Grape Seed Extract and Vitamin C Combination Blocked LPS-Induced Multiple Organ Toxicity in Mice

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ABSTRACT | Gram-negative bacteria mediate multiple organ damage through eliciting systemic inflammatory response and extensive oxidative stress in affected humans and animals. This study was done to evaluate the effect of grape seed extract (GSE) given alone or in combination with vitamin C (Vit C) on organ toxicity in mice treated with lipopolysaccharide (LPS). Mice received intraperitoneal injections of LPS on day 1 (4 mg/kg) and day 8 (2 mg/kg) of the study and starting from the first day were orally treated with GSE (50 and 100 mg/kg), GSE (50 mg/kg) plus Vit C (50 mg/kg) or saline (plus vehicle control) for 15 successive days. The no vehicle control group was treated with saline only. Results indicated that compared to the saline-treated group, LPS injection significantly increased alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, uric acid, and decreased paraoxonase-1 (PON-1) in the serum. Moreover, LPS treatment significantly increased oxidative stress status and decreased the reduced glutathione (GSH) in the liver, kidney, and brain tissues. GSE given at doses of 50 and 100 mg/kg significantly decreased serum markers of liver and kidney tissue injury and decreased oxidative stress in the liver, kidney, and brain of LPS-treated mice. The resultant effect of combined treatment with GSE and Vit C was greater than that of GSE alone. Moreover, immunohistochemical studies of liver, kidney, and brain tissue sections were conducted. The LPS-induced intense immunohistochemical staining of tumor necrosis factor-alpha (TNF-α) and caspase-3 expression was decreased by treatment with GSE or GSE + Vit C in the following manner: Vit C + GSE > GSE 100 mg/kg > GSE 50 mg/kg. Our data indicate that the combination of GSE and Vit C can mitigate multiple organ toxicity in LPS-treated mice.

KEYWORDS | Caspase-3; Endotoxemia; Glutathione; Grape seed extract; Lipopolysaccharide; Hepatotoxicity; Malondialdehyde; Multiple organ toxicity; Nephrotoxicity; Neurotoxicity; Nitric oxide; Sepsis; Tumor necrosis factor-alpha; Vitamin C

ABBREVIATIONS | ALT, alanine aminotransferase; AST, aspartate aminotransferase; GSE, grape seed extract; GSH, reduced glutathione; LPS, lipopolysaccharide; MDA, malondialdehyde; NO, nitric oxide; NOS, nitric oxide synthase; PON-1, paraoxonase-1; TNF-α, tumor necrosis factor-alpha; Vit C, vitamin C
INTRODUCTION

Lipopolysaccharide (LPS) is a constructing component found on the outer cell wall of gram-negative bacteria. It protects the bacteria from harmful environmental insults and maintains cell wall integrity [1]. Systemic injection of LPS induces a proinflammatory response leading to fever, shock, organ failure, and death. This systemic inflammatory response is the cause of septic shock [2]. LPS acts on Toll-like receptor 4 (TLR4) present on inflammatory cells, and induces the release of potent inflammatory molecules, such as tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), monocyte chemoattractant protein-1 (MCP-1), and oxygen free radicals [3–7]. Endotoxemia frequently occurs in patients with liver failure, which is the causative agent in organ pathogenesis [8]. LPS activates hepatic Kupffer cells which subsequently release reactive oxygen species, metabolites of lipid peroxidation [9, 10], and nitric oxide [11]. Thus, LPS endotoxemia depletes the stores of reduced glutathione (GSH) in the liver and many other vital organs, thereby accelerating the susceptibility to organ damage [12, 13].

Grape seed extract (GSE) is a natural extract obtained from Vitis vinifera seed. It is an abundant source of flavonoids and proanthocyanidin oligomers. Proanthocyanidins are a class of phenolic compounds in forms of oligomers or polymers of polyhydroxy falvan-3-ol units, such as (+)-catechin, (−)-epicatechin [14]. These flavonoids exert many health beneficial effects, including their ability to increase intracellular Vit C levels, reduce capillary permeability and fragility, and scavenge free radicals [15]. GSE exerts various efficient pharmacological effects including anti-inflammatory [16], anti-bacterial [17], anti-cancer [18], neuroprotective [19, 20], and antifibrotic effects on the liver tissue [21].

Vit C, a potent water-soluble antioxidant found in the cytosol, is responsible for quenching different types of free radicals, has the capability to regenerate other antioxidants such as GSH and vitamin E, and is highly concentrated in the immune cells and brain [22]. Vit C blocks oxidative stress and helps organ recovery in chronic degenerative diseases [23]. Vit C has an anti-inflammatory effect; it arrests endothelial dysfunction and reduces cardiovascular diseases incidence [24]. Guo et al. [25] suggested that Vit C treatment have potential protective effects on oxidative stress and many hazardous toxicants.

The aim of this study was therefore to evaluate the protective and antioxidant potentials of GSE alone...
and in combination with Vit C against LPS-induced multiorgan (liver, kidney, and brain) toxicity in mice.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Grape seed extract containing ~95% standardized proanthocyanidins was purchased from Arab Company for Pharmaceuticals and Medicinal Plants (MEPACO, Cairo, Egypt). The extract was dissolved in 0.9% NaCl solution to obtain the necessary doses immediately before use. Lipopolysaccharide (LPS) derived from Escherichia coli was purchased from Sigma-Aldrich (St Louis, MO, USA), dissolved in aliquots of sterile saline, and frozen at −20°C. Other chemicals and reagents were purchased from Riedel-de Haën (Germany) and Biodiagnostic (Cairo, Egypt). Kits used for biochemical analyses were purchased from Biodiagnostic. The doses of GSE used in the study was based on that used for humans [26, 27] after conversion to that of mice using Paget and Barnes conversion tables [28].

2.2. Animals

Thirty Swiss albino mice of both sexes, weighing 20–25 g, were used throughout the experiment. Animals were housed under standard environmental conditions (23 ± 1°C, 55 ± 5% humidity, and a 12-h light/12-h dark cycle) and maintained with free access to water and a standard laboratory diet ad libitum. Animal care and the experimental protocols were approved by the National Research Centre Animal Care and Use Committee in accordance with the ethical and standard of Institutional Review Board of author’s institute and the Helsinki Declaration in 1975 (revised in 2000).

2.3. Experimental Design

Mice were randomly divided into five equal groups (6 mice each) and treated for 15 successive days and 2 h prior LPS injection as follows: group 1 was given normal saline 10 ml/kg orally; groups 2–5 were intraperitoneally injected with LPS (4 mg/kg) on the first day and the second dose was 2 mg/kg on the eighth day [29]; group 2 was orally given 10 ml/kg saline; groups 3 and 4 were orally administered GSE 50 and 100 mg/kg, respectively; group 5 was orally given combined treatment of GSE (50 mg/kg) and Vit C (50 mg/kg).

At the end of the experimental period, all animals were subjected under light anesthesia [30] to collect blood samples from retro orbital venous plexuses in plain test tubes. Serum was prepared to determine the activities/levels of aminotransferases, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) [31], creatinine [32], uric acid [33] and paraoxonase-1 (PON-1) [34].

2.4. Preparation of Tissue Homogenates

Liver, kidney, and brain tissues were rapidly removed, washed in ice-cooled saline, plotted dry, and weighed. A weighed part of each tissue was homogenized, using a homogenizer (Medical Instruments, MPW-120, Poland) with ice-cooled saline (0.9% NaCl), to prepare 20% w/v homogenate, then centrifuged at 2,000 g for 5 min at 4°C using a cooling centrifuge (Laborzentrifugen, 2k15, Sigma, Germany) to remove cell debris. The aliquot was divided into three parts to determine malondialdehyde (MDA), nitric oxide (NO), and reduced glutathione (GSH). Lipid peroxidation was assessed by measuring the level of MDA in tissue homogenate according to the method of Ruiz-Larrea et al. [35]. NO was measured as the nitrite using the Griess reagent, according to the method of Moshage et al. [36]. GSH was determined by the Ellman’s method [37].

2.5. Histopathological Examinations

The specimens from liver, kidney and brain tissues were fixed immediately in 10% neutral buffered formalin processed for light microscopy to get 5 μm paraffin sections and stained with Hematoxylin & Eosin (H&E) for histopathological examination. Images were examined and photographed under a digital camera (Microscope Digital Camera DP70, Tokyo) and processed using Adobe Photoshop (version 8.0). The magnification at which the images were captured was ×400.

2.6. Immunohistochemical Assessment of Tumor Necrosis Factor-alpha (TNF-α) and Caspase-3

Immunohistochemical staining of anti-TNF-α and anti-caspase-3 antibodies were performed on liver,
kidney, and brain (cortex and striatum) tissues with 4 μm-thick sections that were deparaffinized and incubated with fresh 0.3% hydrogen peroxide in methanol for 30 min at room temperature. Briefly, deparaffinized tissue slides were incubated with the antibodies against TNF-α (diluted 1:50) and cleaved caspase-3 (diluted 1:100). Positive cells were then determined with streptavidin biotin-peroxidase secondary antibody (Dako/Agilent, Santa Clara, CA, USA). The antibody binding sites were visualized with 3,3′-diaminobenzidine. The sections were then counterstained with hematoxylin, dehydrated using graded alcohols and xylene, and mounted. The immunostaining intensity and cellular localization of TNF-α and cleaved caspase-3 were analyzed by light microscopy.

2.7. Image Analysis for Quantitative Immunohistochemistry

Optical density measurements of TNF-α and caspase-3 immunoreactivity were carried out using the computer-assisted image analysis system, Leica Qwin 500 Image Analyzer system (LEICA Imaging Systems Ltd, Cambridge, England) which consists of a Leica DM-LB microscope with a JVC color video camera attached to a computer system, Leica Q501IW.

2.8. Statistical Analysis

Results were expressed as means ± standard error (SE). Data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test. The level of significance was accepted at p < 0.05.

3. RESULTS

3.1. Biochemical Results

3.1.1. Serum Markers of Liver and Kidney Tissue Injury

LPS treatment induced significant elevations in serum ALT and AST by 138.7% and 136.9%, respectively, compared with the saline-treated control group. Creatinine increased by 157.5%, while uric acid increased by 264.6%. Meanwhile, serum PON-1 activity showed a 37.8% decrease by LPS treatment as compared to the saline control value (Table 1).

In LPS-treated mice, significant decrements in serum ALT by 12.5%, 28.9%, and 41.9%, and in AST by 21.3%, 36.1%, and 48.1% were observed after the administration of GSE at 50 and 100 mg/kg and GSE + Vit C, respectively, as compared to the LPS only treatment group. Serum ALT, however, remained elevated compared to the saline control group by 108.9% and 69.7% after treatment with GSE at 50 and 100 mg/kg, respectively. It reached 38.7% of the saline control value upon GSE + Vit C administration. Meanwhile, serum AST remained elevated compared to the saline control group by 86.4% and 51.4% after treatment with GSE at 50 and 100 mg/kg, respectively. It reached only 22.9% of the saline control value after GSE + Vit C treatment (Table 1).

Serum creatinine decreased by 30.1%, 47.6%, and 57.3% while uric acid decreased by 31.7%, 58.5%, and 68.9% by GSE at 50 and 100 mg/kg and GSE + Vit C, respectively, compared with the LPS control group. Serum creatinine remained elevated by 80.0% and 35.0% and uric acid was elevated by 148.8% and 51.2% compared to the saline control group after treatment with GSE at 50 and 100 mg/kg, respectively. Creatinine and uric acid in the serum of LPS-treated rats were almost normalized following by GSE + Vit C treatment (Table 1).

Moreover, the administration of GSE + Vit C caused a significant increase in serum PON-1 activity by 34.1% compared with the LPS control value. PON-1 was decreased by only 16.6% by GSE + Vit C compared to the saline control group, though this was statistically significant. In contrast, GSE at 50 and 100 mg/kg failed to significantly increase PON-1 activity compared with the LPS-treated group. PON-1 activity remained significantly decreased by 35.4% and 32.9% after GSE at 50 and 100 mg/kg, respectively, compared with the saline control value (Table 1).

3.1.2. Oxidative Stress Markers in Liver, Kidney, and Brain Tissues

Compared with the saline-treated control group, LPS injection induced significant increases in MDA in liver, kidney and brain tissue by 156.9%, 192%, and 121.7%, respectively (Table 2). On the other hand, there were significantly decreased GSH levels in the liver, kidney, and brain by 52.7%, 47.4%, and 72.7%, respectively.
respectively, after LPS exposure (Table 3). There was also a significant increase in the level of NO in the above tissues by 21.8%, 31.0%, and 61.9%, respectively (Table 4).

In LPS-treated mice, the administration of GSE at 50 and 100 mg/kg and the combined treatment of GSE with Vit C significantly decreased the levels of MDA in the liver (by 16.1%, 26%, and 54.7%), kidney (by 13.4%, 29.4%, and 61.0%) and brain (by 15.1%, 26.0%, and 49.3%) compared with the LPS control group. MDA, however, remained significantly elevated by 115.5% and 90.3% in the liver, by 152.7% and 106.2% in the kidney, and by 88.2% and 64.1% in the brain tissue, compared to the respective saline control values after GSE at 50 and 100 mg/kg, respectively. In contrast, after GSE + Vit C, MDA

**TABLE 1. Effect of GSE alone or in combination with Vit C on serum ALT, AST, creatinine, uric acid, and PON-1 in LPS-induced multiple organ toxicity in mice**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Saline</th>
<th>LPS</th>
<th>GSE 50 + LPS</th>
<th>GSE 100 + LPS</th>
<th>GSE 50 + Vit C 50 + LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>23.67 ± 0.88</td>
<td>56.50 ± 1.18*</td>
<td>49.44 ± 0.93*</td>
<td>40.17 ± 1.01*</td>
<td>32.83 ± 0.95*#</td>
</tr>
<tr>
<td>AST</td>
<td>41.50 ± 0.76</td>
<td>98.33 ± 0.88*</td>
<td>77.37 ± 0.84*</td>
<td>62.83 ± 0.95*+</td>
<td>51.00 ± 0.97*++</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.40 ± 0.01</td>
<td>1.03 ± 0.04*</td>
<td>0.72 ± 0.03*+</td>
<td>0.54 ± 0.01*+</td>
<td>0.44 ± 0.01*++</td>
</tr>
<tr>
<td>Uric acid</td>
<td>1.27 ± 0.09</td>
<td>4.63 ± 0.19*</td>
<td>3.16 ± 0.02*+</td>
<td>1.92 ± 0.03*+</td>
<td>1.44 ± 0.05*++</td>
</tr>
<tr>
<td>PON-1</td>
<td>31.31 ± 0.94</td>
<td>19.46 ± 0.96*</td>
<td>20.21 ± 0.76*</td>
<td>21.00 ± 0.72*</td>
<td>26.10 ± 0.96*++</td>
</tr>
</tbody>
</table>

Note: Results are presented as means ± SE (n = 8). *, p < 0.05 vs. saline control; #, p < 0.05 vs. LPS only; +, p < 0.05 vs. GSE 50 mg/kg + LPS; @, p < 0.05 vs. GSE 100 mg/kg + LPS. Units for ALT and AST, IU/l; for creatinine and uric acid, mg/dl; for PON-1, kU/l; for GSC and Vit C, mg/kg body weight.

**TABLE 2. Effect of GSE alone or in combination with Vit C on tissue MDA in LPS-induced multiple organ toxicity in mice**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Saline</th>
<th>LPS</th>
<th>GSE 50 + LPS</th>
<th>GSE 100 + LPS</th>
<th>GSE 50 + Vit C 50 + LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>34.61 ± 1.11</td>
<td>88.94 ± 1.26*</td>
<td>74.58 ± 1.03*</td>
<td>65.86 ± 1.18*</td>
<td>40.25 ± 1.24*#</td>
</tr>
<tr>
<td>Kidney</td>
<td>26.21 ± 1.23</td>
<td>76.54 ± 1.21*</td>
<td>66.23 ± 0.96*</td>
<td>54.05 ± 1.19*#</td>
<td>29.81 ± 1.14*++</td>
</tr>
<tr>
<td>Brain</td>
<td>27.3 ± 1.42</td>
<td>60.53 ± 1.25*</td>
<td>51.38 ± 1.26*</td>
<td>44.8 ± 1.67*#</td>
<td>30.7 ± 1.76*++</td>
</tr>
</tbody>
</table>

Note: Results are presented as means ± SE (n = 8). *, p < 0.05 vs. saline control; #, p < 0.05 vs. LPS only; +, p < 0.05 vs. GSE 50 mg/kg + LPS; @, p < 0.05 vs. GSE 100 mg/kg + LPS. Units for MAD, nmol/g tissue; for GSC and Vit C, mg/kg body weight.

**TABLE 3. Effect of GSE alone or in combination with Vit C on tissue GSH in LPS-induced multiple organ toxicity in mice**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Saline</th>
<th>LPS</th>
<th>GSE 50 + LPS</th>
<th>GSE 100 + LPS</th>
<th>GSE 50 + Vit C 50 + LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>13.54 ± 0.06</td>
<td>6.40 ± 0.04*</td>
<td>7.54 ± 0.02*</td>
<td>8.50 ± 0.01*#</td>
<td>11.80 ± 0.03*#++</td>
</tr>
<tr>
<td>Kidney</td>
<td>4.18 ± 0.01</td>
<td>2.20 ± 0.02*</td>
<td>2.43 ± 0.01*</td>
<td>2.80 ± 0.01*#</td>
<td>3.42 ± 0.06*#++</td>
</tr>
<tr>
<td>Brain</td>
<td>3.74 ± 0.02</td>
<td>1.02 ± 0.04*</td>
<td>1.44 ± 0.02*#</td>
<td>1.89 ± 0.01*#</td>
<td>3.24 ± 0.13*#++</td>
</tr>
</tbody>
</table>

Note: Results are presented as means ± SE (n = 8). *, p < 0.05 vs. saline control; #, p < 0.05 vs. LPS only; +, p < 0.05 vs. GSE 50 mg/kg + LPS; @, p < 0.05 vs. GSE 100 mg/kg + LPS. Units for GSH, µmol/g tissue; for GSC and Vit C, mg/kg body weight.
Levels of ROS in liver, kidney, and brain tissues were only 16.3%, 13.7%, and 12.4% of their respective control saline values. In the kidney tissue, MDA in GSE + treatments was significantly lower than in LPS only. Table 4 shows the effect of GSE alone or in combination with Vit C on tissue NO in LPS-induced multiple organ toxicity in mice. Figure 1 and Figure 2 provide representative photomicrographs of liver and kidney sections, respectively, demonstrating the effects of GSE and Vit C on histology.
Vit C-treatment group was not significantly different from the saline control value (Table 2).

GSE at 50 and 100 mg/kg and the combined treatment of GSE with Vit C resulted in significant increments in the levels of GSH in the liver (by 17.8%, 32.8%, and 84.4%), kidney (by 10.4%, 27.3%, and 55.4%), and brain (by 41.2%, 85.3%, and 21.6%) compared with the LPS control group. It was noted, however, that GSH levels remained significantly decreased after GSE at 50 and 100 mg/kg compared with the saline control values in the liver (by 44.3% and 37.2%), kidney (by 41.9% and 33.0%), and brain tissues (by 61.5% and 49.5%). After GSE + Vit C, GSH values was only 12.8%, 18.2%, and 13.4% lower than their corresponding controls. In the brain tissue, GSH level in GSE + Vit C-treatment group was not significantly different from the saline control group (Table 3).

The levels of NO also significantly decreased by GSE 100 mg/kg and GSE + Vit C in the liver (by 7.9% and 26.4%), kidney (by 14.5% and 27.7%), and brain (by 14.3% and 42.7%). NO, however, remained significantly elevated after GSE 100 mg/kg compared with the saline control group in the liver, kidney, and brain tissues by 12.1%, 11.9%, and 38.8%, respectively. NO levels were brought to their normal saline values in liver, kidney, and brain tissues by GSE + Vit C treatment (Table 4).

3.2. Histopathological Results

3.2.1. Liver

As shown in Figure 1, the liver of the saline control mice had normal histological structure of hepatic lobules. LPS treatment caused loss of hepatic lobular architecture, nuclear vacuolation, and degeneration with increased eosinophilia. Extensive necrosis and
marked infiltration of inflammatory cells were found in periportal and pericentral areas. Moreover, dilatation and congestion in central and portal veins and blood sinusoids were observed. Treatment with GSE alone ameliorated the changes induced by LPS, as evidenced by mild cytoplasmic degeneration. Notably, GSE + Vit C combination resulted in significant improvement in LPS-induced hepatotoxicity; the hepatocytes mostly had normal appearance with few degenerated cells.

### 3.2.2. Kidney

As shown in Figure 2, kidney sections of the saline control mice showed normal nephron structure. LPS treatment caused severe glomerular degeneration including hypercellularity and peripheral infiltration of inflammatory cells. The proximal and distal convoluted tubules showed disruption of the lining epithelium and necrosis with cellular exfoliation into the tubular lumen, scattered apoptotic bodies, irregular basement membrane, and the presence of interstitial hemorrhage. GSE-treatment group (100 mg/Kg) had no remarkable changes except for slight to moderate dilatation of the affected tubules accompanied by mononuclear cell infiltration. The combined effect of GSE + Vit C resulted in obviously further improvement in LPS-induced nephrotoxicity.

### 3.2.3. Brain

As shown in Figures 3 and 4, brain sections from the control group showed normal cortex and striatum structures. LPS-treated mice showed various degrees of degenerated neurons and neuronal loss in both regions with pink shrunken neurons (a sign of neuronal death), pyknotic nuclei surrounded by perineuronal vacuolations, edema, and red blood cell infiltration. GSE-treatment group (100 mg/Kg) caused moderate improvement in LPS-induced histological damage. Notably, the combined treatment group (GSE + Vit C) showed further distinct improvement in neuronal
cell structure with complete membrane integrity and clear nuclei when compared with GSE (100 mg/kg) alone.

3.3. Immunohistochemical Analysis of TNF-α and Caspase-3

As shown in Figures 5–12, faint or no expression of the pro-inflammatory cytokine TNF-α and the apoptotic marker caspase-3 were detected in the liver, kidney, or brain tissue in the saline only control group. LPS induced intense expression of TNF-α and caspase-3 in these organs. TNF-α and caspase-3 immunostaining was markedly decreased in GSE-treated groups and there was an obviously further improvement in GSE + Vit C treated group as shown in Figures 5–12. Tables 5 and 6 show the quantitative optical density measurements of TNF-α and caspase-3 in the liver, kidney, and brain tissues in mice treated with LPS and the effect of GSE alone or in combination with Vit C.

4. DISCUSSION

The present study indicates that treatment with GSE or combined GSE and Vit C could attenuate the biochemical and tissue damage caused by the systemic administration of a septic dose of bacterial endotoxin. LPS induced liver necrosis, glomerular damage, and neuronal loss evidenced by histopathological examination of the affected tissues and by the release of markers such as aminotransferases into the circulation and by the rise in serum creatinine and uric acid indicative of liver and kidney tissue injury. This occurred along with marked oxidative stress indicated by the increase in the lipid peroxidation end product MDA, and by the decrease in the antioxidant molecule GSH in tissues. LPS injection also resulted in increased expression of the proinflammatory cytokine TNF-α and the apoptotic factor caspase-3 in tissues. LPS activates TLR4 on monocytes and macrophages, activates NF-κB, increases expression and release of inflammatory mediators such as IL-1β,
IL-6, TNF-α, MCP-1, cyclooxygenase-2, and reactive oxygen species. This results in the development of systemic inflammatory response and neuroinflammation [2, 4–7, 38]. Qin et al. [4] showed that a single intraperitoneal (ip) injection of LPS of 5 mg/kg in mice activated microglia cells and induced rapid release of TNF-α, and increased the expression IL-1β, TNF-α, and NF-κB. Jeong et al. [5] found that intravenously administered LPS (250 µg/rat) activated microglia and caused infiltration of neutrophils into the brain. LPS given at 200 or 300 µg/kg in rats and mice caused significant increase in MDA and decreased GSH in brain, liver, kidney heart and lung tissues [6, 39, 40], decreased total antioxidant capacity and catalase activity [6], and increased the expression of cyclooxygenase-2 in the brain and liver [7]. Our observations are thus in line with studies indicating increased production of reactive oxygen species and increased expression of TNF-α following the systemic injection of bacterial LPS in rodents.

We have also shown significantly increased tissue levels of NO by LPS. This increase in NO has been shown to be due to increased expression of the inducible nitric oxide (iNOS) by the inflammmogen [38, 39, 41]. These larger amounts of NO, generated for long duration by iNOS to kill pathogens, are responsible for tissue injury observed during endotoxemia. This is because NO can react with superoxide to produce the highly toxic peroxynitrite or react with oxygen yielding nitrogen oxides capable of attacking membrane lipids, enzyme proteins, and nucleic acids. Other researchers have shown increased expression of the endothelial nitric oxide synthase (eNOS) in rat brain astrocytes after systemic LPS injection [42].

This study also provides evidence for an inhibitory effect of LPS on serum PON-1 activity. Similar findings were reported following LPS (300 µg/kg, ip) in rats [43]. LPS injection (200 µg/kg, ip) resulted in a significant decrease in PON-1 activity in mice brain and liver [6, 38]. Paraoxonases are a group of enzymes involved in organophosphates hydrolysis [44]. PON-1 is synthesized by the liver and transported to the plasma. It possesses an antioxidant activity and prevents low-density lipoprotein (LDL) oxidation.

**FIGURE 9.** Representative photomicrographs of TNF-α immunohistochemistry of liver sections. Panel A: saline control group, showing no positive reaction of TNF-α. Panel B: LPS only group, showing marked expression of TNF-α in hepatic cells. Panel C: LPS + GSE (100 mg/kg) group, showing mild expression of TNF-α. Panel D: LPS + GSE (50 mg/kg) and Vit C (50 mg/kg) group, showing weak expression of TNF-α (brown color indicating TNF-α positivity; x400).

**FIGURE 10.** Representative photomicrographs of TNF-α immunohistochemistry of kidney sections. Panel A: saline control group, showing no positive reaction of TNF-α. Panel B: LPS only group, showing marked expression of TNF-α within the glomeruli. Panel C: LPS + GSE (100 mg/kg) group, showing mild expression of TNF-α. Panel D: LPS + GSE (50 mg/kg) and Vit C (50 mg/kg) group, showing weak expression of TNF-α (brown color indicating TNF-α positivity; x400).
Feingold et al. [46] found that enzyme activity decreased in the serum within 24 h of LPS injection along with decreased PON1 mRNA in the liver at 4 h after LPS treatment.

GSE is a dietary antioxidant supplement, rich in vitamins, minerals, and polyphenols (flavonoids, proanthocyanidins, and procyanidins) [47]. In this study, the potential protective effect for GSE given at doses of 50 and 100 mg/kg was investigated in systemic endotoxemia caused by LPS injection. GSE given alone protected against the increased oxidative stress, reduced the expression of TNF-α and caspase-3, and ameliorated tissue injury in a dose-dependent manner. A previous study reported decreased nitric oxides in the plasma, liver, red blood cells, and spleen by GSE (200 mg/kg/day) in LPS-treated rats [48]. GSE contains polyphenolic compounds (procyanidins and proanthocyanidins) which possess potent free radical-scavenging capacity [49, 50]. In vitro, GSE or commercial polyphenols (gallic acid) inhibited nitric acid production from macrophages (RAW 264.7 cells) stimulated with LPS. GSE and [-]-epigallocatechin-3-gallate showed an inhibitory effect on the expression of iNOS in macrophages [51]. In previous studies, GSE prevented hepatic fibrosis produced by arsenic or thioacetamide administrations in rats [21, 52] and attenuated tramadol-alcohol hepatotoxicity, and increased antioxidant status in rats [53]. GSE inhibited hepatic stellate cells (HSCs) activation, subsequently suppressed α1 collagen mRNA level, and thus reduced collagen accumulation [54].

We also investigated the effect of the combined administration of GSE at 50 mg/kg and Vit C at 50 mg/kg during LPS-induced endotoxemia. Vit C is a potent antioxidant agent; it scavenges free radicals, superoxide, hydrogen peroxide, peroxyl, and singlet oxygen species [22] and has anti-inflammatory effect [24]. Our findings revealed that the combined treatment of GSE/Vit C was effective in almost normalizing serum creatinine, uric acid, aminotransferases, PON-1 activity, and also tissue markers of oxidative stress compared to the higher dose of GSE. Moreover, we observed almost total protection against the

**FIGURE 11.** Representative photomicrographs of TNF-α immunohistochemistry of brain cortex sections. Panel A: saline control group, showing no positive reaction of TNF-α. Panel B: LPS only group, showing marked expression of caspase-3. Panel C: LPS + GSE (100 mg/kg) group, showing mild expression of caspase-3. Panel D: LPS + GSE (50 mg/kg) and Vit C (50 mg/kg) group, showing weak expression of TNF-α (brown color indicating TNF-α positivity; ×400).

**FIGURE 12.** Representative photomicrographs of TNF-α immunohistochemistry of brain striatum sections. Panel A: saline control group, showing no positive reaction of TNF-α. Panel B: LPS only group, showing marked expression of TNF-α. Panel C: LPS + GSE (100 mg/kg) group, showing mild expression of TNF-α. Panel D: LPS + GSE (50 mg/kg) and Vit C (50 mg/kg) group, showing weak expression of TNF-α (brown color indicating TNF-α positivity; ×400).
injurious effects of LPS on the liver, kidney, and brain histopathological changes. Collectively, these data suggest that the combined treatment GSE/Vit C was more effective than treatment with GSE alone in diminishing the deleterious effects and multiple organ toxicity produced by LPS in mice, as confirmed by biochemical and histopathological and immunohistochemical investigations.

ACKNOWLEDGMENTS

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REFERENCES


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**TABLE 5.** Optical density measurements of caspase-3 immunoreactivity in the liver, kidney, and brain (cortex and stratum) in LPS-induced multiple organ toxicity in mice and the effect of GSE alone or in combination with Vit C

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Saline</th>
<th>LPS</th>
<th>GSE 50 + LPS</th>
<th>GSE 100 + LPS</th>
<th>GSE 50 + Vit C 50 + LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.041 ± 0.003</td>
<td>0.824 ± 0.014*</td>
<td>0.408 ± 0.006*</td>
<td>0.231 ± 0.005**</td>
<td>0.191 ± 0.002**#</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.036 ± 0.003</td>
<td>0.724 ± 0.010*</td>
<td>0.383 ± 0.006*</td>
<td>0.296 ± 0.016**#</td>
<td>0.217 ± 0.005**#</td>
</tr>
<tr>
<td>Cortex</td>
<td>0.033 ± 0.002</td>
<td>0.753 ± 0.003*</td>
<td>0.313 ± 0.004*</td>
<td>0.260 ± 0.005**#</td>
<td>0.168 ± 0.001**#</td>
</tr>
<tr>
<td>Striatum</td>
<td>0.026 ± 0.001</td>
<td>0.841 ± 0.0013*</td>
<td>0.371 ± 0.005#</td>
<td>0.209 ± 0.01#</td>
<td>0.174 ± 0.009#</td>
</tr>
</tbody>
</table>

Note: Results are presented as means ± SE (%) (n = 8). *, p < 0.05 vs. saline control; #, p < 0.05 vs. LPS only; +, p < 0.05 vs. GSE 50 mg/kg + LPS; @, p < 0.05 vs. GSE 100 mg/kg + LPS. Units for GSC and Vit C, mg/kg body weight.

**TABLE 6.** Optical density measurements of TNF-α immunoreactivity in the liver, kidney, and brain (cortex and stratum) in LPS-induced multiple organ toxicity in mice and the effect of GSE alone or in combination with Vit C

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Saline</th>
<th>LPS</th>
<th>GSE 50 + LPS</th>
<th>GSE 100 + LPS</th>
<th>GSE 50 + Vit C 50 + LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.053 ± 0.002</td>
<td>0.749 ± 0.006*</td>
<td>0.388 ± 0.011**#</td>
<td>0.226 ± 0.004**#</td>
<td>0.202 ± 0.005**#</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.038 ± 0.002</td>
<td>0.862 ± 0.015*</td>
<td>0.372 ± 0.006**#</td>
<td>0.268 ± 0.001**# #</td>
<td>0.213 ± 0.004**# #</td>
</tr>
<tr>
<td>Cortex</td>
<td>0.0341 ± 0.006</td>
<td>0.688 ± 0.007*</td>
<td>0.354 ± 0.004**#</td>
<td>0.272 ± 0.0012**#</td>
<td>0.177 ± 0.0031**# #</td>
</tr>
<tr>
<td>Striatum</td>
<td>0.031 ± 0.003</td>
<td>0.752 ± 0.005*</td>
<td>0.360 ± 0.012**#</td>
<td>0.233 ± 0.005**#</td>
<td>0.198 ± 0.004**# #</td>
</tr>
</tbody>
</table>

Note: Results are presented as means ± SE (%) (n = 8). *, p < 0.05 vs. saline control; #, p < 0.05 vs. LPS only; +, p < 0.05 vs. GSE 50 mg/kg + LPS; @, p < 0.05 vs. GSE 100 mg/kg + LPS. Units for GSC and Vit C, mg/kg body weight.


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