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Impact of Mesenchymal Stem Cells and Quercetin on Protection of Testis Against Cyclophosphamide-Related Damage

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Abstract

Objectives: This study aimed to evaluate the effect of transplantation of mesenchyme stem cells derived from bone marrow and quercetin on the oxidation markers, hormonal profile of testosterone, and morphometric testis and sperm parameters in adult mice poisoned with the antineoplastic drug cyclophosphamide (CTX).

Materials and Methods: All 25 mice were divided into five groups control, CTX group received CTX (150 mg/kg), quercetin + CTX group received IP injections of quercetin (75 mg/kg /daily) for 14 days, and CTX (150 mg/kg), CTX + bone marrow mesenchymal stem cells (BMMSCs) also received CTX and, 24 hours after the administration of CTX, approximately 1×10^6 BMMSCs were injected carefully into each testis of mice. Quercetin + CTX + BMMSCs group received quercetin and CTX (as in group quercetin + CTX), and BMMSCs were injected into each testis of the animals. Thirty-five days after the last injection of BMMSCs, blood samples were taken to gain serum and assess the testosterone level, and histopathological, biochemical, and sperm parameters analyses were performed.

Results: CTX led to testicular tissue damage and reduced the Johnson score and diameters of the seminiferous tube in the CTX group ($P<0.05$). Also, the level of Antioxidant enzyme and testosterone was notably reduced in the CTX group ($P<0.05$). The count and sperm quality were decreased significantly in the CTX group ($P<0.05$). Administration of quercetin led to an increase in the level of testosterone and antioxidant enzymes and improved sperm parameters.

Conclusions: The present study shows the administration of quercetin can protect the testicle against CTX-related damage and improve oxidative stress damage and sperm parameters.

Keywords: cyclophosphamide, Testis damage, Sperm Parameters, Quercetin

Introduction

Cancer is a significant public health problem and a major cause of morbidity and mortality worldwide (1,2). With the emergence of innovative methods for earlier diagnosis and the expansion of anti-neoplastic treatment options, cancer patients have better chances at survival today than they possibly ever did before (3,4). Therefore, with more successful management of malignant disease, addressing the unique needs and challenges of cancer survivors has become increasingly important over the past decades (5).

Cyclophosphamide (CTX) is an antineoplastic chemotherapeutic drug that belongs to the subclass of alkylating agents (6). Despite being one of the oldest anti-cancer treatments available, it is used for the treatment of various subtypes of malignant disease as well as disorders of the immune system frequently in the present day (7).

Male patients treated with CTX frequently develop fertility-related issues later in their lives (8). While the exact molecular mechanism of CTX gonadotoxicity is yet to be elucidated, several in vivo studies have shown evidence of decreased testicular weight, oligospermia, teratospermia, azoospermia, and a decline in the number of spermatogonia in males after administrating CTX (9,10).

Several investigations highlight the role of specific cytopathologic patterns of involvement after exposure to the drug (11). Multiple studies have noted decreased activity of antioxidant enzymes, as well as an increase in reactive oxygen species after exposure to CTX (12-15). These observations suggest a role for oxidative stress in the pathophysiology of gonadal tissue damage due to the drug (16).

Likewise, anti-oxidants are thought to mitigate the gonadotoxic effects of chemotherapeutic drugs (17,18). Pre-treatment of animal models with quercetin, an anti-oxidant flavonoid with anti-inflammatory features, has resulted in attenuation of gonadal tissue damage after the administration of alkylating agents (19,20). However, the

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evidence for administration of anti-oxidant chemotherapy for human patients is inconclusive at best (21).

Mesenchymal stem cells (MSCs) possess the characteristic features of self-renewal and the ability to differentiate into various tissues under the influence of local and systemic molecules, including cytokines and growth factors (22,23). Therapeutic use of MSCs via transplantation has gained much recognition and popularity in the rapidly developing field of regenerative medicine, especially for women (24). Stem cell transplantation for restituting fertility is an area of active research (25). In vitro studies on laboratory animals have demonstrated a potential role for stem cell transplantation in restoring fertility after administering systemic chemotherapeutic medication (26,27).

In response to these findings, we hypothesize that a combination of MSCT and quercetin as an antioxidant would decrease the gonadotoxic effects of CTX and can help preserve fertility in adult male mice treated with the drug.

Materials and Methods

In this research, 25 adult male albino mice (aged 6-8 weeks, weighing 22-25 g) were acquired from the Animal Care Center of Tabriz University of Medical Sciences. They were kept in the standard situations (12 hours light/dark, 24-26 °C, and 45%-50% humidity). Besides this, the mice had free access to food and water.

Study Design

All of the mice were randomly divided into two main groups: control (n = 5), in which the mice received only normal saline vehicle intraperitoneally (IP) (two weeks), and experimental groups, which were separated into four subgroups.

- CTX group (n=5) were given IP injections of CTX (150 mg/kg dissolved in saline) on days 7 and 14; the dose of CTX was chosen based on the previous study (28).
- Quercetin + CTX group (n=5). The mice in this group received IP injections of quercetin (75 mg/kg) for 14 days; the dose of Quercetin was chosen based on a previous study (28,29). They also were given two IP injections of CTX (150 mg/kg) on days 7 and 14.
- Bone marrow mesenchymal stem cells (BMMSCs) + CTX group (n=5). They had gained CTX, as mentioned above, and 24 hours after the final injection of CTX, nearly 1×10^6 BMMSCs were carefully injected into each mouse's testis.
- Quercetin + BMMSCs + CTX group (n=5). The animals in this group were given quercetin and CTX (like the group quercetin + CTX). Subsequently, 24 hours after the last injection of quercetin and CTX, 1×10^6 BMMSCs were injected into each testis of these animals.

To inject BMMSCs into the testis, mice were anesthetized using a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg). Finally, cells were resuspended in normal saline and loaded into a 1 mL syringe for injection into the testes. 1×10^6 BMMSCs in 120 µL of normal saline were injected into each testis.

Thirty-five days after the injection of BMMSCs, all groups were euthanized humanely by dislocation of the cervical vertebra under anesthesia. The testes and blood were harvested for the following experiments.

CTX powder was purchased from (Baxter Healthcare Company, India). Quercetin yellow powder was provided from (Roche, Switzerland). Ketamine and xylazine, respectively, were obtained from (Rotexmedica, Germany) and (Bioveta Company, Czech Republic). Other reagents are described in the text below.

Isolation and Culture of BMMSCs

In brief, mice were first disinfected with 70% ethanol following cervical dislocation. Afterward, tibias and femurs were taken from mice, and then their bones were cut at the median; bone marrow content was flushed out by using a syringe containing 5 mL of low-glucose Dulbecco's modified eagle medium (DMEM/LG, Gibco). The flushed marrow was centrifuged (5 minutes, 1500 rpm), the supernatant was removed, and the pelleted fraction was resuspended in 5 mL DMEM. Then, resultant cells were cultured in a medium with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin and streptomycin (Gibco). The cells were incubated at 37 °C with 5% CO2 and saturated humidity. After 24 hours, non-adherent cells were removed by replacing the medium. The medium was changed every 3 days. When the cells reached 80%∼90% confluence, they were sub-cultured. In our experiments, BMMSCs were used in the third to sixth passages.

Tissue Processing for Histopathological Examination

The animals' testes were removed from all experimental and control groups, fixed in Bouin's solution for 96 hours, and processed for light microscopic examination. Tissues were embedded in paraffin blocks, and 5 µm thick sections were prepared. Sections were deparaffinized with xylene and dyed with hematoxylin and eosin (H&E). A pathologist blinded to the experimental treatments examined the slides. The Johnson scores and seminiferous tubule diameter (STD) and thickness of the germinal epithelium were measured at ×200 by ImageJ software.

Biochemical Analysis of Oxidation

Malondialdehyde (MDA) is a widely used biomarker for assessing lipid peroxidation levels. Traditionally, MDA levels are determined using the thiobarbituric acid reactive substances (TBARS) assay, which involves the reaction of MDA with thiobarbituric acid to produce a colored product. The color intensity is measured spectrophotometrically at 532 nm to quantify MDA levels.

Superoxide dismutase (SOD) activity in samples is typically determined by assessing the ratio of "auto-

oxidation" rates in the presence and absence of the sample. The colorimetric assay involves measuring the color reaction at 480 nm.

Catalase (CAT) activity is assessed "calorimetrically" by monitoring the consumption of hydrogen peroxide (H2O2) in the presence of the sample. The decline in absorbance at 240 nm is measured to determine CAT activity.

Hormone Assays

The hormonal profile of testosterone in blood samples was determined by ELISA kits provided by ALPCO (USA).

Semen Analysis

To check spermatozoa's quality, the epididymis's tail part was placed inside the dishes with 5 mL of phosphate-buffered saline (PBS; pH 7.2) and incubated for 20 minutes. Afterward, 100 µL of this mixed solution was dissolved in 900 µL PBS. The number of sperm cells examined under a light microscope in 8 microscopic fields of 0.1 cm^2 each except the central erythrocyte area. The sperm count and percentage of viable, motile spermatozoa were determined. To evaluate the morphological abnormalities of spermatozoa in the head, neck, and tail parts. The slides were stained with hematoxylin and eosin method and examined under a light microscope (the numbers of 100 spermatozoa of each mouse, at ×400 magnification). Results were expressed as a percentage.

Statistical Analysis

Statistical analysis was performed using SPSS version 22 (USA). All data in the present research are expressed as mean ± standard error of mean (SEM) and were compared using one-way ANOVA and Tukey's post hoc test for comparing the study groups. A value of $P < 0.05$ was considered as significant.

Results

Testis Histological Parameters

Histomorphometric analysis showed a significant decrease in the mean Johnson's score (MJS) in the experimental groups compared to the control group ($P<0.05$). Conversely, treatment with quercetin and BMMSCs resulted in a significant improvement in MJS in the treated groups compared to the CTX group ($P<0.05$).

Furthermore, the STD exhibited a significant reduction in the CTX group compared to the control ($P<0.001$). However, the STD was significantly higher in the quercetin and BMMSCs groups and the combined quercetin + BMMSCs group than in the CTX group ($P<0.05$).

Additionally, the height of the seminiferous epithelium (HE) demonstrated a significant decrease in the CTX group compared to the control ($P=0.02$). Notably, treatment with quercetin and BMMSCs significantly improved the HE in the treated groups compared to the CTX group ($P=0.03$). These findings are illustrated in Figure 1 and Table 1.

Biochemical Parameters

In the experimental groups, serum CAT levels were significantly reduced compared to the control group ($P<0.001$). However, CAT levels were significantly increased in the quercetin and BMMSCs groups, as well as the combined quercetin + BMMSCs group, when compared to the CTX group ($P< 0.001$) (Figure 2A).

Serum SOD levels were significantly lower in the CTX group compared to the control group ($P<0.001$). Conversely, SOD levels were significantly improved in the

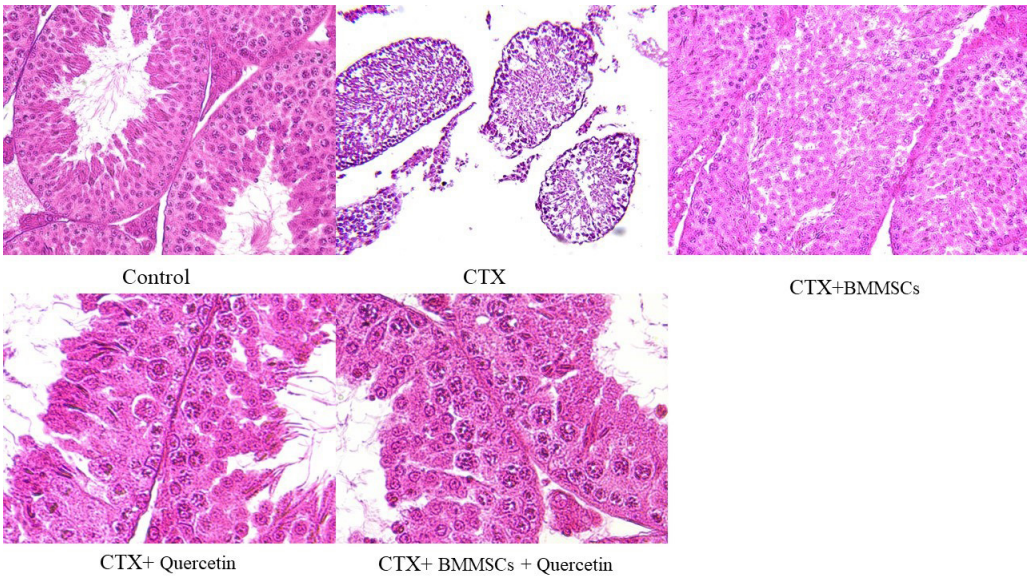

Figure 1. Histological Findings in the Study Group. Control group: Received only normal saline intraperitoneally (IP). CTX group: Administered IP injections of CTX (150 mg/kg dissolved in saline). BMMSCs + CTX Group: Received CTX along with 1 × 10^6 BMMSCs. CTX + Quercetin Group: Received 75 mg/kg of quercetin in addition to CTX. CTX + Quercetin + BMMSCs Group: Animals in this group received both quercetin and CTX, along with BMMSCs.

Table 1. Histological Results in Study Groups

Groups	Johnson Score	STD	HE
Control	9.7±0.15	265.2±8.2	70.25±3.2
CTX	4.35±0.2*	145.5±4.5*	32.5±2.5*
CTX+ BMMSC	6.4±0.25#	185±6.7#	48.5±2.2#
CTX+ Quercetin	6.55±0.15#	198±10.2#	52±3.2#
CTX+ BMMSC+ Quercetin	6.75±0.12#	205±12.5#	55.2±2.5#

Control group: Received only normal saline intraperitoneally (IP). CTX group: Administered IP injections of CTX (150 mg/kg dissolved in saline). BMMSCs + CTX Group: Received CTX along with 1 × 10^6 BMMSCs. CTX + Quercetin Group: Received 75 mg/kg of quercetin in addition to CTX. CTX + Quercetin + BMMSCs Group: Animals in this group received both quercetin and CTX, along with BMMSCs.

*$P < 0.05$ indicates significant differences between the control and other groups, and #$P < 0.05$ indicates significant differences between the CTX and other groups.

treated groups compared to the CTX group ($P<0.001$) (Figure 2B).

Furthermore, serum MDA levels were significantly elevated in the CTX group compared to the control ($P<0.001$). Notably, MDA levels were significantly reduced in the treated groups compared to the CTX group ($P<0.001$) (Figure 2C).

Testosterone Serum Level

Serum testosterone levels were significantly decreased in the CTX group compared to the control group ($P<0.001$). Conversely, testosterone levels were significantly increased in the treatment groups (quercetin, BMMSCs, and quercetin + BMMSCs) compared to the CTX group ($P<0.05$). There was no significant difference in testosterone levels between the various treatment groups ($P<0.05$) (Figure 3).

Result of Sperm Parameters

Examination of semen analyses in this research shows the sperm count was notably reduced in the CTX group compared to the control group ($P<0.05$). On the other hand, in the experimental group with the administration of quercetin, the count of sperm was remarkably higher than in the CTX group ($P<0.05$). Also, the percentage of normal morphological sperm was significantly decreased in the CTX group in compression with control ($P<0.05$). However, the number of sperm with normal morphology was notably higher in the treated group with quercetin and BMMSC than in the CTX group ($P<0.05$). Also, the percentage of motile sperm was significantly decreased in the CTX group in compression with control ($P<0.05$). However, the number of sperm with motility was notably higher in the treated group with quercetin and BMMSC than in the CTX group ($P<0.05$).

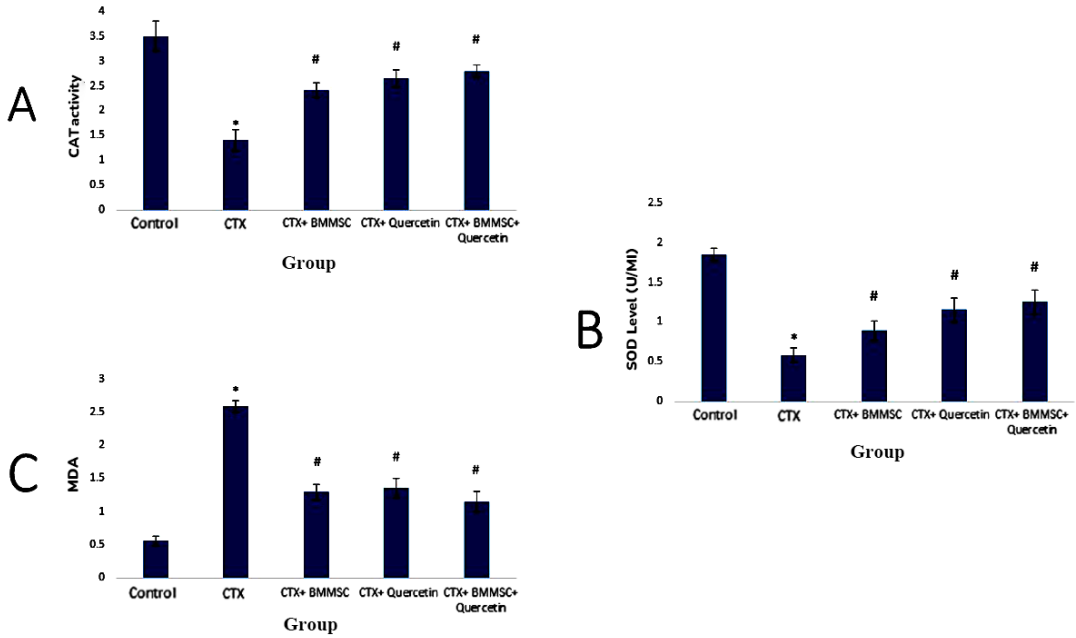

Figure 2. Oxidative Stress Markers in the Study Groups. A: serum level of CAT in study groups; B: serum level of serum SOD in study groups; C: serum level of MDA in study groups. Control group: Received only normal saline intraperitoneally (IP). CTX group: Administered IP injections of CTX (150 mg/kg dissolved in saline). BMMSCs + CTX Group: Received CTX along with 1 × 10^6 BMMSCs. CTX + Quercetin Group: Received 75 mg/kg of quercetin in addition to CTX. CTX + Quercetin + BMMSCs Group: Animals in this group received both quercetin and CTX, along with BMMSCs. *$P < 0.05$ indicates significant differences between the control and other groups, and #$P < 0.05$ indicates significant differences between the CTX and other groups.

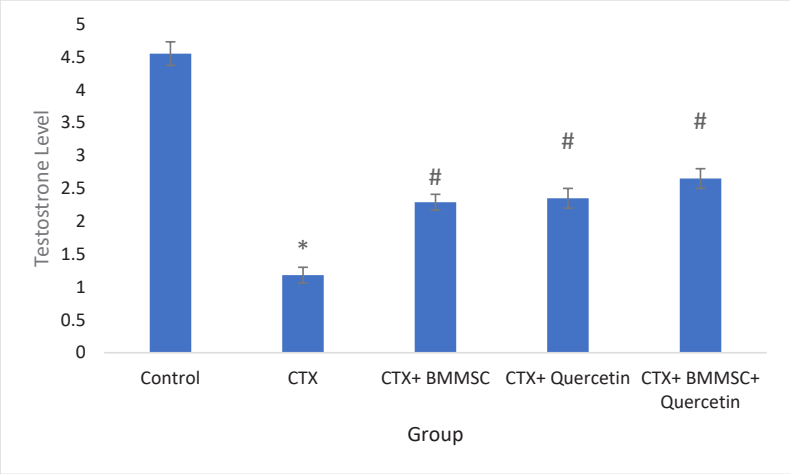

Figure 3. Serum Testosterone Level in the Study Groups. Control group: Received only normal saline intraperitoneally (IP). CTX group: Administered IP injections of CTX (150 mg/kg dissolved in saline). BMMSCs + CTX Group: Received CTX along with 1×10^6 BMMSCs. CTX + Quercetin Group: Received 75 mg/kg of quercetin in addition to CTX. CTX + Quercetin + BMMSCs Group: Animals in this group received both quercetin and CTX, along with BMMSCs. $^*P < 0.05$ indicates significant differences between the control and other groups, and $^{\#}P < 0.05$ indicates significant differences between the CTX and other groups.

Discussion

This study investigates the protective effects of quercetin and BMMSCs on testicular damage induced by CTX, focusing on histological, biochemical, and sperm parameters. The findings provide substantial evidence that both quercetin and BMMSCs, alone or in combination, mitigate the deleterious effects of CTX on testicular structure and function.

The histomorphometric analysis revealed significant alterations in testicular architecture in the CTX group, characterized by a reduction in the MJS, STD, and the height of the seminiferous epithelium (HE). These changes reflect the extensive damage CTX inflicts on spermatogenesis, which is consistent with its known cytotoxic effects, particularly on rapidly dividing cells such as spermatogonia (30). CTX is an alkylating agent that induces DNA cross-linking, leading to apoptosis and a subsequent reduction in germ cell populations within the seminiferous tubules (31,32). In this context, Kim et al demonstrated in a study that administration of CTX in male mice led to testicular damage and toxicity related to oxidative stress induced by CTX (33). Also, Uzun-Goren and Uz presented in their study that CTX can induce testicular inflammation and apoptosis, and administration of antioxidant compound can protect the testicle against CTX-related damage (34).

The observed decrease in MJS indicates a decline in the quality and progression of spermatogenesis, with fewer seminiferous tubules containing complete spermatogenic series. The reduction in STD further corroborates this, as the diameter of seminiferous tubules is directly related to the number of germ cells they contain. A diminished HE height also suggests a loss of Sertoli cell function and a reduction in the support for germ cell development, which is critical for maintaining the integrity of the blood-testis barrier and facilitating the movement of spermatocytes through the seminiferous epithelium (35, 36).

However, individually and in combination, treatment with quercetin and BMMSCs significantly ameliorated these histological alterations. The improvement in MJS, STD, and HE suggests that these treatments enhance the regenerative capacity of the testis, likely through different mechanisms. Quercetin, a flavonoid with potent antioxidant properties (37), may protect against oxidative stress-induced damage, while BMMSCs could contribute to tissue repair through paracrine signaling and possibly by differentiating into supportive cell types, including Sertoli and Leydig cells (38, 39). The combined treatment did not show a statistically significant difference compared to the individual treatments, which might suggest a ceiling effect or that the benefits of each treatment reach a maximum threshold when administered together. In this context, Lu et al demonstrated in research that using MSCs can ameliorate chemotherapy-induced damage in the testis by reducing testis inflammation and apoptosis (40).

The study also examined key biochemical markers of oxidative stress and antioxidant defense mechanisms. In the CTX group, a significant reduction in CAT and SOD levels, coupled with elevated MDA levels, indicates substantial oxidative damage. CAT and SOD are critical enzymes in the antioxidant defense system, catalyzing the conversion of H2O2 and superoxide radicals into less harmful molecules. Their depletion in the CTX group suggests overwhelming oxidative stress, which is a known mechanism of CTX-induced cytotoxicity (37,41).

The increase in MDA, a byproduct of lipid peroxidation, provides further evidence of oxidative stress and damage to cellular membranes, especially in testes involving cells rich in polyunsaturated fatty acids (42). The observed reduction in MDA levels in the treatment groups suggests that quercetin and BMMSCs have significant antioxidative effects, helping to mitigate the oxidative damage caused by

CTX (6,43).

Quercetin's role as an antioxidant is well-documented, where it directly scavenges reactive oxygen species and enhances the activity of endogenous antioxidant enzymes (37). BMMSCs, on the other hand, may exert their effects through the secretion of trophic factors that upregulate antioxidant defenses and facilitate tissue repair and regeneration. The combined treatment group showed similar improvements in these biochemical parameters, underscoring the efficacy of both interventions in restoring redox balance and protecting testicular cells from oxidative damage (44, 45). Uzun-Goren and Uz demonstrated in a study that administration of quercetin led to an increase in antioxidant enzyme activity, enhancement in testosterone levels, and testicular functions (34). As well as Shokoohi et al showed in their research that usage of antioxidants led to testicular protection against oxidative stress-related damages (46).

Testosterone is a key hormone for male reproductive function, influencing spermatogenesis and secondary sexual characteristics (45,47). The significant reduction in serum testosterone levels in the CTX group aligns with the observed histological damage, mainly the impaired function of Leydig cells responsible for testosterone production (32). The restoration of testosterone levels in the treated groups suggests that quercetin and BMMSCs help preserve Leydig cell function or facilitate its recovery post-injury. This hormonal restoration is crucial, as it directly influences the spermatogenic process and the maintenance of male fertility (48,49).

Examining sperm parameters further supports the protective effects of quercetin and BMMSCs. CTX treatment led to a marked reduction in sperm count, motility, and normal morphology, reflecting the extensive damage to the germinal epithelium and the downstream effects on sperm production and quality. Sperm count is particularly sensitive to disruptions in spermatogenesis, and the observed decline in the CTX group suggests significant germ cell loss. The reduction in sperm motility and morphology indicates damage to the sperm maturation process and the structural integrity of sperm cells (50).

In contrast, the treatment groups significantly improved all measured sperm parameters. This suggests that quercetin and BMMSCs not only protect against initial damage but also enhance the recovery of spermatogenesis. Quercetin's antioxidative properties likely play a role in preserving sperm cell integrity and function, while BMMSCs may contribute through regenerative mechanisms, potentially including the replacement of damaged germ cells and the support of spermatogonial stem cell niches.

Limitations of the study

However, this study is not without limitations. Utilizing a rat model CTX-induced related damage, the research provides valuable insights into biological processes; nonetheless, animal models may not fully capture the complexity of human testicular disorders. Therefore, caution is warranted when extrapolating these findings to human populations, as they may not directly apply to all cases of testicular dysfunction.

Conclusions

In summary, the results of this study underscore the protective and restorative potential of quercetin and BMMSCs against CTX-induced testicular damage. By mitigating oxidative stress, preserving testicular architecture, and restoring key reproductive parameters, these treatments offer promising therapeutic avenues for male infertility associated with chemotherapeutic agents like CTX. Further research is warranted to explore the precise mechanisms through which these interventions exert their effects and to determine their potential for clinical application in chemotherapy-induced gonadotoxicity.

Directions for Future Research

Consequently, the efficacy of quercetin and BMMSCs treatment in other testicular dysfunctions remains to be determined. Future research should aim to elucidate the specific molecular targets of quercetin and BMMSCs and optimize their therapeutic efficacy in various contexts of testicular disorders.

Authors' Contribution

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Writing–review & editing: Amirreza Jourabchi, Hamraz Pazoki, Maryam Bilabari, Mahdi Esmaeili, Lila Roshangar.

Conflict of Interests

Authors have no conflict of interest.

Ethical Issues

The present research was based on the International Ethical

Committee of Medical Laboratory Animals guidelines and approved by Tabriz University of Medical Sciences (IR.TBZMED.AEC.1401.028).

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