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Original Article

RNA Sequencing Data Analysis of Oligoasthenoteratozoospermia Patients' Sperms and Effects of Curcumin on the Expression of Some Genes during Sperm Cryopreservation and Freeze-Thawing Process

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Abstract

Background: The cause of oligoasthenoteratozoospermia (OAT) is unclear. In this original study, we examined OAT patients' sperm using RNA-sequencing and studied the impact of curcumin on gene expression during sperm cryopreservation and freeze-thawing.

Methods: RNA-seq was performed using the Galaxy Europe server in IKHC (Imam Khomeini Hospital Complex), Tehran, Iran in 2023. Sperm samples were collected from 30 OAT patients and 30 healthy volunteers. Sperm parameters were analyzed, and samples were frozen with 20 µM curcumin at -196 °C for one week. Thawed samples were assessed for sperm parameters, and the expression levels of *bax*, *bak*, *bcl-2*, *bclw*, *casp9*, *apaf-1*, *SOD*, *cat*, and *GPX4* genes were measured using RT-PCR.

Result: RNA-seq analysis showed increased expression of *NANOS1*, *HSPA6*, and *ALOXE3* genes, while *BHLHE41*, *Hey1*, and *PPM1D* genes were down-regulated in OAT patients' sperm. Curcumin (20 µM) effectively preserves sperm parameters and motility during cryopreservation in healthy subjects and in OAT patients in particular. Cryopreserved sperm from both OAT patients and healthy individuals exhibited reduced expression of pro-apoptotic genes, increased expression of anti-apoptotic genes, and elevated levels of *SOD* and *GPX4* genes.

Conclusion: Altered expressions of *NANOS1*, *HSPA6*, *ALOXE3*, *BHLHE41*, *Hey1*, and *PPM1D* genes likely contribute to OAT development. Additionally, curcumin protects sperm parameters during cryopreservation for both healthy individuals and OAT patients.

Keywords: Oligoasthenoteratozoospermia; Sperm; Cryopreservation; RNA sequencing; Curcumin

Introduction

Oligoasthenoteratozoospermia (OAT) is a condition in which the semen sample has Oligozoospermia, which is low sperm count, Asthenozoospermia, which is poor motility and teratozoospermia, which is abnormal morphology of sperm. OAT is the most common kind of male

infertility. OAT is one of the causes of male infertility, which is accompanied by a decrease in the sperms' concentration (15 x 106 sperm/ml) and motility (<40%) (1).

OAT risk factors include varicocele, family history (genetics), tobacco, alcohol, age (>45), and infections (2). The most important etiology of this disease is defects in spermatogenesis resulting from DNA fragmentation and mainly triggered by oxidative stress, leading to sperm apoptosis (3). Herwig by proteomic analysis of seminal plasma showed upregulation of proteins involved in the anatomical structure development, stress and defense responses, oxidation-reduction, and inflammation (3). However, despite the recent advances in identifying the pathophysiology of OAT, its exact mechanism has not yet been elucidated. Assisted reproductive technique (ART) including 'standard' IVF therapy recommended for OAT patients (4), requires taking sperm from patients and cryopreservation.

Sperm cryopreservation of OAT patients helps fertility (5) and long-term storage of sperm at very low temperatures (-196 °C) by inhibiting metabolic activities (6). However, during the process of freezing and thawing, sperm is damaged. For example, recently cryopreservation of OAT sperm causes a 1.7-fold increase in head defects and the use of these sperm in IVF leads to a decrease in fertility (7). During the freezing-thawing process of sperm, DNA fragmentation is one of the most important reasons for reducing sperm viability (8), and oxidative stress and the activation of the mitochondrial apoptosis pathway play central roles in this phenomenon (9). Therefore, the use of antioxidant compounds in sperm freezing-thawing environments helps to maintain sperm parameters.

Curcumin is a polyphenol isolated from the rhizome of *Curcuma longa* and has a wide range of pharmacological activities such as antioxidant and anti-inflammatory effects. In some studies, the protective effects of this compound on cryopreserved sperms have been reported (10,11). Interestingly, the addition of 20 µM Cur to the freeze-thawing medium resulted in a significant improvement in sperm motility by reducing the levels of reactive oxygen species (ROS) and DNA fragmentation (12). The antioxidant property of Cur plays a role in the effects of semen protection during cryopreservation (13). Considering the improvement of the motility parameter by curcumin in the aforementioned studies, this compound has the potential to be used in the cryopreservation medium of OAT patients' sperm.

Therefore, the current research was carried out with the two objectives of RNA-sequencing of the OAT patients' sperm to identify the molecular pathways involved in the pathogenesis of this disease and to study the effects of Cur on the expression of some genes during sperm cryopreservation and freeze-thawing process.

Methods

Sperms preparation

This study was an interventional experimental type, sperms were taken from 30 OAT patients referred to the Imam Khomeini Hospital, Tehran, Iran in 2023 and 30 healthy volunteers. Each patient is divided to, two test and control groups. The WHO guidelines (https://pmc.ncbi.nlm.nih.gov/articles/PMC10929669/pdf/TAJU_22_2298048.pdf) were used for sperm parameters evaluations. The sperm viability and viability were detected by light microscopy and Eosin-Nigrosin staining, respectively. In addition, sperm morphology was evaluated by Diff Quick staining and all the data were analyzed in Casa software.

The written consent was obtained from all patients before taking semen samples and was approved by the ethics committee of Islamic Azad University, North Tehran branch (IR.IAU.TNB.REC.1403.007). The researchers adhered to the Helsinki Declaration while experimenting.

Inclusion and exclusion criteria

The age between 25 to 45 yr, confirmation of the OAT, and 46. XY normal karyotype were the inclusion criteria and drug addiction, smoking,

intake of antioxidants, alcohol consumption, underlying diseases, and ejaculation less than 3 days or more than 5 days were exclusion criteria.

RNA sequencing and analyzing

The relevant article was selected by searching the NCBI website (14) and the raw data was downloaded from the SRA Run selector and through the Faster Download and Extract Reads tool in FastQ by the Galaxy Europe server. The quality of the raw data was checked by FastQC Read Quality Reports, and the removal of the Illumina Universal Adapter and poly A tail was performed using Trim Galor and confirmed by FastQC Read Quality Reports. In the next step, mapping was done using the HISAT2 tool, and after that counts were done using the Feature Count tool. Finally, using the DESeq2 tool, identified genes were determined and changes in expression were calculated.

Curcumin preparation

Curcumin (C21H20O16) was purchased from Tabshahr Cop. (Tehran, Iran) and then curcumin solutions in the used concentrations (15, 20, and 25µM) were prepared using 96% ethanol as a solvent. First, a 1000 µmol solution of ethanol was prepared, which was used as a stock. Then, a 100 µmol solution was prepared from a 1000 µmol solution. After preparing a 100 µmol solution, dilute solutions of 15, 20 and 25 µmol were prepared. We evaluated sperm parameters before and after adding curcumin. In each group where sperm parameters were better, in that group, to prove the positive effects of curcumin was investigated (12). The concentration of 20 µmol had the best sperm parameters.

Treatment procedure

The sperm sample of each individual was divided into four aliquots (107 sperm/mL) (n=30) and in the presence of Sperm Freeze Medium (KITAZATO, Germany) (ratio 1:1) and curcumin (20 µM) were frozen. Before freezing, the samples were placed on nitrogen vapor for 5 min and then transferred to the nitrogen tank (-196 °C). Seven days after cryopreservation, the microvials containing sperm were placed in a water container at 37 °C for 5 min immediately after being removed from the nitrogen tank, and sperm parameters were measured.

Gene expression analysis

In the present study, the expression levels of *bax, bak, bcl-2, bclw, casp9, apaf-1, SOD, cat, and GPX4* genes in sperm samples were studied. First, RNA extraction was performed using a Trizol RNA extraction kit (Sigma Aldrich) according to the manufacturer's instructions. Quantitative and qualitative evaluations of the extracted RNA were performed. After ensuring the quantity and quality of the extracted RNA, cDNA synthesis was performed using the Yekta Tajhiz kit (Iran) according to the manufacturer's instructions. The sequences of *bax, bak, bcl-2, bclw, casp9, apaf-1, SOD, cat, and GPX4* genes primers were designed by Primer 3 Plus software. To ensure the correctness of the designed primers, the sequences were searched in the human genome sequence using Primer Blast software. In this study, *the GAPDH* gene was used as an internal control gene. The sequence of forward and reverse primers of each study gene is given in Table 1. The reaction mixture for RT-PCR included 7.5 µL of Syber green, 5 µL of Nuclease-free water, 1.5 µL of cDNA, and 1 µL of each of the forward and reverse primers. The temperature-time program of the device included 1 cycle of 95 °C for 15 min, 40 cycles of 95 °C for 15 sec, and 60 °C for 30 sec. Then, the melting curve was checked and finally, Ct was calculated from the obtained data, and graphs were drawn. Moreover, the samples were done duplicate.

Statistical analysis

After ensuring the normal distribution of the data using the One-Sample Kolomogorov-Smirnov test, data analysis was performed based on a one-way analysis of variance (ANOVA) and T-test. The Tukey mean comparison at the probability level of $P<0.05$ was utilized for the mean comparison. The data analysis was carried out in GraphPad Prism V.8 software. The $^{2-\Delta\Delta CT}$ method was used to analyze gene expression data.

Table 1: The primer sequences of *bax, bak, bcl-2, bcl$_w$, casp9, apaf-1, SOD, cat and GPX4* genes

Gene	Sequences [5'-3']
bcl-2-F	ATCGCCCTGTGGATGACTGAGT
bcl-2-R	GCCAGGAGAAATCAAACAGAGGC
bax-F	TCAGGATGCGTCCACCAAGAAG
bax-R	TGTGTCCACGGCGGCAATCATC
bak-F	TTACCGCCATCAGCAGGAACAG
bak-R	GGAACTCTGAGTCATAGCGTCG
Bcl$_w$-F	CAAGGAGATGGAACCACTGGTG
Bcl$_w$-R	CCGTATAGAGCTGTGAACTCCG
casp9-F	GTTTGAGGACCTTCGACCAGCT
casp9-R	CAACGTACCAGGAGCCACTCTT
apaf-1-F	GCCAAGCAGGAGGTCGATAATG
apaf-1-R	GACCATCCTCAGAAAAGCAGGC
SOD-F	CTCACTCTCCAGGAGACCATTGC
SOD-R	CCACAAGCCAAACGACTTCCAG
cat-F	GTGCGGAGATTCAACACTGCCA
cat-R	CGGCAATGTTCTCACACAGACG
GPX4-F	TCAGCAAGATCTGCGTGAAC
GPX4-R	CCGGATGCCATAGTCAGGAT
GAPDH-F	CTTTGGTATCGTGGAAGGAC
GAPDH-R	GCAGGGATGATGTTCTGG

Results

Clinical data and Sperms parameters

There were no significant differences in the mean age of control (32.78±6.42) and case (34.45±5.91) groups as well as BMI (Control: 25.3±6.44; Case: 27.49±3.22). However, sperm parameters including motility, abnormal morphology, and counts showed significant differences between groups (P<0.05). The OAT sperms' motility (22.20±4.81%) and counts (8.6×106 sperms/mL) were lower than healthy individuals (motility: 78±18.93%; counts: 19.34×106+ sperms/mL), but sperms viability showed no significant differences between groups (P>0.05). on the other hand, sperm with abnormal morphology was significantly higher in OAT patients (97.20±1.48%) than in control ones (18.48±7.96%) (Table 2).

Table 2: The clinical data and Sperms' parameters of control (healthy) and case (OTA) subjects

Variables	Control (n=30)	Case (n=30)	*P*-value
Age	32.78±6.42	34.45±5.91	0.638
BMI	25.3±6.44	27.49±3.22	0.614
Sperm viability (%)	97.23±2.48	95.22±4.52	0.243
Sperm motility (%)	78-18.93	22.20±4.81	<0.0001
Sperm abnormal morphology	18.48±7.96	97.20±1.48	<0.0001
Sperm counts (sperm/mL)	19.34×10^6	8.6×10^6	<0.0001

RNA-seq

The differences in gene expressions of healthy individuals and OAT patients were studied and the results indicated the identification of 3852 genes. Among these numbers, 1988 genes were down-regulated and 1864 genes were up-regulated in OAT patients compared to controls. The genes that showed the most overexpressions or downregulations are listed in Table 3. Moreover, based on the obtained data, volcano and heatmap plots were drawn, which are shown in Fig. 1.

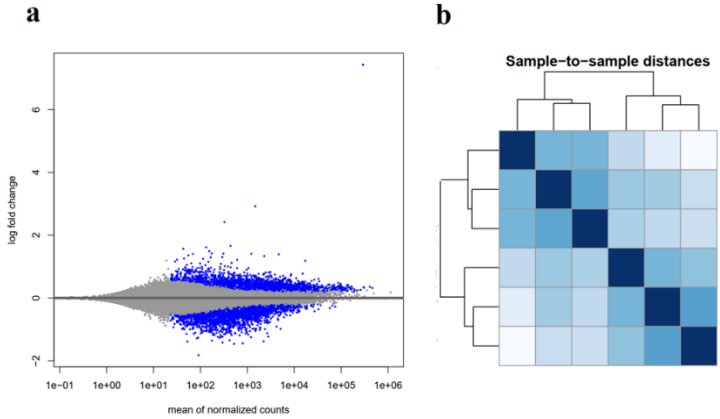

Fig. 1: The differentially expressed genes (DEGs) in OAT and healthy sperms. a) Volcano plot; grey and blue dots show non-significant and significant DEGs, respectively. (b) Heatmap of DEGs

Table 3: The 10 most overexpressed and downregulated genes in OAT patients

Overexpressed				Downregulated		
NO	Gene	LogFC	P-value	Gene	LogFC	P-value
1	NANOSI	7.4286	1.65E-277	BHLHE41	-1.8203	1.29E-14
2	HSPA6	2.9236	1.90E-89	HEY1	-1.4596	6.25E-18
3	ALOXE3	2.4153	8.23E-46	PPMID	-1.4463	8.02E-16
4	AC106774.4	1.6615	3.27E-23	DUSP1	-1.4184	5.55E-13
5	PHRF1	1.6151	1.30E-15	ZNF14	-1.3881	9.81E-09
6	IFNL1	1.5669	2.04E-10	SGK1	-1.3815	4.34E-24
7	CHAC1	1.4927	3.21E-14	PRDM1	1.3531	4.80E-12
8	PRAMEF12	1.4196	6.17E-12	CXCR4	-1.3461	1.89E-13
9	DISP2	1.4137	8.97E-17	FEMIC	-1.2845	2.67E-08
10	ISG15	1.4077	9.21E-22	ZNF608	-1.2667	1.61E-10
LogFC: log fold change						

Curcumin effects on Cryopreserved healthy and OAT sperms

Sperm parameters

Sperm cryopreservation reduced sperm parameters including viability (Fig. 2a) and motility (Fig. 2c) in both healthy and OAT subjects, but abnormal morphology (Fig. 2b) was increased only in control. The supplementation of freeze-thawing medium with 20 μM curcumin prevented the reduction of sperm viability and motility and the increase of abnormal sperms in control. However, this natural product only prevented sperm motility losses in OAT patients (Fig. 2c).

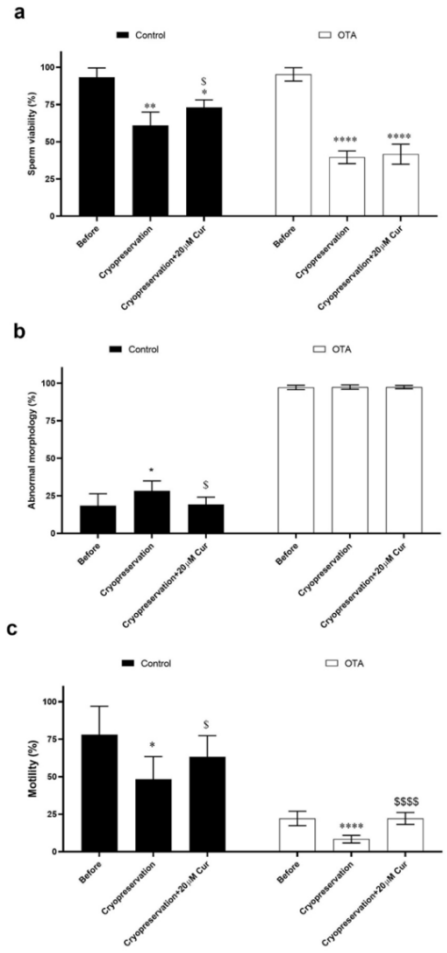

Fig. 2: The effects of supplementation of freeze-thawing medium with 20 μM curcumin on the sperms' viability (a), abnormal morphology (b), and motility (c) of healthy and OTA patients. **P*<0.05^{Befor}; ***P*<0.01^{Befor}; *****P*<0.0001^{Befor}; $*P*<0.05^{cryopreservation}; $$$$*P*<0.0001^{cryopreservation}

Gene expression analysis

Healthy individuals' sperms cryopreservation medium supplemented with 20 μM Cur downregulated significantly *bax* (Fig. 3a, *P*=0.048) and *casp9* (Fig. 3e, *P*=0.022) genes expressions compared with untreated ones. Besides, there were non-significant reductions in *bak* (Fig. 3b) and *apaf-1* (Fig. 3f) gene expression in healthy sperms cryopreserved in a medium supplemented with 20 μM Cur compared to untreated sperms. However, significant overexpression of *bcl-2* was seen in the healthy individuals' sperms cryopreserved in a medium supplemented with 20 μM Cur compared to untreated sperms (Fig. 3c, *P*=0.018). OAT patients' sperms cryopreserved in a medium supplemented with 20 μM Cur showed significant downregulations of *bak* (Fig. 3b, *P*<0.01), *casp9* (Fig. 3e, *P*=0.004) and *apaf-1* (Fig. 3f, *P*=0.036) expressions compared untreated OAT sperms. However, *bcl-2* significantly overexpressed in OAT sperms cryopreserved along with 20 μM Cur (*P*=0.011). There were no significant differences in the *bax* and *bclw* expressions in OAT sperms cryopreserved in a medium supplemented with 20 μM Cur compared to untreated ones (*P*>0.05).

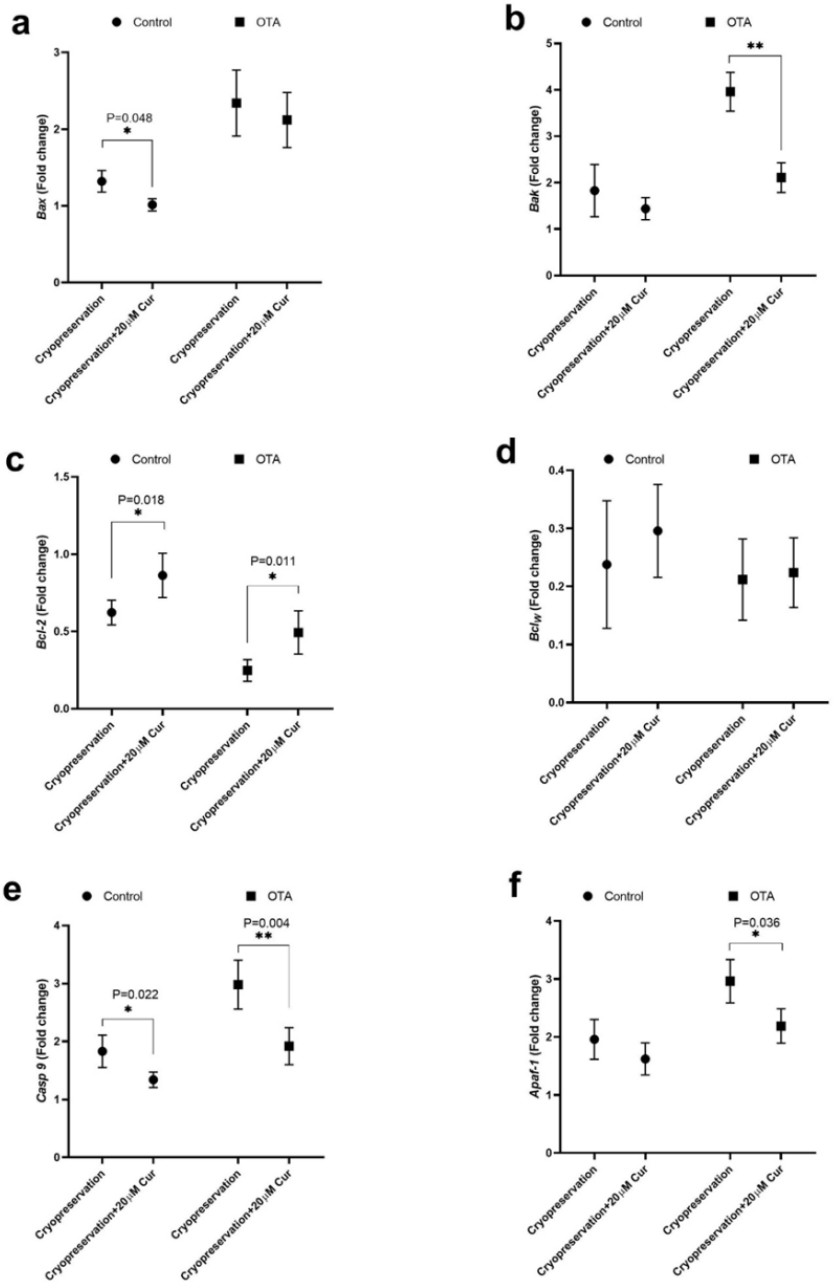

Fig. 3: The effects of supplementation of freeze-thawing medium with 20 μM curcumin (Cur) on the expression levels of *bax* (a), *bak* (b), *bcl-2* (c), *bclw* (d), *cap9* (e), and *apaf-1* (f) genes of healthy and OAT patients' sperms.

We studied the three major genes involved in anti-oxidant defense response, *SOD*, *Cat*, and *GPX4*, in sperms of healthy and OAT patients cryopreserved for 7 days and the results showed overexpression of both *SOD* and *GPX4* in sperm' cryopreserved in medium supplemented with 20 μM Cur compared with untreated ones in healthy and OAT patients (Fig. 4a, c). However, there were no significant differences in *cat* gene expression (Fig. 4b).

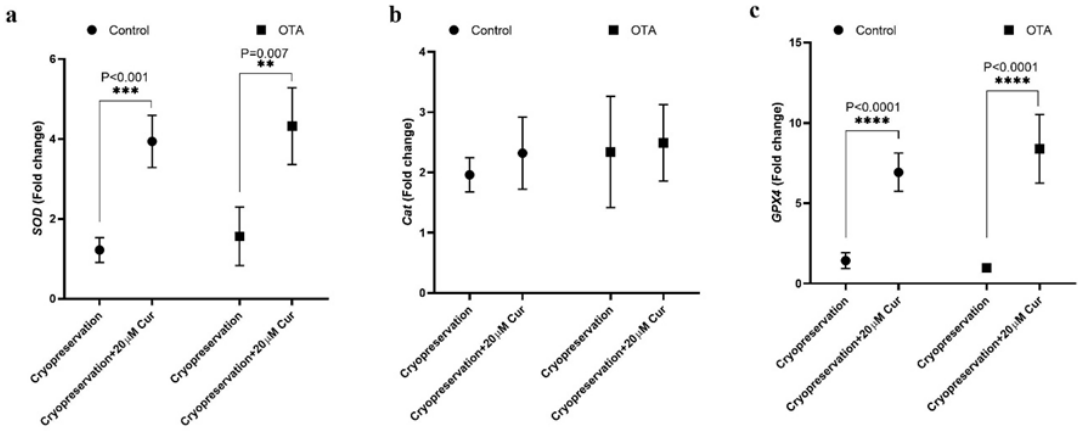

Fig. 4: The effects of supplementation of freeze-thawing medium with 20 μM curcumin (Cur) on the expression levels of *SOD* (a), *cat* (b), and *GPX4* (c) genes of healthy and OAT patients' sperms

Discussion

In the comparison between the studied genes, the increase in the expression of some genes and the decrease in the expression of others can have a significant effect on male spermatogenesis. By knowing these genes and their effect on the process of male infertility and the positive effect of curcumin on maintaining healthy sperms, it is possible to increase the human population. In the sperm of OAT patients, we detected the highest amount of overexpressions in *NANOS1*, *HSPA6*, and *ALOXE3* genes and the highest number of downregulations in *BHLHE41*, *HEY1*, and *PPM1D* genes using RNA-seq bioinformatics tools.

NANOS1 is a key gene in spermatogenesis (15) and it has been found that p.Pro77_Ser78delinsPro and p.Ala173del mutations in this gene are related to the development of severe OAT (16). Although *NANOS2* and *NANOS3* knockouts were associated with infertility (17). However, recently, NANOS has been shown to play an inhibitory role in apoptosis (18). In the present study, the most upregulated gene in RNA-seq analysis in the sperm of OAT patients was *NANOS1*, which seems to prevent the apoptosis of the sperm cells of these patients. Nevertheless, although the expression of this gene was the highest in the OAT patients' sperms, it seems that it could not prevent the decrease in the viability of sperm cells.

The second most upregulated gene in sperms of OAT patients was *HSPA6*. This gene belongs to the heat shock protein member and is activated as a result of heat exposure. *HSPA6* is expressed more under stressful conditions and is not detected under normal conditions (19). Its expression is associated with the improvement of cell viability (20), its high expression in the sperm of OAT patients indicates the defensive response of cells to stressful conditions, especially oxidative stress, which is one of the reasons for the death of sperm cells.

The protein produced by the *ALOX3* gene is arachidonate lipoxygenase 3, which is involved in the lipid peroxidation of the cell membrane (21). The overexpression of this gene in the sperm of OAT patients indicates the strong activation of the lipid peroxidation of the sperm membrane, which leads to the death of sperm cells. *ALOX3* overexpression in the sperm of OAT patients can explain the decrease in sperm viability in these patients compared to healthy subjects in this study.

The *BHLHE41* gene is involved in cell differentiation and any disruption in the expression of this gene results in cell differentiation disorders (22, 23). We, herein, showed the downregulation of *BHLHE41* in the OAT sperms by RNA-seq

analysis, explaining the increases in the abnormal sperm morphology of these patients.

Hey1 plays a central role during the process of spermatogenesis, and the binding of BIOP to the Orange domain of Hey1 is involved in the development of sperm cells (24). The expression of this gene has been reported in all testicular cells (24), and in the present study, its downregulation in the sperm of OAT patients can justify the reduction of sperm parameters in these patients.

PPM1D is one of the key elements in the regulation of spermatogenesis and its defect is associated with defects in this process (25). This gene is expressed in most tissues and its expression reduction is related to defects in immune responses and spermatogenesis (25). Therefore, the downregulation of PPM1D can explain the reduction of sperm parameters in OAT patients in the current study (26,27).

In this study, 20 µM Cur could prevent the decreases in viability and motility and increase in abnormal sperm morphology in cryopreserved sperm of healthy individuals (28). However, in OAT sperms only motility was significantly improved. The improvement of sperm parameters by adding 20 µM Cur was reported previously, which is in line with the current study findings (12). To better understand the action mechanism of the protective effects of Cur on the cryopreserved sperm, we focused on the expression of genes involved in the apoptosis process and antioxidant defense response. The results of this section indicated the effect of Cur on the expression of pro-apoptotic (*bax* and *bak*), anti-apoptotic (*bcl-2*), *casp9*, and *apaf-1* genes. Both Casp9 and Apaf-1 are involved in the formation of the apoptosome complex (29). The release of cytochrome c, which indicates the beginning of the apoptosis process, and its binding to Apaf-1 leads to the formation of apoptosome and the activation of apoptosis (30). The release of cytochrome c from the mitochondrial outer membrane and its entry into the cytoplasm is regulated by bcl-2 family proteins (31). In stress conditions such as oxidative stress leading to DNA damage, BH3-only proteins such as bad and bim are activated and cause inactivation of Bcl-2 and Bclw proteins and activation of pro-apoptotic proteins Bax and Bak (32). This results in the release of cytochrome c and cell apoptosis. Therefore, the decrease in the expression of the pro-apoptotic genes *bax* and *bak*, and the increase in the expression levels of the anti-apoptotic gene *bcl-2* and as a result *casp9* and *apaf-1* in the sperms of OAT patients and healthy individuals after cryopreservation and treated with Cur indicate the prevention of the reduction of cell apoptosis during the freeze-thawing process by this natural product.

Finally, our results showed overexpression of SOD and GPX4 genes in cryopreserved sperms of the healthy and OAT subjects treated with Cur, showing improvement in the anti-oxidant responses for oxidative stress induced by the freeze-thawing process. The overexpression of GPX4 in the cryopreserved sperms by adding 20 µM Cur to the freeze-thawing medium was reported recently (12), which is in line with the current research findings. Cur can reduce oxidative stress during the freeze-thawing process by improving anti-oxidant defense response.

Conclusion

The upregulations of *NANOS1*, *HSPA6*, and *ALOXE3* genes and the downregulations of *BHLHE41*, *HEY1*, and *PPM1D* genes may be involved in the pathogenesis of OAT. Furthermore, the supplementation of freeze-thawing medium with 20 µM Cur is strongly recommended for OAT and healthy subjects sperm cryopreservation. To complete this plan, other genes can also be studied or the same population expanded. And by reducing the male infertility rate, society can be pushed towards the youth of the population and childbearing. In addition, the process of sperm freezing can be a solution to the treatment of some men's diseases. This plan is a way to prevent male infertility and reduce its rate in the human population.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or

falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interests.

References

1. Morgante G, Tosti C, Orvieto R, et al (2011). Metformin improves semen characteristics of oligo-terato-asthenozoospermic men with metabolic syndrome. *Fertil Steril,* 95(6):2150-2152.
2. Okonofua FE, Ntoimo LFC, Omonkhua A, et al (2022). Causes and Risk Factors for Male Infertility. *Int J Gen Med*, 15:5985-5997.
3. Herwig R, Knoll C, Planyavsky M, et al (2013). Proteomic analysis of seminal plasma from infertile patients with oligoasthenoteratozoospermia due to oxidative stress and comparison with fertile volunteers. *Fertil Steril*, 100(2):355-366.e2.
4. Oehninger S (2003). Pathophysiology of oligo-asthenoteratozoo-spermia are we improving in the diagnosis. *Reprod Biomed Online,* 7(4):433-439.
5. Weng H-Y, Lin T-Y, Lin Y-M, et al (2021). The fertility preservation decision-making and testicular sperm retrieval outcome in older adolescents with nonmosaic Klinefelter syndrome and azoospermia. *J Chin Med Assoc,* 84(11):1023-1027.
6. Khan IM, Cao Z, Liu H, et al (2021). Impact of Cryopreservation on Spermatozoa Freeze-Thawed Traits and Relevance OMICS to Assess Sperm Cryo-Tolerance in Farm Animals. *Front Vet Sci*, 8: 609180.
7. Yurchuk T, Petrushko M, Gapon A, et al (2021). The impact of cryopreservation on the morphology of spermatozoa in men with oligo-asthenoteratozoospermia. *Cryobiology*, 100 :117-124.
8. Cankut S, Dinc T, Cincik M, et al (2019). Evaluation of sperm DNA fragmentation via halosperm technique and TUNEL assay before and after cryopreservation. *Reprod Sci,* 26(12):1575-1581.
9. Shaliutina-Loginova A, Loginov DS (2023). Oxidative stress and DNA fragmentation in frozen/thawed common Carp Cyprinus carpio sperm with and without supplemental proteins. *Anim Reprod Sci,* 251:107213.
10. Brugnon F, Ouchchane L, Artonne C, et al (2013). Density gradient centrifugation prior to cryopreservation and antioxydant supplementation by hypotaurine improve post-thaw sperm quality of infertile men with oligo-asthenoteratozoospermia. *Hum Reprod,* 28(8):2045-57.
11. Nur Karakus F, Bulgurcuoglu Kuran S, Solakoglu S (2021). Effect of curcumin on sperm parameters after the cryopreservation. *Eur J Obstet Gynecol Reprod Biol,* 267:161-166.
12. Santonastaso M, Mottola F, Iovine C, et al (2021). Protective Effects of Curcumin on the Outcome of Cryopreservation in Human Sperm. *Reprod Sci,* 28(10):2895-2905.
13. Tvrdá E, Greifová H, Mackovich A, et al (2018). Curcumin offers antioxidant protection to cryopreserved bovine semen. *Czech J Anim Sci,* 63(7):247-255.
14. Janecki DM, Ilaslan E, Smialek MJ, et al (2019). A male infertility mutation reverts NANOS1 activity from anti-apoptotic to pro-apoptotic by disrupting repression of GADD45A, GADD45B, GADD45G and RHOB genes. *bioRxiv,* 28(3):858-869.
15. Ginter-Matuszewska B, Kusz K, Spik A, et al (2011). NANOS1 and PUMILIO2 bind microRNA biogenesis factor GEMIN3, within chromatoid body in human germ cells. *Histochem Cell Biol,* 136(3):279-287.
16. Kusz-Zamelczyk K, Sajek M, Spik A, et al (2013). Mutations of NANOS1, a human homologue of the Drosophila morphogen, are associated with a lack of germ cells in testes or severe oligo-astheno-teratozoospermia. *J Med Genet*, 50(3):187-193.

17. Haraguchi S, Tsuda M, Kitajima S, et al (2003). NONOS1 a mouse nanos gene expressed in the central nervous system is dispensable for normal development. *Mech Dev,* 120(6):721-731.
18. Janecki DM, Ilaslan E, Smialek MJ, et al (2020). Human NANOS1 Represses Apoptosis by Downregulating Pro-Apoptotic Genes in the Male Germ Cell Line. *Int J Mol Sci,* 21(8):3009.
19. Leung T, Rajendran M, Monfries C, et al (1990). The human heat-shock protein family, Expression of a novel heat-inducible HSP70 (HSP70B') and isolation of its cDNA and genomic DNA. *Biochem J,* 267(1):125-132.
20. Deane CA, Brown IR (2018). Knockdown of heat shock proteins HSPA6 (Hsp70B') and HSPA1A (Hsp70-1) sensitizes differentiated human neuronal cells to cellular stress. *Neurochem Res,* 43(2):340-350.
21. Sun Y, Xue Z, Huang T, et al (2022). Lipid metabolism in ferroptosis and ferroptosis-based cancer therapy. *Front Oncol,* 12:941618
22. Kreslavsky T, Vilagos B, Tagoh H, et al (2017). Essential role for the transcription factor Bhlhe41 in regulating the development, self-renewal and BCR repertoire of B-1a cells. *Nat Immunol*, 18(4):442-455.
23. Shen Z, Zhu L, Zhang C, et al (2019). Overexpression of BHLHE41, correlated with DNA hypomethylation in 3'UTR region, promotes the growth of human clear cell renal cell carcinoma. *Oncol Rep,* 41(4):2137-2147.
24. Van Wayenbergh R, Taelman V, Pichon B, F et al (2003). Identification of BOIP, a novel cDNA highly expressed during spermatogenesis that encodes a protein interacting with the orange domain of the hairy-related transcription factor HRT1/Hey1 in Xenopus and mouse. *Dev Dyn,* 228(4):716-725.
25. Chuman Y, Kurihashi W, Mizukami Y, et al (2009).A novel alternative splicing variant of the human PPM1D, can dephosphorylate p53 and exhibits specific tissue expression. *J Biochem*, 145(1):1-12.
26. Zhang X, Lu X, Li J, et al (2019). Mito-Tempo alleviates cryodamage by regulating intracellular oxidative metabolism in spermatozoa from asthenozoospermic patients. *Cryobiology,* 91:18-22.
27. Rezaeian Z, Yazdekhasti H, Nasri S, et al (2016). Effect of selenium on human sperm parameters after freezing and thawing procedures. *Asian Pac J Reprod,* 5(6):462-466.
28. Kopeika J, Thornhill A, Khalaf Y (2015). The effect of cryopreservation on the genome of gametes and embryos: principles of cryobiology and critical appraisal of the evidence. *Hum Reprod Update*, 21(2):209-227.
29. Bratton SB, Walker G, Srinivasula SM, et al (2001). Recruitment, activation and retention of caspases-9 and -3 by Apaf-1 apoptosome and associated XIAP complexes. *EMBO J*, 20(5):998-1009.
30. Zhou M, Li Y, Hu Q, et al (2015). Atomic structure of the apoptosome: mechanism of cytochrome c-and dATP-mediated activation of Apaf-1. *Genes Dev,* 29(22):2349-2361.
31. Shimizu S, Narita M, Tsujimoto Y, et al (1999). Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature,* 399(6735):483-487.
32. Lomonosova E, Chinnadurai G (2008). BH3-only proteins in apoptosis and beyond: an overview. *Oncogene,* 27 Suppl 1(Suppl 1):S2-19.