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Original Article

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Differential Expression of miR-335-5p, miR-424-5p, miR-181a-5p, and hsa-miR-27a-3p and Their Association with Semen Quality Among Infertile Men

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Abstract

Background: Semen analysis alone may not accurately diagnose male infertility. This study investigated four semen miRNAs, miR-335-5p, miR-424-5p, miR-181a-5p, and hsa-miR-27a-3p-as potential non-invasive biomarkers and examined their associations with infertility and sperm quality.

Methods: Semen samples (n=54) were collected from couples who attended an IVF center for male-factor infertility between October 2022 and May 2025. Samples were classified into groups of normozoospermia (Control group; n=8), asthenozoospermia (n=12), oligozoospermia (n=11), teratozoospermia (n=12), and oligoasthenoteratozoospermia (OAT, n=11). Expression levels of selected miRNAs and the genes BCL2, BAX, Caspase-3, PRM1, and PRM2 were assessed using quantitative PCR (qRT-PCR). Sperm quality was evaluated through chromatin integrity, chromatin condensation, viability, acrosome status, and membrane integrity staining assays. One-way ANOVA with Tukey's post hoc test was performed using Prism 8, with $p< 0.05$ considered statistically significant.

Results: miR-424-5p, miR-335-5p, and miR-181a-5p expression levels were significantly reduced in infertility groups ($p<0.01$, $p=0.0003$, and $p=0.001$, respectively), whereas hsa-miR-27a-3p expression was increased ($p=0.0003$). All investigated miRNAs showed significant expression changes in the OAT group compared with controls ($p<0.05$). miR-181a-5p, miR-424-5p, and miR-335-5p were negatively correlated with BAX and Caspase-3 and positively correlated with BCL2, PRM1, and PRM2. The OAT group exhibited the poorest sperm chromatin quality and highest acrosomal damage ($p<0.001$).

Conclusion: These miRNAs are significantly associated with male infertility and sperm quality and may serve as potential non-invasive biomarkers for differential diagnosis of male infertility.

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Introduction

Male infertility is clinically defined as the inability to achieve pregnancy after one year of regular, unprotected sexual intercourse (1). It affects approximately one in six individuals during their reproductive lifespan (2), with male factors contributing to nearly 20–70% of cases. The etiology of male infertility is highly heterogeneous and includes hormonal imbalances, genetic and chromosomal abnormalities, anatomical and sexual dysfunctions, psychological conditions, and lifestyle-related factors. Despite extensive research efforts, the underlying cause remains un

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identified in nearly 70% of cases, which are therefore classified as idiopathic (1).

Given the substantial contribution of male factor and the multifactorial nature of infertility, accurate and reliable diagnostic strategies remain a major clinical challenge. Current diagnostic approaches rely mainly on physical examination, semen analysis, and, in selected cases, testicular biopsy. However, each of these methods has important limitations. Histological evaluation of testicular tissue lacks adequate sensitivity, specificity, and diagnostic accuracy for the classification of spermatogenic disorders (3, 4). Similarly, conventional semen analysis alone is insufficient for definitive diagnosis, as abnormal semen parameters do not necessarily exclude the possibility of natural fertility (5). Moreover, routine semen assessment fails to fully capture sperm functional competence and does not reliably distinguish fertile from infertile men (5, 6). Testicular biopsy, although informative, is an invasive, costly procedure that may damage active spermatogenic foci (1, 7). Therefore, there is a pressing need to identify reliable, non-invasive biomarkers that can improve the diagnosis and classification of male infertility.

In this context, microRNAs (miRNAs) have emerged as promising diagnostic and prognostic biomarkers (3). miRNAs are endogenous, small non-coding RNAs that regulate gene expression at the post-transcriptional level and play essential roles in numerous biological processes, including spermatogenesis. Their remarkable stability in biological fluids such as serum, plasma, and semen, along with their accessibility for quantitative analysis using techniques such as qRT-PCR and microarray platforms, makes them attractive candidates for clinical applications. Importantly, miRNAs are involved in key cellular processes such as proliferation, differentiation, and apoptosis (8). Accumulating evidence indicates that miRNAs play crucial roles in testicular development, spermatogenesis, sperm maturation, motility, metabolism, and early embryonic development. Consequently, dysregulation of specific miRNAs has been associated with impaired spermatogenesis and male infertility (9). In a landmark study, Lian et al. compared miRNA expression profiles in testicular tissue from patients with non-obstructive azoospermia and healthy controls, identifying 173 differentially expressed miRNAs. Among them, 19 miRNAs including miR-302a and miR-491-3P were upregulated, whereas 154 miRNAs such as miR-449a and miR-181a were downregulated, suggesting a potential role of miRNA dysregulation in spermatogenic arrest (10).

Further studies have highlighted specific miRNA–gene regulatory networks involved in sperm dysfunction. In asthenoteratozoospermic patients, increased expression of miR-27a alongside decreased expression of CRISP2 has been reported. Functional analyses demonstrated that miR-27a directly binds to the 3′UTR of CRISP2 mRNA, thereby suppressing its expression and contributing to impaired sperm motility and abnormal morphology (11). Similarly, miR-335-5p targets genes involved in apoptosis and cell survival pathways, including BIRC5, MAPK1, MERTK, PTPRN2, RASA1, and SOX4. Dysregulation of this miRNA may therefore influence sperm concentration and overall spermatogenic efficiency (12).

Alterations in miR-424 expression have also been implicated in male infertility, where its dysregulation may induce double-strand DNA breaks during spermatogenesis, ultimately compromising sperm integrity (13). In addition, miR-181a has been positively associated with sperm concentration, motility, normal morphology, and anti-apoptotic markers such as BCL2, while showing negative correlations with pro-apoptotic BAX and oxidative stress markers such as MDA. In infertile men with oligoasthenozoospermia and varicocele, reduced miR-181a expression has been linked to decreased BCL2 levels and increased BAX and MDA concentrations in seminal plasma (14). Furthermore, miR-181a has been shown to regulate TIMP3 expression, a gene involved in testicular development and differentiation (10).

Despite growing evidence supporting the involvement of miRNAs in male reproductive dysfunction, the high heterogeneity of findings has limited their translation into precise clinical biomarkers for assessing spermatogenesis, fertility potential, and sperm quality. Therefore, the purpose of the present study was to evaluate the expression profiles of miR-335-5p, miR-424-5p, miR-181a-5p, and hsa-miR-27a-3p in semen samples from infertile men and healthy controls. Furthermore, their associations with different types of male infertility, sperm quality parameters, apoptotic pathways, and protamine-encoding genes were investigated. Ultimately, the association of these miRNAs with different patterns of male infertility was investigated, and their potential as

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molecular markers of sperm quality, functional integrity, and chromatin status was explored.

Methods

Sample collection: Semen samples were obtained from 54 men from couples who had presented to IVF center of Alzahra (Rasht, Iran) for infertility treatment between October 2022 and May 2025. The center was selected based on its established collaboration with the research team, the availability of an appropriate study population, and access to the clinical and laboratory facilities required for semen collection and processing. Participants were consecutively recruited during the study period according to the predefined inclusion and exclusion criteria. Although all participants were partners of couples seeking treatment for infertility, male factor infertility was not necessarily present in all cases. Based on semen analysis and the predefined criteria, 46 men were classified into the sperm abnormality groups, whereas 8 men whose semen parameters did not meet the criteria for any of the predefined sperm abnormalities were classified as the normozoospermic control group. Importantly, normal semen parameters were interpreted as the absence of an identifiable male-factor abnormality based on the semen analysis and were not considered definitive evidence that infertility was attributable to the female partner. In these couples, no male-factor abnormality was identified based on routine semen analysis, although other causes, including female factor, could not be excluded. The required sample size was determined a priori using G*Power software. Based on a one-way analysis of variance (ANOVA) design with five study groups, an effect size of 0.50 (Cohen's f), a significance level of 0.05, and a statistical power of 80%, the minimum required sample size was estimated to be approximately 53 participants. To account for potential sample loss or exclusion during processing, 54 participants were ultimately included in the study.

The collected samples were categorized into different sperm quality groups, including normozoospermia (control group; n=8), oligozoospermia (n=11), asthenozoospermia (n=12), teratozoospermia (n=12), and oligoasthenoteratozoospermia (n=11). All samples were washed and processed prior to sperm quality evaluation. The mean age of the participants in the sperm abnormality groups was 35.51 years, while that of the normozoospermic control group was 34.25 years. The study protocol was approved by the Ethics Committee of the University of Guilan (IR.GUILAN.REC.1400.008), and all participants provided written informed consent after receiving a full explanation of the study objectives. Each semen sample was divided into two aliquots: one was analyzed for sperm parameters, and the other was used for miRNA level assessment.

The normozoospermic control group consisted of men with normal semen characteristics according to the WHO reference criteria. These participants were partners of couples undergoing infertility treatment, but no apparent male-factor abnormality was identified based on semen analysis. Therefore, this group should not be interpreted as definitively fertile or as demonstrating that the female partner was the sole cause of infertility. The sperm abnormality groups comprised men diagnosed with asthenozoospermia (total sperm motility <40% and/or progressive motility <32%), oligozoospermia (sperm concentration $<15\times10^6$/*ml*), teratozoospermia (normal sperm morphology <4%), or oligoasthenoteratozoospermia (abnormal sperm concentration, motility, and morphology).

Quantitative PCR (qPCR): This procedure consisted of isolating total RNA from semen specimens, followed by cDNA synthesis and qPCR using SYBR Green as the fluorescent dye to assess the expression levels of several target miRNAs, including hsa-miR-335-5p, hsa-miR-424-5p, hsa-miR-181a-5p, and hsa-miR-27a-3p, as well as the genes BCL2, Caspase-3, BAX, PRM1, and PRM2. Total RNA was extracted from semen specimens using the TRIzol reagent-based phenol-chloroform extraction method. Following RNA extraction, the RNA preparations were treated with DNase I to remove residual genomic DNA contamination prior to reverse transcription and cDNA synthesis. The quantity and purity of the extracted RNA were assessed using a NanoDrop spectrophotometer, while RNA quality was further evaluated by 2% agarose gel electrophoresis. Only RNA samples with acceptable concentration and purity and without apparent degradation were used for subsequent analyses. The DNase-treated RNA was subsequently used for cDNA synthesis and qPCR analysis. Amplification was carried out using LightCycler instrument (Roche, Germany). GAPDH and U6-1 were employed as internal controls for data normalization.

For miRNA quantification, a stem-loop reverse-transcription PCR approach was employed. Total RNA extracted from semen samples was treated with DNase I prior to reverse transcription to min-

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imize genomic DNA contamination. miRNA-specific stem-loop reverse-transcription primers were used to specifically recognize the mature forms of hsa-miR-335-5p, hsa-miR-424-5p, hsa-miR-181a-5p, and hsa-miR-27a-3p. The primers were designed to specifically target mature miRNA sequences rather than precursor or primary miRNA transcripts. No-reverse-transcription (no-RT) controls were included to assess potential amplification arising from residual genomic DNA. For these controls, the reverse transcriptase enzyme was omitted during the reverse-transcription step, while all other reaction components and subsequent qPCR conditions were kept identical to those of the corresponding RT-positive samples. The no-RT controls were subsequently subjected to the same qPCR analysis to verify the absence of amplification attributable to genomic DNA contamination.

The thermal protocol included an initial incubation step at 25°*C* for 5 *min*, followed by 42°*C* for 60 *min* and 70°*C* for 5 *min*. This was succeeded by an initial denaturation phase at 95°*C* for 10 *min*. The qPCR cycling conditions comprised 40 cycles of denaturation at 95°*C* for 15 *s*, annealing at 60°*C* for 30 *s*, and extension at 72°*C* for 30 *s*. To verify amplification specificity, a melting curve analysis was performed ranging from 60°*C* to 95°*C* with incremental increases of 0.5°*C*. Gene expression data were quantified using the ΔΔCt method with the aforementioned housekeeping genes as references. Primer sequences are provided in table 1.

A subset of the collected samples was used for RNA isolation. Following RNA extraction and DNase I treatment, the expression levels of four miRNAs (miR-424-5p, miR-181a-5p, miR-27a-3p, and miR-335-5p) along with several genes (BCL2, Caspase-3, BAX, PRM1, and PRM2) were quantified using qPCR. Relative expression levels were calculated using the comparative threshold cycle (2^-ΔΔCt) method. All qPCR reactions were performed in triplicate.

Analysis of sperm parameters: A portion of the semen specimens was allocated for evaluating sperm quality. This included multiple assays such as assessment of chromatin integrity using Toluidine Blue staining, chromatin condensation using Aniline Blue staining, sperm viability and acrosome reaction through Triple staining, and evaluation of sperm membrane integrity using Eosin–Nigrosin staining.

Aniline Blue (AB) staining: Aniline Blue staining was applied to assess chromatin condensation in sperm cells. Briefly, air-dried semen smears were fixed with 4% formalin (Junsei Chemical Co, Japan). After rinsing with water, the slides were stained with 5% Aniline Blue (Sigma-Aldrich, USA) prepared in 4% acetic acid solution at pH 3.5. Both fixation and staining steps were carried

Table 1. Designed primers for RT-qPCR

Gene name	Primer sequence 5' → 3'	GC%	Tm (°C)
hsa_GAPDH	F CATCACCATCTTCCAGGAGCG	55	58.5
	R GGAGGCATTGCTGATGATCTTG	50	59
U6-1	5´-CTCGCTTCGGCAGCAGCACA -3´	50	57.5
	5´-AACGCTTCACGAATTTGCGT -3´	55	52.2
PRM1	F ACTAGATGCACAGAATAGCAA	45	57.5
	R GTGGCATTGTTCCTTAGCAG	50	57
PRM2	F CAGCCTCAATCCAGAACCTCC	55	60.5
	R CTCGCGTTCATGGTCTTGTCC	55	60
BAX	F TTTTCCGAGTGGCAGCTGAC	55	60.5
	R TTGGTGCACAGGGCCTTGAG	60	61.9
BCL2	F CTTCGCCGAGATGTCCAGC	63	60
	R CAAAGAAGGCCACAATCCTCC	52	59.5
Caspase-3	F TGGAATTGATGCGTGATGTTTC	45	57.9
	R CTTCTACAACGATCCCCTCTG	52	57.3
hsa-miR-27a-3p	F TTCACAGTGGCTAAGTTCCG	50	57.5
	R GCGTCGACTAGTACAACTCAAG	50	58.5
hsa-miR-181a-5p	F AACATTAAACGCTGTCGGTGAGT	45	58.9
	R GCGTCGACTAGTACAACTCAAG	50	58.8
hsa-miR-335-5p	F TCAAGAGCAATAACGAAAAATGT	40	55.5
	R GCGTCGACTAGTACAACTCAAG	50	56
hsa-miR-424-5p	F CAGCAGCAATTAATGTTTTGAAA	40	57.5
	R GCGTCGACTAGTACAACTCAAG	50	58

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out for 5 *min* at room temperature. Following staining, the slides were washed, air-dried, and examined under a light microscope.

Toluidine Blue (TB) staining: To evaluate chromatin integrity, thin smears were prepared on microscope slides and air-dried. The samples were fixed in a 1:1 mixture of 96% ethanol and acetone at 4°*C* for 1 *hr*. Subsequently, slides were treated with 0.1 N HCl at 4°*C* for 5 *min* and washed three times with distilled water. Staining was then performed using 0.05% Toluidine Blue prepared in McIlvaine's citrate-phosphate buffer (pH=3.5; Merck, Germany) for 5 *min* at room temperature. In this method, sperm with intact chromatin appeared light blue, whereas those with damaged or fragmented chromatin showed a dark purple color.

Triple staining: Triple staining was employed to evaluate sperm viability and acrosome reaction. Briefly, equal volumes of sperm suspension and 2% Trypan Blue were mixed and incubated at 37°*C* for 5 *min*. Following incubation, samples were centrifuged at 600 *g* for 5 *min* at room temperature, and the pellet was washed repeatedly with PBS (without human serum albumin) until the supernatant was clear. The pellet was then fixed in 3% glutaraldehyde in PBS (pH=7.4) for 30–60 *min*, after which the suspension was centrifuged, resuspended in distilled water, and centrifuged twice more. The final pellet was resuspended in 50 *µl* of distilled water, and smears were prepared and left to dry overnight at room temperature. The dried smears were stained with 0.8% Bismarck Brown (acidified to pH=1.8) at 40°*C* for 5 *min*, rinsed, and then counterstained with 0.8% Rose Bengal in PBS (pH=3.5) for 20–45 *min*. After a brief wash, slides were dehydrated through a graded alcohol series (25%, 50%, 75%, and 100%), cleared in xylene, and examined under a light microscope at 40× magnification.

Eosin–Nigrosin (E&N) staining: Sperm membrane integrity was assessed using Eosin–Nigrosin staining. In this procedure, 50 *µl* of semen was mixed with an equal volume of stain. After a 30-*s* incubation, 50 *µl* of the mixture was used to prepare a smear. Once air-dried, the slides were analyzed under a light microscope at 40× magnification to determine sperm viability. In Eosin–Nigrosin staining, Eosin penetrates non-viable spermatozoa, producing red-stained heads, while viable sperm exclude the dye and appear white or light pink. Nigrosin provides a dark background, enhancing contrast and visualization of sperm morphology.

Statistical analysis: Statistical analyses were performed using Prism 8 (GraphPad, USA). One-way ANOVA was applied for group comparisons, followed by Tukey's post hoc test for pairwise analysis. All experiments were repeated in triplicate to ensure reliability, and statistical significance was defined as a p-value of less than 0.05.

Results

Sperm parameters: On average, four stained slides were prepared for each semen sample, and approximately 200 spermatozoa were evaluated per slide (Figure 1); results were expressed as

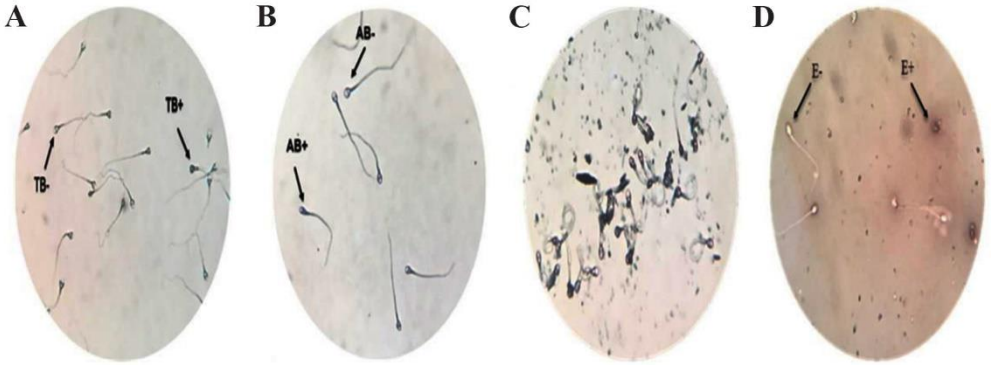


Figure 1. Quality sperm parameters. A) Toluidine Blue (TB) staining was used to evaluate sperm chromatin integrity (×40). Sperm with normal chromatin show a light blue color (TB^-), whereas sperm with abnormal chromatin exhibit a dark blue head (TB^+). B) Aniline Blue (AB) staining was applied to assess chromatin condensation (×40). Sperm with properly condensed chromatin appear light blue (AB^-), while those with incomplete chromatin condensation stain dark blue (AB^+). C) Triple staining (×40) differentiates sperm viability and acrosomal status. Viable sperm exhibit brown nuclei, whereas non-viable sperm show dark blue nuclei. Sperm with intact acrosomes appear pink, while sperm lacking acrosomes are colorless. D) Eosin-Nigrosin (E&N) staining (×40) was used to assess sperm viability. Viable sperm exclude eosin and present white or light pink heads (E^-), whereas non-viable sperm take up eosin and appear red-headed (E^+)

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Table 2. Comparison of sperm quality parameters in normozoospermic and infertile sperm samples

Parameters	Normozoospermia (n=8)	Oligoasthenoterato-zoospermia (n=11)	Teratozoospermia (n=12)	Oligozoospermia (n=11)	Asthenozoospermia (n=12)
Normal chromatin condensation (%)	80.18±6.9	37.97±1.9 [c]	72.65±5.7	71.14±5.3	70.07±5.1
Chromatin integrity (%)	84.86±6.2	45.64±2.1 [b]	61.09±3.7 [a]	88.64±6.5	85.39±6.1
Membrane integrity (%)	91.16±7.1	77.51±5.3 [a]	59.92±2.8 [b]	74.35±5.8 [a]	84.67±6.4
Sperm viability (%)	74.74±5.1	66.17±4.9	63.27±3.9	73.68±4.6	61.23±3.9 [a]
Acrosome integrity (%)	27.71±1.7	10.4±0.7 [b]	20.21±1.01 [a]	23.85±1.4	25±1.5

There are significant differences in sperm parameters in the infertile groups compared to normozoospermia group. p-values: a<0.05, b<0.01, c<0.001, and d< 0.0001

percentages (Table 2). Regarding chromatin condensation, the lowest level was observed in the OAT group (37.97%), which also demonstrated the highest level of chromatin damage. In the present study, no statistically significant correlation was observed between chromatin integrity and sperm motility (asthenozoospermia). However, the OAT group exhibited the highest degree of chromatin damage and the lowest level of chromatin condensation compared to normozoospermia (control) group (p<0.0001). Furthermore, the premature acrosome reaction rate in the OAT group was 17.31% higher than that observed in the control group (p<0.001).

With respect to membrane integrity, a key indicator of sperm viability, the highest level of membrane damage was observed in the teratozoospermia group (p<0.001) in comparison to control group. The highest level of acrosomal damage was also detected in cases with oligoasthenoteratozoospermia (p<0.001) and teratozoospermia (p< 0.05). In addition, asthenozoospermic patients exhibited the highest proportion of dead sperm in Triple staining (p<0.05).

RT-qPCR results: According to table 3, it was determined that overall, the expression of miR-424-5p in the groups of oligoasthenoteratozoospermia (p<0.0001), asthenozoospermia (p<0.001), and oligozoospermia (p<0.01) showed a significant decrease compared to the normal group (normozoospermia). Therefore, it can be concluded that the decrease in this miRNA is likely associated with infertility. It was also found that the difference in miR-424-5p expression between the normal group and patients with oligoasthenoteratozoospermia was significant. Thus, this gene has a positive correlation with sperm concentration. However, in teratozoospermic patients, no signifi-

Table 3. Comparison of gene expression in normozoospermic and infertile sperm samples

Genes	Normozoospermia (n=8)	Oligoasthenoterato-zoospermia (n=11)	Teratozoospermia (n=12)	Oligozoospermia (n=11)	Asthenozoospermia (n=12)
hsa-miR-424-5p	1±0.05	0.45±0.01 [d]	0.79±0.03	0.69±0.05 [a]	0.54±0.1 [c]
hsa-miR-335-5p	1.00±0.08	0.39±0.02 [c]	0.63±0.04 [b]	0.82±0.12	0.68±0.0 [a]
hsa-miR-27a-3p	1.00±0.06	2.03±0.12 [c]	1.64±0.08 [b]	1.40±0.06	1.70±0.06 [b]
hsa-miR-181a-5p	1.02±0.11	0.57±0.02 [b]	0.66±0.03 [b]	0.65±0.02 [a]	0.58±0.01 [b]
BAX	0.98±0.07	1.59±0.03 [c]	1.04±0.04	1.23±0.0 [b]	1.09±0.01
Caspase-3	1.00±0.07	1.70±0.09 [b]	1.4±0.02 [c]	1.62±0.01 [b]	1.22±0.04 [b]
BCL2	0.99±0.02	0.62±0.03 [b]	0.60±0.03 [b]	0.75±0.03 [a]	0.93±0.01
PRM1	1.01±0.05	0.44±0.01 [d]	0.66±0.03 [b]	0.67±0.02 [a]	0.79±0.04
PRM2	1.00±0.06	0.65±0.02 [b]	0.69±0.03 [a]	0.6±0.02 [b]	0.78±0.04

There is significant difference in the gene expression levels in the infertile groups compared to normozoospermic (control) group. P values: a<0.05, b<0.01, c<0.001, and d<0.0001

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cant difference was observed.

It was determined that the expression level of miR-335-5p in all infertile groups, especially in oligoasthenoteratozoospermia (0.39±0.02), showed a significant decrease (p=0.0003). Additionally, in comparisons between the control group and idiopathic groups (expect oligozoospemia), a notable difference in the expression level of this miRNA was observed, particularly in individuals with oligoasthenoteratozoospermia, where the p-value compared to the control group was 0.0001.

The expression of miR-27a-3p, unlike miR-335-5p and miR-424-5p, showed a significant increase in all groups of infertile men (expect oligozoospermia) compared to the control group (p= 0.0003), with the highest increase observable in patients with oligoasthenoteratozoospermia (2.03± 0.12). miR-181a-5p, unlike miR-27a-3p, showed decreased expression in infertile individuals compared to the control group, and this decrease was significant based on p=0.0014.

Additionally, BAX and Caspase-3 showed increased expression in all studied infertile individuals compared to controls, especially in oligoasthenoteratozoospermic group (p<0.0001). On the other hand, miR-181a-5p had the lowest expression in oligoasthenoteratozoospermic group. Therefore, it can be concluded that this biomarker has a negative correlation with these pro-apoptotic factors in semen, meaning that with decreased expression of this miRNA, the expression of BAX increases. On the other hand, BCL2, as an anti-apoptotic factor, showed decreased expression in infertile groups, with the greatest decrease seen in oligoasthenoteratozoospermic group (p<0.001). Given that the biomarker miR-181a-5p also showed decreased expression in oligoasthenoteratozoospermic group, it can be concluded that this biomarker has a positive correlation with BCL2. In addition, the biomarkers miR-335-5p and miR-424-5p also showed a positive correlation with BCL2 in patients with oligoasthenoteratozoospermia.

The expression levels of PRM1 and PRM2, which play essential roles in sperm DNA protamination, were significantly reduced in groups with oligoasthenoteratozoospermia, oligozoospermia, and teratozoospermia (p<0.01). However, this reduction was not statistically significant in patients with asthenozoospermia. These findings indicate that both genes are positively associated with normal sperm concentration and morphology. On the other hand, it was previously demonstrated that miR-27a-3p is negatively correlated with normal sperm morphology. Therefore, this biomarker may contribute to abnormal sperm morphology through disruption of the protamination process. In other words, increased expression of miR-27a-3p in teratozoospermic individuals is associated with reduced PRM1 and PRM2 expression, which ultimately leads to abnormal sperm morphology. It was also shown that miR-424-5p, miR-335-5p, and miR-181a-5p were significantly downregulated in patients with oligoasthenoteratozoospermia and exhibited a positive correlation with sperm concentration. These results suggest that, in addition to potentially reducing sperm count through alterations in apoptotic factors, these biomarkers may also impair sperm integrity and decrease sperm concentration by disrupting the protamination process.

Gene–gene interaction network: The constructed miRNA–gene interaction network demonstrated that the evaluated miRNAs (highlighted in yellow) were closely associated with key genes involved in apoptosis and sperm chromatin remodeling, including BAX, BCL2, CASP3, PRM1, and PRM2 (Figure 2). Among the studied miRNAs, miR-27a-3p showed interactions with both BAX and BCL2, suggesting its possible role in regulating apoptotic pathways involved in male infertility. In addition, miR-181a-5p and miR-424-5p were also connected to BAX and BCL2, indicating their potential contribution to the balance between pro-apoptotic and anti-apoptotic signaling **.** Furthermore, miR-335-5p was linked to Caspase-3 and PRM2, implying that this miRNA may participate not only in apoptosis regulation but also in sperm DNA protamination and chromatin condensation. The interaction of the evaluated miRNAs with PRM1 and PRM2 highlights their possible involvement in sperm maturation and morphology through modulation of protamine-associated pathways. Overall, the network analysis suggests that the studied miRNAs may contribute to male infertility through coordinated regulation of apoptosis-related genes and genes involved in sperm chromatin integrity and protamination.

Discussion

In recent years, increasing attention has been directed toward the role of microRNAs (miRNAs) in the regulation of biological pathways involved in spermatogenesis, sperm function, and male in-

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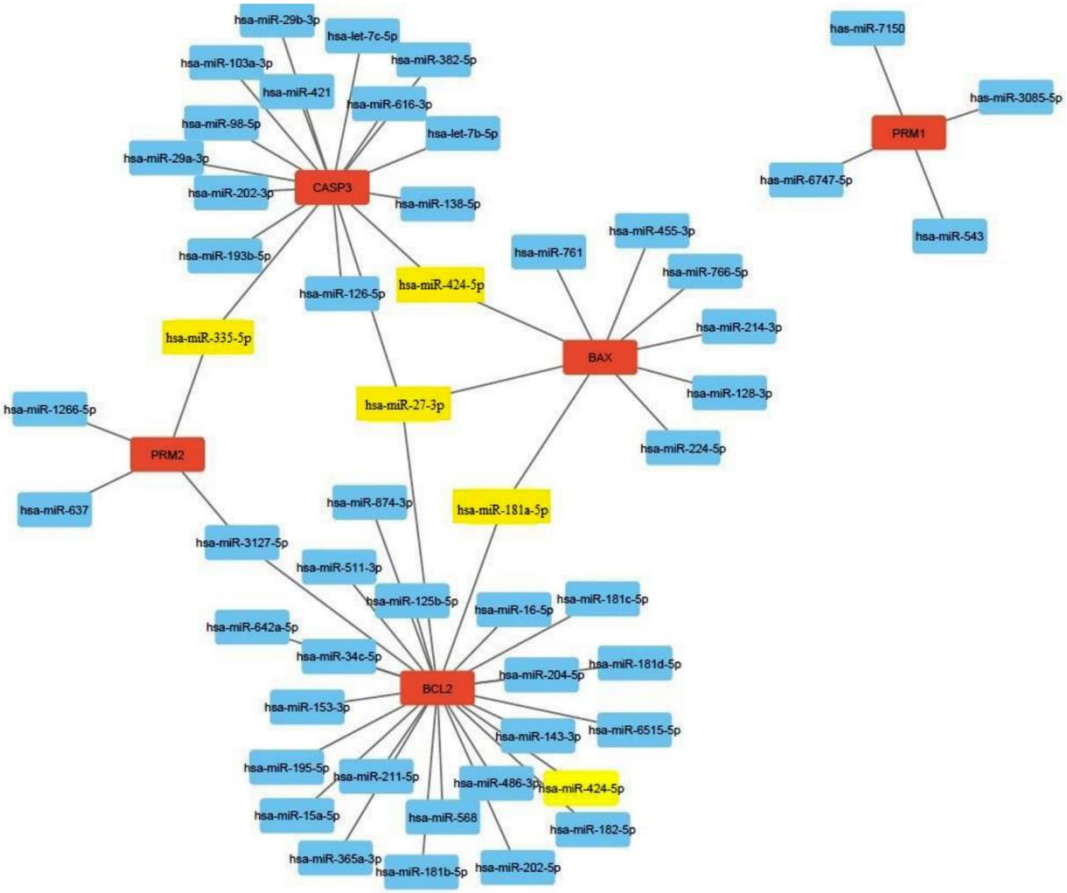


Figure 2. miRNA-gene interaction network illustrating the relationships between the evaluated miRNAs (highlighted in yellow) and target genes involved in apoptosis and sperm chromatin remodeling. The analyzed miRNAs, including miR-27a-3p, miR-181a-5p, miR-335-5p, and miR-424-5p were associated with key infertility-related genes such as *BAX*, *BCL2*, *Caspase-3*, and *PRM2*. Red nodes represent target genes, blue nodes indicate interacting miRNAs, and yellow nodes represent the miRNAs evaluated in the present study

fertility. These small non-coding RNAs regulate gene expression at the post-transcriptional level and are capable of influencing critical processes such as germ cell differentiation, sperm motility, chromatin remodeling, and sperm morphology. In the present study, the expression profiles of four candidate miRNAs, including miR-181a-5p, miR-335-5p, miR-424-5p, and miR-27a-3p, were evaluated using qRT-PCR in different categories of infertile men to investigate their associations with apoptosis, protamination, chromatin status, and sperm quality parameters. Our findings demonstrated that dysregulation of these miRNAs is closely associated with impaired sperm quality and different forms of male infertility, including non-obstructive azoospermia (NOA), asthenozoospermia, teratozoospermia, and OAT. These observations support the growing evidence that miRNAs may serve not only as biomarkers for male infertility but also as potential therapeutic targets.

Among the investigated miRNAs, miR-181a-5p showed a remarkable association with sperm quality and apoptotic pathways. A recent systematic review by Jain et al. identified miR-181a as one of the major regulatory miRNAs involved in spermatogenesis and male infertility through modulation of genes associated with germ cell development (15). Earlier, Lian et al. compared miRNA expression profiles in testicular tissues of NOA patients and fertile controls and reported reduced expression of 154 miRNAs, including miR-181a, in infertile individuals (10). Similarly, Wang et al., following analysis of semen samples from infertile and fertile men, demonstrated decreased miR-181a expression in azoospermic and oligo-

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zoospermic patients, whereas increased expression was observed in cases with asthenozoospermia (16). Mostafa et al. further showed that reduced miR-181a expression in OAT patients was positively correlated with sperm concentration, motility, normal morphology, and BCL2 expression, while negatively correlated with BAX expression (14).

Consistent with these reports, the present study demonstrated significant downregulation of miR-181a-5p in infertile groups, particularly in OAT and azoospermic patients. Moreover, miR-181a-5p exhibited positive correlations with PRM1 and PRM2 expression levels, suggesting a possible regulatory role in sperm protamination and chromatin packaging. Reduced expression of this miRNA may therefore impair chromatin condensation and increase susceptibility to DNA fragmentation. In addition, its inverse relationship with pro-apoptotic markers further supports the hypothesis that miR-181a-5p contributes to maintaining sperm survival and genomic stability. Shi et al. also confirmed significantly reduced expression of miR-181a in azoospermia and elevated levels in asthenozoospermia, highlighting its importance in spermatogenic dysfunction (17).

Another important finding of the current study concerns miR-27a-3p, which demonstrated significantly increased expression in patients with asthenozoospermia and teratozoospermia. Liu et al. initially reported reduced miR-27a expression in abnormal semen samples compared with fertile controls (18). However, subsequent studies by Abu-Halima et al. and Salas-Huetos et al. demonstrated elevated expression of miR-27a in patients with asthenozoospermia (12, 19), findings that are in agreement with our results. Zhou et al. further demonstrated that miR-27a expression is negatively correlated with progressive motility and normal morphology in cases with asthenoteratozoospermia, while showing no significant association with sperm concentration (11). Likewise, Norioun et al. reported overexpression of hsa-miR-27a-3p in NOA patients and suggested that this miRNA may impair spermatogenesis through downregulation of genes involved in germ cell differentiation (20).

Our findings support these observations and indicate that miR-27a-3p is more strongly associated with sperm motility and morphology rather than sperm count. Interestingly, although no significant relationship was observed between miR-27a-3p and apoptotic markers, a negative correlation with PRM1/2 expression was detected in patients with teratozoospermia. This finding suggests that increased miR-27a-3p expression may contribute to defective protamination and abnormal sperm morphology through disruption of chromatin remodeling mechanisms. Therefore, overexpression of miR-27a-3p may represent a compensatory response to impaired spermatogenesis or, alternatively, a pathogenic factor contributing to sperm dysfunction.

Regarding miR-335-5p, previous studies have highlighted its potential role in regulating sperm quality and cell survival. Joshi et al., in an analysis of infertile men with different semen abnormalities, identified hsa-miR-335-5p as one of the consistently dysregulated miRNAs associated with infertility (21). Similarly, Samadi et al. recognized miR-335-5p as a critical regulatory miRNA involved in male infertility-related genetic pathways (22). Salas-Huetos et al. reported reduced expression of hsa-miR-335-5p in cases with oligozoospermia and suggested that its target genes are involved in cellular proliferation, survival, and apoptosis (12). In contrast to the findings of Salas-Huetos et al., our results demonstrated no significant reduction of miR-335-5p expression in patients with oligozoospermia. Instead, significant downregulation was observed in groups with OAT, teratozoospermia, azoospermia, and asthenozoospermia. Furthermore, miR-335-5p showed expression patterns similar to miR-424-5p in relation to apoptotic factors and protamine genes. These findings suggest that miR-335-5p may exert protective effects on sperm chromatin integrity and cell survival, while its decreased expression could facilitate DNA damage and apoptosis.

The present study also demonstrated a significant decrease in miR-424-5p expression, particularly in azoospermic patients. Wang et al. proposed that reduced miR-424 expression in infertile men may promote spermatogenic cell apoptosis and sperm DNA damage (23). Zhao et al. reported lower hsa-miR-424 expression in infertile men with high DNA fragmentation index (DFI), suggesting its involvement in maintaining genomic integrity during spermatogenesis (13). Barceló et al. also found decreased expression of hsa-miR-424-5p in azoospermic individuals compared with normozoospermic controls (24).

Our findings are highly consistent with these studies, as miR-424-5p expression was markedly reduced in azoospermia patients. Moreover, this

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miRNA exhibited negative correlations with pro-apoptotic genes BAX and Caspase-3 and a positive correlation with the anti-apoptotic gene BCL2. These observations indicate that reduced miR-424-5p expression may facilitate activation of apoptotic pathways and subsequent sperm destruction. In addition, the positive association between miR-424-5p and PRM1/2 suggests that this miRNA may contribute to chromatin stabilization and proper protamine replacement during spermiogenesis.

Beyond miRNA dysregulation, increasing evidence supports the importance of chromatin integrity and DNA fragmentation in determining male fertility potential. Liu et al. reported significantly elevated DFI values in patients with asthenozoospermia and oligozoospermia compared with fertile controls and demonstrated negative correlations between DFI and sperm concentration, motility, viability, and morphology (25). Similarly, Stavros et al. and Yang et al. emphasized the strong association between sperm DNA fragmentation and impaired sperm motility and morphology (26, 27). Alahmar additionally demonstrated that antioxidant therapy with selenium can improve sperm parameters and reduce DNA fragmentation in infertile men (28).

Although no significant correlation between chromatin integrity and sperm motility was identified in the current study, the OAT group exhibited the highest degree of chromatin damage and the lowest chromatin condensation, approximately two- to threefold greater than the group with normozoospermia. These findings suggest that severe combined semen abnormalities are more strongly associated with chromatin instability than isolated motility abnormalities. Importantly, all investigated miRNAs showed significant associations with chromatin condensation and integrity, suggesting that these regulatory molecules may participate in maintaining sperm genomic stability.

The role of acrosome reaction and apoptosis in male infertility has also been increasingly emphasized. Orosz demonstrated that absence of the TPPP2 protein disrupts spermatogenesis and reduces acrosome reaction in OAT animal models (29). Rezaie et al. further showed that pentoxifylline and coenzyme Q10 improve sperm motility and acrosome reaction in OAT patients (30). Interestingly, in the present study, the acrosome reaction percentage in the OAT group was approximately 10–17% higher than in controls. This apparently contradictory finding may reflect compensatory premature acrosome activation induced by cellular stress and membrane instability rather than improved sperm function. Since excessive or premature acrosome reaction can impair the fertilization process, increased acrosome-reacted sperm in OAT patients may still contribute to infertility despite elevated percentages.

Moreover, previous studies have highlighted the contribution of apoptosis-related genes to sperm dysfunction. Babaei et al. demonstrated that the BAX/BCL2 ratio is associated with embryo quality and fertility outcomes in OAT patients (31). Similarly, Liu et al. reported that Cep78 knockout induces infertility and OAT through increased apoptosis and structural sperm abnormalities (32), while Olszewska et al. detected elevated Caspase-3 signaling in spermatogenic cells of OAT patients (33). These observations strongly support our findings that increased apoptosis and chromatin damage are major contributors to impaired sperm quality in severe male infertility. Collectively, the present findings suggest that dysregulation of miR-181a-5p, miR-335-5p, miR-424-5p, and miR-27a-3p may contribute to male infertility through multiple interconnected mechanisms, including induction of apoptosis, disruption of protamination, impairment of chromatin condensation, increased DNA fragmentation, and altered acrosome reaction. These miRNAs, therefore, represent promising molecular biomarkers for evaluating sperm quality and may provide novel therapeutic targets for the management of male infertility.

Conclusion

The present study demonstrated distinct alterations in sperm quality, miRNA expression, and the expression of apoptosis and protamination-related genes across different sperm abnormality groups. The OAT group showed the most pronounced abnormalities in sperm chromatin integrity, including reduced chromatin condensation and increased chromatin damage, together with increased premature acrosome reaction.

At the molecular level, miR-424-5p, miR-335-5p, and miR-181a-5p were significantly downregulated, whereas miR-27a-3p was upregulated in several infertile groups, with the most pronounced changes generally observed in the OAT group. These alterations were accompanied by increased expression of the pro-apoptotic genes BAX and Caspase-3 and decreased expression of the anti-apoptotic gene BCL2. In addition, PRM1 and

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PRM2 expression was significantly reduced in cases with OAT, oligozoospermia, and teratozoospermia, consistent with alterations in sperm protamination and chromatin organization.

Overall, the findings indicate that dysregulation of the studied miRNAs is associated with altered apoptosis-related and protamination-related gene expression as well as impaired sperm quality, particularly in cases with OAT. These miRNAs may therefore represent candidate molecular markers associated with sperm dysfunction. However, their diagnostic or mechanistic value requires confirmation in larger, independent cohorts and through functional studies.

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Conflict of Interest

The authors have no financial or non-financial conflicts of interest to declare.

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