Hesperidin protects against diethylnitrosamine-induced nephrotoxicity through modulation of oxidative stress and inflammation

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ABSTRACT

Background: Kidney forms the main controlling organ in sustaining homeostasis and, thus, is vulnerable to toxicity by xenobiotics.

Aims and Objective: To evaluate the possible protective effects of the citrus flavonoid, hesperidin (HES), against diethylnitrosamine (DEN)-induced nephrotoxicity in rats.

Materials and Methods: Rats received a single intraperitoneal dose of DEN (200 mg/kg body weight). Two-weeks after DEN administration, rats received 0.5 g/L phenobarbital in drinking water for 12 weeks. HES (50, 100, and 200 mg/kg body weight) were orally administered from the first day of experiment.

Result: DEN administration induced nephrotoxicity evidenced or DEN-induced nephrotoxicity was evidenced by the histological alterations and significant increase in serum creatinine (P < 0.001), urea (P < 0.01), and uric acid (P < 0.001) levels. DEN-intoxicated rats exhibited a significant (P < 0.001) increase in renal lipid peroxidation levels and reduced glutathione content and activity of superoxide dismutase, glutathione peroxidase, and glutathione-S-transferase. Concomitant supplementation with all the doses of HES markedly prevented DEN-induced biochemical and histopathological alterations.

Conclusion: The study findings provide evidence that HES could protect against DEN-induced renal injury through abolishment of inflammation and oxidative stress and potentiation of the antioxidant defense system.

KEY WORDS: Flavonoids; Renal Injury; Oxidative Stress; Inflammation; Antioxidants

INTRODUCTION

Diethylnitrosamine (DEN), a potent hepatocarcinogen, is produced from the metabolism of some drugs and found in tobacco smoke, processed meats, soybean, cheese, and wide variety of foods.[1] The cytochrome P450-dependent monooxidase systems biotransforms DEN, as reported earlier.

The lethal effects of DEN are initiated by this metabolic activation.[2] During metabolism, DEN induces oxidative stress, resulting in cytotoxicity, mutagenicity, and carcinogenicity.[3,4] Oxidative stress has been reported to play a key role in the pathogenesis of drug-induced renal damage, and reactive oxygen species (ROS) have been implicated in the mechanisms that lead to tubular necrosis.[5] Hence, the use of antioxidants could offer protective effects against drug-induced renal damage.

A growing number of epidemiologic studies consistently reveals a protective effect of polyphenol-rich foods against many diseases.[6,7] The results of multiple studies conducted in animal models[8–14] and in humans[6,7] have provided an evidence about the therapeutic effects of polyphenols. Flavonoids are nonnutritive dietary polyphenolic components widely distributed in plants[12] and possess a wide range of...
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biological effects. Hesperidin (HES), one of the most important flavonoids, is the predominant flavonoid in citrus fruits. The peel and membranous parts of sweet orange and lemon have the highest HES content. HES exhibits numerous biological and pharmacological effects including antioxidant, anti-inflammatory, anti-diabetic, hepatoprotective, and anti-carcinogenic properties. To the best of our knowledge, reports evaluating the protective effects of HES against DEN-induced nephrotoxicity are scarce. Therefore, this study was designed to demonstrate the efficacy of HES in the modulation of oxidative stress, inflammation, and cell damage associated with DEN-induced nephrotoxicity in rats.

MATERIALS AND METHODS

Chemicals
Hesperidin (HES), diethylaminoethylamine (DEN), phenobarbital (PB), pyrogallol, thiojarbituric acid (TBA), glutathione (GSH), and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma (USA). All the other chemicals were of analytical grade and obtained from standard commercial supplies.

Animals and Treatments
Thirty male Wistar rats, weighing 120–140 g, obtained from the animal house of the National Research Centre (El-Giza, Egypt), were included in this investigation. The animals were housed in plastic well-aerated cages (six rats/cage) at normal atmospheric temperature (25 ± 1°C) and normal 12-h light/dark cycle. Rats were provided with free access to water and were supplied daily with laboratory standard diet of known composition ad libitum. All animal procedures were undertaken with the approval of Institutional Animal Ethics Committee of Beni-Suef University (Egypt). Rats were divided to five groups (N = 6) and were subjected to the following treatments:

- **Group 1 (control):** Rats were injected with a single dose of saline (0.9%) and orally administered the vehicle 1% carboxymethylcellulose (CMC).
- **Group 2 (DEN):** Rats were given a single intraperitoneal injection of DEN (200 mg/kg body weight) dissolved in saline and given 1% CMC by gavage daily throughout the experimental period. Two weeks after DEN administration, rats received 0.5 g/L phenobarbital in drinking water for 12 weeks.
- **Group 3 (DEN + 50 mg HES):** DEN/PB-treated animals received 50 mg/kg hesperidin dissolved in 1% CMC by gavage daily throughout the experimental period.
- **Group 4 (DEN + 100 mg HES):** DEN/PB-treated animals received 100 mg/kg hesperidin by gavage daily throughout the experimental period.
- **Group 5 (DEN + 200 mg HES):** DEN/PB-treated animals received 200 mg/kg hesperidin by gavage daily throughout the experimental period.

The doses of HES were balanced consistently as indicated by any change in body weight to keep up the comparable dosage for every kilogram of body weight over the entire period of study. By the end of the experiment, animals were killed, and blood samples were collected, left to coagulate, and centrifuged at 3000 rpm for 15 min to separate the serum. Kidney samples were immediately excised and perfused with ice-cold saline. Frozen samples (10% wt/vol) were homogenized in chilled saline, and the homogenates were centrifuged at 3000 rpm for 10 min. The clear homogenates were collected and used for subsequent assays.

Biochemical Assays
**Determination of serum urea, creatinine and uric acid:** Serum urea, creatinine, and uric acid levels were assayed using reagent kits purchased from Biosystems (Spain), following the methods of Kaplan, Young, and Fossati et al., respectively.

**Determination of serum tumor necrosis factor (TNF-α):** Serum levels of TNF-α were determined by specific ELISA kits purchased from R&D Systems (USA), according to the manufacturer’s instructions. The concentration of TNF-α was determined spectrophotometrically at 450 nm. Standard plot was constructed by using standard cytokine, and the concentrations for unknown samples were calculated from the standard plot.

**Determination of oxidative stress and antioxidant system parameters:** Lipid peroxidation, assayed as malondialdehyde (MDA), was determined in kidney homogenates according to the method of Preuss et al. Reduced glutathione (GSH) content was assayed according to the method of Beutler et al. Activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione-S-transferase (GST) were measured according to the methods of Marklund and Marklund, Matkovic et al., and Mannervik and Gutenberg, respectively.

Histopathological study
The kidney samples were flushed with cold saline and then fixed in 10% buffered formalin for at least 24 h. The specimens were then dehydrated in ascending series of ethanol, cleared in xylene, and embedded in paraffin wax. Blocks were prepared, and 4-μm thick sections were cut by a sledge microtome. The paraffin-embedded sections were deparaffinized, washed, and stained with hematoxylin and eosin (H&E). The stained slides were examined under light microscope.

Statistical Analysis
Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA). Results were expressed as mean ± standard error (SEM), and all the statistical comparisons were made by means of the one-way ANOVA test, followed by Tukey’s test post hoc analysis. A P value < 0.05 was considered significant.

RESULTS

Data summarized in Table 1 show the effect of DEN and HES on renal function markers. The administration of DEN produced marked impairment of kidney function as demonstrated by the
Data are expressed as mean ± SEM.

An image is followed by a table comparing serum levels of TNF-α in different treatment groups. The table shows a significant (P < 0.001) increase in serum TNF-α in DEN-treated rats compared to control rats. Oral administration of HES significantly decreased the serum level of TNF-α compared to DEN-treated rats.

Histopathological examination revealed normal histology of kidney in the control group [Figure 2A]. Treatment with DEN caused renal damage evident by the histological changes including adenoma, dysplastic renal tubules with karyomegalic nuclei, atrophy of glomerular tuft, inflammatory cells infiltration, protein cast in the lumen of renal tubules, and vacuolation of renal tubules [Figure 2B,C]. On the other hand, treatment of the DEN-administered rats with 50 [Figure 2D], 100 [Figure 2E], or 200 mg [Figure 2F] dose of HES protected against the DEN-induced histological alterations. The histopathological alterations are summarized in Table 2.

Concerning renal lipid peroxidation, DEN-administered rats exhibited significant (P < 0.001) elevation in the renal lipid peroxidation marker MDA when compared with the control group of rats [Figure 3]. Oral supplementation of the 50- and 100-mg doses of HES to the DEN-treated rats significantly (P < 0.001) decreased renal MDA content. More or less similar, the higher dose of HES (200 mg) significantly (P < 0.001) prevented the DEN-induced lipid peroxidation in the kidney when compared with the DEN control rats. In addition, the 200 mg HES dose significantly decreased the renal lipid peroxidation when compared with the control (P < 0.05) and the lower HES dose (P < 0.01), as represented in Figure 3.

In contrast, GSH content of the DEN-administered rats showed significant (P < 0.001) decrease when compared with the corresponding control group. Oral supplementation with all the three doses of HES significantly (P < 0.001) ameliorated renal GSH content, as depicted in Figure 4. Similarly, the activity of SOD showed a significant (P < 0.01) decline in the kidney of DEN-intoxicated rats when compared with the control group. The low and high doses of HES significantly (P < 0.001) ameliorated the activity of renal SOD. However, nonsignificant differences exist, and the 100 mg dose of HES produced a less potent (P < 0.05) ameliorative effect on the SOD activity [Figure 5].

The activity of GPx and GST in kidney of the DEN-administered rats showed a significant (P < 0.001) decrease when compared with the control group of rats, as represented in Figures 6 and 7, respectively. The 50-mg HES dose significantly improved the activity of GPx (P < 0.05) and GST (P < 0.001) when compared with the DEN group. Both the 100- and 200-mg doses of HES markedly (P < 0.001) alleviated the activity of GPx and GST.

### Table 1: Serum creatinine, urea, and uric acid levels in control, DEN, and DEN rats treated with hesperidin.

<table>
<thead>
<tr>
<th></th>
<th>Creatinine (mg/dL)</th>
<th>Urea (mg/dL)</th>
<th>Uric acid (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.66 ± 0.10</td>
<td>27.59 ± 2.81</td>
<td>1.80 ± 0.11</td>
</tr>
<tr>
<td>DEN</td>
<td>1.07 ± 0.06***</td>
<td>79.36 ± 6.44</td>
<td>4.67 ± 0.59***</td>
</tr>
<tr>
<td>DEN + 50 mg HES</td>
<td>0.77 ± 0.04*</td>
<td>43.38 ± 5.32</td>
<td>2.32 ± 0.28***</td>
</tr>
<tr>
<td>DEN + 100 mg HES</td>
<td>0.82 ± 0.04*</td>
<td>39.05 ± 1.96</td>
<td>2.26 ± 0.21***</td>
</tr>
<tr>
<td>DEN + 200 mg HES</td>
<td>0.81 ± 0.02*</td>
<td>27.74 ± 3.29</td>
<td>2.29 ± 0.14***</td>
</tr>
</tbody>
</table>

***P < 0.001 vs. control, and *P < 0.05 and ***P < 0.001 vs. DEN. Data are expressed as mean ± SEM.

### Table 2: Histopathological lesions in kidney sections of control, DEN, and DEN rats treated with hesperidin.

<table>
<thead>
<tr>
<th>Histopathological lesions</th>
<th>DEN</th>
<th>DEN + 50 mg HES</th>
<th>DEN + 100 mg HES</th>
<th>DEN + 200 mg HES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenoma</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Karyomegalic nuclei</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Atrophy of glomerular tuft</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Infiltration</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Protein cast in the lumens of renal tubules</td>
<td>++</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vacuolation of renal tubules</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

Figure 1: Serum TNF-α levels in control, DEN, and DEN rats treated with hesperidin. Data are expressed as mean ± SEM. ***P < 0.001 vs. control, and *P < 0.05 and ***P < 0.01 vs. DEN.
This study showed that the administration of DEN induced renal damage, as evidenced by the increased levels of serum creatinine, urea, and uric acid. Serum creatinine level has been reported to reveal glomerular function and its increase is an indicator of renal failure.\[29,30\] These findings are in agreement with the studies of Rezaie et al.\[31\] and Pashmforoosh et al.,\[32\] who demonstrated increased serum creatinine and urea levels in the DEN-administered rats. Renal injury induced by DEN was further confirmed by the observed histological alterations, including adenoma, dysplastic renal tubules with karyomegalic nuclei, atrophy of glomerular tuft, and inflammatory cells infiltration; DEN + 50 mg HES (D), DEN + 100 mg HES (E), and DEN + 200 mg HES (D) showing nearly normal renal tubules and renal corpuscles. (× 400).

**Figure 2:** Photomicrographs of H&E-stained kidney sections of control (A) showing normal histological structure; DEN (B and C) showing several lesions including dysplastic renal tubules with karyomegalic nuclei, atrophy of glomerular tuft, and inflammatory cells infiltration; DEN + 50 mg HES (D), DEN + 100 mg HES (E), and DEN + 200 mg HES (D) showing nearly normal renal tubules and renal corpuscles. (× 400).

**Figure 3:** Lipid peroxidation in kidneys of control, DEN, and DEN rats treated with hesperidin. Data are expressed as mean ± SEM. *P < 0.05 and ***P < 0.001 vs. control, ***P < 0.001 vs. DEN, and **P < 0.01 vs. DEN + 50 mg HES. MDA, malondialdehyde.

**Figure 4:** Reduced glutathione (GSH) content in kidneys of control, DEN, and DEN rats treated with hesperidin. Data are expressed as mean ± SEM. ***P < 0.001 vs. control and **P < 0.01 vs. DEN.
nuclei, atrophy of glomerular tuft, and inflammatory cells infiltration. Concurrent administration of HES, in a dose-dependent manner, significantly decreased the serum levels of creatinine, urea, and uric acid and markedly prevented the DEN-induced renal histological alterations. The nephroprotective effects of HES in DEN-administered rats have not been previously demonstrated. Recently, HES has been demonstrated to protect rats against gentamicin-induced nephrotoxicity.[33]

The proinflammatory cytokine TNF-α showed a significant increase in the serum of DEN-administered rats when compared with the normal control group. The contribution of the immune system to the drug-induced nephrotoxicity has been well recognized.[34] Numerous studies demonstrated that several nephrotoxants could induce an inflammatory response, leading to organ injury.[35] The toxicant-induced generation of inflammatory mediators promotes migration and infiltration of leukocytes and aggravates the primary injury induced by the toxicant.[36] Alleviation of the altered serum TNF-α following HES administration might be attributed to its anti-inflammatory properties. We have previously reported the potent anti-inflammatory effect of HES in diabetic[10] and cyclophosphamide-intoxicated rats.[14]

DEN was suggested to induce generation of ROS and eventually resulting in oxidative stress and cellular injury.[37] ROS have the ability to cause oxidative damage in DNA, proteins, and lipids.[38] The kidneys are susceptible to the injury caused by ROS because of the plenty of long chain polyunsaturated fatty acids found in the composition of renal lipids.[39] In this study, DEN-induced rats exhibited a significant increase in levels of MDA, indicating a serious damage to kidney tissue. Nakae et al.[40] reported that DEN could intercalate with the membrane lipids and form ROS, which increase lipid peroxidation. Increased lipid peroxidation leads to the alteration of the membrane functions through decreasing its fluidity and changing the activity of its binding enzymes and their receptors.[41] Concurrent treatment with HES markedly ameliorated the elevated levels of MDA in a dose-dependent manner. This observation could be attributed to the potent free radical-scavenging activity of HES, which we have confirmed previously.[10,14]

On the other hand, DEN-administered rats exhibited significant decrease in renal GSH content when compared with the control group. GSH, a potent antioxidant, protects the cellular constituents against the damage induced by ROS[42] through its ability to form S-conjugates with the products of lipid peroxidation.[43] Hence, GSH depletion leads to lowered
cellular defenses against free radical-induced cellular injury. More or less similar, reduction in activity of the antioxidant enzymes SOD, GPx, and GST was observed in the kidneys of the DEN-administered rats. SOD and GPx play a significant role in maintaining the body's defense mechanism against the deleterious effects of ROS, and GST is an extra key detoxifying enzyme. The recorded reduction in GSH and the enzymatic antioxidants may be attributed directly to the excessive production of ROS in the DEN-induced rats. Oral administration of all HES doses markedly alleviated renal GSH content and the production of ROS in the DEN-induced rats. Oral administration of all HES doses markedly alleviated renal GSH content and the activity of the antioxidant enzymes. Therefore, we assume that the nephroprotective mechanism of HES against the DEN-induced oxidative stress is partially mediated by preventing GSH decline and potentiation of the enzymatic antioxidant defenses. These findings provide evidence on the radical scavenging and antioxidant activity of HES documented in our previous studies.

**CONCLUSION**

Data of the current study indicate that HES, in a dose-dependent manner, exerts protection against the DEN-induced renal toxicity in albino rats. Their renoprotective effects could be attributed to attenuation of the proinflammatory cytokine production and inhibition of the lipid peroxidative system through prevention of GSH depletion and enhancement of the enzymatic antioxidants.

**REFERENCES**


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