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RESEARCH ARTICLE

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

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CONTENTS

1. 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 ± 1ºC, 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 in to 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 Q 500IW.

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%,

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

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

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

Tissue	Saline	LPS	GSE 50 + LPS	GSE 100 + LPS	GSE 50 + Vit C 50 + LPS
Liver	34.61 ± 1.11	88.94 ± 1.26*	74.58 ± 1.03$^{*\#}$	65.86 ± 1.18$^{*\#+}$	40.25 ± 1.24$^{*\#+@}$
Kidney	26.21 ±1.23	76.54 ± 1.21*	66.23 ± 0.96$^{*\#}$	54.05 ± 1.19$^{*\#+}$	29.81 ± 1.14$^{\#+@}$
Brain	27.3 ± 1.42	60.53 ± 1.25*	51.38 ± 1.26$^{*\#}$	44.8 ± 1.67$^{*\#+}$	30.7 ± 1.76$^{*\#+@}$

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

Tissue	Saline	LPS	GSE 50 + LPS	GSE 100 + LPS	GSE 50 + Vit C 50 + LPS
Liver	13.54± 0.06	6.40 ± 0.04*	7.54 ± 0.02$^{*\#}$	8.50 ± 0.01$^{*\#}$	11.80 ± 0.03$^{*\#+@}$
Kidney	4.18 ± 0.01	2.20 ± 0.02*	2.43 ± 0.01*	2.80 ± 0.01$^{*\#}$	3.42 ± 0.06$^{*\#+@}$
Brain	3.74 ± 0.02	1.02 ± 0.04*	1.44 ± 0.02$^{*\#}$	1.89 ± 0.01$^{*\#}$	3.24 ± 0.13$^{\#+@}$

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.

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 4. Effect of GSE alone or in combination with Vit C on tissue NO in LPS-induced multiple organ toxicity in mice

Tissue	Saline	LPS	GSE 50 + LPS	GSE 100 + LPS	GSE 50 + Vit C 50 + LPS
Liver	57.8 ± 1.02	70.4 ± 1.22*	68.31 ± 1.1*	64.8 ± 1.1$^{*\#}$	51.8 ± 1.42$^{\#+@}$
Kidney	52.1 ± 0.98	68.2 ± 1.01*	64.2 ± 1.44*	58.3 ± 1.21$^{*\#}$	49.3 ± 0.87$^{\#+@}$
Brain	40.2 ± 0.87	65.1 ± 0.89*	60.54 ± 0.53*	55.8 ± 1.1$^{*\#+}$	37.3 ± 0.77$^{\#+@}$

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 NO, µmol/g tissue; for GSC and Vit C, mg/kg body weight.

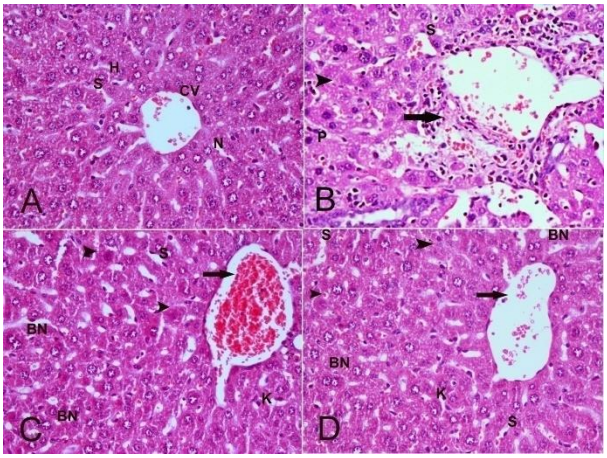

FIGURE 1. Representative photomicrographs of liver sections. Panel A: saline control group, showing a normal hepatic lobule having a central vein (V) and hepatocytes (H) with hepatic sinusoids (S) and prominent nucleus (N). Panel B: LPS only group, showing loss of lobular architecture and presence of necrosis of hepatocytes (arrowhead) and inflammatory cells infiltration (arrow). Central and blood sinusoids were dilated and congested (S) with pyknotic nuclei (P). Panel C: LPS + GSE (100 mg/kg) group, showing moderate ameliorative effect with mild central venous congestion (arrow), few necrotic hepatocytes (arrowhead), binucleated hepatocytes (BN), and activated Kupffer cells (K). Panel D: LPS + GSE (50 mg/kg) and Vit C (50 mg/kg) group, showing nearly normal liver architecture with few of necrotic areas (arrowhead) and binucleated hepatocytes (BN), and less significant dilation of the central vein (arrow) and blood sinusoids, and less activated Kupffer cells (K) (H&E stain, ×400).

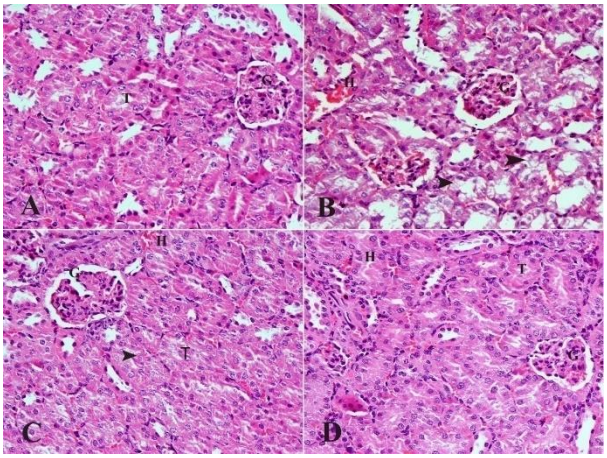

FIGURE 2. Representative photomicrographs of kidney sections. Panel A: saline control group, showing normal glomerulus with urinary space, and proximal and distal tubules with round nuclei (T). Panel B: LPS only group, showing severe degenerated glomeruli (G), the lining epithelium tubules (arrowhead), focal necrosis of tubules (T), and interstitial hemorrhage (H). Panel C: LPS + GSE (100 mg/kg) group, showing moderate degeneration of glomeruli (G) and lining epithelium tubules (arrowhead), little focal necrosis (T) and interstitial hemorrhage (H). Panel D: LPS + GSE (50 mg/kg) and Vit C (50 mg/kg) group, showing restored architecture with few degenerated of glomeruli (G) and little focal necrosis in tubules (T) and interstitial hemorrhage (H) (H&E stain, ×400).

levels 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 +

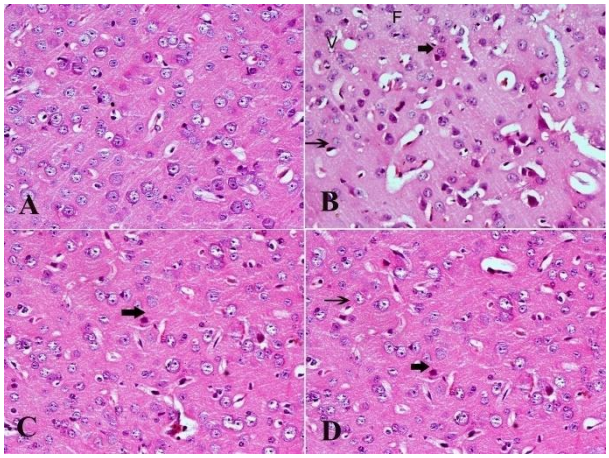

FIGURE 3. Representative photomicrographs of brain cortex sections. Panel A: saline control group, showing that neurons are arranged in neat rows and round nuclei are with basophilic cytoplasm. Panel B: LPS only group, showing marked cerebral necrosis, pyknotic (arrowhead), and apoptotic nuclei (arrow), surrounded by perineuronal vacuolations (V) and fatty changes (F). Panel C: LPS + GSE (100 mg/kg) group, showing less pyknotic nuclei (arrowhead). Panel D: LPS + GSE (50 mg/kg) and Vit C (50 mg/kg) group, showing distinct improvement with much less pyknotic (arrowhead) and apoptotic nuclei (arrow) (H&E stain, ×400).

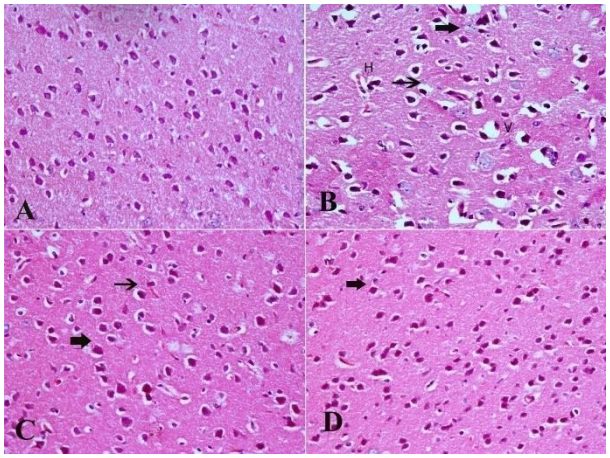

FIGURE 4. Representative photomicrographs of brain striatum sections. Panel A: saline control group, showing normal neuronal appearance. Panel B: LPS only group, showing marked necrosis, pyknotic (arrowhead), and apoptotic nuclei (arrow) were surrounded by perineuronal vacuolations (V) and hemorrhage (H). Panel C: LPS + GSE (100 mg/kg) group, showing less pyknotic nuclei (arrowhead) and apoptotic nuclei (arrow). Panel D: LPS + GSE (50 mg/kg) and Vit C (50 mg/kg) group, showing further distinct improvement with much less pyknotic nuclei (arrowhead) (H&E stain, ×400).

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 eosinophility. Extensive necrosis and

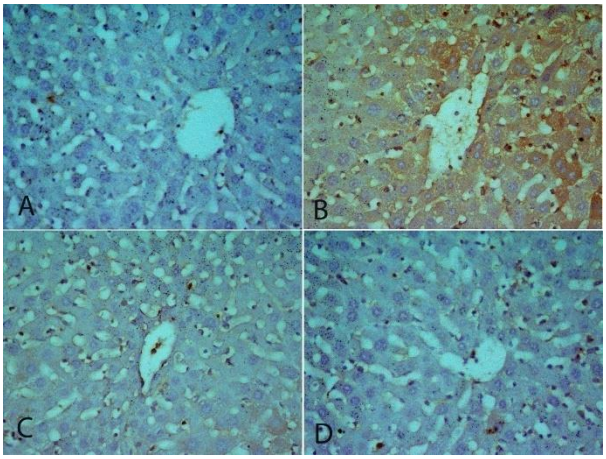

FIGURE 5. Representative photomicrographs of caspase-3 immunohistochemistry of liver sections. Panel A: saline control group, showing no positive reaction of caspase-3. Panel B: LPS only group, showing marked expression of caspase-3 in hepatic cells. Panel C: LPS + GSE (100 mg/kg) group, showing mild expression of caspase-3. Panel D: LPS + GSE (50 mg/kg) and Vit C (50 mg/kg) group, showing weak expression of caspase-3 (brown color indicating caspase-3 positivity; ×400).

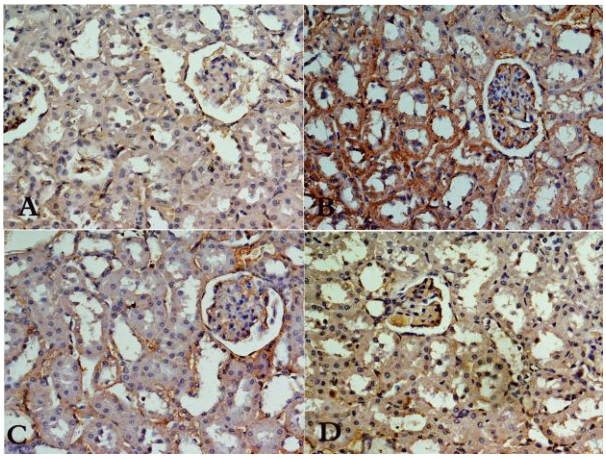

FIGURE 6. Representative photomicrographs of caspase-3 immunohistochemistry of kidney sections. Panel A: saline control group, showing no positive reaction of caspase-3. Panel B: LPS only group, showing marked expression of caspase-3 within the glomeruli. Panel C: LPS + GSE (100 mg/kg) group, showing mild expression of caspase-3. Panel D: LPS + GSE (50 mg/kg) and Vit C (50 mg/kg) group, showing weak expression of caspase-3 (brown color indicating caspase-3 positivity; ×400).

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

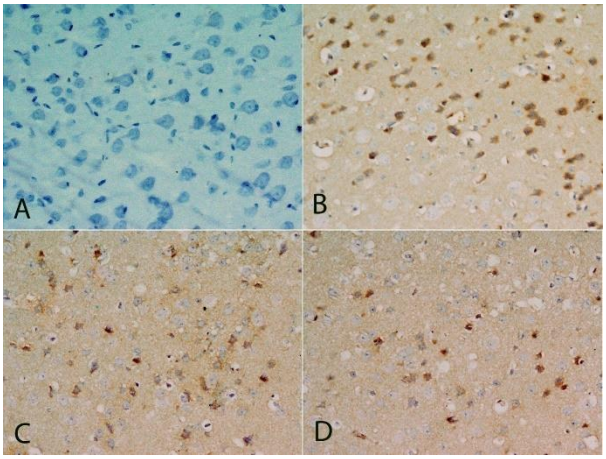

FIGURE 7. Representative photomicrographs of caspase-3 immunohistochemistry of brain cortex sections. Panel A: saline control group, showing no positive reaction of caspase-3. Panel B: LPS only group, showing marked expression of caspase-3. Panel C: LPS + GSE (100 mg/kg) group, showing mild expression of caspase-3. Panel D: LPS + GSE (50 mg/kg) and Vit C (50 mg/kg) group, showing weak expression of caspase-3 (brown color indicating caspase-3 positivity; ×400).

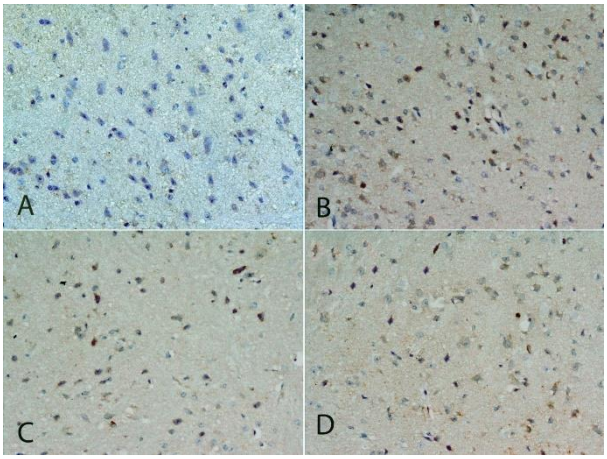

FIGURE 8. Representative photomicrographs of caspase-3 immunohistochemistry of brain striatum sections. Panel A: saline control group, showing no positive reaction of caspase-3. Panel B: LPS only group, showing marked expression of caspase-3. Panel C: LPS + GSE (100 mg/kg) group, showing mild expression of caspase-3. Panel D: LPS + GSE (50 mg/kg) and Vit C (50 mg/kg) group, showing weak expression of caspase-3 (brown color indicating caspase-3 positivity; ×400).

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,

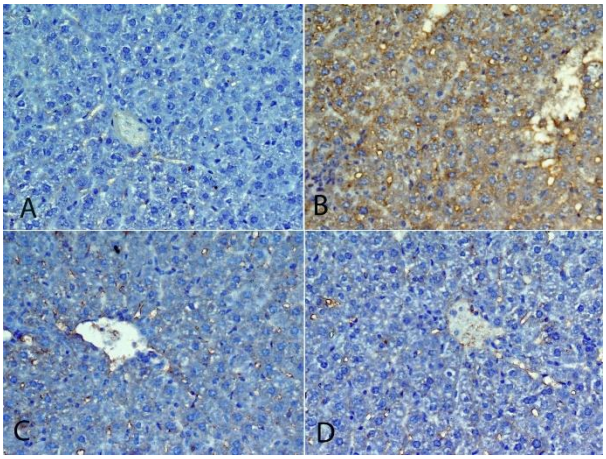

FIGURE 9. Representative photomicrographs of TNF-α immunohistochemistry of liver sections Panel A: saline control group, showing no positive reaction of TNF-α. Panel B: LPS only group, showing marked expression of TNF-α in hepatic cells. Panel C: LPS + GSE (100 mg/kg) group, showing mild expression of 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; ×400).

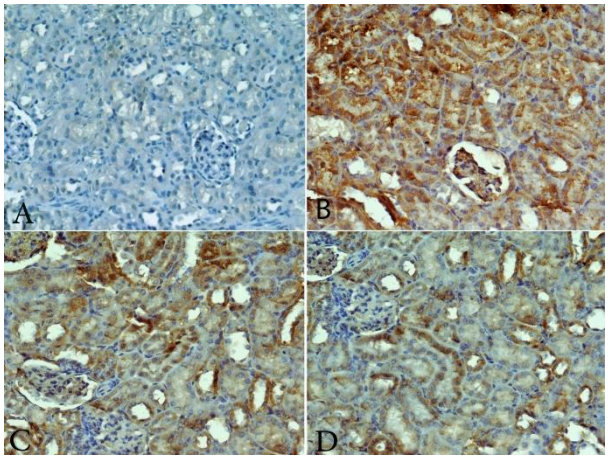

FIGURE 10. Representative photomicrographs of TNF-α immunohistochemistry of kidney sections. Panel A: saline control group, showing no positive reaction of TNF-α. Panel B: LPS only group, showing marked expression of TNF-α within the glomeruli. Panel C: LPS + GSE (100 mg/kg) group, showing mild expression 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; ×400).

IL-6, TNF-α, MCP-1, cycloygenase-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 cyclooygenase-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 inflammogen [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

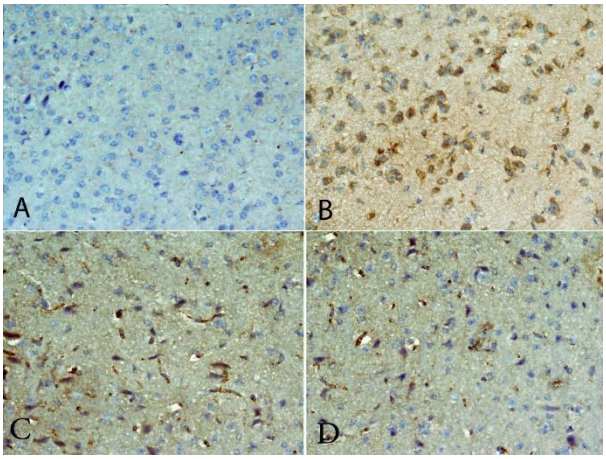

FIGURE 11. Representative photomicrographs of TNF-α immunohistochemistry of brain cortex sections. Panel A: saline control group, showing no positive reaction of TNF-α. Panel B: LPS only group, showing marked expression of caspase-3. Panel C: LPS + GSE (100 mg/kg) group, showing mild expression of caspase-3. 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).

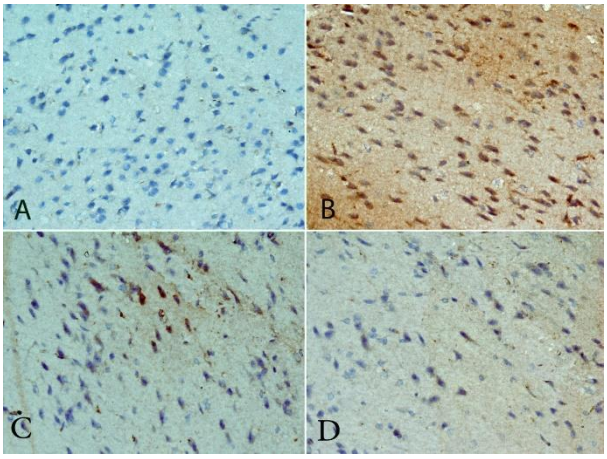

FIGURE 12. Representative photomicrographs of TNF-α immunohistochemistry of brain striatum sections. Panel A: saline control group, showing no positive reaction of TNF-α. Panel B: LPS only group, showing marked expression of TNF-α. Panel C: LPS + GSE (100 mg/kg) group, showing mild expression of TNF-α. 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).

[45]. 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

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

Tissue	Saline	LPS	GSE 50 + LPS	GSE 100 + LPS	GSE 50 + Vit C 50 + LPS
Liver	0.041 ± 0.003	0.824 ± 0.014*	0.408 ± 0.006$^{*\#}$	0.231 ± 0.005$^{*\#+}$	0.191 ± 0.002$^{*\#+}$
Kidney	0.036 ± 0.003	0.724 ±0.010*	0.383 ± 0.006$^{*\#}$	0.296 ± 0.016$^{*\#+}$	0.217 ± 0.005$^{*\#+@}$
Cortex	0.033 ± 0.002	0.753 ± 0.003*	0.313 ± 0.004$^{*\#}$	0.260 ± 0.005$^{*\#+}$	0.168 ± 0.001$^{*\#+@}$
Striatum	0.026 ± 0.001	0.841 ± 0.0013*	0.371 ± 0.005$^{*\#}$	0.209 ± 0.01$^{*\#+}$	0.174 ± 0.009$^{*\#+@}$

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

Tissue	Saline	LPS	GSE 50 + LPS	GSE 100 + LPS	GSE 50 +Vit C 50 + LPS
Liver	0.053 ± 0.002	0.749 ± 0.006*	0.388 ± 0.011$^{*\#}$	0.226 ± 0.004$^{*\#+}$	0.202 ± 0.005$^{*\#+}$
Kidney	0.038 ± 0.002	0.862 ± 0.015*	0.372 ± 0.006$^{*\#}$	0.268 ± 0.001$^{*\#+}$	0.213 ± 0.004$^{*\#+@}$
Cortex	0.0341 ± 0.006	0.688 ± 0.007*	0.354 ± 0.004$^{*\#}$	0.272 ± 0.0012$^{*\#+}$	0.177 ± 0.0031$^{*\#+@}$
Striatum	0.031 ± 0.003	0.752 ± 0.005*	0.360 ± 0.012$^{*\#}$	0.233 ± 0.005$^{*\#+}$	0.198 ± 0.004$^{*\#+@}$

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.

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.

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REFERENCES

1. Muller-Loennies S, Lindner B, Brade H. Structural analysis of oligosaccharides from lipopolysaccharide (LPS) of *Escherichia coli* K12 strain W3100 reveals a link between inner and outer core LPS biosynthesis. *J Biol Chem* 2003; 278(36):34090–101. doi: 10.1074/jbc.M303985200.
2. Lin WJ, Yeh WC. Implication of Toll-like receptor and tumor necrosis factor alpha signaling in septic shock. *Shock* 2005; 24(3):206–9.
3. Hua XY, Chen P, Fox A, Myers RR. Involvement of cytokines in lipopolysaccharide-induced facilitation of CGRP release from capsaicin-sensitive nerves in the trachea: studies with interleukin-1beta and tumor necrosis factor-alpha. *J Neurosci* 1996; 16(15):4742–8.
4. Qin L, Wu X, Block ML, Liu Y, Breese GR, Hong JS, et al. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia* 2007; 55(5):453–62. doi: 10.1002/glia.20467.

5. Jeong HK, Jou I, Joe EH. Systemic LPS administration induces brain inflammation but not dopaminergic neuronal death in the substantia nigra. *Exp Mol Med* 2010; 42(12):823–32. doi: 10.3858/emm.2010.42.12.085.
6. Abdel-Salam OME, Youness ER, Omara EA, Sleem AA. Effect of adipose tissue-derived mesenchymal stem cell treatment on oxidative stress and inflammatory response following Escherichia coli lipopolysaccharide. *Comp Clin Pathol* 2015; 24(2):343–58.
7. Abdel-Salam OME, Youness ER, Omara EA, El-Sayed El-Shamarka M, Sleem AA. Protection by intraperitoneal administration of bone marrow-derived stem cells of lipopolysaccharide-induced brain and liver damage in mice. *Comp Clin Pathol* 2016; 25(1):107–18.
8. Dal-Pizzol F, Tomasi CD, Ritter C. Septic encephalopathy: does inflammation drive the brain crazy? *Braz J Psychiatry* 2014; 36(3):251–8.
9. Decker K. Biologically active products of stimulated liver macrophages (Kupffer cells). *Eur J Biochem* 1990; 192(2):245–61.
10. Jaeschke H, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D, Lemasters JJ. Mechanisms of hepatotoxicity. *Toxicol Sci* 2002; 65(2):166–76.
11. Zhang C, Walker LM, Hinson JA, Mayeux PR. Oxidant stress in rat liver after lipopolysaccharide administration: effect of inducible nitric-oxide synthase inhibition. *J Pharmacol Exp Ther* 2000; 293(3):968–72.
12. Jaeschke H, Ho YS, Fisher MA, Lawson JA, Farhood A. Glutathione peroxidase-deficient mice are more susceptible to neutrophil-mediated hepatic parenchymal cell injury during endotoxemia: importance of an intracellular oxidant stress. *Hepatology* 1999; 29(2):443–50. doi: 10.1002/hep.510290222.
13. Sun S, Zhang H, Xue B, Wu Y, Wang J, Yin Z, et al. Protective effect of glutathione against lipopolysaccharide-induced inflammation and mortality in rats. *Inflamm Res* 2006; 55(11):504–10. doi: 10.1007/s00011-006-6037-7.
14. Yamakoshi J, Saito M, Kataoka S, Kikuchi M. Safety evaluation of proanthocyanidin-rich extract from grape seeds. *Food Chem Toxicol* 2002; 40(5):599–607.
15. Maffei Facino R, Carini M, Aldini G, Bombardelli E, Morazzoni P, Morelli R. Free radicals scavenging action and anti-enzyme activities of procyanidines from *Vitis vinifera*. A mechanism for their capillary protective action. *Arzneimittelforschung* 1994; 44(5):592–601.
16. Terra X, Montagut G, Bustos M, Llopiz N, Ardevol A, Blade C, et al. Grape-seed procyanidins prevent low-grade inflammation by modulating cytokine expression in rats fed a high-fat diet. *J Nutr Biochem* 2009; 20(3):210–8. doi: 10.1016/j.jnutbio.2008.02.005.
17. Nandakumar V, Singh T, Katiyar SK. Multi-targeted prevention and therapy of cancer by proanthocyanidins. *Cancer Lett* 2008; 269(2):378–87. doi: 10.1016/j.canlet.2008.03.049.
18. Mayer R, Stecher G, Wuerzner R, Silva RC, Sultana T, Trojer L, et al. Proanthocyanidins: target compounds as antibacterial agents. *J Agric Food Chem* 2008; 56(16):6959–66. doi: 10.1021/jf800832r.
19. Kong X, Guan J, Gong S, Wang R. Neuroprotective effects of grape seed procyanidin extract on ischemia-reperfusion brain injury. *Chin Med Sci J* 2017; 32(2):92–9. doi: 10.24920/J1001-9294.2017.020.
20. Abdel-Salam OME, Galal AF, Hassanane MM, Salem LM, Nada SA, Morsy FA. Grape seed extract alone or combined with atropine in treatment of malathion induced neuro- and genotoxicity. *J Nanosci Nanotechnol* 2018; 18(1):564–75. doi: 10.1166/jnn.2018.13943.
21. Nada SA, Gowifel AMH, El-Denshary ES, Salama AA, Khalil MG, Ahmed KA. Protective effect of grape seed extract and/or silymarin against thioacetamide-induced hepatic fibrosis in rats. *J Liver* 2015; 4(2):1–7.
22. Harrison FE, Bowman GL, Polidori MC. Ascorbic acid and the brain: rationale for the use against cognitive decline. *Nutrients* 2014; 6(4):1752–81. doi: 10.3390/nu6041752.
23. Mock JT, Chaudhari K, Sidhu A, Sumien N. The influence of vitamins E and C and exercise on brain aging. *Exp Gerontol* 2017; 94:69–72. doi: 10.1016/j.exger.2016.12.008.
24. El-Shafei RA, Saleh RM. Pharmacological effects of vitamin C & E on Diclofenac sodium intoxicated rats. *Biomed Pharmacother* 2016; 84:314–22. doi: 10.1016/j.biopha.2016.09.005.

25. Guo W, Huen K, Park JS, Petreas M, Crispo Smith S, Block G, et al. Vitamin C intervention may lower the levels of persistent organic pollutants in blood of healthy women: a pilot study. *Food Chem Toxicol* 2016; 92:197–204. doi: 10.1016/j.fct.2016.04.006.
26. Nuttall SL, Kendall MJ, Bombardelli E, Morazzoni P. An evaluation of the antioxidant activity of a standardized grape seed extract, Leucoselect. *J Clin Pharm Ther* 1998; 23(5):385–9.
27. Natella F, Belelli F, Gentili V, Ursini F, Scaccini C. Grape seed proanthocyanidins prevent plasma postprandial oxidative stress in humans. *J Agric Food Chem* 2002; 50(26):7720–5.
28. Paget GE, Barnes JM. Toxicity testing. In: *Evaluation of Drug Activities: Pharmacometrics* (DR Laurence, AL Bacharach). Academic Press, London, UK. 1964, pp. 1–135.
29. Mansour DFS, Eldenshary ES, S.A. N, Omara EA, Ibrahim MIM. Therapeutic effectiveness of certain whey proteins on lipopolysaccharide-induced oxidative stress and histopathological changes in rat liver. *J Appl Sci Res*2013; 9(8):4983–92.
30. Cocchetto DM, Bjornsson TD. Methods for vascular access and collection of body fluids from the laboratory rat. *J Pharm Sci* 1983; 72(5):465–92.
31. Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *Am J Clin Pathol* 1957; 28(1):56–63.
32. Bartels H, Bohmer M, Heierli C. [Serum creatinine determination without protein precipitation]. *Clin Chim Acta* 1972; 37:193–7.
33. Fossati P, Prencipe L, Berti G. Use of 3,5-dichloro-2-hydroxybenzenesulfonic acid/4-aminophenazone chromogenic system in direct enzymic assay of uric acid in serum and urine. *Clin Chem* 1980; 26(2):227–31.
34. Aldridge WN. Serum esterases. II. An enzyme hydrolysing diethyl *p*-nitrophenyl phosphate (E600) and its identity with the A-esterase of mammalian sera. *Biochem J* 1953; 53(1):117–24.
35. Ruiz-Larrea MB, Leal AM, Liza M, Lacort M, de Groot H. Antioxidant effects of estradiol and 2-hydroxyestradiol on iron-induced lipid peroxidation of rat liver microsomes. *Steroids* 1994; 59(6):383–8.
36. Moshage H, Kok B, Huizenga JR, Jansen PL. Nitrite and nitrate determinations in plasma: a critical evaluation. *Clin Chem* 1995; 41(6 Pt 1):892–6.
37. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959; 82(1):70–7.
38. Abdel-Salam OME, Omara EA, El-Shamarka ME-S, Hussein JS. Nigrostriatal damage after systemic rotenone and/or lipopolysaccharide and the effect of cannabis. *Comp Clin Pathol* 2014; 23:1343–58.
39. Abdel-Salam OME, Nada SA, Salem NA, El-Shamarka ME-S, Omara E. Effect of *Cannabis sativa* on oxidative stress and organ damage after systemic endotoxin administration in mice. *Comp Clin Pathol* 2014; 23(4):1069–85.
40. Jacewicz M, Czapski GA, Katkowska I, Strosznajder RP. Systemic administration of lipopolysaccharide impairs glutathione redox state and object recognition in male mice: the effect of PARP-1 inhibitor. *Folia Neuropathol* 2009; 47(4):321–8.
41. Wang H, Wu YB, Du XH. Effect of dexamethasone on nitric oxide synthase and caspase-3 gene expressions in endotoxemia in neonate rat brain. *Biomed Environ Sci* 2005; 18(3):181–6.
42. Iwase K, Miyanaka K, Shimizu A, Nagasaki A, Gotoh T, Mori M, et al. Induction of endothelial nitric-oxide synthase in rat brain astrocytes by systemic lipopolysaccharide treatment. *J Biol Chem* 2000; 275(16):11929–33. doi: 10.1074/jbc.275.16.11929.
43. Abdel-Salam OM, Youness ER, Mohammed NA, Abd-Elmoniem M, Omara E, Sleem AA. Neuroprotective and hepatoprotective effects of micronized purified flavonoid fraction (Daflon) in lipopolysaccharide-treated rats. *Drug Discov Ther* 2012; 6(6):306–14.
44. La Du BN, Adkins S, Kuo CL, Lipsig D. Studies on human serum paraoxonase/arylesterase. *Chem Biol Interact* 1993; 87(1–3):25–34.
45. Rajkovic MG, Rumora L, Barisic K. The paraoxonase 1, 2 and 3 in humans. *Biochem Med (Zagreb)* 2011; 21(2):122–30.
46. Feingold KR, Memon RA, Moser AH, Grunfeld C. Paraoxonase activity in the serum and hepatic mRNA levels decrease during the acute phase response. *Atherosclerosis* 1998; 139(2):307–15.
47. Weber HA, Hodges AE, Guthrie JR, O'Brien

BM, Robaugh D, Clark AP, et al. Comparison of proanthocyanidins in commercial antioxidants: grape seed and pine bark extracts. *J Agric Food Chem* 2007; 55(1):148–56. doi: 10.1021/jf063150n.

48. Pallares V, Fernandez-Iglesias A, Cedo L, Castell-Auvi A, Pinent M, Ardevol A, et al. Grape seed procyanidin extract reduces the endotoxic effects induced by lipopolysaccharide in rats. *Free Radic Biol Med* 2013; 60:107–14. doi: 10.1016/j.freeradbiomed.2013.02.007.
49. Bagchi D, Garg A, Krohn RL, Bagchi M, Tran MX, Stohs SJ. Oxygen free radical scavenging abilities of vitamins C and E, and a grape seed proanthocyanidin extract in vitro. *Res Commun Mol Pathol Pharmacol* 1997; 95(2):179–89.
50. Dulundu E, Ozel Y, Topaloglu U, Toklu H, Ercan F, Gedik N, et al. Grape seed extract reduces oxidative stress and fibrosis in experimental biliary obstruction. *J Gastroenterol Hepatol* 2007; 22(6):885–92. doi: 10.1111/j.1440-1746.2007.04875.x.
51. Houde V, Grenier D, Chandad F. Protective effects of grape seed proanthocyanidins against oxidative stress induced by lipopolysaccharides of periodontopathogens. *J Periodontol* 2006; 77(8):1371–9. doi: 10.1902/jop.2006.050419.
52. Pan X, Dai Y, Li X, Niu N, Li W, Liu F, et al. Inhibition of arsenic-induced rat liver injury by grape seed exact through suppression of NADPH oxidase and TGF-beta/Smad activation. *Toxicol Appl Pharmacol* 2011; 254(3):323–31. doi: 10.1016/j.taap.2011.04.022.
53. Nada SA, Eldenshary ES, Abdel Salam OME, Azmy SA, Mahdy T, Galal AF, et al. Grape seed extract attenuate tramadol-alcohol hepatotoxicity and increased antioxidant status in Sprague Dawley rats. *Curr Sci Int* 2014; 3(3):260–70.
54. Li J, Li J, Li S, He B, Mi Y, Cao H, et al. Ameliorative effect of grape seed proanthocyanidin extract on thioacetamide-induced mouse hepatic fibrosis. *Toxicol Lett* 2012; 213(3):353–60. doi: 10.1016/j.toxlet.2012.07.019.