IN VITRO MATURATION OF PORCINE OOCYTE

Effects of lipid manipulation on fertilization, embryo development and cryo-resistance

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Elsa Cristina da Graça Prates

Tese apresentada à Universidade de Évora para
obtenção do Grau de Doutor em Ciências Veterinárias

ORIENTADOR(ES): *Professora Doutora Rosa Maria Lino Neto Pereira*
Professor Doutor José Luís Tirapicos Nunes
CO-ORIENTADOR: *Professor Doutor Carlos E. Plancha dos Santos*

ÉVORA, SETEMBRO DE 2012

INSTITUTO DE INVESTIGAÇÃO E FORMAÇÃO AVANÇADA

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ABSTRACT

In vitro maturation of porcine oocyte: effects of lipid manipulation on fertilization, embryo development and cryo-resistance

The purpose of this thesis was to implement the *in vitro* technique for porcine embryo production and to investigate the effects of lipid content modulation during oocyte *in vitro* maturation (IVM) using *trans*-10,*cis*-12 conjugated linoleic acid (*t*10,*c*12 CLA) and forskolin. Alterations in oocyte morphology and fatty acids (FA)/ plasmalogens profile were induced by chemical modulation, during IVM. After 44 h of *t*10,*c*12 CLA supplementation, a lighter cytoplasmic colour tone was achieved but the blastocyst rate was reduced. Long supplementation with forskolin reduced oocyte growth, nuclear progression, fertilizing ability and interfered with lipid droplet morphology. These disturbances disappeared by reducing supplementation with forskolin to only 2 h. When added simultaneously both supplements improved oocyte maturation rate. Despite modifications in FA/ plasmalogens in treated oocytes, no effects on blastocyst cryo-resistance could be detected.

Key-words: *in vitro* maturation, porcine oocyte, lipid content, *t*10,*c*12 CLA, forskolin, blastocyst.

RESUMO

Maturação *in vitro* do oócito de suíno: efeitos da manipulação lipídica na fertilização, desenvolvimento embrionário e crio-resistência

Os objectivos desta tese foram implementar a técnica de produção *in vitro* de embriões de suíno e investigar os efeitos da modulação lipídica durante a maturação *in vitro* (MIV) usando o isómero *trans*-10,*cis*-12 do ácido linoleico conjugado (*t*10,*c*12 CLA) e a forscolina. A modulação lipídica durante a MIV induziu alterações na morfologia do oócito e perfil em ácidos gordos (AG)/ plasmalogénios dos complexos cumulus-oócito. Após 44 h de suplementação com *t*10,*c*12 CLA ocorreu aclaramento citoplásmico mas a taxa de blastocistos diminuiu. A suplementação em períodos longos com forscolina poderá reduzir o crescimento do oócito, a progressão nuclear e a capacidade fertilizante, ou apenas interferir na morfologia e composição das gotas lipídicas (2 h de suplementação). Os dois suplementos em simultâneo melhoraram a taxa de maturação. Apesar das alterações na composição em AG/ plasmalogénios dos oócitos tratados, não se detetaram efeitos sobre a crio-resistência dos blastocistos.

Palavras-chave: maturação *in vitro*, oócito de suíno, conteúdo lipídico, *t*10,*c*12 CLA forscolina, blastocisto.

EXTENDED ABSTRACT

Swine species has a relevant economical role in livestock and agriculture. In particular, the great ecological importance of same local breeds in the conservation and sustainability of natural environment and resources must be considered. One of the main goals of *in vitro* embryo production (IVP) is to provide viable embryos to be transfered for female and for germoplasm cryopreservation. However, this cryopreservation in swine species has been impaired by the high susceptibility of oocytes and embryos to chilling injury due to their huge lipid contents. Thus interfering with lipid metabolism to reduce and/or modulate the intracellular lipid content seems an important approach to improve assisted reproductive technology (ART) outcome in the pig model.

The main objectives of this thesis were to implement the *in vitro* technique for porcine embryo production and to investigate the effects of two lipid modulators, the *trans*-10, *cis*-12 conjugated linoleic acid (*t*10,*c*12 CLA) and forskolin, supplemented to the maturation media of porcine oocytes, upon maturation, fertilization, embryo development and cryo-resistance. This thesis was composed of a revision work chapter and three sets of experimental chapters.

In the first chapter, the cumulus oocyte complex (COC) was extensively characterized through cellular morphological parameters and molecular mechanisms involved in the maturation process. Particular attention was given to the lipid droplet (LD) as abundant and dynamic cytoplasmic organelle. The lipid-rich porcine oocyte was rendered as an excellent model to study the role of lipids and compared to the oocyte from other species. The mechanisms of action of meiotic inhibitors namely forskolin, the activity and the effects of *t*10,*c*12 CLA and forskolin as lipid modulators, as well as their potential application to oocyte and embryo cryopreservation were described.

In the second chapter the alterations of cytoplasmic lipid content of porcine COC exposed to fixed concentrations of *t*10,*c*12 CLA and forskolin during different incubation time periods were investigated. The effects of these lipid modulators in oocyte maturation and early embryonic development were also evaluated. Results showed that forskolin beyond 2 h of exposition delayed oocyte meiotic progression and competence for fertilization/ cleavage. Simultaneously, forskolin induced a decrease in oocyte and fat areas, affecting oocyte growth and LD cytoplasmic distribution. When forskolin was only added during the first 2 h of IVM, an alteration in LD morphology (measured by their areas) was observed at 22-24 h of IVM (P = 0.006). The *t*10,*c*12 CLA did not interfered with meiotic progression or oocyte competence for fertilization and cleavage. However, by interfering with LD content, *t*10,*c*12 CLA induced a lighter (P = 0.030) cytoplasmic colour tone measured by the gray mean value, indicating interference with the LD composition.

In the third chapter, the objective was to analyse the fatty acid (FA) and plasmalogen (measured by dimethylacetal, DMA) composition of porcine COC during IVM and after culture with *t*10,*c*12 CLA and/or forskolin supplementation. Studied conditions were used according to the best results of the previous experiment and to evaluate a possible synergetic activity. It was found that the maturation process *per se* induces changes in FA and DMA profile in COC, mostly in cumulus cells (CC). During maturation, oocytes were notably rich in saturated fatty acid (SFA), maintaining their FA profile, whereas immature CC were characterized by an abundant monounsaturated fatty acid (MUFA) content. Mature CC presented the highest and diverse polyunsaturated fatty acid (PUFA) concentration, particularly of 20:4n-6 and reduced SFA and MUFA fractions. In both, oocytes and CC, FA and DMA total content tended to increase along maturation (P = 0.056). Moreover, *t*10,*c*12 CLA and/or forskolin supplementation

influenced COC and culture media FA and DMA composition, but their total contents were not modified. This isomer was accumulated by COC, mostly in CC where MUFA and a 20:3 isomer decreased ($P \leq 0.043$). Moreover 20:4n-6 was reduced in *t*10,*c*12 CLA and forskolin suplemented CC and DMA-16:0 in *t*10,*c*12 CLA CC. Forskolin alone increased oocyte *c*9-18:1 ($P \leq 0.043$). Porcine oocyte can thus uptake FA from culture medium to maintain its endogenous reserves. However this uptake is selective, since oocytes incorporate some but not all FA thus maintaining their PUFA concentration and protecting oocytes from oxidation damage associated to unsaturations.

In the forth chapter we aimed to test the influence of the later experimental groups on *in vitro* embryo production and cryo-resistance of blastocysts resulting from treated oocytes. Data showed that addition of *t*10,*c*12 CLA decreased blastocyst rate ($P = 0.008$). When added simultaneously with forskolin to the IVM medium, maturation ($P= 0.040$), monospermic fertilization ($P = 0.003$) and cleavage rates ($P = 0.041$) were affected. However, the cryo-resistance of yielded blastocyst after vitrification-warming and their quality, measured by total cell number, was not affected, suggesting that porcine oocytes and embryos were able to up-regulate the utilization of exogenous and endogenous lipid reserves.

In conclusion, results showed that nuclear and cytoplasmic maturation of porcine oocytes can be manipulated during *in vitro* culture with repercussions on their lipid content, fertilization and embryo development. The utilization of pharmacological substances namely *t*10,*c*12 CLA and forskolin were effective to modulate intracellular FA and DMA composition, and forskolin was also an inhibitor of meiotic progression. These changes were related to modifications of endogenous LD reserves. Furthermore, the supplementation of *t*10,*c*12 CLA and of *t*10,*c*12 CLA plus forskolin during oocyte

IVM interfered with embryonic development though blastocyst survival and quality after cryopreservation were not affected. Alterations on FA porcine COC composition induced by these supplements were shown to be related to oocyte developmental competence.

RESUMO ALARGADO

A espécie suína tem um importante papel económico na agricultura e produção pecuária. Em particular, a grande importância ecológica de algumas raças locais para a conservação e sustentabilidade ambiental devem ser consideradas. Um dos principais objectivos da produção *in vitro* de embriões (PIV) é a transferência embrionária e a criopreservação de germoplasma. Contudo, esta criopreservação na espécie suína tem sido dificultada pela elevada susceptibilidade ao arrefecimento, tendo a causa sido atribuída ao elevado conteúdo lipídico intracelular. Assim, interferir com o metabolismo lipídico para reduzir e/ou modular o conteúdo intracelular lipídico surgiu como uma abordagem importante para melhorar a eficiência das tecnologias da reprodução assistida (ART) no modelo suíno.

Os principais objectivos desta tese foram: implementar a técnica de produção *in vitro* de embriões de suíno e investigar os efeitos de dois moduladores lipídicos, *trans*-10, *cis*-12 ácido linoleico conjugado (*t*10,*c*12 CLA) e a forscolina, através da suplementação dos meios de maturação de oócitos de suíno, sobre a maturação, fertilização, desenvolvimento embrionário e crio-resistência. Esta tese é assim constituída por um capítulo de revisão e três capítulos experimentais.

No primeiro capítulo o complexo cumulus oócito (CCO) foi extensamente caracterizado através de parâmetros celulares morfológicos e pelos mecanismos moleculares envolvidos no processo de maturação. Particular atenção foi dada à gota lipídica (GL) como abundante e dinâmica organela citoplasmática. O oócito de suíno foi apresentado pelo seu elevado teor lipídico como um excelente modelo para estudar o papel dos lipidos e comparado com o oócito de outras espécies. Os mecanismos de ação dos inibidores da meiose, como a forscolina, bem como a atividade e os efeitos do *t*10,*c*12

CLA e da forscolina como moduladores lipídicos foram descritos, assim como o seu potencial de aplicação na criopreservação de oócitos e embriões.

No segundo capítulo as alterações do conteúdo lipídico citoplasmático de CCO expostos a concentrações fixas de *t*10,*c*12 CLA e forscolina foram investigadas durante diferentes períodos de incubação. Igualmente foram avaliados os efeitos destes moduladores lipídicos na maturação do oócito e desenvolvimento embrionário inicial. Os resultados mostraram que apenas a forscolina afetou negativamente a progressão meiótica e a competência oocitária para a fertilização/ clivagem. Simultaneamente, a forscolina induziu um decréscimo nas áreas do oócito e da gordura, afectando o crescimento do oócito e distribuição citoplasmática das GL. Quando a forscolina foi adicionada apenas durante as 2 h iniciais de maturação foi observada uma alteração na morfologia da GL (avaliada pelas suas áreas), após 22-24 h de MIV ($P = 0.006$). Registou-se que o *t*10,*c*12 CLA durante 44-48 h de MIV não interferiu com a progressão meiótica nem com a competência oocitária para a fertilização ou clivagem, mas induziu ao aclaramento do citoplasma medido pela escala de cinzentos ($P = 0.030$), indicando interferência com a composição da GL.

No terceiro capítulo, o objectivo consistiu em analisar a composição em ácidos gordos (AG) e plasmalogénios (medidos por dimetilacetais, DMA), de CCO de suíno antes e após MIV e com suplementação de *t*10,*c*12 CLA e forscolina, de acordo com os melhores resultados obtidos na experiência anterior e para avaliar uma possível atividade sinérgica. Verificou-se que o processo de maturação *per se* induziu a modificações no perfil de AG/ DMA do oócito e célula do cumulus (CC), principalmente nesta. Durante a maturação o oócito caraterizou-se por ser notavelmente rico em ácidos gordos saturados (SFA), mantendo o seu perfil em AG, enquanto a CC imatura foi caracterizada por uma abundante fração de ácidos gordos monoinsaturados

(MUFA), e após a maturação reduziu as frações de SFA e de MUFA, aumentando e diversificando a concentração de ácidos gordos polinsaturados (PUFA), particularmente em 20:4n-6. Em ambos, oócito e CC, o conteúdo em AG/ DMA tendeu a aumentar ao longo da maturação (P = 0.056). Além disso, a suplementação com *t*10,*c*12 CLA e/ou forscolina influenciaram a composição em AG/ DMA do CCO e dos meios de maturação, mas não os conteúdos totais. Este isómero foi acumulado pelo CCO, principalmente na CC onde os MUFA e um isómero 20:3 decresceram ($P \leq 0.043$). Além disso, 20:4n-6 decresceu na CC com suplementação simultânea de *t*10,*c*12 CLA e forscolina (P = 0.025) e o DMA-16:0 na CC com tratamento de CLA (P = 0.008). A forscolina induziu ao aumento de *c*9-18:1 ($P \leq 0.043$). O oócito de suíno pode assim incorporar AG do meio de cultura para manter as reservas endógenas. Contudo, esta absorção é seletiva, dado que o oócito incorpora alguns AG e não todos, mantendo a sua concentração em PUFA, para proteção contra os riscos de oxidação associados a insaturações.

No quarto capítulo o nosso objetivo consistiu em testar a influência dos grupos experimentais anteriores na produção *in vitro* e crio-resistência dos blastocistos resultantes dos oócitos tratados. Os resultados mostraram que a adição de *t*10,*c*12 CLA decresceu a taxa de produção de blastocistos (P = 0.008). Quando adicionado em simultâneo com a forscolina ao meio de maturação, as taxas de maturação (P = 0.003), de penetração monospérmica (P = 0.003) e de clivagem (P = 0.041) foram afetadas. Contudo, a crio-resistência dos blastocistos produzidos após vitrificação-aquecimento e a qualidade dos mesmos, medida pelo número total de células, não foi afectada, sugerindo que o oócito e o embrião de suíno durante o período pré-implantatório regulam a utilização das reservas lipídicas endógenas e exógenas.

Em conclusão, os resultados mostraram que a maturação nuclear e citoplasmática do oócito de suíno podem ser manipuladas durante a cultura *in vitro* com repercursões no seu contéudo lipídico, fertilização e desenvolvimento embrionário. A utilização de substâncias farmacológicas como *t*10,*c*12 CLA e a forscolina foi efectiva na modulação dos AG/ DMA intracelulares, sendo ainda a forscolina inibidora da progressão da meiose. Estas alterações foram relacionadas com modificações das reservas endógenas das GL. Além disso, a utilização de *t*10,*c*12 CLA e de *t*10,*c*12 CLA em simultâneo com a forscolina durante a cultura de oócitos interferiu no desenvolvimento embrionário, apesar da sobrevivência e a qualidade após criopreservação não terem sido afectadas. As alterações morfológicas e da composição de AG no CCO de suíno, induzidas por estes suplementos nos meios de maturação, podem ser relacionadas com a competência oocitária.

LIST OF PUBLICATIONS:

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Prates EG, Marques CC, Baptista MC, Vasques MI, Carolino N, Horta AEM, Charneca R, Nunes JT and Pereira RM 2012. Fat area and lipid droplets morphology of porcine oocytes during *in vitro* maturation with *trans*-10,*cis*-12 conjugated linoleic acid and forskolin. Animal 7, 602-609. DOI: 10.1017/S1751731112001899.

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations

AL	Alentejano
Anaph	Anaphase
ART	Assisted Reproductive Technologies
ATP	Adenosine Triphosphate
BSA	Bovine Seric Albumine
BTS	Beltsville Thawing Solution
cAMP	cyclic Adenosine Monophosphate
CC	Cumulus Cells
Cdc2	Cyclin dependent kinase 2
CO_2	Carbon dioxide
COC	Cumulus Oocyte Complex(es)
CT	Cell Type
CV	Coeficient of variation
DMA	Dimethylacetal
DMSO	Dimethyl Sulfoxide
(m)DPBS / PBS	(modified) [Dulbeco] Physiological Buffer Solution
eCG	equine Corionic Gonadotrophin
EG	Ethylene glycol
EGF	Epidermal Growth Factor
ERK	Extracelular signal-regulated kinase
FA	Fatty Acid
FAME	Fatty Acid Methyl Ester(s)
FCS	Fetal Calf Serum
x *g*	Centrifugal force expressed as product of gravity
GLC-MS	Gas-Liquid Chromatography Mass Spectrometry
GV	Germinal Vesicle

GSH	Glutathione
GVBD	Germinal Vesicle Breakdown
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
FSK	Forskolin
hCG	human Chorionic Gonadotrophin
IVC	*In Vitro* Culture
IVF	*In Vitro* Fertilization
IVM	*In Vitro* Maturation
IVP	*In Vitro* Production
LD	Lipid Droplet(s)
LSM	Least Square Means
MI	Metaphase I
MII	Metaphase II
MS	Maturation Status
mRNA	messenger Ribonucleic Acid
N	Number
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAR	Normal Apical Ridge
NCSU23 (37)	North Carolina State University 23 (37)
nd	not detectable
ni	non identified
ns	non significant
O_2	Oxygen
pFF	porcine Follicular Fluid
PG/PGE_2	Prostaglandin(s)/ Prostaglandin E_2
SD	Standard Deviation
PVA	Polyvinyl Alcoohol
r	regression coefficient
RSD	Residual Standard Deviation
SAS	Statistical Analysis System (Software package)

SEM	Standard Error of the Mean
SOPS	Superfine Open Pulled Straw
SPSS	Statistical Package for the Social Sciences
TCM199	Tissue Culture Medium 199
v	volume
W	Weight
Fatty acids	
AA	Arachidonic Acid; 20:4n-6
FA	Fatty Acid(s)
SFA	Saturated Fatty Acid(s)
MUFA	Monounsaturated Fatty Acid(s)
PUFA	Polyunsaturated Fatty Acid(s)
*t*10, *c*12 CLA	*trans*-10, *cis*-12 Conjugated Linoleic Acid
Neutral Lipids (NL)	
TAG	Triacylglycerol(s)
Symbols	
ºC	Degree Celsius
h	Hour(s)
IU	International units
m	meter(s)
M	Molar
mg	Miligrame
min	Minute(s)
mL	Mililitter
mM	Milimolar
P	Probability
μM	Micromolar
μg	Micrograme
μL	Microlitter
%	Percentage

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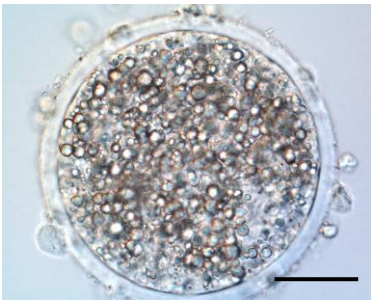
GENERAL INTRODUCTION

In vitro manipulation of porcine oocytes and embryos has an enormous potential of application in assisted reproductive technology (ART) and germoplasm cryopreservation to improve livestock management and genetic conservation. In swine species the intensification of agricultural systems has lead to a strong decrease on extensive system pig herds and in breed biodiversity in past decades (Nunes, 1993 and 2007). Furthermore, the reproductive traits of the Alentejano swine breed (AL) females are characterized by shorter gestation length, smaller litters and higher mortality rate of live-born piglets, comparing to conventional swine genotypes raised under intensive systems (Charneca, 2010). *In vitro* embryo production (IVP) is a potential tool to be applied to the conservation of endangerous breeds or in risk of extinction, namely AL, "Bísaro" ou "Malhado de Alcobaça" (according to "Dados constantes dos Relatórios Anuais 2011_DGAV_PRODER/ Subação 2.2.3.2.; Dados da DRDA – Direção Regional do Desenvolvimento Agrário") or for female transfer or animal genetic resources cryopreservation (Boettcher *et al*., 2005). Adjustments of culture conditions have been referred to allow reaching a maximum efficiency of IVF systems for individual boars (Almiñana *et al*., 2005; Gil *et al*., 2008). Furthermore, the huge availability of porcine oocytes from ovaries obtained in slaughterhouse permits several applications. Thus, the fertilizing ability of boar spermatozoa for artificial insemination purpose can be predicted by the use of zona-intact porcine oocytes in a fertilizing system assay (Matás *et al*., 1996; Sellés *et al*., 2003), while IVP systems can be an effective approach in the production of transgenic pigs, but also for the use in research studies of embryological development and early pregnancy looses (Day and Funahashi, 1995; Kikuchi *et al*., 2002; Suzuki *et al*., 2004; Coy *et al*., 2005). Nevertheless, several problems in the application of IVP technology in porcine have been identified: oocyte

fragmentation (Mateusen *et al*., 2005), incomplete cortical reaction (Coy *et al*., 2002), the aging or the asynchronous meiotic progression (Grupen *et al*., 1997, Wang and Sun, 2007), poor male pronuclear formation (Abeydeera, 2002) or polyspermic fertilization (Coy *et al*., 1999; Funahashi and Romar, 2004). Moreover the high lipid contents of porcine oocyte and embryo have been correlated with their impaired quality after cryopreservation (Dobrinsky *et al*., 1999; Seidel, 2006, Pereira and Marques, 2008). The manipulation of lipid metabolism during oocyte maturation to reduce lipid accumulation and stimulate lipid utilization during culture is thus expected to improve IVP and cryopreservation outcomes in the pig model. Recently, the effects of fatty acid (FA) supplementation to the *in vitro* maturation (IVM) medium has been investigated in the bovine oocyte (Wathes *et al*., 2007; Marei *et al*., 2009; Aardema *et al*., 2011; Marei *et al*., 2010) and in particular the *trans*-10,*cis*-12 conjugated linoleic acid isomer (*t*10,*c*12 CLA, at 100 μM) added during maturation, was found to interfere on lipid metabolism improving bovine oocyte competence to develop into higher quality embryo (Lapa *et al*., 2011). Other reports confirm the reduction of lipid accumulation in *in vitro* embryos (Pereira *et al*., 2007 and 2008) or the decrease in lypogenesis in porcine adipose explants after culture with *t*10,*c*12 CLA (José *et al*., 2008). Furthermore *t*10,*c*12 CLA interferes on eicosanoid pathways , and therefore some effects can be due to CLA direct action or to its metabolites (Banni, 2002) leading to oocyte maturation improvement (Nuttinck *et al*., 2008). Whether lipid content in porcine oocyte and resulting embryo can be reduced or the excessive lipid accumulation prevented by *t*10,*c*12 CLA addition to oocyte maturation medium has not yet been studied. Also, if this CLA isomer may improve porcine oocyte developmental competence acquisition is also unknown.

Moreover, at the time of collection of cumulus-oocyte complexes (COC) for *in vitro* maturation there is a large variation in the germinal vesicle morphology (Grupen *et al*., 1997), that can be synchronized with analogous of cAMP and other pharmalogical tools (Laforest *et al*., 2005; Albuz *et al*., 2010). *In vitro* matured oocytes are often transiently exposed to forskolin to rise up the intracellular level of cAMP, by the activation of adenylyl cyclase and reversibly blocking spontaneous germinal vesicle breakdown (GVBD) during initial IVM (Laforest *et al*., 2005; Sasseville *et al*., 2007). Thus, the use of the adenylyl cyclase stimulator forskolin, till 10 μM concentration, was shown to increase the intracellular level of cAMP delaying meiosis progression while it still can induce progesterone secretion and mass expansion in cumulus cells of porcine oocyte indicating COC maturation (Racowsky, 1985; Xia *et al*., 2000; Fu *et al*., 2011). In addition, cryosurvival of porcine oocyte and embryo was improved by partial delipation through chemical stimulation of lipolysis of triacylglycerols intracellular reserves by forskolin (Men *et al*., 2006; Fu *et al*., 2011). Thus, the possibility of embracing both, meiotic synchronization and lipid content reduction was here investigated.

In the light of the above, the present dissertation intented to contribute to the study of porcine oocyte IVM and the effects of oocyte lipid manipulation with *t*10,*c*12 CLA and forskolin, on oocyte maturation and fertilization, embryo development and cryo-resistance. Furthermore, the mechanisms underlying modifications of lipid metabolism and intracellular lipid content during the porcine cumulus oocyte complexes maturation process were here studied.



*

TECHNICAL BACKGROUND AND RESEARCH OBJECTIVES

*Porcine oocyte observed under a Nomarski microscope. Scale bar 50 µM.

I – *In vitro* embryo production in porcine species

In vitro manipulation of porcine oocytes and embryos is a growing application technique as pigs became important models for biomedical and basic research purposes, and also due to their extensive role in agriculture (Coy *et al*. 1999; Abeydeera, 2002; Men *et al*., 2006). In biomedical research, pigs are important because of their physiological similarities to humans that make them potential xenograft donors and transgenic animals (Abeydeera, 2002). Furthermore, the utilization of ovaries from slaughterhouse animals became crucial to collect large number of oocytes that can be applied in basic research. Moreover, the *in vitro* fertilization techniques can be useful in the prediction of fertilizing capability of boar semen (Martínez *et al*., 1993, Sellés *et al*., 2003), allowing breeders' selection as well as the identification of the best ejaculates for artificial insemination programs. Thus, semen and also embryos can be used as materials for animal genetic resources cryopreservation, to support populations conserved in vivo and for breed reconstruction, this last being considered the maximum guarantee against the risk of breed loss (Boettcher *et al*., 2005). The endangered pig breeds namely the "Alentejano" (n = 6525), "Malhado de Alcobaça" (n = 229) and "Bísaro" breeds (n = 2521), (DGAV-PRODER 2011, "Dados constantes dos Relatórios Anuais 2011_DGAV_PRODER/ Subacção 2.2.3.2.; Dados da DRDA – Direção Regional do Desenvolvimento Agrário") which number of individuals in population has greatly decreased may then benefit from the application of such technologies.

I.1. – The major problems of *in vitro* embryo production in porcine

In porcine species, the inter and intra-boar variability in sperm characteristics have been referred to affect the efficiency of *in vitro* fertilization (IVF) outcomes (Gil *et al*., 2008). The use of frozen-thawed boar sperm from the same semen collection can provide good

reproducible data in IVF experiments, and therefore allows standardizing the variability associated to male factor. However to broad the research results different males must be used.

Another major limitation to porcine embryo production is the polyspermic penetration, and despite the attempts to improve the ability of oocytes to stimulate zona pellucida hardening and male pronuclear formation, following sperm penetration, are being made for long, they have only partially been successful (Coy *et al*., 1999; Abeydeera, 2002; Funahashi and Romar, 2004). Three possible explanations for the low rate of monospermic penetration were advanced: an inadequate *in vitro* maturation of oocytes, an excessive number of spermatozoa reaching the oocyte during IVF and culture conditions affecting sperm-oocyte interaction during IVF (Abeydeera, 2002).

The control of spontaneous meiosis resumption in oocytes selected for *in vitro* maturation by the use of phosphodiesterases (PDE) inhibitors and adenylyl cyclase activators has been reported (Racowsky, 1985; Xia *et al*., 2000; Laforest *et al*., 2005; Albuz *et al*., 2010). A transiently delay in meiotic progression is crucial to allow the oocytes to enrich the cytoplasm with the substances required for normal fertilization and to support subsequent embryonic development (Thomas *et al*., 2004b). For example, to form a male pronucleous inside an oocyte it is necessary to reduce the disulfide bonds of protamine, and to extract or degrade the proteamines in the sperm nucleus and replace them with histones, this processe being dependent upon GSH synthesis during maturation (Eppig, 1996; Cui *et al*., 2009). The intracellular Ca^{2+} mobilization in course of maturation is implicated in triggering cortical granule exocytosis in mammalian oocytes thus interfering with oocyte ability to establish a membrane block to polyspermy (Gardner and Evans, 2006; Cui *et al*., 2009). Therefore, these and other cellular changes namely organelles reorientation, accumulation of mRNA and proteins

in the cytoplasm of the developing oocyte seem to be necessary for a normal fertilization and successful embryonic development (Eppig, 1996; Cui *et al*, 2009; Hao *et al*., 2007).

Regarding IVF conditions, it has been demonstrated that co-incubation time and sperm: oocyte ratio are closely related to achieve the best efficiency in an IVF system: short coincubation times (10 min) require higher sperm : oocyte ratios (1500 or 1000 sperm : oocyte) and long coincubation intervals (2-6 h) should be combined with lower sperm : oocyte ratios (500 sperm : oocyte) (Gil *et al*, 2007). In addition, the spermatozoa bound to the zona pellucida require a maximum of 2 h in an appropriate medium to penetrate the oocytes. Furthermore, caffeine has been used in order to improve oocyte penetration during IVF (Funhashi and Romar, 2004; Almiñana *et al*., 2005). In general, a transient time of exposure to caffeine can induce capacitation and acrosome reaction to achieve penetration, even so spermatozoa concentration should be adjusted to the boar (Funhashi and Romar, 2004; Almiñana *et al*., 2005). Moreover caffeine was referred to induce the increase in cAMP by inhibiting PDE in the spermatozoa, stimulate the capacitation and spontaneous acrosome reaction of boar spermatozoa via the adenylyl cyclase/c AMP pathway (Funhashi and Romar, 2004). Likewise, adenosine and hyaluronic acid, also activators of sperm capacitation, have been tested in IVF. The boar effect was still present and was referred to influence IVF outcomes, demanding preliminary screening of boar sperm for *in vitro* embryo production (Almiñana *et al*., 2005).

Moreover, the high intracellular lipid content of porcine oocytes comparing to those of other species, has been reported (Homa *et al*., 1986; McEvoy *et al*., 2000) and related to impaired cryopreservation resistance of both oocytes and derived embryos (Dobrinsky, 1999; Fujhira *et al*., 2004; Esaki *et al*., 2004; Hara *et al*., 2005; Men *et al*., 2006; Fu *et*

al., 2011), particularly those cultured in the presence of serum (Seidel, 2006). Therefore, metabolic profiles and fatty acid composition of porcine oocytes *in vitro* matured may eventually be modified during *in vitro* culture, though the effect on fertilization and subsequent development are largely unknown. This issue will be further discussed in this section.

I.2. – Techniques and culture media

Porcine *in vitro* production (IVP) systems includes *in vitro* maturation (IVM) and IVF of oocytes and their subsequent *in vitro* culture (IVC). These techniques have been modified by many researchers, to improve the low developmental rates of embryos at the blastocyst stage and their poor qualities (Kikuchi *et al*., 2002; Gil *et al*., 2003; Almiñana *et al*., 2005; Gil *et al*., 2007). In current IVP systems, oocytes are collected from gilts ovaries obtained at slaughterhouses and oocytes with evenly ooplasm and three or more cumulus cells (CC) layers selected.

I.3. – *In vitro* maturation

In vitro maturation of porcine cumulus oocyte complexes (COC) can be achieved in different culture media such as the TCM199 (Schoevers *et al*., 2003; Swain *et al*., 2002; Sturmey and Leese, 2003), North Carolina State University (NCSU) 37 (Kikuchi *et al*., 2002; Coy *et al*., 2005), being the NCSU23 (Petters and Wells, 1993) one of the most extensively based medium used (Almiñana *et al*. 2005; Gil *et al*., 2007; Gadja *et al*., 2008). This is communly supplemented with follicular fluid (pFF, 10%, v/v), cysteine (0.1 mg/mL) and epidermal growth factor (EGF, 10 ng/mL), gonadotropins, equine chorionic gonadotrophin (eCG, 10 IU/mL) and human chorionic gonadotrophin (hCG; 10 IU/mL), in sequential culture, for about 22 h supplemented with hormones and then

for further 22 h, without hormonal additives (Funahashi and Day, 1993; Gil *et al*., 2005). Other supplements can be added to the IVM medium during the first 22 h like meiotic modulators (1mM dibutyryl cAMP, Kikuchi *et al*., 2002) or cyclin B kinase inhibitors such as roscovitine (22 h, 50 μM), or before culture in the conventional two-step IVM system (Coy *et al*., 2005) can be used. The presence of cumulus cells (CC) during maturation has been referred to influence nuclear maturation, intracellular GSH content, penetration rate, pronucleus formation, histone H1 kinase activity and stabilizes the distribution of cortical granules (Abeydeera, 2002). Moreover, cumulus expansion is a good indicator of oocyte maturation (Hurk and Zhao, 2005). Furthermore, nuclear maturation is normally accessed through fixation in acetic acid : ethanol and staining with aceto-lacmoid (1%, Gil *et al*., 2003, Prates *et al*., 2008) or aceto-orcein (1%, Kikuchi *et al*., 2000; Laforest *et al*., 2005) and examination under a phase-contrast microscope. The cytoplasmic maturation can be assessed through oocyte *in vitro* fertilization or by morphological parameters as described below.

I.4. – *In vitro* fertilization

In current systems, IVF of porcine oocytes is being performed in modified Tris-buffered medium (mTBM; Abeydeera and Day, 1997), supplemented with 0.2 % BSA and caffeine. As referred caffeine concentration has to be adjusted to the boar (0.5-2 mM; Almiñana *et al*., 2005; Gil *et al*., 2007; 3.5 mM Swain *et al*., 2002). Besides caffeine, adenosine or hyaluronan can be used (Almiñana *et al*., 2005). The same authors strip of the CC from the oocytes before fertilization and placed them in drops (30 oocytes/50 μL drop) and a 1000-2000 : 1, sperm : oocyte ratio. The gametes co-incubation can be maintained during 3 h (Kikuchi *et al*., 2002) or more, 5-6 h (Funahashi *et al*., 1994; Gil *et al*., 2007). Other IVF procedures are such as placing the oocytes in IVF medium and

incubate them during nearly 30 min before insemination (Gil *et al*., 2005), or pre-incubate the spermatozoa in IVF medium (about 2.5 h) to facilitate capacitation (Swain *et al*., 2002). Furthermore the assessment of monospermic penetration (16–18 after hpi) is crucial to evaluate the efficiency (number of monospermic oocytes/ total inseminated) of the IVF system.

1.5. – *In vitro* embryo culture

Embryo metabolism is an indicator of viability and therefore, efficiency of the culture medium (Gandhi *et al*., 2001). The first two days after IVF seem to be crucial for embryonic genome activation or otherwise at this stage a four-cell block is observed in pigs (Kikuchi *et al*., 2002). Thus, it is probable that adequate chemical or oviductal supplements during the first 2 days may enhance embryonic potential for genome activation and for overcoming cell block (Petters *et al*., 1991; Kikuchi *et al*., 2002). While some authors use the NCSU23 with 0.4% BSA as a single culture medium (Swain *et al*., 2002; Sturmey and Leese, 2003), others use a sequential culture IVC system, according to energy supplementation: in the first 2 days (day 0 = day of fertilization) the IVC medium is the glucose-free NCSU23, containing 0.33 mM pyruvate, 4.5 mM lactate and 0.4% BSA and following, embryos are removed and cultured in NCSU23 medium with 5.5 mM glucose and 0.4 % BSA, until day 6 (Kikuchi *et al*., 2002).

Furthermore, the quality of IVP embryos can be assessed by several methodologies, such as morphological (visual score and blastocyst total cell numbers through fixation and Hoechst staining) (Swain *et al*., 2002; Cuello *et al*., 2008; Lapa *et al*., 2011) and/or cryopreservation resistance. Moreover, the production of piglets derived from *in vitro*-produced blastocysts has yet shown good results, validating IVP systems (Kikuchi *et*

al., 2002; Coy *et al*., 2005), and this was shown by the maintenance of pregnancies in recipients females. Also the higher weights of new-born piglets in derived from day 6 blastocysts were correlated to the higher total cell numbers comparing to those of day 5 (Kikuchi *et al*., 2002). In addition to the increase farrowing rates and number of born piglets derived from IVP blastocysts, new methodologies for embryo transfer that avoid surgical procedures, are being developed (Suzuki *et al*., 2004).

II - Research Objectives

Given the problem of asynchrony of meiosis and cytoplasmic maturation in developing porcine oocytes and its effect on embryo production, their meiotic regulation is currently under investigation. Moreover the abundant lipid content of porcine oocytes and resulting embryos as well as its interference on cryopreservation efficiency, is mandatory for the reduction or modulation of their intracellular lipid content. The main objectives of the present dissertation were to implement IVP technique in swine species and to investigate the effects of lipid modulators during *in vitro* maturation (IVM) on porcine oocyte development potential and embryo cryosurvival. Therefore the following specific objectives were proposed:

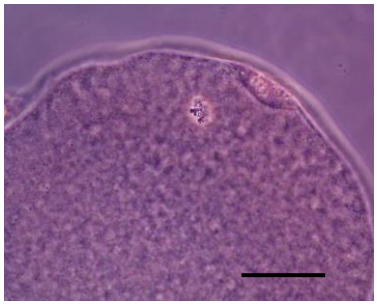
. to review the oocyte morphology and functionality, and the effects of meiotic and lipid manipulation during *in vitro* maturation on ART outcomes in porcine species;

.to implement porcine embryo production techniques;

. to test the alterations of cytoplasmic lipid content of porcine oocytes exposed to lipid modulators, *trans*-10, *cis*-12 CLA (*t*10,*c*12 CLA) and forskolin, under different time periods of IVM supplementation and simultaneously the effect on oocyte nuclear progression, cytoplasmic maturation and competence for fertilization;

. to characterise the FA and DMA content and composition of porcine oocytes and CC before and after maturation and to investigate the effects of *t*10,*c*12 CLA and/or forskolin supplementation to culture media on FA and DMA composition, and maturation rates of porcine COC;

. to test the effect of *t*10,*c*12 CLA and/or forskolin supplementation during oocyte maturation, on maturation and fertilization rates as well as in embryonic development and blastocyst cryo-resistance.



CHAPTER 1

IS THERE A ROLE FOR LIPID MODULATORS IN ASSISTED REPRODUCTIVE TECNHOLOGIES?

E.G. Prates, J.T. Nunes and R.M. Pereira

Adapted from ANIMAL-12-30651 (2012) (Submitted)

* Matured porcine oocyte with chromosomes at metaphase II and an extruded body polar (aceto-lacmoid staining). Scale bar 50 µM.

Is there a role for lipid modulators in assisted reproductive tenhologies (ART)?

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1.1. - Abstract

Oocyte intracellular lipids are mainly stored in lipid droplets (LD) in order to provide energy for growth and development, but they are also important signalling molecules involved in the regulatory mechanisms of maturation and hence in oocyte quality. Recent studies show that LD are highly dynamic organelles changing their shape, volume and location within the ooplasm as well as their association to other organelles during the maturation process. However, high amounts of LD have been correlated with impaired oocyte developmental competence and oocyte/ embryo cryosurvival. This review presents a brief morphological and functional characterization of cumulus-oocyte complexes, the functional and dynamic unit playing a pivotal role in ART outcomes. Particular attention is given to the lipid-rich porcine oocyte, here rendered as an excellent model to understand the role of lipids and fatty acid metabolism during maturation and their implications on subsequent embryo development. Moreover the possibility of applying substances capable of modulating oocyte nuclear and

cytoplasmic maturation, specifically oocyte or embryo lipid content and metabolism to improve ART success is further discussed. In addition, these principles might be applied in germplasm and embryo cryopreservation, in livestock production or in biomedical research.

Keywords: oocyte, lipid droplets, assisted reproduction technologies, maturation, quality.

1.2. - Implications

The effects of lipid modulators in ART are not clearly understood. High amounts of lipid droplets have been correlated with impaired oocyte/embryo developmental competence and cryosurvival. However, this large repository of endogenous lipids is vital for their energy production. Lipids are also important to form membranes or as intracellular messengers. Although modifications of intracellular lipids by chemical stimulation can improve oocyte or embryo quality and cryopreservation, some of the mechanisms by which lipolytic substances interfere with lipid metabolism may affect the metabolic *via* regulating their developmental potential. This review contributes to a better understanding of this intricate issue with repercussions in livestock production and biomedical research.

1.3. - Introduction

Oocyte quality is a key limiting factor in female fertility (Gilchrist *et al*., 2008; Wang and Sun, 2007). The ovarian follicular microenvironment and maternal signals, mediated primarily through granulosa cells and cumulus cells (CC), are responsible for nurturing oocyte growth and its gradual acquisition of developmental competence

(Gilchrist *et al*., 2008). *In vitro* maturation (IVM) of oocytes can generate mature oocytes which are capable of supporting preimplantation embryo development and full development to term (Gilchrist and Thompson, 2007). In livestock production, IVM oocytes can provide a large population of female germ cells (Hirao *et al*., 1994) that can be useful in breeding programmes and animal genetic cryo-conservation. However, the high intracellular lipid content of oocytes and embryos has been reported to impair cryopreservation, with particular relevance in porcine species (Seidel, 2006; Pereira and Marques, 2008). Different strategies can be used to manipulate oocyte or embryo lipid contents. Nevertheless, a role for lipids in energy production during preimplantation development as well as precursors in steroidogenic and eicosanoid pathways has been referred (Sturmey and Leese, 2003; Nuttinck *et al*., 2008; Lapa *et al*., 2011), suggesting that modifications in oocyte intracellular lipids should be carefully estimated. The high intracellular lipid content of porcine oocytes (Prates *et al*., 2012; Prates *et al*., 2013) renders them an excellent model to understand the role of lipids and fatty acid metabolism during maturation and their implications on the decline of female fertility due to age and obesity (Ford and Tavendale, 2010; Purcell and Moley, 2011). Furthermore, mechanisms by which nuclear and cytoplasmic maturation can be synchronised might be related to metabolic pathways that are also involved in the mobilization of intracellular lipid reserves in the oocyte. This review will discuss current knowledge of mechanisms regulating oocyte quality during maturation and highlight recent advances in modifying oocyte lipid metabolism and content to improve assisted reproduction technology (ART) outcome.

1.4. - Morphological and functional characterization of cumulus-oocyte complex

The cumulus oocyte complex (COC) is composed of the feminine gamete and the surrounding CC (Figure 1.1.) and is of primordial importance to the outcomes of ART. This complex forms a complete functional and dynamic unit playing a pivotal role in oocyte metabolism during maturation. Moreover the bidirectional exchanges between oocyte and contiguous CC are important for oocyte competence acquisition, CC expansion and early embryonic development (Sutton *et al*., 2003, Gilchrist and Thompson, 2007; Ouandaogo *et al*., 2011). The oocyte maturation process comprises two aspects, nuclear and cytoplasmic, both essential for normal fertilization and embryonic development, thus being highly coordinated (Hunter, 2000).

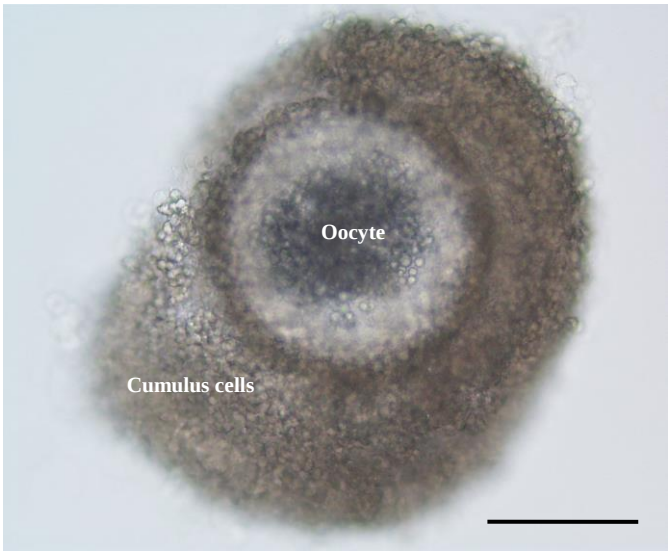

Figure 1.1. – Immature porcine cumulus oocyte complex (COC) observed under a Nomarski microscopy. Scale bar 50 µM.

1.4.1. - *COC maturation*

1.4.1.1. - *Oocyte nuclear maturation*

Oocyte competence to complete nuclear maturation is acquired at least in two steps: firstly, oocytes are able to reinitiate meiosis (Figure 1.2.A.), undergo germinal vesicle breakdown (GVBD) and progress to metaphase I; secondly, oocytes are competent to

advance beyond metaphase I, enter anaphase and proceed to metaphase II (MII) (Eppig, 1996). At the end of the maturation period, the meiotic spindle and chromosomal rearrangement at MII as well as the first polar body can be observed (Figure 1.2.B.).

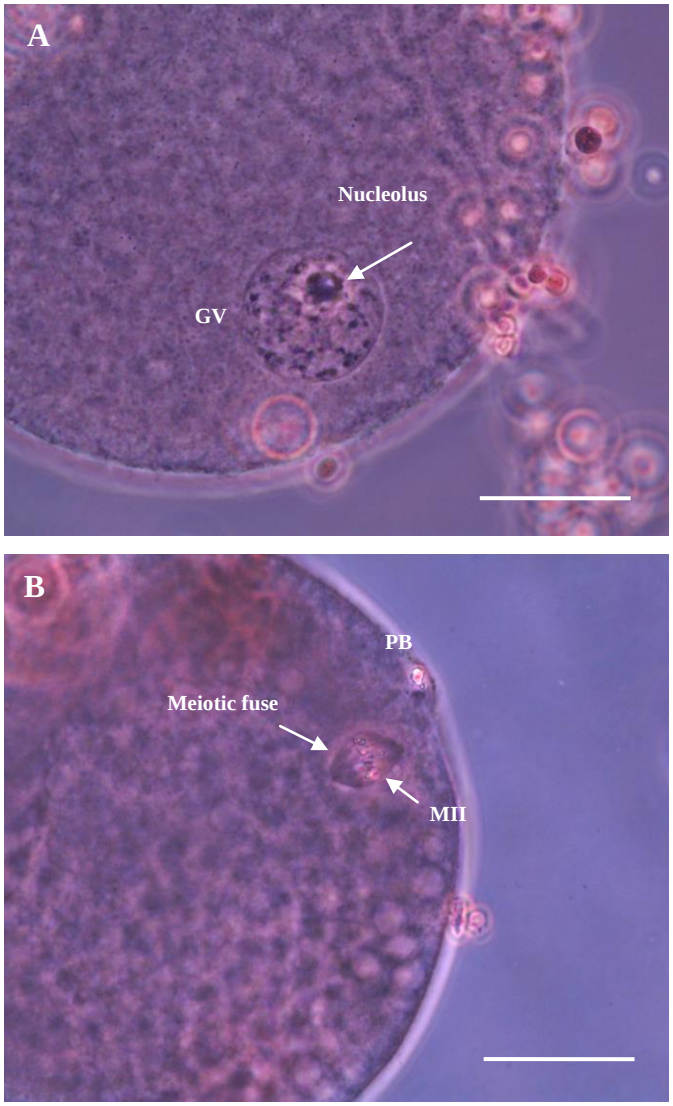

Figure 1.2. - Nuclear configuration of porcine oocytes prior to (A) and after (B) *in vitro* maturation (GV, germinal vesicle: MII and PB, metaphase II with polar body). Scale bar 50 µM.

The oocyte has the largest diameter of any cell in female mammals. Oocyte diameter and area measurements are often used to predict oocyte meiotic competence or maturity (Griffin *et al*., 2006; Prates *et al*., 2008). *In vivo*, oocytes reach their maturity within the antral stage of follicular development presenting different diameters according to

species (mouse, hamster, pig and human) (Griffin *et al*., 2006). In porcine, full meiotic competence of the oocyte is reached in ovarian follicles with a diameter of 3 mm or more (Sun and Nagai, 2003). *In vitro*, porcine oocytes under 90 μm in diameter are unable to resume meiosis, while oocytes measuring 110-115 μm can complete the first meiotic division and acquire MII (Sun and Nagai, 2003; Prates *et al*., 2012). Concurrently, the oocyte grows and its area may be an indicator of cytoplasmic maturation (Prates *et al*., 2012). Furthermore, the size of periviteline space or thickness of zona pellucida are also related to the developmental competence of the oocyte for fertilization (Xia, 1997; Wang and Sun, 2007).

1.4.1.2. - *Oocyte cytoplasmic maturation*

Oocytes are complex cells comprising many organelles and compounds (Wang and Sun, 2007). The large repository of endogenous lipids as an energy source for oocyte and embryo development is mainly stored in the form of an organelle, the lipid droplet (LD) (McEvoy *et al*., 2000; Romeck *et al*., 2010). LD is composed of a core of neutral lipids enveloped by a phospholipid monolayer containing a wide variety of proteins that can be embedded in both the phospholipid monolayer and within the core (Walther and Farese, 2009). The function of these cellular proteins in LD fractions is currently being studied. For instance, perilipins that are located at the LD surface in adipocytes, but also in steroidogenic tissues, were referred as having regulatory functions and as being involved in LD lipolysis (Holm, 2003). These particular organelles can be formed *de novo*, when cells are kept under delipidated conditions and re-fed with free fatty acid (FA) or grow through a coalescence process of existing droplets, mediated by SNARE proteins (Thiele and Spandl, 2008). However, regardless of LD origin, they are highly dynamic, constantly changing shape, volume and location (Watanabe *et al*., 2010). In

particular, the porcine oocyte has been identified as one of the most lipid-rich oocytes among domestic animals (McEvoy *et al*., 2000), in which the cytoplasm is filled with LD. During IVM, a considerable variation in LD areas, from 0.3 μm^2 to about 90 μm^2 (Figure 1.3.), was identified through maturation progression (Prates *et al*., 2012).

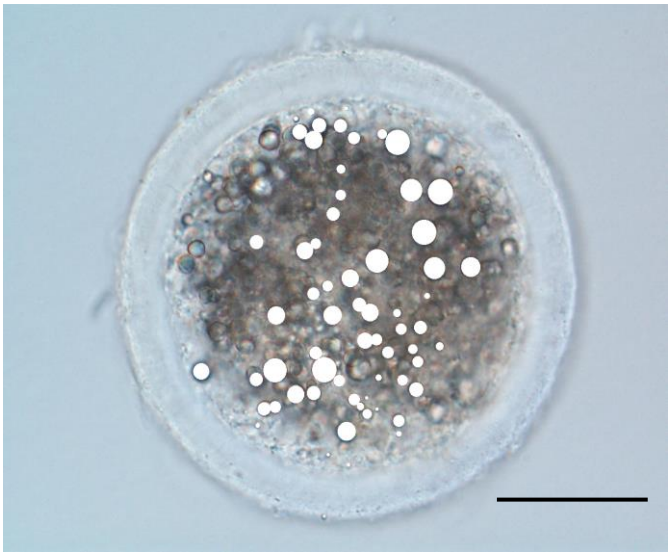

Figure 1.3. – Immature porcine oocyte observed under a Nomarski microscopy, with lipid droplets (LD) highlighted in white colour (LD and oocyte areas were measured using Image J software). Scale bar 50 µM.

Lipid droplets can be found in association with other organelles linked to cellular metabolism such as mitochondrias, endoplasmic reticulum (ER), endossomes, peroximes and cytoskeleton (Sturmey *et al*., 2006; Thiele and Spandl, 2008; Walther and Farese, 2008; Ambruosi *et al*., 2009). During oocyte maturation, the activity and organization of LD-mitochondria are particularly relevant, since oxidative phosphorylation is the main pathway to supply ATP for cellular activities (Sturmey and Leese, 2003; Wang and Sun, 2007). In mature porcine oocytes two distribution patterns were identified for both, LD and mitochondria: an even or homogenous distribution through the ooplasm and an uneven or heterogeneous allocation. The first distribution is more frequently observed (Cui *et al*., 2009). Moreover, the evidence of regions of 'co-

localization' between LD and mitochondria and their relocalization during IVM of porcine oocytes was shown to be linked to intracellular oxygen gradients. Hence, Sturmey *et al*. (2006) observed that the peripheral mitochondrial clustering in porcine oocytes was correlated to higher oxygen availability in this region. In oocytes from other species, such as the equine, the aggregation of LD, which was related to nuclear maturation and cumulus expansion was also observed (Ambruosi *et al*., 2009). Moreover, the morphological changes observed in LD during oocyte maturation may reflect changes in the nature of lipids being stored in those droplets (Silva *et al*., 2011) but can also be a sign of abnormal maturation (Prates *et al*., 2012).

In spite of several efforts, oocyte cytoplasmic maturation remains a key limiting step for ART. The reasons why fully grown oocytes are not capable of becoming viable embryos are still elusive, but incomplete cytoplasmic maturation and/or asynchrony between nuclear and cytoplasmic maturation are certainly among those critically responsible.

1.4.1.3. - *The surrounding investment of cumulus cells*

The simultaneous expansion of compact layers of CC surrounding the oocyte and deposition of mucoelastic material in the extracellular matrix is implicated in supporting of both nuclear maturation to the MII stage and cytoplasmic maturation (Gilchrist and Thompson, 2007; Ambruosi *et al*., 2009; Cui *et al*., 2009). Moreover, the communication between the oocyte and its surrounding cells is essential for normal acquisition of oocyte developmental competence. However fully grown oocyte in vivo is associated with the loss of COC gap junctional communication (Thomas *et al*., 2004). During porcine oocyte IVM, CC interfered with ooplasmic LD-mitochondria distributions, in which LD distribution in oocytes was more sensitive to CC absence

than mitochondria (Cui *et al*., 2009), thus influencing oocyte morphological appearance. The presence of CC during IVM was also found to be effective in regulating the synthesis and concentration of important cytoplasmic factors such as glutathione (GSH) and Ca^{2+} (Hao *et al*., 2007). Denuded mature porcine oocytes present differences in Ca^{2+} homeostasis regarding those matured with CC. Indeed, the duration of Ca^{2+} rise was higher, although its amplitude was lower when compared to oocytes matured in the presence of CC: COC or CC added to culture medium (Cui *et al*., 2009).

Concomitantly with oocyte nuclear maturation, CC underwent a molecular maturation process. Moreover, oocytes actively control the COC microenvironment via oocyte-secreted factors that regulate fundamental aspects of CC function. In turn, the CC gene expression profile varies according to the stages of oocyte maturation and can be useful as predictors of oocyte quality (Gilchrist and Thompson, 2007; Ouandaogo *et al*., 2011). The beneficial effect of CC during oocyte growth to stimulate competence acquisition to further support embryonic development is therefore unequivocal.

1.4.2. - *Molecular mechanisms underlying oocyte maturation*

1.4.2.1. - *The role of cAMP, PKA and PKC in meiotic progression*

The maturation of an oocyte is a multi-pathway process that includes the involvement of several mediator factors (Hurk and Zhao, 2005). A critical signalling compound is the gonadotropin second messenger, cyclic AMP (cAMP), which is synthesized in the oocyte and in adjacent CC through the activation of the constitutively expressed transmembrane G-protein-coupled receptor (Racowsky, 1985; Hurk and Zhao, 2005; Marei *et al*., 2009). The newly synthesized cAMP stimulates the cAMP dependent protein kinase (PKA), which type I mediates the inhibitory action on oocyte GVBD, while type II regulates the meiosis-inducing pulse of cAMP occurring within CC

following hormonal stimulation (Downs *et al*., 2002). Simultaneously to PKA stimulation, protein kinase C (PKC) can also be activated, delaying meiotic progression and enhancing cytoplasmic maturation in porcine and bovine oocytes (Sun and Nagai, 2003; Ali and Sirard, 2005). PKC also participates in cortical granules exocytosis during the fertilization process (Wu *et al*., 2010).

The regulation of cAMP level in the maturating oocyte transiently delays nuclear progression (Racowky, 1985; Xia *et al*., 2000), thus allowing simultaneous cytoplasm enrichment in nutritive substances to support embryonic development. Furthermore, the activity of AMP-phosphodiesterase (PDE) within oocytes hydrolyses cAMP to AMP, inactivating PKA protein, which in turn provides a positive stimulus for oocyte nuclear maturation inducing GVBD (Downs *et al*., 2002) and maturation progression.

1.4.2.2. - *Implications of mitogen-activated protein kinase (MAPK) and maturation promoting factor (MPF) on nuclear maturation*

Other important regulators of oocyte meiosis are MAPK and MPF (Sun and Nagai, 2003). The MAPK pathway is activated by AMP that is synthesized from cAMP or enters the oocyte from the exterior (Downs *et al*., 2002). The MAPK can be activated by growth factors or by gonadotropin stimuli in CC to induce GVBD. This is observed in the porcine species, for which sites of activation can be located both in the GV or in the cytoplasm (Seger and Krebs, 1995; Downs *et al*., 2002; Sun and Nagai, 2003; Hurk and Zhao, 2005). Two isoforms of MAPK proteins were identified (ERK1 and 2) as being involved in the regulation of cell cycle and microtubule dynamics during metaphase organization. Their activity enhances in G1 through S and G2/M phases (Seger and Krebs, 1995; Marei *et al*., 2009). Furthermore, MAPK is implicated: in

retaining MII arrest, in regulating PDE action on cAMP degradation and in the maintenance of MPF activity (Downs *et al*., 2002; Hurk and Zhao, 2005).

The MPF is activated by cyclins and cyclin-dependent kinases following LH-surge. In the absence of the inhibitory influence of cAMP, MPF stimulates the oocyte to enter the M-phase and precede GVBD (Hurk and Zhao, 2005), thus being implicated in chromatin condensation, cytoskeletal reorganization, spindle formation and plasma membrane disassembly (Sun and Nagai, 2003). This complex protein is composed of cyclin B, the regulatory subunit, and p34cdc2 kinase, the catalytic subunit. The activity of MPF is regulated by cyclin B binding to Cyclin dependent kinase 2 (Cdc2), and by phosphorylation of threonine 161, dephosphorylation of tyrosine 15 and threonine 14 (Hurk and Zhao, 2005, Zhang *et al*., 2010). During porcine oocyte maturation, this activity appears initially at GVBD, reaches a high level at metaphase I and disappears transiently at the time of first polar body emission. Then, MPF activity reappears at MII and remains at an elevated level until fertilization (Dekel, 1995). Recent reports showed that the oscillatory activity of MPF during oocyte maturation depends on post-transcriptional regulation of cyclin B1 by cytoplasmic polyadenylation (Zhang *et al*., 2010). Moreover, a decreased expression of maternal cyclin B1 and Cdc2 was identified at mRNA or protein level in developmentally incompetent oocytes.

1.4.2.3. - *GSH, ATP and Ca^{2+} during cytoplasmic maturation*

In maturating oocyte cytoplasm, GSH is one of the most important factors synthesized, since it participates in the regulation of protein and DNA synthesis, in microtubule assembly, protection against oxidative damage and sperm nuclear decondensation and male pronuclear formation following fertilization (Hao *et al*., 2007). GSH can be

formed from precursor amino acids added during IVM, such as cysteine (Cui *et al*., 2009) and cysteamine (Sutton *et al*., 2003).

Simultaneously to GSH utilization, alterations in ATP and Ca^{2+} levels have been related to developmental competence of porcine oocytes. The inhibition of GSH synthesis was shown to induce a decrease in the duration and amplitude of intracellular free Ca^{2+} rise as well as ATP depletion (Cui *et al*., 2009). These alterations in intracellular Ca^{2+} stores are crucial in the regulation of oocyte capacity to undergo activation. Intracellular Ca^{2+} release is highly increased during the progression of nuclear maturation. In fact, the Ca^{2+} signal is required for full activation of MPF. This activation stimulates the oocyte to enter the M-phase, thus overriding the negative effect of cAMP (Krisher, 2004; Hurk and Zhao, 2005). Repetitive Ca^{2+} transients are further generated during fertilization to stimulate the emission of cortical granules released from smooth-membrane vesicles (Eppig, 1996; Kim *et al*., 1997; Krisher, 2004).

1.5. - The role of lipids during oocyte maturation and initial embryo development

Lipids are essential cellular compounds that play an important role as an energy source that is vital for proper mammalian oocyte maturation and succeeding embryo development (Romek *et al*., 2009). The most abundant intracellular lipids stored within porcine oocytes are triacylglycerols, representing approximately 46% (w/w) of total FA in the oocyte (Homa *et al*., 1986; McEvoy *et al*., 2000). These can be utilized in mitochondrial β-oxidation to produce energy during oocyte maturation (Sturmey and Leese, 2003; Sturmey *et al*., 2006). Furthermore, the enrichment in phospholipids and cholesterol during oocyte maturation is crucial to form membranes during rapid cell divisions after oocyte fertilization (McEvoy *et al*., 2000).

Second messengers reflect other phospholipid roles in cell function and, thereby, in oocyte maturation and embryo development. In porcine oocytes, phosphatidylinositol represents 6% of total phospholipids, being rich in arachidonic (20:4n-6) and stearic (18:0) acids and also in palmitic acid (16:0) (Homa *et al*., 1986). Hydrolysis of this membrane phospholipid yields two second messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. IP3 and its derivative (IP4) increase Ca^{2+} concentrations, while diacylglycerol stimulates PKC (McEvoy *et al*., 2000; Wu *et al*., 2006). The importance of Ca^{2+} and PKC in oocyte maturation and fertilization processes is already described above. Plasmalogens are another class of phospholipids identified in oocyte membranes (Homa *et al*., 1986; Prates *et al*., 2013). Moreover their composition might differ according to the oocyte maturation stage (immature and mature). Although reference was made to plasmalogens controlling the dynamics of cellular membranes (Nagan and Zoeller, 2001), their function during oocyte maturation and embryo development remains unknown.

Analysis of FA composition of porcine oocytes during maturation showed that 16:0, followed by oleic (*c*9-18:1), 18:0 were the most abundant FA registered in immature and mature oocytes, followed by n-6 polyunsaturated fatty acids (PUFA), specifically linoleic (18:2n-6) and 20:4n-6, which may indicate that oocytes are capable of synthesizing prostaglandins (PG) and leukotrienes (Homa et al., 1986; McEvoy et al., 2000; Prates *et al*., 2013). Prostaglandins, namely PGE_2 can be critical mediators of CC expansion and of oocyte meiosis resumption and progression (Marques *et al*., 1997, Nuttinck *et al*., 2008). Moreover, FA profile of oocytes can change due to culture media or dietary lipid supplementation and differences in oocyte FA composition were related to oocyte developmental competence (Lapa *et al*., 2011; Warzych *et al*., 2011). Although the role of lipids during oocyte maturation and initial embryo development

are currently under research, their primordial importance in female gamete quality is unequivocal.

1.6. - Oocyte quality and recent evaluation techniques

The use of morphological characteristics and metabolic predictors involved in COC maturation can provide valuable information for the preselection of high-quality oocytes to maximize embryonic developmental outcomes (Wang and Sun, 2007). The huge number of LD as well as their distribution and association to other organelles in the cytoplasm, eventually other cytoplasmic pigments, are responsible for the dark colour tone that characterizes the oocyte of some mammalian species, namely the porcine, bovine, equine or the minke whale (Fujihira *et al*., 2004; Genicot *et al*., 2005; Sturmey *et al*., 2006; Ambruosi *et al*., 2009). In addition, Cui *et al*. (2009) showed that the bright gray and uniform ooplasm in association with an intact plasma membrane were markers of better quality in porcine oocytes. During ageing of oocytes, mitochondrial distribution changed from diffuse to aggregate (Hao *et al*., 2007). Additionally, these authors referred that the shape of mitochondria changed from spherical to elongated and that LD became solidified concomitantly to modifications in oocyte homeostasis, namely in ATP content, Ca^{2+} rise and amplitude. These aged oocytes presented a dark colour and irregular ooplasm. Thus, these morphological characteristics can be used to predict oocyte quality. However, oocytes from different species may present different degrees of cytoplasmic transparency. While a dark granulated ooplasm is a normal characteristic in the pig, is not so in humans, where dark colour and cytoplasmic inclusions are related to low oocyte quality and fertility failure (Xia, 1997; Wang and Sun, 2007).

Moreover when measured by fluorescent light microscopic techniques, bovine, porcine and murine oocytes exhibit different amounts of emitted fluorescent light and which was correlated with the cytoplasmic lipid contents in these species (Genicot *et al*., 2005). Therefore, these microscopic techniques could be used to compare lipid contents of oocytes from different donors, sized follicles or cultured conditions, and also the oocyte quality within species. On the other hand, the gray mean value within the oocyte fat area was suggested as being another appropriate tool to evaluate the lipid content of a single porcine oocyte (Prates *et al*., 2012). This can be a useful non invasive technique, that upon record of oocyte morphology allows for its posterior use, namely for cryopreservation or fertilization. The possibility of posterior use is very important in humans or endangered species due to the limited number of available oocytes.

Other criteria besides morphological characterization, such as metabolic markers may be useful to evaluate oocyte quality and fertilization potential (Sturmey and Leese, 2003). *In vitro*, oocyte competence can be assessed by the brilliant cresyl blue, a dye that can be reduced by the glucose-6-phosphate, which is an enzyme that is characteristic of full grown and more competent oocytes (Antosik *et al*., 2010; Warzych *et al*., 2011). This test has been used in the porcine species to evaluate oocyte quality after modifications were made to maternal diet and thus in the FA profile of follicular fluid (Warzych *et al*., 2011). Interestingly, the FA profile in women changes in early mid-life and these changes have been implicated in the decline of fertility that commences before age 35 (Ford and Tavendal, 2009). Furthermore, the evaluation of other enzyme function, such as delta-9 and delta-5 desaturase, genetic expression monitorization by real-time PCR or gas chromatography analysis are among several other techniques that can be used to complete the information about oocyte quality that

seems to be closely related to its lipid composition (Ford and Tavendal, 2009; Ouandaogo *et al*., 2011; Warzych *et al*., 2011; Prates *et al*., 2012).

1.7. - The manipulation of meiotic and cytoplasmic maturation

1.7.1. - *Meiotic inhibitors*

In order to improve developmental outcomes of oocytes known to prematurely undergo nuclear maturation, pharmacological tools that elevate intracellular cAMP levels and transiently delay spontaneous GVBD, are frequently used (Laforest *et al*., 2005; Gilchrist and Thompson, 2007; Wang *et al*., 2009). These meiotic inhibitors are cAMP analogues, such as dibutyryl cAMP, adenylate cyclase activators like FSH and forskolin, PDE inhibitors as the non-specific inhibitor IBMX, the PDE type 4-specific inhibitor rolipram or the PDE type 3-specific inhibitors milrinone, cilostamide or Org9935 (Laforest *et al*., 2005; Gilchrist and Thompson, 2007). Their dose and effects are species dependent. For example, when compared to other species, porcine oocytes exhibit relatively high sensitivity to forskolin elevating cAMP levels (Racowsky, 1985). On the other hand, inhibitors of p34cdc2/ cyclin B kinase such as roscovitine, can also be used to delay nuclear maturation and thus promoting porcine oocyte competence acquisition (Coy *et al*., 2005).

1.7.2. - *Mechanisms of action of meiotic inhibitors.*

The utilization of meiotic modulators (alone or simultaneously) has proven to induce an effective delay in GVBD and simultaneously an extension in oocyte-CC gap-junctional communication during the meiotic resumption phase, allowing for continued mRNA and protein accumulation within the ooplasm (Xia *et al*., 2000; Laforest *et al*., 2005; Gilchrist and Thompson, 2007). Different incubation times using the meiotic inhibiting

agent are possible, after which the inhibitor is removed and the maturation process proceeds. Forskolin is an adenylate cyclase activator that also has lipolytic properties, interfering with LD morphology and thus with oocyte cytoplasmic maturation (Fu *et al*., 2011; Prates *et al*., 2012). In fact, the meiosis progression of porcine oocytes cultured with 10 µM of forskolin during half or the entire period of IVM was inhibited. Moreover their cytoplasmic morphology resembled that of oocytes in the immature stage, without the redistribution of LD (fat area) characteristic of mature ones (Prates *et al*., 2012). Nevertheless, by decreasing supplementation time to the initial 2 h, oocyte meiotic competence was not affected, although forskolin still interfered with organelles dynamics and thus with cytoplasmic maturation.

1.7.3. - *Synchronization of nuclear and cytoplasmic maturation by meiotic inhibitors.*

Asynchronous or incomplete oocyte maturation has been indicated as a common phenomenon in the porcine species that can predispose oocytes to multiple sperm penetration through the zona pellucida into the cytoplasm before the block to polyspermy (Hunter, 1990; Grupen *et al.*, 1997; Laforest *et al*., 2005). Moreover, *in vitro* culture conditions during fertilization can be a cause for polyspermy (Funhashi and Romar, 2004; Almiñana *et al*., 2005). *In vivo*, this pathological situation leads to the formation of polyploidy embryos that usually die at a very early stage of development (Hunter, 1990). The changes in oviduct fluid composition, spermatozoa concentration, interval between mating and fertilization that includes the period of spermatozoa capacitation as well as the functional state of oocyte cortical granules can all account for polyspermy. Although extensive attempts have been made to reduce the penetration of porcine oocytes by more than a single spermatozoon, the high incidence of polyspermy remains a major obstacle in porcine ART (Hunter, 1990; Almiñana *et al*., 2005). This

problem is also present in aged female gametes of several species, including humans (Kikuchi *et al*., 2002; Hao *et al*., 2007; Wang and Sun, 2007). The utilization of meiotic inhibitors can improve oocyte competence and it can therefore partially resolve the problem of polyspermic fertilization. However, this is also related to *in vitro* culture conditions during IVF. Thus, further research is needed to improve the efficiency of IVF systems, especially in porcine, to produce good quality embryos.

1.8. - *Lipid modulators*

Lipid modulators are substances that are capable of reducing and/or modifying intracellular lipid content of cells that have been successfully applied in ART. The *trans*-10,*cis*-12 conjugated linoleic acid (*t*10,*c*12 CLA) is one of these substances, being capable of interfering with lipid accumulation and metabolism in porcine adipose explants, as well as in bovine oocytes and embryos (José *et al*., 2008; Pereira *et al*., 2007; Lapa *et al*., 2011). Forskolin was also able to reduce lipid content in porcine oocytes and embryos through lipolysis stimulation (Men *et al*., 2006; Fu *et al*., 2011). In a recent experiment, different incubation times (44 h, 22 h and 2 h) with *t*10,*c*12 CLA or forskolin interfered on oocyte lipid content showing that supplementation time affected the distribution and morphology of LD (Prates *et al*., 2012). These authors reported that the fat area became lighter in the presence of *t*10,*c*12 CLA, while in forskolin treated oocytes the fat area was reduced and meiosis delayed beyond 2 h of supplementation. Moreover, as referred, the supplementation time of forskolin during IVM of porcine oocytes can impair fertilization since it delays meiotic progression and oocyte growth (Prates *et al*., 2012). Another lipid modulator that has been used to improve ART results is phenazine ethosulfate (PES). PES supplementation during embryo culture increased

glucose metabolism and tended to increase the PPP flux of glucose, with a clear reduction in the accumulation of lipids (De La Torre-Sanchez *et al*., 2006).

1.8.1. - *Mechanisms of action of lipid modulators*

The use of adenylyl cyclase activators such as forskolin during porcine oocyte culture induces alterations in both, nuclear and cytoplasmic maturation, these latter being related to the reduction of LD containing area (Prates *et al*., 2012). As referred to above, the cytoplasmic maturation in the developing oocyte implies LD movements that can induce LD coalescence and thus morphology modifications. Moreover, by stimulating lipolysis, intracellular LD content may also be modified due to shrinkage (Thiele and Spandl, 2008). Once a lipolytic substance, such as forskolin (Figure 1.4.), binds to the catalytic subunit of the adenylyl cyclase enzyme, the cAMP is hydrolysed from the available ATP in the cytoplasm and the cAMP level is increased, thus activating PKA protein (Xia *et al*., 2000; Holm, 2003). This protein phosphorylates endogenous lipases, as the hormone-sensitive lipase (HSL) and also perilipin protein located at the LD surface (Holm, 2003). Following phosphorilation, HSL is translocated to the cytoplasm where it binds to LD surface protein to induce fragmentation of large droplets in smaller ones, thus increasing accessible droplet surface and the degradation of its core (Holm, 2003; Thiele and Spandl, 2008). In the lipolysis of intracellular lipids, HSL catalyses triglycerides and diglycerides, while monoglyceride lipase is required to obtain complete hydrolysis of monoglycerides (Holm, 2003; Men *et al*., 2006; Thiele and Spandl, 2008; Walther and Farese, 2008). The supplementation of porcine COC culture medium with forskolin, during the initial 2 h of IVM, influenced both oocyte and their CC FA and plasmalogens (measured by dimethylacetal, DMA) composition, although their total contents were not affected (Prates *et al*., 2013). Depending on dose and

exposure time, forskolin treatment may induce a higher modification in intracellular lipids (Fu *et al*., 2011; Prates *et al*., 2012). Besides acylglycerols, HSL can hydrolyse cholesterol and steroid esters (Holm, 2003). The meiosis-activating sterols system was also referred to be stimulated by the MAKP pathway following AMP level rise due to cAMP hydrolysis (Sutton *et al*., 2003). Therefore, sterol metabolism might be affected by the utilization of lipolytic agents during oocyte maturation, such as adenylyl cyclase stimulators.

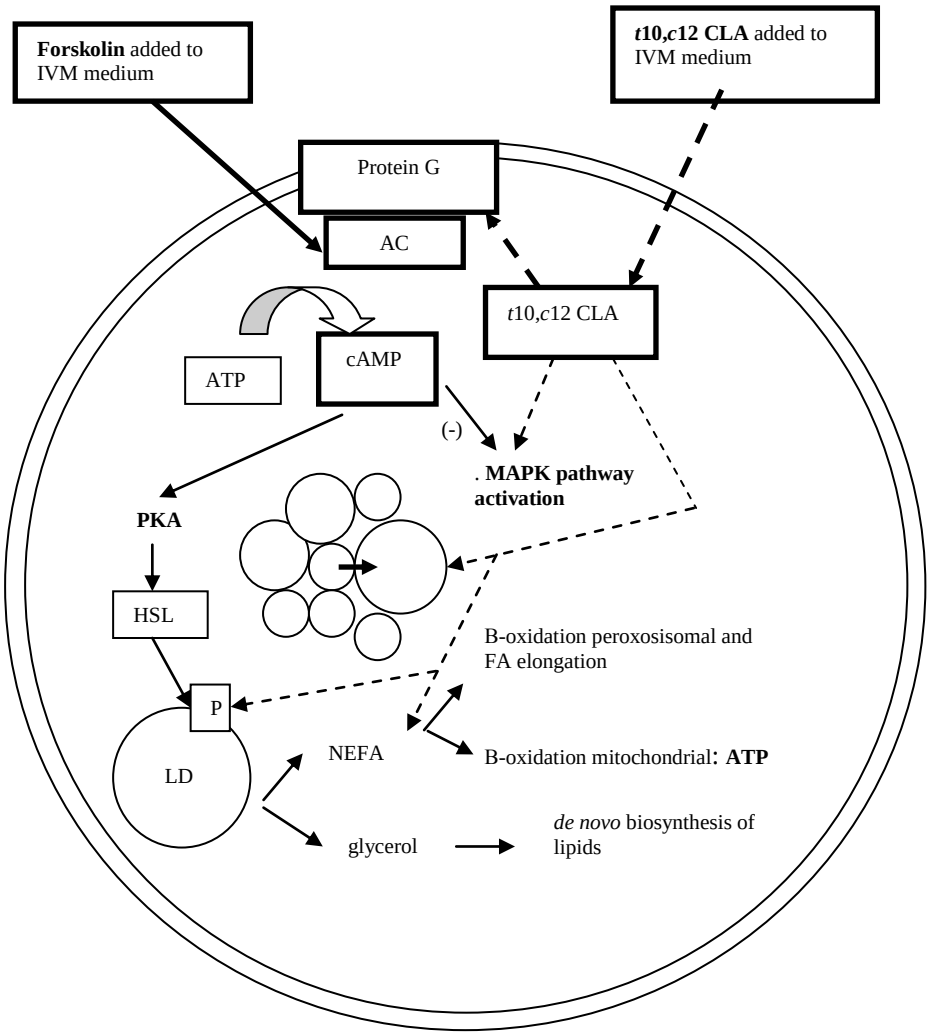

Figure 1.4. – A model for possible effects of forskolin and *trans*-10,*cis*-12 conjugated linoleic acid on regulation of oocyte maturation and metabolic pathways: stimulation of cAMP intracellular levels and PKA or MAPK pathways; other potential mechanisms for CLA action are also illustrated with dashed arrows. PKA, protein kinase A; MAPK, mitogen protein kinase; AC, adenylyl cyclase; HSL, hormone sensitive lipase; MGL, monoglyceride lipase; P, perilipin protein; LD, lipid droplet; NEFA, non-esterified fatty acids. See text for details.

of embryonic development (Men *et al*., 2006; Walther and Farese, 2008). Simultaneously, free FA may be used to produce energy through the β-oxidation pathway, to boost maturation progression as in porcine oocytes (Sturmey and Leese, 2003). On the other hand, *t*10,*c*12 CLA added during the culture of pig adipose tissue interfered with lipogenesis, decreasing the FA synthase activity and the rate of C-labelled glucose incorporation, but had no effect on non-esterified FA release (José *et al*., 2008). However, when porcine COC were matured with 100 μM *t*10,*c*12 CLA, this isomer was accumulated in both, oocyte and CC, changing their FA profiles, mostly in CC (Prates *et al*., 2013). The *t*10,*c*12 CLA appears to affect the PKA signal transduction pathway and thus, the cAMP cascade of reactions (Figure 1.4.) (Ashwell *et al*., 2010). These authors showed that the expression of four genes from the PKA transduction pathway were down regulated while four genes were up regulated by *t*10,*c*12 CLA supplementation. On the other hand, an increase in lipolysis and in cytosolic perilipin associated with smaller LD was identified in human adipocytes cultured in the presence of this isomer (Chung *et al*., 2005). It is then possible that PKA and MAPK/ ERK pathways may be regulated by *t*10,*c*12 CLA, thus interfering with LD lipolysis (Figure 1.4.). The analysis of FA and DMA composition of porcine COC showed that independently from cell type, CLA treatment reduced the proportions of several individual FA and plasmalogens DMA-16:0, 16:1c9, 18:3n-6, and tended to reduce *c*7-16:1, *c*11-18:1 and 20:4n-6 (Prates *et al*., 2013). Furthermore, it increased the concentration of 22:6n-3 in the maturation media at the end of the maturation period, suggesting that this FA might have been elongated during maturation and exported, to maintain oocyte PUFA content to avoid the generation of reactive oxygen species (ROS). Indeed, CLA may follow different pathways once it is available in the cell (Banni, 2002). CLA may then enter in mitochondrial β-oxidation to produce energy for

maturation proceeds or FA synthesis in ER and ER-Golgium complexes (Sturmey and Leese, 2003; Thiele and Spandl, 2008). It is possible that *t*10,*c*12 CLA might have entered the oocyte by pinocytosis and accumulated in LD, thus interfering with its colouration under microscopic observation and LD movements during maturation (Prates *et al*., 2012). The mechanisms of action of *t*10,*c*12 CLA on oocyte and embryos needs to be further studied.

Alternatively, PES is another lipid modulator that stimulates different metabolic pathways. PES increases glucose metabolism through PPP during embryo culture (De La Torres-Sanchez *et al*., 2006). Hence, PES is a strong electron acceptor that readily oxidizes NADPH oxidization to NADP+, thus decreasing NADPH required for the synthesis of numerous lipids, particularly long-chain fatty acids, and reducing intracellular lipid content of embryos (Seidel, 2006).

1.8.2. - *Lipid content reduction applied for cryopreservation of oocytes and embryos*

Considerable progress has been made in improving and simplifying oocyte and embryo cryopreservation procedures to be routinely used in transfer programs. In general, cryopreservation by slow freezing is a process where extracellular water crystallizes, resulting in osmotic gradient that draws water from the intracellular compartment until intracellular vitrification occurs (Pereira and Marques, 2008; Saragusty and Arav, 2011). On the other hand, in cryopreservation through vitrification, both intra and extracellular compartments vitrify after cellular dehydration has already occurred (Saragusty and Arav, 2011). These cryopreservation techniques have been improved to minimise damage and help oocytes and embryos of different developmental stages to regenerate by several strategies: using microsurgical manipulation, cytoskeletal relaxants (such as cytochalasin B or D), membrane and protein stabilisers,

centrifugation, adjusting the concentration of cryoprotectors and/or reducing the cooling volume to a minimum (Esaki *et al*., 2004; Cuello *et al*., 2008; Gerelchimeg *et al*., 2009; Fu *et al*., 2011). Nevertheless, the success rate is still limited particularly in oocytes.

The plasma membrane of oocytes and embryos is the first cellular structure whose integrity is affected by thermotropic phase transition (Nagashima *et al*., 1994; Arav *et al*., 1996). During cooling, irreversible damage occurs shortly after exposure to low, but not freezing temperatures just below 15 ºC (Nagashima *et al*., 1994). In oocytes, the greater cellular volume and higher cytoplasmic lipid content increase chilling sensitivity when compared to embryo cells (Arav and Zvi, 2008). Furthermore, the less submembranous actin microtubules present in oocytes accounts for a less robust membrane and thus, cryopreservation can cause disorganization of cytoskeleton and meiotic spindle, as well as chromosome and DNA abnormalities (Fujihira *et al*., 2004; Arav and Zvi, 2008). Changes in cellular chemical composition and LD association to other organelles or to the cytoskeleton were also identified in GV oocytes and phase separations in the plasma and/or internal membranes in embryos (Nagashima *et al*., 1994; Arav *et al*., 1996; Fujihira *et al*., 2004). Differences in LD colour tone of fresh immature and vitrified-warmed porcine oocytes were identified: gray in fresh and slightly dark in vitrified oocytes (Fujihira *et al*., 2004). However, LD size or distribution was similar. On the contrary Isachenko *et al*. (2003) showed that the two types of LD found in porcine oocytes, dark and 'gray', changed their morphology during cooling into a spherical form with lucent streaks impairing oocyte developmental competence.

As referred above, the high lipid content that has been related to an increased sensitivity to chilling injury during cryopreservation is particularly important in porcine species (Fujihira *et al*., 2004; Romek *et al*., 2009; Fu *et al*., 2011). Changing the lipid content of porcine embryos by removing LD may have a direct effect on embryo survival during

chilling (Nagashima *et al*., 1994; Hara *et al*., 2005). This process can be performed by mechanical delipidation through polarization of the cytoplasmic LD and subsequent physical removal of excess lipid, increasing the survival rates of cryopreserved embryos (Nagashima *et al*., 1994; Esaki *et al*., 2004; Pereira and Marques, 2008). As an alternative to such invasive techniques that can damage the cellular structure, it is possible to improve the success of cryopreservation of *in vitro* produced embryos by eliminating serum from the culture medium, or by inducing chemical delipidation through metabolic manipulation (Seidel 2006; Romeck *et al*., 2009). Indeed, it has been demonstrated that adding *t*10,*c*12 CLA to serum-containing media during *in vitro* culture of bovine embryos reduced lipid accumulation and significantly improved blastocyst survival following cryopreservation (Pereira *et al*., 2007 and 2008). In oocytes, *t*10,*c*12 CLA was shown to interfere with lipid metabolism, both in bovine and porcine species, reducing lipid content in porcine species (Lapa *et al*., 2011; Prates *et al*., 2012). Furthermore, by reducing the lipid content of porcine oocytes and embryos, forskolin was shown to increase cryosurvival following vitrification (Men *et al*., 2006; Fu *et al*., 2011). In the light of the above, other metabolic modulators, namely PES, can be used to reduce cytoplasmic lipid content improving cryosurvival of bovine embryos (Barceló-Fimbres and Seidel, 2007). However, the use of PES during *in vitro* culture had a limited effect on porcine blastocyst survival after vitrification. Nevertheless, PES increased the proportion of morula and blastocyst formation, reducing the index of DNA fragmentation and the cytoplasmic lipid content of cultured blastocysts (Gadja *et al*., 2011). Further studies are needed to broaden the knowledge of this promising approach to improve ART success.

1.9. - Future perspectives

The lipid content of porcine oocyte as well as the asynchrony between nuclear and cytoplasmic maturation renders it a good model in the field of oocyte biology research. Knowledge of the pathways and key molecules regulating these processes may highlight therapeutic possibilities to prevent the excessive accumulation or to modulate lipid composition in cytoplasmic droplets. In the future, lipid modulators may also be applied in germplasm cryopreservation to improve livestock production. Further studies are needed to clarify its potential to avoid disorders in lipid metabolism and its relation to infertility.

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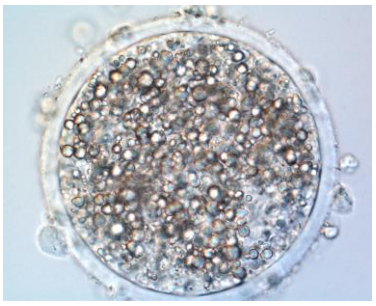

CHAPTER 2

FAT AREA AND LIPID DROPLET MORPHOLOGY OF PORCINE OOCYTES DURING *IN VITRO* MATURATION WITH *TRANS*-10, *CIS*-12 CONJUGATED LINOLEIC ACID AND FORSKOLIN

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Fat area and lipid droplet morphology of porcine oocytes during *in vitro* maturation with *trans*-10, *cis*-12 conjugated linoleic acid (*t*10,*c*12 CLA) and forskolin

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2.1. - Abstract

Lipid droplets (LD) in porcine oocytes form a dark mass reaching almost all cytoplasm. Herein we investigated changes in fat areas, cytoplasmic tone and LD morphology during *in vitro* maturation (IVM) of porcine oocytes cultured with 100 µM *t*10,*c*12 CLA or 10 µM forskolin in different time periods. Four groups were constituted: control, excipient, *t*10,*c*12 CLA and forskolin, drugs being supplemented during 44-48 h and the initial 22-24 h in exp.1 and 2, respectively. In exp.3, forskolin was supplemented for the first 2 h. Matured oocytes were inseminated with frozen-thawed boar semen and cleavage rate recorded. Prior to and during IVM, samples of oocytes were evaluated for LD, total and fat areas and fat gray value or for meiotic progression. Results showed that forskolin supplementation during 44-48 h or 22-24 h inhibits oocyte maturation (exp.1: forskolin = 5.1 ± 8.0%, control = 72.6 ± 5.0%; exp.2: forskolin = 24.3 ± 7.4%,

control = 71.6 ± 5.6%) and cleavage (exp.1: forskolin = 0.0 ± 0.0%, control = 55.4 ± 4.1%; exp.2: forskolin = 8.3 ± 3.3%, control = 54.5 ± 3.0%). Forskolin also reduced oocyte and fat areas. In exp.3, forskolin negative effect on oocyte maturation and cleavage disappeared though minor ($P \leq 0.03$) LD and oocyte fat areas were identified at 22-24 h of IVM. Oocytes supplemented with *t*10,*c*12 CLA during 44-48 h presented a lighter ($P \leq 0.04$) colour tone cytoplasm than those of control and forskolin. In conclusion *t*10,*c*12 CLA and forskolin were capable of modifying the distribution and morphology of cytoplasmic LD during porcine oocyte maturation thus reducing its lipid content in a time dependent way.

Keywords: lipid droplets; porcine oocyte maturation; lipid content; conjugated linoleic acid; forskolin.

2.2. - Implications

Swine is relevant for agriculture and livestock economy. *In vitro* embryo production can be an effective system for boar fertility evaluation and germoplasm cryopreservation but also for biomedical research. However, nuclear and cytoplasmic maturation asynchrony as well as the high intracellular lipid content in porcine oocyte are still limiting factors for the application of such biotechnologies in this species. The present work increases understanding on the effect of two substances, *trans*-10, *cis*-12 conjugated linoleic acid and forskolin, capable of lipid modulating to improve oocyte developmental competence and embryo yield.

2.3. - Introduction

Pigs are important not only as livestock, but also as an experimental model for humans in biomedical research. Obesity and its physiological consequences are increasingly prevalent among women of reproductive age and are associated with infertility being porcine oocytes and embryos excellent models to study this paradigm (Gesink *et al.*, 2007; Wang *et al.*, 2009). The negative effect of an excessive intracellular lipid content of porcine oocytes and embryos on assisted reproductive technologies has been extensively reported (Dobrinsky *et al.*, 1999; Men *et al.*, 2006; Pereira and Marques, 2008). LD are organelles constituted primarily by triacylglycerols and cholesteryl esters surrounded by a monolayer of phospholipids with embedded integral and peripheral proteins, occupying a considerably mass in the eukaryotic cells, particularly in the mammalian oocytes and embryos (Fujihira *et al.*, 2004; Walther and Farese, 2009; Zehmer *et al.*, 2009). LD in porcine oocytes form a dark mass reaching almost all cytoplasm, but their function and importance are scarcely known. This dark appearance of porcine oocytes and embryos, compared to others species, has been attributed to an increased lipid content (McEvoy *et al.*, 2000; Genicot *et al.*, 2005). On the other hand, the intermediate filaments of the cytoskeleton interact directly with these special organelles (Zehmer *et al.*, 2009). Therefore it is not surprising that during gametes and embryos cryopreservation, morphological variations occurring in LD as consequence of physical changes of lipids, lead to irreversible damages in the cytoskeleton (Fujihira *et al.*, 2004; Pereira and Marques, 2008).

Recently, two chemicals, *trans*-10, *cis*-12 conjugated linoleic acid (*t*10,*c*12 CLA) and forskolin, intracellular lipid modulators, were shown to improve porcine and bovine embryos survival after cryopreservation (Men *et al.*, 2006; Pereira *et al.*, 2007, 2008). Indeed, *in vitro* produced bovine embryos cultured with 100 µM *t*10,*c*12 CLA were

characterized by a reduced lipid accumulation and an improved blastocyst cryosurvival (Pereira *et al.*, 2007 and 2008). Moreover, alterations in the lipid profile of bovine oocytes matured with this CLA isomer were associated with an enhanced blastocyst quality (Lapa *et al.*, 2011). In pig, *t*10,*c*12 CLA exerts specific effects on adipocytes by diminishing fat deposition through lipogenesis reduction, lipolysis and fatty acids oxidation increase (Corino *et al.*, 2006). Although this CLA isomer has never been tested in porcine oocytes, it is expected to induce alterations in cytoplasmic lipid content during IVM.

Forskolin, an adenylyl cyclase stimulator, at 10 µM dose is an effective lipolytic substance capable of improving oocyte and embryo cryosurvival (Men *et al.*, 2006; Fu *et al.*, 2011). However, despite the contribution of forskolin to lipid reduction, it can simultaneously arrest meiotic resumption during porcine oocytes IVM in a dose dependent response. At 10 µM dose, the meiotic arrest was fully reversible (Laforest *et al.*, 2005). Moreover by delaying spontaneous meiotic resumption, forskolin can increase oocyte development potential due to a better synchronization of nuclear and cytoplasmic maturation (Thomas *et al.*, 2004). Thus, the possibility of embracing both forskolin effects on meiosis synchronization and lipid content reduction in porcine oocytes should be investigated using different supplementation time periods.

The present study investigates the effect of supplementing maturation media of porcine oocytes with 100 µM *t*10,*c*12 CLA or 10 µM forskolin, during different time periods, on oocyte intracellular lipid content and maturation process. The developmental potential of those oocytes for cleavage was also assessed.

2.4. - Materials and Methods

2.4.1. - *Oocyte recovery and IVM*

Otherwise stated, all products were purchased from Sigma, Sintra, Portugal. Prepubertal gilts ovaries were obtained from a local slaughterhouse. At the laboratory, medium-sized follicles (3–6 mm) were aspirated, cumulus–oocyte complexes (COC) collected and washed in Dulbecco's phosphate-buffered saline medium (mDPBS) (Gil *et al.*, 2003). Oocytes with a dark, evenly granulated cytoplasm and surrounded by a compact cumulus cells mass were selected and washed in maturation medium, the BSA-free North Carolina State University medium (NCSU23; Petters and Wells, 1993) supplemented with 10% (v/v) porcine follicular fluid, 0.1 mg/mL cysteine, 10 ng/mL epidermal growth factor and 100 µM glutathione (GSH). Oocytes were cultured in pre-equilibrated maturation medium (3 mL) containing 10 IU/mL equine chorionic gonadotrophin (eCG; Intervet, Portugal) and 10 IU/mL human chorionic gonadotrophin (hCG; Pregnyl, Organon, Portugal) for 22–24 h and then for another 22–24 h without hormones, at 39ºC in an atmosphere of 5% CO_2 in air.

2.4.2. - *Fertilization and embryo culture*

In vitro fertilization and embryo culture were performed according to Gil *et al.* (2003). Briefly, denuded oocytes were washed in modified Tris-buffered fertilization medium. Oocytes were then placed in 50 µL droplets of this medium under mineral oil (30 oocytes/ drop). Frozen-thawed boar sperm (100 µL) were washed in mDPBS by centrifugation (1900 g/3 min). The sperm pellet was resuspended in fertilization medium to obtain a final concentration of 1×10^6 spz/mL and added to oocytes. After 6 h of co-incubation, presumptive zygotes were washed in embryo culture medium

(NCSU23 with 0.4% BSA) and placed in a 4-well multidish for embryo culture during 48 h at 39ºC and 5% CO_2 in humidified air.

2.4.3. - *Assessment of oocyte maturation and cleavage rates*

Meiotic progression assessment was performed at 0 h, 22-24 h and 44-48 h of IVM. COC were gently pipetted in a 1% sodium citrate solution to remove cumulus cells. Denuded oocytes were mounted on slides, fixed in an acetic acid : ethanol solution (1:3) for 48–72 h, then stained with 1% lacmoid in 45% (v/v) acetic acid prior to examination under a phase-contrast microscope (×400). Oocytes were classified according to their chromosome configuration as: germinal vesicle (GV); germinal vesicle breakdown (GVBD) till metaphase-I (MI); and anaphase to metaphase-II (MII). Oocytes in the latest phase were considered matured. Fixed oocytes presenting a broken oolemma or abnormal cytoplasmic appearance were discharged.

Cleavage rates (cleaved embryos/inseminated oocytes ×100) were assessed at 48 h after oocyte insemination.

2.4.4. - *Lipid content evaluation*

Fresh oocyte samples, prior to IVM (0 h), at 22-24 h and 44-48 h of IVM were examined under Nomarski differential interference contrast microscope for lipid content evaluation (Pereira *et al.*, 2008). Oocytes were photographed using an Olympus camera attached to the microscope and analyzed using ImageJ software (Version 1.44q, National Institutes of Health, USA). One hundred LD randomly distributed at different focal planes were evaluated per oocyte. Total area occupied by LD, denominated fat area, and the gray mean value within fat area of each oocyte were also measured with the same equatorial focal plane, brightness and contrast. This mean value was calculated

by converting each pixel to a gray scale by ImageJ software (Figure 2.1.). Based on the gray mean value within fat area at 44-48 h of IVM, oocytes were classified into two categories: dark (< 112 pixels/μm^2) or light (> 112 pixels/μm^2) gray. In addition, cytoplasmic and total (including zona pellucida) areas of each oocyte were recorded. Fat oocyte index was calculated (fat area/total oocyte area ×100).

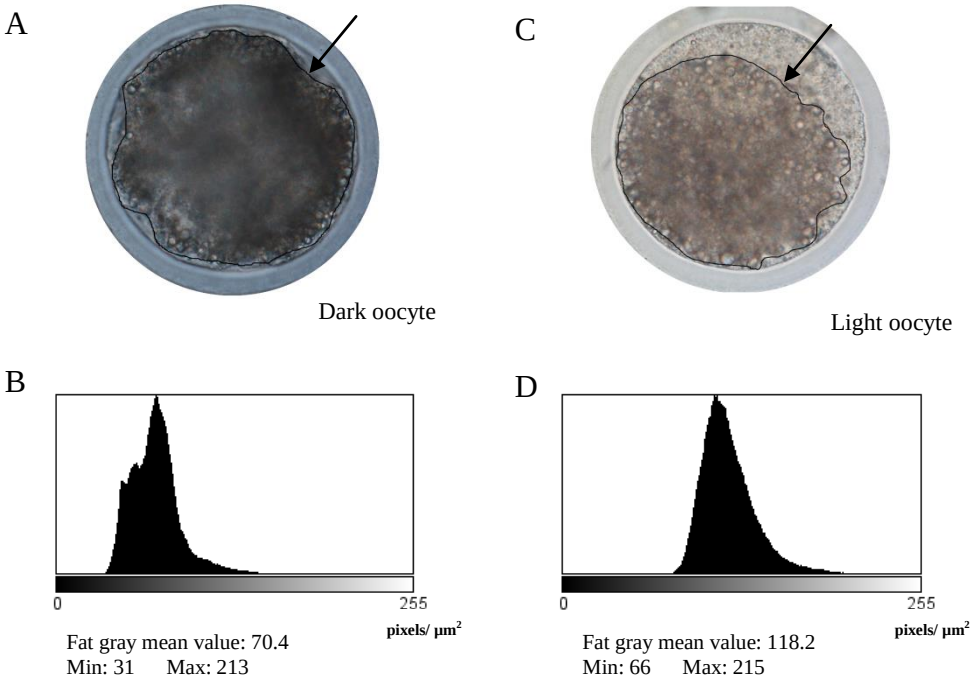

Figure 2.1. - Fat areas (arrows) of porcine oocytes evaluated by their gray mean values after Nomarski microscopy observation at the equatorial plan: a dark oocyte from control group after 44-48 h of *in vitro* maturation (A) and the correspondent gray value histogram (B); a light oocyte from the *trans*-10, *cis*-12 conjugated linoleic acid treatment after 44-48 h of *in vitro* maturation (C) and the correspondent gray value histogram (D). Gray scale: 0 pixels/μm^2– black to 255 pixels/μm^2– white.

2.5. - *Experimental Design*

Three experiments (3 replicates each) were designed. *Trans*-10, *cis*-12 octadecadienoic acid (100 μM *t*10,*c*12 CLA, Matreya, Pleasant Gap, USA; Pereira *et al.*, 2007) and forskolin (10 μM, Men *et al.*, 2006) were first diluted in ethanol due to its hydrophobicity and added to oocytes in the maturation media using different

supplementation times according to the following experiments. The final concentration of ethanol was adjusted to 0.1% in all treatment groups.

2.5.1. - *Experiment 1*

The objective of this experiment was to study the effect of *t*10,*c*12 CLA and forskolin, administered during total IVM period on porcine oocyte lipid content, maturation and developmental potential Four experimental groups were designed: (1) Control– COC (n = 373) were matured during 44-48 h without supplementation; (2) Ethanol– COC (n= 337) were matured during 44-48 h supplemented with 0.1% ethanol; (3) t10,c12 CLA– COC (n = 322) were matured during 44-48 h with 100 μM *t*10,*c*12 CLA, (4) Forskolin– COC (n = 353) were matured during 44-48 h with 10 μM forskolin. After maturation, COC were inseminated (n=816) with frozen-thawed boar semen and cleavage rates recorded. At 22-24 h and 44-48 h of IVM, samples of oocytes were assessed for meiosis progression (n=493) and lipid content evaluation (n = 76). Immature oocytes (n = 97, 87 for meiosis progression and 10 for lipid content evaluation, respectively) were also evaluated.

2.5.2. - *Experiment 2*

In Experiment 2, supplementation period of 100 μM *t*10,*c*12 CLA and 10 μM forskolin was reduced to the first 22-24 h followed by 22-24 h of standard protocol of IVM and their effect on porcine oocyte lipid content, maturation and developmental potential also studied. Four experimental groups were also established: (1) Control– COC (n = 373) were matured during 44-48 h without supplementation; (2) Ethanol– COC (n = 432) were matured during the initial 22-24 h with 0.1% ethanol; (3) *t*10,*c*12 CLA– COC (n = 330) were matured with 100 μM *t*10,*c*12 CLA during the initial 22-24 h; (4) Forskolin–

COC (n = 354) were matured during the initial 22-24 h with 10 μM forskolin. After 44-48 h of maturation, COC were inseminated (n = 908) and cleavage rates recorded. At 22-24 h and 44-48 h of IVM samples of oocytes were assessed for meiosis progression (n = 506) and lipid content evaluation (n = 75). Immature oocytes (n = 97) were also evaluated.

2.5.3. - *Experiment 3*

In the previous experiments, supplementation periods of forskolin showed deleterious effects on oocyte maturation and cleavage rates. A third experiment was performed to investigate if a time reduction in forskolin supplementation up to the initial 2 h of IVM could avoid these effects, simultaneously maintaining its lipolytic effects. *t*10, *c*12 CLA and ethanol were not tested in this trial, since in previous experiments the former did not interfere with oocyte meiotic progression and the latter did interfere neither with oocyte meiotic progression nor with lipid content. Therefore, two experimental groups were performed: (1) Control– COC (n = 291) were matured without supplementation; (2) Forskolin– COC (n = 299) were matured with 10 μM forskolin during the initial 2 h culture. After 44-48 h of maturation, COC (n = 238) were inseminated and cleavage rates recorded. At 22-24 h and 44-48 h of IVM samples of oocytes were assessed for meiosis progression (n = 307) and lipid evaluation (n = 45). Immature oocytes (n = 103) were also evaluated.

2.6. - *Statistical analysis*

All results are expressed as least square means (± standard error of means in text). In each experiment, data representing 3 replicates of cleavage and oocyte nuclear configuration as well as of oocyte lipid content evaluation were analysed using the

MIXED procedure of Statistical Analysis System (SAS Institute, Cary, NC, USA). The mixed linear model included treatment or treatment and IVM time periods (0 h, 22-24 h and 44-48 h) as fixed effects for cleavage and oocyte nuclear configuration evaluation respectively, and replicates as random effect. For lipid content evaluation, treatment and IVM time periods were also considered fixed effects but the oocyte nested in treatment was the random effect. LD were treated as repeated measures within oocyte. When significant effects were identified differences between individual treatments were determined by the PDIFF multiple comparison procedure.

Data from Nomarski records were analysed using a multiple regression analysis (proc Path of SAS), testing if fat gray tones were correlated to other individual or associated variables. An appropriate multicolinearity test (Proc Reg) was performed. The Chi-square test (Proc Freq) was used to compare the distribution of oocytes into categories (dark and light gray) of fat gray value. Differences were considered significant when $P \leq 0.05$.

2.7. - Results

Fat gray value was independent from oocyte total (r = 0.07, $P = 0.48$), fat (r = -0.02, $P = 0.84$) and LD (r = -0.04, $P = 0.58$) areas and fat oocyte index (r = -0.14, $P = 0.10$). Instead, fat area and fat oocyte index, were highly correlated (r = 0.81, $P < 0.0001$). This association explained 4% of fat gray value (tolerance value = 0.35, variance inflation = 2.8). The path coefficient from fat area to fat oocyte index was + 1.50.

2.7.1.- *Experiment 1*

Data from meiosis progression of porcine oocytes supplemented with *t*10,*c*12 CLA and forskolin during 44-48 h of IVM are represented in Table 2.1.. As expected, the GV

configuration was predominant in immature oocytes. At 22-24 h of IVM, 93.4 ± 7.9% ($P < 0.0001$) of oocytes treated with forskolin remained at GV whereas oocytes from control, ethanol and *t*10,*c*12 CLA treatments mainly reached GVBD-MI ($P < 0.0001$). Moreover at the end of IVM, only 5.1 ± 5.1% of forskolin treated oocytes reached Anaph-MII compared to 72.6 ± 5.0%, 65.1 ± 5.1% and 61.0 ± 5.0% from control, ethanol and *t*10,*c*12 CLA treatments ($P < 0.0001$) respectively. At each time period, no differences ($P > 0.05$) were identified in nuclear configuration among oocytes from control, ethanol and *t*10,*c*12 CLA treatments.

Cleavage rate of forskolin matured oocytes was 0.0%, different ($P \leq 0.0001$) from those of control (55.4 ± 4.1%), ethanol (58.9 ± 4.2%) and *t*10,*c*12 CLA (45.6 ± 4.2%) treatments.

At the end of IVM, total areas of oocytes from control ($P = 0.04$), ethanol ($P = 0.008$) and *t*10,*c*12 CLA ($P = 0.006$) treatments were larger than those of immature oocytes (Table 2.1.), whereas areas of forskolin treated oocytes were similar to those of immature.

Table 2.1. Effect of *trans*-10, *cis*-12 conjugated linoleic acid (*t*10,*c*12 CLA) and forskolin supplementation during 44-48 h of *in vitro* maturation, on porcine oocyte meiosis progression, oocyte and lipid droplets areas, in 3 independent replicates.

Treatments		Oocyte meiosis progression				Oocyte areas					Lipid droplets	
		N	GV (%)	GVBD -MI(%)	Anaph.I -MII (%)	N	Total (μm^2)	Cytoplasmic (μm^2)	Fat area (μm^2)	Fat index (%)	N	LD area (μm^2)
Immature	0 h	87	97.7^{a}	2.3^{d}	0.0^{b}	10	18099^{de}	12445^{bc}	9997^{b}	54.8	800	12.4
Control	22-24 h	61	23.1^{cd}	76.9^{a}	0.0^{b}	10	19414^{abcd}	14173^{ab}	12003^{a}	61.3	800	10.8
	44-48 h	58	0.0^{d}	28.0^{bc}	72.6^{a}	10	19963^{ab}	14040^{ab}	10814^{ab}	56.5	800	11.5
Ethanol	22-24 h	61	29.2^{bc}	69.6^{a}	1.3^{b}	9	18806^{b}	13937^{ab}	11416^{ab}	58.2	800	10.6
	44-48 h	61	18.1^{cd}	17.8^{bcd}	65.1^{a}	11	20503^{a}	15533^{a}	12741^{a}	60.0	800	11.0
*t*10,*c*12 CLA	22-24 h	51	23.9^{c}	72.5^{a}	37.4^{b}	9	19791^{abc}	14092^{ab}	11401^{ab}	57.1	800	8.7
	44-48 h	68	19.3^{cd}	20.7^{bcd}	61.0^{a}	9	20736^{a}	14373^{a}	11332^{ab}	55.2	800	11.4
Forskolin	22-24 h	71	93.4^{a}	6.6^{cd}	0.0^{b}	9	17362^{e}	11784^{c}	9759^{b}	55.8	800	10.3
	44-48 h	62	55.6^{b}	40.0^{b}	5.1^{b}	9	17694^{de}	12063^{c}	9886^{b}	54.9	800	10.0
	RSD		34.2	37.7	28.4		2014	2006	2012	7.0		7.1
	P		<0.001	<0.001	<0.001		0.002	0.001	0.015	0.311		0.129

Data were analysed using the MIXED procedure of Statistical Analysis System. Values (least square means) with different superscripts letters in the same column are significantly different ($P \leq 0.05$); RSD, residual standard deviation; GV, germinal vesicle; GVBD-MI, germinal vesicle breakdown up to metaphase I; Anaph.I-MII, anaphase I up to metaphase II; Oocyte areas: total (cytoplasmic plus zona pellucida); cytoplasmic (cytoplasmic, without zona pellucida); fat area (total area occupied by lipid droplets); fat index (fat area/oocyte total area X 100). LD area (lipid droplets area within oocytes). Significant differences were identified on oocyte meiosis progression and oocyte area

Cytoplasmic areas of forskolin treated oocytes were the smallest at 22-24 h ($P \leq 0.03$) and also at 44-48 h ($P \leq 0.05$) of IVM. At the end of maturation, cytoplasmic areas of *t*10,*c*12 CLA and ethanol treated oocytes were larger ($P \leq 0.04$) than those of immature but similar to control. Fat areas were smaller in immature and forskolin treated oocytes (22-24 h and 44-48 h) than in control at 22-24 h ($P \leq 0.03$) and ethanol at 44-48 h ($P \leq 0.0002$) of IVM. No differences were identified among groups in fat oocyte index and LD areas. However after 44-48 h of culture, *t*10,*c*12 CLA oocytes were lighter than those of control ($P = 0.03$) and forskolin ($P = 0.04$) treatments (Figure 2.2.).

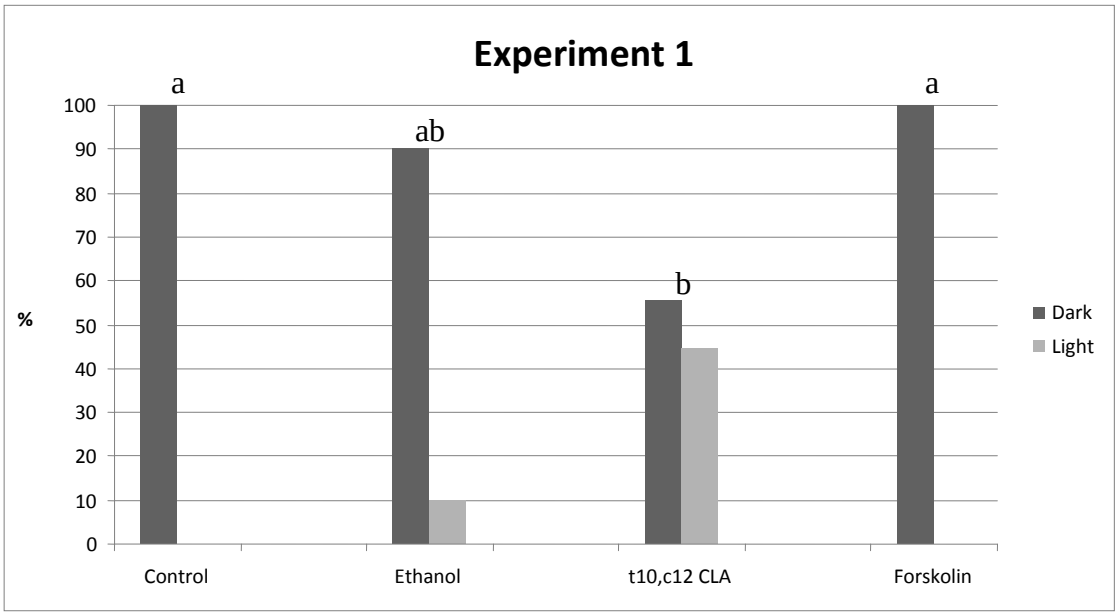

Figure 2.2. - Effect of *trans*-10, *cis*-12 conjugated linoleic acid (*t*10,*c*12 CLA) and forskolin, during 44-48 h of *in vitro* maturation supplementation, on porcine oocytes lipid content. Oocytes were classified into two categories: dark (< 112 pixels/μm^2) or light (> 112 pixels/μm^2) gray according to the gray mean value within fat area.

2.7.2. - *Experiment 2*

Results from meiosis progression of porcine oocytes supplemented with *t*10,*c*12 CLA and forskolin during the initial 22-24 h of IVM are shown in Table 2.2.. In immature oocytes, GV configuration was predominant. Forskolin treated oocytes remained mainly at GV stage at 22-24 h of IVM (91.0 ± 7.3%, $P < 0.0001$). On the contrary, the majority of oocytes from control, ethanol and *t*10,*c*12 CLA treatments was at GVBD-MI ($P < 0.0001$). At 44-48 h of IVM, 24.3±5.4% of oocytes treated with forskolin during 22-24 h reached Anaph-MII. This rate continues lower ($P < 0.0001$) than those from control, ethanol and *t*10,*c*12 CLA treatments. At each time period, no differences ($P > 0.05$) were identified in nuclear configuration among oocytes from control, ethanol and *t*10,*c*12 CLA treatments.

Cleavage rate of oocytes matured with forskolin (8.3 ± 3.3%) was lower ($P \leq 0.0001$) than those of control (54.5 ± 3.0%), ethanol (44.9 ± 3.0%) and *t*10,*c*12 CLA (50.5 ± 4.2%) treatments.

After 44-48 h of maturation, total areas of oocytes from control and *t*10,*c*12 CLA treatments were larger ($P \leq 0.05$) than those of immature and forskolin at 22-24 h of maturation (Table 2.2.). These latter oocytes presented also the smallest cytoplasmic areas ($P \leq 0.02$). After 44-48 h of maturation with forskolin supplementation during 22-24 h, oocyte areas (total and cytoplasmic) were not different from those of other groups at the same time period. At 22-24 h of IVM, oocytes cultured with forskolin presented smaller fat areas than control ($P = 0.006$) and *t*10,*c*12 CLA ($P = 0.05$). However forskolin group showed a larger fat area than control ($P = 0.04$) at 44-48 h of IVM. Oocyte fat areas of immature (0 h) and forskolin groups at 22-24 h were also smaller ($P \leq 0.03$) than those of forskolin and ethanol oocytes at 44-48 h of IVM (Table 2.2.). Fat oocyte index of forskolin treatment was lower ($P = 0.02$) than control at 22-24 h. At 44-

48 h of IVM, the former treatment results became superior to control (P = 0.02) and also to forskolin (P = 0.009) oocytes at 22-24 h. No differences were identified between oocyte categories (dark and light gray) or LD areas among groups.

Table 2.2. Effect of *trans*-10, *cis*-12 conjugated linoleic acid (*t*10,*c*12 CLA) and forskolin supplementation during 22-24 h of *in vitro* maturation, on porcine oocyte meiosis progression, oocyte and lipid droplets areas, in 3 independent replicates.

Treatments		Oocyte meiosis progression				Oocyte areas					Lipid droplets	
		N	GV (%)	GVBD -MI (%)	Anaph.-MII (%)	N	Total (μm^2)	Cytoplami (μm^2)	Fat Area (μm^2)	Fat index (%)	N	LD area (μm^2)
Immature	0 h	87	97.9^{a}	2.2^{e}	0.0^{c}	10	18280^{cd}	12499^{b}	10036^{cd}	54.8^{c}	800	11.9
Control	22-24 h	61	25.0^{bc}	75.8^{a}	0.0^{c}	10	19544^{abc}	14206^{ab}	12147^{ab}	61.9^{ab}	800	10.4
	44-48 h	58	0.0^{d}	28.1^{c}	71.6^{a}	10	20009^{ab}	14054^{ab}	10903^{bcd}	57.0^{c}	800	11.1
Ethanol	22-24 h	61	31.6^{b}	68.8^{ab}	1.3^{c}	9	18863^{bcd}	13961^{ab}	11474^{abcd}	58.5^{abc}	800	10.1
	44-48 h	65	6.0^{cd}	17.7^{cde}	76.7^{a}	8	19747^{abc}	14963^{a}	12141^{ab}	59.4^{abc}	800	12.7
*t*10,*c*12 CLA	22-24 h	51	27.6^{bc}	69.5^{ab}	3.8^{c}	9	19942^{abc}	14137^{ab}	11521^{abc}	57.5^{bc}	800	8.3
	44-48 h	68	6.3^{cd}	25.0^{cd}	68.3^{a}	10	20995^{a}	14628^{a}	11745^{ab}	55.7^{c}	800	9.8
Forskolin	22-24 h	71	91.0^{a}	7.6^{de}	0.0^{c}	9	17456^{d}	11805^{c}	9879^{d}	56.3^{c}	800	9.9
	44-48 h	71	24.8^{bc}	53.0^{b}	24.3^{b}	10	19959^{abc}	14116^{ab}	12573^{a}	62.7^{a}	800	10.3
	RSD		30.2	39.0	30.2		1971	1982	1756	5.1		7.2
	P		<0.001	<0.001	<0.001		0.010	0.024	0.011	0.011		0.152

Data were analysed using the MIXED procedure of Statistical Analysis System. Values (least square means) with different superscripts letters in the same column are significantly different ($P \leq 0.05$); RSD, residual standard deviation; GV, germinal vesicle; GVBD-MI, germinal vesicle breakdown up to metaphase I; Anaph.I-MII, anaphase I up to metaphase II; Oocyte areas: total (cytoplasmic plus zona pellucida); cytoplasmic (cytoplasmic, without zona pellucida); fat area (total area occupied by lipid droplets); fat index (fat area/oocyte total area X 100). LD area (lipid droplets area within oocytes). Significant differences were identified on oocyte meiosis progression and oocyte areas.

2.7.3. - *Experiment 3*

By reducing the time of forskolin exposure to 2 h, oocyte meiotic progression at 22-24 h and 44-48 h was similar ($P > 0.05$) to those of control (Table 2.3.). No differences were identified in cleavage rates between treatments.

At the end of IVM, cytoplasmic and total areas of oocytes from both groups were similar among themselves and larger ($P \leq 0.01$) than those of immature oocytes (Table 2.3.). Exception made for cytoplasmic areas of forskolin oocytes, these measurements at 22-24 h of IVM were also larger ($P \leq 0.04$) than those of immature oocytes. Control oocytes matured during 22-24 h presented the largest ($P \leq 0.03$) fat area (Table 2.3.). Exception made for forskolin oocytes at 22-24 h, similar results ($P \leq 0.03$) were obtained for fat oocyte index. This index presented no differences between treatments at the end of IVM. Also no differences were identified between oocyte categories (dark and light gray). Although reducing the time exposure to forskolin (2 h), at 22-24 h of IVM these oocytes had smaller LD areas than those of control ($P = 0.03$). These LD were also smaller than those of immature ($P = 0.002$) and forskolin oocytes at the end of IVM ($P = 0.03$). LD areas of immature oocytes were larger ($P = 0.02$) than control ones at 44-48 h (Table 2.3.).

Table 2.3. Effect of forskolin supplementation during the initial 2 h of *in vitro* maturation on porcine oocyte meiosis progression, oocyte and lipid droplets areas, in 3 independent replicates.

Treatments		Oocyte meiosis progression				Oocyte areas					Lipid droplets	
		N	GV (%)	GVBD -MI (%)	Anaph.I -MII (%)	N	Total (μm^2)	Cytoplasmic (μm^2)	Fat Area (μm^2)	Fat index (%)	N	LD area (μm^2)
Immature	0 h	91	100.0^{a}	0.0^{c}	0.0^{b}	12	17574^{b}	12280^{b}	10161^{b}	57.6^{bc}	1000	13.3^{a}
Control	22-24 h	77	19.0^{bc}	81.0^{a}	0.0^{b}	11	21336^{a}	16294^{a}	14148^{a}	65.6^{a}	1000	11.5^{ab}
	44-48 h	75	1.1^{c}	29.1^{bc}	71.5^{a}	11	20332^{a}	14977^{a}	11049^{b}	54.1^{c}	1000	10.7^{bc}
Forskolin	22-24 h	84	43.9^{b}	56.2^{ab}	0.0^{b}	11	19740^{a}	14132^{ab}	11896^{b}	59.8^{ab}	1000	8.0^{c}
	44-48 h	71	10.4^{bc}	32.8^{bc}	56.2^{a}	12	20199^{a}	14578^{a}	11054^{b}	55.0^{bc}	1000	11.4^{ab}
	RSD		26.8	34.3	27.0		2402	2609	2375	6.4		7.4
	P		0.002	0.04	<0.001		0.007	0.011	0.003	0.0006		0.006

Data were analysed using the MIXED procedure of Statistical Analysis System. Values (least square means) with different superscripts letters in the same column are significantly different ($P \leq 0.05$); RSD, residual standard deviation; GV, germinal vesicle; GVBD-MI, germinal vesicle breakdown up to metaphase I; Anaph.I-MII, anaphase I up to metaphase II; Oocyte areas: total (cytoplasmic plus zona pellucida); cytoplasmic (cytoplasmic, without zona pellucida); fat area (total area occupied by lipid droplets); fat index (fat area/oocyte total area X 100). LD area (lipid droplets area within oocytes). Significant differences were identified on oocyte meiosis progression and oocyte and LD areas.

2.8. – Discussion

The present study demonstrated that the supplementation of 100 µM *t*10,*c*12 CLA or 10 µM forskolin during different time periods distinctively interfere on maturation progress and lipid content of porcine oocytes. Data presented here suggest for the first time that *t*10,*c*12 CLA did not affect meiosis progression or competence for cleavage of porcine oocytes although matured oocytes were lighter when this CLA isomer was supplemented during the entire maturation period. In opposition, forskolin presence during 44-48 h or 22-24 h impaired oocyte maturation and their competence for cleavage. However by decreasing forskolin supplementation interval to the initial 2 h of IVM, these deleterious effects disappeared though morphological changes in LD and oocyte fat areas were identified during maturation progression.

Previous reports of continuing exposure of porcine oocytes to forskolin along maturation have demonstrated an increase of intracellular level of cAMP thus inhibiting GVBD (Racowsky, 1985; Xia *et al.*, 2000). In accordance, since at 44-48 h of culture, GV configuration continued to be predominant in forskolin treated oocytes, remaining their areas similar to immature ones, these oocytes were unable of normal fertilization and cleavage (0%). In contrast, Fu *et al.* (2011) achieved a cleavage rate of 64.5 ± 3.6% in parthenogenetically activated porcine oocytes cultured with 10 µM forskolin during the entire IVM period. An increase in cytoplasmic calcium during parthenogenic activation was referred to be sufficient to induce low to moderate extents of oocyte activation events such as cell cycle resumption (Gardner and Evans, 2006). Differences between these two mechanisms, parthenogenesis and cleavage, might explain the above discrepancies.

The inhibitory effect of forskolin during maturation seems to be transiently depending on the supplementation time and dose. However at higher doses, above 10 µM,

forskolin presents a toxic effect on COC metabolism (Racowsky, 1985; Xia *et al.*, 2000). Our findings showed that by reducing forskolin exposure to the initial 22-24 h of maturation, GVBD–MI nuclear status became predominant at the end of IVM. Despite of a maturation rate of 24.3 ± 5.4%, only 8.3 ± 3.3% of inseminated oocytes cleaved. Moreover forskolin affected not only nuclear progression, but also oocyte cytoplasmic and total areas. Therefore the transient delay in nuclear meiosis progress of porcine COC matured in the presence of 10 µM forskolin identified initially by Racowsky (1985) might be extended to cytoplasmic maturation. However, when this supplementation was reduced to the first 2 h, meiosis progression, oocyte areas and cleavage rates were similar to those of control group. Nonetheless, several authors suggest that forskolin might be applied during oocytes IVM to improve synchronization of nuclear and cytoplasmic maturation, simultaneously increasing the homogeneity and developmental potential of matured oocytes (Racowsky, 1985; Thomas *et al.*, 2004). Further studies are needed to clarify forskolin positive action on oocyte developmental competence for embryo production and quality.

Porcine oocytes present variations in both morphology and amount of cytoplasmic LD during *in vivo* or *in vitro* maturation (Kikuchi *et al.*, 2002). In the present study, we investigated the extend of these variations by measuring LD areas and total area occupied by LD, the fat areas, as well as the relationship of fat area with total oocyte area through the fat oocyte index, before and during porcine oocyte maturation. Moreover, oocytes exhibit distinct colour tones of cytoplasm due to LD content: dark colour and opaqueness in lipid rich ones contrasting with the brightness and transparency of leaner oocytes (Fujihira *et al.*, 2004). Furthermore Isachenko *et al.* (2003) identified two kinds of lipid droplets in porcine oocytes: homogenous, dark looking vesicles and “gray” looking droplets with electron-lucent streaks. Silva *et al.*

(2011) suggested that dark LD may change to gray after lipid utilization. By measuring the fat gray value of porcine oocytes using a Nomarski microscope and Image J software it was possible to distinguish different colour tones of fat areas reflecting alterations in lipid content. Differences in the gray tones might suggest not only alterations of the composition, amount and morphology of LD but also its aggregation or dispersion in the ooplasm. Several authors confirmed that as maturation progresses, the ooplasm can have different shades of gray caused by uneven distribution of organelles, specifically mitochondria and LD which reside in close proximity (Kikuchi *et al.*, 2002; Ambruosi *et al.*, 2009). Here it was shown that after 44-48 h of culture, *t*10,*c*12 CLA oocytes were lighter (dark gray = 55.6% and light gray = 44.4%) than those of control and forskolin treatments (dark gray = 100%). Reports confirm the reduction of lipid accumulation in *in vitro* embryos (Pereira *et al.*, 2007 and 2008) or the decrease in lipogenesis of porcine adipose explants (José *et al.*, 2008) after culture with this CLA isomer. Moreover the presence of *t*10,*c*12 CLA during maturation improved bovine oocyte developmental competence (Lapa *et al.*, 2011). Although these latter results at the present were not confirmed for porcine oocytes, *t*10,*c*12 CLA during maturation clearly interfered with LD organization and probably with their composition reflected by a lighter colour tone of their cytoplasm. The mechanism of action of *t*10,*c*12 CLA is not clearly known demanding additional investigation.

Even though forskolin stimulates lipolysis (Men *et al.*, 2006), the fat gray value of forskolin treated oocytes were not different from control independently of time exposure or meiosis progression. On the contrary Fu *et al.* (2011) showed that 10 µM forskolin effectively reduced the fluorescence intensity of porcine oocytes stained with Nile Red as well as the number of LD during IVM. Data presented here demonstrated no differences in LD areas when this lipolytic agent was present during the entire

maturation period or till 22-24 h but fat areas were reduced (exp.1, control = 12003 μm^2 vs. forskolin= 9769 μm^2 $P \leq 0.03$; exp.2, control = 12147 μm^2 vs. Forskolin = 9879 μm^2 $P = 0.006$). It seems that by blocking meiosis progression through the cAMP intracellular level increase (Racowsky, 1985; Xia *et al.*, 2000), forskolin interferes also in the characteristic distribution of LD in ooplasm during the maturation process, resembling the immature oocyte organization. Similarly to what happened to the total and cytoplasmic oocyte areas, the fat areas did not grow. Therefore the oocyte fat indexes were similar to control. The mechanism by which forskolin interfered with LD organization is not well understood but its lypolitic activity in porcine oocytes as well as in embryos has already been identified (Men *et al.*, 2006; Fu *et al.*, 2011). Herein, even when forskolin exposure was only at the initial 2 h of culture these oocytes at 22-24 h had smaller LD and fat areas than those of control group, although at 44-48 h of meiosis these measurements were similar to control.

In conclusion, the present study demonstrates that the distribution and morphology of cytoplasmic LD during porcine oocyte maturation can be modified by *t*10,*c*12 CLA or forskolin reducing its lipid content in a time dependent way. However, at the doses tested, whereas *t*10,*c*12 CLA did not seem to interfere with oocyte meiosis progression or developmental competence, forskolin should be supplemented for only 2 h to prevent the negative effects on these processes. On the other hand, fat gray value seems a potential tool to evaluate the lipid content of a single porcine oocyte.

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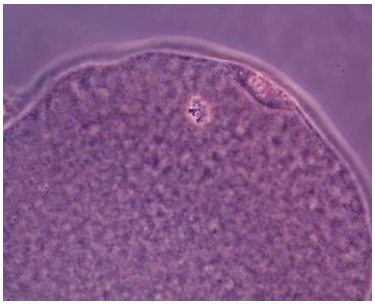

CHAPTER 3

FATTY ACID COMPOSITION OF PORCINE OOCYTE COMPLEXES DURING MATURATION: EFFECT OF THE LIPID MODULATORS *TRANS*-10, *CIS*-12 CLA AND FORSKOLIN

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Fatty acid composition of porcine cumulus oocyte complexes (COC) during maturation: effect of the lipid modulators *trans*-10, *cis*-12 conjugated linoleic acid (*t*10,*c*12 CLA) and forskolin

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3.1. - Abstract

The effect of maturation and of two lipid modulators supplementation along in vitro maturation (IVM) on fatty acid (FA) and dimethylacetal (DMA) composition of porcine cumulus oocyte complexes (COC) were studied. Abattoir derived immature COC were analyzed for FA and DMA or submitted to IVM as follows: control group; *t*10,*c*12 CLA group, *t*10,*c*12 CLA supplementation during 44 h; Forskolin group, forskolin supplementation during the initial 2 h; *t*10,*c*12 CLA+forskolin group, *t*10,*c*12 CLA during 44 h and forskolin just 2 h. Each experimental group had 5 replicates. FA analysis of oocytes, cumulus cells (CC), follicular fluid and culture

media were performed by gas-liquid chromatography. Oocytes and their CC had different FA composition. Oocytes were richer in saturated FA (SFA) preferentially maintaining their FA profile during maturation. Mature CC had the highest polyunsaturated FA (PUFA) content. Five individual and total SFA, and monounsaturated FA (MUFA), notably oleic acid (*c*9-18:1), percentages were lower ($P \leq 0.023$) in mature than in immature CC. *t*10,*c*12 CLA was accumulated by COC from *t*10,*c*12 CLA and *t*10,*c*12 CLA+forskolin groups, mostly in CC where MUFA and a eicosatrienoic isomer decreased ($P \leq 0.043$). Nevertheless PUFA or FA and DMA total content were not affected. Arachidonic acid was reduced in *t*10,*c*12 CLA+forskolin CC and hexadecanal-DMA-16:0 in *t*10,*c*12 CLA CC. Forskolin alone increased ($P \leq 0.043$) c9-18:1 in oocytes. In conclusion, maturation process clearly changed porcine COC FA and DMA profiles, mostly of CC, also more susceptible to modifications induced by *t*10,*c*12 CLA. This possibility of manipulating COC lipid composition during IVM could be used to improve oocyte quality/cryopreservation efficiency.

Key words: porcine oocyte maturation, fatty acids, gas chromatography, *trans*-10,*cis*-12 conjugated linoleic acid, forskolin.

3.2. - Introduction

The bi-directional exchanges between the oocyte and the surrounding cumulus cells (CC) are crucial for the acquisition of oocyte developmental competence (Ouandaogo *et al*., 2011). Nonetheless in terms of lipid metabolism the knowledge of this cross talk is scarce. Previous reports demonstrated that mammalian oocytes

contain a lipid reserve mainly constituted of triacylglycerols and membrane structural phospholipids. Furthermore, the abundance and utilization of this oocyte lipid reserve seems to be species-specific. Undoubtedly porcine oocytes exhibit the highest fatty acid (FA) content among domestic animals (McEvoy *et al.*, 2000; Fujihira *et al.*, 2004), but information about their CC FA composition and function remains obscure.

According to Sturmey and Leese (2003) and Sturmey *et al*. (2006) the intracellular lipids of porcine oocytes are available for energy supply during maturation, but uptake of exogenous lipids from culture media enriched with follicular fluid or serum may also occur (Kim *et al*., 2001; Sturmey *et al*., 2009). Therefore, exogenous FA may be metabolized or accumulate in the ooplasm changing oocyte lipid content and FA composition (Sturmey *et al*., 2009) and probably those of CC. Besides the metabolic role of FA as energy source, lipids play an essential role in cell structure and functions namely as intracellular messengers. Moreover being the major components of cell membranes they have particular relevance in oocyte-spermatozoon interaction (Gardner and Evans, 2006) and in cell resistance to cryopreservation (Kim *et al.*, 2001; Arav and Zvi, 2008; Pereira and Marques, 2008). Thus the clarification of COC lipid composition and contribution to oocyte metabolism during maturation is of primordial importance.

Several studies have reported that triacylglycerols and phospholipids of the porcine reproductive tissues are notably rich in polyunsaturated fatty acids (PUFA), particularly those of the graafian follicles and ovaries (Holman and Hofstetter, 1964) and also of the immature oocytes (Homa *et al.*, 1986; McEvoy *et al.*, 2000). However, in terms of qualitative and quantitative analysis of FA and lipid content, data on porcine oocytes are still limited. To our knowledge the FA and other

membrane constituents such as ether vinylic chains from plasmalogens phospholipids [firstly identified by Homa *et al.* (1986) in immature oocyte and currently analysed as dimethylacetals – DMA] composition of mature porcine oocytes have not yet been reported. Additionally it has not been established whether storage and lipid metabolism influence oocyte maturation and its developmental potential as well as the role of CC along the entire process. Furthermore the possibility of modifying intracellular lipids of oocytes/embryos by changing the composition of the culture medium to increase their quality and cryotolerance is still under investigation (Pereira and Marques, 2008; Sturmey *et al.*, 2009).

Recently, *t*10,*c*12 CLA and forskolin (FSK) both recognized as intracellular lipid modulators were shown to improve porcine and bovine embryos cryosurvival (Men *et al.*, 2006; Pereira *et al.*, 2007; 2008). During maturation, this conjugated dienoic FA interfered on the FA profile of bovine oocytes improving their competence to develop into higher quality embryos (Lapa *et al.*, 2011). Moreover porcine oocytes treated with 10 µM FSK for the initial 2 h or 100 µM *t*10,*c*12 CLA for the entire period of in vitro maturation (IVM), presented modifications on the distribution and morphology of cytoplasmic lipid droplets (Prates *et al.*, 2012). At the doses tested, while oocytes supplemented with *t*10,*c*12 CLA exhibited a lighter cytoplasmic colour tone, oocytes exposed to FSK had smaller lipid droplets. These findings led to the hypothesis that *t*10,*c*12 CLA and/or FSK supplementation during porcine oocyte maturation might be used to change the lipid content and FA composition of these gametes.

The objectives of the present study were firstly to characterize the FA and DMA content and composition of porcine oocytes and CC before and after maturation.

Secondly we investigated if *t*10,*c*12 CLA and/or FSK culture media supplementation might change the FA and DMA composition and maturation rates of porcine COC.

3.3. - Materials and Methods

3.3.1. - *Experimental design*

The effect of IVM and of the supplementation of maturation media of porcine oocytes with *t*10,*c*12 CLA (Matreya, Pleasent Gap, PA, USA; ref. 001249; 100 μM) (Prates *et al.*, 2012) and/or with FSK (10 μM) (Men *et al.*, 2006) on the FA and DMA content and composition of COC were investigated in five replicates as described below.

To study the FA and DMA content and composition of porcine COC during maturation, COC were separated in CC and oocytes and both evaluated as immature and mature. Therefore four groups were considered: (1) immature oocytes, (2) immature CC, (3) mature oocytes and (4) mature CC. The FA and DMA composition of follicular fluid from the pool of ovaries and of the pre-IVM media used in each session were also analyzed.

To investigate the effect of *t*10,*c*12 CLA and FSK on porcine COC maturation four experimental groups were constituted: (1) control group, COC were matured during 44 h without supplementation; (2) *t*10,*c*12 CLA group, COC were matured during 44 h supplemented with 100 μM *t*10,*c*12 CLA, (3) FSK group- COC were supplemented with 10 μM FSK during the initial 2 h of IVM; (4) *t*10,*c*12 CLA+FSK group - COC were matured with 100 μM *t*10,*c*12 CLA during 44 h plus10 μM FSK during the first 2 h of maturation. At the end of the maturation period, samples of mature oocytes were used for lipid content analysis, while others (control group n = 84; *t*10,*c*12 CLA group n = 87; FSK group n = 78; *t*10,*c*12 CLA+FSK group n = 85) were fixed and stained for individual evaluation of maturation rate. As above in each session for FA and DMA

composition analysis, COC from all treatments were divided in CC and oocytes and both evaluated as control, *t*10,*c*12 CLA, FSK and *t*10,*c*12 CLA+FSK, resulting in 10 experimental groups. After COC maturation, the FA and DMA content and composition were also assessed in IVM culture media.

3.3.2. - *Reagents and culture media*

Otherwise stated, all products were purchased from Sigma-Aldrich (Sintra, Portugal). The medium used for the collection and washing of COC was the Dulbecco's phosphate-buffered saline (DPBS) composed of 4 mg/mL bovine serum albumin (BSA; fraction V), 0.34 mmol/L sodium pyruvate, 5.4 mmol/L D-glucose and 70 μg/mL kanamycin (mDPBS) (Gil *et al.*, 2005). Oocyte maturation medium was the BSA-free North Carolina State University (NCSU23) (Petters and Weels, 1993), supplemented with 10% (v/v) porcine follicular fluid, 0.1 mg/mL cysteine, 10 ng/mL epidermal growth factor and 100 μmol/L glutathione.

3.3.3. - *Cumulus oocyte complexes collection*

Ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported at 38 ºC in a 0.9% NaCl solution containing 70 μg/mL kanamycin. At the laboratory, the ovaries were washed several times in mDPBS. Follicles of 3–6 mm in diameter were aspirated and the follicular content placed in a conical flask. The supernatant was centrifuged (3700 g during 30 min) and the resultant follicular fluid retrieved for FA analysis. The remaining COC were washed in mDPBS. Oocytes with a compact cumulus mass and a dark and evenly granulated cytoplasm were selected for maturation rates assessment or FA analysis.

3.3.4. - *In vitro maturation*

Selected oocytes were washed and placed in 3 mL of maturation medium, supplemented with 10 IU/mL equine chorionic gonadotrophin (Folligon; Intervet International, Boxmeer, The Netherlands) and 10 IU/mL human chorionic gonadotrophin (Veterin Corion; Divasa Farmavic, Barcelona, Spain), during 22 h and then for another 22 h in maturation medium without hormones. Oocyte maturation (100-250 oocytes per 3 mL) was performed at 39 ºC in a humidified atmosphere of 5% CO_2 in air.

3.3.5. - *Assessment of oocyte maturation*

At the end of the maturation period, CC were stripped off the oocytes by vortexing in a 1% sodium citrate solution. Denuded oocytes were fixed for 3–4 days in aceto/ ethanol (1 : 3, v/v) then stained with 1% aceto/ lacmoid solution. Oocytes were classified as matured when metaphase II (MII) chromosomes set and the chromatin of the first polar body were present. Fixed oocytes with abnormal appearance were discharged.

3.3.6. - *Fatty acids and dimethylacetal analysis*

For lipid extraction, CC were removed from COC before and after maturation, by vortexing for up to 10 min. Then denuded oocytes and CC were washed three times in 1% sodium citrate. Cell concentration was determined using a haemocytometer camera. In each session, immature and mature oocytes (110 to 200 per pooled group sample) and their CC (7×10^4 to 2×10^6 per pooled group sample) were suspended in citrate solution (5 replicates or sessions in total). Also, 500 µL of culture media before and after maturation, of 1% sodium citrate solution and of follicular fluid from the pool of ovaries used in each session were collected. Then, fatty acid methyl esters (FAME) were prepared by direct transesterification. Briefly, 1 mL of toluene and 200 µL of 19:0

(0.5 mg/mL) as an internal standard were added to each sample in fresh. After the addition of 2 mL of sodium methoxide in methanol (0.5 M), the solution was vortexed and left for about 15 min at 50 ºC. After cooling to room temperature, 3 mL of 10% HCl solution was allowed to react for more 30 min at 80 ºC. Once cooled, 6% potassium carbonate was added in two portions of 2 mL to prevent excessive effervescence, followed by addition of 2 mL of hexane. The solution was vortexed, centrifuged and the organic layer transferred to another tube. The extraction step was repeated twice. The final solution was dried using anhydrous sodium sulphate.

The solvent was then removed under a stream of nitrogen and the final residue dissolved in 100 µL *n*-hexane (GC grade > 99%). The resultant FAME were injected (2 µL), via a Hewlett Packard (Avondale, PA, USA) HP 6890 Series GC System, onto a CP-Sil 88 fused-silica capillary column (100 m length, 0.25 mm internal diameter, 0.20 µm film thickness: Chromopack, Varian Inc., Walnut Creek, CA, USA). Injection was performed using a programmed temperature and a splitless method. The injector and detector temperatures were 250 ºC and 280 ºC, respectively. Initial oven temperature of 45 ºC was held for 5min, increased at 10 ºC to 175 ºC and held for 30 min and increased at 50 ºC/min to 215 ºC and held for 35 min. Helium was used as carrier gas at a flow rate of 1 mL/min. Peaks were identified by comparison with a commercial standard FAME mix 4:0 to 24:0 purchased from Supelco Inc. (Bellefonte, PA, USA) and a bacterial acid methyl esters mix purchased from Matreya LLC (Pleasant Gap, PA, USA). Quantities of each FA present were calculated by reference to internal standard. Additionally, identification of some unknown peaks and confirmation of FAME were performed by GLC-MS using a Varian Saturn 2200 system (Varian Inc., Walnut Creek, CA, USA) equipped with a CP-Sil 88 capillary column.

3.4. - *Statistical Analysis*

All results are expressed as least square means ± standard error. Data representing 5 replicates of oocyte nuclear configuration as well as FA and DMA composition of COC and culture media were analyzed using the MIXED procedure of SAS (SAS Institute, Cary, NC, USA). For FA and DMA composition evaluation of immature and mature COC, the model included the effect of maturation status and cell type (oocytes and CC) and its interaction. For FA and DMA composition evaluation of mature COC treated with *t*10,*c*12 CLA, FSK and FSK+*t*10,*c*12 CLA, the model included the treatment, cell type (oocytes and CC) and its interaction. The cell type was treated as repeated measures within COC assuming a compound symmetry covariance matrix. Homogeneity of variance between groups was tested and when necessary the group statement was included to accommodate variance heterogeneity. The model was simplified when some variable were not detected in one cell type or maturation status. Orthogonal contrasts comparing each treatment with the control were computed. When interactions were significant ($P \leq 0.05$) differences between means were determined by the PDIFF multiple comparison procedure. The mixed linear model used for oocyte nuclear configuration and FA and DMA culture media composition included treatment as fixed effect and session as random effect. Differences were considered significant when $P \leq 0.05$.

3.5. - Results

The FA and DMA composition of porcine follicular fluid and pre-IVM medium analyzed by gas-liquid chromatography is described in table 3.1. The most abundant FA, in decreasing order, were palmitic (16:0), oleic (*c*9-18:1), linoleic (18:2n-6), stearic (18:0) and arachidonic (20:4n-6) acids. The FA composition of follicular fluid and

Table 3.1. Fatty acid (FA) and dimethylacetal (DMA) composition of porcine follicular fluid and maturation media prior to culture (pre-IVM medium). Data are least square means (LSM) ± standard error (SE) percentages (w⁄w) of total fatty acids in five replicates.

Name	Formula	Follicular fluid	Pre-IVM medium
Myristic	14:0	0.56 ± 0.04	0.58 ± 0.06
Myristoleic	*c*9-14:1	0.05 ± 0.01	0.21 ±0.09
Pentadecyclic	15:0	0.24±0.016	0.27±0.024
Palmitic	16:0	24.1±0.40	22.4 ± 0.43
Hexadecenoic	*c*7-16:1	1.02 ± 0.07	0.87 ± 0.05
Palmitoleic	*c*9-16:1	1.27 ± 0.58	1.08 ± 0.07
Heptadecanoic	17:0	0.71 ± 0.10	0.75 ± 0.07
Margaric	*c*9-17:1	0.31 ± 0.07	0.25 ± 0.01
Stearic	18:0	13.40 ± 0.50	14.70 ± 0.58
Oleic	*c*9-18:1	19.40 ± 0.62	20.80 ± 0.33
Cis-Vaccenic	*c*11-18:1	2.37 ± 0.21	1.97 ± 0.10
Linoleic	18:2n-6	17.30 ± 1.12	19.40 ± 0.52
Arachidic	20:0	0.24 ± 0.05	0.38 ± 0.08
γ-Linolenic	18:3n-6	0.42 ± 0.06	0.31 ± 0.03
α-Linolenic	18:3n-3	0.32 ± 0.05	0.28 ± 0.04
Gondoic	*c*11-20:1	0.20 ± 0.02	0.10 ± 0.38
Eicosadienoic	20:2n-6	0.37 ± 0.07	0.29 ± 0.08
Eicosatrienoic[I]	20:3^{niI}	0.62 ± 0.05	0.57 ± 0.05
Eicosatrienoic[II]	20:3 ^{niII}	0.76 ± 0.09	0.56 ± 0.03
Arachidonic	20:4n-6	11.60 ± 0.56	10.40 ± 0.28
Tricosylic	23:0	0.05 ± 0.01	0.23 ± 0.07
Docosadienoic	22:2n-6	0.08 ± 0.03	0.23 ± 0.42
Adrenic	22:4n-6	0.33 ± 0.04	nd
Clupanodonic	22:5n-3	1.34 ± 0.05	1.11 ± 0.16
Cervonic	22:6n-3	0.73 ± 0.09	0.44 ± 0.03
Other FA	Other FA	0.30 ± 0.03	0.33 ± 0.09
Dimethylacetal	**DMA**		
Hexadecanal-	DMA-16:0	0.87 ± 0.06	0.57 ± 0.09
Octadecanal-	DMA-18:0	0.77 ± 0.11	0.57 ± 0.05
Octadecenal-	DMA-18:1	0.34 ± 0.05	0.38 ± 0.05
Partial sums			
SFA		39.30 ± 0.61	39.30 ± 0.88
MUFA		24.90 ± 0.95	25.60 ± 0.42
PUFA		33.80 ± 0.56	33.50 ± 0.69
n6/n3 ratio		12.70 ± 0.70	16.90 ± 1.08
FA (ng/µL)[III]		630.20 ± 14.45	54.00 ± 3.10

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; nd, not detectable; ni[I] and [II], non identified 20:3 isomers; [III] – total FA content ng per µL

maturation media is notably similar, with the major difference being the absence of adrenic acid (22:4n-6) in pre-IVM medium.

3.5.1. - *Effect of the maturation status on the FA and DMA composition of porcine COC*

The FA and DMA composition of COC prior to and after IVM is presented in table 3.2.. Both oocytes and CC tended (P = 0.056) to increase total FA and DMA content during maturation (immature oocytes = 51.1 ± 4.10 *vs*. mature oocytes = 63.7 ± 4.10 ng per oocyte; immature CC = 0.013 ± 0.010 *vs*. mature CC = 0.032 ± 0.010 ng per CC). Differences in FA profile between cell type (C), maturation status (MS) as well as differential effects of maturation status in each cell type (MS×C interaction) were observed (table 2). Oocytes presented higher 16:0 (P < 0.001) and hexadecenoic (*c*7-16:1, P = 0.031) acids, but lower 18:0 (P = 0.001), hexadecanal (DMA-16:0, P = 0.004) and octadecanal (DMA-18:0, P = 0.006) DMAs proportions than CC. *In vitro* maturation increased (P = 0.018) the 18:2n-6 proportion in both oocytes and CC. For the majority of FA displaying a MS×C interaction (myristic, 14:0; pentadecyclic, 15:0; margaric, 17:0; *c*9-18:1; arachidic, 20:0; tricosylic, 23:0; and docosadienoic, 22:2n-6) the general trend was that maturation induced a decrease in its proportions on CC but not in oocytes. The exception was the 20:4n-6 that increased during maturation in CC. Considering the partial sums of FA, the maturation process reduced SFA and MUFA in CC and increased PUFA, whereas the oocytes remained unaffected.

Several lipid compounds were not detected at all in immature or mature oocytes and immature CC (eicosatrienoic, 20:3^{niI}; clupanodocnic, 22:5n-3; and octadecenal-DMA, DMA-18:1). Others, were not detected only in immature oocytes and immature CC (margaric, *c*9-17:1; and γ-linolenic, 18:3n-6) or displayed only an inconsistent occurance in immature oocytes samples (α-linolenic, 18:3n-3; gondoic, *c*11-20:1,

Table 3.2. Effect of the maturation status (immature vs. mature, MS) on fatty acid (FA) and dimethylacetal (DMA) composition of porcine oocytes and cumulus cells. Data are least square means (LSM) ± standard error (SE) percentages (w⁄w) of total fatty acids in five replicates.

	Immature		Mature		*P values*		
	Oocytes	Cumulus cells	Oocytes	Cumulus cells	MS	Cell type (C)	MS x C
14:0	1.66^{ab}±0.529	1.75^{ab}±0.529	2.11^{b}±0.101	1.06^{a}±0.101	0.832	0.004	0.001
15:0	0.57^{ab}±0.126	0.98^{b}±0.126	0.80^{b}±0.045	0.43^{a}±0.045	0.161	0.865	0.004
16:0	44.9±1.81	24.1±0.74	45.2±1.06	26.4±1.20	0.365	<0.001	0.434
*c*7-16:1	1.50±0.097	0.96±0.069	1.95±0.295	1.45±0.216	0.072	0.031	0.898
*c*9-16:1	1.30±0.047	1.33±0.120	1.58±0.117	1.27±0.152	0.390	0.258	0.190
17:0	1.30^{a}±0.258	2.64^{b}±0.258	1.10^{a}±0.147	0.86^{a}±0.147	0.036	0.002	<0.001
*c*9-17:1	nd	nd	0.29±0.031	0.23±0.016	-	0.150	-
18:0	11.6±0.86	15.6±0.86	10.7±0.33	13.4±0.33	0.093	0.001	0.324
*c*9-18:1	17.0^{ab}±0.43	29.2^{c}±1.45	16.5^{a}±0.43	20.4^{b}±1.45	0.012	<0.001	0.005
*c*11-18:1	3.08±0.286	2.19±0.278	2.82±0.119	2.62±0.331	0.765	0.074	0.228
18:2n-6	5.18±0.323	6.59±0.304	6.24±0.277	8.31±0.501	0.018	0.001	0.387
20:0	0.87^{a}±0.333	2.43^{b}±0.333	1.10^{a}±0.333	0.49^{a}±0.333	0.067	0.180	0.010
18:3n-6	nd	nd	0.73±0.206	0.90±0.103	-	0.507	-
18:3n-3	0.19±0.019*	nd	0.29±0.049	0.20±0.021	-	0.221	-
*c*11-20:1	0.30±0.009*	nd	0.29±0.099	0.39±0.042	-	0.438	-
20:2n-6	0.23±0.030*	nd	0.10±0.186	0.71±0.078	-	0.093	-
$20:3^{niI}$	nd	nd	nd	0.42±0022	-	-	-
$20:3^{niII}$	0.69±0.078*	nd	0.43±0.114	0.94±0.066	-	0.018	-
20:4n-6	5.95^{a}±0.768	5.71^{a}±0.768	5.28^{a}±0.444	13.4^{b}±0.44	0.004	<0.001	<0.001
23:0	0.70^{a}±0.234	1.74^{b}±0.234	0.50^{a}±0.062	0.47^{a}±0.062	0.030	0.001	0.001
22:2n-6	1.09^{a}±0.177	3.34^{b}±0.594	0.75^{a}±0.177	0.54^{a}±0.594	0.023	0.048	0.023
22:4n-6	0.68±0.113*	nd	0.70±0.043	0.45±0.025	-	0.007	-
22:5n-3	nd	nd	nd	1.00±0.051	-	-	-
22:6n-3	0.29±0.035*	nd	0.28±0.032	0.45±0.019	-	0.011	-
Other FA	nd	nd	nd	0.60±0.126	-	-	-
DMA							
DMA-16:0	0.71±0.123	0.98±0.123	0.41±0.123	1.27±0.123	0.972	0.004	0.072
DMA-18:0	0.70±0.198	1.12±0.216	0.37±0.134	0.79±0.123	0.219	0.006	0.985
DMA-18:1	nd	nd	nd	0.51±0.027	-	-	-
Partial Sums							
SFA	61.6^{c}±1.45	49.2^{b}±1.45	61.3^{c}±1.45	43.1^{a}±1.45	0.116	<0.001	0.049
MUFA	23.0^{a}±1.09	33.5^{c}±1.09	23.3^{a}±1.09	27.0^{b}±1.09	0.050	<0.001	0.013
PUFA	14.0^{a} ± 0.50	15.7^{a} ± 1.17	14.6^{a} ± 0.50	27.4^{b} ± 1.17	0.002	<0.001	<0.001
FA (ng/un)^{III}	51.1±4.10	63.7±4.10	0.013±0.010	0.032±0.010	0.097	<0.001	0.056

Data within the same row with different superscripts letters are statistically different due to an interaction between cell type and maturation status ($P \leq 0.05$). FA, fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; MS, maturation status; C, cell type; nd, not detectable; ni^{I} and ^{II}, non identified 20:3 isomers; * data not included in statistical analysis; ^{III} – total FA content per individual cell

20:3^{niII}; eicosadienoic, 20:2n-6; 22:4n-6; and cervonic, 22:6n-3). In those cases, although data available for immature oocytes were displayed in table 3.2, the statistical analysis, when possible, involved only the comparison of mature oocytes and CC. Thus, mature CC contained higher proportion of 22:6n-3 and an unidentified 20:3 isomer but lower 22:4n-6 than mature oocytes. Two 20:3 isomers (herein designated as 20:3^{niI} and 20:3^{niII}) were detected in the porcine oocytes and CC. From gas-liquid chromatography-mass spectrometry (GLC-MS) confirmation, they were identified as 20:3 FA however their structures were not completely elucidated. Nevertheless, it was clear from coelution with reference standards, that these 20:3 isomers were not the common 20:3n-6, 20:3n-3 and 20:3n-9 isomers.

3.5.2. - *Effect of t10,c12 CLA and FSK on oocyte maturation and FA and DMA composition of porcine COC and culture media*

In this study the effect of the supplementation of porcine oocyte culture medium with *t*10,*c*12 CLA, FSK or both on oocyte maturation and FA and DMA composition of COC and culture media after 44 h of IVM were evaluated by the observation of oocyte nuclear configuration and gas-liquid chromatography respectively. These supplementations did not interfere ($P > 0.05$) with oocyte maturation rates (MII: control group = 87.99 ± 0.04%, *t*10,*c*12 CLA group = 84.71 ± 0.04%, FSK group = 86.94 ± 0.04%, *t*10,*c*12 CLA+FSK group = 84.98 ± 0.04%) or FA and DMA content expressed as ng/ individual cell (table 3.3.). Nonetheless differences were identified on the FA and DMA profiles of COC and culture media.

The most expressive differences in FA composition are between types of cells (oocytes and CC). Independently of treatment, mature oocytes presented higher proportions of

Table 3.3. Effect of *trans*-10, *cis*-12 conjugated linoleic acid (*t*10,*c*12 CLA), forskolin (FSK) or both (*t*10,*c*12 CLA+FSK) supplementation during in vitro maturation on fatty acid (FA) and dimethylacetal (DMA) composition of mature porcine oocytes and cumulus cells. Data are least square means (LSM) ± standard error (SE) percentages (w⁄w) of total fatty acids in five replicates.

	Control		*t*10,*c*12 CLA		FSK		*t*10,*c*12 CLA+FSK		*P values*				
									Treatment contrasts[1]				
	Oocytes	Cumulus cells	Oocytes	Cumulus cells	Oocytes	Cumulus cells	Oocytes	Cumulus cells	CLA	FSK	CLAFSK	C	T x C
14:0	2.11±0.20	1.06±0.42	1.93±0.20	1.23±0.42	1.95±0.20	1.99±0.42	2.33±0.20	1.49±0.24	0.992	0.269	0.343	0.015	0.402
15:0	0.80^{c}±0.06	0.43^{a} ±0.04	0.73^{bc} ±0.05	0.43^{a} ±0.06	0.60^{b} ±0.01	0.58^{ab} ±0.06	0.65^{bc} ±0.11	0.53^{ab} ±0.09	0.438	0.601	0.722	<0.001	0.010
16:0	45.20±1.43	26.4±0.05	44.5±1.97	25.84±0.66	45.9±1.63	25.9±0.63	44.5±0.98	25. 0±0.50	0.637	0.906	0.266	<0.001	0.926
*c*7-16:1	1.95±0.23	1.46±0.10	1.83±0.23	0.91±0.10	1.94±0.23	1.16±0.10	1.97±0.23	0.95±0.12	0.094	0.422	0.204	<0.001	0.514
*c*9-16:1	1.58±0.09	1.27±0.09	1.39±0.09	1.00±0.09	1.54±0.09	1.22±0.09	1.47±0.09	0.83±0.09	0.010	0.536	0.003	<0.001	0.355
17:0	1.17±0.06	0.93±0.08	1.07±0.06	1.17±0.21	1.11±0.11	1.42±0.15	1.04±0.04	1.33±0.29	0.556	0.072	0.400	0.288	0.070
*c*9-17:1	0.27±0.02	0.23±0.03	0.24±0.08*	nd	0.15±0.02	0.25±0.04	0.24±0.02	0.23±0.04	-	0.178	0.599	0.537	0.085
18:0	10.7±0.37	13.4±2.05	9.98±0.37	17.6±2.05	9.80±0.37	15.4±2.05	10.3±0.37	15.1±2.05	0.266	0.734	0.671	<0.001	0.432
*c*9-18:1	16.5^{a}±0.34	20.4^{cd}±0.97	17.4^{ab}±0.34	18.2^{bc}±0.97	17.8^{b}±0.34	22.4^{d}±0.97	16.8^{ab}±0.34	21.8^{d}±0.97	0.355	0.047	0.272	<0.001	0.043
*c*11-18:1	2.82±0.23	2.62±0.18	2.57±0.13	1.99±0.27	2.77±0.16	2.16±0.21	2.67±0.16	1.90±0.24	0.057	0.212	0.054	0.002	0.557
18:2n-6	6.24±0.34	8.31±0.54	6.13±0.06	8.74±0.62	6.09±0.17	7.56±0.30	5.97±0.14	8.47±0.38	0.729	0.231	0.876	<0.001	0.231
20:0	1.10±0.33	0.49±0.10	0.59±0.14	0.60±0.11	0.69±0.03	1.15±0.19	0.75±0.06	1.13±0.28	0.315	0.541	0.513	0.655	0.067
18:3n-6	0.74±0.19	0.90±0.17	0.35±0.04	0.32±0.01	0.49±0.13	0.76±0.12	0.29±0.06	0.42±0.14	0.003	0.223	0.009	0.163	0.310
18:3n-3	0.32±0.03	0.20±0.02	0.24±0.02	0.22±0.02	0.20±0.03	0.27±0.03	0.24±0.03	0.25±0.03	0.101	0.218	0.411	0.438	0.084
*c*11-20:1	0.29±0.03	0.39±0.03	0.31±0.03	0.27±0.03	0.29±0.03	0.42±0.03	0.30±0.03	0.36±0.03	0.005	0.400	0.525	0.025	0.137
*t*10,*c*12 CLA	nd	nd	1.17±0.29	6.46±2.05	nd	nd	1.25±0.29	6.29±2.05	-	-	-	0.008	0.932
20:2n-6	0.27±0.03	0.71±0.13	0.25±0.02	0.58±0.13	0.19±0.03	0.59±0.15	0.25±0.02	0.56±0.13	0.440	0.328	0.389	<0.001	0.900
20:4n-6	5.28^{a}±0.34	13.4^{c}±0.50	5.18^{a}±0.20	10.30^{c}±1.46	5.44^{a}±0.22	11.4^{c}±0.89	5.04^{a}±0.41	9.55^{b}±1.44	0.062	0.110	0.025	<0.001	0.053
20:3 ni^{I}	nd	0.42±0.143	nd	0.38±0.143	nd	0.97±0.143	nd	0.56±0.143	0.856	0.019	0.492	-	-
20:3 ni^{II}	0.43^{a}±0.11	0.94^{b}±0.10	0.74^{ab}±0.17	0.55^{a}±0.07	0.58^{ab}±0.14	1.00^{b}±0.09	0.75^{ab}±0.12	0.49^{a}±0.07	0.686	0.307	0.504	0.149	0.004
23:0	0.50±0.11	0.47±0.11	0.69±0.11	0.61±0.11	0.62±0.11	0.91±0.12	0.91±0.06*	nd	0.163	0.039	-	0.496	0.246

22:2n-6	0.75±0.21	0.54±0.24	0.79±0.21	0.56±0.24	0.57±0.21	1.13±0.27	1.04±0.21	1.08±0.24	0.898	0.399	0.091	0.804	0.330
22:4n-6	0.70±0.06	0.45±0.03*	0.73±0.06	nd	0.64±0.06	nd	0.74±0.06	nd	0.729	0.440	0.669	-	-
22:5n-3	nd	1.00±0.05	nd	1.00±0.05	nd	0.88±0.05	nd	0.77±0.05	0.981	0.105	0.008	-	-
22:6n-3	0.28±0.02	0.45±0.03	0.27±0.02	0.41±0.05	0.27±0.02	0.43±0.02	0.31±0.02	0.37±0.02	0.529	0.554	0.339	<0.001	0.137
DMA													
DMA-16:0	0.41±0.05	1.27±0.12	0.37±0.05	0.74±0.12	0.46±0.05	1.02±0.12	0.42±0.05	0.82±0.12	0.008	0.299	0.032	<0.001	0.057
DMA-18:0	0.38±0.08	0.79±0.07	0.24±0.20*	nd	0.27±0.09	0.63±0.07	0.44±0.08	0.63±0.08	-	0.139	0.571	<0.001	0.351
DMA-18:1	nd	0.51±0.027	nd	0.25±0.027	nd	nd	nd	nd	0.008	-	-	-	-
Partial sums													
SFA	61.3±0.99	43.1±1.06	59.3±0.56	47.3±4.95	60.5±0.31	46.7±0.77	59.9±0.61	44.3±2.21	0.911	0.209	0.767	<0.001	0.095
MUFA	23.3^{ab} ±0.60	27.0^{c} ±1.05	24.4^{ab} ±0.60	22.4^{a} ±1.05	24.5^{ab} ±0.60	27.4^{c} ±1.05	23.4^{ab} ±0.60	25.7^{bc} ±1.05	0.044	0.535	0.376	0.012	0.017
PUFA	14.6±0.35	27.4±2.32	15.8±0.35	29.5±2.32	14.4±0.35	24.3±2.32	15.8±0.35	28.6±2.32	0.343	0.333	0.483	<0.001	0.696
FA (ng/un)[III]	63.7±6.12	0.03±0.01	65.6±6.12	0.04±0.01	62.7±6.12	0.01±0.01	69.1±6.12	0.02±0.01	0.823	0.916	0.542	<0.001	0.887

Data in the same line with different superscript letters are significantly different ($P \leq 0.05$) due to an interaction between cell type and treatment; [1] Contrasts of each treatment against the control; C, cell type; T, treatment; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids, nd, not detectable; * data not included in statistical analysis; [I] and [II] – non identified 20:3 isomers; [III] – total FA content per individual cell.

saturated (14:0, 15:0, 16:0 but not 18:0) and monounsaturated (*c*7-16:1; palmitoleic, *c*9-16:1; vaccenic, *c*11-18:1 and *c*11-20:1, but not *c*9-18:1) FA than mature CC. Conversely, CC contained higher proportions of 18:0 and most of PUFA (18:2n-6, 20:2n-6, 20:4n-6, 22:6n-3) as well as DMA-16:0 and DMA-18:0. Consistently, mature oocytes presented higher SFA and lower PUFA than mature CC. The *c*9-18:1 was higher in CC than in oocytes in all treatments except on *t*10,*c*12 CLA treatment where was strongly reduced in CC to levels observed in oocytes. Moreover, *t*10,*c*12 CLA treatment seems to drastically eliminate the DMA-18:0 from mature CC, which was not detected only in CC cells treated with *t*10,*c*12 CLA. This effect seems to be reversed when FSK was also included in the maturation medium. On the contrary of other groups ($P = 0.053$), the 20:4n-6 proportion was equal in CC and oocytes of *t*10,*c*12 CLA+FSK group. Forskolin inclusion in maturation media has almost no effect on CC FA profile but slightly reduced the 15:0 ($P = 0.010$) and raised the *c*9-18:1 in oocytes ($P = 0.043$).

Independently of cell type, the CLA treatment reduced ($P < 0.05$) the proportions of DMA-16:0, 18:3n-6 and *c*11-20:1 and tended ($P < 0.10$) to reduce *c*7-16:1, *c*9-16:1, *c*11-18:1 and 20:4n-6. When *t*10,*c*12 CLA was included in the maturation medium with FSK (*t*10,*c*12 CLA+FSK) the proportions of 18:3n-6, 20:4n-6, 22:5n-3 and DMA-16 were also reduced.

The FA and DMA composition of culture media prior to and after COC maturation from all treatments is described in table 3.4.. The *t*10,*c*12 CLA was only detected when it was supplemented to the culture media. Lower concentrations of *c*9-18:1 ($P = 0.013$) and *c*11-18:1 ($P = 0.005$) and consequently of total MUFA ($P = 0.004$) were observed in *t*10,*c*12 CLA group than in pre-IVM, control or FSK groups. Moreover *c*11-18:1 concentration was also lower ($P = 0.004$) in *t*10,*c*12 CLA+FSK group than in *t*10,*c*12 CLA group. However, in the pre-IVM medium the percentage of 18:3n-6 was

Table 3.4. - Effect of *trans*-10, *cis*-12 conjugated linoleic acid (*t*10,*c*12 CLA), forskolin (FSK) or both (*t*10,*c*12 CLA+FSK) supplementation during in vitro maturation of porcine cumulus oocyte complexes on fatty acid (FA) and dimethylacetal (DMA) composition of culture media. Data are least square means (LSM) ± standard error (SE) percentages (w⁄w) of total fatty acids in five replicates.

		Culture media				*P*
	Pre-IVM	Control	*t*10,*c*12 CLA	FSK	*t*10,*c*12 CLA+FSK	*values*
14:0	0.58±0.06	0.82±0.06	0.74±0.06	0.78±0.06	0.78±0.07	0.111
*c*9-14:1	0.21±0.09	0.30±0.09	0.27±0.12	0.43±0.10	0.46±0.12	0.445
15:0	0.27±0.02	0.31±0.02	0.24±0.02	0.31±0.02	0.31±0.03	0.178
16:0	22.4±0.43	21.9±0.43	21.5±0.43	22.3±0.43	21.8±0.43	0.607
*c*7-16:1	0.87±0.05	0.94±0.05	1.03±0.05	0.98±0.05	0.84±0.06	0.118
*c*9-16:1	1.08±0.07	1.22±0.05	1.23±0.05	1.20±0.05	1.05±0.06	0.274
17:0	0.75±0.07	0.85±0.08	0.76±0.08	0.72±0.08	0.74±0.08	0.740
*c*9-17:1	0.25±0.01	0.244±0.01	0.22±0.01	0.24±0.01	0.23±0.02	0.713
18:0	14.70±0.58	13.90±0.58	14.70±0.58	13.40±0.58	14.00±0.58	0.434
*c*9-18:1	20.80^{b} ±0.33	21.00^{b}±0.33	19.40^{a} ±0.33	21.00^{b}±0.33	20.20^{ab}±0.33	0.013
*c*11-18:1	1.97^{b}±0.09	2.07^{b}±0.09	1.56^{a}±0.09	1.97^{b}±0.09	2.16^{b}±0.11	0.005
18:2n-6	19.40±0.52	19.80±0.52	19.20±0.52	20.30±0.52	19.30±0.52	0.532
20:0	0.38±0.08	0.41±0.08	0.51±0.08	0.44±0.08	0.42±0.09	0.800
18:3n-6	0.31^{a} ±0.029	0.41^{ab} ±0.029	0.41^{ab}±0.03	0.44^{ab}±0.03	0.39^{ab}±0.03	0.053
18:3n-3	0.28±0.04	0.28±0.04	0.29±0.04	0.32±0.04	0.36±0.41	0.518
*c*11-20:1	0.10±0.38	0.13±0.33	0.13±0.33	0.20±0.33	0.88±0.33	0.442
*t*10,*c*12 CLA	nd	nd	2.74 ± 1.02	nd	2.35 ±1.02	0.797
20:2n-6	0.29±0.08	0.24±0.08	0.37±0.08	0.34±0.08	0.27±0.09	0.715
$20:3^{niI}$	0.57±0.05	0.50±0.05	0.47±0.05	0.46±0.05	0.46±0.05	0.516
$20:3^{niII}$	0.56^{b}±0.03	0.50^{ab}±0.03	0.43^{a}±0.03	0.47^{ab} ±0.03	0.51^{ab}±0.03	0.042
20:4n-6	10.40±0.28	9.84±0.28	9.89±0.28	10.40±0.28	9.97±0.28	0.439
23:0	0.23±0.07	0.34±0.07	0.45±0.07	0.31±0.07	0.41±0.08	0.276
22:2n-6	0.23±0.42	0.24±0.42	0.45±0.42	0.29±0.42	1.22±0.42	0.428
22:4n-6	nd	0.22±0.12	0.34±0.12	nd	nd	0.520
22:5n-3	1.11±0.16	1.06±0.18	1.15±0.16	1.13±0.16	1.62±0.16	0.139
22:6n-3	0.44±0.03	0.43 ±0.03	0.56±0.03	0.50±0.03	0.53±0.04	0.057
Other FA	1.45^{b}±0.08	1.40^{b}±0.08	1.01^{a}±0.08	0.94^{a}±0.08	0.99^{a}±0.09	<0.001
DMA						
DMA-16:0	0.57±0.09	0.84±0.09	0.59±0.09	0.64±0.09	0.59±0.11	0.256
DMA-18:0	0.57±0.05	0.56±0.05	0.58±0.07	0.50±0.05	0.50±0.06	0.739
DMA-18:1	0.38±0.05	0.38±0.03	0.28±0.05	0.22±0.05	0.26±0.05	0.083
Partial sums						
SFA	39.30±0.88	38.40±0.88	38.80±0.88	38.20±0.88	37.80±0.88	0.754
MUFA	25.60^{b} ±0.42	26.30^{b}±0.42	23.70^{a}±0.42	25.90^{b}±0.42	24.70^{ab}±0.42	0.004
PUFA	33.50^{a}±0.30	33.60^{a}±0.60	36.30^{b}±0.20	34.70^{ab}±1.60	36.40^{ab}±1.44	<0.001
FA (ng/µL)[III]	54.00±3.10	51.30±3.10	50.00±3.10	58.30±3.10	54.30±3.10	0.403

Data within the same row with different superscripts letters are statistically different ($P \leq 0.05$). Pre-IVM, maturation medium prior to culture; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; nd, not detectable; ni[I] and [II], non identified 20:3 isomers; [III] – total FA content ng per µL.

higher (P = 0.038) than in FSK medium and of 20:3^{niII} was lower (P = 0.027) than in *t*10,*c*12 CLA supplemented medium. Finally, PUFA content was lower (P < 0.0001) in pre-IVM and control media than in *t*10,*c*12 CLA supplemented medium after COC maturation.

3.6. - Discussion

Porcine oocytes are for long known to be highly rich in lipids although their nature and function have been largely overlooked (Homa *et al.*, 1986; Sturmey *et al.*, 2006 and 2009). The present study provides new insights on FA and DMA composition of porcine COC prior to and during IVM. Results showed for the first time that immature oocytes and their CC have different FA profiles which change differentially along maturation. While oocytes are notably rich in SFA, preferentially maintaining their FA profile, immature CC are characterized by an abundant MUFA content and their FA profile is greatly modified during maturation. In mature CC, SFA and MUFA are reduced while PUFA content is higher and diverse. However either in oocytes or in CC the FA and DMA content tends to increase along maturation. Moreover, *t*10,*c*12 CLA and/or FSK supplementation influenced COC and culture media FA and DMA composition but their total contents were not modified.

Emerging studies in the field of developmental biology revealed the extremely intricate COC as a complete functional and dynamic unit. The bi-directional exchanges between the oocyte and the surrounding CC are critical for the acquisition of oocyte competence, CC expansion and early embryonic development. Moreover this cross-talk between CC and oocyte plays a pivotal role in oocyte metabolism during maturation (Sutton *et al.*, 2003; Gilchrist and Thompson, 2007; Ouandaogo *et al.*, 2011). In porcine oocytes this process is long and requires different metabolites for energy production (Sturmey and Leese, 2003). Since these gametes cannot use glucose directly, CC catabolise glucose

by the glycolytic pathway, supplying pyruvate or lactate to the oocyte as oxidative substrates (Sutton *et al*., 2003). A metabolic role for triacylglycerols providing ATP through FA mitochondrial β-oxidation which is essential for protein synthesis along cytoplasmic maturation and meiosis was also suggested by Sturmey and Leese (2003) and Sturmey *et al.* (2006). These authors showed that the triacylglycerol content of immature oocytes decreases during maturation with sufficient O_2 consumption to be accounted for this depletion. Additionally Romek *et al*. (2011), also through an indirect method of lipid evaluation using lipid droplets staining, reported that *in vivo* total lipid content in immature porcine oocytes was about 21% higher than in mature. On the contrary, our results determined by internal standard gas-chromatography quantification of FA, showed that FA content of COC, oocytes and CC, tend (P = 0.056) to increase with the maturation process. As herein McEvoy *et al*. (2000) reported that the most abundant FA in immature porcine oocytes was the 16:0, followed by the *c*9-18:1, 18:0, 18:2n-6 and 20:4n-6, although the amount of total FA was lower in the present study. This FA profile was maintained after IVM. Thus in order to support maturation, the oocytes ought to accumulate but also to metabolize their constituent FA and/or utilize those supplied by the CC or from the culture medium enriched with follicular fluid.

Previously it was reported by Cetica *et al*. (2002) that during bovine oocyte maturation, CC may supply FA to the oocytes for further participation on their metabolism. In addition these oocytes were able to perform a selective FA uptake from culture medium during maturation (Kim *et al.*, 2001; Lapa *et al.*, 2011) to maintain their intracellular FA reserves. Eventually FA elongation or *de novo* synthesis may also occur. These mechanisms might also take place during porcine COC maturation. In fact the FA profile of CC changed drastically along this process. The SFA, in particular 14:0, 15:0, 17:0, 20:0, 23:0, as well as MUFA, notably *c*9-18:1, were reduced in mature CC.

Therefore it is possible that during maturation porcine CC can either extensively metabolize SFA and MUFA and/or supply them to the oocyte. Moreover the proportion of mature CC PUFA, composed by eleven individual FA (table 3.2.), was the highest of all COC constituents. The majority of these long chain FA as well as the DMA derived from the vinyl ether chains of plasmalogens are components of membrane phospholipids (Homa *et al.*, 1986; McEvoy *et al.*, 2000). Thus porcine CC might undergo an extensive intracellular remodeling associated with its metabolic differentiation resulting in increased amounts of internal membrane.

On the other hand, oocytes are found to be highly chilling sensitive due to a characteristic high ratio of SFA to PUFA (Arav and Zvi, 2008). This ratio results from the existence of a selective protective process to ensure that PUFA composition of oocytes is kept in a safe minimum level to avoid the risk of cellular damage (Sturmey *et al.*, 2009; Lapa *et al.*, 2011). In accordance, porcine oocytes are mainly constituted by SFA preserving their FA profile during maturation. Interestingly, although oocytes were cultured in a PUFA containing media, mainly 18:2n-6, they did not uptake provisions of all these available FA. The most abundant FA in porcine follicular fluid and thus in IVM media was the 16:0. This SFA may be used by porcine oocytes having a beneficiary effect on their quality (Warzych *et al.*, 2011), in contrast to its harmful action in bovine oocytes (Van Hoeck *et al.*, 2011). Nonetheless when supplemented with *t*10,*c*12 CLA porcine oocytes incorporate this PUFA but in much lower amounts than in CC. Despite that, PUFA percentage was not changed neither in *t*10,*c*12 CLA oocytes nor in CC suggesting that *t*10,*c*12 CLA replaced other PUFA.

Several studies reported the activity of lipid modulators namely *t*10,*c*12 CLA and FSK during oocyte maturation (Lapa *et al.*, 2011; Fu *et al.*, 2011) or embryonic development (Men *et al.*, 2006; Pereira *et al.*, 2007; 2008). According to Prates *et al.* (2012) during

porcine oocyte maturation FSK impaired meiosis beyond 2h of supplementation while *t*10,*c*12 CLA had no effect on meiotic progression. Moreover both substances interfered with the distribution and morphology of lipid droplets. However if these morphological modifications of IVM porcine oocytes (Fu *et al.* 2011, Prates *et al*., 2012) reflect changes of their FA composition was not investigated. Herein we found differences in FA and also in DMA composition of COC supplemented with *t*10,*c*12 CLA, FSK or both. This isomer was accumulated by COC from *t*10,*c*12 CLA and *t*10,*c*12 CLA+FSK groups, mostly in CC where MUFA and a 20:3 isomer decreased ($P \leq 0.043$) though PUFA, total FA and DMA contents were not affected. Thus in the present conditions, the identified *t*10,*c*12 CLA activity in reducing lipid accumulation (Pariza, 2001; Pereira *et al.*, 2007; 2008) was not demonstrated for porcine oocytes. Equally although FSK can induce lipolysis in porcine embryo cells and oocytes reducing their lipid content (Men *et al.*, 2006; Fu *et al.*, 2011), we could not confirm this reduction, since the FA and DMA contents were not different from control in FSK mature oocytes. Nevertheless, when FSK was supplemented alone for 2 h, the 18:1*c*9 increased in mature oocytes.

Although *t*10,*c*12 CLA and FSK did not interfere on nuclear meiosis progression, a possible additive effect of these substances on 20:4n-6 metabolization by CC, probably through eicosanoid cascade (Nuttinck *et al*., 2008, Lapa *et al.*, 2011), seems to occur. In fact during maturation the 20:4n-6 decreases 3.85% in *t*10,*c*12 CLA+FSK CC when compared to control ($P = 0.023$). Both products can affect the protein kinase A and cAMP signaling pathways (Men *et al*., 2006; Ashwell *et al*., 2010) thus exerting a synergic effect. Interestingly, CC of porcine COC treated with *t*10,*c*12 CLA revealed more modifications in the FA profile than those of bovine where only 20:4n-6 were reduced in mature oocytes but not in CC (Lapa *et al.*, 2011). These divergences may be

related to differences in COC acquisition of developmental competence between these species. Furthermore, herein both DMA-16:0 and DMA-18:0 were higher in CC than in oocytes from all groups. However *t*10,*c*12 CLA treatment reduced the DMA-16:0 in COC and the DMA-18:0 (which became undetectable) from CC of *t*10,*c*12 CLA group. These effects of *t*10,*c*12 CLA seem to be mitigated when FSK was also present. A decrease in plasmalogens of cellular membranes might interfere with their dynamics and functions (Nagan *et al.*, 2001). The membranes composition can therefore interfere in the communication between oocytes and CC or CC adherence looseness during CC expansion, being crucial for appropriate oocyte maturation and fertilization (Sutton *et al.*, 2003; Gilchrist and Thompson, 2007). The importance of plasmalogens in porcine COC maturation requires further investigation.

3.7. Conclusions

In conclusion, the findings from the present study indicate that 16:0, 18:0, *c*9-18:1, 18:2n-6 and 20:4n-6 are the major constituents of porcine follicular fluid and COC although in different orders of abundance. Moreover it highlights the distinct FA and DMA profiles of oocytes and CC during maturation. Both immature and mature porcine oocytes are rich in SFA preferentially maintaining their profile as well as their SFA and PUFA ratio during maturation probably to prevent the significant risk of free radical damage currently associated to FA unsaturations. The marked variation in FA profile of CC during this process could indicate a major participation in supplying substrates to the oocyte and an extensive intracellular remodeling resulting in increased amounts of internal membrane and consequently an enrichment in PUFA and DMA at the end of maturation. Besides CC PUFA reservoir, mostly 18:2n-6 and 20:4n-6 might be involved in FA elongation or PG synthesis. On the other hand, porcine COC can uptake and

accumulate *t*10,*c*12 CLA from the culture media, being CC more susceptible to FA and DMA modifications induced by this CLA isomer than oocytes whereas FSK during 2h induced an increase of *c*9-18:1 in oocytes. Therefore, data of FA and DMA composition of porcine COC suggest a pivotal role of both cumulus cells and oocytes in lipid metabolism through maturation. This information can complete previous morphological evaluations and should be used to enhance oocyte quality and the efficiency of *in vitro* embryo production and cryopreservation.

3.8. - Acknowledgment(s)

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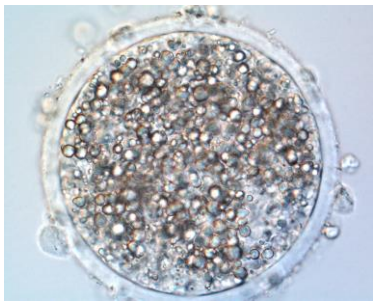

CHAPTER 4

IN VITRO MATURATION OF PORCINE OOCYTE-WITH *TRANS*-10, *CIS*-12 CONJUGATED LINOLEIC ACID AND/OR FORSKOLIN: EFFECT ON FERTILIZATION, EMBRYO DEVELOPMENT AND CRYO-RESISTANCE

4.1. - Introduction

Porcine oocytes and embryos are characterized by their high lipid content (Fujihira *et al.*, 2004; Men *et al.*, 2006; Romek *et al.*, 2009 and 2010). The provision of large endogenous energy repository available to the egg and embryo until it attaches in the uterus, may be one hypothesis for such high lipid content in porcine species (Sturmey *et al.*, 2009). In immature porcine oocytes, triacylglycerols are the major component of intracellular lipids (Homa *et al.*, 1986) and are stored in lipid droplets (LD) that can be associated to mitochondrial metabolism to produce energy during oocyte maturation (Sturmey *et al.*, 2006; Jagerstrom *et al.*, 2009). Also, cholesterol and phospholipids oocyte fractions are prerequisites in the formation of membranes during the rapid cell divisions after fertilization (McEvoy *et al.*, 2000). Analysis of total fatty acids (FA) composition of immature and mature porcine oocytes showed that the palmitic (16:0), the oleic (*c*9-18:1) and the stearic (18:0) acids were the most abundant (Homa *et al.*, 1986; McEvoy *et al.*, 2000; Prates *et al.*, 2013). Moreover, oocyte FA and plasmalogens (measured by dimethyl acethyl, DMA) composition was maintained in course of maturation. Nevertheless, cumulus cells (CC) FA profile was modified during this process, reducing saturated (SFA) and monounsaturated fatty acids (MUFA) contents while those of polyunsaturated (PUFA) increased, particularly due to the raise in arachidonic acid (AA, 20:4n-6) (Prates *et al.*, 2013).

The triacylglycerols and phospholipids of porcine ovarian tissues are rich in PUFA, particularly in linoleic acid (18:2n-6) (Holman and Hofstetter, 1965), suggesting a possible role for those during maturation. As reviewed by Abayasekara and Wathes (1999) PUFA can stimulate ovulation in mammalian species modulating the levels of serum cholesterol and interfering on follicular steroid synthesis. Also they can induce

prostaglandins and other eicosanoid production from AA which precursor is 18:2n-6. Prostaglandins are very important for oocyte maturation namely for meiosis resumption and progression and cumulus cells expansion (Nuttinck *et al.*, 2008).

Previous reports showed that the addition of *trans*-10, *cis*-12 conjugated linoleic acid (*t*10,*c*12 CLA) to culture medium improved bovine oocyte development into higher quality embryo (Lapa *et al.*, 2011). This beneficial effect was proposed to be related to FA modifications during *in vitro* maturation. Moreover, the *t*10,*c*12 CLA isomer is able to induce a decrease in lipogenesis during *in vitro* culture of porcine adipose tissue and to reduce lipid content in bovine blastocysts when added to embryo culture (Pereira *et al.*, 2007; José *et al.*, 2008). In fact, it has been refereed that the cryopreservation of cells may benefit from reduction in excessive lipid accumulation during *in vitro* culture, dimishing chilling injury (Kim *et al.*, 2001; Men *et al.*, 2006, Pereira *et al.*, 2007). Therefore, it seems reasonable to admit that addition of *t*10,*c*12 CLA to porcine IVM media, could enhance oocyte and embryo development, as well as cryosurvival.

The effects of *t*10,*c*12 CLA (100 µM) and also of forskolin (10 µM), an adenylyl cyclase stimulator with lipolytic properties, were previously investigated during porcine oocyte *in vitro* maturation (IVM), showing that oocytes treated with *t*10,*c*12 CLA during the entire period of IVM displayed a lighter cytoplasmic colour tone, whereas oocytes supplemented with forskolin during the first 2 h had smaller LD (Prates *et al.*, 2012). For those doses and exposition times, these cytoplasmic modifications were not accomplished by an interference on oocyte meiotic progression or competence to cleave. Thereafter, Prates *et al.* (2013) correlated the morphological alterations of treated oocytes with modifications in their FA and DMA composition. The *t*10,*c*12 CLA added alone or with forskolin to IVM medium was accumulated in porcine cumulus-oocyte complexes (COC) influencing both oocytes and cumulus cells (CC)

FA/DMA composition. It was shown that in the presence of *t*10,*c*12 CLA and *t*10,*c*12 CLA+forskolin, the MUFA fraction decreased and an interference in several FA and DMA contents was identified, while the effects of forskolin were more subtle with respect to COC and cell type compositions.

A considerable variation in meiotic morphology was identified during the selection of porcine oocytes for IVM that was associated to differences in meiosis progression within oocyte cohort (Grupen *et al.*, 1997; Lucas *et al.*, 2003; Albuz *et al.*, 2010). Therefore, forskolin is used to stimulate the increase in cAMP intracellular levels during IVM to enhance the developmental competence of IVM oocytes that prematurely undergo nuclear maturation (Laforest *et al.*, 2005; Gilchrist and Thompson, 2007). According to Thomas *et al.* (2004) treatments that maintain or elevate cAMP levels in CC, oocytes, or both result in prolonged oocyte-CC communication and delayed meiotic resumption, which can be important to allow the signal flow for a complete oocyte cytoplasmic maturation.

In this work the effects of *t*10,*c*12 CLA (entire period) and/or forskolin (2 h) supplementation to IVM medium on porcine oocyte meiotic and developmental competence for fertilization and embryonic development were studied. Embryo quality was further evaluated by blastocyst total cell number and cryosurvival.

4.2. - Materials and Methods

Unless otherwise stated, all chemicals used in the present study were purchased from Sigma-Aldrich Quimica (Madrid, Spain).

4.2.1. - *Recovery and IVM of COC*

Gilts ovaries were obtained at a local slaughterhouse and transported in 0.9% NaCl, with 70 μg/mL kanamycin, at 37 ºC to the laboratory. Follicles of 3–6 mm in diameter were sectioned with a scalpel and a blade. The follicular content was retrieved in Dulbecco's phosphate-buffered saline (DPBS), composed of 4 mg/mL bovine serum albumin (BSA; fraction V), 0.34 mM sodium pyruvate, 5.4 mM D-glucose and 70 μg/mL kanamycin (mDPBS). Oocytes with a compact cumulus mass and a dark and granulated cytoplasm were selected in mDPBS, then washed in maturation medium, the BSA-free North Carolina State University (NCSU23; Petters and Wells, 1993), supplemented with 10% (v/v) porcine follicular fluid (pFF), 0.1 mg/mL cysteine and 10 ng/mL epidermal growth factor (EGF). After, oocytes were matured in a 4-well multidisc (Nunc, Roskilde, Denmark), containing 80-90 COC per well and 500 μL of IVM medium with 10 IU/mL equine chorionic gonadotrophin (eCG; Folligon; Intervet International, Boxmeer, The Netherlands) and 10 IU/mL human chorionic gonadotrophin (hCG; Veterin Corion; Divasa Farmavic, Barcelona, Spain), for 20–22 h, then for another 20–22 h in IVM medium without hormones. Maturation was performed under mineral oil at 39 ºC in a humidified atmosphere, with 5% CO_2 in air.

4.2.2. - *In vitro fertilization*

After maturation, COC were denuded with 0.1% hyaluronidase in mDPBS, then washed in IVM medium and in pre-equilibrated fertilization medium, the modified Tris-buffered medium (mTBM; Abeydeera and Day, 1997), composed of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris (crystallised free base), 11 mM glucose, 5 mM sodium pyruvate, and supplemented with 0.5 mM caffeine and 0.2% BSA (fraction

V; A7888, initial fractionation by cold alcohol precipitation). Thirty denuded oocytes were placed in 50 μL drops of fertilization medium in a 35 × 10 mm Petri dish (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA) under mineral oil, at 39 ºC in a humidified atmosphere, with 5% CO_2 in air. One hundred μL of thawed sperm were centrifuged for 3 times, in mDPBS, at 1900 *g* during 3 min. The sperm pellet was resuspended in fertilization medium and after appropriate dilution, 50 μL of this sperm suspension was added to the oocytes. The spermatozoa : oocyte ratio was 1000: 1 and co-incubation was performed for 6 h, at 39 ºC in a humidified atmosphere, with 5% CO_2 in air (Gil *et al.*, 2003). IVM media were supplemented with *t*10,*c*12 CLA at 100 μM (Matreya ref. 001249, Pereira *et al.*, 2007) and or 10 μM forskolin during the initial 2 h (Prates *et al.*, 2012).

4.2.3. - *In vitro embryo culture*

Presumptive zygotes were removed from the fertilization medium and washed in pre-equilibrated embryo culture medium and placed in a 4-well multidisc, 35-40 zygotes per well, each containing 500 μL of the same medium. Culture was performed under mineral oil, at 39 ºC in 5% CO_2 in air, either during 16-18 h post insemination (hpi) to assess fertilization parameters, or for 7 days for embryo development evaluations. During the first 2 days (day 0 = day of fertilization), embryo culture medium was the glucose-free NCSU23, with 0.33 mM pyruvate, 4.5 mM lactate and 0.4% BSA. Subsequently, all embryos were removed and cultured in fresh NCSU23 medium containing 5.5 mM glucose and 0.4% BSA until day 7, supplemented with 10% fetal calf serum (FCS) from day 5 onwards.

4.2.4. - *Assessment of maturation, fertilization and embryo development*

For maturation and fertilization evaluations, oocytes and presumptive zygotes were fixed (acetic acid:ethanol, 1:3 v/v) for 48–72 h at room temperature, stained with aceto-lacmoid (1%) and examined under a phase-contrast microscope at a magnification of × 400. Oocytes with abnormal cytoplasmic appearance or degenerated were discharged. Following 40-44 h of IVM, oocytes with chromosomes at MII and an extruded polar body were considered mature oocytes. After 16–18 hpi, oocytes containing one or more swollen sperm heads and/or male pronuclei, with their corresponding sperm tails, and two polar bodies were considered penetrated. The penetration (number of penetrated oocytes/ total of matured oocytes), monospermy (number of oocytes containing only one male pronucleus/ total of penetrated oocytes), number of spermatozoa per oocyte (number of spermatozoa/oocyte penetrated) and efficiency of fertilization (number of monospermic oocytes/ total inseminated) were evaluated.

Embryo development was assessed by the cleavage rate (number of two or four-cell embryos/ total inseminated) on day 2, and blastocyst rate (number of day 7 blastocysts/ total inseminated).

Fresh blastocysts quality was also evaluated by the number of cells. Samples of day 7 blastocysts were fixed using a 4% paraformaldehyde in PBS solution during 20-30 min at room temperature. After, blastocysts were washed in PBS with BSA (3mg/mL) and placed on a slide, in 4 μL Vectashield (Vector, Burlingame, CA, USA) containing 10 μg/mL Hoechst 33342 (H-33342). The total number of nuclei stained with H-33342 and displaying blue fluorescence was counted (Cuello *et al.*, 2008).

4.2.5. - *Evaluation of blastocyst survival and quality following vitrification-warming*

Samples of day 6 blastocysts were vitrified as described by Cuello *et al.* (2004). The basic vitrification and warming medium was the TCM-199–HEPES, with 0.5 mM glutamine and 0.1% polyvinyl alcohol (PVA), equilibrated at 39 ºC and 5% CO_2. Cryoprotectants for vitrification were dimethyl sulfoxide (DMSO) and ethylene glycol (EG). Groups of 4-6 blastocysts were vitrified using a four-well multidisc, firstly washed in TCM, then sequentially equilibrated in TCM + 7.5% DMSO + 7.5% EG for 3 min and then in TCM + 16% DMSO + 16% EG + 0.4 M sucrose for 1 min. During the last step, blastocysts were placed in a 1.5–2 μL drop and loaded into the narrow end of a Superfine Open Pulled Straw (SOPS, Minitüb, Tiefenbach, Germany) by capillary action. Immediately the straw containing the blastocysts was plunged horizontally into liquid nitrogen. After storage blastocysts were warmed by the direct warming method (Cuello *et al.*, 2004) and cultured during 24 h in embryo culture medium (NCSU23 with 5.5 mM glucose and 10% FCS), at 39 °C in 5% CO_2 in air. Blastocysts that fully or partially reformed their blastocoelic cavity, with excellent or good appearance were considered viable and survival rate calculated as the ratio of viable to the total number of cultured blastocysts. Blastocyst total cell number was also evaluated.

4.3. - Experimental Design

The effects of *t*10,*c*12 CLA and forskolin on porcine oocyte competence for maturation, fertilization, embryo development and quality were studied. A total of 3183 immature porcine oocytes were used in 5 replicates to assess maturation and fertilization, embryo development and blastocyst quality. Four experimental groups were constituted as follow: (1) Control (n = 812) - oocytes were subjected to the standard IVM protocol,

(2) *t*10,*c*12 CLA (n = 775) - oocytes were supplemented with 100 μM *t*10,*c*12 CLA during 44 h, (3) forskolin (n = 805) - oocytes were matured with 10 μM forskolin supplementation for the initial 2 h and then subjected to the standard IVM protocol, (4) *t*10,*c*12 CLA+forskolin (n = 791) - oocytes were cultured with *t*10,*c*12 CLA and forskolin supplementation during the initial 2 h, then with *t*10,*c*12 CLA until the end of maturation.

4.4. - Statistical analysis

All data editing and statistical analyses were performed by SPSS, version 11.5 (SPSS Inc., Chicago, III). Data from all replicates were assessed by analysis of variance using the MIXED procedure. Maturation, penetration and monospermic rates, efficiency data, cleavage rates and blastocyst rates and quality were modelled according to the binomial model of variables, as described by Fisz (1980) before analysis. When analysis of variance showed a significant effect, values were compared using the Bonferroni test. The threshold for significance was set at $P < 0.05$. Results are expressed as square means ± standard error of mean (mean ± SEM).

4.5. - Results

The effect of *t*10,*c*12 CLA and forskolin supplementation on nuclear maturation of porcine oocytes are shown in Table 4.1. Although no significant effect of *t*10,*c*12 CLA and forskolin alone were observed, oocytes treated with both supplements, had a higher ($P = 0.040$) maturation rate than oocytes from control group.

Table 4.1. - Oocyte nuclear maturation rates after 44 h of *in vitro* culture with *trans*-10, *cis*-12 conjugated linoleic acid (*t*10, *c*12 CLA)**,** forskolin or both (mean ± SEM) (three independent replicates).

	Matured oocytes (n)	Maturation rate (%)
Control	123	69.9 ± 3.8^{b}
*t*10,*c*12 CLA	141	$82.3 \pm 3.5^{a,b}$
Forskolin	125	$70.4 \pm 3.7^{a,b}$
*t*10,*c*12 CLA + forskolin	117	84.6 ± 3.9^{a}

Data with different letters in the same column are significantly different ($P < 0.05$).

Regarding fertilization parameters there were no differences in the penetration rate, number of spermatozoa per oocyte and efficiency of fertilization among groups. However, a higher ($P = 0.003$) monospermic rate was observed in oocytes of *t*10,*c*12 CLA+forskolin group than of forskolin group (Table 4.2.).

Table 4.3. shows the effect of *in vitro* maturation of porcine oocytes in the presence of *t*10,*c*12 CLA and/or forskolin on cleavage, blastocyst rates and blastocyst total cell number. There was a significant decrease ($P = 0.041$) in cleavage rate of *t*10,*c*12 CLA+forskolin group compared to control group, but at the blastocyst stage this decrease was overcome. On the contrary, the *t*10,*c*12 CLA group presented similar cleavage rate but lower ($P = 0.008$) blastocyst rate than control. No differences were found in the total cell number of blastocysts among groups. The cryosurvival ability and quality of yielded blastocysts are shown in Table 4.4. At 24 h post-warming, there were no significant differences on the re-expansion rates of vitrified-warmed blastocysts among groups. Similarly, no differences were found in the total cell number of vitrified-warmed blastocysts among groups.

Table 4.2. - Effect of *trans*-10, *cis*-12 conjugated linoleic acid (*t*10,*c*12 CLA) during 44 h of *in vitro* maturation, forskolin during 2 h or both supplements, on fertilization parameters (mean ± SEM), in five independent replicates.

Groups	Oocytes (n)	Penetration rates (%)	Monospermy rates (%)	EO (%)	Efficiency (%)
Control	149	70.6 ± 5.8	57.1 ± 7.8[a,b]	1.6 ± 1.2	36.1 ± 4.5
*t*10,*c*12 CLA	134	70.4 ± 6.0	63.2 ± 8.0[a,b]	1.5 ± 1.3	38.6 ± 4.7
Forskolin	153	74.2 ± 5.7	46.2 ± 7.7[b]	1.7 ± 1.2	30.5 ± 4.4
*t*10,*c*12 CLA+forskolin	152	69.8 ± 5.8	69.5 ± 7.8[a]	1.4 ± 1.2	42.5 ± 4.4

Values with different superscripts in the same column are significantly different ($P < 0.05$). Penetration: number of penetrated oocytes/total matured oocytes; monospermy: number of oocytes with only one sperm head or one male pronuclei/ total penetrated oocytes; EO (number of spermatozoa in penetrated oocytes): number of spermatozoa/ oocyte; efficiency of fertilization: number of monospermic oocytes/ total inseminated.

Table 4.3. - Effect of *trans*-10, *cis*-12 conjugated linoleic acid (*t*10,*c*12 CLA) during 44 h of *in vitro* maturation, forskolin during 2 h or both supplements on embryo production (mean ± SEM), in five independent replicates.

Groups	Oocytes (n)	Cleavage rate (%)	Blastocyst rate (%)	Blastocyst total cell number (no. blastocysts sampled)
Control	540	60.9 ± 2.6^{a}	34.9 ± 3.7^{a}	29.8 ± 2.5 (49)
*t*10,*c*12 CLA	500	$57.2 \pm 2.7^{a,b}$	25.7 ± 3.7^{b}	29.6 ± 2.7 (52)
Forskolin	527	$60.1 \pm 2.6^{a,b}$	$32.3 \pm 3.7^{a,b}$	32.5 ± 4.6 (43)
*t*10,*c*12 CLA+forskolin	522	52.8 ± 2.6^{b}	$30.2 \pm 3.7^{a,b}$	29.0 ± 5.3 (40)

Data within the same column with different letters are statistically different ($P < 0.05$). Cleavage rate: number of two or four-cell embryos/total of inseminated oocytes; blastocyst rate: number of day 7 blastocysts/ total of inseminated oocytes

Table 4.4. - Effect of *trans*-10, *cis*-12 conjugated linoleic acid (*t*10,*c*12 CLA) during 44 h of *in vitro* maturation, forskolin during 2 h or both supplements, on blastocyst quality, measured by survival rates and blastocyst cell numbers following 24 h of culture, after vitrification-warming (mean ± SEM), in five independent replicates.

Groups	Blastocysts (n)	Survival rate (%)	Total re-expansion (%)	Partial re-expansion (%)	Blastocyst total cells (no. blastocysts)
Control	125	40.8 ± 4.3	30.4 ± 3.9	10.4 ± 2.9	42.8 ± 3.1 (50)
*t*10,*c*12 CLA	74	35.1 ± 5.6	17.6 ± 5.0	17.6 ± 3.8	34.1 ± 4.4 (24)
Forskolin	107	30.8 ± 4.7	20.6 ± 4.2	10.3 ± 3.2	45.6 ± 4.0 (30)
*t*10,*c*12 CLA+forskolin	95	42.1 ± 5.0	29.5 ± 4.4	12.6 ± 3.4	48.0 ± 3.5 (39)

The set of significance was $P < 0.05$. Blastocyst survival rate was the sum of total (number of fully re-expanded/ total of cultured) and partial (number of partially re-expanded/ total of cultured) re-expansion rates; blastocyst total cells: number of total cells/ number of blastocysts samples.

4.6. - Discussion

A role of endogenous and exogenous lipids in energy provision during *in vitro* maturation of porcine oocytes has been established (Sturmey *et al.*, 2006 and 2009). Recently results of COC morphological and FA and DMA composition analysis showed that the addition of *t*10,*c*12 CLA, forskolin or both to IVM media interfere on porcine oocytes intracellular lipid content (Prates *et al.*, 2012, Prates *et al.*, 2013). However, their effects on blastocyst production and quality have not been investigated. Therefore, in this experiment the effect of those supplements during *in vitro* maturation of porcine oocytes on their nuclear progression and developmental capacity to fertilize and produce embryos as also the cryosurvival ability of yielded blastocysts were studied. Results showed that IVM supplementation with *t*10,*c*12 CLA interfered on embryonic development reducing blastocyst yield. When this isomer was added with forskolin during the first 2 h of IVM an increase in maturation rate was followed by a delay in cleavage. Moreover, monospermy was enhanced in *t*10,*c*12 CLA+forskolin treatment when compared to forskolin group.

Previously analysis of porcine COC FA and DMA composition showed that when *t*10,*c*12 CLA was added to IVM medium, alone or with forskolin it was accumulated in both, oocytes and CC (Prates *et al.*, 2013). Accordingly, *in vitro* matured bovine oocytes were able to select and accumulate *t*10,*c*12 CLA from IVM medium (Lapa *et al.*, 2011). Inside the oocyte this isomer may follow the eicosanoid formation, be subjected to immediate metabolism or incorporated into the cell reserves. However the metabolic fate of CLA can be dependent upon the species (Banni, 2002).

Herein, *t*10,*c*12 CLA supplemented to IVM medium of porcine oocytes did not interfered with meiosis, but when added simultaneously to forskolin (2 h) it increased nuclear maturation. The precursor of PG synthesis is the arachidonic acid (AA, 20:4n-

6), which is elongated from linoleic acid (Abayasekara and Wathes, 1999). In course of IVM, the 20:4n-6 fraction in porcine oocyte did not change during maturation though it has increased in CC (Prates *et al.,* 2013). However, when *t*10,*c*12 CLA and forskolin were added to IVM medium, the 20:4n-6 CC content decreased ($P = 0.025$) comparing to control. In accordance to Lapa *et al.* (2011) a decrease in 20:4n-6 might indicate consumption during eicosanoid generation, namely PG. The AA could thus have a direct effect on PG generation as also on steroidogenesis (Abayasekara and Wathes, 1999; Wathes *et al.*, 2007). These authors referred that gonadotrophins stimulate PG production in the ovarian follicular cells that in turn stimulate ovarian steroidogenesis. Others showed that PGE_2 is synthesized in bovine COC during *in vitro* maturation as well as progesterone (Nuttinck *et al.*, 2008). These authors referred that PGE_2 and progesterone are critical mediators of cumulus expansion and of oocyte meiosis resumption. The beneficial effect of *t*10,*c*12 CLA plus forskolin on oocyte maturation could be closely associated with the mediation of eicosanoid cascade in porcine CC. Therefore FA composition of COC appears to be crucial to maintain its functionality and to enhance oocyte maturation.

In a previous experiment the same *t*10,*c*12 CLA and forskolin doses and supplementation times did not affected meiotic progression when given simultaneously to the IVM medium of porcine oocytes (Prates *et al.,* 2013). This discrepancy can be explained by the differences in the maturation systems. In fact, 100 μM glutathione (GSH) was added to IVM medium in the first trial, though GSH could have been synthesized from the amino acid cysteine (Jeong and Yang, 2001; Sutton *et al.*, 2003) that was available in porcine IVM media of both experiments. The addition of *t*10,*c*12 CLA with GSH in to the IVM medium of bovine oocytes showed interference on oocyte lipid metabolism improving blastocyst quality (Lapa *et al.*, 2011). According to Sutton

et al. (2003) it appears to exist an association between oocyte developmental competence and metabolism, in particular among intracellular GSH levels and subsequent embryo development. In the present experiment, the increase in maturation rate was followed by a delay in cleavage, suggesting that GSH might be implicated in *t*10,*c*12 CLA and forskolin metabolism, during the intial embryo development. Besides, GSH synthesis during oocyte maturation is necessary for sperm decondensation and male pronucleus formation after fertilization (Maedomari *et al.*, 2007). Furthermore, an increase in monospermic rate in *t*10,*c*12 CLA+forskolin treated oocytes was observed in comparation to those of forskolin group, despite no differences were found among forskolin treatment and control. Previously, forskolin inclusion during the initial 2 h of IVM did not show interference on nuclear progression though it induced modifications to LD morphology during IVM (Prates *et al.*, 2012). Eventually these LD modifications could have interfered with other organelles, such as mitochondrias and cortical granules distribution in the ooplasm and thus with oocyte potential to block polyspermy (Sturmey *et al.*, 2006; Wang and Sun, 2007). However no differences were identified in embryo developmental rates and quality.

Data presented here showed that the addition of *t*10,*c*12 CLA during IVM interfered with embryonic development decreasing blastocyst rate. It was refereed that during porcine embryo culture, the oxidative metabolism of triacylglycerols is the major source of ATP production, with oxygen consumption being lower during embryo cleavage and reaching a peak at the blastocyst stage (Sturmey and Leese, 2003). In addition, glycolysis occurs throughout development (Swain and Krisher, 2001; Swain *et al.*, 2002), and the pentose phosphate pathway (PPP) may also be important in the provision of NADPH that generates GSH (Sturmey and Leese, 2003). The energy switch during embryo development is believed to occur in preparation for implantation but also to

afford synthesis of macromolecules from glycolytic intermediates (Tsujii *et al.*, 2001). When *t*10,*c*12 CLA is selected from IVM medium and accumulated in the oocyte cytoplasm (Prates *et al.*, 2013) it might have entered in the β-oxidation pathway to produce energy or in the long chain FA biosynthesis (Weil, 2000). Moreover, once NADP+ is an electron carrier, if delocated preferentially to FA biosynthesis it might decrease the overall ATP production in the β-oxidation pathway, decreasing embryonic developmental up to the blastocyst stage. Nevertheless, forskolin presence was capable of avoiding this deleterious action on blastocyst development. Further research is needed to clarify the mechanisms by which *t*10,*c*12 CLA exerts its activity and how oocytes and embryos can be protected from possible damage effects of lipid peroxidation.

Despite the above, the quality of fresh blastocysts resulting from oocytes treated with *t*10,*c*12 CLA, forskolin or both, measured by total cell number was not affected. Furthermore, blastocyst cryo-resistance, assessed by survival rate and total cell number after vitrification-warming, was not influenced by treatments given during oocyte maturation, which can be related to the fact that total FA and DMA contents of treated oocytes were not modified. However, better results in porcine blastocyst cryo-resistance can be expected if these supplements would be given during embryo culture (Men *et al.*, 2006; Pereira *et al.*, 2007).

In conclusion, the *t*10,*c*12 CLA accumulated in porcine oocytes, when added alone or simultaneously to forskolin, interfered on oocyte developmental competence to cleave and undergo embryonic development. Nevertheless, the quality of resulting blastocysts was not influenced by those supplements added during oocyte maturation. Despite being rich in endogenous lipids since the immature stage, the porcine oocyte can select and accumulate exogenous *t*10,*c*12 CLA during IVM and develop to the blastocyst

stage without impairing blastocyst quality. Further research is needed to identify the mechanisms by which *t*10,*c*12 CLA alone or with other lipid modulators, such as forskolin, can interfere on lipid metabolism during porcine embryonic development.

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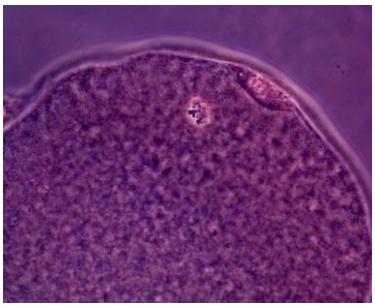


GENERAL DISCUSSION AND CONCLUSIONS

For many years there has been a considerable interest in improving porcine reproductive biotechnologies for both biotechnological and biomedical applications (Gil *et al.*, 2008). Despite the recent advances in IVM-IVF-IVC techniques in alliviating the initial disorders of poor pronuclear formation and the four cell block (Gil *et al.*, 2008), the high intracellular lipid content still limits the efficiency of porcine oocytes and embryos cryopreservation (Esaki *et al.*, 2004; Pereira and Marques, 2008). Moreover, there are several factors that interfering with meiotic process may impair oocyte developmental competence. For instance, while EGF stimulates GVBD, E_2 decreases its incidence when in presence of FSH or LH and FSH or FF (Sing *et al*., 1993). Other substances, such as the diterpene forskolin were shown to have double effect in porcine oocytes interfering with oocyte nuclear maturation and the amount of cytoplasmic lipid stores (Racowsky, 1985; Xia *et al*., 2000; Fu *et al*., 2011). In the present work we demonstrated that long time exposure to forskolin delayed nuclear progression and simultaneously reduced oocyte cytoplasmic growth and fat area, with the morphological appearance of these oocytes resembling that of immature ones without cytoplasmic expansion. Thus it was not suprising that oocyte fertilization/ cleavage were impaired. Nevertheless, by decreasing forskolin incubation time to the initial 2 h, this disturbance was overcome. Furthermore, we innovated in demonstrating that it was possible to modulate porcine oocyte lipid content, by analysing COC FA/DMA composition, after maturation with forskolin supplementation only in the initial 2 h. In these treated oocytes was still possible to detect a reduction in LD areas in course of maturation, which was related to the differences in FA oocyte composition. In addition, no interference was detected on embryonic development in this 2 h supplementation group, thus confirming a time-dependent inhibitory effect of forskolin in meiotic maturation (Racowsky, 1985; Laforest *et al*., 2005). Further, no effects were detected on

blastocysts cryo-resistance, meaning that the FA modifications induced by forskolin supplementation only in the first 2 h were not able to produce effects in the cryo-ability of resulting blastocysts.

The *trans*-10,*cis*-12 conjugated linoleic acid, known by several biological effects, in particular the stimulation of intracellular lipid content reduction (Pariza *et al*., 2001; José *et al*., 2008) was recently recognized by its potential of application in the field of embryology as lipid modulator and for its participation in eicosanoid cascade during oocyte maturation (Pereira *et al*., 2007 and 2008; Lapa *et al*., 2011). Here we tested the *t*10, *c*12 CLA effects during porcine oocyte IVM. The interference of CLA on oocyte lipid content and metabolism was confirmed by the modification in the morphology of the oocyte cytoplasm and in the FA/ DMA COC composition. From the morphological evaluation it was possible to conclude that the modulatory activity of CLA on lipid content was dependent on longer incubation times (44 h vs 22 h). The addition of *t*10,*c*12 CLA during 44 h induced a lighter cytoplasmic tone acquisition, which was correlated to modification in LD cytoplasmic distribution and also in their composition. When supplemented the IVM medium, this CLA isomer was accumulated by the COC, mostly in CC, which were also more susceptible to FA modifications. Contrary to expectations the presence of *t*10,*c*12 CLA during porcine oocyte maturation decreased blastocyst yield. However, no interference was found on embryo quality or blastocyts cryosurvival. These results were different from those obtained in the bovine species (Lapa *et al*., 2011) and they may indicate species related variation in oocyte FA metabolism. However, regardless the species, when this this CLA isomer is present in culture it is accumulated by the COC without changing the total PUFA concentration (in oocytes and CC), indicating regulation of FA storage and utilization.

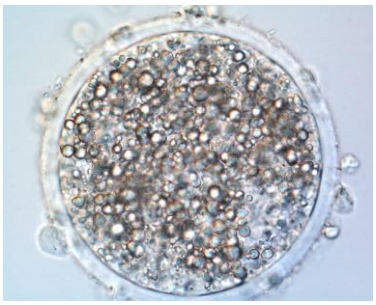
It was previously referred that forskolin increases the cAMP intracellular levels via adenylyl cyclase activation and then stimulates the PKA pathway (Holm, 2003), thus interfering on GVBD and subsequent oocyte development. However, in what concerns the *t*10,*c*12 CLA precise molecular mechanisms of action these are still unknown. Several hypotheses have been postulated. A direct action throught different key enzymes regulating cellular uptake and metabolism of lipids were identified (Chung *et al*., 2005). The protein kinase A and cAMP signaling pathways can also be affected by *t*10,*c*12 CLA. Moreover by acting via a metabolite inside the oocyte, *t*10,*c*12 CLA can be incorporated in lipid stores or/and be further metabolised throught β-oxidation mitochondrial to produce energy or peroxossimal to generate other FA, or participate in eicosanoid formation (Weil, 2000; Ashwell *et al*., 2010; Walther and Farese, 2010).

When forskolin and *t*10,*c*12 CLA were simultaneously added to the maturation media, it was also possible to register changes in FA/DMA profiles of oocytes and CC (Prates *et al*., 2013). This supplementation influenced oocyte maturation, monospermic penetration and cleavage rates, indicating a synergetic activity of both supplements, probably due to interference of these supplements in the cAMP pathway (Holm, 2003; Ashwell *et al*., 2010). However no effect was detected on blastocyst production and cryo-resistance. Futhermore, a tendency to increase the total FA and DMA contents during oocyte maturation as well as differences between oocyte and CC composition were identified during porcine oocyte maturation. Neither forskolin nor *t*10,*c*12 CLA supplementation were able of modifying this tendency (Prates *et al*., 2013).

Based on present results some considerations are here suggested for further studies to improve IVP and cryopreservation efficiency in porcine species: to accurate the time of supplementation and concentrations of these substances and to study their effects during *in vitro* embryo culture and blastocyst cryo-resistance; to test the real impact upon

prolificiency by transfer the cryopreserved blastocysts to recipient females. Moreover, the implementation of such technologies in porcine species might be important in the case of endangered or in risk of extinction breeds. For instance, the utilization of oocytes recovered after female castration, namely in the Alentejano swine breed, might have application for the establishment of germoplasm banks of IVP embryos. Futhermore, the quantification of the insulin and/or IGF1 concentrations in follicular fluid simultaneously to oocyte lipid content evaluation could complete the information of their FA/DMA composition here presented providing a better understanding of lipids role during porcine oocyte maturation and embryo pre-implantatory development.

Finally, the availabity of porcine oocytes from ovaries obtained in slaughterhouse makes them eligible oocytes in the field of transgenesis research.



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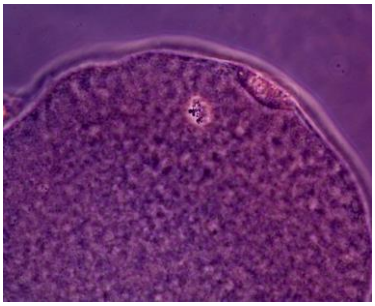

ATTACHMENTS

From: Editorial Office <editorialoffice@animal-journal.eu>
Date: 2012/8/1
Subject: Decision for ANIMAL-12-30079R1
To: Rosa Maria Lino Neto Pereira <rosalnp@gmail.com>

CC: rodhill@uidaho.edu, gary.hausman@ars.usda.gov

Dear Dr Pereira,
Manuscript number: ANIMAL-12-30079R1
Title: Fat area and lipid droplets morphology of porcine oocytes during in vitro maturation with trans-10, cis-12 conjugated linoleic acid (t10,c12 CLA) and forskolin

I am pleased to tell you that your submission has now been accepted for publication in Animal: An International Journal of Animal Bioscience and has been passed to Production for copy-editing and publication.

It was accepted on 01 Aug 2012.

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INRA - Animal consortium
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France

Thank you for submitting your work to Animal: An International Journal of Animal Bioscience.

Kind regards,
Gary Hausman
Editor 3: Physiol & Funct. Biol
On behalf of the Editorial Board Animal: An International Journal of Animal Bioscience

From: Tetsuji Okamoto <tetsuok@hiroshima-u.ac.jp>
Date: 2013/3/28
Subject: IVAN: Your manuscript entitled Fatty acid composition of porcine cumulus oocyte complexes (COC) during maturation: effect of the lipid modulators trans-10, cis-12 conjugated linoleic acid (t10,c12 CLA) and forskolin
To: Rosa Maria Pereira <rosalnp@gmail.com>

Dear Dr Pereira,

Your manuscript "Fatty acid composition of porcine cumulus oocyte complexes (COC) during maturation: effect of the lipid modulators trans-10, cis-12 conjugated linoleic acid (t10,c12 CLA) and forskolin" has received a positive review. However, the reviewers felt that the manuscript would be improved with modifications in response to their suggestions.

If you could make these corrections and satisfactorily reply to the critiques provided below on a point-by-point basis, we should be able to accept your manuscript for publication. For resubmission, please use the link below to upload your revised manuscript file, figure and/or table files, and a detailed response to the review comments. Please include figure captions on a separate page within your manuscript.

Source files in the appropriate formats should be uploaded at this time, as well, if at all possible, in order to expedite publication of your manuscript should it be accepted. Please note: Our printer cannot accept figures embedded in Word documents. See http://www.sivb.org/pubs_a_infoForAuthors.asp for information regarding acceptable source files.

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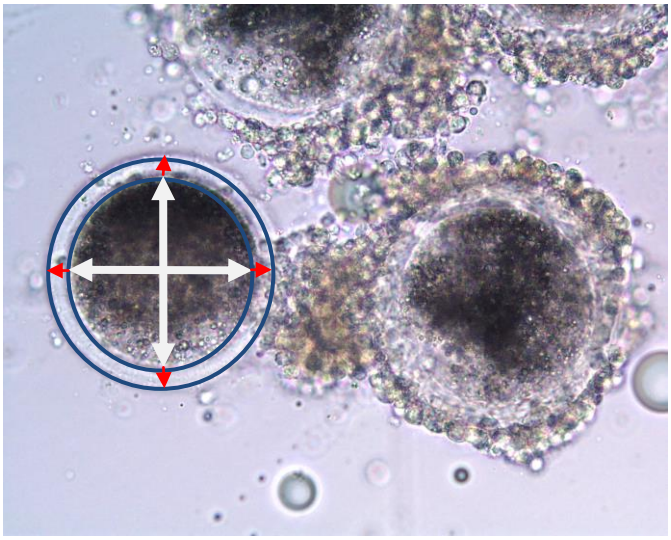
Your revision is due by 27 Apr 2013.

To submit a revision, go to http://ivan.edmgr.com/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Sincerely,

Tetsuji Okamoto, D.D.S., Ph.D.
Editor-in-Chief
In Vitro Cellular & Developmental Biology - Animal



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Prates EG, Marques CC, Baptista MC, Vasques MI, Horta AEM and Pereira RM 2008. Effect of Different Culture Protocols on Porcine Oocyte Nuclear Maturation and Area. In Proceedings of 12th Annual Conference of the European Society of Domestic Animal Reproduction (eds WA King, E Martinez-Garcia, M McGown, JLR Rodrigues, M Taverne and H Rodríguez-Martinez), Vol. 3, pp. 83. Wiley-Blackwell, Utrecht, Netherlands.

*Porcine cumulus oocyte complexes and oocyte observed after 44 h of *in vitro* maturation, under contrast phase microscope. Arrows indicate areas measured in the oocyte. Scale bar 50 µM.

ABSTRACT

Improvement of *in vitro* maturation (IVM) systems has a major interest in the development and application of porcine *in vitro* embryo and stem cells production technologies. We intent to study the effect of different IVM protocols on the oocyte nuclear stages and dimensions. Abattoir porcine oocytes (n = 750) were cultured in TCM199, porcine follicular fluid (10%, v/v) and gentamicine, supplemented or not with E_2 (10µl/mL), cysteamine (10µL/mL) and EGF (10µL/mL), in 4 different times of exposure: Group A, 44 h with supplement; Group B, 22 h with supplement plus 22 h with renewed supplemented medium; group C, 22 h with supplement plus 22 h without supplement; group D, 22 h with supplement. After each IVM session (n = 4), cumulus oocyte complexes (n= 596) were denuded and fixed prior to staining with 1% aceto-lacmoid or measured (n = 151) with a computerized image analysis system. Immature oocytes (n = 29) were also measured. Maturation rate was the lowest (24.7%, $P \leq 0.001$) in group D. This rate was higher ($P \leq 0.001$) in groups A (53.6%) and C (63.1%) than in B (48.5%). Area of matured oocytes with or without zona pellucida was not different among groups. In conclusion, best results were achieved with 44 h of culture despite supplement removal. The negative effect of supplemented medium renewal on oocyte maturation may reflect an inadequate protocol for porcine, namely E_2 whose presence is referred by some authors to delay germinal vesicle breakdown in this species. Moreover oocyte area was not predictive for oocyte maturation. This study was supported by the Foundation for Science and Technology (SFRH-BD-42359-2007).

*

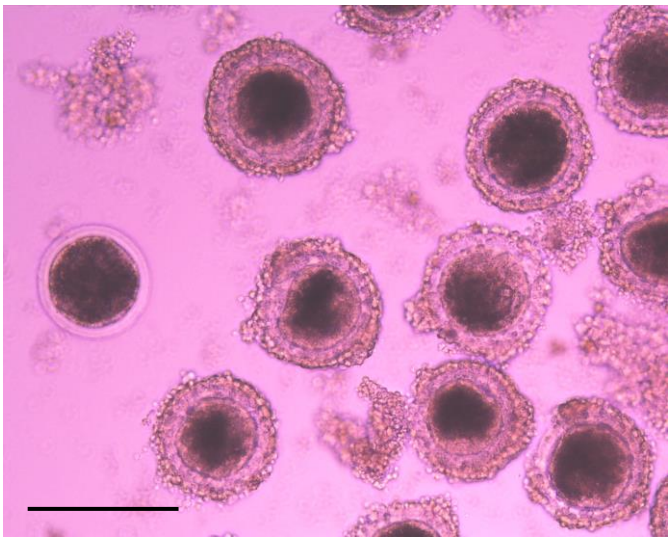
Prates EG, Charneca RMC, Pereira RM and Nunes JT 2011 Does high spermatozoa concentration and low ejaculate volume affect boar sperm cryosurvival and quality? Preliminary results. In V Congresso da Ciências Veterinárias (eds Sociedade Portuguesa de Ciências Veterinárias), pp. 162. INRB IP/L-INIA Fonte Boa, Santarém.

*Boar semen stored in french-straws (0.5 mL) and frozen in nitrogen vapours.

ABSTRACT

Alentejano swine (AL) is an eligible breed for sperm cryopreservation and artificial insemination due to the small size of populations. Sperm sensitivity to cryopreservation is affected by the boar being the cryosurvival variability among ejaculates more important between males than intramale. Ejaculates from AL boars present high sperm concentration and low volume compared to other swine breeds. This study determines whether these characteristics affect boar sperm postthawing survival and quality. Sperm-rich fractions from 3 adult boars (3 ejaculates each), AL, Duroc (DU) and Large White (LW) were collected. Sperm volume and concentration were assessed (in fresh). Progressive motile spermatozoa and normal apical ridges (NAR) were also evaluated (in fresh and postthawing, 3 straws per ejaculate). Sperm was extended in BTS, then in lactose-egg yolk (LEY) and LEY-Glycerol-Orvus ES PASTE (LEYGO) extenders to obtain a $1x10^9$ spz/mL concentration in 0.5 mL straws. Freezing was performed in nitrogen vapours before straws were plunged in liquid nitrogen. Data were analysed using the MIXED procedure, the boar as fixed effect and the threshold of significance as P<0,05. Intraboar variability was estimated by the coefficient of variation (CV). AL boar sperm volume (40.7 ± 9.4 mL) was lower than DU (129.3 ± 9.4 mL, P = 0.002) and LW (193.0 ± 9.4 mL, P = 0.001), but AL showed higher concentration (1495.7 ± 159.6 x 10^6 spz/mL) than DU (581.7 ± 159.6 x 10^6 spz/mL, P = 0.020) or LW (454.2 ± 159.6 x 10^6 spz/mL, P = 0.011). Progressive motility, both in fresh or post-thawed sperm was not different among males. Fresh spermatozoa NAR presented no differences among boars, although DU postthawed NAR was higher (62.9 ± 6.0%) than LW (23.3 ± 6.0%, P = 0.010) or AL (22.9 ± 6.0%, P = 0.010). Volume CV's (AL, 65.5%, DU, 5.0%, LW, 3.2%) and postthawed NAR (AL, 65.9%, LW, 41.7%, DU, 0.6%) were higher in AL and concentration (LW, 47.7%, AL 28.2%, DU, 11.7%) was greater in

LW. Data suggest that the variability in postthawed sperm quality was not affected by the differences of volume and concentration. To improve the cryopreservation efficiency, particularly in AL breed, more ejaculates and boars should be further evaluated. This study was supported by the Foundation for Science and Technology (SFRH-BD-42359-2007).



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Prates EG, Marques CC, Baptista MC, Vasques MI, Charneca RM, Cuello C, Horta AEM, Nunes JT and RM Pereira 2009. *In vitro* fertilization ability of frozen/thawed boar semen: effect of caffeine on monospermic penetration and embryo production. Poster presentation in IV Congresso da Sociedade Científica de Suinicultura, 12-14 Novembro. Estação Zootécnica Nacional, Santarém.

*Selection of immature porcine cumulus oocytes complexes for *in vitro* maturation. Scale bar 50 µM.

ABSTRACT

Porcine breeds stand out for their great genetic and economical importance. However, the commercial application of some reproductive technologies, such as boar semen freezing and *in vitro* embryo production, is still sub-optimal. This late technique has been implemented in Portugal by our team. The purpose of this study was to evaluate the effect of the addition of caffeine to the fertilization medium on the monospermic penetration and subsequent *in vitro* embryo production in pigs. *In vitro* matured porcine oocytes (n = 1099) were inseminated (5 replicates) with 1 x 10^6 spz mL^{-1} of frozen-thawed spermatozoa obtained from Alentejano or Duroc boars. Two different fertilization media were used: mTBM caffeine-free (group 1) or mTBM supplemented with 2 mM caffeine (group 2). Gametes were co-incubated for 6h. After each *in vitro* fertilization session a group of presumptive zygotes were cultured *in vitro* for 12-15 h and then fixed and stained with 1% aceto-lacmoid for the fertilization parameters evaluation (n = 134). The remaining zygotes were cultured for 6-7 days to assess embryo development (n = 965). Data were analysed using SPSS, Version 15.0, software package (SPSS Inc., Chicago, III) and differences were considered significant at $P < 0.05$. Analysis of variance (Mixed procedure) was used. The results showed no significant differences between groups for penetration rate (47.1 ± 9.5 % and 46.9 ± 9.5 %, groups 1 and 2, respectively), monospermy (87.5 ± .3 % for group 1 and 70.2 ± 8.1 % for group 2) and fertilization efficiency, expressed as the percentage of penetrated monospermic oocytes related to the total of fertilized oocytes (36.3 ± 7.9 % and 27.6 ± 7.8 %, groups 1 and 2, respectively). Blastocyst production rates were similar for both experimental groups (8.0 ± 4.0 % for group 1 and 6.0 ± 4.0 % for group 2). In conclusion, the addition of 2 mM caffeine to the *in vitro* fertilization medium did affect neither the fertilization parameters nor the *in vitro* embryo production under our

experimental conditions. This study was supported by the Foundation for Science and Technology (SFRH-BD-42359-2007 and PPTDC/57148/2004).

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