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Key words

Oocyte competence, IVM, n-3 PUFA, cumulus, gene expression

ABSTRACT

The positive effect of n-3 poly-unsaturated fatty acids (FA) on fertility in ruminants seems to be partly mediated through direct effects on the oocyte developmental potential. We aimed to investigate whether supplementation with physiological levels of docosahexaenoic acid (DHA, C22:6 n-3 PUFA) during *in vitro* maturation (IVM) has an effect on oocyte maturation and *in vitro* embryo development in cattle. We showed that DHA (0, 1, 10 or 100 μ M) had no effect on oocyte viability or maturation rate after 22 h IVM. Incubation of oocyte-cumulus complexes (OCC) with 1 μ M DHA during IVM significantly increased ($p < 0.05$) oocyte cleavage rate as compared to control (86.1% vs. 78.8%, respectively) and the >4-cell embryo rate at day 2 after parthenogenetic activation (PA) (39.1% vs. 29.7%, respectively). Supplementation with 1 μ M DHA during IVM also induced a significant increase in the blastocyst rate at day 7 after *in vitro* fertilization (IVF) as compared to control (30.6% vs. 17.6%, respectively) and tended to increase the number of cells in the blastocysts (97.1 ± 4.9 vs. 81.2 ± 5.3 , respectively; $p = 0.08$). On the contrary, 10 μ M DHA had no effects, whereas 100 μ M DHA significantly decreased the cleavage rate compared to control (69.5% vs. 78.8%, respectively) and the >4-cell embryo rate at day 2 after PA (19.5% vs. 29.7%). As was shown by real-time PCR, negative effects of 100 μ M DHA were associated with significant increase of progesterone synthesis by OCCs, a three-fold increase in expression level of FA transporter *CD36* and a two-fold decrease of FA synthase *FASN* genes in cumulus cells (CC) of corresponding oocytes. DHA at 1 and 10 μ M had no effect on expression of those and other key lipid metabolism-related genes in CC. In conclusion, administration of a low physiological dose of DHA (1 μ M) during IVM may have beneficial effects on oocyte developmental competence *in vitro* without affecting lipid metabolism gene expression in surrounding cumulus cells, contrarily to 100 μ M DHA which diminished oocyte quality associated with perturbation of lipid and steroid metabolism in CC.

1. INTRODUCTION

Oocyte competence to develop into viable embryo after fertilization is mainly acquired during final oocyte growth and meiotic maturation. Fatty acids (FA) together with glucose and amino acids are an important source of energy for oocytes, as high amount of energy is required to perform meiosis [1]. Free FA, including non-esterified FA (NEFA), are present in both follicular fluid and in the oocyte-cumulus complex (OCC) and participate in formation of OCC follicular environment directly related to oocyte quality [2, 3]. In fact, an oocyte, surrounded by cumulus cells (CC), is able to rapidly incorporate FA from maturation medium *in vitro*, as shown for oleic and linoleic acids [4, 5]. The oocyte lipid content, in terms of FA composition, has been shown to influence oocyte maturation and developmental competence [6, 7]. Moreover, the highest concentrations of NEFA in the OCC environment, *in vivo* and *in vitro* led to low oocyte quality and embryo developmental rate after *in vitro* fertilization (IVF) [8-10]. FA are essential to oocyte quality as energy source [11, 12], but in the case of elevated FA concentration in the environment (follicular fluid or maturation medium), CC are required to protect the oocyte by incorporating excessive FA and transforming them into storage droplets [13, 14]. Moreover, during oocyte maturation CC ensure active lipid metabolism through modulation of expression of FA metabolism related genes [15].

N-3 polyunsaturated fatty acids (PUFA), namely, alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), belong to a family of biologically active FA and are known to have a variety of health benefits [16]. Mammals cannot produce the precursor of n-3 PUFA, ALA, which is thus considered essential FA. The longest member of this family, DHA, has a 22 carbon chain (C22:6) and can be produced by a series of desaturation and elongation reactions from shorter n-3 PUFA member such as ALA

74 or EPA. Nevertheless, the most extensive source of DHA is the diet and particularly seafood.
 75 DHA was also described as the most biologically active member of this family [17-19].

76 The n-3 PUFA exhibit a wide range of physiological roles in organism development and
 77 diseases [20], via receptors, sensors or cell membrane fatty acid composition (membrane
 78 order, lipid rafts, etc.) (reviewed in [21]). Among their physiological roles, n-3 PUFA have
 79 been reported to affect reproductive parameters in cattle [22, 23]. N-3 PUFA induce a
 80 reduction in prostaglandin F2 alpha (PGF2 α) secretion [24, 25], thus potentially providing a
 81 more favorable uterine environment [26]. Moreover, n-3 PUFA dietary supply in cows was
 82 reported to not only affect uterine environment but also to exert effects on the ovary [27].
 83 Indeed, at the ovarian level, dietary n-3 PUFA leads to larger corpus luteum size and higher
 84 plasma progesterone levels [28, 29] and to increased number or size of follicles [30-32]. In
 85 addition, an n-3 PUFA-enriched diet has also led to an increased cleavage rate or a trend to
 86 increased blastocyst rate after *in vivo* maturation [31, 32]. Overall, the effects of such a diet on
 87 the conception rate are less clear [26]. However, an adequate n-3 PUFA dietary supply in
 88 dairy cows leads to a higher presumptive conception rate [30], or a tendency to an improved
 89 conception rate [33] and to a reduction in pregnancy loss [23, 28, 30]. However, in both dairy
 90 heifers and lactating cows, dietary n-3 PUFA had affected neither follicle numbers nor oocyte
 91 developmental potential and embryo quality [34, 35]. Moreover, potentially unfavorable
 92 effects of dietary n-3 PUFA on plasma progesterone level in dairy cows were reported [36].
 93 These discrepancies in PUFA effects are likely due to differences in n-3 PUFA forms (rumen
 94 protected or unprotected) and/or doses of dietary supplementations suggesting a variety of
 95 effects depending on the concentrations used. Our recent study on n-3 PUFA dietary
 96 supplementation, given during postpartum period as a rumen-protected encapsulated fish oil,
 97 revealed a decrease in the non-fertilization or early embryo mortality rate in high milk
 98 production dairy cows as compared to n-6 PUFA supplementation; that suggested an effect of

99 n-3 PUFA on oocyte quality *in vivo* [37]. Significant enrichment in DHA was confirmed in
100 plasma of n-3 PUFA treated animals, thus it would be interesting to analyze the potential
101 beneficial effect of DHA at follicular level. Moreover, we showed *in vitro* that functionality
102 of bovine follicular cells (cumulus and granulosa cells) is dependent on lipid metabolism [38,
103 39].

104 The first objective of the present study was to analyze whether supplementation with an n-3
105 PUFA, DHA, during *in vitro* maturation (IVM) of bovine oocytes may impact oocyte quality
106 in terms of its competence to embryo development *in vitro*. The discrepancies of PUFA
107 impacts from literature prompted us to investigate the effect of DHA concentrations, ranging
108 from 1 to 100 μM that either covers or overpasses physiological concentrations in follicular
109 fluid and serum (1.5-15 μM) [10]. We therefore performed DHA treatment only during IVM
110 and then analyzed oocyte and CC viability, oocyte meiotic maturation and developmental
111 competence through either parthenogenetic activation or *in vitro* fertilization (IVF). The
112 second objective was to analyze whether lipid metabolism in CC was affected by DHA
113 treatment and could explain its effect. We thus analyzed progesterone production and
114 expression of lipid metabolism related genes in CC.

2. MATERIALS AND METHODS

2.1. Ethics

No experiments with living animals were performed.

2.2. Chemicals

All chemicals were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France) unless otherwise stated.

2.3. Biological material

Bovine immature OCC were retrieved from bovine ovaries collected at a commercial abattoir. Antral follicles from 3 to 6 mm in diameter were punctured using an 18G needle linked to a vacuum pump and to a 50 mL falcon tube. OCC with compact cumulus layers were selected and washed in TCM 199 HEPES medium complemented with 0.04% BSA and 25 µg/mL gentamycin.

2.4. In vitro maturation (IVM)

IVM was performed with groups of 50-60 OCC in 500 µL of either serum-free TCM 199 complemented with EGF (10 ng/mL) and gentamycin (10 µg/mL) or TCM199 supplemented with 10% (v/v) FA-free fetal calf serum (FCS), EGF (5 ng/mL), 17 β-estradiol (1 µg/mL), FSH (10 µg/mL) and LH (12 µg/mL) (both Reprobiol, Liège, Belgium), and gentamycin (5 µg/mL) in the presence or absence of DHA (1 µM, 10 µM or 100 µM) at 38.8 °C in a humidified atmosphere containing 5% CO₂.

After 22 hours of IVM, CC were removed and both cells and spent IVM media were kept at -80°C until analysis. Oocytes were used either for analysis of meiotic maturation or for parthenogenetic activation (PA).

2.5. Analysis of cell viability.

CC viability was assessed using the Bioluminescence Cytotoxicity Assay Kit (Medical and Biological Laboratories, Woburn, USA) to measure adenylate kinase activity in spent culture media according to the supplier's instructions. Adenylate kinase activity is correlated to the dead cell rate; its level was here normalized by the number of OCC in each well. Oocyte viability was assessed using the Live/Dead Viability/Cytotoxicity Kit for mammalian cells (Life Technologies, Cergy Pontoise, France) according to the supplier's instructions. Experiments were repeated five times with 50-60 OCC per conditions.

2.6. Analysis of oocyte nuclear meiotic maturation stages.

After 24 h IVM, oocytes were fixed in paraformaldehyde (4% PFA in PBS 1x) and stained with Hoechst 33342 (10 µg/mL), allowing for the labeling of oocyte chromatin and thus the determination of its meiotic status, as detailed elsewhere [40]. Briefly, prophase oocytes at germinal vesicle (GV), GV breakdown, metaphase-I and anaphase-I stages were considered as immature. The oocytes which had progressed to telophase-I or metaphase-II phases were considered as mature oocytes. Experiments were repeated three times with at least 50 oocytes per each IVM conditions per experiment.

2.7. Parthenogenetic activation (PA)

After 22 hours of IVM, CC were removed by pipette aspiration-ejections in the IVM media, centrifuged 5 min at 3500g, and both CC and spent media were kept at -80°C until analysis. Before PA, denuded oocytes remained in the same IVM conditions for an additional 4 hours. Oocytes were then incubated 5 min in TCM199, ionomycin 5 µM, FA-free FCS 5%, following by 4 hours in TCM199, 6-dimethylaminopurine (6-DMAP) 2 mM, FA-free FCS 5%. *In vitro* development was performed in groups of 25 oocytes in a 25 µL microdrop of

mSOF (modified synthetic oviduct fluid supplemented with 1% estrus cow serum) under mineral oil at 38.8°C in a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂ for seven days. The cleavage rate was assessed at day 2 post activation and embryo development was checked at day 7. Experiments were repeated five times with 50-60 OCC per each IVM conditions per experiment.

2.8. *In vitro* fertilization (IVF)

The mature OCC were transferred for washing in fertilization medium Fert-Talp [41]. Two straws per replicate of semen from the same ejaculate and/or bull were used throughout experiments. Motile sperm were separated by centrifugation (15 minutes at 700g) on 2 mL of Percoll (Pharmacia, Uppsala, Sweden) discontinuous density gradient (45%/90%). Viable sperm were diluted to 1.0 10⁶ sperm/mL in Fert-Talp medium. Sperm and OCC were then coincubated for 18 hours at 38.8 C in a humidified atmosphere of 5% CO₂ in air. Then CC were removed, presumptive zygotes were washed and *in vitro* embryo development was performed in mSOF as described earlier (see section 2.7). The cleavage rate was assessed at day 2 after fertilization and embryo development was checked at day 7 post-IVF. Experiments were repeated three times with 50-60 OCC per each IVM conditions per experiment.

2.9. *Analysis of in vitro* embryo development

At day 2 of *in vitro* development (IVD), embryos were observed using a Zeiss inverted microscope (Zeiss, Germany) in order to count cell number in the cleaved embryos, and then *in vitro* culture was continued. This study being in the context of *in vitro* embryo production optimization, embryos were analyzed 7 days after the beginning of IVF (168 hours). All embryos were fixed in 4% paraformaldehyde and stained with Hoechst 33342 (1 µg/mL). The number of cells in each embryo was counted using an Axioplan Zeiss fluorescent microscope.

Degraded embryos without visible chromatin staining were not taken into account. Blastocyst rate was defined as the total number of blastocysts reported to the number of cleaved embryos.

2.10. Progesterone assay

The progesterone concentration was determined in spent media after 22 hours of IVM in the presence or absence of DHA (1, 10 or 100 μ M) by using an enzyme-linked immunosorbent assay (ELISA) protocol as previously described [42]. For progesterone concentrations ranging from 0.4 to 10 ng/mL, the intra-assay CVs averaged < 10%. Progesterone secreted in each well was normalized to the number of maturing OCC in the same well. The results are presented as the amount of progesterone (ng) secreted per OCC, as means \pm SEM of five independent experiments.

2.11. Gene expression analysis in CC

Total RNA was extracted from CC using TriZol reagent (Invitrogen, Cergy Pontoise, France) following the manufacturer's instructions. RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (Nyxor Biotech, Paris, France). RNA preparations were DNase digested by RQ1 DNase (Promega, Charbonnières, France) following the manufacturer's instructions. Reverse transcription (RT) was performed on 200 ng of total RNA extracted from CC using Moloney Murine Leukemia Virus reverse transcriptase I (Invitrogen, Cergy Pontoise, France) with an oligo dT primer mix (Promega, Charbonnières, France) according to the manufacturer's instructions. Real-time PCR reactions were carried out on a MyiQ Cyclor apparatus (Bio-Rad, Marnes la Coquette, France) in 20 μ l containing primers at a final concentration of 150 nM of each, 5 μ l of the diluted RT reaction (1ng cDNA per reaction) and qPCR Mastermix Plus for Sybr Green I (Bio-Rad, Marnes-la-Coquette,

France) according to the manufacturer's instructions. The efficiency of the primers and standard curve for each gene was deduced from serial dilutions of the correspondent cDNA fragment obtained as a template (Table 1). The relative gene expression levels were calculated in five independent CC samples for each condition. The geometric mean of three housekeeping genes (*RPL19*, *RPS9* and *GAPDH*) was used to normalize gene expression. The relative amounts of gene transcripts (R) were calculated according to the equation:

$$R = \frac{(E_{gene}^{-Ct})}{(\text{geometric mean } (E_{RPS9}^{-Ct}; E_{RPL19}^{-Ct}; E_{GAPDH}^{-Ct}))}$$
, where E is the primer efficiency and Ct the cycle threshold. Five independent CC samples per condition were analyzed.

2.12. Statistical analysis

Statistical analyses taking into account both treatment effect and replica effect were performed for all parameters. The distribution of maturation stages, cleavage rates and blastocyst rates were compared by logistic regression analysis using generalized linear model ((R package Rcmdr, [43]), R version 3.2.1, [44]). Odds ratio and confidence intervals of the logistic regression analyses of oocyte maturation and development rates are provided in supplementary Table 1). Least square means (lsmeans) estimated by the models (R package lsmeans, [45]) were subsequently compared to the control conditions. Results are presented in tables and figures as lsmeans \pm SEM, unless otherwise stated.

Number of blastocyst cells, CC viability, progesterone concentration and mRNA expression level were compared between the groups using either non-parametric one-way ANOVA, when four conditions were compared (R package lmp, [46]) with the Tukey post-hoc test (R package nparcomp, [47]), or Wilcoxon bivariate tests, when 2 conditions were compared ((R package coin, [48]), R version 3.2.1, [44]). A difference with $p \leq 0.05$ was considered significant and with $0.05 < p \leq 0.10$ was considered a tendency. All p-values are given in Supplementary tables 1, 2, and 3.

3. RESULTS

3.1. *In vitro* development of oocytes after IVM with or without DHA

In our experiments, OCC were matured *in vitro* with or without DHA (1, 10, or 100 μ M) and then *in vitro* embryo development was compared between conditions. We chose to assess oocyte developmental competence by using parthenogenetic activation (PA) first, because, in this case, embryo development only relies on oocyte maternal factors. DHA-treated and control oocytes underwent PA, and their cleavage rates were measured 40 hours later. As shown in Figure 1, 1 μ M DHA induced a significant ($p < 0.05$) increase in the oocyte cleavage rate as compared to control ($86.1 \pm 2.1\%$ vs. $78.8 \pm 2.5\%$, respectively). On the contrary, 100 μ M DHA induced a significant decrease in cleaved oocytes compared to control ($69.5 \pm 3.2\%$ vs. $78.8 \pm 2.5\%$, respectively). No difference was seen in the cleavage rate of the oocytes matured with DHA 10 μ M. Cleaved embryos were differentiated as following: i) embryos exhibiting 2-4 cells or ii) embryos exhibiting more than 4 cells. No difference was reported in the 2-4 cell embryo rate in the absence ($44.5 \pm 3.0\%$) or presence of 1 μ M DHA ($43.6 \pm 3.1\%$), 10 μ M ($39.6 \pm 3.0\%$) or 100 μ M ($44.6 \pm 3.4\%$). Concerning the embryos with more than 4 cells (>4-cell embryo), 1 μ M DHA induced a significant increase in the >4-cell embryo rate compared to the control group ($39.1 \pm 3.2\%$ vs. $29.7 \pm 2.9\%$, respectively). The same difference was observed in the 10 μ M DHA group, presenting a higher rate of > 4-cell embryos as compared to control ($38.7 \pm 3.2\%$ vs. $29.7 \pm 2.9\%$, respectively). On the contrary, 100 μ M DHA induced a significant decrease in the >4 cell embryo rate compared to control ($19.5 \pm 2.7\%$ vs. $29.7 \pm 2.9\%$, respectively). At day 7 of *in vitro* development, a rate of more than 16-cell embryos which had to undergo major activation of proper genome [49], was not different between 1 μ M DHA or 10 μ M DHA compared to control (34.1%, 37.3% and 40.0%,

respectively), but was significantly decreased at 100 μ M DHA (13.8%) (Supplementary Table 1). Blastocyst rate was not different between control ($11.0 \pm 3.0\%$), 1 μ M DHA ($15.7 \pm 3.3\%$) and 10 μ M DHA (10.7 ± 2.8) groups. On the contrary, 100 μ M DHA tended to decrease blastocyst rate ($2.9 \pm 2.2\%$) compared to control ($p < 0.07$). In the embryos that have reached blastocyst stage, no differences in cell number per blastocyst were found between control and any DHA group: (58.1 ± 5.1 , 67.2 ± 5.6 , 60.5 ± 3.4 , and 67.5 ± 5.5 cells per blastocyst in control, DHA1, DHA10, DHA100, respectively).

IVF was then performed to confirm our observations with PA on potentially better development of the oocytes matured in the presence of 1 μ M DHA (Figure 2A and supplementary Table 1). At day 2 after IVF, control and 1 μ M DHA groups showed no difference in the cleavage rate (83.4 ± 2.9 vs. 85.0 ± 2.8 , respectively), the 2-4 cell embryo rate (49.4 ± 3.9 vs. 49.8 ± 3.9 , respectively) and the rate of embryos with more than 4 cells (33.2 ± 3.6 vs. 34.0 ± 3.7 , respectively). However, 1 μ M DHA led to a significant increase in the blastocyst rate at day 7 compared to control (30.6 ± 4.1 vs. 17.6 ± 3.3 , respectively). As shown in Figure 2B, the addition of 1 μ M DHA during IVM also tended to increase ($p < 0.08$) the number of cells per blastocyst, as compared to control group (97.1 ± 4.9 vs. 81.2 ± 5.3 , respectively).

Therefore, supplement with 1 μ M DHA during IVM increased developmental capacity of treated oocytes as compared with control, 10 μ M had no effect, whereas 100 μ M DHA reduced embryo development rate.

3.2. Effect of DHA on oocyte meiotic maturation

Taking into account the effect of DHA on the cleavage rate, we aimed to determine whether oocyte nuclear maturation rate was affected by DHA. We thus performed 22 h IVM in the

absence or presence of DHA (1, 10 or 100 μ M) added to the basic culture medium. No difference in the percentage of mature oocytes was found in the absence ($83.5 \pm 3.2\%$) or presence of DHA (1 μ M ($79.2 \pm 3.3\%$), 10 μ M ($84.3 \pm 3.3\%$) or 100 μ M ($84.7 \pm 3.6\%$)). When comparing nuclear meiotic stages, no difference was reported in the ratio of immature, metaphase-I, anaphase-I and metaphase-II oocytes in the absence or presence of DHA (1, 10 or 100 μ M). When considering only telophase-I oocytes, a significant increase ($p < 0.05$) was observed with 100 μ M DHA ($14.0 \pm 3.6\%$) compared to control ($5.0 \pm 1.9\%$) (Supplementary Table 2). Thus, addition of DHA during IVM (1, 10 or 100 μ M) had no effect on the percentage of mature oocytes.

3.3. Oocyte and cumulus cell viability in the presence or absence of DHA during IVM

Oocyte viability showed no difference in terms of number of living oocytes after 22h IVM in the absence or presence of 1, 10, 100 μ M DHA as compared with control (98.8%, 97.0%, 96.3 and 98.7, respectively). In order to investigate whether DHA affected cumulus functioning, the viability of CC was assessed by measuring adenylate kinase activity in spent culture media from corresponding OCC after IVM. No significant difference in adenylate kinase activity level was observed in the absence or presence of DHA (874 ± 44 , 868 ± 47 , 948 ± 40 and 992 ± 18 relative light units for control, 1, 10 and 100 μ M DHA, respectively). Thus, supplement with DHA during IVM affected neither oocytes nor surrounding CC viability.

Gene expression in CC after 22 hours of IVM in the absence or presence of DHA

310 In order to assess whether addition of DHA during IVM affects cellular lipid metabolism,
 311 relative expression of several candidate genes controlling the key steps of FA metabolism was
 312 measured in CC by real-time PCR and compared between the DHA-treated and control
 313 groups. The main processes of lipid metabolism and corresponding candidate genes are shown
 314 in Figure 3A. These include lipogenesis involving FA synthase (*FASN*) and diacylglycerol O-
 315 acyltransferase (*DGAT1*), FA transport (transporters *CD36*, FA binding protein genes *FABP3*
 316 and *FABP5*), lipolysis (phospholipase *PNPLA2*), lipid storage (perilipin *PLIN2*) and
 317 mitochondrial β -oxidation (*CPT1A*, *CPT2*). As shown in Figure 3B, 100 μ M DHA led to a
 318 significant two-fold decrease in FA synthase (*FASN*) expression and a tendency to increase
 319 FA transporter *CD36* expression in CC ($p=0.06$) however lower DHA concentrations did not
 320 affect expression. The genes *FABP3*, *DGAT1*, *PLIN2*, *CPT1A* and *CPT2* were similarly
 321 expressed in CC regardless of the DHA concentration during IVM (Supplementary table 3).
 322 We also analyzed the expression of *PTGS2* gene involved in prostaglandin synthesis, and two
 323 other genes known to be affected by FA in other tissues : *PPARG* (transcription factor) and
 324 *NFKB* (signaling pathway). None of these genes showed a change in expression in DHA
 325 treated CC (Supplementary table 3).

326 Thus, in CC, DHA at 1 μ M and 10 μ M had no effect on expression of candidate genes; *FASN*
 327 was only affected by the highest DHA concentration (100 μ M).

328

329 ***Progesterone secretion by OCC in the presence or absence of DHA during IVM***

330 Progesterone secretion was measured in spent culture media after 22 h IVM (Figure 4). A
 331 significant 2.2-fold increase in progesterone secretion was reported with only 100 μ M DHA
 332 compared to control ($p < 0.0001$). No differences were observed with lower DHA
 333 concentrations as compared to control IVM without DHA.

DISCUSSION

DHA directly affects bovine oocyte quality

In the present study, we analyzed the effect of addition of n-3 PUFA, DHA, during IVM culture, on oocyte developmental competence by following *in vitro* early embryo development in cattle. For the first time, we showed that the addition of a low physiological concentration of DHA (here, 1 μ M) during IVM might improve oocyte proper competence to support embryo development.

In cattle, the concentration of DHA has been reported to be 5-15 μ M in serum and 1.5-3 μ M in follicular fluid from preovulatory dominant follicles [10]. Therefore, in the present study, DHA 1 and 10 μ M were physiological levels, contrary to DHA 100 μ M. While in our study DHA was provided only during IVM, it should directly influence oocyte developmental potential acquired during maturation. Under these conditions, we observed a positive effect of a lower DHA concentration (1 μ M), no effect of DHA at 10 μ M and deleterious effect of 100 μ M dose. Our results are in line with other *in vitro* data obtained in ruminants. Indeed, supplementation with 50 μ M ALA (a shorter member of the n-3 PUFA family) during IVM also led to increases in the cleavage rate, blastocyst rate and number of cells per embryo in bovine [50]. Moreover, 50 μ M ALA treatment during IVM also led to an increased number of cells in sheep embryos after *in vitro* development [51]. Interestingly, when DHA (1,10 or 100 μ M) was supplied during *in vitro* embryo development culture but not during IVM in cattle, no effect on cleavage rate was observed; however such treatment led to reduced blastocyst yield at day 7 when added at 10 μ M and reduced embryo survival at 100 μ M [52].

Our results on the effects of DHA are also relevant to *in vivo* experiments in ruminants. Indeed, an increased cell number was observed in blastocysts obtained in lactating dairy cows *in vivo* after increased n-3 PUFA dietary supply in cattle [53]. Moreover, after n-3 PUFA

358 dietary supplementation of dairy cows, the cleavage rate of recovered oocytes was increased
 359 after IVM/IVF in the n-3 PUFA group and the blastocyst rate also tended to be higher,
 360 highlighting the role of n-3 PUFA on oocyte competence [31]. In addition, fish oil dietary
 361 supplementation (rich in EPA and DHA) improved oocyte quality also in sheep [54] and in
 362 dairy cows [37]. Taken together, all these data confirmed that n-3 PUFA could improve
 363 oocyte quality by acting during oocyte maturation or even before, i.e. during follicular
 364 growth. Nevertheless, a beneficial effect of n-3 PUFA on oocyte quality has not always been
 365 observed *in vivo*. Indeed, n-3 PUFA dietary supply did not improve *in vivo* embryo
 366 production after superovulation in dairy cows [29] or *in vitro* embryo production in dairy
 367 heifers [34]. These discrepancies between ruminant studies are likely due to differences in the
 368 form, dose, period and duration of n-3 PUFA supplementation between the experiments [55].
 369 The PUFA may be differently oxidized, converted in other forms and largely degraded in the
 370 rumen [56] depending on the diet preparation. The absence of n-3 PUFA effects on oocyte
 371 competence in several studies could also be explained by the protective role of ovarian cells,
 372 which were able to absorb most of the lipids in order to protect the oocyte from the
 373 deleterious effects of PUFA excess [14, 57].
 374 According to our *in vitro* data, direct contact of bovine OCC with DHA during 22 hours of
 375 IVM can affect oocyte ability to develop *in vitro*, and the lowest concentration of 1 μ M may
 376 have a beneficial effect on *in vitro* embryo production issues.

377

378 **When used at a high dose, DHA exhibits deleterious effects on oocyte quality**

379 As expected, a high dose of DHA had detrimental effect on developmental potential of bovine
 380 oocytes, nevertheless DHA did not impact their viability at the end of IVM. Indeed, addition
 381 of 100 μ M DHA during IVM significantly decreased the cleavage rate, the >4 cell embryo

rate at day 2, and the rate of embryos with more than 16 cells at day 7. Such embryos had already overcome major embryo genome activation (MGA) occurring at 8-16 cell stage [49], and their lower rate indicates that DHA excessive level (100 μ M) during IVM may have a deleterious effect on oocyte capacity to activate the factors involved in MGA and thus may lead to lower blastocyst rate. These results are relevant to the literature where high doses of n-3 PUFA (100 and 200 μ M ALA) led to negative effects on oocyte maturation and the CC expansion rate in cattle [50] as well as in sheep [51]. Similarly, other PUFA exhibited the same lipotoxic effect at high doses; i.e. 100 μ M linoleic acid decreased the percentage of metaphase-II oocytes compared to lower doses (9 and 43 μ M) and resulted in oocytes arrested at the immature state [5]. Therefore, when used at a high dose, the lipotoxic effects of n-3 PUFA resembled the effects of other FA. Indeed, palmitic acid (150 μ M) or stearic acid (75 μ M) added to bovine IVM media had negative effects on maturation, fertilization, cleavage rate and blastocyst yield [2, 58].

In the present study, 100 μ M DHA significantly increased progesterone secretion by OCC during IVM. Similarly, such an increase has been reported in sheep after treatment of OCC with 100 μ M ALA [51]. However, in sheep this increase in progesterone was not linked to an increase in oocyte or embryo quality. In our study, an increase in progesterone level secreted by OCC coincided with a decrease in blastocyst rate of corresponding oocytes. Indeed, in cattle, it has been shown that oocyte developmental competence was not related to progesterone levels in the follicular fluid [59]. Moreover, it was reported in cattle that although supplement with progesterone during IVM had no effect on the cleavage rate after IVF, the blastocyst rate rather decreased [60]. Therefore, the detrimental effect of 100 μ M DHA on oocyte developmental competence observed here could be partly due to modulations in CC steroid metabolism induced by this treatment.

DHA modulates oocyte quality: what are the mechanisms?

The present study demonstrated that the impact of DHA on oocyte competence to develop was not due to the modulation of meiotic maturation. Indeed, here no differences in maturation rate were reported after any DHA treatment as compared to control, except a slower transition from telophase to metaphase-II in DHA 100 μ M treated oocytes. The discrepancies of n-3 PUFA effect on oocyte maturation occurred also in the literature. Indeed, supplementation with another n-3 PUFA, ALA, at 50 μ M during IVM increased the rate of metaphase-II oocytes in cattle [50]. In contrast, ALA used during IVM at 50 and 100 μ M did not affect nuclear maturation in sheep, and at 200 μ M reduced the maturation rate [51]. In addition, in cattle, when used *in vivo*, ALA also had no effect on oocyte nuclear maturation [35]. Other n-6 PUFA, linoleic acid, have also been reported to exhibit either no effect [5] or an inhibitory effect [61] on nuclear maturation in cows. These results are in line with ours, as we did not find any effect on nuclear maturation in DHA 1 μ M and 10 μ M groups.

Taken together, all these studies and our results inferred that not nuclear maturation but cytoplasm maturation might be affected in oocyte as a result of DHA supply during IVM. Cytoplasm maturation includes accumulation of maternal factors, such as transcripts, proteins and energy storage which are used to support the first cleavages. Cumulus cells play an important role in cytoplasm maturation of oocytes by regulating energy metabolism inside the OCC during maturation [62], and the role of lipid metabolism in CC for oocyte maturation revealed to be important [11, 15, 63]. Addition of DHA in IVM medium was thought to modulate lipid metabolism in OCC and thus be linked to modulation of oocyte quality. Indeed, we investigated the expression of candidate genes from several key lipid metabolism functions: lipogenesis (*FASN*), lipid storage (*DGAT1*), lipolysis (*PLIN2*, *PNPLA2*), FA incorporation (*CD36*) and transport (*FABP3*, *FABP5*), and FA β -oxidation (*CPT1A*, *CPT2*) [64]. While expression was not changed in 1 μ M DHA-treated and control CC, none of these

genes seemed to be involved in the beneficial effect of low DHA concentration on oocyte quality, and therefore lipid metabolism functions might not be critical to promote DHA effects. In contrast, the lipotoxic effect of DHA 100 μ M was relevant to the regulation of gene expression in CC, as reported in the present study, and to the protective role of CC for oocytes against excess of FA in environment [14]. Indeed we observed an increased mRNA expression of *CD36* in CC treated with 100 μ M DHA. *CD36* is responsible of FA incorporation from the extracellular environment [65], and CC protect oocytes from increased free FA levels by massive intracellular lipid storage, this may safeguard the oocyte from lipotoxic effects [14]. Therefore, an increase in the extracellular free FA concentration could up-regulate *CD36* expression in order to increase FA intracellular incorporation in CC, thus protecting the oocyte. Consequently, an increase in the amount of intracellular free FA would down-regulate *FASN* expression, as was here observed in CC, and consequently this may decrease lipogenesis similarly to what was reported in mammary gland [66].

DHA may act in several other ways, as it has been reported, to be able to bind transcription factors such as peroxisome proliferator-activated receptor γ and α (*PPARG* and *PPARA*) and consequently improve insulin sensitivity in adipocytes [67]. *NFkB* was also reported to be increased in adipocytes *in vivo* after DHA supplementation [68] and to be able to interact with *PPARG* and to lead to a decrease in *PTGS2* (alias *COX2*) expression [67]. In our study, expression of *PPARG*, *NFkB* and *PTGS2* was detected in CC, however it did not differ between DHA-treated and control CC, indicating that DHA at the studied doses might not impact mRNA level of these genes. Nevertheless, these factors may act on protein level, being post-translationally modified. Although prostaglandin synthesis in CC does not seem to be affected by DHA in our conditions, n-3 PUFA could decrease eicosanoid production (prostaglandins, thromboxanes, leukotrienes) and can therefore affect cells by regulating these mediators [21].

N-3 PUFA can also bind to surface or intracellular FA receptors or sensors. DHA has been already reported to be able to bind G protein coupled surface receptors such as free fatty acid receptors, thus activating their signaling pathways [21]. In addition, DHA can also affect cells via changes in the composition of cell membrane phospholipids. Indeed, FA in cell membrane can affect protein function by modifying membrane fluidity and lipid raft formation [69]. Lipid rafts appear to act as signaling platforms, as many proteins involved in signal transduction are mostly found in lipid rafts [70], and long-chain n-3 PUFA seem to influence raft formation and function [71]. These mechanisms are not yet investigated in CC and in oocyte and might be considered for further analysis.

Conclusion

In the present study, we have investigated the effect of an n-3 PUFA, DHA, on bovine oocyte quality *in vitro*, by supplementing IVM medium with DHA and assessing both meiotic maturation and embryo development after parthenogenetic activation or IVF. Without effect on meiotic maturation, DHA at 1 μ M could increase embryo development rate *in vitro* and cell number in blastocysts. Such improvement of oocyte developmental quality might be related to oocyte cytoplasm maturation, even if CC gene expression study did not manage to highlight involvement of lipid metabolism in DHA mechanism of action. In contrast, negative effect of 100 μ M DHA on oocyte quality seemed to be related to fatty acid and steroid metabolism perturbations in CC. Further studies are needed to elucidate the mechanisms and pathways in CC involved in the beneficial effects of DHA on oocyte competence.

DECLARATION of INTEREST

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure legends

Figure 1: Effect of DHA during IVM on cleavage rate at day 2 and blastocyst rate at day 7 after parthenogenetic activation

Embryo cleavage and blastocyst rates in control and DHA-treated groups (1, 10 and 100 μ M) were assessed 2 days and 7 days after PA, respectively. Among the cleaved oocytes, embryos >4 cell stage were distinguished. Percentage of blastocysts was reported to the number of cleaved embryos at day 2. Results of five independent experiments are expressed as the *lsmeans* percentage of embryos in each developmental stage \pm SEM. * indicates a significant difference for a given stage compared to the control ($p < 0.05$). # indicates a tendency of a difference compared to the control ($0.05 < p < 0.1$). Total numbers of analyzed parthenogenetically activated oocytes per group are shown between brackets.

Figure 2: Effect of 1 μ M DHA during IVM on embryo development after IVF.

A. Embryo cleavage and blastocyst rates in control and DHA-treated groups (1 μ M) were assessed 2 days and 7 days after IVF, respectively. Among the cleaved oocytes, embryos >4 cell stage were distinguished. Percentage of blastocysts was reported to the number of cleaved embryos at day 2. Results of three independent experiments are expressed as the *lsmeans* percentage of embryos in each developmental stage \pm SEM or as the mean blastocyst cell number \pm SEM. * indicates a significant difference for a given stage compared to the control ($p < 0.05$). Total numbers of analyzed in vitro fertilized oocytes per group are shown between brackets. **B.** Number of cells (mean \pm SEM) in the blastocysts developed from the oocytes treated or not with DHA 1 μ M during IVM. # indicates a tendency of a difference ($0.05 < p < 0.1$) compared to control.

Figure 3: Analysis of lipid metabolism related genes in CC after DHA treatment during IVM.

A. Scheme of disposition of genes involved in lipid metabolism main processes (grey boxes) according to their known functions in different cells, which were analyzed in CC treated with 0, 1, 10 and 100 μ M DHA. Abbreviations: FA: fatty acids. Genes: *FASN* : fatty acid synthase, *FABP3* and *FABP5*: fatty acids binding proteins 3 and 5, *DGAT1* : Diacylglycerol O-acyltransferase 1, *PNPLA2*: Patatin-like phospholipase domain containing 2, *PLIN2*: perilipin 2, *CPT1*: carnitine palmitoyl transferase 1, *CPT2*: carnitine palmitoyl transferase 2, *CD36*: thrombospondin receptor, FA transporter. **B.** Relative expression of *CD36* and *FASN* genes in CC after 22h IVM measured by real time PCR. The geometric mean of three housekeeping genes (*RPL19*, *RPS9* and *GAPDH*) was used to normalize gene expression. Results of five independent cultures with each condition in duplicate are presented as means \pm SEM. * indicates significant difference for a given stage compared to the control ($p < 0.05$). # indicates a tendency of a difference ($0.05 < p < 0.1$) compared to the control. Other genes showed no difference between DHA-treated and control CC.

Figure 4: Effect of DHA on progesterone secretion level by oocyte-cumulus complexes after 22h IVM

Progesterone concentration value was normalized by the number of OCC in each well. The data are expressed as ng of progesterone secreted per OCC. Results of five independent experiments are presented as means \pm SEM. * indicates a significant difference for a given stage compared to the control ($p < 0.05$).

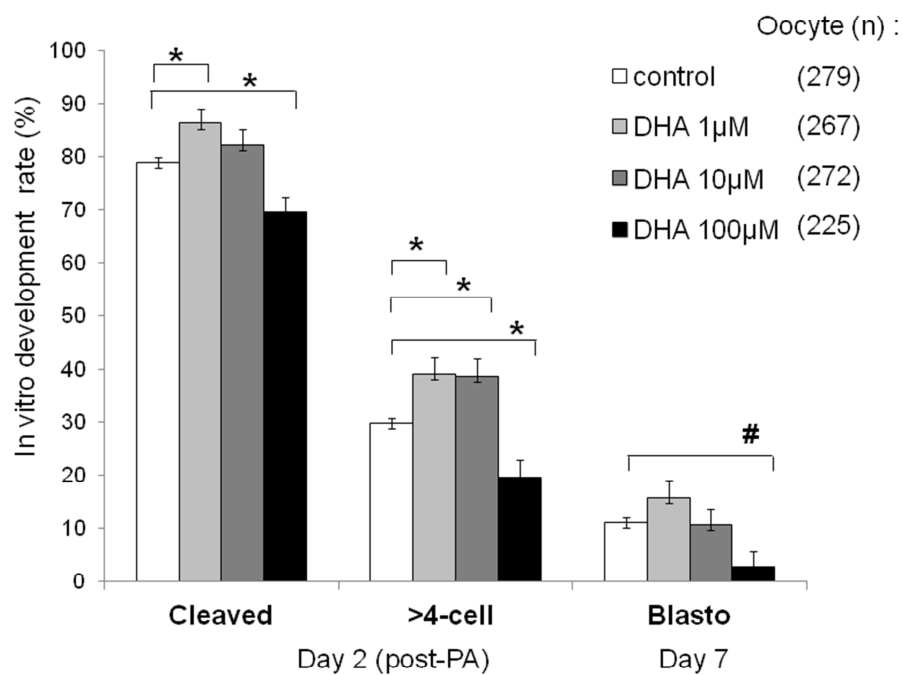
Tables :

Table 1: Oligonucleotide sequences

Abbrev.	Accession number	Gene	Forward primer	Reverse primer	bp	E
<i>CD36</i>	BC103112.1	thrombospondin receptor	GCATTCTGAAAGTGC GTTGA	CGGGTCTGATGAAAGTGGTT	181	1.95
<i>CPT1A</i>	FJ415874	carnitine palmitoyl transferase 1	TCCTGGTGGGCTACCAATTA	TGCGTCTGTAAAGCAGGATG	181	1.98
<i>CPT2</i>	NM_001045889	carnitine palmitoyl transferase 2	TGTGCCTTCCTTCCTGTCTTGG	CGATGGGGTCTGGGTAAACGA	111	1.89
<i>DGAT1</i>	NM_174693.2	Diacylglycerol O-acyltransferase 1	CGCCTTCTTCCACGAGTACC	CCGATGATGAGTGACAGCCA	159	1.96
<i>FABP3</i>	NM_174313	fatty acids binding protein 3	ATCGTGACGCTGGATGGCGG	GCCGAGTCCAGGAGTAGCCCA	210	1.88
<i>FABP5</i>	NM_174315	Fatty acid binding protein 5	TGGCGCATTTGGTTCAACATCAGG	TGAACTGAGCTTGTTTCATCCTCGC	193	1.90
<i>FASN</i>	AY343889	fatty acid synthase	CACTCCATCCTCGCTCTCC	GCCTGTCATCATCTGTCACC	181	1.95
<i>GAPDH</i>	NM_001034034	glyceraldehyde-3-phosphate dehydrogenase	TTCAACGGCACAGTCAAGG	ACATACTCAGCACCAGCATCAC	119	1.95
<i>NFKB</i>	NM_001076409	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	GCACCACTTATGACGGGACT	CCATGTCCAGAGGAGTGGTT	195	1.98
<i>PLIN2</i>	NM_173980	Perilipine 2	ACAACACACCCCTCAACTGG	CTGCCTGCCTACTTCAGACC	211	1.97
<i>PNPLA2</i>	NM_001046005	patatin-like phospholipase domain containing 2	ATGGTGCCCTACACTCTGCC	AGCTTCCTCTTGGCGCGTAT	152	2.02
<i>PPARG</i>	Y12419	peroxisome proliferator activated receptor gamma	CCCTGGCAAAGCATTGTAT	ACTGACACCCCTGGAAGATG	222	1.77
<i>PTGS2</i>	NM_174445	prostaglandin-endoperoxide synthase 2	AGGTGTATGTATGAGTGTAGGA	GTGCTGGGCAAAGAATGCAA	483	2.01
<i>RPL19</i>	BC102223	Ribosomal protein L19	AATCGCCAATGCCAACTC	CCCTTTCGCTTACCTATACC	156	1.85
<i>RPS9</i>	BC148016	Ribosomal protein S9	GGAGACCCTTCGAGAAGTCC	GGGCATTACCTTCGAACAGA	180	1.84

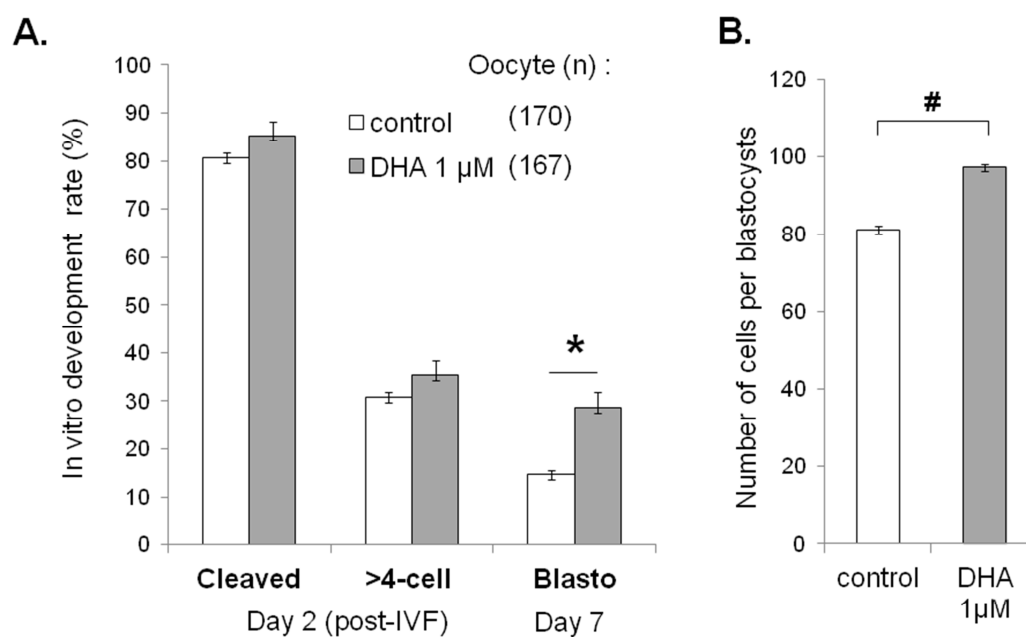
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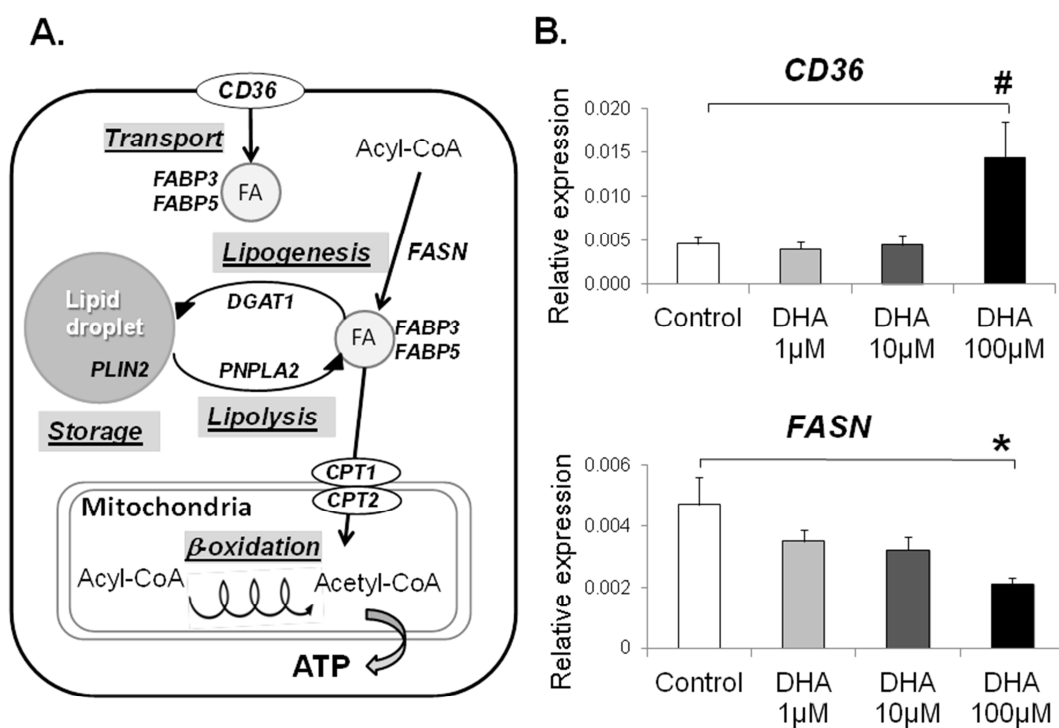


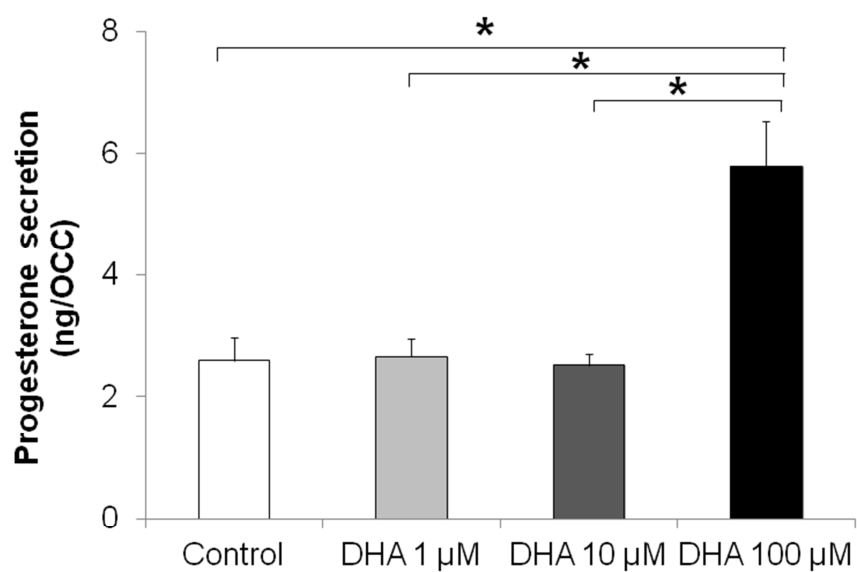
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Highlights

Animal reproductive biotechnologies are required to be more effective in terms of *in vitro* embryo production. The positive effect of n-3 poly-unsaturated fatty acids (FA) on fertility in ruminants seems to be partly mediated through direct effects on the oocyte developmental potential. We aimed to investigate whether supplementation with physiological levels of docosahexaenoic acid (DHA, C22:6 n-3) during *in vitro* maturation (IVM) has an effect on oocyte maturation and early embryo development in bovine. We showed that DHA had no effect on oocyte viability or maturation rate after 22 h IVM with or without 1, 10 or 100 μ M DHA. Incubation of oocyte-cumulus complexes with 1 μ M DHA during IVM significantly increased oocyte cleavage rate as compared to control and the > 4-cell embryo rate at day 2 after parthenogenetic activation. Supplementation with 1 μ M DHA during IVM also induced a significant increase in the blastocyst rate at day 7 after *in vitro* fertilization as compared to control and tended to increase the number of cells in the blastocysts. On the contrary, 100 μ M DHA significantly decreased the cleavage rate compared to control and the > 4-cell embryo rate at day 2 after PA. As was shown by real-time PCR, these effects were associated with a three-fold increase in expression level of FA transporter *CD36* and a two-fold decrease of FA synthase *FASN* genes in cumulus cells (CC) of corresponding oocytes, after IVM in the presence of 100 μ M DHA. Lower DHA concentrations had no effect on expression of those and other key lipid metabolism-related genes in CC. In conclusion, administration of a low physiological dose of DHA (1 μ M) during IVM may have beneficial effects on oocyte developmental competence *in vitro* with no effect on nuclear maturation and without affecting lipid metabolism gene expression in surrounding cumulus cells.

Supplementary Table 1: Effect of DHA during IVM on *in vitro* embryo development after parthenogenetic activation and *in vitro* fertilization

	Control		DHA 1μM		DHA 10μM			DHA 100μM		
	% (lsmeans ± SEM)	% (lsmeans ± SEM)	Logistic regression p-value	OR (CI 2.5% - 97.5%))	% (lsmeans ± SEM)	Logistic regression p-value	OR (CI 2.5% - 97.5%))	% (lsmeans ± SEM)	Logistic regression p-value	OR (CI 2.5% - 97.5%))
Parthenogenetic activation										
Number of oocytes	279	267			272			225		
Cleaved embryo %	78.8 ± 2.5	86.1 ± 2.1	0.024	0.60 (0.39 - 0.93)	82.0 ± 2.4	0.35	0.82 (0.54 - 1.24)	69.5 ± 3.2	0.017	1.63 (1.09 - 2.45)
2-4cell embryo %	44.5 ± 3.0	43.6 ± 3.1	0.83	1.04 (0.74 - 1.46)	39.6 ± 3.0	0.25	1.22 (0.87 - 1.73)	44.6 ± 3.4	0.98	1.00 (0.69 - 1.43)
>4-cell embryo %	29.7 ± 2.9	39.1 ± 3.2	0.030	0.66 (0.45 - 0.96)	38.7 ± 3.2	0.037	0.67 (0.46 - 0.98)	19.5 ± 2.7	0.012	1.74 (1.14 - 2.69)
>16 cell / cleaved embryo %	40.0 ± 4.9	34.1 ± 4.5	0.38	1.28 (0.74 - 2.24)	37.3 ± 4.5	0.69	1.12 (0.65 - 1.94)	13.8 ± 4.2	0.0004	4.15 (1.94 - 9.59)
Blastocyst / cleaved embryo %	11.0 ± 3.0	15.7 ± 3.3	0.30	0.66 (0.30 - 1.43)	10.7 ± 2.8	0.95	1.03 (0.44 - 2.37)	2.9 ± 2.2	0.068	4.14 (1.08 - 27.19)
In vitro fertilization										
n oocyte	170	167								
Cleaved embryo %	83.4 ± 2.9	85.0 ± 2.8	0.68	0.88 (0.49 - 1.59)						
2-4cell embryo %	49.4 ± 3.9	49.8 ± 3.9	0.88	0.97 (0.63 - 1.49)						
>4-cell embryo %	33.2 ± 3.6	34.0 ± 3.7	0.87	0.96 (0.61 - 1.52)						
Blastocyst / cleaved embryo %	17.6 ± 3.3	30.6 ± 4.1	0.013	0.48 (0.27 - 0.85)						

P-values different from the control at $p < 0.05$ are in bold, p-values different from the control with $p \geq 0.05$ and $p < 0.1$ are in italics. OR: odds ratio, CI: confidence interval

Supplementary Table 2: Effect of DHA during IVM on oocyte nuclear maturation

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	Control		DHA 1μM		DHA 10μM			DHA 100μM		
	% (lsmeans ± SEM)	% (lsmeans ± SEM)	Logistic regression		% (lsmeans ± SEM)	Logistic regression		% (lsmeans ± SEM)	Logistic regression	
			p-value	OR (CI 2.5% - 97.5%))		p-value	OR (CI 2.5% - 97.5%))		p-value	OR (CI 2.5% - 97.5%))
In vitro maturation										
Number of oocytes	136	152			119			100		
immature %	3.1 ± 1.5	2.8 ± 1.4	0.89	1.09 (0.30 - 4.02)	3.5 ± 1.7	0.85	0.88 (0.24 - 3.27)	2.6 ± 1.6	0.82	1.19 (0.28 - 5.96)
Meta-1 %	11.3 ± 2.7	12.8 ± 2.7	0.70	0.87 (0.42 - 1.76)	9.1 ± 2.7	0.56	1.28 (0.57 - 2.95)	8.3 ± 2.7	0.43	1.42 (0.61 - 3.50)
Ana-1 %	1.5 ± 1.1	4.6 ± 1.7	0.15	0.31 (0.05 - 1.30)	2.5 ± 1.5	0.55	0.58 (0.08 - 3.56)	4.0 ± 2.1	0.24	0.36 (0.05 - 1.87)
Telo-1 %	5.0 ± 1.9	1.8 ± 1.1	0.15	2.77 (0.75 - 13.09)	1.6 ± 1.2	0.15	3.24 (0.76 - 22.09)	14.0 ± 3.6	0.020	0.32 (0.12 - 0.81)
Meta-2 %	78.1 ± 3.5	77.0 ± 3.4	0.83	1.06 (0.61 - 1.86)	82.4 ± 3.5	0.39	0.76 (0.40 - 1.41)	70.4 ± 4.6	0.18	1.50 (0.83 - 2.71)
mature %	83.5 ± 3.2	79.2 ± 3.3	0.35	1.33 (0.74 - 2.44)	84.3 ± 3.3	0.87	0.95 (0.48 - 1.84)	84.7 ± 3.6	0.80	0.91 (0.45 - 1.83)

Oocyte meiotic stages were assessed after IVM with or without DHA (1 μM, 10 μM or 100 μM). Five meiotic stages were differentiated: prophase oocytes at germinal vesicle (GV), GV breakdown, metaphase-I (Meta-I), and anaphase-I (Ana-I) and telophase-I (Telo-I) and metaphase -II (Meta-II). Results of three independent experiments are expressed as the lsmeans percentage of oocytes in each meiotic stage ± SEM. P-values different from the control at p<0.05 are in bold, p-values different from the control with p ≥ 0.05 and p < 0.1 are in italics. OR: odds ratio, CI: confidence interval

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Supplementary Table 3: Effect of DHA on gene expression in cumulus cells

	Control	DHA 1 μ M	DHA 10 μ M	DHA 100 μ M
<i>FASN</i>	1.00 \pm 0.19	0.74 \pm 0.08	0.69 \pm 0.09	0.44 \pm 0.05
<i>DGAT1</i>	1.00 \pm 0.37	0.76 \pm 0.15	0.85 \pm 0.30	0.45 \pm 0.12
<i>PLIN2</i>	1.00 \pm 0.07	0.62 \pm 0.23	0.95 \pm 0.18	1.39 \pm 0.49
<i>CPT1A</i>	1.00 \pm 0.16	1.07 \pm 0.15	0.80 \pm 0.14	1.16 \pm 0.18
<i>CPT2</i>	1.00 \pm 0.16	0.89 \pm 0.12	0.74 \pm 0.12	0.85 \pm 0.07
<i>PNPLA2</i>	1.00 \pm 0.19	1.01 \pm 0.37	0.41 \pm 0.07	0.49 \pm 0.12
<i>FABP3</i>	1.00 \pm 0.13	0.61 \pm 0.16	0.70 \pm 0.15	0.64 \pm 0.10
<i>FABP5</i>	1.00 \pm 0.36	0.56 \pm 0.19	1.59 \pm 0.98	3.70 \pm 1.81
<i>CD36</i>	1.00 \pm 0.17	0.86 \pm 0.19	0.97 \pm 0.23	3.09 \pm 0.88
<i>NFKB</i>	1.00 \pm 0.20	0.95 \pm 0.11	0.93 \pm 0.12	0.93 \pm 0.22
<i>PTGS2</i>	1.00 \pm 0.36	0.83 \pm 0.27	1.15 \pm 0.34	0.64 \pm 0.15
<i>PPARG</i>	1.00 \pm 0.04	1.13 \pm 0.22	0.93 \pm 0.19	0.84 \pm 0.23

Cumulus cell gene expression was assessed after 22 h IVM of DHA treatment. Genes involved in lipid metabolism (*FASN*, *DGAT1*, *PLIN2*, *CPT1A*, *CPT2*, *PNPLA2*, *FABP3*, *FABP5* and *CD36*) and three genes potentially involved in DHA action pathways (*NFKB*, *PTGS2* and *PPARG*) were analyzed. The geometric mean of three housekeeping genes (*RPL19*, *RPS9* and *GAPDH*) was used to normalize gene expression. Results of five independent cultures with each condition in duplicate are presented as means \pm SEM. Values **in bold** indicate significant difference for a given stage compared to the control ($p < 0.05$). P-value ($0.05 < p < 0.1$) indicates a tendency compared to the control and expression value is in *italic*.