



Effects of grape seed proanthocyanidin extract on side effects of high-dose methylprednisolone administration in male rats

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Abstract

In this study, we investigated the effects of grape seed proanthocyanidin extract (GSPE) against the side effects of high-dose administration of methylprednisolone (MP) in male rats. A total of 32 adult Wistar male albino rats were divided into four groups: (1) control (CON), received standard food only; (2) MP, received standard food + intraperitoneal injection of 60 mg/kg MP on day 7; (3) GSPE, received standard food + 200 mg/kg/day GSPE; and (4) MP + GSPE, received standard food + 200 mg/kg/day of GSPE + intraperitoneal injection of 60 mg/kg MP on day 7. All animals in the GSPE and MP + GSPE groups were treated once a day by oral gavage for 14 consecutive days. The feed intake of rats in the MP and MP + GSPE groups decreased significantly by 24.14% and 13.52%, respectively ($p < 0.05$). Administration of MP resulted in significant increases in serum concentrations of blood urea nitrogen ($p < 0.001$), glucose ($p < 0.01$), alkaline phosphatase, and adrenocorticotrophic hormone ($p < 0.05$). High-dose MP administration significantly reduced catalase ($p < 0.001$) and glutathione peroxidase ($p < 0.05$) concentrations in the liver and kidney tissues of rats, while glutathione concentrations were only reduced in liver tissue ($p < 0.05$). The expression levels of *Bcl-2* and *TNF- α* in liver, kidney, and testicular tissue were significantly increased, while the expression levels of *caspase-3* were reduced ($p < 0.001$). Furthermore, sperm concentration was significantly affected by GSPE in rats induced by high-dose MP, and sperm loss was significantly reduced in MP + GSPE ($p < 0.05$). These findings suggest that GSPE could be useful as a supplement to alleviate MP-induced toxicity in rats.

Keywords Grape seed proanthocyanidin extract · Methylprednisolone · Apoptosis · Biomarker · Sperm characteristic

Introduction

Glucocorticoids (GCs) are effective modulators of inflammation, suppressing the expression of inflammatory mediators, decreasing chemokine levels, promoting the elimination of apoptosis, and fostering macrophage phenotypic changes that suppress inflammation [1]. Therefore, corticosteroids

(CSs), which are synthetic analogs of GCs, are associated with profound changes in the pharmacogenomic and proteomic profiles of various tissues [2]. CSs are potent anti-inflammatory drugs that are used extensively in the treatment of various diseases, including rheumatoid arthritis [3], asthma [4], and some forms of lymphoma [5]. To achieve pharmacologically active levels of drugs at the site

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of inflammation, high doses must be administered systemically, and most of these systems accumulate the drugs in tissues other than those intended for therapy [6]. Therefore, it should be noted that higher doses and prolonged use of CSs may result in significant side effects, including osteoporosis, osteonecrosis, myopathies, immunosuppression, myalgia, atrophy, striae, telangiectasia, hypopigmentation, and delayed healing [7, 8].

Methylprednisolone (MP), a synthetic GC, is one of the most widely used GCs to stimulate immune cell apoptosis, inhibit inflammatory cytokines and neuronal death, promote axonal regeneration, and improve functional outcomes [9]. Several clinical studies have shown that MP has significant neuroprotective effects in the treatment of neurological diseases such as spinal cord injuries [10, 11] and multiple sclerosis [12, 13]. Furthermore, MP has been reported to have an antiapoptotic effect by upregulating the *bcl-X_L* protein, an isoform of the *bcl-x* gene, and activating GC receptors in oligodendrocytes in addition to its antioxidant and anti-inflammatory effects [14]. However, several reports indicate that MP therapy may cause osteonecrosis in patients [15], that high doses of MP have been shown to oxidize rabbit heart lipids [16] and that high doses of MP (over 1000 mg) administered to patients with autoimmune diseases such as multiple sclerosis for 5 days/week for either a week or 3 months may cause some cardiovascular complications in these patients [17]. Furthermore, drug-related toxic hepatitis is an important problem in clinical practice and is a frequent occurrence [18]. Therefore, several studies have suggested that high doses of MP may cause liver damage [18, 19].

Nevertheless, natural compounds in food have received increasing attention in recent years due to their potential health benefits. In addition, the administration of this dietary antioxidant is considered an essential component of traditional and alternative medicine [20]. A variety of antioxidants can be found in vegetables, seeds, and fruits [21]. One of them is grape seed, which is obtained as a byproduct of the juice or wine industry and contains a high level of polyphenols [22]. Depending on the variety, these seeds contain approximately 5–8% polyphenols, including catechin, epicatechin, gallic acid, epigallocatechin, and epicatechin 3-*O*-gallate, as well as procyanidin dimers, trimers, and highly polymerized procyanidins [23]. Polyphenols are known to prevent a number of pathological conditions by inhibiting the overproduction of reactive oxygen and nitrogen species. Consequently, they damage proteins, lipids, and DNA, alter signal transmission pathways, destroy membranes, and damage subcellular organelles, leading to cell death or apoptosis [24].

In particular, seed extracts are commonly used in dietary supplements due to their antioxidant properties [22]. Grape seed proanthocyanidin extract (GSPE), which is derived from grape seeds, is believed to possess a wide range of

biological, pharmacological, and therapeutic properties, including anti-inflammatory, antibacterial, antiviral, anticarcinogenic, antihypertensive, hypolipidemic, cardioprotective, hepatoprotective, and neuroprotective properties [25]. The effects of GSPE on perfluorooctanoic acid-induced hepatotoxicity have been shown to reduce oxidative stress, attenuate the inflammatory response, and inhibit hepatocellular apoptosis, while exposure to perfluorooctanoic acid results in liver damage in mice resulting in oxidative stress, inflammation, and apoptosis [26].

The liver, kidneys, and testicular tissue should be considered in the context of examining the effects of drugs and potential protective agents on vital organ systems. The liver commonly encounters various drugs and their metabolites during detoxification and metabolism, which may result in drug-induced toxicity [13]. Similarly, as vital organs for waste product filtering, excess fluid removal, and electrolyte management, kidneys play a critical role in maintaining systemic homeostasis, rendering them vulnerable to drug-induced toxicity that might impair renal function [27]. Testicular tissue also plays an important role in reproduction, and drug-induced toxicity may have a severe effect on fertility due to changes in sperm quality [28]. Therefore, it is imperative to assess the influence of such drugs on these interconnected organ systems, given the significant implications and potential consequences that may arise from their use. On the other hand, previous research has primarily focused on the biological effects of MP [11, 19, 29, 30], whereas the current study explores the mitigating properties of GSPE on vital organ systems, particularly in relation to the oxidative stress, hepatotoxicity, protein expression, and sperm characteristics associated with high-dose MP treatment. In addition, little information is available regarding the effects of GSPE on oxidative stress, hepatotoxicity, protein expression, and sperm characteristics when high-dose MP is administered. Therefore, the aim of this study is to address the current knowledge gaps by investigating the potential of GSPE in reducing oxidative stress, inhibiting hepatocellular apoptosis and inflammation, and preserving sperm functions in the context of high-dose MP-induced toxicity, thereby shedding light on its possible protective effects on liver, kidney, and testicular tissues.

Materials and methods

Ethics statement

All animal experiments were carried out with the approval of the Local Ethics Committee of Firat University (no. 2020/7-2) according to Directive 2010/63/EU on the protection of animals used for scientific purposes and the 1986 Animals Act of the United Kingdom (Scientific Procedures).

Animals

Adult Wistar male albino rats (20–30 weeks old; 413 ± 27.2 g) were housed in individual cages, temperature (21 ± 2 °C) and humidity controlled ($50 \pm 10\%$) animal facility with an artificial 12 h light–dark cycle in a standard housing environment with free access to food [commercial standard food for rats (88.0% dry matter, 23.5% crude protein, 3.3% ether extract, 6.1% crude fiber, 5.3% ash and 2800 kcal/kg metabolizable energy), Korkutelim Food Company, Antalya, Turkey] and filtered tap water.

Experimental design and groups

A total of 32 adult Wistar male albino rats were provided by the Balikesir University Experimental Research Center, Balikesir, Turkey. Animals were acclimatized for one week before being assigned to one of the following groups: (1) Control group, received standard food only; (2) MP group, received standard food + intraperitoneal injection of 60 mg/kg MP on day 7; (3) GSPE group, received standard food + 200 mg/kg/day GSPE; (4) MP + GSPE group, received standard food + 200 mg/kg/day GSPE + intraperitoneal injection of 60 mg/kg MP on day 7. All animals in the GSPE and GSPE + MP groups were treated once daily by oral gavage for 14 consecutive days. It should be noted here that the MP dose utilized in this investigation was in accordance with the "EULAR Standing Committee on International Clinical Studies including Therapeutic Trials" and was double the minimal amount specified for the "high dose" [31]. MP (methylprednisolone sodium succinate) and GSPE (95% proanthocyanidin) were obtained from Gensenta Ilac Sanayi ve Ticaret A.S. (Istanbul, Turkey) and Ari Muhendislik Ltd. Sti. (Ankara, Turkey), respectively.

Sample collection

Blood samples were collected from the deceased animals on day 14, the last day of the experiment. After centrifuging the blood samples at 2500 rpm and 4 °C for 15 min, they were stored at -20 °C until analysis was performed. For analyses, liver, kidney, and testicular tissue samples were also removed and stored at -80 °C.

Assessment of hepatoprotective activity

Using an automated biochemistry analyzer (RX Monaco, Randox Laboratories Ltd., Crumlin, UK), alanine aminotransferase (ALT, U/L), aspartate aminotransferase (AST, U/L), alkaline phosphatase (ALP, U/L), blood urea nitrogen (BUN, mg/dL), total protein (TP, g/dL), adrenocorticotrophic hormone (ACTH, ng/L), glucose (mg/dL), albumin (g/dL), creatine (mg/dL), and cortisol (ng/mL) were measured.

Biological markers of oxidative stress

Samples of the liver and kidney were homogenized separately in a Teflon-glass homogenizer with Tris-buffer (pH 7.4) to obtain 1:10 (w/v) whole homogenates. Following centrifugation for 45 min at 4 °C at 3500 rpm, the supernatants of these homogenates were used to quantify malondialdehyde (MDA), glutathione (GSH), glutathione peroxidase (GSH-Px), and catalase (CAT) activity. In tissue samples, the levels of MDA and GSH were measured using spectrophotometric methods described by Placer et al. [32] and Sedlak and Lindsay [33], respectively, and the results were expressed as nmol/g, whereas GSH-Px activity was measured using Lawrence and Burk [34]'s method and expressed as IU/g protein. The CAT activity of tissue samples was determined by measuring the rate at which H_2O_2 decomposes at 240 nm, following the method of Aebi [35], and was expressed as k U/g protein, where k denotes the first-order rate constant.

Western blot analysis

The tissue homogenization procedure was carried out according to the method described by Aslan et al. [36]. Using SDS–PAGE, equal amounts of liver, kidney, and testicular tissue protein samples were analyzed after determining the protein concentration of tissue samples [37]. Total proteins were then transferred to the nitrocellulose membrane, and the expression levels of *Bcl-2*, *caspase-3*, *TNF- α* and beta-actin proteins were determined [38]. A density determination analysis system (ImageJ; National Institutes of Health, Bethesda, USA) was used to determine the protein levels [39].

Assessment of sperm characteristics

Cauda epididymides of male rats were excised in a 35 mm culture plate containing TL-HEPES solution supplemented with 3.5 mg/mL BSA (fraction V) to collect sperm. Following dissection of the cauda epididymides with fine scissors, the sperm were allowed to swim out at room temperature for up to 15 min. Using a plastic transfer pipette, the sperm suspension was transferred from the pipette to a 5-mL tube for further experiments [40]. The number of spermatozoa in the right epididymis was determined using the method described by Sönmez et al. [41]. Using the standard method, sperm motility was determined by removing fluid from the cauda epididymis and diluting it with Tris buffer solution to 2 mL [41]. A phase-contrast microscope was used to assess the percent motility of the slides by placing an aliquot of this solution on each slide at a magnification of 400 \times . Samples for motility evaluation were maintained at a temperature of 35 °C, and the motility score was calculated by averaging

estimates from three different fields in each sample. On each slide, 200 sperm cells were examined for each animal [42].

Statistical analysis

Statistical analysis was conducted using the SPSS packet program (Version 21.0; SPSS, Armonk, NY, USA). Data are reported as the mean \pm standard error of means (SEM), with a significance level of $p < 0.05$. The values were compared using one-way variance analysis (ANOVA) and post hoc Tukey-HSD tests to determine the differences between all groups.

Results

Figure 1 illustrates the effects of GSPE on performance parameters in rats induced by high-dose MP. There were no significant differences between the groups with respect to body weight (BW) ($p > 0.05$). Following intraperitoneal injection of high-dose MP on day 7, the feed intake (FI) of rats in the MP and MP + GSPE groups decreased significantly by 24.14% and 13.52%, respectively ($p < 0.05$). A significant decrease in FI (0–14 d) was also observed in the high-dose MP-administered groups ($p < 0.05$), resulting in significant weight loss in the MP (− 1.38 g) and MP + GSPE (− 1.23 g) groups compared to the CON group (0.25 g) ($p < 0.01$).

ALT, AST, TP, albumin, and cortisol concentrations in rats induced by high-dose MP were not affected by GSPE ($p > 0.05$). In contrast, high-dose MP administration resulted in significant increases in serum concentrations of BUN (31.75%; $p < 0.001$), glucose (22.37%; $p < 0.01$), ALP (36.19%; $p < 0.05$), and ACTH (53.23%; $p < 0.05$) compared to the CON group. Furthermore, it is clear from Fig. 2 that

MP combined with GSPE was associated with significant reductions in serum BUN (12.84%; $p < 0.001$), glucose (9.70%; $p < 0.01$), ALP (18.91%; $p < 0.05$), and creatine (4.76%; $p < 0.05$) in comparison to MP alone.

As shown in Fig. 3, GSPE had a significant impact on the antioxidant parameters of the liver and kidney in rats induced by a high dose of MP. The concentrations of MDA in the kidney and liver increased after high-dose MP administration, but only the kidney showed statistical significance ($p < 0.001$). High-dose MP administration significantly reduced the concentrations of CAT ($p < 0.001$) and GSH-Px ($p < 0.05$) in the liver and kidney tissues of rats, while the concentrations of GSH were only reduced in the liver tissue ($p < 0.05$). Compared to the MP group, MP + GSPE had the same effect on CAT concentrations in the liver and GSH concentrations in the kidney. Furthermore, in the MP + GSPE group, kidney MDA concentrations were reduced, while GSH-Px concentrations increased, and no differences in CAT concentrations were observed in the MP + GSPE group compared to the MP group.

In Fig. 4, the effects of GSPE on sperm characteristics are shown in rats induced by high-dose MP. Of the sperm characteristics, only sperm were significantly affected by GSPE in rats induced by a high-dose MP ($p < 0.05$). As a result of high-dose MP administration, the sperm concentration decreased, while MP + GSPE maintained the level of the sperm concentration at the CON group.

In all types of studied tissue, the expression levels of *Bcl-2* and *TNF- α* were significantly increased after high-dose MP administration, while the expression levels of *caspase-3* were reduced (Fig. 5; $p < 0.001$). On the other hand, GSPE supplementation increased the level of *Bcl-2* expression in the liver, kidney, and testicular tissues of rats administered high-dose MP, while the expression of *caspase-3* in these tissues decreased. Furthermore, MP + GSPE maintained *TNF- α*

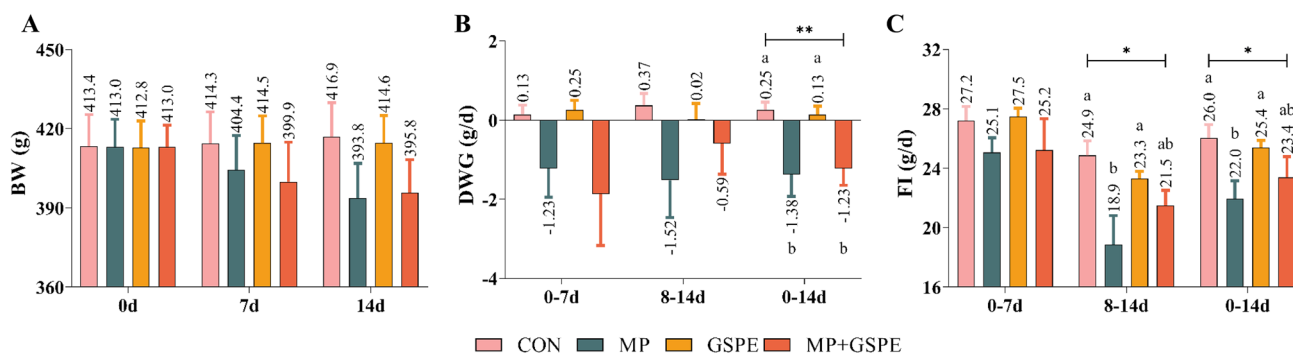


Fig. 1 Effects of grape seed proanthocyanidin extract on performance parameters in rats induced by high-dose methylprednisolone: **A** changes in body weight [BW]; **B** changes in daily weight gain [DWG]; **C** changes in feed intake [FI]. CON: control; MP: received standard food + intraperitoneal injection of 60 mg/kg methylprednisolone (MP) on day 7; GSPE received standard food + 200 mg/kg/day

grape seed proanthocyanidin extract (GSPE), GSPE+MP received standard food + 200 mg/kg/day GSPE + intraperitoneal injection of 60 mg/kg MP on day 7; * $p < 0.05$; ** $p < 0.01$. The asterisks highlight the importance levels of each parameter within the groups for the specified time period, with * indicating a statistically significant difference at $p < 0.05$ and ** indicating $p < 0.01$

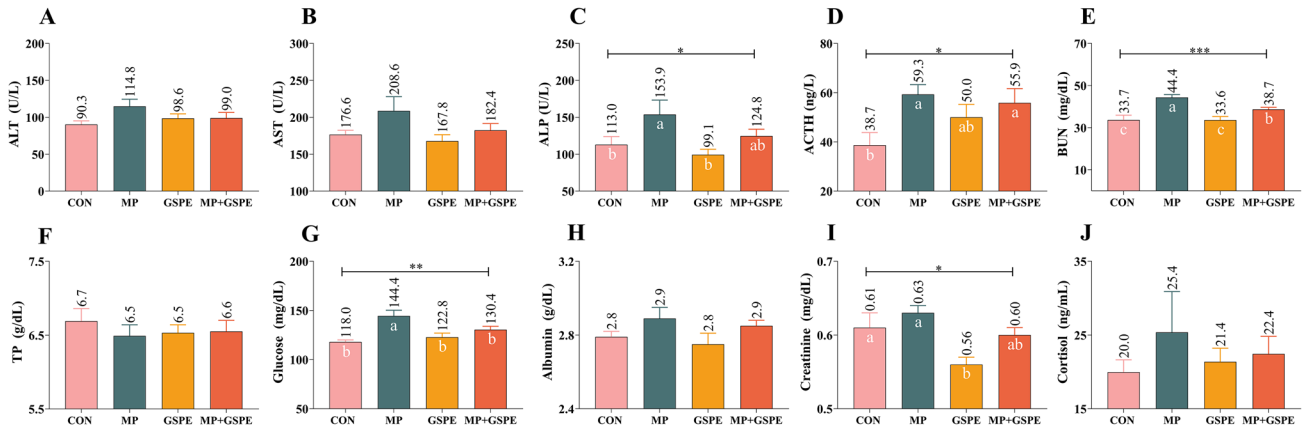


Fig. 2 Effects of grape seed proanthocyanidin extract on blood biochemistry in rats induced by high-dose methylprednisolone: **A** alanine aminotransferase (ALT); **B** aspartate aminotransferase (AST); **C** alkaline phosphatase (ALP); **D** adrenocorticotropic hormone (ACTH); **E** blood urea nitrogen (BUN); **F** total protein (TP); **G** glucose; **H** albumin; **I** creatinine; **J** cortisol. *CON* control, *MP* received standard food+intraperitoneal injection of 60 mg/

kg methylprednisolone (MP) on day 7, *GSPE* received standard food+200 mg/kg/day grape seed proanthocyanidin extract (GSPE), *GSPE+MP* received standard food+200 mg/kg/day GSPE+intraperitoneal injection of 60 mg/kg MP on day 7. The asterisks highlight the significance levels of each parameter within groups, with * indicating a statistically significant difference at $p < 0.05$, ** indicating a statistically significant difference at $p < 0.01$, and *** indicating a higher level of significance at $p < 0.001$

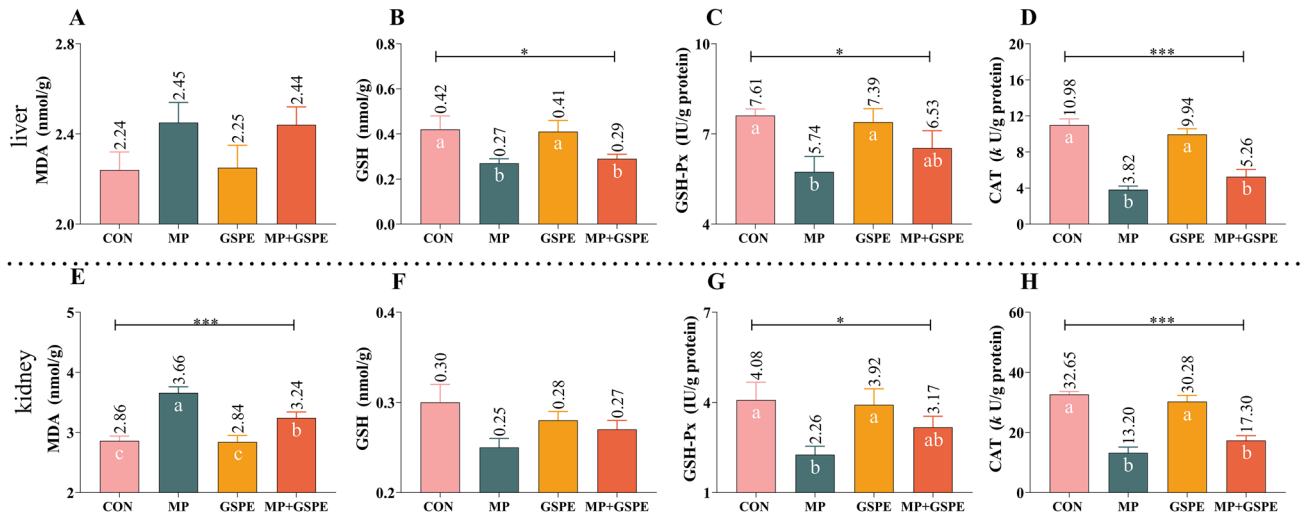


Fig. 3 Effects of grape seed proanthocyanidin extract on liver and kidney antioxidant parameters in rats induced by high-dose methylprednisolone: **A, E** malondialdehyde (MDA); **B, F** glutathione (GSH); **C, G** glutathione peroxidase (GSH-Px); **D, H** catalase (CAT); *CON* control, *MP* received standard food+intraperitoneal injection of 60 mg/kg of methylprednisolone (MP) on day 7, *GSPE* received

standard food+200 mg/kg/day of grape seed extract (GSPE), *GSPE+MP* received standard food+200 mg/kg/day of GSPE+intraperitoneal injection of 60 mg/kg of MP on day 7. The asterisks highlight the significance levels of each parameter within groups, with * indicating a statistically significant difference at $p < 0.05$ and *** indicating a higher level of significance at $p < 0.001$

expression in the kidney in the CON group and increased the expression level in the liver and testicular tissue.

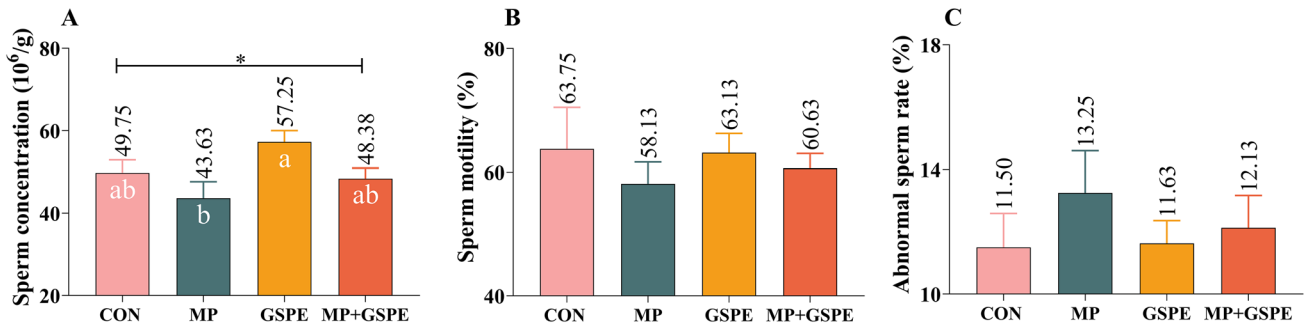


Fig. 4 Effects of grape seed proanthocyanidin extract on sperm characteristics in rats induced by high-dose methylprednisolone: **A** sperm concentration; **B** sperm motility; **C** abnormal sperm rate. *CON* control, *MP* received standard food + intraperitoneal injection of 60 mg/kg methylprednisolone (MP) on day 7. *GSPE* received standard

food + 200 mg/kg/day grape seed proanthocyanidin extract (GSPE), *GSPE+MP* received standard food + 200 mg/kg/day GSPE + intraperitoneal injection of 60 mg/kg MP on day 7. The asterisks highlight the significance level of each parameter within groups, with * indicating a statistically significant difference at $p < 0.05$

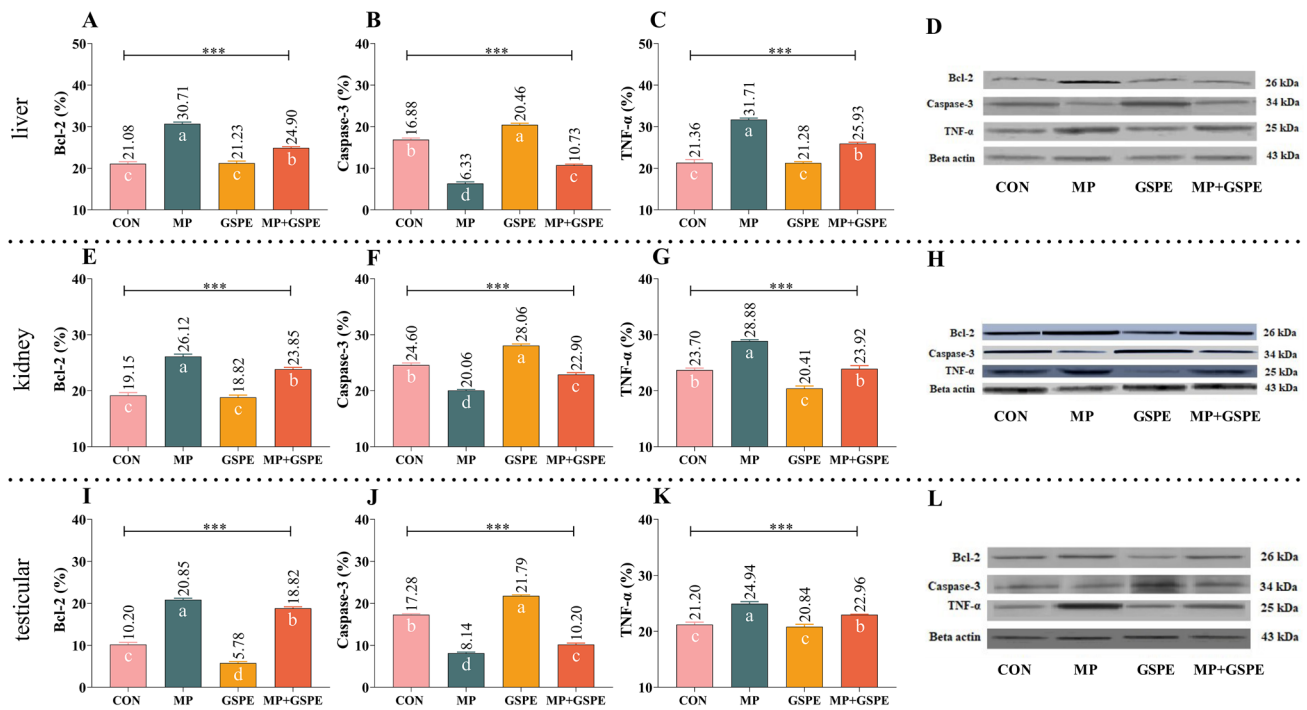


Fig. 5 Effects of grape seed proanthocyanidin extract on apoptosis and inflammation biomarkers and their expression levels in rats induced by high doses of methylprednisolone: *Bcl-2* (**A**, **E**, **I**); *caspase-3* (**B**, **F**, **J**); *TNF-α* (**C**, **G**, **K**); the expression levels of *Bcl-2*, *caspase-3*, *TNF-α* and beta-actin proteins (**D**, **H**, **L**). *CON* control, *MP* received standard food + intraperitoneal injection of 60 mg/kg methylprednisolone (MP) on day 7, *GSPE* received standard

food + 200 mg/kg/day grape seed proanthocyanidin extract (GSPE), *GSPE+MP* received standard food + 200 mg/kg/day GSPE + intraperitoneal injection of 60 mg/kg MP on day 7. The asterisks highlight the significance levels of each parameter within groups, with * indicating a statistically significant difference at $p < 0.05$, ** indicating $p < 0.01$, and *** indicating a higher level of significance at $p < 0.001$

Discussion

The use of GSPE, a natural source of antioxidants, as a supplement to lessen the negative effects of GC consumption has been extensively researched, and dexamethasone

(dex) was frequently used as a GC in these studies. Hasona et al. [43] showed that dex-induced oxidative stress may be mitigated and antioxidant defenses restored by administering GSPE at 400 mg/kg/day for four weeks after treatment and as preventive therapy for seven days. Another study indicated that when 7 mg/kg of dex was given daily and

100 mg/kg of GSPE was taken as a preventative, GSPE substantially reduced serum follicle-stimulating and luteinizing hormones, which had risen with dex treatment, and raised testosterone levels [44]. Compared to dex alone, the authors also demonstrated a rise in sperm and a decrease in abnormal sperm count [44]. Another study found that when 0.1 mg/kg of dex was administered three times a week for four weeks in a row, together with a preventive dose of 200–400 mg/kg of GSPE, GSPE reduced liver enzyme activities, alleviated cholesterol, triglycerides and LDL cholesterol levels, and restored superoxide dismutase and CAT enzyme activities, leading to a decrease in the level of MDA [20]. In this study, MP was used as a GC and administered intraperitoneally at a single dose of 60 mg/kg on day seven. To mitigate the negative impact of MP, we opted to administer a single oral dose of 200 mg/kg GSPE over a 14-day period. Our study focused on elucidating the potential adverse effects of MP on vital organs such as the liver, kidney, and testicular tissue while also assessing the potential ameliorative effects of GSPE on these vital organs and the biochemical alterations induced by MP in albino rats.

There is a strong correlation between food intake and stress responses, so each system can influence the other in eliciting a response. Feeding responses to stress are known to fluctuate in a bidirectional manner, with both increases and decreases in intake observed in response to stress. Several factors have been shown to contribute to stress-induced bidirectional feeding responses, including GC levels (depending on how severe the stressor is), GC interaction with feeding-related neuropeptides, such as neuropeptide Y (NPY), α -melanocyte stimulating hormone, agouti-related protein, melanocortins, urocortin, CRH, and peripheral signals [45]. In addition to bidirectional feeding responses, GCs also regulate glucose metabolism [46].

In the current study, after intraperitoneal injection of high-dose MP, the FI of rats in both the MP and MP + GSPE groups decreased significantly, as did weight loss. These results are in line with those obtained by Novelli et al. [47] and Hasona et al. [48]. GC therapy can have adverse effects, including insulin resistance and certain metabolic disorders, loss of appetite, and weight loss associated with elevated blood glucose levels and triglycerides [18, 47]. The present results support previous research that suggests that a reduction in FI in animals treated with different doses of GC can be attributed to the induction of leptin expression and the reduction of NPY, which is a potent inhibitor of FI [46, 47]. However, the FI of the MP + GSPE group was improved compared to that of the MP group with GSPE supplementation without any significant effect on BW. In contrast to this study, Hasona et al. [48] found that grape seed extract significantly increased BW compared to the control group in a study

with dexamethasone as the GC. An increased sensitivity to insulin, resulting in an increase in glucose uptake, may be responsible for this phenomenon [49, 50].

Hepatocellular injuries may be diagnosed by histopathological analysis of the liver or by biochemical markers found in the serum. The most sensitive biochemical markers for evaluating liver function are serum levels of AST, ALT, and ALP [51], and low levels are generally indicative of a healthy liver [52, 53]. In the present study, high-dose MP administration was associated with significant increases in serum ALP compared to the CON group. Additionally, MP combined with GSPE resulted in significantly lower serum ALP levels (18.91%) than MP alone. ALP is an important marker of bone turnover, as it dephosphorylates phosphates from a variety of molecules, including nucleotides, proteins, and alkaloids [54]. Furthermore, serum ALP levels serve as an indicator of excessive bone activity, with elevated levels indicating increased bone activity, resulting in increased bone loss in the future [55]. The alleviating effect observed in the MP + GSPE group could be related to the powerful antioxidant properties of GSPE, which possess a free radical scavenging effect and provide a protective effect against both internal and external free oxygen radicals. These results are in line with those obtained by Osuntokun et al. [56] and Yalçın and Çavuşoğlu [57].

ACTH secreted by the pituitary gland, one of the components of the hypothalamic-pituitary adrenal (HPA) axis, is controlled by corticotropin-releasing hormone (CRH), inhibited by adrenocortical hormone, and plays a crucial role in maintaining the balance of the neuroimmune endocrine system [58]. In addition to its role in the regulation of adrenocortical hormone, ACTH has also been recognized as an important physiological agonist of the melanocortin system [59]. ACTH performs a variety of physiological functions in addition to its role as an adrenal gland hormone, as evidenced by its affinity for melanocortin receptors. ACTH is believed to promote lipid breakdown, lower blood lipid levels, reduce pigmentation, maintain body energy balance, regulate the immune system, maintain sexual function, and secrete exocrine glands, thus protecting the kidney through both steroidal and nonsteroidal mechanisms [59]. The level of cortisol in the blood is also believed to be responsible for maintaining normal levels of GCs in the circulation through a negative feedback mechanism on the HPA axis [50]. The results of this study showed that despite a significant increase in the serum ACTH levels in groups using MP, GSPE and their combination, cortisol levels remained unchanged. ACTH has been shown to have a strong anti-inflammatory effect and directly protect the kidneys by inhibiting proliferation, infiltration, and expression of inflammatory cytokines in inflammatory cells [60]. Therefore, increased ACTH levels without a negative effect on cortisol concentration in blood serum and reduced pro-inflammatory cytokines in

the kidney suggest that GSPE may be useful in protecting against the side effects of MP toxicity.

In the current study, we also measured the main markers of kidney function, creatinine and BUN, to observe the effects of GSPE upon high-dose MP administration. A comparison of the serum concentrations of the kidney markers creatinine and BUN with those of MP alone revealed reductions of 4.76% and 12.84%, respectively. The results are consistent with those reported by Hasona et al. [43] and Kour et al. [21]. Meanwhile, it is well known that GCs regulate glucose metabolism either directly by affecting glucose production or indirectly by promoting insulin resistance in peripheral tissues [46]. Furthermore, as GC doses and durations increase, their ability to alter protein, carbohydrate, and lipid metabolism increases, leading to diabetes [61]. The glucose concentrations in the MP + GSPE groups decreased significantly by 9.70% compared to the MP group in the present study. The results of this study are consistent with those of other studies that have employed MP and GSPE [27, 48, 62].

The relevance of oxidative stress to nephrotoxicity and hepatotoxicity has been demonstrated in previous research [26, 42]. A well-known symptom of oxidative stress is an imbalance between reactive oxygen species (ROS) that exhibit systemic manifestations in a biological system and their ability to easily detoxify and repair intermediates. Since ROS play a significant role in redox signaling, oxidative stress can disrupt normal mechanisms of cell communication [63]. It is believed that changes in the redox state of a cell are believed to have toxic effects through the production of peroxides and free radicals that are capable of oxidizing biological molecules such as unsaturated lipids, proteins, and DNA [64].

Oxidative stress parameters such as MDA, GSH, GSH-Px, and CAT are often used to identify the extent of oxidative damage to tissues and organs. After high-dose MP administration, MDA, an end product of lipid peroxidation, increased significantly in kidney tissue but not in liver tissue. A significant decrease in MDA concentration was observed in the MP + GSPE group in response to GSPE supplementation by 11.48%. However, a significant increase in GSH-Px activity was observed in both liver and kidney tissue after GSPE supplementation, which protects the cell membrane against oxidative damage by maintaining the redox status of the proteins in the membrane. GSPE supplementation also significantly increased endogenous antioxidant CAT in the liver and kidney of rats in the MP + GSPE group compared to those in the MP group. Thus, GSPE supplementation is speculated to significantly reduce nephrotic and hepatic oxidative stress caused by high-dose MP administration by suppressing lipid peroxidation and increasing antioxidant enzyme activity. According to this study, GSPE exerts nephroprotective and hepatoprotective effects as

an antioxidant on high-dose MP-induced oxidative kidney and liver damage. These results are in agreement with those obtained by Shin et al. [65], Li et al. [63], and Liu et al. [26].

An energy-consuming process of cell death with DNA fragmentation, apoptosis is initiated by intrinsic and extrinsic mediators that are interconnected and affect one another [66]. Extrinsic pathways activate death receptors (*Fas* and *TNF- α*), which in turn activate *caspase-8* [67]. On the other hand, the intrinsic apoptotic pathway works directly without the involvement of a receptor mediator and disrupts the balance between antiapoptotic and proapoptotic proteins within cells, resulting in the activation of *caspase-3* and *caspase-9*, leading to the release of mitochondrial cytochrome C from cells [68]. As a result of the intrinsic apoptosis pathway, DNA fragmentation is also promoted, resulting in cell death. This pathway is also accompanied by activation of the *Bcl-2* protein, which alters the release of cytochrome C, causing cell death. Therefore, *Bcl-2* is also expressed in the outer membrane and regulates other intrinsic apoptosis cascades [66]. The application of GCs may also produce excessive amounts of ROS, which may result in apoptosis due to oxidative stress [69].

In this study, the expression levels of *Bcl-2* and *caspase-3* were measured as apoptosis markers, and the expression level of *TNF- α* was measured as an inflammation biomarker. Upon administration of high-dose MP, our studies showed an increase in the expression of *Bcl-2* and *TNF- α* in all liver, kidney, and testicular tissues. With GSPE supplementation, significant reductions in *Bcl-2* and *TNF- α* were observed in the MP + GSPE group. Contrary to expectations, *caspase-3* was significantly downregulated in the MP group, while GSPE supplementation significantly increased the upregulation of *caspase-3* in the MP + GSPE group compared with the MP group. In the MP group, low *caspase-3* expression is likely due to the suppression of *caspase-3* gene expression by MP. These results are in line with those obtained by Wang et al. [70], Bashir et al. [28] and Mohi-ud-din et al. [71] but differ from those obtained by Kandhare et al. [72].

Apoptosis and several hormones, such as testosterone, luteinizing hormone, and follicle stimulating hormone, significantly influence the evolution of normal spermatogenesis and the dynamic process of germ cell turnover in the testes [73]. Therefore, the process of spermatogenesis requires close coordination between apoptosis and hormonal control to maintain a balanced number of sperm cells. Furthermore, adequate levels of GCs are essential for the proper functioning of the testes [74]. In previous studies, GCs have been shown to have a direct effect on Sertoli cells, which provide structure and nutrition to all types of spermatogenic cells, as well as germ cells, thus restricting testicular development [75]. Furthermore, GC compounds are capable of disrupting spermatogenesis and inducing apoptosis by altering proapoptotic proteins such as *Fasl* and *Bax*, and upregulation

of these proteins is considered a sign of apoptosis in cells [76]. Excess apoptosis of germ cells results in loss of sperm [77]. The present study found that high-dose MP administration increased *Bcl-2* expression in testicular tissue; however, GSPE supplementation reduced sperm loss. In this regard, the antiapoptotic effect might be responsible for the reduction in apoptosis [28].

Overall, this study demonstrated that GSPE is effective in enhancing endogenous antioxidant enzyme activity, decreasing lipid peroxidation, minimizing inflammatory biomarkers, and inhibiting free radical generation and apoptosis. In male rats subjected to MP toxicity, GSPE was found to have nephroprotective, hepatoprotective, and reproductive effects. However, further studies are needed to understand the time- and dose-dependent processes underlying the protective characteristics. It has been shown to be useful in reducing MP-induced toxicity, but it is important to note that these findings may not be generalized to other GCs. The efficacy of GSPE may differ from one glucocorticoid to the next because of its varying adverse effects and tissue-specific activities. Consequently, more studies are required to evaluate the possible advantages of supplementation with GSPE when taking various GCs.

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Data availability The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

Ethics approval The study was conducted in accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes and the 1986 Animals Act of the United Kingdom (Scientific Procedures) and approved by the Ethics Committee of Firat University (no. 2020/7-2).

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