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Importance of lipid metabolism on oocyte maturation and early embryo development: Can we apply what we know to buffalo?

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ABSTRACT

The knowledge about the biological events that regulate lipid metabolism in oocytes and embryos in buffalo is scarce. Lipogenesis, lipolysis, transport and oxidation of fatty acids (FAs) occur in gametes and embryonic cells of all mammalian species, as an intrinsic component of energy metabolism. In oocytes and cumulus cells, degradation of lipids is responsible for the production of ATP that is essential for the metabolic processes that lead to oocyte maturation in *in vivo* and *in vitro* culture conditions. Similarly, throughout embryo development, blastomeres have the capacity to use exogenous and/or endogenous lipid reserves to serve as an energy source necessary for early embryonic development. In addition, supplementation of culture media with L-carnitine to promote lipid metabolism during *in vitro* oocyte maturation and early embryonic development leads to an improved embryo quality. The limited scientific evidence available in buffalo indicates there is relatively greater oocyte lipid content as compared with many other species that undergoes a dynamic distribution during folliculogenesis and follicle maturation and that has a positive effect on oocyte maturation and embryo development when there is L-carnitine supplementation of the media. Advances in the understanding of the biological peculiarities of lipid metabolism, and the consequences of its alteration on the quality of buffalo gametes and embryos, therefore, are necessary to design specific culture media and laboratory procedures as a strategy to increase *in vitro*-derived embryo production rates.

1. Introduction

Even with the great advances in regards to the capacity to achieve *in vitro* mammalian oocyte maturation and embryo development, knowledge about the biological regulatory processes of embryo development is still scarce. The absence of suitable *in vivo* models to perform studies is a factor limiting the progress in this area (Ménézo et al., 2013). It is well accepted that the *in vitro* culture conditions have marked effects on oocyte and embryo metabolic, transcriptional and epigenetic processes that also may have profound effects on embryo quality and survival (Kues et al., 2008; Tesfaye et al., 2009; del Collado et al., 2017a). Through cytoplasmic and nuclear maturation, oocytes must gain the competence to sustain mono-spermic fertilization, undergo preimplantation development, and develop to the blastocyst stage (Palomba et al., 2017). Unfortunately, the quantity and quality of embryos produced from *in vitro*-matured oocytes is much less than those developing *in vivo* (Coticchio et al., 2015; Arias-Álvarez et al., 2017). In buffalo,

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research developments with assisted reproduction technologies (ART) are further hindered by the lack of availability of biological material for study.

Lipids are hydrophobic substances very important for cell metabolism and signaling (Welte and Gould, 2017). Lipids are precursors in the steroidogenic and eicosanoid synthesis pathway in cumulus oocyte complexes (COCs) (Nuttinck et al., 2008), mediators in cell signaling, essential components for membrane formation, as well as a primary energy source for metabolic processes (Prates et al., 2014). The functions of lipids in supporting metabolic processes of gametes, follicular cells and embryos have been investigated (Boccia et al., 2005; Marei et al., 2010; Sudano et al., 2016). In this regard, the intra-cytoplasmic lipid composition affects *in vitro* oocyte maturation, embryo development and quality, as well as embryo and oocyte cryo-tolerance in several species (Accorsi et al., 2016; Arcarons et al., 2017; Barrera et al., 2018). Furthermore, mechanical removal of lipids from pig oocytes (Ren et al., 2015) and embryos (Nagashima et al., 1994), addition of inhibitors of fatty acid synthesis in culture medium for cattle embryos (Sudano et al., 2011), or addition of the fatty acid oxidation inducer L-carnitine in the medium for oocyte IVM and/or embryo culture in several species, enhance embryonic development and/or cryosurvival (Takahashi et al., 2013; Zare et al., 2015; Mishra et al., 2016; Fathi and El-Shahat, 2017). The lipid composition of the extracellular medium during culture affects lipid composition within oocytes (Lim et al., 2007). Lipid metabolism, therefore, is a promising research area with the prospect of improving *in vitro* embryo yield and quality in species of production value.

The recognition of the importance of some genetic variables to productivity such as rusticity, longevity, adaptability, prolificacy and precocity, has resulted in buffalo breeding being an emerging economic endeavor with great potential, especially in South American countries (Bastianetto, 2009). An added value to buffalo production has stemmed from the emerging use of buffalo milk for direct dietary consumption by humans and particularly for making mozzarella cheese because of its relative greater total solid concentration as compared with cattle milk (Ahmad et al., 2013). Some assisted reproductive biotechnologies currently used in cattle, namely *in vitro* embryo production (IVEP), have been successfully utilized in buffalo herds to increase genetic gain (Salina et al., 2013; Ferraz et al., 2015), albeit with inconsistent results (Neglia et al., 2004; Vecchio et al., 2010; Ohashi et al., 2017).

Information regarding the relationship between lipid metabolism and IVEP in buffalo is rather limited. With the present review, the aims were to evaluate and present the current knowledge regarding lipid metabolism in COCs and embryos, as well as how the modulation of this metabolism during *in vitro* culture may be used to improve oocyte maturation and/or embryo development. When applicable, there was a focus on information from the few reports in which buffalo were the species studied with the goal to provide the background for future studies aimed at improving IVEP in this species.

2. Neutral lipids in cumulus-oocyte complexes (COCs) and embryos

Lipids are organic molecules that have important functions in the processes of oocyte maturation and early embryonic development (Hillman and Flynn, 1980; Ferguson and Leese, 2006). Lipids are present inside cumulus cells, oocytes and blastomeres in the form of triglycerides that are subsequently stored within the cytosol in structures known as lipid droplets (LDs). It is the large number of LDs together with interactions with other organelles that confers the typical dark color characterizing the oocyte cytoplasm in some species (Genicot et al., 2005; Ambruosi et al., 2009), including buffalo (Boni et al., 1992; Mondadori et al., 2010b). The total content of triglycerides in immature oocytes has been estimated at approximately 58–59 ng for cattle oocytes (Ferguson and Leese, 1999; Kim et al., 2001), and 135 ng for pig oocytes (Sturmey and Leese, 2003). Information available regarding the content of neutral lipids in horses (Grøndahl et al., 1995, 1997; Ambruosi et al., 2009) and buffalo (Boni et al., 1992; Mondadori et al., 2010b) oocytes is limited to the mere qualitative description of the large amount of LDs in oocytes of these species. These LDs are structurally a hydrophobic core wrapped by a membrane composed of a phospholipid monolayer and the droplet being coated with proteins termed peripilins (Yang et al., 2010). These proteins have been implicated in the regulation of the metabolism of lipids stored inside the LDs of the cells in which peripilins are produced (Yang et al., 2010; Sastre et al., 2014).

Lipid droplets have highly dynamic functions throughout oocyte maturation and embryonic development. The LDs, therefore, can be synthesized *de novo,* degraded, or may change in size by coalescing of the LDs. For example, in buffalo oocytes and embryos, LDs and mitochondria undergo active structural reorganization and distribution throughout maturation and development with these changes being associated with the metabolic requirements of the cells (Mondadori et al., 2010a, b; Zhuang et al., 2012). Regardless of the intracellular distribution, LDs are associated with the endoplasmic reticulum and mitochondria (Nagano et al., 2006). This association has led to the hypothesis that these organelles form a metabolic unit (Kruip et al., 1983) that results in mitochondrial utilization of lipids as an energy substrate for the generation of adenosine triphosphate (ATP) (Dunning and Robker, 2012). This finding is supported by results from other studies where triglycerides within LDs were found to be an ample source of energy for oocytes and embryos of several mammalian species (Ferguson and Leese, 1999; McEvoy et al., 2000). A potential explanation for the larger abundance of LDs in oocytes as compared to many other cells and in embryos is the reliance on LDs as an energy source for the period between fertilization and placentation in some species, such as pigs, cattle and horses (McEvoy et al., 2000; Sturmey et al., 2006; Ambruosi et al., 2009; Assis Neto et al., 2010). Notably, in buffalo, placentation is not complete until 30 to 35 days after fertilization (Neglia et al., 2009).

Diet has a direct effect on lipid contents and fatty acid profiles in oocytes and embryos while, in turn, affecting oocyte competence and subsequent embryo development (Zachut et al., 2010; Aardema et al., 2011; Freret et al., 2019). It, therefore, is logical to conclude that diet has an effect on reproductive performance at least as it relates to processes requiring lipid metabolism (Leroy et al., 2013). Because buffalo are able to utilize poor quality forage (Zicarelli, 1994), it is plausible to assume that buffalo can more effectively adapt to a reduced energy intake than cattle (Campanile et al., 2010). When there are negative energy balance (NEB) conditions, even when estrous-cyclic ovarian activity is observed, both the number of dominant follicles present during waves of

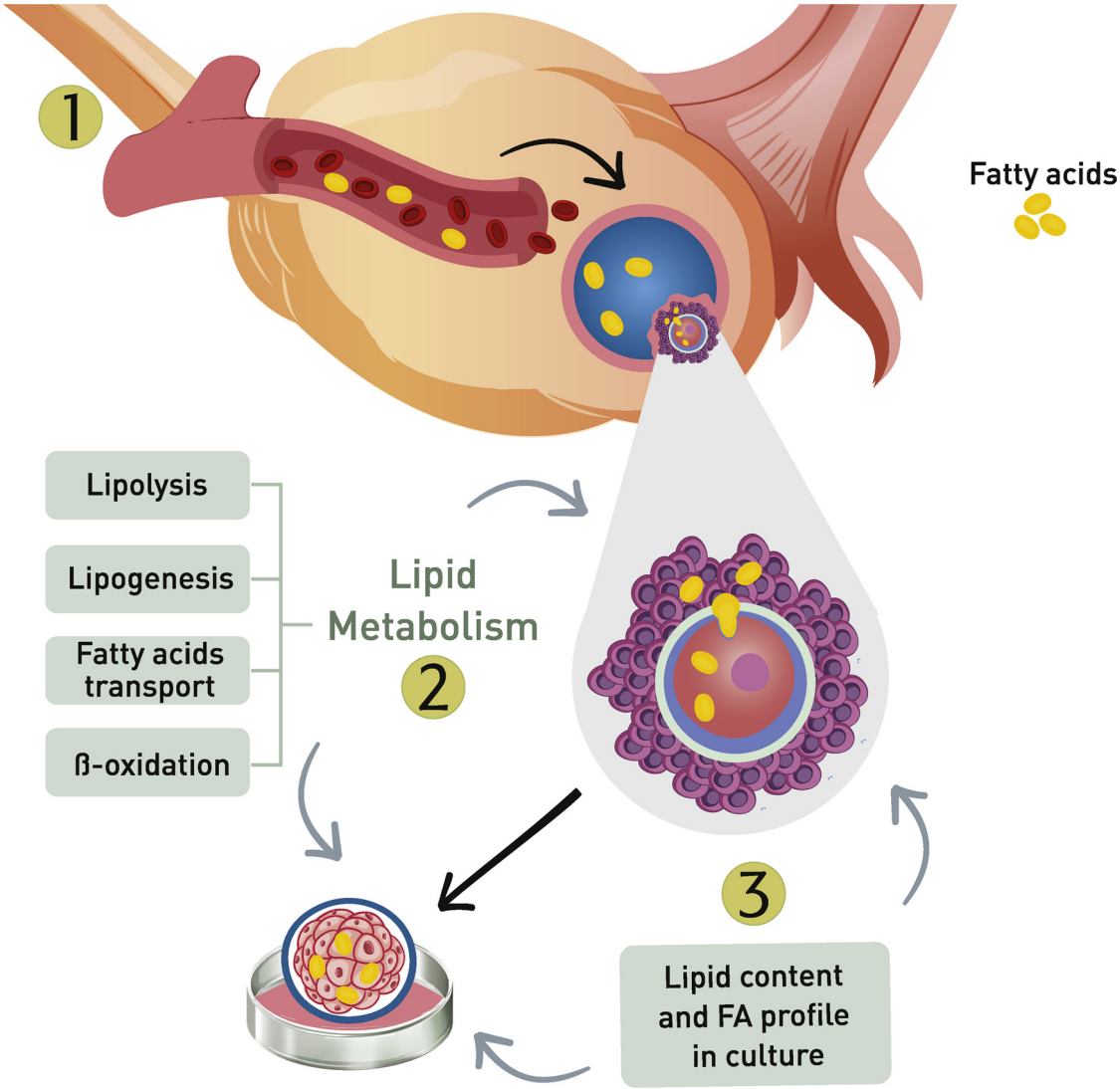

Fig. 1. Depictions of primary pathways that determine the amount and profile of fatty acids in mammalian oocytes and embryos; 1) *In vivo*, free fatty acids in the bloodstream affect lipid content and profile in follicular fluid, from where fatty acids are incorporated in cumulus cells, subsequently into the oocyte; and ultimately into the resulting embryo; 2) Lipolysis, lipogenesis, fatty acid transport and fatty acid oxidation are pathways involved in lipid metabolism in follicular cells, oocytes and embryos; 3) *In vitro*, lipids in the culture media can markedly effect lipid content and profile in oocytes and embryos.

ovarian follicular development and the number of high quality oocytes are less as compared with when there are not a NEB (Campanile et al., 2001, 2010; Khan et al., 2011). Notably, when post-pubertal buffalo are fed a diet lacking in energy, there are lesser blood glucose and insulin concentrations than in females fed a diet with greater energy content. Furthermore, in this study there was greater glucagon concentrations in post-pubertal females where there was a NEB, presumably because of a greater activity of the lipolytic pathway and utilization of lipid reserves for the production of energy (Campanile et al., 2010). In cattle, when there is greater lipolysis, there is a greater amount of non-esterified fatty acids (NEFA) in the bloodstream (Delfino et al., 2018), follicular fluid, and uterine tissues that lead to greater NEFA concentrations in follicular cells, oocytes and embryos (Fouladi-Nashta et al., 2007; Leroy et al., 2008) (Fig. 1). In buffalo, when there were relatively greater NEFA concentrations in follicles, there was a negative effect on theca and granulosa cell proliferation and, therefore, on follicular development (Khan et al., 2011). Consistent with results from these studies, in cattle that had greater than optimal NEFA concentrations both in serum (Leroy et al., 2005) and in *in vitro* culture media (Leroy et al., 2010; Van Hoeck et al., 2011, 2013; De Bie et al., 2017) there was an apparent lipid toxic effect that negatively affected oocyte and embryo survival. Results from studies with several species, including humans, indicate the effects of greater than optimal NEFA concentrations led to stress effects at the endoplasmic reticulum that resulted in oxidative stress (Yang et al., 2012; Van Hoeck et al., 2013), or mitochondrial dysfunction (Sutton-McDowall et al., 2015) within cumulus cells, oocytes and embryos. In addition, oxidative stress in COCs of cattle matured *in vitro* when there were high concentrations of palmitic acid was less

in oocytes as compared with cumulus cells, indicating oocytes had more effective compensatory mechanisms (Marei et al., 2019). Accordingly, it was proposed that with lipid-toxic conditions, cumulus cells function as a defense barrier for oocytes by accumulating larger amounts of fatty acids than oocytes from the external milieu, and consequently cumulus cells have a greater amount of oxidative damage (Aardema et al., 2013).

Furthermore, as occurs with the total neutral lipid content, the fatty acid profile in oocytes and embryos is also dynamic and responds to variations in the fatty acid profile of the external environment (Wonnacott et al., 2010; Zachut et al., 2010) (Fig. 1). Palmitic, stearic, oleic and linoleic acids have been reported to be the main fatty acids in follicular fluid (Renaville et al., 2010; Pantasri et al., 2015; Forde et al., 2016). Fatty acid diet composition, however, may favor the increase of a particular fatty acid, thus affecting COC quality in cattle, humans, and sheep (Zeron et al., 2002; Hammiche et al., 2011; Guardieiro et al., 2014). These findings are further supported by results from *in vitro* experiments. For example, when COCs of cattle were cultured with saturated fatty acids (palmitic, stearic), the oocytes of the COC had a lesser intra-cytoplasmic lipid storage capacity. Conversely, with culture with unsaturated fatty acids (oleic), there was greater lipid storage and improved oocyte developmental competence, along with less deleterious effects of saturated fatty acids (Aardema et al., 2011). Similarly, with supplementation of the COC maturation medium with linoleic acid, there was lesser oocyte quality and embryo development rates (Marei et al., 2010), while supplementation with linolenic acid resulted in greater maturation and embryo production rates, as well as embryo quality (Marei et al., 2009). Overall, evidence indicates there are beneficial effects on oocytes and embryos when culture media are supplemented with unsaturated but not saturated fatty acids.

In conclusion, while the oocyte and embryo lipid content and profile are species-specific, these can be modulated by external conditions. Alterations in both lipid content and profile can result in either deleterious or beneficial effects, depending upon the extent of variation from the optimal amount and type of fatty acid. Available information for buffalo is limited, but results from studies with this species indicate lipid content in oocytes and embryos can be quantitatively and qualitatively affected by the same pathways regulating cellular lipid content of other mammalian species.

2.1. Lipid metabolism during oocyte maturation

The degradation of organic compounds in the form of carbohydrates, amino acids or lipids provides the ATP necessary for the metabolic processes that lead to the completion of oocyte maturation in both *in vivo* and *in vitro* culture conditions (Collado-Fernandez et al., 2012). The importance of lipids as an energy source during these processes has been widely documented in several mammalian species. Lipid metabolism during maturation of pig oocytes *in vivo* is a complex and highly dynamic process, comprising the interaction of the follicular environment, follicular cells, and oocytes (Uzbekova et al., 2015). The amount and composition of lipid in the follicular fluid affects the proportion and content of LDs within oocytes and cumulus cells (Fig. 1), and there is a close correlation with the extent of expression of the genes involved in the synthesis, oxidation and transport of fatty acids in follicular wall cells and in COCs (Warzych et al., 2017). *In vitro*, multiple studies have been conducted to examine effects of inhibition of β-oxidation on nuclear and cytoplasmic oocyte maturation (Downs et al., 2009; Dunning et al., 2010; Ferguson and Leese, 2006; Sturmey and Leese, 2003). Furthermore, effects of increased rates of fatty acid oxidation have been studied with the aim to improve oocyte developmental competence during maturation in both laboratory and livestock species (Dunning et al., 2010; Somfai et al., 2011; Wu et al., 2011; You et al., 2012; Takahashi et al., 2013).

Lipid content (McEvoy et al., 2000) and mitochondrial activity (Stojkovic et al., 2001) have been proposed as potential markers of oocyte competence. For example, amount of brilliant cresyl blue (BCB) dye staining is correlated with a greater developmental competence of cattle and buffalo oocytes, as compared to BCB-negative counterparts and the greater lipid content is correlated with greater mitochondrial activity (Castaneda et al., 2013; Bhardwaj et al., 2016; Pandey et al., 2018). Furthermore, mitochondrial β-oxidation activity was greater during the period of oocyte maturation (Cetica et al., 2002; Tarazona et al., 2006). Consistent with this finding, chemical stimulation of β-oxidation with L-carnitine or acetyl-L-carnitine during *in vitro* maturation of buffalo oocytes had a beneficial effect on oocyte quality (Phongmitr et al., 2013; Hui-Yan et al., 2018), and there were enhanced rates of nuclear and cytoplasmic oocyte maturation (Ocampo et al., 2013). The mechanism of action and activity of pharmacological β-oxidation induction with L-carnitine will subsequently be described in greater detail in this manuscript.

While there are no studies in which there was direct assessment of the importance of lipid as energy sources during maturation of buffalo COCs, it is well documented that both the oocytes and cumulus cells store large amounts of lipids in the form of LDs (Boni et al., 1992; Mondadori et al., 2010a, b). Sensitivity to the detrimental effects of β-oxidation inhibition on *in vitro* maturation of mouse, cattle and pig COCs is proportional to the lipid content of the oocytes contained within the respective COCs (Paczkowski et al., 2013). These results indicate oocytes with relatively greater lipid concentrations have a greater dependence on this metabolic pathway for the production of energy during the maturation process.

There are inconsistent results regarding how lipid intracellular stores change during oocyte maturation. There was a decrease in triglyceride content during maturation of both cattle and pig oocytes, consistent with the activation of lipolytic pathways (Ferguson and Leese, 1999; Kim et al., 2001; Cetica et al., 2002; Sturmey and Leese, 2003; Romek et al., 2011). Conversely, results of other studies indicate there is an increase in storage of lipid reserves from the germinal vesicle through the metaphase II (MII) developmental stage in *in vitro* matured cattle oocytes (Rizos et al., 2002; Aardema et al., 2011; del Collado et al., 2017b). This outcome, however, may have resulted from adaptations of the oocyte to the composition of the culture medium (Fig. 1). The COCs cultured with blood serum (i.e., fetal calf serum or FCS) had greater stores of fatty acids when compared to those cultured with fatty acid-free bovine serum albumin (BSA-FAF) (del Collado et al., 2016). Importantly, blood serum has greater concentrations of lipids (Cagnone and Sirard, 2014), which are internalized by the cumulus cells, and from there transported into the oocyte (Adamiak et al., 2006; del

Collado et al., 2017b). When added to the culture medium, the greater accumulation of fatty acids within oocytes may have deleterious effects on mitochondrial activity and migration, thus affecting lipid metabolism (del Collado et al., 2016). The presence of FCS also enhances the acquisition of oocyte competence (del Collado et al., 2017a) which could be, at least partially due, to the stimulation of fatty acid metabolism. Consistent with when COCs were matured in the presence of BSA-FAF and without FCS, there was a lesser proportion of oocytes developing to the metaphase II stage (Ali and Sirard, 2002) or there being dysfunction in the process of cortical granule migration at the end of the *in vitro* maturation process (del Collado et al., 2016). Interestingly, mouse COCs have greater β-oxidation when cultured in FCS as compared with when there is culturing with BSA-FAF (Dunning et al., 2010). Blood serum is a potential source of, not only fatty acids but also L-carnitine, further indicating there are functions of fatty acid oxidation on oocyte maturation.

Cumulus cells have an important function on COC lipid metabolism and, indirectly, on the acquisition of oocyte competence. Indeed, with use of MALDI mass spectrometry, it was observed that cumulus cells modulated the lipid profile of cattle oocytes. As a consequence of this modulation, the lipid profile depended on the composition of the *in vitro* culture medium (Vireque et al., 2017). Furthermore, supplementation of the i*n vitro* maturation medium with etomoxir, an inhibitor of CPT-1 enzyme activity, had a detrimental effect on cattle oocyte maturation rates that occurred to a greater extent when oocytes were matured with cumulus cells rather than when oocytes were denuded. The supplementation of culture media with etomoxir resulted in a suppression of gene expression for proteins involved in lipid metabolism, in particular in cumulus cells, hence decreasing the viability in a greater proportion of COCs compared with denuded oocytes (Sanchez-Lazo et al., 2014). Similarly, *in vitro* culture of denuded cattle oocytes resulted in inadequate cytoplasmic maturation and alterations in fatty acid metabolism (Auclair et al., 2013). Hence, in the absence of cumulus cells, there is a lack of oocyte regulation of lipid metabolism, partially explaining the limited competence of denuded oocytes compared to COCs, as reported in cattle, goats and pigs (Wongsrikeao et al., 2005; Dey et al., 2012; Souza-Fabjan et al., 2016). When results of all these studies are considered, there is considerable evidence that cumulus cells have a regulatory function regarding oocyte lipogenesis or lipolysis in regulating oocyte maturation.

2.2. Lipid metabolism during early embryonic development

In general, most of the factors that affect lipid metabolism in oocytes and cumulus cells, that have been previously described in this manuscript, also affect lipid metabolism in embryos. The lipid profile in early embryos is a reflection of the energy metabolism that occurred during oocyte maturation (del Collado et al., 2016) (Fig. 1). Lipid metabolism is also apparently important as a source of energy during the early stages of embryo development in several species (Dunning et al., 2010; Sutton-McDowall et al., 2012; Sudano et al., 2016). For example, inhibition of β-oxidation during mouse and cattle zygote culture had deleterious effects on cleavage and particularly blastocyst development rates (Hewitson et al., 1996; Ferguson and Leese, 2006; Dunning et al., 2010). *In vitro* cultured pig embryos, therefore, were not affected when there was inclusion of the β-oxidation inhibitor, methylpamoxirate, in the medium; instead, there was an increase in glucose metabolism indicating there was a compensatory effect in the absence of energy from fatty acid metabolism (Sturmey and Leese, 2008). When pig (Lowe et al., 2017) and cattle (Sutton-McDowall et al., 2012) embryos were cultured, without inclusion of a carbohydrate source, rates of cleavage and development to the blastocyst stage were greater when fatty acid oxidation was stimulated with L-carnitine medium supplementation. These results indicate that in species with relatively greater lipid content in the embryo, oxidation of endogenous fatty acids may partially compensate for the absence of carbohydrates as exogenous energy sources. Interestingly, addition of L-carnitine in mouse zygote culture medium devoid of glucose, lactate, or pyruvate, increased the proportion of embryos at the two-cell stage, indicating that even in species where oocytes have a relatively lesser lipid content, fatty acid oxidation can occur as a compensatory metabolic pathway in some circumstances (Dunning et al., 2010).

Lipid metabolism in embryos also appears to be stage-specific. With *in vitro* produced cattle embryos, the upregulation of genes related to lipid metabolism correlated with the LD content which increased at the morula and decreased again at the blastocyst stages of development, indicating these modifications occurred in a direct response in biological changes during development (Sudano et al., 2016). Similarly, manipulating lipid metabolism of cattle embryos cultured *in vitro* with substances that inhibit fatty acid synthesis and favor the pentose phosphate pathway (phenazine ethosulfate) or stimulate fatty acid oxidation (L-carnitine), as expected, decreased cytoplasmic lipid content and increased mitochondrial activity (Ghanem et al., 2014). Furthermore, there was also a change in the gene expression profile relating to lipid metabolism that resulted in an increased embryo quality and cryotolerance (Ghanem et al., 2014). When results of all these studies are considered, there were indications that lipid metabolism has an important function on energy metabolism during embryo development in both laboratory and livestock species. Furthermore, there are differences in lipid metabolism depending on the culture conditions, regardless of initial lipid content, and there are consequent effects on embryo development and quality. For example, inclusion of blood serum or lipid fractions derived from serum in the culture medium resulted in increases in the lipid content of cattle embryos in a dose-dependent manner, and decreased resistance to cryopreservation processes (Ferguson and Leese, 1999; Rizos et al., 2003; Sudano et al., 2012). Results with use of molecular analysis techniques indicated that when there were greater concentrations of lipids, there was an enhanced expression of genes related to oxidative stress, and genes involved in the cellular inflammatory response, which could subsequently affect embryonic development and maternal recognition of pregnancy (Cagnone and Sirard, 2014). Furthermore, the inclusion of serum in culture medium leads to molecular alterations with sustained effects on fetal development, such as the large offspring syndrome (LOS) in sheep and cattle (Thompson et al., 1995; Lazzari et al., 2002). From results of these studies, it can be inferred that concentration of blood serum in media, which may function as a source of lipids and other embryotrophic factors, must be carefully titrated in the embryo culture medium (del Collado et al., 2016). Medium supplementation with L-carnitine or similar compounds leads to stimulation of β-oxidation of fatty

acids, serving as an additional source of energy from endogenous lipids but also as a strategy to reduce excess lipid content (Sutton-McDowall et al., 2012). For *in vitro* maturation of buffalo oocytes, supplementation of L-carnitine led to a reduction in reactive oxygen species (ROS) of mitochondrial origin, hence mitigating oxidative stress, and increasing ATP content (Hui-Yan et al., 2018). With these media supplementations, there are greater proportions of IVF-produced embryos developing to the blastocyst stage. Ultimately, strategies to modulate intracellular lipid concentrations and metabolism may be used to increase embryo production rates, as well as the quality and freezing capacity of embryos in different species, including buffalo (Gasparrini et al., 2013).

3. L-carnitine

Carnitine is a quaternary amine synthesized primarily in the liver from the amino acids lysine and methionine (Mishra et al., 2016). This compound is an essential cofactor required for the transport of long-chain fatty acids into the mitochondria for the generation of energy (Dunning et al., 2010, 2014). In addition, carnitine has been identified as an antioxidant with the capacity to neutralize free radicals derived from oxygen (ROS), especially the superoxide anion, and thus has the capacity to protect cellular organelles such as mitochondria against the deleterious effects of oxidative stress (Ye et al., 2010). There is not expression for carnitine synthesis in human COCs indicating in *in vivo* conditions the amounts of this compound in both cumulus cells and oocytes would depend on its concentration in follicular fluid which would ultimately be affected by bloodstream concentrations (Dunning and Robker, 2012; Montjean et al., 2012). In oocytes, the amount of fatty acid β-oxidation is less *in vitro* than *in vivo*. The difference in L-carnitine bioavailability *in vitro* and *in vivo* could partially explain this difference in fatty acid β-oxidation (Montjean et al., 2012). Notably, L-carnitine is transported into cells from the extracellular milieu as a result of actions of the organic/carnitine transport membrane proteins (OCTNs). After transport into the cytoplasm of gametes and embryonic cells, L-carnitine can have actions through the following proposed pathways: (i) increasing energy sources as a result of palmitate transfer into the mitochondria to maintain the acetyl CoA/CoA ratio; (ii) protecting cells from oxidative stress and lipid toxicity, by removing free radicals, and reducing excess palmitate from the endoplasmic reticulum; and (iii) promoting cellular growth and proliferation, by decreasing the rate of apoptosis (Agarwal et al., 2018).

Supplementation of culture media for IVEP during oocyte maturation and/or early embryonic development with L-carnitine in cattle (Ghanem, 2015), swine (Lowe et al., 2017), mice (Khanmohammadi et al., 2016), buffalo (Verma et al., 2018) and other species (Table 1) has been associated with improved oocyte competence, increased rates of embryo development to the blastocyst stage, and embryos with greater inner cell mass numbers that have a greater capacity to withstand the cryopreservation process. Specifically in buffalo, Verma et al (2018) evaluated the effects of L-carnitine addition to the *in vitro* embryo culture medium, on lipid metabolism and resistance to cryopreservation. At 1.5 mM, L-carnitine induced an increase in the development rate to the blastocyst stage, stimulated expression of genes related to fatty acid metabolism, and decreased lipid synthesis and formation of LDs. Additionally, at this concentration L-carnitine supplementation of media increased cryotolerance and improved the quality of cryopreserved embryos. Furthermore, frozen-thawed embryos had an increase in the expression of genes associated with energy metabolism and maternal recognition of pregnancy (Verma et al., 2018). Regarding the response of *in vitro* culture medium supplementation with L-carnitine, it should be noted that changes in lipid content, gene expression and mitochondrial activity were not as marked in Jersey as in Holstein cow embryos (Baldoceda et al., 2015a). The authors suggested that those results could be a reflection of the reported differences

Table 1
Studies in which there was evaluation of effects of L-carnitine during *in vitro* embryo production (IVEP) in different animal species.

Species	Concentration with best effect	Stage of IVEP	Effect observed	Reference
Buffalo	0.3 mg/mL	IVM	Improved nuclear maturation	(Phongmitr et al., 2013)
	1.5 mM	IVC	Improved blastocyst rates, quality and cryotolerance of embryos	(Verma et al., 2018)
	0.25 mM	IVC	Increased resistance to cryopreservation	(Boccia et al., 2013)
	5–10 mg/mL	IVM	Improved oocyte competence from aged donor	(Ocampo et al., 2013)
Bovine	3.03 mM	IVC	Increased rates of blastocysts and cryotolerance	(Takahashi et al., 2013)
	2.5 mM	IVC	Higher survival rates after slow cryopreservation	(Held-Hoelker et al., 2017)
	1.5 mM	*IVM/IVF /IVC	Increased blastocyst rate, with higher cell numbers and less apoptotic cells	(Ghanem, 2015)
	2.5 mM	IVM	Improved embryo development from less competent oocytes	(Knitlova et al., 2017)
	0.3 - 0.6 mg/mL	IVM/IVF /IVC	Improved oocyte nuclear maturation and embryo development	(Phongnimitr et al., 2013)
Mouse	0,6 mg/mL	IVM	Better nuclear and cytoplasmic oocyte maturation	(Zare et al., 2015)
	0.5 mg/mL	IVC	Increased number of blastocyst cells and hatching rates	(Khanmohammadi et al., 2016)
	0.3–0.6 mg/mL	IVC	Decreased the level of DNA damage in embryos	(Abdelrazik et al., 2009)
Camel	0.5 mg/mL	IVM/IVC	Better oocyte maturation and embryo development	(Fathi and El-Shahat, 2017)
Pig	0.6–5 mg/mL	IVM	Improved oocyte maturation and embryo cleavage	(Somfai et al., 2011)
	3 mM	IVC	Increased cleavage rates and higher cryotolerance	(Lowe et al., 2017)
	0.5 mg/mL	IVM/IVC	Increased nuclear maturation in oocytes, and blastocysts rates after parthenogenetic activation	(Wu et al., 2011)
Rabbit	10 mM	IVM/IVC	Reduced oxidative stress, increased maturation, cleavage and embryo rates	(Mishra et al., 2016)

IVM: *In vitro* maturation; IVF: *In vitro* fertilization; IVC: *In vitro* culture.

between Jersey and Holstein embryos, with Jersey embryos having a greater LD content and lesser mitochondrial activity, as well as a different lipid profile characterized by a greater content of phospholipids (Baldoceda et al., 2015b). Notably, the characteristics described for Jersey embryos in regards to color and lipid content are similar to those observed in buffalo embryos. Considering the differences, not only between species, but also breeds within species, the concentrations of L-carnitine used in buffalo oocyte maturation and embryo culture media should be carefully evaluated according to the species-specific lipid content and profile. More research is needed to evaluate how these factors affect *in vitro* embryo development in buffalo.

Greater than optimal concentrations of L-carnitine may induce deleterious effects on cells. For example, greater than optimal concentrations of L-carnitine for embryo development, results in a greater binding of L-carnitine to Ca^{++} which could lead to inhibition of the activity of enzymes and metabolic processes that are Ca^{++} dependent as the second messenger, and ultimately alter cellular homeostasis (Mongioi et al., 2016). Furthermore, an excess in L-carnitine availability could induce a greater rate of fatty acid oxidation, thus increasing the cellular energetic metabolism because of greater mitochondrial activity. Greater than optimal metabolic activity may contribute to generation of ROSs as a byproduct in relatively greater amounts (Harvey et al., 2002), such that there is an exceeding of the antioxidant capacity of L-carnitine (Takahashi et al., 2013). This metabolic compound milieu would undoubtedly affect embryo viability because of damage induced by oxidative stress. There, therefore, is a necessity to use L-carnitine cautiously in cell culture, titrating the appropriate concentrations based on cell type and animal species.

4. Conclusion

In conclusion, information is scarce regarding the biochemical, metabolic and molecular pathways that involve lipids and associations with the processes of oocyte maturation and early embryonic development, in particular in buffalo. The limited scientific evidence indicates that there is a relatively greater cytoplasmic lipid content in the oocytes of buffalo as compared with many other species. These lipids undergo a dynamic distribution during folliculogenesis and maturation, and confer a positive effect on oocyte maturation and embryo development after lipid metabolism stimulation with substances such as L-carnitine. Meanwhile, details of these metabolic pathways and the function on these important reproductive processes should be inferred from findings in closely related species (i.e., cattle) or those in which oocytes and embryos contain comparable amounts of intra-cytoplasmic lipids (i.e., pigs).

Long term, it will be necessary to perform studies on the particular biological characteristics of lipid metabolism, and the consequences of alterations in oxidation, transport, lipolysis or lipogenesis during culture of buffalo oocytes and embryos. These studies should allow for development of culture media formulations and specific laboratory procedures aimed at increasing the quantity and quality of embryos produced to confer commercial viability of this bio-technique for culturing of buffalo oocytes and embryos.

Declaration of Competing Interest

The authors declare that there is not conflict of interest in publishing this review.

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