Advantages of vitrification preservation in assisted reproduction and potential influences on imprinted genes

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Abstract
Cryopreservation has important application in assisted reproductive technology (ART). The vitrification technique has been widely used in the cryopreservation of oocytes and embryos, as a large number of clinical results and experimental studies have shown that vitrification can achieve a higher cell survival rate and preimplantation development rate and better pregnancy outcomes. Ovarian tissue vitrification is an alternative method to slow freezing that causes comparatively less damage to the original follicular DNA. At present, sperm preservation mainly adopts slow freezing or rapid freezing (LN2 vapor method), although the vitrification method can achieve higher sperm motility after warming. However, due to the use of high-concentration cryoprotectants and ultra-rapid cooling, vitrification may cause strong stress to gametes, embryos and tissue cells, resulting in potentially adverse effects. Imprinted genes are regulated by epigenetic modifications, including DNA methylation, and show single allele expression. Their accurate regulation and correct expression are very important for the placenta, fetal development and offspring health. Considering that genome imprinting is very sensitive to changes in the external environment, we comprehensively summarized the effect of cryopreservation—especially the vitrification method in ART—on imprinted genes. Animal studies have found that the vitrification of oocytes and embryos can have a significant impact on some imprinted genes and DNA methylation, but the few studies in humans have reported almost no influence, which need to be further explored. This review provides useful information for the safety assessment and further optimization of the current cryopreservation techniques in ART.

Keywords: Assisted reproduction, Vitrification, Epigenetics, Imprinted genes, DNA methylation
osmotic pressure and temperature changes. In addition, the high-concentration cryoprotectants used have cytotoxic effects. The possible adverse effects have been investigated by embryologists and reproductive specialists, including the impact of vitrification on epigenetic modifications [12–15]. The epigenetic modification of the genome is very important for regulating gene expression, cell differentiation and function.

An imprinted gene describes one where two alleles from parents are subject to different epigenetic modifications, resulting in the silencing of one parent allele—that is, the state of single allele expression. One hundred and forty-nine imprinted genes are known in mouse, and 128 imprinted genes were found in humans. The presence of imprinted genes explains why both paternal and maternal genomes are required for eutherian and marsupial mammals [16–18]. The single-parent expression of imprinted genes is essential for normal embryonic development, placental differentiation and prenatal and postpartum growth, in addition to normal neurobehavioral processes and metabolism [19–22]. Imprinting abnormalities are associated with human imprinting syndromes such as Beckwith–Wiedemann syndrome (BWS), Prader–Willi syndrome (PWS) and Angelman syndrome (AS), as well as cancers [23, 24]. The establishment and maintenance of genome imprinting depend on normal epigenetic modifications (DNA methylation, post-translational histone modification, chromatin structure and non-coding RNAs) and their interactions. DNA methylation is the most typical epigenetic modification that controls genomic imprinting [25, 26]. There are differentially methylated regions (DMRs) between the two alleles of imprinted genes, that is, a specific gene sequence has DNA methylation modification in one allele, while the other allele is not modified. The methylation difference between alleles is acquired at gamete stage, and most imprinted DMRs are methylated on the maternally inherited allele [27]. The imprinting of mature gametes is maintained by a specific mechanism during fertilization and the whole preimplantation development [28, 29]. However, a recent study showed that DMRs regulating some imprinted genes exist demethylation and rapid imprinting stabilization during preimplantation embryo development of pigs [30]. In mammals, after the primordial germ cells (PGCs) arrive at the embryonic gonad, the imprinting marks are removed and then reestablished in a sex-specific manner during subsequent gamete development [31, 32]. The timeline and acquisition of DNA methylation at male and female differ considerably. Imprints are established prenatally in pro spermatogonia, whereas, in female gametes, imprint establishment occurs after birth in the growing oocyte as in de novo methylation of other regions [33].

The majority of imprinted genes in human are largely conserved with respect to methylation status and allelic expression in mouse although there are some exceptions. The protection of DNA methylation at imprinting control regions (ICRs) is required in both human and mouse, but the epigenetic modifiers implicated in this maintenance have evolved distinct roles between species [34].

Genome imprinting is sensitive to changes in environmental factors [35]. A number of conventional assisted reproductive technologies, such as hormone stimulation, in vitro culture, cryopreservation and embryo transfer, cause abnormal methylation of imprinted genes [36, 37]. Genomic imprinting can reflect the impact of assisted reproductive technology (ART) on epigenetic stability, to a certain extent [38, 39]. Recent researches show that ART may be associated with an increased incidence rate of human imprinting syndromes AS and BWS, and most ART patients with AS also showed imprinting defect [40–42]. Although the children born with ART had a normal clinical phenotype, they may have carried abnormal methylation in the imprinting control region [43]. At present, the relationship between ART and imprinting disorder is still controversial, which may be related to the low detection rate of imprint disorder, different assisted reproduction schemes and the lack of appropriate control [44, 45].

Cryopreservation of gametes, embryos and tissues, especially by the vitrification method, has been widely used in the field of assisted reproduction, but related safety assessments remain to be conducted. They may interfere with the normal establishment and maintenance of genomic imprinting, lead to abnormal expression of imprinted genes, affect the normal development and function of fetus and placenta, cause adverse pregnancy outcomes and may pose a potential threat to the health of postnatal offspring. Therefore, the relationship between cryopreservation techniques and imprinting disorder should be paid more attention and studied further. This review mainly summarizes the application and advantages of vitrification preservation in assisted reproduction, and its influences on imprinted genes according to the current reports.

**Development of vitrification technique**

In assisted reproduction, cryopreservation techniques include slow cooling and rapid cooling. The slow freezing method uses programmable freezers to achieve controlled freezing rates. It relies on the balance between the formation rate of ice crystals and the dehydration rate of cells to prevent the formation of ice crystals in cells. The rapid cooling method requires the use of high concentration of cryoprotectants and additives to quickly dehydrate cells, and then, the device containing cells is...
Sperm cryopreservation is an important part of ART and male fertility preservation. It can avoid repeated biopsy in patients with azoospermia and oligozoospermia [52]. Cancer patients can also use this technique to maintain their fertility before receiving chemotherapy or radiotherapy. Due to the small volume and large number of mature sperm and good tolerance to freezing conditions, the conventional freezing technique can obtain an acceptable survival rate. E. Isachenko and V. Isachenko et al. have repeatedly explored the vitrification of sperm. It was not until the improvement in plastic containers and the use of sucrose as a cryoprotectant in 2008 that an acceptable survival rate after warming was achieved [53]. In recent years, the vitrification method of sperm has been continuously optimized and can now obtain a higher survival rate and reduce the degree of DNA damage compared with slow-freezing [54, 55]. High-concentration permeable cryoprotectants are cytotoxic and cannot be used for sperm vitrification due to the uniqueness of sperm [56]. The nonpermeable protectant sucrose can be used for rapid freezing and vitrification of sperm, and vitrification can achieve higher sperm motility [57, 58]. At present, studies discussing sperm vitrification are focused on the selection of cryoprotectants and optimization of the cooling process [59]. In the future, we can expect that the vitrification technique is applied to sperm cryopreservation effectively and safely.

Oocyte and embryo cryopreservation

The vitrification technique has been widely used in oocyte and embryo cryopreservation in recent years and can achieve better clinical outcomes compared with slow-freezing [60, 61]. The method of vitrification and warming of human oocytes was first in the theoretical and highly experimental stage at the end of the last century and entered the stage of practice and experimental test after 2000. It has been applied in assisted reproduction as a standard therapy to preserve fertility since 2013 [62]. Because the growing number of children is conceived by oocyte vitrification without birth defects [63, 64], it appears to be the gold standard technique of preserving fertility in young women [65]. People have recognized many clinical uses and practical advantages of oocyte vitrification, including the following: (1) the fertility preservation of women at risk of fertility loss due to chronic diseases and/or treatment (including Turner syndrome treated with gonadal toxin or radiation, autoimmune diseases and cancer); (2) in the process of assisted reproduction—if appropriate sperm is not obtained, the treatment cycle can be carried out flexibly [66]; (3) reducing the management burden during the treatment cycle of oocyte donation; (4) providing possibilities for patients who choose or need to postpone childbearing age [67]; (5) reducing the cost of infertility treatment; and (6) providing options for patients who are concerned about ethical or legal issues of embryo cryopreservation. Embryo vitrification is the most commonly used method of fertility preservation in ART [68]. If extra embryos are obtained and are of good quality, or when the patient is not suitable for fresh embryo transfer (poor uterine environment, chemotherapy, etc.), the remaining embryos can be cryopreserved and directly transferred after thawing in the next cycle, so as to avoid embryo waste and increase the chance of pregnancy. Embryo vitrification also helps to reduce the rate of multiple births and avoid the occurrence of ovarian hyperstimulation syndrome (OHSS).

Ovarian tissue cryopreservation (OTC)

According to the GLOBOCAN 2020 database, there were 19.3 million new cancer patients in world in 2020. The
new rate of breast cancer has exceeded that of lung cancer, with an estimated 2.3 million new cases, becoming the first cancer to do so, and the mortality rate of breast cancer and cervical cancer is high [69]. With continuous improvements in the medical field, the survival rate of cancer patients has significantly improved [70], but radiotherapy and chemotherapy cause serious fertility damage [71, 72]. How to meet the reproductive needs of such patients has become a global concern. Compared with oocyte and embryo cryopreservation, ovarian tissue cryopreservation does not require ovulation induction and can be directly used before cancer treatment without delaying the treatment time. The cryopreservation of ovarian tissue is not to remove the whole ovary, but generally to cut part of ovarian tissue. Ovarian cortical slices are then cryopreserved through slow freezing or vitrification, which is similar to the principle of oocyte or embryo cryopreservation. Currently, slow freezing is the most frequently used method for cryopreserving human ovarian tissue. OTC is the only fertility preservation method for prepubertal girls, women who cannot delay radiotherapy and chemotherapy and patients with hormone-sensitive tumors [73, 74]. After the transplantation of cryopreserved ovarian tissue, most patients can restore endocrine function. However, only one quarter of women can obtain live birth and the fertilization rate for the obtained oocytes is low. Therefore, relevant cryopreservation methods must be further studied and optimized [75].

Compared with slow freezing, ovarian tissue vitrification causes less damage to the original follicular DNA [76] and has simple operation, low cost and high efficiency. At present, there is no standard method and solution formula for vitrification of ovarian tissue; the size of ovarian tissue, the type and concentration of cryoprotectant and equilibrium time are important factors affecting its efficiency [77]. By 2019, more than 130 infants had obtained live birth through transplantation after ovarian tissue cryopreservation [78], of which two were obtained after vitrification [79, 80]. The effect of vitrification was evaluated by observing the ovarian tissue structure, follicular morphology, intracellular ultrastructure (nucleus, mitochondria, lysosome, etc.), DNA fragment rate of primordial follicles and endocrine function. It was found that the vitrification and transplantation of ovarian tissue were feasible and effective. The specific improvement effect requires further research and long-term follow-up [6].

Influences of the vitrification technique on imprinted genes in animals and human

Effects of sperm cryopreservation on imprinted genes

It is very important to establish correct methylation markers at imprinted gene sites during spermatogenesis; abnormal methylation will affect spermatogenesis [81–83]. At present, the main techniques applied to sperm cryopreservation are slow freezing and rapid freezing (LN2 vapor method). Both rapid freezing and slow freezing of porcine sperm can lead to changes in the expression of imprinted gene Igf2 and DNA-methylation-related genes Dnmt3a and Dnmt3b, and the addition of cryoprotectants can improve them [84]. The slow freezing of porcine sperm affected the structure of the nucleoprotein and its binding with DNA [85]. It has been found that rapid freezing of mouse sperm hinders the normal DNA demethylation process of fertilized embryos, resulting in abnormal methylation level higher than that in the control group [86]. There was no significant difference in DNA methylation in early embryos obtained by ICSI fertilization of fresh mouse sperm and frozen sperm (without cryoprotectant and placed at −20 °C) [87]. The short-term refrigeration of horse sperm has little effect on DNA methylation [88], but the DNA methylation level increases abnormally after slow freezing and can be used as a potential index for sperm quality evaluation [89]. Therefore, although non-vitrification techniques are widely applied to sperm cryopreservation, they can have adverse impacts on the imprinted gene and DNA methylation, which need to be further clarified. It is necessary to further optimize the sperm vitrification technique as an alternative to traditional freezing methods.

There are few studies on the effect of human sperm freezing on imprinted genes. Slow freezing of human sperm does not affect the DNA methylation pattern of DMRs related to imprinted genes, including maternal imprinted genes KCNQ1OT1, SNRPN and MEST, and paternal imprinted genes MEG3 and H19 [90]. Rapid freezing of human sperm had no significant effect on the methylation of DMRs related to imprinted genes SNURF, SNRPN and UBE3A contained in chromosome 15q11-q13 [91]. In short, no adverse effect of sperm freezing on imprinted genes was found and there are no relevant studies on sperm vitrification.

Effects of oocyte vitrification on imprinted genes

Although the survival rate of mature oocytes after vitrification is very high [60, 92], there are still some problems, including the depolymerization of microtubules and spindles [93, 94], premature release of cortical particles [95, 96] and polyspermy fertilization [97]. In recent years, some reports have discussed the effects of oocyte vitrification on imprinted genes. It was found that oocyte vitrification can significantly affect the imprinted genes of oocytes and embryos in mouse, bovine and other animals (Table 1). After the vitrification and warming of mouse MII oocytes, the expression of the imprinted gene Kcnq1ot1 decreased significantly [98]. Our previous study
found that after vitrification of bovine MII oocytes, the expression of imprinted genes Peg10, Kcnq1ot1 and Xist in blastocysts obtained by ICSI increased abnormally [99]. We also found that vitrification of mouse MII oocytes affected the expression of some imprinted genes, including maternal imprinted genes Peg3 and Peg10 and paternal imprinted gene Gtl2 in IVF 2-cell embryos [100]. However, some studies have reported no significant change in the expression of imprinted genes Igf2r and Gtl2 in 2-cell embryos and blastocysts obtained by parthenogenetic activation after vitrification and warming of mouse MII oocytes [101].

DNA methylation is an important modification to regulate imprinted genes. A study has found that DMRs methylation of imprinted genes H19, Peg3 and Snrpn is decreased in blastocysts obtained by in vitro fertilization of vitrified mouse oocytes [102]. Single-cell genome-wide methylation sequencing identified 151 DMRs between the control IVF blastocysts and those obtained after vitrification of bovine MII oocytes, including a DMR located in the maternal imprinted gene Peg3, whose methylation level was abnormally reduced by oocyte vitrification [103]. Studies have shown that vitrification of mouse oocytes leads to a decrease in the overall DNA methylation level in oocytes and early embryos [104]. Single-cell whole-genome methylation sequencing (scWGBS) combined with single-cell RNA sequencing (scRNA-seq) also showed that, after the vitrification and warming of mouse MII oocytes, the overall methylation level of the genome decreased abnormally and the expression of the DNA methyltransferase Dnmt1 decreased significantly [98]. DNMTs expression in vitrified oocytes and the expression of Dnm3b in blastocysts derived from vitrified oocytes were significantly reduced [102]. Therefore, oocyte vitrification may affect the normal expression of imprinted genes by changing the DNA methylation level of the whole and the regulatory region of imprinted genes.

| Table 1 | Effect of gamete cryopreservation on imprinted genes |
|---------|-----------------------------------------------------|
| Reference | Species | Materials | Technology of assessment | Targeted imprinted genes | Conclusions |
| Kläver et al. [90] | Human | Spermatozoa | Bisulfite conversion | MEG3, H19, Kcnq1ot1, SNRPN, MEST, ALU, LINE1, VASA, MTHFR | No significant differences |
| Khosravizadeh et al. [91] | Human | Spermatozoa | Quantitative methylation specific PCR | SNURF, SNRP and UBE3A | No significant differences |
| Al-Khtib et al. [105] | Human | MII oocytes that were vitrified at the GV stage, warmed and matured in vitro | Bisulfite mutagenesis and sequencing | H19 and Kcnq1ot1 | Oocyte vitrification at the GV stage does not affect the methylation profiles of H19-DMR and Kcnq1ot1-DMR of the in vitro matured oocytes |
| Zeng et al. [84] | pPig | Spermatozoa | q-PCR and ELISA | Igf2 | Igf2 was significantly decreased after vitrification |
| Zhao et al. [103] | Bovine | Vitrified MII oocytes from matured in vitro | Single-cell whole-genome methylation sequencing | Global analysis | Peg3 methylation level was significantly decreased in derived blastocysts |
| Chen et al. [99] | Bovine | Vitrified MII oocytes from matured in vitro | q-PCR | Peg3, Peg10, Kcnq1ot1, Xist, Igf2r | Peg3, Peg10, Kcnq1ot1, Xist were significantly increased after vitrification |
| Chen et al. [100] | Mouse | MII oocytes and 2-cell embryos | q-PCR and bisulfite sequencing | Gtl2, H19, Igf2, Peg3, Peg10, Igf2r | Peg3, Peg10, Igf2r were significantly different in MII oocytes and 2-cell embryos after vitrification |
| Cantatore et al. [101] | Mouse | 2-cell and blastocyst from vitrified metaphase II oocytes | q-PCR | Igf2r and Gtl2 | No significant differences |
| Cheng et al. [102] | Mouse | Blastocysts from vitrified MII oocytes | Bisulfite sequencing | H19, Peg3, Snrpn | No significant differences in oocytes. Decrease in blastocysts after oocyte vitrification |
| Ma et al. [98] | Mouse | Mature metaphase II (MII) oocytes | WGBS combined with RNA-seq | Kcnq1ot1 was significantly down regulated in the vitrified oocytes |
Different from animal research results, few studies have investigated the effect of human oocyte vitrification on imprinted genes, mainly due to the limited number of human oocytes for research and ethical restrictions on fertilization and embryo processing, which affect the scope and depth of detection. Current studies on vitrification of human oocytes suggest that imprinted genes and DNA methylation are not significantly affected (Table 1). The pyrosequencing method found that the methylation of imprinted genes \( H19 \) and \( KCNQ1OT1 \) was not affected in in vitro matured MII oocytes derived from vitrified GV oocytes [105]. Immunofluorescence using anti-5-methylcytosine antibody has shown that vitrification and warming of MII oocytes matured in vitro did not affect the overall methylation level of oocytes [106]; further, it found that there was no significant change in the overall DNA methylation level of in vitro cultured 8-cell embryos derived from vitrified oocytes [107]. In a word, the current detection methods have low accuracy and resolution and further research is required.

**Effects of embryo vitrification on imprinted genes**

In recent years, many studies have found that the vitrification of embryos has a great impact on imprinted genes in preimplantation embryos, placenta and fetal tissues (Table 2). Porcine blastocyst vitrification leads to a decrease in the expression of imprinted genes \( Igf2 \) and \( Igf2r \) [108]. In the blastocysts obtained after mouse 2-cell embryo vitrification, the expression of \( Gtl2 \) was down-regulated and the expression of \( Dlk1, H19 \) and \( Mest \) were up-regulated [109, 110]. After the mouse 8-cell embryo’s vitrification, the transcription and ICR methylation levels of the imprinted gene \( Grb10 \) were both reduced [111]. \( Grb10 \) is an imprinted gene that regulates social behavior [112]; it can encode growth factor receptor binding protein 10, and its expression is very important for

### Table 2 Effects of embryo cryopreservation on imprinted genes

| Reference          | Species  | Materials                        | Technology of assessment       | Targeted imprinted genes | Conclusions                                      |
|--------------------|----------|----------------------------------|--------------------------------|--------------------------|--------------------------------------------------|
| Derakhshan-Horeh et al. [115] | Human    | Blastocysts                      | Bisulfite sequencing PCR       | \( H19 \) and \( IGF2 \)  | No significant differences                        |
| Yao et al. [116]   | Human    | Placenta from vitrified embryos  | q-PCR, western blot and pyrosequencing | \( SNRPN \)              | The expression level of \( SNRPN \) increased after vitrification |
| Barberet et al. [118] | Human    | Placenta                         | Pyrosequencing and q-PCR       | \( H19, IGF2, KCNQ1OT1, SNURF \) | The placental DNA methylation levels of \( H19/IGF2 \) were lower in the fresh embryo transfer group than in the control (\( H19/IGF2\)-seq1) and frozen embryo transfer (\( H19/IGF2\)-seq2) groups |
| Bartolac et al. [108] | Pig      | Blastocysts                      | q-PCR                          | \( Igf2 \) and \( Igf2r \) | \( Igf2 \) and \( Igf2r \) were significantly decreased after vitrification |
| Movahed et al. [109] | Mouse    | Blastocysts from vitrified 2-cell embryos | q-PCR                         | \( Gtl2 \) and \( Dlk1 \) | \( Gtl2 \) was down-regulated and \( Dlk1 \) was up-regulated after vitrification |
| Jahangiri et al. [110] | Mouse    | Blastocysts from vitrified 2-cell embryos | q-PCR                         | \( H19 \) and \( Mest \) | Significantly increased after vitrification |
| Yao et al. [111]   | Mouse    | 8-cell embryos                   | q-PCR and bisulfite sequencing PCR | \( Grb10 \)              | The methylation level and gene expression of \( Grb10 \) decreased |
| Ma et al. [113]    | Mouse    | Fetuses and placentas from vitrified eight-cell embryos | q-PCR                         | \( Dlk1, Igf2, KCNQ1OT1, Mest, Ndn, Peg3, Plag1, Sgce, Snrpn, Cad81, Cadknc, Dcen, Gatm, Gnin, Grb10, Gtl2, H19, Igf2r, Mash2, Osbp15, Phlda2, Sic22a18, Ube3a, Zim1 \) | A majority of maternally expressed genes were upregulated in fetuses |
| Wang et al. [114]  | Mouse    | Fetuses and placentas from vitrified embryos | q-PCR and pyrosequencing       | \( H19 \) and \( Igf2 \) | The expression of \( H19 \) was significantly increased after vitrification, whereas \( Igf2 \) was significantly decreased. Methylation levels were decreased |


fetal growth. After vitrified mouse embryo transfer, the expression of imprinted genes in the E9.5-day fetus and placenta were detected. It was found that the expression of imprinted genes in fetus (Sgce, Dcn, Gatm, Gtl2) and placenta (Kcnq1ot1, Sgce, Gatm) had changed, and the methylation level of the KvDMR1 in fetus and placenta was changed [113]. Another study also detected the effect of mouse embryo vitrification on H19/Igf2 in the fetus and placenta; they found that the expression of H19 was abnormally increased and the expression of Igf2 was abnormally decreased [114]. After the mouse 8-cell embryo’s vitrification, it was found that the overall DNA methylation level decreased abnormally in the warmed 8-cell embryos and the developed blastocysts [111]. After vitrified mouse embryo transfer, the expression of DNA-methylation-related enzymes (Dnmt1, Dnmt3b, Tet2, Tet3) was abnormal in the E9.5-day fetus and placenta [113]. Therefore, animal embryo vitrification affects the whole DNA methylation, which may lead to abnormal expression and methylation of some imprinted genes in preimplantation and postimplantation fetus and placenta.

Due to the small number of human embryos and ethical restrictions, it is difficult to obtain enough embryos and tissue materials for research purposes, which hinders study of the impact of human embryo vitrification on imprinted genes. Studies have shown that the vitrification of day-3 human embryos has no significant effect on the DNA methylation status of H19/IGF2 DMR at the blastocyst stage [115], but the vitrification of human embryos leads to an abnormal increase in the expression of SNRPN in placenta [116]. A follow-up study from France showed that, compared with the natural pregnant group, fresh embryo transfer (Fresh-ET) significantly increased the risk of imprint-related diseases (1.6 times higher), but frozen embryo transfer (FET) reduced this risk, which was not statistically different from the natural pregnant group (1.2 times higher) [117]; this may be because the FET cycle can avoid endometrial exposure to high concentrations of gonadotropins, thereby reducing placental dysfunction and pathological changes. However, it is a study based on an epidemiological study. The cases counted as imprinting abnormalities in this study include uniparental disomy, chromosomal structural abnormalities and epimutations. In addition, it is unclear whether the results are based on genetic testing. Uniparental disomy is a chromosomal abnormality, and the usage of controlled ovarian stimulation may increase the frequency of uniparental disomy. A recent study showed that, compared with the natural pregnancy group, the methylation levels of imprinted gene H19/IGF2 and transposon element LINE-1 in the placenta of the fresh embryo transfer group were abnormally reduced, but there was no significant abnormality in the FET group [118]. In the future, we need to obtain more preimplantation embryos, placental tissues and aborted fetuses within the scope of ethics without affecting the reproductive needs of patients to clarify the potential adverse effects of embryo vitrification on genomic imprinting through microsample analysis.

**Effects of ovarian tissue cryopreservation on imprinted genes**

At present, a few studies on the effects of ovarian tissue cryopreservation on imprinted genes exist (Table 3). After slow freezing and transplantation of mouse ovarian tissue, the ICRs methylation of imprinted genes H19 and Kcnq1ot1 in offspring tissue is not affected [119]. After vitrification and warming of ovarian tissues from 10-day-old female mouse, the expression of DNA methyltransferase Dnmt1 was decreased while imprinted gene Grb10 was increased [120]. Allogeneic heterotopic transplantation after the vitrification and warming of prepubertal mouse ovarian tissue can restore puberty and fertility, and this process does not affect the DMR methylation of imprinted gene Snrpn in GV oocytes [121]. After vitrification and warming of mouse follicles, the DMR

| Reference     | Species | Materials     | Technology of assessment | Targeted imprinted genes | Conclusions                                      |
|---------------|---------|---------------|---------------------------|--------------------------|--------------------------------------------------|
| Sauvat et al. [119] | mouse   | ovarian tissue | Southern blotting         | H19 and Kcnq1ot1       | No significant differences                       |
| He et al. [120]  | mouse   | ovarian tissue | Western blot and q-PCR    | Grb10                    | Grb10 was significantly increased after vitrification |
| Wang et al. [121] | mouse   | ovarian tissue | Bisulfite sequencing PCR  | Snrpn                   | Freezing of ovarian tissue did not affect the methylation status of Snrpn-DMR of imprinted genes in GV oocytes |
| Trapphoff et al. [122] | mouse | pre-antral follicles | pyrosequencing          | H19, Igf2 and Snrpn  | The methylation of DMR of imprinted gene H19 and Igf2 in mature oocytes was not affected, and Snrpn changed slightly |
methylcytosine of imprinted genes $H19$ and $Igfl2$ in mature oocytes was not affected, and Snrpn changed slightly [122]. Vitrification and warming of mouse ovarian tissue affected follicular growth, and the promoter methylation level of Inhba in granulosa cells decreased [123]. In summary, there are limited studies investigating the influences of ovarian tissue vitrification on imprinted genes in mouse and there is still a lack of research on human ovarian tissue.

**Conclusion**

At present, oocyte and embryo vitrification is widely used in ART. Although ovarian tissue cryopreservation is developed late and slowly, the vitrification method has developed rapidly. Currently, strategies for sperm cryopreservation are still mainly slow or rapid freezing, and the vitrification technique requires further development and evaluation. Compared with traditional freezing methods, the vitrification process is time-consuming and convenient. Most importantly, it can minimize the damage to the ultrastructure and cellular DNA in gametes, embryos and follicles and improve the survival and clinical outcomes.

Vitrification can significantly affect the expression and methylation of some imprinted genes in animal oocytes and embryos, and the few studies in humans have reported almost no influence. The slow or rapid freezing of human sperm does not affect the DNA methylation related to imprinted genes, but the effect of vitrification is unknown. There is still a lack of research on the effect of vitrification on imprinted genes in human ovarian tissue. In summary, vitrification can adversely affect imprinted genes in animals, but the impacts of vitrification on human imprinted genes have not been fully studied. It is necessary to strengthen the detection of the effects of vitrification on imprinted genes in human gametes, embryos and tissues and to conduct a long-term follow-up study to evaluate the safety for their offspring.

**Abbreviations**

ART: Assisted reproductive technology; BWS: Beckwith–Wiedemann; PWS: Prader–Willi syndrome; AS: Angelman syndrome; PGCs: Primordial germ cells; ICRs: Imprinting control regions; DMRs: Differentially methylated regions; OTC: Ovarian tissue cryopreservation; scWGBS: Single-cell whole-genome methylation sequencing; scRNA-seq: Single-cell RNA SEQ; FET: Frozen embryo transfer; SmIC: 5-Methylcytosine.

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**Author contributions**

HC undertook the initial systematic search and screening of the literature. LL summarized the findings from articles. LZ drafted the manuscript. LM and CZ substantively contributed to revisions to manuscript drafts. All authors read and approved the final manuscript.
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