DOCOSAHEXAENOIC ACID IMPAIRS *IN VITRO* MATURATION OF SWINE OOCYTES

JOSÉ VICTOR CARDOSO BRAGA[1]; ANDREZ PASTORELLO BOHN[2]; LUANA PESCKE[3]; ELIZA ROSSI KOMNINOU[4]; ARNALDO DINIZ VIEIRA [5]; RAFAEL GIANELLA MONDADORI[6]

[1]*Universidade Federal de Pelotas, ReproPel– bragavictor93@gmail.com*
[2]*Universidade Federal de Pelotas, ReproPel– andrezbohn@gmail.com*
[3]*Universidade Federal de Pelotas, ReproPel– luapeske@gmail.com*
[4]*Universidade Federal de Pelotas, ReproPel– elizarossikom@gmail.com*
[5]*Universidade Federal de Pelotas, ReproPel– vieira_ad@yahoo.com.br*
[6]*Universidade Federal de Pelotas, ReproPel– rgmondadori@gmail.com*

1. INTRODUCTION

Oocyte maturation is a process of either meiotic progression from an arrested diplotene stage of the first prophase until a fertilizable gamete at the second metaphase and cytoplasmatic organelle rearrangement (FERREIRA et al., 2009). Within cytoplasmatic changes, lipid droplets (LD) are formed near the endoplasmic reticulum through the utilization of fatty acids (FA) that are metabolized for lipid synthesis by cumulus cells during oocytes follicular growth and development (PAULINI et al., 2014), resulting in energy storage that requires β-oxidation in order to support the maturation process (DUNNING; RUSSELL; ROBKER, 2014).

When compared to other domestic species, one of the most limiting factors for swine *in* vitro production (IVP) is the high lipid content and accumulation of LD in the cytoplasm of both oocytes and embryos (GAJDA, 2009; MCEVOY et al., 2000).

Many recent researches focus on swine IVP improvement by lipid reduction through the addition of lipid metabolizers (reviewed by BRAGA et al., 2019). Among them, FA supplementation has shown to be beneficial as it acts as energy supply and forces lipid metabolism.

Docosahexaenoic acid (DHA), a polyunsaturated fatty acid (PUFA), has crucial involvement during oocyte maturation by acting as energy reservoir, precursor of steroidogenesis (KHAJEH et al., 2017) and reduction of genes related to lipid metabolism (BARBER; SINCLAIR; CAMERON-SMITH, 2013). Its usage during bovine (OSEIKRIA et al., 2016) and swine (HOYOS-MARULANDA et al., 2019) has already improved oocyte maturation and embryo development. However, it has been applied with follicular fluid, which is known to already have DHA as one of its components (MCEVOY et al., 2000).

The objective of this study was to evaluate oocyte maturation and embryo development after addition of DHA during IVM with and without follicular fluid supply.

2. METODOLOGY

Ovaries were collected at local abattoir and transported to the laboratory in saline solution (0,9%) at 30°C to 35°C. Follicles with a diameter ranging from 3mm to 6mm were aspirated using a vacuum pump attached to a 19 G needle (10 - 15 mL/min). The cumulus oophorus complexes (COCs) were searched under stereomicroscopy in 35mm Petri dishes filled with centrifuged porcine follicular fluid (pFF) and subsequently placed in a dish containing TCM-HEPES medium (TCM-199 with 0.1% PVA, 2.0mM $C_3H_4O_3$ and 2.5mM $NaHCO_3$) for selection. Oocytes

with homogenous cytoplasm and at least three layers of cumulus cells were then selected and washed again in TCM-HEPES before maturation.

Thirty to forty COCs were matured for 44h at 38.5°C in 5% CO_2 atmosphere with saturated humidity in 4-well plates (Nunc) containing 400µL of media, according the treatment groups: 1) Negative Control (IVM); 2) Positive Control (IVM + 25% pFF); 3) DHA A (negative control + 50µM DHA); and 4) DHA B (positive control + 50µM DHA). A total of 268 COCs were matured in Negative Control, 246 in Positive Control, 231 in DHA A and 257 in DHA B. The basal IVM medium consisted of TCM-199 supplemented with 0.1% PVA and 2.5mM $NaHCO_3$, 0.57mM cysteine, 0.91mM $C_3H_3NaO_3$, 0.001g/ml EGF, 0.05g/ml streptomycin, 0.065g/ml penicillin, with gonadotrophins (20µg/µL FSH, 5µg/µL LH) for the first 22h of maturation. DHA was prepared according to HOYOS-MARULANDA et al. (2019).

After maturation for 44h, COCs were denuded by vortex for 2 to 3 minutes in their own maturation media. Part of the denuded oocytes (DO) were washed in PBS, fixed in 4% paraformaldehyde for 30 minutes and stored in PBS at 4°C until staining for maturation assessment. The other remaining DO were washed in TCM IVM plus bovine serum albumin fatty acid free (BSA-FAF) and subjected to chemical activation by exposure to 15µM ionomycin for 5 minutes, washed again, incubated for 4h in PZM (-) medium (YOSHIOKA et al., 2002) containing 10mM strontium, 7.5mM cytochalasin B and 10µg/mL cycloheximide (CHE; LALONDE; BORDIGNON, 2007). After activation, potential parthenotes were cultured in 60µL droplets of PZM (+) (YOSHIOKA et al, 2002) under mineral oil. Cleavage rates were obtained at 24h and 48h and blastocyst rates at day 7. At day 5, feeding was done by addition of 10% estrus mare serum (EMS).

Denuded oocytes were stained with 7.5µg/ml Hoechst 333342 for 10 minutes and placed on slides with 10µL Mowiol with coverslips for maturation evaluation using epifluorescence microscopy (Nikon Eclipse-TS100).

For statistical analysis, maturation and cleavage rates were compared among treatments by chi-square tests and comparison of means was done by LSD test through Statistix 10 software.

3. RESULTS AND DISCUSSION

Table 1 shows that maturation, cleavage and blastocyst rates were lower in the DHA A group ($p < 0.05$), whereas all the other groups had similar behavior. However, when blastocyst per 48h cleaved oocytes rates were evaluated, there is no difference among the groups, showing that matured and cleaved oocytes from DHA A group could develop equally as the other groups until the blastocyst stage.

Interestingly, our control groups did not differ from each other as expected since Positive Control has pFF presence while it is absent in the Negative Control. Although, it is of note that our preliminary results are lacking blastomere and apoptotic cell count as well as lipid content evaluation of these developed embryos, a data that could explain if the quality of our controls is not affected by the presence of pFF such as their development rates.

Also, these data can demonstrate why DHA lacking follicular fluid had such negative impact during the maturation and cleavage stages, especially since DHA oil is widely administrated in dietary studies and has shown to have no toxic effects on reproductive tract and offspring malformation in rats (BLUM et al., 2007; FALK et al., 2017) and has benefits for male and female swine in regards to animal growth, fertility, immunity and bone strength (reviewed by LEE; WHENHAM; BEDFORD, 2019).

In fact, HOYOS-MARULANDA et al. (2019) found better response in blastocyst development and lipid content reduction with the same 50µM DHA dose administrated in our study. Also, ELIS et al., (2017), demonstrated that 4h exposure of 1uM DHA supplemented with 10% fetal calf serum (FCS) during IVM improved bovine embryo development. However, both studies had a lower fatty concentration (10% vs 25%) and did not isolate DHA from fatty with a negative control lacking both DHA and fatty supply, suggesting that DHA supplementation during porcine IVM is not only dependent of the presence of fatty source, but with the concentration of this fatty source in the maturation medium.

Table 1. Maturation rates, cleavage rates, blastocyst development rates and blastocyst per cleavage after maturation with or without DHA and/or pFF.

	Treatments			
	Negative Control	Positive Control	DHA A	DHA B
Maturation (%)	42.96 (55/128)[a]	56.73 (59/104)[a]	9.73 (11/113)[b]	65.45 (72/110)[a]
Cleavage 24h (%)	27.86 (39/140)[a]	42.25 (60/142)[a]	8.47 (10/118)[b]	37.41 (55/147)[a]
Cleavage 48h (%)	55.00 (77/140)[a]	57.75 (82/142)[a]	17.80 (21/118)[b]	51.02 (75/147)[a]
Blastocyst (%)	22.14 (31/140)[a]	28.17 (40/142)[a]	9.32 (11/118)[b]	17.01 (25/147)[a]
Blastocyst/48h Cleavage (%)	40.26 (31/77)	48.48 (40/82)	52.38 (11/21)	33.33 (25/75)

Frequencies with different superscripts (a, b) differ by at least $P < 0.05$.
DHA A – negative control + 50µM DHA
DHA B – positive control + 50µM DHA

4. CONCLUSION

In conclusion, supplementation of DHA at 50uM without pFF negatively impacts oocyte maturation, embryo initial cleavage, total blastocyst rates.

5. REFERÊNCIAS BIBLIOGRÁFICAS

BARBER, E.; SINCLAIR, A. J.; CAMERON-SMITH, D. Comparative actions of omega-3 fatty acids on in-vitro lipid droplet formation. **Prostaglandins Leukotrienes and Essential Fatty Acids**, v. 89, n. 5, p. 359–366, 2013.

BLUM, R. et al. One-generation reproductive toxicity study of DHA-rich oil in rats. **Regulatory Toxicology and Pharmacology**, v. 49, n. 3, p. 260–270, 2007.

BRAGA, J. V. et al. Approaches to reduce lipids : a review of its impacts on in vitro embryo production. **Revista Brasileira de Reprodução Animal**, p. 3–7, 2019.

CHE, L.; LALONDE, A.; BORDIGNON, V. Chemical activation of parthenogenetic and nuclear transfer porcine oocytes using ionomycin and strontium chloride. **Theriogenology**, v. 67, n. 7, p. 1297–1304, 2007.

DUNNING, K. R.; RUSSELL, D. L.; ROBKER, R. L. Lipids and oocyte

developmental competence: The role of fatty acids and β-oxidation. **Reproduction**, v. 148, n. 1, 2014.

ELIS, S. et al. Docosahexaenoic acid mechanisms of action on the bovine oocyte-cumulus complex. **Journal of Ovarian Research**, v. 10, n. 1, p. 1–14, 2017.

FALK, M. C. et al. Developmental and reproductive toxicological evaluation of arachidonic acid (ARA)-Rich oil and docosahexaenoic acid (DHA)-Rich oil. **Food and Chemical Toxicology**, v. 103, p. 270–278, 2017.

FERREIRA, E. M. et al. Cytoplasmic maturation of bovine oocytes: Structural and biochemical modifications and acquisition of developmental competence. **Theriogenology**, v. 71, n. 5, p. 836–848, 2009.

GAJDA, B. Factors and methods of pig oocyte and embryo quality improvement and their application in reproductive biotechnology. **Reproductive biology**, v. 9, n. 2, p. 97–112, 2009.

HOYOS-MARULANDA, V. et al. Effects of polyunsaturated fatty acids on the development of pig oocytes in vitro following parthenogenetic activation and on the lipid content of oocytes and embryos. **Animal Reproduction Science**, v. 205, n. April, p. 150–155, 2019.

KHAJEH, M. et al. Potential role of polyunsaturated fatty acids, with particular regard to the signaling pathways of arachidonic acid and its derivatives in the process of maturation of the oocytes: Contemporary review. **Biomedicine and Pharmacotherapy**, v. 94, p. 458–467, 2017.

LEE, S. A.; WHENHAM, N.; BEDFORD, M. R. Review on docosahexaenoic acid in poultry and swine nutrition: Consequence of enriched animal products on performance and health characteristics. **Animal Nutrition**, v. 5, n. 1, p. 11–21, 2019.

MCEVOY, T. G. et al. Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida. **J Reprod Fertil**, v. 118, n. 1, p. 163–170, 2000.

OSEIKRIA, M. et al. N-3 polyunsaturated fatty acid DHA during IVM affected oocyte developmental competence in cattle. **Theriogenology**, v. 85, n. 9, p. 1625-1634.e2, 2016.

PAULINI, F. et al. Ultrastructural changes in oocytes during folliculogenesis in domestic mammals. **Journal of Ovarian Research**, v. 7, n. 1, p. 1–12, 2014.

YOSHIOKA, K. et al. Birth of Piglets Derived from Porcine Zygotes Cultured in a Chemically Defined Medium1. **Biology of Reproduction**, v. 66, n. 1, p. 112–119, 2002.