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Adipose Tissue-Derived Mesenchymal Stem Cells Rescue Spermatogenesis and Alleviate Testicular Damage in a Rat Model of Chemotherapy-Induced Infertility

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ABSTRACT

Stem cell therapy is one of the most promising fields of biomedical research, as enthusiasm for its applications and effectiveness have been proven in different therapeutic approaches. Therefore, we aimed to appraise the therapeutic role of adipose tissue-derived mesenchymal stem cells (AT-MSCs) in mitigating cyclophosphamide (CTX)-induced infertility in a rat model. The study included three experimental groups: control, CTX, and stem cell treatment groups. Testes were collected for histopathological analysis and gene expression assessment of protamine, prohibitin, and StAR genes. The results showed that cyclophosphamide impaired spermatogenesis and caused testicular damage. However, the transplantation of AT-MSCs facilitated the restoration of the seminiferous epithelium and enhanced the regeneration of spermatogenesis. The expression of protamine, prohibitin, and StAR genes was upregulated in the AT-MSCs group compared to the CTX group. This study provides further evidence for the relationship between the expression of protamine and prohibitin genes and male infertility. In conclusion, AT-MSCs were able to recover the spermatogenesis process and regenerate the damaged testicular elements in cyclophosphamide-treated rats.

Key words: Stem cells, Cyclophosphamide, Infertility, Spermatogenesis, Gene expression

INTRODUCTION

Spermatogenesis is the process whereby male germ cells are transformed into mature spermatozoa within the testes. Disruptions in this process can lead to infertility, which accounts for 70-90% of human infertility cases, making it a significant health and social concern (Ibtisham and Honaramooz 2020; Adriansyah et al. 2023). Several factors affect spermatogenesis (Chao et al. 2023). a) Genetically: genetic abnormalities can cause spermatogenic disorders. b) Endocrine factors: serum hormone imbalance can disrupt spermatogenesis and lead to infertility. c) Environmentally and pollutants: adverse environmental factors can negatively impact sperm quality and cause infertility (Chao et al. 2023).

One factor that can disrupt spermatogenesis is chemotherapeutic drugs, such as Cyclophosphamide (CTX). CTX's mutagenic and cytotoxic effects can induce testicular damage, leading to oligospermia (low sperm count) or azoospermia (absence of sperm), and ultimately, male infertility (Aliakbari et al. 2016; Salimnejad et al. 2018).

Stem cell therapies have emerged as a promising approach for treating infertility and different diseases such as spinal cord injury (Abdallah et al. 2021), diabetes (Algan et al. 2023) skin wounds (Bahr et al. 2021) and multiple sclerosis (Abdallah et al. 2019 and 2024).

Mesenchymal stem cells (MSCs), in particular, have garnered interest because of their capacity for self-renewal, differentiation, proliferation, and immunomodulation (Adriansyah et al. 2023). Previous research has shown that MSCs can improve spermatogenesis, sperm motility,

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fertilization, and inhibit apoptosis. They also have the potential to scavenge free radicals, enhance the testicular antioxidant defense system, and promote tissue remodeling and cell division (Liu et al. 2020; Zickri et al. 2021; Stavely and Nurgali 2020; Sagaradze et al. 2020; Adriansyah et al. 2023).

Adipose tissue-derived mesenchymal stem cells (AT-MSCs) provide several advantages, including easy and safe extraction, low morbidity, and minimal invasiveness (Gabr et al. 2017). Researchers have reported the potential of AT-MSCs to produce male germ cells necessary for maintaining sperm production, as well as their potential for differentiation into male germ cells, Leydig cells and Sertoli cells (Hou et al. 2016; Kurek et al. 2020; Zhankina et al. 2021; Cui et al. 2023).

In this research, we aim to appraise the therapeutic role of Adipose Tissue-Derived Mesenchymal Stem Cells (AT-MSCs) in ameliorating Cyclophosphamide (CTX)-induced infertility in a rat model, with the goal of improving male fertility.

MATERIALS AND METHODS

Ethical approval

All animal-related procedures conducted for this study were performed in accordance with the ethical guidelines set forth by the Institutional Animals Ethics Committee of the Faculty of Veterinary Science at Suez Canal University (approval number SCU 2023076). This ensures that the use of the animal subjects in this research adhered to the established ethical standards and protocols for the human treatment and handling of laboratory animals.

Isolation of adipose tissue derived mesenchymal stem cells (AT-MSCs)

The experiment began by sterilizing a laminar flow cabinet with 70% alcohol and placing all necessary equipment and disposables inside. An anesthetized rat was used, and subcutaneous fat tissue was collected from the animal into a sterile 50mL falcon tube. The collected fat pads were then washed 3-4 times with PBS while shaking, and then transferred to a new falcon tube. Addition of the digestion solution (0.5mL Collagenase type 1 + 50mL PBS) were added, then incubation of the falcon in shaking incubator (at 37°C in incubator for 1 hr), centrifugation of the falcon (3500rpm for 10min). The pellet was re-suspended in complete DMEM (low glucose with stable glutamine DMEM,10%FBS and 1% antibiotic mix) after the supernatant was discarded. The solution was transferred containing cells into sterile flasks. The media was changed after 24 hours (Zuk et al. 2001). This process allowed for the isolation and initial culture of adipose tissue-derived mesenchymal stem cells (AT-MSCs) from the rat subcutaneous fat tissue, which were later used in the stem cell therapy experiments.

Culturing for AT-MSCs

Cells were incubated in CO2 incubator; the media was replaced every 2 days. When the confluences of the flasks reached 75%, sub-culturing of the flasks was done using Trypsin-EDTA. The same process was repeated each time the cell confluences reached 75% from passage number 0 till reaching passage number 3.

AT-MSCs of characterization

The morphology and adherence of AT-MSCs to the tissue culture flask served as markers for their identification. Following that, the surface markers of cultivated AT-MSCs were analyzed flow cytometrically utilizing CD105, CD90, and CD73 (Dominici et al. 2006).

Animal experimental model

The study utilized adult, healthy, male albino rats aged between 8 and 10 weeks, weighing 200-250 grams. The rats were acquired from the Animal Experiment Center of VACSERA, the Holding Company for Biological Products & Vaccines in Egypt.

Experimental design

Forty rats were allocated into four random groups of ten male albino rats:

a) Control group, which contains normal male rats served as normal control, received phosphate buffer saline (PBS),

b) CTX Group, contains male rats with induced testicular damage using intraperitoneally (Ip) injected cyclophosphamide (CTX) and left untreated. CTX was administrated IP at a dose of 50mg/kg body weight on one day, followed by two weeks of alternating doses of 8mg/kg body weight with Endoxan 1g (Baxter Co., Germany) dissolved in PBS (Neosar 2013; Meistrich 2013),

c) Stem cells Group, Rats with induced testicular failure using CTX and treated with adipose tissue derived mesenchymal stem cells (AT-MSCs). The rats were treated with AT-MSCs (1×10^6 cells/rat/week) dissolved in saline (phosphate buffer saline) by intravenous tail injection for 7 days, after their final CTX injection and

d) Donation Group, for Adipose tissue donation.

Histological and histopathology analysis and examination

All rats were slaughtered under anesthesia using Thiopental 30mg/kg after three weeks since the final CTX injection (Ahiskalioglu et al. 2018) and all testes isolated. Following their fixation in 10% neutral buffer formalin, The testes underwent various procedures including trimming, water washing, ethyl alcohol dehydration in ascending grades, xylene clearing and paraffin embedding. For processing, a thin section (4-6µm) was stained with Hematoxylin and Eosin stain (Bancroft 2008).

Additional sections on the IHC techniques use of markers for the integrity status of testes component using avidin biotin-peroxidase complex (ABC) approach for positively charged coated slides were as follows: i) Caspase 3 Polyclonal Antibody (Servicebio, Cat. No. GB11532, dilution 1:800), and ii) Androgen Receptor Monoclonal Antibody, (Biocare medical, Cat. No. ACI 109 A, dilution 1:100).

Following an incubation period with these antibodies, Paraffin sections from each group were treated with required reagents for ABC method (Vectastain ABC-HRP kit, Vector laboratories). Marker expression conjugated with peroxidase and stained with diaminobenzidine (DAB, Sigma) were used to identify the antigen-antibody complex. Non-immune serum was substituted for primary or secondary antibodies in order to create negative controls. Olympus microscope (BX-53) was used to examine IHC stained sections.

Real-time PCR for gene expression

An extract of total RNA was made from the final sample combination using a RNeasy Plus Universalmini Kit (ID: 73404: Qiagen, Hilden, Germany), as directed by the manufacturer.

To summarize, RNA was extracted from each tissue to yield 40µL of RNA solution. The quality of the RNA was determined using a Nanodrop Spectrophotometer (A260/280 ratio). The purified RNA was immediately used to synthesis cDNA. Following the manufacturer's instruction, The ReverAid RT Kit (ThermoFisher Scientific, Waltham, USA) was used to reverse transcribe 5µL of RNA. Real-Time PCR Amplification on the Rotor Gene Q platform was used to measure mRNA expression using the following primer sets (Table 1).

In a final volume of 20µL, the cDNA was mixed with Maxima SYBER Green Master Mix (Thermo Fisher Scientific, Waltham, USA). The reactions were run at 95°C for 5 min., then 40 cycles of 15s. at 95°C, and 30s. at 60°C were performed. In order to report the expression, the cycle threshold (CT) values were determined, which was computed by deducting the housekeeping gene (GAPDH) CT values from the target genes CT values.

Statistical Analysis

With the IBM SPSS statistics 20 program, One-way ANOVA with Dunnett's posttest were used for the statistical analyses. The evaluation of the histopathological scores were conducted by the one-way ANOVA (nonparametric Kruskal–Wallis test) and Dunn's multiple comparison test.

RESULTS

Morphological analysis of AT-MSCs

Using an inverted microscope, AT-MSCs in the third passage were observed and identified and they had the proper morphological characteristics. According to the (Fig. 1) they were adherent and spindle-shaped.

Characterization of AT-MSCs using flowcytometry

Flow cytometry analysis of CD markers revealed positive expressions of the examined CD markers (CD105, CD90 and CD73 as it indicated by approximately 91%, 87%, 90 % of the samples cells, respectively, as Fig. 2 illustrates.

Table 1: Forward and reverse primers for Protamine, Prohibtin and StAR genes

Gene	Forward Primer	Reverse Primer
Protamine	5'-GGCCACCACCACCACAGACACAGGCG-3'	5'-TTAGTGATGGTGCCTCCTACATTTCC-3'
Prohibitin	5'-GTGGCGTACAGGACATTGTG-3'	5'-AGCTCTCGCTGGGTAATCAA-3'
StAR	5'-GCAGCAGGCAACCTGGTG-3'	5'-TGATTGTCTTCGGCAGCC-3'
GAPDH (Housekeeping)	5'-TGTCGTGGAGTCTACTGGTGTCTT-3'	5'-CGGTGGTCACACCCATCACAA-3'

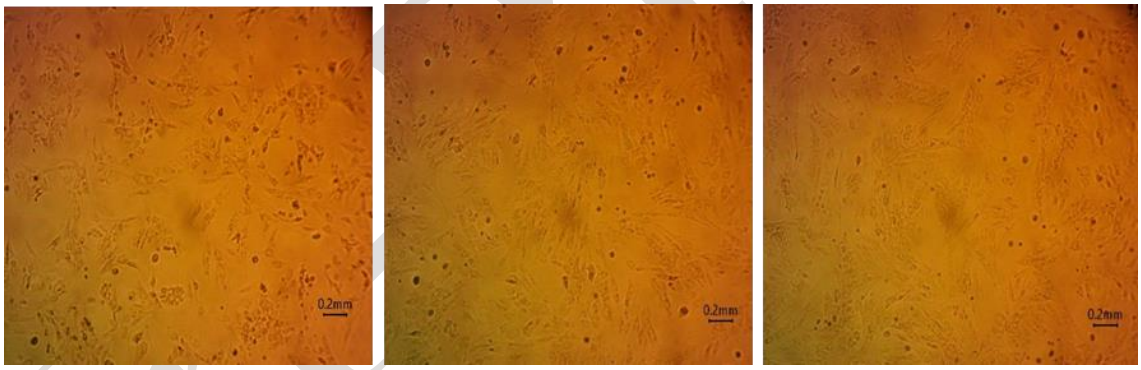

Fig. 1: Morphological characterization of AT-MSCs. Representative phase-contrast images at passage 3 that show the morphology of the adapted MSC spindle-shaped. Scale bar =0.2 mm.

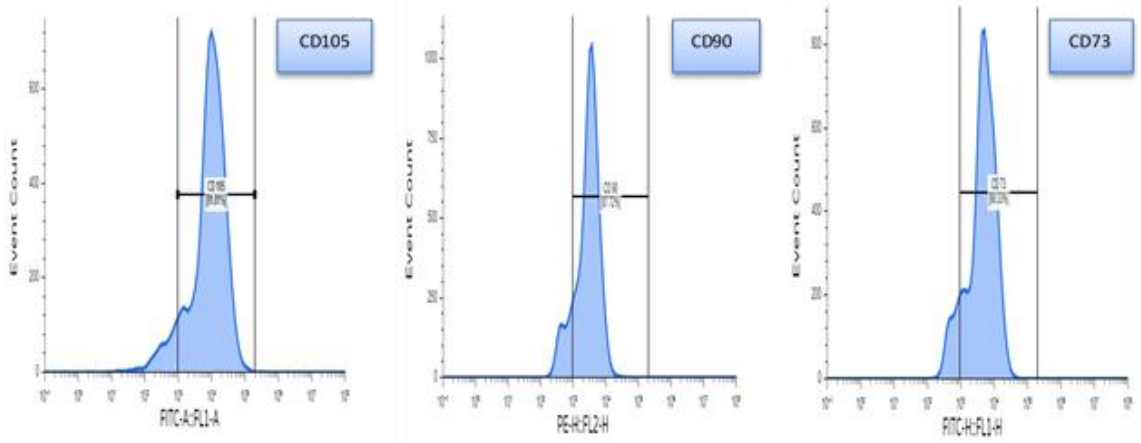

Fig. 2: Detection of Adipose Tissue-derived Mesenchymal Stem Cells markers expression CD105+, 91%; CD90+, 87%; CD73+, 90% using flowcytometry

Histopathological findings

The control group sections showed a normal testes architecture, including typical seminiferous tubules and normal spermatogenesis (Fig. 3A). Cyclophosphamides effects on the rat testis were demonstrated by CTX group histology, which revealed necrotic alteration in spermatogenic cells and aberrant seminiferous tubules (Fig. 3B). The rat testis from the stem cells group displayed the histological effects of stem cells in the form of significantly preserved spermatogenesis, regular seminiferous tubules with average diameters, more layers of germ cells and more spermatids resembling normal (Fig. 3C, D).

Testicular immunohistochemistry observation

Caspase 3 expression: In the control group, no Caspase 3 expression was seen (Fig. 4A). Rats treated CTX exhibited Caspase 3 expression (Fig. 4B); positive Caspase 3 protein expression was shown by brown staining in the apoptotic cells cytoplasm. Caspase 3 expression in the AT-MSCs group ranged from mild to moderate (Fig. 4C). These changes are scored in Fig. 6.I.

Androgen receptor (AR) expression

In the control normal group (5A) androgen receptor expression showed a significant response to Androgen-Antibody in Sertoli cells, while in CTX group (5B), the damage group, there was no response at all in Sertoli cells to Androgen-Antibody. Furthermore, in Stem Cells group (5C), re-expression and response to Androgen (Fig. 5). These changes are scored in Fig. 6.II.

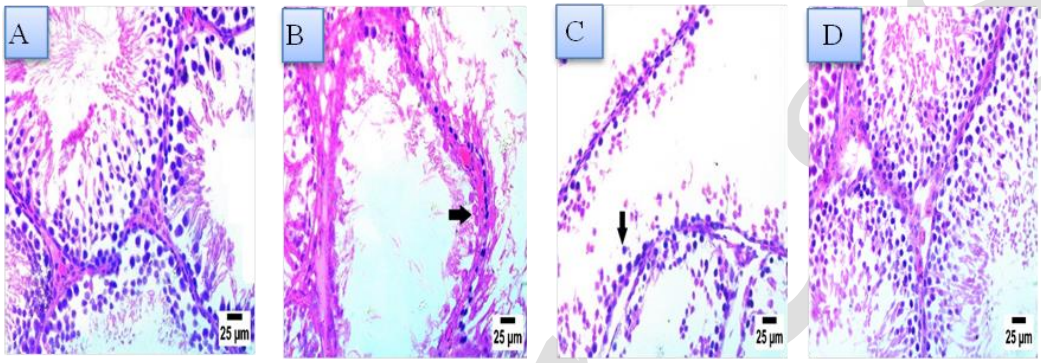

Fig. 3: A) showing seminiferous tubules and Leydig cells with their usual histological structure, B) showing testicular damage and necrotic changes in spermatogenic cells after CTX (arrow) while, (C and D) represent Stem cell group, C) showing few numbers of pyknotic spermatogonia (arrow) in seminiferous tubules whereas D) showing more layers of germ cells layers and more spermatids resembling normal. (Hematoxylin and Eosin stain) Bar=(25µm)(

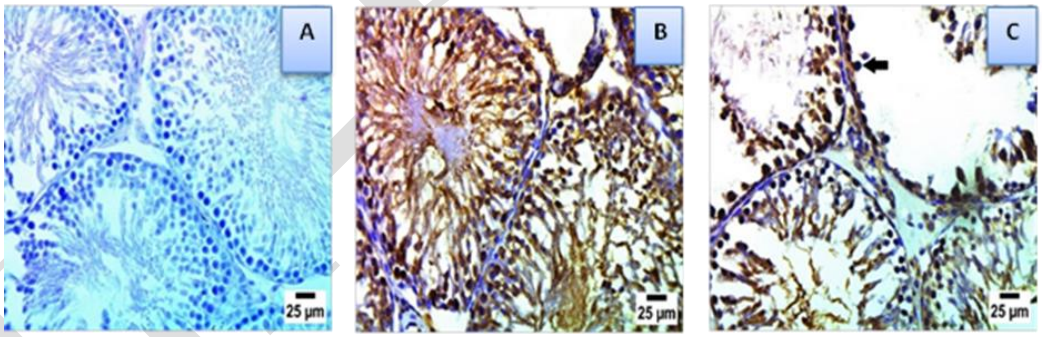

Fig. 4: A) showing negative expression for caspase 3 in spermatogenic cells, B) demonstrated that all spermatogenic cells in seminiferous tubules have caspase 3 strongly expressed in their nucleus after CTX and C) demonstrated that caspase 3 is moderately expressed in spermatogonia nuclei after AT-MSCs transplantation (IHC-peroxidase-DAB). Bar=25µm.

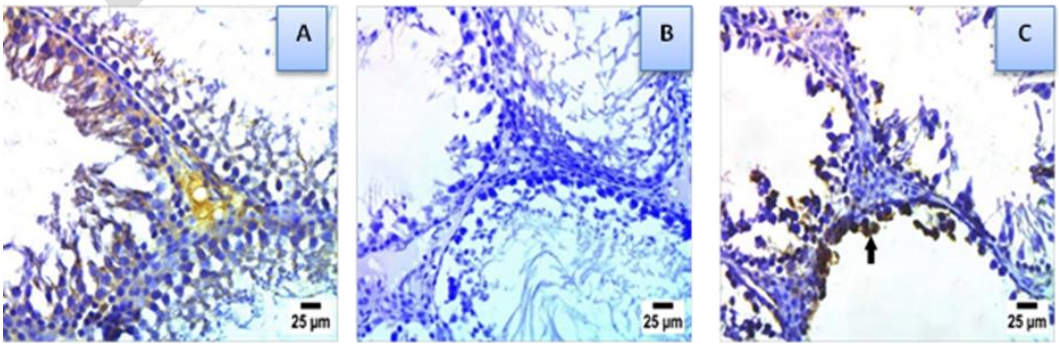

Fig. 5: A) showing positive expression in Leydig cells and positive expression in cytoplasm of spermatogenic cells, B) showing negative expression for androgen in seminiferous tubules and Leydig cells and C) showing sever positive expression in nuclei of spermatogonia after AT-MSCs transplantation (arrow) (IHC-peroxidase-DAB). Bar=25µm.

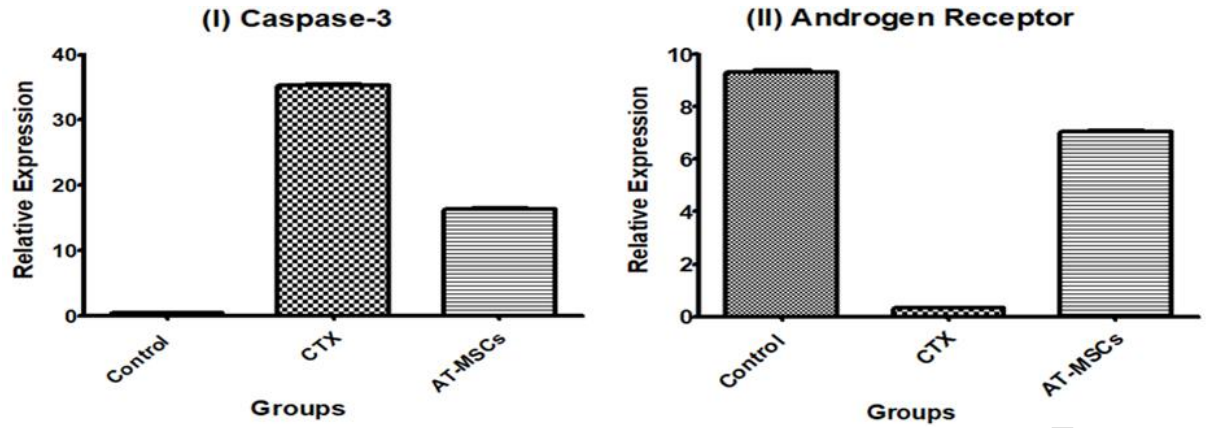

Fig. 6: Showing reaction area percent of caspase 3 (6.I) and Androgen receptor (6.II) and data represented as a mean±SE (n=10), values indicate that mean of CTX group were significantly variable than other groups (P≤0.0001) according to one-way ANOVA and Tukey tests.

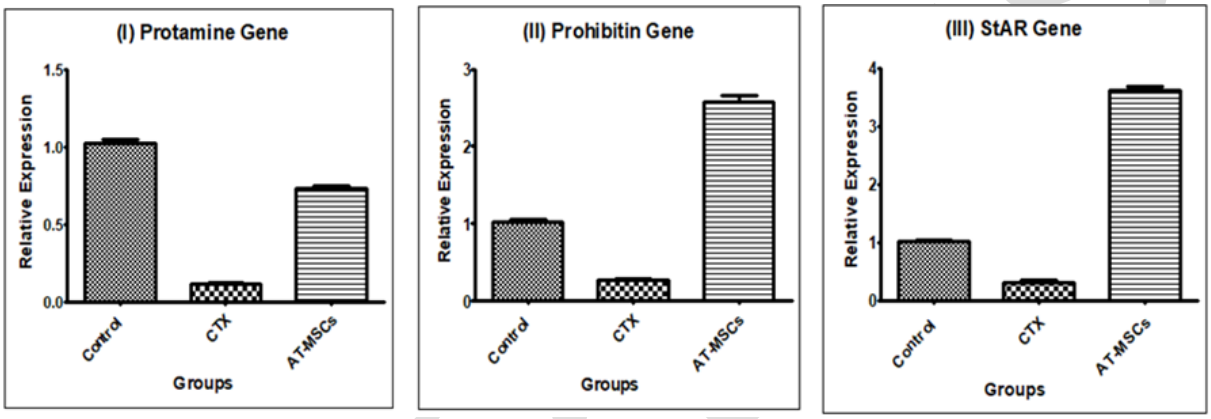

Fig. 7: Histogram for gene expression of protamine (7.I), Prohibitin (7.II) and StAR (7.III) in response to the treatments; the control; CTX and AT-MSCs. All groups provided have statistically significant interrelationship (P≤0.05). Data were displayed as mean±SD.

Gene expression results

Three genes (Protamine, Prohibitin and StAR genes) had their mRNA expression levels measured in the current investigation using real-time PCR; the housekeeping reference gene was glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

To assess the role of AT-MSCs in spermatozoa morphology and function, we measured the expressions of protamine gene (Fig. 7.I) and prohibitin gene (Fig. 7.II) in testes and observed the influence of AT-MSCs. When comparing CTX-treated rats to normal control rats, there was a noticeable decrease in the testicular expression of protamine and prohibitin genes. Compared to rats treated with CTX, rats treated AT-MSCs exhibited a significant elevation of testicular protamine and prohibitin expressions. Our findings revealed that when compared to CTX group, AT-MSCs can increase protamine and prohibitin testicular expressions.

Moreover, we measured the gene expression of StAR gene (The Biomarker of fertility), StAR gene (Fig. 7.III) showed a significant downregulation of gene expression after CTX injection. While, revealed that testicular expressions were significantly up-regulated (by 2.5-fold) after AT-MSCs treatment.

DISCUSSION

The successful use of stem cells in treating various diseases, such as chondral defects (Abdallah et al. 2016), skin wounds (Bahr et al. 2021), spinal cord injury (Abdallah et al. 2021), and multiple sclerosis (Abdallah et al. 2019 and 2024), has encouraged us to investigate the therapeutic role of adipose-derived mesenchymal stem cells (AT-MSCs) against cyclophosphamide (CTX)-induced infertility in a rat model. Our findings were evaluated through histopathological analysis and gene expression to improve understanding of male fertility.

Numerous laboratory investigations have demonstrated the protective role of MSCs in testicular injuries caused by various substances (Zhankina et al. 2021; Adriansyah et al. 2023). Most often, this effects is mainly achieved by mostly directly replacing damaged tissues and stimulating cell differentiation. Alternatively, it can be achieved indirectly by stimulating cell regeneration via paracrine signaling, which entails the production of diverse growth factors and mediators that regulate cellular connections (Kurek et al. 2020; Zhankina et al. 2021; Adriansyah et al. 2023).

Stem cells may contribute to the restoration of spermatogenesis by either maintaining already-existing spermatogonial stem cells (SSCs) or transdifferentiating to generate spermatocytes by transforming into SSC-like cells (Ibtisham and Honaramooz 2020; Diao et al. 2022). This improved impact was ascribed to stem cell secreting cytokines and growth factors, which results in antiapoptotic and immunomodulatory actions (Mohamed et al. 2023; Adriansyah et al. 2023).

Caspases are enzymes that have a role in apoptosis. Caspase-3 initiates an apoptotic cascade when activated (Asadi et al. 2022). A higher level of caspase activity in the testis is linked to infertility and impaired spermatogenesis (Yu et al. 2024). MSCs have been proven to protect against testicular tissue apoptotic damage through release of several substances with antiapoptotic, anti-inflammatory and antioxidative properties (Liu et al. 2022), which aligns with results in rats (Meligy et al. 2019; Liu et al. 2022) and rabbits (Ismail et al. 2023).

Androgens are sexual hormones that are essential for the growth and operation of reproductive organs. It is vital for the development and function of reproductive organs (Edelsztein and Rey 2019). Androgen regulation of spermatogenesis relies on sertoli cells. Lack of androgen recptor in sertoli cells can disrupt sertoli cells activities resulting in spermatogenesis arrest (Alemany 2022). Immunohistochemical analysis revealed that the control group had the highest expression of androgen receptor (AR), the CTX group had the lowest level, and the AT-MSCs group had significantly higher expression. The lack of androgen receptors in the CTX group is due to the adverse effects of CTX on the testes, causing histological changes. The transplantation of AT-MSCs significantly improved the seminiferous tubules, thereby restoring normal testicular function, consistent with previous findings (Chen et al. 2021).

Regarding gene expression, the protamine gene is essential for chromatin condensation during spermatogenesis, protecting the paternal genetic code in spermatozoa (Ren et al. 2021). Protamine insufficiency can negatively impact male fertility and sperm morphology (Elango et al. 2022). Furthermore, there is proof that changed protamine levels could make spermatozoa DNA more vulnerable to damage, which could lead to infertility or poor results with assisted reproduction (Bibi et al. 2022). Our research revealed a correlation between infertility and alterations in protamine expression, which was improved after stem cell treatment.

Prohibitin gene is one of the elements of the sperm mitochondrial membrane that is ubiquitinated during spermatogenesis (Rodriguez and Sanz 2018). Sperm quality can be determined by measuring the expression of Prohibitin gene, which is necessary for the mitochondrial integrity of germ cells (Li et al. 2020). Decreased levels of prohibitin may result in impaired mitochondrial function and loss of sperm motility (Zhang et al. 2020). In our study, there are significant positive correlations between prohibitin expression and the recovery of spermatogenesis in the treated groups.

The steroidogenic acute regulatory (StAR) gene, a biomarker of fertility, regulates steroid hormone production in the testis (Matsuyama and DeFalco 2024). Testicular stress is associated with decreased StAR gene expression, which may impact the testis's effectiveness. (Hassen et al. 2021). Our findings indicate that stem cell treatment improved testicular efficiency.

Overall, the therapeutic potential of AT-MSCs in mitigating CTX-induced infertility has been demonstrated through histopathological analysis and the modulation of key genes involved in spermatogenesis and testicular function. These results provide valuable insights into the mechanisms by which stem cell therapy can restore male fertility and pave the way for further investigations in this promising field.

Conclusion

The findings of this study provide compelling evidence that adipose tissue-derived mesenchymal stem cell (AT-MSC) therapy can effectively mitigate the detrimental impact of cyclophosphamide (CTX) on male fertility in a rat model. The key conclusions are:

A) Cyclophosphamide treatment significantly impaired spermatogenesis and induced structural damage to the testes, as observed through histopathological analysis.

B) Transplantation of AT-MSCs facilitated the restoration of the seminiferous epithelium and enhanced the regeneration of spermatogenesis in the CTX-treated testes.

C) Protamine, prohibitin, and StAR are example of important spermatogenesis-related genes whose expression was significantly upregulated in the testes of the AT-MSC treatment group compared to the CTX group.

D) These results provide additional evidence linking alterations in protamine and prohibitin gene expression to the development of male infertility.

Author's contribution: AMM and ANA implemented the study design and the animal injection, the isolation and characterization of the stem cells, follow up, blood and tissue sampling, Carried out the biochemical and immunological assays. AKA carried out RT PCR experiment, WSM data analysis, YAD supervision. All authors read and approved the final manuscript.

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