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REVIEW

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# Targeting Lipid Metabolism to Improve Oocyte Cryopreservation (OCP) in Domestic Animals

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## ABSTRACT

Oocyte cryopreservation (OCP) lags behind other advancements that have recently been made in many areas of assisted reproductive technology. One of the risk factors that increases the chilling sensitivity of oocytes, especieally in domestic animals, is the high lipid content. Many studies have demonstrated that reducing the lipid content improves cryosurvival of oocytes. The methods that have been used to reduce the lipid content include mechanical delipidation by centrifugation of the oocytes and polarizing lipid droplets, enhancing lipid catabolism and inhibiting lipogenesis. Targets of the latter include incude fatty acids that are synthesized *de novo* and those taken up from culture media by the oocytes. In the present review, we provide an overview of studies and approaches on delipidation and suggestions for future studies in the area.

**Keywords:** *Oocyte, Cryopreservation, Lipogenesis, Lipid metabolism, Reproductive technology*

## INTRODUCTION

The application of reproductive technologies in mammals saw significant advancements over the years. Major milestones include the discovery of spermatozoa and their use for artificial insemination (AI) and production of canine pups by Spallanzani in 1770 [reviewed by [1]], the first successful embryo transfer (ET) in the rabbit in 1890 [2], production of bovine offspring after AI with cryopreserved spermatozoa in 1951 [3], production of rabbit offspring after *in vitro* fertilization (IVF) and ET in 1959 [4], successful cryopreservation and ET of mouse embryos in 1972 [5], birth of mouse pups after using cryopreserved oocytes for IVF and ET in 1977 [6], and the use of somatic cell nuclear transfer or cloning to produce offspring in sheep in 1996 [7]. These advances have been replicated in different genera of domestic and nondomestic mammalian species. Thus, a wide array of tools is available to us to conserve and propagate certain genotypes, and to treat many forms of infertility.

Cryopreservation of spermatozoa is routinely used in animals and humans; however, oocyte cryopreservation (OCP), although the first success was reported more than 35 years ago, is poorly developed. Difficulties with OCP are due to inherent properties of the oocytes such as its large size; the high lipid content that varies among species also increases the chilling sensitivity of oocytes [8]. As a result, the use of OCP for embryo production and subsequent ET and live birth reports are sporadic. To date, no such offspring has been reported in pigs and canids, two domestic mammalian species with the highest lipid content in oocytes. Thus, the flexibility to conserve and propagate valuable genotypes of domestic animals such as pigs is limited.

It is unclear why and how oocytes of different species accumulate varying amounts of lipids, although it is likely to be associated with peculiar metabolic needs of the oocytes during maturation and development into early embryos. Nevertheless,

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there is a broad consensus among reproductive physiologists that in order to improve the cryosurvival of oocytes of many mammalian species there is a need for reduction (delipidation) of oocyte lipid content. In the present paper, we provide an overview of currently used delipidation approaches and suggest on future directions for delipidation that target pathways of lipid metabolism.

## Importance and Current Status of Oocyte Cryopreservation

Currently, advanced reproductive technologies provide us with multiple tools to facilitate the breeding management of animals. In humans, they are widely used for treating infertility and more than 5 million babies have so far been born with such technologies involving oocyte handling such as IVF and sperm injection. The cryopreservation of gametes (spermatozoa and oocytes) improves the reproductive management of animals by removing limitations of time and space for pairing males and females that will breed with each other. For example, to breed an animal with another one across or within a continent, it is much easier to transport frozen (cryopreserved) gametes than to transport animals. It also enables breeding and propagation of a genotype long after the death of either parent. Success in OCP will advance our ability and efficiency to preserve and propagate genetically valuable animals (e.g. high performers, genetically modified) and endangered species.

Offspring have been produced after using cryopreserved oocytes for embryo production and ET in mouse, rabbit, cat, and cattle, sheep, and horses [9,10]. However, success rates in domestic animals are poor and the technique has not been successful in species such as pigs in spite of many efforts [11-14]. The difficulties to successfully cryopreserve oocytes are associated with some unique structural and physiological properties of the oocyte as described below.

Cryopreservation induces multiple changes, including the formation of intracellular ice crystals and alterations in the content/structure of cells [10,15]. The oocyte is the largest cell in the body with low surface area to volume ratio that reduces permeability to water and cryoprotectant agents. Additionally, the large spindles and cortical granules of oocytes are susceptible to cryoinjury. These features are common among oocytes of different species; however, oocyte lipid content, which varies among species (Figures 1 and 2) and is higher in domestic animals also increases the chilling sensitivity of oocytes [8]. Paradoxically, because of their greater potential to develop further, oocytes with high lipid content are selected for reproductive technology procedures. Enhancing lipid catabolism also appears to be beneficial for the development of oocytes [16,17]. The present review focuses on lipid metabolism but many studies have also targeted non-lipid associated risk factors to improve OCP, which include using multiple cryoprotectant agents, minimal volumes for vitrification, cytoskeleton stabilizers, enhancers of heat shock protein function and freezing of oocytes at advanced maturation stages [12,13,18].

## Targeting Lipid Content to Improve OCP

High lipid content of oocytes and embryos is one of the well characterized factors associated with poor cryosurvival in pigs, cattle and other species [13,19-22]. For example, pig oocytes contain 161 ng of lipid per oocyte which is ~2.5-fold that of bovine oocytes which in turn contains ~17 times as much lipid as mouse oocytes [23]. Species with more lipid content have oocytes that are more susceptible to chilling sensitivity [12,13,23,24]. Thus, the lipid content is an attractive target in attempts to improve methods of OCP. Large amount of lipid is also carried over to embryos negatively affecting embryo cryosurvival.

Many studies have demonstrated that reducing the lipid content is a practical option to improve the cryotolerance of oocytes and embryos [22,25-28]. Early attempts to improve cryosurvival of oocytes and embryos evaluated **mechanical removal (delipidation)** of lipid droplets [26,27,29-31]. This technique requires centrifugation of oocytes at high speed (≥12,000 xg) to polarize lipid droplets, which are either aspirated or left in the zona pellucida. This approach improves cryosurvival and subsequent development; however, it is time-consuming and negatively affects developmental potential [32]. Lipid droplets are also biologically important organelles and not just lipid aggregates [33]; hence, mechanical delipidation removes vital droplet components from oocytesThus, there is a need for mild **pharmacological approaches** of depleting the lipid droplets by enhancing catabolism and inhibiting synthesis of lipids. Aspects of the latter approaches are being investigated by different groups; here, we provide an overview of lipid metabolism with emphasis on potential targets for pharmacological reduction of lipid content during *in vitro* maturation (IVM) of oocytes.

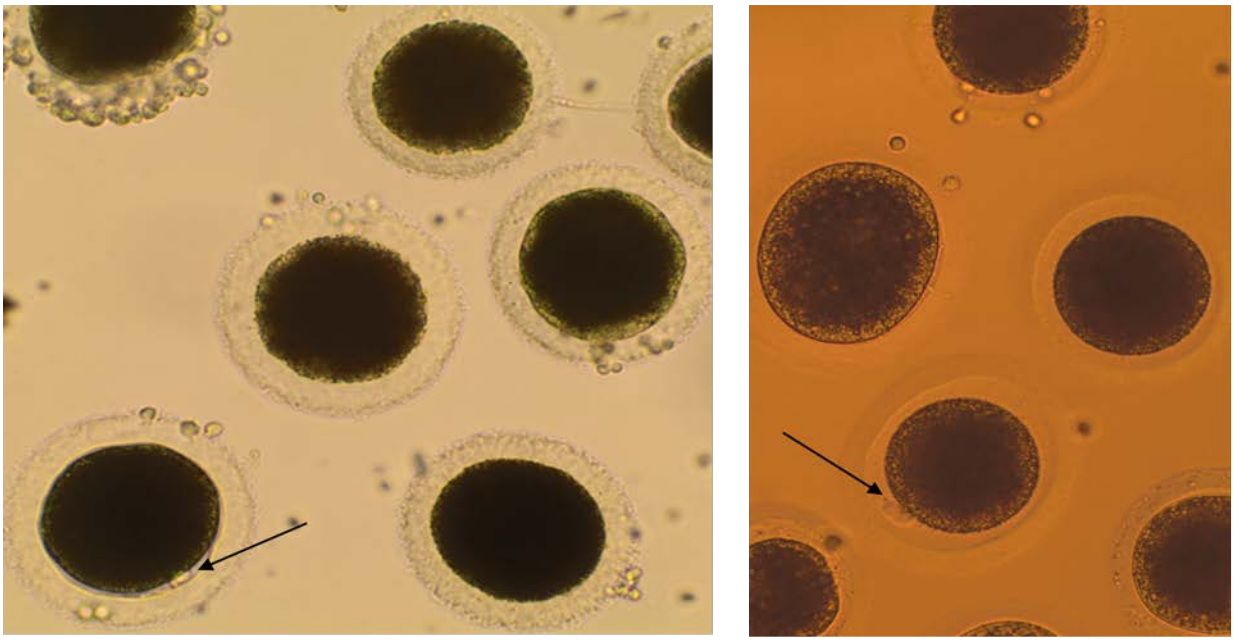
## Lipid Types in Oocytes and Targets for Improving OCP

The targets of delipidation are cytoplasmic lipid droplets (Figure 2). A viable delipidation process should spare structural lipids such as the phospholipid bilayer of plasma and organelle membranes. Studies characterizing the lipid content of oocytes involve analysis of oocyte lysates; hence, data are lacking on the specific contribution to fatty acids of plasma and organelle membranes vs. lipid droplets. Nevertheless, oocyte lipids contain both saturated and unsaturated fatty acids (FA), the most abundant ones accounting for >50% of the FA being oleic, palmitic and stearic acids in ovine, bovine and porcine oocytes [23,34]. Most of the FAs occur as triglycerides [35].

The lipids form during the growth of oocytes in the ovarian follicles; hence, they are already present in oocytes recovered from antral follicles [36]; however, gene expression and metabolic studies suggest that oocytes can also take up and synthesize the FAs *de novo* [37-39]. Additionally, oocytes actively utilize the stored lipid during IVM. For example, the triglyceride content of pig oocytes decreased by ~10% during a two day culture period [40] and that of bovine oocytes declined progressively during maturation and early embryonic

**Figure 1:** *Canine (left) and feline (right) oocytes subjected to in vitro maturation (IVM) for 96 and 24 h, respectively (Standard oocyte IVM duration is 3 to 4 days in dogs but 24 hours for cats). Arrow points to a polar body of a mature oocyte. The evenly darker appearance of canine oocytes as compared to the more clear periphery of feline oocytes suggests the presence of more cytoplasmic lipids in canine oocytes (original magnification: 100x).*



development [41]. Thus, pharmacological options to decrease oocyte lipid can involve inhibition of lipogenesis or stimulation of lipolysis, and similar strategies have been evaluated in many reports, some of which are descriebed next.

Culturing bovine [42,43] and porcine [44,45] embryos in media containing phenazine ethosulphate (PES), an NADPH-depleting agent, causes a reduction of intracellular lipid. NADPH is a reducing agent and serves as a hydrogen donor during cholesterol synthesis and elongation of fatty acids. Thus, its depletion is expected to interfere with *de novo* lipogenesis. However, NADPH is also an important reducing agent for other processes and its depletion induces oxidative stress [46,47] and compromises the development of porcine oocytes [48]. Nevertheless, bovine embryos cultured in PES-supplemented media have been used for ET with normal offspring produced [49] suggesting that pharmacological reduction of lipid content is compatible with normal in vivo development of embryos.

Enhancing lipid catabolism (β-oxidation), by supplementing the

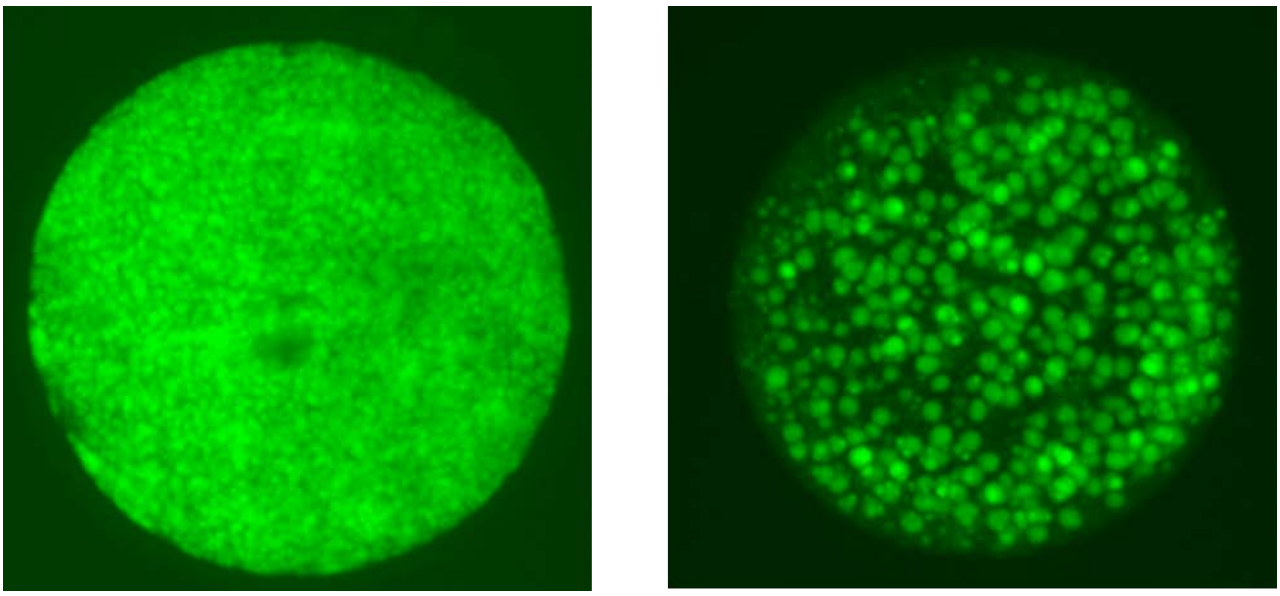


**Figure 2:** *Fluorescent images (FITC filter) of Nile red-stained canine (left) and feline (right) oocytes subjected to in vitro maturation for 96 and 24 h, respectively. Note the more numerous but smaller in size lipid droplets in the canine oocyte (original magnification: 200x). Oocytes were fixed in formalin for at least 30 min and stained in 1 μg/ml nile red before being mounted for evaluation using fluorescent microscopy.*

culture media with L-carnitine, also reduced the lipid content and improved the potential of oocytes and embryos for development [14,17,50,51]. Similarly, forskolin-a cAMP-inducer and lipase activator, increased lipolysis in porcine oocytes and embryos [52,53], with positive effects on embryo cryosurvival [52]. Nevertheless, hyperstimulation of β-oxidation may not be compatible with optimal development of embryos [54].

Restriction of nutrient supply in IVM media (i.e. use of serum-free culture system) also reduces the lipid content of oocytes and embryos, presumably by enhancing utilization of endogenous lipids, and thereby improves cryosurvival [41,55]. However, developmental competence is usually suboptimal when serum is excluded from culture media.

Enzymes of *de novo* lipid synthesis such as acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS) offer other potential targets for pharmacological reduction of oocyte lipid content. Many drugs that target this pathway are in various stages of development as potential chemotherapeutic agents against cancer and obesity [56-59].

ACC is considered a rate-limiting enzyme and is expressed in oocytes of multiple species [37,38,60]. In somatic cells, inhibition of ACC reduces *de novo* lipid synthesis with specific declines in the formation and elongation of FAs and their esterification to di- or triglycerides [57]. Further consequences of ACC inhibition are a decline in malonyl-CoA, and up regulation of carnitine palmitoyl transferase (CPT)-1 and β-oxidation. Although data are lacking for other species, mouse [37] and canine (Simon and Wirtu, personal observation) oocytes express the active (ACC) and inactive (phospho-ACC, pACC) forms of the enzyme suggesting that pharmacological modulation of ACC:pACC ratio is possible and could be used to influence lipogenesis. In a preliminary study in our laboratory, supplementation of IVM media with an ACC inhibitor for ~24 h had minimal effect on canine oocyte lipid content as evaluated by using Nile red fluorescence intensity [61]; the lack of marked effect may be related to the inherently poor potential for IVM of canine oocytes [62]. Additionally, genes in this pathway are crucial for development [63], with complete ACC1 and FAS knockout but not that of ACC2 being embryo lethal in mice. Thus, caution should be used to prevent potential embryo toxic effects of pharmacological inhibitors.

FAS is also expressed in oocytes [64]. Two FAS inhibitors had positive effects on IVM rates of mouse oocytes [65] although effects on lipid content remain to be investigated. AMPK agonists have also been evaluated for their effects on mouse and bovine oocytes with mixed effects [65,66].

Various cells, including oocytes [38] express fatty acid transporter proteins such as FAT/CD36 that facilitate uptake of extracellular FAs. Recent data indicate FA uptake is accomplished primarily by facilitated diffusion using protein transporters, with simple diffusion across the phospholipid bilayer having a minor role [67]. The effects of inhibiting these proteins in oocytes have not been reported. Embryos cultured in the presence of serum, which is rich in FA, accumulate large amounts of lipids [41,55,68]; thus, selective inhibition of FA uptake could allow oocytes and embryos utilize other beneficial components of serum-supplemented media with minimal concern about the negative effects of lipid accumulation resulting from serum components.

## Conclusion Remarks

The reduction of oocyte lipid will remain an integral component of protocols for optimizing OCP in domestic animals. It is well established that oocytes are active in lipolysis (β-oxidation). On the other hand, although mRNA and protein expression data suggest the existence of local lipogenic machinery, the significance of lipogenesis to the overall lipid content and metabolic requirements of oocytes needs to be characterized. Nevertheless, studies conducted so far indicate that pharmacological upregulation of lipolysis and inhibition of lipogenesis may be used to delipidate oocytes. The proven targets and additional steps in lipolytic and lipogenetic pathways should be evaluated further and optimized for their potential use in improving OCP.

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Gemechu Wirtu studied veterinary medicine at Addis Ababa University, where he also worked as a faculty member. He then obtained MS and PhD degrees from Virginia Tech and Louisiana State University, respectively. His graduate and postdoctoral research was on assisted reproductive technologies of domestic and nondomestic ungulates. He is currently an assistant professor of veterinary physiology at Tuskegee University. His research interest includes characterizing factors that affect developmental potential of oocytes, specifically how lipid metabolism affects maturation rates, cryosurvival and subsequent embryonic development. Further area of interest is understanding the role of lipid metabolism in mammary tumor invasiveness.