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Chapter

Beyond Survival Effects of Vitrification-Warming on Epigenetic Modification and Maternal Transcripts of Oocytes

Yu-bing Liu, Ju Chen and Ri-Cheng Chian

Abstract

Oocyte vitrification-warming is a worldwide used technique for human fertility preservation. The question of whether the potential risk is associated with this specific procedure remains unresolved. As a fundamental factor of development, oocytes play an important role in early embryonic development, including epigenetic reprogramming and maternal-to-zygotic transition (MZT), that can develop to term. Vitrification, as a significant stressor, appears to have a significant impact on epigenetic modifiers and maternal transcripts of the oocyte, which ultimately results in lower developmental potential. Due to the rapidly evolving single-cell multi-omics sequencing, there have been many advances in this field. We will discuss recent progress in the impact of oocyte vitrification on epigenetic modification and maternal transcripts in this manuscript, hoping to provide a theoretical basis for the optimization and improvement of vitrification-warming technology.

Keywords: oocyte, Vitrification-warming, epigenetics, maternal transcripts

1. Introduction

Oocyte survival and pregnancy rates have increased significantly since the development of vitrification technology. Many studies have proven that rates of survival, fertilization, and cleavage as well as embryo quality after vitrification are equivalent to those of fresh oocytes [1, 2]. In 2013, the American Society for Reproductive Medicine listed “freezing of human mature oocytes” as a technology that could be widely used in women undergoing gonadotoxic therapy [3]. The scope of clinical application of oocyte vitrification cryopreservation is gradually expanding, including fertility preservation before radiotherapy and chemotherapy for malignant tumors, ART remedy for sperm collection failure on the day of oocyte retrieval, donation of oocytes, and female fertility preservation for non-medical reasons, with an increasing number of babies born through this technology [4–6].

It is acknowledged that oocyte vitrification-warming is still a developing technique, though widely used. Pregnancy outcomes from vitrified oocytes vary from the quality

of oocytes before freezing and the operators, who have different knowledge levels and use different technologies [7, 8]. In addition, several studies have reported that vitrification of mammalian oocytes can induce the generation of abnormal reactive oxygen species (ROS), accumulation of histone γ -H2AX, and increase apoptosis rate, as a result of lowering developmental potential of both early embryo and fetal [9–11].

Beyond survival and embryo development, it is essential to study the impact of oocyte vitrification-warming on the long-term safety of offspring from the perspective of epigenetics. Epigenetic modifications during oocyte genesis are crucial in subsequent embryonic development as well as individual development. Epigenetics refers to the process leading to the heritable changes of gene functions and phenotypic changes without modifying the nucleotide sequences, mainly, including DNA methylation, histone modification, and regulation of non-coding RNA, which can directly or indirectly influence the state of chromatin to activate or repress gene expression [12].

In primordial germ cells (PGCs), epigenetic reprogramming leads to germ cell-specific characteristics, such as meiosis, spermatogenesis, and oogenesis. Upon fertilization, the totipotency acquisition and development of embryos also rely on epigenetic reprogramming. In addition, toward the end of oogenesis, condensed chromatin prevents gene transcription, resulting in transcriptional silencing. As a consequence, the oocyte must synthesize and preserve sufficient transcripts to fulfill protein requirements during the period of meiosis completion, fertilization, and maternal-to-zygotic transition (MZT) [13].

Vitrification-warming procedures may affect oocyte epigenetic modifications and the composition of the maternal transcripts, which in turn affect epigenetic reprogramming, may have a significant impact on the biological processes of subsequent embryonic development [14]. This review will discuss the effects of vitrification-warming on epigenetic reprogramming and maternal transcripts of oocytes.

2. Effects of vitrification-warming on DNA methylation of oocytes

DNA methylation is a major epigenetic mark in DNA, which is essentially a covalent modification. DNA methylation is controlled by a family of DNA methyltransferases (Dnmts), which can transfer the methyl group of S-adenosyl methionine (SAM) to the carbon-5 position of cytosine (5mC) of cytosine-guanine (CpG) dinucleotide. DNA methylation can control gene expression by altering chromatin structure, DNA conformation, DNA stability, and the interaction between DNA and protein [15]. In mammals, CpG sites in gene bodies and intergenic regions are generally highly methylated, except for gene regulatory sequences, such as promoters and enhancers, which are usually low or intermediate DNA methylated [16]. Several studies have suggested that DNA methylation in promoter regions plays a regulatory role in the genome by interfering with transcription factor binding, recruiting methyl-CpG binding proteins (MeCP1, MeCP2, and other MBD proteins) and repressor complexes, leading to stable transcriptional repression [17, 18].

Mammals undergo two massive waves of global DNA demethylation and remethylation during the intergenerational transmission of life. The first major wave of genome-wide DNA demethylation occurs in PGCs, which are the progenitors of oocytes and sperm, where the paternal and maternal somatic programs are erased. Subsequent to this, DNA methylation was reestablished during spermatogenesis and oogenesis [19]. Different from male germ cells, which can rapidly initiate and

complete DNA remethylation before birth, oocytes need to gradually remethylate through de novo DNA methyltransferases (DNMT3A, DNMT3L, etc.) over a long period of development, from the primary stage to the antral follicle stage.

During fertilization and early embryogenesis, parental genomes undergo a second wave of demethylation and remethylation for epigenetic reprogramming. It is well known that DNA methylation reprogramming prior to the blastocyst stage is critical for early embryo development and subsequent ontogeny [20]. It is generally believed that the DNA methylation status of oocytes and early embryos is very sensitive to external stimulus, which potentially may lead to the low developmental competence and quality of embryos. Vitrification-warming is such a strong stimulus that its effects on the DNA methylation pattern of oocytes cannot be ignored.

Most research on the effect of oocyte vitrification-warming is performed with murine and bovine animals. It was found that the overall DNA methylation level of bovine oocytes was decreased after vitrification-warming [21, 22]. In mouse oocytes, vitrification-warming significantly reduced the methylation levels in the promoter regions of pluripotency gene Oct4 and Sox2, in addition to the decrease in the methylation levels of imprinted genes H19, Peg3, and Snrpn in blastocysts [23, 24]. However, vitrification-warming did not significantly alter the methylation levels of CpG islands in the promoter regions of Dnmt1o, Hat1, and Hdac1 [25].

Few studies have been conducted on the DNA methylation of vitrification-warming on human oocytes. In 2015, a study analyzed the effects of oocyte vitrification-warming on methylation levels in Day 3 embryos with immunofluorescence staining of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC), which showed that there was no significant difference in DNA methylation between embryos derived from young donors vitrified and fresh oocytes [26]. Two other studies analyzed that oocyte vitrification-warming at the GV stage had no significant effect on DNA methylation of oocytes after *in vitro* maturation, using 5-methyl cytosine (5mC) immunofluorescence staining and imprinted genes H19 and KCNQ1OT1 bisulfite sequencing [27, 28]. However, as we know, immunofluorescence staining can only reflect the global DNA methylation level, and cannot measure the methylation levels at single-base resolution. Bisulfite sequencing can allow for the determination of methylation patterns at single-base resolution. However, as it is limited by throughput, only a few genes can be selected for detection.

In conclusion, oocyte vitrification-warming may decrease the DNA methylation level of oocytes or embryos, especially the promoter regions. However, most studies analyze DNA methylation of different gene regions, and lack of relevance between each other requires more experimental studies to be confirmed in the future. Limited by the resources and analytical methods, there are few studies about the effects of human oocyte vitrification-warming on DNA methylation, and the results are not consistent with that in animal models. Furthermore, it remains unclear whether oocyte vitrification-warming has different effects on DNA methylation differ among species. In recent years, the rapid development of whole-genome DNA sequencing of single cells (scWGS) enables the detection of global DNA methylation from the single base level, which makes us believe that more in-depth investigation will soon be available.

3. Effects of vitrification-warming on histone modification of oocytes

Histones are the core components of nucleosomes, the structural unit of chromatin. The nucleosome consists mainly of an octamer of four histones (two copies each

of H2A, H2B, H3, and H4) and 147 base pairs of DNA fragments around the outside. The free N-terminus of histones can undergo a variety of modifications, including acetylation, methylation, phosphorylation, ubiquitination, ADP ribosylation, and so on, which can control the state of chromosomes to produce different biological effects, such as transcriptional repression or activation [29, 30].

Histone modifications often cooperate with DNA methylation to control the state of chromosomes to activate or inhibit genes. Basically, DNA methylation in promoter regions is related to transcriptional inhibition, while histone acetylation is related to transcriptional activation. Actually, when DNA is methylated, lysine 9 of histone H3 is demethylated or trimethylated (H3K9me₂/me₃), chromatin is compressed, and transcription is blocked. Conversely, when DNA is demethylated, lysine 9 of histone H3 is acetylated, lysine 4 of histone H3 is demethylated or trimethylated (H3K4me₂/ME₃), chromatin is relaxed, and transcription is facilitated [31].

Similar to DNA methylation, histone modifications are highly dynamic during oogenesis and preimplantation embryo development. For example, in the early growth stage of mouse oocytes, H3K4me₃ appears as a canonical pattern (Canonical H3K4me₃) at promoter regions, and then in the later stage of oocyte maturation, non-canonical H3K4me₃ (ncH3K4me₃) gradually replaces canonical H3K4me₃, covering broad domains in both promoters and distal regions, which makes up approximately 22% of the oocyte genome. In 2-cell embryos, the coverage of ncH3K4me₃ decreases rapidly and is replaced by canonical H3K4me₃, which is gradually restricted to the transcription start site regions, instead of being far away from the transcription start site (TSS). It is believed that the broad establishment of ncH3K4me₃ in oocytes and timely deletion at the 2-cell stage are essential for embryo development. The broad establishment of H3K4me₃ in the early stage can maintain DNA hypomethylation, which is associated with gene silencing, while the gradual removal of H3K4me₃ and its restriction to promoters in later stages contribute to the activation of the zygotic genome and embryonic development [12, 32].

In addition, H3K27me₃ has been shown to be intergenerationally inherited from the maternal genome during early embryogenesis, involved in regulating the activation of enhancers and lineage-specific genes [12, 33]. During the early embryonic development of mice, H3K9me₃ has been found to be mainly enriched in long terminal repeats (LTR), which inhibits the expression of LTR. The abnormal H3K9me₃ reprogramming is deemed to directly cause ZGA failure [33].

Therefore, the histone modifications of the oocyte are so vital to zygote and subsequent embryo development that we have to take into account the effect of oocyte vitrification-warming on histone modifications. By now, the studies that analyzed the effect of oocyte vitrification-warming on histone modification have only been conducted in animals. Due to different animal species and histone modifications studied, the reliability of the results needs to be further verified.

The study has shown that vitrification-warming could significantly reduce the H3K9me₃ level of bovine oocytes and cleavage-stage embryos, significantly increasing the H3K9 acetylation level of cleavage-stage embryos, while significantly decreasing the H3K9 acetylation level of the blastocyst-stage trophectoderm [22]. Besides, oocyte vitrification-warming has been reported that could increase histone H4 acetylation in porcine and murine oocytes, and increase H3K9 methylation in murine oocytes [34–36]. Overall, oocyte vitrification-warming may lead to significant changes in histone modifications, but the results are inconsistent with each other. As the most commonly used detection method, the immunofluorescence method has the problems of low sensitivity and strong subjectivity, which may be

the reason for the inconsistent results. In addition, current studies mainly focus on histones H3 and H4, while there are few studies on histones H2A and H2B. The application of single-cell epigenetic detection technology in oocytes contributes to a more comprehensive study of the effect of vitrification-warming on histone modifications.

4. Effects of vitrification-warming on non-coding RNA of oocytes

The vast majority of human genes can be transcribed into RNA, but only about 2% of the genome codes for protein. RNA without protein-coding potential is called non-coding RNAs (ncRNAs), and most of the RNA is ncRNAs. According to the number of nucleotides, ncRNAs can be divided into small RNAs (small ncRNAs, less than 200 nucleotides) and long non-coding RNAs (lncRNAs, more than 200 nucleotides), and there is also covalently closed circular RNAs (circ-RNAs) [37].

In mammals, lncRNAs are involved in the regulation of transcription, which is related to cell pluripotency and differentiation. For example, Xist lncRNA and its antisense sequence Tsix, associate with the complex regulation of X chromosome inactivation (XCI), thus silence most of the genes on one X chromosome in female mammals to correct for a double number of X chromosome genes [38]. Small non-coding RNAs as a class of important post-transcriptional regulators, mainly contain micro RNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs), and Piwi-interacting RNAs (piRNAs) [39]. The miRNAs can interact with the 3' untranslated region of target mRNA to initiate mRNA degradation and inhibit gene expression, which plays an important role in the development of mammalian oocytes and embryos [40]. Studies have shown that aberrant miRNA expression in female germ cells and embryos is associated with infertility and embryogenesis defects [41]. In addition, it has been suggested that RNase III Dicer is important in siRNA processing, and knocking out Dicer in oocytes can lead to meiosis arrest, accompanied by severe spindle and chromosome segregation defects, therefore, endo-siRNAs may be crucial to oocyte meiosis [39].

Non-coding RNAs are so sensitive to the environment that we need to consider whether they are affected by the vitrification-warming procedure. In 2019, a study about the comparison of miRNAs in mouse fresh oocytes and vitrified oocytes found that 22 miRNAs were differentially expressed between the two groups, and most of the target genes regulated by these miRNAs were closely related to metabolic pathways. Real-time quantitative PCR and sequencing results showed that Mir-134-5p, Mir-210-5p, and Mir-21-3p were significantly up-regulated in vitrified oocytes, while Mir-465C-5p was significantly down-regulated [42]. In addition, as a negative regulator of the PI3K/AKT signaling pathway, PTEN plays an important role in coordinating primordial follicular activation and oocyte DNA damage repair [43]. However, the expression of potential target PTEN in vitrified oocytes was significantly decreased at both the transcriptional level and the post-transcriptional level (protein level). Another study on vitrified human oocytes with RNA-seq also found the down-regulation of lncRNAs (CTB-180A7.6, AP000320.7, OOEP-AS1, RP11-59H7.3) [44]. Therefore, oocyte vitrification-warming may result in a decrease in the expression of certain specific non-coding RNAs.

At present, there are few studies on lncRNAs and other types of short coding RNAs in oocyte and early embryonic development, and their functions have not been fully explored. It is also rarely studied whether oocyte vitrification-warming has an impact on these non-coding RNAs.

5. Effects of vitrification-warming on oocyte maternal transcripts

The effect of vitrification-warming on gene expression of human oocytes is still in the preliminary stage. A study using the microarray method to compare gene expression between fresh and vitrified human mature oocytes showed significant differences in gene expression between the two groups, especially the downregulation of many genes in the ubiquitination pathway [45]. Another study, using transcriptome sequencing, also found that vitrification-warming reduced transcription levels of genes closely related to human oocyte genesis and development, involving multiple biological processes, such as cell cycle (NCAPD2 and TUBGCP5 significantly down-regulated), meiosis process (NCAPD2 and TUBB4B significantly down-regulated), multiple metabolic pathways (tricarboxylate cycle, amino acid metabolism, oxidative phosphorylation, etc.), DNA methylation, and DNA damage repair etc., among which HSPA1A and HSPA1B are the most representative ones [44]. HSPA1A and HSPA1B, as members of the heat shock protein family A (HSP70), can reverse or inhibit the denaturation or unfolding of cellular proteins under stress or high temperature, which are an important anti-stress defense system, participating in several cell functions, including regulation of mitosis, ubiquitin-proteasome pathway, regulation of lysosomal membrane stability, etc. It has strong resistance against abnormal apoptosis in cells and plays an important role in immune regulation outside cells. In addition, in early embryo development, the expression levels of HSPA1A and HSPA1B are closely related to the heat shock response ability of the embryo [46]. The downregulation of HSPA1A and HSPA1B gene expression goes against protein synthesis in biological processes, thus lowering the developmental potential of oocytes.

Additionally, one study, analyzing the effect of vitrification-warming on the expression of specific genes by real-time PCR, found that the overall mRNA content of oocytes decreased significantly (63.3% retained) after vitrification-warming, and the expression levels of genes relevant to DNA tissue structure, mitochondrial energetic pathway, cell cycle regulation (NAP1L1, TOP1, H1FOH1, SMC, SCC3, PAD21, SMC1A, SMC1B, etc.) were significantly decreased [47]. In contrast, several studies suggested that vitrification-warming had no significant effect on the expression levels of oocyte cytokinesis-related genes (DCTN1, DCTN12, DCTN13, DCTN16, and PLK1) and oocyte development-related genes (HPRT, GAPDH, CYCLOPHILIN, BMP15, GDF9, etc.) in oocytes [48, 49]. In brief, most studies have concluded that oocyte vitrification-warming results in a decrease in the transcription levels of genes involved in important biological processes in human oocytes.

In animals, there were four studies conducted in murine, bovine, and pig models to investigate the effect of vitrification-warming on gene expression levels, using transcriptome sequencing [50–53]. It was found that vitrification-warming had no effect on the transcriptome of mouse oocytes [50]. On the contrary, differentially expressed genes have been found in bovine and porcine vitrified oocytes, especially genes related to transcriptional regulation, cell division and differentiation, and apoptosis pathways [51–53]. In addition, the expression levels of epigenetic modification related genes (Mad1, Mad2, BubR1, Peg3, Igf2R, Sirt1, Dnmts, CD9, CD81, HMG3a, etc.) were significantly down-regulated after vitrification-warming, using real-time quantitative PCR [24, 25, 54–57]. Vitrification-warming could up-regulate the expression of pro-apoptotic genes BAX and P53 and down-regulate the anti-apoptotic gene BCL2 in bovine and porcine oocytes [58–61].

Vitrification-warming also caused overexpression of Eg5, a gene promoting cell division, in bovine oocytes [60]. It was also found that vitrification-warming decreased the expression of CD9 in mouse oocytes, which may lead to difficulties in fertilization [62].

In conclusion, most studies believe that vitrification-warming can lead to down-regulation of gene expression levels in animal and human oocytes, which may account for the decreased developmental potential of vitrification-warming oocytes.

6. Summary and outlook

Compared with slow freezing, vitrification has become an important means of the oocyte *in vitro* preservation as an important part of assisted reproductive technology (ART), which can improve the survival rate of oocytes. It is worthy of studying and discussing the impacts that oocyte vitrification-warming has on subsequent embryonic development and the later generations. This review mainly summarizes the effects of oocyte vitrification-warming on epigenetic modifications and transcriptional levels.

Generally, vitrification-warming decreased the level of global DNA methylation in oocytes or embryos of the mouse, cattle, and pig models, especially the methylation level in promoter regions [21–24]. Few studies that assessed the effect of human oocyte vitrification-warming on epigenetic modification have found that vitrification has no significant effect on DNA methylation [26–28]. The results are inconsistent with that in animal oocytes. The differences may be due to the low accuracy of immunofluorescence staining, the short sequence length of bisulfite sequencing, or the differences between species, which need to be confirmed by further studies.

In addition, there are a few studies on the effect of vitrification-warming on the transcriptome of human oocytes. Two studies have found no significant difference in the transcriptome characteristics of fresh oocytes and vitrified oocytes [48, 49], while the other three have suggested that vitrification-warming reduces the transcription levels of genes closely related to ovulation and developmental processes, involving multiple biological processes [44, 45, 47]. Therefore, the effect of vitrification-warming on the transcriptome of human oocytes is still inconclusive. The reserve of oocyte maternal transcripts is so essential for fertilization and maternal-to-zygotic transition (MZT) that we cannot ignore the effects of vitrification-warming on human oocyte maternal transcripts, which needs further study.

In animal models, oocyte vitrification resulted in a decrease in the global DNA methylation, changes in histone modifications, differential expression of miRNAs, and a decrease in gene expression levels, thereby reducing the rates of oocyte cleavage and blastocyst formation and the development potential of the early embryo. Another study has found that the maturation rate of vitrified immature human oocytes decreases after thawing, though most people believe that vitrification-warming has little effect on epigenetic modification and maternal transcripts of human oocytes [63]. In addition, there is still a lack of reliable data on whether the epigenetic or expressional changes possibly resulting from oocyte vitrification-warming have any effect on the subsequent offspring, which needs to be further studied.

Mitochondrial activity and redox homeostasis in oocytes are vital for proper embryonic development. It is well known that ROS is an unavoidable byproduct of mitochondrial respiration. Oxidative stress (OS) in oocytes, stemming from the imbalance of ROS production and their neutralization, is related to low rates of

fertilization and embryo developmental potential [64, 65]. Mitochondrial activity and ROS levels are most often affected by extreme conditions imposed by vitrification [66]. Studies found that vitrification significantly affects mitochondrial distribution and mitochondrial potential in bovine, human, rabbit, murine, and porcine oocytes [66–68]. However, it has been suggested that mitochondrial membrane potential reduction caused by vitrification in human oocytes is temporary and would be fully recovered after 4 h culture. In addition, evidence indicates that supplementation of antioxidants in a cryopreservation medium could enhance the developmental competence of vitrified oocytes by preventing mitochondrial damage and reducing oxidative stress [69–71].

In conclusion, there are few studies on the effects of vitrification-warming on epigenetic modification and maternal transcripts of human oocytes, which is still in their infancy. In order to get conclusive evidence, new technologies to analyze the methylation and transcription levels with a small amount of material should be applied. In addition, we need to examine the effects of vitrification variables (type and concentration of refrigerant, cryostorage duration, oocytes at different stages, etc.) on biological processes more thoroughly, so as to continuously optimize the safety and sustainability of the vitrification process.

Conflict of interest

The authors declare no conflict of interest.

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