Hindawi
Journal of Food Biochemistry
Volume 2023, Article ID 5562120, 11 pages
https://doi.org/10.1155/2023/5562120

Research Article

Exploring the Impact of Ketogenic Diet and Intermittent Fasting on Male Rats' Testicular Health: An Analysis of Hormonal Regulation, Oxidative Stress, and Spermatogenesis

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Received 22 June 2023; Revised 5 September 2023; Accepted 9 September 2023; Published 4 October 2023

Academic Editor: Duygu Ağagündüz

This study aimed to investigate the effects of ketogenic diet (KD), intermittent fasting (IF), and their combination on testicular tissue and hormonal changes in adult male rats. Twenty-eight adult male rats were randomly divided into four groups: control diet (CD), KD, IF, and KD combined with IF (IF + KD). Body and testicular weights were recorded, serum estradiol and testosterone levels were analyzed, and oxidative stress parameters and nitric oxide (NO) in testicular tissue were assessed. Furthermore, histopathological analysis of testicular tissue was conducted. KD notably enhanced body and testis weights and testosterone levels and reduced estradiol levels. Additionally, KD and IF + KD improved oxidative status by decreasing malondialdehyde and myeloperoxidase and increasing glutathione, catalase, and NO in testicular tissue. Histopathological examination showed enhanced spermatogenesis in KD and IF + KD groups. These findings suggest KD and IF may enhance male fertility via hormonal, oxidative, and histological improvements.

1. Introduction

The ketogenic diet (KD) is a dietary regimen high in fats and low in protein [1]. Initially implemented in the 1920s, KD influences glucose metabolism and insulin resistance, contributing significantly to the treatment of metabolic diseases [2]. Despite the demonstrated contributions of KD to heightened energy metabolism and potential enhancements in sexual activity, the clarity around its direct influence on fertility is still emerging [3]. However, recent evidence suggests that KD may have beneficial effects on male reproductive health. Specifically, studies have indicated that KD can significantly improve sperm quality, increasing the percentage of sperm exhibiting normal motility and morphology, thereby potentially enhancing fertility [3–5]. These intriguing observations highlight the need for further research to fully elucidate the potential fertility benefits of the ketogenic diet.

Intermittent fasting (IF) is a dietary strategy that involves cycles of restricted or completely absent caloric intake. IF is increasingly recognized for its potential to influence metabolic physiology, leading to the improvement of glucose tolerance [6]. Extensive studies have validated the positive effects of IF on diabetes mellitus, positioning it as an effective

intervention in the disease management [7]. Significantly, IF not only impacts metabolic disorders but also shows promise in improving male reproductive health. Research suggests that IF may enhance sperm parameters, including sperm count and quality, as well as testicular weight, thereby potentially bolstering fertility [8, 9].

It is well established that the type and quantity of fats consumed in the diet can affect semen quality through changes in metabolism [10]. These metabolic changes can lead to increased oxidative stress, contributing to DNA damage, lipid peroxidation, and disruption of antioxidant balance in the male reproductive system [11]. This oxidative stress can result in apoptosis, inflammation, and conditions often associated with infertility. Infertility linked to these changes typically involves impaired spermatogenesis, altered glucose metabolism in the blood-testis barrier, and a reduction in testosterone synthesis and secretion [12, 13]. Several biochemical markers are often used to assess these changes: malondialdehyde (MDA) in the testicular tissue indicates oxidative stress and myeloperoxidase (MPO) represents both oxidative stress and inflammation, while superoxide dismutase (SOD), glutathione (GSH), and catalase (CAT) represent the antioxidant defense mechanisms [14]. Interestingly, in vitro studies have shown that ketone bodies are utilized as an energy source for sperm motility and weight loss induced by intermittent fasting has been proven to reduce sperm DNA damage and improve semen quality [15, 16].

Our study addresses a notable research gap by investigating the combined effects of the KD and IF on the male reproductive system. Despite the potential of each approach individually, their synergy remains underexplored. With this knowledge, our study aims to analyze the acute impact of the KD and IF combination on oxidative stress, histopathological alterations, hormonal equilibrium, and NO levels within testicular tissue.

2. Materials and Methods

2.1. Chemicals and Animals. Twenty-eight mature male Wistar-albino rats, between 10 and 12 weeks of age and with an average weight of 220–250 grams, were procured from Sakarya University Medical Faculty's Experimental Animal Center (SÜDETAM). These rats were randomly divided into four distinct groups, each comprising of seven rats. The study was conducted in accordance with the ethical approval granted by the Sakarya University Animal Experiments Local Ethics Committee (Decision No: 08, Date: 22.02.2023).

The experimental design was set to last four weeks, with each of the twenty-eight male rats assigned to one of four distinct dietary groups. Both the standard feed and the high-fat diet feed used in the study were provided by Arden Research and Experiment, Ankara, Turkey.

2.1.1. Control Diet Group (CD; n = 7). Rats in this group were freely fed with standard pellet food throughout the experiment. The food was designed to offer 70.5% of energy from carbohydrates, 24% from proteins, and the remaining 5.5% from fats, for a total caloric value of 3000 kcal/kg.

2.1.2. Ketogenic Diet Group (KD; n = 7). This group was freely fed a high-fat, low carbohydrate special feed throughout the experiment. This diet provided 74.24% of energy from fats, 19.53% from proteins, and a minimal 4.95% from carbohydrates, yielding a total caloric value of 4095 kcal/kg.

2.1.3. Intermittent Fasting + Control Diet Group (IF + CD; n = 7). Rats in this group followed an alternate-day fasting regimen throughout the experiment, which involved fasting for 24 hours on two nonconsecutive days per week. During these fasting periods, they had unrestricted access to water, while on nonfasting days, they were freely fed with standard pellet food.

2.1.4. Intermittent Fasting + Ketogenic Diet Group (IF + KD; n = 7). Similar to the IF + CD group, these rats also followed a regimen of fasting for 24 hours on two nonconsecutive days per week throughout the experiment. They had unrestricted access to water during their fasting periods but were freely fed a high-fat, low-carbohydrate special feed on nonfasting days.

Throughout the experiment, we ensured that the dietary intake of the experimental animals in all groups maintained an isocaloric balance. This was achieved by considering the average calorie requirements of the rats and adjusting their food intake accordingly.

2.2. Establishing an Isocaloric Balance. In our study, we achieved this balance by closely monitoring and adjusting the amount of feed consumed by each group of rats. Following an initial ad libitum feeding trial, we determined the amount of food consumed and the equivalent caloric intake for both the standard and ketogenic diet groups. Based on these measurements, we were then able to adjust the quantities of each diet to ensure an isocaloric balance, thereby ensuring that all rats received the same amount of total daily calories [17, 18].

2.3. Collection of Samples. Following the completion of the four-week experiment, general anesthesia was administered to the experimental animals using a combination of ketamine (100 mg/kg) and xylazine (15 mg/kg) [19]. Once anesthetized, eight milliliters of intracardiac blood samples were collected. Subsequently, the animals were euthanized using the exsanguination method, and the testicular tissues were retrieved for further examination.

The harvested blood samples were then transferred into dry biochemistry tubes and centrifuged at 4000 rpm for 15 minutes to separate the serum. Both the retrieved organs and the separated serum were preserved at −20°C until further analysis. This ensured that there were no variations or distortions in the collected samples prior to their measurement and analysis.

3. Biochemical Analysis

3.1. Homogenization of Testicular Tissues. Equal portions of the harvested testicular tissues were selected and transferred to Eppendorf tubes. These samples were then complemented with a buffer solution, calibrated to a pH of 7.4, to maintain physiological conditions during homogenization. The tissue samples were homogenized using a suitable homogenizing device, operated at 6000 rotations per minute (rpm) for a duration of 40 seconds to ensure a thorough breakdown of the tissue structure. After homogenization, the samples were centrifuged at 3000 rpm for a period of 20 minutes at +4°C. This procedure facilitated the separation of the supernatant from the pellet, preparing the samples for subsequent analysis. The supernatant, which contained the soluble proteins and other intracellular components, was carefully transferred to separate tubes for further biochemical analyses. Throughout the process, care was taken to perform all steps at low temperatures to prevent the degradation of sensitive components. The homogenized tissue samples were preserved at −80°C until required for the subsequent stages of the investigation.

3.2. Analysis of Oxidant and Antioxidant Enzyme Activities in Testicular Tissue. The activities of oxidant and antioxidant enzymes within the testicular tissue were assessed using the supernatant obtained from the tissue homogenization process. Specific enzyme assays were used to quantify the activity levels of key oxidant and antioxidant enzymes.

MDA measurement was performed according to the method of Ohkawa et al. [20] and the modification by Kurt et al. [21]. The principle of the method is based on the spectrophotometric measurement of the absorbance of the pink-colored complex formed by thiobarbituric acid (TBA) and MDA at 532 nm. A 1.5 mL Eppendorf tube was filled with 25 μL of tissue homogenate, 25 μL of sodium dodecyl sulfate (80 mg/mL), and 1 mL of a mixture solution (20 g/L acetic acid + 1.06 g 2-thiobarbituric acid + 180 mL of distilled water). The mixture was incubated at 95°C for 60 minutes. After cooling, the mixture was centrifuged at 1,200 ×g for 10 minutes. The absorbance of the supernatant was measured at 532 nm. A standard curve was generated using 1,1,3,3-tetramethoxypropane.

MPO measurement was performed according to the method of Bradley et al. [22]. The principle is based on the kinetic measurement of the absorbance of the yellowish-orange complex formed by the oxidation of o-dianisidine with MPO in the presence of hydrogen peroxide at 460 nm. MPO activity was calculated using a standard curve and expressed as U/g protein.

SOD activity was measured according to the method of Sun et al. [23]. The measurement method is based on the principle that the superoxide anion reduces nitro blue tetrazolium (NBT) to produce a blue-colored formazan compound, which gives absorbance at a wavelength of 560 nm. SOD activity was calculated using a standard curve and expressed as U/mg protein.

GSH levels were determined using Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid) [24]. The supernatant was mixed with Ellman's reagent and phosphate buffer, and the absorbance was measured at 412 nm. GSH levels were calculated using a standard curve and expressed as μmol/g protein.

The activity of glutathione peroxidase (GPx) was evaluated following the method described by Beutler [25]. GPx activity was assessed by measuring the rate of oxidation of NADPH to NADP+. The oxidation process was monitored by reading the absorbance difference at a wavelength of 340 nm.

CAT enzyme activity was determined spectrophotometrically at 240 nm using hydrogen peroxide (H2O2) as a substrate, according to the Aebi method [26]. CAT activity was calculated using a standard curve and expressed as U/mg protein.

3.3. Nitrate/Nitrite Concentration. Since nitric oxide (NO) is an unstable compound, it undergoes a series of reactions with several molecules present in biological fluids. These reactions are

$$\begin{aligned} 2\,NO + O_2^- &> 2NO_2 \longleftrightarrow N_2O_4, \\ N_2O_4 + 2OH^- &> NO2^- + NO_3^- + H_2O, \\ NO_2 + NO^- &> N_2O_3, \\ N_2O_3 + 2OH^- &-> 2NO_2^- + H_2O. \end{aligned} \tag{1}$$

The final products of NO are nitrite (NO_2^-) and nitrate (NO_3^-). The relative ratios of NO_2 and NO_3 are variable and cannot be precisely predicted; therefore, the best indicator of total NO production is the sum of both NO_2^- and NO_3^- concentrations.

NO levels were measured using a commercial colorimetric analysis kit (Cayman, Nitrate/Nitrite Colorimetric Assay kit, Item No: 780001). According to the protocol of the kit used for determining $NO_3^- + NO_2^-$ concentrations, NO_3^- is reduced to NO_2^- by the enzyme nitrate reductase, and a total NO_2^- measurement is performed. The coefficient of variation (CV) for the measurements of the kit used is reported to be 2.7% for intra-assay and 3.4% for interassay variations.

3.4. Measurement of Testosterone and Estradiol Levels. Serum testosterone and estradiol measurements were performed using the chemiluminescent immunoassay method on the Beckman Coulter DXI 800 Immunoassay system (Beckman Coulter Inc., Brea, USA), with appropriate commercial kits.

3.5. Histopathological Procedures. Testis tissue samples taken from rats were subjected to fixation for 24–48 hours in a 10% buffered formaldehyde solution. Following fixation, the tissues were washed under running tap water for 6 hours. Subsequently, these tissues were passed through graded alcohol and xylene series and embedded in paraffin blocks. Sections of 5 μm thickness were obtained from the paraffin

blocks using a Leica RM2125RT microtome (Leica Microsystems, Wetzlar, Germany). Sections from all groups were stained with Mallory's trichrome stain as modified by Crossman for histopathological evaluations. Microscopic imaging of the sections was performed with a microscope equipped with a camera (Zeiss AXIO Scope.A1, Germany). Histological damage and spermatogenesis in the testis sections were evaluated using Johnsen's average testicular biopsy score [27]. This evaluation is based on the presence or absence of types of germ cells in the testis seminiferous tubules. For each testis, 30 tubules were graded according to factors such as spermatozoa, spermatids, spermatocytes, spermatogonia, germ cells, and Sertoli cells, with a score of 1 to 10 given for each tubule. A higher Johnsen score indicates a healthier spermatogenesis situation, while a lower score indicates a more serious dysfunction. The scoring points are presented in Table 1.

Table 1: Histological classification of seminiferous tubular sections according to Johnsen's scoring system.

Score	Definition
10	Full spermatogenesis is observed with many spermatozoa, the germinal epithelium has a regular thickness
9	Many spermatozoa are present, but the germinal epithelium appears irregular
8	Only a few spermatozoa are found in the section
7	No spermatozoa, but many spermatids are present
6	No spermatozoa and only a few spermatids are present
5	No spermatozoa or spermatids, but a few or many spermatocytes are present
4	Only a few spermatocyte cells are present, no spermatids or spermatozoa
3	Only spermatogonia cells are present, other germ cells are absent
2	No germ cells, but Sertoli cells are observed
1	No germ cells or Sertoli cells are found in the tubular section

3.6. Statistical Analysis. The extent to which the experimental intervention differentiated the parameter and biomarker values was calculated with the effect size (Eta-$\eta 2$). In this study, statistical power analysis was performed by taking the effect size of 0.7 (calculated with $\eta 2$), alpha = 0.05, and power = 0.80, and the total required minimum number of subjects was determined as 28. While the assumption of normality of the data was examined with the Shapiro–Wilk test, the homogeneity of the group variances was checked with the Levene test. The distribution of all parameters in the groups provided the assumption of normality. The mean, standard error, and confidence intervals of the mean were given for the descriptive statistics of the parameters. Paired samples t-test was used to compare the body weights of the groups at the beginning of the study and at the end of the study. Bootstrap one-way ANOVA linear contrast analysis was used to compare antioxidant, oxidant, and hormone levels between control and experimental groups. In the bootstrap ANOVA analysis using the simple sampling method, bootstrapping performance was obtained according to the number of 1000 samples. Post hoc tests (Tukey HSD or Dunnett's test) were used to examine significant differences between subgroups. Post hoc results are shown in lowercase letters in tables and graphs.

While there was no significant difference between groups symbolized with a common letter, there was a significant difference between groups not symbolized with a common letter. Box-plot graphics were used to show the distribution of measurement results between groups. Error bars are calculated at 95% confidence level. SPSS (version 26.0, SPSS Inc., Chicago) package program was used for statistical analysis of the data. Asymptotic significances (2-sided tests) are displayed. $p < 0.05$ was considered statistically significant.

4. Results

4.1. Effects of KD and IF on Average Testicular and Body Weights. The data presented in Table 2 show that at the beginning of the study, the body weights of the rats were quite similar across all four groups (CD, IF + CD, KD, and IF + KD) as indicated by a nonsignificant p value (0.737). However, at the end of the study, significant differences were observed in the body weights across the four groups ($p = 0.003$). Rats in the CD group showed a significant increase in body weight (from 330.86 ± 10.68 g to 368.71 ± 9.81 g, $p < 0.001$). Similarly, the KD group also showed a significant increase in body weight (from 325.00 ± 4.86 g to 378.00 ± 6.55 g, $p < 0.001$). In contrast, rats in the IF + CD group showed no significant change in body weight (from 339.14 ± 11.88 g to 332.71 ± 11.49 g, $p = 0.119$), while rats in the IF + KD group showed a slight, but statistically significant, increase in body weight (from 330.86 ± 6.29 g to 340.86 ± 5.20 g, $p = 0.004$). These results suggest that the CD and KD diets led to an increase in body weight over the course of the study, whereas the addition of IF to these diets mitigated this weight gain. Further investigation is needed to understand the underlying mechanisms for these observed differences in body weight changes across the different dietary interventions.

Regarding the testis weight shown Table 3, a significant difference among groups was observed with a p value of 0.001. The KD group had a noticeably higher mean testis weight compared to the other groups. In contrast, the CD, IF + CD, and IF + KD groups had similar testis weights. These findings suggest that a high-fat diet may influence testicular weight. However, this increase in testis weight in the KD group does not translate into improved spermatogenesis or male reproductive health, as our histopathological findings suggest.

4.2. Effects of KD and IF on Testicular Oxidative Stress and Nitric Oxide Levels. This study also ventured into assessing the effects of KD and IF on oxidative stress and NO levels in testicular tissue, which are critical parameters for male reproductive health. The balance of reactive oxygen species and antioxidants in the testes is integral to the normal functioning of spermatogenesis. Additionally, NO plays a significant role in male reproductive physiology, contributing to processes such as spermatogenesis, steroidogenesis, and penile erection.

Table 2: Descriptive statistics for body weight results.

Groups	At the beginning of the study		At the end of the study		
	Mean ± SEM	95.0% lower-upper CL for mean	Mean ± SEM	95.0% lower-upper CL for mean	$^{*}p$
CD	330.86 ± 10.68	304.72–356.99	368.71^{ab} ± 9.81	344.72–392.71	<0.001
IF + CD	339.14 ± 11.88	310.09–368.20	332.71^{c} ± 11.49	304.61–360.82	0.119
KD	325.00 ± 4.86	313.11–336.89	378.00^{a} ± 6.55	361.96–394.04	<0.001
IF + KD	330.86 ± 6.29	315.47–346.24	340.86^{bc} ± 5.20	328.14–353.58	0.004
$^{**}p$	0.737		0.003		

$^{*}p$ values indicate paired samples t-test results. $^{**}p$ values show the results of one-way ANOVA. Differences in parameters between groups were represented by lowercase letters. There is no significant difference between groups with a common letter, but there is a significant difference between groups without a common letter. CD: control diet; IF: intermittent fasting diet; KD: ketogenic diet. SEM: standard error of mean. CL: confidence level.

Table 3: Descriptive statistics for testicular weight results.

Groups	Mean ± SEM	95.0% lower-upper CL for mean
CD	4.27^{b} ± 0.13	3.96–4.59
IF + CD	4.22^{b} ± 0.09	4.00–4.43
KD	5.01^{a} ± 0.24	4.43–5.59
IF + KD	4.17^{b} ± 0.06	4.01–4.32
$^{*}p$	0.001	

$^{*}p$ values indicate the results of the one-way ANOVA. Groups denoted with the same superscript letter (a or b) do not significantly differ from each other. However, groups labeled with different letters (a vs. b) demonstrate statistically significant differences. In this context, "a" and "b" denote different groups based on post hoc comparisons following one-way ANOVA. CD: control diet; IF: intermittent fasting diet; KD: ketogenic diet. SEM: standard error of mean. CL: confidence level.

In our analysis of oxidative stress markers, we noted significant differences across groups ($p < 0.001$). For MDA, a marker of lipid peroxidation, the IF + CD group exhibited the lowest. The KD group showed a reduction in MDA compared to the CD group, but not to the extent observed in the IF + CD group. The IF + KD group's MDA levels were intermediate between the IF + CD and KD groups, indicating the potential mitigating effect of IF on oxidative stress induced by KD (Figure 1).

The pattern was somewhat similar for MPO, a marker of neutrophil infiltration and oxidative stress. The IF + CD group displayed the lowest MPO levels in inter-group comparison. The KD group displayed reduced MPO compared to the CD group, but levels in the IF + KD group were lower than those in the KD group, implying that IF might have some protective effect against oxidative stress in testicular tissue (Figure 1).

We further observed significant differences among groups ($p < 0.001$) for SOD, CAT, GPx, and GSH, crucial components of the antioxidant defense system. The CD and KD groups showed relatively lower levels of antioxidants than the IF + CD and IF + KD groups, suggesting that IF may boost the antioxidant capacity in testicular tissue (Figure 2).

Specifically for SOD, there was no significant difference in activity between the KD and IF + KD groups. This implies that adding intermittent fasting to a ketogenic diet (IF + KD) did not significantly change SOD activity as compared to a standalone ketogenic diet (KD). In other words, the ketogenic diet's effect on SOD activity does not appear to be boosted by intermittent fasting.

Lastly, we found significant differences in NO levels among groups ($p < 0.001$). The IF + CD group showed the highest levels, followed by the IF + KD, KD, and CD groups. The elevated NO levels in IF groups align with previous research showing that IF can stimulate NO production, which is beneficial for spermatogenesis and overall male reproductive health (Figure 3).

4.3. Effects of KD and IF on Spermatological Parameters. Our investigation into the effects of KD and IF on spermatological parameters revealed a significant impact on hormonal levels in serum. Specifically, we examined levels of estradiol and testosterone, two hormones critical for normal spermatogenesis and male reproductive health.

In examining estradiol levels, we found significant differences among the four groups ($p < 0.001$). The CD group exhibited the highest serum estradiol levels with a mean value of 6.67 ± 0.61 pg/mL, while the IF + KD group showed the lowest levels at 2.40 ± 0.12 pg/mL. This indicates that the combination of a KD and IF regimen results in the greatest decrease in estradiol levels. Meanwhile, the KD group also showed lower estradiol levels (3.07 ± 0.13 pg/mL) than the CD group, but higher levels than the IF + KD group. The IF + CD group displayed intermediate levels at 4.52 ± 0.25 pg/mL. These results suggest that both a ketogenic diet and intermittent fasting, individually or combined, can contribute to a reduction in estradiol levels (Figure 4).

In terms of testosterone, a crucial hormone for the production and development of sperm cells, we also noted statistically significant differences among groups ($p < 0.001$). The KD group exhibited the highest serum testosterone levels (7.81 ± 0.19 ng/mL), significantly more than the CD, IF + CD, and IF + KD groups (Figure 4). This elevated testosterone level in the KD group aligns with prior research indicating that ketogenic diets can enhance testosterone production. However, in the context of our study, this increased testosterone level does not appear to contribute to improved spermatogenesis, as suggested by our histopathological findings.

The IF + CD group showed higher testosterone levels than the CD group but lower than the KD group. Importantly, the IF + KD group exhibited a marked reduction in testosterone levels compared to the KD group, suggesting that intermittent fasting might counteract the testosterone-enhancing effects of the ketogenic diet.

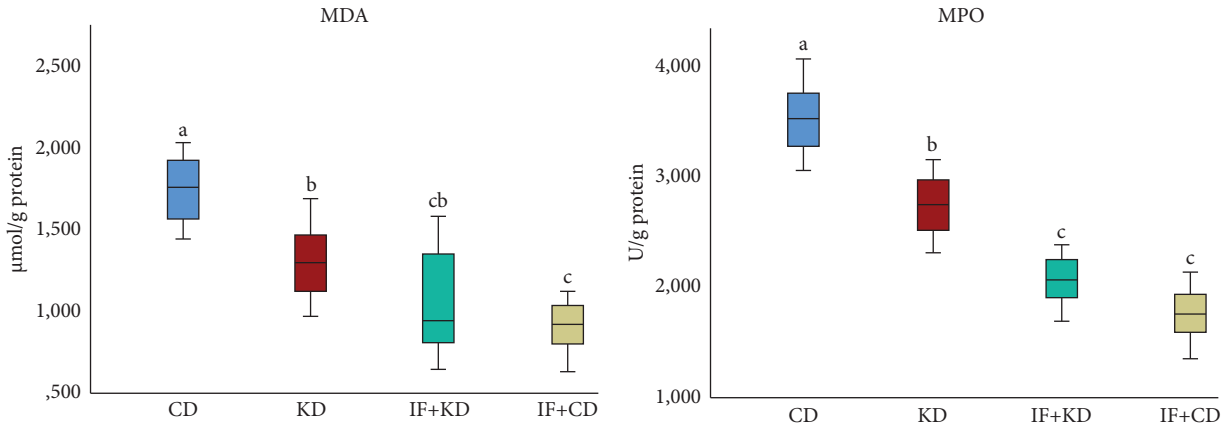

Figure 1: Distribution of MDA and MPO oxidant parameter values between control and experimental groups. [a] $p < 0.001$ compared with CD group; [b] $p < 0.001$ compared with KD and IF + KD groups; [c] $p < 0.001$ compared with IF + KD and IF + CD groups. There is no significant difference between groups with a common letter, but there is a significant difference between groups without a common letter. Error bars were calculated with 95% confidence level.

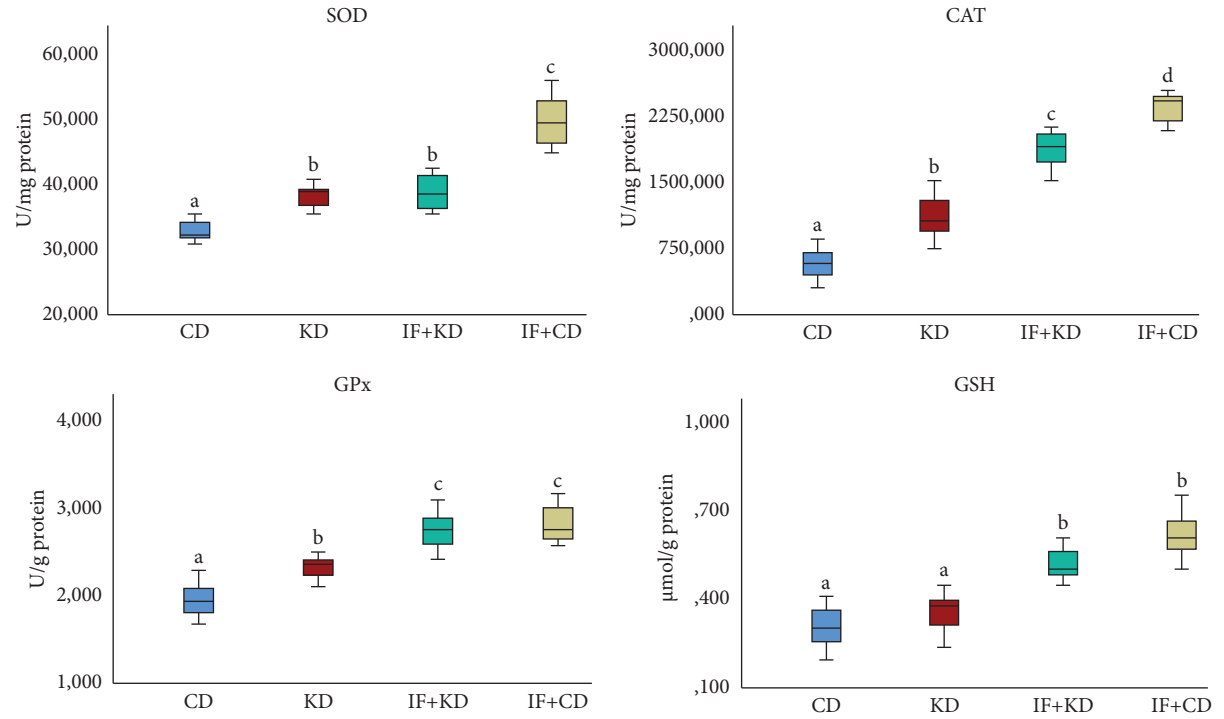

Figure 2: Distribution of SOD, CAT, GPx, and GSH antioxidant parameter values between control and experimental groups. [a] $p < 0.001$ compared with CD group; [b] $p < 0.001$ compared with KD and IF + KD groups; [c] $p < 0.001$ compared with IF + KD and IF + CD groups; [d] $p < 0.001$ compared with IF + CD group. There is no significant difference between groups with a common letter, but there is a significant difference between groups without a common letter. Error bars were calculated with 95% confidence level.

4.4. Histopathological Findings. In the testis tissue of the CD group, a normal structure was observed. The testes were seen to be divided into lobules by the tunica albuginea, a dense connective tissue characteristic of the testes, along with inwardly extending septa. It was determined that the seminiferous tubules and interstitial spaces within the lobules were normal. It was seen that the seminiferous tubules contained dark and light spermatogonia seated on the basal membrane, primary spermatocytes, and round and elongating spermatids towards the center of the tubules (Figures 5(A) and 5(B)).

In the testis sections of the group where the CD and IF diet were applied together; seminiferous tubules of normal structure were observed. The process of spermatogenesis was ongoing. Leydig cells of normal structure were observed in

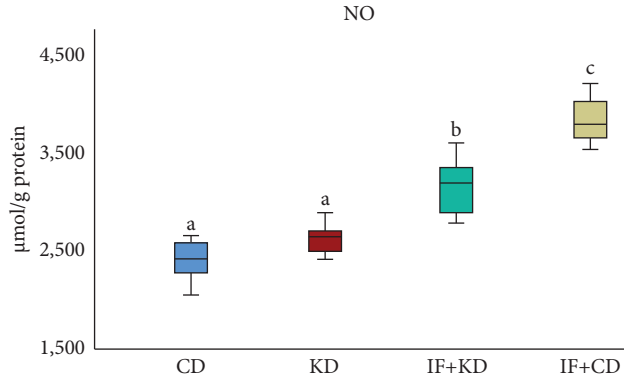

Figure 3: Distribution of NO parameter values between control and experimental groups. [a] $p < 0.001$ compared with CD and KD groups; [b] $p < 0.001$ compared with KD, IF + KD, and IF + CD groups; [c] $p < 0.001$ compared with IF + KD and IF + CD groups; [d] $p < 0.001$ compared with IF + CD group. There is no significant difference between groups with a common letter, but there is a significant difference between groups without a common letter. Error bars were calculated with 95% confidence level.

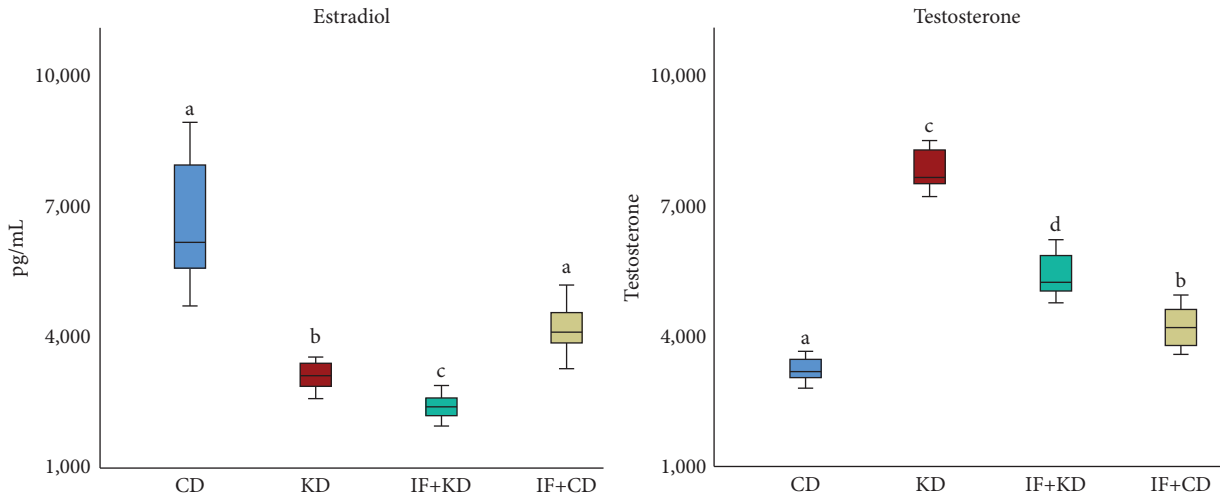

Figure 4: Distribution of serum estradiol and testosterone values between control and experimental groups. [a] $p < 0.001$ compared with CD and IF + CD groups; [b] $p < 0.001$ compared with KD and IF + CD groups; [c] $p < 0.001$ compared with KD and IF + KD groups; [d] $p < 0.001$ compared with IF + KD group. There is no significant difference between groups with a common letter, but there is a significant difference between groups without a common letter. Error bars were calculated with 95% confidence level.

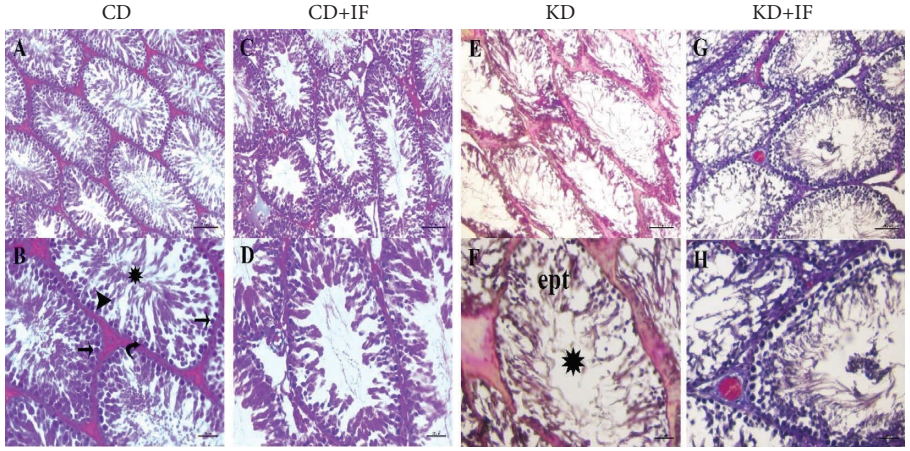

Figure 5: Seminiferous tubules of the testis stained with Mallory's triple stain as modified by Crossman. Star: seminiferous tubule lumen and spermatozoa; arrow: spermatogonia; curved arrow: Sertoli cell; arrowhead: spermatid; ept: testis epithelium. Magnification: (A, C, E, G) 200X; (B, D, F, H) 400X.

the interstitial area. It was seen that the seminiferous tubules maintained their normal structure. This indicates that spermatogenesis is continuing similarly to the testes of the CD group (Figures 5(C) and 5(D)).

In the testes of the KD group, degenerative changes and cellular losses in the spermatogenic cells located in the wall of the seminiferous tubules were observed along with intense tubular damage and noticeable dilatation and degeneration in some tubules. Separations in the interstitial area and the basal membrane structure separated from the tubule wall were also detected. The process of spermatogenesis was greatly affected, and adverse changes were observed in the interstitial tissue (Figures 5(E) and 5(F)).

In the testis sections of the group where the KD and IF diet were applied together, although tubular damage was still observed, generally decreased tubular damage and relatively normal seminiferous tubules were observed. The process of spermatogenesis was still ongoing. In the interstitial area, decreased edema and Leydig cells with a near-normal appearance were detected. This situation indicates a sign of some recovery compared to the sections of the KD group, and that spermatogenesis is partially ongoing (Figures 5(G) and 5(H)).

According to the histopathological evaluations, it was observed that the score in the CD group was 9 according to Johnsen's scoring system, and the score in the CD + IF group was 8. While the score of the KD group was observed to be 2, it was determined that the score of the KD + IF group was 8 (Table 4).

Table 4: Evaluation of testicular histology according to Johnsen's scoring system.

Parameters	CD	CD + IF	KD	KD + IF
Johnsen's score	8.66 ± 0.47	8.16 ± 0.68	2.16 ± 0.68	7.50 ± 0.95

5. Discussion

The KD and IF are dietary strategies that have gained significant attention in recent years for their potential effects on various health parameters. The KD is a high-fat, low-carbohydrate diet that is designed to mimic the metabolic effects of fasting, forcing the body to utilize fats rather than carbohydrates as its primary energy source. This diet has been proposed to have several health benefits, including weight loss, improved blood sugar control, and neuroprotective effects [28, 29]. On the other hand, IF involves periods of eating and fasting, with several different methods, such as the 16/8 method, which involves fasting for 16 hours a day, or the 5 : 2 method, which involves fasting for two days a week. IF has been reported to improve various health outcomes, including weight loss, metabolic health, and possibly even extending lifespan [30, 31].

As we continue to deepen our understanding of the interplay between diet and health, the focus has increasingly shifted to include aspects of reproductive health. In the context of male reproductive health, and specifically testicular health, the roles of dietary strategies such as KD and IF have been relatively understudied. Given the growing popularity of KD and IF for health and wellness, our study aimed to explore their potential effects on testicular tissue and related parameters.

Among the diverse array of factors influencing hormonal dynamics, dietary selections have gained prominence due to their potential to modulate pivotal hormones crucial for male reproductive health. Estradiol and testosterone play integral roles in processes like spermatogenesis and male sexual function [32, 33]. Although estradiol is traditionally considered a female hormone, it also contributes significantly to male reproductive health. Conversely, testosterone, as the primary male sex hormone, has a critical role in upkeeping male reproductive health. In our study, we found that KD resulted in a substantial increase in testosterone levels and decrease in estradiol levels compared to both the CD and IF + CD diets. These findings align with previous studies that suggest high-fat diets, akin to KD, can significantly alter sex hormone levels [34]. This shift in hormonal balance may have profound implications. An investigation by Pilz et al. [35] proposed that testosterone levels could impact body composition, influencing muscle mass and strength. This interplay might provide an explanation for the increased testicular weight observed in the KD group in our study. Furthermore, it was intriguing to note that the group subjected to both IF and KD exhibited an intermediate hormone profile, falling between the isolated KD and IF + CD diets. This observation suggests a potential synergistic effect of KD and IF on hormonal regulation. Therefore, our findings emphasize a complex interplay between diet, particularly KD and IF, and hormonal regulation in the context of male fertility.

Our study also investigated the impact of KD and IF on oxidative stress parameters in testicular tissue. Oxidative stress, resulting from an imbalance between reactive oxygen species (ROS) production and the antioxidant defense system, can induce tissue damage and impair cellular functions. In the testes, oxidative stress can lead to sperm dysfunction, contributing to male infertility [36–38]. Our results showed an increase in MDA, a marker of lipid peroxidation and oxidative stress, in the KD group compared to the CD group. This finding suggests that KD may increase oxidative stress in testicular tissue, consistent with previous studies indicating that high-fat diets could induce oxidative stress [39]. Interestingly, we observed a reduction in MDA levels in the IF + CD and IF + KD groups, indicating that IF might mitigate KD-induced oxidative stress. This aligns with the findings of He et al. [40], who reported that IF could attenuate oxidative stress and improve antioxidant defense mechanisms. Furthermore, the changes in GSH levels and catalase activity observed in our study support the potential protective role of IF against oxidative stress.

In light of the histopathological evaluations, our findings indicate that diet has a significant impact on testicular health and spermatogenesis. The CD group, indicative of a balanced diet, showcased healthy spermatogenesis and testicular architecture, corroborating previous research endorsing balanced diets for reproductive health [41]. Conversely, the KD group showed severe damage to spermatogenic cells and testicular tubules, resulting in compromised spermatogenesis, similar to findings from studies reporting detriments of high-

fat diets on testicular function potentially due to induced oxidative stress and inflammation [42–44].

Interestingly, the combination of KD and IF mitigated the deleterious effects observed in the KD group, indicating a protective potential of IF. This aligns with research highlighting the health benefits of IF, such as anti-inflammatory effects and reduced oxidative stress [45, 46]. Similarly, the combination of CD and IF did not adversely affect spermatogenesis, further reinforcing the potential benefits of IF in conjunction with balanced nutrition.

Lastly, we explored the impact of KD and IF on NO levels in testicular tissue. NO is a gaseous molecule involved in various physiological processes, including vasodilation, neurotransmission, and modulation of the immune response [47]. In the testes, NO plays a critical role in spermatogenesis [48]. Our results indicated that IF, either alone or in combination with KD, could enhance NO production. This suggests a potential beneficial effect of IF on spermatogenesis and fertility.

5.1. Limitations. While our study offers valuable insights into the acute effects of ketogenic diet and intermittent fasting on male reproductive parameters, it is important to acknowledge certain limitations. Firstly, our experimental design utilized an animal model, which may not perfectly replicate human physiological responses to these dietary interventions. Secondly, the short-term nature of our study prevents us from drawing conclusions about the long-term effects and sustained benefits of these diets. Additionally, we did not delve into the precise cellular and molecular mechanisms underpinning the observed changes, leaving room for further investigation in this area. Lastly, our model did not account for variables such as age, genetic factors, and preexisting metabolic conditions, which can influence individual responses to dietary alterations.

In the light of these limitations, we propose several future directions for research. While our results demonstrate the positive effects of short-term application of the ketogenic diet and intermittent fasting, the long-term efficacy, safety, and overall health benefits of these interventions remain less understood due to the limited existing literature. Therefore, it is crucial for individuals to undertake these diets under the supervision of healthcare professionals. Furthermore, it is important to acknowledge that our study was conducted on animals rather than humans, highlighting the necessity for more comprehensive and expansive experimental trials that provide evidence of their effectiveness in both human and animal models. The field would greatly benefit from more randomized controlled clinical trials with extended durations, considering the potential adverse health effects associated with these dietary strategies.

6. Conclusion

In summary, this study investigated the combined effects of a KD and IF on male reproductive health using an animal model. Our findings shed light on the intricate relationship between these dietary interventions and various parameters of testicular health.

The study demonstrated that the KD and IF interventions had discernible impacts on body and testicular weights. Notably, the addition of IF appeared to mitigate the weight gain associated with the KD. This points to the potential synergistic effects of these interventions on body weight regulation. Our examination of oxidative stress markers and NO levels revealed intriguing patterns. IF seemed to mitigate the oxidative stress induced by the KD, potentially suggesting a protective role of IF against the adverse effects of a high-fat diet. Moreover, the elevated NO levels in the IF groups highlight the potential positive impact of IF on male reproductive health. Hormonal analysis indicated that both KD and IF have intricate effects on estradiol and testosterone levels. The combined KD and IF regimen led to a significant reduction in estradiol levels and distinct testosterone responses. These results emphasize the dynamic interplay of these dietary strategies on male hormonal dynamics. Histopathological findings indicated that while KD could negatively influence spermatogenesis, the addition of IF to KD exhibited some ameliorative effects. This highlights the potential of IF in mitigating the adverse impacts of a high-fat diet on testicular health.

In conclusion, our study provides valuable insights into the intricate interplay between KD and IF on male reproductive health parameters. These findings not only lay a foundation for further investigations but also emphasize the importance of tailored dietary approaches for addressing male fertility concerns. Yet, for the translation of these outcomes into clinical practice, the necessity for rigorous randomized controlled clinical trials involving human subjects becomes evident. Understanding the potential long-term effects, sustainability, and possible adverse outcomes is crucial.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

SD, BÖ, FK, and HÜ were responsible for research design. SD, BÖ, and FK were responsible for data collection. HÜ, NK, NAC, MTH, and FBÖ were responsible for analysis. HÜ and SD were responsible for original draft preparation and critical review.

Acknowledgments

This study was supported by Sakarya University Scientific Research Projects Coordinatorship (Project no: 2022-7-24-91).

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