Accepted Manuscript published as REP-18-0561.R2. Accepted for publication: 25-Apr-2019
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1 # Gene expression and metabolic response of bovine oviduct epithelial cells to the
2 # early embryo

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23 **Short title**: Embryo effect on oviduct epithelial cells
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26

27 **Abstract**

28 During its journey through the oviduct, the bovine embryo may induce
29 transcriptomic and metabolic responses, via direct or indirect contact, from bovine
30 oviduct epithelial cells (BOEC). An *in vitro* model using polyester mesh was established,
31 allowing the study of the local contact during 48 h between a BOEC monolayer and early
32 embryos (2- or 8-cell stage) or their respective conditioned media (CM). The
33 transcriptomic response of BOEC to early embryos was assessed by analyzing the
34 transcript abundance of *SMAD6, TDGF1, ROCK1, ROCK2, SOCS3, PRELP* and *AGR3*
35 selected from previous *in vivo* studies and *GPX4, NFE2L2, SCN9A, EPSTI1, IGFBP3*
36 selected from *in vitro* studies. Moreover, metabolic analyses were performed on the media
37 obtained from the co-culture. Results revealed that presence of early embryos or their CM
38 altered the BOEC expression of *NFE2L2, GPX4, SMAD6, IGFBP3, ROCK2 and SCN9A.*
39 However, the response of BOEC to 2-cell embryos or their CM was different from that
40 observed to 8-cell embryos or their CM. Analysis of energy substrates and amino acids
41 revealed that BOEC metabolism was not affected by the presence of early embryos or by
42 their CM. Interestingly, embryo metabolism before embryo genome activation (EGA)
43 seems to be independent of exogenous sources of energy. In conclusion, this study
44 confirms that early embryos affect BOEC transcriptome and BOEC response was embryo
45 stage-specific. Moreover, embryo affects BOEC via a direct contact or via its secretions.
46 However transcriptomic response of BOEC to the embryo did not manifest as an
47 observable metabolic response.

48 **Introduction:**

49 During its four-day journey through the oviduct (Hackett *et al.* 1993), the bovine
50 embryo undergoes important morphological and transcriptional events such as the first

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51 mitotic cell divisions, the timing of which is reflective of developmental competence
52 (Lonergan *et al.* 1999) and embryonic genome activation (EGA) involving a switch from
53 dependence on transcripts stored in the oocyte to *de novo* transcription (Memili and First
54 2000). There is convincing evidence demonstrating a positive influence of the oviduct on
55 embryo development in terms of embryo cryotolerance, gene expression and
56 developmental competence (Enright *et al.* 2000; Lazzari *et al.* 2002; Rizos *et al.* 2002;
57 Lonergan *et al.* 2003). However, the embryo effect on the oviduct is less studied. In mice,
58 the presence of embryos in the oviduct increased the expression of specific genes such as
59 thymosin beta-4, ribosomal protein L41 and nonmuscle myosin light chain 3 (Lee *et al.*
60 2002). In pigs, Almiñana *et al.* (2012) reported that the presence of embryos reduced the
61 expression of genes related with immune function. Also, transforming growth factor-
62 alpha, transforming growth factor-beta-binding protein 2 and atrial natriuretic factor
63 receptor-like were up regulated in the presence of 4-cell porcine embryos (Chang *et al.*
64 2000).

65 It is likely that the effect of any putative embryo-derived signals on the cells of
66 the oviduct would be magnified in litter-bearing species due to the presence of multiple
67 embryos in the oviduct. That notion is supported by a previous study from our group in
68 cattle, a mono-ovulatory species, which failed to detect a transcriptomic response of the
69 oviduct to the presence of a single embryo, while a response was observed after the
70 transfer of multiple embryos (Maillo *et al.* 2015). In the horse, the presence of a single
71 embryo altered the oviduct transcriptome (Smits *et al.* 2016), when epithelial cells were
72 collected locally from the ampullary-isthmic junction. Combined, these findings suggest
73 that the presence of a single embryo in the oviduct may induce a very local effect on
74 oviduct transcriptome which is difficult to detect. Furthermore, *in vivo* studies remain
75 challenging, due to the relative inaccessibility of the oviduct. Bovine oviduct epithelial

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76 cells (BOEC) have been cultured *in vitro* in many systems, as monolayers, in perfusion
77 chambers, in suspension, in polarized or in three-dimensional (3D) systems (reviewed by
78 Maillo *et al.* 2016). Recent *in vitro* culture strategies have mimicked the morphological
79 and functional changes occurring in the oviduct epithelium by using an air-liquid interface
80 (Chen *et al.* 2017; Chen *et al.* 2018) or by using the state-of-the-art oviduct-on-a-chip
81 platform (Ferraz *et al.*, 2018). However, considering the complexity of studies aiming to
82 understand the regulatory mechanisms controlling embryo-maternal communication, the
83 simple BOEC *in vitro,* cultured as a monolayer might provide a good starting point to
84 study embryo signals. For instance, Schmaltz-Panneau *et al.* (2014) identified
85 differentially expressed genes related to the immune system and interferon signaling by
86 co-culturing early embryos for 8 days on a 13-day old BOEC monolayer (Schmaltz-
87 Panneau *et al.* 2014). Also, in a recent study carried out by our group using the BOEC
88 monolayer model, we reported that bovine embryo-oviduct interaction *in vitro* reveals an
89 early cross talk mediated by bone morphogenetic protein (BMP) signaling (García *et al.*
90 2017).

91 In order to test the hypothesis that the early embryo induces transcriptional and
92 metabolic changes in BOEC, we established an *in vitro* co-culture system allowing both
93 local and temporal contact between early bovine embryos and oviduct isthmus epithelial
94 cells, where development occurs *in vivo*. The objectives were: (i) to assess the gene
95 expression response of BOEC to early embryos and to test whether it is due to a contact-
96 dependent signal or the result of BOEC interaction with embryo secretions; and (ii) to
97 examine the metabolic changes of BOEC in the presence or absence of early embryos.

98 **Materials and methods**

99 ***Chemicals***

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100 All reagents were purchased from Sigma Chemical Química S.A Company (Madrid,
101 Spain) unless otherwise stated.

102 ***Bovine oviduct epithelial cell isolation and in vitro culture***

103 Bovine oviducts at the early luteal phase (corresponding to Days 3–5 of the estrous cycle)
104 were obtained from slaughtered heifers and selected based on ovarian morphology,
105 according to Ireland *et al*. (1980). Oviduct epithelial cell isolation was performed as
106 described previously (García *et al.* 2017). Cells were cultured at a final concentration of
107 2 x 10^6 cells/mL with TCM 199 supplemented with 10% fetal calf serum (FCS), 2.5%
108 gentamycin and 1% amphotericin, in four-well plates at 38.5 ºC in an atmosphere of 5%
109 CO_2, 20% O_2 and saturated humidity until confluence (6-7 Days). Half of the media was
110 renewed every 48 h. Twenty-four hours before starting the co-culture with embryos or
111 their conditioned medium (CM), the cell culture media was replaced with Synthetic
112 Oviduct Fluid supplemented with amino acids (SOFaa) + 5% FCS (see experimental
113 design section for more details and Fig 2).

114 ***Immunocytochemistry***

115 The epithelial nature of the cultured cells was confirmed by immunocytochemical
116 analysis using antibodies anti-bovine-pancadherin (C1821), anti-bovine-pancytokeratin
117 (C2931) and anti-bovine-vimentin (V2258), as previously described (Lopera-Vasquez *et*
118 *al.* 2016) (Fig 1A).

119

120 ***In vitro production of embryos and their conditioned media***

121 Immature cumulus oocyte complexes recovered by aspirating follicles (2-8 mm in
122 diameter) from ovaries of heifers slaughtered at a local abattoir were submitted to *in vitro*
123 maturation and fertilization, as described previously (García *et al.* 2017). Approximately
124 18 to 22 h after insemination, presumptive zygotes were completely denuded of cumulus

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125 cells by vortexing and groups of 50 were initially cultured in 500 μL of SOFaa
126 supplemented with 5% of FCS in a four-well dish under an atmosphere of 5% CO_2, 5%
127 O_2 and 90% N_2 at 38.5 °C. Embryos at the 2- and 8- cell stage were selected at 31 and 52
128 hours post-insemination (hpi), respectively, and then randomly cultured either in groups
129 of 50 for 48 h to produce embryo CM or co-culture with BOEC (see Experimental design,
130 Fig 2).

131 ***Mesh preparation and co-culture condition***

132 In order to limit the area of contact between the embryos and the BOEC monolayer, a
133 nontoxic woven polyester mesh (Sefar Petex; Sefar, Bury, Lancashire, UK) was used as
134 described previously (García *et al.* 2017). Grids with size of 41x41 openings covering an
135 area of 121 mm^2, fitting perfectly inside the well of a Nunc 4-well dish, were used. First,
136 a template grid containing delimited marked areas of 50 openings [(7x7)+1] was placed
137 outside underneath each well of a four-well dish (Fig. 1B). A second mesh square of the
138 same size was washed once with 70% ethanol and three times with PBS followed by
139 SOFaa media, and then it was introduced into the well, so that the external and internal
140 meshes overlapped (Fig. 1B). During the co-culture, 50 embryos (at the 2- or 8- cell stage)
141 were placed inside the BOEC well, over the central marked area. After 48 h of co-culture,
142 BOEC were collected from the marked area directly beneath the embryos and as well as
143 from other areas of the well that were not in direct contact with the embryos (Fig 1B) by
144 mechanical scraping with a micropipette tip and snap frozen in liquid nitrogen and
145 maintained at -80°C until gene expression analysis.

146

147 ***RNA isolation and reverse transcription***

148 mRNA from each experimental group of BOEC was extracted from 4 biological
149 replicates using Dynabeads mRNA Direct Extraction Kit (Dynal Biotech, Oslo, Norway),

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150 following the manufacturer's instructions with minor modifications as described
151 previously (Bermejo-Alvarez *et al.* 2008). After 10 min of incubation in lysis buffer with
152 Dynabeads, poly(A) RNA attached to the Dynabeads was extracted with a magnet and
153 washed twice in washing buffer A and washing buffer B. RNA was eluted with Tris-HCl.
154 Immediately after extraction, the reverse transcription reaction was performed as
155 recommended by the manufacturer (Epicentre Technologies Corp, Madison, WI, USA).
156 Briefly, oligo-dT (0.2 μM) and random primers (0.5 μM) were added to the RNA and
157 were then heated for 5 min at 70ºC to denature the secondary RNA structure. Next, the
158 tubes were incubated at 25°C for 10 min to promote the annealing of random primers.
159 Then, the RNA was reverse-transcribed for 60 min at 37ºC in a final volume of 40 μl
160 containing 0.375mM dNTPs (Biotools, Madrid, Spain), 6.25U RNasin RNAse inhibitor
161 (Promega, Madison,WI, USA), 10X MMLV-RT buffer with 8mM dithiothreitol, and 5U
162 MMLV high performance reverse transcriptase (Epicentre Technologies Corp, Madison,
163 WI, USA), followed by incubation at 85 °C for 5 min to inactivate the RT enzyme.

164 ***Gene expression analysis by quantitative real-time polymerase chain***

165 The mRNA expression levels of the selected genes were determined by real-time
166 quantitative reverse transcription polymerase chain reaction (RT-qPCR) using specific
167 primers designed with Primer-BLAST software
168 (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to span exon-exon boundaries when
169 possible (Table 1). Primers were previously validated for adequate primer efficiency and
170 specificity of their PCR products was confirmed by electrophoresis on a 2% agarose gel.
171 All target genes showed efficiencies between 95 and 100% and correlation coefficients
172 close to 1.0.

173 All mRNA transcripts were quantified in duplicate. Each RT-qPCR reaction was
174 performed in a final volume of 20 μL, containing 0.25 mM of forward and reverse

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175 primers, 10 μL of GoTaq RT-qPCR Master Mix (Promega) and 2 μL of each cDNA
176 sample derived from BOEC (≈60ng/μL) using a Rotorgene 6000 Real Time Cycler
177 (Corbett Research, Sydney, Australia) and SYBR Green as double stranded DNA-specific
178 fluorescent dye.
179 Relative expression levels were quantified by the comparative cycle threshold (ΔΔCT)
180 method (Schmittgen and Livak 2008). Values were normalized using two housekeeping
181 genes (*H2AFZ* and *ACTG1*). Fluorescence was acquired in each cycle to determine the
182 threshold cycle during the log-linear phase of the reaction at which fluorescence increased
183 above background for each sample. According to the comparative CT method, the ΔCT
184 value was determined by subtracting the mean CT value of the two housekeeping genes
185 for each sample from each gene CT value of the sample. Calculation of ΔΔCT involved
186 using the highest sample ΔCT value (i.e. the sample with the lowest target expression) as
187 an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in the
188 relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT}$ (Livak
189 and Schmittgen 2001).

190 ***Metabolic Analysis***

191 *Glucose Lactate and Pyruvate consumption:*
192 Concentrations of glucose, lactate and pyruvate in spent medium following culture of
193 BOEC in the presence or absence of embryos were measured using fluorometric assays
194 based on those described by (Gardner and Leese 1990), but with modifications (Guerif *et*
195 *al.* 2013). All values were expressed as mM and were relative to a blank control to
196 calculate consumption rate.

197 *Amino Acid Profiling:*
198 The analysis of 18 amino acids in spent medium was performed using high-performance
199 liquid chromatography (HPLC) as previously described (Houghton *et al.* 2002). All

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200 values were expressed in μM and were relativized to blank control to calculate
201 consumption rate.

202 ***Experimental design***

203 *Experiment 1*: *Direct and indirect effects of the embryo on the BOEC gene expression*

204 An *in vitro* co-culture system was established to allow a localized and temporal contact
205 between BOEC and early bovine embryos. Isthmic epithelial cells obtained from a pool
206 of three post-ovulatory stage oviducts on each day (day=replicate, 4 replicates) were
207 cultured until confluence/monolayer for 6 days. Based on our previous studies (García *et*
208 *al*. 2017; Barrera *et al*. 2017), where the highest proportions of 2- and 8-cell embryos
209 were observed at 31 and 52 hpi respectively, in this experiment embryos at the 2- and 8-
210 cell stages were generated by IVF and removed from culture at 31 or 52 hpi respectively
211 to be cultured during 48h to either generate fresh CM (4 replicates) or co-cultured with
212 BOEC (4 replicates) (Fig 2A). BOEC were cultured for 48 h as follows: (1) directly
213 beneath and in direct contact with 50 embryos at the 2- or 8- cell stage ($BOEC^{+}$); (2) in
214 the same well as 50 embryos at the 2- or 8-cell stage but not in direct contact with the
215 embryos ($BOEC^{-}$); (3) with CM from embryos at the 2- or 8-cell stage ($BOEC^{CM}$); (4)
216 without embryos or CM (control, BOEC) (Fig. 2B). Following 48 h of culture, BOEC
217 were collected from the marked area from all groups by mechanical scraping to perform
218 RT-qPCR. The mRNA abundance of genes that had previously been shown to be affected
219 by the presence of embryos from an *in vivo* study (Maillo, *et al*., 2015: *SMAD6, TDGF1*,
220 *ROCK1*, *ROCK2, SOCS3*, *PRELP*, *AGR3, NFE2L2*) and from *in vitro* studies (Schmaltz-
221 Panneau *et al.* 2014; 2015*: GPX4*, *SCN9A*, *EPSTI1, IGFBP3*) were analyzed as described
222 above.

223 *Experiment 2*: *Effect of early embryo on BOEC metabolism*

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224 After 48 h of co-culture, the media obtained from the previous experimental groups
225 (BOEC^{+}, BOEC^{CM}, BOEC) as well as the spent media obtained from embryos cultured
226 alone (EMB) were collected to assess the metabolic changes that may result from
227 interaction of BOEC with the embryos or with their CM, by analyzing the energy
228 substrate and amino acid composition. As blank control, embryo/BOEC-free media was
229 treated in the same manner as the experimental groups (Fig. 2C).

230 ***Statistical analysis***

231 Statistical analysis was performed with the SigmaStat software package (Jandel
232 Scientific, San Rafael, CA). The Kolmogorov-Smirnov test was applied to assess data
233 normality. Differences in embryo development were analyzed using chi-square.
234 Differences in the relative mRNA abundance levels and metabolic results were analyzed
235 using one-way analysis of variance (ANOVA) followed by Holm–Sidak post hoc test,
236 when applicable, to determine statistical differences between the experimental groups.
237 When normality test failed Kruskal-Wallis, a non-parametric test was conducted. Values
238 were considered significantly different when the *P* value was lower than 0.05

239 **Results**

240 ***Effect of BOEC co-culture on early embryo development in vitro:***
241 Cleavage rates at 31 hpi (2-cell=774/1177) and 52 hpi (8-cell=652/1197) were
242 65.5%±2.5%; 54.0±7.4% respectively. After 48 h of co-culture, no differences were
243 found in the percentage of 2-cell embryos developed to 8-cell stage between embryos co-
244 cultured with (57.0±1.4%) or without BOEC (65.0± 2.6%). Similarly, the percentage of
245 8-cell embryos developed to 16-cell stage was not different between both groups (51.0±
246 4.2% vs 57.7± 1.9% for embryos cultured with or without BOEC respectively).

247 ***Experiment 1:*** *Direct and indirect effects of the embryo on the BOEC gene expression*

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248 The presence of 50 2-cell embryos, in direct contact, or their CM decreased the relative
249 abundance of *GPX4*, *ROCK2* and *SCN9A* in BOEC in comparison with the control group
250 (P<0.05). Abundance of *SMAD6* was decreased in the presence of embryos, irrespective
251 of direct contact, but was not altered following exposure to CM. The only difference
252 between cells cultured in direct or indirect contact with embryos was observed for
253 *NFE2L2* gene, which decreased in cells directly cultured with embryo or their CM in
254 comparison with the culture in indirect contact or control groups. Culture of BOEC with
255 CM increased expression of *IGFBP3* (P<0.05) compared with the control, but no
256 difference was observed with the rest of groups (Fig 3A). The remaining genes were
257 unaffected by treatment (P>0.05) (Fig 3A).
258 Co-culture in the presence of 8-cell embryos or their CM, revealed a different expression
259 pattern of many of the analyzed genes to that observed with 2-cell embryos. *PRELP* was
260 decreased (P<0.05) in the direct or indirect presence of 8-cell embryos or their CM in
261 comparison with the control group. Also, BOEC directly exposed to 8-cell embryos or
262 their CM exhibited decreased expression of *SOCS3* and increased expression of *SCN9A*
263 in comparison with BOEC cultured in indirect contact with embryos and with the control
264 (P<0.05). *GPX4* was only decreased in BOEC cultured directly with embryos in
265 comparison with the other groups. Furthermore, the expression level of *NFE2L2* was
266 increased (P<0.05) in cells cultured with CM compared to the control but no differences
267 were observed with the rest of groups. Also, abundance of *ROCK1* and *ROCK2* was
268 increased (P<0.05) in cells cultured with CM in comparison to the control or to the direct
269 culture with embryos but it was similar to the indirect contact group. *SMAD6* is the only
270 gene that maintained the pattern of expression between BOEC co-cultured with either 2-
271 or 8- cell embryos, where it decreased in BOEC cultured in direct or indirect contact with

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272 embryos in comparison with the rest of groups (P< 0.05) (Fig 3B). The remaining genes
273 did not exhibit an alteration of their expression due to treatment (P> 0.05) (Fig 3B)

274 ***Experiment 2:*** *Effect of early embryo on BOEC metabolism*

275 *Glucose, Lactate and Pyruvate consumption:*

276 Glucose, lactate and pyruvate consumption by BOEC was not affected by the presence of
277 2- cell or 8-cell embryos (Fig 4 A and B). Indeed, a similar consumption of glucose and
278 pyruvate by BOEC was observed whether they were cultured independently with or
279 without embryos or CM. However, BOEC consumption of those substrates was higher
280 than embryo consumption (P<0.001 for 2-cell embryos; P<0.005 for 8-cell embryos). In
281 contrast, lactate production was elevated in BOEC cultured with CM from 2-cell embryos
282 and in media from 2-cell embryos cultured alone in comparison with the other groups
283 (P<0.001) (Fig 4A). Lactate consumption was also higher in BOEC cultured alone or with
284 8-cell embryos and in media from 8-cell embryos cultured alone in comparison with
285 BOEC cultured with CM (P<0.005) (Fig 4B). Regarding embryo metabolism, 2-cell
286 embryos cultured without BOEC did not reveal a consumption of energy substrates when
287 they were compared to the blank control (data of blank media were not included in the
288 graphs).

289 *Amino Acid Profiling*

290 The results of HPLC revealed that among the 18 amino acids analyzed, the concentration
291 of 10 was affected in BOEC co-cultured with 2-cell embryos and 12 in the BOEC co-
292 cultured with 8-cell embryos. These amino acids were significantly highly depleted in
293 BOEC cultured alone or with embryos or with CM compared with both stage embryos
294 cultured alone, except for alanine having the opposite pattern (Fig 5A and B) (P<0.005).
295 Similar to alanine, serine had the same tendency of accumulation, but significant
296 differences were observed only for 8-cell embryos in BOEC cultured alone or with

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297 embryos or CM compared with 8-cell embryos cultured alone (*P*<0.005) (Fig 5B).
298 Regarding embryo metabolism, 2-cell embryos cultured without BOEC did not reveal a
299 consumption of amino acids substrates when they were compared to the blank control
300 (data of blank media was not included in the graphs).

301 **Discussion**

302 We have previously reported that oviduct transcriptome changes produced by the
303 presence of an embryo may be very local in nature (Maillo et al., 2015). The present
304 study aimed to validate these data by using an *in vitro* model which offered the double
305 benefit of immobilizing the embryo on the surface of the BOEC monolayer during co-
306 culture, enabling the analysis of the local interaction between BOEC and embryos. In this
307 model, we chose to use 50 embryos as we wanted to amplify any putative signal, also this
308 number corresponds to the number of embryos transferred in the study of Maillo et al.
309 (2015).

310 The response of BOEC to 2- and 8-cell embryos or their CM was evaluated at
311 gene expression and metabolic levels. The overall transcriptional profile of a panel of
312 candidate genes between BOEC co-cultured with 2-cell embryos or with their CM was
313 different from that observed after co-culture with 8-cell embryos or their CM. In cattle,
314 the major activation of transcription occurs during the 8- to 16-cell stage, (Reviewed by
315 Graf *et al.* 2014). These events perhaps are accompanied by specific embryo signaling.
316 Evidence is emerging of a dynamic mutual paracrine communication between both the
317 embryonic and the maternal environments, through extracellular vesicles (EVs), at the
318 early stages of preimplantation embryo development (Saadeldin *et al.* 2015; Almiñana *et*
319 *al.* 2017). For example, the secretome of *in vitro* cultured human embryos contains EVs
320 that are taken up by the maternal side (Giacomini *et al.* 2017). In support of the existence
321 of stage-specific effects of the embryo on the reproductive tract, data from Passaro *et al.*

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322 (2018) have shown that bovine endometrial explants respond to the presence of 8-day old
323 blastocysts and not to earlier stages (oocytes, 2-cell, Day 5 morula).
324 Serum has been associated with reduced blastocyst quality, although it is still used
325 for embryo culture (e.g., 5 to 10%) (reviewed by Rizos *et al.* 2017). We have reported
326 that 10% FCS accelerates blastocyst development, with more blastocysts appearing at
327 Day 6 after IVF than when it is absent and that this accelerated development was
328 associated with poorer survival following cryopreservation (Rizos et al., 2003). However,
329 others working in commercial IVF/ET in Brazil routinely use low concentrations (e.g.,
330 2.5%) of FCS and report no difference in survival following cryopreservation or
331 conception rates after transfer (Sanches *et al.* 2016). Also, BOEC cultures typically
332 require the presence of serum for normal development, cell attachment, growth and
333 proliferation (Brunner *et al.* 2010). Thus, FCS was supplemented to all experimental
334 groups.
335 Results indicate that BOEC mRNA abundance was affected directly by embryos
336 or by their secretions, depending on the transcript analyzed, which indicates the
337 complexity of the mechanisms implicated in embryo-maternal communication. The
338 oviductal mRNA abundance of *SMAD6* decreased in the presence of both 2- and 8-cell
339 embryos, consistent with our previous data indicating the relevance of *SMAD6* as a
340 component of the BMP signaling pathway (García *et al.* 2017).
341 *SOCS3, PRELP* and *SCN9A* expression was affected in BOEC cultured in direct
342 contact with 8-cell embryos and their CM. The fact that 8-cell embryos decreased the
343 expression of *SOCS3* and *PRELP*, implicated in immune defense, supports the idea that
344 embryo may avoid the maternal immune response by decreasing inflammation (Maillo *et*
345 *al.* 2015). This agrees with data obtained from the comparison of ipsilateral oviducts from
346 pregnant and cyclic mares, showing that the embryo modifies the expression of immune

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347 response-related genes with marked upregulation of interferon-associated genes (Smits *et*
348 *al.* 2016). This is also consistent with recent data indicating that BOEC stimulate
349 embryos to produce IFNT, which then acts on immune cells to promote an anti-
350 inflammatory response in the oviduct (Talukder et al., 2018). Expression of *SCN9A* was
351 up-regulated in BOEC co-cultured with 8-cell embryos or their CM. This is consistent
352 with the study of Schmaltz-Panneau *et al*. (2014), who hypothesized that there is
353 neurotrophin signaling by the embryo at the time of blastocyst formation (Schmaltz-
354 Panneau *et al.* 2014). Furthermore, *SCN9A* mediates the voltage-dependent sodium ion
355 permeability of excitable cell membrane. (Chan *et al.* 2012). Taking this information into
356 account, our results indicate that during its passage through the oviduct the early embryo
357 might affect the epithelial ion channels modulating the luminal fluid volume and
358 composition according to its needs.

359 The *IGFBP3* transcript was also increased in BOEC cultured with conditioned
360 media of 2-cell embryos, a finding consistent with previous reports of an increase of
361 *IGFBP3* in BOEC co-cultured for 8 days with 20 early embryos (Schmaltz-Panneau *et al.*
362 2014). IGFBP3 belongs to a family of six binding proteins that bind insulin-like growth
363 factor 1 and 2 (IGF-1 and –2). It was demonstrated that the bovine oviduct expresses
364 *IGFBP-3* in a region-specific manner in the isthmus epithelium. This pattern of
365 distribution might help to create an IGF-1 gradient along the oviduct as the embryo travels
366 through it (Pushpakumara *et al.* 2002).

367 Recently, changes in the oviduct microenvironment in its metabolite levels related
368 to the stage of the cycle and the proximity of ovulation have been reported (Lamy *et al.*
369 2018). However, whether the embryo can elicit similar responses is largely unknown.
370 Consequently, we sought to evaluate the effect of the early embryo on the metabolism of
371 energy substrates and amino acids of BOEC cultured *in vitro*. Results indicate that BOEC

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372 metabolism was not affected by the presence of early embryos (2-cell or 8-cell stage) or
373 by their CM. Furthermore, results indicate that BOEC metabolism is different to that of
374 embryos, suggesting that BOEC and embryos have metabolic requirements that may be
375 independently satisfied *in vitro* by the culture media. BOEC consumed glucose, lactate
376 and pyruvate as well as amino acids, except for serine and alanine, which were produced
377 by cells. It has previously been proposed that BOEC altered the composition of culture
378 medium in a manner consistent with embryo development; indeed, BOEC depleted
379 glucose concentrations whilst there was an accumulation of lactate and pyruvate, two key
380 metabolites required for successful development of the early embryo indicating that
381 BOEC might be attempting to create an environment favourable for embryo development
382 (Edwards *et al.* 1997). However, it is important to note that in their experiment, Edwards
383 *et al* (1997) performed the co-culture in SOFaa media supplemented with high levels of
384 carbohydrates, mimicking those present in TCM-199. In contrast, our study used SOFaa
385 that was free of carbohydrates, following the composition previously described (Holm *et*
386 *al.* 1999) wich may explain the discrepancies in results.

387 Interestingly, embryo metabolism before EGA seems to be independent of the
388 environment. The results indicated no consumption of energy substrate or amino acids by
389 2- to 8-cell embryos. However, this was not the case for embryos at the 8- to 16-cell stage
390 which displayed an active metabolism. The lack of metabolism by 2- to 8-cell embryos
391 may suggest that the embryo has sufficient endogenous energy sources, most likely fat
392 but possibly protein, to support its early development (Sturmey *et al.* 2009; Leese 2015).

393 The amino acid analysis revealed the production of serine and alanine by BOEC
394 independent of the presence of embryos. The release of alanine amino acid in the
395 extracellular medium is witness of ammonium detoxification mediated by pyruvate
396 metabolism (reviewed by de Souza *et al.* 2015). Furthermore, alanine is involved in the

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397 regulation of intracellular pH and may have a role in protecting the embryo from osmotic
398 stress (Van Winkle 2001) and it has shown to promote bovine pre-implantation
399 development *in vitro* (Moore and Bondioli 1993). Thus, BOEC could be predisposed to
400 increase alanine concentration in the media to sustain embryo needs.

401 In conclusion, using an *in vitro* approach allowing a local and temporal interaction
402 between the embryo and oviduct epithelial cells, this study confirms that the early embryo
403 affects the gene expression of BOEC. The effect was embryo stage-specific and resulted
404 from a direct contact with BOEC or from embryo secretions released into the media.
405 Under our experimental conditions the early embryo did not affect BOEC metabolism
406 indicating that during its short passage through the oviduct it might elicit changes at
407 signaling level necessary for its development without inducing metabolic changes.

408

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410

411 **Declaration of interest**

412 The authors declare that there is no conflict of interest that could be perceived as
413 prejudicing the impartiality of the research reported.

414 **Funding**

415 This work was funded by the Spanish Ministry of Economy and Competitiveness
416 (AGL2015-70140-R to D. Rizos and AGL2015-66145-R to A. Gutierrez-Adan). M.
417 Hamdi was supported by a fellowship from the Spanish Ministry of Economy and
418 Competitiveness (BES-2013-066767). The authors are also funded by the European
419 Union H2020 Marie Sklodowska-Curie (MSCA) Innovative Training Network (ITN)
420 project “Biology and Technology of Reproductive Health - REP-BIOTECH -675526”.

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421 The authors are members of the COST Action 16119. *In vitro* 3-D total cell guidance and
422 fitness (Cellfit).

423 **Acknowledgments**

424 The authors would like to thank Almansa Ordóñez A and Barroso Sáenz A for their help.
425 Special thanks are extended to the abattoirs (Transformación Ganadera De Leganés SA;
426 Matadero Madrid Norte, San Agustín de Guadalix; and Cárnica Colmenar SC, in Madrid,
427 Spain) for providing access to the biological material (ovaries) and to the Spanish
428 Association of Breeders of Selected Cattle of the Asturian Valley Breed (ASEAVA) for
429 providing the semen.

430

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582

583 **Figure legends**

584 **Figure 1:** (A) Immunofluorescence analysis of BOEC monolayers showing positive
585 staining for the epithelial markers, cadherin and cytokeratin, and negative staining for the
586 fibroblast marker, vimentin. Cell nuclei were counterstained with Hoechst (blue). (B)
587 Representative image of the polyester meshes employed to establish a direct and indirect
588 contact area between the embryos and the BOEC monolayer. A guide mesh of 41x41
589 openings, covering an area of 121 mm^2, containing delimited marked areas of 50 openings
590 [(7x7)+1] was placed outside underneath each well of a four-well dish and was used as a
591 guide to localize the co-culture area. Another mesh square of the same size, fitting exactly
592 into the well, was introduced in a way that the external and internal meshes overlapped
593 during the co-culture.

594 **Figure 2: Experimental design.** (A) Replicates of BOEC (n=4), embryo-CM (n=4) and
595 embryo (n=4) production were carried out in a synchronized way. BOEC were cultured
596 until confluence for 6 days. Half of the media was renewed every 48 h, and 24 h before
597 starting the co-culture, it was replaced with SOFaa + 5% FCS. Embryos at the 2- and 8-
598 cell stages were generated by IVF (0 h) and selected at 31 or 52 h post insemination (hpi)
599 respectively to be cultured during 48 h to either generate fresh CM or co-cultured with
600 BOEC. The following experimental groups were established: (1) BOEC directly beneath
601 to 50 embryos at 2- or 8- cell stage ($BOEC^{+}$); (2) BOEC in the same well as 2- or 8-cell
602 embryos but not in direct contact ($BOEC^{-}$); (3) BOEC cultured with CM produced from
603 50 embryos at 2- or 8-cell stage ($BOEC^{CM}$); (4) BOEC cultured without embryos

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604 (BOEC); (5) embryos at 2- or 8-cell stage cultured alone (EMB); and (6) embryo/BOEC-
605 free media as control (BLANK MEDIA). After 48h of co-culture, BOEC were recovered
606 from the marked area from groups 1, 2, 3 and 4 to perform gene expression analysis (B),
607 while the media obtained from groups 1, 3, 4, 5 and 6 were collected to assess metabolic
608 analysis of energy substrate and amino acid composition (C).
609

610 **Figure 3**: Relative mRNA abundance of genes assessed in BOEC cultured with 2-cell
611 (A) or 8-cell (B) stage embryos. $BOEC^{+}$: BOEC directly beneath to embryos. $BOEC^{-}$:
612 BOEC in the same well as embryos but not in direct contact. $BOEC^{CM}$: BOEC cultured
613 with embryo CM. BOEC: cultured without embryos or CM. Bars represent the relative
614 abundance of the transcripts analyzed and normalized to *H2AFZ* and *ACTG1* as
615 housekeeping genes. Results are expressed as means ± SEM. Different superscripts
616 indicate significant differences ($P< 0.05$) between groups. Data were obtained from four
617 replicates of independent BOEC samples per experimental group.

618 **Figure 4**: Energy substrate analysis (Glucose, Lactate and Pyruvate) in BOEC cultured
619 with 2-cell (A) or 8-cell (B) stage embryos. Depletion/production data are expressed
620 relative to a control blank media. Values are expressed as means ± SEM. $BOEC^{+}$: BOEC
621 co-cultured with embryos. $BOEC^{CM}$: BOEC cultured with embryo CM. BOEC: BOEC
622 cultured neither with embryos nor with their CM. EMB: embryos cultured alone.
623 Different superscripts indicate significant differences between groups (A, $P<0.001$); (B,
624 $P<0.005$). All the groups included under the brackets are statistically similar between each
625 other, but they are different to the other group(s)

626 **Figure 5**: Amino acid metabolism in BOEC co-cultured with 2-cell (A) or 8-cell (B)
627 stage embryos. Depletion/production data are expressed relative to a control blank media.
628 Values are expressed as means ± SEM. $BOEC^{+}$: BOEC co-cultured with embryos.

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629 BOEC^{CM}: BOEC cultured with embryo CM. BOEC: BOEC cultured neither with
630 embryos nor with their CM. EMB: embryos cultured without BOEC. Different
631 superscripts indicate differences between groups (P<0.005). All the groups included
632 under the brackets are statistically similar between each other, but they are different to
633 the other group(s)

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Table 1. Set of primers used for qRT-PCR assays

Gene	Primer sequence (5´-3´)*		Fragment Size, bp	Gene Bank Accession
	Forward	Reverse		
H2AFZ	AGGACGACTAGCCATGGACGTGTG	CCACCACCAGCAATTGTAGCCTTG	209	NM_174809
ACTG1	GAGAAGCTCTGCTACGTCG	CCAGACAGCACCGTGTTGG	255	NM_001033618.1
GPX4	TGTGGTGAAGCGGTATGGTC	TATTCCCACAAGGCAGCCAG	266	NM_174770.4
NFE2L2	GCTCAGCATGATGGACTTGGAG	GGGAATGTCTCTGCCAAAAGC	390	NM_001011678.2
ROCK1	ACGTGACCTAGTGCCTTGTG	CCTCAGTGTGCTTTTGTGCC	164	XM_002697789.5
ROCK2	CTTGGCTGCTCAACTGGAGA	TGCTCTTGGGCTTCCTTCAG	276	NM_174452.2
AGR3	TGTCACACTCAGTTCTGGTCC	GTCATCTCCCCACCCTCTTGA	119	NM_001191502.1
SMAD6	GGAGAAATTCGCTCCAAGTGC	CCCTGCCTTTAAAACCCAAGC	242	NM_001206145.1
TDGF1	ATGGTGAGAGACGGGCTGCTAG	GCCCTTGTCTCATACAGCTTCC	201	NM_001080358.1
SCN9A	GTTGATAACCCTGTGCCTGGA	CTTCAAAAGCCAGAGCACCAC	250	XM_005202453.3
SOCS3	GCGAGAAGATCCCTCTGGTG	CTAAAGCGGGGCATCGTACT	167	NM_174466.2
IGFBP3	GAGTCCAAGCGTGAGACAGAA	GCGGCACTGCTTTTTCTTGTA	150	NM_174556.1
PRELP	CAGCATCGAGAAAATCAATGGGA	AGCACATCATGAGGTCCAGC	158	NM_174434.3
EPSTI1	AAACGACAGCAACAGGAGGAA	CCTTGGAGTCGGTCCAGAAAA	89	XM_002700904.4

*All the primers were designed with NCBI Primer-BLAST online tool of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/tools/primer-blast/)

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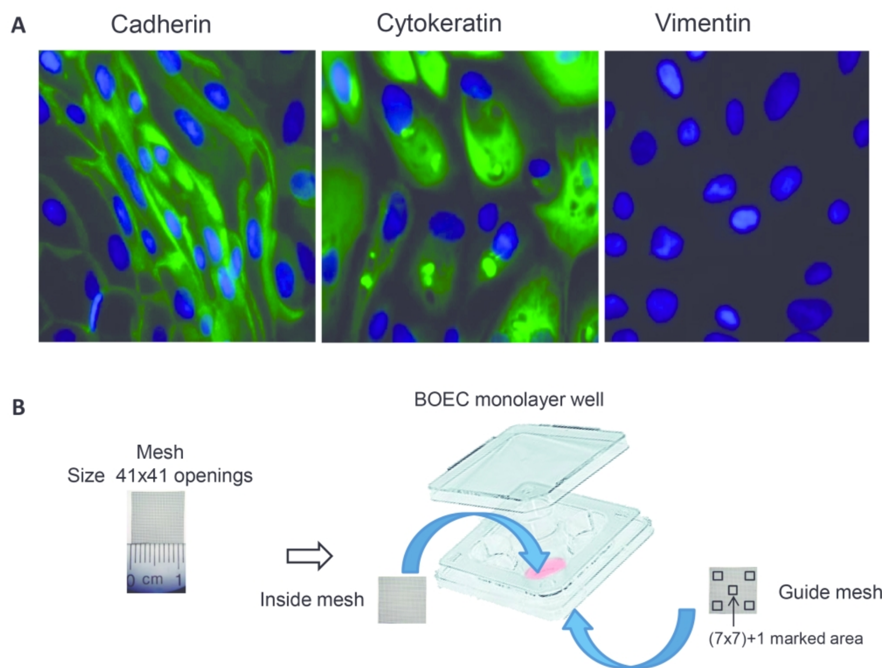

Figure 1: (A) Immunofluorescence analysis of BOEC monolayers showing positive staining for the epithelial markers, cadherin and cytokeratin, and negative staining for the fibroblast marker, vimentin. Cell nuclei were counterstained with Hoechst (blue). (B) Representative image of the polyester meshes employed to establish a direct and indirect contact area between the embryos and the BOEC monolayer. A guide mesh of 41x41 openings, covering an area of 121 mm2, containing delimited marked areas of 50 openings [(7x7)+1] was placed outside underneath each well of a four-well dish and was used as a guide to localize the co-culture area. Another mesh square of the same size, fitting exactly into the well, was introduced in a way that the external and internal meshes overlapped during the co-culture.

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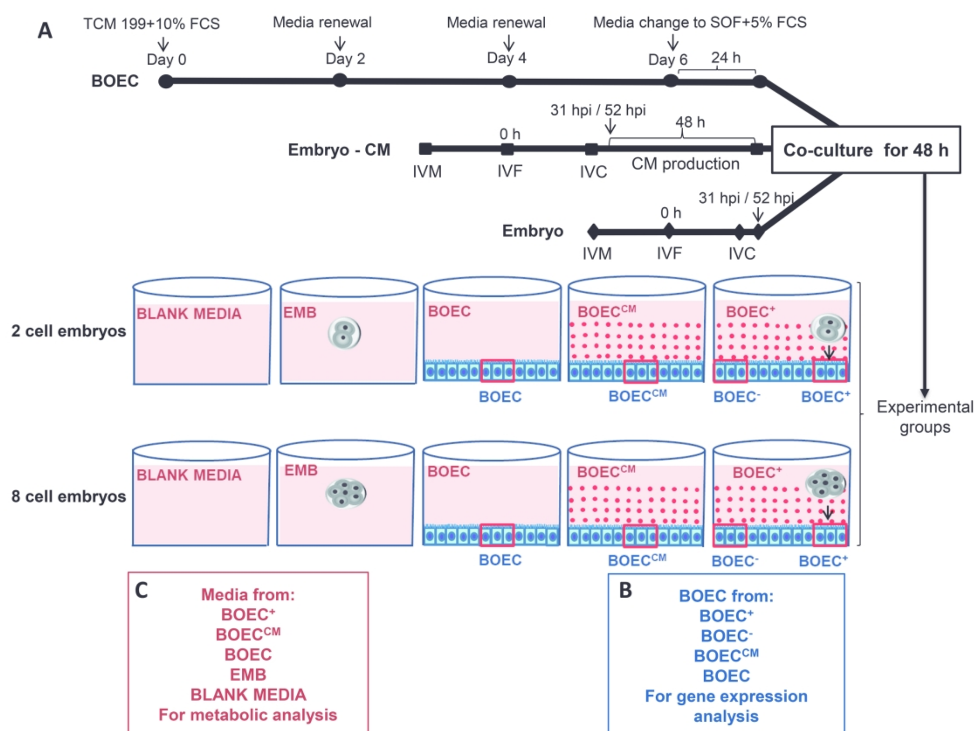

Figure 2: Experimental design. (A) Replicates of BOEC (n=4), embryo-CM (n=4) and embryo (n=4) production were carried out in a synchronized way. BOEC were cultured until confluence for 6 days. Half of the media was renewed every 48 h, and 24 h before starting the co-culture, it was replaced with SOFaa + 5% FCS. Embryos at the 2- and 8-cell stages were generated by IVF (0 h) and selected at 31 or 52 h post insemination (hpi) respectively to be cultured during 48 h to either generate fresh CM or co-cultured with BOEC. The following experimental groups were established: (1) BOEC directly beneath to 50 embryos at 2- or 8- cell stage (BOEC+); (2) BOEC in the same well as 2- or 8-cell embryos but not in direct contact (BOEC-); (3) BOEC cultured with CM produced from 50 embryos at 2- or 8-cell stage (BOECCM); (4) BOEC cultured without embryos (BOEC); (5) embryos at 2- or 8-cell stage cultured alone (EMB); and (6) embryo/BOEC-free media as control (BLANK MEDIA). After 48h of co-culture, BOEC were recovered from the marked area from groups 1, 2, 3 and 4 to perform gene expression analysis (B), while the media obtained from groups 1, 3, 4, 5 and 6 were collected to assess metabolic analysis of energy substrate and amino acid composition (C).

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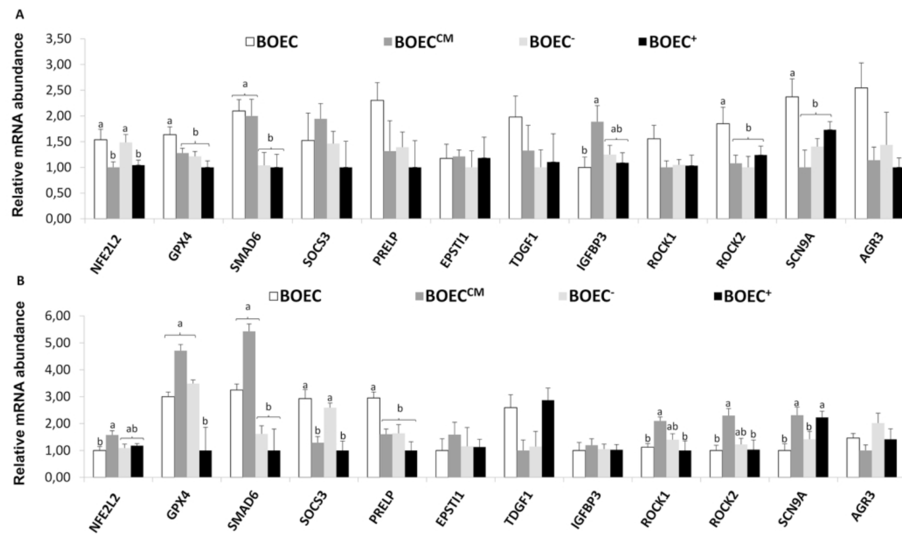

Figure 3: Relative mRNA abundance of genes assessed in BOEC cultured with 2-cell (A) or 8-cell (B) stage embryos. BOEC+: BOEC directly beneath to embryos. BOEC-: BOEC in the same well as embryos but not in direct contact. BOECCM: BOEC cultured with embryo CM. BOEC: cultured without embryos or CM. Bars represent the relative abundance of the transcripts analyzed and normalized to H2AFZ and ACTG1 as housekeeping genes. Results are expressed as means ± SEM. Different superscripts indicate significant differences ($P < 0.05$) between groups. Data were obtained from four replicates of independent BOEC samples per experimental group.

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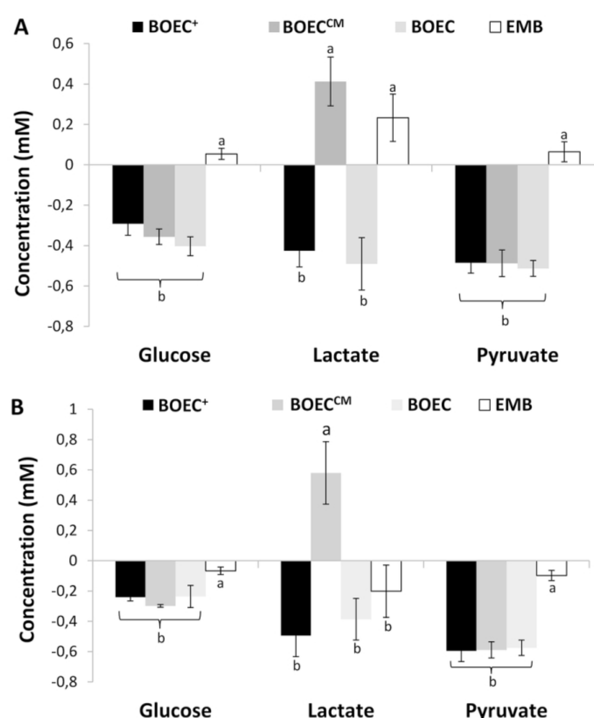

Figure 4: Energy substrate analysis (Glucose, Lactate and Pyruvate) in BOEC cultured with 2-cell (A) or 8-cell (B) stage embryos. Depletion/production data are expressed relative to a control blank media. Values are expressed as means ± SEM. BOEC+: BOEC co-cultured with embryos. BOECCM: BOEC cultured with embryo CM. BOEC: BOEC cultured neither with embryos nor with their CM. EMB: embryos cultured alone. Different superscripts indicate significant differences between groups (A, P<0.001); (B, P<0.005). All the groups included under the brackets are statistically similar between each other, but they are different to the other group(s).

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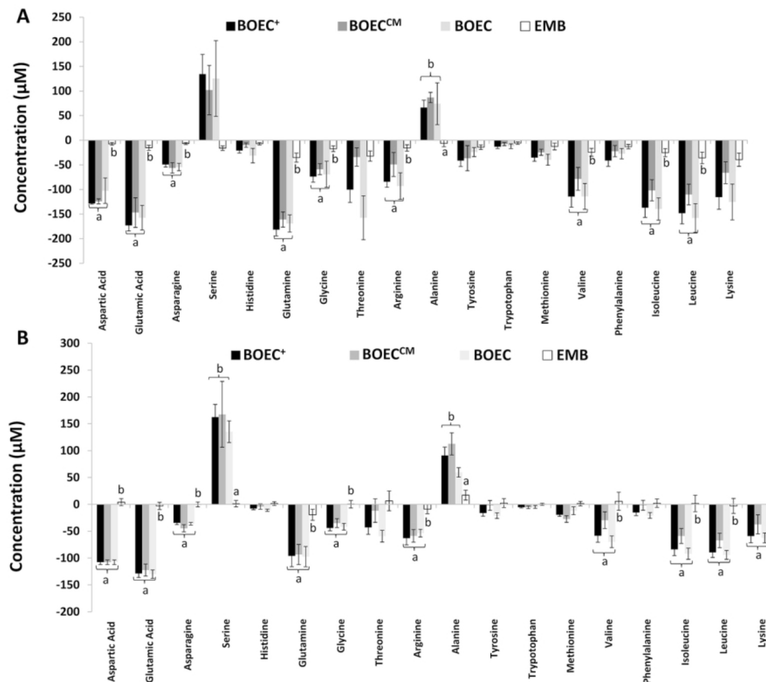

Figure 5: Amino acid metabolism in BOEC co-cultured with 2-cell (A) or 8-cell (B) stage embryos. Depletion/production data are expressed relative to a control blank media. Values are expressed as means ± SEM. BOEC+: BOEC co-cultured with embryos. BOECCM: BOEC cultured with embryo CM. BOEC: BOEC cultured neither with embryos nor with their CM. EMB: embryos cultured without BOEC. Different superscripts indicate differences between groups (P<0.005). All the groups included under the brackets are statistically similar between each other, but they are different to the other group(s).

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