CryoLetters 42(3), 120-128 (2021)

PERSPECTIVE

CRYOPRESERVATION OF HUMAN OOCYTES AND THE 'CARRYOVER' EFFECT ON EARLY EMBRYO DEVELOPMENT

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Abstract

Worldwide women are increasingly facing the issue of delayed child-bearing and fertility decline. Oocyte cryopreservation provides an option for fertility preservation, especially for women with diseases and other special needs to conceive babies later. In this review we examine the effect of oocyte cryopreservation on early development of human embryos. Databases (Medline, PubMed and Web of Science) were searched for relevant clinical studies published between 1999 and 2020. A total of 27 studies on oocyte cryopreservation and embryo development were identified, and data in those studies are retrieved for meta-analysis on the outcomes of oocyte survival, fertilization and early embryo development. In comparison to the slow freezing technique, vitrification yields significantly better oocyte survival (84.7% ± 0.6% vs 58.0% ± 0.5%), and subsequently higher rates of fertilization (65.5% ± 0.9% vs 40.0% ± 0.6%), cleavage (58.8% ± 0.9% vs 34.6% ± 0.8%), as well as embryo implantation (5.9% ± 0.3% vs 2.9% ± 0.2%). This analysis reveals a negative 'carryover' effect of oocyte cryopreservation on early development of embryos after oocyte fertilization (i.e., cleavage and implantation). This 'carryover' effect is greater for slowly-frozen oocytes than for vitrified oocytes, and may represent subtle functional or molecular alterations that are not severe enough to affect cell survival and fertilization, but sufficient to impair later development. The nature of the 'carryover' effect is unknown. Hypothermia, membrane ion channels, bioenergy metabolism and epigenetic modifications are likely involved. In conclusion, oocyte cryopreservation can negatively affect early development of human embryos. Future studies should go beyond oocyte survival and look further into the effects on epigenetic changes.

Keywords: cryopreservation; embryo development; fertility preservation; oocyte; vitrification.

INTRODUCTION

Worldwide women are increasingly facing two issues, delayed child-bearing and fertility decline, which results in the reduction in birth rate. Cryopreservation of oocyte, embryo and ovarian tissues has become a hot topic again for female fertility preservation (1-5). For young women with diseases whose undergoing therapies will affect fertility, women who face the fertility decline problem, and/or women who wish to delay child-bearing, it is possibly the best way to conceive later offspring of their own genetic background (6). For various reasons (age, disease, legal issue or religion), fertility

Table 1. The effect of cryopreservation method on human oocyte survival.

Cryopreservation method	Slow freezing technique	Vitrification
Number of studies	14	14
Number of oocytes	> 45,000	> 18,600
Freezing methods	Programmed cooling	Liquid nitrogen plunge
Cryoprotectants	DMSO, glycerin, EG, PROH, sucrose	EG, DMSO, sucrose
Median survival (range)	62.7% (37.0% - 88.1%)	87.7% (68.6%-99.4%)
Mean survival	58.0 % ± 0.6%	84.7% ± 0.9%

preservation is an important means to reduce the social impact of infertility.

Oocyte cryopreservation is well-accepted for fertility preservation since it avoids ethical and legal issues associated with embryo storage. But oocyte cryopreservation is more challenging than embryo cryopreservation. There are more obstacles to overcome to retain oocyte development potential. The problem is now addressed by optimizing process variables (cryoprotectant formulation, loading/ unloading method, solution volume, ice seeding, and cooling/rewarming rate. etc.) (7-11). Oocyte preservation is performed by either slow freezing or vitrification. The former uses lower cryoprotectant concentration and cools slowly to avoid intracellular ice formation, whereas the latter uses high cryoprotectant concentrations, minimal liquid volume and an ultra-fast cooling method to eliminate ice formation completely and achieve vitrification (12).

However, oocyte cryopreservation is not without risks. Cryopreservation is a multi-step process that involves loading and unloading of cryoprotectants (cell dehydration), cooling and rewarming, as well as ultra-low temperature storage. Unlike other cells, human oocytes have high water content, low membrane permeability and a small surface-to volume ratio, which makes cryopreservation much more challenging. The pregnancy with embryos derived from frozen oocytes has a higher risk of miscarriage during the first 12 weeks, and babies tend to have higher birthweight (13). It is reported that assisted reproductive technology (ART) may affect DNA methylation in imprinted regions, causing imprinting disorders and fetal epigenetic changes (14). ART may also increase the risk of leukemia and sympathetic nerve tumors (15). It raises a serious concern on the development potential of cryopreserved oocytes and embryos. The present study reviews effects of oocyte cryopreservation on early development of human embryos and recent advances in understanding cryo-damages.

METHOD AND DATA EXTRACTION

Databases (Medline, PubMed and Web of Science) were searched for studies of human oocyte cryopreservation published from 1999 to 2020, with keywords “fertility preservation, human (donor) oocyte / egg cryo-preservation, slow freeze and/or vitrification”. The search retrieved 133 articles, of which 45 articles were excluded due to repetition and irrelevance. The remaining 88 articles were reviewed. Twenty-seven clinical studies on oocyte cryopreservation and embryo development (16-42) were identified. Data in those studies were then retrieved for the meta-analysis.

OOCYTE SURVIVAL AND EARLY EMBRYO DEVELOPMENT

Table 1 shows the result of the overall meta-analysis on human oocyte survival upon slow freezing cryopreservation and rapid vitrification. These clinical studies involved >45,000 slowly frozen oocytes and >18,600 vitrified oocytes. The vitrification method, in comparison to slow freezing technique, obtained significantly higher immediate oocyte survival (84.7% ± 0.6% *vs* 58.0% ± 0.5%). Vitrified oocytes also had significantly higher fertilization rate (65.5% ± 0.9% *vs* 40.0% ± 0.6%), cleavage rate (58.8% ± 0.9% *vs* 34.6% ± 0.8%), and embryo implantation (5.9% ± 0.3% *vs* 2.9% ± 0.2%) than slowly frozen oocytes (Fig 1A). Compared to fresh oocytes, however, vitrified oocytes still yielded only about half the level of embryo implantation (11.9% ± 0.4% *vs* 5.9% ± 0.3%) (Fig 1A). The analysis clearly shows that oocyte cryopreservation can negatively affect early embryo development, and the conventional slow freezing technique has greater impact.

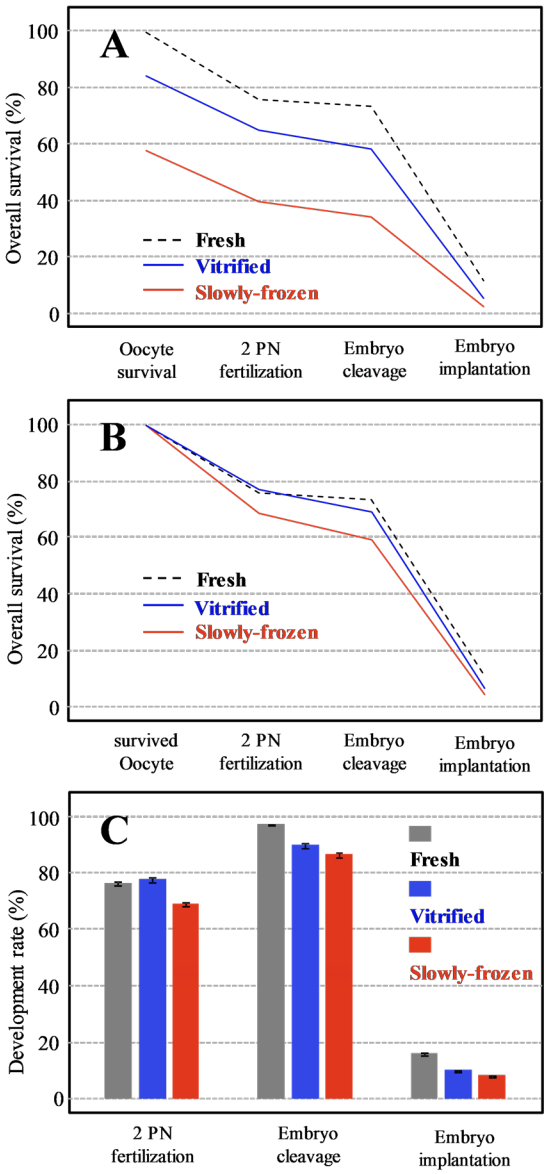

Figure 1. Survival rate and subsequent development potential of cryopreserved human oocytes. **A**, the survival curves of slowly-frozen and vitrified oocytes at different stages; **B**, the survival curves normalized by the survival rate immediately after rewarming; **C**, the development rate/potential of fresh, slowly-frozen and vitrified oocytes to 2PN fertilization, embryo cleavage and implantation (calculated according to the number of oocytes or embryos in the earlier development stage.

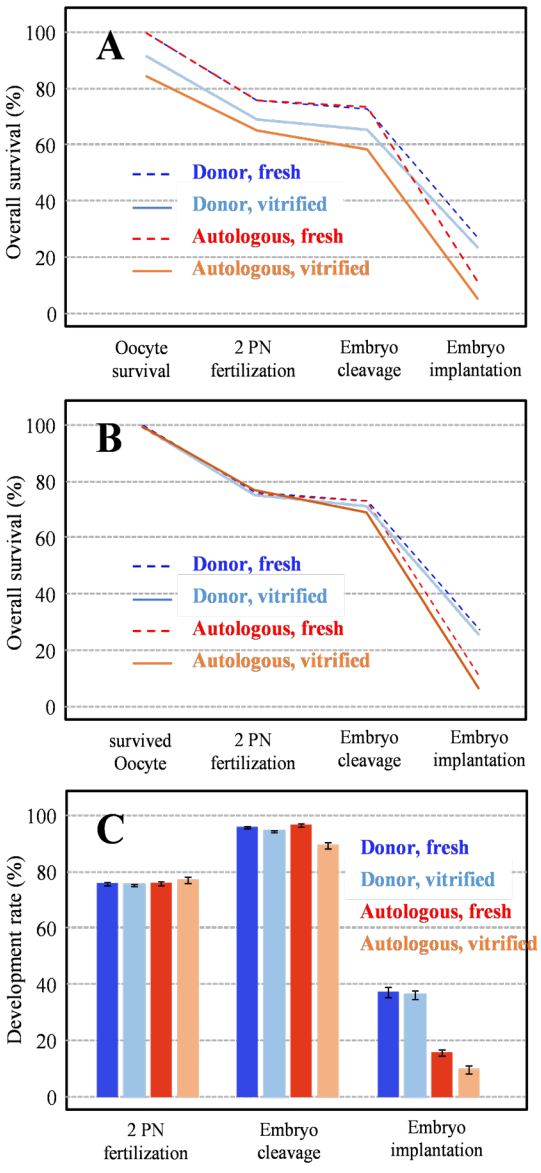

Figure 2. Survival rate and subsequent development potential of vitrified human oocytes. **A**, the survival curves of fresh oocytes and vitrified oocytes at different stages; **B**, the survival curves normalized by the survival rate immediately after rewarming; **C**, the development rate/potential of fresh and vitrified donor/autologous oocytes to 2PN fertilization, embryo cleavage and implantation (calculated according to the number of oocytes or embryos in the earlier stage.

To examine precisely the cryopreservation impact, the survival curve of oocytes (Fig 1A) is normalized by the survival rate after rewarming to track the further development of oocytes that have survived upon cryopreservation. Fig 1B shows the development curve of surviving oocytes. Fig 1C shows the development rate of oocytes or embryos at an earlier stage to the next stage (i.e., 2PN fertilization, embryo cleavage and embryo implantation). It is apparent that there is a 'carryover' effect of cryopreservation on the early development of embryos. The effect is significantly greater for conventional slowly-frozen oocytes than for vitrified oocytes. The nature of the 'carryover' cryo-damage remains unknown. It may represent some kind of subtle

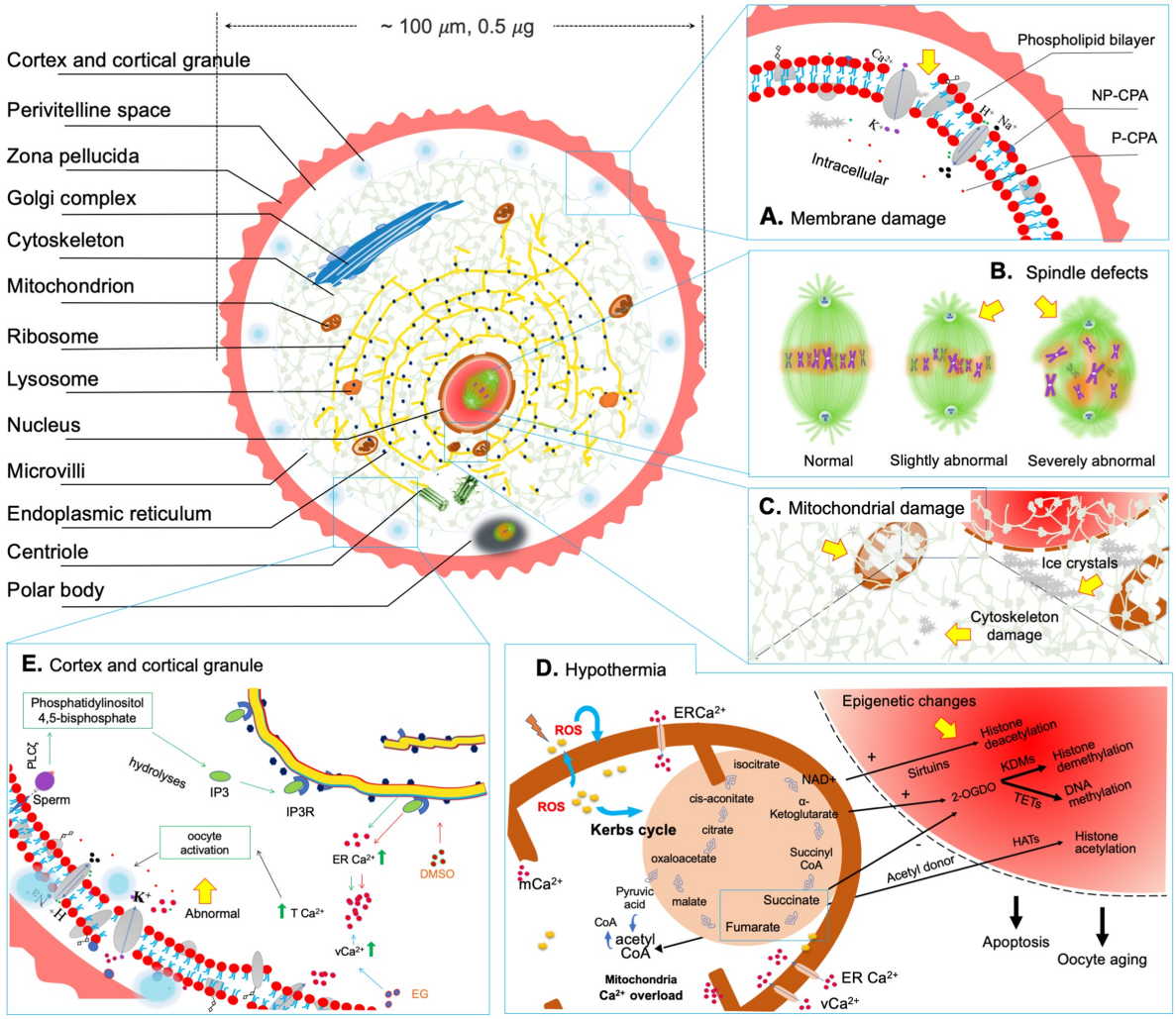

Figure 3. An overview of the physical and biological criticalities associated with the potential mechanisms of oocyte cryoinjuries. **A**) membrane disruption; **B**) spindle defects; **C**) mitochondrial damage; **D**) hypothermia effect; **E**) changes in cortex and cortical granule. Calcium ion regulation: vCa^{2+} from vitrification medium, ER Ca^{2+} from endoplasmic reticulum, and mCa^{2+} from mitochondria. See the text for the detailed discussion.

functional or molecular alterations that are not severe enough to affect normal cell survival, but sufficiently to impair certain developmental and functional expression later.

The meta-analysis of clinical related studies often has its pitfalls. Human oocytes used for the assisted reproduction come from two sources, donors and patients themselves (autologous). These two groups have distinctive demographic differences. Oocyte donors are younger, healthier and more fertile females, whereas patients who seek aid by assisted reproduction are generally older and/or have declined fertility. Based on the data we retrieved from 14 studies, the average age of oocyte donors are 27 years old, and autologous patients are 38 to 45 years old. Therefore, we further analyzed subsets of data, which separate younger donor oocytes (7 studies, 22,181 oocytes) from older autologous oocytes (7 studies, 8,066 oocytes). This analysis shows that, in comparison to fresh donor oocytes, surviving vitrified donor oocytes in fact yields similar fertilization rates (fresh 76.1% ± 0.6% vs vitrified 75.6% ± 0.6%), similar cleavage rates (fresh 96.1% ± 0.4% vs vitrified 94.7% ± 0.4%), as well as comparable embryo implantation rates (fresh 37.6% ± 1.8% vs vitrified 36.6% ± 1.5%) (Figs 2B, 2C). But the outcomes are quite different for the vitrified autologous oocytes. The fresh and surviving vitrified autologous oocytes yields similar fertilization rates (fresh 76.2.% ± 0.7% *vs* vitrified 77.4.% ± 1.1%). However, the embryo cleavage rate of vitrified autologous oocytes is lower than that of autologous fresh oocytes (fresh 96.9%±0.6% *vs* vitrified 89.7.% ± 1.1%). The embryo implantation rate of vitrified autologous oocytes is even worse (fresh 16.1% ± 1.1% *vs* vitrified 10.1% ± 1.4%) (Figs 2B, 2C).

Overall, the implantation rate of using vitrified donor and autologous oocytes is 24.1 ± 1.0% and 5.9 ± 0.9%, respectively. This analysis suggests that the oocyte source plays an important role in the successful outcome, and autologous oocytes of older patients are more prone to freeze damage. The quality of oocytes is a key factor for ensuring the development potential after thawing (43, 44). The decline of oocyte quality as females get older is associated with increased chromosomal abnormality and decreased live birth rate (6, 45, 46).

In summary, cryopreservation of oocytes could affect the early development of embryos. The slow freeze technique has greater negative impact on the embryo development potential, whereas vitrification can minimize such negative impact. Vitrification does not appear to affect the early development of embryos derived from cryopreserved young donor oocytes, but affects the early development of embryos derived from cryopreserved older autologous oocytes.

POSSIBLE NATURE OF THE 'CARRYOVER' CRYO-DAMAGE

The oocyte is the largest human cell with unique structures for specialized functions. The zona pellucida controls the combination of sperm and oocyte, the membrane maintains the cellular homeostasis, the cytoskeleton supports cell structure with a network of filamentous and tubular protein structures, various organelles (e.g., centrosome, endoplasmic reticulum, Golgi body, lysosome, mitochondria and ribosomes) maintain metabolic functions, and the nucleus regulates and coordinates various highly ordered activities in response to a changing environment.

Cryo-damage can occur in a number of critical physical and biological sites of oocytes due to the formation of ice crystals, the toxicity of cryoprotectants, the overproduction of reactive oxygen species (ROS), excessive dehydration, hypothermia, ion imbalance, and altered gene expression. Since the "carryover" effect of oocyte cryopreservation does not appear to affect normal cell survival (Figs 1 and 2), this cryo-damage may represent subtle functional or molecular alterations that would only impair certain developmental expressions at later stages of growth. We focus on the effects of cryopreservation on regulatory mechanisms that are likely relevant to the early development of embryos (Fig 3).

Hypothermia effect

Hypothermia could affect the development of cryopreserved oocytes and derived embryos via several regulatory pathways. First, low temperature may alter the structure and function of the cell membrane, leading to phase transition and protein dislocation (47, 48). Such changes include separation of oocyte membrane lipid and protein, and phase transition of the phospholipid bilayer to cause bending or breakage (49). These changes may cause the abnormal function of ion channel proteins in the cell membrane structure, resulting an imbalance of intracellular and extracellular ions, as well as an abnormal membrane potential. The hypothermia-induced disruption of the intra-cellular Ca^{2+} homeostasis can in turn reduce the development potential of pre- and post-implantation embryos by starting the apoptosis process (Fig 3A). The interruption of intra-cellular Ca^{2+} homeostasis decreases the rate of embryo implantation, and the oocyte over-stimulation by calcium signals influences the development of the post-implantation embryo (50).

Secondly, hypothermia can also change the carbohydrate composition and secondary structure of surface proteins in the zona pellucida of the oocytes, resulting in non-physiological hardening, abnormal recognition and information exchange with the endometrium and ultimately implantation failure (51).

Thirdly, the oocyte's spindle is very temperature-sensitive. It has been reported that the meiotic spindle is not retained during slow freezing, whereas vitrification does not affect the meiotic spindle (52). The spindle depolymerizes at low temperature upon cooling and is rebuilt upon rewarming. The dispersion of spindle microtubules could cause minor irreversible injuries with the consequence of unequal chromosome division and polyploid generation (53-55). Severe spindle damage is likely to activate the spindle checkpoint to arrest the cell cycle (Fig 3B).

Membrane ion channels

The ion homeostasis (particularly Ca^{2+}) is crucial for oocyte activation and further embryo development. The exposure to cryoprotectants causes osmotic contraction, and increases the intercellular Ca^{2+} level, which could induce early oocyte activation and release of cortical particles, affecting normal fertilization (56-59). Ethylene glycol increases the intercellular Ca^{2+} level via extracellular Ca^{2+} influx, and dimethyl sulfoxide induces the Ca^{2+} release from intracellular calcium stores such as the endoplasmic reticulum

(ER Ca^{2+}) (Fig 3E). Cryopreservation affects the oocyte's calcium signaling during human fertilization (60), and lowers the oocyte's sensitivity to calcium ionophore A23187 (61). It has been hypothesized that the reduction of the normal fertilization rate of vitrified oocytes, when compared to the fresh oocytes, might be related to the imbalance of intracellular Ca^{2+} level that leads to an impaired oocyte activation (62-64). Sperm-oocyte fusion causes the influx of Na^{+} ions in the process of normal fertilization, which changes membrane potential to hinder polyspermy. The sperm-released phospholipase C zeta hydrolyzes the phosphatidylinositol 4,5-diphosphate molecule to inositol 1,4,5 triphosphate (IP3), activating the receptor IP3R on the endoplasmic reticulum, resulting in an increase in intracellular Ca^{2+} level and the hardening of zona pellucida (Fig 3E). Oocyte activation depends on intracellular free Ca^{2+}, and only when the total Ca^{2+} level exceeds the threshold value the oocyte can be activated to release cortical particles, to form pronucleus and to begin embryo development (65, 66).

Bioenergy metabolism

Several studies observed the ultrastructural changes in cryopreserved oocytes with low development potential, including mitochondrial swelling, pale coloring, unclear crest and cavitation (67, 68) (Fig 3C). Cryopreservation-caused mitochondrial malfunction and abnormal energy metabolism decreases the mitochondrial NAD+ level and increases the production of reactive oxygen species (ROS) (69-71) (Fig 3D). Since embryo cleavage is strictly controlled by the cell cycle, ROS over-production may cause embryo division abnormalities by damaging mitochondrial DNA and nuclear DNA (72). When nuclear DNA is damaged, P53 phosphorylates and dissociates from Mdm2. The increased P53 level regulates the expression of the Cdk2 kinase inhibitor P21 in the nucleus and leads to the inactivation of Cdk2/cyclin E complex, preventing the G1 phase to S phase transition. P53 could induce apoptosis in the case of severe DNA damage. It should be pointed out that there are very few reports on DNA damage in thawed human oocytes. The results of DNA damage in animal studies vary from species to species (73-75).

Epigenetic change

Epigenetic changes are implicated in the 'carryover' effect of cryopreservation on early embryo development by several workers. Cryopreservation has down-regulated expression of mitochondrial genes (AT5G3, ATPG, COX7R, GBRL2, FRIH and CYB5B), transmembrane proteins (TM163; GP160; RABL3; PIGP; GBG5; RSU1; RHG20; GPC5D) and calcium signaling regulation (CALM and SSRG). Abnormal fertilization for oocytes may be related to the upregulation of GRP78 expression (76). The lower development potential of frozen oocytes is related to the down-regulation of gene expression related to chromosomal structure maintenance (KIF2C and KIF3A) and cell cycle regulation (CHEK2 and CDKN1B) (77). Epigenetic modifications are also found to decrease the expression of HDAC1 and increases expression of DNMT3B, STAT3 and SAARCAL1 (78). More research is needed to pinpoint whether and how cryopreservation may induce epigenetic modifications in human oocytes.

PERSPECTIVE

The biological integrity of frozen oocytes, normal embryo development and offspring safety are of great importance. Oocyte cryopreservation is associated with the increased risks of miscarriage, large for gestational age, imprinting disorders and fetal epigenetic changes, high birth weight, as well as leukemia and sympathetic tumors. However, these risks may also be related to other factors such as donor age and oocyte source as illustrated in Fig 2. In perspective, the future study of human oocyte cryopreservation should go beyond the protocol optimization for oocyte survival, and look further into the effects on molecular expression and epigenetic changes. The advances in new technologies, such as microfluidics and single cell genetic sequencing have provided powerful tools for studying the effect of oocyte cryopreservation on epigenetic modifications of nuclei and mitochondrial DNA.

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