Secretome derived from different cell lines in bovine embryo production *in vitro*

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28

29 **ABSTRACT**

30 The effect of the conditioned medium (CM), composed of microvesicles (MVs) and soluble factors
31 present in the supernatant (SN), of bovine endometrial and amniotic cells on embryo quality and
32 rate of production was investigated. Presumptive zygotes were randomly assigned on day 1, 3 and 5
33 post fertilization in SOFaa (control, CTR) or in the same medium supplemented with 20%
34 endometrial or amniotic CM or 20% SN or $100x10^6$ MVs/mL. Embryos were evaluated on day 7.
35 On day 1, embryos developed in MVs in both cell lines but at a lower rate than in CTR. On day 3,
36 amniotic and endometrial CM and MVs produced blastocysts, but the rate of production in CM was
37 statistically lower than that obtained by MVs in both cell lines. On day 5, embryos were produced
38 by all the secretome fractions of both cell lines but only amniotic MVs produced embryos at a rate
39 comparable to CTR. Qualitatively, only amniotic CM and MVs on day 5 provided inner cell mass
40 values statistically higher than CTR. These data were confirmed by evaluation of genes involved in
41 apoptosis and reactive oxygen species protection. In conclusion, only amniotic MVs gave results
42 better than CTR.

43

44 **Key words:** embryo quality, microvesicles, conditioned *medium*, amniotic cells, endometrial cells

45

46 **INTRODUCTION**

47 As already stated, embryos produced *in vitro* are generally of poorer quality compared to those
48 developing *in vivo* (Wrenzycki and Stinshoff, 2013). The embryo conceived *in vitro* is manipulated
49 and cultured in very different conditions and many factors involved in this technique, such as
50 superovulation, characteristics of female and male gametes, *medium* of embryo culture and the
51 absence of maternal embryo-communication, can influence the quality of *in vitro* embryo (Ventura-
52 Junca et al. 2015). In cattle, it has been suggested that the absence of these maternal-embryo signals
53 could be an important cause of the low efficiency of *in vitro* fertilization (IVF) (Thatcher et al.
54 2001; Ventura-Junca et al. 2015).

55 To mimic the *in vivo* cross-talk environment and to improve the *in vitro* development of embryos,
56 co-culture systems with somatic cells are widely used in *in vitro* embryo production (IVP) in many
57 species (White et al. 1989; Rexroad and Powell 1993; Park et al. 2000; Li et al. 2001). Indeed, a co-
58 culture system with bovine oviduct epithelial cells, to produce bovine *in vitro* zygotes, enhanced
59 blastocyst formation, the quality of the resulting embryos, and induced specific transcriptomic
60 changes (Schmaltz-Panneau et al. 2015). This suggests that paracrine mechanisms of
61 communication between monolayer cells ("helper" cells) and embryos exist. In fact, a variety of
62 growth factors, receptors and binding proteins are secreted, not only by cells in the *medium*, but also
63 by the embryos themselves (Van Langendonckt et al. 1996; Izquierdo et al. 1999). This *medium*,
64 called conditioned media (CM), is composed of factors secreted by cells during culture and it is
65 cell-free. The CM, produced by culturing granulosa, cumulus or oviductal cells, is used in bovine
66 embryo IVP and has similar properties in embryonic development to effects produced by co-culture
67 systems (Rieger et al. 1995). Recent studies have identified the existence of MVs released by cells
68 into the extracellular environment (Ratajczak et al. 2006; Camussi et al. 2010). Thus, CM can be
69 defined as a composition of soluble factors plus MVs. These MVs have been shown to contain
70 proteins, lipids (specifically high levels of sphingomyelins), a variety of RNA species, including
71 microRNAs (miRNAs) and mRNA fragments (Thery et al. 2009). The MVs could serve as vehicles
72 for the transfer of these molecules (Zhang et al. 2009; Raposo and Stoorvogel 2013; Machatinger et
73 al. 2016). Many studies demonstrated that MVs are secreted by most cells and can be found in
74 organic fluids such as, for example, in the uterine luminal fluid of cycling and pregnant sheep
75 (Burns et al. 2014; Machatinger et al. 2016). These findings suggest that these MVs have a
76 biological role in the interaction between the embryo and the microenvironments that, *in vivo* is
77 represented by endometrium (Machatinger et al. 2016) and, *in vitro* by different culture conditions.
78 The *in vitro* use of feeder cells or CM probably provides the MVs required for this cross talk. The
79 effect of different components of feeder cell secretome has not been investigated and, since it is
80 difficult to study these communications *in vivo,* we tested these interactions *in vitro*. Endometrium

81 and amnion have a role in the establishment of pregnancy. Endometrial cells were studied at the
82 early luteal phase because only in this phase (up to the blastocyst stage) are these cells in contact
83 with the early embryo *in vivo*. The amnion was chosen because Lange-Consiglio et al. (2010) used
84 the monolayer of equine amniotic progenitor epithelial cells as feeder cells to culture bovine
85 embryos and showed an increase in blastocyst rate compared to bone marrow derived cells and
86 control cells, represented by monolayer of cumulus cells. These authors suggested that EGF,
87 produced by amniotic epithelial cells, may have a positive role on embryonic development. Indeed,
88 EGF is produced *in vivo* by the endometrial cells and the embryo possesses receptors for it (Pohland
89 and Tiemann, 1994; Lonergan et al. 1996). In this study, we compared the effects of the secretome
90 from amniotic progenitor cells and endometrial cells. The secretomes, composed of CM *in toto*, or
91 MVs or supernatant (SN), collected after removal of MVs, were investigated to identify which of
92 these components had demonstrable effects on embryo production, in terms of blastocyst quality
93 and formation rate.
94
95 **MATERIALS AND METHODS**
96 Chemicals were obtained from Sigma-Aldrich Chemical (Milan, Italy) unless stated otherwise, and
97 tissue culture plastic dishes were purchased from Euroclone (Milan, Italy).
98 Samples of endometrial tissue and ovaries were collected from cows slaughtered in a
99 slaughterhouse (INALCA, Ospedaletto Lodigiano, Lodi, Italy) under national food hygiene
100 regulations.
101 Allanto-amniotic membranes were obtained at term of normal pregnancies and parturitions from
102 three cows (*Bos taurus*). All procedures were performed according to approved animal care and use
103 protocols of the institutional ethics committee and to good veterinary practice for animal welfare as
104 to European directive 2010/63/UE. Moreover, written consent was obtained from farmers at the
105 beginning of the study.
106

107 **Cell isolation and culture**

108 Portions of allanto-amnion were kept at 4°C in phosphate-buffered saline (PBS; EuroClone, Milan,
109 Italy) with 100 U/mL penicillin-100 mg/mL streptomycin and amphotericin B and were processed
110 within 12 h. The amniotic membrane was stripped from the overlying allantois and cut into small
111 pieces (about 9 cm^2 each) before enzymatic digestion.

112 Amniotic progenitor epithelial derived cells (AMCs) were isolated and characterized as previously
113 reported (Corradetti et al. 2013). Briefly, amnion fragments were digested with 0.05%
114 trypsin/EDTA for 45 min at 38.5°C for approximately 45 min. Then, the amnion fragments were
115 removed and mobilized cells were passed through a 100 μm cell strainer before being collected by
116 centrifugation at 250 x g for 10 min.

117 Epithelial endometrial cells (EDCs) at early luteal phase (day 4-8, considering day 0 = estrus) were
118 isolated according to Donofrio et al. (2008). Pieces of endometrium deep 1 mm, large 5 mm and
119 long 10 mm were collected (overall 3.5 g). These pieces were deeply cut with scissors and digested
120 in sterile filtered Hank's buffered salt solution supplemented with 2 mg/mL collagenase II, 2
121 mg/mL trypsin III (Roche), 4 mg/mL bovine serum albumin, and 0.4 mg/mL DNase I for 90
122 minutes at 38.5°C in a shaking bath. To obtain separate stromal and epithelial cell populations, the
123 cell suspension was removed 18 h after plating, which allowed selective attachment of stromal cells.
124 The removed cell suspension was then re-seeded and incubated allowing epithelial cells to adhere.
125 Stromal and epithelial cell populations were distinguished by cell morphology. Only epithelial
126 endometrial cells were used in this study.

127 Amniotic and endometrial cells were cultured in a *medium* composed of HG-DMEM supplemented
128 with 10% fetal calf serum (FCS), 1% penicillin (100 UI/mL)–streptomycin (100 mg/mL), 0.25
129 mg/mL amphotericin B and 2 mM L-glutamine. Both cell lines were incubated at 38.5°C in a
130 humidified atmosphere of 5% CO_2, until they reached passage (P) 3. Endometrial cells reached P3
131 in eight days while amniotic cells in ten days.

132

133 **Immunocytochemical detection of pancytocheratin**

134 Amniotic and endometrial epithelial cells were tested for immunoreactivity against pancytokeratin
135 (mouse monoclonal, clone A1E; Santa Cruz Biotechnology, Santa Cruz, CA, USA) using primary
136 antibody (anti PanCytokeratin: 1/200) diluted in TBST and secondary antibody conjugated with
137 horseradish peroxidase (HRP) as described by Lange-Consiglio et al. (2015).

138

139 **Conditioned *medium*, microvesicles and supernatant production**

140 For CM production, cells at P3 at confluence were cultured for 5 days in a serum-free *medium*
141 (Ultraculture, Lonza, Milan, Italy). Conditioned *media* of three samples from each cell line were
142 collected, pooled, centrifuged at 3500 x g for 20 min to remove cellular debris. Control *medium*,
143 (non-conditioned *medium;* no-CM), was generated in the same way as above, except that no cells
144 were cultured in the plates.

145 A portion of CM *in toto* was stored at -80°C. To obtain MVs and SN, another portion of CM was
146 ultra-centrifuged (Beckman Coulter Optima L - 100K) at 100,000 x g for 1h at 4°C. SN was
147 collected and stored at – 80°C for later use. The pellet was washed in serum-free *medium* 199
148 containing N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) 25 mM and submitted to
149 a second ultra-centrifugation under the same conditions. The resulting pellet, composed of MVs,
150 was fractionated for MVs analysis or used for the *in vitro* test.

151

152 **Measurements of MVs**

153 Size and concentration of MVs were evaluated by NanoSight LM10 instrument (Nanoparticle
154 tracking analysis, NTA, Nano-Sight Ltd., Amesbuty, U.K.), which permits discrimination of
155 microparticles less than 1 μm in diameter, as described byBruno et al. (2009).

156

157 **MVs labeling with PKH-26**

158 To trace MVs *in vitro* by fluorescence microscopy, MVs from AMCs and ECs were labeled with the

159 red fluorescence aliphatic chromophore intercalating into lipid bilayers PKH-26 dye. Briefly, after
160 ultra-centrifugation, the MV pellet was diluted to 1 mL with PKH-26 kit solution and 2 µL of
161 fluorochrome were added to this suspension and incubated for 30 min at 38.5°C. Thereafter, 7 mL
162 of serum free DMEM were added to the suspension that was ultra-centrifuged again at 100,000 x g
163 for 1 h at 4°C. The final pellet was immediately cryopreserved at -80°C.

164

165 ***In vitro* embryo production in different IVC *media***

166 *Collection of oocytes and in vitro maturation (IVM)*

167 Ovaries were collected from slaughtered Holstein-Friesian cows (*Bos taurus*) whose age,
168 genealogy, and physiological status were unknown. Ovaries were transported in sterile saline
169 solution (0.9% NaCl) supplemented with 150 mg/L kanamycin and maintained at 30°C. About 5940
170 oocytes, in twenty replications, were used to produce embryos to submit to IVC with different
171 culture systems. Oocytes were retrieved by aspiration of 3-5 mm diameter follicles with 18 G
172 needles. Cumulus–oocyte complexes (COCs) were selected and washed three times in pre-
173 incubated TCM 199-Hepes buffered supplemented with 10% FCS.

174 *In vitro* maturation was performed for 24 h in TCM 199 Earl's Salt *medium* supplemented with 10%
175 FCS, 5 µg/mL LH (Lutropin, Vetoquinol, France), 0.5 µg/mL FSH (Folltropin, Vetoquinol), 0.2 mM
176 sodium pyruvate, 5 µg/mL gentamycin and 1 mg/mL estradiol 17β. Cultures were performed in 70
177 µL droplets (up to 20 oocytes/droplet) of the *medium* under mineral oil, at 38.5°C in 5% CO_2.

178

179 *In vitro fertilization (IVF)*

180 *In vitro* fertilization was performed in Tyrode's-albumin-lactate-pyruvate (TALP) *medium*
181 containing 2 mM penicillamine, 1 mM hypotaurine, 250 mM epinephrine, 20 µg/mL heparin, 114
182 mM NaCl, 3.2 mM KCl, 0.4 mM NaH2PO4, 10 mM sodium lactate, 25 mM NaHCO3, 0.5 mM
183 MgCl2-6H20, 2.0 mM CaCl2-2H2O, 6 mg/mL bovine serum albumin (BSA,), 5 µl /mL gentamicin,
184 0.2 mM sodium pyruvate. Frozen-thawed semen of a single bull of proven fertility was prepared by

185 Percoll density gradient (Amersham Pharmacia Biotec) (45/90%). Semen was thawed at 37°C for
186 30 s, placed on the top of the Percoll gradient and centrifuged for 30 min at 300 x g. The semen
187 suspension was diluted in the appropriate volume of fertilization medium to obtain a final
188 concentration of 10^7 spermatozoa per mL. An aliquot of 10 µL of semen was co-incubated with
189 matured oocytes for 18 h at 38.5°C in 5% CO_2. Cultures were performed in 70 µl droplets (up to 20
190 oocytes/droplet) of the *medium* under mineral oil. At the end of gamete co-culture, cumulus cells
191 were completely removed and cumulus-free presumptive zygotes were randomly transferred into
192 different culture system and cultured up to day 7.
193
194 *In vitro culture (IVC)*
195 The standard *medium* for IVC was synthetic oviductal fluid with aminoacids (SOFaa; Holm et al.
196 1999) composed of 1.1 M NaCl, 72 mM KCl, 12 mM KH_2PO_4, 7.4 mM $MgSO_4$, 50 mM DL-
197 lactate, 250 mM $NaHCO_3$, 260 mM phenol red, 100 mM sodium pyruvate, 178 mM $CaCl_2$-$2H_2O$,
198 125 mM Hepes sodium salt, 30.8 mM glutamine, 500 mM glycine, 84.2 mM alanine, 100X MEM
199 non-essential, 100X BME, 2.8 mM Myo-Inositol, 340 mM trisodium citrate, 2% FCS, 0.005 gr/mL
200 BSA, 0.2mM sodium pyruvate, 5 µL/mL gentamicin.
201 At the beginning of the culture in SOFaa, presumptive zygotes were randomly assigned to a control
202 group (CTR, no supplementation), or to one of the experimental groups in which SOFaa was
203 supplemented with one of the a-cellular extracts on day 1 or 3 or 5. Experimental groups were
204 resumed in table 1
205 In every culture condition, IVC was performed for 7 days in 5% O_2, 5% CO_2 and 90% N_2 in
206 humidified atmosphere at 38.5°C. Cultures were performed in 70 µl droplets (up to 20
207 oocytes/droplet) of the *medium*. In the standard protocol, the *medium* is renewed on days 3 and 6
208 during the culture period. In this study, to avoid stress to the embryos and to allow the action of
209 secretome, the *medium* was renewed on days 3 and 5, and these time points were chosen to add
210 different components of the secretome to SOF. In the same days, also the control group was

211 renewed with SOF only.

212

213 **Evaluation of MVs uptake by blastocysts**

214 To confirm MVs incorporation into blastocysts, a group of IVC embryos was cultured in SOF
215 supplemented with AMCs- and EDCs-MVs previously stained with PKH-26. mLOn day 7,
216 blastocysts were stained with Hoechst 33343 (10 μg/mL) and MVs incorporation was evaluated by
217 intensity of fluorescence under fluorescent microscopy BX71 (Olympus). Labeled MVs were
218 excited at 550 nm while emission wavelength was set at 567 nm. Hoechst 33342 dye was excited at
219 353-365 nm while the emission wavelength was set at 460 nm. The intensity of fluorescence was
220 detected by semiquantitative analysis by the microscope BX71 equipped with a Scion Corporation
221 1394 video camera interfaced with a computer provided with software for image acquisition and
222 analysis (Image-Pro Plus 5.1-Media Cybernetics; Immagini & Computer), as described by Lange-
223 Consiglio et al. (2016).
224 In addition, confocal microscopy analysis to assess internalization of MVs was performed using a
225 Leica SP2 laser scanning confocal microscope (Leica Microsystems Srl, Italy) equipped with a PL
226 Fluotar 20x AN 0.5 Dry objective.

227

228 **Blastocyst production rate and viability evaluation**

229 On day 7, for the evaluation of blastocyst formation rate, embryos from control and experimental
230 groups were counted. A set of the blastocysts was used to test viability, which was assessed by
231 Hoechst 33342 and propidium iodide staining. Blastocysts were retrieved from the IVC drops,
232 washed with PBS supplemented with 2% BSA and incubated with Hoechst 33342 (10 μg/mL) and
233 propidium iodide (1 μg/mL) for 15 min at 38.5°C. Then, they were observed under fluorescent
234 Olympus BX51 microscope at a magnification of 40x, analyzing the images with Image-Pro Plus
235 5.1-Media Cybernetics software. Hoechst 33342 dye was excited at 353-365 nm while the emission
236 wavelength was set at 460 nm. Propidium iodide was excited at 535 nm while the emission

237 wavelength was set at 617 nm.

238

239 **Differential staining**

240 Another set of control and experimental group embryos was used for differential staining.
241 Differential staining was performed with propidium iodide and Hoechst 33342 after disrupting the
242 membrane integrity of the surface trophectoderm cells by means of a detergent (Triton X-100),
243 which permits penetration of the propidium iodide into the trophectoderm cells, but not into the
244 inner mass cells (ICM). Since all the cells were stained with Hoechst 33342, in this way the ICM
245 stained blue and the trophectoderm purple. The staining was performed according to Thouas et al.
246 (2001) with some modifications. Briefly, prior to differential staining, embryos were washed in PBS
247 supplemented with 5% FCS, then were permeabilized for 30 seconds in PBS containing 0.04%
248 Triton X-100 and 0.1 mg/mL of propidium iodide. After that, embryos were stained with 10 µg/mL
249 of Hoechst for 15 min at 38.5°C and mounted on a glass slide in a small drop of PBS. Four small
250 pillars of wax were applied and a cover glass was laid over the drop applying a suitable pressure to
251 visualize the cells for counting. Cell counting was performed from digital photographs obtained by
252 a fluorescent Olympus BX51 microscope at a magnification of 40x at the wavelength previously
253 described. The images were analysed, using Image Pro-Plus software, by a single operator in a
254 blinded manner.

255

256 **Gene expression assessment in IVP bovine embryos on day 5 of treatment**

257 Genes involved in apoptosis (*BAX;* Bcl-2–associated X protein) and reactive oxygen species (ROS)
258 protection (*GPX1;* glutathione peroxidase 1) were analyzed by reverse transcription-quantitative
259 PCR (RT-qPCR) in embryos from both cell lines treated on day 5 with CM, MVs, and SN. The
260 mRNA expression levels of all the cited genes were measured in three samples (biological
261 replicates). Fifteen blastocyst for each group were collected on day 7 of culture, washed in sterile
262 PBS and immediately placed in sterile RNAse DNAse–free Eppendorf tubes. Samples were kept at

263 -80°C until RNA extraction. RNA extraction was performed using Trizol followed by DNase
264 treatment. The concentration of RNAs was evaluated three times using a NanoDrop ND-1000
265 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNAs quality was checked
266 using the Agilent Bioanalyser 2100 (Agilent, Santa Clara, CA). RT-qPCRs were performed with the
267 High Capacity cDNA Reverse Trascription Kit (Applied Biosystems/Life Technologies, Carlsbad,
268 CA, USA) using 100 ng of RNA per reaction. All the qPCR experiments were run in triplicates
269 (technical replicates) using the qPCR protocol described by TaqMan Fast Gene Expression Assays
270 (Life Tecnologies™) on 7500 Fast Real-time PCR System instrument (Applied Biosystems by Life
271 Technologies™). To assess gene expression, each target gene and the *GAPDH*, as the housekeeping
272 control gene, were co-amplified. Conditions of amplifications were an initial step of 95°C (3
273 minutes), followed by 40 cycles of 95°C (30 seconds), 60°C (30 seconds), and 72°C (20 seconds),
274 followed by the acquisition of the melting curve (fluorescence acquisition every 0.5°C). Efficiency
275 of amplification for each primer was monitored through the analysis of serial dilution. Additional
276 dissociation curve analysis was performed and in all cases showed a single peak.
277 Average target gene threshold cycle (ΔCt_g) for each sample (calculated using the CT values of the
278 technical replicates within each experimental conditions), were normalized to the average *GAPDH*
279 values (ΔCt_{GAPDH}) of the same cDNA sample. Then, the expression variations calculated were
280 normalized to internal control (i.e. CTR) using the ΔΔCt method. Finally, the fold change
281 expression of each gene was calculated as $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). Primers from each
282 gene were designed using Primer3 web interface (www. primer3plus.com). The assay primers were
283 available and synthesized by Life technologies™.
284 ***BAX*:** Forward AGAGGATGATCGCAGCTGTGGA;
285 Reverse CAAAGATGGTCACTGTCTGCCATGT; (bp = 300); Gene Bank access NM_173894.1
286 ***GPX1***: Forward GCAACCAGTTTGGGCATCA; Reverse CTCGCACTTTTCGAAGAGCATA; (bp
287 = 116); Gene Bank access NM_174076.3.
288 ***GAPDH*:** Forward TTCAACGGCACAGTCAAGG; Reverse ACATACTCAGCACCAGCATCA;

289 (bp = 119); Gene Bank access NM_001034034.2

290

291 **Statistical analysis**

292 Statistical analysis was performed using full factorial model, with polynomial contrasts for within-
293 subjects factor and between-subjects factor.

294 For RT-qPCR expression, Kruskal–Wallis and Mann–Whitney nonparametric tests were performed
295 to analyze relative mRNA abundance. Differences were considered statistically significant at the
296 95% confidence level and $P<0.05$. Data were analyzed with GraphPad Instat 3.00 for Windows
297 (GraphPad software, Inc. La Jolla, CA, USA).

298

299 **RESULTS**

300 **Cell isolation**

301 The initial viability of EDCs and AMCs was >90%. EDCs and AMCs showed ability to adhere to
302 flask and typical polygonal epithelial morphology (Fig 1 A,B). Immunopositivity to
303 pancytocheratin confirmed that both cells lines were of epithelial origin (C,D). Previous molecular
304 biological analyses on AMCs at P3 showed that these cells displayed a typical stem cell phenotype,
305 with the expression of markers, such as CD29, CD44, CD106, CD105, and MHCI, but not CD34
306 and MHCII, as reported by Corradetti et al. (2013).

307 Previous molecular biology studies on EDCs have confirmed their endometrial nature due to the
308 expression of *ER-A*, *ER-B*, *PR* and *TP53* (Marini et al. 2016).

309

310 **MVs identification and uptake in bovine blastocyst**

311 The 100,000 g fractions isolated in this study and measured using NanoSight Instruments showed a
312 major peak in each preparation between 226-363 nm for EDCs (Fig 2A) and 220-295 nm for AMCs
313 (Fig 2B). NanoSight analysis determined that amniotic MVs had a dimension between 50 nm and
314 670 nm, with a mean of 258±55 nm. Considering the seeding density and the amount of MVs

315 obtained it possible to speculate that AMCs produced about 800 to 4700 MVs/cell, with a mean of
316 2550±71 MVs/cell, corresponding to 540x10^6 MVs/mL of CM. Endometrial cells instead produced
317 MVs with a dimension between 199 nm and 337 nm with a mean of 238±40 nm. Endometrial cells
318 produced about 2762 to 3414 MVs/cell with a mean of 3166±329 MVs/cell, corresponding to
319 670x10^6 MVs/mL of CM.

320 Fluorescent microscope analysis demonstrated that both amniotic and endometrial MVs were
321 internalized into blastomeres. Blastomere nuclei were stained in blue by Hoechst 33342 (Fig 3A)
322 and MVs in red by PKH-26 (Fig 3B). MVs incorporation was better evaluated by the merging of
323 blue and red images (Fig 3C). Previously, a dose–response curve was studied with different
324 concentration of MVs (10-20-40-60-80-100-150 x10^6 MVs/mL) revealing that the concentration of
325 100x10^6 MVs/mL provided the more intense fluorescence signal (data not shown). We calculated
326 that 100x10^6 MVs are present in about 20% of CM of both cell lines and, for this reason, SOF was
327 supplemented with 20% of CM or SN.

328 As seen by confocal microscopy, after incubation with labeled MVs, blastomeres showed a fine
329 granular fluorescent pattern within their cytoplasm at the same nuclei plane, indicating
330 incorporation of MVs (Fig 3D).

331

332 **Blastocyst rate**

333 A total of 5840 oocytes were fertilized over the course of this experiment that consisted of twenty
334 replicates. The number of embryos obtained in all the different experimental conditions was 1453.

335 Embryo morphology was evaluated on day 7 after fertilization under a stereomicroscope (Leica)
336 and the embryos were grouped according to stage (morula, compact morula and blastocyst). Poor
337 quality morulas/compact morulas were classified as degenerate if there was loss of plasma
338 membrane integrity (lysis) and/or generalized loss of cell forms.

339 Blastocyst formation rates obtained from the different fractions of secretome are showed in Fig 4. In
340 CTR, blastocyst formation rate was 35.45±2.53%.

341 No blastocysts were produced in either CM and SN by either cell line on day 1. Amniotic and
342 endometrial MVs on day 1 produced 24.24±2.75% and 26.19±2.82% of blastocysts, respectively.
343 These results were not statistically different (P>0.05) from each other but different (P<0.05)
344 compared to the CTR.
345 On day 3, CM and MVs, but not SN, of both cell lines provided embryos. Only amniotic MVs gave
346 a rate of embryos comparable to CTR, while the other experimental conditions provided numbers
347 that were statistically lower (P<0.05) when compared to CTR.
348 On day 5, amniotic CM and MVs provided blastocyst formation of 34.17±3.29% and 34.85±3.66%
349 respectively, and there was no statistical difference (P<0.05) between these results and CTR. The
350 25.80±2.83% rate produced by amniotic SN on day 5 was statistically lower (P<0.05) than all the
351 other groups previously described. All the blastocyst rates obtained by endometrial CM, SN or MVs
352 used on day 5 were statistically lower than amniotic secretome and CTR (21.69±1.87, 13.70±2.05
353 and 29.27±2.44%, respectively).
354

355 **Embryo viability evaluation**

356 The number of embryos used for viability evaluation in all experimental conditions, was 652. The
357 viability results of embryos obtained on exposure to different fractions of secretome are shown in
358 Fig 5. Figure 6 A shows an embryo on day 7. Figures 6 B,C and 6 D,E show embryos treated
359 respectively by amniotic derived MVs and endometrial derived MVs on day 5. Their corresponding
360 stainingswere performed on day 7. In embryo treated by endometrial MVs, a reduced inner cell
361 mass and a greater number of non viable blastomers were observed.
362 The rate of blastomeres viability in CTR was 92.36±4.48%.
363 Almost all experimental conditions that produced embryos provided a viability rate that was not
364 statistically different (P<0.05) compared to the CTR. Three conditions, namely endometrial CM
365 added on day 3, (83.61±5.18), endometrial SN added on day 5 (84.29±2.71) and endometrial MVs
366 added on day 5 (85.98±2.77) provided statistically significant lower viability compared to CTR.

367 The only treatments that statistically (P<0.05) enhanced viable blastomere production more than the
368 CTR were amniotic CM and MVs added on day 5 (99.4±4.83and 98.56±3.27% respectively).
369
370 **Embryo quality evaluation**
371 The number of embryos of all experimental conditions used for differential staining was 666.
372 Quality of embryo obtained by different fractions of secretome is showed in Table 2, 3, 4. Figure. 6
373 C shows the differential staining in an embryo on day 7, while Fig 7 displays the number of ICM.
374 The rate of ICM/trophoblast in CTR was 29.65±2.03.
375 When blastocysts were differentially stained to permit the counting of the inner cell mass and
376 trophectoderm, the rate of ICM/trophoblast was statistically similar (P>0.05) to CTR for all the
377 experimental condition with two exceptions. Namely, amniotic and endometrial MVs, added on day
378 1, gave a lower rate (P<0.05) of ICM/trophoblast than the CTR, while amniotic CM and MVs added
379 on day 5, provided a quality of embryos that was statistically better (P<0.05) than the control and
380 the other groups. This increase is due to the statistically higher number (P<0.05) of ICM cells in the
381 amniotic CM and MVs treated embryos compared to the CTR (33.66±1.93 and 34.42±1.27 vs
382 27.6±1.44, respectively). On the contrary, the number of trophoblast cells in these experimental
383 conditions was similar.
384
385 **Embryo gene expression**
386 With regard to genes related to apoptosis, *BAX* was significantly (P<0.05) downregulated in groups
387 treated with CM and MVs secreted by AMCs (0.5±0.12 and 0.5±0.13, respectively) but up-
388 regulated with SN of both cell lines compared to CTR group (P<0.05, Fig 8 A). *GPX1* (Fig 8 B),
389 was significantly upregulated (P<0.05) for treatments with CM and MVs secreted by AMCs
390 compared to CTR group (3.2±0.27 and 4.0±0.37). *GPX1* expression in AMC-MVs was statistically
391 different (P<0.05) compared to AMC-CM.
392

393 **DISCUSSION**

394 As is well know, IVP embryos have a lower quality, a lower number of blastomeres and a higher
395 apoptosis rate compared to those produced *in vivo* in different animal species (Booth et al. 2005;
396 Gjorret et al. 2005; Pomar et al. 2005). In addition, it is known that their implantation rate after
397 transfer is low (Kikuchi et al. 2002; Berg et al. 2010; Bakri et al. 2016).

398 To improve embryo quality, usually, *in vitro* blastocyst production is performed with feeder cells,
399 mainly oviduct and uterine epithelial cells, to mimic *in vivo* conditions. This suggests that paracrine
400 or autocrine communication between helper cells and embryos is present. During *in vitro* culture,
401 helper cells secrete many growth factors, receptors and binding proteins into the culture *medium*
402 (Van Langendonckt et al. 1996; Zquierdo et al. 1999). The potential of this *medium*, called CM, was
403 already established by Ijaz et al. (1994) and Zhu et al. (1994) that defined the CM as a complex
404 matrix of growth factors, cytokines and glycoproteins, secreted from cells, that may influence the
405 development of early embryos pre-implantation. In some experiments, the use of CM provided
406 embryo developmental rates similar to those obtained in the co-culture systems (Kobayashi et al.
407 1992; Mermillod et al. 1993; Lee et al. 2003), with the benefit of not having animal cells in the
408 embryo culture. To date, the individual effects of the components of CM on *in vitro* embryo
409 development has not been investigated. Lopera-Vásquez et al. (2016) evaluated the effect of CM
410 and extracellular vesicles (EVs) derived from bovine oviduct epithelial suggesting that EVs provide
411 functional communication between the oviduct and the embryo in the early stage of development.
412 These authors did not study the effect of soluble factors (SN) only and the incorporation of MVs
413 inside the blastomeres. Since the embryo interacts both with the uterus, for implantation, and with
414 the placenta, this study investigated the effect of different components of endometrial and amniotic
415 cell secretome on the IVP embryos development.

416 In this study, we first identified the presence of MVs in CM produced by both amniotic and
417 endometrial cell culture. By their size, these MVs could be classified as shedding vesicles, also
418 known as ectosomes or microparticles, that originate from direct budding and 'blebbing' of the

419 plasma membrane. These shedding vesicles are different from exosomes that arise from the cell
420 membrane endosomal compartment and are released into the extracellular space after fusion of
421 multivesicular bodies with the plasma membrane. Exosomes tend to be homogeneous in size (30-
422 120 nm) while shedding vesicles are more heterogeneous (ranging from 100 nm to 1 µm). MVs
423 found in amniotic and endometrial CM are similar in size (258±55 nm for AMCs and 238±40 nm
424 for EDCs) even if, in the same culture conditions and density of seeding, the EDCs produced more
425 MVs than AMCs ($670x10^6$ MVs/mL vs $540x10^6$ MVs/mL). Obviously, these data refer to an *in*
426 *vitro* study and no information about their production in *in vivo* conditions are available.
427 In this study, the control *medium* (no-CM) was tested to verify whether components of the original
428 *medium* used for culture had any effect on *in vitro* embryo production. No blastocysts were obtained
429 and development was arrested at early morula stage (data not shown). This is probably due to the
430 high level of glucose contained in HG-DMEM. Indeed, some results on post-fertilization embryo
431 development confirm that glucose has a detrimental effect on the course of *in vitro* embryo culture
432 (Kim et al. 1993; Gutiérrez-Adán et al. 2001). Conversely, during cell metabolism, the level of
433 glucose in CM decreases because it is converted to lactate and pyruvate (Pereira et al. 2014).
434 Next, the CM *in toto* and its different components, MVs and SN, were used on different days during
435 embryo culture to study the temporal effect, considering the changing nutritional requirements of
436 the embryo during its development.
437 The results of our study suggest that the exposure of bovine embryos at different times of culture to
438 the various fractions of secretome of both cell lines provides dissimilar rates of blastocyst
439 formation. Among the different experimental conditions, the best results were obtained when
440 supplementation started on day 5, corresponding also, in the case of amniotic CM and MVs, to the
441 better culture conditions for embryo quality. Indeed, this condition did not improve the blastocyst
442 rate compared to the CTR but enhanced the quality of embryos as observed by the increase of
443 ICM/trophoblast rate and by the proportion of viable blastomeres produced. In all the other
444 conditions, the rate of blastocyst production was lower than CTR and, in the main, the results

445 obtained by endometrial CM, SN or MVs used on day 5 were lower than those obtained by
446 amniotic secretome and CTR groups. In almost all the experimental conditions that reduced the
447 total number of cells, or that of the inner mass cells, a decrease in embryo viability was noted.
448 Moreover, the data obtained by addition of amniotic CM *in toto* or MVs were equivalent while
449 blastocysts number and quality provided by SN were always scarce or insignificant.
450 These data were confirmed by gene expression evaluation performed only on day five of treatment.
451 This time point was chosen because on day 5 embryos were produced in all the fractions of
452 secretome from both cell lines and, qualitatively, only amniotic CM and MVs produced statistically
453 greater inner cell mass than CTR. There is still not full agreement between studies on the set of
454 genes that will reflect accurately the effect of different culture conditions on embryo quality
455 (Cordova et al. 2014). Therefore, we selected two genes related to apoptosis and protection against
456 ROS, respectively. Melka et al. (2010) reported that *Bax* pro-apoptotic gene expression could be a
457 potential quality marker as they found an upregulation of this gene in poor quality 4-cell
458 preimplantation embryos with a higher DNA fragmentation, compared with morphologically good
459 quality embryos at the same stage. Our study, indeed, found a downregulation of this gene in groups
460 treated by CM and MVs secreted by AMCs compared to CTR group ($P<0.05$) and up-regulation in
461 groups treated by SN of both cell lines.
462 The embryo relies on the activity of certain antioxidant enzymes to protect itself from oxidative
463 stress (Harvey et al. 1995), so we evaluated mRNA expression of *GPX1* gene to investigate the
464 effect of different components of the secretome on embryo defense mechanisms against ROS.
465 GPX1 deficiencies can make cells more susceptible to possible stressors leading to an increase in
466 apoptosis. In support of this, the CTR group in our study shows less abundant *GPX1* mRNA
467 compared to groups exposed to CM and MVs secreted by AMCs, suggesting its susceptibility to
468 ROS damage. Moreover, the expression of *GPX1* in amniotic MVs is higher than in amniotic CM.
469 Probably, the MVs exert an anti-apoptotic effect on embryo, down-regulating the *Bax* gene and up-
470 regulating the *GPX1* gene, in the same way of MVs derived by stem cells, as demonstrated *in vitro*

471 by Bruno et al. (2012). These authors demonstrated that bone-marrow derived MVs up-regulated
472 anti-apoptotic genes, such as B-cell lymphoma-extra large (*Bcl-xL)*, B-cell lymphoma 2 (*Bcl2*) and
473 baculoviral IAP repeat containing 8 (*BIRC8*), and down-regulated genes that have a central role in
474 the execution-phase of cell apoptosis such as caspase-1 (*Casp1*), caspase-8 (*Casp8*) and
475 limphotoxin alpha (*LTA*), in cisplatin-treated human tubular epithelial cells. This effect is, perhaps,
476 attributable to the transfer of microRNA from MVs to target cells (in our case, the embryos).
477 The reasons for the good results on day 5 of supplementation are not clear, and can only be
478 hypothesized. In bovine species, activation of the genome starts between the 8 and 16 cell stage,
479 corresponding, to the embryonic stage attained in *vitro,* between days 2 and 4 of culture. The
480 embryo at this stage may be more susceptible to the influence of culture conditions compared to
481 other developmental stages (Xu et al. 2001). Environmental changes at this stage could disturb the
482 activation of the embryonic genome and this would explain why amniotic and endometrial
483 secretomes do not favor embryonic development. In fact, the supplementations on the 5th day
484 increased the development of blastocysts. Moreover, these findings mirror those in mouse studies
485 where two periods (between 48 and 72 hours, and between 72 and 120 hours), during which
486 embryos are more responsive to culture conditions, are recognized (Xu et al. 2001). Furthermore,
487 day 5 mimics the time at which the bovine embryo enters the uterus following fertilization. It is
488 important to underline that amniotic secretome appears more suitable than the endometrial one. This
489 result was surprising but it is likely that endometrial epithelial cells de-differentiate during culture
490 in monolayers. Some authors (Rottmayer et al. 2006; Sostaric et al. 2008; Gualtieri et al. 2009;
491 Ulbrich et al. 2010) reported that these cells lose cell polarity, cell height, ciliation, secretory
492 activity, and responsiveness to hormones, showing signs of alteration after 24-48 h post confluence.
493 In this way, the secretions produced by these cells *in vitro* are different compared to the *in vivo*
494 secretions. Moreover, it is probably that amniotic and endometrial secretomes contain different
495 components and that these differences cause different degrees of development. It is also probable
496 that a different secretome of amniotic and endometrial cells is attributable to the different age of

497 these tissue (adult for endometrium and fetal for amnion) but no data in literature support this
498 hypothesis. At this stage, we have not investigated the composition of secretome but it is known
499 that CM contain many factors secreted by somatic cells. Indeed, some studies have shown that
500 many factors, such as EGF, PDGF, LIF, IL6, IL1, bFGF, VEGF (Kobayashi et al. 1992; Baranao et
501 al. 1997; Ishiwata et al. 2000), and embryotrophic factors (Xu et al. 2001) are secreted from the
502 cells in co-culture and, therefore, are also present in the CM. It is the authors' belief, as supported
503 by the present study, that soluble factors do not affect the quality of embryos produced because
504 embryos developed in SN (that only contains soluble factors) did not show the benefits. As a matter
505 of fact, MVs produced the best results when added, either alone, or as components of CM *in toto,*
506 while positive benefits were not seen when soluble factors in CM deprived of MVs were provided.
507 Moreover, we demonstrated that embryos can uptake labeled MVs and it is possible there is a
508 mechanism of transfer of the MVs contents within the blastomeres.

509 To our knowledge no published literature relating to the incorporation of MVs of any origin into the
510 blastocyst exists. An unanswered question is which of the molecules contained in MVs are involved
511 in this communication. Recently, several studies have focused on miRNAs. These small, non-
512 coding, RNA molecules are present in many biological fluids such as follicular fluid and culture
513 *media* (Rosenbluth et al. 2014). While total miRNAs in human bio-fluids or supernatants from cell
514 cultures may be released from apoptotic cells or cell debris, MVs miRNAs are actively released by
515 viable cells and are thought to represent an active means of communication between cells and
516 tissues both locally or systemically. In particular, MV-encapsulated miRNAs are shielded from
517 degradation and are remarkably stable in biological fluids. Specifically, miRNAs that are
518 encapsulated in MVs might have a different role compared with miRNAs in bio-fluids as they can
519 transfer biological information to recipient cells (Machtinger et al. 2016).

520 Certainly, the presence of amniotic MVs improved embryo quality, compared to endometrial MVs,
521 by transfer of specific amniotic progenitor cell signals or material. We did not investigate the
522 different expression profile of miRNAs between amniotic and endometrial MVs. In our study, we

523 can only see their functional influence on embryo development. MiRNAs play an essential
524 regulatory role during development, indeed, aberration of blastocyst miRNA expression is
525 associated with human infertility (McCallie et al. 2010). It could be that miRNAs, transferred by
526 MVs within the blastocyst, contribute to the miRNA content of the blastocyst itself promoting their
527 development and quality. Moreover, it is likely that amniotic secretome promotes embryo
528 development in a more physiological way than endometrial secretomes, but further experiments are
529 planned to better explain these data and to better understand the cargo of MVs in both cell lines.
530 In conclusion, it is known that co-culture systems improve the *in vitro* development of embryos, but
531 our results suggest that MVs (along with their miRNA contents) are probably involved in this effect.
532 Indeed, the present study demonstrated that the brief *in vitro* exposure of embryos to amniotic CM
533 and MVs, but not to soluble factors (SN), improved embryo quality. It is essential to achieve a good
534 blastocyst formation rate in IVP and, in this study, culture with amniotic MVs gave a similar
535 formation rate to CTR. On the other hand, it is mandatory that IVP embryos should be of the
536 highest possible quality at the blastocyst stage to ensure optimal pregnancy rates after transfer,
537 especially after freezing and thawing. Amniotic MVs induced an increase of ICM and changes in
538 the profile of expression of some genes known to be related to embryo quality, suggesting reduced
539 apoptosis and increased capacity to survive oxidative stress. If relevant miRNAs and functional
540 targets can be identified, a possible clinical use for these molecules will represent the next front line
541 and may lead to novel strategies for better enhancing or manipulating reproductive efficiency.
542 Moreover, this study may provide a useful starting point for further studies related to paracrine
543 mechanisms of communications between embryos and culture *medium*.
544

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552

553 **CONFLICT OF INTEREST**

554 The authors declare that no conflict of interest and no competing financial interests exist in relation
555 to this manuscript.

556

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Table 1: Different experimental conditions of embryo culture and days of supplementation

Experimental conditions	days
SOF (CTR)	1
SOF + 20% CM-AMCs	1 or 3 or 5
SOF + 20% SN-AMCs	1 or 3 or 5
SOF + $100x10^6$ MVs-AMCs	1 or 3 or 5
	1 or 3 or 5
SOF + 20% CM-EDCs	
SOF + 20% SN-EDCs	1 or 3 or 5
SOF + $100x10^6$ MVs-EDCs	1 or 3 or 5

Legend: CTR, control; AMCs, amniotic mesenchymal cells; EDCs, endometrial cells; CM, conditioned medium; SN, supernatant; MVs, microvesicles.

739 **Table 2: Effect of conditioned *medium* (CM) secreted by amniotic or endometrial cells on**
740 **embryo quality evaluated by differential staining**

Conditioned *media* (CM)	day	Differential staining			
		Total cells	**ICM**	**TE**	**Ratio ICM:TE**
SOF (CTR)		120.69±5.43	27.6±1.44[a]	93.09±5.62	29.65±2.03[a]
SOF + CM-AMCs	1	-	-	-	-
SOF + CM-AMCs	3	115.43±8.82	25.6±2.72[a]	86.12±4.55	29.75±3.48[a]
SOF + CM-AMCs	5	128.66±3.52	32.4±1.83[b]	96.26±5.82	33.66±1.93[b]
SOF + CM-EDCs	1	-	-	-	-
SOF + CM-EDCs	3	101.66±7.55	20.82±2.98[c]	76.44±8.37	26.7±5.67[b]
SOF + CM-EDCs	5	102.91±3.58	25.41±1.03[a]	87.5±7.54	29.04±2.88[a]

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754 All values represent mean±standard error
755 Different small letters superscripts (a,b,c) indicate statistically different comparisons ($P<0.05$).
756 Legend: CTR, control; AMCs, amniotic mesenchymal cells; EDCs, endometrial cells; ICM, inner cell mass; TE,
757 trophectoderm cells.

758 **Table 3: Effect of supernatant (SN) secreted by amniotic or endometrial cells on embryo**
759 **quality evaluated by differential staining**

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Supernatant (SN)	day	Total cells	Differential staining		
			ICM	TE	Ratio ICM:TE
SOF (CTR)		120.69±5.43	27.6±1.44[a]	93.09±5.62	29.65±2.03 [a]
SOF + SN-AMCs	1	-	-	-	-
SOF + SN-AMCs	3	-	-	-	-
SOF + SN-AMCs	5	105.54±2.51	22.33±2.58[b]	83.21±5.86	26.84±2.89 [b]
SOF + SN-EDCs	1	-	-	-	-
SOF + SN-EDCs	3	-	-	-	-
SOF + SN-EDCs	5	98.98±2.26	20.29±1.17[b]	78.69±5.56	25.78±2.83 [b]

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772
773 All values represent mean±standard error
774 Different small letters superscripts (a,b,c) indicate statistically different comparisons (*P*<0.05).
775 Legend: CTR, control, AMCs, amniotic mesenchymal cells; EDCs, endometrial cells; ICM, inner cell mass; TE,
776 trophectoderm cells.
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778 **Table 4: Effect of microvesicles (MVs) secreted by amniotic or endometrial cells on embryo**
779 **quality evaluated by differential staining**

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Microvesicles (MVs)	day	Total cells	Differential staining		
			ICM	TE	Ratio ICM:TE
SOF (CTR)		120.69±5.43	27.6±1.44 [a]	93.09±5.62	29.65±2.03[a]
SOF + MVs-AMCs	1	108.15±8.49	20.87±1.64 [b]	87.28±3.23	23.91±2.41[b]
SOF + MVs-AMCs	3	107.83±8.41	25.71±2.16 [a]	82.12±4.37	31.31±3.33[a]
SOF + MVs-AMCs	5	132.98±8.42	34.42±1.27 [c]	98.56±4.66	34.92±2.98[c]
SOF + MVs-EDCs	1	115.72±7.55	23.92±2.66 [a]	91.78±3.95	26.06±2.37[a]
SOF + MVs-EDCs	3	105.77±8.57	20.98±1.14 [b]	84.79±6.12	24.74±2.85[b]
SOF + MVs-EDCs	5	104.42±7.21	23.18±2.83 [a]	81.22±6.65	28.54±2.51[a]

791
792 All values represent mean±standard error
793 Different small letters superscripts (a,b,c) indicate statistically different comparisons (P<0.05).
794 Legend: CTR, control; MVs, microvesicles; AMCs, amniotic mesenchymal cells; EDCs, endometrial cells; ICM, inner
795 cell mass; TE, trophectoderm cells.

796 **FIGURE LEGENDS**
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798 **Figure 1.** Cell morphology. Monolayer of epithelial endometrial (A) and amniotic epithelial cells
799 (B). Scale bar = 20μm. Original magnification x 20. Immunolocalization of pancytocheratin in
800 endometrial (C) and amniotic (D) cells. Scale bar = 15 μm. Original magnification 40 x.
801
802 **Figure 2.** NanoSight analysis. Results from analysis of MVs purified from endometrial (A) and
803 amniotic epithelial cells (B). The mean size and particle concentration values were calculated by the
804 Nanoparticle Tracking Analysis software that allows the analysis of video images of the particle
805 movement. The curve describes the relationship between particle number distribution (left Y-axis)
806 and particle size (X-axis).
807
808 **Figure 3.** Incorporation of MVs. Representative micrographs of internalization of MVs labeled with
809 PKH-26by blastomeres. Under a fluorescent microscope, the blastomeric nuclei are blue (A) and
810 the MVs are red (B). Representative merged image and z-stack orthogonal projection micrographs
811 showing the internalization of MVs as detected by confocal microscopy in blastocyst co-cultured
812 with MVs for 24 h (C). The images were taken at different planes scanned every 5 μm from top to
813 bottom of the blastocyst. Scale bar = 50 μm.
814
815 **Figure 4.** Graphic representation of effect of either amniotic or endometrial cell secretomes on
816 blastocyst formation rate. Different small letter superscripts (a,b,c) indicate statistically different
817 comparisons ($P<0.05$).
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819 **Figure 5.** Graphic representation of effect of amniotic or endometrial cell secretomes on embryo
820 viability. Different small letter superscripts (a,b,c) indicate statistically different comparisons
821 ($P<0.05$).

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823 **Figure 6.** Evaluation of blastocyst. Blastocyst at 7 day of culture observed under optical
824 microscopy (A). Viability staining (B) and differential staining (C) of embryos cultured with
825 amniotic derived MVs supplemented on day 5. Viability staining (D) and differential staining (E) of
826 embryos cultured with endometrial derived MVs supplemented on day 5. Scale bar = 50 μm.

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828 **Figure 7.** Graphic representation of effect of amniotic or endometrial cell secretomes on inner cell
829 mass. Different small letter superscripts (a,b,c) indicate statistically different comparisons ($P<0.05$).

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831 **Figure 8.** Quantitative PCR analysis for the expression *BAX* and *GPX1* in blastocysts treated on day
832 5 with secretomes from both cell lines. Expression levels normalized to the reference gene *GAPDH*.
833 Data are represented as fold-change compared with expression observed in CTR blastocysts. Values
834 are mean ± SD (n=3). Different letters depict significant ($P<0.05$) differences between treatments.