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N-3 polyunsaturated fatty acid DHA during IVM affected oocyte developmental competence in cattle.

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Key words

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

26 **ABSTRACT**

27 The positive effect of n-3 poly-unsaturated fatty acids (FA) on fertility in ruminants seems to
28 be partly mediated through direct effects on the oocyte developmental potential. We aimed to
29 investigate whether supplementation with physiological levels of docosahexaenoic acid
30 (DHA, C22:6 n-3 PUFA) during *in vitro* maturation (IVM) has an effect on oocyte maturation
31 and *in vitro* embryo development in cattle. We showed that DHA (0, 1, 10 or 100 μ M) had no
32 effect on oocyte viability or maturation rate after 22 h IVM. Incubation of oocyte-cumulus
33 complexes (OCC) with 1 μ M DHA during IVM significantly increased ($p < 0.05$) oocyte
34 cleavage rate as compared to control (86.1% vs. 78.8%, respectively) and the >4 -cell embryo
35 rate at day 2 after parthenogenetic activation (PA) (39.1% vs. 29.7%, respectively).
36 Supplementation with 1 μ M DHA during IVM also induced a significant increase in the
37 blastocyst rate at day 7 after *in vitro* fertilization (IVF) as compared to control (30.6% vs.
38 17.6%, respectively) and tended to increase the number of cells in the blastocysts (97.1 ± 4.9
39 vs. 81.2 ± 5.3 , respectively; $p = 0.08$). On the contrary, 10 μ M DHA had no effects, whereas
40 100 μ M DHA significantly decreased the cleavage rate compared to control (69.5% vs. 78.8%,
41 respectively) and the >4 -cell embryo rate at day 2 after PA (19.5% vs. 29.7%). As was shown
42 by real-time PCR, negative effects of 100 μ M DHA were associated with significant increase
43 of progesterone synthesis by OCCs, a three-fold increase in expression level of FA transporter
44 *CD36* and a two-fold decrease of FA synthase *FASN* genes in cumulus cells (CC) of
45 corresponding oocytes. DHA at 1 and 10 μ M had no effect on expression of those and other
46 key lipid metabolism-related genes in CC. In conclusion, administration of a low
47 physiological dose of DHA (1 μ M) during IVM may have beneficial effects on oocyte
48 developmental competence *in vitro* without affecting lipid metabolism gene expression in
49 surrounding cumulus cells, contrarily to 100 μ M DHA which diminished oocyte quality
50 associated with perturbation of lipid and steroid metabolism in CC.

51

1. INTRODUCTION

52 Oocyte competence to develop into viable embryo after fertilization is mainly acquired during
53 final oocyte growth and meiotic maturation. Fatty acids (FA) together with glucose and amino
54 acids are an important source of energy for oocytes, as high amount of energy is required to
55 perform meiosis [1]. Free FA, including non-esterified FA (NEFA), are present in both
56 follicular fluid and in the oocyte-cumulus complex (OCC) and participate in formation of
57 OCC follicular environment directly related to oocyte quality [2, 3]. In fact, an oocyte,
58 surrounded by cumulus cells (CC), is able to rapidly incorporate FA from maturation medium
59 *in vitro*, as shown for oleic and linoleic acids [4, 5]. The oocyte lipid content, in terms of FA
60 composition, has been shown to influence oocyte maturation and developmental competence
61 [6, 7]. Moreover, the highest concentrations of NEFA in the OCC environment, *in vivo* and *in*
62 *vitro* led to low oocyte quality and embryo developmental rate after *in vitro* fertilization (IVF)
63 [8-10]. FA are essential to oocyte quality as energy source [11, 12], but in the case of elevated
64 FA concentration in the environment (follicular fluid or maturation medium), CC are required
65 to protect the oocyte by incorporating excessive FA and transforming them into storage
66 droplets [13, 14]. Moreover, during oocyte maturation CC ensure active lipid metabolism
67 through modulation of expression of FA metabolism related genes [15].

68 N-3 polyunsaturated fatty acids (PUFA), namely, alpha-linolenic acid (ALA),
69 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), belong to a family of
70 biologically active FA and are known to have a variety of health benefits [16]. Mammals
71 cannot produce the precursor of n-3 PUFA, ALA, which is thus considered essential FA. The
72 longest member of this family, DHA, has a 22 carbon chain (C22:6) and can be produced by a
73 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 (PGF₂α) 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.

115

2. MATERIALS AND METHODS

116 **2.1. Ethics**

117 No experiments with living animals were performed.

118

119 **2.2. Chemicals**

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

122

123 **2.3. Biological material**

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

129

130 **2.4. In vitro maturation (IVM)**

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

137 After 22 hours of IVM, CC were removed and both cells and spent IVM media were kept at -
138 80°C until analysis. Oocytes were used either for analysis of meiotic maturation or for
139 parthenogenetic activation (PA).

140 2.5. Analysis of cell viability.

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

148

149 2.6. Analysis of oocyte nuclear meiotic maturation stages.

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

157

158 2.7. Parthenogenetic activation (PA)

159 After 22 hours of IVM, CC were removed by pipette aspiration-ejections in the IVM media,
160 centrifuged 5 min at 3500g, and both CC and spent media were kept at -80°C until analysis.
161 Before PA, denuded oocytes remained in the same IVM conditions for an additional 4 hours.
162 Oocytes were then incubated 5 min in TCM199, ionomycin 5 µM, FA-free FCS 5%,
163 following by 4 hours in TCM199, 6-dimethylaminopurine (6-DMAP) 2 mM, FA-free FCS
164 5%. *In vitro* development was performed in groups of 25 oocytes in a 25 µL microdrop of

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

170

171 **2.8. *In vitro* fertilization (IVF)**

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

182

183 **2.9. Analysis of *in vitro* embryo development**

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

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

193

194 **2.10. Progesterone assay**

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

202

203 **2.11. Gene expression analysis in CC**

204 Total RNA was extracted from CC using TriZol reagent (Invitrogen, Cergy Pontoise, France)
205 following the manufacturer's instructions. RNA concentration was determined using a
206 NanoDrop ND-1000 spectrophotometer (Nyxor Biotech, Paris, France). RNA preparations
207 were DNase digested by RQ1 DNase (Promega, Charbonnières, France) following the
208 manufacturer's instructions. Reverse transcription (RT) was performed on 200 ng of total
209 RNA extracted from CC using Moloney Murine Leukemia Virus reverse transcriptase I
210 (Invitrogen, Cergy Pontoise, France) with an oligo dT primer mix (Promega, Charbonnières,
211 France) according to the manufacturer's instructions. Real-time PCR reactions were carried
212 out on a MyiQ Cyclor apparatus (Bio-Rad, Marnes la Coquette, France) in 20 μ l containing
213 primers at a final concentration of 150 nM of each, 5 μ l of the diluted RT reaction (1ng cDNA
214 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:

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

223

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.

240 3. RESULTS

241

242 3.1. *In vitro* development of oocytes after IVM with or without DHA

243 In our experiments, OCC were matured *in vitro* with or without DHA (1, 10, or 100 μ M) and
244 then *in vitro* embryo development was compared between conditions. We chose to assess
245 oocyte developmental competence by using parthenogenetic activation (PA) first, because, in
246 this case, embryo development only relies on oocyte maternal factors. DHA-treated and
247 control oocytes underwent PA, and their cleavage rates were measured 40 hours later. As
248 shown in Figure 1, 1 μ M DHA induced a significant ($p < 0.05$) increase in the oocyte cleavage
249 rate as compared to control ($86.1 \pm 2.1\%$ vs. $78.8 \pm 2.5\%$, respectively). On the contrary, 100
250 μ M DHA induced a significant decrease in cleaved oocytes compared to control ($69.5 \pm 3.2\%$
251 vs. $78.8 \pm 2.5\%$, respectively). No difference was seen in the cleavage rate of the oocytes
252 matured with DHA 10 μ M. Cleaved embryos were differentiated as following: i) embryos
253 exhibiting 2-4 cells or ii) embryos exhibiting more than 4 cells. No difference was reported in
254 the 2-4 cell embryo rate in the absence ($44.5 \pm 3.0\%$) or presence of 1 μ M DHA ($43.6 \pm$
255 3.1%), 10 μ M ($39.6 \pm 3.0\%$) or 100 μ M ($44.6 \pm 3.4\%$). Concerning the embryos with more
256 than 4 cells (>4-cell embryo), 1 μ M DHA induced a significant increase in the >4-cell embryo
257 rate compared to the control group ($39.1 \pm 3.2\%$ vs. $29.7 \pm 2.9\%$, respectively). The same
258 difference was observed in the 10 μ M DHA group, presenting a higher rate of > 4-cell
259 embryos as compared to control ($38.7 \pm 3.2\%$ vs. $29.7 \pm 2.9\%$, respectively). On the contrary,
260 100 μ M DHA induced a significant decrease in the >4 cell embryo rate compared to control
261 ($19.5 \pm 2.7\%$ vs. $29.7 \pm 2.9\%$, respectively). At day 7 of *in vitro* development, a rate of more
262 than 16-cell embryos which had to undergo major activation of proper genome [49], was not
263 different between 1 μ M DHA or 10 μ M DHA compared to control (34.1%, 37.3% and 40.0%,

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

271 IVF was then performed to confirm our observations with PA on potentially better
272 development of the oocytes matured in the presence of 1 μ M DHA (Figure 2A and
273 supplementary Table 1). At day 2 after IVF, control and 1 μ M DHA groups showed no
274 difference in the cleavage rate (83.4 ± 2.9 vs. 85.0 ± 2.8 , respectively), the 2-4 cell embryo
275 rate (49.4 ± 3.9 vs. 49.8 ± 3.9 , respectively) and the rate of embryos with more than 4 cells
276 (33.2 ± 3.6 vs. 34.0 ± 3.7 , respectively). However, 1 μ M DHA led to a significant increase in
277 the blastocyst rate at day 7 compared to control (30.6 ± 4.1 vs. 17.6 ± 3.3 , respectively). As
278 shown in Figure 2B, the addition of 1 μ M DHA during IVM also tended to increase ($p < 0.08$)
279 the number of cells per blastocyst, as compared to control group (97.1 ± 4.9 vs. 81.2 ± 5.3 ,
280 respectively).

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

284

285 **3.2. Effect of DHA on oocyte meiotic maturation**

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

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

297

298 **3.3. Oocyte and cumulus cell viability in the presence or absence of DHA during IVM**

299 Oocyte viability showed no difference in terms of number of living oocytes after 22h IVM in
300 the absence or presence of 1, 10, 100 μ M DHA as compared with control (98.8%, 97.0%,
301 96.3 and 98.7, respectively). In order to investigate whether DHA affected cumulus
302 functioning, the viability of CC was assessed by measuring adenylate kinase activity in spent
303 culture media from corresponding OCC after IVM. No significant difference in adenylate
304 kinase activity level was observed in the absence or presence of DHA (874 ± 44 , 868 ± 47 ,
305 948 ± 40 and 992 ± 18 relative light units for control, 1, 10 and 100 μ M DHA, respectively).
306 Thus, supplement with DHA during IVM affected neither oocytes nor surrounding CC
307 viability.

308

309 **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.

334 **DISCUSSION**335 **DHA directly affects bovine oocyte quality**

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

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

355 Our results on the effects of DHA are also relevant to *in vivo* experiments in ruminants.
356 Indeed, an increased cell number was observed in blastocysts obtained in lactating dairy cows
357 *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 μM 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 μM DHA during IVM significantly decreased the cleavage rate, the >4 cell embryo

382 rate at day 2, and the rate of embryos with more than 16 cells at day 7. Such embryos had
383 already overcame major embryo genome activation (MGA) occurring at 8-16 cell stage [49],
384 and their lower rate indicates that DHA excessive level (100 μ M) during IVM may have a
385 deleterious effect on oocyte capacity to activate the factors involved in MGA and thus may
386 lead to lower blastocyst rate. These results are relevant to the literature where high doses of n-
387 3 PUFA (100 and 200 μ M ALA) led to negative effects on oocyte maturation and the CC
388 expansion rate in cattle [50] as well as in sheep [51]. Similarly, other PUFA exhibited the
389 same lipotoxic effect at high doses; i.e. 100 μ M linoleic acid decreased the percentage of
390 metaphase-II oocytes compared to lower doses (9 and 43 μ M) and resulted in oocytes arrested
391 at the immature state [5]. Therefore, when used at a high dose, the lipotoxic effects of n-3
392 PUFA resembled the effects of other FA. Indeed, palmitic acid (150 μ M) or stearic acid (75
393 μ M) added to bovine IVM media had negative effects on maturation, fertilization, cleavage
394 rate and blastocyst yield [2, 58].

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

406

407 **DHA modulates oocyte quality: what are the mechanisms?**

408 The present study demonstrated that the impact of DHA on oocyte competence to develop
409 was not due to the modulation of meiotic maturation. Indeed, here no differences in
410 maturation rate were reported after any DHA treatment as compared to control, except a
411 slower transition from telophase to metaphase-II in DHA 100 μM treated oocytes. The
412 discrepancies of n-3 PUFA effect on oocyte maturation occurred also in the literature. Indeed,
413 supplementation with another n-3 PUFA, ALA, at 50 μM during IVM increased the rate of
414 metaphase-II oocytes in cattle [50]. In contrast, ALA used during IVM at 50 and 100 μM did
415 not affect nuclear maturation in sheep, and at 200 μM reduced the maturation rate [51]. In
416 addition, in cattle, when used *in vivo*, ALA also had no effect on oocyte nuclear maturation
417 [35]. Other n-6 PUFA, linoleic acid, have also been reported to exhibit either no effect [5] or
418 an inhibitory effect [61] on nuclear maturation in cows. These results are in line with ours, as
419 we did not find any effect on nuclear maturation in DHA 1 μM and 10 μM groups.

420 Taken together, all these studies and our results inferred that not nuclear maturation but
421 cytoplasm maturation might be affected in oocyte as a result of DHA supply during IVM.
422 Cytoplasm maturation includes accumulation of maternal factors, such as transcripts, proteins
423 and energy storage which are used to support the first cleavages. Cumulus cells play an
424 important role in cytoplasm maturation of oocytes by regulating energy metabolism inside the
425 OCC during maturation [62], and the role of lipid metabolism in CC for oocyte maturation
426 revealed to be important [11, 15, 63]. Addition of DHA in IVM medium was thought to
427 modulate lipid metabolism in OCC and thus be linked to modulation of oocyte quality.
428 Indeed, we investigated the expression of candidate genes from several key lipid metabolism
429 functions: lipogenesis (*FASN*), lipid storage (*DGAT1*), lipolysis (*PLIN2*, *PNPLA2*), FA
430 incorporation (*CD36*) and transport (*FABP3*, *FABP5*), and FA β -oxidation (*CPT1A*, *CPT2*)
431 [64]. While expression was not changed in 1 μM DHA-treated and control CC, none of these

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

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

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

466

467 **Conclusion**

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

478

479

480

481 **DECLARATION of INTEREST**

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

484

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489

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493

494

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686 **Figure legends**

687

688 **Figure 1: Effect of DHA during IVM on cleavage rate at day 2 and blastocyst rate at day**
689 **7 after parthenogenetic activation**

690 Embryo cleavage and blastocyst rates in control and DHA-treated groups (1, 10 and 100 μ M)
691 were assessed 2 days and 7 days after PA, respectively. Among the cleaved oocytes, embryos
692 >4 cell stage were distinguished. Percentage of blastocysts was reported to the number of
693 cleaved embryos at day 2. Results of five independent experiments are expressed as the
694 lsmeans percentage of embryos in each developmental stage \pm SEM. * indicates a significant
695 difference for a given stage compared to the control ($p < 0.05$). # indicates a tendency of a
696 difference compared to the control ($0.05 < p < 0.1$). Total numbers of analyzed
697 parthenogenetically activated oocytes per group are shown between brackets.

698

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

700 **A.** Embryo cleavage and blastocyst rates in control and DHA-treated groups (1 μ M) were
701 assessed 2 days and 7 days after IVF, respectively. Among the cleaved oocytes, embryos >4
702 cell stage were distinguished. Percentage of blastocysts was reported to the number of cleaved
703 embryos at day 2. Results of three independent experiments are expressed as the lsmeans
704 percentage of embryos in each developmental stage \pm SEM or as the mean blastocyst cell
705 number \pm SEM. * indicates a significant difference for a given stage compared to the control
706 ($p < 0.05$). Total numbers of analyzed in vitro fertilized oocytes per group are shown between
707 brackets. **B.** Number of cells (mean \pm SEM) in the blastocysts developed from the oocytes
708 treated or not with DHA 1 μ M during IVM. # indicates a tendency of a difference ($0.05 < p <$
709 0.1) compared to control.

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

712 **A.** Scheme of disposition of genes involved in lipid metabolism main processes (grey boxes)
713 according to their known functions in different cells, which were analyzed in CC treated with
714 0, 1, 10 and 100 μ M DHA. Abbreviations: FA: fatty acids. Genes: *FASN* : fatty acid synthase,
715 *FABP3* and *FABP5*: fatty acids binding proteins 3 and 5, *DGAT1* : Diacylglycerol O-
716 acyltransferase 1, *PNPLA2*: Patatin-like phospholipase domain containing 2, *PLIN2*: perilipin
717 2, *CPT1*: carnitine palmitoyl transferase 1, *CPT2*: carnitine palmitoyl transferase 2, *CD36*:
718 thrombospondin receptor, FA transporter. **B.** Relative expression of *CD36* and *FASN* genes in
719 CC after 22h IVM measured by real time PCR. The geometric mean of three housekeeping
720 genes (*RPL19*, *RPS9* and *GAPDH*) was used to normalize gene expression. Results of five
721 independent cultures with each condition in duplicate are presented as means \pm SEM. *
722 indicates significant difference for a given stage compared to the control ($p < 0.05$). #
723 indicates a tendency of a difference ($0.05 < p < 0.1$) compared to the control. Other genes
724 showed no difference between DHA-treated and control CC.

725

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

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

732

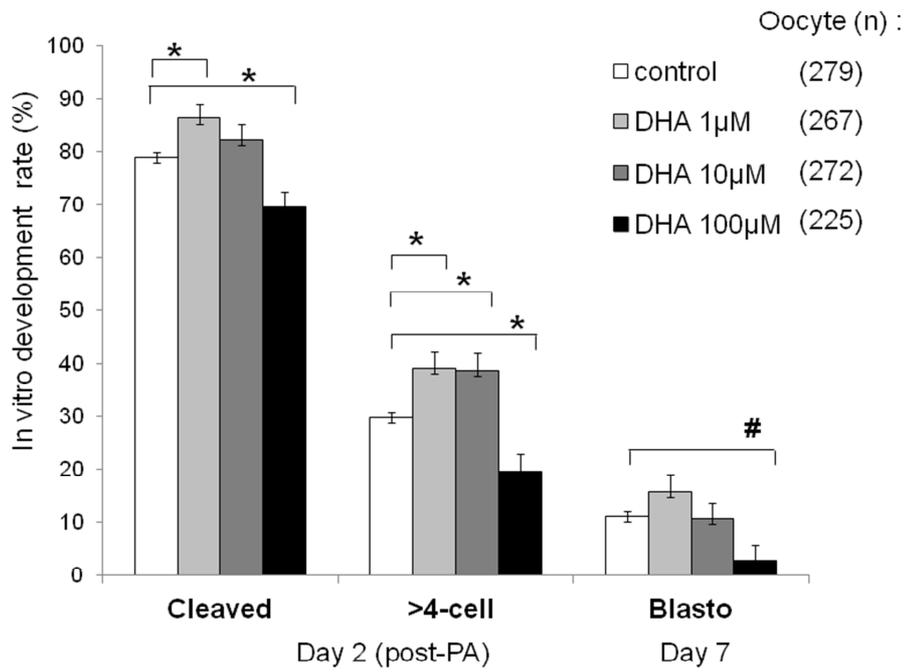
Tables :

Table 1: Oligonucleotide sequences

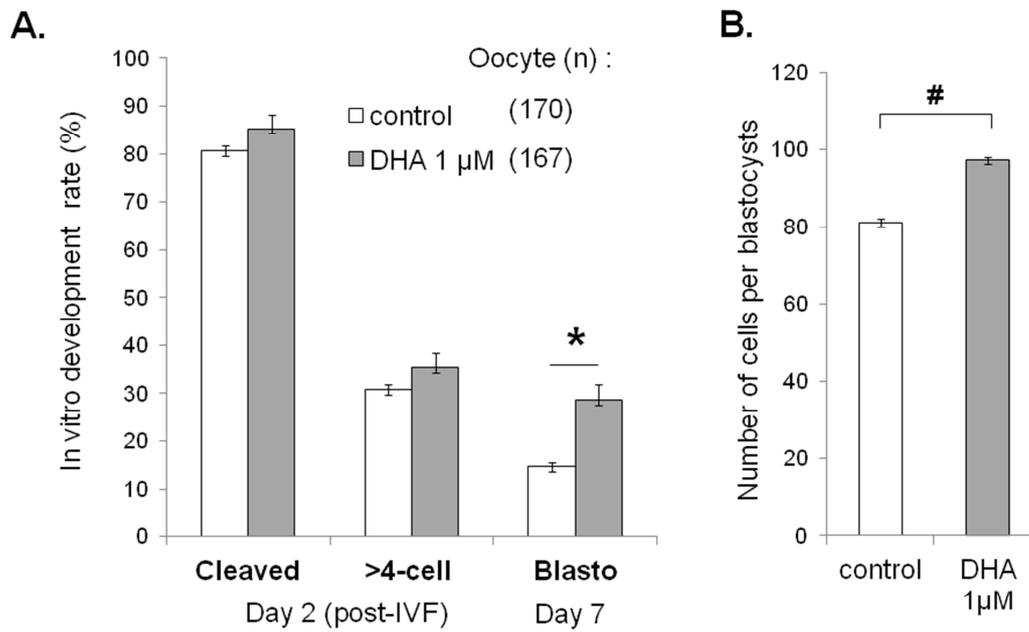
Abbrev.	Accession number	Gene	Forward primer	Reverse primer	bp	E
<i>CD36</i>	BC103112.1	thrombospondin receptor	GCATTCTGAAAGTGC GTTGA	CGGGTCTGATGAAAGTGGTT	181	1.95
<i>CPT1A</i>	FJ415874	carnitine palmitoyl transferase 1	TCCTGGTGGGCTACCAATTA	TGCGTCTGTAAAGCAGGATG	181	1.98
<i>CPT2</i>	NM_001045889	carnitine 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	TGGCGCATTGGTTCAACATCAGG	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	CCCTTTCGCTTACCTATAACC	156	1.85
<i>RPS9</i>	BC148016	Ribosomal protein S9	GGAGACCCTTCGAGAAGTCC	GGGCATTACCTTCGAACAGA	180	1.84

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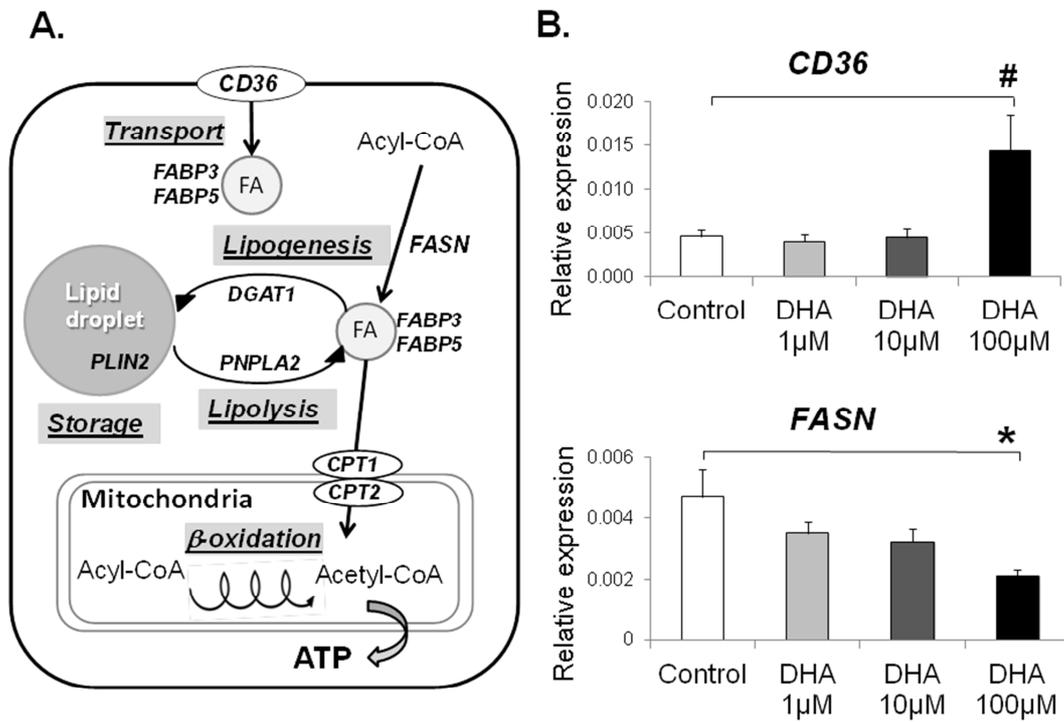
Oseikria, M., Elis, S., Maillard, V., Corbin, E., Uzbekova, S. (2016). N-3 polyunsaturated fatty acid DHA during IVM affected oocyte developmental competence in cattle. *Theriogenology*, 85 (9), 1625-1634. DOI : 10.1016/j.theriogenology.2016.01.019



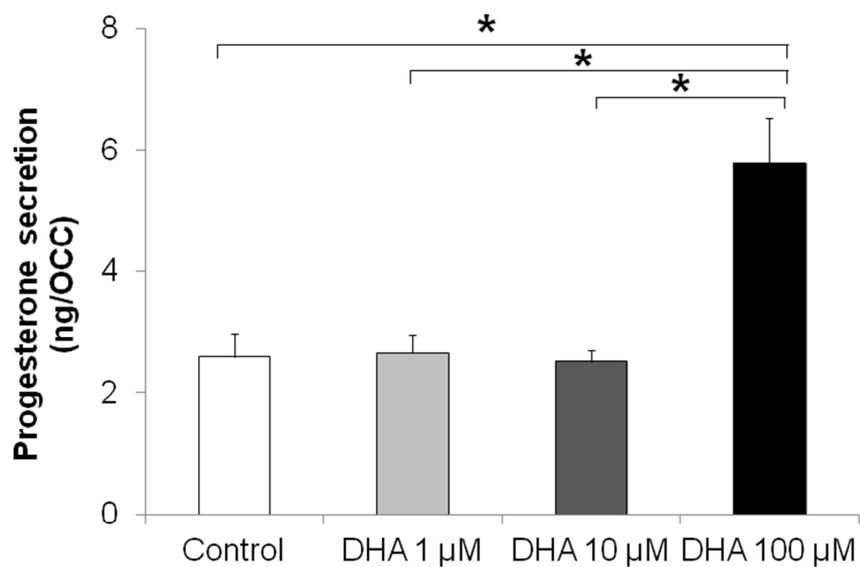
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Highlights

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 1 μ M		DHA 10 μ M		DHA 100 μ M			
	% (smeans \pm SEM)	% (smeans \pm SEM)	p-value	Logistic regression OR (CI 2.5% - 97.5%)	% (smeans \pm SEM)	p-value	Logistic regression OR (CI 2.5% - 97.5%)	% (smeans \pm SEM)	p-value	Logistic regression OR (CI 2.5% - 97.5%)
Parthenogenetic activation										
Number of oocytes	279	267			272			225		
Cleaved embryo %	78.8 \pm 2.5	86.1 \pm 2.1	0.024	0.60 (0.39 - 0.93)	82.0 \pm 2.4	0.35	0.82 (0.54 - 1.24)	69.5 \pm 3.2	0.017	1.63 (1.09 - 2.45)
2-4cell embryo %	44.5 \pm 3.0	43.6 \pm 3.1	0.83	1.04 (0.74 - 1.46)	39.6 \pm 3.0	0.25	1.22 (0.87 - 1.73)	44.6 \pm 3.4	0.98	1.00 (0.69 - 1.43)
>4-cell embryo %	29.7 \pm 2.9	39.1 \pm 3.2	0.030	0.66 (0.45 - 0.96)	38.7 \pm 3.2	0.037	0.67 (0.46 - 0.98)	19.5 \pm 2.7	0.012	1.74 (1.14 - 2.69)
>16 cell / cleaved embryo %	40.0 \pm 4.9	34.1 \pm 4.5	0.38	1.28 (0.74 - 2.24)	37.3 \pm 4.5	0.69	1.12 (0.65 - 1.94)	13.8 \pm 4.2	0.0004	4.15 (1.94 - 9.59)
Blastocyst / cleaved embryo %	11.0 \pm 3.0	15.7 \pm 3.3	0.30	0.66 (0.30 - 1.43)	10.7 \pm 2.8	0.95	1.03 (0.44 - 2.37)	2.9 \pm 2.2	<i>0.068</i>	4.14 (1.08 - 27.19)
In vitro fertilization										
n oocyte	170	167								
Cleaved embryo %	83.4 \pm 2.9	85.0 \pm 2.8	0.68	0.88 (0.49 - 1.59)						
2-4cell embryo %	49.4 \pm 3.9	49.8 \pm 3.9	0.88	0.97 (0.63 - 1.49)						
>4-cell embryo %	33.2 \pm 3.6	34.0 \pm 3.7	0.87	0.96 (0.61 - 1.52)						
Blastocyst / cleaved embryo %	17.6 \pm 3.3	30.6 \pm 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 1 μ M		DHA 10 μ M		DHA 100 μ M			
	% (lsmeans \pm SEM)	% (lsmeans \pm SEM)	p-value	Logistic regression OR (CI 2.5% - 97.5%)	% (lsmeans \pm SEM)	p-value	Logistic regression OR (CI 2.5% - 97.5%)	% (lsmeans \pm SEM)	p-value	Logistic regression OR (CI 2.5% - 97.5%)
In vitro maturation										
Number of oocytes	136	152			119			100		
immature %	3.1 \pm 1.5	2.8 \pm 1.4	0.89	1.09 (0.30 - 4.02)	3.5 \pm 1.7	0.85	0.88 (0.24 - 3.27)	2.6 \pm 1.6	0.82	1.19 (0.28 - 5.96)
Meta-1 %	11.3 \pm 2.7	12.8 \pm 2.7	0.70	0.87 (0.42 - 1.76)	9.1 \pm 2.7	0.56	1.28 (0.57 - 2.95)	8.3 \pm 2.7	0.43	1.42 (0.61 - 3.50)
Ana-1 %	1.5 \pm 1.1	4.6 \pm 1.7	0.15	0.31 (0.05 - 1.30)	2.5 \pm 1.5	0.55	0.58 (0.08 - 3.56)	4.0 \pm 2.1	0.24	0.36 (0.05 - 1.87)
Telo-1 %	5.0 \pm 1.9	1.8 \pm 1.1	0.15	2.77 (0.75 - 13.09)	1.6 \pm 1.2	0.15	3.24 (0.76 - 22.09)	14.0 \pm 3.6	0.020	0.32 (0.12 - 0.81)
Meta-2 %	78.1 \pm 3.5	77.0 \pm 3.4	0.83	1.06 (0.61 - 1.86)	82.4 \pm 3.5	0.39	0.76 (0.40 - 1.41)	70.4 \pm 4.6	0.18	1.50 (0.83 - 2.71)
mature %	83.5 \pm 3.2	79.2 \pm 3.3	0.35	1.33 (0.74 - 2.44)	84.3 \pm 3.3	0.87	0.95 (0.48 - 1.84)	84.7 \pm 3.6	0.80	0.91 (0.45 - 1.83)

Oocyte meiotic stages were assessed after IVM with or without DHA (1 μ M, 10 μ M or 100 μ M). 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 \pm SEM. 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

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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*.

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