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Tese

Estudos celulares e moleculares visando o aumento da eficiência de biotécnicas reprodutivas em suínos

Verónica Hoyos Marulanda

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5

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Para os meus pais, Eduardo e Fabiola. Eles, meu tudo

Dedico

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“Não te rendas, por favor não cedas,
Mesmo que o frio queime,
Mesmo que o medo morda,
Mesmo que o sol se ponha e se cale o vento,
Ainda há fogo na tua alma,
Ainda há vida nos teus sonhos
Porque cada dia é um começo novo,
Porque esta é a hora e o melhor momento.”

Mario Benedetti

Resumo

HOYOS-MARULANDA, Verónica. **Estudos celulares e moleculares visando o aumento da eficiência de biotécnicas reprodutivas em suínos.** 2019. 63f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

As taxas de produção *in vitro* de embriões (PIV) suínos melhoraram significativamente na última década. Porém, a qualidade e o desenvolvimento embrionários ainda devem ser aperfeiçoados. As altas concentrações de lipídios intracelulares e de polispermia são os principais obstáculos. Este estudo buscou entender os aspectos moleculares e celulares na reprodução de fêmeas suínas para melhorar biotécnicas reprodutivas utilizadas nesta espécie. Foi avaliada a regulação da expressão de enzimas esteroidogênicas e de membros da superfamília TGFβ durante a foliculogênese de leitoas pré-púberes a partir da simulação de dois perfis endócrinos por indução hormonal. O perfil de pró-estro foi simulado através do tratamento com eCG, enquanto o perfil pré-ovulatório foi simulado pelo tratamento com eCG, seguido de hCG. O grupo controle foi composto de fêmeas que não receberam tratamento. Amostras de sangue e ovários de 30 fêmeas foram colhidos no momento do abate. A expressão gênica relativa foi determinada por PCR em tempo real, enquanto as dosagens hormonais determinadas por quimioluminiscência. Os níveis séricos de progesterona foram maiores no perfil periovulatório do que nos demais perfis ($P<0.01$), enquanto os níveis de estradiol não foram afetados ($P>0.05$). Os perfis endócrinos dos animais não influenciaram a expressão dos genes BMP15, BMPR1A, BMPR2, FSHR, GDF9, LHCGR e TGFBR1 ($P>0.05$), não encontrando diferenças entre os grupos ($P<0.05$). O grupo no perfil de pró-estro apresentou maior expressão relativa de CYP11A1 ($P=0.08$) comparadas ao controle, e de CYP19A1 ($P<0.05$) em comparação aos demais grupos. No grupo com perfil periovulatório, a expressão relativa de BMPR1B foi menor ($P<0.05$) do que nas leitoas do grupo controle. Outros experimentos foram conduzidos para avaliar a eficiência de inclusão do ácido eicosapentaenóico (EPA) e do ácido docosahexaenóico (DHA) no meio de MIV suíno, sobre as taxas de maturação de ovócitos, de desenvolvimento de blastocistos após ativação partenogenética, e de conteúdo lipídico de ovócitos maturados e blastocistos produzidos *in vitro*. Taxas de clivagem foram menores em meios contendo 12.5 μM de EPA e 50 μM de DHA, assim como as taxas de desenvolvimento de blastocistos, igualmente inferiores nos grupos em que os meios receberam qualquer concentração de EPA ($P<0.05$). Uma significativa redução do acúmulo de conteúdo lipídico ($P<0.05$) tanto nos ovócitos maturados, quanto em embriões cultivados por 7 dias, foi observada no grupo em que os ovócitos foram maturados em meio contendo 50 μM de DHA. Estes estudos concluem que o tratamento de fêmeas suínas pré-púberes com gonadotrofinas foi associado com a maior expressão de *CYP19A1* e de *CYP11A1* e à menor expressão de *BMPR1B*, sendo o efeito sobre a esteroidogênese evidente apenas no grupo com perfil endócrino periovulatório, com aumento nos níveis séricos de progesterona. Também, os resultados indicam um efeito prejudicial do meio de maturação *in vitro* contendo EPA sobre ovócitos suínos, e demonstram que a inclusão de 50 μM de DHA no meio MIV de suínos provoca a redução do conteúdo de lipídeo

citoplasmático tanto durante a maturação ovocitária, quanto em blastocistos, demonstrando o sucesso de uma nova alternativa para potencializar a eficiência de biotécnicas reprodutivas em suínos.

Palavras-chave: maturação *in vitro*, ácidos graxos, foliculogênese, expressão gênica, suínos.

Abstract

HOYOS-MARULANDA, Verónica. **Cellular and molecular studies aimed to improve the efficiency of reproductive techniques in pigs.** 2019. 63f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Although greater rates of *in vitro* embryo production (IVP) were achieved over the last decade, embryo quality and development still need to be improved. High concentrations of intracellular lipids and polyspermia are the main obstacles. This thesis aimed to investigate molecular and cellular approaches to improve the efficiency of some reproductive techniques in swine. The regulation of the expression of steroidogenic enzymes and members of the TGFβ superfamily during folliculogenesis of prepubertal sows was evaluated from the simulation of two endocrine profiles by hormonal induction. The pro-estrus profile was simulated by treatment with eCG, while the pre-ovulatory profile was simulated by eCG treatment, followed by hCG. The control group consisted of females that did not receive treatment. Samples of blood and ovaries from 30 females were collected at the time of slaughter. Relative gene expression was determined by real-time PCR, while hormonal dosages determined by chemiluminescence. Serum progesterone levels were greater in the periovulatory profile than in the other profiles ($P<0.01$), whereas estradiol levels were not affected ($P>0.05$). The endocrine profiles did not influence the expression of the BMP15, BMPR1A, BMPR2, FSHR, GDF9, LHCGR and TGFBR1 genes ($P>0.05$). The pro-estrus profile presented slightly greater relative expression of CYP11A1 ($P=0.08$) compared to the control, and CYP19A1 ($P<0.05$) compared to the other groups. In the periovulatory profile, the relative expression of BMPR1B was lower ($P<0.05$) than in control group. Other experiments were conducted to evaluate the efficiency of inclusion of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the IVM medium, on embryo development after parthenogenetic activation and on the lipid content of pig oocytes and blastocysts matured in vitro. Cleavage rates were lower in media containing 12.5 μM of EPA and 50 μM of DHA, and blastocysts development rates were reduced in media including any concentration of EPA ($P<0.05$). A reduction in the accumulation of lipid content ($P<0.05$) in oocytes and embryos cultured for 7 days was observed in the medium containing 50 μM DHA. These studies concluded that the treatment of prepubertal swine females with gonadotrophins was associated with greater expression of CYP19A1 and CYP11A1 and lower expression of BMPR1B, but effects on steroidogenesis were evident only in the periovulatory endocrine profile, which presented increased progesterone levels. There was a deleterious effect of including EPA in the IVM medium for porcine oocytes, but inclusion of 50 μM DHA was related to reduction in the cytoplasmic lipid content both during oocyte maturation and in blastocysts, which may consist of an alternative to enhance the efficiency of IVM in in pigs.

Key words: in vitro maturation, fatty acids, folliculogenesis,, gene expression, swine.

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Lista de Abreviaturas

ALA - *α-Linolenic acid* (ácido graxo α-linolênico).

AMP - *Adenosine monophosphate* (adenosina monofosfato).

BMP - *Bone morphogenetic protein* (proteína morfogenética do osso).

BMP15 - *Bone morphogenetic protein* 15 (proteína morfogenética do osso 15).

CLA - *Conjugated Linoleic* Acid (ácido graxo linoleico conjugado).

COX - *Cyclooxygenase* (cicloxigenase).

DHA - *Docosahexaenoic* acid (ácido docosaexaenoico).

EFA - *Essential fatty acid* (ácidos graxos essenciais).

EPA - *Eicosapentaenoic* acid (ácido graxo eicosapentaenoico).

FSH - *Follicle-stimulating hormone* (hormônio folículo estimulante).

BMPR1B - *Bone morphogenetic protein receptor, type* 1B (receptor das proteínas morfogenéticas do osso, tipo 1B).

GDF9 - *Growth differentiation factor* 9 (fator de crescimento e diferenciação 9).

LH - *Luteinizing hormone* (hormônio luteinizante).

MAPK - *Mitogen-activated protein kinase* (proteína quinase ativada por mitôgeno).

PIV - Produção *in vitro*.

PUFA - *Polyunsaturated fatty acid* (ácido graxo poli-insaturado).

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1 INTRODUÇÃO GERAL

No momento de falar sobre a produção *in vitro* de embriões (PIV) suínos os pesquisadores encontram-se com três dificuldades básicas que afetam um melhor sucesso nas taxas de desenvolvimento de blastocistos. Em primeiro lugar, em uma espécie multiovulatória como a suína, a foliculogênese ainda não foi bem descrita e entendida; em segundo lugar em termos da reprodução *in vivo*, o oviduto exerce um papel fundamental no processo de maturação ovocitária que tem sido difícil de imitar em ambientes *in vitro*, o que nos leva ao terceiro ponto, que é que a maturação *in vitro* ainda tem falhas pois os ovócitos imaturos apresentam altas concentrações de lipídios. Uma vez ocorrendo uma maturação insatisfatória, o processo de fertilização *in vitro* é prejudicado pela dificuldade do ovócito de bloquear a polispermia. Por este motivo, esta tese inclui estudos com duas abordagens: *in vitro*; e *in* vivo. Na primeira, tenta-se entender como é a regulação e função de fatores ovocitários em espécies multiovulatórias como a suína, avaliando a expressão de genes que poderiam estar relacionados com este quesito. Para a avaliação *in vitro*, parte-se de relatos de estudos anteriores, onde é destacado que uma grande quantidade de gotículas lipídicas presentes nos ovócitos suínos pode prejudicar a subsequente maturação *in vitro* por causa da oxidação de ácidos graxos. Neste contexto, tem sido observado que os ácidos graxos poli-insaturados de cadeia longa possuem papel na adipogênese celular, na manutenção da fluidez da membrana lipídica e talvez na deposição de lipídios , possivelmente melhorando taxas de produção de embriões *in vitro*. Assim, a presente tese teve por objetivos avançar no conhecimento sobre: o efeito da adição de ácidos graxos poli-insaturados no meio de maturação *in* vitro sobre a maturação de ovócitos e a produção de embriões partenotos suínos; a regulação de genes pelas gonadotrofinas em células foliculares de fêmeas suínas sob diferentes perfis endócrinos, visando o estabelecimento de protocolos que tornem mais eficientes as biotecnologias aplicadas à reprodução de suínos.

2 REVISÃO BIBLIOGRÁFICA

2.1 Fatores locais na foliculogênese em espécies mono e multiovulatórias

Sob o ponto de vista endócrino, o desenvolvimento folicular é regulado principalmente pelas gonadotrofinas FSH e LH e por esteróides ovarianos. Uma elevação nos níveis de FSH estimula o crescimento de um grupo de pequenos folículos antrais, fenômeno que é denominado emergência folicular (Adams et al., 1992). Em espécies monovulares, como os bovinos, apenas um dos folículos recrutados é selecionado para continuar crescendo (folículo dominante), o que ocorre mesmo quando os níveis de FSH declinam, levando os demais folículos recrutados a entrarem em atresia (tornando-se subordinados), processo que caracteriza a divergência folicular (Ginther et al., 1996). Já em espécies com múltiplas ovulações, como os suínos, não ocorre divergência folicular, embora existam mecanismos ativos de seleção folicular. Portanto, fica evidente que os processos de seleção de folículos ovulatórios e atresia dos subordinados são regulados por fatores ovarianos (Fortune et al., 2004).

Em espécies monovulares, o controle autócrino/parácrino da foliculogênese desempenha um papel essencial na modulação do desenvolvimento folicular. Fatores produzidos pelo ovócito são cruciais para o crescimento e a diferenciação folicular. Entre as principais proteínas ovocitárias, destacam-se as proteínas morfogenéticas ósseas (BMPs) e seus receptores. Em ovelhas, mutações inativadoras nas proteínas BMP15 (Galloway et al., 2000) e GDF9 (fator de crescimento e diferenciação) (Hanrahan et al., 2004) estão associadas à infertilidade, quando em homozigose, ou com aumento da taxa ovulatória, quando em heterozigose. Isto se deve ao fato dessas proteínas exercerem funções essenciais durante o desenvolvimento folicular pré-antral (Hanrahan et al., 2004; McNatty et al., 2007) e, ao mesmo tempo, atuarem na regulação da diferenciação em folículos antrais (McNatty et al., 2005). Postula-se que, com um alelo do gene inativado, a reduzida atividade desses fatores de crescimento induziria a diferenciação precoce dos folículos em desenvolvimento (com menos células da

granulosa). De acordo com esta hipótese, células da granulosa de ovelhas heterozigotas para a mutação inativadora da BMP15 são mais responsivas ao LH (McNatty et al., 2009).

Estudos funcionais *in vivo* comprovam a relevância do sistema BMP nas diferentes fases do desenvolvimento folicular. Juengel et al. (2009) realizaram imunização ativa contra as proteínas GDF9 e BMP15 em vacas e obtiveram superovulação em alguns animais, sugerindo que esses fatores são envolvidos na seleção do folículo dominante e na determinação da taxa ovulatória. Em ovelhas, observou-se um incremento na taxa de ovulação após um curto período de imunização contra BMP15 ou GDF9, mas não houve efeito sobre a fecundação dos ovócitos, o desenvolvimento embrionário e a gestação (Juengel et al., 2004). Por outro lado, a imunização contra essas proteínas por períodos prolongados bloqueou o desenvolvimento folicular em ovelhas (McNatty et al., 2007). Coletivamente, esses resultados indicam um grande potencial de utilização da regulação das proteínas ovocitárias BMP15 e GDF9 para incrementar a taxa ovulatória ou como contraceptivos em animais domésticos e humanos.

Em espécies com ovulações múltiplas, as informações sobre a regulação e função dos fatores produzidos localmente no ovário, incluindo as BMPs e seus receptores são escassas. A comparação dos padrões de expressão e da forma como o sistema é regulado por gonadotrofinas poderá fornecer informações relevantes para o entendimento da determinação da taxa ovulatória nas diferentes espécies. Os fenótipos observados após mutações nos ligantes e receptores em ovinos sugerem que o sistema BMP15/GDF9/BMPR1B atua regulando negativamente a diferenciação folicular. Por outro lado, em suínos, a expressão de BMPR1B está correlacionada positivamente aos níveis de estradiol no fluído folicular (Paradis et al., 2009), o que contraria os dados obtidos nos modelos monovulares. Portanto, as diferenças na taxa de ovulação das espécies podem parcialmente ser atribuídas a variações na regulação da expressão e/ou função dos fatores ovocitários.

A maioria dos estudos sobre os fatores envolvidos nos processos de diferenciação, esteroidogênese e atresia foi realizada com folículos provenientes de

ovários colhidos de fêmeas abatidas em fases aleatórias do ciclo estral. Este fato dificulta a identificação exata do estágio de desenvolvimento folicular, pois os fatores de diferenciação são regulados temporalmente durante a onda de crescimento dos folículos, antecedendo as mudanças nos níveis de estradiol e progesterona no fluído folicular. Modelos de cultivo de células da granulosa têm sido amplamente utilizados em estudos funcionais, mas a interação entre as células da teca, da granulosa e do ovócito ainda não foram consideradas em modelos *in vitro*. Além disso, apenas modelos *in vivo* com um criterioso controle da fase de desenvolvimento folicular podem proporcionar o ambiente endócrino fisiológico. O modelo suíno possibilita o estudo da regulação de fatores ovocitários, uma vez que é possível obter dezenas de ovócitos de um mesmo animal, possibilitando avaliar a regulação dos fatores em células germinativas inclusas em folículos de diferentes diâmetros, ou ovócitos com graus distintos de capacitação. Além do sistema BMP, diversos genes expressos localmente podem ser avaliados depois da coleta dos tecidos nos momentos adequados.

2.2 Estrutura e função dos ácidos graxos

Os ácidos graxos poli-insaturados (PUFAs) possuem uma base metileno entre carbonos de ligações duplas, e com configuração *cis*, que é uma característica estrutural dos ácidos graxos essenciais. Por outro lado, existem os ácidos graxos conjugados, que têm ligações duplas conjugadas em múltiplas posições e isômeros geométricos (Koba & Yanagita, 2014)**.** No ambiente natural, os PUFA são encontrados em gorduras derivadas de animais ruminantes, como por exemplo, da gordura do leite e do sebo (Sehat et al., 1999). Dentre os diversos tipos de PUFA, o ácido linoleico conjugado (CLA), o ácido α-linolênico (ALA) e o ácido graxo conjugado eicosapentaenóico (EPA) são encontrados em maiores quantidades (Pariza et al., 2001).Existem relatos de que o CLA traz benefícios para a saúde humana, como no tratamento do câncer, diabete, aterosclerose, redução e redistribuição da gordura corporal e modulação do sistema imune, sendo que, em suínos, o seu principal efeito seria como promotor de crescimento (Saavedra et al., 2015).

Em humanos e em animais, alguns ácidos graxos essenciais (EFAs) não podem ser obtidos endogenamente, necessitando de suplementação exógena através da dieta (Beare-Rogers et al., 2001). Dentre eles, existem duas famílias: ômega 3 (ácido linoléico) e o ômega 6 (ácido linolênico). O ácido linoléico é o precursor dos ácidos eicosapentaenóico (EPA) e docosaexaenoico (DHA) (Holub, 2002; Rooke et al., 2003), que estão entre os PUFA mais importantes (Kurlak & Stephenson, 1999). O EPA e o DHA podem ser suplementados a partir de várias fontes, como óleos de peixe e alguns tipos de algas. Na nutrição de suínos, estes ácidos graxos têm sido testados para determinar a modulação da resposta inflamatória, o melhoramento do desempenho reprodutivo e de características nutricionais da carne e seus derivados (Rossi, et al., 2010). A síntese dos PUFA ômega-6 e ômega-3 a partir dos seus precursores é esquematizada na Figura 1.

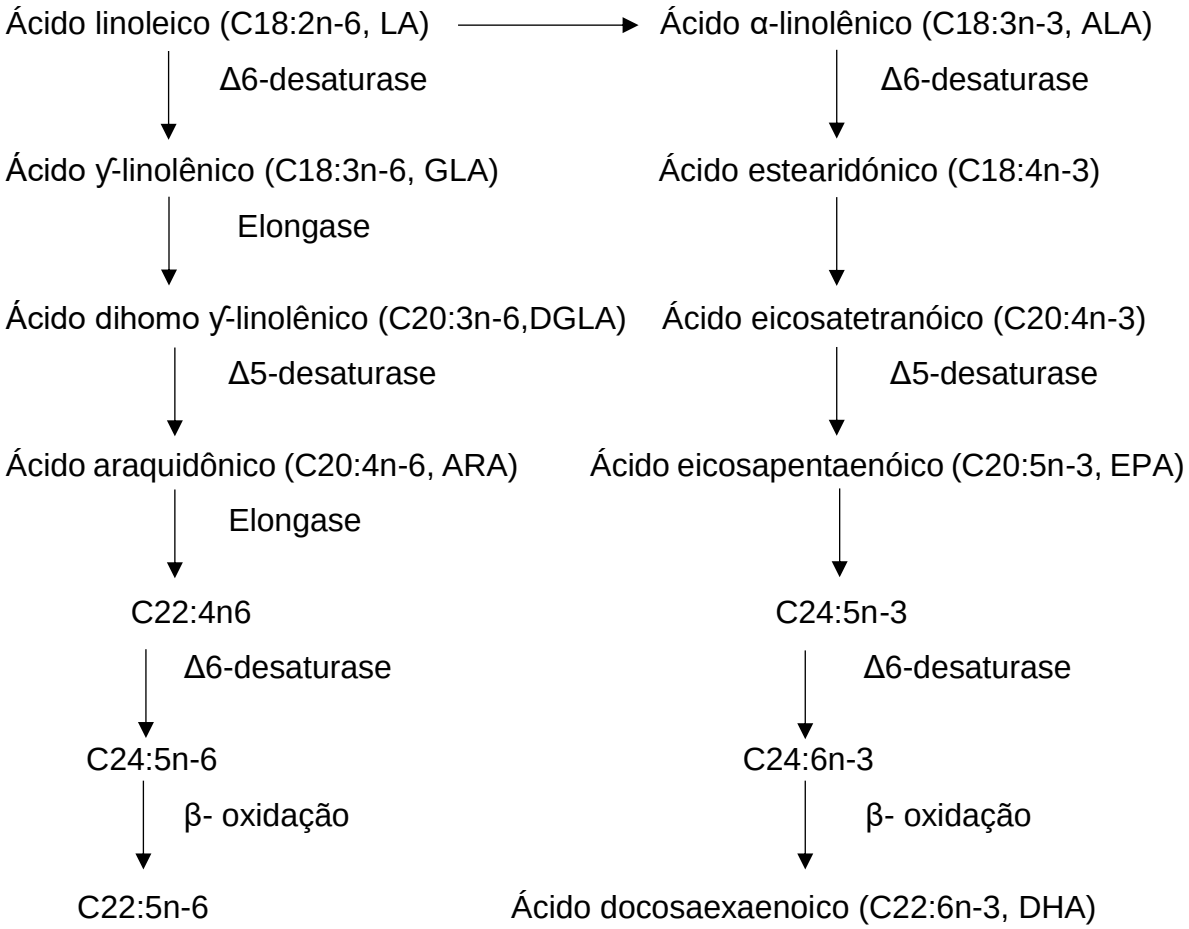

Figura 1. Síntese dos ácidos graxos poli-insaturados n-6 e n-3 a partir dos seus ácidos graxos precursores ácido linoleico e ácido α-linolênico.

Segundo Tanghe & De Smet (2013), muitos estudos já foram conduzidos para avaliar efeitos da inclusão de PUFA sobre o desempenho reprodutivo de suínos. Os PUFA influenciam na fertilidade da fêmea suína, na sobrevivência embrionária e no desenvolvimento fetal (Henman, 2006), possuindo também efeito sobre o tamanho da leitegada (Quiniou et al., 2002). Além disso, os PUFA podem influenciar no desempenho reprodutivo ao serem incorporados nas membranas celulares do ovócito, modulando a expressão de padrões de enzimas-chave envolvidas no metabolismo de prostaglandinas e de esteróides (Wathes et al., 2007). Porém, os mecanismos envolvidos nestas ações ainda não foram determinados. Adicionalmente, a suplementação com ácido linolênico poderia ter efeitos positivos sobre o número de folículos e a qualidade ovocitária, como já observado em éguas (Zeron et al., 2002).

2.3 Ácidos graxos e maturação *in vitro*

Considerando os efeitos dos PUFA sobre a qualidade ovocitária, acredita-se que estes produtos possam trazer benefícios para a produção *in vitro* de embriões. Os PUFA são encontrados em altas concentrações nos ovócitos e espermatozoides (Aitken & Baker, 1995) e no fluido folicular (Homa & Brown, 1992). Porém, pouco se sabe sobre o metabolismo lipídico nos ovócitos, em especial como o alto conteúdo intracelular de lipídeos presente nos ovócitos suínos poderia influenciar a assincronia na maturação citoplasmática (Prates et al., 2012;2013).

Existem estudos nos quais a adição de CLA *in vitro* é avaliada para melhorar a subsequente maturação de ovócitos suínos (Jia et al., 2014). Este tipo de ácido graxo pode interferir na regulação de vias que envolvem proteínas e receptores de proteínas, os quais têm um papel principal na regulação da maturação ovocitária, tais como as proteínas quinases ativadas por mitôgenos (MAPK), as proteínas quinases ativadas por AMP, o receptor acoplado à proteína G, a cicloxigenase (COX) e a lipoxigenase, entre outras (Hsu & Ip, 2011).

Os ácidos graxos são os principais componentes das membranas celulares, desempenhando funções relevantes na interação ovócito-espermatozoide (Gardner & Evans, 2006), bem como na resistência celular à criopreservação (Pereira & Marques, 2008). Segundo Ouandaogo et al (2011), existem trocas bidirecionais entre os ovócitos e as células do *cumulus oophorus.* Estas trocas são cruciais para a aquisição da competência ovocitária. Portanto, os ácidos graxos podem ser metabolizados ou acumulados no ovoplasma, mudando tanto o conteúdo de lipídeos quanto a composição dos ácidos graxos no ovócito (Sturmey et al., 2009), assim como provavelmente, a composição das células do *cumulus oophorus* (Prates et al., 2013). Conforme Prates et al., (2013), os ácidos graxos presentes no fluido folicular suíno e no meio antes da MIV, dentre eles o EPA e o DHA, são mostrados na Tabela 1.

Tabela 1. Ácidos graxos presentes no fluído folicular suíno e no meio de maturação prévio ao cultivo (meio pré-MIV) (Prates et al., 2013).

Ácido graxo	Fórmula	Fluído folicular	Meio pré-MIV
Miristico	14:0	0.56 ± 0.038	0.58 ± 0.060
Miristoléico	C9- 14:1	0.053 ± 0.010	0.210 ± 0.087
Pentadeciclico	15:0	0.24 ± 0.016	0.27 ± 0.024
Palmítico	16:0	24.1 ± 0.40	22.4 ± 0.43
Hexadecenóico	C7- 16:1	1.02 ± 0.074	0.87 ± 0.050
Palmitoléico	C9- 16:1	1.27 ± 0.58	1.08 ± 0.069
Heptadecanóico	17:0	0.71 ± 0.098	0.75 ± 0.065
Margárico	C9- 17:1	0.31 ± 0.067	0.25 ± 0.011
Esteárico	18:0	13.4 ± 0.50	14.7 ± 0.58
Oleico	C9- 18:1	19.4 ± 0.62	20.8 ± 0.33
Cis-Vacénico	C11- 18:1	2.37 ± 0.206	1.97 ± 0.09
Linoleico	18:2 n-6	17.3 ± 1.12	19.4 ± 0.52
Araquídico	20:0	0.24 ± 0.045	0.38 ± 0.52
ɣ- Linolênico	18:3 n-6	0.42 ± 0.059	0.31 ± 0.029
α- Linolênico	18:3 n-3	0.32 ± 0.048	0.28 ± 0.035
Gondóico	C11- 20:1	0.20 ± 0.022	0.10 ± 0.379
Eicosadienóico	20:2 n-6	0.37 ± 0.066	0.29 ± 0.075
Eicosadienóico (I)	20:3 ni II	0.62 ± 0.050	0.57 ± 0.045
Eicosatrienóico (II)	20:3 ni I	0.76 ± 0.087	0.56 ± 0.022
Araquidônico	20:4 n-6	11.6 ±0.56	10.4 ± 0.028
Tricosilico	23:0	0.05 ± 0.012	0.23 ± 0.071
Docosadienóico	22:2 n-6	0.08 ± 0.032	0.23 ± 0.417
Adrénico	22:4 n-6	0.33 ± 0.036	ND
Clupanodónico	22:5 n-3	1.34 ± 0.048	1.11 ± 0.159
Cervónico	22:6 n-3	0.73 ± 0.089	0.44 ± 0.032
Outros AG	Outros AG	0.30 ± 0.031	1.45 ± 0.075
Dimetilacetal	DMA		
Hecadecanal	DMA- 16:0	0.87 ± 0.062	0.57 ± 0.091
Octadecanal	DMA- 18:0	0.77 ± 0.111	0.57 ± 0.051
Octadecenal	DMA- 18:1	0.34 ± 0.049	0.38 ± 0.045
Somas parciais			
Ácidos graxos saturados		39.3 ± 0.61	39.3 ± 0.88
Ácidos graxos monoinsaturados		24.9 ± 0.95	25.6 ± 0.42
Ácidos graxos poli-insaturados		33.8 ± 0.56	33.5 ± 0.69
Relação n6/n3		12.7 ± 0.70	16.9 ± 1.08
AG (ng/μL) [a]		630.2 ± 14.45	54.0 ± 3.10

Os dados são percentuais (w/w) de médias ± EPM (5 repetições).
ND (não detectável); ni I e ni II (isômeros 20:3 não identificados).
[a] Conteúdo de AG total (ng/μL).

Desta forma, a presente tese procura realizar estudos celulares e moleculares visando o aumento da eficiência de biotécnicas reprodutivas em suínos; investigando a expressão de genes em células foliculares de fêmeas suínas pré-púberes em grupos com diferentes perfis endócrinos objetivando o melhor entendimento reprodutivo de uma espécie multiovulatória como a suína. Também, pretende-se determinar se o uso de ácidos graxos poli-insaturados de cadeia longa, como o EPA e o DHA, inclusos no meio de maturação *in vitro*, melhoram as taxas de clivagem, desenvolvimento de blastocistos, número de células embrionárias, assim como diminuem a concentração lipídica de ovócitos e embriões suínos, isto como alternativa à melhora de uma biotécnica como é a produção *in vitro* de embriões em suínos.

3 HIPÓTESE E OBJETIVOS

3.1 Hipóteses

- A concentração de lipídeos em ovócitos suínos pode diminuir com a adição dos ácidos graxos EPA e DHA no meio de MIV, melhorando as taxas de clivagem, blastocistos e qualidade embrionária.

- Os genes relacionados com a foliculogênese, a ovulação e a fecundação se alteram em células foliculares de fêmeas suínas pré-púberes em função de diferentes perfis endócrinos.

3.2 Objetivo geral

Estudar eventos em nível celular e molecular envolvidos nos processos de foliculogênese in vivo e embriogênese in vitro, afim de melhorar a eficiência de biotécnicas reprodutivas utilizadas na espécie suína.

3.3 Objetivos específicos

- Avaliar a presença e concentração de gotículas lipídicas em ovócitos suínos antes, durante e depois da maturação *in vitro*, e em embriões em desenvolvimento, após a inclusão de ácidos graxos no meio utilizado na maturação e sem a presença deles.

- Comparar as taxas de clivagem, blastocistos e qualidade embrionária, a partir da adição dos ácidos graxos EPA e DHA em diferentes concentrações na maturação *in vitro* de ovócitos suínos.

- Comparar a expressão de genes relacionados à esteroidogênese, proliferação e

diferenciação celular.

- Investigar a regulação da expressão de fatores produzidos por ovócitos inclusos em folículos provenientes de fêmeas pré-púberes estimuladas ou não com gonadotrofinas.

4 CAPÍTULOS

4.1 Manuscrito 1 – Expression of components of the transforming growth factor beta (TGF-β) in follicular cells of gilts under distinct endocrine profiles

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Abstract

The transforming growth factors beta (TGFβ) superfamily have important functions during ovulation, but their role on regulating the expression of steroidogenesis-related genes in follicular cells of swine is not fully known. This study evaluated the regulation of the expression of steroidogenic enzymes and members of the TGFβ superfamily during folliculogenesis in prepubertal gilts with known endocrine profiles. Two endocrine profiles were mimicked in prepubertal gilts from a commercial farm through treatments with gonadotrophins: 1,200 IU eCG at D -3 (proestrus profile); 1,200 IUI eCG at D -6 plus 500 IU hCG at D -3 (periovulatory profile). Untreated gilts composed the prepubertal profile (control). At slaughter, blood samples and both ovaries were collected from each gilt. *Cumulus oophorus*-oocytes complexes and follicular cells were recovered. Relative gene expression was determined by real time PCR. Serum progesterone levels were greater for gilts in the periovulatory profile than for those in the other profiles ($P < 0.01$), but estradiol levels were unaffected ($P > 0.05$). No difference across treatments were observed for several genes: *BMP15*, *BMPR1A*, *BMPR2*, *FSHR*, *GDF9*, *LHCGR* and *TGFBR1* ($P > 0.05$). Gilts in the proestrus profile presented greater relative expression of *CYP11A1* ($P = 0.08$) compared to control gilts and of *CYP19A1* than gilts in the other two profiles ($P < 0.05$). For gilts in the

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periovulatory profile, relative expression of *BMPR1B* was lower than for control gilts ($P < 0.05$). Gonadotrophin treatment increased the expression of both *CYP19A1* and *CYP11A1* but declined the expression of *BMPR1B*. Direct effect on steroidogenesis was observed only for serum progesterone levels, which were increased for gilts treated with eCG and hCG.
Key words: folliculogenesis, steroidogenesis, gonadotrophins, gene expression, gilts.

Introduction

Follicular development is a complex process, mainly regulated by FSH, LH and ovarian steroid hormones (LaVoie, 2017), resulting in the ovulation of oocytes potentially competent to be fertilized and to sustain embryo development. Members of the transforming growth factors β (TGFβ) superfamily, which are secreted by the oocytes, play relevant roles in the folliculogenesis of mammals (Gilchrist et al., 2008; Juengel and McNatty, 2005). Among them, the bone morphogenetic protein 15 (BMP15), the growth differentiation factor 9 (GDF9) and their receptors are involved in key events, such as proliferation and differentiation of follicular cells, steroidogenesis, ovulation and luteinization (Su et al., 2008; Orisaka et al., 2009; Peng et al., 2010). Moreover, BMP15 is involved in regulating the apoptosis of *cumulus oophorous* cells (Zhai et al., 2013).

In sheep, increased ovulation rates were observed after a short period of immunization against BMP15 and GDF9, although there were no effects on oocyte fecundation, embryo development and pregnancy rates (Juengel et al., 2004). In contrast, prolonged immunization against those same proteins impaired follicular development (McNatty et al., 2007). On the other hand, in swine, expression of BMPR1B is positively correlated with estradiol levels in the follicular fluid (Paradis et al., 2009), which contradicts data reported for cattle (Gasperin et al., 2014). Thus, although there are species-specific differences in folliculogenesis, limited knowledge is available about the regulation and function of local oocyte factors in multi-ovulatory species.

Some enzymes are involved in the action of steroid hormones in folliculogenesis. The CYP11A1 converts cholesterol to pregnenolone (Hanukoglu, 1992), whereas the CYP19A1 is responsible for synthesizing estrogen from androgens at granulosa cells (Kandiel et al., 2010; Tosca et al. 2006). At ovarian level, the CYP19A1 acts on follicular development and atresia in cattle (Bao and Garverick, 1998) and pigs (Pan et al., 2012) and on luteogenesis

during diestrus in mares (Albrecht and Daels, 1997). After the preovulatory LH surge, expression of *CYP11A1* raises quickly, while expression of *CYP19A1* declines drastically (Wissing et al., 2014), resulting in decreased estrogen circulating levels and accelerating the synthesis of progesterone (Lydon et al., 1995).

The use of swine as an experimental model for multi-ovulatory species allows the evaluation of many oocyte factors, since several oocytes are released during ovulation, from follicles with different diameters. To our knowledge, only one study evaluated the regulation of members of the TGF superfamily during follicular development in swine (Paradis et al., 2009), although the female's endocrine status was not well characterized. The objective of this study was to evaluate the expression of members of the TGFβ superfamily and of steroidogenic enzymes during antral folliculogenesis in prepubertal gilts with endocrine profiles controlled by gonadotrophins.

4.1.1 Material and methods

Sample were obtained from thirty prepubertal gilts that were destined to slaughter, housed in finishing barns from a commercial farm. Gilts were randomly assigned to three treatments (n = 10 each). Since the control group was designed to be representative of the physiological prepubertal endocrine profile, gilts in such group received no treatment. The proestrus endocrine profile was mimicked through treatment with 1,200 IU eCG (Folligon®, MSD Animal Health) three days prior to the scheduled date of slaughter (D -3). Gilts in the group representing the periovulatory endocrine profile received 1,200 IU eCG (Folligon®) six days before slaughter (D -6), followed by 500 IU hCG (Chorulon®, MSD Animal Health) at D -3.

At slaughter (D0), gilts were 130 d-old and weighted 100 kg, in average. Blood samples were collected during slaughter. Thereafter, samples were centrifuged at 3,000 rpm for 10 min. The serum was split in two aliquots, which were stored at -20°C. To validate the three desired endocrine profiles, serum samples were sent to a commercial laboratory to determine estradiol and progesterone concentrations through chemiluminescence. Samples with progesterone levels equal or inferior to 3.0 ng/ml were censored (four from the prepubertal profile treatment and three from the proestrus profile treatment). Thus, for further

analyses, sample sizes were unequal among treatments: prepubertal profile, n = 6; proestrus profile, n = 7; and periovulatory profile, n = 10.

Both ovaries of each gilt were collected at the slaughterhouse, placed in individual flasks and conditioned in a thermal box at 5°C, during transportation. At the laboratory, *cumulus oophorus*-oocyte complexes (COC) and follicular cells were aspirated using a 19 G needle attached to a vacuum pump. After sedimentation, the aspirate was placed in Petri dishes for collection of COC in a binocular stereomicroscope (Meiji Techno®). The content was centrifuged at 8,000 rpm for 6 min, to separate follicular cells from the fluid. Samples of follicular cells were stored at -196°C.

Total RNA was extracted from follicular cells with TRIzol® (Invitrogen), according to the manufacturer's guidelines. The extracted RNA was quantified through a NanoDrop® spectrophotometer (Thermo Scientific) with 260 wave length. The purity of the extracted RNA was evaluated by the absorption rate of the OD260/OD280 ratio, standardized to values equal or greater than 1.8. Residues of contaminant DNA were digested by treating the total RNA with DNAse (Promega) at 37°C for 5 min. After inactivating the DNAse at 65°C for 10 min, the reverse transcription reaction was conducted using the iScriptc® DNA synthesis kit (BioRad), following the manufacturer's recommendations. The relative gene expression was evaluated through real-time PCR (CFF 384®, BioRad), for steroidogenic enzymes (CYP19A1, CYP11A1), gonadotrophin receptors (FSHR and LHCGR) and for members of the TGFβ superfamily (GDF9, BMP15, TGFβR1, BMPR2, BMPR1A and BMPR1B). The variability in the amount of mRNA was corrected by amplification of the constitutive genes GAPDH and Cyclophilin. All evaluated genes are shown in Table 1.

For statistical analyses, all response variables were tested for normality using the Shapiro-Wilk test. Whenever necessary, log transformations were applied. Responses were compared among treatments using analyses of variance, with comparisons of means by the Tukey test.

4.1.2 Results

As expected, progesterone serum levels were greater in the periovulatory profile ($P < 0.01$) than in the control and proestrus profiles (Figure 1). However, estradiol serum levels did not differ among treatments ($P > 0.05$).

Gilts from the proestrus profile presented greater relative expression of *CYP19A1* ($P < 0.05$) than gilts from the other profiles and a marginal increase in the relative expression of *CYP11A1* ($P = 0.08$) compared to control gilts (Figure 2). Gilts from the periovulatory profile presented lower relative expression of *BMPR1B* ($P < 0.05$) than control gilts (Figure 3).

No differences in relative expression across treatments ($P > 0.05$) were observed for *BMP15, BMPR1A, BMPR2, FSHR, GDF9, LHCGR* and *TGFBR1* (Figures 2 and 3).

4.1.3 Discussion

In the proestrus profile, expression of *CYP19A1* in follicular cells was increased for gilts treated with eCG compared to both gilts treated with the eCG/hCG combination and to untreated gilts. The *CYP19A1* is more frequent in pre-ovulatory follicles, as a marker of terminal differentiation of granulosa cells (Estienne et al., 2015). As the aromatase activity at the theca and granulosa cells during steroidogenesis is influenced by FSH (Slomczynska et al., 2003), expression of *CYP19A1* decreases as the FSH concentration declines (Wang et al., 2011; Grzesiak et al., 2012), which agrees with our findings. Increased relative expression of *CYP19A1* was also observed in granulosa cells of mice treated with eCG (Samardzija et al., 2016). On the other hand, initial antral follicles of female swine present low (although detectable) levels of mRNA of *CYP19A1* (Slomczynska and Tabarowski, 2001), indicating limited capacity of estrogen production. Additionally, after treatment with eCG, gilts from the proestrus profile presented slightly increased expression of *CYP11A1*, which processes cholesterol into pregnenolone, a precursor of all steroid hormones (Mast et 2011) that commonly increases as follicles mature (Gillio-Meina et al., 2005). Nonetheless, such an increase was marginal, likely due to sample size limitations. In mice, eCG treatment was related to reduced expression of *CYP11A1* in granulosa cells, which contrasts with the results of the present study.

The ovulation is influenced by endocrine and paracrine processes involving local growth factors secreted by granulosa cells and growing oocytes (Manabe et al., 2004). Among the TGFβ superfamily, the BMP are involved in several actions related to apoptosis and steroidogenesis (Zhao et al., 2014), linking to the TGFβR1 receptor, which is potentially related to the selection and maintenance of the preovulatory oocyte population in the ovaries of swine (Foxcroft et al., 2016). However, the gonadotrophin treatments used in the present

study did not result in changes on the relative expression of such genes across distinct endocrine profiles. That may have occurred because BMP15 and GDF9 are both expressed in all stages of the estrous cycle (Orisaka et al., 2009; Peng et al., 2010; Su et al., 2008). Our results corroborate the lack of effect of GDF9 in the growth of cattle follicles (Haas et al., 2016), although there is evidence that BMP15 may be more relevant during the periovulatory period than in other stages of the estrous cycle in swine (Paradis et al., 2009). Data for cattle indicated that granulosa cells of small follicles (3-6 mm) may be more sensitive to the effects of GDF9 on their proliferation and on steroidogenesis (Spicer et al., 2008). Nonetheless, although the abundance of mRNA for TGβR1 is positively correlated with follicular size (Paradis et al., 2009), no effect was observed in the present study after gonadotrophin treatment, since the size of the follicles were similar across treatments.

In the present study, expression of *BMPR1B* was greater in control gilts than for those in the periovulatory profile. That contradicts a study that reported greater expression of *BMPR1B* in large porcine follicles than in intermediate and small follicles (Zhao et al., 2014), which would be more common as follicular development advances. However, in that study, ovaries were collected at slaughterhouses from females with unknown endocrine profiles. In the present study, although no gilts from any group presented either large follicles or corpora lutea, it is possible that untreated control gilts already presented atretic follicles. Yet, such findings agree with reports of greater expression of *BMPR1B* in granulosa cells of atretic cattle follicles than in healthy dominant follicles (Gasperin et al., 2014). The *BMPR1B* is a type-I receptor present in oocytes of ewes (Wilson et al., 2001) and sows (Quinn et al., 2004), with a key role on signaling to members of the TGFβ superfamily (Lavery et al., 2008), acting on the proliferation and differentiation of granulosa cells (Edson et al., 2010) and on follicular development (Reader et al., 2012). Nevertheless, the functions and regulation of *BMPR1B* in distinct stages of folliculogenesis in swine still require further research.

The presence of receptors for FSH and LH in follicular cells at different stages of the estrous cycle suggests that such follicles are sensitive to gonadotrophins. Thus, the *FSHR* may be detected in the granulosa cells of primordial follicles during the estrous cycle even at low concentrations (Liu et al., 1998), and it would have relevant roles for follicular development and steroidogenesis (Bramble et al., 2016). Antral follicles would also have detectable mRNA for *LHCGR* in the theca (Yuan and Lucy, 1996). Although the *LHCGR* are

confined to external thecal layer and to the granulosa cells of the ovarian wall, the LH initiates a cascade of events that will also affect *cumulus oophorous* cells and oocytes (Hsieh et al., 2007; Park et al., 2004). Increased levels of LH would be related to reduced abundance of mRNA for *LHCGR* in pre-ovulatory follicles, concurrent with a decrease in estradiol levels as luteinization starts after ovulation (Agca et al., 2006). In cattle, expression of mRNA for *LHCGR* was not observed in follicular cells of heifers, but was detected in similar cells of adult cows, only in large follicles of at least 8 mm diameter (Simoes et al., 2012). In the present study, the distinct endocrine profiles induced by gonadotrophin treatments did not affect the expression of both *FSHR* and *LHCGR*. That may be due to the fact such genes are regulated more characteristically in the ovaries of adult females than in those from prepubertal females, which may present neither large follicles nor evidence of corpora lutea (Du et al., 2016).

The only detectable change in the serum levels of steroid hormones occurred for progesterone, which was increased for gilts from the periovulatory endocrine, following treatment with eCG and hCG. Such an effect may be expected, since progesterone levels normally start to decrease prior to estrous onset, when the luteal function declines, and raise after the LH surge (Parvizi et al., 1976; Shille et al., 1979). Although low serum estradiol levels would be expected in prepubertal females (Esbenshade et al., 1982), such levels did not differ for gilts from the three evaluated endocrine profiles. That may have occurred because those gilts generally presented acceptable body weight for their age, which suggests that at least some of them might be cycling.

Conclusions

After treatment with eCG, relative expression of *CYP19A1* and *CYP11A1* increased in prepubertal gilts with endocrine profile consistent with proestrus, but relative expression of *BMPR1B* declined in the periovulatory profile. Although gonadotrophin treatment may potentially enhance folliculogenesis in prepubertal gilts, effects on steroidogenesis were restricted to raised serum progesterone levels in the periovulatory profile.

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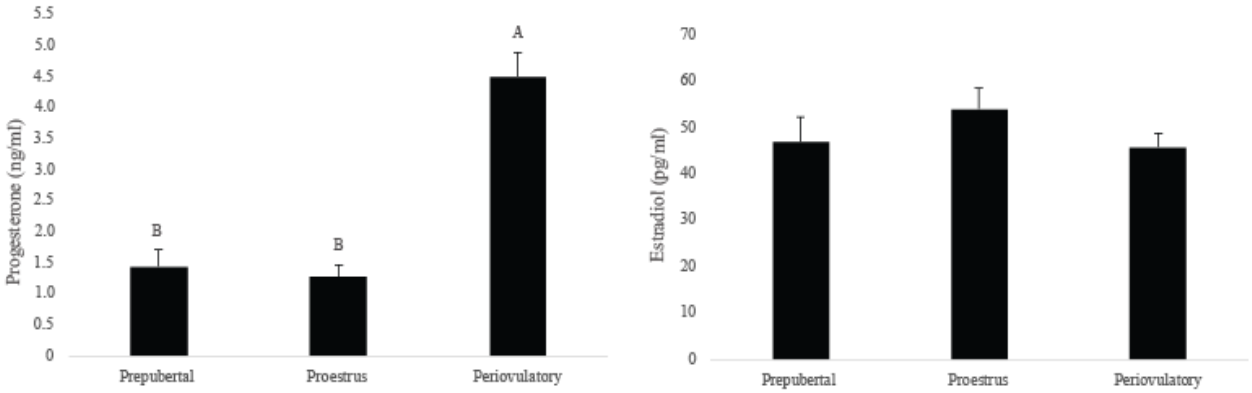

Figure 1: Serum progesterone and estradiol levels in prepubertal gilts with distinct endocrine profiles controlled by treatments with gonadotrophins*

*Prepubertal profile (control): no treatment (n = 7);

Proestrus profile; 1,200 IU eCG at D -3 (n = 6);

Periovulatory profile; 1,200 IU eCG at D -6 and 500 UI hCG at D -3 (n = 10).

^{A,B}Means ± SEM with distinct superscripts differ by at least $P < 0.01$

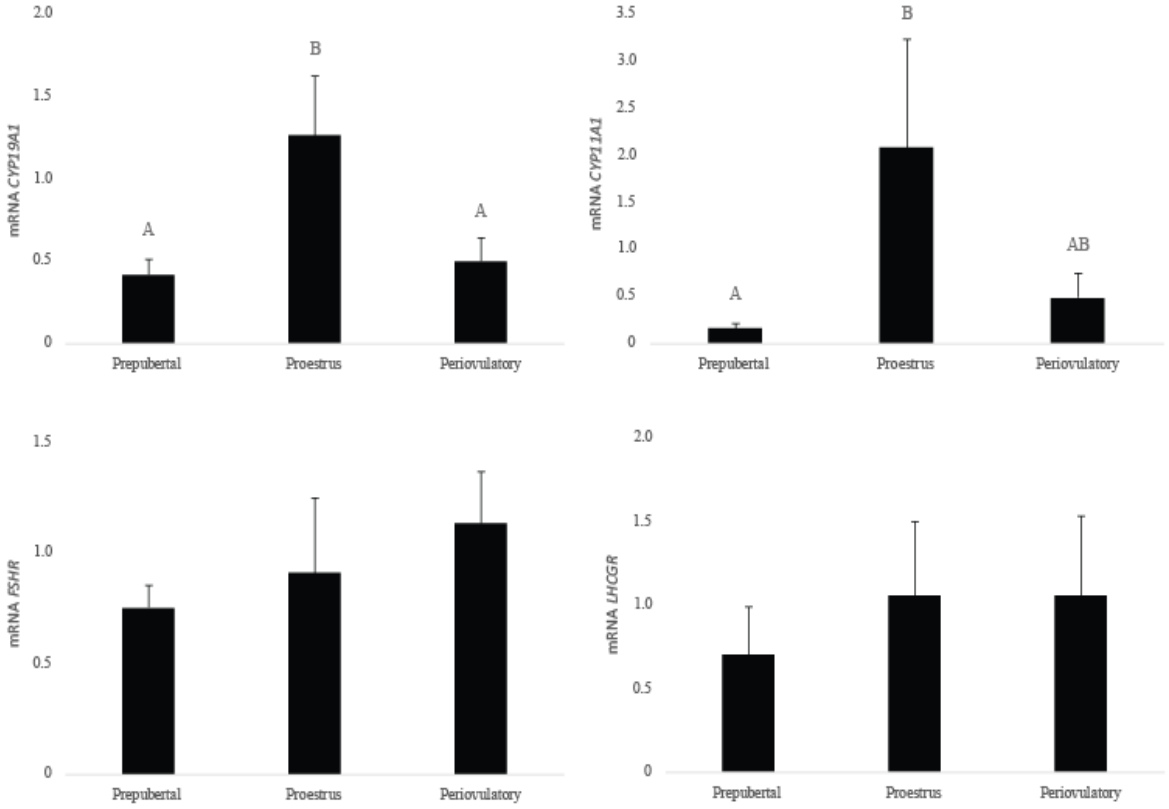

Figure 2: Relative abundance of mRNA for the genes *CYP19A1*, *CYP11A1*, *FSHR* and *LHCGR* in prepubertal gilts with distinct endocrine profiles controlled by treatments with gonadotrophins*

*Prepubertal profile (control): no treatment (n = 7);

Proestrus profile; 1,200 IU eCG at D -3 (n = 6);

Periovulatory profile; 1,200 IU eCG at D -6 + 500 UI hCG at D -3 (n = 10).

^{A,B}Means ± SEM with distinct superscripts differ by at least $P < 0.05$

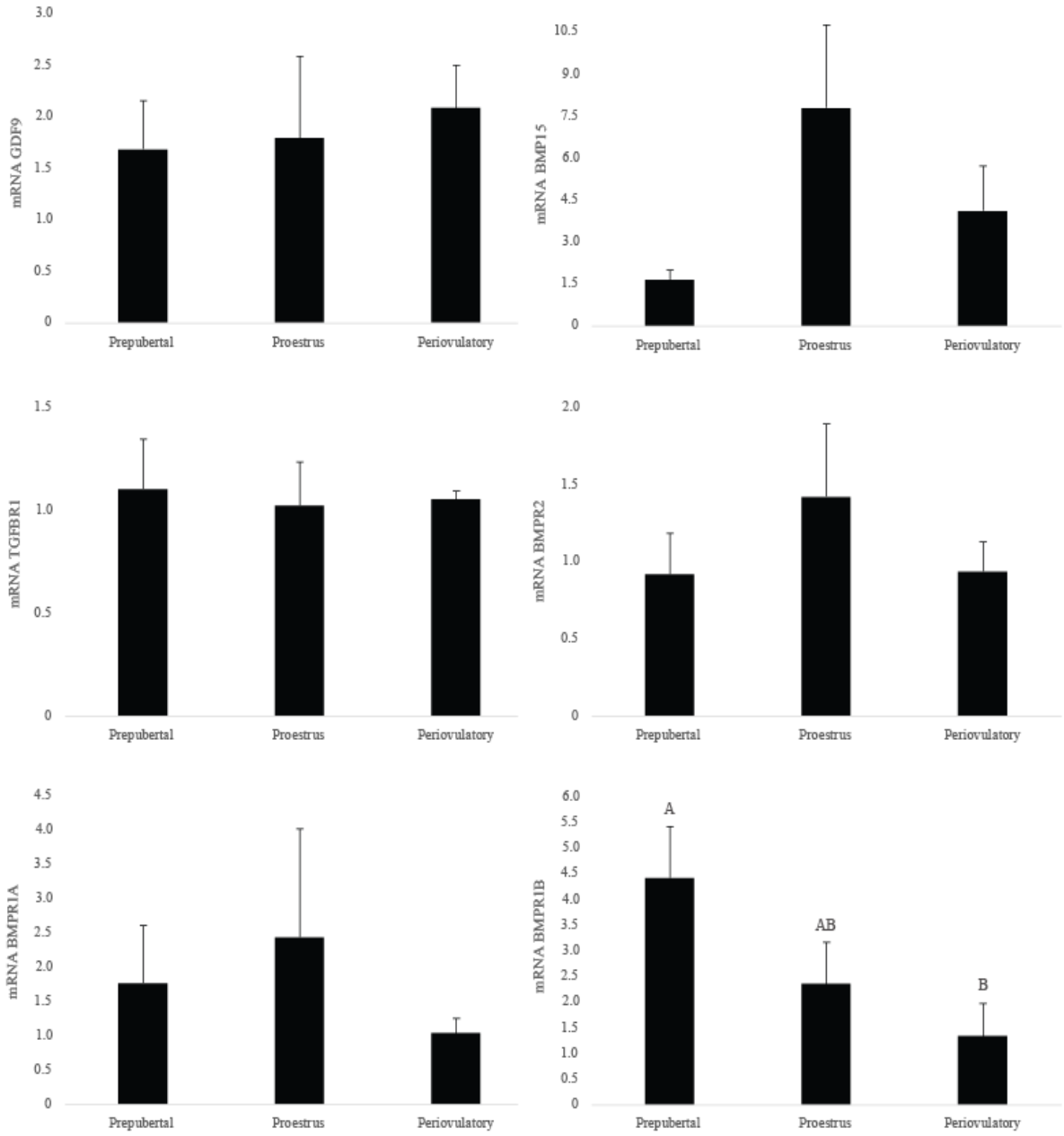

Figure 3: Relative abundance of mRNA for the genes *GDF9, BMP15, TGFBR1, BMPR2, BMPR1A* and *BMPR1B* in prepubertal gilts with distinct endocrine profiles controlled by treatments with gonadotrophins*

*Prepubertal profile (control): no treatment (n = 7);

Proestrus profile; 1,200 IU eCG at D -3 (n = 6);

Periovulatory profile; 1,200 IU eCG at D -6 + 500 UI hCG at D -3 (n = 10).

[A,B]Means ± SEM with distinct superscripts differ by at least $P < 0.05$

Table 1: Genes and sequence of initiators

Gene	Sense initiator	Anti-sense initiator	Reference
GAPDH	ATTGCCCTCAACGACCACTT	ACATGACGAAGGCAGGTCTCC	(Diaz, 2004)
Cyclophilin	AATGCTGGCCCCAACACA	TCAGTCTTGGCAGTGCAAATG	(Paradis, 2009)
BMP15	TTCCCAGAGGCCTGGAAGA	GCCTTCCGCAAAAGAAGAGA	(Paradis, 2009)
BMPR1A	GTGGATCTGGACTACCCTTGTTG	TTGCCGAACCATCTGTATCTGT	(Paradis, 2009)
BMPR1B	TGGTTCCGAGAGACAGAAATATATCA	GCAATGAAGCCCAAAATGTTTT	(Paradis, 2009)
BMPR2	GGGTCGGGTGAAAAGATCAA	GCGCCACCGCTTAAGAGA	(Paradis, 2009)
CYP11A1	TGCAATTGGTCCCACTCCTC	TTTGAGAAGAAGGCGGGGTC	(Anciuti, 2018)
CYP19A1	GCTAATTGCAGCACCAGACA	TGTTGGTTCCCTTTTTCACC	(Ebeling, 2011)
FSHR	CCAAGCTTCGAGTCATCCCA	GAAGGCATCAGGGTCGATGT	(Anciuti, 2018)
GDF9	ATGTGACGGCCATCCTTCAG	CGATGGACATGTGAATCTCTCTCT	(Paradis, 2009)
LHCGR	CAGCCACTGCTGTGCTTTTA	GAGTGTCTTGGGTGAGCAGA	(Anciuti, 2018)
TGFBR1	GTCTGCATCTCACTCATGTTGATG	GCACTCGATGGTGAATGACTGT	(Paradis, 2009)

4.2 Manuscrito 2 – Inclusion of long-chain polyunsaturated fatty acids on *in vitro* maturation media of swine oocytes: effects on maturation, embryo development and lipid content

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Abstract

As swine oocytes and embryos have great lipid content in their cytoplasm, supplementation of medium for *in vitro* maturation (IVM) of oocytes with omega-3 polyunsaturated fatty acids (PUFA) may help to improve embryo development. This study evaluated the effects of adding 12.5 µM, 25.0 µM and 50.0 µM of docosaexaenoic (DHA) and 12.5 µM, 25.0 µM and 50.0 µM of eicosapentaenoic acid (EPA) to the IVM medium on embryo development of porcine oocytes, and on the lipid content of porcine oocytes as well as in the lipid content of oocytes and embryos. In Experiment 1, cleavage rates were lower with 12.5 µM EPA and blastocyst development rates were inferior with any tested EPA concentration ($P < 0.05$) compared to the control. With 50 µM DHA cleavage rates were greater than for the control in Experiment 1 ($P < 0.05$), and the lipid content was lower in oocytes after 22 h and 44 h and on D7 embryos compared to the control ($P < 0.05$), in Experiment 2. In both experiments, control media included porcine follicular fluid. Thus, although swine oocytes appears to be negatively influenced by EPA, inclusion of DHA potentially improve the efficiency of IVM of swine oocytes due to their effects on lipid metabolism.

Key words: nuclear maturation; DHA; lipid; Nile Red; oocytes; swine.

Introduction

Despite of the successful birth of piglets through *in vitro* production (IVP) of swine embryos [1,2], such process is still considered inefficient [3,4], yielding quite variable blastocyst development rates. That may be due to the greater amount of lipid droplets present in the cytoplasm of swine oocytes and embryos compared to other mammal species [5]. As their triglyceride content decreases during *in vitro* maturation (IVM) [6], the oxidation of fatty acids can impair embryo development [7]. Therefore, lipid metabolism plays a relevant role on the developmental competence of swine oocytes.

Besides serving as energy sources, long-chain polyunsaturated fatty acids (PUFA), especially those of the omega-3 series, play a structural role in the phospholipid bilayer of cell membranes, influencing their fluidity and intracellular signal transmission mechanisms [8]. Among the omega-3 PUFA, the eicosapentaenoic (C20:5n-3, EPA) and the docosahexaenoic (C22:6n-3, DHA) acids are those with greatest physiological relevance [9]. As omega-3 PUFA may be incorporated into oocytes, embryos and fetuses [10,11], they modulate the expression of enzymes that play key roles on the metabolism of prostaglandins and steroid hormones [12]. Thus, supplementation of omega-PUFA in diets for swine is related to enhanced neural embryo development [11], embryo survival and fetal development [13] and increased litter size [14], potentially stimulating puberty in gilts [15]. However, the mechanisms involved in such processes are still undetermined.

Omega-3 PUFA also act on adipogeneses and other physiological events, regulating gene transcription [16], resulting in reduced expression of genes that code many enzymes involved in lipid metabolism [17]. As such fatty acids are present in fairly high concentrations in oocytes and on the follicular fluid [18,19], effects on embryo IVP can be expected. Thus, including omega-3 PUFA in culture media may alter the pattern of lipid deposition in adipocytes [20]. However, there is limited knowledge about the lipid metabolism in swine oocytes [21,22], especially how their high intracellular lipid content influences cytoplasmic maturation. The objective of this study was to evaluate the effects of adding EPA and DHA to IVM media for porcine oocytes on their nuclear maturation, embryo development and lipid content.

4.2.1. Material and methods

Except when specially mentioned differently, all media were prepared with reagents from Sigma-Aldrich Chemical Company and ultrapure Milli-Q water.

4.2.1.1. Harvesting and processing of cumulus oophorus-oocyte complexes (COC)

Ovaries from prepubertal gilts were collected at a local slaughterhouse and transported to the laboratory in a saline solution at 30 ◦C. Thereafter, 3-6 mm follicles were aspirated using a vacuum pump attached to a 19 G needle (15 mL/min). The COC were searched under stereomicroscopy into 35 mm Petri dishes and subsequently moved to a dish containing TCM-HEPES medium (TCM199 with 0.1 % PVA and 2.5 mM NaHCO3), for morphological evaluation [23]. Selected oocytes had homogenous ooplasm and at least three layers of compact *cumulus oophorus* cells.

4.2.1.2 Experiment 1 - Cleavage and embryo development

COC were randomly distributed into eight groups (Fig. 1). Such groups represented alcoholic solutions of both EPA (Sigma E-2011) and DHA (Sigma D-2534) added to the IVM medium at three concentrations (12.5, 25.0 and 50.0 μM) and one control group for each PUFA, containing 10% follicular fluid from pubertal sows. The IVM medium was TCM-HEPES, including FSH (Folltropin□-V, Vetoquinol N.-A. Inc. Lavaltrie, QC, Canada) and LH (Lutropin- ®-V, Vetoquinol N.-A.Inc) during the first 22 h of incubation [24]. Four replicates were conducted, each including 30 COC per treatment, matured in 0.1 mL droplets of the IVM medium kept under mineral oil in Nunc® 4-well dishes, at 38,5°C with 5% CO2 and maximum humidity.

After 44 h of IVM, *cumulus oophorus* cells were mechanically removed and oocytes were submitted to parthenogenetic activation. Initially, oocytes were washed twice in TCM-HEPES including bovine serum albumin fatty acid free (BSA-FAF). After incubation in a well containing 15 μM ionomycin for 4 min, oocytes were washed and incubated for 4 h in porcine zygote medium (PZM) supplemented with calcium, hypotaurine, glutamine, BSA-FAF, strontium, cytochalasin B and cycloheximide, as reported by Che et al. [26] with modifications. After activation, potential parthenotes from both groups were cultured in droplets containing 0.1 ml calcium free PZM medium [27], under mineral oil.

Structures were evaluated at 24 h and 48 h, to determine cleavage rates, and at 168 h, to evaluate embryo development rates. Thereafter, embryos were fixed in 4% paraformaldehyde for 15 min and stored until evaluation. Embryos were stained with Hoescht

33342 [25] as described by Uhm et al. [25] and evaluated under epifluorescence microscopy (Nikon 80i), to determine the number of cells.

4.2.1.3. Experiment 2 – Determination of lipid content

Based on the observed cleavage and embryo development rates, COC were submitted to IVM in a medium including 50 μM DHA and in a control medium, with the same composition described above. Lipid content was evaluated at 22 h and 44 h of IVM and in day 7 embryos. Embryos and denuded oocytes were washed three times in PBS including 0.1% PVA (PBS-PVA), fixed in 4% paraformaldehyde and simultaneously permeabilized in 0.5% Triton 100X in PBS-PVA, for 20 min at room temperature. After another triple wash, samples were stained with 1.0 μg/mL Nile Red in PBS-PVA during 30 min, protected from natural light, at room temperature. Then, samples were once again triple washed in PBS-PVA. Stained oocytes and embryos were placed in slides containing 7.0 μl Mowiol, covered with coverslips and evaluated by epifluorescence microscopy (Nikon Eclipse- TS100), as reported by Romek et al. [5], with modifications. Images were captured using an G2A filter, with fast resolution (1280X1024, fine normal and 5,44 ms exposure). The fluorescence intensity was determined using Image J® [28].

4.2.1.4. Statistical analyses

Cleavage and blastocyst development rates were compared among treatments by chi-square tests. After transformation to the logarithmic scale due to lack of normality, the number of embryo cells and the fluorescence intensity of lipid droplets were compared by analyses of variance, with comparisons of means using the LSD test [29].

4.2.2. Results

4.2.2.1. Experiment 1 - Cleavage and embryo development

Cleavage rates were lower in the medium with 12.5 μM EPA than in the control ($P < 0.05$), at 24 and 48 h (Table 1). Cleavage rates did not differ across media including EPA ($P > 0.05$). No differences in embryo development rates occurred among treatments including EPA ($P > 0.05$), which were all inferior to those of the control ($P < 0.05$). The number of embryo cells was similar across EPA-supplemented media ($P > 0.05$). With 50 μM DHA, cleavage rates were greater at both 24 and 48 h ($P < 0.05$) compared to other treatments (Table 2). Blastocyst development rates were similar in all media including DHA ($P > 0.05$).

Embryos from COC matured with 12.5 μM and 50 μM DHA had more blastomeres than those in the control group ($P < 0.05$).

4.2.2.2. Experiment 2 - Determination of lipid content

Swine oocytes matured with 50 μM DHA had lower lipid content at 22 h and 44 h ($P < 0.05$) than those matured in the control medium (Fig. 1). Reduced lipid content was also observed in D7 embryos in DHA-supplemented IVM medium ($P < 0.05$).

4.2.3. Discussion

This is the first study to evaluate supplementation of IVM medium for swine oocytes with DHA and EPA. In Experiments 1 and 2, both PUFA were tested at concentrations of at most 50 μM. In Experiment 1, supplementation of IVM media with 50 μM DHA increased cleavage rates at 24 h and 48 h after parthenogenetic activation. Although blastocyst development rates were unaffected, further positive effects may occur, such as the increased cell number observed in blastocysts from oocytes matured in DHA-supplemented media, as also reported for cattle oocytes matured in medium including 1.0 μM DHA [42]. Improved development of swine embryos can occur with supplementation with other PUFA, such as the alpha-linolenic acid, due to positive effects on nuclear and cytoplasmic maturation [43,44]. However, as also observed in the present study, greater DHA concentrations resulted in declined cleavage and blastocyst development rates, which suggests negative effects on fatty acid metabolism and possibly accumulation of reactive oxygen species.

The reduced lipid content observed in Experiment 3 for oocytes and D7 embryos cultured in medium supplied with 50 μM DHA is particularly relevant for swine oocytes, which accumulate greater lipid content than most domestic mammal species [5]. Most of such content is included in droplets inside the cytoplasm, which are available intracellularly during maturation as energy substrates [7] and are mainly required for meiosis resumption [45]. Although both the lipid and the DHA content are similar in immature and mature oocytes, DHA content is greater in mature *cumulus oophorus* cells than in immature cells, which present no such content [21]. Furthermore, as reported after culture of fibroblasts, DHA may be incorporated into cell membranes [46], inhibiting the synthesis of phospholipids and leading to an increase in basal lipolysis. In rodents, that resulted in the presence of smaller lipid droplets deposited in differentiated adipocytes [47,48]. In swine

oocytes, with greater intracytoplasmic lipid content, DHA may affect the phospholipid composition and the fluidity of cell membranes [49], improving embryo development and potentially boosting their cryotolerance [50,51]. Further studies should test DHA in media for *in vitro* embryo culture, investigating potential effects on genes
involved in lipid metabolism.

On the other hand, at all tested concentrations, inclusion of EPA in the IVM medium impaired the development of swine oocytes. That suggests that EPA is potentially toxic for swine oocytes during IVM, since there was apparently no effect on the number of blastomeres in embryos that eventually developed to the blastocyst stage. These findings agree with reports for cattle oocytes, which presented decreased maturation rate and embryo development after IVM in medium including other fatty acids [19] and increased frequency of cells with DNA lesions, apoptosis and necrosis when cultured in medium including EPA [52]. Such cytotoxicity may be attributed to an increased sensitivity of omega-3 PUFA to the action of free radicals, generating lipid peroxides [52]. Nonetheless, a selective protection mechanism may guarantee that the concentration of omega-3 PUFA in oocytes remains at a minimum secure threshold [45,53], to prevent cellular damages that may occur when such fatty acids are at increased concentrations [54]. The EPA concentrations tested in the present study may have extrapolated such threshold, resulting in cellular lesions that harmed oocytes' subsequent development. When omega-3 PUFA are at increased concentrations in the follicular fluid *in vivo*, COC interactions mediated by fatty acid-binding proteins and transzonal projections act on lipid metabolism, preventing excessive storage of fatty acids in the cytoplasm of oocytes [32,33]. Nonetheless, during IVM, deregulating mechanisms may disrupt oocytes' metabolism, inducing lipotoxicity [33,34]. Swine oocytes could be more sensitive to lipid peroxidation because of their great intracytoplasmic concentration of fatty acids [18,40].

In conclusion, the docosaexaenoic acid may be a promising additive for IVM medium, since its inclusion at 50.0 μM was associated with increased cleavage rates and embryo cell number and with reduced lipid concentration in both oocytes and embryos. The inclusion of eicosapentaenoic acid in IVM medium is not recommended, since it resulted in decreased nuclear maturation, cleavage and blastocyst development rates.

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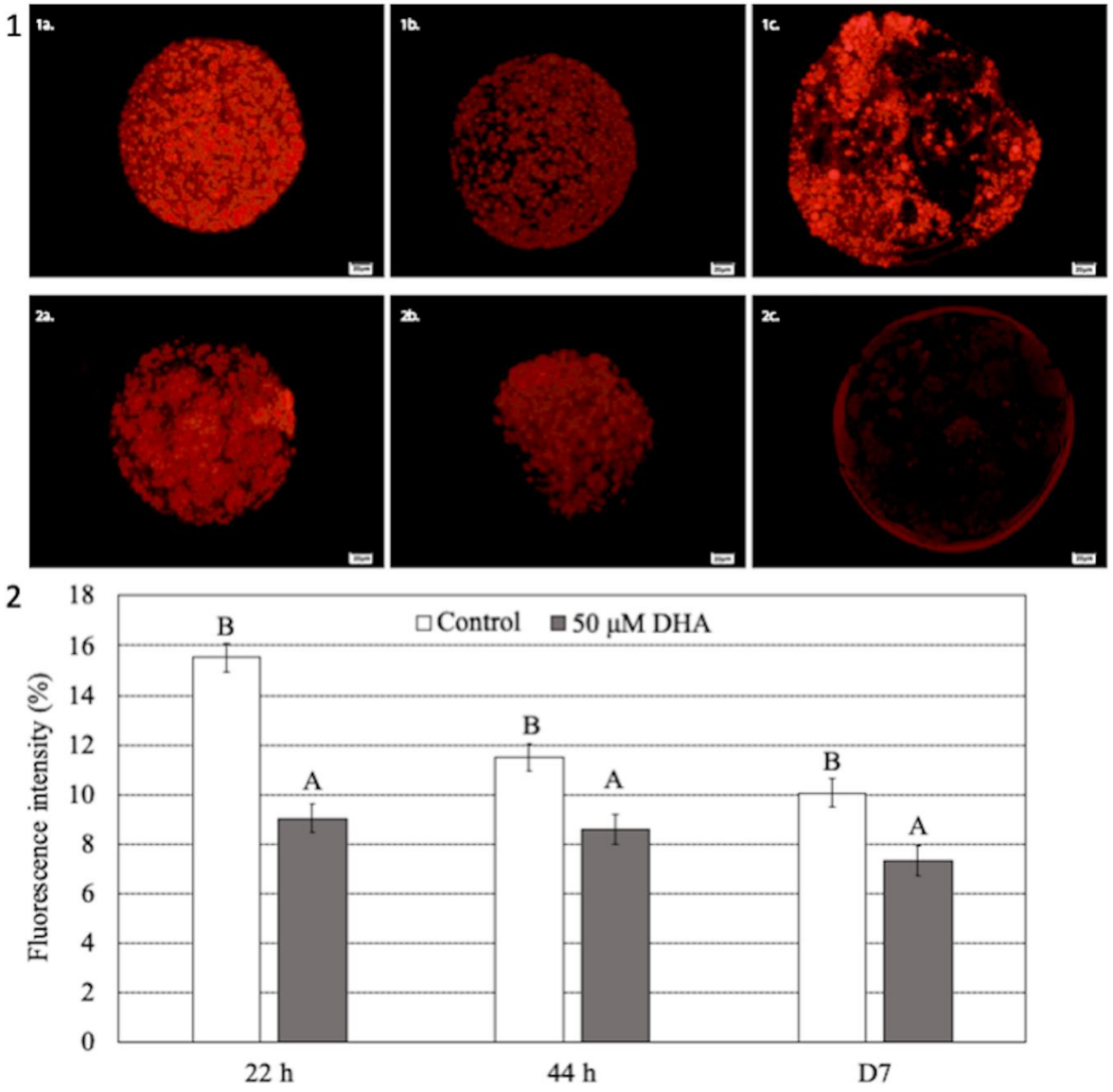

Figure 1: Fluorescence intensity of lipid droplets (%) on swine oocytes after 22 h and 44 h and on D7 embryos stained with Nile Red, after *in vitro* maturation in medium including 50 µM docosahexaenoic acid (DHA) - Experiment 2

a: Oocytes after IVM for 22 h; b: Oocytes after IVM for 44 h; c: D7 embryos

1: Control (follicular fluid): n = 94 (22 h); n = 100 (44 h); n = 90 D7 embryos

2: 50 µM DHA: n = 101 (22 h); n = 88 (44 h); and n = 76 D7 embryos

^{A,B}Means ± SEM with distinct superscripts differ by at least $P < 0.05$

Table 1: Cleavage rate, embryo development rate and embryo cell number after *in vitro* maturation of swine oocytes with distinct concentrations of eicosapentaenoic acid (EPA)

EPA (μM)	Cleavage (%)		Blastocyst development (%)	Cells (n)
	24 h	48 h		
0	43.7 (52/119)[a]	72.3 (86/119)[a]	37.8 (45/119)[a]	33.5 ± 2.5
12.5	29.2 (33/113)[b]	60.2 (68/113)[b]	15.9 (18/113)[b]	29.1 ± 3.9
25.0	34.5 (40/116)[ab]	63.8 (74/116)[ab]	22.4 (26/116)[b]	35.6 ± 3.0
50.0	33.1 (39/118)[ab]	66.1 (78/118)[ab]	25.4 (30/118)[b]	36.1 ± 3.9

[a,b]Frequencies with distinct superscripts differ by at least $P < 0.05$

Table 2: Cleavage rate, embryo development rate and embryo cell number after *in vitro* maturation of swine oocytes in media with distinct concentrations of docosahexaenoic acid (DHA)

DHA (µM)	Cleavage (%)		Blastocyst development (%)	Cells (n)
	24 h	48 h		
0	32.2 (46/143)[b]	65.7 (94/143)[b]	27.3 (39/143)	26.1 ± 3.3[C]
12.5	42.9 (60/140)[b]	72.9 (102/140)[b]	30.7 (43/140)	35.7 ± 2.3[A]
25.0	47.6 (68/143)[ab]	72.7 (104/143)[b]	36.4 (52/143)	28.7 ± 2.9[BC]
50.0	54.5 (78/143)[a]	76.9 (110/143)[a]	30.1 (43/143)	34.2 ± 3.4[AB]

[a,b]Frequencies with distinct superscripts differ by at least $P < 0.05$

[A,B]Means ± SEM with distinct superscripts differ by at least $P < 0.05$

5 DISCUSSÃO GERAL E PERSPECTIVAS

Segundo nosso entendimento este é o primeiro estudo realizado em suínos que identificou melhora nas taxas de clivagem e número de células embrionárias com inclusão de DHA no meio de maturação *in vitro*, assim como a comprovação de que este ácido graxo poli-insaturado pode também diminuir a concentração de gotículas lipídicas, favorecendo o processo de capacitação ovocitária assim como a competência do desenvolvimento embrionário subsequente. Porém, a inclusão de EPA no meio provocou uma diminuição das taxas de clivagem, blastocistos e células embrionárias em todas as concentrações testadas, o que nos levou a pensar que talvez este ácido graxo pode ter um efeito deletério no processo de maturação nuclear dos ovócitos.

Por outro lado, quando fêmeas suínas pré-púberes foram submetidas a diferentes tratamentos com gonadotrofinas, o seu perfil hormonal foi alterado apenas quanto aos níveis séricos progesterona, que aumentaram no perfil preovulatório; enquanto a expressão relativa de *CYP19A1 e CYP11A1* foi elevada no perfil de pró-estro, com redução na expressão de *BMPR1B* no perfil periovulatório.

Com os resultados destes estudos foi possível avançar no conhecimento relacionado a algumas biotécnicas reprodutivas utilizadas na espécie suína, incluindo abordagens que visaram entender aspectos moleculares na reprodução da fêmea suína, assim como identificar novos métodos para melhorar as taxas de desenvolvimento embrionário nesta espécie. Assim, nossas perspectivas são a avaliação de genes que possam estar relacionados e expressos em ovócitos e embriões previamente submetidos à inclusão de ácidos graxos no meio de maturação; assim como avaliar ovários e ovidutos no campo histológico, de fêmeas pré-púberes com diferentes perfis endócrinos e a determinação da expressão gênica em células do oviduto. Assim, após entender todos os mecanismos relacionados com o processo de fertilização do ovócito, do ponto de vista aplicado, busca-se diminuir o tempo de fertilização *in vitro* de ovócitos suínos, encontrar um

método melhor para seleção espermática para assim finalmente determinar um método eficiente para evitar a polispermia na fecundação *in vitro* de suínos.

6 CONCLUSÃO GERAL

As hipóteses geradas neste projeto foram parcialmente comprovadas, pois no trabalho com a inclusão de ácidos graxos no meio de maturação *in vitro* foi corroborado que, efetivamente, a adição de DHA na maior concentração (50 µM) melhorou as taxas de clivagem e número de células embrionárias, assim como diminuiu a concentração de gotículas lipídicas de ovócitos e embriões quando comparado com o controle, mas não aconteceu o mesmo com o EPA em nenhuma das concentrações testadas. Já para o segundo estudo, foi confirmado que houve expressão de genes relacionados com o desenvolvimento de folículos, a ovulação e a fecundação em células foliculares de fêmeas suínas pré-púberes, em função de diferentes perfis endócrinos reprodutivos, porém, dos genes utilizados os que expressaram em maior quantidade foram *CYP19A1* e *CYP11A1* nas fêmeas durante o proestro, e houve uma diminuição da expressão relativa do *BMPR1B* durante o período periovulatório, sendo assim encontrado que o tratamento com gonadotrofinas pode sim potencializar a foliculogênese em fêmeas suínas pré-púberes, mas os efeitos sobre a esteroidogênese foram restritos aos níveis elevados de progesterona sérica no perfil periovulatório.

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