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The role of L-carnitine during oocyte *in vitro* maturation: essential co-factor?

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Abstract

In vitro maturation (IVM) of oocytes is a promising technology for both the treatment of human infertility and in animal production as a means of improving genetic gain. However, IVM derived oocytes remain inferior to those matured *in vivo* with reduced developmental potential. The environment in which an oocyte matures *in vitro* is vastly different to *in vivo* where maturation takes place within the ovarian follicle. The *in vitro* environment differs in oxygen concentration, exposure to light, and metabolite composition of culture media *vs*. follicle fluid, to name a few. Human follicle fluid contains the metabolite L-carnitine and has shown to be associated with human fertility. L-carnitine has known biological functions as an essential co-factor for beta-oxidation, regulating ATP production from lipids, and as a potent antioxidant. Importantly, it appears that cumulus cells and the oocyte lack the machinery to synthesize L-carnitine *de novo*. The inability for local production of L-carnitine during IVM and its importance in human fertility warrants investigation of its affects during IVM. The potential to improve oocyte quality by inclusion of L-carnitine in the culture media thus increasing the capacity for beta-oxidation and/or antioxidant activity of the culture media is receiving increased attention. This review summarizes studies to date investigating the developmental importance of L-carnitine during IVM and the mechanisms by which improved developmental potential is elicited. Overall, the inclusion of L-carnitine during IVM of several species results in improved oocyte quality with increased development to blastocyst. This is likely due to the antioxidant capacity of L-carnitine and its ability to increase ATP production from intracellular lipid stores.

Keywords: antioxidant, beta-oxidation, *in vitro* maturation, L-carnitine, oocyte,

Introduction

The final stages of oocyte maturation *in vivo* occur once folliculogenesis has reached the antral stage. The periovulatory antral follicle is capable of responding to the LH-surge from the pituitary which initiates the ovulatory cascade. The LH-surge acts on the granulosa cells of the follicle which transmit the ovulatory signal to the cumulus oocyte complex (COC) via secretion of EGF-like peptides. This initiates nuclear and cytoplasmic maturation in the oocyte and the cumulus cells to undergo expansion via the production of vast amounts of extracellular matrix. Following this, ovulation ensues with the COC then picked up by the fimbria of the oviduct and transported into the oviduct where fertilization can occur. Following fertilization, meiosis is completed and the one-cell zygote develops to the blastocyst stage whilst transcending through the oviduct to the uterus where the blastocyst embryo implants and pregnancy commences.

The ability of a zygote to successfully complete preimplantation embryo development, implant in the uterus and develop into a healthy offspring is dependent on the quality, or developmental competence, of the oocyte. The environment in which the oocyte matures *in vivo* is modified by maternal diet and metabolic status, which can negatively affect oocyte quality and the health of the offspring (Lane *et al*., 2014). Similarly, *in vitro* maturation of oocytes occurs in a sub-optimal environment drastically different from that which occurs *in vivo*. For *in vitro* maturation the COC is removed from the follicle prior to the LH-surge and the final stages of maturation occur *in vitro* in the absence of the follicle and follicular fluid.

Oocyte *in vitro* maturation (IVM) is a potentially useful assisted reproductive technology both clinically and in animal production (Gilchrist and Thompson, 2007). Developmental potential of IVM oocytes following fertilization is however, lower than their *in vivo* matured counterparts (Rizos *et al*., 2002; Gilchrist and Thompson, 2007). Oocytes matured *in vitro* have lower rates of development to the blastocyst stage and have increased rates of miscarriage (Buckett *et al*., 2008). The direct mechanisms responsible for the poorer oocyte quality are the topic of continued investigation and not as yet, clear. As discussed above, the environment in which the oocyte matures *in vitro* is dramatically different to *in vivo* maturation. *In vitro* maturation differs in the exposure to light, oxygen concentration and different metabolite composition of culture media *vs*. follicular fluid, among many other factors.

The composition of the IVM culture media is known to affect COC metabolism and metabolic rate (reviewed in Brown *et al*., 2017). Further, oocyte developmental competence is affected by metabolism with generation of sufficient levels of ATP required for oocyte growth, meiosis, fertilization and the early stages

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of preimplantation embryo development (Van Blerkom *et al*., 1995). The metabolism of carbohydrates, glucose, pyruvate and lactate for the production of ATP, have been well characterized in the COC (Sutton *et al*., 2003; Brown *et al*., 2017). Metabolism of fatty acids via beta-oxidation is a potent source of energy, several fold more energy dense than glucose, and the developmental importance of this form of metabolism for the COC is gaining recognition. The use of oocyte intracellular stores for energy production during IVM is of particular interest in species with high levels of stored lipid such as bovine, sheep and porcine (reviewed in Dunning *et al*., 2014b). Even in species with comparatively low levels of oocyte lipid (mouse and human; Dunning *et al*., 2014b), beta-oxidation during oocyte maturation appears essential for subsequent developmental competence (Dunning *et al*., 2010, 2011, 2014b) Beta-oxidation of fatty acids requires the essential co-factor L-carnitine to shuttle activated fatty acids from the cytosol into mitochondria. The addition of L-carnitine to the *in vitro* culture of cell lines in known to increase beta-oxidation (Nada *et al*., 1995; Huynh *et al*., 2014). L-carnitine also acts as a powerful antioxidant shown in other *in vitro* systems to quench reactive oxygen species (ROS) and in turn protect cells from oxidant injury (Ye *et al*., 2010). Oocytes matured *in vitro* are exposed to increased levels of ROS (reviewed in Combelles *et al*., 2009), thus inclusion of L-carnitine may be of benefit. The role of L-carnitine during IVM has gained interest in recent years as evidenced by a growing body of literature. This review will highlight studies utilizing L-carnitine during IVM, the resultant impact on oocyte quality and the mechanism(s) by which it acts.

Carnitine and fertility

Carnitine is a naturally occurring quaternary amine with the L-stereoisomer (3R)-3-hydroxy-4-(trimethylazaniumyl)butanoate having potent bioactivity. Synthesis of carnitine from lysine and methionine occurs mainly in the liver with highest levels found in skeletal muscle and heart (Vaz and Wanders, 2002). Dietary sources high in carnitine include meat and dairy (Rebouche, 1992). L-carnitine is essential for the transport of activated long chain fatty acids from the cytoplasm into mitochondria where, via beta-oxidation, ATP can be generated. Carnitine is also responsible for the transfer of peroxisomal beta-oxidation metabolites to the mitochondria for completion of oxidation to CO_2 and H_2O via the TCA cycle (reviewed in Vaz and Wanders, 2002). Other functions of carnitine include the maintenance of the acyl-CoA:CoA ratio and as a means of storing cellular energy in the form of acetylcarnitine (Vaz and Wanders, 2002). Additionally, L-carnitine has been used during *in vitro* culture and animal studies as a scavenger of free radicals, in turn protecting antioxidant enzymes from oxidant injury (Bremer, 1983; Sener *et al*., 2004; Gulcin, 2006; Kolodziejczyk *et al*., 2011).

Carnitine is present in human follicular fluid (Dunning and Robker, 2012; Montjean *et al*., 2012; Valckx *et al*., 2012; Varnagy *et al*., 2013; Zhao *et al*., 2015; Giorgi *et al*., 2016). While follicular fluid from these studies was sampled following ovulation induction, we can infer from the chemical properties of carnitine and its presence in serum, that carnitine would be present during oocyte maturation *in vivo*. In relation to oocyte quality and human fertility, it was shown that decreased carnitine (total and free) and acylcarnitine metabolites in follicular fluid was associated with increased numbers of oocytes (>9) and embryos (>6) from women undergoing IVF (Varnagy *et al*., 2013). We can speculate that decreased levels of carnitine and acylcarnitines were associated with improved reproductive potential as a result of their increased utilization via beta-oxidation during oocyte maturation (Varnagy *et al*., 2013). A similar association was not seen in in a separate study, however, only total carnitine was measured and compared with pregnant and non-pregnant cycles (Montjean *et al*., 2012). Replication of the association between carnitine (total and free) and the acylcarnitine metabolites with oocyte quality and fertility outcomes, in particular live birth, would be of interest.

Current evidence suggests that cumulus cells and oocytes do not have the capacity to biosynthesize L-carnitine from precursor amino acids as they lack the metabolic machinery (Montjean *et al*., 2012). This study examined human cumulus cells and oocytes at the end of maturation and only mRNA for two of the enzymes in the biosynthesis pathway. Whether regulation of these genes or the presence of the enzymes themselves occurs earlier in maturation of COCs is yet to be determined.

The potential developmental importance of carnitine and its acylcarnitine derivatives for oocyte quality following *in vivo* maturation of human oocytes and the apparent inability of COCs to synthesize carnitine points to the importance of investigating the role of carnitine during *in vitro* maturation. There is a growing number of studies that have investigated the role of L-carnitine during IVM in various species including mouse (Dunning *et al*., 2010, 2011; Paczkowski *et al*., 2014), bovine (Ferguson and Leese, 2006; Phongnimitr *et al*., 2013; Giorgi *et al*., 2016; Sovernigo *et al*., 2017), porcine (Hashimoto, 2009; Somfai *et al*., 2011; Wu *et al*., 2011; You *et al*., 2012) and sheep (Reader *et al*., 2015; Mishra *et al*., 2016a). The effects of L-carnitine on oocyte quality and the associated mechanism(s) are detailed in the following sections.

Importance of beta-oxidation and its modulation by L-carnitine during IVM

There is a growing body of literature describing the importance of fatty acid beta-oxidation as a source of energy during oocyte IVM (Ferguson and Leese, 2006; Sturmey *et al*., 2006; Downs *et al*., 2009; Dunning *et al*., 2010; Dunning and Robker, 2012; Valsangkar and Downs, 2013; Dunning *et al*., 2014b; Paczkowski *et al*., 2014). This is not surprising when one considers the energy dense nature of fatty acids with the potential to produce 106 ATP molecules from a

single fatty acid, greater than 3.5-fold the capacity of a single glucose molecule.

The metabolism of fatty acids for the production of ATP occurs within mitochondria. This requires entry of activated fatty acids into the mitochondrial matrix, a process catalyzed by carnitine palmitoyl-transferase 1 (CPT1) and also requiring carnitine. CPT2 then removes carnitine from the fatty acid which can then enter the mitochondrial matrix for catabolism via beta-oxidation (McGarry and Brown, 1997).

Beta-oxidation appears to be important for oocyte nuclear maturation; meiotic maturation to metaphase II and extrusion of the first polar body (Downs *et al*., 2009; Valsangkar and Downs, 2013; Paczkowski *et al*., 2014). Further, it appears that beta-oxidation is important in the acquisition of oocyte developmental competence (reviewed in McKeegan and Sturmey, 2011; Dunning *et al*., 2014b). Of particular interest here are studies demonstrating a reduction in beta-oxidation in both the oocyte and cumulus cell compartments of the COC when maturation occurs *in vitro* (Lee *et al*., 2011; Dunning *et al*., 2014a), demonstrating the importance of the ovarian follicular environment in appropriate regulation and/or the supply of metabolic factors that support this metabolic pathway (including supply of carnitine). The expression of genes involved in the beta-oxidation pathway are dysregulated in non-human primate cumulus cells and mouse COCs that have undergone maturation *in vitro* compared to *in vivo* maturation (Lee *et al*., 2011; Dunning *et al*., 2014a). In functional assays, both mouse and cat oocytes have decreased levels of beta-oxidation compared to those matured *in vivo* (Spindler *et al*., 2000; Dunning *et al*., 2014a). Specifically, we have shown that the rate of beta-oxidation in mouse COCs is significantly reduced, more than fifty percent, during IVM (Dunning *et al*., 2014a).

The importance of beta-oxidation is further exemplified in studies using inhibitors of this pathway during *in vitro* maturation. These studies demonstrate a requirement for beta-oxidation in nuclear maturation (Downs *et al*., 2009; Valsangkar and Downs, 2013; Paczkowski *et al*., 2014; Sanchez-Lazo *et al*., 2014), as described above, and in the acquisition of oocyte developmental competence in mouse, bovine and porcine oocytes (Ferguson and Leese, 2006; Sturmey *et al*., 2006; Dunning *et al*., 2010; Paczkowski *et al*., 2014).

We and others have shown that supplementation of IVM culture media with L-carnitine significantly increases beta-oxidation in mouse COCs (Dunning *et al*., 2010; Valsangkar and Downs, 2013). The inclusion of L-carnitine during maturation also results in a significant increase in oocytes reaching metaphase II in mouse (Dunning *et al*., 2011) and porcine (Somfai *et al*., 2011) and significantly more blastocyst embryos following fertilization (Dunning et al., 2010). The inclusion of L-carnitine has also been shown to increase oocyte mitochondrial activity in mouse (Wu *et al*., 2012), bovine (Hashimoto, 2009), and porcine (Somfai *et al*., 2011) and decrease intracellular lipid stores in porcine oocytes (Somfai *et al*., 2011). Further, our work has demonstrated the ability for L-carnitine to facilitate metabolism of intracellular lipid stores in preimplantation mouse and bovine embryos, significantly improving development (Dunning *et al*., 2010; Sutton-McDowall *et al*., 2012). Collectively, these studies illustrate the ability of L-carnitine to upregulate beta-oxidation during IVM and utilize intracellular lipid stores. The beneficial effects of L-carnitine supplementation on oocyte quality can at least be partially ascribed to its effects on stimulating fatty acid beta-oxidation. The role of supplying exogenous fatty acid to IVM for utilization via beta-oxidation, at least in the mouse, appears detrimental (Paczkowski *et al*., 2014). This may be due to the concentration or type of fatty acid used in this study. Elucidation of the oocytes preferential fatty acid type(s) and concentration and the detrimental effects of excess lipid in relation to oocyte developmental competence, is ongoing in a number of models and agriculturally important species (reviewed in Dunning *et al*., 2014b).

Anti-oxidant capacity of L-carnitine during IVM

Reactive oxygen species (ROS) are naturally formed during cellular metabolism, but kept in check by enzymatic and non-enzymatic antioxidants. If the production of ROS outweighs the cells antioxidant capacity this leads to oxidative stress (cellular damage via lipid peroxidation, DNA and protein damage). The process of *in vitro* culture itself generates increased oxidative stress, in addition to that generated from cellular metabolism, by exposure of cells to light, increased oxygen concentration and atmospheric conditions during handling (reviewed in Combelles *et al*., 2009). Specifically, *in vitro* maturation of oocytes has been shown to result in excessive production of ROS compared to *in vivo* matured oocytes (Banwell *et al*., 2007; Zeng *et al*., 2014). Further, the *in vitro* matured oocytes may be less equipped to attenuate deleterious effects of increased ROS compared to those matured *in vivo* (Uppangala *et al*., 2015). Collectively, this may contribute to the poorer developmental competence of IVM derived oocytes.

In vitro assays have demonstrated the ability of L-carnitine to act as an effective scavenger of superoxide anions, hydrogen peroxide and inhibit lipid peroxidation (Gulcin, 2006). The anti-oxidant property of L-carnitine is supported by *in vitro* studies demonstrating its capacity to prevent oxidant injury in various somatic cells (Silva-Adaya *et al*., 2008; Ribas *et al*., 2010). Thus, investigation of L-carnitines capacity to scavenge free radicles during IVM and its resultant effect on oocyte quality warrants investigation.

There are a number of studies demonstrating a link between inclusion of L-carnitine during IVM on reduced intracellular ROS and increased antioxidant enzymes within the oocyte of a number of species including mouse (Zare *et al*., 2015), bovine (Sovernigo *et al*., 2017), porcine (Somfai *et al*., 2011; You *et al*., 2012) and sheep (Mishra *et al*., 2016b). IVM of mouse oocytes in the presence of carnitine decreased ROS and increased the antioxidant glutathione (GSH) within

oocytes and was associated with significantly increased rates of maturation to MII, cleavage and blastocyst embryos, but no change in blastocyst cell number (Zare *et al*., 2015). Similarly, the inclusion of L-carnitine during bovine IVM, in addition to reduced oxidative stress, resulted in significantly more blastocyst embryos with these embryos containing significantly increased number of cells (Sovernigo *et al*., 2017). Decreased ROS and increased GSH within porcine oocytes matured with L-carnitine also resulted in significantly improved oocyte developmental competence with increased rates of blastocysts following parthenogenetic activation (Wu *et al*., 2011; You *et al*., 2012) or somatic cell nuclear transfer (You *et al*., 2012).

Challenging the *in vitro* maturation environment by inclusion of hydrogen peroxide (H_2O_2) or TNFα during oocyte maturation *in vitro* leads to a significant reduction in the number of mature oocytes and the number of blastocyst embryos post fertilization in both sheep and pig (Yazaki *et al*., 2013, Mishra *et al*., 2016a, b). The inclusion of L-carnitine has shown to reverse the effects of these exogenous insults on nuclear maturation (Yazaki *et al*., 2013; Mishra *et al*., 2016a) and capacity of the fertilized zygote to develop to blastocyst (Yazaki *et al*., 2013; Mishra *et al*., 2016a, b). The ability of L-carnitine to attenuate the detrimental effects of H_2O_2 during IVM has been shown to be associated with a reduction in ROS and increase in intracellular GSH within oocytes (Mishra *et al*., 2016b). To investigate the impact of increased ROS in human follicular fluid on oocyte quality following maturation, one study matured mouse oocytes in the presence of control follicular fluid and follicular fluid from women with endometriosis and suffering infertility (Giorgi *et al*., 2016). Oocyte maturation in the presence of follicular fluid from women with endometriosis resulted in a significant increase in meiotic spindle abnormalities that were reversed to control levels when 0.6 mg/ml L-carnitine was included in the maturation media (Giorgi *et al*., 2016).

Decreased oxidative stress and improved oocyte developmental competence in response to L-carnitine has been associated with increased expression of cell cycle machinery in mouse oocytes (Zare *et al*., 2015); however the activity of these factors are regulated at the level of phosphorylation which was not investigated, likely due to the number of oocytes required to perform such experiments being prohibitive. A direct causal link between L-carnitine treatment and cell cycle machinery via carnitine's anti-oxidant capacity seems unlikely. Similarly, in porcine oocytes decreased ROS and improved developmental competence was associated with increased expression of genes involved in nuclear reprogramming (You *et al*., 2012) but again, a causal link between these pathways was not determined.

Effects of L-carnitine during IVM on the cryotolerance of oocytes

Oocyte cryopreservation would preserve fertility for women undergoing treatment for cancer and be useful in avoiding the effects of aging by preserving oocytes earlier in life. While human and mouse MII oocytes have been successfully used following cryopreservation, MII oocytes are susceptible to cryoinjury including spindle damage (Gomes *et al*., 2008; Huang *et al*., 2008). Cryopreservation of GV oocytes would be advantageous as they are not susceptible to damage inflicted by cryopreservation due to the chromosomes being at interphase, and utilizing oocytes at this stage would avoid the need for ovarian hyperstimulation and its associated risks. However, cryopreserved GV oocytes are developmentally compromised with significantly reduced number of blastocysts post-thawing and fertilization (Aono *et al*., 2003, 2005). Cryopreservation of oocytes in agriculturally important species also has advantages with the ability to bank oocytes from genetically important animals. However, cryopreserved bovine oocytes have poorer developmental competence on thawing. Supplementation of IVM and/or vitrification media with L-carnitine has resulted improvements in oocyte quality in mouse GV oocytes (Moawad *et al*., 2013, 2014) and bovine MII oocytes following cryopreservation and thawing (Spricigo *et al*., 2017). Specifically, the inclusion of L-carnitine during IVM and vitrification of mouse GV oocytes significantly increased the number of blastocyst embryos, comparable to the number of blastocysts developed from oocytes that were not vitrified or matured *in vivo* and vitrified (Moawad *et al*., 2013, 2014). L-carnitine treatment during IVM and vitrification was associated with improved meiotic spindle formation and distribution and increased activity of mitochondria (Moawad *et al*., 2014).

Conflicting evidence is seen in studies investigating the effects of L-carnitine on bovine oocyte developmental competence post vitrification/thawing. L-carnitine inclusion during bovine IVM has been shown to result in significantly increased numbers of blastocysts following cryopreservation and thawing of oocytes, which was associated with redistribution of lipid within oocytes (Chankitisakul *et al*., 2013). In contrast, Phongnimitr *et al*. (2013) showed that L-carnitine supplementation during IVM significantly improved subsequent development to blastocyst, but this effect was lost following vitrification. Similarly, inclusion of L-carnitine during IVM of prepubertal bovine oocytes had no beneficial effect on oocyte developmental competence post thawing (Spricigo *et al*., 2017). Thus, further research is required to determine the effects of L-carnitine on bovine oocyte cryotolerance.

Summary and future directions

There is mounting evidence in the literature that L-carnitine has beneficial effects during the *in vitro* maturation of oocytes. The mechanism by which L-carnitine improves oocyte quality is likely to be via both increased beta-oxidation and reducing oxidative stress via is ability to scavenge free radicals. Future studies could interrogate the relative contribution of these

actions to improved oocyte developmental competence.

Differential effects of L-carnitine in various studies may be a result of different animal strains/breeds used and different laboratory practices, including culture media composition or the use of fully grown oocytes or oocytes collected from smaller follicles or prepubertal animals.

Based on the studies discussed here, future improvements in IVM culture media may benefit from the inclusion of L-carnitine. However, as promotion of fatty acid beta-oxidation impacts glucose metabolism in the COC, it is important to consider the appropriate balance between these metabolic pathways (Paczkowski *et al*., 2014), and utilization of *in vivo* maturation as the *gold standard* would assist in optimization.

As yet, there are no studies demonstrating impact of L-carnitine during IVM on offspring health. Use of L-carnitine during mouse preimplantation embryo development *in vitro* demonstrates safety (Truong *et al*., 2016). However, the effect of L-carnitine supplementation specifically during IVM on offspring health is yet to be studied in any species. Demonstration of safety in several mouse strains and animal models would be advisable before implementation in the fertility clinic.

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