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PRINT: ISSN 0972-3757 ONLINE: ISSN 2456-6330
Int J Hum Genet, 26(3): 169-180 (2026)
DOI: 10.31901/24566330.2026/26.03.945

DNA Methylation and miRNA Regulation in Spermatogenesis: Epigenetic Mechanisms and Analytical Approaches in Male Infertility

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KEYWORDS Biomarkers. DNA Methylation. Epigenetics. Male Reproductive Health. MicroRNA. Spermatogenesis

ABSTRACT Male infertility is a complex disorder caused by genetic, epigenetic, environmental, and lifestyle factors. Among epigenetic mechanisms, DNA methylation and microRNA (miRNA) biogenesis serve critical roles in regulating gene expression during spermatogenesis and preserving sperm function. Differential methylation patterns in the MTHFR, MEST, and RHOX genes are linked to idiopathic male infertility, influencing sperm quality, motility, and function. Similarly, dysregulation of specific miRNAs, including the miR-34 family, has been linked to impaired sperm parameters and defective spermatogenesis. This review investigates how male fertility is influenced by miRNAs and DNA methylation and discusses advanced analysis techniques, including methylation-specific PCR, MethyLight, Oxford Nanopore sequencing, RT-qPCR, and sequencing-based approaches. These epigenetic mechanisms could be used as valuable potential therapeutic targets and biomarkers in male infertility.

INTRODUCTION

Male Infertility: An Overview

The inability to conceive following a year of regular, unprotected sexual activity is known as infertility, and it is a major global health concern, affecting 15 percent of couples worldwide (WHO 2024). It can impact both men and women and has a varied number of causes. Infertility is thought to impact around 48.5 million couples trying to conceive worldwide. Twenty to thirty percent of infertility cases are determined to be exclusively male, while men account for fifty percent of cases overall (Agarwal et al. 2015; Mazzilli et al. 2023). Male infertility, defined as the incapability of a reproductively mature man to fecundate a clinically fertile woman, has multifactorial causes (Kumar and Singh 2015). It has been said that male infertility is a type of infertility that does not respond well to basic treatment. Only a small percentage of male factor infertility can be resolved using main treatment procedures, according to reports (Okonofua et al. 2022).

Male infertility has a variety of causes, encompassing anatomical and structural defects, like ejaculatory and erectile dysfunctions, obstructive problems, varicocele, semen morphology, motility and viscosity abnormalities, endocrinological disorders, genital tract infections, and testicular failures (Rambhatla et al. 2025). Additionally, the presence of anti-sperm antibodies can impair fertility potential (Kumar and Singh 2015). When considering the molecular genetic basis, chromosomal abnormalities, point mutations such as those in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene, mitochondrial genome mutations, and imprinting disorders are recognised as contributors of male infertility (Anjankar et al. 2025).

Lifestyle and occupational exposures also significantly influence gamete health. Opiate use, smoking, pesticide toxicity and radiation exposure have an important part in contributing to male infertility (Sharma et al. 2013). Recent research highlights the crucial function of epigenetic modifications in the pathogenesis of male infertility relating to the development of the male reproductive system, sperm production, and

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male sexual behaviour (Hallak et al. 2026). Ineffective epigenetic reprogramming in male germ cells may result in defective sperm production, representing a potential cause of infertility that is of significant scientific interest (Agarwal and Prabakaran 2005). Alteration of the epigenetic make up, such as DNA methylation abnormalities, are implicated in unexplained infertility cases (Kumar and Singh 2015). Various researches explore the diverse causes of male infertility, emphasising the cross-link between genetic mutations, epigenetic modifications, oxidative stress, and environmental influences. Infertility in men is thus a complex disorder of the reproductive system, where both genetic and epigenetic factors synergistically contribute to compromised fertility outcomes.

Numerous genetic factors responsible for male infertility have been discovered so far, however, a considerable number of cases are still considered to be of unknown origin. It is now believed that one of the reasons behind male infertility may be due to the involvement of some epigenetic regulatory processes such as DNA methylation and miRNA expression (Malini et al. 2026). The comprehension of these regulatory factors will thus help discover new biomarkers or provide personalised treatments. However, there is a lack of integration of these aspects into male infertility studies.

Objectives

The present review aimed to evaluate the role of differential DNA methylation patterns in spermatogenesis defects and in turn male infertility, understand the contribution of microRNA (miRNA)-driven gene expression in sperm development, summarise the current analytical approaches used in epigenetic studies, and assess the possibility of epigenetic markers as therapeutic and diagnostic targets.

Epigenetic Modifications

Epigenetics refers to alterations in the phenotype that does not involve alterations in the DNA sequence itself (Ashe et al. 2021). DNA wounds around histones to form nucleosome structures, which organise into more complex structures called chromatin (Raman 2022). Epigenetic processes are driven by covalent changes of the histone proteins and DNA components of chromatin. These include post-translational modifications of histones, DNA methylation, chromatin remodelling and non-coding RNA biogenesis (Bure et al. 2022). These changes maintain gene regulation by modifying the structure of chromatin and DNA accessibility. These modifications play a crucial role in the development and differentiation of all cells, particularly spermatozoa (Carrell and Hammoud 2009). Although various genetic causes for male infertility have been identified, increasing evidence points toward the significant contribution of epigenetic changes in male reproductive defects. This review focuses on how spermatogenesis is regulated through DNA methylation-mediated and microRNA-regulated gene expression.

DNA Methylation

The most prevalent form of DNA modification in somatic cells is DNA methylation (Shiota et al. 2002). The biological process of DNA methylation entails adding a methyl group to the cytosine pyrimidine ring at 5' position, usually in a CpG dinucleotide, which is found upstream of over 40 percent of the genes in mammals (Singal and Gindar 1999). CpG islands are regions of DNA that contain more CpG dinucleotides than the rest of the genome. They are linked to genes specific to particular tissues (Shiota et al. 2002). Cytosine residues in the C5 position mostly undergo methylation to produce 5-methyl cytosine. S-adenosyl methionine is the source of the methyl group in the process (Singal and Gindar 1999). By preventing transcription factors from binding or inducing the binding of several transcriptional repressors, hypermethylation of DNA can inhibit transcription. Different DNA methyltransferases (DNMTs), such as DNMT1, DNMT3a, and DNMT3b, regulate DNA methylation (Loaeza-Loaeza et al. 2020). The development and management of methylation marks are controlled by DNMT enzymes.

A crucial process that controls the expression of genes is the methylation/demethylation of CpG sites, which influences transcription factor binding and therefore inhibits the expression of developmental and imprinted genes (Fitzpatrick and Wilson 2003). Accurate methylation

of DNA plays crucial functions in many essential physiological processes such as chromatin stability and X- chromosomal inactivation (Li and Zhang 2014).

DNA Methylation Mediated Sperm Development

The complex process of spermatogenesis is how spermatogonial stem cells (SSCs) that are diploid mature into haploid spermatozoa and spermatocytes. Somatic cells in the testes, such as peritubular myoid cells, Leydig cells, and Sertoli cells, work together in this process (O'Donnell et al. 2022). It is a dynamic process controlled by both genetic and epigenetic factors (Kubota and Brinster 2018) DNA methylation is necessary for both embryonic development and fertility (Shacfe et al. 2023). During gametogenesis, DNA methylation patterns are initially learned. A greater number of data has demonstrated the significance of DNA methylation for the development of male germ cells in animal models (Kubo et al. 2015). Gene targeting enzymes that cause DNA methylation has been demonstrated to result in meiosis deficit, male infertility, and loss of germ cell methylation (Cui et al. 2016). DNA methylation thus regulates gene expression during spermatogenesis, and its disruption can adversely affect sperm function. Idiopathic infertile males with low semen quality have been found to exhibit an aberrant DNA methylation pattern in their spermatozoa (Chi et al. 2020). According to certain research, the sperm of fertile men has a single, stable CpG methylation pattern, whereas the sperm of sub fertile men exhibits a substantially different pattern (Vieweg et al. 2015). Sperm motility, chromatin, and DNA integrity are all correlated with DNA methylation level according to reports and it can potentially be the basis of male infertility due to the changing lifestyle patterns (Guns and Estevez 2021).

For example, it has been shown that low quality of sperm and an elevated chance of infertility are associated with promoter hypermethylation of genes, such as H19, MTHFR, PLAG1, SNRPN and IGF2 (Cannarella et al. 2021). Sperm counts, motility, and normal morphology can all be negatively impacted by hypermethylation of DNA, and these are particularly in the upstream promoter regions of the PAX8, PLAG1, MEST, NTF3, SFN, HRAS and DIRAS3 genes (Houshdaran et al. 2007). Amjadian et al. (2023) looked into the methylation patterns of MEST gene in male patients undergoing intracytoplasmic sperm injection assisted fertility treatment. They found a negative relationship between MEST methylation and sperm parameters indicating it to be strong predictors for assessing the male fertility potential.

Another important gene cluster regulating gene expression maintaining spermatogenesis is the Reproductive Homeobox gene (RHOX) clusters. These genes are necessary for normal germ cell survival and spermatogenesis and are only found in the reproductive system (Tan et al. 2021). In the human genome, three RHOX genes have been found, namely, RHOXF1, RHOXF2 and RHOXF2B (Le Beulze et al. 2023). They are selectively expressed in female and male reproductive tissues in a region-specific cell type manner. RHOXF1 is expressed in the brain, testis, and ovary throughout foetal development. Expression of RHOXF1 is ubiquitous in adults but is predominantly expressed in the testis (Richardson et al. 2014). But RHOXF2 is solely expressed in the testes in both adult and foetal stages. RHOX genes are a suitable biomarker for idiopathic male infertility cases. Hypermethylation of RHOX gene clusters is highly associated with abnormal sperm parameters (Le Beulze et al. 2023; Richardson et al. 2014). The principal genes reported to undergo abnormal DNA methylation in male infertility and their functional significance are summarised in Table 1.

Table 1 summarises genes reported to exhibit altered DNA methylation patterns in infertile males and their associated effects on sperm quality, fertility potential and spermatogenesis.

miRNA Biogenesis and Function

Small non-coding RNAs (sncRNAs) consist of piwi-interacting RNAs (piRNAs), tRNA, rRNA, small-interfering RNAs (siRNAs) and microRNAs (miRNAs) (Costa et al. 2014). They are roughly 20-30 nucleotides long. Epigenetic changes can affect non-coding RNAs like miRNAs, piRNAs and miRNAs, in particular, make up the majority of RNAs in a cell and control gene expression on several levels (Bure et al. 2022). At the post-transcriptional stage, miRNAs

Table 1: DNA methylation alterations in genes associated with male infertility

Gene	*Epigenetic alteration*	*Effect*
MTHFR	Hypermethylation	Reduced sperm quality
MEST	Hypermethylation	Reduced motility
H19	Aberrant methylation	Impaired fertility
IGF2	Aberrant methylation	Sperm defects
RHOXF1	Hypermethylation	Spermatogenesis defects
RHOXF2	Hypermethylation	Germ cell dysfunction
PLAG1	Hypermethylation	Low sperm count

also adversely control the pattern of expression of certain genes (Panni and Pizzolotto 2025). Through a variety of epigenetic processes, miRNAs play crucial post-transcriptional regulatory roles in gene expression. By binding to mRNA and either degrading or repressing translation, miRNAs downregulate the expression of genes (Wei et al. 2017).

Role of miRNAs in Spermatogenesis

According to recent studies, miRNAs have significant roles in controlling the development, differentiation, and progression of a number of diseases (Li et al. 2023; Kalayinia et al. 2021; Das and Rao 2022). At the post-transcriptional stage, miRNAs negatively control the expression pattern of a number of genes. Alterations in expression patterns of miRNAs in sperm cells can be linked to serious malformations in these cells and in the coming subsequent generations (Harchegani et al. 2018). Furthermore, the role of SnRNAs, especially miRNAs, is of increasing attention as critical post-transcriptional regulators involved in spermatogenesis and sperm function. Dysregulation of specific miRNAs is now put-forward as an important factor contributing to unexplained male infertility (Kumar and Singh 2015). Specific miRNAs found in sperm that seem to be crucial for fertility and embryogenesis has always been the core of different studies, some sperm miRNAs have been repeatedly identified and linked to different types of male infertility. The most prominent of these single miRNAs has been miR-34C (Patel et al. 2020). Evidence suggests that dysregulation of miR-34-c can potentially cause impaired spermatogenesis (Heidary et al. 2019). Chencheng Yao et al. (2017) examined specific microRNA expression profiles in round spermatids, pachytene spermatocytes and human spermatogonia from non-obstructive azoospermia (NOA) and obstructive azoospermia (OA) patients, revealing 396, 395, and 378 differentially expressed microRNAs, respectively. This highlights the potential of epigenetic regulators in normal and aberrant spermatogenesis, offering insights into azoospermia etiology (Yao et al. 2017).

When expression of miRNAs was analysed from testicular samples of azoospermic men with different histopathological patterns who underwent biopsies for diagnostic or therapeutic purposes, the microarray and qRT-PCR analyses revealed 46, 197, and 68 variably expressed miRNAs in germ cell arrest patterns, Sertoli cell-only and mixed atrophy, and germ cell arrest patterns, respectively, when compared to normal spermatogenesis. In silico analysis identified four key miRNAs (hsa-mir-34b, hsa-mir-34c-5p, hsa-mir-449a, hsa-mir-449b) involved in cell proliferation, differentiation, apoptosis and spermatogenesis (Abu-Halima et al. 2014).

When the etiology of hypospermatogenesis were explored using whole exome and miRNA sequencing, it identified novel gene variants (CDC27, TUBB8, MTOR, TLK1) and four previously unreported microRNAs, alongside established ones like the let-7 and miR-34 families, which are strongly linked to spermatogenesis-related pathogenesis. These findings confirm the association of these microRNAs with hypospermatogenesis and suggest their potential as biomarkers for differentiating obstructive from non-obstructive azoospermia (Sharma et al. 2022). Key miRNAs involved in spermatogenesis and their association with male infertility are summarised in Table 2.

MATERIAL AND METHODS

The researchers performed a comprehensive literature search on Google Scholar and PubMed database. All studies without limiting on the dates of publication were reviewed and signifi-

Table 2: Major miRNAs implicated in spermatogenesis and male infertility

miRNA	*Biological function*	*Associated infertility condition*
miR-34c	Germ cell differentiation and spermatid maturation	Azoospermia
miR-34a	Regulation of spermatogenesis	Impaired sperm production
miR-449a	Cell cycle regulation and germ cell development	Germ cell arrest
miR-449b	Regulation of spermatogenesis	Azoospermia
let-7 family	Germ cell maturation and differentiation	Hypospermatogenesis

cant studies were included. The following search terms alone and in combination were employed for an extensive search of the literature review, that is, 'epigenetics', 'epigenome', 'CpG islands', 'epigenetic modifications', 'methylation', 'DNA methylation', 'methylome', 'hypermethylation', 'hypomethylation', 'miRNA', 'miRNA expression', 'gene silencing', 'gene regulation', 'genomic imprinting', 'imprinted genes', 'sperm', 'sperm cells', 'spermatozoa', 'semen parameters', 'spermatogenesis', 'infertility', 'subfertility', 'primary infertility', and 'male infertility'. Studies were selected for review that was written in English, focused on DNA methylation and miRNA-based regulation of genes associated with male infertility.

Reversibility of DNA Methylation

DNA methylation was thought to be a permanent mark for over a long period of time. But discoveries over the years have shown that this process is actually reversible (Ramchandani et al. 1999). DNA demethylases are enzymes that can remove these methyl groups, allowing genes to be turned back on. These enzymes are selective, often targeting specific CpG sites, and can work on both fully and partially methylated DNA (Ramchandani et al. 1999). This discovery has major implications for reproductive health. DNA methylation plays a significant role in various reproductive processes, like the development of sperm and eggs, embryo growth, and placenta function (Bashiri et al. 2021). Thus, aberrant methylation patterns in different stages of spermatogenesis can lead to infertility.

The promising aspect of methylation being a reversible process is that it can play key functions in correcting epigenetic abnormalities linked with reproductive disorders. Treatments that target the enzymes responsible for adding or removing methyl groups could help reset abnormal gene expression patterns (Pappalardi et al. 2021). Even lifestyle changes, like quitting smoking or improving diet, may gradually reverse harmful methylation marks and improve reproductive outcomes (Heikkinen et al. 2022). Understanding the reversible nature of DNA methylation opens up new possibilities for treatment of reproductive disorders and improving fertility through both medical and lifestyle interventions.

Techniques to Evaluate DNA Methylation

The mechanism of epigenetic gene regulation that has been explored the most is DNA methylation. The control of gene expression in mammalian cells is influenced by DNA methylation patterns in CpG-rich promoter regions, or CpG islands. An in-depth investigation of DNA methylation patterns is essential to comprehending the epigenetic basis of sperm disorders. Hundreds of researches are published every year focusing on PCR based DNA methylation analysis, yet choosing and implementing the right technique can be daunting for molecular genetics researchers not yet familiar with methylation analysis (Li and Zhang 2014). Almost all of the PCR based methylation analysis techniques require a step of bisulfite conversion of DNA sequence of interest (Hernández et al. 2013). Bisulfite conversion serves as the foundation for detecting methylation status. This process involves treating DNA with sodium bisulfite, which leaves methylated cytosines unaltered while changing unmethylated cytosines into uracil. This chemical distinction between unmethylated and methylated cytosines enables subsequent analyses using techniques like combined bisulfite restriction analysis (COBRA), methylation-specific PCR (MS-PCR) and MethyLight. These techniques, while sharing a common ini-

tial point, differ in their methods, applications, and sensitivity.

Methylation Specific PCR (MS-PCR)

MS-PCR is among the most widely applied techniques for methylation analysis due to its ease of use and specificity. After bisulfite conversion, this method employs primers specifically created to distinguish between methylated and unmethylated DNA sequences (Herman and Baylin 2003). PCR amplification occurs only when the primer matches the methylation pattern at the target locus. The results are typically visualised using gel electrophoresis to confirm the presence or absence of amplification for either the methylated or unmethylated sequence.

MS-PCR is particularly useful for locus-specific studies and is often employed as a preliminary screening tool. Its low cost and ease of use make it accessible to laboratories with limited resources (Frommer et al. 1992). However, MS-PCR is a qualitative technique and cannot provide information on the degree of methylation (Jeddi et al. 2024).

Combined Bisulfite Restriction Analysis (COBRA)

COBRA is a semi-quantitative method that combines PCR amplification of bisulfite-treated DNA with restriction enzyme digestion. In this technique, PCR primers amplify both methylated and unmethylated DNA (Xiong and Laird 1997). The amplified product is then digested with methylation-sensitive restriction enzymes, which cut only at methylated recognition sites. The digestion products are analysed using gel electrophoresis, and the relative intensities of the fragments gives information if the sequence is methylated and levels of methylation at specific CpG sites.

COBRA offers the advantage of being quantitative while still being relatively simple to perform. It is particularly useful for studying methylation heterogeneity within a sample (Ehrich et al. 2005*)*. However, the technique is more labour-intensive compared to MS-PCR and is limited by the availability of restriction enzymes that recognise methylation-sensitive sites and is less adequate for high-throughput studies.

MethyLight

MethyLight is a fluorescence-based, quantitative PCR technique designed for high-throughput methylation analysis. This method uses sequence-specific probes and primers that target methylated DNA sequences after bisulfite treatment. During PCR amplification, fluorescence signals generated by probe hybridisation are measured in real-time, which gives quantitative data on the methylation levels at specific loci.

MethyLight is highly sensitive and specific, making it suitable for genome-wide methylation studies and clinical applications. This technique's potential to analyse many loci simultaneously allows for efficient high-throughput workflows. Though expensive comparatively, MethyLight is widely used in epigenetic research and diagnostics due to its scalability and precision (Weisenberger et al. 2005; Esteller 2007).

DNA Methylation Analysis Using Oxford Nanopore Sequencing

Oxford Nanopore Sequencing (ONS) is revolutionising DNA methylation analysis by surpassing traditional PCR-based methods. Unlike PCR-based techniques, which require chemical bisulfite conversion and are often limited in resolution, ONS provides direct, real-time detection of methylated cytosines (Simpson et al. 2017). Its unique ability to sequence native DNA enables concurrent review of genomic and epigenetic data, offering a holistic view of the genome. Additionally, ONS overcomes limitations in studying repetitive regions, which are often inaccessible to short-read PCR-based methods.

ONS is particularly advantageous in identifying complex methylation patterns over long genomic stretches and regions with structural variations. Studies have demonstrated that ONS achieves comparable accuracy to conventional methods while providing superior efficiency and resolution for genome-wide methylation profiling (Liu et al. 2019*)*. These attributes make ONS a preferred tool for epigenomic research in fields such as oncology, developmental biology, and germline epigenetics. The comparative overview and ease of use od common DNA Methylation analysis techniques is discussed in Table 3.

Table 3: Comparative overview of commonly used DNA methylation analysis techniques

Technique	*Principle*	*Type*	*Advantages*	*Limitations*
MS-PCR (Methylation-Specific PCR)	Uses methylation-specific primers post-bisulfite conversion to detect methylated vs. unmethylated sequences.	Qualitative	- Simple and cost-effective - High specificity- Good for preliminary, locus-specific analysis	- Not quantitative - Cannot detect methylation percentage
COBRA (Combined Bisulfite Restriction Analysis)	PCR of bisulfite-converted DNA followed by digestion with methylation-sensitive restriction enzymes.	Semi-quantitative	- Provides relative methylation levels - Detects heterogeneity - Simple methodology	- Labour-intensive - Dependent on availability of restriction sites - Limited throughput
MethyLight	Fluorescence-based real-time PCR using methylation-specific probes and primers.	Quantitative	- High sensitivity and specificity - Suitable for high - throughput analysis - Real-time detection	- Expensive- Requires specialised equipment
Oxford Nanopore Sequencing (ONS)	Direct detection of methylated cytosines using native DNA and long-read sequencing.	Quantitative (High-resolution)	- No bisulfite treatment needed - Genome-wide and long-read capability - Detects structural variation and repeats	- Requires high-quality DNA - Expensive instruments and data processing

Techniques for miRNA Analysis in Male Infertility

Identifying miRNA biomarkers in spermatogenesis involves generating miRNA expression profiles using high-throughput and sensitive techniques. Two of the most widely used approaches are reverse transcription quantitative PCR (RT-qPCR) and sequencing based approaches.

Reverse Transcription Quantitative PCR (RT-qPCR)

RT-qPCR is a widely used technique for miRNA expression profiling due to its sensitivity, specificity, and cost-effectiveness. RT-qPCR is ideal for targeted analysis of a small number of miRNAs, making it particularly suitable for validating potential miRNA biomarkers identified through high-throughput screening. It provides highly quantitative data with a broad dynamic range. However, its specificity depends on the design of primers and probes, and its throughput is restricted to the number of miRNAs that can be reviewed simultaneously (Hunt et al. 2015). Despite these disadvantages, RT-qPCR remains a gold standard for miRNA quantification in studies of spermatogenesis.

Sequencing Based Approaches

Sequencing-based approaches are particularly valuable in spermatogenesis research, where they can capture the complexity of miRNA dynamics at various stages. For example, studies using NGS have revealed stage-specific miRNA expression profiles in spermatogonia, spermatocytes, and spermatids, highlighting their role in regulating meiotic and post-meiotic processes (Zheng et al. 2020; Moritz et al. 2018). Additionally, sequencing makes it possible to identify miRNAs with low abundance, which may escape detection in PCR-based methods. Sequencing provides a global view, revealing novel miRNAs and complex expression patterns. Combining these approaches offers a comprehensive strategy for identifying miRNA biomarkers, paving the way for deeper insights into spermatogenesis and male infertility. The comparative overview and ease of most common miRNA expression profiling techniques are discussed in Table 4.

RESULTS

The literature search conducted across Google Scholar and PubMed databases, using the specified search terms individually and in com-

Table 4: Comparative overview of commonly miRNA expression profiling techniques

Technique	*Principle*	*Type*	*Advantages*	*Limitations*
Reverse Transcription Quantitative PCR (RT-qPCR)	Quantitative	Measures miRNA expression levels by reverse transcribing RNA into complementary DNA and amplifying it with specific primers.	- High sensitivity and specificity - Cost-effective - Ideal for targeted analysis of a small number of miRNAs - Provides highly quantitative data	- Limited throughput (can analyse only a small number of miRNAs) - Dependent on primer and probe design
Sequencing-based Approaches (for example, NGS)	Global Profiling	Uses next-generation sequencing (NGS) to obtain miRNA expression profiles across different stages of spermatogenesis.	- Comprehensive, global view of miRNA expression - Detects low-abundance miRNAs - Can identify novel miRNAs and complex expression patterns	- More expensive and complex than RT - qPCR - Requires more data analysis and bioinformatics

bination, yielded a broad range of studies relevant to the epigenetic regulation of male infertility. Since no restrictions were placed on publication date, the retrieved studies spanned several decades, reflecting the evolving understanding of epigenetics in reproductive biology. After screening for relevance and restricting inclusion to English-language publications, studies were selected based on their focus on DNA methylation and miRNA-mediated regulation of genes implicated in male infertility, encompassing aspects such as sperm quality, spermatogenesis, semen parameters, and genomic imprinting. The final body of literature included a mix of original research and review articles addressing mechanisms such as CpG island methylation, gene silencing, hypermethylation and hypomethylation patterns, and miRNA expression profiles, collectively providing a comprehensive evidence base for examining the epigenetic underpinnings of primary and secondary male infertility.

DISCUSSION

Male infertility being a complex, multifactorial condition is influenced by genetic, environmental, lifestyle and epigenetic factors (Tesarik 2025). Among the emerging epigenetic mechanisms, MicroRNAs (miRNAs) and DNA methylation are essential for the regulation of spermatogenesis and sperm function. Male infertility has been associated with their dysregulation in a number of ways. This review discussed the roles of DNA methylation and miRNAs in male infertility, highlighting their role as biomarkers for therapeutic and diagnostic targets.

DNA methylation is a significant epigenetic mechanism that regulates gene expression during spermatogenesis. Aberrant methylation patterns in specific genes have been associated with male infertility, particularly in cases of idiopathic infertility, where no clear genetic cause can be identified. Studies have shown that genes such as MTHFR, MEST, and RHOX exhibit abnormal methylation patterns in infertile males, leading to impaired sperm quality, motility, and function (Rotondo et al. 2021; Richardson et al. 2014). However, the reported methylation patterns are not always consistent across different populations, suggesting that environmental exposures, lifestyle factors, genetic background, and methodological differences may influence epigenetic profiles (Liao et al. 2025). Recent research has highlighted the significance of validating methylation biomarkers in larger and more diverse cohorts before their routine clinical application (Zuo et al. 2026).

The reversibility of DNA methylation marks provides an exciting opportunity for therapeutic interventions. Lifestyle modifications, coupled with targeted epigenetic therapies, may potentially restore normal methylation patterns, improving fertility outcomes in affected men (Youvan 2024). Moreover, advanced techniques such as MethyLight and Oxford Nanopore se-

quencing have proven instrumental in profiling DNA methylation patterns in sperm (Trinh et al. 2001; Liu et al. 2021). These methods allow for a thorough comprehension of methylation changes across the genome, providing insightful information about the fundamental causes of infertility. However, despite their high sensitivity and accuracy, factors such as cost, technical complexity, and lack of standardised analytical pipelines may currently limit their widespread implementation in routine clinical settings. Future research should focus on refining these techniques and integrating them into clinical practice, potentially enabling the identification of methylation-based biomarkers for male infertility.

Small, non-coding RNAs known as microRNAs (miRNAs) control post-transcriptional gene regulation. Their role in spermatogenesis has gained increasing attention, as miRNA dysregulation has been associated with various forms of male infertility. Specific miRNAs, such as miR-34C, miR-34a, miR-449a, and miR-449b, have been implicated in regulating crucial processes in sperm development and function. For example, the dysregulation of miR-34C is linked to altered spermatogenesis, resulting in conditions like azoospermia and hypospermatogenesis (Bouhallier et al. 2010). MiRNA profiling has been used to identify differential expression of miRNAs in various forms of male infertility, such as non-obstructive azoospermia (NOA) and obstructive azoospermia (OA). Studies have highlighted key miRNAs, including the miR-34 and miR-449 families that regulate spermatogenesis by influencing germ cell development, differentiation, and apoptosis (Pantos et al. 2021). These outcomes demonstrate how miRNAs can be used as biomarkers for determining male infertility and distinguishing between different etiological factors. Nevertheless, variability in miRNA expression profiles has been reported among studies, largely due to differences in sample sources, patient populations, RNA extraction procedures, and analytical platforms. Consequently, further validation studies are required to establish clinically reliable miRNA signatures for male infertility (Al Smadi et al. 2026).

Moreover, sequencing-based approaches, such as Next-Generation Sequencing (NGS), have provided comprehensive miRNA expression profiles at various stages of spermatogenesis. This has revealed stage-specific miRNA dynamics in spermatogonia, spermatocytes, and spermatids, offering insights into their role in regulating meiotic and post-meiotic processes. NGS also allows the detection of low-abundance miRNAs, which might otherwise be missed using PCR-based methods (Martinez- Dominguez et al. 2021*)*. However, the high cost of sequencing technologies and the requirement for extensive bioinformatic analysis remain significant challenges for their routine clinical use. NGS findings often require validation using complementary approaches such as RT-qPCR to ensure reproducibility and accuracy. The integration of RT-qPCR and NGS techniques holds promise for advancing the understanding of miRNA regulation during spermatogenesis and improving male infertility diagnostics.

Despite the promising findings, several challenges remain in the field of miRNA and DNA methylation research in male infertility. The complexity of miRNA regulation, with its potential interactions with multiple genes and pathways, makes it difficult to pinpoint specific miRNAs as reliable biomarkers (Ray et al. 2026). Additionally, the sensitivity of techniques like RT-qPCR and NGS is crucial for detecting low-abundance miRNAs, but the high cost and complexity of sequencing technologies remain limitations in clinical practice. Furthermore, while DNA methylation is a reversible process, the ability to restore normal methylation patterns in sperm cells remains an ongoing challenge. The current lack of robust clinical trials evaluating the efficacy of epigenetic therapies in male infertility means that these approaches have yet to be fully translated into practice.

Future research should focus on expanding the understanding of how DNA methylation and miRNA dysregulation contribute to male infertility. Large-scale studies involving diverse populations of infertile men are necessary to identify specific miRNA and methylation biomarkers associated with different forms of infertility. Additionally, integrating multi-omics approaches, combining miRNA profiling, DNA methylation analysis, and other genomic methods, could provide a more comprehensive understanding of the molecular mechanisms involved in male infertility. In the clinical context, the development of miRNA-based therapeutics, such as miR-

NA mimics or inhibitors, could offer novel treatment options for men with infertility. These therapies, combined with DNA methylation restoration techniques, potentially result in more individualised, successful male infertility therapies.

CONCLUSION

DNA methylation and miRNA-mediated regulation are fundamental epigenetic mechanisms governing spermatogenesis. Aberrant epigenetic modifications contribute significantly to male infertility and may serve as valuable diagnostic biomarkers. Emerging technologies such as Oxford Nanopore sequencing and next-generation miRNA profiling provide powerful tools for identifying infertility-associated epigenetic alterations. Future translational studies are needed to validate these biomarkers and develop targeted therapeutic interventions.

RECOMMENDATIONS

Future studies should be designed as large-scale studies to validate DNA methylation and microRNA-based biomarkers in different populations. Standardisation of analytical methods will be required to improve the reproducibility of results. In addition, longitudinal studies will be required to assess the reversibility of epigenetic changes and to explore the potential of epigenetics-based therapeutic strategies in the management of male infertility.

LIMITATIONS

The review is based on the literature and does not include experimental and clinical data. The design and the sample used, as well as the population and the methodology employed, differ from one study to another. Moreover, though various epigenetic markers have been proposed, clinical validation and therapeutic data are still limited.

AUTHOR CONTRIBUTIONS

Hasna Shajahan: Literature review, conceptualisation, manuscript drafting

Aneesh Nair: Supervision, manuscript revision and approval

Poornima Prasad A: Literature collection and data interpretation

Ramgopal M Pillai: Clinical expertise, critical revision and approval

ACKNOWLEDGMENTS

The authors would like to express their sincere gratitude to Noorul Islam Centre for Higher Education for providing the necessary facilities and a supportive academic environment to carry out this review. The researchers also thank their colleagues and peers at the institution for their valuable suggestions and encouragement throughout the preparation of the manuscript.

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Paper received for publication in March, 2026
Paper accepted for publication in August, 2026