

Mitigating High-Fat Diet-Induced Testicular Oxidative Stress and Fibrosis with Bromelain

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ABSTRACT

Objectives: Obesity induced by a high-fat diet (HFD) is closely associated with impaired male fertility, primarily through oxidative stress, inflammation, and testicular dysfunction. Natural compounds with antioxidant and anti-inflammatory properties have gained increasing attention for their potential therapeutic effects. Bromelain, a proteolytic enzyme complex derived from *Ananas comosus*, exhibits antioxidant, anti-inflammatory, and antifibrotic activities, suggesting potential protective effects against obesity-related reproductive impairments.

Methods: Male Wistar rats were randomly assigned to four groups (n=9 per group). Animals were fed an HFD for 12 weeks to induce obesity, followed by one month of bromelain supplementation. Testicular tissues were histologically assessed using hematoxylin & eosin (H&E) and Masson's trichrome staining. Serum and testicular samples were analyzed for antioxidant and oxidative stress markers, including paraoxonase-1 (PON1), arylesterase (ARE), total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI).

Results: The HFD group showed significant testicular alterations, including thickening of the tunica albuginea, perivascular collagen accumulation, germ cell loss, and disrupted seminiferous tubule architecture (P<0.0001). Biochemically, TAS (P<0.0001) and PON1 (P=0.0041) levels were significantly decreased, whereas TOS (P<0.0001) and OSI (P<0.0001) levels were elevated. Bromelain supplementation mitigated histopathological changes, reducing collagen deposition and tunica albuginea thickness. Moreover, Bromelain significantly restored TAS and PON1 levels while decreasing TOS and OSI. No significant differences were observed in ARE (P=0.0002) activity between the groups.

Conclusions: Bromelain supplementation attenuated HFD-induced oxidative stress and fibrotic alterations in testicular tissue, improving both histological and biochemical parameters. These findings suggest that Br may be a potentially beneficial natural supplement for alleviating obesity-induced impairments in male reproductive functions and associated metabolic dysfunctions.

Keywords: Bromelain, Fibrosis, Oxidative Stress, Testis, High-Fat Diet

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Long-term consumption of a high-fat diet (HFD) leads to obesity, increased body weight, and disturbances in glucose and lipid metabolism, contributing to the development of various metabolic disorders [1, 2]. Accumulating evidence indicates that diet-induced obesity negatively impacts male fertility [3, 4]. High-calorie diets and obesity can directly or indirectly impair sperm quality through multiple mechanisms, including inflammatory responses, oxidative stress, alterations in gut microbiota, and sperm DNA damage and remodeling. The cumulative effects of these mechanisms may result in irreversible structural and functional alterations in sperm cells [5]. Moreover, obesity adversely affects spermatogenesis via hormonal imbalances, elevated leptin and estrogen levels, increased testicular temperature, and oxidative stress [6]. Oxidative stress, resulting from the oxidation of various cellular components, increases reactive oxygen species (ROS) levels, which can cause testicular atrophy, structural disruption of the seminiferous epithelium, germ cell loss, apoptosis in Sertoli and Leydig cells, reduced steroidogenesis, and impaired sperm production and motility [7]. Excess adipose tissue in obesity triggers systemic inflammatory responses and promotes oxidative stress through adipose-derived proinflammatory mediators, further compromising semen parameters [8]. Spermatozoa are particularly vulnerable to oxidative stress due to limited antioxidant defenses and insufficient DNA damage detection and repair capacity [9]. Additionally, studies have reported that HFD-induced endoplasmic reticulum (ER) stress contributes to reduced sperm motility and male reproductive dysfunction through mitochondrial signaling pathways [10, 11]. Nutritional supplements and plant-derived (phytochemical) preparations with antioxidant capacity have shown beneficial effects by modulating apoptosis and inflammatory processes. Therefore, these natural compounds are considered promising strategies for managing male infertility, particularly obesity-associated infertility, as antioxidant therapies and lifestyle modifications effectively mitigate oxidative stress-related reproductive impairments [12]. Bromelain (Br), a crude extract derived from the stem and fruit of *Ananas comosus*, consists of a group of thiol endopeptidases and other bioactive components, exhibiting diverse biological functions as a natural anti-inflammatory enzyme [13]. Its medicinal properties are primarily attributed to pro-

teolytic enzymes (proteases) and sulfur-containing protein-digesting enzymes, along with minor components such as peroxidases, acid phosphatases, protease inhibitors, and calcium ions. The biological activity of bromelain relies on the synergistic effects of these enzymes and proteins in modulating biochemical reactions within the organism [14]. Due to its anti-inflammatory, antioxidant, antiangiogenic, and analgesic properties, Br is considered a potential therapeutic and protective agent against inflammatory and oxidative stress-related pathologies, including testicular and nephrotoxicity [15-17]. Previous studies have demonstrated the protective potential of Br against various testicular impairments induced by different stressors. For instance, Jebur *et al.* [16] reported that aluminum chloride exposure causes oxidative stress, hormonal imbalance, and deterioration of sperm quality, whereas pretreatment with bromelain alleviated these harmful effects by enhancing antioxidant defense, suppressing ROS production, and improving testicular function. Similarly, Hosseinpour *et al.* [18] showed that bromelain exerts protective effects against Rapid Eye Movement (REM) sleep deprivation-induced testicular dysfunction through its strong antioxidant and anti-inflammatory properties. These findings collectively suggest that bromelain may play a beneficial role in preserving testicular integrity under conditions of oxidative and inflammatory stress.

HFD is a widely used model for inducing obesity. Obesity can negatively affect testicular function, causing decreased testosterone levels, increased oxidative stress, and impaired sperm production. Therefore, the HFD model provides a suitable experimental approach for studying obesity-related male reproductive disorders. In this study, we aimed to evaluate the protective and therapeutic effects of Br on testicular structure and biochemical changes in rats with HFD-induced obesity, and to clarify its possible role in reducing obesity-related reproductive disorders.

METHODS

Animals and Experimental Design

A total of 36 adult male Wistar Albino rats weighing 250-300 g were used in this study. All experimental procedures were conducted in accordance with the ARRIVE guidelines and approved by the Local Ethics

Committee of Necmettin Erbakan University (decision no: 2024-117, dated 28 November 2024). Animal care and use complied with the Guide for the Care and Use of Laboratory Animals. Rats were obtained from the Experimental Medicine Application and Research Center (KONÜDAM) of Necmettin Erbakan University. Animals were randomly assigned to four groups (n=9 per group): (1) standard diet control (SD), (2) standard diet + bromelain (SD+Br), (3) high-fat diet (HFD), and (4) high-fat diet + bromelain (HFD+Br). Animals were housed under controlled conditions at 21 ± 2 °C with a 12 h light/12 h dark photoperiod. SD and SD+Br groups received standard chow and water ad libitum for 12 weeks, whereas HFD and HFD+Br groups were fed a high-fat diet ad libitum for the same period. Body weights were monitored regularly.

Obesity Induction and Bromelain Administration

Obesity was induced in the HFD and HFD+Br groups over a 3-month period using a high-fat feed (Arden Research & Experiment Company, Ankara, Turkey). According to the manufacturer's nutritional analysis, this diet consisted of 24% protein, 30% carbohydrate, and 35% fat (primarily from vegetable oils), providing a total energy content of 5.2 kcal/g, approximately 45% of which was derived from fat. Rats in the SD and SD+Br groups received standard rat chow. During the establishment of the obesity model, body weights of all rats were monitored regularly [7]. Baseline weights were recorded, and subsequent measurements were taken approximately every 10 days. After the obesity model was established, rats in the SD+Br and HFD+Br groups were administered bromelain via oral gavage at a dose of 200 mg/kg/day for 30 days [19]. The bromelain preparation, with a digestive activity of 2400 GDU/g (Meteoric Biopharmaceutical, batch number: BM2403332), was freshly dissolved in physiological saline for daily administration. Each rat received 1 mL/100 g body weight (200 mg/kg) using a 16-gauge flexible feeding needle. Dosing was performed each morning (09:00–10:00) after absorption. A single researcher gently restrained each rat, inserted the gavage needle, and visually confirmed swallowing to ensure full administration of the dose. Any regurgitation events were immediately addressed with repeated dosing, and all incidents were recorded. Rats in the SD and HFD groups were similarly han-

dled and administered 1 mL/100 g body weight of physiological saline via oral gavage to control for handling and vehicle effects.

Histological Analyses

Testicular tissues were surgically excised and fixed in 10% formalin, dehydrated in graded alcohol series (70-100%), cleared in xylene, and embedded in paraffin. Sections of 5 μ m thickness were cut and stained with hematoxylin–eosin (H&E) and Masson's trichrome. All histological evaluations were performed in a blinded manner by two independent observers to minimize observer bias. For each specimen, systematic random sampling was employed to select microscopic fields, ensuring representative and unbiased assessment of the tissue sections.

Hematoxylin & Eosin (H&E) Staining

Tissue sections obtained from paraffin-embedded blocks were deparaffinized by immersion in xylene for three consecutive 20-minute intervals. The sections were then sequentially rehydrated through a graded ethanol series (100%, 90%, 80%, 70%, and 50%) and rinsed with distilled water to remove residual alcohol. For nuclear staining, sections were immersed in hematoxylin solution, followed by thorough washing under running tap water. Differentiation was performed using acid alcohol to remove excess stain, and sections were subsequently blued in alkaline water. Eosin staining was then applied to visualize cytoplasmic components. The sections were dehydrated through an ascending ethanol series (70%, 80%, 90%, and 100%), cleared in xylene, and mounted with a coverslip using an appropriate mounting medium for microscopic examination.

In H&E stained testicular sections, the histological structure of the seminiferous tubules and interstitial areas was evaluated in detail. For each experimental group, ten randomly selected seminiferous tubule areas were examined under $\times 40$ objective magnification using a light microscope (AxioCam Erc 5s, Carl Zeiss AG, Germany). Histopathological changes in the seminiferous tubules and disruptions in spermatogenesis were scored according to Johnsen's spermatogenesis evaluation criteria. Scoring was performed in a blinded manner, and the collected data were subjected to statistical analysis (Table 1) [20].

TABLE 1. Johnsen Scoring System for the Evaluation of Spermatogenesis.

Score	Evaluation of spermatogenesis
1	No cells visualized in tubular cross section
2	Sertoli cells only
3	Only spermatogonia present
4	No sperm cells or spermatids, few spermatocytes (<5)
5	No sperm cells or spermatids, presence of spermatocytes
6	No sperm cells, few spermatids (<5 to 10)
7	No sperm cells, presence of spermatids
8	Presence of few sperm cells (<5 to 10)
9	Some sperm cells, with a disorganized epithelium
10	Compete spermatogenesis with mature sperm cells

Masson's Trichrome Staining

Sections of 5 μm thickness were obtained from the testis blocks of experimental groups to assess fibrosis. Deparaffinization was conducted by passing the sections through xylene and a descending alcohol series (90%, 80%, 70%, and 50%). Masson's Trichrome Stain Kit (ChemBio, CB6095.0200, İstanbul, Türkiye) was then applied. The sections were covered with Entellan[®] after final rinsing with the alcohol series and xylene.

Biochemical Analyses

At the end of the experimental period, 10 mL of blood was collected from all animals into gel-coated biochemical tubes. The collected blood samples were centrifuged at $1500 \times g$ for 10 minutes to obtain the serum fraction. The serum was aliquoted into labeled Eppendorf tubes and stored at -80°C until analysis. On the day of analysis, all serum samples were thawed simultaneously to room temperature and vortexed to ensure homogeneity before biochemical measurements. Tissue samples obtained post-anesthesia were homogenized, sonicated, centrifuged, and the resulting supernatants were used for biochemical assays. Oxidative stress parameters analyzed in serum included paraoxonase-1 (PON1) and arylesterase (ARE). Serum PON1 (Rel Assay Diagnostics Kits, Cat. no:

RL0031, Mega Tıp San., Gaziantep, Turkey) and ARE (Rel Assay Diagnostics Kits, Cat. no: RL0055, Mega Tıp San., Gaziantep, Turkey) activities were measured according to the manufacturer's protocols. In tissue samples, total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI) were determined.

Measurement of PON1 Activity

Serum PON1 activity was assessed using a fully automated method developed by Rel Assay Diagnostics (Cat. no: RL0031). The assay evaluates paraoxonase activity based on the hydrolysis of paraoxon (diethyl p-nitrophenyl phosphate) in the presence of NaCl (basal and salt-stimulated activity). The formation of p-nitrophenol was monitored by absorbance increase at 412 nm at 37°C . The amount of p-nitrophenol was calculated using a molar extinction coefficient of $17,000 \text{ M}^{-1} \text{ cm}^{-1}$ at pH 8. Net enzymatic activity was calculated by subtracting basal activity from salt-stimulated activity and expressed in units per liter, where 1 unit corresponds to the hydrolysis of 1 μmol of substrate per minute per liter [21].

Measurement of ARE Activity

Serum ARE activity was determined using a fully automated method (Rel Assay Diagnostics, Cat. no: RL0055). Phenyl acetate was used as a substrate and hydrolyzed to produce phenol and acetic acid. The resulting phenol reacted with 4-aminoantipyrine and potassium ferricyanide to form a colored complex, measured spectrophotometrically. ARE activity was calculated using the molar absorption coefficient of the colored complex and expressed as units per liter, with 1 unit defined as the hydrolysis of 1 μmol of phenyl acetate per minute per liter [22].

Measurement of TAS

TAS was measured using commercially available kits (Rel Assay Diagnostics Kits, Cat. no: RL0017). The fully automated method is based on the suppression of the characteristic color of the ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical cation by antioxidants in the sample. The assay has an ideal deviation of less than 3%, and results were expressed in mmol Trolox equivalents per liter [23].

Measurement of TOS

TOS was measured using commercially available kits (Rel Assay Diagnostics Kits, Cat. no: RL0024). In this method, oxidants in the sample oxidize the ferrous ion–o-dianisidine complex to ferric ion, which in an acidic medium reacts with xylenol orange to form a colored complex. The color intensity, measured spectrophotometrically, is proportional to the total oxidant molecules present. Results were expressed in μmol hydrogen peroxide equivalents per liter ($\mu\text{mol H}_2\text{O}_2$ equiv/L) [24].

Calculation of OSI

The oxidative stress index (OSI) was calculated as the ratio of TOS to TAS. TAS values were converted to $\mu\text{mol/L}$, and OSI was determined using the following formula:

$$\text{OSI (arbitrary unit)} = \frac{\text{TOS } (\mu\text{mol H}_2\text{O}_2 \text{ equivalent/L})}{\text{TAS (mmol Trolox equivalent/L)}} \times 100 \text{ [25, 26].}$$

Statistical Analysis

Normality was assessed using the Shapiro–Wilk test. Intergroup differences were analyzed using one-way ANOVA followed by Tukey's post hoc test when appropriate. Data are presented as mean \pm standard deviation (SD), and $P < 0.05$ was considered statistically significant. Statistical analyses were performed using Microsoft Office 365 and GraphPad Prism 8.

RESULTS

Hematoxylin-Eosin Staining Findings

Microscopic examination of H&E-stained testicular sections revealed that the SD and SD+Br groups exhibited normal seminiferous tubule architecture, characterized by an intact basal membrane with spermatogonia, dispersed Sertoli cells, and well-organized germinal cells, including spermatids and spermatozoa.

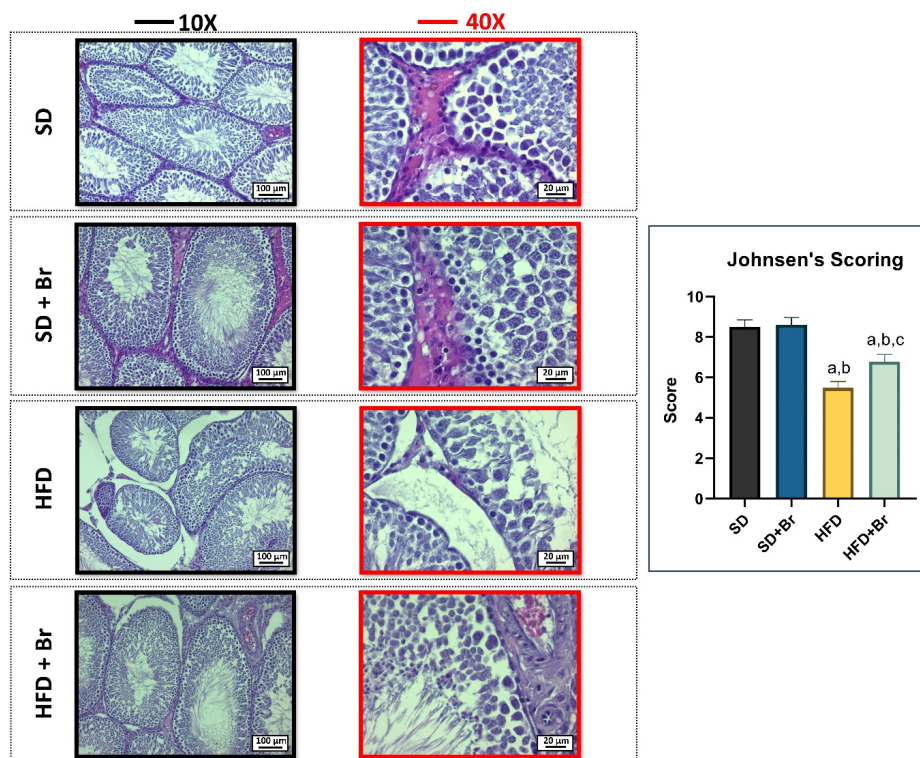


FIGURE 1. Hematoxylin–Eosin (H&E) staining of testicular tissue sections. Each experimental group consisted of 9 rats. In the standard diet (SD) and standard diet+Bromelain (SD+Br) groups, seminiferous tubules exhibited normal architecture with spermatogonia in the basal membrane, dispersed Sertoli cells, well-organized germinal cells, and morphologically normal Leydig cells. In the high-fat diet (HFD) group, seminiferous tubules displayed germ cell degeneration, vacuolization, atrophy, disorganization, and a reduction in the interstitial area. In the high-fat diet+Bromelain (HFD+Br) group, focal atrophy was observed; however, the overall seminiferous tubular structure was largely preserved, and the interstitial area appeared normal. Statistical analysis of Johnsen's scores is presented as mean \pm SD. ^a($P < 0.05$ vs. SD), ^b($P < 0.05$ vs. SD+Br), ^c($P < 0.05$ vs. HFD).

The interstitial connective tissue and Leydig cells appeared structurally intact. In contrast, testicular sections from the HFD group demonstrated marked histopathological alterations in germ cells, including degeneration, vacuolization, atrophy, and disorganization of the seminiferous epithelium. The interstitial space was also reduced in this group. In the HFD+Br group, focal atrophy of seminiferous tubules was observed; however, the overall tubular architecture was largely preserved, and the interstitial area maintained normal morphology. Mean Johnsen scores were calculated for 10 randomly selected seminiferous tubules per animal according to the criteria outlined in Table 1. Statistical analysis revealed that the HFD and HFD+Br groups differed significantly from the SD and SD+Br groups. While the HFD+Br group had significantly lower scores than the SD and SD+Br

groups, it showed significantly higher scores compared to the HFD group ($P < 0.0001$) (Figure 1).

Masson's Trichrome Staining of Testicular Tissue

Masson's Trichrome staining was performed to visualize collagen fibers and fibrosis in the connective tissue. In the SD group, the tunica albuginea layers of the testes exhibited normal thickness, whereas in the SD+Br group, the tunica albuginea was slightly thinner. In the HFD group, the tunica albuginea layers were observed to be thicker compared to the SD and SD+Br groups. In the HFD+Br group, the tunica albuginea thickness resembled that of the SD group. Examination of collagen fibers in the perivascular area revealed an increase in the HFD group relative to the other groups (Figure 2).

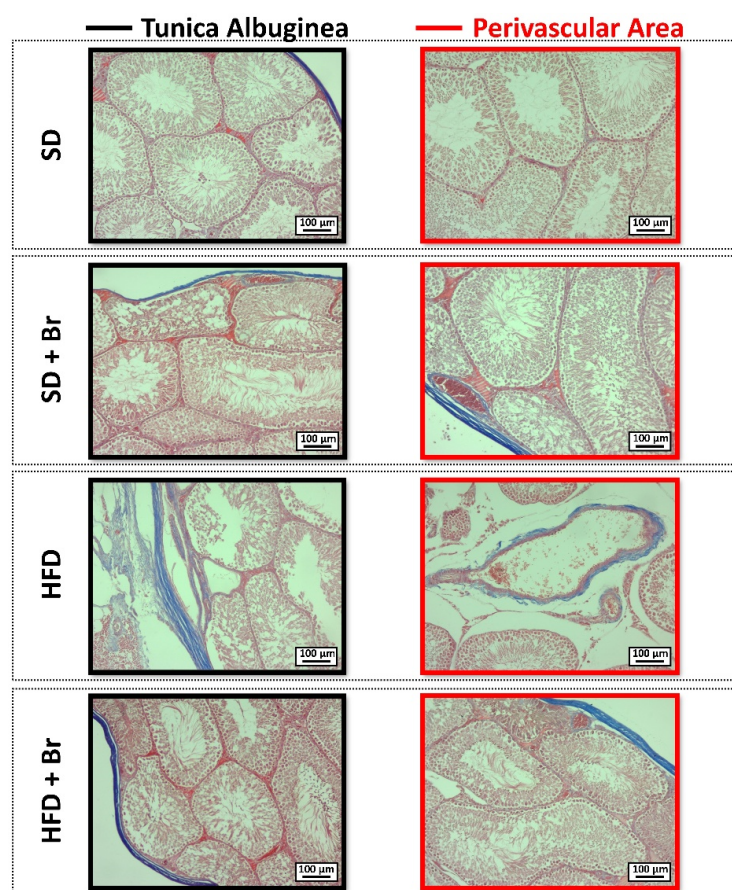


FIGURE 2. Masson's Trichrome staining of testicular tissue. Each experimental group consisted of 9 rats. In the standard diet (SD) group, the tunica albuginea exhibited normal thickness, while in the standard diet + Bromelain (SD+Br) group, slight thinning was observed. In the high-fat diet (HFD) group, the tunica albuginea was markedly thickened, and an increased amount of collagen fibers was observed in the perivascular area. In the high-fat diet+Bromelain (HFD+Br) group, the tunica albuginea thickness was similar to that of the SD group. Areas stained blue indicate the density of collagen fibers.

Biochemical Findings

Serum PON1 and ARE Results

PON1 enzyme activity, which decreases under conditions of elevated oxidative stress and organ damage, was found to be high and comparable in the SD and SD+Br groups, whereas it was lower in HFD group. In the HFD+Br group, PON1 activity was lower than in the SD and SD+Br groups but slightly higher than in the HFD group. Statistical analysis re-

vealed that PON1 levels in the HFD group were significantly different from those in the SD, SD+Br, and HFD+Br groups ($P=0.0041$). When the ARE enzyme activities were examined across all groups, the activity in the HFD group was found to be significantly decreased compared to the SD+Br group ($P=0.0002$).

Tissue TAS, TOS, and OSI Results

TAS, an important biochemical parameter reflect-

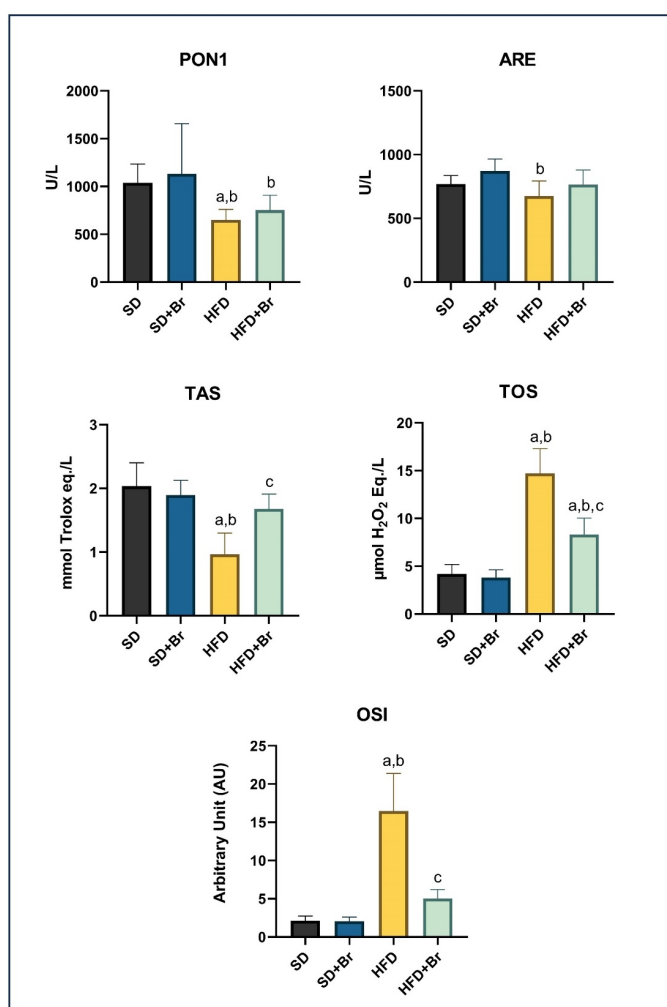


FIGURE 3. Biochemical parameters in serum and testicular tissues across all experimental groups. Each experimental group consisted of 9 rats. Serum paraoxonase-1 (PON1) and arylesterase (ARE) enzyme activities are shown. PON1 activity was high in the standard diet (SD) and standard diet + Bromelain (SD+Br) groups, whereas a significant decrease was observed in the high-fat diet (HFD) group. In the high-fat diet+Bromelain (HFD+Br) group, PON1 activity was partially higher than in the HFD group but remained lower than in the SD and SD+Br groups. There was a statistically significant difference in ARE activity between SD+Br and HFD groups. In testicular tissue, total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI) levels are presented. TAS levels were high in the SD and SD+Br groups, significantly decreased in the HFD group, and increased in the HFD+Br group. TOS levels were low in the SD and SD+Br groups, elevated in the HFD group, and reduced in the HFD+Br group. OSI values were low in the SD and SD+Br groups, high in the HFD group, and significantly decreased in the HFD+Br group. Data are presented as mean±SD. Statistical significance is indicated by ^a($P<0.05$ vs. SD), ^b($P<0.05$ vs. SD+Br), ^c($P<0.05$ vs. HFD).

ing the overall antioxidant capacity of the organism, was evaluated in testicular tissue. TAS levels were high and similar in the SD and SD+Br groups, while a decrease was observed in the HFD group. In the HFD+Br group, TAS levels increased. Statistical analysis indicated that the HFD group differed significantly from the SD, SD+Br, and HFD+Br groups ($P < 0.0001$).

TOS was measured to assess the intensity of oxidative stress and monitor the efficacy of antioxidant interventions. TOS levels were low in the SD and SD+Br groups, whereas a significant increase was observed in the HFD group. In the HFD+Br group, TOS levels decreased but remained higher than in the SD group ($P < 0.0001$).

OSI, reflecting the balance between oxidant and antioxidant systems, was low in the SD and SD+Br groups. Conversely, OSI values were markedly elevated in the HFD group. In the HFD+Br group, a significant reduction and improvement in OSI were observed ($P < 0.0001$) (Figure 3).

DISCUSSION

Approximately 20% of reproductive problems in couples worldwide are increasingly attributed solely to male infertility, which can arise from multiple factors, including metabolic disorders such as obesity [27]. Obesity is a systemic metabolic disorder affecting numerous major organs, with the testes being among the most severely impacted [7]. In this context, the present study employed a HFD for 12 weeks to establish an obesity model in rats. Following confirmation of obesity, animals received Br treatment for 30 days to investigate whether Br could mitigate obesity-induced testicular impairments through histopathological assessment, oxidative stress parameters, and fibrosis evaluation.

The present findings are consistent with previous studies indicating that prolonged exposure to HFD leads to reproductive dysfunction. For instance, Demirci and Şahin [28] demonstrated that HFD altered testicular histology and negatively affected spermatogenesis, as assessed using Johnsen scores. Similarly, Mohammadi Roushandeh *et al.* [29] reported that oxidative stress induced by HFD and obesity adversely affected sperm motility, morphology,

and viability, directly impacting spermatogenesis. In line with our findings, they observed histological alterations in testicular tubules, including vacuolization, reduced mature sperm counts, depletion of sperm from the epididymal lumen, and basal membrane disruption. The role of oxidative stress in testicular fibrosis and collagen synthesis is also supported by the literature. Cultured human fibroblasts exposed to oxidative stress have been shown to exhibit increased collagen synthesis [30]. Masson-Trichrome staining allows for the quantitative evaluation of collagen fibers in interstitial edema and peritubular fibrosis [31]. In our study, HFD exposure led to increased collagen fiber content in both the tunica albuginea and perivascular regions of the testicular tissue, confirming prior reports of collagen up-regulation in mammalian testes [32].

Antioxidant dietary supplements and phytochemicals may provide beneficial effects in the treatment of obesity-related reproductive disorders by reducing apoptosis and inflammation, thereby preserving testicular function [12]. In particular, Br, a proteolytic enzyme derived from *Ananas comosus*, enhances antioxidant capacity by reducing lipid peroxidation and other oxidative stress markers. Bromelain exhibits antioxidant properties by scavenging free radicals and ROS [33–37]. Supplementation with Br has been reported to improve sperm count and morphology, elevate testosterone levels, and enhance antioxidant enzyme activities [18]. Additionally, ethanolic extracts of *Ananas comosus* and Br protect cellular components against oxidative damage via ROS detoxification and the glutathione redox cycle [38]. Previous studies demonstrated that Br activates the Nrf2 antioxidant pathway and suppresses NF- κ B-mediated inflammatory responses in non-reproductive tissues (e.g., liver, lung) and cell models [39, 40]. In light of these data, the protective effects observed in our HFD-induced testicular dysfunction model may be mediated, at least in part, via similar modulation of Nrf2 and NF- κ B signalling. Consistent with these findings, our study demonstrated that Br treatment histologically ameliorated HFD-induced testicular damage.

PON1 protects low-density lipoprotein (LDL) from oxidation and neutralizes hydrogen peroxide and peroxidized phospholipids in oxidized LDL [41]. Due to structural similarities between LDL and cellular membranes, some authors have suggested that HDL-associated PON1 antioxidant activity provides general

protection against membrane lipid peroxidation [42]. Serum PON1 activity has been inversely correlated with oxidative stress in serum and macrophages, and PON1 deficiency has been reported to exacerbate oxidative stress. Elkiran *et al.* [43] found that serum PON1 and ARE activities were significantly lower in smoking lung cancer patients compared to smoking healthy controls. Similarly, in our study, the partial restoration of PON1 activity following Br supplementation suggests that Br not only suppresses oxidative load but may also positively modulate lipid metabolism and enzymatic antioxidant defense mechanisms. The absence of significant changes in ARE activity indicates that this enzyme may be more resistant to diet-induced oxidative challenges relative to other parameters [44]. Regarding ARE activity, the absence of significant changes may be due to cell type-specific or substrate-specific responses, meaning that the measured antioxidant response might not fully reflect the actual intracellular changes [45, 46]. Additionally, some enzymes may be influenced by adaptive regulatory mechanisms; under prolonged oxidative stress or elevated antioxidant load, certain enzymes can be downregulated or maintained at a steady activity via feedback mechanisms [47]. Therefore, unchanged ARE activity does not necessarily indicate ineffective antioxidant defense but may reflect substrate specificity or adaptive enzyme regulation.

In our study, the HFD-induced decreases in PON1 and TAS levels, together with increases in TOS and OSI, indicate that a high-fat diet suppresses endogenous antioxidant defenses while increasing oxidant load. Br supplementation exerted strong antioxidant effects, evidenced by increased TAS and significant reductions in TOS and OSI. Moreover, the strong negative correlation between TOS and TAS suggests that elevated oxidant burden reduces antioxidant capacity, shifting the oxidative/antioxidative balance toward oxidants [23]. Similarly, Akkoca *et al.* [48] demonstrated that ischemia-reperfusion (IR) decreases TAS, increases TOS, and that MitoTEMPO exerts protective effects against these changes. Bromelain has also been shown to reduce AGE receptor (RAGE)-mediated inflammation and tissue damage by disrupting advanced glycation end products (AGE)-associated pathways [49–51].

Several studies have demonstrated that HFD induces oxidative stress and testicular dysfunction in rat models. For instance, micronutrient-based antioxidant interventions have been shown to ameliorate HFD-induced sperm and testicular oxidative damage [52], while quercetin supplementation reduced oxidative stress and improved spermatogenesis in HFD-fed Wistar rats [53]. These studies provide relevant context and support the role of oxidative mechanisms in HFD-induced reproductive impairment, reinforcing the rationale for investigating Br's protective effects.

Overall, our results indicate that Br supplementation can partially restore testicular structure and oxidative balance in HFD-induced obese rats, highlighting its potential as a protective agent against obesity-associated reproductive dysfunction. Future studies should further investigate the protective effects of Br on HFD-induced testicular dysfunction at a detailed molecular level. Direct assessment of Nrf2 and NF- κ B signalling pathways, evaluation of different doses and treatment durations, and long-term safety profiling are warranted. Additionally, the effects of Br on other reproductive parameters and hormone levels, as well as its clinical applicability, should be explored. Such investigations would provide a clearer understanding of the potential therapeutic role of bromelain in male reproductive health.

Strengths and Limitations

Strengths of the study include the use of a well-controlled experimental design, the incorporation of both biochemical and histological evaluations to provide comprehensive insight into tissue alterations, and the application of standardized methods for assessing oxidative stress and reproductive parameters. The study also contributes valuable preliminary data regarding the potential protective effects of Br against diet-induced testicular damage, thereby establishing a foundation for future mechanistic and translational research. However, it should be noted that this study was conducted in an experimental rat model, which may not fully replicate human pathophysiology. The sample size was relatively limited, and only short-term Br treatment was evaluated. In addition, molecular pathways underlying the protective effects of Br were not investigated in depth. Therefore, further studies involving larger cohorts, longer treatment periods, and

mechanistic analyses are required to confirm the translational relevance of these findings. Another limitation of this study is that, although sham gavage was performed in the SD group, the potential adverse effects of repeated gavage-induced stress on oxidative and reproductive parameters were not elucidated. Additionally, firstly no effect size calculations were reported, which limits the interpretation of the magnitude of the observed differences; secondly, a priori power analysis was not performed, and therefore the risk of type II errors cannot be excluded.

CONCLUSION

Our findings indicate that the increase in oxidative stress parameters induced by HFD can be alleviated by Br administration, leading to improvements in the biochemical and histological structure of rat testicular tissue. Consequently, Br treatment may be considered a protective and therapeutic agent against diet-induced testicular damage.

Ethics Approval and Consent to Participate

This study was approved by the Necmettin Erbakan University KONUDAM Experimental Medicine Application and Research Center Animal Experiments Local Ethics Committee (Decision No: 2024-117; date: 28.11.2024). All experimental procedures involving animals were conducted in accordance with the ethical standards of the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Data Availability

All data generated or analyzed during this study are included in this published article. The data that support the findings of this study are available on request from the corresponding author, upon reasonable request.

Authors' Contribution

Study Conception: BG, RÖK, MBB; Study Design: BG, HTC; Supervision: BG, RÖK; Funding: BG, HTC, MBB; Materials: MBB, RÖK; Data Collection and/or Processing: BG, HBS, ZISG; Statistical Analysis and/or Data Interpretation: ZISG, HBS; Literature

Review: BG, HTC, RÖK; Manuscript Preparation: BG, MBB, HBS; and Critical Review: BG, HTC, ZSIG.

Conflict of Interest

The author(s) disclosed no conflict of interest during the preparation or publication of this manuscript.

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Generative Artificial Intelligence Statement

The entire content of this study was produced by the author(s) in full compliance with scientific research methodologies and academic ethical standards. The author(s) affirm that all scientific components of the manuscript were independently generated by themselves, and explicitly state that ChatGPT 4.0 AI was used solely for English language editing, without contributing to the scientific interpretation, analysis, or conclusions of the study.

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