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Review

Epigenetics modification among vitrified oocytes and early embryos derived from them: a narrative review

Mostafa Saeedinia[1#], Jiayi Chen[2#], Omid Kohandel Gargari[3], Kyana Jafarabady[3], Majid Zaki-Dizaji[4], Alireza Ghorbankhanloo[5], Zohreh Heidary[6*]

[1] *Shahid Beheshti University of Medical Sciences, Tehran, Iran*
[2] *Jiading Branch of Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, 800 Huangjiahuayuan Road, Shanghai 201803, P.R.China*
[3] *Student Research Committee, School of Medicine, Alborz University of Medical Sciences, Karaj, Iran*
[4] *Human Genetics Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran*
[5] *Cancer Biology Research Center, Cancer Institute, Tehran University of Medical Sciences, Tehran, Iran*
[6] *Vali-E-Asr Reproductive Health Research Center, Family Health Research Institute, Tehran University of Medical Sciences, Tehran, Iran*

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Abstract

Vitrification has important application in assisted reproductive technology (ART) and this technique has been widely used in the cryopreservation of oocytes and embryos. However, due to susceptibility of epigenetic modifications to environmental changes induced by cryopreservation procedures, there are concerns about the potential epigenetic consequences of oocyte and embryo vitrification. This review comprehensively summarized the effect of cryopreservation—especially the vitrification method in ART-on oocytes and embryos. Various studies have reported changes in different aspects of genomic status which directly affect the quality of fertilized embryos. The objective of this review is to assess existing literature on the epigenetic modifications that occur in vitrified oocytes and early embryos resulting from oocyte vitrification, including DNA modifications, RNA methylation, histone modification and microRNAs related to ART.

Keywords: Assisted reproductive technology, Oocyte vitrification, Epigenetic, Cryopreservation.

1. Introduction

Assisted reproductive technologies (ARTs) have revolutionized the field of reproductive medicine and have successfully helped millions of women worldwide to conceive. In the United States alone, 330,773 ART cycles were performed in 2019, resulting in 77,998 live births [1]. While ART has undoubtedly brought hope to many couples struggling with infertility, it is essential to study the cellular and molecular changes associated with these procedures to ensure their safety and efficacy. Cryopreservation and oocyte vitrification are integral parts of ART, and they effectively preserve the fertility of women undergoing cancer treatments or those who wish to delay pregnancy [2]. Epigenetic modifications, such as DNA methylation, histone modifications, and microRNA expression, play a crucial role in determining gene expression and cellular differentiation. Histones are structural and functional proteins located in the core of the nucleosome, and they regulate chromatin structure and function. Histone modifications, including phosphorylation, ubiquitination, acetylation, and methylation, play a critical role in chromatin remodeling and gene expression [3]. In this study, we will review the current literature on epigenetic changes in vitrified oocytes and the early embryos derived from them. Understanding these changes is crucial to improving the safety and efficacy of ART and ensuring the long-term health of the offspring.

2. DNA methylation

Recently, the role of epigenetics in the field of reproduction medicine has increased and includes different aspects including DNA modification. (Figure 1) DNA methylation is regarded as one of the modification methods and most commonly occurs as the methylation of the

* Corresponding author.
E-mail address: z.heidary2016@gmail.com (Z. Heidary).
# These authors contributed equally
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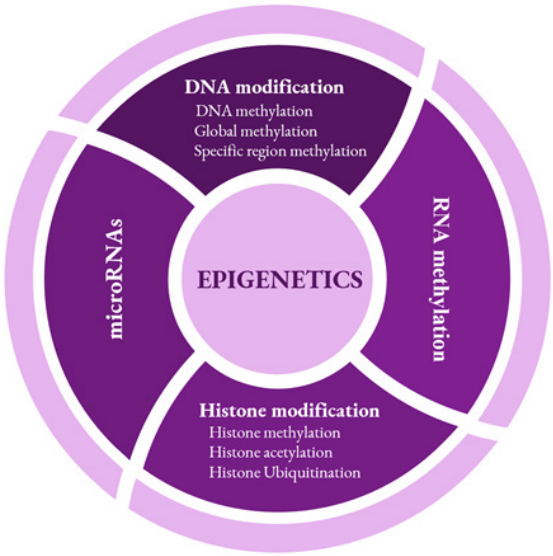

Fig. 1. Epigenetic modifications including different DNA, RNA and Histone modification or non-coding RNAs such as microRNAs.

cytosine base at the 5-Carbon position by DNA methyltransferases (DNMTs). The methylated cytosine is called 5mc(5-methylcytosine). 5mc is mostly present at CpG sites, where a cytosine base is followed by a guanine. CpG islands or CGIs are regions of genomes in which CpG sites are most frequently located. DNA methylation could have various effects on cells; the methylation of the promotor region with high CpG level is associated with gene silencing. DNA methylation is critical for normal development, genomic imprinting, X-chromosome inactivation, repression of transposable elements, aging, and carcinogenesis. Methylation can change the activity of a DNA segment without altering the sequence. Cytosine methylation can repress gene transcription and is the most extensively studied form of DNA methylation, while adenine methylation has received less attention. DNA methylation is required to establish and maintain cell identity and regulates gene expression through various mechanisms [4, 5]. Methylations are present in both intra-genic and extra-genic regions.

2.1 Global methylation

Global methylation is defined as the level of 5mc relative to the total cytosine level. The method used for this measurement is immunofluorescence staining which is done by visualization of antibodies against 5 mM. Oocytes were vitrified at either the MII or GV stage, and global methylation was measured at different developmental stages, including GV oocytes, MII oocytes and early embryos. Overall, both human and animal studies did not report a significant difference in global methylation at different stages of development.

Liang et al. [6] reported a significant reduction in global methylation among vitrified MII oocytes and early embryos derived from them (2-cell, 4-cell, 8-cell), this difference was resolved at the morula and blastocyst stages. In another study, Moulavi et al. [7] vitrified oocytes at the GV stage and measured global methylation in 2-cell embryos and blastocyst; they observed a significant reduction in general methylation among 2-cell embryos, but the difference was not significant in case of blastocysts. Ma et al. [8] study is the latest published paper on this matter, they reported a significant reduction in the level of global methylation among vitrified mouse oocytes compared to fresh oocytes. They also reported that there was no significant difference in methylation levels of exons, $3'$ UTR and $5'$ UTR, but methylation was significantly reduced in introns and promotors of vitrified oocytes compared to fresh ones.

In vitro maturation (IVM) after vitrification is suggested to be a culprit for changes in global methylation. In a relatively large mice study by Yodrug et al. [9] and another study by Yan et al. [10], global methylation of vitrified MII oocytes and MII oocytes derived from vitrified GV oocytes, which underwent IVM, was compared and no significant difference was reported by them. A meta-analysis is needed to make a certain statement about the effect of IVM after vitrification on global methylation, but in a general overview of included studies, it does not seem to be significantly associated with changes in global methylation.

These studies also suggest that the stage of vitrification either at the MII or the GV stage does not seem to affect global methylation. Nevertheless, more studies, especially human studies, must make a concrete conclusion.

Two of the studies that reported a reduction in global methylation shared an interesting result; significance and amount of difference decrease further through the development at the blastocyst stage [6, 7]. In contrast, in a study by Yodrug et al. although no significant differences between fresh and vitrified oocytes global methylation was significantly reduced in blastocysts derived from vitrified oocytes compared to controls.

The systematic review conducted by Barberet et al. [1] has shown that the differences in DNA methylation observed after ART conceptions are not significant, and their functional relevance in adult tissues is still unclear. There is a high controversy among studies reporting global methylation, which indicates the complex nature of epigenetic studies, overall, it could be concluded that vitrification either has no effect or decreases the level of global methylation among vitrified oocytes and early embryos derived from them.

2.2. Methylation of specific regions

2.2.1. Dnmt1o, Hat1, and Hdac1

In a study by Zhao et al. [11] methylation patterns of the CPG Islands in the DNA methyltransferase 1o (*Dnmt1o*), histone acetyltransferase 1, and histone deacetylase 1 (*Hat1, and Hdac1*) promotors were compared between 2495 vitrified and 2218 fresh mouse MII oocytes. They reported no significant difference in the methylation pattern of *Dnmt1o, Hat1, and Hdac1* promotors between vitrified and fresh MII oocytes.

2.2.2. Oct4 and Sox2

Milory et al. [12] compared the methylation pattern of *the Oct4* gene promotor between in vivo matured oocytes and vitrified in vitro matured mouse oocytes. All oocytes were vitrified at the MII stage in their study. Overall methylation of *Oc4* was significantly lower among vitrified oocytes compared with in vivo mature oocytes. 25% to 62.5% respectively($P<0.05$). They also reported the methylation status of three CPGs in *Oct;* their findings are summarized in Table 1. They also reported the methylation status of *the Sox2 promotor, and* again a significant

Table 1. Methylation percentage of three CPGs of Oct4 promotor

CPG	In-vivo matured oocyte	Vitrified in-vitro matured oocytes	P-value
CPG1	50%	25%	0.206
CPG2	100%	33.3%	0.001
CPG3	100%	33.3%	0.001

reduction in promotor methylation was reported. Overall *Sox2* promotor methylation was 8.5% for in vivo matured oocytes and 4.5% for vitrified in vitro matured oocytes (P<0.05).

2.2.3. Nanog and Foxd3

Milory et al. [12] also worked on Nanog and Foxd3 promotor methylation, despite two previous sequences, they reported no significant difference in the overall methylation of *Nanog* and *Foxd3* promotors between in vivo matured oocytes and vitrified in vitro matured mouse oocytes. [12]

2.2.4. H19, Peg3, IGF2 and C

Cheng et al. [13] analyzed the methylation pattern of CPGs located in differentially methylated regions (DMR) of *H19, Peg3,* and *Snrpn*, including 15 CPGs in the *H19* DMR, 18 CPGs in the *Peg3* DMR, and 16 CPGs in the *Snrpn* DMR. They reported no significant difference between fresh and vitrified mouse oocytes and interestingly, reported that methylation level of these three genes was reduced among blastocysts derived from vitrified oocytes compared to fresh oocytes. This difference could be due to reduced expression of *Dnmt3b* among blastocysts derived from vitrified oocytes. (Figure 2) [8] The manipulation and culture of preimplantation embryos can result in abnormal methylation of histones in the H19/Igf2 promoter region, leading to changes in gene expression patterns. This alteration of H3K4me3 and H3K9me3 methylation has been observed to affect H19/Igf2 expression in chimeric blastocysts. In mice embryos, H19/Igf2 imprinting genes are more sensitive to culture supplements and micromanipulations than other imprinted genes and play an essential role in embryo development, placental organization, and fetal growth [14-16]. Previous studies have linked abnormal imprinting of the H19/Igf2 genes to atypical DNA methylation and histone modifications at the imprinting control region [17-19]. H19 and Igf2 are critical regulatory genes involved in various aspects of embryo development, fetoplacental growth, and postnatal behavior [20]. In vitro manipulations such as in-vitro fertilization (IVF) and SCNT have been shown to influence H19 expression [21].

According to a recent investigation, the fresh embryo transfer group exhibited an abnormal reduction in the methylation levels of the imprinted gene H19/IGF2 and transposon element LINE-1 in the placenta, in comparison with the natural pregnancy group. However, no significant abnormality was observed in the FET group. Following the vitrification and warming of mouse follicles, there was no impact on the DMR methylation of imprinted genes H19 and Igf2r in mature oocytes, but Snrpn underwent a slight alteration [13].

2.2.5. GRB10

The Grb10 is an important gene that is parentally imprinted and plays a role in development and glucose metabolism [22, 23]. It acts as a negative regulator of the insulin and IGF-1 signaling pathways, making it a vital component of the insulin signaling cascade [24] In mice, overexpression of Grb10 leads to postnatal insulin resistance by negatively modulating the IGF1R and IR cascades, linking growth and glucose metabolism in postnatal life. Grb10 negatively regulates insulin/IGF-1 and Erk/MAPK signaling pathways, inhibiting IGF-1/IGF-1R signaling and affecting insulin-regulated glucose transport. Its overexpression can reduce DNA synthesis, inhibit cell division and proliferation, and is associated with insulin resistance, type 2 diabetes mellitus, and individual growth and development [23, 25]. The study conducted by Zhou et al. [26] on cryopreserved ovary transplantation and fresh group revealed that the overexpression of Grb10 in mice causes postnatal insulin resistance via negative modulation of the IGF1R and IR cascades, suppressing IGF1R, IRS2, and AKT phosphorylation.

2.2.6. MEST

The presence of differentially methylated regions (DMRs) in CpG islands and exons of imprinted genes like MEST and GNAS in early-postpartum oocytes indicates that metabolic stress during early lactation could impact the acquisition of imprinting, which may contribute to embryo loss. MEST is a gene that is expressed from the paternal alleles in the mesoderm and its differentiated lineages, and it undergoes dynamic methylation in mice in the upstream promoter region of a CpG island, similar

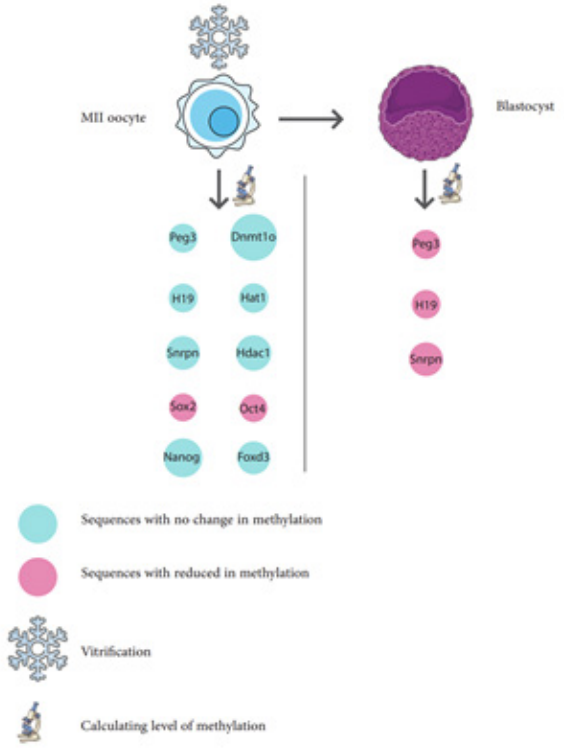

Fig. 2. Changes in the level of vitrification of certain sequences after vitrification at the MII stage.

to what was observed in the overlapping DMRs. While MEST is typically fully methylated in the maternal germline, it was differentially methylated in fully grown and freshly ovulated oocytes, but hypermethylated in oocytes cultured in vitro, demonstrating the dynamic nature of imprint acquisition and its susceptibility to varying growth conditions [27].

2.2.7. KCNQ1

The formation of the IKs channel complex involves the co-assembly of Kv7.1 (KCNQ1), a voltage-gated potassium channel, with its β-subunit, KCNE1, as well as the interaction with various accessory regulatory molecules, such as PIP2, calmodulin, and yotiao [28]. The inactivation of this complex is more evident when KCNQ1 is expressed in mammalian cells [29, 30], as opposed to oocytes, where it is less apparent [31]. This current has a low conductance and a fast activation and deactivation rate, but its association with endogenous currents in the body has not been positively identified [32]. The stoichiometry of KCNE1 forming complexes with KCNQ1 varies among different mammals, including humans, and remains unclear [28].

2.2.8. SNRPN and PLAGL1

Although the methylation pattern in SNRPN remained unchanged, changes were observed in Igf2r, H19, and PLAGL1 in the brain and liver tissue of the offspring compared to the natural controls. These changes were accompanied by varying levels of gene expression for Igf2r, H19, and PLAGL1, but did not result in any significant physical or functional differences (such as birth defects, weight gain, exercise capacity, or anti-fatigue ability) between the offspring from the cryopreserved and non-cryopreserved groups [33].

2.2.9. GTL2 and DLK-1

The study of Wilkinson et al. [34] indicated that the expression of lncRNA Gtl2 and its corresponding imprinted gene, Dlk1, in mouse blastocysts are crucial for the proper development of various tissues in the embryo, including the brain and bones, as well as regulating genes in the TGF-b signaling pathway. Gtl2 also has anti-tumor properties in humans through different pathways [35, 36]. Dlk1 codes for a transmembrane protein that plays a role in cellular differentiation and carcinogenesis. The maternal allele of Dlk1/Gtl2 has an unmethylated IG-DMR and expresses Gtl2, while the paternal allele has a methylated IG-DMR and expresses Dlk1 [37]. It was found that IVF and vitrification led to decreased Gtl2 expression and increased Dlk1 expression in mouse blastocysts [38].

3. Gene interaction analysis

The Gene Ontology enrichment and pathway analysis of the related genes described in this article show the importance of IRS activation R-HSA-74713 pathways in embryo modification. (Figure 3) The IRS molecule plays a role in insulin signaling pathways and is activated through phosphorylation. This leads to a series of cascades that involve PI3K, SOS, RAF, and MAP kinases. Through studies on mutated receptors, it has been determined that IRS1 binds to the insulin receptor at tyrosine 972 in the juxtamembrane region through its PTB domain. The interaction is stabilized by the PH domain of IRS1, which interacts with phospholipids on the plasma membrane. This allows for up to 13 tyrosine residues on IRS1 to be phosphorylated by the receptor. Once phosphorylated, IRS1 separates from the receptor and becomes activated, allowing other proteins to interact with it [39].

The Gene-Gene interaction of important genes was found in the article and was performed using GeneMANIA tool [40]. The report of predicted analysis is presented in Supplementary Figure 1 (Figure S1.).

4. RNA methylation

The involvement of N6-methyladenosine (m6A) in oogenesis, embryonic growth and reproduction has been increasingly supported by growing evidence [41]. This modification, which is the most prevalent, and conserved internal modification in eukaryotic RNAs, particularly in higher eukaryotic cells, is subject to dynamic and reversible regulation by specific enzymes. These enzymes include methyltransferases (writers), such as METTL3/14/16, RBM15/15B, ZC3H3, VIRMA, CBLL1, WTAP, and KIAA1429, which modify m6A modification, and demethylases (erasers), including FTO and ALKBH5, which remove it. The modification is recognized by m6A-binding proteins (readers), including YTHDF1/2/3, YTHDC1/2, IGF2BP1/2/3, and HNRNPA2B1 [42]. The study of Zhang et al. [43] revealed that the maturation process of oocytes was delayed due to the disruption of spindle organization and chromosome alignment by the use of cycloleucine as an inhibitor of RNA m6A methylation. It is also shown that m6A mRNA methylation can play a crucial role during meiotic maturation and maternal-to-zygotic transition in the mouse model [44]. Despite this, there is requirement for future study, helping to understand the role of RNA m6A methylation in human oocytes.

5. Histon modifications

5.1. Histone acetylation

Changing the chromatin architecture and regulating gene expression by opening and closing the chromatin structure following the epigenetic modification of nuclear histone acetylation plays an essential role in various cellular functions [45, 46]. The involvement of histone acetylation in cell functions other than gene expression has recently been noticed. There are very limited sources regarding the role of histone acetylation in meiosis, but its role in mitosis is very prominent [46].

In a study on mouse oocytes by Kim et al. [46], changes in acetylation patterns of different histone lysine residues were investigated in meiotic oocytes and compared with post-fertilization embryos during mitosis. Because of this research, the reduction in acetylation levels of different ly-

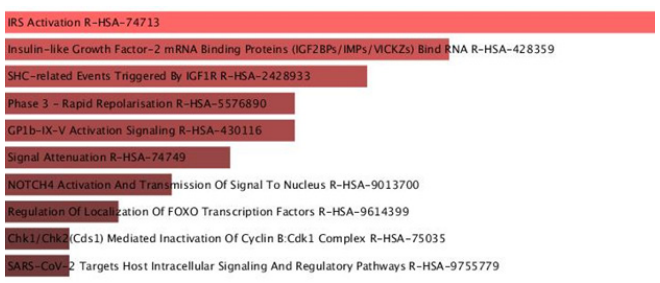

Fig. 3. Gene Ontology enrichment and pathway analysis of vitrified oocyte-related genes.

sine residues on histones H3 and H4 was observed both in oocytes during meiosis and in somatic nuclei transferred to nucleated oocytes. In a study by Moulvi et al. [7], in line with the possible effects of oocyte cryopreservation on the epigenetic status of the resulting embryos, the effects of oocyte cryopreservation in the 2-cell and blastocyst stages on epigenetic re-transcripts were investigated in Dromedary camels. Because of this study, the possibility of interfering with the vital steps of epigenetic reprogramming during embryo development before implantation due to oocyte cryopreservation was suggested, they reported a significant reduction in the acetylation level of H3K9 among 2-cell embryos derived from vitrified oocytes. In another study by Suo et al. [47], it was observed that cryopreservation affected the distribution of chromatin and AcH4K12, which is the result of changing the patterns of AcH4K12 in oocytes.

5.2. Histone acetylation and methylation

Among the important epigenetic changes that usually occur on lysine residues located in core histones are histone acetylation and methylation. Acetylation and methylation are related to activation of gene transcription and silencing of euchromatin along with the formation of heterochromatin, respectively, and they influence each other's effects [48].

In a study by Spinaci et al. [48], the status of H4 acetylation and H3K9 methylation were investigated. Because of this research, which was done on the changes caused by pig oocyte cryopreservation, an important role was suggested for histone H4 acetylation along with H3K9 methylation, because the change in these two items due to oocyte cryopreservation probably leads to inappropriate epigenetic alternation in the female chromatin present in fertilization, and as a result, the qualification of pig oocytes is reduced in cryopreservation. In a study by Yodrug et al. [9], taking into account that oocytes that are vitrificated by the Cryotop method have less ability to grow than fresh oocytes, the effect of vitrification on the epigenetic characteristics of bovine oocytes at the stage of metaphase II and subsequently in developing embryos was investigated. A significant decrease in the growth of blastocysts after in vitro fertilization due to oocyte vitrification was observed in this study, whereas the vitrification in the GV stage had a higher blastocyst development than the vitrification in the metaphase II stage. Finally, oocyte vitrification had a lack of effect on the intensity of H3K9me3, and acH3K9 immunostaining was determined [9].

5.3. Histone ubiquitination

Histone ubiquitination is a countless modification compared to other histone modifications, and it differs substantially from them. In a study by Ma et al [8], the molecular level changes, oocyte cryopreservation, and subsequent heating were investigated, and it was concluded that, unlike down-regulated genes, differentially regulated genes are mainly related to histone ubiquitination and in many cellular events including transcription initiation and elongation, silencing, and repairing of DNA, they play important roles. These roles are mainly related to chromatin and cytoskeleton structures such as histone H2A and histone mono-ubiquitination.

6. Micro-RNAs

The micro-RNA (miRNA) biogenesis pathway could also be influenced by the vitrification of oocytes. miRNA is a single-stranded noncoding RNA molecule that has a transcriptional/posttranscriptional function in the gene expression [49]. This molecule can be regulated by genetic and environmental conditions, so it is considered a target to be affected by vitrification [50]. miRNAs have a vital role in the regulation of embryo implantation. 270 miRNAs have been identified so far in this pathway [51]. miR-15a and miR-16-1 are responsible for apoptosis by repression of Bcl2, and miR-Let-7a suppresses the implantation by decreasing Itgβ3, Vav3, and Dicer expression [52].

The study's results show the decreased expression of miR-Let-7a, which leads to the suppression of implantation [52]. The development of the study performed by Daneshvar et al. [50] also assessed the expression of miR-16 and miR-let7 and their target gene; the comparison between the vitrified group and fresh group showed a significant decrease in miR-let7, but miR-16 has not changed significantly between these two groups. mmu-miR-199a-5p, mmu-miR-329-3p, mmu-miR-136-5p, mmu-miR-16-1-3p, and mmu-miR-212-3p are five other miRNAs that have been determined in the comparison between vitrified and fresh mouse blastocysts; the result shows downregulation of mmu-miR-212-3p and upregulation of mmu-miR-199a-5p, mmu-miR-329-3p, mmu-miR-136-5p, and mmu-miR-16-1-3p, which means miRNA transcriptome is affected by vitrification in mouse blastocysts [51]. Also, 22 miRNAs out of 520 were reported to be significantly different between the vitrified oocyte and fresh group in the Li et al. study [53], downregulation of the expression of miR465c-5p and upregulation of the expression of miR-21-3p, miR-210-5p, and miR-134-5p were reported.

6.1. MicroRNA and embryo quality

There is increasing evidence that miRNAs found in the bloodstream and other biological fluids can act as biomarkers for various diseases, including cancer. Recent research has shown that the miRNA profiles in the culture media of human blastocysts can be linked to factors such as fertilization method, chromosomal status, and pregnancy outcome. [54, 55] This suggests that miRNAs could be used to select embryos non-invasively in IVF cycles. According to research using droplet digital polymerase chain reaction (ddPCR), the levels of hsa-miR-26b-5p and hsa-miR-21-5p in the culture media of cleavage embryos were found to be significantly lower in embryos with successful pregnancies compared to those with failed pregnancies. The study of also revealed that these miRNAs could potentially be used as biomarkers for predicting reproductive outcomes [56].

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