Renoprotective Influence of Pomegranate Juice on Nephrotoxicity Induced by Methotrexate

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Author’s contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

ABSTRACT

Methotrexate (Metho) is cytotoxic drug widely used to treat malignant (lymphoma, leukemia, breast cancer) and non-malignant (rheumatic arthritis) diseases. It mediates nephrotoxicity via cellular oxidative stress. Pomegranate juice (POJ) has a potent antioxidant property. This research aimed to assess the potential protective effect of POJ against Metho-induced renal damage in rats. Renal toxicity was induced through intraperitoneal (ip) injection with a single dose of Metho (20 mg/kg). Forty male rats were randomly segregated into 4 groups; each group contained 10 rats. Control (Cont); Metho: rats on the 23rd day injected ip with Metho; POJ (2 ml/kg) + Metho: rats given POJ (2 ml/kg) orally once a day, and on the 23rd day injected with Metho ip; and POJ (4 ml/kg) + Metho: rats given POJ (4 ml/kg) orally once a day, and on the 23rd day rats were injected with Metho ip. After 5 days of Metho ip. injection, blood samples and renal tissue were obtained. Serum renal functions, ionic electrolytes (sodium and potassium), and pro-inflammatory cytokines were analyzed. Renal oxidative stress and antioxidant enzymes were also measured. Renal tissue were examined microscopically. Metho caused a significant increase in serum renal functions and disturbance in ionic electrolytes. As well as, there was a significant increase in pro-inflammatory cytokines and oxidative stress parameters, with detectable degenerative alteration in glomerulus and renal tissue changes compared with the Cont group. Pretreatment with POJ resulted in preventing biochemical and histopathological alterations induced by Metho. The high dose of POJ...
1. INTRODUCTION

The kidney is susceptible to damage by many drugs [1]. Experimental findings pointed to the reality that many drugs induce nephrotoxicity [2]. They found in laboratory animals that the application of certain medicines such as gentamicin, cisplatin and methotrexate causes reductions in kidneys blood flow and glomerular filtration along with increased vascular resistance [3]. Drugs can induce oxidative stress through the production of free radicals, which can cause damage to nucleic acids membrane and tissue when generated excessively at the cellular level [4]. As by-products or aerobic metabolic products, the drugs are available [5]. Methotrexate (Metho), a structural equivalent of folic acid and a powerful inhibitor of the dihydrofolate reductase enzyme, is commonly used as a chemotherapeutic agent [6]. It is now also used for inflammatory intestinal diseases, sarcoidosis, arthritis, vasculitis, and acute refractory asthma [7]. However, its use is limited because of the high occurrence of extreme dose-dependent toxicity, including hepatotoxicity, nephrotoxicity, bovine disease, etc. Due to the generation of reactive oxygen species that are associated with tissue injury, Metho induces oxidative stress [8].

Management of nephrotoxicity is still a challenge to the modern scientific community [9]. Unfortunately, drugs have little to offer alleviation of kidney ailments [10]. Thus, given rise to research involved in identification of safe, inexpensive and available alternatives from natural resources [11]. It is commonly recognized that the health benefits of several fruits and vegetables are related to their bioactive compounds [12]. Numerous bioactive components are found in pomegranate fruits including flavonoids, phenolic acids, and tannins, which play an essential role in the avoidance and treatment of many diseases [13]. The pomegranate fruit is regarded as a functional food since it contains rich components that have functional and therapeutic effects in different portions of the fruit [14]. These compounds can act as antihepatotoxic [15], antinephrototoxic [16], antioxidants [17], antidiabetic [18], anti-inflammatory [19], anticancer [20], and cardiovascular health boosters [21].

The aim of the research is to determine the possible preventive role of pomegranate juice (POJ) against Metho-induced renal damage in rat models.

2. MATERIALS AND METHODS

2.1 Drug, Plant, and Chemicals

Fresh pomegranate (PO) fruits in this research were bought from Panda market, city of Jeddah, KSA. Methotrexate (Metho) (20 mg/1ml) was purchased from local pharmacy, KSA. Other chemicals with high analytical quality were bought from Sigma (Aldrich Chemical Co.), USA.

2.2 Pomegranate Juice Preparation (POJ)

For the preparation process, fresh fruits were washed and peeled manually. In order to extract the juice, a commercial blender was used, then the juice was filtered with a Buchner funnel [22].

2.3 Induction of Renal Toxicity

Renal toxicity was induced through intraperitoneal (ip) injection with a single dose of Metho (20 mg/kg), then rats were sacrificed after 5 days [23].

2.4 Animals’ Protocol

Forty male rats (200 -220 g) were taken from the animal unit located in King Fahd Medical Research Center, KAU, KSA. The experimental protocol was carried according to rules approved by the Committee for the Care of Animals, King Abdulaziz University, Jeddah, Saudi Arabia. Rats (n=40) after acclimatization periods of one week under the standard lab condition were randomly segregated to four groups ten each. GI (Cont); rats given orally saline until end of experiment (28 days). GII (Metho); rats given orally saline, then rats on the 23th day injected ip with a single dose of Metho (20 mg/kg). GIII (POJ 2 ml/kg +Metho) rats given pomegranate juice (2 ml/kg) orally once a day [24], and on the 23th day injected with Metho ip. GIV (POJ 4 ml/kg +...
Metho) rats given pomegranate juice (4 ml/kg) orally once a day, and on the 23th day rats were injected with Metho ip as in GII. On the 28th day, after 5 days of Metho ip injection, blood samples obtained, serum was divided, and stored at temperature of -80°C up until the time for biochemical analysis. Kidneys sample collected and prepared for biochemical and histopathological studies.

2.5 Biochemical Nephrotoxicity Indices

Colorimetric kits obtained from Abcan, USA, were used to measure kidneys' functions of uric acid (UA), creatinine (Cr), blood urea nitrogen (BUN), ionic sodium, and potassium concentrations according to the instructions of manufacturer.

2.6 Serum Pro-inflammatory Cytokines

ELISA kits have been used to measure serum tumor necrosis factor alpha (TNF-α) and interleukin 1beta (IL-1β) from Abbexa, Cambridge, UK.

2.7 Estimation of Renal Oxidative Stress and Enzymatic Antioxidants

Enzymatic antioxidants (superoxide dismutase (SOD) and catalase (CAT) activities); and lipid peroxidation by measuring malondialdehyde (MDA) level were assessed in kidneys tissue homogenate following ELISA kits’ instructions. The used kits obtained from MyBioSource, USA. Kidneys histopathological studies.

Sections of kidney were stained after prepared with hematoxylin and eosin. After that, they were inspected under a microscope to detect the changes in kidneys tissue.

2.8 Statistic

All results were analyzed by SPSS version 25, by using ANOVA test. The values were expressed as mean ± SMD, and p-value ≤ 0.05 considered significance.

3. RESULTS

3.1 Effect of POJ on Serum Renal Function Parameters in Nephrotoxic Rats

Table 1 shows the impact of pretreatment with POJ (2 ml/kg) and (4 ml/kg) on renal function parameters in Metho-induced nephrotoxicity in rats. Injection of rats with Metho induced a marked renal toxicity as evidenced by significantly increased (p ≤ 0.001) in UA, Cr, and BUN concentrations versus the Cont group. Pretreatment of rats with POJ (2 ml/kg) and (4 ml/kg) markedly prevented the increase in serum levels of renal function parameters induced by Metho. There were significant decreases in renal function parameters (UA, Cr, and BUN concentrations) (p ≤ 0.001) versus the Metho injected rats. Furthermore, the pretreatment with POJ 4 ml/kg was more effective than the pretreatment with POJ 2 ml/kg. There was a significant difference (p≤0.05) between the POJ 4 ml/kg + Metho group compared with the POJ 2 ml/kg + Metho group on renal functions (UA, Cr, and BUN concentrations).

3.2 Effect of POJ on Serum Ionic Electrolytes in Nephrotoxic Rats

Fig. 1 shows the impact of pretreatment with POJ 2 ml/kg and 4 ml/kg on serum ionic electrolytes (Na+ and K+) concentration in Metho-induced nephrotoxicity in rats. Injection of rats with Metho induced a significant decrease (p≤ 0.001) in serum sodium (Na+) level concurrent with a significant elevation (p≤ 0.001) in serum potassium (K+) level versus the Cont group. Oral administration of rats with POJ 2 ml/kg and 4 ml/kg exhibited a significant rise in serum Na+ concurrent with a significant reduction in serum K+ levels (p ≤ 0.001) versus the Metho injected rats in a dose-dependent. Furthermore, there was a significant difference (p≤ 0.05) between the POJ 4 ml/kg + Metho group versus the POJ 2 ml/kg + Metho group in tested serum ionic electrolytes (Na+ and K+).

3.3 Effect of POJ on Serum Pro-inflammatory Cytokines in Nephrotoxic Rats

Table 2 illustrates the impact of pretreatment with POJ 2 ml/kg and 4 ml/kg on serum pro-inflammatory cytokines (TNF-α and IL-1β) concentrations in Metho-induced nephrotoxicity in rats. Injection of rats with Metho induced a marked inflammation as evidenced by significant increase (p≤ 0.001) in TNF-α and IL-1β levels as compared with the Cont group. Pretreatment of rats with POJ 2 ml/kg and 4 ml/kg significantly (p≤ 0.001) prevented the increase in serum TNF-α and IL-1β levels induced by Metho in a dose-dependent. Besides, there was a significant difference (p≤ 0.05) between the POJ 4 ml/kg +...
Metho group versus the POJ 2 ml/kg + Metho group in serum TNF-α and IL-1β levels.

3.4 Effect of POJ on Renal Oxidative Stress and Enzymatic Antioxidants in Nephrotoxic Rats

Injection of rats with Metho induced a marked renal oxidative stress as showed by significantly elevated ($p \leq 0.001$) in renal MDA level, concurrent with significantly decreased in the renal SOD and CAT concentrations versus the Cont group. Pretreatment of rats with POJ 2 ml/kg and 4 ml/kg markedly prevented the renal oxidative stress induced by Metho. There was a significant reduction in renal MDA level, concurrent with significant increases in the renal SOD and CAT concentrations ($p \leq 0.001$) versus the Metho injected rats. Furthermore, the pretreatment with POJ 4 ml/kg was more effective than the pretreatment with POJ 2 ml/kg. There was a significant difference ($p \leq 0.05$) between the POJ 4 ml/kg + Metho group versus the POJ 2 ml/kg + Metho group in all tested enzymatic antioxidants and oxidative stress indices (Fig. 2).

Table 1. Effect of POJ on renal function parameters in Metho-induced nephrotoxicity in rats

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>UA (mg/dl)</th>
<th>Cr (mg/dl)</th>
<th>BUN (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont</td>
<td>0.978 ± 0.025</td>
<td>0.364 ± 0.032</td>
<td>40.98 ± 2.467</td>
</tr>
<tr>
<td>Metho</td>
<td>1.734 ± 0.055</td>
<td>1.001 ± 0.061</td>
<td>84.84 ± 3.245</td>
</tr>
<tr>
<td>POJ (2 ml/kg) + Metho</td>
<td>1.383 ± 0.052</td>
<td>0.559 ± 0.041</td>
<td>51.84 ± 2.459</td>
</tr>
<tr>
<td>POJ (4 ml/kg) + Metho</td>
<td>1.081 ± 0.047</td>
<td>0.426 ± 0.020</td>
<td>43.38 ± 2.174</td>
</tr>
</tbody>
</table>

Values were presented as mean ± SE (n=10). Results were significantly varied ($p \leq 0.05^*, p \leq 0.01^&, and p \leq 0.001^#$) from a: Cont group. b: Metho group. c: POJ (2ml/kg) + Metho group.

POJ: Pomegranate juice; UA: Uric acid; Cr: Creatinine; BUN: Blood urea nitrogen

Fig. 1. Effect of POJ on ionic electrolytes [Na⁺ (A) and K⁺ (B)] in Metho-induced nephrotoxicity in rats

Values were presented as mean ± SE (n=10). Results were significantly varied ($p \leq 0.05^*, p \leq 0.01^&, and p \leq 0.001^#$) from a: Cont group. b: Metho group. c: POJ (2ml/kg) + Metho group.

POJ: Pomegranate juice; Na⁺: Sodium, K⁺: Potassium

Table 2. Effect of POJ on serum pro-inflammatory cytokines in Metho-induced nephrotoxicity in rats

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>TNF-α (pg/mL)</th>
<th>IL-1β (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont</td>
<td>30.43 ± 2.52</td>
<td>82.72 ± 2.72</td>
</tr>
<tr>
<td>Metho</td>
<td>84.97 ± 3.04</td>
<td>152.26 ± 3.84</td>
</tr>
<tr>
<td>POJ (2 ml/kg) + Metho</td>
<td>43.40 ± 2.25</td>
<td>95.02 ± 3.37</td>
</tr>
<tr>
<td>POJ (4 ml/kg) + Metho</td>
<td>35.16 ± 1.81</td>
<td>85.32 ± 2.77</td>
</tr>
</tbody>
</table>

Values were presented as mean ± SE (n=10). Results were significantly varied ($p \leq 0.05^*, p \leq 0.01^&, and p \leq 0.001^#$) from a: Cont group. b: Metho group. c: POJ (2ml/kg) + Metho group.

POJ: Pomegranate juice; TNF-α: Tumor necrosis factor alpha; IL-1β: Interleukin 1beta
**Fig. 2.** Effect of POJ on renal content of antioxidants indices (MDA [A], SOD [B], and CAT [C]) in Metho-induced nephrotoxicity in rats

Values were presented as mean ± SE (n=10). Results were significantly varied (p ≤ 0.05*, p≤ 0.01&, and p≤ 0.001#) from a: Cont group. b: Metho group. c: POJ (2ml/kg)+ Metho group.

POJ: Pomegranate juice; MDA: Malondialdehyde; SOD: Superoxide dismutase, CAT: Catalase

**Fig. 3.** Impact of POJ on renal histopathology changes in nephrotoxic rats (H & E X 100, scale bar 100 um). Cont (Fig. A): Renal tissues of rat exhibited the regular histological feature of renal parenchyma, normal glomeruli (G) and renal tubules (T). Metho (Fig. B): Renal tissues of rat showed interstitial nephritis (black arrow) and cystic dilatation of renal tubules (red arrow).

POJ (2 ml/kg) + Metho (Fig. C): Renal tissues of rat showed mild interstitial nephritis (black arrow) and congestion of renal blood vessel (red arrow). POJ (4 ml/kg) + Metho (Fig. D): Renal tissues of rat showed apparent no histopathological alterations
3.5 Effect of POJ on Renal Histopathological Alteration in Nephrotoxic Rats

The control renal sections presented a normal histological feature of renal parenchyma, normal renal tubules and glomeruli (Fig. 3.A and Fig. 4 A). The Metho rats showed cystic dilatation of renal tubules, and interstitial nephritis (Fig. 3.B and Fig. 4 B). The POJ (2 ml/kg) + Metho rats showed mild interstitial nephritis, congestion of renal blood vessel, few periglomerular inflammatory cells infiltration, slight thickening of the parietal layer of Bowman’s capsule and slight dilatation of Bowman’s space (Fig. 3.C and Fig. 4 C). The POJ (4 ml/kg) + Metho rats presented nearly normal histological feature of renal parenchyma, normal glomeruli (G) and renal tubules (Fig. 3.D and Fig. 4 D).

4. DISCUSSION

Metho application is utilized to treat many types of malignance and autoimmune diseases [25,26]. However, the usage of Metho is limited due to its toxicity caused by peroxidative damage to renal, hepatic, and splenic cell membranes and nucleic damage in these organs [27]. The main causes of Metho toxicity include excessive production of free radicals, inflammation, and depletion of antioxidant enzymes [28]. The metabolites of Metho are eliminated by the kidney, and then precipitate in the renal tubule causing renal toxicity and failure due to high levels of creatinine and blood urea nitrogen [29,30]. Systemic toxicity can result from nephrotoxicity in which it reduces the ability to excrete body wastes; maintain a balance of body fluids and electrolytes; and synthesis of essential hormones like erythropoietin [31]. Therefore, the utilization of natural medicinal plants containing bioactive compounds such as antioxidants can to be used as a complementary form in health care programs is encouraged to reduce drugs complication [32,33]. Pomegranate is one of the highest nutritional value fruit due to the presence of many valuable compounds such as flavonoids, phenolic acids, proanthocyanidin, and tannins [28,34-36].

The current research was implemented to illustrate the preventive role of the pretreatment...
with pomegranate juice at two dosage levels against Metho-induced renal damage in rats during the experimental period of 28 days on serum renal functions, ionic electrolytes, pro-inflammatory cytokines, renal oxidative stress, enzymatic antioxidants as well as the histopathological examination of renal tissue.

Metho induced disturbances in the renal functions, rats group injected with Metho revealed appreciably renal toxicity as detected through significant elevations in serum renal function parameters of creatinine, uric acid, and blood urea nitrogen concentrations. In addition, there was a significant rise in ionic K+ with a significant decline in serum ionic Na+. These results were in accordance with those obtained by Asvadi et al. [37] and Gaies et al. [38] who established current findings.

The mechanism behind the increased levels of the biochemical parameters might be due to the Metho induced changes in the permeability in the cell membrane and compromised structural integrity causing the enzymes leak into the circulation [39]. Histopathological examination of the renal tissue corroborated these findings. Current findings were in agreement with earlier studies [40-42].

Oral given POJ of 2 ml/kg and 4 ml/kg to rats induced significant reduction in renal function parameters, and serum ionic K+ as well as a significant elevation of the ionic Na+ when compared to the Metho injected group. The pretreatment with POJ 4ml/kg caused more effect than the pretreatment with POJ 2ml/kg. Pomegranate may help with cell membrane permeability and clearance of creatinine and urea in the kidneys [43]. The fact that histopathological examination of kidney tissues revealed less damage confirmed the protective effect of pomegranate.

Results of this research demonstrated a high incidence of kidney inflammation evidenced by the significant elevations in serum TNF-α and IL-1β in the Metho group versus the Cont group. With regard to the effects of pomegranate juice at the two doses, the results showed preventive action against the inflammation response via significantly reducing levels of pro-inflammatory cytokines. Pomegranate juice application significantly reversed the Metho-induced alterations in the inflammation markers suggesting its promising preventive effect against the Metho-induced inflammatory activity. Several preceding studies [16,44] indicated similar results regarding the ability of pomegranate to decline serum inflammatory cytokines TNF-α and IL-1β levels.

Beside, Metho led to a marked oxidative stress via unbalancing the state between the oxidants and antioxidants expressed by the reduction in renal antioxidant defense capacity including SOD and CAT with the elevation in renal lipid peroxidation (MDA) Level. Oxidative stress causes numerous pathological destructions as evidenced in the histopathological of the renal tissues. These findings were matched those obtained by Kolli, et al. [45] and Vardi et al. [46] who corroborated the research results.

In the current study, pretreatment with pomegranate juice in doses of 2 and 4 mg/kg produced significant decline in renal MDA levels with increase in activities of renal SOD and CAT enzymes versus the Metho injected group. The doses of POJ at 4 mg/kg showed more significant effect than POJ 2 mg/kg. The increased activity of the CAT and SOD enzymes could be elucidated by the antioxidant properties of POJ, which may be attributed to the varied phenolic and flavonoid compounds present in POJ that help to scavenge free radicals and prevent the damage in DNA [47,48].

The current study’s biochemical findings were confirmed by histopathological examination in kidney sections. The Metho group exhibited interstitial nephritis and cystic dilation of tubules. The histological findings of the rat pretreated with pomegranate juice revealed improvement in histological structure. The renal section of the Metho rats treated with POJ 2mg/kg exhibited mild changes in the renal tubules, mild interstitial nephritis, congestion of renal blood vessel, few periglomerular inflammatory cells infiltration, slight thickening of the parietal layer of Bowman’s capsule and minor dilatation of Bowman’s space. The pretreatment with POJ 4mg/kg restored the normal histological feature of kidney paraenchyma, normal glomeruli, and tubules. The present results are consistent with preceding studies demonstrating similar findings of the free radicals scavenging actions of pomegranate juice that appear to attenuate histopathological alterations related to oxidative damage and inflammation in nephrotoxicity [49-51].

5. CONCLUSION

Current study concluded that pretreatment with POJ at two dosage levels to Metho-induced
nephrotoxicity in rats lowered the elevated serum levels of renal function markers and potassium; improved renal level of antioxidant enzymes; and attenuated inflammatory cytokines parameters. Higher dose of POJ exhibited more effective in protecting the kidney damage. These effects are linked to enhancement of degenerative histopathological alterations in kidney induced by Metho. Our findings confirmed that pomegranate juice application significantly reversed all the Metho-induced alterations in the measured markers proposing its possible protective role against Metho-induced nephrotoxicity.

CONSENT
It is not applicable.

ETHICAL APPROVAL
Animal Ethic committee approval has been taken to carry out this study.

DISCLAIMER
The author has declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the author and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by the personal efforts of the author.

COMPETING INTERESTS
Author has declared that no competing interests exist.

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https://www.sdiarticle4.com/review-history/71390