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Original Article

Protective Effects of Curcumin on Cryopreserved Human Sperm Through Mitochondrial Apoptosis Regulation

Masoumeh Masoumi; Ph.D.[1], Maryam Bagheri; Ph.D.[2], Sedighe Hantoushzadeh; M.D.[1], Mamak Shariat ; M.D.[3], Mina Jafarabadi; M.D.[1], Marjan Ghaemi; M.D.[1], Masoumeh Dehghan Tarazjanid; Ph.D.[4], Zohreh Heidary; Ph.D.[1], Fedyeh Haghollahi; M.Sc.[1], Sara Zabihzadeh; M.Sc.[4]

1 Vali-E-Asr Reproductive Health Research Center, Family Health Research Institute, Tehran University of Medical Sciences, Tehran, Iran
2 Breastfeeding Research Center, Family Health Research Institute, Tehran University of Medical Sciences, Tehran, Iran
3 Maternal, Fetal & Neonatal Research Center, Family Health Research Institute, Tehran University of Medical Sciences, Tehran, Iran
4 Infertility Department, Imam Khomeini Hospital Complex, Tehran University of Medical Sciences, Tehran, Iran

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Abstract

Objective: Antioxidants have shown positive effects on semen quality by improving sperm parameters such as motility and viability. This study investigate the effects of curcumin on sperm parameters, apoptosis, and DNA fragmentation index (DFI) following freezing in oligoteratoasthenospermia (OAT) patients.

Materials and methods: In this experimental study, a total of 40 semen samples obtained from 10 men aged 25–42 years with OAT according to WHO guidelines were treated with different concentrations of curcumin (0, 10, 20, and 30 µM) in a freezing medium. Following the freeze-thaw process, sperm parameters were evaluated. At the optimal dose, DNA fragmentation index (DFI) was assessed using the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay, and the expression levels of BAX (BCL2-associated X) and BCL2 (B-cell lymphoma-2) genes were measured by real-time PCR in both the optimal dose group and the control group.

Results: The cryopreservation had a significant detrimental effect on sperm parameters. Curcumin treatment, particularly at the 20 µM dose, showed improvements in sperm motility, although these improvements did not reach statistical significance. Also in optimal dose (20 µM dose), there was a significant decrease ($p < 0.001$) in the level of DFI, in BAX gene expression and BAX/BCL2 ratio, as well as a significant increase in BCL2 gene expression, which It indicates a decrease in apoptosis.

Conclusion: It seems that the addition of curcumin to the sperm- freezing medium has a positive impact on sperm motility. This improvement can be attributed to the reduction in apoptosis and the protective effects on sperm DNA. By mitigating apoptosis, curcumin helps preserve the viability and functionality of sperm cells.

Keywords: Spermatozoa; Apoptosis; Cryopreservation; Curcumin

Introduction

Infertility is a significant global health issue, affecting approximately 15-20% of couples worldwide, with male factors contributing to about half of the cases (1). In such cases, sperm freezing is often recommended for individuals with conditions like oligozoospermia, azoospermia, and for sperm extraction from testicular tissue (TESE). Sperm

Correspondence:
Dr. Maryam Bagheri
Email: mbagheri58@yahoo.com

freezing is also commonly employed in sperm donation programs (2). However, it is important to note that the freeze-thaw process can potentially cause damage to spermatozoa (3), affecting the mitochondria and plasma membrane of sperm and resulting in reduced sperm motility (4). This process also leads to an increase in reactive oxygen species (ROS), alterations in cytokine levels, production of free radicals, and activation of oxidative stress pathways, ultimately leading to increased apoptosis and DNA fragmentation (5).

Oxidative stress and subsequent apoptosis have a significant impact on normal sperm function and, consequently, on reproduction and fertility (6). The extent of apoptosis in sperm is influenced by the choice of freezing medium and the specific protocols used during the freeze-thaw process (5). To minimize oxidative damage during this process, the use of antioxidant supplements is crucial to protect the sperm. Antioxidants act as scavengers of free radicals and help counteract the harmful effects of ROS on spermatozoa (7).

Curcumin, derived from the rhizome of the turmeric plant, is the active ingredient and a polyphenol compound known for its antioxidant properties. Its unique structure, which includes 2-methoxyphenol and enol diketene, contributes to its antioxidant effects (8). Curcumin has been demonstrated to prevent DNA fragmentation and apoptosis, which can ultimately enhance sperm motility and viability (9).

Previous studies have primarily focused on evaluating the antioxidant effect of curcumin in the freeze-thaw process using normal sperm samples (8-11). However, research specifically investigating the impact of curcumin on (oligoteratoasthenospermia) OAT samples remains limited. Given that a significant proportion of infertility cases are attributed to male factors, this study was conducted to investigate the potential of curcumin in reducing complications associated with the freezing and thawing process in OAT samples.

Materials and methods

Study design: This study is an experimental study involving 40 semen samples obtained from 10 men who visited Imam Khomeini Hospital in Tehran between August and December 2023. Inclusion criteria were age 25 to 42 years, with OAT according to WHO guidelines (12), Sperm ejaculated less than 3 or more than 5 days and exclusion criteria included history of smoking, alcohol consumption, or previous drug abuse, Underlying diseases and used any antioxidants within the last three months.

Sample size: In this study, we employed a formula to compare quantitative traits between two independent groups, using a 95% confidence level. Our research focused on examining the differences in gene expression changes and sperm Parameters between a control group and an intervention group (10). While the minimum sample size calculated for this study was 5 individuals in each group, we opted to analyze 10 individuals in each group to enhance the reliability of our findings. Therefore, in the present study, 40 samples obtained from ten patients and treated with different doses of curcumin were used (Figure 1).

Preparation of curcumin: Curcumin powder with the chemical formula $C_{21}H_{20}O_6$ (Merck, Germany) was obtained from Teb Shahr Company for use in the study. To create a curcumin stock solution with a concentration of 0.1 M, 96% ethanol was used as the solvent (10).

Semen Collection and preparation

Semen analysis: Semen samples were collected from OAT patients following a period of 3-5 days of abstinence from ejaculation. After a 30-minute liquefaction period at room temperature, various parameters were assessed according to the 2010 WHO guidelines (12). These parameters included seminal fluid volume, sperm concentration, viability, motility, and sperm morphology. Sperm count was measured according to WHO criteria (12). To determine sperm viability, 20 µl of the sperm solution was mixed with 10 µl of 0.05% Eosin-Nigrosine dye. After incubating the mixture at room temperature for 2 minutes, the sperm were observed under a light microscope at 400x magnification. Nonviable sperm with compromised plasma membrane integrity appeared pink, while live sperm remained unstained. Sperm motility was assessed using an optical microscope at 400x magnification. For this, 10 µl of the sperm solution was placed on a microscope slide, and the percentage of motile sperm was calculated.

Sperm morphology was evaluated using the standard Papanicolaou staining technique. The stained sperm were observed under an optical microscope at 400x magnification. A total of 200 sperm were evaluated for the presence of abnormal morphology, which included characteristics such as two heads, large head, small head, round head, no acrosome, no head, long or short tail, no tail, twisted tail, and cytoplasmic droplets (13). All experiments were performed in triplicate.

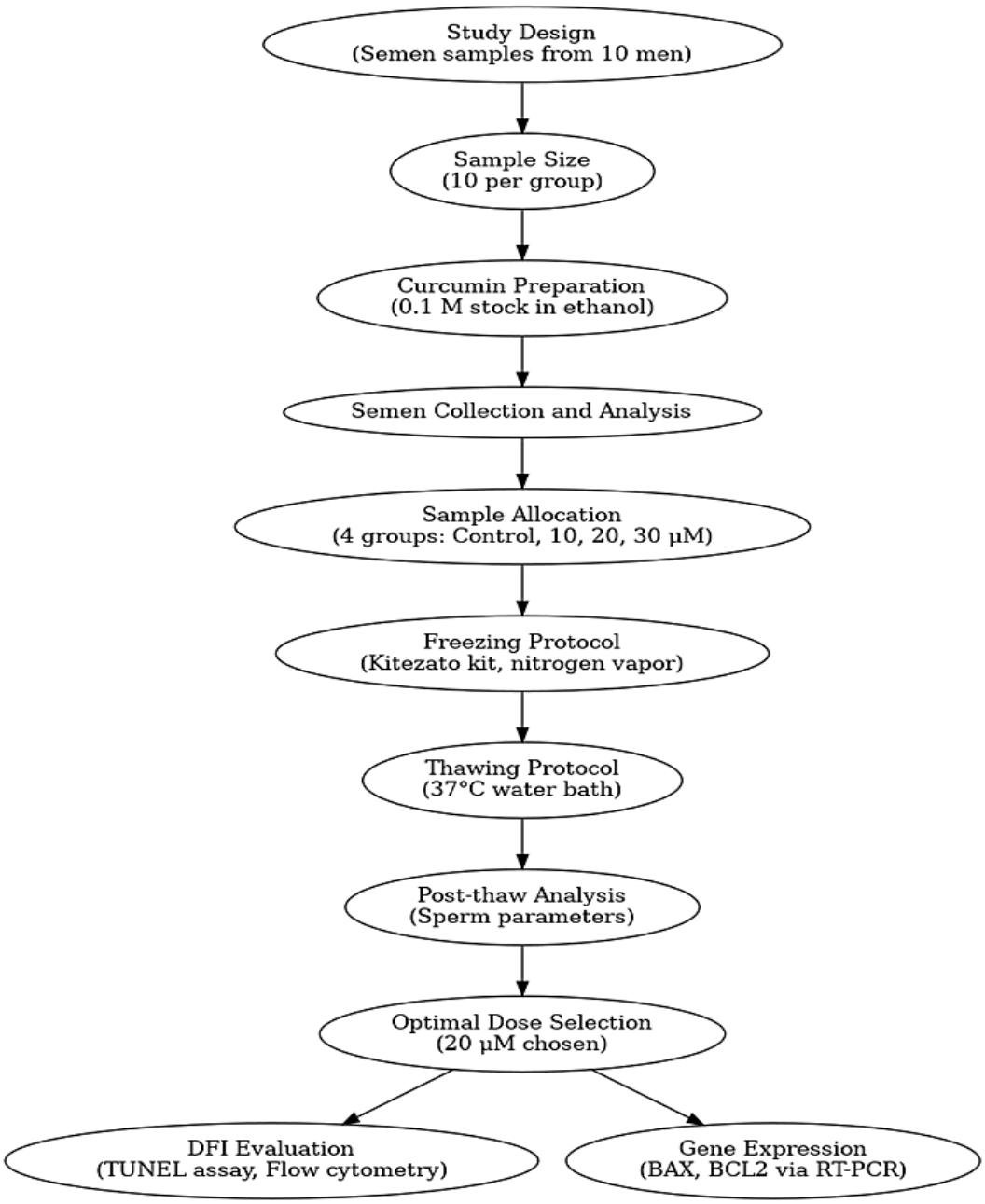

Figure 1: Schematic diagram of experimental workflow including semen analysis, cryopreservation, and gene expression studies

Sample allocations: In this study the semen samples were divided into four groups: three intervention groups supplemented with different concentrations of curcumin (10, 20, and 30 µM), and one control group. In the control group, ethanol was added to the semen samples at the same concentration used to dissolve curcumin in the intervention groups. For the intervention groups, curcumin stock solution was added at specific doses. For the 10 µM group, 1.7 µL of curcumin stock solution was added to a mixture of 0.85 mL of sample and freezing medium. Similarly, for the 20 µM group, 3.4 µL of curcumin stock solution was added to a mixture of 1.7 mL of sample and freezing medium. Finally, for the 30 µM group, 5.1 µL of curcumin stock solution was added to a mixture of 2.55 mL of sample and freezing medium (10).

Sperm freezing protocol: Sperm thawing and freezing were performed using the kit (Kitazato brand) and according to the kit instructions. Which

are mentioned in the text of the article.

Specifically, 0.5 ml of frozen material and 0.5 ml of sperm were combined by pouring them into a microvial. The mixture was then gently mixed using a head sampler. To maintain the integrity of the samples during the freezing process, the mixture was placed under nitrogen vapor for a duration of 5 minutes. After the 5-minute nitrogen vapor exposure, the mixture was transferred to a nitrogen tank for further storage. This protocol was followed consistently for all the intervention and control groups in the study, ensuring standardized freezing conditions for the samples.

Sperm thawing protocol: After a minimum of 7 days of freezing, the samples were thawed, and the sperm parameters were re-evaluated. To initiate the thawing process, the micro vial was removed from the nitrogen tank and immediately placed in 37-degree water. After 5 minutes of thawing in the water, the contents of the micro vial were transferred to a 5cc tube, and semen analysis was conducted to assess various sperm parameters.

After evaluating sperm parameters, the optimal dose of Curcumin is determined**.** In the optimal dose group of Curcumin (Which was a dose of 20 µM), DFI was assessed using the TUNEL assay, while the expression levels of BAX and BCL2 genes were measured using real-time PCR.

Evaluation of DNA Fragmentation: In this study, the TUNEL method was used to evaluate the amount of DNA breakage. After fixing the cells using 4% paraformaldehyde and washing with phosphate buffered saline (PBS), proteinase K treatment was performed. Then the cells were stained using the TUNEL kit and the fluorescent dye fluorescein-12-dUTP and the presence of DNA strand breaks was evaluated using a fluorescence microscope. Finally, the results were analyzed using a flow cytometry device, (Partec PAS model Denmark) (14).

Evaluation of *BAX* and *BCL2* gene expression

RNA extraction and cDNA synthesis: RNA extraction from the semen samples was performed using an RNA Extraction Kit (Yekta Tajhiz Azma, Tehran, Iran) following the manufacturer's instructions. The extracted RNA was then assessed for both quality and quantity. The quality of the extracted RNA was evaluated using gel electrophoresis and the quantity of the extracted RNA was determined using a Nanodrop device. For cDNA synthesis, a cDNA Synthesis Kit (Yekta Tajhiz Azma, Tehran, Iran) was employed, following the instructions provided by the manufacturer. To quantitatively measure DNA, a Nanodrop device was utilized.

Primers: The primers for the *Bax* (Gene ID: 581) and *Bcl-2* (Gene ID: 596) genes were designed using Gene Runner software. The primer sequences are presented in Table 1.

Table 1. *BAX*, *BCL-2* and *GAPDH* genes primer sequence

Gene Name	Primer sequence
BAX-F	CGGCGAATTGGAGATGAACTG
BAX-R	GCAAAGTAGAAGAGGGCAACC
BCL2-F	TGGTCTTCTTTGAGTTCGG
BCL2-R	GGCTGTACAGTTCCACAA
GAPDH-F	CTTTGGTATCGTGGAAGGAC
GAPDH-R	GCAGGGATGATGTTCTGG

2.5.3. RT-PCR: According to the previous study, the RT-PCR program on the ABI 7300 instrument followed a defined time and temperature protocol for cDNA amplification. The procedure began with an initial denaturation step at 95 °C for 60 seconds to separate the DNA strands and prepare the template for amplification. This was followed by 40 amplification cycles, each consisting of a denaturation step at 95 °C for 15 seconds and an annealing/extension step at 60 °C for 31 seconds. After completing the amplification cycles, a melting curve analysis was performed (13).

Ethical Considerations: Ethical approval for this study was obtained from the ethics review board of the Tehran University of Medical Sciences, with the ethics codes (IR.TUMS.IKHC.REC.1401.063). Prior to their participation, all participants provided written informed consent, indicating their voluntary agreement to take part in the study.

Statistical analysis: Statistical analysis was conducted using SPSS Statistics 23.0 (IBM Corp., Armonk, NY, USA)**.** One-way analysis of variance (ANOVA) followed by Tukey's test was used to detect significant differences between groups. A significance level of p-value <0.05 was considered for determining statistical significance. GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA) was used to create graphs and perform statistical analyses.

Results

Sperm parameters: This experimental study involved the participation of 10 males aged 25 to 42 years who had OAT.

Table 2. Sperm parameters before and after Intervention with curcumin

Group	Count (× 106 sperm/mL) (mean ± SD)	Motility (%) (mean ± SD)	Abnormality (%) (mean ± SD)
Before freezing	7.9±5.7	21.9±7.54	96.5±1.18
Control*	3.06±2.20	7±5.54	96.5±1.60
Intervention**			
10µM	3.2±2.3	8.7±06.13	96.5±1.44
20µM	3.2±2.3	9.65±6.57	96.5±1.40
30µM	3.1±2.2	7.65±6.12	96.5±1.40

*0µM curcumin, **Intervention (Three groups of 10, 20, and 30 µM curcumin)

The participants were randomly assigned to three intervention groups, where their semen samples were supplemented with curcumin at concentrations of 10, 20, and 30 µM, respectively (Table 2). Additionally, there was a control group consisting of 10 samples. The results of the study revealed that the process of freezing and thawing sperm led to a significant decrease in sperm parameters ($p < 0.001$). However, treatment with curcumin at various doses showed improvements in sperm parameters. Notably, the parameter of sperm motility exhibited more pronounced improvement, particularly at the dose of 20 µM. However, this increase in mobility did not reach statistical significance (Figure 2).

After assessing the sperm parameters, a comparison was made between the control group and the intervention group treated with 20 µM of curcumin (the optimal dose group of curcumin). The evaluation focused on two aspects: the measurement of DFI and the expression levels of the *BAX* and *BCL2* genes.

Sperm DNA Fragmentation Evaluation: The results of the TUNEL assay, as depicted in Figure 3, demonstrated a notable and statistically significant reduction in DFI after treatment with 20 µM of curcumin ($P < 0.001$).

Gene expression analysis: Figure 4, which compares the expression levels of the *BAX* and *BCL-2* genes between the control group and the intervention group treated with 20 µM of curcumin, reveals significant findings. Specifically, the intervention group demonstrated a significant decrease in the expression of the *BAX* gene (P value < 0.001) and a significant increase in the expression of the *BCL-2* gene ($P < 0.001$) when compared to the control group.

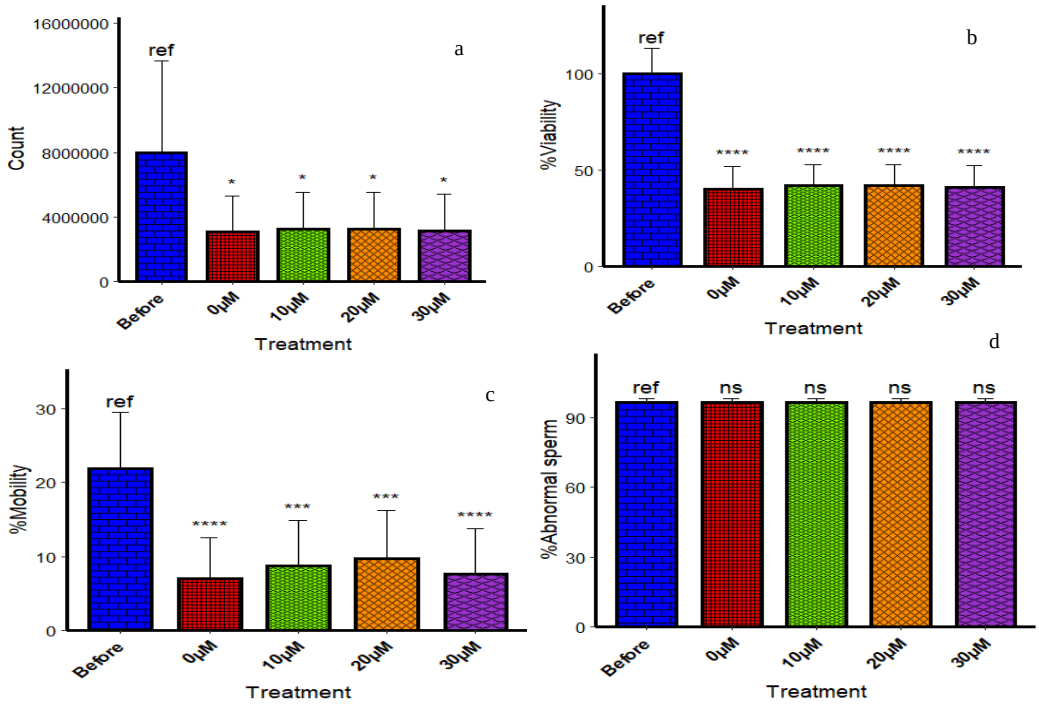

Figure 2: The effects of different concentrations of curcumin (0, 10, 20 and 30 µM) on sperm parameters after freezing and thawing

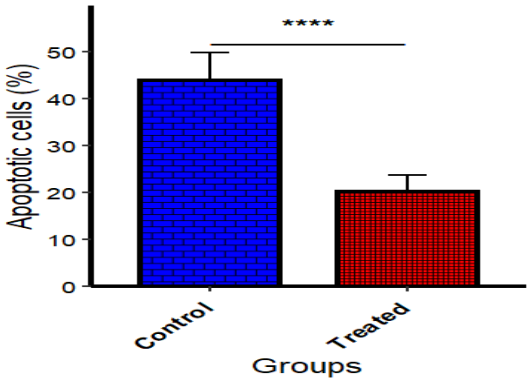

Figure 3: The effects of curcumin (20 µM) on sperm DFI following the thawing and freezing

Discussion

The findings of this experimental study show a significant decrease in the number, motility and viability of sperm in OAT samples after the freezing and thawing process (Table 2). the treatment of frozen sperm samples with different doses of curcumin, particularly at a dose of 20 µM, resulted in improvements in sperm count, motility, and viability (Table 2), although these improvements did not reach statistical significance (Figure 2).

Freezing is a widely used method for long-term sperm storage in order to preserve fertility (15). However, it is known to have deleterious effects on sperm and mitochondrial DNA, plasma membrane integrity, sperm motility, viability, and intracellular enzyme activity (4, 20, 21) and increases DFI (16-18). These effects can lead to reduced sperm count, increased abnormal morphology, and elevated DFI (11, 13, 19).

In this experimental study, freezing and thawing procedures resulted in a significant decrease in sperm count ($P < 0.001$), motility ($P < 0.001$), and viability ($P < 0.001$) (Figure 2). These findings align with previous research reports (16, 19-21). It is important to note that fertilization using sperm with damaged DNA increases the risk of genetic disorders in offspring (22). Several studies have also reported an increase in DFI as a result of freezing and thawing processes (16, 17, 18).Curcumin, known for its anti-inflammatory properties (23), exhibits noteworthy characteristics. Previous research has shown that in vitro administration of 5 µM curcumin increases sperm motility, viability, and membrane integrity (7, 24). In another study, the addition of 10 µM curcumin to sperm freezing medium improved sperm motility and reduced DFI (8, 10, 11).

In another study, it was demonstrated that the administration of curcumin at doses ranging from 50 to 100 mg/kg for a duration of 2 weeks in a digalactose-induced aging model in mice led to improvements in sperm parameters and a reduction in oxidative stress (25). Similarly, another study revealed that exposure to fluorescent lamps for 45 days followed by treatment with 16 µM of curcumin resulted in improved sperm motility and increased follicle-stimulating hormone (FSH) levels (26).

Curcumin exhibits protective effects against ROS and reduces DFI (13). It has also been observed that curcumin can act as a protective agent against DNA damage induced by titanium dioxide nanoparticles (27). Additionally, curcumin has shown protective effects against arsenic-induced toxicity in testicular Sertoli cells (28).

In the present study, treatment of frozen sperm samples with 20 µM curcumin resulted in improvements in sperm parameters including sperm count, motility, and viability, although these improvements did not reach statistical significance (Figure 2) and also significantly reduced DFI (Figure 3).

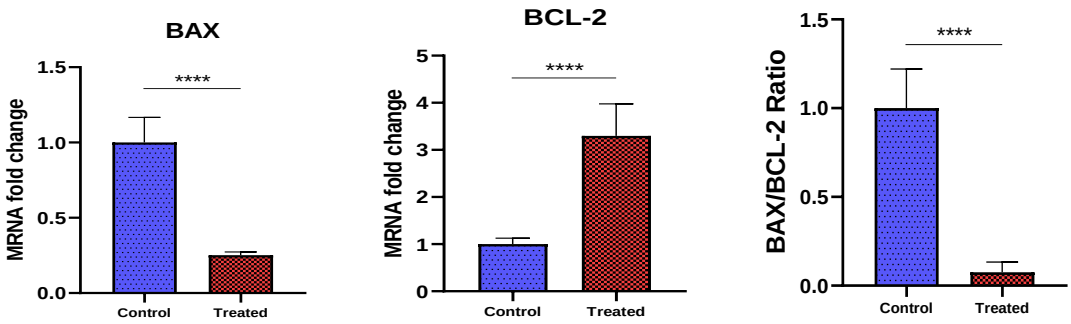

Figure 4: The effects of curcumin (20 µM) on sperm BCL2, BAX and BAX/BCL2 gene expression following the freezing and thawing

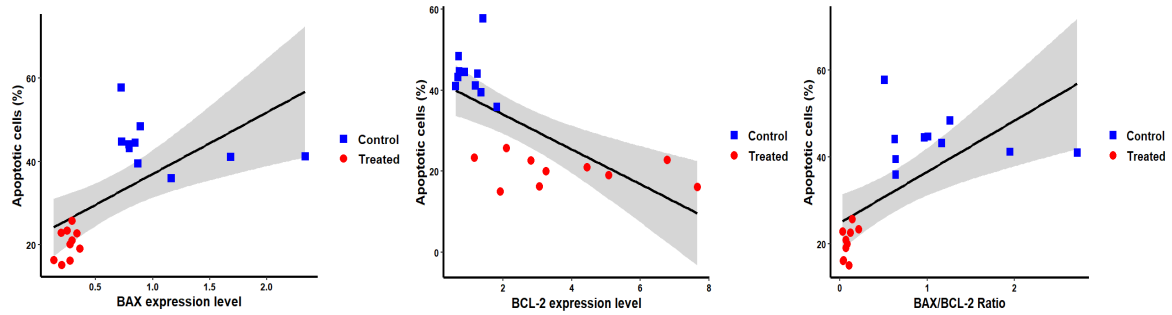

Figure 5: Investigating the relationship between BAX, BCL-2, BAX/BCL-2 ratio and apoptotic cells (%) with regression analysis

It is noteworthy that even small improvements in sperm motility are crucial in the intracytoplasmic sperm injection (ICSI) process since sperm motility is a key factor.

Numerous studies have highlighted the significance of oxidative stress and subsequent apoptosis in relation to normal sperm function, reproduction, and fertility (6). It has been demonstrated that curcumin can effectively restore the structure and function of sperm cells in a dose-dependent manner (10, 29). Curcumin has been found to reduce oxidative stress, lipid peroxidation, and increase the levels of anti-apoptotic proteins in sperm following the freezing and thawing process (30). In a study involving diabetic rats, researchers observed that treatment with curcumin in the testes resulted in decreased levels of malondialdehyde (MDA), reduced *BAX* gene expression, increased *BCL2* gene expression, and a subsequent decrease in apoptosis and cell death (31). Another study demonstrated that curcumin reduced apoptosis in fat cells by modulating the *BAX/BCL2* ratio (32). Curcumin has also been shown to protect testicular structure against the damaging effects of gamma radiation, promoting spermatogenesis and reducing DNA breakage by inhibiting the apoptosis process (27).

In the present study, the addition of 20 µM of curcumin to the sperm freezing medium led to a significant decrease in *BAX* gene expression, an increase in *BCL2* gene expression, a reduced *BAX/BCL2* ratio, and subsequently a decrease in apoptosis ($P<0.001$) (Figure 4). These findings underscore the antioxidant effects of curcumin in mitigating apoptosis (Figure 5). Therefore, to minimize the adverse effects of cryopreservation in cases of OAT, it is suggested to add 20 µM of curcumin to the freezing medium. The limitation of this study was the small sample size. So, it is recommended to conduct a study with a larger sample size to confirm the results. Increasing the sample size can strengthen the reliability and validity of the findings.

Conclusion

Indeed, curcumin has shown its potential to reduce the side effects of cryopreservation in cases of OAT and to protect sperm from cryopreservative damage. In this study, curcumin, through its antioxidant properties, helps to increase the level of anti-apoptotic proteins in sperm following the freezing and thawing process. Curcumin effectively reduces apoptosis and promotes cell survival by modulating gene expression and *BAX/BCL2* ratio.It seems that this protective effect of curcumin on sperm during cryopreservation strengthens its potential as a useful compound to maintain sperm quality and fertility in cases of OAT.

Conflict of Interests

Authors declare no conflict of interests.

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