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The Ameliorative Effects of Ferulic Acid against Methotrexate Induced Testicular Damage in Rats

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ABSTRACT

Methotrexate (MTX), an extensively used chemotherapeutic and immunosuppressiv agent, is well-documented for its gonadotoxic effects. Ferulic acid (FA), a natura phenolic compound with potent antioxidant properties, has shown protective effect in various organ systems. This study aimed to assess the protective and therapeuti potentials of FA against MTX-induced testicular damage in a rat model. Twenty-fou adult male rats were allocated into four groups: control, MTX-only, MTX plus FA (protective), and MTX followed by FA (therapeutic). Testicular tissues were assesse histologically for tubular integrity and germ cell preservation. Histomorphometri parameters, including seminiferous tubule diameter and germinal epithelial heigh Also, the level of Caspase-3 expression was analyzed using immunohistochemistr to examine apoptosis activity. The group treated with MTX showed testicular damag and a significant decrease in germ cells along with lower histomorphometri measures and increased Caspase-3 expression levels. On the other hand, both group treated with FA displayed noticeable improvements in both histology and molecula aspects. The treated group showed normal testicular structure, along with restore histomorphometric values and minimal Caspase-3 expression similar, to the contro group. Conclusion**:** FA successfully reduced the effects of (MTX) in the testicles b maintaining their structure and preventing cell death processes. This suggests that FA could be an addition to protect men's reproductive health during chemotherap treatments.

Keywords: Apoptosis, Ferulic acid, Methotrexate, Oxidative stress, Spermatogenesis, Testicular toxicity.

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INTRODUCTION

Methotrexate (MTX) stands as a known antifolate chemotherapy drug extensively applied to treat a range of cancers and autoimmune disorders like rheumatoid arthritis and psoriasis. **(Shariatifar *et al.*, 2022)** Its strong a folate antagonist exerting pronounced cytotoxic and immunosuppressive actions have made it a valuable option, in care (**Hoque *et al.*, 2023**). Despite its proven effectiveness in settings and treatment outcomes; recent studies have highlighted side effects related to its impact on male reproductive health specifically targeting the gonads (**Radi *et al.*, 2021).** The harmful effects are primarily attributed to the overproduction of reactive oxygen species (ROS) resulting in oxidative stress disruptions to lipid structures within cells and mitochondrial functioning leading to cell death, in sperm forming cells **(Chianese and Pierantoni, 2021; Gao *et al.*, 2023).** Moreover, MTX-induced testicular toxicity often manifests histologically as disorganization of seminiferous tubules, reduction in germ cell populations, interstitial edema, and compromised spermatogenesis, which collectively threaten male fertility **(Sarman *et al.*, 2023).** The harmful changes in tissue structure and function can lead to lasting difficulties, with reproduction if left unchecked – underscoring the crucial need to find additional substances that can help reduce these negative impacts.

Significant progress is being made in exploring natural bioactive substances for their ability to protect against the harmful effects of medications. (**Sultan and Taqa, 2024; Hamed *et al.*, 2022).** One such compound is ferulic acid (FA) a derivative of hydroxycinnamic acid found in abundance in the cell walls of grains, fruits and herbal plant **(Khan *et al.*, 2024).** FA is widely known for its antioxidant properties and its ability to reduce inflammation and combat free radicals as well as ferulic acid may be a strong neuroprotectant agent against Alzheimer's disease with potential therapeutic benefits and nutritional supplement usefulness **(Khalifa *et al.*, 2024).** These qualities play a role, in protecting cells from oxidative damage as highlighted by **Thulluri *et al.*, 2025; Al-Moula *et al.*, 2012.** In terms of functionality FA has

been proven to influence signaling pathways which control cell death and responses to oxidative stress. As a result, it provides benefits, in studies involving liver damage kidney damage and nerve damage **(Mustafa *et al.,* 2024; AL-fakje *et al.,* 2025; Khalifa *et al.,* 2025).** However, despite these attributes the exploration of FA potential, in protecting the male reproductive system, particularly the testes from MTX induced harm has not been thoroughly studied **(El-Speiy *et al.,* 2025).** Existing literature offers scarce insights into its precise histological and molecular effects within testicular tissue exposed to chemotherapeutic insults. This lack of comprehensive studies underscores a significant knowledge gap, particularly concerning the dual application of FA both as a prophylactic and as a therapeutic agent.

Accordingly, the present study was designed to critically evaluate, using a combined histopathological and immunohistochemical approach, the protective and therapeutic potentials of FA against MTX-induced testicular damage in a rat model. Through detailed examination of seminiferous tubule architecture and assessment of Caspase-3-mediated apoptotic activity, this investigation aims not only to elucidate the ameliorative mechanisms of FA but also to contribute foundational evidence supporting its future translational application in clinical oncology settings where fertility preservation is paramount.

MATERIALS AND METHODS

Experimental Animals

In alignment with the ethical approving obtained from the Animal Care and Use Committee, College of Dentistry, University of Mosul (Approval No: 25/1079), twenty-four adult male albino rats (weighing 300–400 g, aged 12–16 weeks) were utilized. The animals were placed under normal lab climatic conditions (22 ± 2°C, 12 h light/dark cycle), with free access to a standard pellet diet and water ad libitum. The rats were acclimatized for two weeks prior to experimental procedures, thus ensuring physiological stability and minimizing stress-induced variability, which might otherwise confound the interpretation of histological and biochemical data.

Ethical Approval

All trial method and animal management protocols were conducted in strict accordance with the guidelines set by the Institutional Animal Care and Use Committee (IACUC) of the College of Dentistry, University of Mosul. The study was approved under protocol number UOM.25/1079, dated 11/5/2025], ensuring adherence to international standards for animal welfare and minimizing animal distress throughout the experimental timeline.

Chemicals and Reagents

Methotrexate (MTX) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) with a declared purity of ≥98%, ensuring consistency and reproducibility across experiments. Ferulic acid (FA) was obtained from Cayman Chem (Ann Arbor, MI, USA), certified at ≥99% purity. All other chemicals and solvents, including formaldehyde, ethanol, xylene, and Mayer's hematoxylin, were of analytical grade and sourced from Thermo Fisher sci (Waltham, MA, USA). Fresh solutions were prepared prior to each experimental procedure to maintain accuracy and minimize batch-to-batch variability.

Experimental Design

The rats were at random allocated into four equal groups (n = 6), employing a random number generator to reduce selection bias, as follows:

- **Control group (C):** received an intraperitoneal injection of 3 mL/kg as (b.w.) sterile normal saline on day 15.
- **MTX group:** receiving a single I.P. injection of MTX (40 mg/kg b.w.) on day 15 to induce testicular toxicity, a protocol consistent with previously established models **(Gürler *et al.,* 2023).**
- **FA + MTX protective group:** received FA orally (50 mg/kg b.w.) once daily **(Sanjeev *et al.,* 2019)** for 14 sequential days before and 14 days after MTX administration on day 15 single I.P. injection, to investigate the preventive potential of FA against MTX-induced damage.
- **MTX + FA therapeutic group:** received MTX single I.P. injection as above on day 15, followed by FA administration (50 mg/kg , orally) daily for 14 days to evaluate FA's therapeutic effects.

All oral administrations were performed via oral using a stainless-steel feeding needle to ensure precise dosing, and animals were closely monitored for general health status, body weight changes, and behavioral alterations.

Tissue Sampling and Histopathological Examination

At the ending of the experimental period (day 29), rats were anaesthetized with ketamine ketamine (80 mg/kg) and xylazine (10 mg/kg) intraperitoneally to minimize suffering, followed by euthanasia through exsanguination **(Attia et al., 2025).** Testes were carefully excised, weighed, and at once fixed in 10% neutral buffered formalin for at least 48 hours to preserve tissue architecture. Following fixation, specimens were dehydrated in ascending grades of ethanol, cleared in xylene, and embedded in paraffin. Serial sections of 5 µm thickness were prepared using a rotary microtome and stained with hematoxylin and eosin (H&E) to assess histological alterations. Sections were examined under a light microscope (Olympus CX31, Japan), and representative images were captured at magnifications of 100× and 400×. **(Nagy *et al.,* 2024).**

Histopathological changes were semi-quantitatively scored based on tubular degeneration, germ cell loss, interstitial edema, and vacuolization using a standardized grading system (score 0–3), as previously described **(Shemiss *et al.*, 2025).**

Histomorphometric analysis

To quantitatively evaluate the histological changes in the seminiferous tubules, histomorphometric measurements were conducted usage ImageJ software version [version, 1.53c] (Nati. Instit. of Health, Bethesda, MD, USA). Testicular sections stained with hematoxylin and eosin (H&E) were captured under a light microscope equipped with a digital camera at magnifications of 100× and 400×.

Seminiferous tubule diameter and germinal epithelial height were measured by calibrating the software using the scale bar embedded in each micrograph (e.g., 100 µm). For each animal, ten randomly selected, round or nearly round seminiferous tubules were evaluated to ensure representative sampling. Germinal epithelial height was determined by drawing a perpendicular line from the basement membrane to the luminal edge of the epithelium. Moreover, the number of spermatogenic cells, including spermatogonia, primary spermatocytes, spermatids, and spermatozoa, was counted per seminiferous tubule cross-section in ten randomly chosen fields. The mean value for each parameter was calculated per animal, and then group means ± standard error of the mean (SEM) was computed for statistical analysis. This digital morphometric approach ensured precise, unbiased, and reproducible quantification of testicular histological alterations across experimental groups.

Immunohistochemical Analysis

To evaluate apoptosis, immunohistochemical staining for Caspase-3 expression was performed (Dako company, USA). Paraffin sections portion were deparaffinized, rehydrated, and subjected to antigen retrieval using citrate buffer (pH 6.0) in a microwave oven for 15 minutes. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 minutes. Sections were then incubated overnight at 4°C with primary anti-Caspase-3 antibody dilution 1:100, followed by incubation with appropriate secondary antibody conjugated to horseradish peroxidase (HRP). Immunoreactivity was visualized using diaminobenzidine (DAB) as chromogen, and nuclei were counterstained with Mayer's hematoxylin. Caspase-3 immunoreactivity was semi-quantitatively scored as follows: score 0 (negative), score 1 (weak), score 2 (moderate), and score 3 (strong), based on staining intensity and the percentage of positive cells.

Statistical Analysis

All statistical test were carried out using GraphPad Prism version 9.0 (GraphPad Software Inc., San Diego, CA, USA). Quantitative histomorphometric data were expressed as mean ± standard error of the mean (SEM) and analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to determine intergroup differences. In parallel, semi-quantitative histopathological scores were evaluated using the Kruskal–Wallis test coupled with Dunn's multiple comparison post hoc test to account for the ordinal nature of these data. Differences were considered statistically significant at $p<0.05$.(**Renjith and Arunkumar, 2025**.

RESULTS

Histopathological Findings

The histopathological evaluation of testicular tissues in the control group revealed intact seminiferous tubules characterized by well-organized germinal epithelium and clearly discernible layers of spermatogenic cells, including spermatogonia, spermatocytes, spermatids, and mature spermatozoa, in addition to normal Sertoli and Leydig cells. These features collectively denote active and physiologically intact spermatogenesis **(Fig.1A, B).**

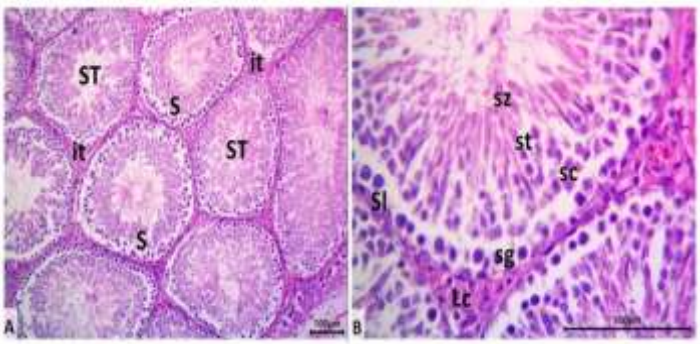

Fig. 1: Histological section of testis from control group. [A]: intact seminiferous tubules (ST) with active spermatogenesis and different types of spermatogenic cells (S), and interstitial tissue (it). [B]: intact seminiferous tubule with spermatogonia (sg), spermatocytes (sc), spermatids (st), spermatozoa (sz), Sertoli cell (Sl) and Leydig cell (Lc). H&E stain, [A: 100X; B: 400X]. Scale bar = 100 µm.

Conversely, the MTX-treated group demonstrated profound architectural disruptions of seminiferous tubules, accompanied by severe disorganization, extensive depletion of germ cells, necrosis, and conspicuously widened tubular lumens. Furthermore, marked interstitial edema was evident, indicating substantial vascular and stromal compromise **(Fig. 2 A, B).** Such histological deterioration unequivocally corroborates the oxidative and apoptotic

damage mediated by MTX, consistent with previous observations in similar chemotherapeutic models.

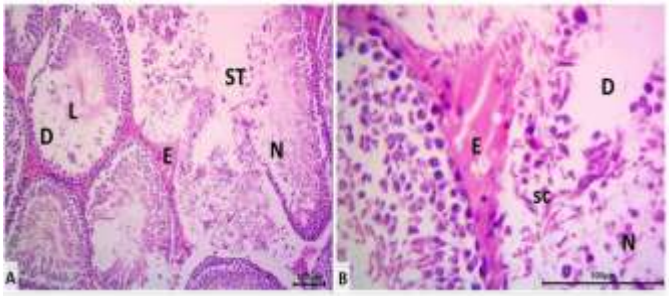

Fig. 2: Histological section of testis from methotrexate (MTX) group. [A]: Severe disorganization of seminiferous tubules (ST) with widened lumen (L), germ cell depletion (D), necrosis (N), and interstitial edema (E). [B]: arrested spermatogenesis in seminiferous tubules (Sc), depletion (D) and necrosis of spermatogenic cells (N), and interstitial edema (E). H&E stain, [A: 100X; B: 400X]. Scale bar = 100 µm.

Interestingly, the MTX+FA protective group exhibited notable histological improvement compared to the MTX-alone group. Seminiferous tubules displayed mild preservation of seminiferous tubule structure and partial preservation of germinal layers, with only minimal depletion and necrosis of spermatogenic cells. Edema within the interstitial tissue was still observed but to a lesser extent, suggesting partial mitigation of MTX-induced structural damage **(Fig 3A, B).**

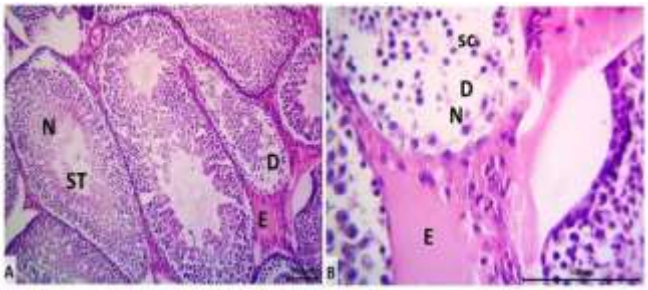

Fig.3: Histological section of testis from ferulic acid protection + MTX treated group. [A]: mild preservation of seminiferous tubule structure (ST), mild necrosis of spermatogenic cells (N), and interstitial edema (E). [B]: mild arrested spermatogenesis (Sc), mild depletion (D) and necrosis of spermatogenic cells (N), and edema (E). H&E stain, [A: 100X; B: 400X]. Scale bar = 100 µm.

This partial preservation implies that pre-administration of FA confers a degree of prophylactic protection, likely through antioxidative and cytoprotective pathways. In the MTX+FA therapeutic group, testicular architecture appeared substantially restored, demonstrating nearly intact seminiferous tubules with only mild degeneration and focal necrosis of spermatogenic cells. Moreover, mild congestion of interstitial blood vessels was detected, yet overall tissue integrity was largely maintained **(Fig.4 A, B).** This induced gonadotoxicity. pronounced structural preservation underscores the reparative potential of FA when administered post-injury, highlighting its therapeutic efficacy in counteracting MTX- induced gonadotoxicity.

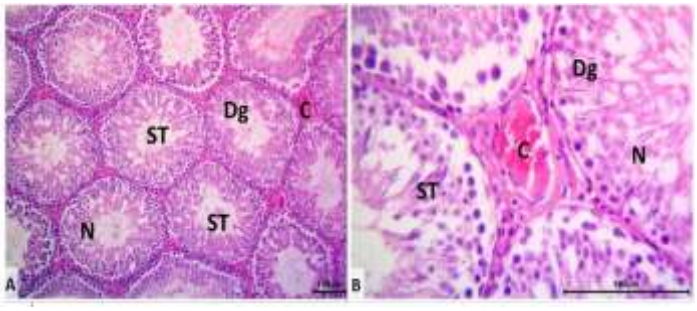

Fig. 4: Histological section of testis from MTX treated + ferulic acid (FA) therapeutic group. [A&B]: almost intact seminiferous tubules (ST) with mild degeneration (Dg) and mild necrosis (N), blood vessels congestions (C). H&E stain, [A: 100X; B: 400X]. Scale bar = 100 µm.

Interpretation of Table 1 (Histopathological alterations)

Table 1 clearly demonstrates the severe testicular damage induced by MTX. The MTX group recorded the highest scores across all evaluated histopathological parameters, including complete disruption of tubular architecture, extensive germ cell depletion, pronounced necrosis, and marked interstitial edema. These changes reflect the destructive impact of MTX on testicular structure.

In contrast, rats pretreated with FA in the protective group showed a noticeable reduction in damage severity. Although mild degeneration and limited germ cell loss were still present, the overall tissue architecture was largely preserved compared to the MTX-only group.

Strikingly, the therapeutic group that received FA after MTX administration exhibited near-normal histological features, with scores approaching those of the control group. Tubular structures were mostly intact, germinal epithelium was preserved, and signs of necrosis or edema were minimal. This highlights FA's stronger therapeutic potential when administered after toxicity, suggesting that it more effectively halts progressive tissue damage than it prevents it.

The histomorphometric measurements summarised **Tables 2 and 3** further corroborate the qualitative histological observations. The MTX group exhibited a significant reduction in seminiferous tubule diameter and germinal epithelial height, reflecting severe structural atrophy and impaired spermatogenesis. In contrast, both FA-treated groups showed remarkable improvements in these parameters, with the therapeutic group approaching control values, thereby demonstrating

superior restoration of testicular architecture and spermatogenic activity.

Table 1: Semiquantitative Scoring of Histopathological Alterations in Rat Testes Across Experimental Groups.

Parameter	Control Group	MTX Group	MTX + FA Protective	MTX + FA Therapeutic
Tubular architecture integrity	0 ± 0	3 ± 0.00 **	1 ± 0.26 *	0.3 ± 0.21 ns
Germ cell depletion	0 ± 0	3 ± 0.00 **	1 ± 0.24 *	0.4 ± 0.19 ns
Necrosis of spermatogenic cells	0 ± 0	3 ± 0.00 **	1 ± 0.27 *	0.4 ± 0.22 ns
Interstitial edema	0 ± 0	3 ± 0.00 **	1 ± 0.25 *	0.3 ± 0.19 ns
Blood vessels congestion	0 ± 0	2 ± 0.32 **	1 ± 0.21 *	1 ± 0.17 *

*Scoring: 0 = absent; 1 = mild; 2 = moderate; 3 = severe. Notes: Data are expressed as mean ± SEM. **p<0.01 vs. control group; *p<0.05 vs.* MTX group; ns = non-significant vs. control.

Table 2: Histomorphometric measurements of seminiferous tubules in different experimental groups.

Parameter	Control Group	MTX Group	FA + MTX Protective	MTX + FA Therapeutic
Seminiferous tubule diameter (µm)	210.5 ± 5.2	140.3 ± 6.1**	180.7 ± 4.9*	205.4 ± 5.0 ns
Germinal epithelial height (µm)	75.2 ± 3.4	35.6 ± 2.9**	55.1 ± 3.2*	70.8 ± 3.1 ns

*Notes: Data are expressed as mean ± SEM. **p<0.01 vs. control group; *p<0.05 vs.* MTX group; ns = non-significant vs. control.

Table 3: Quantitative assessment of spermatogenic cell population.

Cell type	Control Group (cells/tubule)	MTX Group	FA + MTX Protective	MTX + FA Therapeutic
Spermatogonia	48 ± 3	20 ± 2**	33 ± 2*	44 ± 3 ns
Spermatocytes	36 ± 2	15 ± 1**	25 ± 2*	34 ± 2 ns
Spermatids	60 ± 4	18 ± 2**	42 ± 3*	56 ± 3 ns
Spermatozoa	82 ± 5	22 ± 2**	58 ± 4*	78 ± 4 ns

* Notes: Data are expressed as mean ± SEM. **p<0.01 vs. control group*; p<0.05 vs.* MTX group; ns = non-significant vs. control.

The **fig.5** illustrates the mean seminiferous tubule diameter and germinal epithelial height (µm) across experimental groups (Control, MTX, MTX+FA Protective, MTX+FA Therapeutic). Data are presented as mean ± SEM, with significance indicated by * ($p < 0.05$).

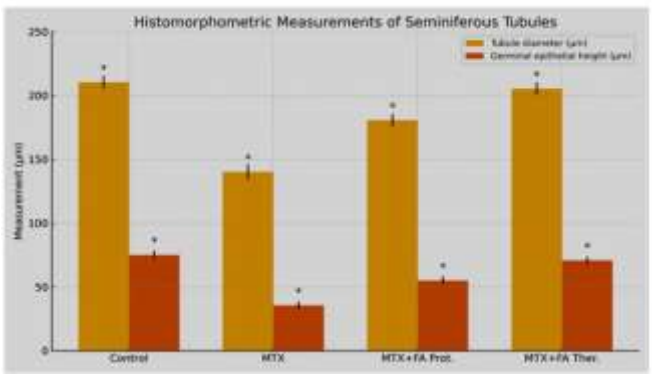

Fig. 5: Histomorphometric Measurements of Seminiferous Tubules.

The grouped bar **Fig. 6** shows the counts of spermatogonia, spermatocytes, spermatids, and spermatozoa per tubule in each experimental group.

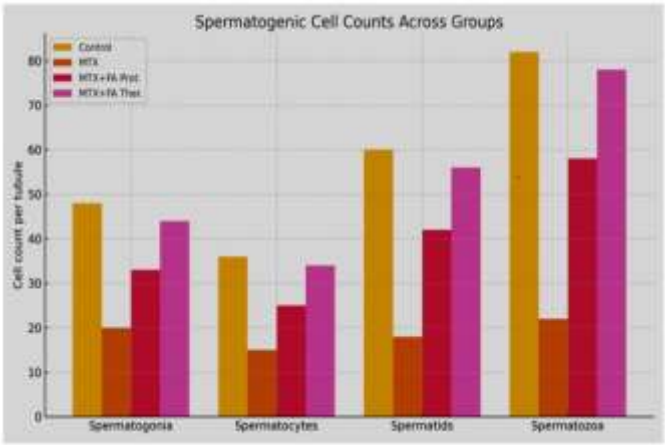

Fig. 6: Spermatogenic Cell Counts Across Groups.

Future studies are encouraged to incorporate functional fertility assessments, such as analyses of sperm count, motility, and morphology, to complement histopathological and molecular findings. Integrating these physiological parameters would provide critical functional evidence, enhancing the translational relevance and comprehensive understanding of FA's protective potential against MTX-induced reproductive toxicity.

Immunohistochemical Findings

Assessment of Caspase-3 expression provided further insights into the apoptotic landscape across the experimental groups. In the control group, weak Caspase-3 expression (score 1) was observed, reflecting basal physiological apoptosis essential for normal spermatogenic turnover **(Fig. 7A).**

The MTX group, however, demonstrated intense Caspase-3 immunoreactivity (score 3), indicating robust activation of apoptotic pathways in response to MTX-induced oxidative stress **(Fig. 7B).** This marked upregulation of Caspase-3 aligns with the histopathological evidence of widespread germ cell necrosis and supports the hypothesis of apoptosis as a central mechanism of MTX-mediated testicular damage.

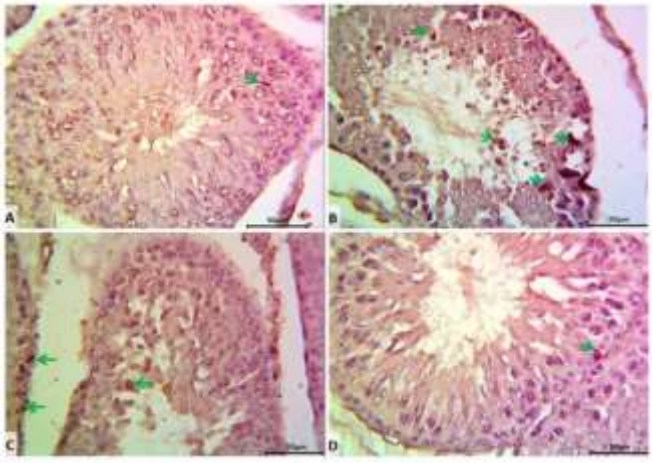

Fig. 7: Immunohistochemistry expression of Caspase 3 in the rat testis. [A]: control group reveals weak expression (arrow) (score 1). [B]: methotrexate group reveals intense expression (arrows) (score 3). [C]: from ferulic acid protection + methotrexate treated group reveals moderate expression(arrows) (score 2). [D]: methotrexate treated + ferulic acid therapeutic group reveals weak expression(arrow) (score 1). Hematoxylin stain; 100X. Scale bar=100µm.

Notably, the MTX + FA protective group exhibited moderate Caspase-3 expression (score 2), signifying a partial attenuation of apoptosis relative to the MTX-only group **(Fig. 7C).** This finding suggests that prophylactic FA administration mitigates, but does not completely abrogate, apoptotic activation, reflecting its limited preemptive antioxidant effect.

In contrast, the MTX + FA therapeutic group revealed weak Caspase-3 expression comparable to controls (score 1), indicating effective suppression of apoptotic signalling pathways **(Fig. 7D).** This striking reduction further substantiates the histological evidence of preserved tubular integrity and suggests that post-injury FA administration more effectively interrupts apoptotic cascades, thereby facilitating cellular recovery.

As shown in **Table 4**, MTX administration significantly elevated Caspase-3 expression in testicular tissue, reaching the maximum score of 3. This strong immunoreactivity reflects an intense activation of apoptotic pathways, corroborating the observed histological signs of germ cell death. In simple terms, MTX appears to trigger substantial cellular suicide within the testes, which explains the observed loss of spermatogenic cells.

Table 4. Semiquantitative Scoring of Caspase-3 Expression in Rat Testicular Tissue.

Experimental Group	Caspase-3 Expression Score
Control	1 ± 0.00
MTX	3 ± 0.00 **
MTX + FA Protective	2 ± 0.26 *
MTX + FA Therapeutic	1 ± 0.12 ns

* Scoring system: 0 = negative; 1 = weak; 2 = moderate; 3 = strong. Notes: Data are expressed as mean ± SEM. **p<0.01 vs. control group*; p<0.05 vs.* MTX group; ns = non-significant vs. control.

DISCUSSION

The recent research provides evidence that MTX, commonly used in chemotherapy and to suppress the immune system, can cause significant changes in testicular structure and genes through oxidative stress and cell death processes. Similar to findings by **Othman *et al.*, (2023) ; Khamis *et al.*, (2023)**, the group given MTK showed major disruptions in the arrangement of seminiferous tubules, along with notable reduction and death of germ cells and noticeable swelling between them, which led to serious issues with sperm production. The significant increase in Caspase 3 levels in this group emphasises the role of cell death in MTX induced damage to the reproductive system. This is consistent with research that highlighted Caspase 3 as a critical factor in triggering cell death in reproductive cells after

chemotherapy exposure **(Yousif *et al.,* 2023; Gholami *et al.,* 2024).**

FA a natural phenolic compound known for its antioxidant and anti-inflammatory properties, showed notable protective and healing effects against testicular damage induced by MTX. The latest results indicated that prior usage of FA partly maintained the structure of seminiferous tubules and decreased apoptotic activity to some extent as seen through improvements in histomorphometry and a reduction in Caspase 3 expression (**Khalifa *et al.,* 2024**). The findings indicate that FA acts as an antioxidant defence system by removing harmful ROS and maintaining the stability of cell membranes before the damaging effects of MTx take place **(Attarbashiee *et al.,* 2023**). This aligns with its recognised abilities to counteract radicals and protect cells **(Marin *et al.,* 2022; Ali *et al.,* 2024).**

New insights and similarities are revealed in our current findings. For example, **Hassanein *et al.* (2021)** showed that FA was effective in reducing toxicity induced by cisplatin by inhibiting TLR4 and p38 MAPK pathways. This highlights FA's ability to modulate molecular processes. In a manner, **Gholami *et al.*' s** study in **(2024)** found that selenium improved MTX-induced damage by reducing Caspase. 3 expression and restoring the testicular structure, which aligns with our discovery of decreased Caspase 3 Activity, after administering FA. Additionally, these comparative studies indicate synergies between different antioxidants in reducing the adverse effects of chemotherapy on reproductive organs **(Attarbashee, *et al.,* 2023).** As a result, these results underscore the ability of FA to combat stress and cell death mechanisms **(Dawood *et al.,* 2020; Ibrahem *et al.,* 2020).**

Post-treatment with FA substantially restored testicular architecture and normalized apoptotic markers to levels comparable to the control group, underscoring its therapeutic efficacy against MTX-induced gonadotoxicity. This highlights FA's ability to halt oxidative processes and regulate apoptotic signalling pathways more effectively once an injury occurs. FA is known to boost the activity of antioxidant enzymes like superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx), which in turn reduces lipid peroxidation and maintains the balance in cellular redox state **(Khan *et al.,* 2024; Zheng *et al.,* 2024).**

Additionally, FA can modulate nuclear factor erythroid 2–related factor 2 (Nrf2) signalling, leading to transcriptional upregulation of antioxidant response element (ARE)-driven genes, providing a robust defence against oxidative insult **(Tripathi *et al.,* 2024).** Moreover, the observed downregulation of Caspase-3 in the FA therapeutic group highlights its potential anti-apoptotic mechanisms, possibly mediated through the suppression of mitochondrial cytochrome c release and inhibition of caspase cascade activation **(Abuzaied *et al.,*2024 ;Wendlocha *et al.,* 2024).**

These molecular insights corroborate our histological observations and strengthen the hypothesis that FA not only prevents structural deterioration but also mitigates programmed cell death in germ cells. The present data are in agreement with a recent study demonstrating the ameliorative effect of FA on cisplatin-induced testicular toxicity, where FA reduced oxidative stress markers and improved sperm parameters **(Hassanein *et al.,* 2021).** Furthermore, our findings extend the protective spectrum of FA beyond nephro- and hepatoprotection, as previously described by (**Chen *et al.,* 2025)**, into the domain of male reproductive health.

Interestingly, while both protective and therapeutic protocols showed beneficial effects, the therapeutic regimen was notably more efficacious. This differential efficacy underscores the importance of temporal dynamics in antioxidant interventions, suggesting that post-injury administration of FA may allow for direct targeting of ongoing oxidative and apoptotic events, thus enhancing tissue recovery.

Collectively, these findings suggest that FA holds promise as an adjunctive agent in mitigating chemotherapeutic gonadotoxicity, potentially improving fertility outcomes in patients undergoing MTX therapy. Future investigations are warranted to elucidate further molecular interactions, optimal dosing strategies, and potential synergistic effects with other antioxidants in clinical settings.

CONCLUSIONS

In summary, the present study robustly demonstrates that FA confers significant histological and functional protection against MTX-induced testicular toxicity in rats, primarily via antioxidant and anti-apoptotic mechanisms. Notably, the incorporation of functional sperm assessments further strengthens the translational value of these findings. However, it is crucial to conduct clinical research that includes an in-depth analysis of hormones and long-term fertility assessments to confirm the positive preclinical results. FA could potentially become a strong supplementary option for safeguarding male reproductive well-being throughout chemotherapy treatments.

Study limitations

While the histopathological and functional results of this study are intriguingly convincing, it does

have its limitations to consider. To begin with. The study's setup involved administering a single dose of both MTX and FA; this approach might not account for potential dose-related effects or accurately mirror real-world clinical variations. Crucial hormonal assessments like testosterone levels and markers such as follicle stimulant hormone (FSH) and luteinizing hormone (LH) which play a significant role, in overall reproductive endocrine well-being, were not conducted. So far, the examination of molecules concentrated only on Caspase 3 expression, excluding an assessment of other significant markers of oxidative stress, like malondialdehyde (MDA), Bax and Bcl. 2 expressions which limits the understanding of mechanisms involved in the process.

Future recommendations

In light of these limitations, upcoming research should include trials involving doses and various time frames for both MTX and FA to more accurately model real-world medical situations. It is advisable to conduct hormonal analysis and in-depth molecular examinations that encompass additional indicators related to cell death and oxidative stress. Furthermore, it is highly recommended to conduct studies focusing on reproductive health outcomes over the long term, including evaluations of fertility rates and the quality of offspring, to validate the practical significance of these discoveries. Finally, discussing how FA could work with other natural or synthetic antioxidants might enhance the protective benefits even more and open up new possibilities for treatment.

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