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Protective effects of royal jelly on testicular tissue damage in rats treated with methotrexate: the relationship between oxidative stress and autophagy

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Abstract

The present study evaluated the protective effects of royal jelly (RJ) on methotrexate (MTX)-induced testicular damage in rats, focusing on oxidative stress and autophagy. Methotrexate, a folic acid analogue used in cancer and autoimmune treatments, impairs spermatogenesis via oxidative stress and apoptosis. Twenty-four male Wistar rats were randomized into four groups: Control (normal saline, 35 days), MTX (0.30 mg kg^{-1}, gavage, three times *per* week, 35 days), MTX + RJ (0.30 mg kg^{-1} MTX + 0.10 mg kg^{-1} RJ, gavage, three times *per* week, 35 days), and RJ (0.10 mg kg^{-1}, gavage, three times *per* week, 35 days). After 35 days, rats were euthanized and testicular tissue was analyzed via histopathology, immunohistochemistry for LC3-I/II expression in germ cells and qRT-PCR for mRNA expression of autophagy-related genes (*Beclin-1*, *Atg7*, *LC3-I*). Histopathological findings revealed that MTX caused severe interstitial edema, coagulative necrosis and disrupted spermatogenesis with reduced seminiferous tubule diameter, epithelial thickness, tubular differentiation index (TDI) and spermiogenesis index. Co-administration of RJ significantly improved seminiferous tubule morphology, diameter, epithelial thickness, TDI and spermiogenesis index. Immunohistochemistry showed a significant increase in LC3-I/II+ germ cells (spermatogonia, spermatocytes, spermatids) in the MTX group which was markedly reduced in the MTX + RJ group. Similarly, qRT-PCR analysis demonstrated elevated mRNA levels of *Beclin-1*, *Atg7*, and *LC3-I* in the MTX group which were significantly reduced in the MTX + RJ group. These findings suggested that RJ mitigated MTX-induced testicular damage by reducing oxidative stress and autophagy, thereby, preserving spermatogenesis and testicular integrity.

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Introduction

Recent advancements in cancer treatment have significantly improved long-term survival rates for patients, yet fertility preservation remains a critical concern, particularly for young cancer patients undergoing chemotherapy.[1] Methotrexate (MTX), a folic acid analogue, is a fundamental chemotherapeutic agent used in the management of various malignancies such as leukemias and lymphomas as well as autoimmune conditions including rheumatoid arthritis and psoriasis.[2,3] Despite its therapeutic efficacy, MTX is associated with significant adverse effects, notably testicular toxicity, which poses a critical challenge to male patients who rely on long-term fertility.[4] MTX is transported into cells, similarly to folinic acid, via the reduced folate carrier 1 (RFC1/SLC19A1).[5] Within the cell, MTX is converted by folylpolyglutamate synthase into its active polyglutamated forms, known as MTX polyglutamates. MTX polyglutamates competitively inhibit dihydrofolate reductase, a key enzyme in tetrahydrofolate synthesis, which is essential for DNA and RNA production.[6] The depletion of tetrahydrofolate impairs de novo synthesis of purines and pyrimidines, disrupting cellular proliferation.[7-9] This leads to oxidative stress, resulting in seminiferous tubule (ST) damage, sperm DNA mutations, spermatocyte apoptosis and elevated levels of reactive oxygen species (ROS).[10] While physiological ROS levels support sperm capacitation, hyperactivation and sperm-oocyte fusion,[11] excessive ROS due to MTX-induced oxidative stress, disrupting the oxidant-antioxidant balance, impairing spermatogenesis and causing testicular damage.[12]

Recent research has implicated autophagy, a conserved cellular process responsible for the lysosomal degradation

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and recycling of damaged organelles and proteins, in the pathogenesis of MTX-induced cellular injury.[13] In spermatogenesis, autophagy maintains tissue homeostasis under physiological conditions, supporting germ cell differentiation and energy supply, particularly in spermatogonia.[14] During germ cell division, elevated mitochondrial oxygen consumption increases free radical production, which supports cell division under physiological conditions. Notably, glutathione levels, crucial for controlling free radical production, are closely linked to the initiation, maintenance and enhancement of autophagy.[15] These findings highlight oxidative stress as a key trigger for autophagy induction. At the onset of autophagy, Beclin-1 and p62 proteins serve as primary markers for autophagosome formation and the identification of autophagic cargo. Beclin-1 exhibits behavior similar to LC3-II, while p62 expression inversely correlates with LC3-II.[16] Additionally, E2-like enzymes, particularly Atg7, a homolog of the ubiquitin-activating enzyme, initiate both specific and non-specific autophagy in somatic and testicular germ cells.[17] LC3 is activated by Atg7 and subsequently transferred to Atg3, another E2-like enzyme, before integrating into the autophagosome lipid membrane. To fully understand autophagy role in maintaining spermatogenesis integrity, it is essential to recognize that necrosis, apoptosis and autophagy collectively regulate germ cell proliferation and differentiation as inducers of cell death in testicular tissue. Beyond its protective effects, autophagy can, under specific metabolic conditions, also induce cell death.[18] While high levels of autophagy may promote cell death, physiological levels maintain cytoplasmic homeostasis, thereby, enhancing cell survival.[18]

Royal jelly (RJ), a secretion from the cephalic glands of nurse honeybees, comprises a unique blend of proteins (12.00 - 15.00%), sugars (10.00 - 12.00%), lipids (3.00 - 7.00%), minerals, amino acids and vitamins (A, C, E) with potent anti-inflammatory, anti-apoptotic and ROS-scavenging properties.[19-21] Previous studies have demonstrated RJ protective effects against oxidative stress-related damage in various tissues, including the testes, in models of varicocele,[22] cisplatin toxicity,[23] and diabetes-induced reproductive dysfunction.[24] The RJ has been shown to enhance antioxidant enzyme activity (e.g., superoxide dismutase, catalase, glutathione peroxidase), reduce lipid peroxidation, and improve sperm parameters and testicular histopathology.[22-24] However, its potential to counteract MTX-induced testicular toxicity, particularly through modulation of oxidative stress and autophagy, remains underexplored. Thus, this study investigated the protective effects of RJ against MTX-induced testicular damage in rats focusing on the interplay between oxidative stress and autophagy to elucidate potential therapeutic mechanisms for preserving male fertility.

Materials and Methods

Animal preparation and housing. Twenty-four healthy adult male Wistar rats, weighing 150 - 200 g, were obtained from the animal resource center for laboratory models at the Faculty of Veterinary Medicine, Urmia University. The rats were housed at a controlled temperature of 25.00 ˚C with a 12-hr light/12-hr dark cycle and fed on a standard diet. After a 1-week acclimatization period, the rats were randomized into four group: Control Group (normal saline for 35 days), MTX (Sigma, USA) Group (0.30 mg kg^{-1} body weight of MTX via gavage three times *per* week for 35 days),[25] MTX + RJ (Merck, Germany) Group (0.30 mg kg^{-1} body weight of MTX combined with 0.10 mg kg^{-1} body weight of RJ via gavage three times *per* week for 35 days),[25,26] and RJ Group (0.10 mg kg^{-1} body weight of RJ via gavage three times *per* week for 35 days).[26] We would like to clarify that all protocols used in this study were rigorously reviewed and approved by the Ethics Committee University of Tabriz, Iran, under the approval number (d/43/12007).

Tissue collection and histopathological analysis. After 35 days, the rats were euthanized using an overdose of ketamine and xylazine (Alfasan, Woerden, The Netherlands).[27] The left testis was removed, washed with normal saline and fixed in Bouin's solution (Sigma-Aldrich, St. Louis, USA) for 72 hr. The samples underwent standard histological processing, were embedded in paraffin and sectioned using an automatic rotary microtome. Sections were stained with Hematoxylin-Eosin. The following parameters were evaluated by light microscope (BX60; Olympus, Tokyo, Japan) and compared among groups: spermiogenesis index (SPI), tubular differentiation index (TDI), total germ cell count *per* ST, seminiferous epithelium thickness and ST diameter. Seminiferous tubules with more than three layers of germ cells and active spermatogenesis were classified as TDI- and SPI-positive.[28] For histopathological analysis, 20 cross-sections of STs *per* slide (120 sections *per* group) were examined. The right testis was also collected, washed with normal saline and stored at – 70.00 ˚C for further molecular studies.

Immunohistochemical analysis. Three cross-sections from each rat testis (18 sections *per* group) were prepared and stained. The number of LC3-I/II+ germ cells (spermatogonia, spermatocytes and spermatids) *per* ST was counted and compared among groups. Seminiferous tubules with similar characteristics were selected for germ cell counting. Briefly, tissue sections (5.00 - 6.00 µm thick) were incubated at 56.00 ˚C for 25 min, deparaffinized in xylene and rehydrated in graded alcohol concentrations (5 min *per* concentration). Antigen retrieval was performed using 10.00 mM sodium citrate buffer (pH 7.20; Sigma-Aldrich). Endogenous peroxidase activity was blocked with 1.50% hydrogen peroxide in phosphate buffer for 20

min at room temperature followed by incubation in a superblock solution for 10 min. Control slides (without primary antibody) and other group slides were incubated overnight at 4.00 ˚C with either blocking solution alone or with LC3-II primary antibodies, respectively. Slides were then incubated for 60 min at room temperature with goat anti-rabbit immunoglobulin G secondary antibody conjugated with peroxidase/HRP (1: 500; Elabscience, Houston, USA). Protein detection was performed using 3,3'-Diaminobenzidine chromogen (Sigma, St. Louis, USA) for 5 min followed by Hematoxylin-Eosin staining for 10 sec.

Molecular studies (RNA extraction, cDNA synthesis and qRT-PCR). Total mRNA was extracted from samples using TRIzol reagent (GeneAll, Seoul, South Korea). The concentration and quality of extracted mRNA were assessed using a spectrophotometer (Maestrogen, Hukou, Taiwan) at 260 nm and a 260/280 ratio of 1.80 - 2.00. One microliter of mRNA was used for cDNA synthesis in a 20.00 µL reaction mixture containing 1.00 µL oligo primer, 4.00 µL reaction buffer, 1.00 µL RNase inhibitor, 2.00 µL dNTP mix (10.00 mM), 1 µL MuLV reverse transcriptase and nuclease-free water. For qRT-PCR, cDNA templates 0.50 µL containing 5.00 - 10.00 ng cDNA were mixed with 10.00 µL 1.00 X SYBR Green master mix (High ROX, Noavaran Teb-International, Tehran, Iran), 0.60 µL forward and reverse primers for target genes and sufficient nuclease-free water (total volume 20.00 µL). The PCR protocol included denaturation (1 cycle, 600 sec at 95.00 ˚C), annealing (45 cycles, 20 sec at 95.00 ˚C) and extension (45 cycles, 60 sec at 72.00 ˚C) followed by a final extension cycle (600 sec at 72.00 ˚C). Each sample was run in triplicate and data were normalized using GAPDH. Primer sequences are provided in Table 1.

Image processing and presentation. Images were resized, processed and presented using Adobe Photoshop CS10 Software (Adobe Systems Inc., Mountain View, USA).

Statistical analysis. To ensure data normality and homogeneity, Kolmogorov-Smirnov and Levene's tests were applied. One-way ANOVA followed by Tukey's multiple comparisons post hoc test was performed using SPSS Software (version 11.00, SPSS, Inc., Chicago, USA) for statistical and quantitative analysis of tissue and molecular data.

Table 1. Sequences of the primers used in the present study.

Genes	Sequences 3'-5'	References
Atg7	F: AGCAGTGATGACCGCATGAA R: TCAGCAGCTTGGGTCTCTTG	29
Beclin-1	F: GGCCAATAAGATGGGTCTGA R:GCTGCACACAGTCCAGAAAA	30
LC3-II	F: GATGTCCGACTTATTCGAGAGC R:TTGAGCTGTAAGCGCCTTCTA	31
GAPDH	F: AAGGTCATCCATGACAACTT R:GGCCATCCACAGTCTTCTGG	32

Results

Histopathological evaluation of testicular tissue. Histopathological analysis of testicular tissue sections revealed that the control group exhibited normal testicular structure with healthy germ cells and STs, normal cell density in the tubule walls, fully differentiated cells and visible spermatozoa in the lumen. The RJ group displayed a testicular structure similar to the control group. In contrast, the MTX -treated group showed significant structural changes including severe interstitial edema, coagulative necrosis in spermatogenic tubules and germ cells, reduced cell density, irregular and sparse cellular arrangement in the tubule walls and empty spaces within the wall thickness. Spermatogenesis was severely impaired. In the MTX + RJ group, mild interstitial edema and coagulative necrosis were observed, with STs appearing relatively healthy, reduced pyknosis, fewer empty spaces and improved cellular density and organization in most tubule walls. Statistical analysis indicated a significant difference ($p < 0.05$) between the MTX group and other groups (Fig. 1).

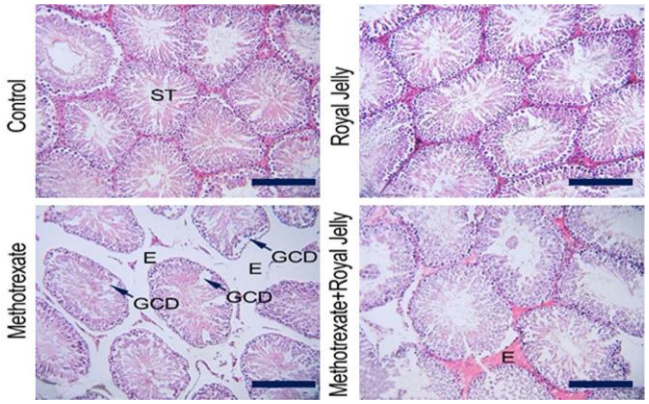

Fig. 1. Histopathological changes in cross-sections of seminiferous tubules of rat testicular tissue (Hematoxylin-Eosin staining). The control group exhibits normal testicular tissue structure with intact germ cells and healthy tubules. The royal jelly group also displays normal testicular tissue structure. The single-dose methotrexate group shows severe interstitial edema (E), coagulative necrosis in sperm-producing tubules and germ cell deficiency (GCD). The methotrexate + royal jelly group exhibits mild tissue damage in the testicular tissue. Normal spermatogenesis and spermiogenesis are observed in the control and royal jelly groups while spermatogenesis is halted in the methotrexate group (Scale bars = 250 µm).

Effects of royal jelly (RJ) on MTX -induced histomorphometric changes in testicular tissue. Cross-sections from the MTX -treated group showed deformed STs, reduced tubule diameter and decreased seminiferous epithelium thickness. Additionally, the percentage of STs with positive TDI and SPI was significantly reduced in this group. Conversely, rats treated with RJ exhibited significant improvements in

histological characteristics, including ST diameter and seminiferous epithelium thickness. Furthermore, a higher percentage of STs with positive TDI and SPI was observed in the RJ -treated group (Fig. 2).

Effects of RJ on MTX -induced upregulation of LC3-I/II+ protein expression. Immunohistochemical staining was used to evaluate the effect of RJ on MTX -induced changes in LC3-I/II expression (Fig. 3). Testicular cross-sections from the MTX group showed a significant increase ($p < 0.05$) in LC3-I/II+ expression in germ cells (spermatogonia, spermatocytes, spermatids) within a single ST (Fig. 4). In contrast, LC3-I/II expression in the MTX + RJ group was significantly reduced compared to the MTX -only group.

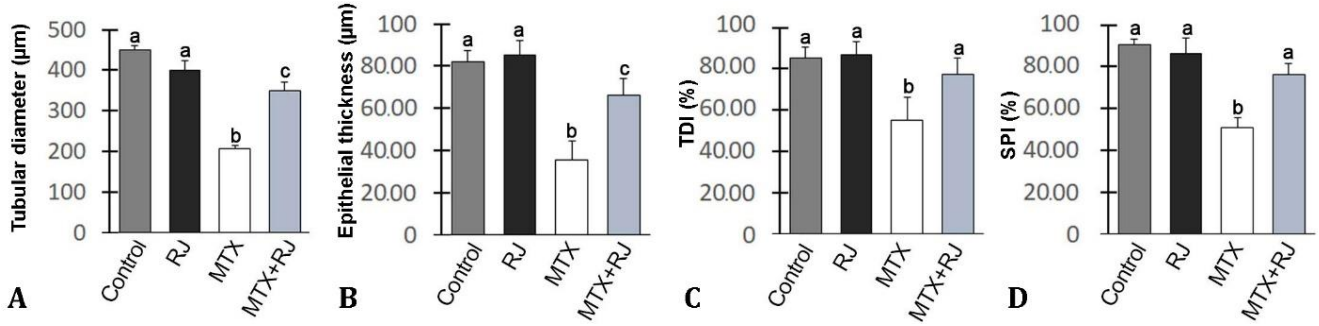

Fig. 2. Effects of royal jelly (RJ) on methotrexate (MTX)-induced changes in testicular histomorphometry. Histomorphometric analysis of: **A)** tubular diameter, **B)** epithelial thickness, **C)** tubular differentiation index (TDI), and **D)** spermiogenesis index (SPI) in seminiferous tubules. Data are presented as mean ± SD with $p < 0.05$ indicating statistical significance.

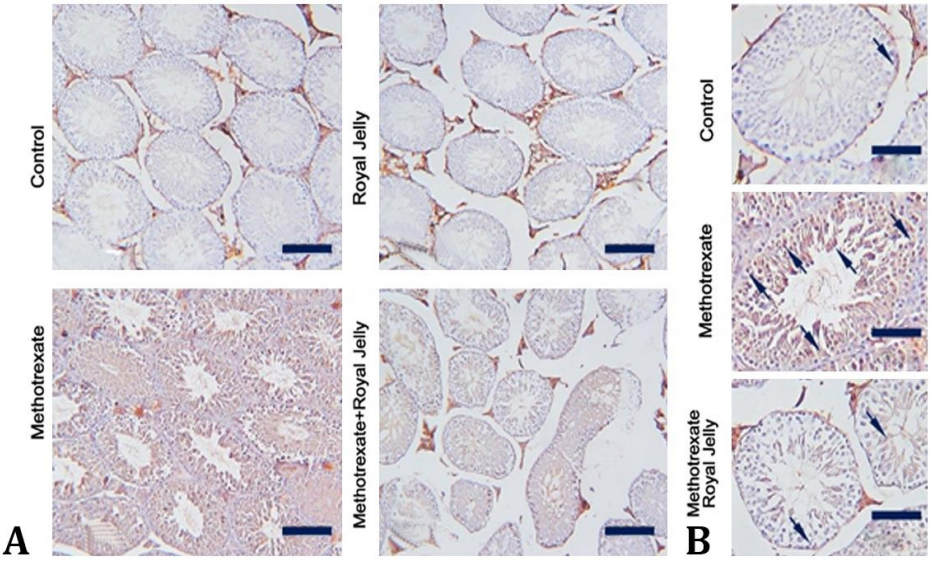

Fig. 3. Effects of royal jelly (RJ) on increased LC3-I/II protein expression following methotrexate (MTX) treatment in testicular tissue. **A** and **B)** Immunohistochemical staining for LC3-I/II. LC3-I/II+ cells are indicated by arrows. Note the significant increase in LC3-I/II+ cells in the MTX group which is reduced in the RJ-treated group (Scale bars A = 250 µm, B = 100 µm).

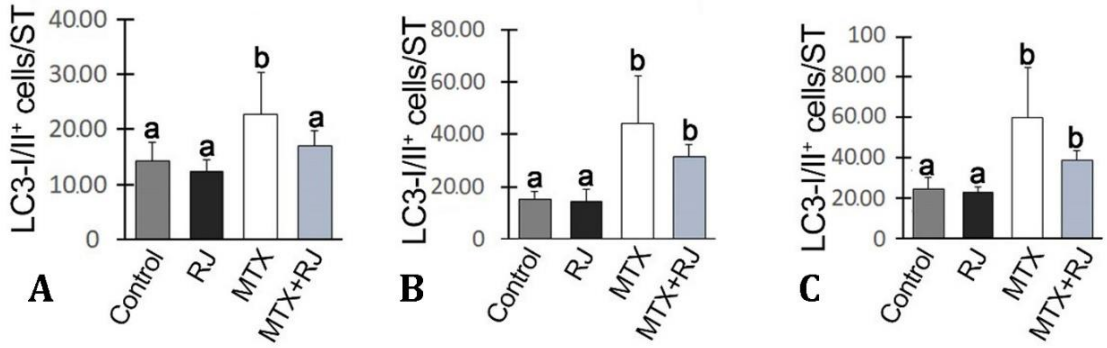

Fig. 4. The average number of germ cells in **A)** spermatogonia, **B)** spermatocytes, and **C)** spermatids with positive LC3-I/II+ staining in a single seminiferous tubule is reported. All data are expressed as Mean ± SD with $p < 0.05$ indicating statistical significance.

Effects of RJ on MTX -induced upregulation of *Beclin-1*, *Atg7*, and *LC3-I* gene expression. The qRT-PCR was used to assess the effects of RJ on MTX -induced changes in *Beclin-1*, *Atg7*, and *LC3-I* expression. The mRNA levels of *Beclin-1*, *Atg7* and *LC3-I* were significantly elevated ($p < 0.05$) in the MTX group (Fig. 5). In contrast, the RJ -treated group exhibited significantly lower expression of these genes compared to the MTX group. Rats treated with RJ showed a significant reduction ($p < 0.05$) in mRNA levels of *Beclin-1*, *Atg7*, and *LC3-I*.

Discussion

Methotrexate, an immunosuppressive drug widely used in the treatment of cancer, rheumatic diseases and systemic inflammation, exerts its effects by inhibiting dihydrofolate reductase, thereby, disrupting thymidylate, serine and methionine synthesis. This interference impairs DNA, RNA and protein synthesis, ultimately leading to cell death.[33] Numerous studies have reported MTX's potential to cause gonadal damage, particularly affecting spermatogenesis.[34] MTX induces sperm abnormalities, ST disruption (disorganization and vacuolation), DNA damage and reduced sperm count.[10] The germinal epithelium of the testis, characterized by high mitotic activity is particularly susceptible to cytotoxic damage from chemotherapeutic agents like MTX.[35]

The MTX can cross the blood-testis barrier and chronic administration has been shown to reduce the size of primary and secondary spermatocytes, spermatids and Leydig and Sertoli cells compared to controls. Additionally, MTX alters chromatin structure in spermatocytes from spherical to elliptical and increases interstitial space while reducing ST diameter.[36] Vardi *et al.*, reported that MTX causes testicular damage through ST disorganization, germ cell shedding, atrophy and apoptosis.[37] Hess and Nakai, suggested that germ cell shedding and tubular atrophy result from chemical effects on microtubules and intermediate filaments in Sertoli cells, potentially due to downregulated expression of adhesion proteins like cadherin.[38] These effects propagate to dividing germ cells, leading to tubular atrophy, as consistently observed in multiple studies.[39,40] Our findings were consistent with these observations, demonstrating that MTX administration resulted in disorganization, reduced cellular density, vacuolation in ST walls, decreased tubule diameter and epithelial thickness, and germ cell shedding.

The MTX also significantly reduces antioxidant enzyme levels (superoxide dismutase, catalase, glutathione peroxidase), which normally maintain oxidant-antioxidant balance, thereby, exacerbating oxidative stress and cellular damage.[37] This imbalance, driven by MTX inhibition of cytosolic NAD(P)-dependent dehydrogenase and NADP malic enzyme limits Nicotinamide adenine dinucleotide phosphate (NADPH), availability for glutathione synthesis, a critical cytosolic antioxidant protecting against ROS.[41] The resultant glutathione depletion and elevated ROS levels exacerbate oxidative stress, rendering testicular cells, particularly the germinal epithelium, highly susceptible to damage due to their high mitotic activity and lipid-rich membranes.[35]

Autophagy, a conserved cellular process, plays a dual role in testicular function by maintaining homeostasis under physiological conditions while potentially inducing cell death under stress.[13] Oxidative stress, particularly glutathione depletion, is a key trigger for autophagy in germ cells, disrupting spermatogenesis.[42]

More than 30 distinct genes play a crucial role in the regulation of autophagy. These genes participate in various phases of autophagosome formation, including phagophore induction, cargo capture, vesicle expansion and closure as well as the transport of vesicles to the vacuole.[43] Beclin-1, essential for autophagosome formation and early embryonic development, is often downregulated in cancers, suggesting its role as a tumor suppressor.[44] The LC3, a reliable autophagy marker, exists in two forms: The cytosolic LC3-I (18.00 kDa) and the lipidated LC3-II (16.00 kDa). Among its three isoforms (LC3A, LC3B, LC3C) in mammalian tissues, LC3B is particularly significant in autophagy with LC3B-II being directly incorporated into autophagosomes. Various cellular stresses upregulate LC3 expression, promoting the conjugation of LC3-I to

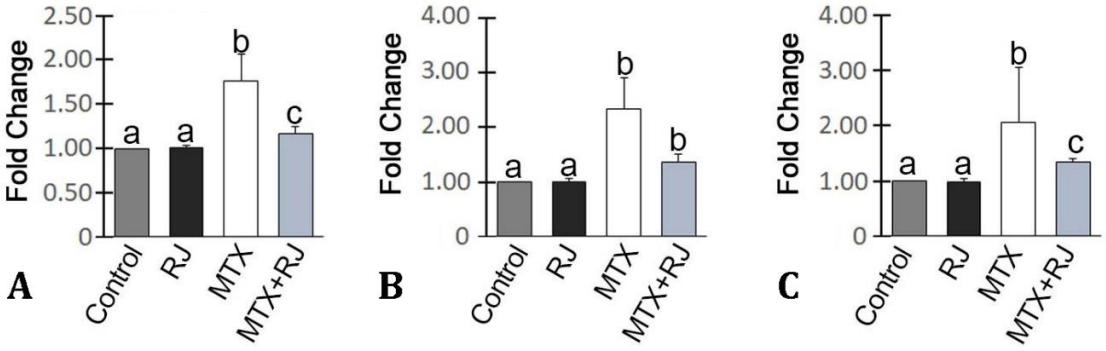

Fig. 5. Effects of royal jelly (RJ) on methotrexate (MTX)-induced expression of **A)** *Beclin-1*, **B)** *Atg7*, and **C)** *LC3-1* in testicular tissue. Data are presented as Mean ± SD, with $p < 0.05$ indicating statistical significance.

phosphatidyl ethanolamine to form LC3-II.[43] Autophagosome elongation relies on two ubiquitin-like conjugation systems. The first involves Atg12 binding to Atg5, mediated by Atg7, an E1-like ubiquitin-activating enzyme, forming an Atg5-Atg12 complex that associates with ATG16L to facilitate membrane elongation. This complex dissociates during autophagosome maturation.[17] The second system involves LC3 processing, where Atg4B cleaves the LC3 precursor to produce LC3-I, exposing a C-terminal glycine for lipidation. Sequential actions of Atg7 and Atg3 (E2-like) conjugate LC3-I to phosphatidyl ethanolamine, yielding LC3-II, which integrates into the autophagosomal membrane. In mammals, the transition from LC3-I to LC3-II is a critical step in autophagosome formation, with LC3-II redistribution serving as a key indicator.[45] Autophagy can be quantitatively assessed by counting autophagosomes via electron microscopy or measuring steady-state levels of components like LC3-II, providing insights into autophagic activity.[45] In this study, MTX significantly upregulated autophagy-related genes (*Beclin-1*, *Atg7*, *LC3-I*) and LC3-I/II+ protein expression in germ cells (spermatogonia, spermatocytes, spermatids), indicating enhanced autophagic activity.

Royal jelly, known for its antioxidant properties has shown protective effects against testicular damage in various models. In a study conducted in 2016, Anbara *et al.*, investigated the protective effects of vitamin C and RJ on sex hormones and testicular histochemistry in mice suffering from experimental hemolytic anemia induced by phenylhydrazine. The results demonstrated that vitamin C and RJ improved phenylhydrazine-induced testicular damage. RJ prevented disruption of the germinal epithelium in the STs and enhanced its thickness.[46] Najafi *et al.*, explored RJ protective role against oxymetholone-induced oxidative damage in adult mouse testes. Co-administration of RJ with oxymetholone resulted in increased ST diameter and significant prevention of germ cell loss compared to the oxymetholone-only group.[47] Furthermore, Azad *et al.,* examined the protective effect of RJ on testicular tissue and sperm characteristics in nicotine-treated adult mice. Morphometric analysis revealed that RJ significantly increased the thickness of the germinal epithelium, ST diameter and the count of spermatocytes and Sertoli cells compared to the nicotine-only group. Histological assessments confirmed that RJ-treated groups, including controls, displayed normal ST structure and reduced testicular damage when co-administered with nicotine.[48] Raafat and Hamam, investigated RJ protective role against cisplatin-induced testicular damage in adult albino rats. The RJ and cisplatin co-treatment group showed increased ST diameter and reproductive epithelium thickness compared to the cisplatin-only group with no significant differences from the control group.[49] Similarly, our study found that RJ co-administration with MTX significantly improved ST histology, diameter, epithelial thickness, TDI and SPI. Notably, RJ reduced vacuolation, pyknosis and germ cell shedding, restoring cellular density and organization. Furthermore, RJ significantly downregulated MTX-induced *Beclin-1*, *Atg7*, and *LC3-I* mRNA expression and LC3-I/II+ protein levels in germ cells ($p < 0.05$) likely due to its antioxidant capacity which mitigated ROS-mediated autophagy activation.

It is clearly established from the current study that MTX disrupted the oxidant-antioxidant balance, inducing oxidative stress and upregulating autophagy in germ cells, which impaired spermatogenesis. Royal jelly counteracted these effects by reducing ROS levels, thereby, reducing testicular damage and autophagic activity. Consequently, RJ significantly decreased the expression of autophagy-related genes (*Beclin-1*, *Atg7*, *LC3-I*) and LC3-I/II protein levels. These findings suggested that RJ held potential as a protective agent against MTX-induced testicular toxicity, offering a promising strategy to mitigate chemotherapy-related reproductive side effects and support fertility preservation.

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Conflicts of interest

There is no conflict of interest.

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