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The role of L-carnitine in the control of oxidative stress and lipid β-oxidation during *in vitro* follicle growth, oocyte maturation, embryonic development and cryopreservation: a review

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Review Article

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Abstract

L-carnitine has an important role in the control of oxidative stress and lipid β-oxidation during *in vitro* culture and cryopreservation of ovarian follicles, oocytes and embryos. This substance balances the acetyl-CoA/CoA ratio, maintains glucose metabolism and increases energy production in mitochondria. It also plays a key role in reducing endoplasmic reticulum stress, by transferring palmitate to mitochondria or eliminating it to avoid toxicity. By eliminating reactive oxygen species, L-carnitine increases the percentages of mature oocytes with uniform mitochondrial distribution and improves embryo post-thaw cryotolerance. Therefore, L-carnitine controls lipid β-oxidation and oxidative stress during *in vitro* culture of ovarian follicles, oocyte maturation, embryonic development and cryopreservation.

Introduction

The improvements in *in vitro* culture systems for ovarian follicles, oocytes and embryos are of great relevance to increase efficiency of assisted reproduction techniques in mammalian species. However, lipid peroxidation and imbalance in the production and elimination of reactive oxygen species (ROS) represent the main barriers to having healthy oocytes and embryos after *in vitro* culture and cryopreservation (Soto-Heras and Paramio, 2020). In this sense, the addition of natural substances to the culture media has been an alternative to control the damages caused by excessive ROS (Paulino *et al*., 2022). L-carnitine is a water-soluble vitamin-like compound that is naturally produced and synthesized primarily from lysine and methionine in the liver to improve lipid breakdown and generate metabolic (Modak *et al*., 2022). According to Carrillo-González *et al*. (2023), lipids are the most abundant reservoir of energy in bovine embryos, and triacylglycerol-containing lipid droplets represent the main stocks of fatty acids in oocytes. These authors showed that L-carnitine mobilizes fatty acids from oocyte cytoplasm to mitochondria, which results in β-oxidation and generation of energy. Additionally, acetyl-L-carnitine exhibits antioxidant effects and has beneficial effects on reproductive functions (Liu *et al*., 2004; Cheng and Chen, 2008; Aliabadi *et al*., 2012; Agarwal *et al*., 2018). When administered exogenously, acetyl-L-carnitine has higher bioavailability than L-carnitine and regulates even the production of reproduction-associated hormones (Agarwal *et al*., 2018).

This review aims to show the role of L-carnitine on hypothalamus-pituitary-gonad-axis and to discuss its influence on lipid β-oxidation and oxidative stress during *in vitro* culture of ovarian follicles, oocyte maturation, embryo development and cryopreservation.

Oxidative stress

Free radicals are chemical specimens that have at least one unpaired electron in their outer orbitals, being highly reactive (Prevedello and Comachio, 2021). This characteristic enables the transfer of electrons between neighbouring molecules, causing changes in the molecular environment (Ferreira *et al*., 2020; Martelli and Nunes, 2014). The ROS are naturally produced by cellular metabolism and play an important physiological role, being involved in several processes, such as energy production, phagocytosis, intercellular signalling, regulation of cell growth, immunity, cell defence and synthesis of biological substances (Prevedello and Comachio, 2021). However, when ROS production exceeds its degradation, it causes oxidative stress, being responsible for various damages to DNA, proteins and phospholipids in different cell types (Simas *et al*., 2019). Controlling the production and neutralization of ROS is crucial for

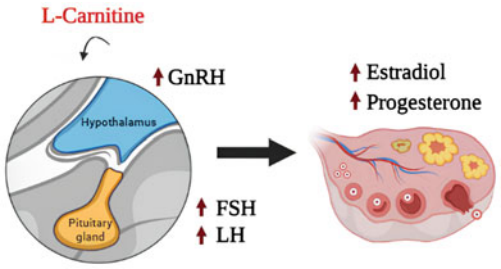

Figure 1. Effects of L-carnitine on hypothalamus-pituitary gonad axis.

maintaining cellular integrity. *In vivo*, this control is performed through enzymatic and non-enzymatic antioxidant systems. Various endogenous enzymes, like catalase (CAT), peroxiredoxins (PRDX), superoxide dismutase (SOD) and glutathione reductase/peroxidase (GPX) constitute the endogenous antioxidant system (Souza *et al.*, 2020), which are capable of inactivating the harmful effects of free radicals. The non-enzymatic system includes low molecular weight compounds such as L-carnitine, ascorbic acid, tocopherol, selenium, zinc, taurine, hypotaurine, carotene, lipoic acid and other thiol compounds such as cystine, cysteine, cysteamine and beta-mercaptoethanol (Crocomo *et al.*, 2012).

During *in vitro* culture of different types of cells, the reduction of endogenous antioxidant protection linked to other factors, such as exposure to light and high concentrations of oxygen, favours a significant increase in ROS production (Alves *et al.*, 2019; Sadeesh *et al.*, 2014) and oxidative stress, which has been reported as one of the main limitations of *in vitro* culture of various types of cells (Del Collado *et al.*, 2017; Soto-Heras and Paramio, 2020). In excess, oxidative stress in granulosa cells results in follicular atresia (Saeed-Zidane *et al.*, 2017) and has been reported as one of the main factors associated with poor quality of cultured ovarian follicles (Sá *et al.*, 2018; Paulino *et al.*, 2022). Due to damages caused by oxidative stress during *in vitro* culture, several studies have sought to develop protocols to minimize it (Cordeiro *et al.*, 2023; Nascimento *et al.*, 2022).

Effects of L-carnitine on hypothalamus-pituitary-gonadal axis

The L-carnitine influences the hypothalamus-pituitary-gonad axis and upregulates gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus and, consequently, induce depolarization of hypothalamic neuronal cells to increase secretory activity (Agarwal *et al.*, 2018; Krsmanovic, *et al.*, 1994). Also, L-carnitine has been reported to increase the levels of other hormones, like luteinizing hormone (LH), progesterone and oestradiol, while it decreases prolactin secretion in mammalian species (Agarwal *et al.*, 2018; Genazzani *et al.*, 2011; Krsmanovic *et al.*, 1992). Figure 1 illustrates the effects of L-carnitine on the reproductive system of mammalian females.

The L-carnitine and its primary ester have direct effects against oxidative stress, minimizing cell death by apoptosis and maintaining cellular energy (Agarwal *et al.*, 2018; Abdelrazik *et al.*, 2009; Infante *et al.*, 2002; Vanella *et al.*, 2000). To minimize oxidative stress, L-carnitine can also be used in combination with other antioxidant commonly known for quenching free radicals such as vitamins (C, E and β-carotene), and some metalloenzymes, including GPx, CAT and SOD (Nimse and Pal, 2015). Thus, due to its energy generation property combined with its antioxidant property, L-carnitine has been studied for use in reproductive technologies, including *in vitro* culture of ovarian follicles, *in vitro* maturation, *in vitro* embryo production and cryopreservation.

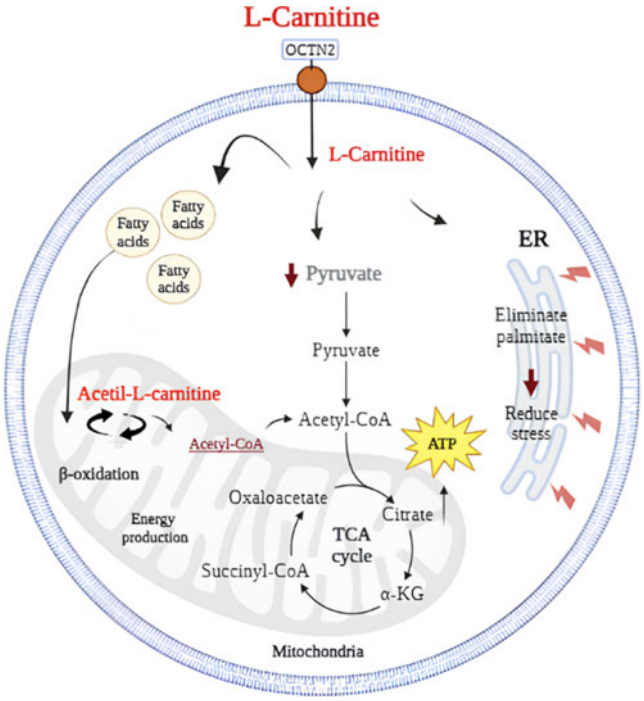

Figure 2. Direct effects of L-carnitine on oocytes of mammals.

Effects of L-carnitine on in vitro *follicle development and oocyte maturation*

The role of L-carnitine in ovarian follicles *in vitro* is still little explored. Dunning and Robker (2012) reported that L-carnitine did not alter survival, growth or differentiation of mouse secondary follicles *in vitro*. However, it significantly increased β-oxidation and markedly improved fertilization rate and blastocyst development. Recently, Modak *et al.* (2022) reported that L-carnitine increased the rate of oocytes in metaphase II (MII) stage from early antral follicles cultured *in vitro*. Furthermore, the presence of L-carnitine decreased the rate of degeneration and even promoted the formation of structures similar to antrum after the *in vitro* culture of buffalo oocyte granulosa complexes.

In mouse oocytes, L-carnitine acts through the electrogenic force of voltage-gated Na^+ channels and it is transported by Na^+/organic cationic transporter-2 (OCTN-2) to oocytes (Infante *et al.* 2002; Dunning and Robker 2012). In the oocyte, L-carnitine is converted to acetyl-L-carnitine by carnitine palmitoyltransferase-I (CPT-I) in the mitochondria and can act on the endoplasmic reticulum, mitochondria and even in ooplasm (Mingorance *et al.*, 2011) (Figure 2). Various studies have shown that L-carnitine optimizes glucose metabolism by transferring fatty acids to the mitochondria and facilitating β-oxidation since lipid metabolism is one of the primary regulators of oocyte maturation (Stojkovic *et al.*, 2001; Dunning *et al.*, 2010; Agarwal *et al.*, 2018). Within the oocyte, L-carnitine is converted to acetyl-L-carnitine and keeps glucose metabolism through the citric acid cycle and, consequently, increases energy production (Infante *et al.*, 2002; Agarwal *et al.*, 2018) (Figure 2). The L-carnitine reduces pyruvate entry into the citric acid cycle and transports palmitate and other long-chain fatty acids to facilitate their utilization through β-oxidation

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(Dunning *et al*., 2010) (Figure 2). The L-carnitine reduces the levels of palmitate in the endoplasmic reticulum by transferring it to mitochondria or by eliminating it from where it can cause oocyte lipotoxicity by oxidative stress (Agarwal *et al*., 2018). L-carnitine increases the proportion of mature oocytes with uniform mitochondrial distribution and supports *in vitro* oocyte maturation and embryonic development in mice and pigs (Zare *et al*., 2015; Somfai *et al*., 2011). Chankitisakul *et al*. (2013) showed that L-carnitine increases the rate of bovine embryo production after *in vitro* maturation and subsequent vitrification of oocytes. Marin *et al*. (2020) also reported that L-carnitine increased oocyte competence during buffalo oocyte maturation in the absence of foetal bovine serum.

In vivo, L-carnitine can stabilize the mitochondrial membrane and protect DNA against ROS-induced damage in oocytes of women with polycystic ovary syndrome (PCOS) (Mohd Shukri et al., 2022; Ismail *et al*., 2014; Fenkci, *et al*., 2008). Using a mouse model of PCOS, oral administration of acetyl-L-carnitine alleviated ovarian dysfunction associated with that syndrome through its antioxidant/glycation activity and mitochondria potentiation (Di Emidio *et al*., 2020).

Effects of L-carnitine on in vitro *embryo development*

The ROS may originate in the embryo itself or from exogenous sources. During oocyte *in vitro* fertilization (IVF), strategies to reduce ROS production, such as addition of free radical scavengers and lowering the oxygen tension are important for improving the fertility potential in assisted reproductive technologies (Agarwal *et al*., 2014). The ROS are involved in defective embryo development and retardation of embryo growth and induce cell membrane damage, DNA damage and apoptosis (Volpe *et al*., 2018). Apoptosis results in fragmented embryos, which have limited potential to implant and therefore, result in poor fertility rates (Agarwal *et al*., 2014).

The antioxidant capacity of L-carnitine might account for its preferential use to improve *in vitro* oocyte and embryo development. The treatment of porcine embryos with antioxidants improved blastocyst production (Castillo-Martín, *et al*., 2014). Moreover, the presence of L-carnitine in the culture medium was associated with increased cleavage and blastocyst rates in porcine species (Lowe *et al*., 2017). Finally, the supplementation of L-carnitine to bovine embryo culture medium has been shown to scavenge ROS within two-cell stage embryos (Takahashi *et al*., 2013).

Effects of L-carnitine on cryopreservation of ovarian follicles, oocytes and embryos

The cryopreservation technique allows the sub-zero storage of tissues or cells by dramatically reducing natural cellular biochemical processes for extended periods of time (Vining *et al*., 2021). Currently, cryopreservation is a modern and safe method which assists in the preservation of genetic material from follicles, oocytes and embryos in human and animals (Sekhon *et al*., 2018), but the cells can still be seriously damaged during cryopreservation (Truong, *et al*., 2022; Spijkers *et al*., 2017; Barsky *et al*., 2016; Beyer and Griesinger, 2016). Unfortunately, frozen follicles, oocytes and embryos are still reported to contain a higher proportion of apoptotic cells compared to their non-frozen counterparts, with freezing procedures generally associated with triggering apoptotic cell death (Vining, *et al*., 2021). Exposure to high concentrations of cryoprotectants, osmolarity and rapid temperature changes during cryopreservation have been shown to affect gamete and embryo physiology (Somoskoi *et al*., 2015; Dalcin *et al*., 2013), as well as their gene expression (Sahraei *et al*., 2018; Monzo *et al*., 2012). These deleterious effects are strongly associated with the occurrence of oxidative stress during cryopreservation.

Production of ROS during the vitrification of gametes may be a crucial mediator of damage to proteins and DNA (Costa *et al*., 2022; Zhang *et al*., 2020). Disturbances in the oxidative metabolism and damage in cell membranes are other important stress factors related to vitrification. Together, these effects decrease glutathione (GSH) levels, alter expression of regulatory genes and are associated with decreasing maturation rate and developmental competence of follicles, oocytes and embryos after cryopreservation (Berteli *et al*., 2022; Zare *et al*., 2022; Costa *et al*., 2022; Wu *et al*., 2019; Pan *et al*., 2018). Furthermore, cryopreservation of oocytes or embryos has been reported to cause mitochondrial dysfunction, such as changes in membrane potential and reduced adenosine triphosphate (ATP) production (Gualtieri *et al*., 2021; Iwata, 2021). However, the detrimental effects of cumulative stress have been shown to be partly improved by adding antioxidants in vitrification media, such as L-carnitine. Some reports have already demonstrated that L-carnitine plays an important role in attenuating the deleterious effects of oxidative stress on cryopreserved follicles. For instance, Zhang *et al*. (2015) observed lower rates of apoptosis and malondialdehyde, as well as higher levels of oestradiol in mice ovarian follicles cryopreserved in situ. These results were translated into increased follicular survival. Zolini *et al*. (2019) demonstrated that the addition of L-carnitine in embryo culture medium improved post-thaw cryotolerance but had no effect on pregnancy and implantation rate after transfer of cryopreserved bovine embryos.

The L-carnitine is well known for its role in β-oxidation, ATP production and decreasing the lipid content during embryo development, providing improved cryo-survivability (Truong *et al*., 2016). In buffaloes, the addition of L-carnitine to the medium significantly benefits embryonic developmental competence after vitrification, as evidenced by the high cleavage rate and the formation of morulae and blastocysts. Improving the cryotolerance of buffalo embryos directly after thawing may be through increased lipid metabolism (El-Sokary *et al*., 2021). Furthermore, L-carnitine acts as an antioxidant blocking degenerative changes arising from oxidative stress during embryonic development (Bhakty *et al*., 2021). Lowe *et al*. (2017) reported that the antioxidant capacity of L-carnitine was associated with the increased cleavage rate and the improved cryotolerance of resultant porcine blastocysts. Supplementation of L-carnitine to bovine embryo culture medium has been shown to scavenge ROS within two-cell stage embryos, antagonize the cryodamage and enhance the cryotolerance of blastocysts (Takahashi *et al*., 2013). It is plausible to suggest that L-carnitine may be used to improve the freezing survival of oocytes or embryos (Li *et al*., 2023). Table 1 shows some effects of L-carnitine during *in vitro* culture of follicles, oocytes and embryos in different species.

Final considerations

Many factors inherent to the oocyte itself and *in vitro* culture environment determine the chance of having a complete follicular development with success in the acquisition of oocyte competence *in vitro*. A culture system, with different combinations of hormones, growth factors and mainly antioxidant factors, at each

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Table 1. Effects of L-carnitine during *in vitro* culture of follicles, oocytes and embryos in different species

Effects	Species	Reference
Ovarian follicles		
Inhibits apoptosis, alleviates oxidative damage and increases the survival and function of follicles cryopreserved *in situ*.	Murine	Zhang *et al*., 2015.
Oocytes		
Exposure of oocyte to L-carnitine prior to insemination increase cleavage and improve cryotolerance of the resulting blastocysts.	Porcine	Lowe *et al*., 2017.
Increases the percentage of oocytes reaching metaphase II.	Ovine	Bhakty *et al*., 2021
Supports *in vitro* oocyte maturation and embryonic development.	Porcine	Hashimoto, 2008
Increases the proportion of mature oocytes with uniform mitochondrial distribution during *in vitro* maturation.	Murine	Zare *et al*., 2015; Somfai *et al*., 2011
L-carnitine during *in vitro* maturation and subsequent oocyte vitrification improves the *in vitro* production rate of embryos.	Bovine	Chankitisakul *et al*., 2013
Increases the rate of nuclear maturation up to metaphase II (MII) stage, decreases the rate of degeneration and promotes the antrum formation.	Buffalo	Modak *et al*., 2022
Increases oocyte competence during *in vitro* maturation and in the absence of FBS.	Buffalo	Marin *et al*., 2020
Embryos		
Increases lipid metabolism, improves development and cryo- tolerance.	Bovine	Takahashi *et al*., 2013.
Improves post-thawing cryotolerance in cryopreserved embryos.	Bovine	Zolini *et al*., 2019.
In combination with N-acetylcysteine and α-lipoic acid, L-carnitine increases the number of cells in blastocyst.	Murine	Truong *et al*., 2016.
Increases the cleavage rate, as well as the formation of morulae and blastocysts.	Buffalo	El-Sokary *et al*., 2021.

stage of growth, is necessary to allow the follicles to present an adequate size in a long-term culture period. This review shows that L-carnitine can be used to regulate oxidative stress and lipid β-oxidation during *in vitro* culture of ovarian follicles, oocyte maturation, embryo production and cryopreservation, especially due to stimulation energy generation combined with its antioxidant properties.

Competing interests. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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