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Dissertation

Lipid modulators during *in vitro* maturation of porcine oocytes

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I dedicate this work to those who believed in a little boy playing with his microscope.

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To Faith, where we can always find strength.

To Resilience, the greatest thing this work taught me.

To Family, Friends and Colleagues, always supportive.

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It matters not how strait the gate,
How charged with punishments the scroll,
I am the master of my fate:
I am the captain of my soul.

Invictus, William Ernest

Abstract

BRAGA, José Victor Cardoso Braga. **Lipid modulators during *in vitro* maturation of porcine oocytes.** 2020. 65f. Dissertation (Master degree in Sciences) – Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2020.

Swine *in vitro* embryo production (IVP) has limitations for better outcomes in reproductive biotechnologies. Among factors impairing IVP, high lipid content in both oocytes and embryos, insufficient media composition and consequent reactive oxygen species (ROS) production are the most prominent. The 1st paper of this dissertation contains a review regarding metabolizers included into the IVP system in order to sustain nuclear and cytoplasmic maturation, as well as improvement of lipid droplet (LD) consumption. The 2nd paper reports a study where 50µM DHA was added during the maturation period, with and without porcine follicular fluid (pFF) supplementation. The results demonstrated that DHA without pFF impairs maturation and embryo development. Also, there was no reduction of the lipid content in oocytes treated with DHA, a finding that might be related to metabolic disorders in the cumulus-oocyte complexes (COC). During maturation period, porcine oocytes prefer glucose as substrate for energy consumption. Phenazine Ethosulfate (PES) is an electron receptor that converts NADPH to NADP, inducing glucose utilization through the pentose phosphate pathway (PPP), influencing lipid droplets (LD) metabolism. Forskolin (FSK) is another chemical modulator that stimulates lipolysis through cAMP activation, being also able to synchronize cytoplasmic and nuclear maturation of oocytes. In the 3rd paper, two concentrations of PES (0.5µM and 0.05µM) and one of FSK (10 µM) were used during the entire *in vitro* maturation period, for two different experiments. In Experiment 1, oocyte maturation and lipid content were evaluated; and embryo development, embryo cell count and lipid content of blastocysts on day 7 were evaluated in Experiment 2. The concentration of 0.5µM of PES had a negative impact on most of the evaluated parameters, while the lowest PES dose was similar to the negative control (NC) and FSK control in both data of Experiment 1, but also had lower cleavage rates when compared to NC in Experiment 2. Taken together, our results demonstrated impairment of embryo development due to possible disharmony on oocyte maturation and metabolism disorder caused by either addition of DHA or PES. Also, FSK did not improve maturation rates or reduced lipid content.

Keywords: lipid droplets; *in vitro* culture; metabolism inducer

Resumo

BRAGA, José Victor Cardoso Braga. **Moduladores lipídicos durante a maturação *in vitro* de oócitos suínos.** 2020. 65f. Dissertação (Mestrado em Ciências) – Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2020.

A produção de embriões suínos *in vitro* (PIVE) possui limitações para melhores resultados em biotecnologias reprodutivas. Dentre os fatores prejudiciais na PIVE, o alto teor lipídico em oócitos e embriões, a composição insuficiente do meio e a consequente produção de espécies reativas de oxigênio (EROS) são as mais proeminentes. O 1º artigo desta dissertação revisa sobre metabolizadores incluídos no sistema de PIVE, a fim de sustentar a maturação nuclear e citoplasmática, bem como a melhoria do consumo de gotículas lipídicas (GL). O 2º artigo relata um estudo onde 50μM DHA foi adicionado durante o período de maturação, com e sem suplementação de fluído folicular suíno (FFp). Os resultados demonstraram que DHA sem FFp prejudica a maturação e o desenvolvimento embrionário. Além disso, não houve redução do conteúdo lipídico em oócitos tratados com DHA, o que pode estar relacionado a distúrbios metabólicos no complexo cumulus-oócito (CCO). Durante a maturação, oócitos suínos preferem glicose como substrato para consumo de energia. O etosulfato de fenazina (PES) é um receptor de elétrons capaz de converter NADPH em NADP, induzindo a utilização de glicose através da via de pentose fosfato (PPP), influenciando o metabolismo lipídico. Forskolin (FSK) é outro modulador químico que estimula a lipólise através da ativação do AMPc, podendo também sincronizar a maturação citoplasmática e nuclear de oócitos. No último artigo, duas concentrações de PES (0,5μM e 0,05μM) e uma de FSK (10μM) foram utilizadas durante todo o período de maturação *in vitro,* para dois experimentos diferentes. No Experimento 1, a maturação oócitária e o conteúdo lipídico foram avaliados; o desenvolvimento embrionário, a contagem de células embrionárias e o conteúdo lipídico de blastocistos no dia 7 foram avaliados no Experimento 2. A concentração de 0,5μM de PES teve um impacto negativo na maioria dos parâmetros avaliados, enquanto a menor dose de PES foi semelhante ao controle negativo (CN) e ao controle de FSK em ambos os dados do Experimento 1, obtendo também taxas menores de clivagem quando comparada ao CN no Experimento 2. Em conjunto, nossos resultados demonstraram comprometimento do desenvolvimento embrionário devido à possível desarmonia na maturação oócito e transtorno do metabolismo causada pela adição de DHA ou PES. Além disso, FSK não melhorou as taxas de maturação ou reduziu o conteúdo lipídico.

Palavras-chave: gotículas lipídicas; cultivo *in vitro;* indutor de metabolismo

Figure List

Table List

Abbreviation list

ACAT	cholesterol acyltransferase
Acyl-CoA	Acyl-coenzyme A
ATP	Adenosine triphosphate
BAT	Brown adipocyte tissue
cAMP	Cyclic adenosine monophosphate
CDC25B	Cell division cycle 25B
CDK1	Cyclin-dependent kinase 1
CLA	Conjugated linoleic acid isomer
COC	Cumulus-oocyte complex
DGAT	Diacylglycerol acyltransferase
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
FA	Fatty acid
FASN	Fatty acid synthase
FIT/FITM	Fat storage-inducing transmembrane protein
FSK	Forskolin
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* production
LD	Lipid droplet
LH	Luteinizing hormone
MPF	Maturation promoting factor
NADP	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate
NEFA	Non-esterified fatty acid

PDM	Peridroplet mitochondria
PES	Phenazine ethosulfate
PKA	Protein kinase A
PLIN	Perilipin
PPAR	Peroxisome proliferator-activated receptor
PPP	Pentose phosphate pathway
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SA	Stearic acid
TAG	Triacylglycerol
Thr14/Tyr15	Cyclin-dependent kinase 1 (phosphorylated)

Symbol list

β	Beta
µM	Micromolar
mM	Millimolar
mm	Millimeters
%	Percentage
°C	Celsius degree
<	Minor

Summary

1 Introduction

Oocyte maturation is a complex event dependent on the synchrony of chromosome separation, redistribution of cytoplasmic organelles and storage of transcripts and proteins needed for embryonic genome activation (CONTI; FRANCIOSI, 2018; FERREIRA et al., 2009). In pigs, the first meiotic division occurs 36h to 40h after luteinizing hormone (LH) surge and interactions with cumulus cells via gap junctions are essential to delivery of substrates for energy metabolism, and paracrine signals from granulosa cells to regulate oocyte development (HUNTER, 2000). Antral follicular somatic cells produce cyclic adenosine monophosphate (cAMP) and consecutively transfer into the oocyte, maintaining Maturation Promoting Factor (MPF) in its inactive form. Briefly, high cAMP level within the oocyte activates protein kinase A (PKA), and, in turn, inactivate cell division cycle 25B (CDC25B), inactivating MPF by promoting the phosphorylation of cyclin-dependent kinase 1 (CDK1) on Thr14 and Tyr15 (PAN; LI, 2019). Decrease in cAMP levels during oocyte maturation leads to the dephosphorylation of CDK1 on Thr14 and Tyr15, and the MPF complex becomes active, such that the oocyte can re-enter meiosis (MEHLMANN, 2005). Thus, removal of cumulus oocyte-complexes (COCs) from the follicles for *in vitro* embryo production (IVP) causes spontaneous resumption of meiosis (APPELTANT et al., 2016).

IVP system is based on *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) of oocytes, and *in vitro* culture (IVC) of presumable zygotes (SOMFAI; HIRAO, 2011). Until the late 70's, IVP research were focused in lab animal models, however, it's commercial application in farm animals expanded the technique (GRUPEN, 2014). Moreover, embryo need for research and transgenic animal production lead to swine IVP development in the early 90's due to similar genetic structure as humans (GRUPEN, 2014), making swine a good model for biomedical research (SUN; NAGAI, 2003). However, swine IVP has lower embryo production rates (APPELTANT et al., 2016), especially when compared to bovine (KIKUCHI et al., 2016)**.** The first step to successful IVP is to provide the oocyte with a favorable environment, able to support both cytoplasmatic and nuclear maturation (ZHANG et al., 2012), since disharmony between these events is a major obstacle during IVP (PRATES; NUNES; PEREIRA,

2014), also an excessive accumulation of lipid droplets (LD) within the cytoplasm of both oocytes and embryos negatively impacts IVP development rates and biotechniques such as cryopreservation, cloning and transgene (GAJDA, 2009).

Lipids have metabolic functions and are presents in mammals as triglycerides, phospholipids, steroids, and their metabolic products such as long-chain fatty acids, glycerol and ketone bodies (TSUJII; KHANDOKER; HAMANO, 2001). Lipid profile of swine, ovine and bovine oocytes has already been described (HOMA; RACOWSKY; MCGAUGHEY, 1986; MCEVOY et al., 2000), demonstrating that swine oocytes possess 1.8 to 2.5 times more fatty acids then ovine and bovine counterparts, respectively (MCEVOY et al., 2000)**.** As related by PRATES; NUNES; PEREIRA. (2014), it is plausible to say that this discrepancy in the lipid content could be related to litter size, since the pig is a polytocous mammal, this energy reservoir can be favorable in the embryo competition for implantation in the uterus. Moreover, swine embryos have different lipid profiles according to their development stages, to their lipid classes, their location in the cytoplasm and among embryos produced *in vivo* versus *in vitro* (ROMEK et al., 2010).

LD act as cells energy source, since they are composed by a monolayer of phospholipids, possessing triacylglycerol (TAG) and esters as main storage lipids (THIELE; PENNO, 2015; WALTHER; FARESE JR, 2012; WELTE; GOULD, 2017), being synthesized by acyl-coenzyme A (Acyl-CoA), diacylglycerol acyltransferase (DGAT) and cholesterol acyltransferase (ACAT) through fatty acids. These droplets are formed near the endoplasmic reticulum (ER) by neutral lipid synthesis, exchange of lipid content among droplets and by usage of fatty acids for lipid synthesis (THIELE; PENNO, 2015).

Since LD has been identified by electron microscopy of oocytes from many species, they have been often associated with mitochondria (DUNNING; RUSSELL; ROBKER, 2014). In fact, porcine oocytes stained with Nile Red and analysed by florescence resonance energy transfer demonstrated a proximity of that 10nm between lipid droplets and mitochondria (STURMEY; O'TOOLE; LEESE, 2006). BENADOR et al. (2018) found that peridroplet mitochondria (PDM) is specialized LD growth, since mitochondria-LD association was shown to be highly regulated by Perilipin5 (Plin5) in brown adipocyte tissue (BAT) and that PDM promotes free fatty acids incorporation into TAG in a mitochondrial adenosine triphosphate (ATP) synthesis dependent

manner, supporting that PDM enhance LD expansion through the provision of ATP to acyl-CoA synthesis and lipid cycling.

Research focused in identification of genes related to lipid storage in yeast, adipocytes, hepatocytes and zebrafish (*Danio rerio*) embryos revealed specific LD markers, including fat storage-inducing transmembrane proteins (FIT or FITM) and perilipins (PLIN) (WALTHER; FARESE JR, 2012). Proteins FIT1, FIT2, PLIN1, PLIN2, PLIN3 and PLIN5 are regulated by peroxisome proliferator-activated receptors (PPAR), important regulators of lipid metabolism (ITABE et al., 2017; KADEREIT et al., 2008). Indeed, KADEREIT et al. (2008) were the first to identify FIT genes as genes of a unique family, with exclusive presence in the ER. Moreover, overexpression of FIT2 lead to a higher amount of LD while its depletion lead to reduction of LD, TAG and PLIN and PPAR expression (KADEREIT et al., 2008).

Therefore, our objective in the present dissertation is to reduce LD content in porcine oocytes and embryos, by using lipid modulators during IVM. Our hypothesis is based on the following published review (Paper 1), where supplementation with lipid modulators either during IVM or IVC reduce LD and improves embryo development. Thus, in the second and in the third manuscripts of this document, the reader will encounter results regarding the effects of docosaehaenoic acid (DHA) and phenazine ethosulfate (PES) during IVM and posterior embryo stages, respectively.

2 Papers

2.1 Paper 1

Approaches to reduce lipids: a review of its impacts on *in vitro* embryo production

J.V. Braga, E.R. Komninou, A.D. Vieira, R.G. Mondadori

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Approaches to reduce lipids: a review of its impacts on *in vitro* embryo production

Abordagens para reduzir lipídeos: uma revisão sobre seus impactos na produção de embriões *in vitro*

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Abstract

In vitro embryo production (IVP) has limitations for better outcomes in blastocyst production, cryopreservation efficiency and pregnancy rates. Among the factors impairing IVP, high lipid content in both oocytes and embryos, and consequent reactive oxygen species (ROS) production, have a negative impact in embryo development and further biotechnologies. The present review aims to address techniques and strategies that collaborate to improve embryo development rates through reduction of lipid content either in the oocyte or in the embryo.

Keywords: delipidation, in vitro supplementation, lipid droplets, oxidative stress, early embryo metabolism.

Resumo

A produção in vitro de embriões (PIVE) possui limitações para melhoras na produção de blastocistos, eficiência da criopreservação e taxas de prenhez. Dentre os fatores

limitantes à PIVE, o alto conteúdo lipídico tanto em oócitos quanto em embriões e a consequente produção de espécies reativas a oxigênio (ROS), possuem impacto negativo no desenvolvimento embrionário e subsequentes biotecnologias. A presente revisão visa tratar sobre tecnologias e estratégias capazes de colaborar com a melhora nas taxas de desenvolvimento embrionário através da redução do conteúdo lipídico tanto em oócitos quanto em embriões.

Palavras-chave: *delipidação, suplementação in vitro, gotículas de lipídeo, estresse oxidativo, metabolismo embrionário inicial.*

Introduction

In vitro embryo production (IVP) system is based on *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) (Somfai e Hirao, 2011). Two important limiting factors in this system, for both oocytes and embryos, are: (1) the high lipid content and accumulation of lipid droplets (LD) that occurs in some domestic species, mainly in pigs (McEvoy et al., 2000; Gajda, 2009) and; (2) the high levels of reactive oxygen species (ROS) produced in response to fatty acids β-oxidation and culturing conditions. Together these factors negatively impacts embryo development rates (Romek et al., 2017), as well as cryopreservation efficiency (Grupen, 2014). Therefore, reduction of lipid content could be beneficial, however the total removal is not indicated, since they have metabolic functions (Tsujii; Khandoker; Hamano, 2001), and LD act as energy source to the cells (Walther; Farese Jr, 2012; Thiele; Penno, 2015; Welte; Gould, 2017) by forming metabolic units together with mitochondria and the endoplasmic reticulum (Warzych et al., 2017).

In oocytes, during follicular growth and development (Paulini et al., 2014) cumulus-oocyte complex (COC) modulates the expression of genes that regulate fatty

acid (FA) metabolism (Sanchez-Lazo et al., 2014). As a result, during final development, oocytes already contain LD with triacylglycerol and sterol esters as main stored lipids (Walther; Farese Jr, 2012; Warzych et al., 2017) that requires β-oxidation in order to support the maturation process (Dunning; Russell; Robker, 2014). On the other hand, in embryos, during first cleavage, the energy is obtained preferably via pentose phosphate pathway (PPP), while, for further embryo development, glycolysis and β-oxidation are more prominent (reviewed by Prates et al. 2014).

ROS are by products of the cellular oxidative metabolism, a process where ATP is synthesized by reduction of oxygen in the mitochondria, though proton and electron transfer reactions (Fu et al., 2014). ROS production seems also be related to lipid accumulation (Furukawa et al., 2004). This production, associated with the reduction of antioxidant enzymes, can result in oxidative stress, with serious detrimental effects such as: (1) lipid peroxidation, (2) breaking of DNA double strand, and (3) mitochondrial DNA mutation, which result in decrease in embryo and oocytes viability (Guérin; El Mouatassim; Ménézo, 2001).

IVP embryos had higher lipid content and produce higher ROS levels than their *in vivo* counterparts, ROS production also varies according to the stage of development (Guérin; El Mouatassim; Ménézo, 2001). Culture media composition also has influence over these factors (Romek et al., 2010). Thus, it is necessary to optimize the IVP system aiming to reduce lipid content and to increase protection against ROS.

This review aims to gather information about the approaches that have been recently used to reduce lipids levels during IVP. As it will be observed, most of the results use pig as model, because it presents oocytes and embryos with a high lipid content when compared to other species (McEvoy et al., 2000).

Strategies to reduce lipids levels

Mechanic delipidation techniques

Mechanical delipidation includes physical methods to remove or disrupt LD from the cytoplasm. One of the first to attempt this approach was Nagashima et al. in 1994, which used centrifugation and micromanipulation as a combined alternative to reduce LD in porcine oocytes. Their results showed improvement on chilling resistance, but no significant differences in embryo development for delipidated or partially delipidated oocytes. More recently, porcine embryos were used as model to test different centrifugation protocols and their benefits in embryo vitrification, with generally better results for the delipidated ones (Kawakami; Kato; Tsunoda, 2008). Delipidation also reduced the triglyceride content and the expression of GPAT1, AGPAT1, AGPAT2, LIPIN1, DGAT, genes related to triacylglycerol synthesis (Zeng et al., 2017), which are essential for LD structure and formation (Walther; Farese Jr, 2012). However, in one study, mechanical delipidation have not improved total blastocyst survival after cryopreservation, but, in the other hand, those that survived and were transferred to recipients resulted in pregnancies and piglets' birth (Men et al., 2011).

Similar behavior was observed in sheep embryos, were Romão et al. (2015) found that centrifugation plus exposure to cytochalasin D or the use of *trans*-10 *cis*-12-conjugated linoleic acid isomer (CLA) resulted in higher blastocyst rate and survival after vitrification and warming, possibly by reduction of the lipid content, although no lipid evaluation was performed. Briefly, besides being a time-consuming practice, mechanical delipidation, improves the efficiency of IVP system.

Fatty acids supplementation

FA are a class of lipids that form structural components of membranes and are an energy source as they are stored as triacylglycerol inside LD. They are metabolized by β-oxidation, influencing oocyte development potential (Dunning; Russell; Robker, 2014). Although the addition of lipids may seem controversial, once oocytes and embryo already have a high lipid content, recent studies have shown that supplementation with FA may be beneficial as it forces lipid metabolism and contributes to energy supply. Sanchez-Lazo et al (2014) described that FA synthesis, lipolytic activity and fatty acid oxidation (FAO) have direct influence in the oocyte maturation process. Likewise, the expression of genes related to FA metabolism is consonant with the modifications of lipid content in cumullus cells, since they communicate with the oocyte through paracrine signals and tranzonal projections (reviewed by Clarke, 2018).

In bovines, the addition of *trans-10, cis-12 octadeca-dienoic acid* (*t10, c12* CLA), a conjugated isomer of the linoneic acid, to the IVC medium, had no effect on cleavage or blastocyst rates, but reduced lipid content and increased survival rate of vitrified-warmed bovine embryos (Pereira et al., 2008). Controversially, addition of high levels of both non-esterified fatty acids (NEFA) or stearic acid (SA) in the maturation media of bovine COC, lead to different effects: (1) the first, significantly reduced blastocyst development and also increased the LD content; (2) and the second, reduced LD accumulation and lowered re-expansion rates after the cryopreservation-warming process (Van Hoeck et al., 2015).

Such as high content of NEFA, an elevated dose of polyunsaturated fatty acids (PUFA) in the maturation media impaired the development of bovine (Oseikria et al., 2016) and porcine (Hoyos-Marulanda et al., 2017) embryos. However, Oseikria et al

(2016) found that a lower dose -1µM - of docosahexaenoic acid (DHA), an omega-3 essential fatty acid, during IVM, improved cleavage rates of both parthenogenetic or fertilized bovine embryos, although no differences in the expression of FA metabolism related genes such as FA synthase (FASN), diacylglycerol O-acyl-transferase (DGAT1), FA transport (transporters CD36, FA binding protein genes FABP3 and FABP5), lipolysis (phospholipase PNPLA2), lipid storage (perilipin PLIN2), and mitochondrial b-oxidation (CPT1A, CPT2) were found. In our lab, IVM supplementation with eicosapentaenoic acid (EPA), also an omega-3 fatty acid, reduced cleavage rates, while 50µM of DHA improved cleavage rates and reduced blastocysts lipid content (Hoyos-Marulanda et al., 2017).

By modifying maturation media of mice oocytes, Paczkowski et al (2014) found that carnitine, a lipid modulator, alone or in combination with 1µM palmitic acid, common saturated fatty acid, reduced lipid content, while 100µM palmitic acid made the opposite; nevertheless, the use of these agents resulted in alterations of gene expression that directly affected the lipid content (Paczkowski; Schoolcraft; Krisher, 2014).

Therefore, the use of fatty acids during IVP modulates lipid metabolism in oocytes and embryos. However, they either improve or impair embryo production, depending on the concentration and nature of fatty acid used.

Lipid modulators

Lipid modulators are substances that can reduce and/or modify lipid content within cells through several mechanisms (Prates; Nunes; Pereira, 2014). Among them, carnitine, phenazine ethosulfate (PES) and forskolin are examples utilized during IVP procedures.

Carnitine

Carnitine carries FA into the mitochondria for ATP generation via β-oxidation and binds to Acetyl-CoA going back to the cytoplasm, influencing glucose metabolism (Dunning; Robker, 2012) and reducing lipid peroxidation (Somfai et al., 2011). The addition of carnitine during IVM enhanced oocyte maturation, embryo development, mitochondrial activity, while reducing lipid content and ROS levels in porcine (Somfai et al., 2011; Wu et al., 2011; You et al., 2012), buffalo (Verma et al., 2018) and bovine (Ghanem et al., 2014).

Although being mostly advantageous, Wu et al (2011) found that porcine COC exposed to 2 mg/mL of carnitine presented reduction in maturation and the blastocyst rates, whereas lower (0.5 mg/mL) concentrations were beneficial for parthenote embryo development, reducing ROS levels. On the other hand, You et al (2012) obtained better blastocyst development when pig oocytes were matured with 1.98 mg/mL of carnitine, increasing glutathione and reducing ROS levels. Interestingly, Somfai et al (2011), also added lower concentrations of carnitine (1.25 mg/mL) during IVM, resulting in higher maturation rates, although no differences on blastocyst rate were observed. Authors also described reduction on lipid content and ROS production, as also found by Wu et al (2011) and You et al (2012).

Verma et al (2018) added different doses of carnitine, 48h after buffalo IVF, resulting in lipid droplets reduction, and altered expression of metabolism related genes DGAT1 and DGAT2, as well as blastocyst competence markers GLUT1, OCT4 and INFτ, leading to a higher developmental rates of blastocysts after vitrification. Similarly, Ghanem et al (2014) related that bovine embryos supplemented with carnitine and/or phenazine ethosulfate (PES) presented reduced lipid content, increased mitochondria

density and cryotolerance, presenting also modification in the expression profile of genes related to FA transport (SLC27A1 and SLC22A5), FA oxidation (CPT1B and CPT2), FA synthesis (ACCα), LD formation (DGAT1, DGAT2 and PLIN2) and embryo competency (SOD2, NADH and GLUT8). Therefore, both lipid modulators improved embryo quality and cryotolerance, but carnitine supplementation seems to be more beneficial.

Finally, supplementation of IVP media with lower carnitine concentration is beneficial in reducing lipid content, and increasing cleavage and blastocyst rates. Carnitine is also capable to alter the expression profile of different genes related to energy and lipid metabolism, as well as embryo quality.

Phenazine Ethosulfate (PES)

PES is a metabolic regulator that inhibits fatty acids synthesis by NADH oxidation to NADP, favoring glucose metabolism through phosphate pentose pathway (PPP) (Ghanem et al, 2014). De La Torre-Sanchez et al (2006) compared metabolic regulators on bovine embryos and found that 0.3μM PES during IVC accelerate glucose metabolism by a higher glucose flux through PPP and reduced the amount of large lipid droplets in blastomere cytoplasm. The same PES concentration during IVC had no detrimental effect on cattle pregnancy and parturition after embryo transfer (Barceló-Fimbres; Brink; Seidel, 2009). For pigs, 0.05 μM PES supplementation during IVC had no effect on cleavage rate but increased morula and blastocyst production, also, this concentration was able to reduce lipid content by 23% but the survival rate after vitrification was similar than the control (Gajda et al., 2011).

Even though PES seems promising, few researches demonstrated its potential for lipid reduction. Furthermore, it is interestingly that a lower concentration significantly

reduced lipids in the porcine COCs, which has highest lipid content in comparison to bovines (McEvoy et al., 2000) that needed higher concentrations to improve lipid metabolism.

Forskolin

Forskolin is a chemical stimulator of lipolysis through the activation of adenyl cyclase (cAMP) (Prates; Nunes; Pereira, 2014). This substance is also capable of synchronize cytoplasmic and nuclear maturation of oocytes by arresting meiotic progression (Park et al., 2016).

According to Fu et al. (2011), 10μM of forskolin reduced lipid content and improved survival rate of porcine COC after cryopreservation. However, independently of the period of exposure to forskolin, cleavage rates were lower than the control. Going further, Park et al (2016) exposed pig COC to 50μM forskolin prior to IVM, resulting in higher blastocyst rates and reduction of lipid levels in parthenogenetically activated embryos, although lower cleavage rates was also observed. However, when compared with cilostamide, another cAMP modulator, forskolin couldn't maintain the same maturation levels for pig COC derived from small antral follicles and seems to inhibit cilostamide effects when both were supplemented (Park et al., 2016). Forskolin was also beneficial to lipid reduction in buffalo and bovine embryos exposed to 10μM during IVC and had progressive lipid reduction from day 2 to day 7 embryos, besides no effect on embryo cleavage or in freezing ability (Panyaboriban et al., 2018).

As can be observed, forskolin is efficient in reducing lipid content, increasing cryotolerance and blastocyst rate, besides this beneficial characteristics, this substance seems to impair cleavage rates.

Conclusion

This review showed alternative ways to deal with high lipid content in oocytes and embryo derived from IVP systems. Significant progress has been made, especially regarding cryoresistance and blastocyst development in different species. On the other hand, there is still no well determined pattern related to the best strategy for lipid reduction, since diverse modulators and concentrations were used in different species. In conclusion, additional studies should be conducted to find the optimal combination of substances and strategies, as well as the concentrations and phases of IVP to be used, to avoid that the accumulation of lipid observed in oocytes and embryos does not become an obstacle to optimize embryonic development and cryotolerance.

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2.2 Paper 2

Docosahexaenoic acid impairs *in vitro* maturation of swine oocytes

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DOCOSAHEXAENOIC ACID IMPAIRS *IN VITRO* MATURATION OF SWINE OOCYTES

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Contents

This study reports the addition of 50µM DHA during maturation period of swine oocytes, with and without porcine follicular fluid (pFF) supplementation. DHA individually negatively impacted maturation, cleavage and blastocyst rates ($p < 0.05$) However, when evaluating blastocyst per 48h cleaved embryos rates, there is no difference among the groups, showing that the negative influence of DHA during maturation does not last during early embryonic development. Oocytes matures in the absence of DHA and pFF accumulated less lipid during IVM ($p < 0.05$) while the other groups did not differ from each other after 44h of maturation. In conclusion, supplementation of DHA at 50µM without pFF negatively impacts oocyte maturation, embryo cleavage, total blastocyst rates and did not interfere in the lipid content regardless of the presence or absence of pFF.

Keywords: embryo development; lipid droplets; PUFA.

1. INTRODUCTION

One of the most limiting factors for swine *in vitro* embryo production (IVP) and further biotechnologies is the high lipid content and accumulation of lipid droplets (LD) in the cytoplasm of both oocytes and embryos (Gajda, Bryla, & Smorag, 2008). LD are formed near the endoplasmic reticulum through the utilization of fatty acids (FA) that are metabolized and transported to the oocyte, for lipid synthesis, by cumulus cells during follicular growth and development, as well as, during oocyte maturation (Paulini, Silva, De Paula Rôlo, & Lucci, 2014), resulting in energy storage that requires β-oxidation to support the maturation process (Dunning, Russell, & Robker, 2014).

Therefore, many recent researches focus on swine IVP improvement by lipid reduction through the addition of lipid metabolizers (reviewed by Braga, Komninou, Vieira, & Mondadori, 2019). Among them, docosahexaenoic acid (DHA) has crucial involvement during oocyte maturation by acting as energy reservoir, precursor of steroidogenesis (Khajeh, Rahbarghazi, Nouri, & Darabi, 2017) and altering genes related to lipid metabolism (Barber, Sinclair, &

Cameron-Smith, 2013). Also, DHA is a natural ligand of retinoid X receptor and peroxisome proliferator-activated receptors (PPARs) family (Suzuki et al., 2009). The PPARs are central in the regulation of lipid homeostasis in several tissues by regulating adipose differentiation-related protein (ADRP), which is part of the PAT protein family that includes Perilipins, essential in LD formation (Walther & Farese Jr, 2012)**.**

It was already shown that DHA supplementation *in vitro* reduced the lipid content of bovine (Elis et al., 2017) and swine (Hoyos-Marulanda et al., 2019) oocytes and embryos, without impairing their development. However, in these IVP studies, DHA has been applied with follicular fluid (FF) during *in vitro* maturation (IVM), turning the media undefined and the results less reproductible.

Therefore, we aimed to evaluate swine oocyte maturation and lipid content, as well as its embryo development after DHA addition during IVM, with and without follicular fluid supply.

2. MATERIAL AND METHODS

Given that the experiments carried out involved the collection of oocytes from ovaries collected from a slaughterhouse supervised by the Brazilian Ministry of Agriculture, Livestock and Supply, there is no need for approval of the animal ethics committee. Ovaries from prepuberal gilts were collected at local abattoir and transported to the laboratory in saline solution (0,9%) at 30°C to 35°C. Follicles from 3mm to 6mm were aspirated using a vacuum pump (10 - 15 mL/min) attached to a 19 G needle. The cumulus-oocyte complexes (COCs) were searched under stereomicroscopy and subsequently placed in a dish containing TCM-HEPES medium (TCM-199 with 0.1% PVA, 2.0mM $C_3H_4O_3$ and 2.5mM $NaHCO_3$). Oocytes with homogenous cytoplasm and at least three layers of cumulus cells were selected and washed again in TCM-HEPES before maturation.

Thirty to forty COCs were matured in six replicates for 44h at 38.5°C in 5% CO_2 atmosphere with saturated humidity in 4-well plates (Nunc, Roskilde, Denmark) containing 400µL of media, in four different treatments: 1) Negative Control (NC - IVM - TCM-199 supplemented with 0.1% PVA and 2.5mM $NaHCO_3$, 0.57mM cysteine, 0.91mM $C_3H_3NaO_3$, 0.001g/ml EGF, 0.05g/ml streptomycin, 0.065g/ml penicillin, with gonadotrophins (20µg/uL FSH, 5µg/uL LH) for the first 22h of maturation); 2) Positive Control (PC - IVM + 25% pFF); 3) DHA NC (negative control + 50µM DHA); and 4) DHA PC (positive control + 50µM DHA). DHA was prepared according to Hoyos-Marulanda et al. (2019).

After maturation, COCs were denuded by vortex for 3 minutes. Half of the denuded oocytes were washed in PBS, fixed in 4% paraformaldehyde for 30 minutes and stored in PBS at 4°C until staining for maturation and lipid assessment. A total of 547 oocytes were activated as previously described by (Che, Lalonde, & Bordignon, 2007). After activation, potential parthenotes were cultured in 60µL droplets of PZM (+) (Yoshioka, Suzuki, Tanaka, Anas, & Iwamura, 2002) under mineral oil. Cleavage rates were evaluated at 24h and 48h and blastocyst rates at day 7. At day 5, feeding was performed by addition of 10% estrus mare serum (EMS).

For maturation evaluation, 455 fixed oocytes were stained with 7.5µg/ml Hoechst 333342 for 10 minutes. In parallel, 184 fixed oocytes were stained with 1µg/mL Nile Red for 30 minutes for lipid content measurements. Both stained

oocytes were placed on slides containing a single Mowiol droplet covered with coverslips and evaluated using epifluorescence microscopy (Nikon 80i). Oocyte maturation was evaluated according to (Uhm, Gupta, Kim, & Lee, 2007). For lipid measurements, as previously described by Barceló-Fimbres & Seidel (2011), images were obtained using a G2A filter with a 1920 x 1080 resolution and 80ms exposure and fluorescence intensity was determined by selecting only the cytoplasm of oocytes with ImageJ free-hand drawing tool, where the values were adjusted by Corrected Total Cell Fluorescence (CTCF): CTCF = Integrated Density - (Area of selected cell X Mean fluorescence of background readings) (Fitzpatrick, 2014).

For statistical analysis, maturation, cleavage, blastocyst and blastocysts per 48h cleavage rates were compared among treatments by chi-square tests while the CTCF, due the lack of normality, was transformed into the logarithmic scale and treatments compared by ANOVA. For both data, comparison of means was done by LSD test through Statistix 10 software.

3. RESULTS

Table 1 shows that DHA individualy negatively impacted maturation, cleavage and blastocyst rates ($p < 0.05$). However, when evaluating blastocyst per 48h cleaved embryos rates, there is no difference among the groups, showing that the negative influence of DHA during maturation does not last during early embryonic development.

Oocytes from NC accumulated less lipid during IVM ($p < 0.05$) while the other groups did not differ from each other after 44h of maturation (Figure 1), showing that DHA is not able to reduce lipid accumulation during IVM.

4. DISCUSSION

Our results showed that 50µM DHA without follicular fluid support negatively impacted maturation, cleavage and blastocyst rates. One possible explanation for the low development in DHA NC group is that cumulus cells showed an unattached aspect after maturation period (personal observation), and COC communication via gap junctions is fundamental for oocyte maturation (Sanchez-Lazo et al., 2014)**.** Regarding blastocyst development, our results may not represent an actual equality among the groups due to the lower crude number of blastocysts per 48h cleaved embryos in DHA NC group.

In fact, Hoyos-Marulanda et al. (2019) found better response in blastocyst development and lipid content reduction with the same DHA dose administrated in our study. However, they had a lower pFF concentration (10% vs 25%) and did not isolate DHA from pFF with a negative control lacking both DHA and pFF, suggesting that DHA supplementation during porcine IVM is not only dependent of the presence of pFF, but with the concentration of pFF in the maturation medium. Higher DHA doses were also described as toxic for bovine (Oseikria, Elis, Maillard, Corbin, & Uzbekova, 2016) and pig oocytes (Hoyos-Marulanda et al. 2019).

Nevertheless, developmental rates in NC group was surprisingly, since COCs had no external energy source to metabolize and intake (Sanchez-Lazo et

al., 2014), relaying only on internal LD content for further development. This result was interesting, because, besides the use of a chemically defined medium, oocytes developed into blastocysts at the same rate of the undefined medium, i. e., even with no unknown external fatty acid as energy supply or any metabolizer to intake, leading only internal LD to be consumed for further development. However, in the future, the gene expression analysis of perilipin, ADRP, and TIP-47 (PAT family) and fat-storage-inducing transmembrane (FIT family) proteins should be performed, for better results comprehension.

Taken together, our results suggest that DHA might impair oocyte maturation process, but embryo development was not affected. On the other hand, time of exposure, DHA concentration and presence or absence of protein source must be observed as variables impacting maturation and further embryo development. In conclusion, supplementation of DHA at 50µM without pFF negatively impacts oocyte maturation, embryo cleavage, total blastocyst rates and did not interfere in the lipid content regardless of the presence or absence of pFF.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Table 1. Nuclear maturation, cleavage, blastocyst development and blastocyst per 48h cleavage rates after maturation with or without 50µM DHA and/or pFF.

	Treatments			
	NC*	PC**	DHA NC***	DHA PC****
Maturation (%)	42,96 (55/128)^{c}	56,73 (59/104)^{bc}	9,73 (11/113)^{d}	65,45 (72/110)^{ab}
Cleavage 24h (%)	27,86 (39/140)^{a}	42,25 (60/142)^{a}	8,47 (10/118)^{b}	37,41 (55/147)^{a}
Cleavage 48h (%)	55,00 (77/140)^{a}	57,75 (82/142)^{a}	17,80 (21/118)^{b}	51,02 (75/147)^{a}
Blastocyst (%)	22,14 (31/140)^{a}	28,17 (40/142)^{a}	9,32 (11/118)^{b}	17,01 (25/147)^{ab}
Blastocyst/48h Cleavage (%)	40,26 (31/77)	48,48 (40/82)	52,38 (11/21)	33,33 (25/75)

Frequencies with different superscripts (a, b) in the row, differ by at least $P < 0.05$.

* NC (Negative Control IVM - TCM-199 supplemented with 0.1% PVA and 2.5mM $NaHCO_3$, 0.57mM cysteine, 0.91mM $C_3H_3NaO_3$, 0.001g/ml EGF, 0.05g/ml streptomycin, 0.065g/ml penicillin, with gonadotrophins (20µg/µL FSH, 5µg/µL LH) for the first 22h of maturation);

**PC (Positive Control - IVM + 25% pFF);

***DHA NC (negative control + 50µM DHA)

****DHA PC (positive control + 50µM DHA).

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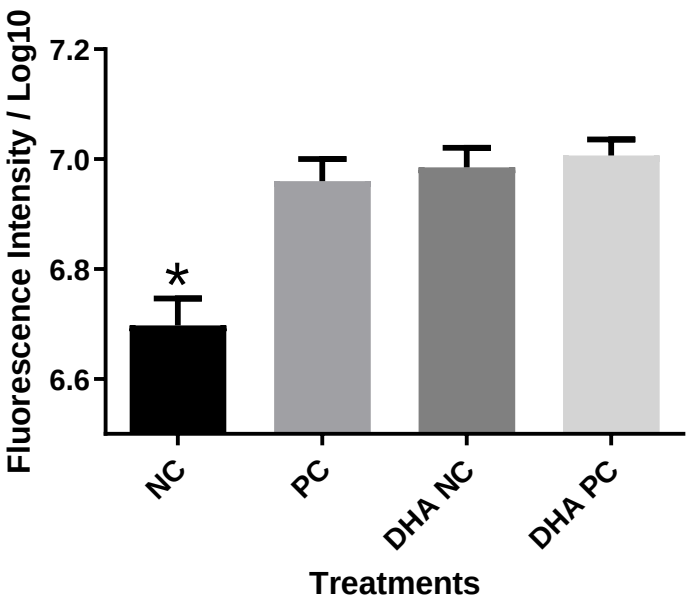

Figure 1. Fluorescence intensity of lipid droplets (Log10) of oocytes stained with Nile Red after maturation in Negative Control (NC), Positive Control (PC), 50µM DHA + Negative Control (DHA NC) and 50µM DHA + Positive Control (PC).
NC (n = 30 oocytes); PC (n = 53 oocytes); DHA NC (n= 54 oocytes) and DHA PC (n= 47 oocytes)

* Mean frequencies ± SEM with different superscripts differ by at least p <0.005

2.3 Paper 3

***In vitro* production of swine embryos matured with phenazine ethosulfate**

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Manuscript will be submitted to Animal Reproduction Science

In vitro production of swine embryos matured with phenazine ethosulfate

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Abstract

Glucose is the preferred substrate for porcine oocytes during maturation period. Phenazine Ethosulfate (PES) is an electron acceptor which can induce utilization of glucose by the pentose phosphate pathway (PPP) through conversion of NADPH to NADP and could influence lipid droplets (LD) metabolism. Here, we added two concentrations of PES (0.5µM and 0.05µM) during the entire *in vitro* maturation period evaluating oocyte maturation and lipid content in Experiment 1, and embryo development and lipid content of blastocysts on day 7 in Experiment 2. Addition of 0.5µM of PES negatively impacted most of the evaluated parameters, i.e., maturation ($P < 0.05$) and increased lipid content ($P < 0.05$), cleavage ($P < 0.05$) and blastocyst rates ($P < 0.05$), reduced total blastomere count ($P < 0.05$) and also increased embryo lipid content ($P < 0.05$) while the lowest PES dose was similar to the negative control (NC) and Forskolin control in both data of Experiment 1, but also had lower cleavage rates when compared to NC in Experiment 2. Unlike already observed in other species, addition of PES

during the entire maturation period was not beneficial to embryo development and lipid reduction.

Keywords: Lipid content, embryo development, metabolism inducer.

1. Introduction

Lipids are hydrophobic molecules that serves as a rich source of energy, cell signaling mediators and the foundation of plasma and organelle membranes (DUNNING; RUSSELL; ROBKER, 2014). Lipid droplets (LD) are formed by a phospholipid monolayer filled by a set of key enzymes related to size, stability and lipid storage with a neutral lipid core (BARNEDA; CHRISTIAN, 2017). LD are believed to be formed near the endoplasmic reticulum (ER) by neutral and fatty acid lipid synthesis which results in synthesis and storage of triacylglycerol (TAG) into LD (WALTHER; FARESE JR, 2012) by acyl-coenzyme A (Acyl-CoA), diacylglycerol acyltransferase (DGAT) and cholesterol acyltransferase (ACAT) through fatty acids, as well as exchange of lipid content between LD of variable size (THIELE; PENNO, 2015). Also, LD growth is tightly regulated by Perilipin5 (Plin5) in brown adipocyte tissue (BAT) in a close association of peridroplet mitochondria (PDM) and LD (BENADOR et al., 2018). Swine oocytes have more LD when compared to other domestic species (MCEVOY et al., 2000), and it represents an species-specific energy storage supply for oocytes to develop into embryos (PRATES; NUNES; PEREIRA, 2014), leading to different lipid profiles along oocyte/embryo development (ROMEK et al., 2010). Also, this high lipid content impairs biotechnologies such as *in vitro* embryo production (IVP), cloning, transgene and cryopreservation due to reactive oxygen species (ROS) production (GAJDA, 2009).

Throughout maturation, the oocyte undergoes nuclear and cytoplasmatic changes in order to support embryo development (MEHLMANN, 2005) and swine oocytes have preference for glucose over pyruvate in a balanced utilization either by the pentose phosphate pathway (PPP) or by glycolysis (KRISHER et al., 2007). However, *in vitro* maturation (IVM) procedure causes disharmony between these events due to the lack of some substrates in the media

composition (PRATES et al., 2014) leading to an unbalanced glucose utilization, with glycolysis being preferred over PPP (KRISHER et al., 2007). In fact, only about 20% to 30% porcine oocytes develop until the blastocyst stage *in vitro* (APPELTANT et al., 2016). Possible strategies related to synchronize nuclear and cytoplasmic maturation and, by doing so, reduce LD, have been reviewed elsewhere (BRAGA et al., 2019; PRATES; NUNES; PEREIRA, 2014).

Follicular cells produce cAMP, transferring it to the oocyte and keeping Maturation Promoting Factor (MPF) inactive. Thus, spontaneous maturation happens when oocytes are removed from their follicles, leading to meiosis resumption (CONTI; FRANCIOSI, 2018). The addition of Forskolin (FSK), a cAMP modulator, had provided better oocyte maturation and lipid reduction by keeping MPF inactive until cytoplasmic and nuclear maturation synchronizes, as reported in domestic species (CAO et al., 2018; FU et al., 2011; HASHIMOTO et al., 2019; LEE et al., 2017; PANYABORIBAN et al., 2018; PASCHOAL et al., 2016; RAZZA et al., 2018).

Phenazine ethosulfate (PES) is an electron acceptor that can induce a balanced utilization of glucose by the pentose phosphate pathway (PPP) (DOWNS; HUMPHERSON; LEESE, 1998) by converting NADPH to NADP, inhibiting fatty acid synthesis, and, therefore, improve LD utilization by mitochondria (BARCELÓ-FIMBRES; SEIDEL, 2011; DE LA TORRE-SANCHEZ; PREIS; SEIDEL, 2006; SUDANO et al., 2011). In fact, PES had improved blastocyst quality and reduced the lipid content of bovine (BARCELÓ-FIMBRES; SEIDEL, 2011; DE LA TORRE-SANCHEZ; PREIS; SEIDEL, 2006; SUDANO et al., 2011) and swine (GAJDA et al., 2011; GAJDA; BRYLA; SMORAG, 2008; ROMEK; KEPCZYNSKI; GAJDA, 2011) embryos. Although being able to reduce lipid content and improve embryo quality in these studies, PES was only added at the embryonic stage. To our knowledge, PES was only added during the maturation stage in the studies of Downs et al. (1998) and Correia. (2019), with mice and bovine oocytes, respectively.

Thus, our objective is to test the hypothesis that addition of PES during the entire maturation period can improve maturation by favoring the PPP utilization, leading to better embryo development and LD reduction as well.

2. Materials and Methods

2.1 Cumulus-oocyte complexes collection and in vitro culture.

Ovaries collected at local abattoir were transported to the laboratory in saline solution (0,9% NaCl) at 30°C to 35°C. With a vacuum pump (10 - 15 mL/min) attached to a 19 G needle, follicles from 3mm to 6mm were aspirated and their cumulus-oocyte complexes (COCs) were searched under stereomicroscopy and subsequently placed on TCM-HEPES medium (TCM-199 with 0.1% PVA, 2.0mM $C_3H_4O_3$ and 2.5mM $NaHCO_3$) for stabilization. Oocytes with homogenous cytoplasm and at least three layers of cumulus cells were selected and washed again in TCM-HEPES before maturation. Thirty to forty COCs were matured per well in 4-well plates (Nunc, Roskilde, Denmark) containing 400µL of TCM-IVM (TCM-199 supplemented with 0.1% PVA and 2.5mM $NaHCO_3$, 0.57mM cysteine, 0.91mM $C_3H_3NaO_3$, 0.001g/ml EGF, 0.05g/ml streptomycin, 0.065g/ml penicillin and 25% pFF) for 44h at 38.5°C in 5% CO_2 atmosphere with saturated humidity, with gonadotrophins (20µg/µL FSH, 5µg/µL LH) for the first 22h of maturation.

After maturation, COCs were denuded by vortex for 5 minutes. Oocytes were parthenogenetically activated as previously described (CHE; LALONDE; BORDIGNON, 2007) and potential parthenotes were cultured in 60µL droplets of PZM (+) (YOSHIOKA et al., 2002) under mineral oil. Cleavage rates were evaluated at 24h and 48h and blastocyst rates at day 7. At day 5, feeding was performed by changing 50% of the drop, with the addition of 10% estrus mare serum (EMS).

2.2 FSK and PES preparation

FSK (Sigma F6886) was prepared according to Fu et al. (2011) and used as a positive control, since it has already reduced cytoplasmatic lipid content in other studies (reviewed by BRAGA et al, 2019). Briefly, a mother solution was diluted in DMSO to final concentration of 10mM. Mother solution was re-diluted with TCM-IVM to a concentration of 0.9mM, aliquoted and kept at -20°C until use.

PES (Sigma P4544) was prepared according to De la Torre-Sanchez et al. (2006). Briefly, a mother solution was diluted in MiliQ Water to final concentration of 0.5mM. Mother solution was re-diluted with TCM-IVM to concentrations of 0.025mM and 0.0025mM, aliquoted and kept at -20°C until use.

2.3 Experimental design

COCs were matured as described above, in the following groups: 1) Control- TCM-IVM; 2) FSK - TCM-IVM + 10µM FSK during the last 22h (FU et al, 2011); 3) H-PES – TCM-IVM + 0.5µM PES and 4) L-PES - TCM-IVM + 0.05µM PES.

For experiment 1, oocytes were denuded after maturation by vortex for 5 min. Denuded oocytes were washed in PBS, fixed in 4% paraformaldehyde for 30 minutes and stored in PBS at 4°C until staining for maturation and lipid assessment. For experiment 2, oocytes were matured and denuded as experiment 1, being activated and cultivated as described in section 2.1. At D7, embryos were fixed and stored as experiment 1 until staining for blastomere quantification and lipid assessment.

2.4 Maturation evaluation and cell count

For maturation evaluation, 281 fixed oocytes from five replicates (experiment 1) and 104 embryos from six replicates (experiment 2) were stained with 7.5µg/ml Hoechst 33342 for 10 minutes. Stained structures were placed on slides containing a single Mowiol droplet covered with coverslips and evaluated using epifluorescence microscopy (Nikon 80i). Oocyte maturation was evaluated according to (UHM et al., 2007) and cell count was performed by the same technician.

2.5 Lipid content measurements

To measure lipid content, 263 fixed oocytes from five replicates (experiment 1) were stained with 1µg/mL Nile Red for 30 minutes (HOYOS-MARULANDA et al., 2019) and 129 fixed embryos from six replicates were stained overnight (GENICOT et al., 2005). After staining, both

oocytes and embryos were washed in PBS and placed on slides containing a single Mowiol droplet covered with coverslips. For lipid measurements, as previously described by Barceló-Fimbres and Seidel (2011), images were obtained by epifluorescence microscopy (Nikon 80i) using a G2A filter with a 1920 x 1080 resolution and 80ms exposure and fluorescence intensity was determined by selecting only the cytoplasm of oocytes and embryos with ImageJ free-hand drawing tool, where the values were adjusted by Corrected Total Cell Fluorescence (CTCF): CTCF = Integrated Density - (Area of selected cell X Mean fluorescence of background readings) (Fitzpatrick, 2014).

2.6 Statistical analysis

All tests were performed through Statistix 10 software. Maturation, cleavage, blastocyst and blastocysts per 48h cleavage rates were compared among treatments by chi-square tests. For cell count, ANOVA was performed and means compared by Dunn's all-pairwise comparisons. CTCF was normalized into the logarithmic scale and treatments were compared by ANOVA. For oocytes, comparison of means was done by Tukey while for embryos Dunn's test were applied.

3. Results

3.1 – Experiment 1

Maturation rates (n=557), i.e. progression to MII stage, for Control (55.2%), FSK (50.5%) and L-PES (51.5%) were similar and higher ($P<0.05$) than for H-PES group (42.0%). As demonstrated in Figure 1, oocytes from H-PES group had the highest lipid content at the end of maturation period.

3.2 – Experiment 2

Data from embryo development is summarized in table 1. Cleavage was evaluated at 24h and 48h, showing lower cleavage rates in both PES groups when compared to the Control but similar to FSK group. When blastocyst rates were evaluated, H-PES group had lower rates

then Control, but similar to L-PES and FSK. Similar differences were observed regarding cell number. When lipid content was measured, Control had lower lipid content than FSK and H-PES, while L-PES was similar to all groups (Fig 2).

4. Discussion

According to our results, addition of PES during swine oocyte maturation period was not beneficial to embryo development and lipid content reduction, especially when compared to Control, free of exogenous metabolic inducers. Indeed, the high dose of PES used in this study led to low maturation, low blastocyst development and lower cell number. De la Torre-Sanchez et al (2006a) also described the deleterious effect of 0.9µM to bovine embryos. Evaluating the available literature, seem that PES is beneficial during embryo development stage, when a similar dosage used in our study was administrated (DE LA TORRE-SANCHEZ et al., 2006), PES enhanced glucose metabolism through PPP and reduced the lipid content without impairing embryo development and quality. However, in cited studies, PES was added after selecting embryos from 8 to 16-cell stages, and by doing so, it improved glucose metabolism of already developing embryos in the compaction and post-compaction stages, excluding possible benefits regarding initial cleavage stages. Also, different doses of PES during initial embryo culture of *in vivo* matured oocytes, lead to better cleavage (GAJDA; BRYLA; SMORAG, 2008), morula and blastocyst production (GAJDA et al., 2011) as well as reducing lipid content (ROMEK et al., 2011). However, oocytes matured *in vivo* are known to have better synchrony between molecular and cytoplasmic maturation, favoring its development when transferred to an *in vitro* system (KRISHER et al., 2007) and it could explain why PES addition was so beneficial, especially since glucose is the primary energy substrate for porcine oocytes either via PPP or the glycolytic pathway (KRISHER et al., 2007).

When PES was used to study the PPP in mice denuded oocytes, Downs et al. (1998) demonstrated that germinal vesicle breakdown (GVBD) and glucose consumption are dose-

dependent events. Interestingly, Correia (2019) found development rates of bovine embryos matured with PES to be reduced when compared to the control but similar among themselves, which is similar to our results. However, in that study, PES treated oocytes had lower lipid content after maturation and higher lipid content after embryo culture, when compared to the control. In our study, total lipid content in oocytes was higher in H-PES, while the other groups had similar lipid content, a behavior that was not observed in embryos since PES groups were similar among themselves and FSK, with L-PES being also similar to the Control group, which had lower lipid content. High lipid content and low development rates for PES treatments, especially H-PES group, could be explained by the fact that PPP is involved in fatty acid synthesis, and, even when PES specific mechanism of action is involved in conversion of NADPH to $NADP^+$ (DE LA TORRE-SANCHEZ; PREIS; SEIDEL, 2006), high levels of PES might have acted as NADPH oxidase, increasing ROS due to NADPH oxidation by altering oxidation-reduction equilibrium (SUDANO et al., 2011), impairing maturation and keeping high lipid levels of H-PES group. Moreover, Sudano et al. (2011) demonstrated that a great reduction of lipid content by longer exposure to PES impaired embryo development.

Here, we added Forskolin as a positive control for comparisons since this cAMP modulator influences meiotic arrest and leads to a better synchrony between nuclear and cytoplasmic maturation. Indeed, addition of dibutyryl cAMP (dbcAMP) in the first half of *in vitro* maturation increased blastocyst rates (GRUPEN, 2014). According to Appeltant et al. (2016), dbcAMP is the only cAMP-elevating treatment that improves porcine oocyte development, and it should be administrated together with gonadotrophins. However, FSK inhibits meiosis resumption in a dose-dependent manner (SUN; NAGAI, 2003), increases mitochondrial function, oxygen consumption and ATP levels (HASHIMOTO et al., 2018), favoring both nuclear and cytoplasmic maturation.

Fu et al. (2011) treated swine oocytes with FSK for the entire maturation period and for the second half of it. It was found that when oocytes are exposed to FSK for 42h, their lipid content was drastically reduced, and their blastocyst formation was reduced too, possibly caused by alterations in cytoplasmic maturation. In FSK oocytes treated during the second half, lipid content was reduced, and embryo development was not compromised (FU et al., 2011). In bovine oocytes, cAMP triggers hydrolysis of triglycerides inside LD, generating fatty acids and glycerol for lipolysis which, in turn, are metabolized during embryo energy demands (RAZZA et al., 2017). In our study, FSK oocytes had lower lipid content when compared to H-PES but was similar to Control. Also, embryo development was not affected by FSK treatment, even when embryo lipid content was higher than Control. This contrasts with the study of Razza et al. (2017) where pre-IVM of bovine oocytes unexpectedly elevated oocyte lipid caused by downregulation of genes involved in TAG lipolysis and upregulation of genes involved in LD formation content, but reduced lipid content in embryos. Perilipins are involved in storage and stabilization of neutral lipids inside the LD monolayer (FU et al., 2011). PLIN1 is associated with LD growth and is phosphorylated in a cAMP-dependent manner (ITABE et al., 2017), which could be an explanation of the increased lipid content found in blastocyst in our study, although gene expression analysis would be necessary to confirm this hypothesis.

Hence, it is possible that long exposure of PES during maturation could not have balanced the glycolysis/PPP ratio during IVM, as it would be expected if compared to an *in vivo* matured porcine oocyte, leading to low developmental rates and low cell number. Also, an increase in oocytes LD in high levels of PES could be due to ER stress and ROS production by unbalance of the NADH/NADPH ratio (WELTE; GOULD, 2017), since LD growth is a form of ROS protection within cells (BRASAEMLE; WOLINS, 2012). The same defense mechanism could have happened in embryos of the FSK group; however, their development capacity was similar to the control at all levels and criteria evaluated in this study. Perhaps FSK should indeed be administrated during pre-IVM or at the first half of porcine IVM, as suggested by other authors

(APPELTANT et al., 2016; GRUPEN, 2014), for better synchrony of nuclear and cytoplasmic maturation. Another possibility would be the combination of FSK and PES during maturation, since the first causes meiotic arrest (PASCHOAL et al., 2016) and the second influences glucose consumption (DOWNS et al., 1998).

5. Conclusions

According to this study, PES impairs oocyte maturation by disturbing lipid metabolism. Further studies are still necessary to understand the results obtained here.

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Conflicts of interest

The authors declare no conflicts of interest.

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Table 1. Embryo development rates and embryo cell count of structures derived from pig oocytes matured *in vitro* in the presence of Forskolin (FSK) or Phenazine ethosulfate (PES) - Experiment 2.

	Groups			
	Control[†]	FSK[‡]	H-PES[§]	L-PES[¥]
Cleavage 24h (%)	52.1 (162/311)[a]	48.8 (158/324)[ab]	43.1 (140/325)[bc]	44.2 (136/308)[bc]
Cleavage 48h (%)	76.5 (238/311)[a]	70.4 (228/324)[ab]	68.3 (222/325)[bc]	66.9 (206/308)[bc]
Blastocyst (%)	38.6 (120/311)[a]	31.8 (103/324)[ab]	30.2 (98/325)[b]	31.8 (98/308)[ab]
Embryo Cells (n)	57.8 ± 4.7[A]	53.9 ± 6.7[AB]	40.0 ± 4.5[B]	43.5 ± 3.6[AB]

a,b,c Frequencies with different superscripts differ by at least $P < 0.05$.
A,B Means ± SE with different superscripts differ by at least $P < 0.05$.
[†]Control - TCM-IVM - TCM-199 supplemented with 0.1% PVA and 2.5mM NaHCO3, 0.57mM cysteine, 0.91mM C3H3NaO3, 0.001g/ml EGF, 0.05g/ml streptomycin, 0.065g/ml penicillin, with gonadotrophins (20µg/µL FSH, 5µg/µL LH) for the first 22h of maturation;
[‡]FSK - Forskolin (TCM-IVM + 10µM FSK during the last 22h) (Fu et al, 2011);
[§]H-PES - TCM-IVM + 0.5µM PES;
[¥]L-PES - TCM-IVM + 0.05µM PES.

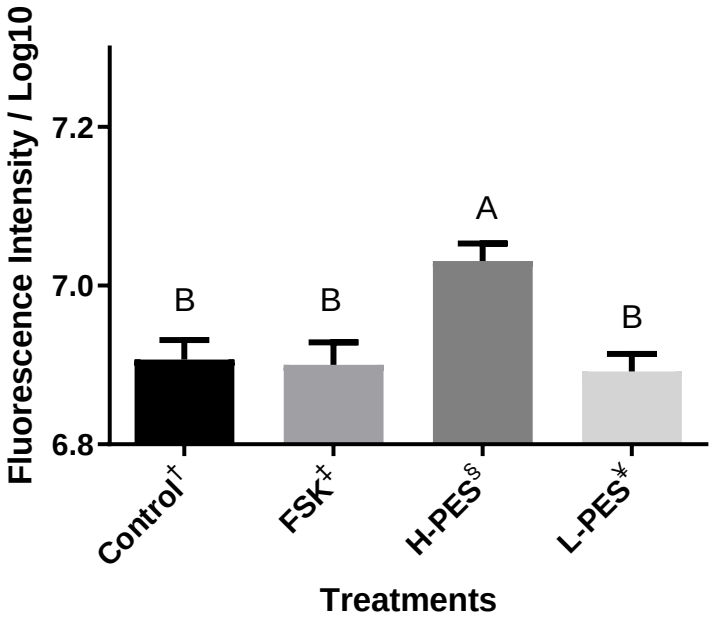
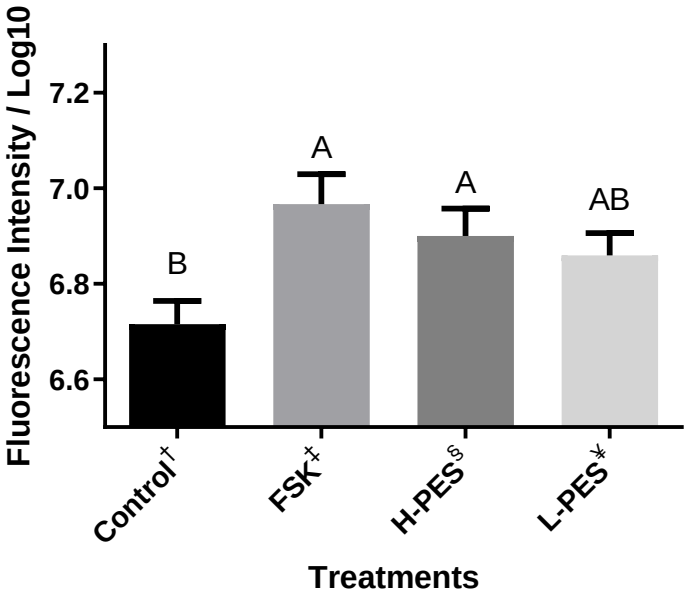
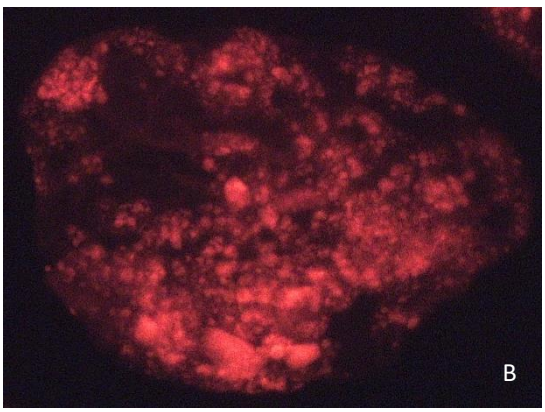


Figure 1. Fluorescence intensity of lipid droplets (Log10) of oocytes stained with Nile Red after maturation in Control, Forskolin (FSK), 0.5µM PES (H-PES) and 0.05µM PES (L-PES). A,B Mean frequencies ± SEM with different superscripts differ by at least p <0.005.

†Control (n = 68) - TCM-IVM - TCM-199 supplemented with 0.1% PVA and 2.5mM NaHCO3, 0.57mM cysteine, 0.91mM C3H3NaO3, 0.001g/ml EGF, 0.05g/ml streptomycin, 0.065g/ml penicillin, with gonadotrophins (20µg/µL FSH, 5µg/µL LH) for the first 22h of maturation;
‡FSK (n = 62) - Forskolin (TCM-IVM + 10µM FSK during the last 22h) (Fu et al, 2011);
§H-PES (n= 67) - TCM-IVM + 0.5µM PES;
¥L-PES (n= 67)- TCM-IVM + 0.05µM PES.





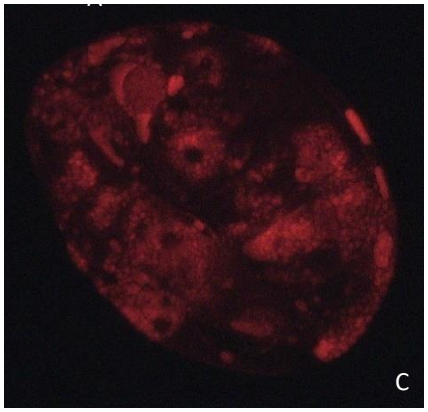


Figure 2. (A) Fluorescence intensity of lipid droplets (Log10) of embryos stained with Nile Red after maturation in Control, Forskolin (FSK), 0.5µM PES (H-PES) and 0.05µM PES (L-PES). A,B Mean frequencies ± SEM with different superscripts differ by at least p <0.005. Representative images of blastocyst with high (B) and low (C) lipid content.

†Control (n = 41) - TCM-IVM - TCM-199 supplemented with 0.1% PVA and 2.5mM NaHCO3, 0.57mM cysteine, 0.91mM C3H3NaO3, 0.001g/ml EGF, 0.05g/ml streptomycin, 0.065g/ml penicillin, with gonadotrophins (20µg/µL FSH, 5µg/µL LH) for the first 22h of maturation;
‡FSK (n = 33) - Forskolin (TCM-IVM + 10µM FSK during the last 22h) (Fu et al, 2011);
§H-PES (n= 24) - TCM-IVM + 0.5µM PES;
¥L-PES (n= 31)- TCM-IVM + 0.05µM PES.

3 Final considerations

As reviewed in the 1st paper, our hypothesis for the following experiments was based in other studies where addition of these metabolizers leads to better embryo development and reduction in lipid content.

DHA could lower LD content through modulation of PPAR receptors. However, DHA needs support of pFF in order to sustain cumulus expansion and COCs communication throughout the maturation period. It was observed when DHA was applied without pFF, and cumulus cells showed a loosen aspect that impaired maturation, oocyte lipid content reduction and embryo development thereafter. Remarkably, oocytes matured in the negative control, with a chemically defined medium, had lesser lipid content and similar maturation and embryo development rates as the control with pFF.

In our second paper, PES was added during maturation since PES could equilibrate the glucose consumption through its influence over the PPP, favoring the unbalance seen in glycolysis/PPP when porcine oocytes are matured *in vitro*. Although L-PES showed similar results to the Control, H-PES was harmful during maturation and embryo development, as well as having a high lipid content in both structures.

Therefore, based on our studies, both supplementation of DHA and PES were detrimental to embryo development. This opens a window for further research regarding gene expression and metabolic analysis to acquire new insights on the mechanisms by which these metabolizers impaired embryo development. Nevertheless, there is a possibility of induced toxicity in a dose/time dependent manner, and future studies should be administrated to test this hypothesis.

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