REPRODUCTION

REVIEW

Lipids and oocyte developmental competence: the role of fatty acids and β-oxidation

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Abstract

Metabolism and ATP levels within the oocyte and adjacent cumulus cells are associated with quality of oocyte and optimal development of a healthy embryo. Lipid metabolism provides a potent source of energy and its importance during oocyte maturation is being increasingly recognised. The triglyceride and fatty acid composition of ovarian follicular fluid has been characterised for many species and is influenced by nutritional status (i.e. dietary fat, fasting, obesity and season) as well as lactation in cows. Lipid in oocytes is a primarily triglyceride of specific fatty acids which differ by species, stored in distinct droplet organelles that re-localise during oocyte maturation. The presence of lipids, particularly saturated vs unsaturated fatty acids, in *in vitro* maturation systems affects oocyte lipid content as well as developmental competence. Triglycerides are metabolised by lipases that have been localised to cumulus cells as well as oocytes. Fatty acids generated by lipolysis are further metabolised by β-oxidation in mitochondria for the production of ATP. β-oxidation is induced in cumulus–oocyte complexes (COCs) by the LH surge, and pharmacological inhibition of β-oxidation impairs oocyte maturation and embryo development. Promoting β-oxidation with L-carnitine improves embryo development in many species. Thus, fatty acid metabolism in the mammalian COC is regulated by maternal physiological and *in vitro* environmental conditions; and is important for oocyte developmental competence.

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Introduction

Successful reproduction is dependent on the ovulation of an oocyte capable of undergoing fertilisation and subsequent embryo and foetal development, and requires the co-ordinated and stepwise growth and maturation of the oocyte and its companion somatic cells known collectively as the ovarian follicle. Ovarian follicles grow from the dormant primordial stage in which the oocyte is surrounded by a single layer of granulosa cells to a preovulatory stage follicle. By this stage, the fully grown oocyte has amassed nutrient stores, mRNA, proteins and organelles, including large numbers of mitochondria, and is surrounded by specialised cumulus cells, a fluid-filled antral cavity and a stratified epithelial layer of granulosa cells. Ovulation of the cumulus–oocyte complex (or COC) and the final stages of oocyte maturation are initiated by a surge of luteinising hormone (LH), which signals to the oocyte via the granulosa and cumulus cells to resume meiosis and complete nuclear maturation to the metaphase II (MII) stage of meiosis in preparation for fertilisation. These rapid and tightly synchronised events, which include granulosa cell proliferation, cumulus cell matrix production and chromosome segregation, are energy-consuming processes and require adequate generation of ATP from cellular energy stores.

The *in vitro* maturation of oocytes (IVM) involves removal of the COC from the antral follicle before the LH surge and stimulation of the final stages of maturation *in vitro*, i.e. in the absence of the *in vivo* follicular signals. Oocytes matured by IVM are however less likely to develop to the blastocyst stage (Rizos *et al.* 2002, Gilchrist & Thompson 2007) and result in higher rates of miscarriage compared with oocytes that mature in follicles *in vivo* (Buckett *et al.* 2008). The causal mechanisms responsible for this poor oocyte quality following IVM are not clear; however, oocyte developmental competence is associated with the metabolism and metabolic rate of the oocyte and its surrounding cumulus cells (Biggers *et al.* 1967, Downs 1995, Sugiura & Eppig 2005, Preis *et al.* 2007, Thompson *et al.* 2007). Adequate levels of intracellular ATP are also required for optimal oocyte developmental potential (Van Blerkom *et al.* 1995) and therefore energy substrate supply to the COC via the follicular fluid or culture medium during *in vivo* maturation or IVM, respectively, is likely to affect oocyte quality.

Lipids are hydrophobic or amphipathic molecules with diverse biological roles that include being a rich

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source of energy, cell signalling mediators and the foundation of plasma and organelle membranes. Fatty acids are a class of lipid and function as structural components of membranes, precursors for prostaglandin synthesis and to anchor proteins to cell membranes. Fatty acids are also stored intracellularly as triacylglycerides within lipid droplets, providing a potent source of energy upon demand; for instance, oxidation of the fatty acid palmitate can generate 106 ATP molecules compared with glucose oxidation which yields ~30 ATP molecules.

Important roles for fatty acids in the promotion of embryo development have been clearly demonstrated and comprehensively reviewed (McKeegan & Sturmey 2011); however, accumulating evidence indicates that the metabolism of lipids by β-oxidation in the COC before fertilisation also influences subsequent oocyte developmental potential. Oocytes in particular but also cumulus cells are well known to contain lipid droplets, but how these are utilised during oocyte maturation is only just emerging. Similarly, although lipid utilisation by oocytes has been demonstrated primarily by indirect methods (reviewed in Sturmey *et al*. (2009)), β-oxidation within the whole COC has been directly documented more recently and appears to be occurring in large part in cumulus cells. Understanding these pre-conceptional roles of fatty acids in both cumulus cells and oocytes is essential in order to understand how physiological alterations in follicular lipids, as well as the supply of lipids as substrates for metabolism during IVM, impacts the earliest stages of embryo development.

Fatty acid supply to the COC

Lipid composition of ovarian follicular fluid and relationship to serum

Free fatty acids (or non-esterified fatty acids (NEFA)) are attached to serum albumin which acts as a carrier protein rendering the insoluble fatty acid suitable for transport through the circulation to tissues. However, the majority of circulating fatty acids are in the form of carboxylic acid derivatives including esters or amides. Fatty acids are also stored as triacylglycerol molecules, or triglycerides, in which three fatty acid molecules are attached to a glycerol backbone; and these are carried throughout the blood in lipoprotein particles, i.e. HDLs, LDLs and VLDLs. Triacylglycerols and fatty acids are present in the follicular fluid of numerous species and there is emerging interest in understanding how these substrates are ultimately utilised by ovarian somatic cells and oocytes for energy production. Analyses of lipoproteins in human follicular fluid in several studies corroborate that HDLs but little or no LDL or VLDL are present (Simpson *et al*. 1980, Perret *et al*. 1985, Volpe *et al*. 1991, Jaspard *et al*. 1997). Follicular fluid HDL cholesterol is positively correlated with serum HDL cholesterol (Gautier *et al*. 2010, Valckx *et al*. 2012), indicating that HDL particles are serum derived and passively equilibrated; however, there is no similar correlation for VLDL cholesterol in follicular fluid and serum (Gautier *et al*. 2010). Thus, it is generally accepted that in mammals HDL is the sole lipoprotein present in follicular fluid due to the porosity of the follicle basement membrane which is permeable to serum proteins up to 300 kDa in size (Shalgi *et al*. 1973), thus excluding LDL and VLDL. Interestingly, although LDL and/or VLDL are detected in follicular fluid of some women (Von Wald *et al*. 2010) and it is also reported that human granulosa-lutein cells express lipoprotein marker ApoB-100 and assemble and secrete *in vitro*-native VLDL particles similar to those in serum, except with slightly higher triglyceride content and less cholesterol (Gautier *et al*. 2010). Thus, LDL and VLDL particles detected in follicular fluid may in fact be generated by ovarian cells.

It is well understood that HDL particles have important functions in granulosa and theca cell steroidogenesis, serving as the predominant source of cholesterol (Azhar *et al*. 1998, Hughes *et al*. 2011); yet studies examining the effects of lipoproteins on oocyte developmental competence are contradictory. Studies in the mouse demonstrate the importance of HDL integrity for oocyte competence; as knockout mice lacking the HDL receptor scavenger receptor class B, member 1 (SRBI) are infertile due to fertilised oocytes arresting before the morula stage (Miettinen *et al*. 2001). These mice have abnormally large circulating HDL particles and, interestingly, restoration of SRBI expression in the liver alone is sufficient to normalise HDL particle size and restore fertility (Yesilaltay *et al*. 2006). Increased HDL in human follicular fluid was associated with low embryo fragmentation (Browne *et al*. 2008, 2009), suggesting that HDL components play a cytoprotective role for the oocyte. Similarly, high ApoB in human follicular fluid is associated with better quality embryos and higher pregnancy rates (Gautier *et al*. 2010). However, two recent studies have shown that increased HDL and ApoAI levels were associated with failure of oocytes to cleave and decreased the numbers of good quality embryos (Valckx *et al*. 2012, Wallace *et al*. 2012). Furthermore, it is unclear whether lipoproteins found in follicular fluid influence oocyte quality via their ability to deliver lipid substrates such as triacylglycerol or whether other components of these particles, namely the surrounding apolipoproteins, which are known to have scavenging properties, protect cells from oxidative stress.

Numerous studies have compared triacylglycerol and free fatty acid levels between serum and follicular fluid. In dairy cows, follicular fluid triacylglycerol and free fatty acid (i.e. NEFA) levels were lower than but significantly correlated with those in serum (Leroy *et al*. 2004*a*,*b*). Linoleic, palmitic and oleic acid (see Table 1) predominate in the total fatty acid fraction of bovine follicular fluid (Homa & Brown 1992,

Table 1 The common name and structure of fatty acids.

Common name	Structure		Saturation
Myristic acid	14:0		Saturated
Palmitic acid	16:0		Saturated
Stearic acid	18:0		Saturated
Oleic acid	18:1 (9)		Monounsaturated
Linoleic acid	18:2 (9, 12)	n-6	Polyunsaturated (PUFA)
α-linolenic acid	18:3 (9, 12, 15)	n-3	Polyunsaturated (PUFA)
Arachidonic acid	20:4 (5, 8, 1, 14)	n-6	Polyunsaturated (PUFA)
Adrenic acid	22:4 (7, 10, 13, 16)	n-6	Polyunsaturated (PUFA)

These varying structures play important biological roles, but for the purpose of energy production via oxidation, longer saturated fatty acids yield more energy than shorter or unsaturated fatty acids.

Tsujii *et al*. 2001). Oleic, palmitic and stearic acid were the most prevalent free fatty acids in follicular fluid from lactating cows, and were ∼40% of the levels in serum (Leroy *et al*. 2005*a*). Free fatty acid levels may be related to follicle maturation, as follicles with higher oestrogen: progesterone (E_2:P_4) ratio have more palmitic and oleic acids and less stearic and linoleic acids (Renaville *et al*. 2010). In human follicular fluid, recent analyses have demonstrated that most serum metabolites including triacylglycerol and free fatty acids are reflected in follicular fluid, but at lower levels (Valckx *et al*. 2012). Oleic, linoleic and palmitic acids are the most prevalent free fatty acids in human follicular fluid and exhibit weak but significant correlations with levels in serum (Jungheim *et al*. 2011).

Thus, there is extensive information about the prevalence of lipoproteins, triacylglycerol and free fatty acids in the microenvironment of the COC, namely the follicular fluid. However, whether lipoproteins act as a major delivery system of lipids or whether other specific lipid transport systems are required in cumulus cells and/or the oocyte is not known. Furthermore, although the fatty acid composition of follicular fluid of many species has been characterised, it is unclear whether or to what extent the metabolism of follicular fluid fatty acids provides ATP to the cumulus cells and/or oocyte. Importantly, no studies to date have demonstrated a correlation of any specific follicular fluid fatty acids with fertility outcomes.

Lipid droplets in oocytes

Oocytes are renowned as large cells containing lipid stores and numerous studies have observed differences in the darkness of the oocyte cytoplasm, even in oocytes from the same ovary, and deemed it lipid. Bovine oocytes classified into different 'darkness categories' were found to have differences in cleavage and blastocyst development rates *in vitro* (Jeong *et al*. 2009). Comparison of bovine oocytes with light homogeneous cytoplasm and those with course dark cytoplasm found that palmitic acid was equally prevalent in both types, but that light oocytes contained a higher percentage of oleic and linoleic (18:2) fatty acids, while the darker oocytes had more saturated stearic acid (Kim *et al*. 2001). Thus the optical density of the ooplasm may indeed reflect its lipid, i.e. fatty acid, content, and if so, suggests that lipid content contributes to oocyte developmental competence.

Using a variety of techniques, many studies have quantified lipids in oocytes and determined that triacylglycerol is the major constituent (reviewed in Sturmey *et al*. (2009)). An early study determined that 'ova' isolated from the oviducts of mated mice contain 3.25 ng of lipid or 12.5% of the dry mass based on sample weight before and after chloroform extraction (Loewenstein & Cohen 1964). Since then lipid storage in intracellular droplets has been identified by electron microscopy of oocytes from many species, including rabbit (Zamboni & Mastroianni 1966), cat (Martins *et al*. 2009), pig (Kikuchi *et al*. 2002) and cow (Kruip *et al*. 1983), with the lipid droplets often associated with mitochondria. Lipophilic Nile red dye is also commonly used to visualise lipid in oocytes; oocytes that have darker cytoplasm have greater Nile red staining (Leroy *et al*. 2005*b*). Decomposition of Nile red spectra was used to quantify triacylglycerol, phospholipid and cholesterol in oocytes and embryos (Romek *et al*. 2011); Nile Red staining of porcine oocytes followed by florescence resonance energy transfer was used to demonstrate that lipid droplets and mitochondria are within ∼10 nm of each other (Sturmey *et al*. 2006). BODIPY 493/503 is a neutral lipid dye that has more recently been used to detect lipid droplets in oocytes and demonstrate they are bounded by the lipid droplet protein ADRP in both mouse and bovine (Yang *et al*. 2010, Aardema *et al*. 2011). Examination of lipid droplets in an individual mouse oocytes by matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) (Ferreira *et al*. 2010) and syncotron Fourier transform-infrared (FT-IR) and Raman microspectroscopy demonstrated that the molecular composition of the lipid deposits exhibits spatial organisation within the droplet (Wood *et al*. 2008) and that mature oocytes which have high developmental competence have a distinct lipid profile compared with oocytes with immature chromatin structures that are unable to complete meiosis and form blastocysts (Ami *et al*. 2011). Overall, however, our understanding of the mechanisms that control lipid deposition in oocytes is in its infancy.

Interestingly, oocytes of different species exhibit marked variations in lipid content. We have used BODIPY 493/503 neutral lipid stain to illustrate these differences in oocyte lipid content between several species: mouse, cow, sheep, pig and humans (Fig. 1). Pig oocytes are known to be lipid laden, with the majority stored as neutral lipids, mainly triacylglycerol

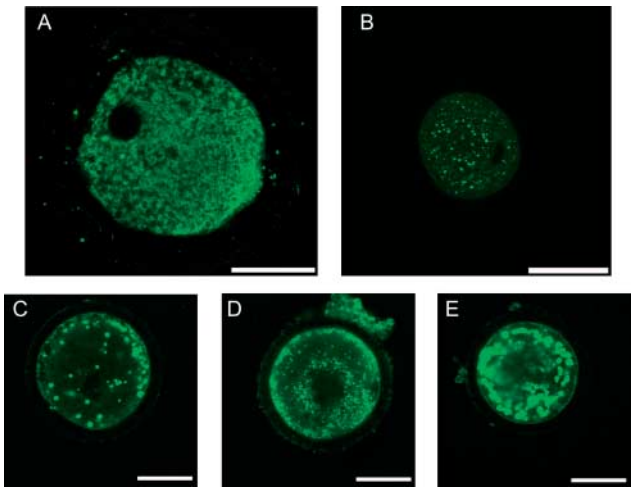

Figure 1 Lipid droplet localisation in mammalian oocytes. Images of immature oocytes from humans (A), mouse (B), sheep (C), cow (D) and pig (E) stained with BODIPY 493/503 neutral lipid dye. All procedures were carried out in serum-free polyvinylpyrrolidone-(PVP; 0.2% w/v)-containing medium or PBS. Cumulus–oocyte complexes were denuded and oocytes fixed in 4% paraformaldehyde. Oocytes were permeabilised for 30 min in 0.1% (w/v) saponin/0.1 M glycine in PBS and intracellular neutral lipid stained with BODIPY 493/503 (Molecular Probes) 1 μg/ml in PBS for 1 h in the dark. Oocytes were imaged by confocal microscopy (FluoView FV10i; Olympus), using fixed, predetermined magnification and exposure settings. Scale bar = 60 μm. The human oocyte was obtained during routine IVF procedures, and fixed and stained following its failure to fertilise. Diffuse staining and morphology is likely due to the 24 h of *in vitro* culture. Mouse oocyte was collected 48 h post-eCG (5 IU) stimulation. Cow, sheep and pig oocytes for staining were collected from abattoir material.

and cholesterol, which were threefold more abundant than phospholipids (Homa *et al*. 1986). Analysis of pig oocytes revealed that palmitic acid followed by oleic acid were the most abundant fatty acids, in terms of both total lipids and neutral lipids; however, n-6 PUFAs such as linoleic, arachidonic and adrenic acids also represent a significant proportion of fatty acids (Homa *et al*. 1986). Comparison of cow, pig and sheep oocytes revealed that pig had the most triglyceride with ~74 ng per oocyte, about three times more than both cow and sheep (McEvoy *et al*. 2000). Pig also had the most total fatty acids (~160 ng per oocyte), which was 2.5-fold more than cow and 1.8 times more than sheep. Palmitic, stearic and oleic acids were the most abundant in oocytes of cow, pig and sheep, but pig had higher palmitic than oleic acids whereas cow and sheep had greater relative oleic acid. Although oocyte size was not taken into consideration, Nile red staining has also been used to show that porcine oocytes have 2.4 times more lipid than bovine oocytes which in turn have 2.8-fold more than mouse oocytes (Genicot *et al*. 2005).

There has been relatively little analysis of lipid content of human oocytes. One study analysed lipid content of fertilisation-failed human oocytes pooled into groups of ten and found that the major fatty acids were stearic (38% of total fatty acids), palmitic (33%), oleic (10%), myristic (4%) and linoleic (4%) acids. Saturated fatty acids represented 79%, mono-unsaturated were 14%, n-6 PUFA 5% and n-3 PUFA 1% (Matorras *et al*. 1998). This study indicates that the types of lipids present in human oocytes are relatively similar to those in the oocytes of other species. Interestingly, the fatty acid composition of the human oocytes was very different to that of comparable female adipose tissue which was just 27% saturated fatty acid (Matorras *et al*. 1998). Similarly, bovine oocytes (Wonnacott *et al*. 2010) and whole COCs (Adamiak *et al*. 2006) are enriched in saturated fatty acids compared with granulosa cells and plasma from the same animals, suggesting selective uptake and/or *de novo* fatty acid synthesis in oocytes as well as specific energy storage and metabolism needs.

During the course of oocyte maturation, the intracellular lipid stores undergo dramatic changes. In pig oocytes, lipid droplets exhibit a pronounced peripheral distribution pattern following maturation *in vitro* (Sturmey *et al*. 2006). Bovine oocytes matured *in vitro* exhibit a small but significant increase in the number of lipid droplets compared with germinal vesicle stage (GV) oocytes (Aardema *et al*. 2011). In mouse oocytes, lipid droplets undergo structural reorganisation, aggregating centrally during the course of maturation *in vitro* (Yang *et al*. 2010) or *in vivo* (Wood *et al*. 2008, Wu *et al*. 2010).

Importantly, oocyte lipid content can be altered by the environment in which the oocyte matures, particularly by serum and lipid supplements used for IVM. Bovine oocytes matured in 10% FCS have more triacylglycerol and more cholesterol than those matured in serum-free conditions (Kim *et al*. 2001). Similarly, mouse COC matured in 5% serum contain more neutral lipid in the oocytes than those matured in fatty acid-free media (Yang *et al*. 2010). From these studies it is concluded that the increased oocyte lipids in response to serum is due to simple diffusion. However, serum also contains a number of growth factors, cytokines and metabolites, which could increase intracellular lipid via inducing transporter-mediated fatty acid uptake and/or triglyceride biosynthesis. Furthermore, these experiments utilised whole COCs and little is known about how maturation in different *in vitro* environments impacts cumulus cell lipid content. It is also likely that cumulus cells directly influence oocyte triacylglycerol and/or fatty acid deposition, similar to their role in controlling oocyte cholesterol content (Su *et al*. 2008). In support of this, IVM of bovine oocytes in the absence of cumulus cells results in decreased intracellular lipid stores (Auclair *et al*. 2013), suggesting that in the absence of cumulus cell-supplied metabolites the oocyte has less capacity for lipid storage or may more heavily utilise intracellular lipid stores for its energy requirements.

There is surprisingly little direct evidence for how oocyte lipid content might influence developmental

competence under normal physiological circumstances. There are examples of mouse oocytes, with poor developmental competence having more lipid droplets compared with developmentally competent oocytes; however, the poor oocytes also exhibit other fundamental cytoplasmic differences (Kim *et al*. 2001, Monti *et al*. 2013). Thus, further work is clearly needed to understand whether oocyte (or cumulus cell) lipid content may indeed be a biomarker of competence and, more importantly, how physiological or *in vitro* alterations to COC lipid content may impact embryo development. It is notable that palmitic, stearic, oleic and linoleic fatty acids are consistently the most prevalent in oocytes across species, with saturated fatty acids generally accounting for the vast majority of stores. The variations in oocyte lipid content across different species are perhaps indicative of distinct physiological requirements during early embryo development. Certainly, the long-chain fatty acids prevalent in mammalian oocytes are energy rich and differing levels of unsaturated fatty acids, such as oleic acid, could influence cellular membrane fluidity. This is illustrated by the fact that lipid content influences oocyte viability following cryopreservation by changing membrane integrity (reviewed in Zhang *et al*. (2012)).

Influences of physiological conditions on follicular fluid and oocyte lipid content

Diet and body composition

As circulating lipid metabolites are often similarly reflected in follicular fluid, it stands to reason that physiological conditions in which serum lipids are altered would affect lipid levels in the follicle and oocyte. Indeed, there are many examples of diet and body composition influencing the lipid content of follicular fluid and ovarian cells, including oocytes. For instance, beef heifers given dietary fish oil supplementation for 46 days exhibited dose-dependent alterations in specific n-3 and n-6 fatty acids in follicular fluid (Childs *et al*. 2008). Ewes fed a diet supplemented with fish oil for 13 weeks had significantly more 18:2 and 22:6 in follicular fluid and more 18:2, 18:3, 20:4, 22:4 and 22:6 in cumulus cells (Zeron *et al*. 2002). There were no differences detected in oocyte lipid content, but oocytes from animals that fed fish oil had better morphology and improved membrane integrity in response to chilling (Zeron *et al*. 2002). Ewes fed a combination of fish and vegetable oils containing n-3 and n-6 PUFAs had increased n-3 and n-6 fatty acids in granulosa cells and oocytes respectively (Wonnacott *et al*. 2010). Heifers with moderate body condition score had less fatty acids in aspirated COCs than heifers with low body condition score; and the inclusion of a lipid supplement in the diet for 35 days increased the total fatty acid content of whole COCs (Adamiak *et al*. 2006). In contrast to experiments that increased or altered fat in the diet, cows that were fasted for 4 days also exhibited increased NEFA in both plasma and follicular fluid (Jorritsma *et al*. 2003, Aardema *et al*. 2013). Interestingly, there are also seasonal changes in the fatty acid composition of bovine ovarian follicles. Namely, the levels of all fatty acids analysed were increased in follicular fluid in winter compared with summer (Zeron *et al*. 2001). Furthermore, granulosa cells and oocytes isolated in summer had a higher percentage of saturated membrane phospholipids, particularly palmitic acid, while in winter they had a greater percentage of unsaturated phospholipids, particularly 16:1, 18:1, 18:2 (Zeron *et al*. 2001). These differences significantly alter the biophysical behaviour of the oocytes, but it is unclear how this may be related to seasonal differences in diet and the observed better developmental competence of oocytes isolated in winter.

In addition to the large literature on ruminants, studies on mice also demonstrate that diets rich in specific lipids are associated with changes in the lipid content of ovarian cells. Mice fed a high n-3 diet for 4 weeks had increased n-3 fatty acid content in the ovary and this was associated with altered oocyte mitochondrial distribution, increased ROS levels and poorer embryo morphology and development into blastocyst following fertilisation *in vivo* (Wakefield *et al*. 2008). Mice fed a diet high in saturated fat for 4 weeks exhibited marked lipid accumulation in both oocytes and cumulus cells, as well as evidence of lipotoxicity responses, which were associated with impaired fertilisation *in vivo* (Wu *et al*. 2010). Overall, these studies on animals show that dietary lipids can alter the fatty acid composition of not only blood and follicular fluid but also cumulus cells and oocytes. How these changes relate to the observed changes in conception rates and fertility in response to different dietary paradigms is an important area of investigation. It is likely that alterations in cellular fatty acid levels cause structural changes in membranes that influence fluidity. Furthermore, exposure to excessive dietary saturated fat is associated with oocyte mitochondrial damage, which is in turn related to induction of oxidative stress (Igosheva *et al*. 2010) and endoplasmic reticulum (ER) stress (Wu *et al*. 2012); however, whether the alterations in mitochondrial activity in these cases change ATP generation has not been definitively shown.

Very little is known about how dietary lipids in humans may affect follicular fluid or ovarian cell lipid composition; however, differences in body composition are associated with distinct alterations. In women attending a fertility clinic, increased BMI (kg/m^2) was associated with increased levels of follicular fluid triglyceride (Robker *et al*. 2009) that mirrors changes in blood (Valckx *et al*. 2012). Free fatty acids in follicular fluid do not seem to be tightly correlated with BMI (Jungheim *et al*. 2011, Valckx *et al*. 2012), yet elevated follicular free fatty acids have been associated with poor cumulus–oocyte morphology (Jungheim *et al*. 2011),

providing evidence that alterations in ovarian lipid profile can impact the human COC during its maturation. Furthermore, mouse oocytes exposed to human follicular fluid that is high in free fatty acids and triglycerides during maturation had increased oocyte lipid content and impaired nuclear maturation (Yang *et al*. 2010). Thus, it is becoming increasingly important to better understand how the follicular environment is affected by specific metabolic conditions; not only obesity but also polycystic ovary syndrome (PCOS) which is also associated with a more atherogenic lipid profile, i.e. higher circulating triglycerides, cholesterol and LDL and lower HDL (Valkenburg *et al*. 2008).

Lactation in dairy cows

With the onset of lactation, dairy cows experience systemic negative energy balance that is associated with dramatic elevations in circulating lipids as well as follicular fluid lipid levels and this is emerging as a significant negative influence on their fertility (Leroy *et al*. 2008*a*,*b*). The follicular fluid of both heifers and lactating cows was shown to contain primarily linoleic, oleic, stearic and palmitic acids: however, compared with heifers, the cows had higher levels of at least 24 different fatty acids in follicular fluid, particularly palmitic acid and stearic acid and lower levels of docosahexaeonic acid (Bender *et al*. 2010). In dairy cows, circulating triglyceride levels decrease at parturition, while NEFA levels increase dramatically, relative changes that are also reflected in the follicular fluid (Leroy *et al*. 2004*b*). By day 44 *post-partum*, follicular fluid NEFA levels have increased to match that in serum, yet follicular fluid is selectively enriched with palmitic, oleic and linoleic fatty acids (Leroy *et al*. 2005*a*). These studies highlight that serum NEFA levels are dynamic, increasing dramatically at parturition; and that during lactation follicular fluid NEFA also increase but display a distinct fatty acid profile compared with serum (Leroy *et al*. 2005*a*, Bender *et al*. 2010). Oocytes of repeat breeder dairy cows were also shown to contain significantly more lipid than oocytes from virgin heifers, even though the COCs isolated from both types of animals were classified as normal good quality (Awasthi *et al*. 2010). Importantly, however, the dairy cows in this study were also significantly older and had a higher body condition score which could also affect oocyte lipid content.

There is of interest in using different dietary supplements, particularly those enriched with specific fatty acids to improve fertility in dairy cows (Leroy *et al*. 2011). For instance, in addition to studies described earlier, dairy cows fed a diet high in linoleic acid had an increased proportion of this fatty acid in follicular fluid and COCs, while cows fed a diet high in a-linolenic acid had an increased proportion of a-linolenic acid in follicular fluid, granulosa cells and aspirated COCs, as well as improved cleavage rates following IVF (Zachut *et al*. 2010). Thus dietary fat supplementation, which is known to influence reproductive performance in ruminants, particularly during lactation, may do so by influencing follicular fluid and cellular lipid levels.

Effects of lipid supplementation during oocyte IVM

In vitro experiments examining the direct effects of specific lipids on oocytes and early embryos are beginning to shed light on the mechanisms by which physiological conditions that alter circulating lipids influence fertility. In particular, a large body of work conducted in bovine oocytes is elucidating the mechanisms by which elevations in specific fatty acids contributes to reduced conception rates in dairy cows. These experiments also emphasise how lipid substrates in various *in vitro* oocyte maturation culture systems might differentially affect oocyte maturation and embryo development.

Although increased body fat is associated with higher oocyte and neutral lipid content, there are conflicting reports about whether oocyte IVM in the presence of high lipid directly impacts oocyte lipid content. Mouse COC exposed to follicular fluid that was high in both triglycerides and free fatty acids had increased oocyte neutral lipid content at the end of IVM compared with those matured in lipid-poor follicular fluid (Yang *et al*. 2012). In contrast, treatment of bovine oocytes with either stearic acid or palmitic acid during maturation did not increase oocyte lipid content (Leroy *et al*. 2005*a*). A more recent study has shown that bovine COCs exposed to palmitic acid and stearic acid during maturation had decreased oocyte lipid droplet size and number compared with controls; and also that oleic acid reversed these effects and at highest concentrations even promoted oocyte lipid storage, i.e. increased oocyte droplet size and number (Aardema *et al*. 2011). However, when bovine COCs were treated with a mixture of these three NEFAs (palmitic/stearic/oleic acids) during IVM, oocyte lipid content was not affected even though cumulus cells exhibited lipid accumulation (Aardema *et al*. 2013). Addition of conjugated linoleic acid (t10,c12CLA) to bovine COCs during maturation increased t10,c12CLA content in both cumulus cells and oocytes, but did not alter total fatty acid levels (Lapa *et al*. 2011). These studies illustrate that further work is needed to understand in which contexts lipid exposure modifies oocyte lipid content.

Individual fatty acids clearly have distinct effects on oocyte maturation and developmental competence. Treatment of bovine COCs with either stearic acid or palmitic acid (at relatively high doses based on levels in dairy cow follicular fluid) during maturation inhibited cumulus expansion, increased cumulus apoptosis and delayed progression to MII (Leroy *et al*. 2005*a*). Treatment of bovine COCs with a mixture of these NEFA (palmitic/stearic/oleic acid) during IVM upregulated genes involved

in energy metabolism and oxidative stress (lactate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase and glutathione peroxidase) in oocytes; interestingly, these same genes were downregulated in cumulus cells (Van Hoeck *et al*. 2013). Fertilisation of these oocytes gives rise to blastocysts, with significantly lower cell number and increased apoptosis (Van Hoeck *et al*. 2011). Experiments on mouse indicate that saturated fatty acids, namely palmitic acid, induce ER stress in COCs and mitochondrial dysfunction in oocytes (Wu *et al*. 2012). Mouse COCs exposed to lipid-rich follicular fluid had similarly increased expression of ER stress genes in association with impaired nuclear maturation (Yang *et al*. 2012). Importantly, co-treatment with an ER stress inhibitor during maturation reverses impaired cumulus expansion, altered oocyte mitochondrial activity and poor embryo development induced by high doses of palmitic acid (Wu *et al*. 2012), demonstrating that the detrimental effects of the high lipid environment are mediated through a classic ER stress pathway. Whether fatty acids are saturated or unsaturated also influences oocyte maturation, with unsaturated fatty acids generally having beneficial effects on subsequent embryo development. IVM in the presence of conjugated linoleic acid (t10,c12CLA) improves the morphology of bovine blastocysts (Lapa *et al*. 2011). Similarly, treatment of bovine COCs with linolenic acid improved maturation to MII and promoted embryo development, effects that seem to be mediated by its ability to influence prostaglandin production (Marei *et al*. 2009) and increase active MAPK signalling (Marei *et al*. 2010). Oleic acid reverses the detrimental effects of palmitic acid and stearic acid on cleavage, 8-cell and blastocyst rates (Aardema *et al*. 2011). It appears, however, that even unsaturated fatty acids are detrimental at high levels because linolenic acid at increasing doses reduces cumulus expansion and impaired maturation (Marei *et al*. 2009, 2010). Similarly, exposure of maturing bovine oocytes to high dose of oleic acid (1 mM in the presence of 10% serum) delayed progression to MII and reduced subsequent fertilisation, cleavage and embryo development (Jorritsma *et al*. 2004).

Importantly, blastocysts generated from oocytes exposed to high lipid *in vitro* display distinct phenotypes, particularly relating to metabolism. Bovine embryos generated from oocytes that matured in high levels of combination NEFA (palmitic/stearic/oleic acid) have altered transcriptional activity of several genes including higher glucose transporter *SLC2A1* (Van Hoeck *et al*. 2011) and increased expression of two genes involved in fatty acid synthesis, *ACSL1* and *GPR3* (*ACCA*) (Van Hoeck *et al*. 2013). They do not consume more glucose but exhibit altered amino acid turnover and compromised oxidative metabolism; indicators of lower embryo quality and viability (Van Hoeck *et al*. 2011).

Thus, specific fatty acids have distinct effects on oocyte maturation and it is emerging that in general saturated fatty acids, particularly palmitic acid and stearic acid, which are elevated in follicular fluid in some metabolic contexts are detrimental, while the presence of unsaturated NEFA such as oleic acid and linoleic acid can, at least in some *in vitro* contexts, counteract these detrimental effects and promote developmental competence.

Triacylglycerol and fatty acid metabolism in the COC

Lipolytic metabolism of triacylglycerols

The utilisation of fatty acids for metabolic fuel necessitates their hydrolysis of triacylglycerol from the glycerol backbone, which is catalysed by lipases. Lipoprotein lipase, an extracellular lipase tethered to capillary walls by heparin sulphate proteoglycans and activated by apolipoprotein ApoC-II on the surface of circulating lipoproteins, hydrolyses lipoprotein triacylglycerol-releasing free fatty acids, which can then be taken up by cells (Fig. 2). Lipolysis of intracellular triacylglycerol within lipid droplets is catalysed by intracellular lipases including LIPE (formerly known as hormone-sensitive lipase (HSL)) and adipocyte triglyceride lipase (ATGL). Intracellular triacylglycerol stored within lipid droplets is surrounded by a phospholipid monolayer and lipid droplet coat proteins of the perilipin family, which regulate droplet size and variously restrict access of intracellular lipases to the neutral core or promote lipolytic activity under the appropriate metabolic or hormonal conditions. The intracellular lipases, Lipe and

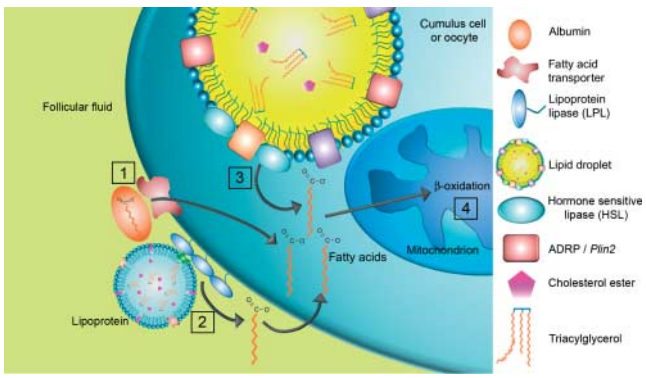

Figure 2 Schematic of proposed mechanisms of mobilisation and catabolism of free fatty acids in the cumulus–oocyte complex. (1) Free fatty acids (FFAs) in follicular fluid are bound to albumin and likely enter cells via fatty acid transporters or directly diffuse the lipid bilayer; however, the exact mechanisms are not known. (2) The mobilisation of triacylglycerol from lipoproteins in follicular fluid may occur via extracellular lipoprotein lipase liberating FFAs, which are then available for cellular uptake. (3) Intracellular triacylglycerol is stored in cumulus cells and oocytes within lipid droplets surrounded by coat proteins including Perilipin2 in oocytes. Upon activation, lipid droplet proteins facilitate lipase-mediated hydrolysis of triacylglycerol and release of FFAs. (4) Intracellular FFAs generated via either transport or lipolysis are then available for metabolism via β-oxidation in mitochondria.

Lipc, have been detected in rat ovaries (Hixenbaugh *et al.* 1989, Lobo *et al.* 2009), with Lipe localising to the oocyte and cumulus and granulosa cells of large antral follicles (Lobo *et al.* 2009). Mice lacking the *Lipe* gene exhibit a significant reduction in ovulation rate and increased numbers of oocytes trapped in corpora lutea (Wade *et al.* 2002). The ovaries of these mice have no difference in the number of mature antral follicles but decreased numbers of corpora lutea that contribute to reduced P_4 levels and decreased fecundity (Wade *et al.* 2002).

Lipase activity has been measured in bovine oocyte and cumulus cells during IVM and was found to be higher in the oocyte compared with cumulus cells (Cetica *et al.* 2002). During maturation, lipase activity remained constant in the oocyte while decreased in the cumulus cells (Cetica *et al.* 2002). Whether these cell-specific patterns of lipolysis hold true in species other than bovine is yet to be determined. Further evidence for triacylglycerol catabolism within the oocyte is the reduction in total intracellular lipid content that is observed during the course of maturation in porcine (Sturmey & Leese 2003, Alvarez *et al.* 2012) and bovine oocytes (Ferguson & Leese 1999, Kim *et al.* 2001).

Thus, lipase enzymes and activity are detected in COCs and likely to be regulated during the course of maturation. Based on their roles in other cells, it is proposed that extracellular lipases may mediate the transfer of fatty acids from follicular fluid lipoproteins into cumulus cells and that intracellular lipases via interactions with lipid droplet proteins, such as Perilipin-2, which is expressed in oocytes (Yang *et al.* 2010, Aardema *et al.* 2011), release fatty acids from stores in the oocyte (Fig. 2). Mechanistic studies are needed, however, to clarify this as well as to demonstrate whether modulation of triacylglycerol metabolism in the COC would have a significant impact on oocyte developmental competence and embryo development.

Metabolism of fatty acids for the production of ATP

Fatty acids for energy production are transported into the cells from circulation via fatty acid transporter proteins or direct diffusion through the lipid bilayer; thus, NEFA in follicular fluid may also enter cumulus cells and oocytes via similar mechanisms (Fig. 2). However, whether cumulus cells and oocytes preferentially uptake fatty acids from the extracellular milieu or generate fatty acids intracellularly from lipid droplet stores is not known and almost certainly would be differentially regulated in different hormonal and nutritional contexts. Subsequent to either mechanism, however, the catabolism of fatty acids to yield ATP occurs in the mitochondrial matrix via β-oxidation. The oxidation of fatty acids within ovulated mouse oocytes was first implied in a study by Hillman & Flynn (1980), in which incorporation of ^{14}C-palmitic acid into oocytes was reported. This was confirmed by a study demonstrating that β-oxidation, measured as metabolism of ^{3}H-palmitic acid, dramatically increases during COC maturation *in vitro* with a significant proportion of this metabolism occurring in the oocyte, although the majority is within the cumulus cells (Dunning *et al.* 2010).

Only a few studies to date have investigated the expression profile of genes involved in the regulation of β-oxidation and how these change during oocyte development and nuclear maturation. *Cpt1b*, whose gene product is responsible for the entry of long-chain fatty acids into the mitochondria, is significantly induced in mouse COCs in the peri-ovulatory maturation phase *in vivo* (Dunning *et al.* 2010). The expression of *Cpt1b* within the mouse oocyte itself at the MII stage has also been reported (Gentile *et al.* 2004). Recently, gene transcripts involved in β-oxidation, including acyl-CoA synthetases, acyl-CoA dehydrogenases and enoyl-CoA hydratase, have been detected in human oocytes and cumulus cells (Montjean *et al.* 2012). Activity of these enzymes involved in the β-oxidation spiral have also been detected in human oocytes and, interestingly, found to be negatively associated with maternal age (Yazigi *et al.* 1993).

Necessity for lipid metabolism in oocyte developmental potential

β-oxidation is likely to be important in the acquisition of oocyte developmental competence and female fertility. Ablation of *Acox1*, a gene involved in β-oxidation of very long-chain fatty acids, leads to sterility in female mice associated with smaller ovaries (Fan *et al.* 1996); however, the physiological mechanism linking *Acox1* function and female sterility is yet to be investigated. There is also an interesting case study of a female patient with a functional mutation in the *CPTII* gene, seeking fertility treatment (Hull *et al.* 2009). The oocytes and embryos of this patient were predicted to have CPTII enzyme deficiency until activation of the embryonic genome at the 8-cell stage and as such the culture media were adjusted to include more glucose for the culture of intact COCs and increased levels of pyruvate for cleavage stage embryos (Hull *et al.* 2009). While two previous intrauterine insemination cycles had failed, ICSI using carbohydrate-supplemented media resulted in the birth of a singleton baby, suggesting that plasticity in embryo metabolism allows for increased glycolysis to support development when β-oxidation is compromised.

β-oxidation is reduced in both oocytes and cumulus cells that mature *in vitro* compared with COCs matured *in vivo*, demonstrating that this metabolic pathway is acutely regulated by follicular factors at the time of ovulation. β-oxidation (oxidation of ^{14}C-palmitic acid) in cat oocytes maturing *in vitro* was significantly less than oocytes that had matured *in vivo* (Spindler *et al.* 2000). In rhesus monkey, microarray analysis found that several lipid metabolism genes, including some involved in β-oxidation, were dysregulated in cumulus cells

following IVM compared with those matured *in vivo* (Lee *et al*. 2011). In porcine oocytes, levels of *ACSL3* and *ACADL*, which activate long-chain fatty acids before their entry into mitochondria and catalyse the first step in the β-oxidation spiral respectively, were found to be dysregulated in another model of poor oocyte developmental competence (Yuan *et al*. 2011). Recently we have found that mouse COCs matured *in vitro* metabolise fatty acid at less than half the rate of COCs matured *in vivo*, in association with dysregulated expression of at least 15 genes involved in fatty acid activation, transport and oxidation (Dunning *et al*. 2014).

Studies using pharmacological inhibitors have confirmed the essential role of β-oxidation in both oocyte nuclear maturation and the acquisition of developmental competence. β-oxidation is required for the resumption of meiosis and nuclear maturation in the mouse (Downs *et al*. 2009, Paczkowski *et al*. 2013, Valsangkar & Downs 2013), bovine and porcine oocyte (Paczkowski *et al*. 2013). Furthermore, inhibition of β-oxidation during oocyte maturation in numerous species demonstrates that this form of metabolism is important for subsequent embryo development. Immature bovine oocytes exposed to methyl palmoxirate, which inhibits CPT1, exhibit reduced oxygen consumption and impaired capacity to develop to the blastocyst stage (Ferguson & Leese 2006). Similarly, treatment of porcine oocytes during maturation with methyl palmoxirate or mercaptoacetate, an inhibitor of 3-hydroxyl CoA dehydrogenase in the β-oxidation spiral, resulted in impaired fertilisation and decreased blastocyst development (Sturmey *et al*. 2006). We have shown similar sensitivities of mouse oocytes to the inhibition of β-oxidation, in which treatment of COCs with etomoxir, an inhibitor of *Cpt1*, results in a significant decrease in β-oxidation and significantly fewer blastocyst embryos following fertilisation (Dunning *et al*. 2010).

The requirement for β-oxidation during oocyte maturation for subsequent embryo development in the mouse is interesting in light of their comparatively low levels of intracellular lipid stores compared with bovine and porcine oocytes (reviewed in Sturmey *et al*. (2009) and see Fig. 1). However, triacylglycerol stores are energy dense and metabolism of a relatively small amount of lipid produces large amounts of ATP; thus despite the low levels of stored lipid in the mouse oocyte, it appears to be essential for oocyte maturation and quality.

Promoting β-oxidation to improve oocyte developmental outcomes

Upregulation of β-oxidation in COCs *in vitro* has also been used to demonstrate the importance of this metabolic pathway for developmental competence. Carnitine is an essential co-factor required for the entry of long-chain fatty acids into the mitochondrion. Supplementation of culture medium with L-carnitine significantly increases β-oxidation in mouse COCs maturing *in vitro* and in follicles grown *in vitro* (Dunning *et al*. 2010, 2011). This increased level of β-oxidation was associated with a significant improvement in oocyte quality as demonstrated by the ability of fertilised oocytes to reach the blastocyst stage of development (Dunning *et al*. 2010, 2011) and a higher number of cells allocated to the inner cell mass (Dunning *et al*. 2010). Others have also demonstrated a clear benefit of L-carnitine on oocyte maturation and developmental competence during IVM of porcine oocytes (Hashimoto 2009, Somfai *et al*. 2011, Wu *et al*. 2011, You *et al*. 2012) which are heavily laden with intracellular lipid. L-carnitine supplementation is associated with redistribution of intracellular lipid droplet in bovine oocytes (Chankitisakul *et al*. 2013), a reduction in intracellular lipid content in porcine oocytes (Somfai *et al*. 2011) and a significant increase in oocyte mitochondrial activity in mouse (Wu *et al*. 2012), bovine (Hashimoto 2009) and porcine (Somfai *et al*. 2011) oocytes. In addition, we have shown that L-carnitine, in the absence of other energy substrates, significantly improves embryo development in both mouse (Dunning *et al*. 2010) and bovine (Sutton-McDowall *et al*. 2012) pre-implantation embryos, indicating that L-carnitine stimulates metabolism of intracellular lipid stores. In other studies, L-carnitine supplementation was associated with decreased oocyte cytoskeletal damage (Mansour *et al*. 2009) and reversed the negative effects of repeated superovulation, i.e. the decreased number and abnormal distribution of mitochondria and impaired development to blastocyst (Miyamoto *et al*. 2010).

While L-carnitine has known anti-oxidant properties, its ability to upregulate β-oxidation and improve oocyte quality suggests that the beneficial effects of L-carnitine during oocyte maturation are attributable, at least in part, to its role in promoting lipid metabolism. Furthermore, human oocytes and cumulus cells lack transcripts for the genes involved in the biosynthetic pathway for carnitine production (Montjean *et al*. 2012). *In vivo* carnitine is most likely to be sourced from the follicular fluid from which levels are abundant and reflect those that found in serum (Dunning & Robker 2012, Montjean *et al*. 2012, Valckx *et al*. 2012); however, *in vitro* cultures of COCs would lack carnitine and are likely to be deficient in β-oxidation. Thus the potential to improve human oocyte quality during IVM by modulating β-oxidation, including with L-carnitine warrants further investigation.

Summary

Lipid metabolism is induced in COCs during maturation and beneficial to oocyte developmental potential; however, to date the characterisation of lipases and key β-oxidation enzymes involved has been limited. Inhibitor studies have demonstrated that lipolysis and β-oxidation within the maturing COC significantly influence

subsequent embryo development. Yet it is not clear which are the essential regulatory gene products associated with oocyte developmental competence and optimal pre-implantation embryo development. Thus, further studies are required to elucidate the complete expression profile of metabolic regulatory genes during folliculogenesis, particularly during the final stages of COC maturation which will also help to identify the lipid types most important to the peri-conception oocyte.

Lipoproteins derived from blood are prevalent in follicular fluid and may contribute to oocyte development. Whether lipoproteins transport triacylglycerides to ovarian cells for energy production similar to their roles in other cells is less clear, but there is mounting evidence for this as an important metabolic pathway in maturing ovarian follicles. Within cells, triacylglycerides are stored in lipid droplets and these are prevalent and being characterised molecularly in the oocytes and cumulus cells of many species. Much remains to be determined about how the metabolism of triacylglycerides by lipolysis and fatty acids by β-oxidation is regulated in cumulus cells and oocytes and the relative importance of this form of energy production for female fertility.

Specific fatty acids, particularly saturated vs unsaturated, have clearly distinct effects on oocyte maturation and developmental competence. Thus, oocyte and embryo development *in vitro* may be optimised through the provision of appropriate energy substrates and essential co-factors. Similarly, the influence of physiological conditions, such as diet, on the supply of fatty acids to the COC *in vivo* warrants further investigation. Through greater understanding of the *in vivo* regulation of lipid levels, lipid metabolism and essential co-factors, strategies may be developed to improve oocyte developmental potential in domestic animals and alleviate sub-fertility in women.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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