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Alterations in the expression pattern of some epigenetic-related genes and microRNAs subsequent to oocyte cryopreservation

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Review Article

Cite this article: Shadmanesh A and Nazari H (2023) Alterations in the expression pattern of some epigenetic-related genes and microRNAs subsequent to oocyte cryopreservation. *Zygote*. **31**: 411–419. doi: 10.1017/S0967199423000321

Received: 21 November 2022
Revised: 16 April 2023
Accepted: 23 May 2023
First published online: 20 June 2023

Keyword:
Cryopreservation; Epigenetic; Mammalian; MicroRNA; Oocyte

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Summary

MicroRNAs (miRNAs) are small non-encoding RNAs that actively regulate biological and physiological processes, and play an important role in regulating gene expression in all cells, especially in most animal cells, including oocytes and embryos. The expression of miRNAs at the right time and place is crucial for the oocyte's maturation and the embryo's subsequent development. Although assisted reproductive techniques (ART) have helped to solve many infertility problems, they cause changes in the expression of miRNA and genes in oocytes and preimplantation embryos, and the effect of these changes on the future of offspring is unknown, and has caused concerns. The relevant genomic alterations commonly imposed on embryos during cryopreservation may have potential epigenetic risks. Understanding the biological functions of miRNAs in frozen maturated oocytes may provide a better understanding of embryonic development and a comparison of fertility conservation in female mammals. With the development of new techniques for genomic evaluation of preimplantation embryos, it has been possible to better understand the effects of ART. The results of various articles have shown that freezing of oocytes and the cryopreservation method are effective for the expression of miRNAs and, in some cases, cause changes in the expression of miRNAs and epigenetic changes in the resulting embryo. This literature review study aimed to investigate the effects of oocyte cryopreservation in both pre-maturation and post-maturation stages, the cryopreservation method and the type of cryoprotectants (CPA) used on the expression of some epigenetic-related genes and miRNAs.

Introduction

Cryopreservation is a very important technique that exposes the oocyte, embryo and various cells to different physical and chemical conditions, and today is an essential assisted reproductive technology (ART). According to studies, vitrification affects oocyte physiology to a lesser extent than slow freezing (Kuwayama *et al*., 2005; Cao *et al*., 2009; Borini and Bianchi, 2010). In general, survival and fertilization, as well as the rate of cleavage and implantation of embryos, were higher in vitrified human mature oocytes than in slowly frozen counterparts (Dessolle *et al*., 2009). However, high concentrations of cryoprotectants and rapid freezing rates may affect ultrastructure such as spindles and chromosomes (Trapphoff *et al*., 2016), mitochondrial function (Nohales-Córcoles *et al*., 2016), gene expression patterns, microRNAs (miRNAs), and epigenetic stabilities of the oocytes and the resulting embryos (Monzo *et al*., 2012). A study of the Danish population showed an increased risk of childhood cancer after frozen/thawed embryo transfer (FET) in a population of 174,881 children conceived through ART compared with 910,291 children naturally conceived (Hargreave *et al*., 2019). Data from four counties (Denmark, Finland, Norway, and Sweden) showed that children born after FET had a higher risk of childhood cancer than children born after fresh embryo transfer and spontaneous conception (Sargisian *et al*., 2022). Therefore, it is important to determine the effect of cryopreservation protocols on miRNA and especially gene expression, as one of the negative modulators on epigenetics.

miRNAs, a subset of the non-coding RNA class, have been identified as central regulators of gene expression (Kim *et al*., 2005). Studies have reported significant changes in miRNA expression patterns in frozen blastocysts (Zhao *et al*., 2015) and frozen sperm (Capra *et al*., 2017). Conversely, suppression of miR-762 has been reported to be beneficial for the survival and developmental capacity of frozen oocytes (Wang *et al*., 2018). One study found that the miR-21 level in pig MII oocytes was enhanced compared with germinal vesicle (GV) oocytes (Song *et al*., 2016). and also reported a significant increase in miR-21 levels in *in vitro* maturation of vitrified bovine oocytes, although the pre-miR-21 transcript was not statistically changed between immature and *in vitro*-matured oocytes (Tscherner *et al*., 2018). Several pieces of molecular evidence on the effect of cryopreservation on oocyte gene expression profile have

been presented (Liu *et al*., 2003; Lee *et al*., 2008; Anchamparuthy *et al*., 2010; Di Pietro *et al*., 2010; Chamayou *et al*., 2011). Most of this evidence is connected to mammalian species, and has shown that, in metaphase II (MII) oocytes, cryopreservation mainly affected the expression of genes connected with oxidative stress (members of the heat shock protein family, superoxide dismutase 1), apoptosis (members of the BCL2 family, death receptors) and cell cycle (cyclin B, members of the histone family, polymerases; Liu *et al*., 2003; Succu *et al*., 2007; Lee *et al*., 2008; Anchamparuthy *et al*., 2010). Some studies did not report the negative effects of cryopreservation at the molecular level (such as DNA damage, altered gene expression and miRNAs) in pigs, humans (Di Pietro *et al*., 2010), mice (Liu *et al*., 2003; Gao *et al*., 2017), and canine (Turathum *et al*., 2010), However other studies have shown signs of adverse effects in ovine (Succu *et al*., 2007) murine (Lee *et al*., 2008) bovine (Wang *et al*., 2017a; Anchamparuthy *et al*., 2010) and human (Chamayou *et al*., 2011; Monzo *et al*., 2012; Shahedi *et al*., 2017) species. It is apparent that these adverse effects might subsequently be corrected or repaired during further growth and development (Succu *et al*., 2007). Any changes in gene expression because of cryopreservation may explain some of the differences in viability between fresh and post-thaw cells. Moreover, it has been shown that changes in gene expression may also lead to significant defects in the brain, ear, eye and kidneys. In a study by Azizi *et al*. (2021) on mouse embryos it was shown that let-7a expression significantly decreased in IVF-driven blastocysts versus *in vivo* ones (Azizi *et al*., 2021). Zhao *et al*. (2015) showed that vitrification of mouse blastocysts resulted in significant changes in the miRNA transcriptome, which may affect the implantation potential of vitrified blastocysts. In one study, a significant decrease in the expression of miR-16, as well as a significant increase in the expression level of the *BCL2* gene (as one of the targets of miR-16), was reported in the vitrified and re-vitrified human blastocysts (groups) compared with the fresh group (Daneshvar *et al*., 2021). The aim of this review was to discuss the effects of cryopreservation on miRNA and the expression of some genes in oocytes and embryos of vertebrate species.

Oocyte cryopreservation

Effect of method cryopreservation

In recent years, many studies have revealed the superiority of vitrification over slow freeze protocols in reducing chilling damage to oocytes during cryopreservation (Saragusty and Arav, 2011). Fadini *et al*. (2009) reported higher pregnancy rates for vitrification (18.2 vs. 7.6%). In addition, vitrification has been proposed as a cheaper and more reasonable strategy for the cryopreservation of human and animal oocytes instead of the slow freezing technique using a higher concentration of cryoprotectants and a rapid cooling rate to prevent the formation of intracellular ice crystals (Antinori *et al*., 2007). Furthermore, the vitrification method has also been applied to cryopreserve oocytes or embryos produced from IVM programmes (Mogas, 2019). Unlike the slow freezing method, vitrification presents the capacity to control solute penetration, the dehydration rate, and the maintenance of physiological temperature during the equilibration process. Breaking the zona pellucida and formation of growing ice crystals are possible during the slow freezing process, but not with vitrification (Alcolak *et al*., 2011).

Results showed that the addition of melatonin to the freezing medium can effectively improve the development ability of frozen human oocytes by increasing mitochondrial function, reducing oxidative damage (by expressing proteins related to the phosphorylation pathway), maintaining reactive oxygen species (ROS)/GSH homeostasis and, as a result, normal ATP production and the ROS/GSH ratio were improved (Zhu *et al*., 2022). Limited numbers of studies on oocytes have provided controversial results regarding the effect on DNA fragmentation, sister chromatid exchange (SCE), and aneuploidy. A study of human embryos has shown that vitrification affects DNA integrity much less than slow freezing. Animal studies have shown increased mitochondrial DNA mutations in embryos after cryopreservation (Kopeika *et al*., 2015). Studies have shown that slow freezing and vitrification alter the distribution of mitochondria in mouse embryos. Also, in the vitrification method, ROS are higher in the morula stages. Therefore, in addition to the freezing method, the embryonic stage also determines the changes in the cells caused by freezing (Somoskoi *et al*., 2015; Hayashi *et al*., 2019). A study on rabbit embryos showed that there was no significant difference in the expression of genes (*SCGB1A1*, *EMP1*, *C1QTNF1*, *ANXA3*, *EGFLAM* and *TNFAIP6*) between embryos frozen with the two techniques (slow freezing and vitrification; Saenz-de-Juano *et al*., 2014). A comparison between the transcriptional profiles of two vitrified oocyte techniques (semi-automated and manual vitrification) using single-cell RNA sequencing showed that the transcriptional changes between the two techniques were very low and not significant, but specific adverse effects of oocyte freezing on genomic expression were prominent compared with fresh oocytes. Given that fertility preservation through egg freezing will be developed worldwide in the coming years, More studies in this field are needed for the health of children born from frozen oocytes (Barberet *et al*., 2022).

Type of CPA and miRNAs

Results of the assessment of IVM bovine oocytes submitted to vitrification showed that DMSO had a significant effect on the incidence of abnormal microtubule distribution when compared with fresh oocytes (Verheijen *et al*., 2019). Frequencies found when comparing the DMSO group to glycerol were similar ($P > 0.05$). Additionally, no differences were found when comparing fresh oocytes to those submitted to vitrification using the combination of ethylene glycol (EG) and glycerol ($P > 0.05$). No differences between oocytes vitrified using EG and glycerol and fresh oocytes were found. Conversely, oocytes vitrified using the combination of EG and glycerol presented a higher incidence of normal microtubule configuration. It has been widely suggested that the incubation process allows the reorganization of microtubules. The chromosomal arrangement was not affected by vitrification and the incubation process did not affect it (Verheijen *et al*., 2019). In a recent study, DMSO effects were assessed with complete transcriptome analysis (using ribodepleted total RNA sequencing and microRNA sequencing), whole-genome methylation profiling (using MeDIP-seq) and proteomics analysis (using mass spectrometry). The results demonstrated that DMSO could not be considered biologically inert, but induced significant changes in miRNAs and the epigenetic landscape in cells in culture even at very low concentrations, especially in the maturing cardiac model. Zhang *et al*. (2021) evaluated the effects of EG as a cryoprotectant in open vitrification medium in human oocytes. Global gene expression studies based on fragments per kilobase million (FPKM) cluster analyses showed that 3740 genes were upregulated

https://doi.org/10.1017/S0967199423000321 Published online by Cambridge University Press

and 956 genes were downregulated in vitrified donor oocytes vs. fresh donor oocytes.

Stage of oocyte cryopreservation

Despite the proven superiority of the vitrification method over slow freezing, there is still debate about the vitrification stage of the oocyte (before and after *in vitro* maturation). Some studies in species such as pigs (Martin and Zhang, 2007) and humans (Ma *et al*., 2012; Gao *et al*., 2017) have reported that vitrified oocytes at the GV stage have a lower survival rate and efficiency than at the metaphase II stage (MII). Conversely, one study reported that vitrification of mouse oocytes in the MII stage was more suitable for transcription (Gao *et al*., 2017). Also, a study using the same cohort of oocytes (oocytes from the same donors) reported that vitrified oocytes did not show any statistically significant differences in fertilization, embryo quality, or clinical outcome compared with fresh oocytes (Solé *et al*., 2013). A study using RNA-seq showed that vitrification of *in vitro*-matured bovine oocytes caused changes in the expression of some genes (Wang *et al*., 2017a, 2018). Song *et al*. (2016) also used GV-stage and GVBD (MI)-stage oocytes generated from IVF cycles. A comparison between matured oocytes *in vivo* without vitrification with groups of vitrified oocytes before and after maturation *in vitro* showed that, after thawing, the cleavage rate in vitrified oocytes at the MII stage after IVM was higher than in vitrified immature oocytes before IVM.

One study evaluated the expression of DNMT1, apoptosis-related (*Bcl-2* and *Bax*) and stress-related *(Sod1* and *HSP70*) genes in fresh IVM oocytes, vitrified GV (vGV) and vitrification of *in vitro*-matured oocytes (vIVM). The results showed that the expression of *Bcl-2*, *Bax*, *Sod1*, and *HSP70* genes in the vIVM group was significantly increased, and also the *DNMT1* gene in both groups [vitrification before (vGV) and after (vIVM) maturation]. But the rate of expression was reduced in vitrified IVM (vIVM) oocytes (Shahedi *et al*., 2017). Therefore, vitrification after maturation caused more stress on the oocytes, and higher expression of stress-related genes in the oocyte. The major problem associated with freezing mature oocytes is meiotic spindle sensitivity to low temperatures and cryoprotectants. To solve this problem, GV oocytes were used instead of mature oocytes. However, poor maturation, fertilization, and embryo development were the main disadvantages associated with the cryostorage of immature oocytes (Walls *et al*., 2012).

One study found that vitrification acted as a stressor and altered the expression of these genes, thereby reducing the developmental competence of mouse zygotes (Borini and Bianchi, 2010). In addition, it should be noted that various factors in the freezing process, such as high concentrations of cryoprotectants, freezing, and osmotic stress, changed the pattern of gene expression as well as the onset of apoptosis, which threatened the viability and development of oocytes (Men *et al*., 2003). Men *et al*. (2003) showed that the degeneration mechanism of cryopreserved oocytes was apoptosis. The results showed that the percentage of oocyte maturation in the oocytes matured *in vitro* before vitrification was significantly higher than in immature oocytes, which were vitrified, and then matured *in vitro*. Cao *et al*. (2009) similarly announced that the oocyte maturation rate in oocytes that were vitrified at the GV stage and afterwards matured *in vitro*, compared with oocytes that were first matured *in vitro* and followed by vitrification, was significantly reduced.

Epigenetic effects of cryopreservation on oocytes (such as non-coding RNAs; miRNAs)

Vitrification of porcine oocytes prompted changes in histone H4 acetylation and histone H3 methylation of lysine 9 (Spinaci *et al*., 2012). Also, vitrification significantly reduced the expression of *DNMT1* mRNA in mouse metaphase II oocytes (Zhao *et al*., 2013; Cheng *et al*., 2014). The results of experimental studies showed that the expression level of *DNMT1* decreased in the vitrified IVM group. Decreased *DNMT1* gene expression usually alters DNA methylation patterns, leading to impaired gene expression, genomic printing, and genome stabilization, ultimately leading to cell death. Various studies have shown that, following the freezing and thawing of oocytes, H3K9 methylation and H4K5 acetylation increased sharply. Usually, no H3K14 acetylation occurs in fresh or frozen MII oocytes. Vitrified oocytes are highly sensitive to changes in H3K9 and H4K5 (Yan *et al*., 2010; Rasti and Vaquero, 2018). Histone methylation involves arginine and lysine residues and plays an important role in regulating transcription, genome integrity and epigenetic inheritance. Overall, the results showed that cryopreservation may be associated with changes in physiological epigenetic marks with putative long-term effects on the cells and/or their derivatives.

According to some reports, vitrification of oocytes did not affect zygote cleavage rates, but reduced the blastocyst rate. Chen *et al*. (2016) reported that the degrees of DNA methylation and H3K9me3 in vitrified oocytes and early cleavage embryos were lower than in the control group (non-vitrified group), albeit in the vitrified group the level of acH3K9 increased in the early cleavage stages. These changes in methylation may be due to changes in the expression of micoRNAs in vitrified oocytes. The expression levels of putative-imprinted genes (*PEG10*, *XIST*, and *KCNQ1O1T*) were upregulated in blastocysts. These epigenetic abnormalities may be partially explained by the altered expression of genes associated with epigenetic regulations. DNA methylation and H3K9 modification suggested that oocyte vitrification may excessively relax the chromosomes of oocytes and early cleavage embryos. Consequently, these epigenetic indexes can be used as damage markers of oocyte vitrification during early embryonic development (Chen *et al*., 2016). Overall, methylation in bovine MII oocytes has been reported to decrease significantly after vitrification with EG and DMSO as cryoprotectants and 0.25-ml straws as a container (Hu *et al*., 2012). In mice, DNA methylation was also significantly decreased in vitrified MII oocytes and in 2-cell- to 8-cell-stage embryos compared with the control. The data indicated that vitrification of the oocyte seemed to always results in the reduction or loss of DNA methylation in the oocyte or embryo, which may lead to impaired embryonic development and qualities in later stages (Hu *et al*., 2012).

miRNAs

Specific characteristics of miRNAs in oocytes (oocyte maturation)

miRNAs are an important class of small non-coding RNAs of ~22 nucleoids that act as effectors of post-transcriptional gene silencing, and also act as a potent regulator of gene expression in most biological systems and cellular contexts. In eukaryotic organisms, miRNA genes are highly conserved, affect specific biological processes such as amplification, differentiation, and signal transduction, and are widely involved in development, physiology, and pathology (Flynt and Lai, 2008; Sun and Lai, 2013).

https://doi.org/10.1017/S0967199423000321 Published online by Cambridge University Press

miRNAs typically suppress the expression of 'target' genes by antisense base-pairing interactions with the 3′ untranslated region (3′UTR). An active miRNA molecule binds to the 'target' mRNA through Watson–Crick base pairs using an essential seven-nucleotide seed sequence (Sun and Lai, 2013). Due to this short pairing, a miRNA has the potential to bind to hundreds of 3′UTR targets. Hundreds of miRNAs have been examined based on *in silico* analysis; miRNAs were initially reported to regulate ~30% of the expressed mammalian genes (Sevignani *et al*., 2006), which increased to more than 60% (Friedman *et al*., 2009). Given the potential for these small molecules to dramatically alter cellular behaviour and function, it continues to be crucial to understand how miRNAs are themselves regulated. miRNA biosynthesis is a sequential process that provides ample opportunity for complex regulation.

General and specific studies of miRNA profiles have shown that changes in the expression pattern of biologically relevant miRNAs occur during the growth and development of mammalian oocytes (Tesfaye *et al*., 2009; Xu *et al*., 2011). For example, miR-21 is a miRNA that potentially targets genes involved in fatty acid metabolism and fatty acid biosynthesis, and is expressed in different patterns during oocyte maturation (Song *et al*., 2016). Inhibition of miR-21 by an anti-miR21 peptide nucleic acid leads to a reduced ratio of porcine oocytes to the MII stage and further embryo development (Wright *et al*., 2016). Similar results by inhibiting the activity of let-7c, miR-27a and miR-322 in mouse oocytes (Kim *et al*., 2013), miR-15/16 in *Xenopus* oocytes (Wilczynska *et al*., 2016) and miR-378 in the porcine oocyte (Pan *et al*., 2015) have been reported. Conversely, increasing the expression of miR-224 in cumulus cells reduced the rate of oocyte maturation and blastocyst growth by regulating PTX3 expression (Li *et al*., 2017).

The results showed that specific changes in miRNA expression were associated with significant changes in the function and morphology of aged oocytes. Experimental studies on the function of key proteins predicted by KEGG analysis and injection of miR-98 mimics or inhibitors have confirmed that miRNAs have stimulatory/inhibitory roles in post-ovulatory oocyte ageing (Wang *et al*., 2017b). Whereas miR-21 acts as an anti-apoptotic, miR-29b, miR-15a and miR-16, miR-128, and miR-98 act as pro-apoptotics (Xia *et al*., 2018). However, all these six apoptosis-related miRNAs significantly increased their expression from 18 to 24 h after hCG injection. The study showed that miR-98 upregulated the expression of caspase-3, which increased the release of Ca^{2+} from the endoplasmic reticulum into the oocyte, leading to increased spontaneous ovulation activity (SA). Therefore, the results suggested that increased oocytes in SA were associated with increased cytoplasmic Ca^{2+} (Zhang *et al*., 2014). Experimental results showed that suppressed expression of miR-224 in cumulus cells (CC) was essential for oocyte maturation. Overexpression of miR-224 stopped or delayed the progression of oocyte maturation, whereas inhibition of miR-224 induced oocyte development. As a result, overexpression of miRNA-224 led to the decreased expression of genes and proteins associated with oocyte maturation such as GDF9, BMP15, CX37, and ZP3 in CC and oocytes (Li *et al*., 2017).

Oocyte cryopreservation and miRNAs

In general, growth and development in oocytes occurred as a result of various cellular and molecular changes during ovarian follicular development. These changes are controlled by endocrine and paracrine factors coordinated by the oocyte and surrounding somatic cells. These processes are regulated by the precise expression and interaction of many genes in different parts of the ovary (oocytes, granulosa, and theca cells) to achieve oocyte development (Bonnet *et al*., 2007) and are possibly regulated by miRNAs. Studies on miRNAs have shown that miRNAs are involved in the regulation of oocyte cross-communication and CC (Assou *et al*., 2013). Therefore, altering the expression of miRNAs at different stages of oocyte development can have a negative effect on the maturation and competence of the oocyte and the resulting embryo.

In most mammals, the process of meiotic division in oocytes arrests at the stage of the dictate of prophase I until the resumption of meiosis. Despite the extensive transcript turnover in the oocyte, it is almost accepted that the meiotic maturity of the oocyte is transcriptionally quiescent. Therefore, the fertilization and early development of the embryo depend on the maternal transcripts that have accumulated during the development of the oocyte. This mother-to-zygotic transition occurs in different species at different times in the embryonic stage. Recent studies have detailed conflicting reports on the occurrence of transcription following the resumption of oocyte meiotic maturation. Increased expression of some transcripts during oocyte maturation has been reported between immature (GV) and *in vivo* matured oocytes (MII) in mice, cattle, and humans (Assou *et al*., 2006). The comparison of results between GV and MII oocytes showed an increased expression of four miRNAs (hsa-miR-193a-5p, hsa-miR-297, hsa-miR-625, and hsa-miR-602), and decreased expression of 11 miRNAs (hsa-miR-888, hsa-miR-212, hsa-miR-662, hsa-miR-299–5p, hsa-miR-339–5p, hsa-miR-20a, hsa-miR-486–5p, hsa-miR-141, hsa-miR-768–5p, hsa-miR-376a, and hsa-miR-15a; Xu *et al*., 2011). Expression of specific miRNAs in human oocytes showed dynamic changes during meiosis. High concentrations of FSH in IVM medium had the opposite effect on the expression of hsa-miR-15a and hsa-miR-20a. A study to confirm miRNA dynamic changes in bovine GV, MII oocytes, and presumptive zygotes (PZ) showed that bta-miR-155, bta-miR-222, bta-miR-21, bta-let-7d, bta-let-7i, and bta-miR-190a had progressive changes between stages from GV to PZ. pri-miR-155 and pri-miR-222 are not normally detected in GV oocytes, but pri-miR-155 is present in MII oocytes, indicating transcription during maturation. miR-148a was found in large amounts with stable expression at all stages (Gilchrist *et al*., 2016).

However, in the maturing oocyte, several significant pathways have the strong potential for repression by miRNAs that are expressed, including phosphatidyl inositol phosphate binding (miR-222), transcriptional regulatory region DNA binding (miR-21), and regulation of the protein kinase cascade activity (let-7d). Studies of miRNAs in other cells, such as cancer tissue, have shown that *p53* is targeted by the let-7 family of miRNAs. p53 is known to be widely involved in cell cycle checkpoints and is present in early bovine embryos (Tomek and Smiljakovic, 2005). In addition, miR-155 targets the mRNA of inositol 5-phosphatase 1 (*INPP5D*) and reduces inositol 5-phosphate 1 (O'Connell *et al*., 2009). A decrease in INPP5D has been shown to increase AKT activity, a pathway involved in bovine oocyte maturation (Yamanaka *et al*., 2009). The data showed that let-7 (Takahashi *et al*., 2012) and miR-27a (Kim *et al*., 2013) regulate cell cycle signalling pathways. Studies have shown that miR-322 modulates ovarian development, folliculogenesis, and cell differentiation (Leem *et al*., 2011; Kim *et al*., 2013).

The results showed that several miRNAs were expressed in human oocytes and blastocysts, targeting key genes involved in

https://doi.org/10.1017/S0967199423000321 Published online by Cambridge University Press

DNA repair and cell cycle checkpoints. Most miRNAs are generally expressed at lower levels in the blastocyst than in the oocyte. The results of the correlation analysis showed that there was an inverse and direct relationship between miRNAs and their target mRNAs (Tulay *et al.*, 2015). This study investigated the expression of 10 mRNAs and 20 miRNAs in human oocytes and blastocysts. The results showed that all mRNAs studied and 11 miRNAs were expressed in both oocytes and blastocysts. However, mRNA expression levels in oocytes were higher relative to blastocysts. This result appeared normal because the oocyte must transfer enough mRNAs to support the resulting zygote's early development and maintain survival and development until embryonic genome activation. The results of the molecular analysis showed that miRNA can be involved in various processes of oocyte maturation. The importance of miRNA in oocyte maturation has initially been demonstrated in knockdown or knockout studies, and it has become clear that Dicer is required for meiotic spindle integrity and completion of meiosis I (Murchison *et al.*, 2007). Many factors, such as oocyte maturation (*in vitro* or *in vivo*), cryopreservation methods and cryopreservation procedures (slow freezing or vitrification), oocyte vitrification stages (GV or MII oocyte), maternal age etc., can adversely cause changes in the expression pattern of miRNAs in the oocyte (Monzo *et al.*, 2012; Wang *et al.*, 2017a).

According to some studies, ~520 miRNAs have been detected in oocytes, of which only 22 miRNAs were significantly expressed in vitrified oocytes compared with fresh oocytes (Li *et al.*, 2019). Changes in miRNA expression are dynamic during oocyte vitrification. The data showed that miR-21–3p expression increased after cryopreservation. It has been identified that miR-21–3p target genes are involved in physiological and pathological processes (Yan *et al.*, 2015; Xia *et al.*, 2018). This suggested that miR-21–3p suppressed *PTEN* gene expression in umbilical cord blood plasma exosomes (Hu *et al.*, 2018). It also proved that miR-21–3p reduced *PTEN* gene expression by targeting its 3′UTR. PTEN always acts towards the PI3K/AKT pathway, important signalling in regulating cell apoptosis through the regulation of oxidative stress (Matsuda *et al.*, 2018). Studies have shown that decreased PTEN expression is associated with increased levels of ROS (Li *et al.*, 2013; Noh *et al.*, 2016), which acts as a factor in reducing the developmental potential of vitrified oocytes (Pan *et al.*, 2015; Wang *et al.*, 2018). In the study, ultrastructural malformations such as increased vacuolation, aberrant dynamic variations in mitochondria–smooth endoplasmic reticulum and mitochondria–vesicle complexes, and scarce cortical granules were observed in vitrified oocytes (Nottola *et al.*, 2016). In addition, disturbed mitochondrial localization has been reported in vitrified mouse oocytes (Yan *et al.*, 2010, 2015). Also, vitrification causes damage to microtubules, actin filaments, and chromosome integrity in the oocytes (Wen *et al.*, 2014).

Studies have shown that storage duration did not affect the gene expression pattern in frozen oocytes and did not alter their expression (Goldman *et al.*, 2015; Stigliani *et al.*, 2015). Oocyte developmental competence has been shown to depend on the accumulation of maternal RNAs and proteins during oogenesis. Conversely, reduced developmental competence in the oocyte is one of the main reasons for IVF failure. Therefore, this proves that the developmental potential of frozen/thawed oocytes is not related to their retention time in liquid nitrogen. Therefore, the possible damage to frozen/thawed oocytes is only due to the method and procedure of cryopreservation, which seems to be more important than the storage time. In support of this, clinical studies have been previously performed on controlled-rate cryopreserved and vitrified human oocytes (Goldman *et al.*, 2015).

Under *in vitro* conditions, the dynamic miRNA profile changes are partly attributed to the *in vitro* maturation environment or ingredients used under *in vivo* conditions. The miRNA profiles could be affected by physiological conditions such as the animal's age. For example, in humans, treatment of metaphase I (MI) human oocytes with insulin-like growth factor 1 activated the expression of miR-133a, miR-205–5p and 145 miRNAs and suppressed 200 others, including miR-152 and miR-142–5p (Xiao *et al.*, 2014). Conversely, altered expression of 12 miRNAs (including let-7b-5p and let-7e-5p) in oocytes derived from older women compared with young women suggested that the *in vivo* miRNA profile in the oocytes was affected by oocyte ageing (Battaglia *et al.*, 2016).

A study in mice showed that, although the expression pattern of the miRNA biogenesis pathway varied between 8-cell embryos and blastocysts, vitrification did not affect the expression level of these genes in preimplantation embryos. Expression levels of miR-21 and let-7a were significantly reduced in vitrified 8-cell embryos and fresh blastocysts compared with fresh 8-cell embryos. STAT-3 expression in blastocysts decreased significantly after vitrification (Azizi *et al.*, 2021). However, changes in the expression pattern of miRNAs in the oocyte and multicellular embryos were compensated in the blastocyst stage. These changes were very small in vitrified and fresh embryos in the blastocyst stage. The results indicated that re-vitrification of embryos changed the expression of miR-16, miR-let-7a and their target genes. These changes caused increased expression of *BCl-2* and *ITGβ3* genes which play important roles in embryo survival and implantation, respectively (Daneshvar *et al.*, 2021).

Method of preservation (slow freezing or vitrification) and miRNAs

Vitrification is relatively simple, does not require expensive programmable freezing equipment, and uses a small amount of liquid nitrogen for freezing. In addition, cryopreservation of oocytes using vitrification has been suggested to maintain female fertility by servicing and freezing their oocytes at optimal times. Experimental data showed that both cryopreservation methods (slow freezing and glass vitrification) had a negative effect on miRNAs and the pattern and gene expression of human MII oocytes compared with non-frozen MII oocytes, but the effects of vitrification on oocyte physiology (developmental competence of oocyte) was less in slow freezing (Monzo *et al.*, 2012; Quan *et al.*, 2017). However, slow-frozen and vitrified MI oocytes showed signatures for specific gene expression. Slow freezing with downregulation of genes that maintain chromosomal structure (*KIF2C* and *KIF3A*) and cell cycle regulation (*CHEK2* and *CDKN1B*) may reduce oocyte developmental competence. In vitrified oocytes, many genes of the ubiquitination pathway are downregulated (including members of the ubiquitin-specific peptidase family and subunits of the 26S proteasome; Monzo *et al.*, 2012). The controlled-rate cryopreservation method (slow freezing) seriously alters specific transcript levels, leading to loss of transcript content (Chamayou *et al.*, 2011; Monzo *et al.*, 2012; Stigliani *et al.*, 2015).

Experimental data showed that the expression profiles of cryopreserved MII oocytes significantly differed from those of non-cryopreserved oocytes in 107 probe sets corresponding to 73 downregulated and 29 upregulated unique transcripts. Gene

https://doi.org/10.1017/S0967199423000321 Published online by Cambridge University Press

Ontology analysis using the DAVID bioinformatics resource disclosed that cryopreservation deregulates genes involved in oocyte function and early embryo development (such as chromosome organization, RNA splicing and processing, cell cycle, cellular response to DNA damage and to stress, DNA repair, calcium ion binding, malate dehydrogenase activity, and mitochondrial activity; Stigliani *et al*., 2015). A study on mice indicated that the influence of vitrification on the transcriptome of oocytes was negligible as no differentially expressed genes were found between vitrified and fresh oocytes. The MII stage is more suitable for oocyte vitrification with respect to the transcriptome (Gao *et al*., 2017).

Comparison of IVF results from slow-frozen and vitrified oocytes showed that vitrification improved survival, fertilization, and pregnancy rates (Cao *et al*., 2009; Fadini *et al*., 2009), although only Fadini *et al*. (2009) reported significantly higher pregnancy rates (18.2 vs. 7.6%). Increasing evidence on the effectiveness of IVF with vitrified oocytes showed that it could achieve similar results to IVF using fresh oocytes, with an oocyte survival rate of more than 84% (Rienzi *et al*., 2012). A meta-analysis of five studies showed that the fertilization rate, embryo cleavage, high-quality embryos and ongoing pregnancy progress did not differ between the vitrification and fresh oocyte groups (Cobo and Diaz, 2011). Some IVF programmes now use vitrification to cryopreserve oocytes (Brison *et al*., 2012; Glujovsky *et al*., 2014).

Applications of miRNAs in assisted reproduction technology

Due to the important role of miRNAs in the suppression or expression of post-translational mRNAs, in addition to treating some diseases, they can be used to improve the maturity of oocytes, *in vitro*-produced embryos and determine the non-invasive quality of oocytes. Data showed that increased expression of *HDAC6* by miR-762 suppression may improve the current protocol for oocyte vitrification. Overexpression of HDAC6 or reduction of miR-762 improved the survival rate, cleavage rate, and blastocyst rate of oocytes after oocyte vitrification (Wang *et al*., 2018). The results showed that fresh ovine embryos expressed the *HDAC1* gene at a higher level than their vitrified counterpart embryos (Bahr *et al*., 2016). Histone acetylation is critical for proper cellular functions, including chromosome condensation, DNA double-stranded breakage repair, and mRNA transcription, but there is no evidence for the effect of HDAC6 on *in vivo* development of the blastocysts from cryopreserved oocytes. The interpretation of this issue will depend on future works.

Conclusion

Oocyte vitrification has been proven to be a successful approach for the long-term storage of oocytes and maintaining women's fertility. Various molecular studies have shown that cryopreservation can alter the expression of miRNAs in vitrified oocytes compared with fresh oocytes. These changes can affect the expression of some genes and lead to physiological and metabolic changes in the vitrified oocytes and early embryo development, which can reduce the final competence of the frozen oocytes and the resulting embryos (Liu *et al*., 2003; Di Pietro *et al*., 2010; Gao *et al*., 2017). However, the rate of change resulting from cryopreservation is much lower than *in vitro* maturation of oocytes. Conversely, several studies have reported that cryopreservation did not alter gene and miRNA expression (Anchamparuthy *et al*., 2010; Monzo *et al*., 2012; Stigliani *et al*., 2015; Shahedi *et al*., 2017; Wang *et al*., 2017a). Therefore, further research is needed to investigate the expression of genes and miRNAs in vitrified oocytes and their embryos. It seems that these changes in the expression of miRNAs and genes can be compensated for in other stages of development, because these minor changes may be resolved due to 'embryo adaptability' and may not significantly affect embryo competence.

Availability of data and material. All data searched in this study are included in this publication.

Acknowledgements. We are thankful to the anonymous reviewers for their critical reading of the manuscript and suggestions for improvement.

Funding. This research received no specific grant from any funding agency, commercial or not-for-profit sectors.

Code availability. Not applicable.

Consent to participate. Not applicable.

Competing interests. The authors declare no conflict of interest. All authors approved the final manuscript for publication.

Ethics approval. This is a literature review article that examines past studies and the references used in the text.

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https://doi.org/10.1017/S0967199423000321 Published online by Cambridge University Press