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Association of Circular RNAs with Sperm Fertility Potential: Implications for Male Infertility Diagnosis

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Abstract

Background: Circular RNAs (circRNAs) are biomarkers that may play a role in the pathological processes underlying abnormal spermatogenesis. Four circRNAs present in human semen, namely STK31-hsa-circ-0133980, LRWD1-hsa-circ-0003327, circRNF17-chr13:25341410-25356082, and circBRDT-chr1:92428275-92433817, were investigated in this study and their relationship with sperm parameters was evaluated.

Methods: In this analytical observational study, 45 semen samples were analyzed according to World Health Organization criteria, and were classified into the following groups: normozoospermia (control, n=9), asthenozoospermia (n=10), oligozoospermia (n=9), teratozoospermia (n=9), and oligoasthenoteratozoospermia (n=8). The expression levels of four circRNAs, along with genes related to apoptosis (BCL2, BAX, and Caspase3) and protamination (PRM1 and PRM2), were assessed using real-time polymerase chain reaction (RT-PCR). Sperm quality was additionally assessed on another portion of each semen sample using triple staining, aniline blue, toluidine blue, and eosin–nigrosin. Data were analyzed using Prism software, and the significance of the observed differences was evaluated by one-way ANOVA.

Results: The results showed that changes in circRNA expression levels were significantly related to sperm morphology (all circRNAs, $p<0.05$), motility (circ-RNF17 and-STK31) ($p<0.01$), and concentration (circBRDT, $p<0.05$) compared to the normozoospermia group. Apoptosis and abnormal protamination were increased in all infertile groups, except the asthenozoospermia, as evidenced by upregulation of BAX and Caspase 3 and downregulation of BCL2 and PRM1&2 genes, compared to the normozoospermic group ($p<0.001$).

Conclusion: The circRNA levels were different in the semen samples with different sperm quality. Our results provide new insights into evaluating sperm functions, supporting the potential role of circRNAs as biomarkers of male fertility.

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Introduction

Infertility is defined as the inability to conceive after a year or more of regular unprotected intercourse without the use of contraceptive methods. Infertility is a problem that affects between 10% and 15% of couples around the world, and almost half of all infertility cases are caused by male factors. Many cases of male infertility are attributed to genetic disorders and lifestyle factors; however, approximately 30% of cases remain unexplained and are classified as idio

pathic (1).

Spermatogenesis is a complex process in which spermatogonial stem cells differentiate into spermatogonia, spermatocytes, round spermatids, and ultimately mature spermatozoa. Based on the World Health Organization (WHO) criteria, semen samples are classified as normooospermia (sperm concentration >15 *million/ml*, progressive motility >32%, and normal morphology >4%), oligozoospermia (sperm concentration <15 *million/ml*), asthenozoospermia (progressive motility <32%), teratozoospermia (normal morphology <4%), and oligoasthenoteratozoospermia (sperm concentration <15 *million/ml*, progressive motility <32%, and normal morphology <4%) (2).

CircRNAs are non-coding RNAs (ncRNAs) that are generally produced from precursor mRNA with conventional splicing, reverse splicing, or head-to-tail back splicing (3). The circRNAs are new molecules which may also play an important role in the regulation of gene expression. The expression and function of circRNAs in human reproductive system are not yet known. It is estimated that there are over 15,000 circRNAs in the human testis, 67% of which are novel. It has also been suggested that genes producing circRNAs are usually associated with spermatogenesis, sperm motility, and fertilization (4, 5). Exon-derived circRNAs (ecRNAs) are predominantly located in the cytoplasm, where they function as sponges for miRNAs. Exon–intron circRNAs (eI-circRNAs) and circular intronic RNAs (ciRNAs) act as effective cis-and trans-regulators, with their parent genes functioning in the nucleus (3, 4, 6).

The possible role of hsa_circ_0000116 in patients with non-obstructive azoospermia (NOA) was investigated and determined the relationship between its expression and clinical-pathological parameters such as age, testicular volume, serum hormone level, and pathological pattern (7). The results showed that the expression of hsa_circ_0000116 in testicular tissue samples of NOA subjects was significantly higher than the one in individuals with obstructive azoospermia (7, 8). In asthenozoospermic men, biomarkers include circPAPPA2 and circFABP6 in spermatozoa, whereas circUSP54 and circEPS15 act downstream in these individuals (9). Despite several studies, many aspects of the relationship among these biomarkers and male fertility potential and spermatogenesis remain unknown.

Previous studies have demonstrated that genes such as STK31, BRDT, RNF17, and LRWD1 play essential roles in spermatogenesis and sperm function. Accordingly, their corresponding circRNAs (STK31-hsa-circ-0133980, LRWD1-hsa-circ-0003327, circRNF17, and circBRDT) were selected as potential biomarkers for evaluating sperm quality and male fertility potential (SRA database under accession number SRX22540414). Gene ontology (GO) analysis further indicates that these genes are associated with several biological processes, including DNA replication, spermatogonia development, histone displacement, androgen receptor interaction, meiotic cell cycle regulation, male meiosis, and spermatogenesis (4). To the best of our knowledge, only a few studies have explored the association between circRNA expression and various factors of male infertility. Consequently, our current understanding of the role and effect of circRNAs on the regulation of spermatogenesis and sperm fertility potential remains limited. Therefore, an attempt was made to investigate whether apoptosis and abnormal sperm protamination are associated with specific circRNA expression pattern.

Therefore, the roles of selected circular RNAs (*e.g.*, STK31-hsa-circ-0133980, LRWD1-hsa-circ-0003327, circRNF17-chr13:25341410-25356082, and circBRDT-chr1:92428275-92433817) were investigated with sperm quantity parameters, including morphology, concentration, motility, and sperm quality indicators such as apoptosis, protamination, chromatin condensation and integrity, and viability, using semen samples representing different infertility factors.

Methods

Semen samples: In this analytical observational study, 76 semen samples were obtained from couples attending the Alzahra Educational and Remedial Center (IVF center) who had a history of infertility for more than 12 months, between October 2022 and June 2023. The semen samples were collected after 3-7 days of sexual abstinence via masturbation in the sterile sample containers, which were transferred to the laboratory within 1 hour after ejaculation. Men with certain medical conditions or a history of factors known to affect fertility were excluded from the study. The semen samples were assessed for sperm parameters based on WHO criteria after liquefaction at 37°*C* for 30 *min* (2). From the original sample group, 45 patients aged 25-39 were included in the study according to G*Power analysis and were divided into five groups based on their semen analysis:

normozoospermia (control, n=9), asthenozoospermia (n=10), oligozoospermia (n=9), teratozoospermia (n=9), and oligoasthenoteratozoospermia (n=8). The study was approved by the Ethics Committee of University of Guilan, Rasht (IR.GUILAN.REC.1400.008;2021), and written informed consent was obtained from all participants after the study's purpose was explained to them. One portion of each sample was used to evaluate sperm parameters, while the remaining portion was used to measure the level of circRNAs.

The fertile/control group comprised men with normozoospermia and normal semen parameters according to WHO criteria, whose partners had female factor infertility. The "infertile groups" included men diagnosed with asthenozoospermia, oligozoospermia, teratozoospermia, and oligoasthenoteratozoospermia.

Evaluation of sperm parameters

Triple staining: Triple staining was employed to evaluate acrosome reaction, encompassing both acrosome integrity and acrosome-reacted samples. The spermatozoa were mixed with 2% trypan blue in a 1:1 ratio and then incubated at 37°*C* for 15 *min*. After that, the spermatozoa were fixed with glutaraldehyde (3% in 0.1 *M* cacodylate buffer, pH=7.4) for 30-60 *min*. Following centrifugation, a smear was prepared from the fixed spermatozoa and stained with Bismarck Brown Y at 40°*C* for 5 *min*. Next, the slides were stained with Rose Bengal at 24°*C* for 20-45 *min*. Finally, the slides were washed and dehydrated in a series of water and alcohol, respectively. A minimum of 100 spermatozoa were examined per slide using a light microscope.

Aniline blue staining: The chromatin condensation of sperm samples was assessed using aniline blue staining. Sperm heads exhibiting condensed chromatin appeared bright blue, whereas those with abnormal chromatin condensation appeared dark blue. It should be noted that a minimum of 100 spermatozoa were counted for each staining procedure in all groups. To carry out this procedure, smears were prepared and air-dried before being fixed with 4% formalin. Next, they were rinsed with water and stained using a 5% aniline blue solution in acetic acid (pH=3.5). Each fixation and staining step lasted for 5 *min* at room temperature. The slides were subsequently rinsed with water, air-dried, and evaluated under a light microscope.

Toluidin blue staining: To assess the quality of chromatin, the technique of toluidine blue staining was utilized. This method involves the examination of sperm heads under a microscope, where those with intact chromatin appear bright blue, while those with fragmented or abnormal chromatin appear dark purple. At least 100 sperm cells were counted in each group. The process involved preparing a thin smear of semen on a slide, followed by fixation in an ethanol–acetone (1:1) solution at room temperature for 1 *hr*. Subsequently, the slides were soaked in 0.1 *N* HCl at 4°*C* for 5 *min*, washed three times in distilled water for 2 *min*, and then stained with 0.05% toluidine blue (TB, 50% Mcllvaine citrate phosphate buffer, pH=3.5; Merck, Germany) at room temperature for 5 *min*. After washing the slide with distilled water, it was air-dried and evaluated using a light microscope.

Eosin-nigrosin staining: This staining technique distinguishes live and dead sperm by selectively staining damaged sperm cells. Live sperm cells appear white or light pink, while dead cells appear bright red or pink due to the staining with eosin dye. To ensure accuracy, at least 100 sperm were counted in each group. The staining process uses two dyes: eosin, which is absorbed by dead sperm cells, and negrosin, which acts as a background dye to enhance the visualization of stained sperm. For staining, a solution of 0.67% (w/v) eosin and 0.9% (w/v) NaCl was prepared, followed by the addition of 10% (w/v) nigrosin dye. The semen sample was then stained with the eosin-nigrosin (1:1) for 30 *s*, air-dried at room temperature, and examined under a light microscope.

Polymerase chain reaction (PCR) primer design: The online tool PrimerBLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design primers for validating and amplifying selected circRNAs in human sperm samples. To ensure amplification specificity for circular RNA isoforms, primers targeting the back-splice junction were designed. Additionally, primers specific to the housekeeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) were designed for normalization purposes. Table 1 displays the primers for human genes.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR): Real-time PCR was utilized to measure gene expression in semen samples. The RNA was first extracted from the samples

Table 1. Characteristics of primers designed for genes

Gene primers	Primer pair sequence (5'-3')	Annealing temperature (°C)	PCR product length (bp)
BAX	F5′ TTTTCCGAGTGGCAGCTGAC 3′	60.5	109
	R 5′ TTGGTGCACAGGGCCTTGAG 3′	61.9	
BCL2	F5′ CTTCGCCGAGATGTCCAGC 3′	60.88	122
	R 5′ CAAAGAAGGCCACAATCCTCC 3′	59.18	
Caspase 3	F5′ TGGAATTGATGCGTGATGTTTC 3′	57.90	160
	R 5′ CTTCTACAACGATCCCCTCTG 3′	57.56	
Protamine 1	F5′ ACTAGATGCACAGAATAGCAA 3′	54.0	134
	R 5′ GTGGCATTGTTCCTTAGCAGS 3′	54.3	
Protamine 2	F5′ CAGCCTCAATCCAGAACCTCC 3′	60.5	108
	R 5′ CTCGCGTTCATGGTCTTGTCC 3′	60.6	
STK31-hsa-circ-0133980	F5′ GCAACGGAAAGTGTGAGGAATTG 3′	60.85	158
	R 5′ CAGACAGTGAGCAACCAATCTTC 3′	59.81	
LRWD1-hsa-circ-0003327	F5′ CAGACAGTGAGCAACCAATCTTC 3′	66.15	142
	R 5′ TGATCCCCCACCCATCCAGGC 3′	66.12	
circRNF17-chr13:25341410-25356082	F5′ AAACTTCCAGCCACAGAAAGACG 3′	61.55	137
	R 5′ GATCATCTGTGCCGCATGTTTCC 3′	62.62	
circBRDT-chr1:92428275-92433817	F5′ TCACATAAGTCCGGGCAGTTGAG 3′	62.49	163
	R 5′ CCACTTGCAGAATAAACACAAAATGCC 3′	62.36	
GAPDH (housekeeping gene)	F5′ CATCACCATCTTCCAGGAGCG 3′	60.81	235
	R 5′ GGAGGCATTGCTGATGATCTTG 3′	59.71	

and then treated with DNase I to eliminate any contaminating DNA. The quality and quantity of RNA were assessed through electrophoresis in a 2% agarose gel and NanoDrop spectrophotometer, respectively. The average RNA concentration in the samples ranged from 500 to 2000 *ng/µl*, with an absorption ratio of 1.8–2. A cDNA synthesis kit (Thermo Fisher Scientific, USA) was used for reverse transcription, which included an RNase inhibitor, 5× reaction buffer, reverse transcriptase, and 10 *mM* dNTPs. Specific primers were designed for each target gene.

Real-time PCR reactions were performed in a total reaction volume of 20 *µl*, containing 10 *µl* of 1×PCR Master Mix (Thermo Fisher Scientific, USA), 1 *µl* of forward primer and 1 *µl* of reverse primer (for STK31, LRWD1, RNF17, BCL2, BAX, and GAPDH at 10 *pmol/µl*; for Caspase3, BRDT, and PRM1 & 2 at 7.5 *pmol/µl*), 1 *µl* of cDNA template (1 *ng/µl*), and 7 *µl* of nuclease-free water. The PCR thermal cycling protocol consisted of an initial denaturation at 94°*C* for 5 *min*, followed by denaturation at 94°*C* for 30 *s*; gene-specific annealing at 59°*C* for STK31, BRDT, BCL2, and PRM1/2, 61°*C* for LRWD1 and RNF17, and 60°*C* for BAX and Caspase 3; extension at 72°*C* for 30 *s*; a final extension at 72°*C* for 5 *min*; and an indefinite hold at 4°*C*.

For RT-qPCR, amplification was performed for 45 cycles under the following conditions: an initial denaturation at 95°*C* for 15 *min*; denaturation at 95°*C* for 30 *s*; annealing and extension at the gene-specific temperatures described above for 30 *s*; followed by a final melting curve analysis from 65 to 97°*C* to confirm primer specificity and the absence of nonspecific amplification. The presence of nonspecific fragments in the PCR product was also checked by plotting a melting curve using SYBR green fluorescent dye. Relative expression levels were calculated using the ΔΔCt method, with GAPDH as the internal control. All reactions were performed in triplicate, and each sample was analyzed three times to ensure data accuracy.

Statistical analysis: The statistical analysis of real-time PCR data was conducted using Prism soft-

ware (GraphPad, USA), and the significance of the observed differences was evaluated through one-way ANOVA. Before performing the one-way ANOVA, the normality of data distribution was assessed using the Shapiro–Wilk test, and homogeneity of variances was evaluated using Levene's test. One-way ANOVA was performed to assess the genetic and treatment effects, and Tukey's multiple comparisons test was used to compare the means. A p-value of less than 0.05 was considered to indicate a significant difference.

Results

Sperm parameters: A significant decrease in normal chromatin condensation, as observed by aniline blue staining, in the teratozoospermia, oligozoospermia, asthenozoospermia, and oligoasthenoteratozoospermia groups compared to the normozoospermia group ($p<0.0001$) (Table 2). Oligoasthenoteratozoospermia exhibited the highest abnormal chromatin condensation compared to the other groups. Additionally, the assessment of chromatin integrity using toluidine blue staining showed a significant difference in the oligoasthenoteratozoospermia and teratozoospermia groups compared to normozoospermia ($p<0.0001$). Moreover, sperm viability was significantly decreased in the teratozoospermia, oligozoospermia, asthenozoospermia, and oligoasthenoteratozoospermia groups compared to the normozoospermia group ($p<0.0001$). The triple staining evaluation of sperm acrosome showed a significant increase in the rate of premature acrosome reaction in all groups compared to the normozoospermia group ($p<0.0001$).

Circular RNA expression in infertile groups: The statistical analysis results (Table 3) indicate a notable variance in the circRNF17-chr13:25341410-25356082 gene level in oligoasthenoteratozoospermia, asthenozoospermia, and teratozoospermia groups when compared to the control group (normozoospermia; $p<0.05$). The abnormal groups (except oligozoospermia) showed a lower circRNF17 expression level in comparison to the control group. The outcomes suggest that the decline in circRNF17 expression may be associated with the rise in the abnormalities of sperm mor-

Table 2. Sperm function assay results in men with different fertility profiles

Samples	Normozoospermia (%)	Oligozoospermia (%)	Asthenozoospermia (%)	Teratozoospermia (%)	Oligoasthenoteratozoospermia (%)
Chromatin condensation	80.19±3.09	71.15±2.5 [d]	70.07±3.01 [d]	72.65±2.67 [d]	37.97±2.34 [d]
Chromatin integrity	84.87±3.2	88.65±2.7	85.39±2.76	61.09±2.4 [d]	45.64±2.81 [d]
Sperm vitality	74.74±2.6	73.68±2.8 [d]	61.23±1.89 [d]	63.27±1.7 [d]	66.18±1.56 [d]
Acrosome integrity	27.71±1.9 [d]	43.85±1.84 [d]	25±1.2 [d]	20.22±1.1 [d]	10.04±0.98 [d]

Significant differences in sperm parameters were observed between the infertile groups and the fertile (normozoospermic) group ($p<0.0001$)

Table 3. Results of gene expression analysis in the control and infertile groups

Gene expression	Normozoospermia	Oligozoospermia	Asthenozoospermia	Teratozoospermia	Oligoasthenoteratozoospermia
RNF17	1.008±0.07	0.8240±0.04	0.7875±0.08 [a]	0.6968±0.05 [b]	0.6097±0.20 [c]
LRWD1	0.9999±0.04	0.7507±0.04 [c]	0.7221±0.002 [c]	0.7576±0.05 [c]	0.6735±0.01 [d]
STK31	0.9961±0.04	0.9449±0.03	1.5767±0.06 [d]	1.2038±0.03 [b]	1.6818±0.04 [d]
BRDT	1.0057±0.08	0.6721±0.06 [b]	0.8034±0.05	0.7464±0.06 [a]	0.7566±0.02 [a]
BAX	0.9871±0.07	1.2362±0.01 [d]	1.3905±0.0	1.4145±0.04 [b]	1.5931±0.03 [d]
Caspase 3	1.0053±0.07	1.6214±0.01 [d]	1.2220±0.04	1.4008±0.02 [b]	1.7003±0.09 [d]
BCL2	0.9995±0.02	0.7563±0.03 [c]	0.9349±0.01	0.6087±0.03 [d]	0.6299±0.03 [d]

The relative expression of genes in all infertile groups was significantly different compared with control (normozoospermia) group, with p-values of a <0.05, b <0.01, c <0.001 and d <0.0001

phology and motility. The LRWD1-hsa-circ-0003327 expression demonstrated a substantial difference in all teratozoospermia (p=0.0009), oligozoospermia (p=0.0008), asthenozoospermia (0.0003) groups and particularly in oligoasthenoteratozoospermia (p<0.0001) when compared to the control group (normozoospermia). Therefore, the reduced level of this gene might be connected to a decrease in various sperm parameters, such as morphology, motility, and concentration. Furthermore, STK31-has-circ-0133980 expression displayed a significant difference in oligoasthenoteratozoospermia (p<0.0001), asthenozoospermia (p<0.0001), and teratozoospermia (p=0.0027) groups compared to the control group (normozoospermia). However, the difference between the oligozoospermia and normozoospermia groups was not statistically significant. According to the results, it can be stated that the expression level of this gene may be inversely related to sperm motility and morphology. In addition, the evaluation of other circRNAs showed a significant decrease in the circBRDT-chr1:92428275-92433817 expression in oligoasthenoteratozoospermia (p=0.015), teratozoospermia (0.0172), and oligozoospermia groups (p=0.0023) compared to the control group (normozoospermia). However, asthenozoospermia showed no significant association with normozoospermia. Based on the above results, it can be mentioned that a reduced level of this gene may be associated with sperm morphological abnormalities and decreased concentration.

CircularRNAs, apoptosis, and male infertility: The statistical analysis revealed a significant difference in proapoptotic BAX gene expression in all groups except asthenozoospermia. Specifically, oligoasthenoteratozoospermia (p<0.0001), teratozoospermia (p=0.0013), and oligozoospermia (p< 0.0001) showed significantly higher levels in comparison with the control group (normozoospermia). The upregulation of BAX gene is indicative of a decrease in sperm count due to the induction of apoptosis (Table 3). Similarly, a significant difference in BCL2 gene expression was observed in the oligoasthenoteratozoospermia (p< 0.0001), teratozoospermia (p<0.0001), and oligozoospermia (p=0.0004) groups compared to the control group (normozoospermia). In contrast, no significant difference in BAX and BCL2 gene expression was found in the asthenozoospermia group compared to the control group (p=0.3311 and p=0.3358, respectively).

Furthermore, the results showed a significant difference in Caspase 3 gene expression in all groups of oligoasthenoteratozoospermia (p< 0.0001), oligozoospermia (p<0.0001), and teratozoospermia (p=0.0028) compared to the control group (normozoospermia). However, no significant difference was observed between the asthenozoospermia group and the control group. This study suggests an increase in the expression of Caspase 3 gene in abnormal groups, particularly in oligoasthenoteratozoospermia and oligozoospermia, compared to the control group. In all infertile groups (except asthenozoospermia), BAX and Caspase 3 levels were elevated compared to the control group. Additionally, based on the results of circRNA analysis, the expression of the circBRDT gene increased in all infertile groups (except asthenozoospermia). Thus, the circBRDT gene may be directly related to BAX and Caspase 3 gene expression and could serve as a potential biomarker for meiotic and/or meiosis cell cycle in semen samples.

Protamination and circular RNAs: According to the results of the statistical analysis (Table 3), significant differences were found in the levels of PRM1 and PRM2 genes among the oligoasthenoteratozoospermia (p=0.0001 and p=0.0031, respectively), teratozoospermia (p=0.0052 and p= 0.009, respectively), and oligozoospermia (p= 0.0058 and p=0.0011, respectively) groups when compared to the control group (normozoospermia). Conversely, the asthenozoospermia group did not show any significant differences when compared to the control group. These findings indicate that altered LRWD1-and BRDT-circRNA expression is associated with sperm concentration and morphological abnormalities, potentially leading to structural defects via impaired protamination

Discussion

To our knowledge, this study is the first to investigate the association between circRNAs (circRNF17, circSTK31, circBRDT, and circLRWD1) and sperm quality and quantity in men with various infertility factors. The findings revealed that circRNF17 expression was decreased in the oligoasthenoteratozoospermia, teratozoospermia, and azoospermia groups, with fold changes of 0.6, 0.7, and 0.5, respectively. In contrast, circSTK31 expression was increased in the oligoasthenoteratozoospermia, asthenozoosper-mia, and azoosper-

mia groups, showing fold changes of 1.68, 1.58, and 1.73, respectively, which may influence sperm morphology, count, and motility. Similarly, circBRDT expression was reduced in the oligoasthenoteratozoospermia, teratozoospermia, oligozoospermia, and azoospermia groups, with fold changes of 0.75, 0.74, 0.66, and 0.49, respectively, suggesting its strong association with sperm count and morphology. Additionally, circLRWD1 expression was decreased across all infertile groups (approximately 0.7-fold), indicating its potential impact on sperm motility, morphology, and concentration.

Furthermore, the study demonstrated that these circRNAs are related to sperm protamination status and apoptosis rate, underscoring their critical role in the regulation of spermatogenesis and overall sperm quality.

CircRNAs, a class of non-coding RNAs, are widely expressed in eukaryotic transcripts and have a unique closed-loop structure that makes them more stable than linear RNAs and less susceptible to endonuclease-mediated degradation. The role of circRNAs in reproduction is currently under investigation. CircRNAs are expressed in human testes, spermatozoa, and seminal plasma (10). Their functions have been explored in various contexts. For example, Dong et al. identified 10,792 novel testis-derived circRNAs in human seminal plasma, which were associated with 5,928 host genes (4). These genes were found to be related to reproductive functions, such as sperm motility, spermatogenesis, and gamete formation. The study identified hsa-circ-0049356 as being associated with CARM1 gene, which is crucial for sperm epigenome stability. CircRNAs were also suggested as novel biomarkers for male infertility diseases and were found to provide valuable information about spermatogenesis (4, 7).

Our study found that circBRDT levels significantly changed in all abnormal groups (except asthenozoospermia), indicating a possible association with cell cycle and sperm concentration. Circ-STK31 and -RNF17 levels were also different in all abnormal groups (except oligozoospermia), possibly indicating an association with spermatogonia development, sperm morphology, and motility. CircSTK31 has been identified as a potential gene involved in spermatogenic defects, with its deficiency reported to cause delayed mitotic progression, unsuccessful mitotic exit, and apoptosis (11). CircLRWD1, a testicular-specific gene highly expressed in the sperm neck where the centrosome is located, was suggested to influence spermatogenesis (12). Studies on semen and testicular tissue from patients with various spermatogenic defects showed reduced expression levels of circLRWD1 (13), which supports our study's findings. Other studies have also shown that certain circRNAs primarily influence pathways related to mitochondrial function and sperm motility (14-15). Yue et al. (16) investigated the circRNA profile of exosomes derived from the seminal plasma of patients with oligoasthenozoospermia, noting that exosomes have recently been proven to affect all sperm functions necessary for effective fertilization.

Thereore, based on the constructed circRNA-microRNA-mRNA networks, they suggest that circRNAs exert a negative feedback effect on gene expression, thereby impacting sperm motility and the spermatogenesis process. However, the precise mechanism of circRNAs in oligoasthenozoospermia remains unclear.

The assessment of several sperm quality parameters, including protamination and apoptosis, was performed on groups with different levels of circRNA expression. The expression levels of BAX, BCL2, Caspase 3, and PRM1&2 varied in all abnormal groups except asthenozoospermia. The observed changes in apoptotic and protamination gene expression were similar to those of circBRDT, indicating a correlation between circBRDT and the meiotic cell cycle via apoptosis and post-meiotic processes such as protamination. Previous studies have reported that BRDT is a key regulator of both meiotic divisions and post-meiotic genome repackaging (17), and that circBRDT is a chromatin-dependent protein that is expressed in round spermatocytes and spermatids, making it tissue-specific (18). These results align with previous studies (4, 17), highlighting a relationship between circBRDT alterations, sperm count, and meiotic cell cycle processes, and suggesting a role for these biomarkers in the pathological mechanisms of impaired spermatogenesis.

This study's limitation is its focus on a subset of circRNAs in semen samples, highlighting the need for further research on other circRNAs and their impact on embryo development. Further studies on circRNA mechanisms, especially their regulation of oxidative stress and gene expression, are crucial for a complete understanding. Although studies of circRNAs in male infertility are still in their early stages, and despite the limitations mentioned earlier, our study has several key

strengths. To our knowledge, this is the first experimental research to evaluate the changes in specific circRNAs in semen samples associated with various infertility factors. Therefore, this finding highlights the role of circRNAs in sperm fertility potential.

Conclusion

The current research suggest that certain circRNAs and genes related to apoptosis and protamination play a significant role in male infertility. The expression levels of circRNF17, LRWD1-hsa-circ-0003327, and STK31-has-circ-0133980 were significantly lower in abnormal sperm groups, indicating a potential link between decreased circRNA levels and decreased sperm motility, morphology, and concentration. Additionally, the upregulation of BAX and Caspase 3 genes in all abnormal sperm groups, except for asthenozoospermia, suggests that increased apoptosis may contribute to reduced sperm counts. On the other hand, the expression levels of circBRDT were significantly higher in all abnormal sperm groups (except for asthenozoospermia), potentially serving as a biomarker for meiotic and/or meiosis cell cycle in semen samples. Moreover, the study revealed significant differences in PRM1 and PRM2 levels among abnormal sperm groups, suggesting a potential role for circRNAs in regulating sperm chromatin structure and stability.

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Conflict of Interest

There was no conflict of interest.

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