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Modulation of Caspase-3 Expression and Spermatogenic Cells by *Urtica dioica* Extract in Obesity-Induced Male Rats

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ABSTRACT

Obesity is associated with impaired steroidogenesis and spermatogenesis through mechanisms involving hypogonadism, inflammation, and oxidative stress. Pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) contribute to apoptotic signaling pathways, including caspase-3 activation, leading to germ cell loss. *Urtica dioica* contains bioactive compounds with reported antioxidant and anti-apoptotic properties. This study aimed to evaluate the effects of *U. dioica* extract on TNF-α and caspase-3 mRNA expression as well as spermatogenic cell counts of the testes of obese male Sprague Dawley rats. This experimental study employed a post-test-only control group design using 25 rats divided into five groups: healthy control (C1), obese control induced by a high-fat and fructose diet (C2), and three intervention groups receiving *U. dioica* extract at doses of 125 mg/kg (D1), 250 mg/kg (D2), and 500 mg/kg (D3) for four weeks. The results showed no significant differences in TNF-α mRNA expression were observed between the intervention groups and the obese control. In contrast, caspase-3 mRNA expression was significantly reduced in all *U. dioica*–treated groups compared with the obese control. No significant differences were observed in the number of primary or secondary spermatocytes among groups. However, spermatid counts were significantly higher in D2 and D3 groups compared with the obese control. In conclusion, *U. dioica* extract demonstrated potential anti-apoptotic effects and was associated with improved post-meiotic spermatogenic outcomes in obese rats.

ABSTRAK

Obesitas berhubungan dengan gangguan steroidogenesis dan spermatogenesis melalui mekanisme yang melibatkan hipogonadisme, inflamasi, dan stres oksidatif. Sitokin proinflamasi seperti tumor necrosis factor-α (TNF-α) berperan dalam jalur pensinyalan apoptosis, termasuk aktivasi kaspase-3, yang berujung pada kehilangan sel germinal. *Urtica dioica* mengandung senyawa bioaktif yang dilaporkan memiliki sifat antioksidan dan antiapoptotik. Penelitian ini bertujuan untuk mengevaluasi pengaruh ekstrak *U. dioica* terhadap ekspresi mRNA TNF-α dan kaspase-3 serta jumlah sel spermatogenik pada testis tikus jantan Sprague Dawley obesitas. Penelitian eksperimental ini menggunakan desain *post-test-only control group* dengan 25 ekor tikus yang dibagi ke dalam lima kelompok, yaitu kontrol sehat (C1), kontrol obesitas yang diinduksi dengan diet tinggi lemak dan fruktosa (C2), serta tiga kelompok intervensi yang menerima ekstrak *U. dioica* dengan dosis 125 mg/kg (D1), 250 mg/kg (D2), dan 500 mg/kg (D3) selama empat minggu. Hasilnya tidak terdapat perbedaan bermakna pada ekspresi mRNA TNF-α antara kelompok intervensi dan kontrol obesitas. Sebaliknya, ekspresi mRNA kaspase-3 lebih rendah secara signifikan pada seluruh kelompok intervensi dibandingkan dengan kontrol obesitas. Tidak ditemukan perbedaan bermakna pada jumlah spermatosit primer maupun sekunder antar kelompok. Namun, jumlah spermatid secara signifikan lebih tinggi pada kelompok D2 dan D3 dibandingkan dengan kontrol obesitas. Sebagai simpulan, ekstrak *U. dioica* menunjukkan potensi efek antiapoptotik dan berhubungan dengan perbaikan luaran spermatogenesis tahap pascameiotik pada tikus obesitas.

Keywords:
apoptosis;
caspase-3;
obesity;
spermatogenesis;
Urtica dioica

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INTRODUCTION

Obesity is a major global health problem with a rapidly increasing prevalence and has been associated not only with metabolic disorders but also with male infertility.[1–3] Excess visceral adiposity in obesity disrupts spermatogenesis through multiple mechanisms, including chronic low-grade inflammation, oxidative stress, and hormonal imbalance.[4] These alterations impair the hypothalamic–pituitary–gonadal axis, reduce androgen levels, and negatively affect testicular function.[5]

Inflammatory processes in obesity are characterized by increased infiltration of immune cells into adipose tissue and elevated production of proinflammatory cytokines, particularly tumor necrosis factor-α (TNF-α).[6] Tumor necrosis factor-α plays a crucial role in activating nuclear factor-κB (NF-κB), inducing oxidative stress, and promoting apoptosis through the activation of caspase-3. These pathways contribute to Sertoli and Leydig cell dysfunction, germ cell loss, and impaired spermatogenesis in obese males.[7–9] The inflammatory and apoptotic milieu associated with obesity highlights the need for therapeutic agents with antioxidant and anti-inflammatory properties. *Urtica dioica* (stinging nettle), a plant belonging to the Urticaceae family, contains various bioactive compounds, including flavonoids, polyphenols, vitamins, and minerals.[10] Extracts of its leaves and stems demonstrated antioxidant and anti-inflammatory activities and have been traditionally used for various medicinal purposes.[11,12] Previous studies have shown that *U. dioica* extract exerts protective effects on male reproductive parameters, including sperm motility, count, morphology, seminiferous tubule diameter, testosterone levels, and testicular histology in nicotine-induced animal models.[13]

However, evidence regarding the effects of *U. dioica* on testicular inflammation, apoptosis, and spermatogenic cells under obesity conditions remains limited. This study aimed to evaluate the effects of *U. dioica* extract on TNF-α and caspase-3 mRNA expression, as well as on spermatogenic cell populations, in obese male Sprague Dawley rats induced by a high-fat and fructose diet.

MATERIAL AND METHODS

Study subjects

This experimental study used 25 male Sprague Dawley rats aged 7–8 weeks with an initial body weight of 170–200 g. All experimental procedures were approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta (No. KE/FK/0956/EC/2023). *Urtica dioica* was taxonomically identified at the Faculty of Biology, Universitas Gadjah Mada, Yogyakarta (No. 0172/STb/XI/2022).

Preparation of *U. dioica* extract

Leaves and stems of *U. dioica* were dried at approximately 45°C and ground into a fine powder. Extraction was carried out by maceration using 70% ethanol at room temperature for 3 × 24 hr, with solvent replacement every 24 hr. The combined filtrates were concentrated using evaporation to obtain a crude extract.

Animal design and treatment

After a 7-day acclimatization period, rats were randomly divided into five

groups (n = 5 per group): healthy control (C1), obese control receiving a high-fat and fructose diet (C2), and intervention groups receiving *U. dioica* extract at doses of 125 mg/kgBW (D1), 250 mg/kgBW (D2), and 500 mg/kgBW (D3). Obesity was induced for 6 weeks, followed by oral administration of *U. dioica* extract in the intervention groups for 4 weeks.

Obesity induction and confirmation

Obesity was induced using a high-fat and fructose diet (HFFD) formulated according to Murwani,[14] consisting of standard feed (50%), wheat starch (25%), lard (10%), pure cholesterol (2%), cholic acid (0.2%), and distilled water (12.8%), provided *ad libitum*. In addition, pure fructose at 1% of body weight was administered daily via oral gavage, following Sunarti.[15]

Body weight and naso-anal length were measured weekly. Obesity was confirmed using the Lee index, calculated as the cube root of body weight (g) divided by naso-anal length (cm) x 1000. Rats with a Lee index greater than 300 were classified as obese.[16]

Intervention with *U. dioica* extract

Following obesity induction, rats in groups D1–D3 received *U. dioica* extract dissolved in 2 mL of distilled water and administered orally via gavage once daily for 4 weeks. Control groups received standard feed without extract supplementation.

Termination and organ collection

At the end of the experimental period (week 10), rats were fasted for approximately 10 hours and anesthetized intramuscularly with ketamine (100 mg/kgBW). Testes were collected through surgical procedures for subsequent molecular and histological analyses.

Semi-quantitative RT-PCR analysis

Approximately 30 mg of testicular tissue was used for total RNA extraction using a Favorgen RNA extraction kit, followed by RNA purity and concentration assessment using NanoVue Plus. Complementary DNA (cDNA) was synthesized using ReverTra Ace® qPCR RT Kit II (Toyobo®).

Gene expression of TNF-α and caspase-3 was analyzed using semi-quantitative reverse transcription polymerase chain reaction (sqRT-PCR) with specific primers (TABLE 1). PCR amplification was performed for 40 cycles, consisting of denaturation at 94°C, annealing at 62°C, and extension at 72°C. PCR products were separated on 2% agarose gel electrophoresis at 100 V, visualized using a Syngene G:BOX gel documentation system, and analyzed for band density using ImageJ software. Relative mRNA expression was calculated using the following formula:
Relative expression = Target gene band density/GAPDH band density

TABLE 1. Target gene primers for research

Gen	Primer forward (5'–3')	Primer reverse (5'–3')
Caspase-3	CCGACTTCCTCTATGCTTACTC	CGTACAGTTTCAGCATGGC
TNF-α	AAATGGGCTCCCTCTCATCAGTTC	TCTGCTTGGTGGTTTGCTACGAC
GAPDH	AGTGCCAGCCTCGTCTCATA	ATGAAGGGGTCGTTGATGGC

Histological examination

Testicular tissues were fixed in 10% buffered neutral formalin (BNF) for 24 hours and processed for paraffin embedding, including trimming (2 mm), graded dehydration (70–100%), xylene clearing, embedding, and sectioning at a thickness of 3–5 µm using a rotary microtome. Sections were stained with Hematoxylin–Eosin (HE) following standard histological procedures.

Spermatogenic cell populations (primary spermatocytes, secondary spermatocytes, and spermatids) were evaluated by a single experienced pathologist. For each animal, three randomly selected seminiferous tubules were examined at 400 x magnification. Each assessment was repeated three times per sample, and the mean value was used for statistical analysis.

Statistical analysis

Data were analyzed using SPSS version 24. Normality was assessed with the Shapiro–Wilk test and homogeneity of variance with Levene's test. Normally distributed data ($p \geq 0.05$) were analyzed using one-way ANOVA followed by LSD post-hoc test, while non-normally distributed data were analyzed using the Kruskal–Wallis test followed by the Mann–Whitney test. Results were considered statistically significant at $p \leq 0.05$.

RESULTS

The mRNA expression of TNF-α

The mRNA expression of TNF-α (98 bp) was normalized to GAPDH (133 bp) (FIGURE 1). The data were normally distributed and homogeneous ($p > 0.05$). One-way ANOVA demonstrated a significant difference in TNF-α expression among groups ($p = 0.018$).

Post-hoc LSD analysis revealed that TNF-α expression in the obese control group (C2= 1.20 ± 1.14) was significantly higher than in the healthy control group ($C1 = 1.02 \pm 0.04$; $p = 0.002$). Administration of *U. dioica* extract in the intervention groups D1 (1.18 ± 0.08), D2 (1.16 ± 0.05), and D3 (1.15 ± 0.03) resulted in lower mean TNF-α expression compared with C2; however, these differences did not reach statistical significance ($p > 0.05$). No significant differences were observed among the intervention groups (FIGURE 2).

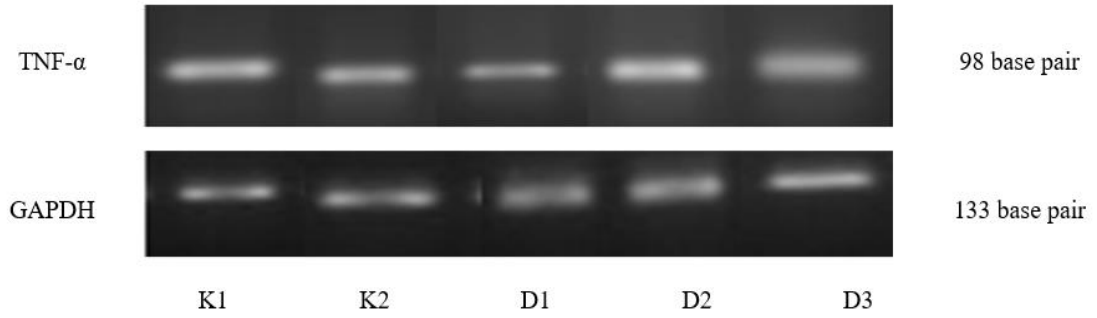

FIGURE 1. Representative electrophoresis image of RT-PCR products of TNF-α/ GAPDH

The mRNA expression of caspase-3

Caspase-3 mRNA expression (108 bp) was normalized to GAPDH (133 bp) (FIGURE 3). The data were not normally distributed ($p < 0.05$), and Kruskal–Wallis analysis showed a significant difference among groups ($p = 0.003$).

Pairwise analysis using the Mann–Whitney test indicated that caspase-3 expression in the obese control group (C2= 1.05 ± 0.08) was higher than in the healthy control group (C1= 1.01 ± 0.20), although the difference was not statistically significant ($p = 0.917$). In contrast, administration of *U. dioica* extract significantly reduced caspase-3 expression in all intervention groups, including D1 (0.79 ± 0.15; $p = 0.016$), D2 (0.63 ± 0.06; $p = 0.009$), and D3 (0.59 ± 0.02; $p = 0.009$), compared with the obese control group. Significant differences were also observed among the intervention groups ($p = 0.017$) (FIGURE 4).

Spermatogenic cell counts

Primary spermatocytes were identified by their relatively large cell and nuclear size with abundant cytoplasm, whereas secondary spermatocytes appeared smaller. Spermatids were characterized by elongated morphology, smaller nuclei, scant cytoplasm, and darker staining.[17] Spermatogenic cells were quantified from hematoxylin–eosin (HE)–stained testicular sections at 400× magnification in three randomly selected seminiferous tubules (FIGURE 5).

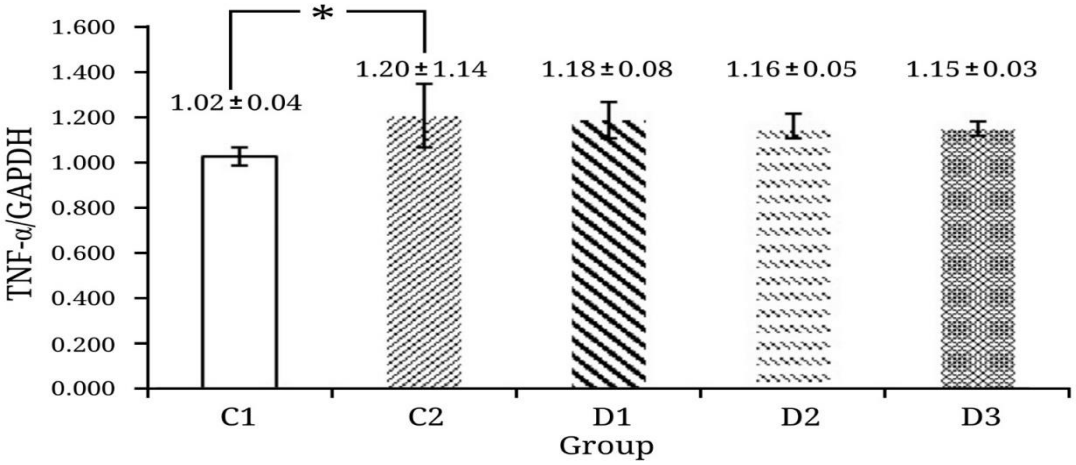

FIGURE 2. Comparison of mean ± SD mRNA expression of TNF-α/ GAPDH across groups

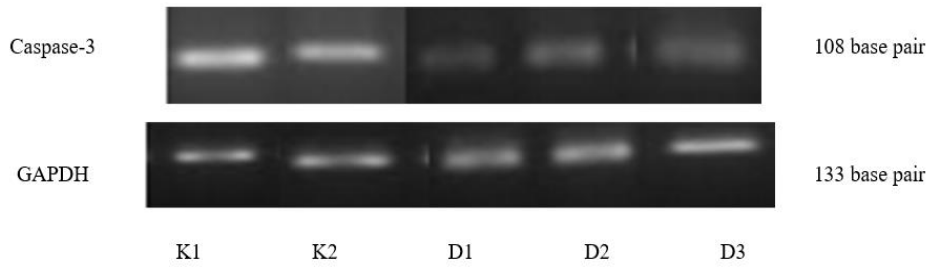

FIGURE 3. Representative electrophoresis image of RT-PCR products of caspase-3/GAPDH

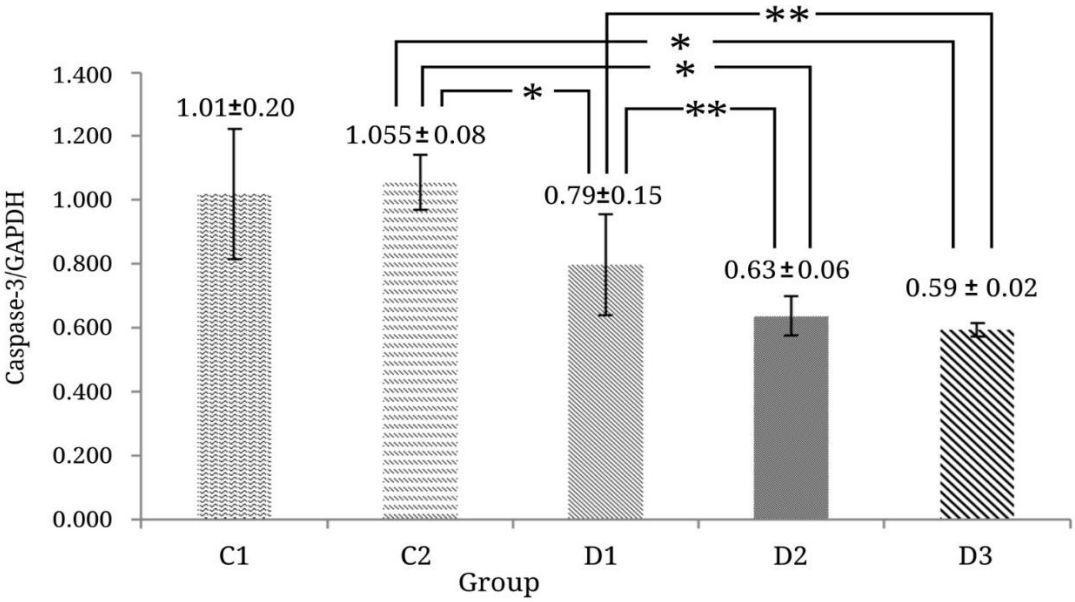

FIGURE 4. Comparison of mRNA expression of caspase-3/GAPDH across groups (mean ± SD)

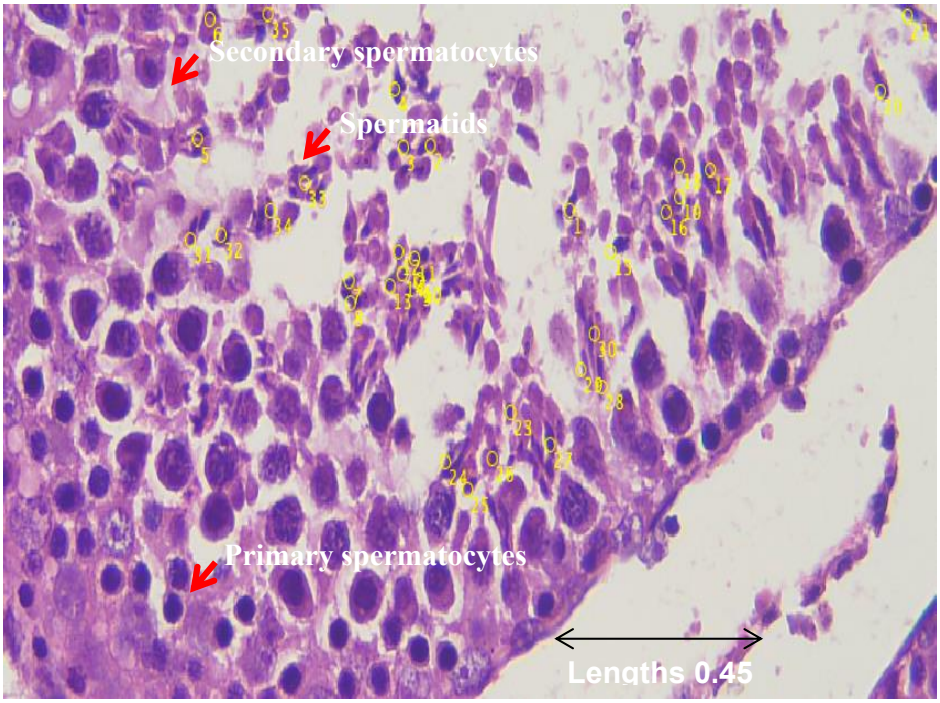

FIGURE 5. Histological images of spermatogenic cells in testicular samples stained with Hematoxylin–Eosin (HE) at 400× magnification

TABLE 2. Mean number (mean ± SD; n=5) of spermatogenic cells with and without *U. dioica* extract administration.

Group	Primary spermatocyte count	Number of secondary spermatocytes	Number of spermatids
C1	55.60±15.20	94.40±28.90	78.40±19.93
C2	34.60±4.03[a]	68.20±11.71	40.60±13.70[a]
D1	36.20±7.32[a]	76.60±14.90	55.00±10.90
D2	33.60±11.05[a]	84.80±19.50	60.60±6.76[b]
D3	40.40±11.86[a]	93.60±13.57	68.20±3.90[b,c]
p	0.022	0.170	0.015

Notes: (a) $p<0.05$ compared with C1; (b) $p<0.05$ compared with C2; (c) $p<0.05$ compared with D1.

The numbers of primary and secondary spermatocytes were normally distributed ($p > 0.05$). One-way ANOVA demonstrated a significant difference in the number of primary spermatocytes among groups ($p = 0.022$), whereas no significant difference was observed for secondary spermatocytes ($p = 0.170$). Post-hoc analysis showed that the obese control group (C2= 34.60 ± 4.03) had significantly fewer primary spermatocytes compared with the healthy control group (C1= 55.60 ± 15.20; $p = 0.005$). Although *U. dioica* administration increased the mean number of primary spermatocytes in D1 (36.20 ± 7.32) and D3 (40.40 ± 11.86) compared with C2, these increases were not statistically significant ($p > 0.05$). No significant differences were observed among the intervention groups ($p>0.05$).

The number of spermatids was not normally distributed ($p < 0.05$). Kruskal–Wallis analysis revealed a significant difference among groups ($p = 0.015$). Mann–Whitney analysis showed that the obese control group (C2= 40.60 ± 13.70) had significantly fewer spermatids compared with the healthy control group (C1= 78.40 ± 19.93; $p = 0.028$). Administration of *U. dioica* extract significantly increased spermatid counts in D2 (60.60 ± 6.76; $p = 0.027$) and D3 (68.20 ± 3.90; $p = 0.009$) compared with C2, whereas the increase observed in D1 (55.00 ± 10.90) was not statistically significant ($p = 0.173$). Significant differences were observed among the intervention groups ($p = 0.015$).

Overall, caspase-3 mRNA expression was significantly lower, and spermatid numbers were significantly higher, in obese male rats treated with *U. dioica* extract. In contrast, its effects on TNF-α expression and primary spermatocyte counts were modest and did not reach statistical significance, while secondary spermatocyte counts remained unchanged.

DISCUSSION

Effect of *U. dioica* extract on TNF-α mRNA expression

Obesity is associated with chronic low-grade inflammation, hormonal alterations, and increased reactive oxygen species (ROS), all of which adversely affect the male reproductive system. In this study, TNF-α mRNA expression differed significantly among groups, with the obese group (C2) showing higher expression compared with the healthy control group (C1). This finding is consistent with Kolb *et al.*,[18]

who reported that obesity activates JNK and NF-κB signaling pathways, thereby promoting inflammatory responses.

Administration of *U. dioica* extract in the intervention groups (D1, D2, and D3) did not significantly alter TNF-α mRNA expression compared with the obese control group. Although the mean TNF-α values in the intervention groups were numerically lower than those in C2, these differences were not statistically significant. This finding differs from previous studies reporting significant TNF-α suppression following *U. dioica* supplementation, which may be attributed to longer intervention durations in those studies.[19,20] The relatively high variability observed in TNF-α expression in the present study may also have limited the ability to detect statistically significant differences. Nevertheless, the known anti-inflammatory mechanisms of *U. dioica*, including inhibition of NF-κB activation, suppression of NADPH oxidase–mediated ROS production, and enhancement of antioxidant enzyme activity, remain biologically plausible.[12,21]

Effect of *U. dioica* extract on caspase-3 mRNA expression

Oxidative stress and inflammation associated with obesity have been implicated in disruption of the hypothalamic–pituitary–gonadal (HPG) axis and induction of germ cell apoptosis. In the present study, caspase-3 mRNA expression did not differ significantly between the obese control (C2) and healthy control (C1) groups, indicating that obesity induction under the current experimental conditions did not result in a detectable increase in caspase-3 transcription at baseline.

Despite the absence of a significant baseline difference, administration of *U. dioica* extract significantly reduced caspase-3 mRNA expression in all intervention groups compared with the obese control group. This finding suggests that *U. dioica* may exert an anti-apoptotic effect in the testis independent of marked baseline apoptotic activation. This effect is likely related to the flavonoid content of *U. dioica*, particularly quercetin, which has been shown to attenuate apoptosis by suppressing caspase-3 activity. Supporting evidence from Hossain *et al.*[22] demonstrated that quercetin reduced caspase-3 expression and apoptosis in pancreatic β-cells of hyperglycemic rats. Additionally, reduced ROS levels and enhanced antioxidant enzyme activity may contribute to inhibition of the intrinsic apoptotic pathway in germ cells.[23,24]

Effect of *U. dioica* extract on spermatogenic cells

This study identified significant differences in spermatogenic cell populations, particularly at the spermatid stage, while earlier germ cell stages were less affected. Obese rats exhibited significantly lower numbers of spermatids compared with healthy controls, consistent with previous reports that obesity increases testicular germ cell apoptosis.[25]

In the intervention groups, there was no significant difference in the number of primary or secondary spermatocytes compared with the obese control group. In contrast, spermatid counts were significantly higher in the D2 and D3 groups. These findings indicate that *U. dioica* extract was associated with increased spermatid numbers rather than generalized enhancement of spermatogenesis across all developmental stages. A possible explanation for this stage-specific effect is that post-meiotic germ cells, such as spermatids, are more susceptible to oxidative stress and apoptosis, and therefore may benefit more readily from antioxidant and anti-apoptotic interventions. Similar improvements

in spermatogenic outcomes following *U. dioica* administration have been reported by Jalili *et al.*,[13] in nicotine-induced testicular injury models.

Several limitations should be acknowledged. First, the lack of a significant difference in caspase-3 expression between the healthy and obese control groups suggests that the obesity model or its duration may not have induced robust baseline apoptotic activation at the transcriptional level. Second, the high variability observed in TNF-α expression may have limited statistical power to detect treatment-related differences. Third, the absence of protein-level confirmation or histological apoptosis markers restricts interpretation to mRNA expression alone. Future studies incorporating longer intervention periods and additional molecular and histological endpoints are warranted.

Overall, this study demonstrates that *U. dioica* extract was associated with reduced caspase-3 mRNA expression and increased spermatid counts in obese male rats, while its effects on TNF-α expression and earlier spermatogenic cell populations were limited under the present experimental conditions.

CONCLUSION

Administration of *U. dioica* extract in obese rats significantly reduced testicular caspase-3 mRNA expression and increased spermatid counts, while TNF-α expression and earlier spermatogenic stages were not significantly affected. These findings suggest a potential anti-apoptotic role of *U. dioica* associated with improved post-meiotic spermatogenic outcomes under obese conditions. Further studies using larger sample sizes, longer intervention periods, and standardized extract compositions are warranted to confirm and extend these findings.

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