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

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22   **Running Title**: Secretome and *in vitro* embryo production

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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).

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

Cell isolation and culture

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

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

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

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

Immunocytochemical detection of pancytocheratin

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

Conditioned *medium*, microvesicles and supernatant production

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

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

Measurements of MVs

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

MVs labeling with PKH-26

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 $\Delta\Delta Ct$ 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

Statistical analysis

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

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

RESULTS

Cell isolation

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

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

MVs identification and uptake in bovine blastocyst

The 100,000 g fractions isolated in this study and measured using NanoSight Instruments showed a major peak in each preparation between 226-363 nm for EDCs (Fig 2A) and 220-295 nm for AMCs (Fig 2B). NanoSight analysis determined that amniotic MVs had a dimension between 50 nm and 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

DISCUSSION

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

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

In this study, we first identified the presence of MVs in CM produced by both amniotic and endometrial cell culture. By their size, these MVs could be classified as shedding vesicles, also 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 (670×10^6 MVs/mL vs 540×10^6 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

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

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

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

can only see their functional influence on embryo development. MiRNAs play an essential regulatory role during development, indeed, aberration of blastocyst miRNA expression is associated with human infertility (McCallie et al. 2010). It could be that miRNAs, transferred by MVs within the blastocyst, contribute to the miRNA content of the blastocyst itself promoting their development and quality. Moreover, it is likely that amniotic secretome promotes embryo development in a more physiological way than endometrial secretomes, but further experiments are planned to better explain these data and to better understand the cargo of MVs in both cell lines.

In conclusion, it is known that co-culture systems improve the *in vitro* development of embryos, but our results suggest that MVs (along with their miRNA contents) are probably involved in this effect. Indeed, the present study demonstrated that the brief *in vitro* exposure of embryos to amniotic CM and MVs, but not to soluble factors (SN), improved embryo quality. It is essential to achieve a good blastocyst formation rate in IVP and, in this study, culture with amniotic MVs gave a similar formation rate to CTR. On the other hand, it is mandatory that IVP embryos should be of the highest possible quality at the blastocyst stage to ensure optimal pregnancy rates after transfer, especially after freezing and thawing. Amniotic MVs induced an increase of ICM and changes in the profile of expression of some genes known to be related to embryo quality, suggesting reduced apoptosis and increased capacity to survive oxidative stress. If relevant miRNAs and functional targets can be identified, a possible clinical use for these molecules will represent the next front line and may lead to novel strategies for better enhancing or manipulating reproductive efficiency. Moreover, this study may provide a useful starting point for further studies related to paracrine mechanisms of communications between embryos and culture *medium*.

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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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726

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

728

729

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

735 Legend: CTR, control; AMCs, amniotic mesenchymal cells; EDCs, endometrial cells; CM, conditioned medium; SN,
736 supernatant; MVs, microvesicles.
737

738

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

Conditioned *media* (CM)	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 + 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]

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

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

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]

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

Table 4: Effect of microvesicles (MVs) secreted by amniotic or endometrial cells on embryo quality evaluated by differential staining

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]

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

796 **FIGURE LEGENDS**

797

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).

818

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).

822

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

827

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

830

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