Microbiology, Metabolites and Biotechnology
Journal home page:
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Quercetin Ameliorates Methotrexate-Induced Reproductive Toxicity in Sertoli Cells

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Article Info

Document Type:
Research Paper

Received 10/12/2025
Received in revised form 14/02/2026
Accepted 21/02/2026

Published 26/02/2026

Keywords:
Methotrexate,
Quercetin,
Sertoli,
TM4,
ROS

Abstract

Methotrexate (MTX) is a chemotherapeutic and immunosuppressive drug that can impair spermatogenesis and lead to male infertility. This study examined the effects of different MTX concentrations on TM4 Sertoli cells and evaluated the protective role of quercetin (QT), a natural antioxidant, against MTX-induced oxidative stress.TM4 cells were treated with MTX at the following concentrations: 1 µg/mL /mL, 5 µg/mL /mL, and 10 µg/mL /mL for 24 hours, 48 hours, and 72 hours. Our results show that an IC_{50} value of MTX in these cells was 7 µg/mL. Quercetin was then applied by itself to the cultures at the following concentrations: 10 µg/mL /mL, 25 µg/mL /mL, and 50 µg/mL /mL, and in combination with MTX at its IC_{50} concentration, for 24 hours. MTX significantly reduced TM4 cell viability, whereas quercetin treatment markedly alleviated this cytotoxic effect. Levels of malondialdehyde (MDA) and reactive oxygen species (ROS) were significantly higher in MTX-treated cells compared with controls ($p < 0.001$ and $p < 0.01$, respectively). In contrast, MDA and ROS levels were significantly decreased in quercetin-treated cells ($p < 0.05$ and $p < 0.01$), further supporting its antioxidant activity. In addition, cotreatment with quercetin significantly restored the total antioxidant capacity suppressed by MTX ($p < 0.01$). These findings indicate that quercetin effectively protects TM4 Sertoli cells against MTX-induced oxidative damage.

1. Introduction

It is a fact that infertility poses innumerable problems worldwide, usually resulting in emotional stress, social problems, and high financial burdens on the health systems in most parts of the world. Among all reproductive tissues, testicular cells are by far the most vulnerable to oxidative damage and harmful substances since they have very limited antioxidant defenses and insufficient repair. Reactive oxygen species (ROS) are involved in several cellular pathways, including regulation of apoptosis (Bisht *et al.*, 2017; Sharma & Shrivastava, 2022; Walczak-Jedrzejowska *et al.*, 2013). Natural compounds found in plants have been studied for the purpose of enhancing reproductive health for humans and animals for a long time (Roozbeh *et al.*, 2021). Phytochemical studies have gained tremendous momentum with increasing demand for natural therapeutic agents (Chorosho *et al.*, 2023). Quercetin (QT) is one of the naturally occurring flavonoids that is present widely in many fruits and vegetables, including apples, strawberries, grapes, tomatoes, onions, and broccoli (Beazley & Nurminskaya, 2016). Many studies have investigated both the beneficial and adverse effects of quercetin and other polyphenolic compounds on male reproductive health.

Most findings highlight quercetin's antioxidant activity and its potential as a protective modality against male infertility. Among its properties, quercetin has anti-inflammatory effects, acts against toxic agents and heavy metals, and fights the effects of environmental pollutants. The compound also exerts its antioxidant effect through partial modulation of Nrf2-dependent signaling pathways (Poudineh *et al.*, 2023). Methotrexate (MTX), a drug used to treat malignancies, rheumatoid arthritis, and other autoimmune disorders, is a very often prescribed chemotherapeutic and immunosuppressive agent. It acts as an antagonist of folate by inhibiting dihydrofolate reductase (DHFR), thereby interfering with DNA synthesis and cell proliferation (Elango *et al.*, 2014). Conversely, while a relatively effective therapeutic agent, MTX also interferes with gametogenesis, thereby constituting a hazard to fertility in either sex. Prior studies on quercetin and related polyphenols have belied the complexity of their interaction with reproductive function, producing both positive and negative effects (Wasfey *et al.*, 2023). Quercetin is a well-known antioxidant that scavenges ROS, so it would probably have a protective effect against MTX reproductive toxicity. Through quercetin as a strong free radical quencher, oxidative and inflammatory responses are lessened. Numerous studies

In Press

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DOI: 10.22104/mmb.2026.8048.1192 https://mmb.irost.ir/article_1695.html

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have suggested that quercetin may afford some protection to Sertoli cells against oxidative injury caused by several toxic agents. Rat models, for example, have demonstrated the protective effects of quercetin on Sertoli-germ cell co-cultures exposed to atrazine-induced oxidative stress by reducing ROS generation and lipid peroxidation, thus maintaining integrity within the cell (Gutierrez & Hwang, 2017; Liu *et al.*, 2022; Ma *et al.*, 2024; Ranawat, Kaushik, *et al.*, 2013; Ranawat, Pathak, *et al.*, 2013). Quercetin has protective effects against methotrexate-induced oxidative stress and reproductive toxicity. The present study aimed to investigate the protective effects of quercetin, a natural antioxidant, on TM4 Sertoli cells against methotrexate (MTX)-induced reproductive toxicity. MTX is a widely used chemotherapeutic and immunosuppressive drug known to impair spermatogenesis by reducing sperm concentration and quality, primarily through oxidative stress. Given the crucial role of Sertoli cells in supporting and nourishing developing sperm, understanding how quercetin can mitigate MTX-induced cytotoxicity and oxidative damage in these cells may provide valuable insights into potential strategies for preserving male fertility during chemotherapy. This study, therefore, focused on evaluating cell viability, oxidative stress markers, and antioxidant capacity in TM4 Sertoli cells treated with MTX, with and without quercetin supplementation.

2. Materials and Methods

2.1. Experimental Design

The experimental procedures conforming to ethical research principles were carried out on the mouse Sertoli TM4 cell line bought from the National Center for Genetic and Biological Resources of Iran. The study was approved by the institutional ethics committee (Approval Code: IR.AJUMS.REC.1396.759). TM4 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Cells were maintained at 37 °C in a humidified incubator with 5% CO_2. In addition, TM4 cells were subjected to different concentrations of methotrexate (MTX) from Sigma-Aldrich (St. Louis, MO, USA) at various time points. The experimental design included four varied groups as follows:

1. **Control:** cells treated only with standard culture medium.
2. **MTX (1):** cells treated with 1 µg/mL MTX for 24, 48, and 72 hours.
3. **MTX (5):** cells treated with 5 µg/mL MTX for 24, 48, and 72 hours.
4. **MTX (10):** cells treated with 10 µg/mL MTX for 24, 48, and 72 hours.

The IC_{50} was determined using cell viability data from 24 hours post-exposure. Percentages of growth inhibition were plotted against MTX concentrations to create a dose-response curve, and the IC_{50} value was calculated by a nonlinear regression model. This analysis identifies 7 µg/mL as the IC_{50} value for MTX in TM4 cells. In order to evaluate the modulatory effect of quercetin (QT) on MTX-induced cytotoxicity, cells were treated with three different concentrations of QT from Sigma-Aldrich (St. Louis, MO, USA): 10, 25, and 50 µg/mL alone or together with the IC_{50} concentration of MTX for 24 and 48 hours (cotreatment). The experimental design consisted of five groups:

- **Control:** cells exposed only to the basal culture medium.
- **MTX:** cells treated with 7 µg/mL MTX (IC_{50}).
- **QT:** cells treated with 25 µg/mL quercetin.
- **QT + MTX:** cells co-treated with quercetin (10, 25, or 50 µg/mL) and MTX (IC_{50}) for 24 and 48 hours.

Methotrexate (MTX) was obtained from Sigma-Aldrich (St. Louis, MO, USA), dissolved at 0.1% in dimethyl sulfoxide (DMSO), and then further diluted in the culture medium. Quercetin was obtained through the same Sigma-Aldrich route and prepared similarly. TM4 cells were treated with 1% DMSO for 24 hours to confirm the safety of the solvent, and this was checked for cell viability by MTT assay. Then, TM4 cell viability was assessed at 0.1% DMSO for 24 hours. The untreated control group was 100 ± 0.0% viable, whereas TM4 cells treated with 0.1% DMSO were 100.01 ± 1.4% viable. The results are expressed as mean ± standard deviation (SD), based on six independent samples (n = 6).

2.2. MTT Assay

Cell viability was determined by an MTT assay. MTT powder was provided by Sigma-Aldrich (St. Louis, MO, USA). Cells were subsequently incubated with the MTT solution (0.5 mg/mL) for 4 h at 37 °C. The formazan crystals were dissolved in DMSO, followed by measuring absorbance at 570 nm.

2.3. Determination of MDA Content, ROS, and Antioxidant Levels

TM4 cells were lysed at the end of the treatment, and total protein was quantified by BCA assay (Biotechnology, USA). The supernatants were used to measure MDA, CAT, SOD, GSH, and GPx according to the manufacturer's manual (ZellBio, Germany). ROS production was assessed using the DCFH-DA kit (Sigma-Aldrich, USA), and fluorescence was measured at 490/570 nm.

2.4. Statistical Analyses

All data were represented as mean ± standard error (SE). ANOVA for one-way analysis followed by Tukey's post hoc test was used to assess the differences among the experimental groups. The nonparametric Kruskal-Wallis test was applied, when appropriate, using GraphPad Prism software version 10.4.2. Statistical significance was defined as $p < 0.05$.

3. Results and Discussion

3.1. Microscopic Examination of Cellular Changes

Methotrexate (MTX) is a potent inhibitor of folic acid reductase and is widely used in the treatment of autoimmune diseases and various neoplasms. Despite clinical utility, a major drawback of MTX is its

considerable testicular toxicity, which can lead to male infertility. This study assessed the potential protective role of quercetin (QT) against MTX-induced reproductive toxicity in TM4 Sertoli cells. In our study, the morphology of normal TM4 cells treated with quercetin (QT) was microscopically observed to be the same as that of the control untreated group, without any observable macroscopic alterations. The morphological changes observed with methotrexate (MTX) were pronounced and clearly concentration-dependent. At higher concentrations and longer durations, MTX caused more visible deformations of the cells: cell growth inhibition, star-shaped morphology, cell atrophy, cytoplasmic shrinkage, formation of vacuoles, pigmentation of the nucleus, etc. Notably, atrophy and nuclear pigmentation are well-known indicators of late-stage apoptotic processes. Other morphological changes, such as vacuolation and nuclear condensation, probably represent metabolic derangements that were initiated by MTX exposure, implicating putative molecular alterations within TM4 cells. The cell-intrinsic and nuclear alterations observed support the cytotoxic property of MTX towards these cells (Figure 1). Mostly in association with quercetin and MTX (QT + MTX), some structural integrity was preserved in TM4 cells when treated. This could be explained by the fact that this mitigation of MTX-induced cellular impairment was due to the protective effect of quercetin against MTX toxicity in Sertoli cells (Figure 1).

3.2. MTT Assay

The MTT assay results confirmed the cytotoxic effect of methotrexate (MTX) on TM4 Sertoli cells. At 1 µg/mL, MTX caused a moderately toxic effect in that a reduction in viable cells was noted; however, at 5 and 10 µg/mL, cell survival drastically fell during the 24 h period. In all cases, MTX-treated groups appear to have significantly reduced cell viability in comparison to the control group, $p < 0.01$ (Table 1). The cells treated with quercetin (QT) alone showed an increase in viability slightly above the level of control cells, suggesting that, in itself, QT was not cytotoxic. In the MTX + QT cotreatment groups, the cell viability was significantly higher compared to MTX-only treated cells ($p < 0.05$), indicating the protective action of QT. Among all tested concentrations, 25 µg/mL quercetin had the most potent cytoprotective effect. It can be implied from these results that quercetin improved TM4 cell viability and inhibited MTX-induced cytotoxicity, demonstrating a significant difference between QT-treated and control groups, as shown in Tables 2 and 3.

3.3. Determination of MDA Content

Several studies have confirmed that quercetin enhances oxidative defense in testicular tissues through upregulation of antioxidant enzymes and by decreasing markers of oxidative stress, such as malondialdehyde (MDA) (Rotimi *et al.*, 2022; Taepongsorat *et al.*, 2008). In our study, a notable increase in MDA concentration was observed in TM4 cells treated with methotrexate (MTX) compared to the control group ($p < 0.001$), implying increased lipid peroxidation. However, in quercetin and methotrexate co-treated groups (QT + MTX), the MDA levels were significantly reduced as compared to the MTX alone-treated cells ($p < 0.05$), demonstrating the antioxidant and protective role of quercetin (Table 3).

3.4. Determination of ROS Levels

Quercetin can fit into the categories of a flavonoid and phytoestrogen. Due to its diverse biological properties, such as acting as an antioxidant, anticancer, anti-inflammatory, antiviral, anti-obesity, and antibacterial agent, the compound has gained increased prominence.

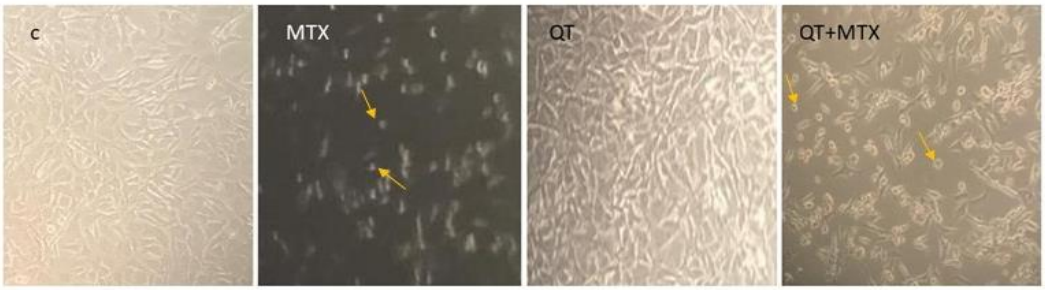

Figure 1. Morphological Changes in TM4 Cells Treated with MTX and QT. (**Note.** C: control, MTX: Methotrexate, QT: TM4 cells treated with quercetin, and QT+ MTX: TM4 cells treated with (IC50) MTX + QT; Arrows indicate apoptotic morphology.)

Table 1. Average Survival Rate of TM4 Cells Treated with Different Concentrations of MTX at 24, 48, and 72 Hours and Concentrations of 1, 5, 10 µg/mL

MTX (10)	MTX (5)	MTX (1)	Control	Time(hours)
67/57%	60.03%	79.39%	97.701%	24
29.35%	32.28%	37.38%	98.167%	48
10/49%	12.68%	16.80%	98.194%	72

Table 2. Mean Survival Rate of TM4 Cells under Treatment with (IC50) MTX + QT with Pretreatment at 24 and 48 Hours and Concentrations of 10, 25, and 50 µg/mL

(ic50) MTX QT (50) +	(ic50) MTX QT (25) +	(ic50) MTX QT (10) +	QT (25)	Control	Time(hours)
98.708%	98.927%	98.01%	-	98.512%	Cotreatment
98.928%	98.957%	98.837%	99.70%	98.344%	24

Table 3. Levels of Malondialdehyde (MDA), Reactive Oxygen Species (ROS, measured as DCF fluorescence), and Antioxidant Enzymes in TM4 Cells

	DCF formation (% of control)	*MDA (nmol/mg Protein)*	*GSH (nmol/mg Protein)*	*SOD (U/mg Protein)*	*GPX (U/mg Protein)*	*CAT (U/mg Protein)*
Control	160.2 ± 2.8	0.2±0.04	10±0.5	14±2.05	7±2.3	150±5.09
MTX	296.8±26.22**	0.76±0.07***	5.2±0.4**	5±1.0***	3±1.60**	60±6.0***
QT	150.5±10.2	0.1±0.04*	13±0.4*	16±3.00*	9±2.9*	159±7.09
MTX-QT	221.3±17.1$^{*\#}$	0.5±0.05$^{**\#}$	8±0.4$^{*\#}$	11±4.2$^{*\#\#}$	6±3.0$^{\#\#}$	120±9.01$^{*\#\#}$

Note. Data are presented as mean ± SD. Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; # $p < 0.05$. Symbols *, # indicate comparisons with the control and MTX groups, respectively.

Quercetin was proven in previous studies to enhance sperm quality and longevity by preventing the generation of reactive oxygen species (ROS) by both enzymic and non-enzymic pathways (Banday *et al.*, 2017). In this study, exposure to MTX resulted in a dramatic increase in intracellular levels of reactive oxygen species (ROS) within TM4 cells ($p < 0.01$) compared with controls. Conversely, cells co-treated with quercetin (QT + MTX) showed much less ROS generation compared to the MTX-only group ($p < 0.05$). This finding suggests that quercetin may effectively inhibit MTX-induced oxidative stress (Table 3).

3.5. Determination of Antioxidant Levels

Quercetin's extreme capacity as an antioxidant is attributable to the molecular site, with three potent active rings that serve effectively to neutralize free radicals (Gomes *et al.*, 2015). Additionally, quercetin is widely known to prevent the oxidation of DNA and enhance the antioxidant defense of sperm cells by reducing hydrogen peroxide production. Moretti *et al.* reported that quercetin and quercetin-loaded liposomes improved the viability and motility of sperm in a dose-dependent manner. It was also found that 50 μM of quercetin supplemented to human sperm during cryopreservation significantly increased motility and survival and reduced DNA fragmentation upon thawing (Moretti *et al.*, 2016; Zribi *et al.*, 2012).

In this study, the antioxidant defense ability of TM4 cells has been considerably compromised through MTX treatment ($p < 0.01$), which is further reflected by the decreased statuses in catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), and glutathione peroxidase (GPx) activities. Conversely, the QT + MTX cotreatment groups had strikingly higher activity of each of these antioxidant enzymes, as compared to the MTX group ($p < 0.05$). These results imply that quercetin supplementation addresses the improper balance in antioxidant status caused by MTX exposure (Table 3).

4. Conclusion

Here, we show that MTX significantly reduced TM4 Sertoli cell viability, increased MDA and ROS levels, and decreased cellular antioxidant activity. However, treatment with quercetin, particularly as a cotreatment, significantly ameliorated these effects, leading to increased cell viability, reduced oxidative stress, and increased antioxidant enzyme levels compared to MTX-alone-treated cells. Collectively, our findings suggest that quercetin may provide significant protection against MTX-induced cytotoxicity in TM4 cells primarily by exhibiting good antioxidant properties.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

The authors wish to express their sincerest appreciation to Iranian Research Organization for Science and Technology (IROST) for its high-value science support and providing some background enabling the authors to prepare this review.

Ethical approval

This research was conducted in accordance with ethical standards and was approved by the relevant ethics committee (Approval Code: IR.AJUMS.REC.1396.759).

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Funding

None declared.

Authors' Contributions

Marzieh Zeinvand Lorestani developed the concept, conducted the experiments and performed the data analysis. Hamed Zeinvand Lorestani and Nooshin Asadmasjedi analyzed the data, wrote, and revised the manuscript. All authors have read and approved the final version of the manuscript.

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How to cite this paper:

Zeinvand-lorestani, M., Zeinvand lorestani, H. & Asadmasjedi, N. (2025). Quercetin Ameliorates Methotrexate-Induced Reproductive Toxicity in Sertoli Cells. *Microbiology, Metabolites and Biotechnology, 7*(2), 109-114.
DOI: 10.22104/mmb.2026.8048.1192