The Ameliorative Effects of Ferulic Acid Against Methotrexate Induced Testicular Damage in Rats as a Model

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WORD COUNT 4612
TIME SUBMITTED 13-AUG-2025 10:02AM
PAPER ID 117709303

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Abstract

Methotrexate (MTX), an extensively used chemotherapeutic and immunosuppressive agent, is well-documented for its gonadotoxic effects, primarily mediated through oxidative stress and apoptotic pathways. Ferulic acid (FA), a natural phenolic compound with potent antioxidant properties, has shown protective effects in various organ systems; however, its role in ameliorating MTX-induced testicular toxicity remains inadequately explored. This study aimed to assess the protective and therapeutic potentials of FA against MTX-induced testicular damage in a rat model, using integrative histopathological, histomorphometric, and immunohistochemical analyses. Twenty-four adult male rats were allocated into four groups: control, MTX-only, MTX plus FA (protective), and MTX followed by FA (therapeutic). Testicular tissues were assessed histologically for tubular integrity and germ cell preservation. Histomorphometric parameters, including seminiferous tubule diameter and germinal epithelial height, were quantified using ImageJ software. The level of Caspase-3 expression was analyzed using immunohistochemistry to examine apoptosis activity. The group treated with MTX showed damage to the structure of the testicles and a significant decrease in germ cells along with lower histomorphometric measures and increased Caspase-3 expression levels. On the other hand, both groups treated with FA displayed noticeable improvements in both histology and molecular aspects. The group receiving therapy showed normal testicular structure restored histomorphometric values and minimal Caspase-3 expression similar, to the control group. These results indicate that FA is more effective when given after an injury occurs. Conclusion**:**(FA) successfully reduced the effects of methotrexate (MTx) in the testicles by maintaining their structure and preventing cell death processes. This suggests that FA could be an addition to protect men's reproductive health during chemotherapy treatments. More research in settings is required to confirm these findings and establish the best dosage plans, for practical use.

Keywords

Methotrexate; Ferulic acid; Testicular toxicity; Spermatogenesis; Oxidative stress; Apoptosis; Histomorphometry

INTRODUCTION

Methotrexate stands as a known antifolate chemotherapy drug extensively applied to treat a range of cancers and autoimmune disorders like rheumatoid arthritis and psoriasis. 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 concerning 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 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 are not just temporary. 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.

In the few decades investigation has higher focused on 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 acid (FA) a derivative of hydroxycinnamic acid found in abundance in the cell walls of grains, fruits and herbal which has captured the attention of many scientists **(Khan *et al.,* 2024).** FA is widely known for its antioxidant properties and its ability to reduce inflammation and combat free radicals. 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 folic acids potential, in protecting the male reproductive system, particularly the testes from methotrexate induced harm has not been thoroughly studied. 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 (preventive) and as a therapeutic (curative) agent.

Accordingly, the present study was meticulously 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 gained 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 (20 mg/kg b.w.) on day 15 to induce testicular toxicity, a protocol consistent with previously established models (El-Sayed et al., 2022).
- **MTX + FA protective group:** received FA orally (20 mg/kg b.w.) once daily for 14 sequential days before and 14 days after MTX administration on day 15, to investigate the preventive potential of FA against MTX-induced damage.
- **MTX + FA therapeutic group:** received MTX as above on day 15, followed by FA administration (20 mg/kg , orally) daily for 14 days to evaluate FA's reparative effects post-injury.

All oral administrations were performed via gavage 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 (80 mg/kg) and xylazine (10 mg/kg) intraperitoneally to minimize suffering, followed by euthanasia through exsanguination. 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×.

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) were 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 $p < 0.05$.

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.1 A,B).**

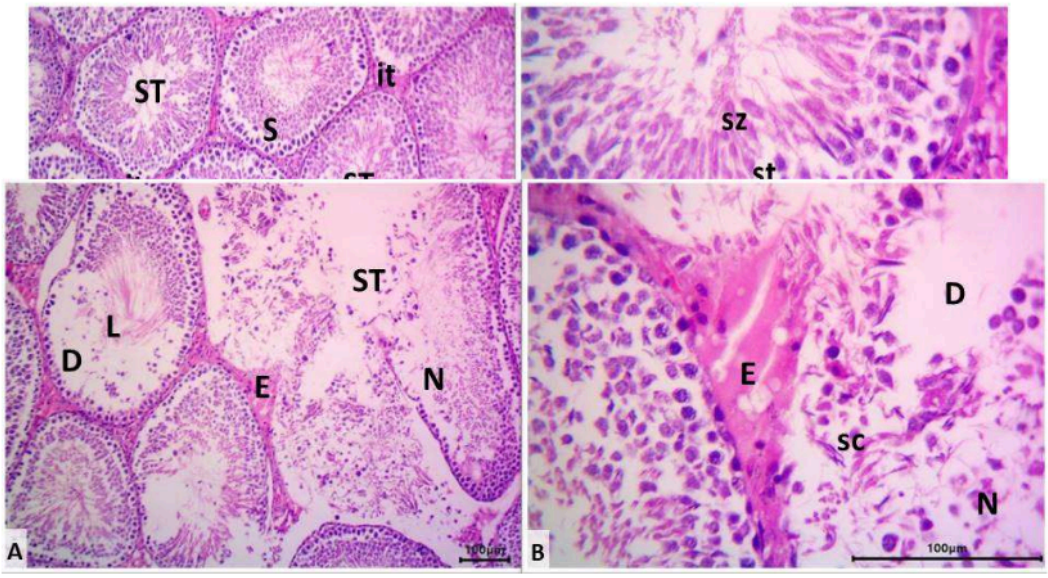
Conversely, the MTX-treated group demonstrated profound architectural disruptions of seminiferous tubules, accompanied by severe disorganization, extensive depletion of germ cells, necrosis, and conspicuously widened tubular lumens. Furthermore, marked interstitial edema was evident, indicating substantial 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.

Interestingly, the MTX+FA protective group exhibited notable histological improvement compared to the MTX-alone group. Seminiferous tubules displayed mild elongation 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).** 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 pronounced structural preservation underscores the reparative potential of FA when

administered post-injury, highlighting its therapeutic efficacy in counteracting MTX-induced gonadotoxicity.

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=100µm.

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

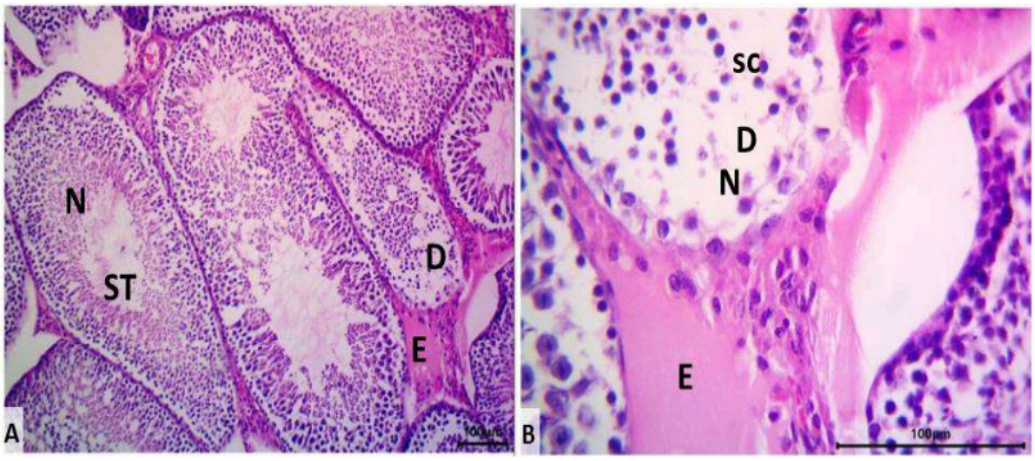


Fig.3: Histological section of testis from ferulic acid protection + methotrexate treated group. [A]: mild elongation of seminiferous tubules (ST), mild necrosis of spermatogenic cells (N), and edema in the interstitial tissue (E). [B]: mild arrested spermatogenesis in seminiferous tubules (Sc), mild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

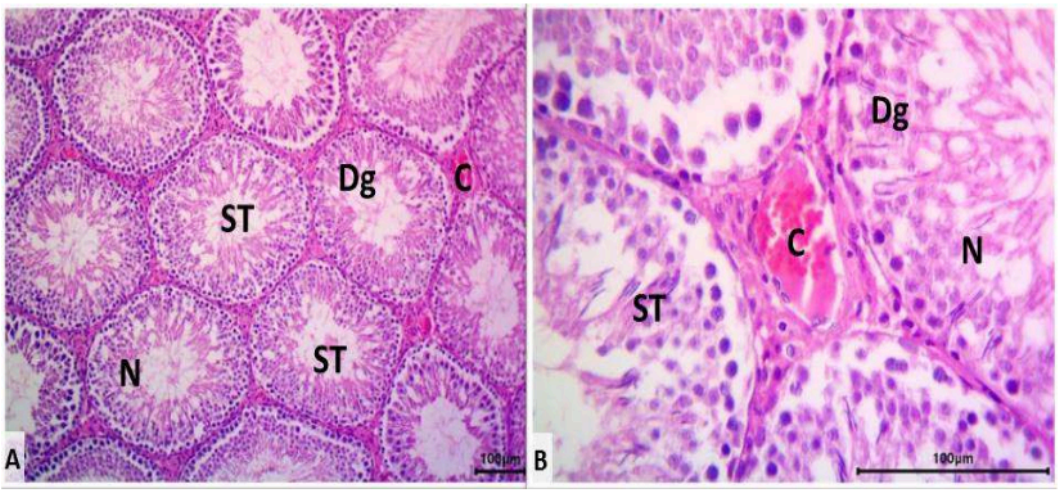


Fig. 4: Histological section of testis from methotrexate treated + ferulic acid therapeutic group. [A&B]: almost intact seminiferous tubules (ST) with mild degeneration (Dg) and mild necrosis (N) of spermatogenic cells, and congestion of blood vessels in the interstitial tissue (C). H&E stain, [A: 100X;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

Interpretation of Table 1 (Histopathological alterations)

Table 1 clearly demonstrates the severe testicular damage induced by methotrexate (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, most likely mediated by oxidative stress and apoptotic mechanisms.

In contrast, rats pretreated with ferulic acid (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. This suggests that FA, when administered prior to MTX exposure, confers partial protection—likely through its antioxidant and cytoprotective actions.

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 injury, suggesting that it more effectively halts progressive tissue damage than it prevents it.

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
Data expressed as mean ± SEM.
Statistical significance:
$p<0.01$ vs. control group (), *$p<0.05$ vs. MTX group, ns = non-significant vs. control.

The histomorphometric measurements summarized in **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 2. Histomorphometric measurements of seminiferous tubules in different experimental groups.

Parameter	Control Group	MTX Group	MTX + FA 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 as mean ± SEM.
**$p<0.01$ vs. control group;
*$p<0.05$ vs. MTX group;
ns: non-significant difference vs. control group.

Table 3. Quantitative assessment of spermatogenic cell population.

Cell type	Control Group (cells/tubule)	MTX Group	MTX + FA 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 as mean ± SEM.
**p < 0.01 vs. control group;
*p < 0.05 vs. MTX group;
ns: non-significant difference vs. control group.

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 * ($p < 0.05$).

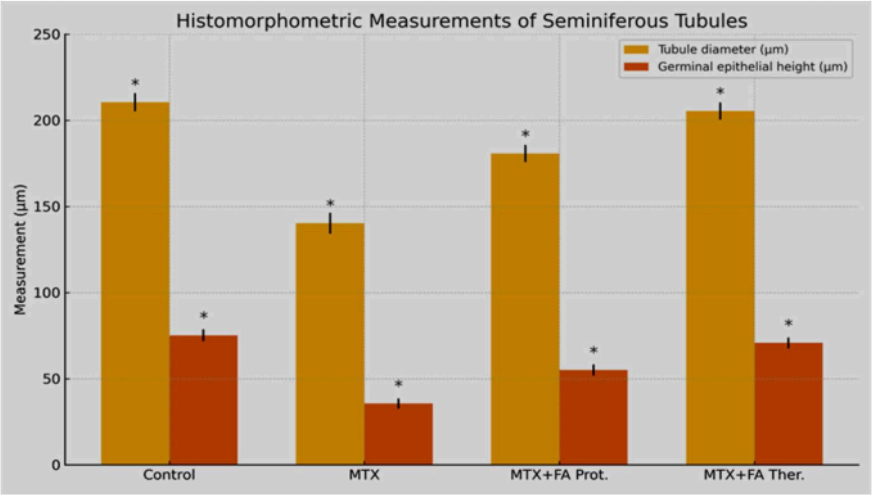


Fig. 5. Histomorphometric Measurements of Seminiferous Tubules.

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

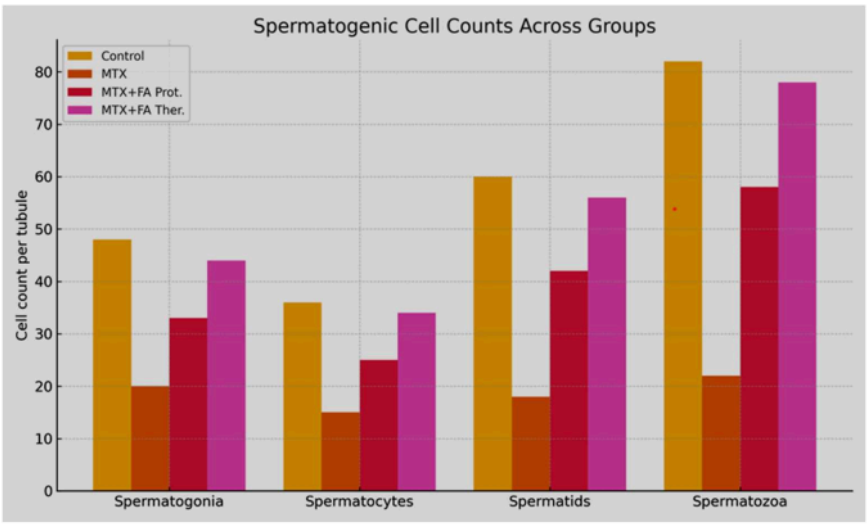


Fig. 6. Spermatogenic Cell Counts Across Groups.

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. 5A).**

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. 5B).** 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.

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. 5C).** 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 signaling pathways **(Fig. 5D).** 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.

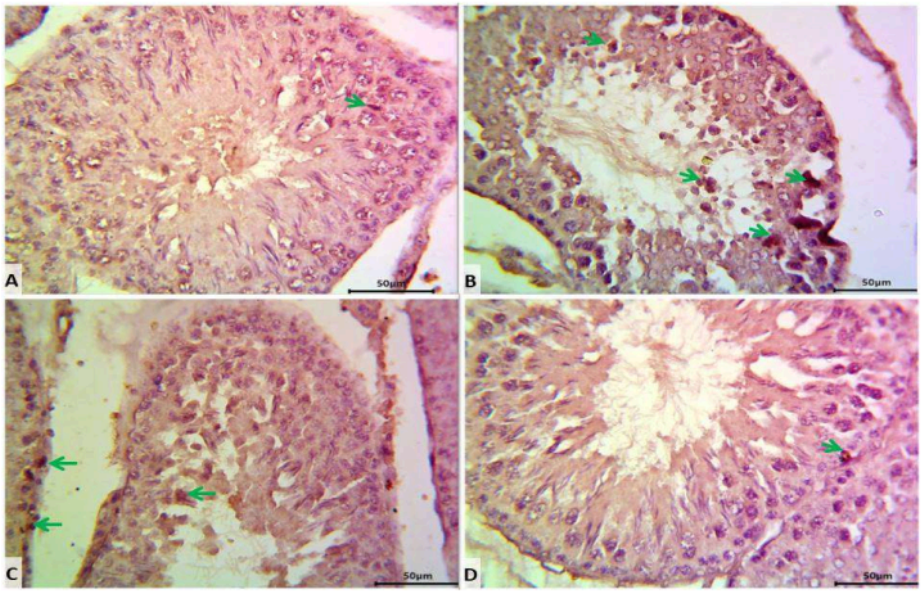


Fig. 7: Immunohistochemistry expression of Caspase 3 in the rat testis. [A]: control group reveals weak expression (arrow) (score 1). [B]: methotrexate group reveals intense expression (arrows) (score 3). [C]: from ferulic acid protection + methotrexate treated group reveals moderate expression(arrows)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

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.

The protective FA group showed a moderate reduction in Caspase-3 levels, indicating that pre-treatment with FA can dampen—but not completely block—apoptotic activity. While this partial reduction is promising, it does not fully prevent the molecular cascade initiated by MTX.

Notably, the therapeutic FA group displayed weak Caspase-3 expression, comparable to that of the control group. This significant reduction suggests that FA, when administered after MTX-induced injury, is capable of halting or reversing apoptotic processes. These results provide molecular confirmation of the histological recovery observed in this group, underscoring the effectiveness of post-injury intervention with FA in mitigating testicular apoptosis.

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
Data expressed as mean ± SEM.
Statistical significance:
$p<0.01$ vs. control group (), *$p<0.05$ vs. MTX group, ns = non-significant vs. control.

DISCUSSION

The recent research provides evidence that methotrexate (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 mostly. 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 emphasizes 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).**

Ferulic acid (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 in contrast to the original text. The latest results indicated that prior usage of FA (in the group) 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 defense system by removing harmful reactive oxygen species (ROS) and maintaining the stability of cell membranes before the damaging effects of MTx take place **(Attarbashiee *et al.*, 2023**). This aligns, with its recognized abilities to counteract radicals and protect cells. **(Marin *et al.*, 2022; ; Ali *et al.*, 2024).**

When compared to studies in this field of research new insights and similarities are revealed in our current findings. For example, **Hassanein *et al*,**.' s work in **(2021)** showed that FA was effective in reducing toxicity induced by cisplatin by inhibiting TLR4 and p38 MAPK pathways. This highlights FAs 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 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 simultaneously providing **(Dawood *et al.*, 2020; Ibrahem *et al.*, 2020).**

After treating MTX exposure with FA therapy remarkably restored testicular structure almost completely and normalized the apoptotic markers to levels similar to those in control samples. This highlights FAs ability to halt oxidative processes and regulate apoptotic signaling 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 (GP x) 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) signaling, leading to transcriptional upregulation of antioxidant response element (ARE)-driven genes, providing a robust defense 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 **(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.

Conclusion

In summary, the present study robustly demonstrates that ferulic acid 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 in depth analysis of hormones and long-term fertility assessments to confirm the positive preclinical results. In the run 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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Acknowledgments

The authors would like to thank the team at the histology lab in the College of Dentistry at the University of Mosul for their support with gratitude towards their contributions. The animal care staff are also recognized for their help, in taking care of the animals during the study period. Thanks are extended to colleagues who provided feedback and guidance during the researchers experimental and analytical stages.

Conflicted interest: None.

Funding: Self-funding.

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