Ameliorative effects of pomegranate on carbon tetrachloride hepatotoxicity in rats: A molecular and histopathological study

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Abstract. The present study aimed to investigate the molecular mechanism underlying the hepatoprotective effects of pomegranate (POM) against oxidative stress in a rat model of carbon tetrachloride (CCl4)-induced liver damage. Injection of rats with CCl4 resulted in hepatic inflammation and lipid accumulation via the upregulation of interleukin (IL)-6 and sterol regulatory element-binding protein 1c (SREBP-1c) mRNA expression. CCl4 induced downregulation of the anti-inflammatory factors alpha 2-macroglobulin (α-2M) and IL-10 in comparison with the POM treated group. In addition, CCl4 induced downregulation of superoxide dismutase (SOD), glutathione S-transferase (GST) and catalase (CAT) expression. Conversely, prior administration of POM counteracted the deleterious alterations induced by CCl4. POM downregulated CCl4-induced IL-6 upregulation, normalized the increase in SREBP-1c expression, and prevented CCl4-induced α-2M downregulation. POM counteracted CCl4-induced alterations via immunosuppressive, anti-inflammatory and regenerative effects by upregulating transforming growth factor-β1, HSP70 and IL-10 mRNA expression. In addition, POM increased reactive oxygen species scavenging activity by augmenting the antioxidant defense mechanism against CCl4 hepatotoxicity, as demonstrated by detecting SOD, CAT and GST expression. These results confirm that, at the molecular level, POM exerts hepatoprotective effects against CCl4-induced oxidative stress and liver tissue damage.

Introduction

Oxidative stress occurs due to the inability of various antioxidant mechanisms to scavenge excessive levels of reactive oxygen species (ROS) and/or a reduction in antioxidant defense mechanisms. Consequently, degenerative diseases, including hepatopathies (1) and nephropathies (2) may occur. The liver and kidneys are the first tissues to be affected by oxidative stress produced by infectious agents, alcohol, drugs, toxic industrial chemicals, food additives, and pollutants in the air and water. Furthermore, free radicals and ROS have a crucial role in the initiation and progression of liver disease and cancer (3).

Carbon tetrachloride (CCl4) is an industrial solvent, which is extensively used as a xenobiotic to induce chemical liver injury. CCl4-induced oxidative stress is commonly used in rodent models to determine the protective effects of synthetic or natural products against drug-associated hepatotoxicity and nephrotoxicity (4,5). CCl4 is metabolized by hepatic microsomal cytochrome P450 into trichloromethyl free radicals. Trichloromethyl can react with sulphhydryl groups (glutathione and protein thiols) and antioxidant enzymes, including catalase (CAT) and superoxide dismutase (SOD) (4). Overproduction of trichloromethyl free radicals may initiate membrane lipid peroxidation, eventually leading to various pathological alterations (6). ROS have an important role in the development and progression of human disease, including liver disorders, lung and kidney damage, diabetes mellitus, atherosclerosis and aging (7), via free radical-induced lipid peroxidation and cell membrane damage (8). Furthermore, CCl4 causes tissue damage during inflammation, cancer and aging (9). Parallel to oxidative stress, CCl4 may induce alterations in various pathways, which affect the metabolic and healthy state of subjects, including changes in the gene expression of acute phase proteins, cytokines (inflammatory and anti-inflammatory), and genes associated with lipid metabolism.

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Abbreviations: α-2M, alpha 2-macroglobulin; CAT, catalase; GST, glutathione S-transferase; HSP70, heat shock protein 70; i.p., intraperitoneal injection; IL-6, interleukin-6; IL-10, interleukin-10; SOD, superoxide dismutase; ROS, reactive oxygen species; POM, pomegranate; SREBP-1c, sterol regulatory element-binding protein 1c; TGF-β1, transforming growth factor-β1; RT-PCR, reverse transcription-polymerase chain reaction

Key words: pomegranate, carbon tetrachloride, protection, gene expression, hepatic toxicity
It has previously been demonstrated that various natural products can protect organs against CCl₄-induced oxidative stress by enhancing the activities of antioxidant enzymes, including CAT, glutathione S-transferase (GST) and SOD (10). Pomegranate (Punica granatum; POM) is widely renowned in the Middle East due to its health benefits (11). POM fruit, juice and peel possess a marked antioxidant capacity, exert anti-obesity and antihypertensive effects, and may be used to treat prostate cancer (12-14). POM contains high levels of polyphenols, particularly ellagitannins, condensed tannins and anthocyanins (15). POM juice consumption has been reported to significantly increase sperm quality, spermatogenic cell density, antioxidant activity and testosterone levels in male rats (16). In addition, POM juice has been proposed to exert chemopreventive, chemotherapeutic, antiatherosclerotic and anti-inflammatory effects (14,17); therefore, its consumption has markedly increased (18,19). It is well known that the liver is the main organ responsible for detoxification and drug metabolism; therefore, the present study aimed to investigate the effects of POM on CCl₄-induced hepatotoxicity. In addition, the molecular mechanisms underlying the effects of POM on CCl₄-induced alterations in the expression of antioxidants, cytokines, inflammatory markers and genes associated with lipid metabolism, as well as hepatic histopathology, were examined.

Materials and methods

Materials. CCl₄ was purchased from Sigma-Aldrich (St. Louis, MO, USA). The DNA ladder (100 bp) was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). QiAzoL for RNA extraction and oligo-dT primers were purchased from Qiagen, Inc. (Valencia, CA, USA). Rabbit anti-rat heat shock protein 70 (HSP70) primary antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Experimental animals. Male Wistar rats, weighing 200-250 g, were purchased from the Animal House, King Abdulaziz University (Jeddah, Saudi Arabia). The adult male Wistar rats were divided into four equal groups (n=6/group) in the present study. The rats were kept under observation for ~1 week, in order to acclimate to the environment prior to onset of the experiment. The rats were maintained in stainless steel cages at normal atmospheric temperature (27±5˚C), under good ventilation and a 12 h/12 h light/dark cycle. The rats had access to food and water ad libitum. All animal procedures were approved by the Ethics Committee of the Dean of Scientific Affairs of Taif University (Taif, Saudi Arabia).

Experimental design and treatments. Group 1 served as a control group, in which the rats were injected intraperitoneally (i.p.) with corn oil (0.5 ml/kg) once daily. Group 2 were administered water daily for 25 days and on days 18 and 20 were injected with 1 ml/kg CCl₄ (50% in corn oil; i.p.). Group 3 were administered POM juice daily [30 ml/kg body weight (BW), and were injected with corn oil (i.p.) on days 18 and 20. Group 4 were administered POM juice (30 ml/kg BW) daily for 25 days, and were injected with 1 ml/kg CCl₄ (50% in corn oil; i.p.) on days 18 and 20. POM juice was administered to the rats in the morning, 2 h after water deprivation, and the rats consumed the given dose within 2 h, in order to assure no changes due to environmental conditions. The dose of POM juice was chosen based on a previous study (20).

Sampling. At the end of the experiment, the rats were sacrificed by cervical dislocation following anesthetization by diethyl ether (Sigma-Aldrich) inhalation. The liver tissues of all groups were harvested, homogenized and maintained in either formalin or QiAzoL reagent for histopathology and RNA extraction, respectively.

Analysis of gene expression
RNA extraction and cDNA synthesis. Total RNA was extracted from 100 mg tissue samples using QiAzoL reagent, according to the manufacturer’s instructions. The integrity of the prepared RNA was determined by electrophoresis. RNA concentration and purity were determined spectrophotometrically at 260 and 280 nm (Bio-Rad SmartSpec Spectrophotometer; Bio-Rab Laboratories, Inc., Hercules, CA, USA). The 260/280 optical density ratio of all RNA samples was 1.7-1.9. A total of 2 µg RNA was reverse transcribed using oligo-dT primers and Moloney murine leukaemia virus (M-MuLV) reverse transcriptase (SibEnzyme Ltd., Novosibirsk, Russia). For cDNA synthesis, a mixture of 2 µg total RNA and 0.5 ng oligo dT primer in a total volume of 11 µl sterilized diethylpyrocarbonate (DEPC) water was incubated in a PeX 0.5 thermal Cycler (Bio-Rad Laboratories, Inc.) at 65°C for 10 min for denaturation. Then, 4 µl 5X RT-buffer, 2 µl 10 mM dNTPs and 100 units M-MuLV Reverse Transcriptase (all purchased from SibEnzyme Ltd., Novosibirsk, Russia) was added in a total volume of 20 µl by DEPC water. The mixture was re-incubated in the thermal cycler at 37˚C for 1 h, then at 90˚C for 10 min in order to inactivate the enzyme.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). RNA from hepatic tissues was analyzed by semi-quantitative RT-PCR using corresponding specific primers for the indicated genes (Table I). The primers were designed using Oligo-4 computer program (version 7.0; Molecular Biology Insights, Cascade, CO, USA) according to the nucleotide sequences published in GenBank (http://www.ncbi.nlm.nih.gov/genbank/; Table I), and were synthesized by Macrogen Korea (Seoul, South Korea). PCR was conducted in a final volume of 25 µl consisting of 1 µl cDNA, 1 µl (10 picomoles) of each primer (forward and reverse), and 12.5 µl PCR Master Mix (Promega Corporation, Madison, WI, USA). The final volume was brought to 25 µl using sterilized, nuclease-free deionized water. PCR was carried out using a PeX 0.5 Thermal Cycler, and the following cycling conditions were used: Denaturation at 94°C for 5 min for one cycle, followed by 27 cycles of denaturation at 94°C for 1 min, annealing at the specific temperature corresponding to each primer (Table I) for 1 min, and extension at 72°C for 1 min, followed by a final extension step at 72°C for 5 min. As an internal reference, glyceraldehyde 3-phosphate dehydrogenase mRNA expression was detected using specific primers (Table I). PCR products subsequently underwent 1.5% agarose gel electrophoresis (Bio Basic Inc., Markham, ON, Canada) in Tris-Borate-ethylenediaminetetraacetic acid buffer at 100 V for 30 min with ethidium bromide staining (Sigma-Aldrich). PCR products were visualized under ultraviolet (UV) light and images
were captured using an InGenius 3.0 gel documentation system (Syngene, Frederick, MD, USA). Band intensities from the various rats from each group were quantified densitometrically using ImageJ software version 1.47 (http://imagej.en.softonic.com/).

**Immunohistochemical staining of HSP70.** Tissue sections were deparaffinized and were then treated with 3% H2O2 for 10 min, in order to inactivate peroxidases. Subsequently, the sections were heated in 10 mM citrate buffer at 121˚C for 30 min for antigen retrieval, blocked in 5% normal serum for 20 min, and were incubated with primary polyclonal anti-HSP70 [1:100 in phosphate-buffered saline (PBS); sc-33575; Santa Cruz Biotechnology, Inc.] overnight at 4˚C. After three extensive washes with PBS, sections were incubated with a biotin-conjugated goat anti-rabbit secondary antibody (1:2,000 in PBS; sc-2040; Santa Cruz Biotechnology, Inc.) for 20 min at 32˚C. Following a further incubation with horseradish peroxidase-labeled streptavidin (Santa Cruz Biotechnology, Inc.) according to the manufacturer's instructions, antibody binding was visualized with diaminobenzidine (Sigma-Aldrich) and sections were counterstained with hematoxylin (Sigma-Aldrich). Tissue slides were visualized using a Wolfe S9-0982 microscope (Carolina Biological Supply Co., Burlington, NC, USA) and photos were captured using a Canon Power Shot SX500 IS digital camera (Canon, Tokyo, Japan).

**Table I. Sequences and conditions of polymerase chain reaction primers.**

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<tr>
<th>Gene</th>
<th>Product size (bp)</th>
<th>Annealing temp. (˚C)</th>
<th>Number of PCR cycles</th>
<th>Direction</th>
<th>Sequence (5'-3')</th>
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<tr>
<td>GST</td>
<td>575</td>
<td>55</td>
<td>29</td>
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<td></td>
<td>Antisense</td>
<td>GTCTGACCACGTCAACATAG</td>
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<tr>
<td>SOD</td>
<td>410</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Antisense</td>
<td>TCTACAGTTGCAAGGGCCAGCAG</td>
</tr>
<tr>
<td>Catalase</td>
<td>652</td>
<td>55.5</td>
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<td>Sense</td>
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<td>56</td>
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<tr>
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<td>Antisense</td>
<td>TCCCTCAAGATTGTCAAGCA</td>
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</table>

GST, glutathione S-transferase; SOD, superoxide dismutase; AGP, α-2M, alpha 2-macroglobulin; IL, interleukin; TGF-β1, transforming growth factor-β1; FAS, SREBP-1c, sterol regulatory element-binding protein 1c; G3PDH, glyceraldehyde 3-phosphate dehydrogenase.

**Results**

**Effects of POM on the hepatic mRNA expression levels of interleukin (IL)-6, sterol regulatory element-binding protein 1c (SREBP-1c) and alpha 2-macroglobulin (α-2M).** To examine whether POM was able to modulate cytokine expression in order to exert its hepatoprotective effects, the mRNA expression levels of IL-6 were detected (Fig. 1A). The mRNA expression levels of IL-6 were upregulated following CCl4 injection. Furthermore, when administered alongside CCl4, POM completely normalized SREBP-1c mRNA expression (Fig. 1B). α-2M is known to stabilize target protein and enhance its regeneration. In the present study, α-2M expression was significantly downregulated in the CCl4 group compared with the control (P<0.05). However, treatment with POM increased α-2M mRNA expression. Concurrent POM administration in the CCl4-injected group resulted in a further upregulation in α-2M expression (Fig. 1C).
Effects of POM on the mRNA expression levels of transforming growth factor (TGF-β1) and IL-10. The mRNA expression levels of TGF-β1 were significantly upregulated in the CCl₄-treated group compared with in the control group (P<0.05). POM treatment alone only slightly upregulated TGF-β1 mRNA expression, as compared with the control group. However, when the rats were co-treated with CCl₄ and POM the mRNA expression levels of TGF-β1 were further significantly upregulated compared with in the CCl₄ group (P<0.05; Fig. 2A). Treatment of the rats with CCl₄ significantly suppressed the mRNA expression levels of the anti-inflammatory cytokine IL-10, as compared with the control (P<0.05). However, co-treatment with POM and CCl₄ significantly upregulated interleukin-10 mRNA expression compared with the control and CCl₄-treated groups (P<0.05; Fig. 2B).

Effects of POM on hepatic antioxidant enzyme expression. CCl₄ induced the downregulation of the expression of CAT, GST
and SOD, which were normalized following co-administration of POM with CCl₄. Following co-treatment with CCl₄ and POM, the mRNA expression levels of the antioxidant enzymes were completely restored, compared with in the CCl₄ group (Fig. 3A-C).

**Effects of POM on hepatic HSP70, as revealed by immunohistochemical staining.** Immunostaining of liver samples from control rats with HSP70 antibodies indicated normal hepatic architecture, with normal hepatic cords, hepatic sinusoids and normal portal area, and an absence of HSP70 expression (Fig. 4A). Liver samples from the CCl₄ group exhibited moderate HSP70 expression in the hepatocytes with increased fat infiltration (Fig. 4B). Liver samples from the POM-supplemented group exhibited a lack of HSP70 expression in hepatocytes, alongside normal hepatic architecture (Fig. 4C). Liver samples from the rats co-treated with CCl₄ and POM exhibited significantly increased HSP70 expression in the hepatocytes (P<0.05), alongside a decrease in the aberrant alterations detected in the CCl₄ group (Fig. 4D).

**Discussion**

The present study demonstrated that POM exerted a protective effect on the liver against CCl₄-induced oxidative stress. CCl₄ is one of the most commonly used hepatotoxins, and its metabolites trichloromethyl radical (CCl₃) and trichloromethyl peroxy radical (Cl₃COO•) are involved in the pathogenesis of liver and kidney damage (7,21). Both radicals are able to covalently bind macromolecules and induce peroxidative degradation of the cell membrane of liver cells.

IL-6 is produced in the liver by several types of cells, including biliary epithelial cells and cholangiocytes, in response to inflammatory mediators (22,23). IL-6 is a commonly used marker of inflammation, the expression of which has been demonstrated to be markedly increased in the liver of patients with non-alcoholic fatty liver disease and non-alcoholic steatohepatitis (24). In the present study, CCl₄ induced inflammation, as demonstrated by an upregulation in hepatic IL-6 expression. However, downregulation of basal IL-6 mRNA expression levels, and attenuation of the CCl₄-induced upregulation of IL-6 by POM, indicates its anti-inflammatory effects. Parallel with these results, increased levels of the proinflammatory cytokine IL-6 in the ileum of a rat model of necrotizing enterocolitis were normalized following treatment with POM seed oil (25).

SREBP-1c is an important transcription factor, which regulates the hepatic expression of enzymes associated with the de novo synthesis of fatty acids (26). Upregulation of the gene expression levels of SREBP-1c has previously been shown to accompany ethanol-induced fatty liver (27). In the present study, CCl₄ induced inflammation, as demonstrated by an upregulation in hepatic IL-6 expression. However, downregulation of basal IL-6 mRNA expression levels, and attenuation of the CCl₄-induced upregulation of IL-6 by POM, indicates its anti-inflammatory effects. Parallel with these results, increased levels of the proinflammatory cytokine IL-6 in the ileum of a rat model of necrotizing enterocolitis were normalized following treatment with POM seed oil (25).

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α-2M is the largest major non-immunoglobulin plasma protein, which is synthesized by numerous cell lineages, including lung fibroblasts, monocytes, macrophages, hepatocytes, astrocytes and adrenocortical cells (29). α-2M is expressed in hepatocytes where it has an important role in the regulation of proteolytic activity in the tissue and pericellular space, and contributes to the clearance of α-2M-proteinase complexes.
In addition, α-2M exerts an inhibitory effect on various types of nonspecific proteases and possesses pronounced immunosuppressive activity (31). The results of the present study demonstrated that treatment with CCl₄ resulted in downregulation of hepatic α-2M mRNA expression compared with the control group. The ability of POM to upregulate basal and CCl₄-suppressed α-2M mRNA expression indicated that the hepatoprotective effects of POM may operate via upregulation of α-2M expression. Notably, increased α-2M levels have been reported to have an important role in radioprotection via anti-fibrotic, anti-inflammatory, antioxidant, homeostatic, and repair and remodeling mechanisms (32).

TGF-β₁ is a powerful pleiotropic cytokine that possesses immune-suppressing and anti-inflammatory properties (33). In the present study, treatment with POM alone elevated TGF-β₁ mRNA expression levels, thus indicating that the modulatory effects of POM on immune status may be caused by TGF-β₁ upregulation. Treatment with CCl₄ induced the upregulation of TGF-β₁ mRNA expression, the expression of which was further upregulated in the rats co-treated with POM and CCl₄. These results suggested that the anti-inflammatory effects of POM may be due to TGF-β₁ upregulation. In patient-tolerated kidney or liver allografts, type 1 regulatory T cells were demonstrated to exist, and were capable of suppressing naïve T-cell activation and producing high levels of both IL-10 and TGF-β₁ (34).

IL-10 has been reported to exert both pro- and antitumoral effects through the inhibition of nuclear factor-κB signaling; therefore, it is able to downregulate proinflammatory cytokine expression (35). IL-10 configures development of the immune response and suppresses proinflammatory cytokine expression (36). In addition, IL-10 induces the downregulation of T helper 1 cytokine mRNA expression, and inhibits IL-1 and IL-6 production (37,38). In the present study, CCl₄-induced downregulation of IL-10 is consistent with the findings of a previous study, which reported that CCl₄ reduced hepatic IL-10 expression, as compared with in the control group (39). In the present study, POM upregulated IL-10 expression, thus suggesting that POM exerts an anti-inflammatory effect, which protects the liver from CCl₄-induced inflammation. Notably, previous studies have reported that IL-10 may exert antifibrotic effects during CCl₄-induced hepatic fibrosis (40,41). Therefore, POM may protect the liver from CCl₄-induced inflammation and fibrosis by upregulating the expression of IL-10 (42).

Delaying or inhibiting the oxidation of easily oxidizable macromolecules, such as lipids, is accomplished by antioxidants, including SOD, CAT and GST, which have a major role in protecting these molecules from the actions of free radicals or ROS (43). Conversion of superoxide to less toxic H₂O₂ is catalyzed by SOD, whereas conversion of H₂O₂ into nontoxic H₂O is catalyzed by CAT (44). The present study demonstrated that administration of POM in CCl₄-administered rats significantly restored mRNA expression of the antioxidants examined, thus suggesting that POM possesses anti-lipid peroxidation and antioxidative properties. Parallel with these results, Ocimum sanctum extract has been shown to cause a significant decrease in lipid peroxidation, coupled with a
significant increase in SOD and CAT expression in the liver homogenates of rats exposed to oxidative stress (45). The present study suggested that CCl4-induced suppression of SOD mRNA expression may be caused by enhancement of lipid peroxidation or inactivation of antioxidant enzymes, which leads to increased accumulation of superoxide radicals and lipid peroxidation acceleration. However, when administered alongside CCl4, POM prevented CCl4-induced antioxidant suppression. These results indicated that the ROS scavenging activities of POM juice may protect the liver from CCl4-induced oxidative stress. A similar protective effect of flavonoids has been reported in a previous study, which detected the ability of flavonoids to scavenge oxidative radicals in the liver of mice and rats following exposure to CCl4 (46). In accordance with the present results, POM has been reported to exhibit a promising antioxidant capacity, and is an effective scavenger for several ROS, primarily due to its high levels of phenolic acids, flavonoids and other polyphenolic compounds (47), through which POM may protect against free radical-mediated oxidative stress and attenuate CCl4-induced antioxidant depletion (48).

HSPs are induced in response to various stressors, in order to protect cells from such effects (49). HSP70 has been reported to protect cells from tumor necrosis factor-α, prostaglandin, hydrogen peroxide, ethanol and UV (50). In the present study, HSP70 expression was slightly increased following treatment with CCl4, which is parallel to the previously reported induction of HSP70 in acute liver damage by CCl4 (51). POM administration, together with CCl4, induced strong HSP70 immunoreactivity, thus preventing protein denaturation, which may act as a mechanism to overcome CCl4-induced hepatic oxidative stress. Parallel with this assumption, CCl4-induced liver damage has been shown to be attenuated by pre-induction of chaperones by heat treatment (52).

In conclusion, the present study confirmed that POM exerts hepatoprotective effects against CCl4-induced oxidative stress. POM was demonstrated to possess anti-inflammatory effects by suppressing CCl4-induced IL-6 expression, and was able to normalize lipid peroxidation by decreasing CCl4-induced SREBP-1c expression and increasing CCl4-suppressed α-2M mRNA expression. In addition, POM may exert hepatoprotective activity by its immunosuppressive, anti-inflammatory and regenerative effects via upregulation of TGF-β1, HSP70 and IL-10, and may increase ROS scavenging activities by augmenting the antioxidant defense mechanism against CCl4-induced hepatotoxicity and preventing CCl4-induced SOD, CAT and GST downregulation.

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References

against cadmium:


