Animal Reproduction Science 205 (2019) 150–155

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

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

V. Hoyos-Marulanda[a,c], B.S. Alves[a,c], P.R.A. Rosa[a,b], A.D. Vieira[a,b], B.G. Gasperin[a,b], R.G. Mondadori[a,d], T. Lucia Jr.[a,b,*]

[a] *ReproPel, Universidade Federal de Pelotas, 96010-900, Pelotas, RS, Brazil*
[b] *Faculdade de Veterinária, Universidade Federal de Pelotas, 96010-900, Pelotas, RS, Brazil*
[c] *Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, 96010-900, Pelotas, RS, Brazil*
[d] *Instituto de Biologia, Universidade Federal de Pelotas, 96010-900, Pelotas, RS, Brazil*

ARTICLE INFO

Keywords:
DHA
Nile Red
Lipid
Porcine
Oocytes

ABSTRACT

As oocytes and embryos of pigs have greater lipid content in the cytoplasm than those of other species, supplementation of the medium for *in vitro* maturation (IVM) of oocytes with omega-3 polyunsaturated fatty acids (PUFA) may help to improve embryo development. This study was conducted to evaluate effects of the inclusion of the docosaexaenoic (DHA) and of the eicosapentaenoic acids (EPA) in the IVM medium on the development of pig oocytes and on the lipid content of oocytes and embryos. In all experiments, control media consisted of porcine follicular fluid and oocytes were activated through parthenogenesis. In Experiment 1, there were four treatments for each PUFA: one control; and three treatments including EPA or DHA in the IVM medium at 12.5 μM, 25.0 μM and 50.0 μM). In Experiment 2, inclusion of 50 μM DHA was compared against the control. Cleavage rates in the IVM medium including 12.5 μM EPA and blastocyst development rates in media at any EPA concentration were less than for the control in Experiment 1 ($P < 0.05$). Compared to the control, inclusion of 50 μM DHA in the IVM medium was related to greater cleavage rates and greater number of embryo cells, in Experiment 1, and lesser lipid content in oocytes after 22 and 44 h and in embryos after 7 days, in Experiment 2 (both $P < 0.05$). Addition of DHA in the IVM medium may benefit the development of pig oocytes, but EPA appears to be cytotoxic.

1. Introduction

Although the birth of piglets has been successfully achieved with pig embryos produced *in vitro* (Somfai et al., 2014; Mito et al., 2015), such processes still require improvement, because blastocyst development rates are quite variable (Dang-Nguyen et al., 2011; Hwang et al., 2016). These differences in outcomes may be due to the greater amount of lipid droplets present in the cytoplasm of pig oocytes and embryos compared to other mammalian species (Romek et al., 2011). As the triglyceride content of pig oocytes decreases during *in vitro* maturation (IVM) (Sturmey and Leese, 2003), the oxidation of fatty acids can impair embryo development (Sturmey et al., 2006). Lipid metabolism, therefore, has a relevant function on the developmental competence of pig oocytes.

* Corresponding author at: ReproPel, Universidade Federal de Pelotas, 96010-900, Pelotas, RS, Brazil.
E-mail addresses: thomaz@ufpel.edu.br, thomaz@pq.cnpq.br (T. Lucia).

https://doi.org/10.1016/j.anireprosci.2019.05.003
Received 13 March 2019; Received in revised form 15 April 2019; Accepted 4 May 2019
Available online 06 May 2019
0378-4320/

Long-chain polyunsaturated fatty acids (PUFA), especially those of the omega-3 series, are components of the structure of the phospholipid bilayer of cell membranes, affected membrane fluidity and intracellular signal transmission mechanisms (Calder, 2016). The eicosapentaenoic (C20:5n-3, EPA) and the docosahexaenoic (C22:6n-3, DHA) acids are considered the most important omega-3 PUFA (reviewed by Rossi et al., 2010). Omega-3 PUFA may be incorporated into oocytes, embryos and fetuses (Wakefield et al., 2008; Brazle et al., 2009), modulating the actions of enzymes that have important functions on the metabolism of prostaglandins and steroid hormones (Wathes et al., 2007). Thus, supplementation of omega-PUFA in diets for swine is related to enhanced neural embryo development (Brazle et al., 2009), improved embryo survival and fetal development (Henman, 2006) and increased litter size (Smits et al., 2011), also potentially stimulating puberty in gilts (Moreira et al., 2016).

Omega-3 PUFA also functions in adipogenesis processes and other physiological processes, regulating gene transcription (Jump, 2008), which results in reduced expression of genes that code for many enzymes involved in lipid metabolism (Barber et al., 2013). Fatty acids, therefore, alter lipid accumulation in adipocytes, reducing the formation and the size of lipid droplets (Manickam et al., 2010). Furthermore, fatty acids are present in large amounts in oocytes and in the follicular fluid (McEvoy et al., 2000; Dunning et al., 2014). The hypothesis for the present study, therefore, was that the inclusion of omega 3-PUFA in the culture medium would have positive effects on the lipid metabolism in pig oocytes, potentially improving development *in vitro*. The objective of this study was to evaluate the effects of the supplementation of the IVM medium with EPA and DHA on the development of pig oocytes activated through parthenogenesis and on the lipid content of oocytes and embryos.

2. Material and methods

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

2.1. Harvesting and processing of cumulus oophorus-oocyte complexes (COCs)

Ovaries from prepubertal gilts were collected at a slaughterhouse and transported to the laboratory in a saline solution at 30 °C. Follicles that were 3–6 mm in diameter were subsequently aspirated using a vacuum pump attached to a 19 G needle (15 ml/min). The COCs were located using stereomicroscopy and placed in 35 mm Petri dishes and subsequently placed in a dish containing TCM-HEPES medium (TCM199 with 0.1% PVA and 2.5 mM $NaHCO_3$), for morphological evaluation (Magamage et al., 2011). The selected COCs had a homogenous ooplasm and at least three layers of compact cumulus oophorus cells.

Prior to initiation of the experiments, a pilot study was conducted to evaluate the nuclear maturation of pig oocytes in TCM-HEPES including FSH (Folltropin®-V) and LH (Lutropin-®-V), both from Vetoquinol N.-A. Inc. (Lavaltrie, QC, Canada), during the first 22 h of incubation (Liu et al., 2015). Alcoholic solutions of EPA (Sigma E-2011) and DHA (Sigma D-2534) were added at concentrations previously used for fibroblast culture (Barber et al., 2013): 50 μM; 100 μM; and 150 μM. The control treatments consisted of 10% follicular fluid from pubertal sows. The MII rates in the medium with 50 μM were similar to that for the control group, whereas MII rates with 100–150 μM DHA and EPA were much less (data not shown). Those findings confirmed that alcohol as a solvent had no negative effect on the responses of interest and indicated that 50 μM would be the maximum concentration of both PUFA to be evaluated in the subsequent experiments.

2.2. Experiment 1 - cleavage and embryo development

Each PUFA was assessed in four treatment groups: one control; and three treatment groups with alcoholic solutions of either EPA or DHA being added to the IVM medium at different concentrations (12.5 μM, 25.0 μM and 50.0 μM). The composition of the control and of the IVM media were the same as those previously described in this manuscript. For each fatty acid, four replicates were conducted, each with approximately 30 COCs randomly allocated per treatment group, imposing the same IVM conditions described previously.

After 44 h of IVM, cumulus oophorus cells were mechanically removed and the oocytes were activated through parthenogenesis. Initially, the oocytes were washed twice in TCM-HEPES including bovine serum albumin fatty acid free (BSA-FAF). After incubation in 15 μM ionomycin for 4 min, the oocytes were washed and incubated for 4 h in PZM medium supplemented with calcium, hypotaurine, glutamine, BSA-FAF, strontium, cytochalasin B and cycloheximide, as reported by Che et al. (2007), with modifications. After activation, potential parthenotes were cultured in droplets containing 0.1 ml calcium free PZM medium (Yoshioka et al., 2002), under mineral oil. Cleavage rates were determined at 24 and 48 h and blastocyst development rates were determined at 168 h of incubation. Embryos were subsequently fixed in 4% paraformaldehyde for 15 min and stored until evaluation. Embryos were stained as described by Uhm et al. (2007) and evaluated using an epifluorescence microscopy, to determine the number of cells.

2.3. Experiment 2 – determination of lipid content

Considering the rates of cleavage and development to the blastocyst stage in Experiment 1, only 50 μM DHA was evaluated in Experiment 2, included to the IVM medium with the same composition as previously described in this manuscript. The control medium consisted of 10% follicular fluid from pubertal sows. For this experiment, five replications conducted.

Denuded oocytes and embryos 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,

Table 1
Cleavage rate, blastocyst development rate and number of embryonic cells after maturation of pig oocytes *in vitro* in medium including different concentrations of eicosapentaenoic acid (EPA) - Experiment 1.

EPA (μM)	Cleavage (%)		Blastocyst development (%)	Embryonic 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,bFrequencies with different superscripts differ by at least $P < 0.05$.

samples were stained with 1.0 μg/mL Nile Red in PBS-PVA for 30 min, protected from natural light, at room temperature. Samples were again triple washed using PBS-PVA. Stained oocytes and embryos were placed on slides with 7.0 μl Mowiol, with coverslips being applied, and there was evaluation of oocytes using epifluorescence microscopy (Nikon Eclipse-TS100), as reported by Romek et al. (2011). Images were obtained using a G2A filter, with fast resolution (1280 × 1024, fine normal and 5.44 ms exposure). The fluorescence intensity was determined using Image J®. Lipid content was evaluated at 22 and 44 h of IVM for oocytes and after 7 d (D7) for embryos.

2.4. Statistical analyses

In Experiment 1, cleavage and blastocyst development rates were compared among treatments using the chi-square tests, whereas the number of embryo cells was compared using an analysis of variance, after logarithmic transformation were conducted due to lack of normality. In Experiment 2, the data for mean fluorescence intensity of lipid droplets were also transformed to the logarithmic scale and compared among treatments using an analysis of variance. For both experiments, comparisons of means were conducted using the LSD test (Statistix®, 2013).

3. Results

In Experiment 1, cleavage rates in the medium with 12.5 μM EPA were less than in the control ($P < 0.05$) at both 24 and 48 h (Table 1), but there were no differences among the media including EPA ($P > 0.05$). Blastocyst development rates in the media including EPA were all less than those with the control conditions ($P < 0.05$) but did not differ among treatment groups ($P > 0.05$). The number of embryonic cells was similar with use of all the different media including EPA ($P > 0.05$).

With the medium including 50 μM DHA, cleavage rates were greater at both 24 and 48 h ($P < 0.05$) compared to rates with other treatments (Table 2). Although blastocyst development rates did not differ with the different media treatments ($P > 0.05$), embryos resulting from development of COCs matured during the culture periods in the media with 12.5 μM and 50 μM DHA and had more blastomeres than the embryos of the control group ($P < 0.05$).

In Experiment 2, oocytes in the medium including 50 μM DHA had a lesser lipid content at 22 and 44 h ($P < 0.05$) of culture than those in the control medium (Fig. 1). There was also a lesser lipid content compared to the control for D7 embryos in the IVM medium supplemented with 50 μM DHA ($P < 0.05$).

4. Discussion

This is the first study in which there was evaluation of the effects of the supplementation of IVM medium for pig oocytes with DHA and EPA. The reduced lipid content observed in Experiment 2 of oocytes and D7 embryos cultured in the medium with 50 μM DHA is particularly relevant for pig oocytes, which accumulate greater lipid content than those of most mammalian species (Romek et al., 2011). Most of such content is included in droplets inside the cytoplasm, which are available intracellularly during maturation as energy substrates and are mainly required for meiosis resumption (Sturmey et al., 2009). Although both the lipid content and the

Table 2
Cleavage rate, blastocyst development rate and number of embryonic cells after maturation of pig oocytes *in vitro* in medium including distinct concentrations of docosahexaenoic acid (DHA) - Experiment 1.

DHA (μM)	Cleavage (%)		Blastocyst development (%)	Embryonic 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,bFrequencies with different superscripts differ by at least P < 0.05.
A,B,CMeans ± SEM with different superscripts differ by at least P < 0.05.

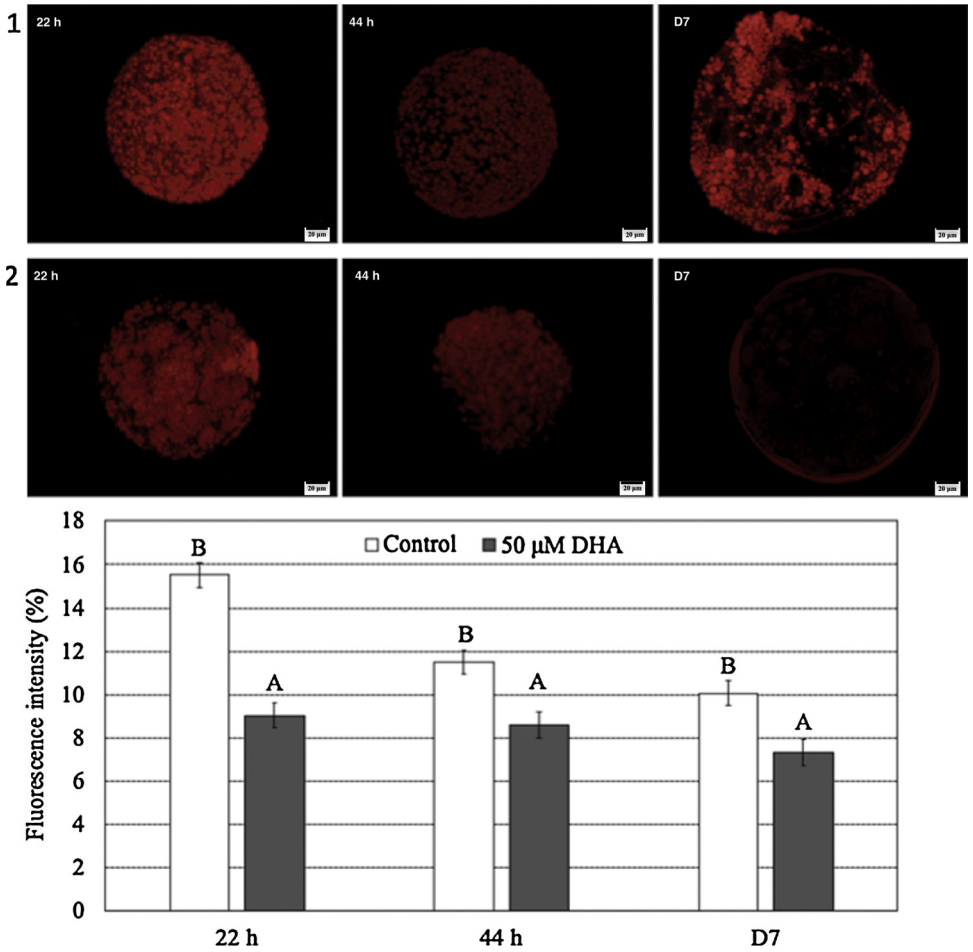

Fig. 1. Fluorescence intensity of lipid droplets (%) on pig oocytes after 22 and 44 h and on D7 embryos stained with Nile Red, after maturation *in vitro* in medium including 50 µM docosahexaenoic acid (DHA) - Experiment 2*.
1. : Control (follicular fluid): 22 h (n = 94 oocytes); 44 h (n = 100 oocytes); D7 (n = 90 embryos)
2: 50 µM DHA: 22 h (n = 101 oocytes); 44 h (n = 88 oocytes); D7 (n = 76 embryos)
^{A,B}Mean frequencies ± SEM with distinct superscripts differ by at least $P < 0.05$
*Scale bar: 20 µm

DHA content are similar in immature and mature oocytes, the DHA content is greater in mature cumulus oophorus cells than in immature cells, which have no lipid content (Prates et al., 2013). Furthermore, as reported after culture of fibroblasts, DHA may be incorporated into cell membranes (Brown and Subbaiah, 1994), inhibiting the synthesis of phospholipids and leading to an increase in basal lipolysis. In rodents, these conditions resulted in the presence of smaller lipid droplets deposited in differentiated adipocytes (Kobatake et al., 1984; Kim et al., 2006). In pig oocytes, which have greater intracytoplasmic lipid content, DHA may affect the phospholipid composition and the fluidity of cell membranes (Calder, 2011), improving embryo development and potentially enhancing embryo cryotolerance (Pereira and Marques, 2008; Zhou and Li, 2009). In further studies there should be assessments of the inclusion of DHA in media for *in vitro* embryo culture, investigating potential effects on genes involved in lipid metabolism.

In Experiment 1, supplementation of the IVM medium with 50 µM DHA was related to increased cleavage rates at 24 and 48 h after parthenogenetic activation. Although blastocyst development rates were unaffected, further positive effects may occur, because the number of cells were greater in blastocysts from oocytes matured in the medium supplemented with DHA, which was also reported for cattle oocytes matured in medium including 1.0 µM DHA (Oseikria et al., 2016). Improved development of pig embryos can occur with supplementation with other PUFAs due to positive effects on nuclear and cytoplasmic maturation (Lee et al., 2016, 2017). Furthermore, in the present study, the greater DHA concentrations were associated with lesser cleavage and blastocyst development rates, which indicates there are negative effects on fatty acid metabolism.

At all PUFA concentrations evaluated in the present study, EPA appeared to be potentially toxic for pig oocytes during IVM, even though there was no effect on the number of blastomeres in embryos that eventually developed to the blastocyst stage. These findings are consistent with results of studies with cattle oocytes where there was a lesser maturation rate and embryo development after IVM in a medium including other PUFA, such as stearic and palmitic acid (Leroy et al., 2005; Van Hoeck et al., 2011). Furthermore, there

was an increased frequency of DNA lesions, apoptosis and necrosis after culture in a medium including EPA (Nikoloff et al., 2017). Such toxicity may be due to the sensitivity of omega-3 PUFA to the action of free radicals, which generates lipid peroxides (Nikoloff et al., 2017). Nevertheless, a selective protection mechanism may ensure that the concentration of omega-3 PUFA in oocytes remains at a minimum and safe threshold (Sturmey et al., 2009; Lapa et al., 2011), to prevent cellular damage that may occur when fatty acids are at relatively greater concentrations (Arav and Zvi, 2008). The EPA concentrations assessed in the present study may have exceeded this threshold, leading to cellular lesions that impaired subsequent development of the oocytes.

5. Conclusions

The inclusion of 50.0 μM docosaexaenoic acid in the medium for maturation of pig oocytes *in vitro* was associated with increased cleavage rates, increased number of embryo cells and reduced lipid concentration of both oocytes and embryos. The inclusion of eicosapentaenoic acid in the medium is not recommended because this inclusion resulted in lesser cleavage and blastocyst development rates as compared with control values.

Acknowledgements

The authors thank CNPq for providing financial support for this research project.

References

Arav, A., Zvi, R., 2008. Do chilling injury and heat stress share the same mechanism of injury in oocytes? Mol. Cell. Endocrinol. 282, 150–152.
Barber, E., Sinclair, A.J., Cameron-Smith, D., 2013. Comparative actions of omega-3 fatty acids on *in-vitro* lipid droplet formation. Prostaglandins Leukot. Ess. Fatty Acids 89, 359–366.
Brazle, A.E., Johnson, B.J., Webel, S.K., Rathbun, T.J., Davis, D.L., 2009. Omega-3 fatty acids in the gravid pig uterus as affected by maternal supplementation with omega-3 fatty acids. J. Anim. Sci. 87, 994–1002.
Brown, E.R., Subbaiah, P.V., 1994. Differential effects of eicosapentaenoic acid and docosahexaenoic acid on human skin fibroblasts. Lipids 29, 825–829.
Calder, P.C., 2011. Fatty acids and inflammation: the cutting edge between food and pharma. Eur. J. Pharmacol. 668 (Suppl 1), S50–58.
Calder, P.C., 2016. The role of DHA in the first 1,000 days. Ann. Nutr. Metab. 69 (Suppl 1), 8–21.
Che, L., Lalonde, A., Bordignon, V., 2007. Chemical activation of parthenogenetic and nuclear transfer porcine oocytes using ionomycin and strontium chloride. Theriogenology 67, 1297–1304.
Dang-Nguyen, T.Q., Somfai, T., Haraguchi, S., Kikuchi, K., Tajima, A., Kanai, Y., Nagai, T., 2011. *In vitro* production of porcine embryos: current status, future perspectives and alternative applications. Anim. Sci. J. 82, 374–382.
Dunning, K.R., Russell, D.L., Robker, R.L., 2014. Lipids and oocyte developmental competence: the role of fatty acids and beta-oxidation. Reproduction 148, R15–27.
Henman, D., 2006. Nutritional management of reproduction. In: Taylor-Pickard, J.A., Nollet, L. (Eds.), Nutritional Approaches to Arresting the Decline in Fertility of Pigs and Poultry. Wageningen Academic Publishers, The Netherlands, pp. 73–92.
Hwang, J., Zhang, H., Qin, G., Wang, F., Wang, X., Luo, A., Zheng, Q., Cao, C., Zhao, J., 2016. BIX-01294 increases pig cloning efficiency by improving epigenetic reprograming of somatic cell nuclei. Reproduction 151, 39–49.
Jump, D.B., 2008. N-3 polyunsaturated fatty acid regulation of hepatic gene transcription. Curr. Opin. Lipidol. 9, 242–247.
Kim, S.-J., Lee, S.-A., Yun, S.J., Kim, J.-K., Park, J.-S., Jeong, H.-S., Lee, J.-H., Moon, S.-J., Won, Y.-H., 2006. Expression of vanilloid receptor 1 in cultured fibroblast. Exp. Dermatol. 15, 362–367.
Kobatake, Y., Kuroda, K., Jinnouchi, H., Nishide, E., Innami, S., 1984. Differential effects of dietary eicosapentaenoic and docosahexaenoic fatty acids on lowering of tri-glyceride and cholesterol levels in the serum of rats on hypercholesterolemic diet. J. Nutr. Sci. Vitaminol. 30, 357–372.
Lapa, M., Marques, C.C., Alves, S.P., Vasques, M.I., Baptista, M.C., Carvalhais, I., Silva Pereira, M., Horta, A.E., Bessa, R., Pereira, R.M., 2011. Effect of trans-10 cis-12 conjugated linoleic acid on bovine oocyte competence and fatty acid composition. Reprod. Dom. Anim. 46, 904–910.
Lee, Y., Lee, H., Park, B., Elahi, F., Lee, J., Lee, S.T., Park, C.K., Hyun, S.H., Lee, E., 2016. Alpha-linolenic acid treatment during oocyte maturation enhances embryonic development by influencing mitogen-activated protein kinase activity and intraoocyte glutathione content in pigs. J. Anim. Sci. 94, 3255–3263.
Lee, J.E., Yong, H., Kim, H.Y., Lee, W.H., Lee, H.T., Cheong, H.T., Yang, B.K., Park, C.K., 2017. Effect of alpha-linolenic acid on oocyte maturation and embryo development in pigs. Dev. Reprod. 21, 205–213.
Leroy, J.L., Vanholder, T., Mateusen, B., Christophe, A., Opsomer, G., de Kruif, A., Genicot, G., Van Soom, A., 2005. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. Reproduction 130, 485–495.
Liu, C., Wu, G.-Q., Fu, X.W., Mo, X.H., Zhao, L.H., Hu, H.M., Zhu, S.E., Hou, Y.P., 2015. The extracellular calcium-sensing receptor (CASR) regulates gonadotropins-induced meiotic maturation of porcine oocytes. Biol. Reprod. 93, 131–139.
Magamage, M.P., Zengyo, M., Moniruzzaman, M., Miyano, T., 2011. Testosterone induces activation of porcine primordial follicles in vitro. Reprod. Med. Biol. 10, 21–30.
Manickam, E., Sinclair, A.J., Cameron-Smith, D., 2010. Suppressive actions of eicosapentaenoic acid on lipid droplet formation in 3T3-L1 adipocytes. Lipids Health Dis. 9, 57.
McEvoy, T.G., Coull, G.D., Broadbent, P.J., Hutchinson, J.S., Speake, B.K., 2000. Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida. J. Reprod. Fert. 18, 163–170.
Mito, T., Yoshioka, K., Noguchi, M., Yamashita, S., Misumi, K., Hoshi, T., Hoshi, H., 2015. Birth of piglets from *in vitro*-produced porcine blastocysts vitrified and warmed in a chemically defined medium. Theriogenology 84, 1314–1320.
Moreira, F., Cheuiche, Z.M.G., Rizzoto, G., Santos, M.Q., Schuch, M.S., Flach, M.J., Gasperin, B.G., Bianchi, I., Lucia Jr., T., 2016. Metabolic and reproductive parameters in prepubertal gilts after omega-3 supplementation in the diet. Anim. Reprod. Sci. 170, 178–183.
Nikoloff, N., Pascua, A.M., Anchordoquy, J.M., Anchordoquy, J.P., Sirini, M.A., Seoane, A., Furnus, C.C., 2017. Effect of eicosapentaenoic acid on bovine cumulus–oocyte complex in vitro. Cell Biol. Int. 41, 505–513.
Oseikria, M., Elis, S., Maillard, V., Corbin, E., Uzbekova, S., 2016. N-3 polyunsaturated fatty acid DHA during IVM affected oocyte developmental competence in cattle. Theriogenology 85, 1625–1634.
Pereira, R.M., Marques, C.C., 2008. Animal oocyte and embryo cryopreservation. Cell Tissue Bank 9, 267–277.
Prates, E.G., Alves, S.P., Marques, C.C., Baptista, M.C., Horta, A.E., Bessa, R.J., Pereira, R.M., 2013. 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. In vitro Cell Dev. Biol. Anim. 49, 335–345.
Romek, M., Gajda, B., Krzysztofowicz, E., Kepczynski, M., Smorag, Z., 2011. New technique to quantify the lipid composition of lipid droplets in porcine oocytes and pre-implantation embryos using Nile Red fluorescent probe. Theriogenology 75, 42–54.
Rossi, R., Pastorelli, G., Cannata, S., Corino, C., 2010. Recent advances in the use of fatty acids as supplements in pig diets: a review. Anim. Feed Sci. Technol. 162, 1–11.

Smits, R.J., Luxford, B.G., Mitchell, M., Nottle, M.B., 2011. Sow litter size is increased in the subsequent parity when lactating sows are fed diets containing n-3 fatty acids from fish oil. J. Anim. Sci. 89, 2731–2738.
Somfai, T., Yoshioka, K., Tanihara, F., Kaneko, H., Noguchi, J., Kashiwazaki, N., Nagai, T., Kikuchi, K., 2014. Generation of live piglets from cryopreserved oocytes for the first time using a defined system for in vitro embryo production. PLoS One 9, e97731.
Statistix®, 2013. Statistix® 10 Analytical Software. Tallahassee, FL, USA.
Sturmey, R.G., Leese, H.J., 2003. Energy metabolism in pig oocytes and early embryos. Reproduction 126, 197–204.
Sturmey, R.G., O'Toole, P.J., Leese, H.J., 2006. Fluorescence resonance energy transfer analysis of mitochondrial:lipid association in the porcine oocyte. Reproduction 132, 829–837.
Sturmey, R.G., Reis, A., Leese, H.J., McEvoy, T.G., 2009. Role of fatty acids in energy provision during oocyte maturation and early embryo development. Reprod. Dom. Anim. 44 (Suppl 3), 50–58.
Uhm, S.J., Gupta, M.K., Kim, T., Lee, H.T., 2007. Expression of enhanced green fluorescent protein in porcine- and bovine-cloned embryos following interspecies somatic cell nuclear transfer of fibroblasts transfected by retrovirus vector. Mol. Reprod. Dev. 74, 1538–1547.
Van Hoeck, V., Sturmey, R.G., Bermejo-Alvarez, P., Rizos, D., Gutierrez-Adan, A., Leese, H.J., Bols, P.E., Leroy, J.L., 2011. Elevated non-esterified fatty acid concentrations during bovine oocyte maturation compromise early embryo physiology. PLoS One, 6e23183.
Wakefield, S.L., Lane, M., Schulz, S.J., Hebart, M.L., Thompson, J.G., Mitchell, M., 2008. Maternal supply of omega-3 polyunsaturated fatty acids alter mechanisms involved in oocyte and early embryo development in the mouse. Am. J. Physiol. Endocrinol. Metab. 294, E425–434.
Wathes, D.C., Abayasekara, D.R., Aitken, R.J., 2007. Polyunsaturated fatty acids in male and female reproduction. Biol. Reprod. 77, 190–201.
Yoshioka, K., Suzuki, C., Tanaka, A., Anas, I.M.-K., Iwamura, S., 2002. Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. Biol. Reprod. 66, 112–119.
Zhou, G.B., Li, N., 2009. Cryopreservation of porcine oocytes: recent advances. Mol. Hum. Reprod. 5, 279–285.