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Effect of DHA on the quality of In vitro Produced Bovine Embryos

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Abstract:	<p>Docosahexaenoic acid (DHA) is an n-3 polyunsaturated fatty acid (PUFA) that improves fertility by increasing membrane fluidity. Moreover, embryos produced by donor females supplied with n-3 PUFA did not show any difference in terms of the lipid profile after 7 days of culture. The present study aimed to investigate the effects of DHA (20 and 100μM) coupled with carnosine (5mg/mL), an antioxidant, during oocyte maturation and embryo development on the developmental and cryosurvival rates and the number of pluripotent cells. Free fatty acid receptor-4 (FFAR4), which is able to bind DHA, was visualised by immunostaining. The addition of DHA in the in vitro development (IVD) medium decreased the percentage of pluripotent SOX2 positive cells compared with the control (8.4% vs. 10.9%) without affecting the number of cells (196.7 vs. 191.6 cells) or the developmental (20.9% vs. 23.9% blastocysts rate on D7) and cryosurvival rates (86.3% vs 86.2%). Such a decrease in pluripotent cells, relevant to the differentiation of the first lineage within the inner cell mass, represents an improvement in the embryo quality. On the contrary, embryos without any pluripotent SOX2-positive cells would not be able to achieve gestation. Future studies should follow up these results by carrying out embryo transfers to assess the beneficial effects of DHA supplementation.</p>

1 Effect of DHA on the quality of *In vitro* Produced Bovine Embryos

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15 fertility by increasing membrane fluidity. Moreover, embryos produced by donor females supplied
16 with n-3 PUFA did not show any difference in terms of the lipid profile after 7 days of culture. The
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19 and cryosurvival rates and the number of pluripotent cells. Free fatty acid receptor-4 (FFAR4), which is
20 able to bind DHA, was visualised by immunostaining. The addition of DHA in the *in vitro* development
21 (IVD) medium decreased the percentage of pluripotent SOX2 positive cells compared with the control
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24 pluripotent cells, relevant to the differentiation of the first lineage within the inner cell mass,
25 represents an improvement in the embryo quality. On the contrary, embryos without any pluripotent
26 SOX2-positive cells would not be able to achieve gestation. Future studies should follow up these
27 results by carrying out embryo transfers to assess the beneficial effects of DHA supplementation.

28

29 1. Introduction

30 The ability of oocytes to develop *in vitro* into viable embryos after fertilisation is mainly acquired during
31 final oocyte growth and meiotic maturation [1]. However, while the intrinsic quality of the oocyte is
32 the main factor affecting the embryo developmental rate, the main factor affecting the quality of
33 blastocysts is the post-fertilisation culture condition [2]. Freezing impairs the developmental potential
34 of bovine embryos especially ICM cells [3]. In addition, membrane damage and DNA fragmentation
35 induced by cryopreservation, reduce embryonic competence [4]. Thus, post-thaw establishment of
36 ICM and TE and expression of the markers for their development competence could be relevant
37 indicators of the quality of bovine embryos prior to embryo transfer.

38 The lipid composition of oocytes and embryos affects their development and their cryotolerance [5].
39 Lipid supplementations was used to increase embryo developmental rates in cattle. Docosahexaenoic
40 acid (DHA) is an n-3 polyunsaturated fatty acid (PUFA) with a 22 carbon chain (C22:6) [6, 7]. DHA
41 supplied in the cow diet modified the lipid composition of bovine oocytes [1]. DHA also increase
42 membrane fluidity: its incorporation into the membrane facilitate membrane deformation [8].
43 Moreover, DHA supplementation in the culture medium (1 to 20 μ M) increases the proliferation and
44 steroidogenesis of granulosa cells [9] and increases oocyte quality *in vitro* and blastocyst rate on day 7
45 [7]. Furthermore, because lipids and especially n-3 PUFA are easily oxidised, compensatory antioxidant
46 supplementation were also assessed *in vitro*. Supplementation with carnosine, a reactive oxygen
47 species scavenger, into the *in vitro* development (IVD) medium accelerated the kinetics from the 4-cell
48 stage to compaction of the morula, and it also increased the post-thaw viability [10].

49 We hypothesized that DHA increases the success rate of frozen embryo transfer by improving embryo
50 quality as well as by reducing their cryosensitivity due to its incorporation into the membrane. Thus,
51 we evaluated the effect of DHA and carnosine supplementation in *in vitro* maturation (IVM) and/or
52 IVD media, in the absence of serum, on embryonic quality using both the cryosurvival rate and a marker
53 of pluripotency (SOX2) of bovine expanded grade-1 blastocysts, after thawing.

54 2. Materials and Methods

55 No experiments with living animals were performed. All chemicals were obtained from Sigma-Aldrich
56 (Saint Quentin Fallavier, France), unless otherwise stated in the text.

57

58 *2.1. In vitro embryo production*

59 COCs were retrieved from bovine ovaries collected at a local slaughterhouse. Follicles from 2 to 8 mm
60 in diameter were punctured with an 18G needle linked to a vacuum pump and added to a 50 mL Falcon
61 tube containing 1 mL of flushing medium (Euroflush, IMV Technologies, L'Aigle, France) maintained at
62 37 °C.

63 Only COCs with at least three layers of compact cumulus cells, with homogenous and non-granular
64 cytoplasm, were selected [11] and washed two times in Euroflush at 37 °C and then a third time in
65 serum-free IVM medium before undergoing maturation for 22 h. The maturation medium comprised
66 TCM-199, supplemented with 4g/L fatty acid free (FAF) bovine serum albumin (BSA), 10 ng/mL
67 epidermal growth factor (EGF), 10 µg/mL porcine follicle-stimulating hormone (pFSH)/porcine
68 luteinising hormone (pLH), 1 µg/mL 17β-oestradiol and 5 µg/mL gentamycin. All COCs were incubated
69 at 38.5 °C for 22 h under a maximally humidity atmosphere with 5% CO₂.

70 IVF was performed as described previously [12]. The same semen from the same bull and the same
71 ejaculate were used for all the experiments. Therefore, there is no male effect among the experiments.
72 Eighteen hours after fertilisation, all presumptive zygotes were cleared of cumulus cells and of
73 spermatozoa attached to the zona pellucida by centrifugation. Zygotes were washed twice in Euroflush
74 then twice in IVD medium. Zygotes were cultured in a 30 µL micro-drop of IVD medium at 38.5 °C in a
75 maximally humidified atmosphere of 5% O₂, 5% CO₂ and 90% N₂ and covered with mineral oil (Liquid
76 Paraffin, Origio, Måløv, Denmark). IVD_c medium consisted of a serum-free, synthetic oviductal fluid
77 control (SOF_c) whose composition is described in [13], supplemented with 6 g/L of FAF BSA.

78 2.2. Embryo quality assessment

79 The zygote cleavage rate was assessed under a stereoscopic microscope at 20× magnification 48 h
80 post-fertilisation (D2). The total developmental rate at D6 was evaluated by the numbers of
81 morula/blastocysts. Additionally, the blastocyst developmental rate and embryo quality were
82 recorded on D6–D8 (D0 = day of IVF), according to the International Embryo Technology Society (IETS)
83 morphological criteria (Chapter 9 and Annex D, IETS Manual, 3rd edition). Only expanded grade-1
84 blastocysts were used for the quality assessment experiment – that is, expanded blastocysts with a
85 compact inner cell mass, uniformly coloured blastomeres, few irregularities or excluded cells and an
86 intact and smooth zona pellucida. After 7 days of development, the expanded grade-1 blastocysts were
87 frozen. Those that were not completely expanded were frozen after 24h of additional culture, i.e. on
88 day 8. To indicate the development of D8 expanded grade-1 blastocysts, the rate of expanded grade-
89 1 blastocysts in Figure 2 and 3 include those frozen on D7 and those newly appeared on D8 since it was
90 considered that an expanded blastocyst on D7 will still be present on D8.

91 2.3. Embryo Freezing and Thawing

92 Embryos were frozen slowly in ethylene glycol sucrose solution, following a protocol described
93 previously [12]. Briefly, expanded grade-1 blastocysts were washed in embryo-holding medium (EHM,
94 IMV Technologies, L'Aigle, France). Then, embryos were placed in 1.5 M ethylene glycol freezing
95 medium (IMV Technologies) supplemented with 0.1 M sucrose for 10 min at room temperature.
96 Embryos were mounted in groups of up to 9 embryos in 250 µL straws. The straws were placed in the
97 cryochamber of the freezer (Freeze Control®, Cryologic, Melbourne, Australia), previously equilibrated
98 at -6 °C. After 2 min at -6 °C, the seeding was induced manually. The temperature was lowered at a
99 rate of 0.3 °C/min, down to -32 °C. When this temperature was reached, the straws were directly
100 immersed in liquid nitrogen at -196 °C, before being stored.
101 For post-thawed experiments, straws containing the embryos were thawed for 3 s in ambient air and
102 then immersed in a water bath at 35 °C for 30 s. Their contents were poured into a Petri dish, thus
103 mixing the different phases. After 15 min at room temperature, the embryos were washed for 5 min

104 in EHM at room temperature. Finally, the embryos were washed and then cultured in SOF (Minitüb,
105 Gmbh, Tiefenbach, Germany), supplemented with 2% of minimum essential media (MEM) and 1% of
106 basal medium eagle (BME), 0.33 mg/mL sodium pyruvate, 1% oestrus cow serum and 6g/L FAF BSA, in
107 the incubator under 5% CO₂, 5% O₂ at 38.5 °C for 48 h. The cryosurvival of the embryos was evaluated
108 at 24 and 48 h post-thawing, by recording the re-expansion and hatching rates.

109

110 *2.4. Immunostaining*

111 The presence of FFAR4 (also known as GPR120), a receptor able to bind DHA, was determined by
112 immunofluorescence on COCs, embryos at the 2-cell stage and expanded blastocysts, as described
113 previously [14]. Briefly, COCs, 2-cell stage embryos and expanded blastocysts were fixed in 4%
114 paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. Following 15 min of permeabilization
115 in 0.5% Triton in PBS, oocytes and embryos were incubated in PBS with 10% FCS for 3 h and in PBS with
116 5% FCS and 2% BSA for 2 h. The FFAR4 antibody was produced in rabbits (homemade antibody).
117 Samples were then incubated at 4 °C overnight with rabbit anti-FFAR4 (9.5 ng/mL) or rabbit IgG before
118 immunisation for the negative control (same concentration), both diluted in PBS with 2% BSA. After
119 four washes in PBS with 0.2% BSA, samples were incubated in the dark with secondary antibody, Alexa
120 Fluor488–conjugated goat anti-rabbit IgG (Life Technologies, Saint Aubin, France) diluted 3:1000 for 3
121 h at ambient temperature. Another four washes in PBS with 0.2% BSA were performed, and the nuclei
122 of the samples were counter-labelled with Hoechst 33342 (5 µg/mL) (Thermo Fisher Scientific, Illkirch-
123 Graffenstaden, France) for 6 min at 37 °C.

124 After the cryosurvival evaluation the presence of SOX2 and CDX2 was determined by
125 immunofluorescence. All surviving embryos were co-immunolabeled with SOX2 and CDX2 to assess
126 the state of pluripotency (SOX2) as well as the proliferation of the trophectoderm (CDX2) [15]. Embryos
127 were fixed in 4% paraformaldehyde in PBS for 15 min. The blastocysts were then washed twice in a
128 solution of TCM199 HEPES, supplemented with 0.1% Triton and 2% FCS, for 5 min. Embryos were
129 permeabilised in PBS, 0.5% Triton, 1% BSA and 10% FCS, for 1 h at 37 °C. Embryos were incubated with

130 two primary antibodies, goat polyclonal anti-SOX2 (AF2018, R&D Systems, Minneapolis, Minnesota,
131 USA) and mouse monoclonal anti-CDX2 IgG (sc-166830, Santa Cruz Biotechnology, Dallas, Texas, USA),
132 diluted 1/250 and 1/200, respectively, in PBS supplemented with 0.1% Triton and 1% BSA for 1 h at 37
133 °C. The embryos were then rinsed for 45 min in the rinsing solution at room temperature. Then, they
134 were incubated with two polyclonal secondary antibodies, anti-goat IgG conjugated to
135 indocarbocyanine Cy3 (Jackson ImmunoResearch, West Grove, Pennsylvania, USA) and anti-mouse IgG
136 conjugated to Alexa Fluor 488 (Abcam, Cambridge, United Kingdom), diluted 1:1500 in PBS containing
137 0.1% Triton and 1% BSA for 1 h at 37 °C. After 45 min of washing, the nuclei of the embryos were
138 counter-labelled with Hoechst 33342 (5 µg/mL) (Thermo Fisher Scientific) for 6 min at 37 °C. After
139 rinsing, the embryos were mounted on slides in mounting medium (Fluoromount-G™, Invitrogen™,
140 Thermo Fisher Scientific, Illkirch-Graffenstaden, France).

141

142 *2.5. Confocal Microscopy and Image Processing*

143 A confocal laser scanning microscope (LSM700, Carl Zeiss Microscopy GmbH, Munich, Germany) was
144 used to examine the immunofluorescence. This microscope is driven by the Zen black acquisition
145 software (ZEISS). The acquisition parameters were as follows: objective 20×0.8; gain between 700 and
146 800 depending on the channel; laser power between 0.3% and 4.5%; excitation of Alexa Fluor 488 at
147 488 nm, Cy3 at 555 nm and Hoechst 33342 at 405 nm; optimal resolution of 2048×2048 pixels/image;
148 scanner speed at 7 (scanning time 0.79 µs/pixel, or 23.23s/image); acquisition interval of 1.00 µm for
149 z-images.

150 To acquire the images of FFAR4 immunofluorescence, 'Snap' using the ZEN black software (release
151 2009) was performed. Z-images of SOX2/CDX2 immunofluorescence were acquired with 'Z-stack' using
152 the ZEN black software. This process compiles several images with varying focal planes to produce an
153 image with a greater depth of field. To exploit and extract information concerning the number of cells,
154 the following image treatments have been applied: 'Maximum intensity projection', which allows
155 projecting the pixel with the highest value on the z-axis so that in a single two-dimensional image all

156 the dense structures of a given volume are observed, and 'Filter median', to reduce the noise of the
157 image. Cell counting was then performed with Fiji software (Image J, release 2.3.0, National Institutes
158 of Health, Bethesda, MD) using the 'Multi-point Tool', which allows manual counting.

159

160 *2.6. Experimental Design*

161 **Experiment 1.** *Evolution of the allocation of SOX2 within grade-1 expanded blastocysts between J7 and*
162 *J9.*

163 In the following experiments (experiment 3 and 4), the grade-1 expanded blastocysts were selected
164 on Day 7 or Day 8 and then frozen and then their cryosurvival was evaluated over 48h, before the SOX2
165 immunostaining was performed. To know the evolution of the allocation of SOX2 during the whole
166 process, grade-1 expanded blastocysts were selected on Day 7, half was immunolabelled for SOX2,
167 while the other half was cultured for 48h to mimic the cryosurvival period, before being
168 immunolabelled for SOX2.

169 **Experiment 2.** *Analysis of the expression of free fatty acid receptor-4 (FFAR4) in COCs, 2-cell stage*
170 *embryos and expanded blastocysts.*

171 DHA is a ligand of FFAR4 and exerts some of its physiological effects through this receptor. To confirm
172 that DHA was able to act through FFAR4 during early embryo development, the presence of this
173 receptor was determined on COCs, 2-cell stage embryos and expanded blastocysts using
174 immunofluorescence.

175

176 **Experiment 3.** *The effects of adding DHA to the oocyte maturation medium on the development and*
177 *quality of in vitro produced bovine embryos.*

178 To determine which DHA concentration is beneficial for embryo development, COC were matured in
179 IVM medium supplemented with either 20 μM DHA (IVM_{DHA20}) or 100 μM DHA (IVM_{DHA100}). DHA has

180 been diluted in DMSO and, therefore, the controls also had the same DMSO level, either 0.02% or
181 0.1%. To avoid lipid oxidation, following the addition of DHA, 5 µg/mL carnosine was added. All oocytes
182 were *in vitro* fertilised and cultured in serum-free medium (IVD_c). After 7 days of *in vitro* culture, all
183 grade-1 blastocysts were frozen slowly. After thawing, the cryosurvival of embryos was evaluated by
184 recording the re-expansion and hatching rates after *in vitro* culture for additional 48h. At the end of
185 the cryosurvival evaluation, to assess the embryo quality, SOX2 immunostaining was performed on the
186 viable embryos, i.e. expanded/hatched blastocysts. Six experimental groups were constituted
187 corresponding to 11 independent experiments:

- 188 - IVM_c: without supplementation (n = 800 COCs)
- 189 - IVM_{DHA20}: IVM_c + 20 µM DHA in 0.02% DMSO + 5 µg/mL carnosine (n = 773 COCs)
- 190 - IVM_{DHA100}: IVM_c + 100 µM DHA in 0.1% DMSO + 5 µg/mL carnosine (n = 492 COCs)
- 191 - IVM_{DMSO 0.02}: IVM_c + 0.02% DMSO (n = 297 COCs)
- 192 - IVM_{DMSO 0.1}: IVM_c + 0.1% DMSO (n = 499 COCs)
- 193 - IVM_{CARN}: IVM_c + 5 µg/mL carnosine (n = 458 COCs)

194

195 **Experiment 4.** *The effects of adding 20µM DHA to the oocyte maturation medium and/or to embryo*
196 *development medium on the development and quality of bovine in vitro produced embryos.*

197 To assess the effects of DHA supplementation throughout embryo culture, COCs were matured in IVM
198 medium in the absence (IVMC) or the presence of 20 µM DHA and 5 µg/mL carnosine (IVMDHA20) and
199 *in vitro* cultured in IVD medium in the absence (IVDC) or the presence of 20 µM DHA and 5 µg/mL
200 carnosine (IVDDHA20) until the freezing day (D7 or D8) (Figure 4). The period of culture is a period
201 where embryo will undergo cell membrane production. Indeed, cells will proliferate from one to
202 several hundred cells, suggesting a significant need for lipids involved in membrane composition. We
203 hypothesized that the lipid requirement could increase the incorporation of DHA into the membranes
204 and thus increase membrane fluidity.

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Four experimental groups were formed:

- IVM_C / IVD_C (n = 463 COCs)
- IVM_C / IVD_{DHA20} (n = 475 COCs)
- IVM_{DHA20} / IVD_C (n = 482 COCs)
- IVM_{DHA20} / IVD_{DHA20} (n = 480 COCs)

After 7 or 8 days of *in vitro* culture, all grade-1 blastocysts were frozen slowly. After thawing, the cryosurvival of embryos was evaluated by reculturing embryos for 48 h and by recording the re-expansion and hatching rates. At the end of the cryosurvival evaluation, to assess the embryo quality, SOX2 immunostaining was performed, at D9 or D10 depending on the freezing day. To prevent DHA from interfering with sperm and impacting the fertilisation rate, the IVF medium has not been supplemented.

2.7. Statistical Analysis

Data analysis was performed by using SAS software (Statistical Analysis Software release 9.4, SAS, Cary, North Carolina, United states). For qualitative data, multivariate analyses were performed by using logistic regression mixed models with post hoc Tukey–Kramer correction (GLIMMIX procedure), including the fixed effect of the experimental group and the random effect of the embryo production session. For the cryosurvival and immunostaining data, the fixed effect of the freezing day was added to the model as well as its interaction with the experimental group. For quantitative data, the MIXED procedure was performed with post hoc Tukey–Kramer correction, the fixed effect of the experimental condition and the random effect of embryo production session. For the immunostaining data, the fixed effect of the freezing day was added to the model as well as its interaction with the experimental group. The differences were considered significant when *p*-adjusted < 0.05. The data are presented in the tables and figures as the adjusted least squares mean ± standard error of the mean are presented.

231 3. Results and Discussion

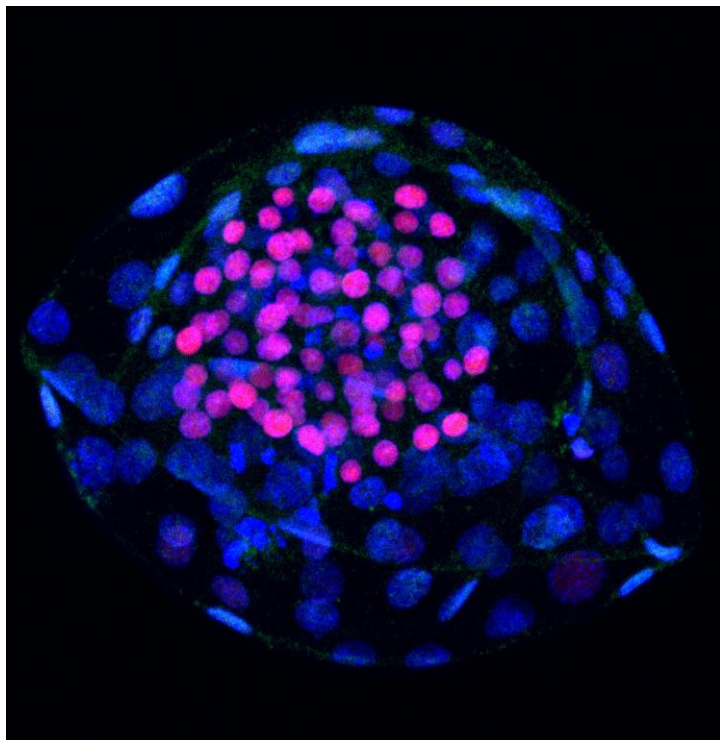
232 3.1. Evolution of the allocation of SOX2 within grade-1 expanded blastocysts between J7 233 and J9.

234 Between Day 7 and Day 9, the number of total cells was 1.8-fold increased. On Day 7, the grade-1
235 expanded blastocysts averaged 183.3 total cells versus 334.6 cells 48h later on Day 9 ($p < 0.0001$).
236 Regarding the number of SOX2 cells, a decrease was observed between Day 7 and Day 9, respectively
237 76.4 cells vs. 50.9 cells, $p = 0.003$ (Figure 1). The increase in the number of total cells and the decrease
238 in the number of SOX2 cells resulted in a decrease in the ratio of SOX2 cells / total cells. Indeed, SOX2
239 cells represented 41.6% of the grade-1 expanded blastocysts on Day 7 and represented only 15.1% of
240 the blastocysts on Day 9. This preliminary experiment highlighted that between Day 7 and Day 9 a
241 strong proliferation of the trophectoderm and a decrease in the number of SOX2 cells is expected.

242 Although SOX2, therefore the presence of pluripotent cells, is required in the embryo so that a
243 gestation is possible, the decrease in the number of cells expressing SOX2 is expected and correspond
244 to the differentiation of the first lineage within the inner cell mass [16].

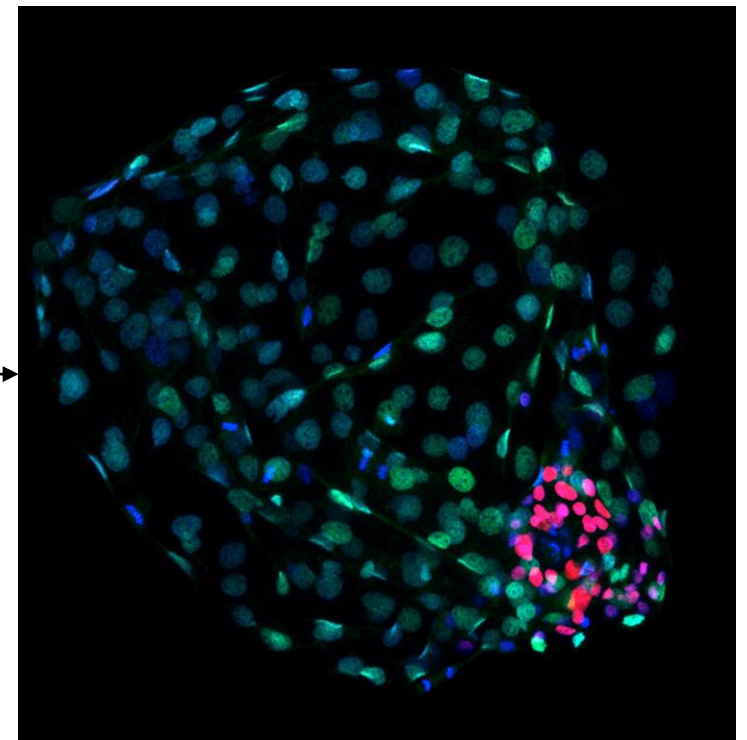
245 The establishment of cell lines during pre-implantation development should be related to a decrease
246 in the expression of some pluripotency factors because some of the cells engage in a developmentally
247 restricted process (TE and the hypoblast). Thus, the decrease in the number of SOX2-positive cells is
248 linked to its restriction to the primary epiblast [16]. In addition, in line with these results, researchers
249 have shown that in mice, the expression of GATA-binding protein 6 (GATA6), which is a known marker
250 of the hypoblast [17], seems to repress SOX2 expression [18]. The decrease in the number of SOX2
251 cells between Day 7 and Day 9 therefore seems physiological. The establishment of these tissues and
252 their allocation during early stages of development in cattle is therefore a quality criterion [3, 19].

MIV_c / CIV_c	Number of BE Q1 at D7	BE with SOX2 positive cells	Total Cell Number	SOX2 positive Cell Number	SOX2 positive /Total Cell (%)
D7	19	100%	183.3 ± 16.9^a	$76,4 \pm 6.7^a$	$41,6 \pm 2.0^a$
D9	18	100%	334.6 ± 17.3^b	$50.9 \pm 6,8^b$	15.1 ± 1.0^b
<i>p value of day effect</i>			<0.0001	0.0028	<0.0001



BEQ1 on D7

48h of culture



BEQ1 on D9

253 3.2. *Free Fatty Acid Receptor 4 (FFAR4) Expression in Cumulus Oocyte Complexes (COCs),*
254 *2-Cell Stage Embryos and Expanded Blastocysts.*

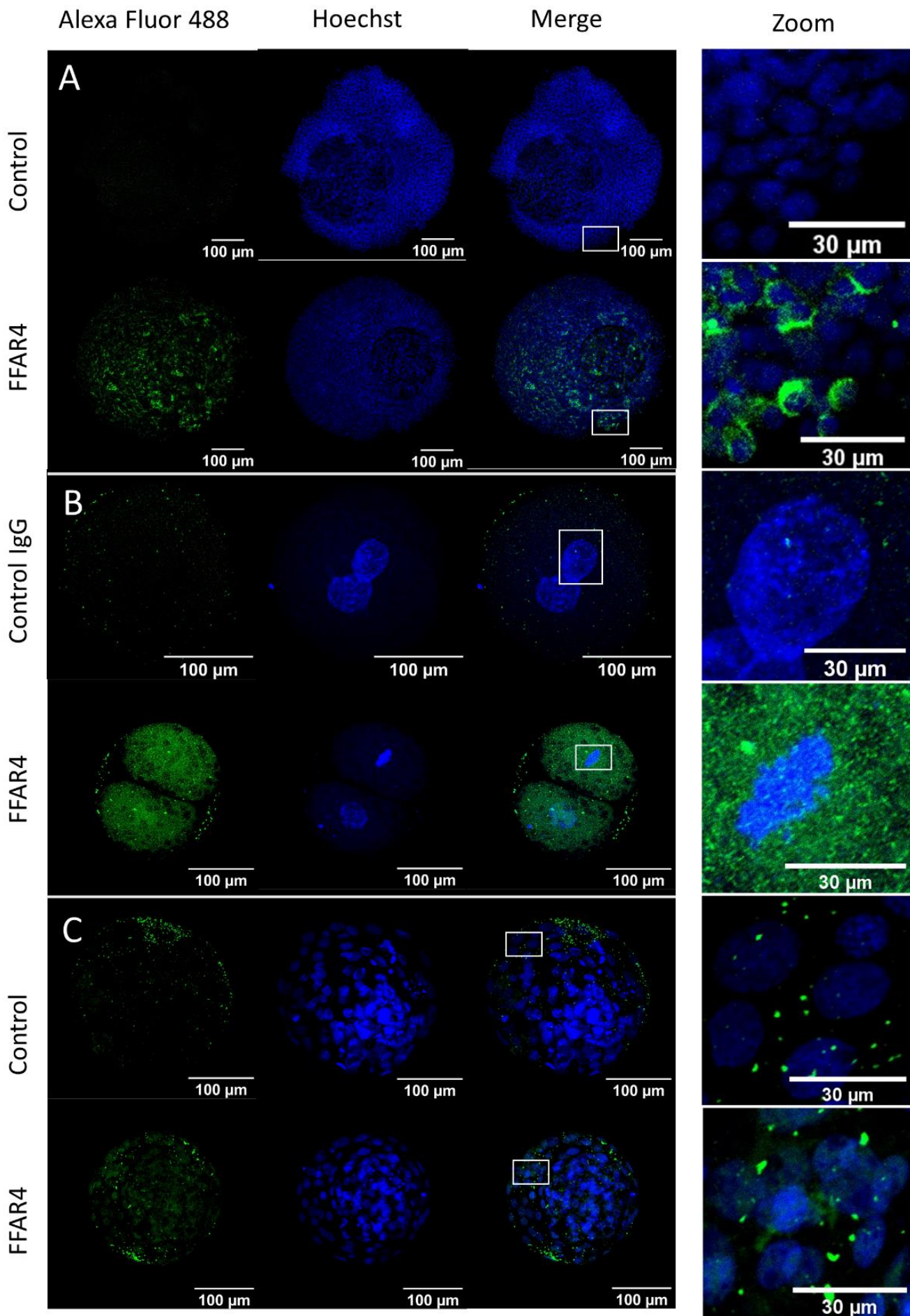
255
256 FFAR4 protein was detected by immunofluorescence in COCs, 2-cell stage embryos and
257 expanded blastocysts by using a customized specific antibody against the bovine FFAR4 protein (**Figure**
258 **2**). The green fluorescence corresponding to FFAR4 was localised preferentially at the periphery of
259 cumulus cells, and cytoplasmic but not nuclear staining was observed (**Figure 2A**). Concerning the 2-
260 cell stage embryos, the staining level was high and evenly distributed between the cytoplasm and
261 nuclei (**Figure 2B**). Regarding the expanded blastocysts, the immunostaining seemed to be localised
262 similarly to the Hoechst labelling, which indicates that FFAR4 was localised in the nucleus, although
263 there was some cytoplasmic staining (**Figure 2C**).

264 We have reported the presence of FFAR4 not only in COCs, as previously described [14], but also in 2-
265 cell embryos and D7 blastocysts. The presence of FFAR4, a membrane receptor able to bind DHA,
266 suggests that DHA might exert an effect throughout oocyte maturation to the early steps of embryo
267 development. It has been reported that FFAR4 is present on bovine oocytes and cumulus cells [14] and
268 that its pharmacological activation by the agonist TUG891 would reproduce in part the effects of DHA
269 supplementation, by increasing the developmental rate [14]. These results suggest that in addition to
270 exerting a mechanical effect on membrane fluidity, DHA could also exert signalling effects up to the
271 blastocyst stage. It is therefore interesting to assess the DHA effects not only during oocyte maturation
272 but also during IVD of bovine embryos to assess its beneficial effect.

273

274 3.3. *Effect of DHA During Oocyte Maturation on the Development and Quality of Bovine In*
275 *vitro Produced Embryos.*

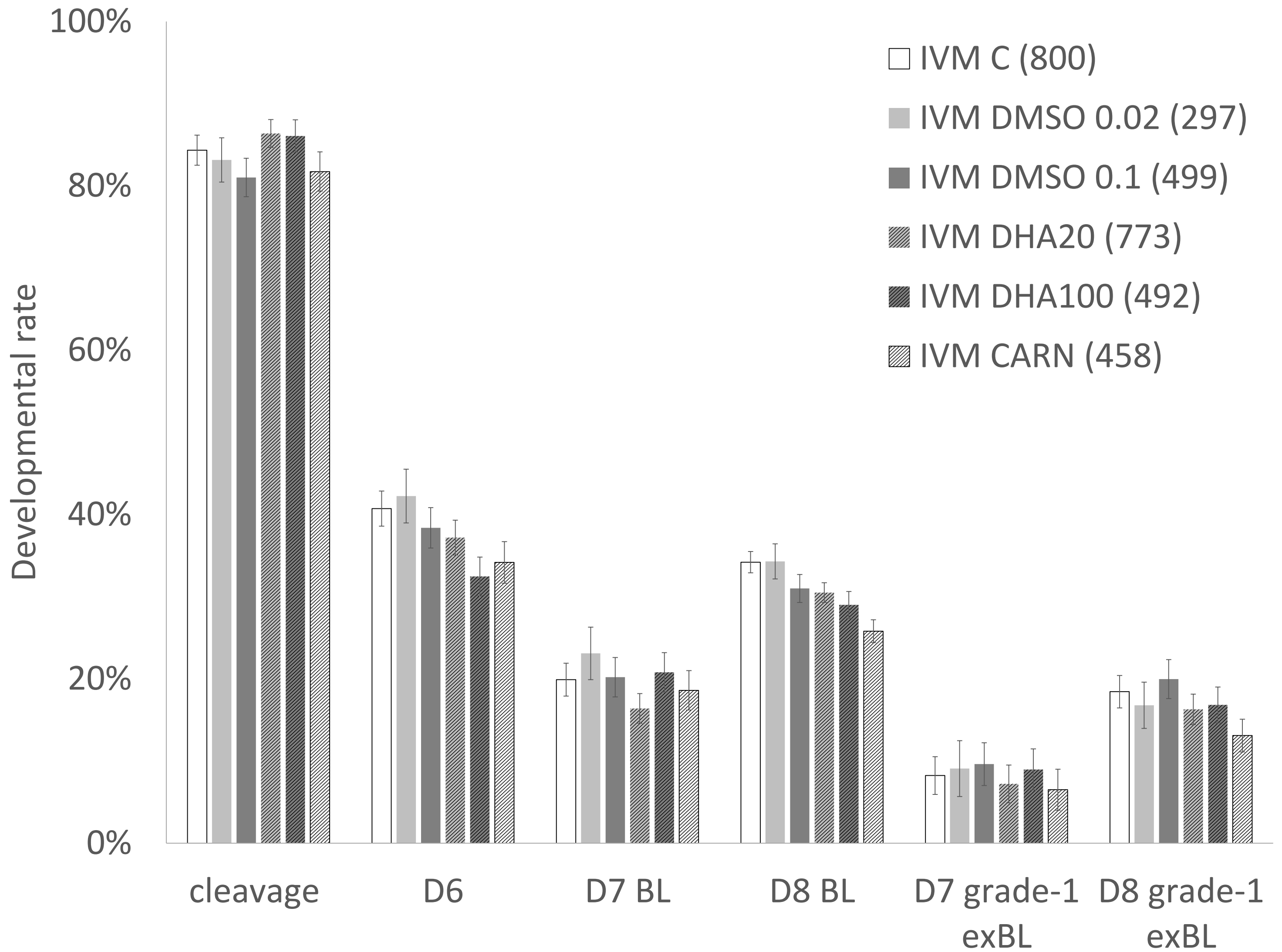
276 The addition of 20 or 100 μ M DHA in the IVM medium did not affect the developmental rate and the
277 quality of expanded blastocysts at D7 and D8. The addition of 5 μ g/mL carnosine or 0.02% or 0.1%



278 dimethyl sulphoxide (DMSO) in the maturation medium did not induce a change in the developmental
279 rate compared with the control (**Figure 3**).

280 These results are partly in agreement with the literature [7, 20]. In fact, concerning the developmental
281 rate, the addition of 12.5 or 25 μM DHA to the IVM of porcine oocytes did not alter the cleavage and
282 blastocyst rates compared with the control [20]. When 1 or 10 μM DHA was added to the IVM medium
283 of bovine oocytes, the cleavage and the blastocyst rate were increased. A higher DHA dose, 100 μM ,
284 decreased both the cleavage and blastocyst rates [7], contrary to the present study. These
285 discrepancies among studies could be partly due to differences between species and in medium
286 compositions such as the use of serum-free media in the present study, as serum is known to
287 accelerate the development kinetics of *in vitro*-produced embryos [21]. In contrast to the literature,
288 the antioxidant used in the present study, carnosine – which is physiologically present in bovine
289 oviductal fluid after ovulation [22], – did not affect the developmental kinetics of embryos. It had been
290 reported that embryos cultured *in vitro* with 5 $\mu\text{g}/\text{mL}$ carnosine showed increased developmental
291 kinetics, particularly between the first cleavage and the compaction of the morula [10]. In fact, the
292 kinetic from 4-cell stage to compaction was shorter when embryos were cultured in the presence of
293 carnosine compared with control (59.9 vs 51.8 hours). We therefore expected to observe an increased
294 developmental rate at D6 (morulae + blastocysts). However, we did not observe an increase at D6 or
295 after. Contrary to our study, carnosine was only added in the IVD medium containing serum; this could
296 suggest that the carnosine effect depends on serum. It would be possible that carnosine and DHA
297 exhibit antagonistic effects since the development rates were always lower when the maturation
298 medium was supplemented with carnosine alone. The addition of an antioxidant to culture media, to
299 counter the oxidative effect of long-chain fatty acids, is used frequently and allows maintaining
300 antioxidant stability in culture system by reducing the intracellular levels of oxygen reactive species
301 [23].

302



303 After thawing, the re-expansion and hatching rates did not differ between groups (**Table 1**). The
 304 presence of carnosine or DMSO at different concentrations did not affect the cryosurvival rate
 305 compared with the control.

306

307 **Table 1.** The re-expansion and hatching rates of expanded grade-1 blastocysts matured *in vitro* in the
 308 presence of 20 or 100 μ M docosahexaenoic acid (DHA), after warming and 24 and 48 h of culture.

	Thawed embryos	Reexpansion rate		Hatched embryos
	n	After 24h	After 48h	After 48h
Groups (<i>p</i> adjusted)		0.338	0.219	0.304
IVM _C	49	91.9 \pm 3.9	91.8 \pm 3.9	69.4 \pm 6.6
IVM _{DMSO 0.02}	32	95.3 \pm 4.6	90.5 \pm 6.4	66.7 \pm 10.3
IVM _{DMSO 0.1}	21	93.7 \pm 4.3	96.9 \pm 3.1	87.5 \pm 5.8
IVM _{DHA20}	52	84.8 \pm 5.0	78.9 \pm 5.7	69.2 \pm 6.4
IVM _{DHA100}	32	77.9 \pm 7.5	78.1 \pm 7.3	59.4 \pm 8.7
IVM _{CARN}	21	90.4 \pm 6.5	85.7 \pm 7.6	76.2 \pm 9.3

309 Please see the Materials and Methods for full details of the groups. The data are presented as the
 310 least squares mean \pm standard error of the mean. Data within the same column and experiment with
 311 different superscripts are statistically different (*p*-adjusted < 0.05).

312

313 There were no differences in the cryosurvival rate, indicating that 20 or 100 μ M of DHA did not affect
 314 the cryosurvival of the expanded grade-1 blastocysts frozen slowly on D7. The addition of carnosine
 315 did not affect the cryosurvival rate, a finding in contrast to the literature. Indeed, authors reported
 316 that 5 μ g/mL carnosine in the culture medium increased the cryosurvival rate 48 h after thawing
 317 compared with the control condition (70.4% vs 40.0%) [10]. It is possible that when combined with
 318 serum, carnosine would allow obtaining better quality embryos, more capable of withstanding the
 319 freezing process. Similar results were obtained when embryos were cultured in medium containing
 320 serum and in the presence of 0.5 μ M of resveratrol, a flavonoid antioxidant. Indeed, the authors
 321 demonstrated that the addition of resveratrol increased the cryosurvival rate (54.2% vs. 68.3%) [24].

322

323 The addition of DHA, DMSO or carnosine in IVM medium did not affect the proportion of embryos with
 324 SOX2-positive cells (*p* = 0.885). The number of cells and SOX2-positive cells did not differ between the

325 groups (**Table 2**). Only the percentage of SOX2-positive cells, i.e. the ratio of SOX2-positive cells to the
 326 number of total cells, differed without an impairment of the total cell number. By performing
 327 immunostaining on D9 embryos, we expected to obtain embryos with more cells and a reduced ratio
 328 of SOX2-positive cells to total cells (see above, Results and Discussion 3.1).

329

330 **Table 2.** The number of cells and SOX2-positive cells in expanded grade-1 blastocysts at day 9 (D9) of
 331 development, 48 h post-thawing.

	n	% Embryos with SOX2-positive cells	Number of cells		Mean ratio
			Total cells	SOX2-positive cells	SOX2-positive cell/total cells
Groups (<i>p</i> adjusted)		0.885	0.263	0.887	<0.0001
IVM _C	35	78.7 ± 9.2	143.1 ± 12.9	19.2 ± 3.4	13.7 ± 1.2 ^a
IVM _{DMSO 0.02}	27	81.5 ± 10.7	165.9 ± 15.9	17.1 ± 4.1	11.2 ± 1.1 ^{a,b}
IVM _{DMSO 0.1}	43	83.8 ± 7.7	162.4 ± 11.4	17.5 ± 3.0	11.0 ± 8.4 ^b
IVM _{DHA20}	23	86.7 ± 8.4	193.8 ± 15.4	16.5 ± 3.9	8.4 ± 0.8 ^c
IVM _{DHA100}	24	71.4 ± 12.5	157.6 ± 17.1	16.4 ± 4.4	10.4 ± 1.1 ^{b,c}
IVM _{CARN}	17	82.8 ± 13.0	169.5 ± 21.8	23.9 ± 5.5	13.3 ± 1.4 ^{a,b}

332 Please see the Materials and Methods for full details of the groups. The data are presented as the least
 333 squares mean ± standard error of the mean. Data within the same column and experiment with
 334 different superscripts are statistically different (p-adjusted < 0.05).

335

336 Regarding the cryosurvival rate, the addition of DHA, DMSO and carnosine in IVM medium did not
 337 show a significant effect on the number of cells and SOX2-positive cells in blastocysts after thawing.
 338 Even though an increase in the number of cells at D9 and a corresponding decrease in the number of
 339 SOX2-positive cells was expected, compared with D7 expanded grade-1 blastocysts (**Figure 1**), no
 340 difference in the number of cells were observed.

341 The addition of 20 μM DHA in the IVM medium appears to be beneficial to embryo development,
 342 allowing a reduction in the percentage of SOX2-positive cells without impairment in the total number
 343 of cells, probably at the origin of cell allocation of the first embryo cell lines.

344 DHA has already been reported to affect cellular functions through several mechanisms: transcription
 345 factor regulation, arachidonic acid production, membrane lipid composition modification or through

346 receptor binding [8]. Indeed, DHA is not only a component of membranes but it can activate receptors
347 such as FFAR4 and peroxisome proliferator-activated receptor (PPAR, part of the β -oxidation pathway)
348 [14, 25].

349 The lower percentage of SOX2-positive cells in DHA-treated embryos could be related to the higher
350 number of cells and, therefore, to a DHA effect on cell proliferation. Indeed, 20 μ M DHA stimulated
351 cell proliferation and steroidogenesis in bovine granulosa cells [9], through FFAR4 and not PPAR for
352 cell proliferation. TUG-891, a FFAR4 agonist, mirrored the effects of DHA on granulosa cell
353 proliferation. Thus, FFAR4 could activate the extracellular signal-regulating kinase (ERK)1/2 pathway
354 and activate the transcription factors linked to cell proliferation [14, 25].

355 Hence, DHA treatment leading to higher rate of SOX2-positive embryos could also suggest that when
356 treated with DHA, embryos have a better transfer potential.

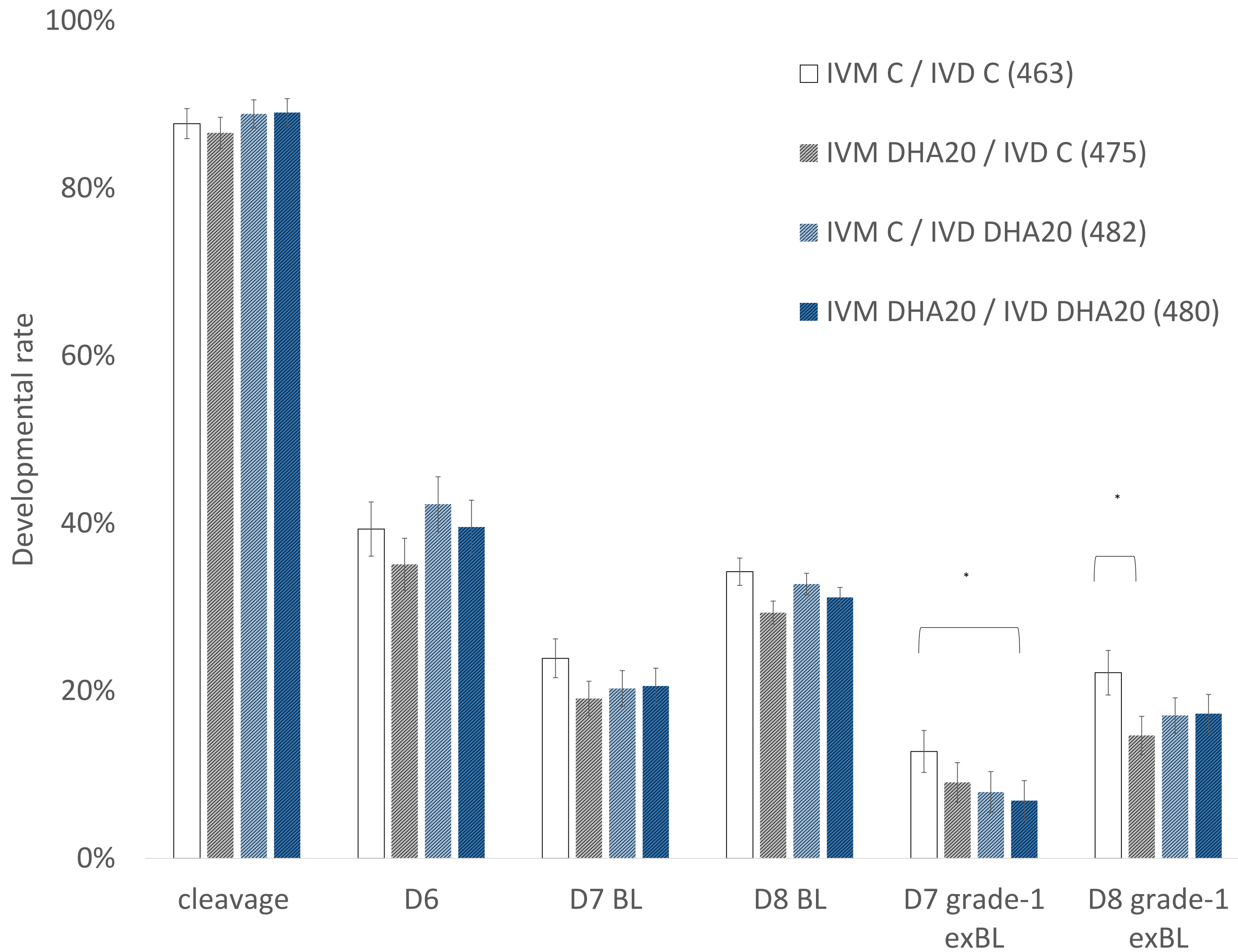
357 Considering all the results, we hypothesize that despite the absence of change in the developmental
358 rate, the more favourable percentage of SOX2-positive cells observed in embryos treated with 20 μ M
359 DHA during IVM could result in a higher pregnancy rate. Future studies should thus focus on embryo
360 transfer of such embryos to confirm this hypothesis.

361

362 *3.4. Effect of Adding DHA to Oocyte Maturation Medium and/or to Embryo Culture*

363 *Medium on the Development and Quality of Bovine In vitro–Produced Embryos*

364 The addition of 20 μ M DHA to oocyte maturation medium and/or to the embryo culture medium did
365 not affect the cleavage rate at D2 or the developmental rate of D6 morulae and blastocysts as well as
366 D7 and D8 blastocysts (**Figure 4**). However, 20 μ M DHA affected embryonic quality at D7 and D8.
367 Indeed, the addition of 20 μ M DHA during the entire development (IVM + IVD) decreased the expanded
368 grade-1 blastocyst rate at D7 compared with the control ($6.9\% \pm 1.2\%$ vs $12.8\% \pm 1.6\%$, p -adjusted =
369 0.041) (**Figure 4**). When added only in the IVM medium, it decreased the expanded grade-1 blastocyst
370 rate at D8 compared with the control ($14.7\% \pm 2.3\%$ vs $22.2\% \pm 2.7\%$, p -adjusted = 0.045) (**Figure 4**).
371 No difference was observed when 20 μ M DHA was only added to the IVD medium.



372 When added during the entire development (IVM + IVD), 20 μ M DHA decreased the quality of the
373 embryos, corresponding to a lower developmental rate of expanded grade-1 blastocysts at D7, without
374 a change on D8, and therefore not explained by a developmental delay. When added during IVM only,
375 20 μ M DHA reduced the embryo quality by decreasing the developmental rate of expanded grade-1
376 blastocysts on D8, without a change on D7. As these results differed from those obtained in the
377 previous experiment (Figure 3) that was performed with more embryos, other experiments would be
378 needed to demonstrate the decreasing effect of DHA on the blastocyst rate. Hence, the difference
379 observed here could be due to the lower number of embryos per groups (475 vs. 773 COCs).

380 Even though DHA acts through several mechanisms, some of which are potentially beneficial to the
381 oocyte or embryo, the concomitant activation of PPAR γ by n-3 PUFA during IVM would induce lipid
382 storage in lipid droplets of COCs and compromise the developmental competence of embryos [25, 26].
383 Thus, PPAR activation could potentially nullify other mechanisms that would potentially mediate the
384 beneficial effects of DHA on embryos.

385 When DHA was added during IVD only, there was no effect on the quality of the embryos, which is in
386 line with previous studies in which 1, 10 or 100 μ M DHA added to the IVD medium of bovine embryos
387 changed neither the cleavage rate nor the D8 blastocyst developmental rate [27]. It is therefore
388 possible that the difference in the results comparing the DHA effects during IVM or IVD might be due
389 to the presence of cumulus cells expressing PPAR. To confirm these hypotheses, it would be interesting
390 to measure the expression of PPAR in expanded blastocysts and to assess the DHA effects while
391 blocking PPAR activation.

392

393 After thawing, the re-expansion and hatching rates did not differ between the groups, indicating that
394 20 μ M DHA in the oocyte maturation medium and/or the embryo culture medium did not affect the
395 cryosurvival of the expanded grade-1 blastocysts at D9 or D10 depending on the freezing day. There
396 was no effect of the freezing day and the experimental group on re-expansion and hatching rates
397 (**Table 3**). However, there was a tendency for a decreased hatching rate in embryos frozen on D8

398 compared with embryos frozen on D7 (61.4% vs 71.5%, $p = 0.079$). A significant interaction between
 399 freezing day and groups was also reported on the re-expansion rate on D10 ($p = 0.045$), likely related
 400 to the fact that IVM_{DHA20} / IVD_{DHA20} corresponded to the lowest re-expansion rate on D7 embryos while
 401 it corresponded to the highest re-expansion rate on D8 embryos.

402

403 **Table 3.** The effects of adding 20 μ M docosahexaenoic acid (DHA) to oocyte maturation medium
 404 and/or to the embryo culture medium on the cryosurvival rate of expanded grade-1 blastocysts, 24
 405 and 48 h post-thawing.

	Freezing day	Thawed embryos n	Re-expansion rate		Hatched embryos
			After 24h	After 48h	After 48h
<i>p value freezing day</i>			0.492	0.856	0.079
D7		161	88.0 \pm 2.6	84.1 \pm 3.7	71.5 \pm 4.3
D8		153	85.3 \pm 2.9	83.3 \pm 3.9	61.4 \pm 4.9
<i>p value groups</i>			0.799	0.626	0.244
IVM _C / IVD _C		85	89.4 \pm 3.2	86.2 \pm 4.1	74.5 \pm 5.0
IVM _{DHA20} / IVD _C		65	84.0 \pm 4.7	79.1 \pm 5.9	63.2 \pm 6.8
IVM _C / IVD _{DHA20}		77	86.2 \pm 4.0	86.3 \pm 4.4	68.0 \pm 6.0
IVM _{DHA20} / IVD _{DHA20}		77	86.7 \pm 3.9	82.4 \pm 5.3	59.9 \pm 6.3
<i>p value freezing day x groups interactions</i>			0.679	0.045	0.169
IVM _C / IVD _C	D7	53	90.6 \pm 4.0	88.6 \pm 4.7	79.4 \pm 5.8
IVM _{DHA20} / IVD _C	D7	41	87.8 \pm 5.1	85.5 \pm 5.9	73.7 \pm 7.2
IVM _C / IVD _{DHA20}	D7	36	88.9 \pm 5.2	89.1 \pm 5.4	75.9 \pm 7.4
IVM _{DHA20} / IVD _{DHA20}	D7	31	83.9 \pm 6.6	67.8 \pm 9.2	53.8 \pm 9.5
IVM _C / IVD _C	D8	42	88.1 \pm 5.0	83.4 \pm 6.2	68.9 \pm 7.5
IVM _{DHA20} / IVD _C	D8	24	79.2 \pm 8.3	70.9 \pm 10.0	51.3 \pm 10.6
IVM _C / IVD _{DHA20}	D8	41	82.9 \pm 5.9	83.0 \pm 6.4	58.9 \pm 8.2
IVM _{DHA20} / IVD _{DHA20}	D8	46	89.1 \pm 4.6	91.3 \pm 4.4	65.7 \pm 7.4

406 Please see the Materials and Methods for full details of the groups. The data are presented as the least
 407 squares mean \pm standard error of the mean. Data within the same columns and experiment with
 408 different superscripts are statistically different (p -adjusted < 0.05).

409

410 In the absence of serum, embryos exhibit slower development [2], which is why, in the present study,
 411 expanded grade-1 blastocysts were frozen on both D7 and D8. There was no difference in cryosurvival
 412 according to the group or the freezing day, except for a tendency for a lower hatching rate in embryos
 413 frozen on D8. This study confirmed that the expanded grade-1 blastocysts at D8 were of lower quality

414 [28, 29] or suggested that they could be more sensitive to the freezing/thawing process compared with
415 morphologically similar embryos observed one day earlier (D7). These results are in line with the
416 literature. Indeed, the best quality embryos (grade 1) would exhibit similar survival rates whether they
417 were frozen on D7 or D8 [30]. Therefore, we did not expect a cryosurvival difference between D7 and
418 D8 expanded grade-1 blastocysts. For this reason, we focused on the hatching rate, which provides
419 insights on further development, because without hatching, pregnancy is impossible. Taken together,
420 these data confirmed that D7 or D8 grade-1 blastocysts would survive similarly [30]. DHA treatment
421 had no effect on cryosurvival when used during IVM, IVD or both.

422 Neither the freezing day nor the experimental group affected the proportion of SOX2-positive embryos
423 (experimental group effect $p = 0.322$, freezing day effect $p = 0.597$) (**Table 4**). While the experimental
424 group did not affect the number of cells and SOX2-positive cells ($p = 0.238$ and $p = 0.358$, respectively),
425 the freezing day has a significant effect (**Table 4**). In fact, D7 embryos exhibited more cells 48 h after
426 thawing compared with D8 embryos (194.4 ± 9.0 vs 166.4 ± 10.0 , $p = 0.039$). Similarly, D7 embryos had
427 more SOX2-positive cells 48 h after thawing compared with D8 embryos (26.5 ± 2.8 vs 16.2 ± 3.0 , $p =$
428 0.006). The percentage of SOX2-positive cells was affected by both the freezing day and the
429 experimental group. D7 embryos exhibited a higher SOX2-positive cell percentage compared with D8
430 embryos ($13.06\% \pm 1.27\%$ vs $9.55\% \pm 1.0\%$, $p < 0.0001$). Regarding the experimental group effect, the
431 percentage of SOX2-positive cells was reduced in the IVM_c / IVD_{DhA20} group compared with the control
432 ($8.4\% \pm 0.9\%$ vs $10.9\% \pm 1.1\%$, $p < 0.0001$). When DHA was supplemented during IVM or the entire
433 culture process (IVM + IVD), the percentage of SOX2-positive cells was higher compared with the
434 control group ($13.1\% \pm 1.4\%$, $p = 0.002$, and $13.3\% \pm 1.4\%$, $p = 0.0004$, respectively) (**Table 4**). There
435 was a significant interaction between the freezing day and the experimental group effect for the SOX2-
436 positive cell percentage, meaning that the results could vary among experimental groups depending
437 on the freezing day. This significant interaction means that depending on the experimental batch or
438 the freezing day, the results do not vary in the same way. The post-thaw culture may be suboptimal
439 and not suitable for the development of more demanding epiblast cells than trophectoderm cells [31-

440 33]. Indeed it would seem that the addition of 10% serum and 0.5% of glucose is beneficial for the
 441 proper development of hatched embryos [33] whose glycolytic activity and glucose uptake increase
 442 [34, 35]. The addition of DHA into the IVD medium of these embryos could be a way to protect ICM
 443 cells from the freezing/thawing process.

444 **Table 4.** The number of cells and SOX2-positive cells in expanded grade-1 blastocysts at day 9 (D9) or
 445 D10 of development, depending on the freezing day (D7 or D8), 48 h post-thawing.

	n	Freezing day	Number of cells		mean ratio	
			% SOX2-positive Embryos	Total cells	SOX2-positive cells	SOX2/Total cells
Freezing day (<i>p adjusted</i>)			0.597	0.039	0.006	< 0.0001
D7	111		66.1 ± 7.4	194.4 ± 9.0 ^a	26.5 ± 2.8 ^a	13.06 ± 1.27 ^a
D8	92		62.1 ± 8.1	166.4 ± 10.0 ^b	16.2 ± 3.0 ^b	9.55 ± 1.0 ^b
Groups (<i>p adjusted</i>)			0.322	0.238	0.358	< 0.0001
IVM _C / IVD _C	66		63.3 ± 8.4	191.6 ± 10.6	20.6 ± 3.2	10.9 ± 1.1 ^b
IVM _{DHA20} / IVD _C	46		59.7 ± 10.3	169.0 ± 15.7	23.2 ± 4.5	13.1 ± 1.4 ^a
IVM _C / IVD _{DHA20}	54		75.3 ± 7.6	196.7 ± 10.9	16.6 ± 3.3	8.4 ± 0.9 ^c
IVM _{DHA20} / IVD _{DHA20}	37		56.7 ± 10.7	164.4 ± 15.7	25.0 ± 4.5	13.3 ± 1.4 ^a
Freezing day x Groups interactions (<i>p adjusted</i>)			0.193	0.157	0.134	< 0.0001
IVM _C / IVD _C	38	D7	62.8 ± 10.0	191.2 ± 13.2	23.3 ± 3.9	12.0 ± 1.2 ^b
IVM _{DHA20} / IVD _C	32	D7	74.8 ± 9.2	197.8 ± 14.0	25.9 ± 4.0	12.0 ± 1.2 ^b
IVM _C / IVD _{DHA20}	27	D7	68.0 ± 10.6	194.2 ± 16.2	18.4 ± 4.6	9.6 ± 1.1 ^c
IVM _{DHA20} / IVD _{DHA20}	14	D7	57.5 ± 15.0	194.7 ± 26.0	38.4 ± 7.2	20.5 ± 2.1 ^a
IVM _C / IVD _C	28	D8	63.8 ± 10.9	192.0 ± 16.7	18.0 ± 4.7	9.3 ± 1.0 ^{c,d}
IVM _{DHA20} / IVD _C	14	D8	42.5 ± 14.9	140.2 ± 28.1	20.4 ± 7.7	14.2 ± 1.8 ^b
IVM _C / IVD _{DHA20}	27	D8	81.4 ± 8.6	199.2 ± 14.7	14.8 ± 4.2	7.4 ± 0.8 ^d
IVM _{DHA20} / IVD _{DHA20}	23	D8	55.8 ± 12.7	134.1 ± 17.8	11.6 ± 5.0	8.4 ± 1.0 ^d

446 Please see the Materials and Methods for full details of the groups. The data are presented as the least
 447 squares mean ± standard error of the mean. Data within the same columns and experiment with
 448 different superscripts are statistically different (*p*-adjusted < 0.05).
 449

450 The freezing day affected the quality of embryos. Indeed, embryos frozen on D7 contained more cells,
 451 suggesting a higher quality embryo, alongside more SOX2-positive cells. In this case, the reduced SOX2-
 452 positive cell number and percentage reported in D8 embryos was associated with a reduced number
 453 of cells, despite being one day older, and were therefore not indicative of better quality. These D8

454 embryos could be of lower quality and could have more difficulties facing the freezing protocol
455 compared with D7 embryos. Fewer cells could be the consequence of the destruction of some of the
456 cells both in the trophectoderm and ICM [31].

457 Considering both D7 and D8 embryos, although the number of total cells and SOX2 cells were not
458 different between the control and the group with DHA supplementation during only IVD, respectively
459 (20.6 vs. 16.6 cells and 191.6 vs. 196.7 cells), the percentage of SOX2-positive cells was significantly
460 decreased, suggesting accelerated development [15, 36]. On the contrary, when embryos were
461 supplemented with DHA during IVM or during IVM + IVD, embryos exhibited fewer cells and an
462 increased percentage of SOX2-positive cells, suggesting slower development. SOX2 overexpression
463 after D9 could reflect a suboptimal embryo development [36]. Therefore, DHA supplementation during
464 the entire culture process (IVM + IVD) could be detrimental to proper development. An embryo
465 without SOX2 expression would not be able to lead to a gestation, and future studies should focus on
466 a transfer study to assess this issue. Even though the developmental rates are similar, DHA could
467 increase the percentage of embryos expressing SOX2 and, consequently, the percentage of embryos
468 that would lead to gestation. Failure of epiblast development has been described *in vitro* and *in vivo*,
469 and several authors reported that between 23% and 65% of *in vitro* produced and transferred embryos
470 no longer had an embryonic disc, as reviewed by Ramos-Ibeas et al. [37]. In the present study,
471 regardless of the freezing day, 27.4% to 43.3% of the embryos lacked SOX2 cells and therefore epiblast,
472 after thawing and 48h of culture (Table 4).

473

474 3.5. Limitations of the study

475 We assessed the effect of DHA supplementation on *in vitro* oocyte and embryo quality. Nevertheless,
476 DHA was combined with both DMSO and carnosine, an antioxidant to limit the oxidation of DHA and
477 potentiate its effects, and thus we have reported the effect of this combination in the present study.
478 DMSO is regularly used in media for the production and the cryopreservation of bovine embryos [9,
479 14, 38, 39]. Due to its cellular toxicity and its impact on the maintenance of pluripotency [40], DMSO

480 should only be used at a low concentration [41], which was the case in the present study (0.02 or 0.1%).
481 As mentioned before, the real embryo quality assessment is performed through embryo transfer
482 studies. It would have been interesting to carry out these transfers to verify whether the improvement
483 suggested by SOX2 positive cells staining in the present study would lead to an improvement in the
484 pregnancy rates. Moreover, the numbers of embryos, especially in the last experiment were too low
485 to enable to observe significant difference when comparing SOX2 positive embryo % in the different
486 conditions. The major difficulty is to have enough statistical power to highlight a 12% difference (63%
487 vs. 75%), requiring at least 130 embryos per experimental group. Nevertheless, It is important to
488 further study the addition of DHA in IVM-IVD medium to assess whether it could enhance the % of
489 SOX2 positive embryos (embryos containing pluripotent cells) and, therefore, the pregnancy rate.

490

491 4. Conclusion

492 In this study we used SOX2 immunostaining to study embryonic quality after exposure of oocytes and
493 embryos to DHA, during IVM and/or IVD. SOX2-positive cell evaluation of grade-1 expanded blastocysts
494 suggested that DHA might improve the embryo quality as well as the percentage of embryos that
495 express SOX2, and therefore the embryos able to achieve a gestation. Future studies should confirm
496 these results by carrying out embryo transfers to assess the beneficial effects of DHA supplementation.

497

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504

505 **Conflicts of Interest:** The authors declare no competing interests.

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631

632 **Figure Legends**

633

634 **Figure 1.** Evolution of the allocation of SOX2 within grade 1 blastocysts between J7 and J9. SOX2 was
635 immunolabelled in pink, CDX2 in green and all nuclei in blue.

636

637 **Figure 2.** Intracellular localisation of the free fatty acid receptor-4 (FFAR4) protein based on
638 immunofluorescence of (A) a cumulus oocyte complex, (B) a 2-cell embryo and (C) an expanded
639 blastocyst. Rabbit IgG was used as the negative control (Control IgG) for each developmental stage.
640 Hoechst staining (in blue) indicates nuclei.

641

642 **Figure 3.** The developmental rate of bovine oocytes treated with 20 or 100 μ M docosahexaenoic acid
643 (DHA). The cleavage rate was checked on day 2 (D2) and the developmental rate on D6 (morulae +
644 blastocysts [BL]), D7 (BL and expanded grade-1 blastocysts [exBL]) and D8 (BL and exBL). The
645 developmental rate is presented as the least squares mean \pm standard error of the mean of 11
646 independent experiments. The total number of cumulus oocyte complexes used per condition is noted
647 in the figure key. Please see the Materials and Methods for full details of the groups. Differences were
648 considered significant when p -adjusted < 0.05 .

649

650 **Figure 4.** The effects of adding docosahexaenoic acid (DHA) to the oocyte maturation medium and/or
651 to the embryo culture medium on the developmental rate. The cleavage rate was checked at day 2
652 (D2), and the developmental rate was checked at D6 (morulae + blastocysts [BL]), D7 (BL as well as
653 expanded grade-1 blastocysts [exBL]) and D8 (BL as well as exBL). The developmental rate is presented
654 as the least squares mean \pm standard error of the mean of 11 independent experiments. The number
655 of COCs used is been noted in the figure legend. Please see the Materials and Methods for full details
656 of the groups. Differences were considered significant at p -adjusted < 0.05 .