Updating the Impact of Lipid Metabolism Modulation and Lipidomic Profiling on Oocyte Cryopreservation

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Disclosure: The authors have declared no conflicts of interest.

Received: 25.04.18

Accepted: 04.09.18

Keywords: Cryopreservation, lipid metabolism, lipid profile, mass spectrometry, oocyte.

Citation: EMJ. 2019;4[1]:79-87.

Abstract

Oocyte cryopreservation has drastically improved in recent years and is receiving widespread clinical use with increasing demand for fertility preservation and assisted reproduction treatments. However, there are still several points to be reviewed in terms of suppressing sub-lethal damages and improving overall safety, especially when trying to preserve oocytes at the germinal vesicle stage or oocytes matured *in vitro*. The lipid content of oocytes is highly associated with both their competence and cryotolerance. Differences in lipid content are observed not just between different species but also at different developmental stages and when the oocytes are kept under different conditions, including cryopreservation. Many efforts have been made to understand how physiological or *in vitro* alterations in the lipid profile of oocytes impacts cryotolerance and vice-versa; however, the dynamics of cytosolic and membrane lipid involvement in the cryopreservation process remains poorly clarified in the human female gamete. This review presents an updated overview of the current state of cryopreservation techniques and oocyte lipidomics and highlights possible ways to improve cryotolerance, focussing on lipid content modulation.

INTRODUCTION

Oocyte cryopreservation has improved in recent years and is receiving widespread clinical use with increasing demand for fertility preservation and assisted reproduction treatments.[1] From the birth of healthy mouse pups using cryopreserved oocytes for *in vitro* fertilisation (IVF) and embryo transfer in 1977 to now, many advances have been achieved and a wide array of tools made available to preserve genetic material, evaluate oocyte viability biomarkers, and treat many forms of infertility.[2,3] In many mammals, however, oocyte cryopreservation techniques are still poorly developed and, despite advances in oocyte vitrification methods, there are still several points to be reviewed in terms of suppressing sub-lethal damage to oocytes and improving overall safety, especially when trying to preserve immature oocytes or oocytes matured *in vitro*. While cryopreservation of oocytes at metaphase II (MII) (mature oocytes)

is approved for clinical use, cryopreservation of germinal vesicle-stage oocytes, or oocytes matured *in vitro*, are still considered experimental without consistent results.[2-4]

The lipid content of oocytes is highly associated with gamete competence and cryotolerance.[5,6] The oocyte is the largest mammalian cell and has an extensive amount of cytoplasm containing abundant lipid storage. Differences in the lipid content of oocytes are observed not only between different species but also in oocytes from the same ovary and are even noted in the same oocyte under different conditions or at developmental stages.[7] Differential lipid profiling occurs throughout the gamete and its companion cumulus cells under all stages of follicular development. Noticeable changes also occur between immature and matured oocytes,[8] as well as after cryopreservation.[9] Additionally, the presence of various lipid sources in *in vitro* maturation (IVM) systems effects oocyte lipid profile and developmental competence.[8,10,11]

Many efforts have been made to understand how physiological or *in vitro* alterations in the lipid profile of oocytes impact cryotolerance and vice-versa, including studies on the principles of plasma membrane properties[12] and the characterisation of the lipid profile of a single human oocyte.[13] However, the dynamics of cytosolic and membrane lipids involved in the cryopreservation process remain poorly clarified in the human female gamete. This review presents an overview of cryopreservation techniques and oocyte lipidomics, and highlights possible ways to improve cryotolerance, focussing on lipid content modulation.

CRYOPRESERVATION TECHNIQUES

Since its inception 30 years ago, cryopreservation of human oocytes has become an important component of assisted reproduction technology. The increasing success rates of MII oocyte vitrification led to its implementation in IVF clinics worldwide, with the American Society of Reproduction Medicine (ASRM) lifting the experimental status of the practice in 2013.[14] The ascending popularity is being followed by a noted increase in demand, especially among women trying to overcome age-related fertility issues.[15] The rise of 'social egg-freezing' is pushing reproduction scientists to review recent cryopreservation protocols and outcomes, and to pursue new ways of improving the process, to achieve maximum safety.[15-17]

If carried out correctly, the alterations caused by cryopreservation, especially the vitrification technique in MII-oocytes, do not impair gamete function and development. However, the oocyte's unique structure with a large amount of cytoplasm and low hydraulic conductivity, and the sub-lethal damages caused to it by freezing and thawing (temperature, exposure time, and cooling rates) are responsible for the increased difficulty in the cryopreservation of these cells and might explain the observed lower rates of live births when compared to fresh oocytes.[18] In the following section, the authors briefly discuss the known damages caused to the oocyte and the current state of cryopreservation techniques.

Sub-Lethal Damages Caused to the Post-Thaw Oocyte

Cryopreservation-induced stress is a complex and multifaceted mechanism in which different stressors and stress cell responses appear to play important roles. The chilling process affects membrane structure. During freezing, cells are exposed to hyperosmotic solutions and equilibrated by the movement of water across the membranes. During storage and thawing, oocytes are subject to ice recrystallisation episodes.[17,19] Susceptibility of cells to biochemical damage by oxidative stress is also reported during the thawing process.[20]

The most affected structures within the cell are the microtubules, cytoskeleton, lipid droplets, and the membrane system.[21] Studies by Ghetler et al.[12] showed that the oocyte membranes are far more sensitive to the effects of chilling than the membranes of embryos and even zygotes, which are visually very similar to the female mammalian gametes. The membrane of oocytes has a high melting temperature, which means that the lipids are more easily affected by the drop to low temperatures, causing a loss of membrane function. This is a possible explanation for the relatively poor survival rates of cryopreserved human oocytes compared to embryos using the slow freezing method.[12] It has also been observed that lipid droplets are

affected differently between immature oocytes surrounded by cumulus cells and mature oocytes. According to a study by Okotrub et al.,[22] lipid crystallisation occurs gradually in lipid droplets of embryos and mature oocytes and more abruptly in immature oocytes, possibly explaining one of the reasons why vitrification of the latter is less successful in general.

Recently, Xu et al.[23] demonstrated that the vitrification process can also alter phospholipids composition. Comparing fresh and vitrified buffalo oocytes, they observed that five phospholipids were less abundant in the vitrified group. This finding suggests that lipid composition might be a good indicator of the quality of vitrified oocytes.

Cryopreservation also affects communication between oocytes and the surrounding cumulus cells through the disruption of tranzonal processes, microfilaments that maintain the meiotic spindle in the right position during the maturation process.[24] It was recently observed that the number of cumulus cells attached to the oocyte is associated with gamete energy sufficiency, affecting its lipid and ATP content.[25,26] This is thought to be the main cause of low survival and development rates of cryopreserved immature and *in vitro*-matured oocytes.[24]

Current State

Cryopreservation of oocytes can be performed by both slow freezing and vitrification. While the rapid freezing used in vitrification protects the cell from most damages associated with the chilling process, including membrane damages, it requires the use of higher concentrations of cryoprotectant solutions (CPA) that are toxic. In their 2011 review, Saragusty and Arav[27] concluded that due to its lower cost and ease-of-use, vitrification should be the go-to cryopreservation method for human oocytes. More recently, there has been debate as to whether the 'closed-system' vitrification, which prevents the direct contact of gametes with liquid nitrogen, is safer and at least as effective as the traditional 'open-system', but current data are inconclusive regarding safety issues and indicate that the traditional system has better results.[15,17]

There is also discussion as to whether the cryopreservation of oocytes in the germinal vesicle state is advantageous in comparison to oocytes in the MII stage. So far, this debate has not been resolved due to the lack of data on the viability of immature oocytes post-vitrification. It is, however, an undoubtedly useful resource to avoid the risks associated with ovarian stimulation.[24] In these cases, updated protocols suggest that the oocyte should be vitrified in the company of its surrounding cumulus cells for a better chance of surviving and developing after the thaw process.[28]

The adjustment of current protocols, the refinement of CPA composition and delivery, as well as the upgrade of equipment have been identified as possible next steps to further develop the cryopreservation technique. These should take the 'weak links' observed in oocytes (e.g., cellular membrane and intracellular lipid content) into account to achieve maximum effectiveness.[27,29]

Marques et al.[29] recently tested the effects of different cryoprotectants and calcium in the vitrification media on bovine oocytes. Oocytes were exposed to CPA containing either ethylene glycol, dimethyl sulfoxide, and sucrose (EGDMSO), or 1,2-propanediol and sucrose in the presence or absence of calcium. EGDMSO had the best results, independently of Ca^{2+} concentration in the media. The fatty acid (FA) composition of oocytes and cumulus cells was also assessed. Independently of cell type, concentration of vaccenic acid (c11-18:1) was highest in cumulus–oocyte complexes exposed to EGDMSO with Ca^{2+}, while the lowest was present in cumulus–oocyte complex exposed to 1,2-propanediol, sucrose, and Ca^{2+}.[29] This confirms the influence of CPA solutions in oocyte metabolism; however, more studies are needed to understand the precise effects of technical components on oocyte lipid profile and subsequent development.

As for devices, recent studies point to automated systems, such as microfluidic platforms, which keep the oocyte stationary and exposed to an automatic and gradual flow of CPA, as a promising approach capable of lessening many of the known negative effects on the cell. This would be achieved, however, at the expense of important steps that require visual control, such as morphological selection after the equilibration phase.[2,3,17,30]

ALTERING LIPID METABOLISM AND MEMBRANE COMPOSITION OF OOCYTES: A PROMISING ALTERNATIVE TO IMPROVE CRYOTOLERANCE

Apart from the aforementioned changes in current cryopreservation protocols and equipment, another approach to improve cryosurvivability is to induce changes in the properties of the structures subject to freezing. In the case of oocytes, these are usually made through supplementation of IVM media and/or freezing solutions.[31] The intracellular lipid quantities and the lipid composition of membrane systems are two of the main targets of modulation, since it is known that the susceptibility of gametes to freezing is directly associated to membrane properties and lipid content.[2,32]

The use of lipid metabolism regulators appears to be one of the most promising methods to achieve progress in oocyte vitrification, mainly in domestic mammalian species.[33] Centrifugation and delipidation of oocytes,[34] as well as reducing the concentration of fetal bovine serum used in the culture media and concomitant addition of phenazine ethosulfate, a metabolic regulator,[34-36] improved cryosurvivability reducing lipid accumulation without interfering with embryo quality in species with naturally high concentration of lipids like pigs and cattle.[31,37,38]

Options to decrease oocyte lipid content can involve inhibition of lipogenesis or stimulation of lipolysis processes.[33] The ability to control lipid metabolism is valuable for improving cryotolerance of oocytes as well as to improve the quality of the embryo developing *in vitro*.[39,40] Since endogenous lipids participate in energy metabolism and other important functions, lipid content alterations should be managed with caution, especially if conducted through invasive methods such as delipidation.[41] Just as with the cryopreservation technique and *in vitro* manipulation itself, the use of artificial inducers of lipid metabolism may also have a negative impact on oocyte competence. Many of the negative effects sometimes observed are related to an imbalance of reactive oxygen species (ROS) production and endogenous antioxidants, leading to oxidative stress, which is the cause of many conditions related to female fertility.

The superoxide anion, the product of the one-electron reduction of dioxygen, is the precursor of most ROS. Its dismutation reaction produces hydrogen peroxide, which in turn can be cleaved by catalase in water and oxygen or within the Fenton reaction, partially reduced to the extremely reactive hydroxyl radical.[42] Studies have reported that an increase in hydrogen peroxide levels, as well as ROS and a decrease in catalase activity, trigger the meiotic resumption of oocytes in rats, suggesting the importance of ROS in oocyte maturation.[43,44] There are increasing data on the activity of ROS and antioxidants as a driver of oocyte development and maturation; however, extensive research is needed to identify the safe and physiological concentrations of ROS and antioxidants suitable for oocyte development and cryopreservation. There are currently no studies quantifying how much oxidative stress can affect lipid composition via analysis of the lipid profile, but the use of antioxidant supplementation is recommended.[38]

Further research is needed to comprehend the precise mechanisms of lipid modulation.[41] In the subsequent sections, cases where lipid modulators have positively impacted the outcome of cryopreservation are discussed. In addition to phenazine ethosulfate, other examples of lipid modulators include: carnitine, forskolin, and isomers of linoleic acid. Updated information on the use of these compounds is presented.

Forskolin

Forskolin is a labdane diterpene that activates adenylate cyclase and increases intracellular levels of cAMP. Forskolin has a retarding effect on spontaneous maturation of oocytes and, therefore, this substance is being used to achieve greater synchronicity between nuclear and cytoplasmic maturation *in vitro* leading to improvements of development rates.[45] Monteiro et al.[46] showed the ability of forskolin and other cAMP modulators to minimise the damaging effects of vitrification on the cytoskeleton of immature oocytes after exposure in short term culture; however, it was not enough to improve blastocyst and embryo development.

Forskolin also stimulates lipolysis and was shown to have positive effects on the cryopreservation

of oocytes matured *in vitro*.[47] Paschoal et al.[48] confirmed its lipolytic action on tests with bovine embryos but it failed to improve their cryosurvivability and development potential. Recently, Meneghel et al.[49] reported interesting results. While forskolin supplementation on culture media had no effect on blastocyst yield, pre-treatment with 5.0 µM forskolin for 24 hours before vitrification was shown to decrease intracytoplasmic lipid content and improve the cryotolerance of bovine embryos. Its precise mechanism of action and how it affects cryotolerance is currently unknown.[50]

L-Carnitine

In animal cells, the ammonium compound L-carnitine acts as an enhancer of lipid metabolism due to its primary role in FA transport from the cytoplasm to the mitochondria. Triacyglycerols are metabolised by lipases from both cumulus cells and the oocyte. Therefore, FA generated by lipolysis are metabolised by β-oxidation in the mitochondria for the production of ATP.[39] The use of L-carnitine led to greater development of blastocysts in mice,[51] increased mitochondrial activity, and reduced intracellular lipid content and levels of ROS, which improved nuclear maturation and cleavage of porcine oocytes.[52] A brief exposure of porcine MII oocytes to 3 mM L-carnitine shortly before IVF improved post-warming survival rate of blastocysts.[53] In cattle, this led to higher hatchability rates on fresh oocytes and protected vitrified oocytes from spindle damage; however, L-carnitine exposure failed to improve the development of either fresh or vitrified oocytes.[54] The effects were attributed to the capacity of the compound to reduce cellular lipid content and provide antioxidant protection. Despite the incongruent results, it is a candidate reagent for non-invasive improvement of oocyte cryotolerance and developmental competence in oocytes of domestic animals with high lipid concentration. Its action is likely caused by an improvement of mitochondrial function.[23] Acetyl-L-carnitine, an ester of L-carnitine, has been used in some studies with similar results. Further studies are needed to determine the precise mechanism of action of carnitine compounds and the optimal protocol to replicate the improvements on human oocytes.[23,53,55,56]

Conjugated Isomers of Linoleic Acid

The conjugated isomers of linoleic acid (CLA) have been identified as promising lipid modulators. Tests with CLA in cattle demonstrated its ability to improve cryotolerance of embryos cultured in medium with the addition of 100 µM CLA, and improve the overall quality of the blastocyst.[57] These effects were able to overcome the possible negative effects caused by the presence of fetal bovine serum in culture media.[57] The positive effects of CLA were also observed in sheep embryos when used in a lower concentration.[38] The addition of CLA in bovine oocyte maturation medium was able to reduce its lipid content without interfering with the progression of meiosis.[58,59] The precise mechanism of action of CLA has not yet been fully clarified; it is currently believed that CLA undergoes β-oxidation in the mitochondria for energy production or synthesis of FA used in development, similar to other polyunsaturated FA. This increased mitochondrial activity may cause excess ROS production, leading to oxidative stress. An alternative to overcome the negative effects of ROS is the addition of antioxidants to production media.[38] A study by Leão et al.[60] showed a significant improvement in post-vitrification re-expansion of bovine embryos produced in media with CLA supplementation, especially when used in both maturation and culture media. In addition, this study helped to elucidate the mechanism of action of CLA analysing the lipid content of embryonic plasma membrane with a matrix-assisted laser desorption/ionisation-mass spectrometry technique (MALDI-MS). It was observed that CLA not only reduces intracellular lipid content, but it may also cause changes in the composition of membrane phospholipids (PL), particularly phosphatidylcholines (PC), increasing unsaturation levels and, hence, its fluidity.

The effects of dietary delivery of rumen-protected supplementation of CLA was also assessed; the results indicate that CLA may be used to prevent aberrant accumulation of saturated FA, frequently observed in *in vitro* production,[61] and confirms the influence of dietary supplementation on lipid profile. Nevertheless, recent data showed that, although CLA was able to reduce intracytoplasmic lipid

content, CLA addition to IVP media had no effect on post-thaw viability of embryos.[62] Similarly, there was no beneficial effect of supplementing IVM medium with both CLA and L-carnitine on embryo development or post-thaw cryosurvival.[63] Therefore, further studies are necessary to confirm the possible effects on the oocyte and the precise mechanism of CLA action.

Cholesterol

Ideally, a viable cryopreservation process should spare structural lipids present in the bilayer of organelles and plasma membranes. However, it is known to affect all membrane systems within the cell, with full recovery after post-thaw rehydration being an exception.[64] Membrane systems mostly suffer with decreased fluidity; therefore, the most desirable change in cell membranes exposed to low temperatures is to avoid an increase in the viscosity of the membrane lipid bilayer.

Apart from the CLA and other FA supplementations mentioned previously, another strategy used to enhance membrane fluidity to protect the oocyte from cryoinjuries is the addition of cholesterol. The increase in cholesterol content of sperm and oocyte membranes seems to improve cryotolerance by making them more fluid at low temperatures.[65] Arcarons et al.[66] observed that pre-treatment with cholesterol-loaded methyl-β-cyclodextrin, which transfers the cholesterol into cellular membranes, before the vitrification of bovine oocytes did not affect cleavage and embryo development rates. However, it did improve the quality of embryos derived from vitrified oocytes and altered the gene expression related to lipid metabolism.

LIPID PROFILE: ASSESSING THE CHANGES IN LIPID CONTENT

Knowledge of what is really changing on the lipid profile of oocytes is just as important as knowing the impact of metabolism regulation and lipid supplementation on the outcomes of cryopreservation and development.

Mass spectrometry has revolutionised the field of lipidomics; current advances allow for precise and quick analyses with little or no preparation of gamete cells.[67] Examination of the lipid profile of individual and/or pools of up to five oocytes have been performed by distinct mass spectrometry ionisation methods, including MALDI-MS,[13] electrospray-ionisation mass spectrometry,[68] and desorption electrospray-ionisation mass spectrometry.[66] These innovative tools confirmed that the lipid profile is indeed dynamic and showed great potential of such techniques for detailed lipidomic studies in the reproductive field. Using a variety of techniques, studies have successfully quantified lipids in oocytes and determined distinct lipid profiles due to the physiologic or *in vitro* environment that oocytes were exposed to.[8,11,39,69]

Impact of Lipid Profile on Oocyte Cryopreservation

While most studies in the field had mainly observed changes in zygotes and embryos, a few studies presented important findings regarding the alterations on oocyte lipid profile. The aforementioned study by Xu et al.[23] used mass spectrometry to show that phospholipids m/z 728.7 (phosphatidylcholine [PC] 32:3), 746.9 (PC 32:5), 760.6 (PC 34:1), 768.8 (PC P-36:3), and 782.6 (PC 36:4) were more abundant in fresh oocytes than in vitrified oocytes. Lapa et al.[70] tested the effect of CLA on bovine oocyte competence and FA composition. Total lipids and FA profiles were determined by gas chromatography after CLA supplementation to IVM medium. Besides CLA presence, lower levels of arachidonic acid (C20:4n-6) in FA profile of mature oocytes was observed, suggesting oocyte consumption and improving bovine oocyte competence to develop into higher quality embryos. Despite lower levels of arachidonic acid, the total level of polyunsaturated FA was maintained. Cryosurvivability was not assessed, but these results indicate a possible mechanism in which CLA compensates for the polyunsaturated FA used during maturation process, maintaining membrane fluidity.

Supplementation with acetyl-L-carnitine altered the composition of three phospholipids (m/z 734.6 [PC 32:0], 760.6 [PC 34:1], and 782.6 [PC 36:4]) of vitrified buffalo oocytes, all of which were more abundant in the supplemented group.[23]

Further confirming the influence of short-term exposure of lipid supplementation on

the composition of the lipid profile of oocytes, Pitangui-Molina et al.[10] showed that the addition of soybean PC to maturation media resulted in greater relative abundances of PL PC (32:1), PC (34:2), PC (36:6), PC (36:4), and PC (38:6) in oocyte membrane, and did so without compromising development rates.

The incorporation of so many polyunsaturated PL is a remarkable finding. Recently, a report suggested that preserving PL during cryostorage is of utmost importance and that PL monitored by MALDI-MS could even be used as possible biomarkers for healthy oocytes after cryopreservation procedure.[68]

Another major step for lipid modulation and its influence on cryopreservation is the possibility of using non-invasive techniques. Besides mass spectrometry, quantification of oocyte lipid is also being explored by Raman microspectroscopy.[71] Interestingly, this technique has been used to non-invasively examine the actin cytoskeleton of sheep oocytes following vitrification and and the structural changes of lipids within lipid droplets during the process.[22,70] Coherent anti-Raman stokes microscopy has been successfully used to quantify lipid content of oocytes from species with known differences, including mouse, human, and pig.[69,71] Differences in the chemical composition of lipid droplets in living oocytes matured in media supplemented with different saturated and unsaturated FA can also be detected using coherent anti-Raman stokes microscopy.[72]

CONCLUSION

Knowledge about the precise changes in lipid profile induced by the addition of different biomolecules, such as FA, PL, and lipid modulators, in the culture medium is of great importance to assess improvements in the quality of oocytes and have direct application on the understanding of oocytes' metabolism and membranes response to freezing and, consequently, to the possible refinement of cryopreservation techniques. Mass spectrometry has been an undeniably valuable tool in this process. The advances of non-invasive techniques for lipid profile assessment and the refinement of equipment and CPA used in oocyte vitrification to better monitor and manage metabolism and cell membranes composition are seen as promising steps to improve overall success rates of female gamete storage. However, it might not be enough since there are many gaps in the knowledge of oocyte lipid dynamics and oocyte metabolism whether applied to cryoresistance or not.

For instance, the lack of studies using human oocytes is strikingly evident. Ethical and procedural barriers are responsible, in part, for this shortage. However, the use of discarded immature oocytes after IVF cycles is a clear viable alternative that is not being explored. While there are plenty of other species being studied, there is a need to define the best animal model for translational studies of oocyte lipidomics and oxidative metabolism.

In addition, accumulated data shows that few groups have directed their attention to the basis of oocyte metabolism and lipid and/or protein dynamics, while many studies focus on finding substances that improve individual aspects of gametes storage and development *in vitro* with disregard to their precise mechanism of action in culture medium and/or freezing solutions.

The identification of classes of lipids of interest is a good start. Important work has been done to single out possible candidates for further research, but now that the influence of lipids on oocyte development has been well-established, the authors believe that forthcoming studies should help to contextualise the results obtained so far. More co-ordinated studies that integrate genetic, metabolomic, and morphologic aspects are needed to deepen our understanding of oocyte lipid metabolism and cryobiology.

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