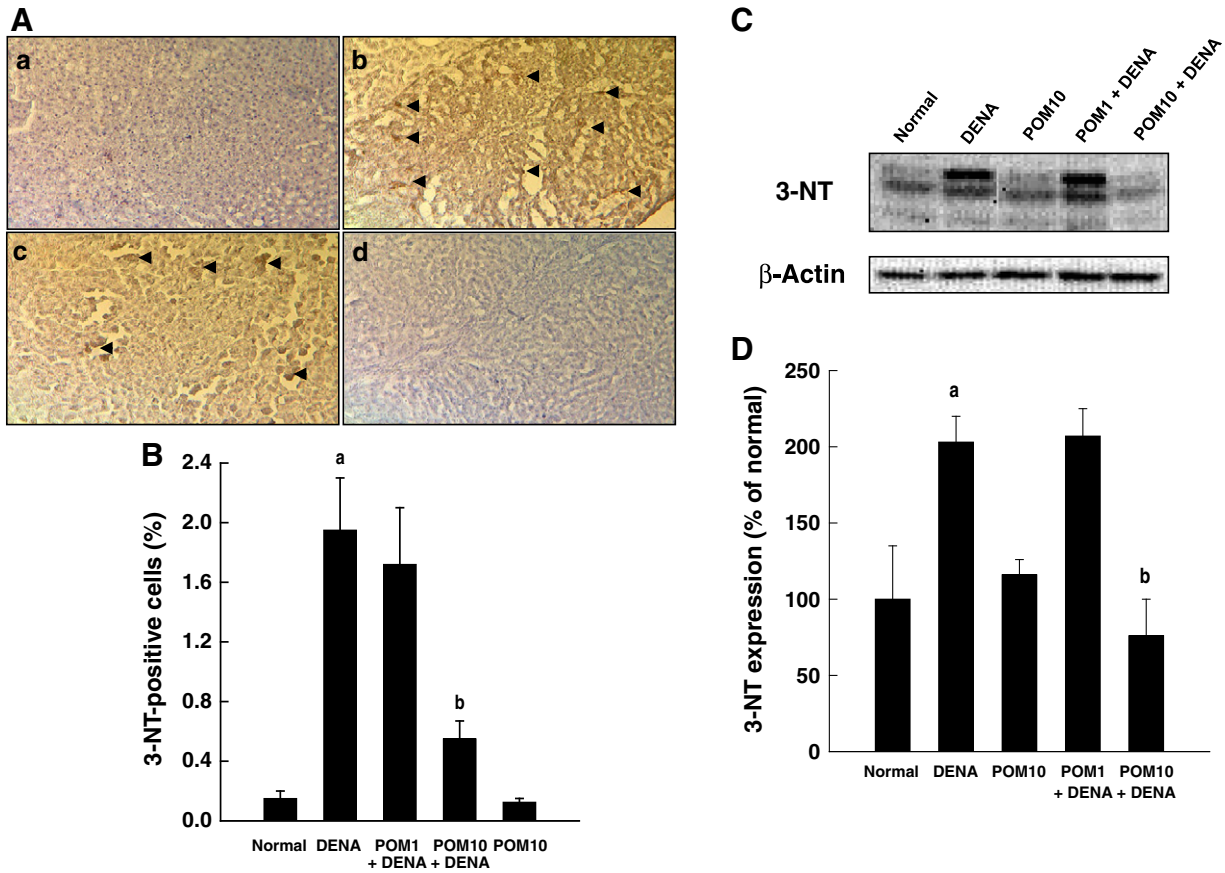

Fig. 2. Effects of PE on hepatic 3-NT expression during DENA-initiated hepatocarcinogenesis in rats. (A) Representative immunohistochemical localization of 3-NT (magnification: 100×) in various groups: (a) normal, (b) DENA control, (c) PE at 1 g/kg + DENA and (d) PE at 10 g/kg. Arrows indicate immunohistochemical staining of 3-NT. (B) Quantification of 3-NT-positive cells based on 1000 hepatocytes per animal and 4 animals per group. Each bar represents the mean±S.E.M. ($n=4$). [a]$P<.001$ as compared with normal group; [b]$P<.01$ as compared with DENA control. (C) Representative Western blot and (D) densitometric analysis of hepatic 3-NT expression. Each bar represents the mean±S.E.M. ($n=4$–6). [a]$P<.01$ as compared with normal group; [b]$P<.05$ as compared with DENA control.

3.3. PE abrogates increased expressions of inflammatory-stress-induced HSP70

Hepatic sections from the normal control animals do not show any expression of HSP70 (Fig. 3A-a). HSP70-positive cells are clearly not detected in the sections obtained from experimental group of animals exposed to PE alone (figure not shown). The frequency of cells expressing HSP70 is highly increased in the animals exposed to DENA alone (Fig. 3A-b), which is then found to be markedly reduced in the DENA-administered animals treated with PE at 1 g/kg (Fig. 3A-c) and 10 mg/kg (Fig. 3A-d). Quantitative analysis (Fig. 3B) reveals the percentage of HSP70 immunopositive cells in the rats subjected to DENA alone to be significantly ($P<.001$) elevated in comparison to the normal controls. Treatment with PE (both doses) in conjunction with DENA with respect to the DENA-control animals is seen to be effective in reducing the elevated levels of HSP70, although a significant ($P<.01$) result is achieved only with PE treatment at 10 g/kg. Fig. 3D represents the Western blot performed to quantify HSP70 protein expression in liver samples isolated from the various experimental groups. Quantification of the blots elucidates a significant ($P<.01$) rise in protein expression level of HSP70 in the DENA-subjected rats compared to the normal control animals which is significantly ($P<.01$) reduced with PE treatment at a dose of 10 g/kg with respect to the animals exposed to DENA alone.

3.4. PE reduces the increased expression of inflammatory-stress-induced HSP90

From the immunohistochemical data, another inflammatory stress-related protein, HSP90, is up-regulated in the liver sections obtained from the rats exposed to DENA alone (Fig. 3A-f). This is in stark contrast to sections procured from the normal control animals as no HSP90-immunopositive cells are identified (Fig. 3A-e). Similar results are also observed in rats subjected to PE treatment alone (figure not shown). Sections from rats treated with both doses of PE (1 g/kg and 10 g/kg) in conjunction with DENA (Fig. 3A-g and Fig. 3A-h, respectively) have decreased numbers of HSP90-positive cells. Quantitative analysis indicates a significant ($P<.001$) increase in the percentage of immunopositive cells in the DENA-challenged group compared to that of rats from the normal control group (Fig. 3C). Though treatment with PE at 1 g/kg reduced the expression of HSP90, the data are not significant. Nevertheless, a significant ($P<.01$) result is obtained with PE treatment at the highest dose (10 g/kg) with respect to the DENA control. Liver samples harvested from the various experimental groups were then used to perform Western blotting (Fig. 3D) which concurs with our immunohistochemical data. Expression of the protein HSP90 is significantly ($P<.01$) induced in rats administered with the carcinogen DENA in comparison to the normal control group of rats (Fig. 3F). HSP90 protein levels is greatly reduced following treatment with PE at both doses; however, a significant ($P<.01$) decrease in the protein expression is achieved with

the highest dose of PE treatment (10 g/kg) when compared to the DENA-challenged animals.

3.5. PE inhibits the elevated levels of COX-2 expression

Immunohistochemistry data from our study clearly indicates variations in the expression of COX-2-positive cells in liver sections obtained from different groups. Almost no expression of immunopositive cells is observed in the normal control animals (Fig. 4A-a) as well as in the rats treated with only PE (figure not shown). Rats belonging to the DENA group show highly elevated numbers of COX-2-positive cells compared to those of normal counterparts (Fig. 4A-b); this is clearly inhibited in the groups of animals treated with different concentrations of PE (1 g/kg and 10 g/kg) (Fig. 4A-c and A-d), with the latter group (Fig. 4A-d) bearing little to no immunopositivity to COX-2. The quantitative results on immunohistochemistry are presented in Fig. 4B. This inhibition of COX-2 expression with PE treatment in animals challenged with DENA was further confirmed using Western blotting techniques (Fig. 4C), and the quantitative analysis is represented in Fig. 4D. In comparison to the normal control group, DENA-exposed animals have significantly ($P<.001$) elevated levels of COX-2 protein, while treatment with PE at both doses is found to attenuate the elevated COX-2 expression significantly ($P<.001$) compared to the DENA-control group.

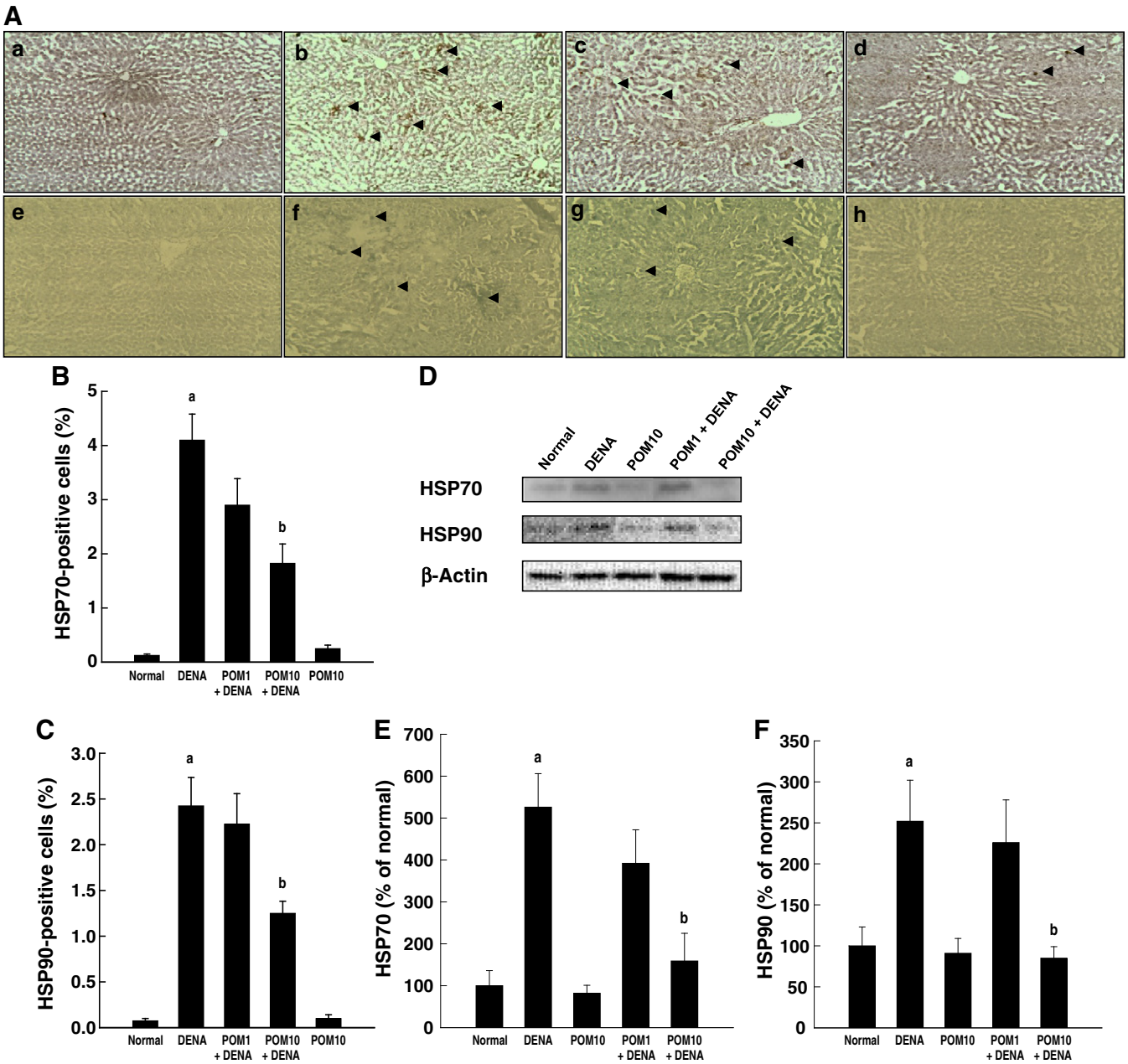

Fig. 3. Effects of PE on hepatic HSP70 and HSP90 expression during DENA-initiated hepatocarcinogenesis in rats. (A) Representative immunohistochemical localization of HSP70 (a–d) or HSP90 (e–h) (magnification: 100×). Various treatment groups are as follows: (a and e) normal, (b and f) DENA control, (c and g) PE at 1 g/kg + DENA and (d and h) PE at 10 g/kg. Arrows indicate immunohistochemical staining of HSP70 or HSP90. (B and C) Quantification of immunopositive cells based on 1000 hepatocytes per animal and 4 animals per group. Each bar represents the mean±S.E.M. ($n=4$). [a]$P<.001$ as compared with normal group; [b]$P<.01$ as compared with DENA control. (D) Representative Western blot and (E and F) densitometric analysis of hepatic HSP70 and HSP90 expression. Each bar represents the mean±S.E.M. ($n=4$–6). [a]$P<.01$ as compared with normal group; [b]$P<.01$ as compared with DENA control.

3.6. PE suppresses NF-κB expression at both transcriptional and translational levels

NF-κB, a widely expressed protein, has been well referred to being the potential link between inflammation and cancer, largely known to play a central role in inflammatory and immune responses [22,23]. Under inflammatory and other similar stressful stimuli, NF-κB (usually sequestered in the cytoplasm by IκB) translocates to the nucleus to activate the expression of various proinflammatory gene [24]. Represented in Fig. 5A-a–d is the immunohistochemical staining of cytosolic and nuclear NF-κB, while Fig. 5A-e–h identifies the staining for cytosolic expression of IκBα in rat liver sections from the various experimental groups. The normal control group demonstrates very low expression of NF-κB in the nucleus and moderate expression of the same in the cytosol (Fig. 5A-a). DENA control demonstrates an increase in the expression of nuclear NF-κB and almost disappearance of cytosolic NF-κB (Fig. 5A-b). The treatment with PE at 1 g/kg shows a slight decrease in the number of immunopositive cells for NF-κB in the nucleus and marginal increase in cytoplasmic NF-κB-immunopositive cells (Fig. 5A-c), whereas the highest treatment dose of PE (10 g/kg) clearly indicate a substantial reduction of nuclear NF-κB expression and marked elevation of cytosolic NF-κB expression (Fig. 5A-d) indicating limited translocation of NF-κB from the cytosol to the nucleus. The cytosolic IκBα demonstrates a considerable expression in normal control (Fig. 5A-e), almost no expression in DENA control (Fig. 5A-f) and limited expression in low-dose PE (1 g/kg) group (Fig. 5A-g), while substantial expression is observed with the high-dose PE (10 g/kg) (Fig. 5A-h). Fig. 5B represents the immunohistochemical analysis of percentage of immunopositive cells. The expression of nuclear p65 is significantly ($P<.001$) greater in DENA control than that in the normal group, but treatment with PE at 10 g/kg significantly ($P<.001$) inhibited the translocation of NF-κB to the nucleus, with less numbers of NF-κB immunopositive cells in nucleus when compared to DENA control. Simultaneously, cytosolic expression of NF-κB in DENA control is significantly ($P<.01$) lower in comparison to the normal control, whilst treatment with the highest dose of PE (10 g/kg) significantly ($P<.05$) elevated the expression of cytosolic NF-κB. Cytosolic IκBα was significantly ($P<.01$) lower in DENA control with respect to the normal control group, and PE treatment at 10 g/kg showed significant ($P<.05$) elevation in the expression of this protein.

Fig. 5C represents the gene expression data determining the mRNA levels of NF-κB p65. There is a substantial increase in the gene expression of NF-κB *p65* in the DENA-challenged control compared to that in the normal group. PE dose-dependently reduced DENA-induced up-regulation of NF-κB *p65* gene, and a substantial abrogation has been achieved with the highest dose of PE (10 g/kg), supporting our immunohistochemical findings.

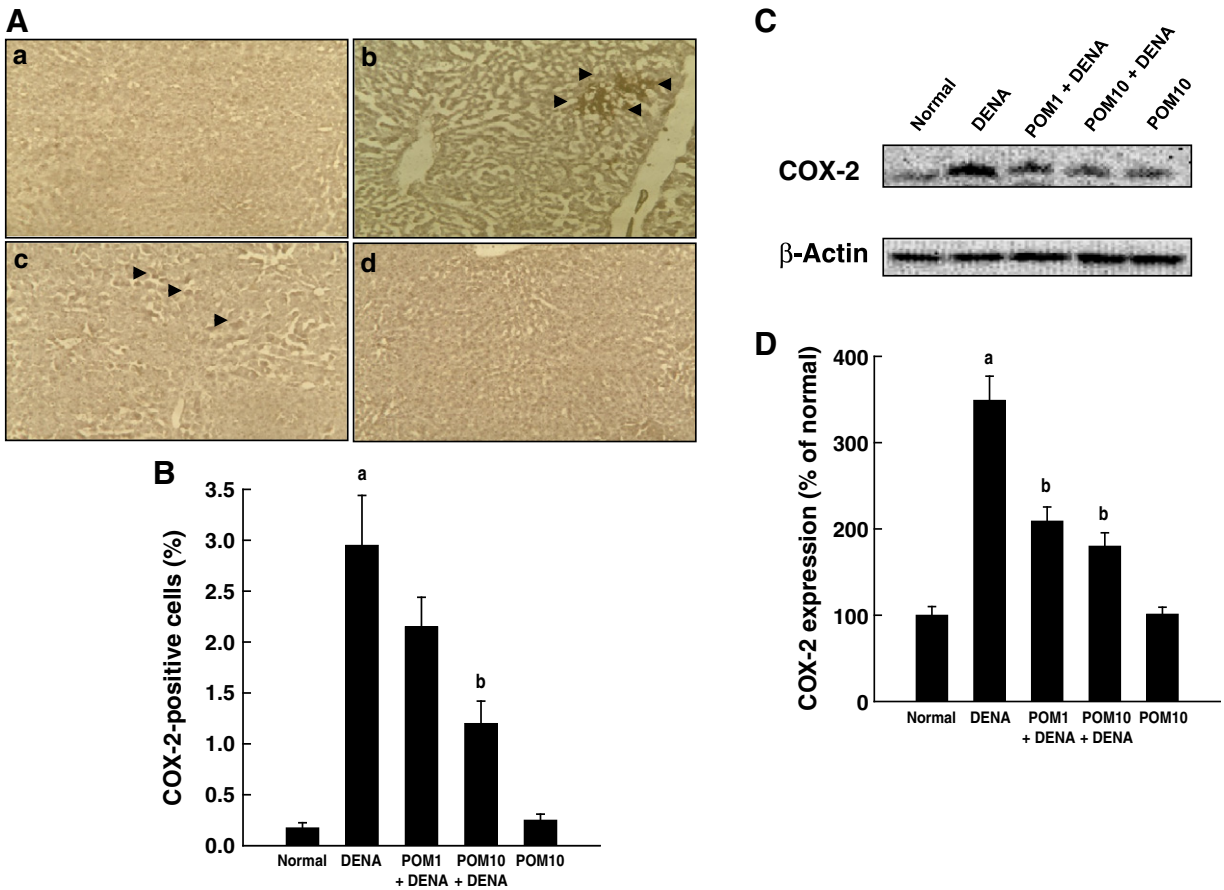

Fig. 4. Effects of PE on hepatic COX-2 expression during DENA-initiated hepatocarcinogenesis in rats. (A) Representative immunohistochemical localization of COX-2 (magnification: 100×) in various groups: (a) normal, (b) DENA control, (c) PE at 1 g/kg + DENA and (d) PE at 10 g/kg. Arrows indicate immunohistochemical staining of COX-2. (B) Quantification of COX-2-positive cells based on 1000 hepatocytes per animal and 4 animals per group. Each bar represents the mean±S.E.M. ($n=4$). [a]$P<.001$ as compared with normal group; [b]$P<.01$ as compared with DENA control. (C) Representative Western blot and (D) densitometric analysis of hepatic COX-2 expression. Each bar represents the mean±S.E.M. ($n=4$–6). [a]$P<.001$ as compared with normal group; [b]$P<.001$ as compared with DENA control.

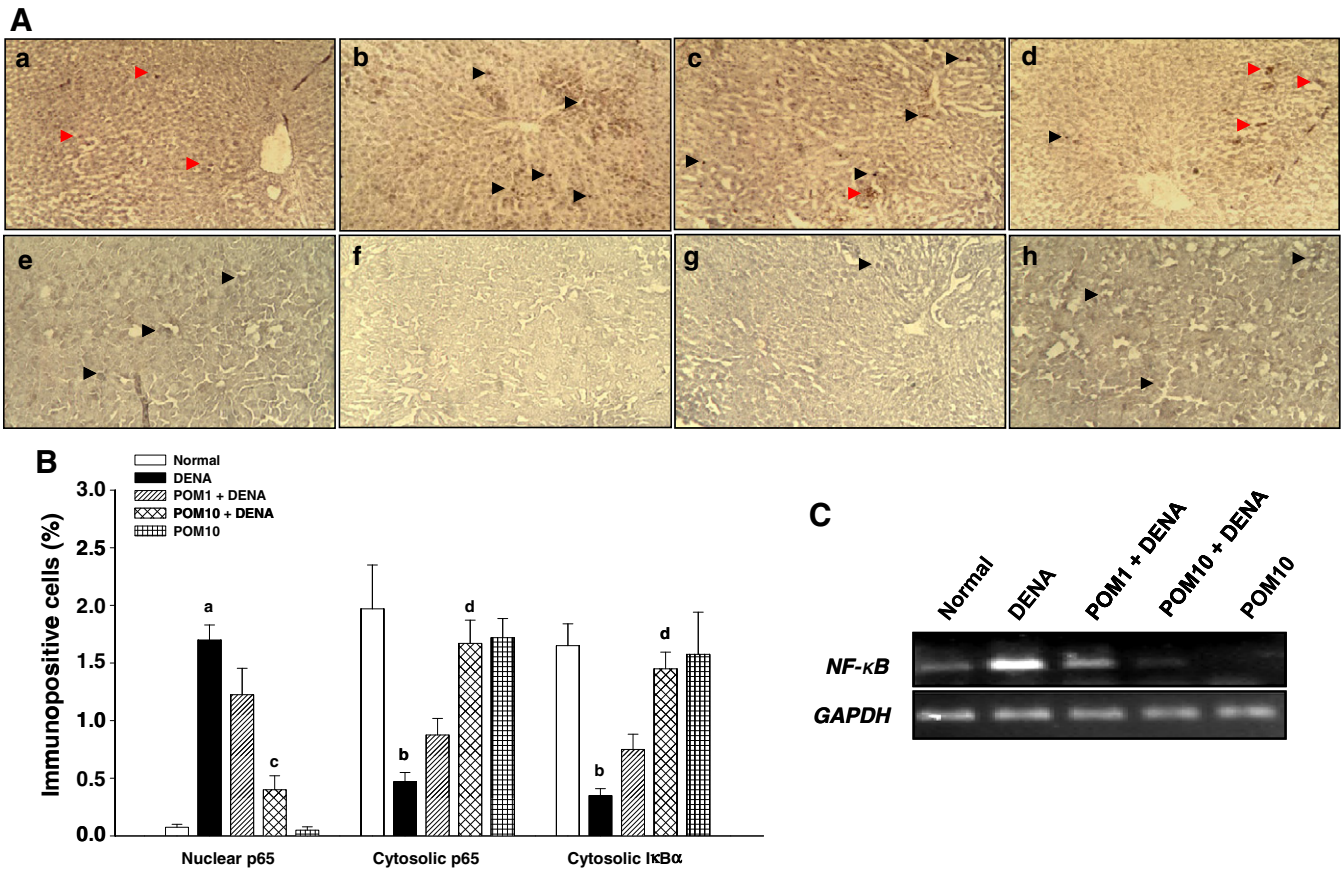

Fig. 5. Effects of PE on hepatic NF-κB p65 translocation and IκBα degradation during DENA-evoked hepatic preneoplasia in rats. (A) Representative immunohistochemical localization of NF-κB p65 in cytosol (a, c and d; red arrows) and nucleus (b, c and d; black arrows) and IκBα in cytosol (e, g and h; black arrows) (magnification: 100×). Various treatment groups are as follows: (a and e) normal, (b and f) DENA control, (c and g) PE at 1 g/kg + DENA and (d and h) PE at 10 g/kg. (B) Quantification of NF-κB p65- and IκBα-immunopositive cells in rat livers of several experimental groups. One thousand hepatocytes were counted per animal, and the results were based on four animals per group. Each bar represents the mean ±S.E.M. ($n=4$). [a]$P<.001$ and [b]$P<.01$ as compared to normal group; [c]$P<.001$ and [d]$P<.05$ as compared to DENA control. (C) Representative reverse transcriptase (RT)-PCR analysis of hepatic NF-κB p65 expression in various groups of rats. Total hepatic RNA was isolated and subjected to reverse transcription, and resulting cDNA was subjected to RT-PCR analysis using specific primer sequence. The *GAPDH* was used as the housekeeping gene.

3.7. PE does not affect cardiac performance based on echocardiography

Transthoracic echocardiography was performed at the end of the PE treatment just prior to sacrifice of various animal groups to assess the impact of PE administration on cardiac functions. Several indices of cardiac performance including ejection fraction and fractional shortening, cardiac dimensions or remodeling such as left ventricular volume and mass, and blood flow based on mitral valve early to late atrial flow ratio were monitored noninvasively on anesthetized animals. The results are summarized in Table 1, which shows no significant difference in the aforementioned cardiac function parameters among the various experimental groups. Our results indicate that the experimental doses of PE (1 or 10 g/kg) do not induce cardiotoxicity under the conditions of the present study.

4. Discussion

Our initial studies with PE provide substantial evidence that PE exerts a remarkable chemopreventive activity against DENA-induced hepatocarcinogenesis [16]. We have also previously shown that pomegranate phytoconstituents utilize Nrf2-mediated antioxidant mechanisms to abrogate the oxidative stress generated during DENA hepatocarcinogenesis [16]. Interestingly, Nrf2 may also act as a key modulator of inflammatory pathways through its coordinated interaction with NF-κB, a master regulator of genes of inflammatory signatures [25]. Several studies have established the strong relationship between inflammation and cancer with over 20% of all cancer-related deaths being attributed to inflammation that ensues and recurs with an ongoing infection, as in the case of HCC [26,27]. Targeting anti-inflammatory mechanisms by means of chemopreventive strategies could deter and halt the progress of preneoplastic lesions into a full-blown state of hepatic malignancy. Accordingly, our present study essentially aims to investigate if PE can abrogate NF-κB-mediated inflammatory pathways as well as attenuate the expression of various inflammation-associated markers, such as iNOS, 3-NT, HSP70, HSP90 and COX-2, during an early event of rodent liver carcinogenesis.

One of the major contributors of chronic inflammatory reactions is nitric oxide (NO), produced by hepatic cells from L-arginine and generated by iNOS which plays an important role in the development and progression of HCC. iNOS is found to be overexpressed in rodent as well as human HCC [28,29]. Recent studies have shown convincingly that suppression of iNOS leads to the abrogation of HCC growth, indicating that iNOS signaling could be a pertinent target for the prevention of hepatocarcinogenesis [29]. In confirmation with results from prior studies [28–30], our current study has clearly demonstrated elevated levels of hepatic iNOS expression in DENA-exposed animals. Increased iNOS was significantly reversed with PE treatment, thereby indicating a clear anti-inflammatory effect exerted by PE through iNOS down-regulation. Our findings provide ample support to the notion that iNOS may be a potential target in the chemoprevention of HCC.

Table 1
Transthoracic echocardiographic parameters of various rat groups at the end of the study

Parameters	Normal	DENA	POM1 + DENA	POM10 + DENA	POM10
Ejection fraction (%)	72.73±0.89	73.07±2.60	72.27±1.29	68.33±2.74	66.03±1.85
Fractional shortening (%)	43.26±0.86	43.64±2.37	43.16±1.21	40.70±2.41	37.83±1.43
LV volume (diastolic) (μl)	313.33±20.39	294.77±12.09	367.59±28.20	336.06±46.05	327.28±40.77
LV mass (corrected) (mg)	969.85±27.84	1006.00±48.60	1070.20±22.15	974.89±68.91	1001.94±66.19
Mitral valve E/A ratio	1.08±0.03	1.06±0.05	1.12±0.06	1.08±0.05	0.99±0.01

Values are presented as means±S.D. ($n=5$).
Abbreviations: E/A ratio, mitral valve early to late atrial flow ratio; LV, left ventricular.

The uncontrolled production of NO has been shown to trigger inflammation and promote development of tumors [31]. Harmful effects of NO are attributed to its nonenzymatic reaction with the superoxide anion yielding peroxynitrite ($ONOO^-$), better known as reactive nitrogen species (RNS). RNS reacts with certain susceptible amino acids, namely, arginine, cysteine, histidine and lysine, and causes the carbonylation of proteins. Another potentially damaging effect of $ONOO^-$ is its ability to oxidize nuclear DNA and cause nitration of tyrosine to generate 3-NT. The presence of 3-NT is recognized as an important marker of oxidative damage and inflammation inflicted by NO-generated RNS [32]. A ponderous increase in protein nitration was detected in the form of increased 3-NT expression in rats that were subjected to the DENA regimen alone, suggesting that oxidative damage was induced by RNS during the progression of hepatocarcinogenesis in the rodent liver. The data represented in our current study concur with those of previous communications which reported the presence of elevated levels of 3-NT expression in DENA-exposed rat liver [20,28]. The treatment with PE was found to significantly mediate the suppression of the up-regulated levels of 3-NT expression, suggesting the possible reduction in RNS generation through mechanisms that pertain to limiting NO production. So far, no study with pomegranate or its bioactive constituents has shown such effects. Thus, our findings may be viewed as a novel chemopreventive strategy in curbing the incidence of HCC among humans.

Induced under conditions of physiological stress, HSPs are generally ubiquitous and highly conserved molecules in cells, functioning as defense mechanisms against various environmental stresses [33]. HSPs, divided into six families based on their molecular weight, namely, HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs including HSP27 [34,35], are widely expressed under conditions such as carcinogenesis [33,36,37]. Several studies have demonstrated HSPs to be involved in modulating p53 function, cell cycle progression and apoptosis during the carcinogenic progression [38,39]. Two major contributors of HCC development and tumor progression include HSP70 and HSP90 [36,40]. Various malignant cells have been shown to overexpress HSP70, thereby being directly involved in promoting the proliferation of tumor cells [36,37,40], while HSP90 has been shown to regulate the expression and function of a variety of hepatocarcinogenic factors. A recent study revealed that the inhibition of HSP90 may induce cell cycle arrest and apoptosis in HCC cells [41]. Inhibiting HSP expression seems to be a pertinent approach which can possibly lead to the potential reduction in inflammatory stress and therefore an ideal method to decrease the incidence of HCC. In agreement with other studies [42,43], our present findings clearly indicated elevated expression levels of both HSP70 and HSP90 in the DENA-challenged rats. Treatment with dietary PE was demonstrated to suppress these up-regulated protein levels of HSP70 and HSP90, indicating a significant alleviation of inflammation-mediated stressful situation during DENA-evoked hepatocarcinogenesis.

Inflammation-related HCC has been well suggested to be caused by the COX-2 enzyme, responsible for catalyzing the conversion of arachidonic acid to prostaglandins (PGs) [44]. Williams et al. [45] have reported that COX-2 expression is highly induced by proinflammatory cytokines, mitogens and tumor promoters. The increased expression of COX-2 in tumors has been shown in a recent study to increase PG levels, known to affect multiple mechanisms involved in carcinogenesis, including induction of angiogenesis, inhibition of apoptosis and promotion of cell proliferation [19]. Studies have also shown selective COX-2 inhibition to decrease proliferation of HCC cells [46–49] as well as prevent experimental hepatocarcinogenesis in rodents [50]. A very interesting and exciting approach from a chemopreventive standpoint involves the inhibition of COX-2 utilizing dietary agents. The results of our current study, in line with other studies [46,51], demonstrated that the DENA-administered rats alone were found to have up-regulated COX-2 levels. PE treatment had abrogated these high levels of COX-2, indicating anti-inflammatory mechanisms to be in play by pomegranate phytoconstituents. An absence of cardiotoxicity following long-term PE consumption as opposed to various synthetic inhibitors of COX-2, evidenced from our noninvasive echocardiographic assessment, represents an important finding of our current study. Several laboratories have reported the protective effects of pomegranate juice or extract against experimentally induced cardiotoxicity in rats [52,53].

The canonical IκB kinase-beta (IKKβ)-dependent classical NF-κB pathway has been implicated in liver damage and the associated ensuing inflammatory cascade [54]. NF-κB, a eukaryotic transcription factor, has been cited as one of the most critical in initiating the expressions of various inflammatory molecules, including enzymes such as COX-2 and iNOS, all of which are then involved in the downstream generation of inflammatory mediators, such as NO and PGE2 [55]. In normal cells, NF-κB dimers are found in the cytoplasm, bound to inhibitory proteins, primarily IκB. Activation of the NF-κB signaling pathway usually occurs under conditions of stress, especially by proinflammatory molecules that act as signals which contribute towards the degradation of IκB. The degradation of IκB is facilitated by the phosphorylation of IKK, the activation of which occurs mainly through IKKβ. This consequently liberates NF-κB from IκB, allowing it to translocate to the nucleus where it functions to activate the transcription of various target genes [24]. In response to carcinogenic stress, the IκB-dependent NF-κB pathway has been known to activate genes encoding proteins that promote cell survival and antiapoptosis [55]. Results from various laboratories have shown that NF-κB induces the activation of COX-2, a primary mediator of the inflammatory cascade, which ensues right after hepatocarcinogenesis-mediated liver injury [29,56,57]. In the present study, the results are in concurrence with previous findings [50,58] where the DENA-challenged animals demonstrated attenuated levels of IκB protein expression in the cytosol, while the expression of NF-κB p65 was found to be highly elevated in the nucleus. The former observation is highly suggestive of the degradation of IκB in the cytosol, while the latter clearly indicates the translocation of NF-κB to the nucleus to have occurred. Treatment with PE in the DENA-exposed rats was shown to block this degradation of IκB and therefore prevent the translocation of NF-κB to the nucleus. This, in turn, may disable gene transcription and subsequent expression of several proteins known to be participants of the inflammatory cascade during DENA-induced

hepatocarcinogenesis. These observations support pomegranate constituents to be ideal chemopreventive agents that may well suppress the inflammatory insult most detrimental in the treatment and recovery of liver cancer patients.

In conclusion, the results of our present investigation clearly demonstrate that pomegranate bioactive constituents are capable of suppressing DENA-mediated inflammatory cascade by reversing the increased expression of inflammatory mediators, namely, iNOS, 3-NT, HSP70, HSP90, COX-2 and NF-κB, as well as hindering the nuclear translocation of NF-κB during an early stage of experimental hepatic neoplasia. An earlier study from our laboratory has shown that PE exerts potent chemopreventive activity against DENA-mediated hepatocarcinogenesis by mechanisms that involve the stimulation of the Nrf2-mediated antioxidant pathway [16]. The results of our current study thus coincide with our previous findings and thereby establish antioxidative and anti-inflammatory effects as the underlying mechanisms by which pomegranate phytochemicals exert powerful chemopreventive action. Data presented here in conjunction with those of our earlier communication [16] undoubtedly underline the importance of simultaneously targeting two interconnected molecular circuits, namely, the Nrf2-mediated redox signaling and NF-κB-regulated inflammatory pathway, to achieve chemoprevention of hepatocellular carcinogenesis. These interesting attributes supplanted with a safety profile highlight the chemopreventive and therapeutic potential of pomegranate in the management of liver cancer.

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