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Oviduct extracellular vesicles protein content and their role during oviduct-embryo cross-talk

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1 **TITLE**

2 Oviduct extracellular vesicles protein content and their role during oviduct-embryo

3 cross-talk

4

5 **RUNNING TITLE**

6 EVs in oviduct-embryo dialogue

7

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45 **KEYWORDS:** Extracellular vesicles, Oviduct, Embryo development, Embryo-
46 maternal interactions, proteomic.

47

48 **ABSTRACT**

49 Successful pregnancy requires an appropriate communication between the mother and
50 the embryo. Recently, exosomes and microvesicles, both membrane-bound extracellular
51 vesicles (EVs) present in the oviduct fluid have been proposed as key modulators of this
52 unique cross-talk. However, little is known about their content and their role during
53 oviduct-embryo dialogue. Given the known differences in secretions by *in vivo* and *in*
54 *vitro* oviduct epithelial cells (OEC), we aimed at deciphering the oviduct EVs protein
55 content from both sources. Moreover, we analyzed their functional effect on embryo
56 development. Our study demonstrated for first time substantial differences between *in*
57 *vivo* and *in vitro* oviduct EVs secretion/content. Mass spectrometry analysis identified
58 319 proteins in EVs, from which 186 were differentially expressed when *in vivo* and *in*
59 *vitro* EVs were compared (p-value<0.01). Interestingly, 97 were exclusively expressed
60 in *in vivo* EVs, 47 were present only in *in vitro* and 175 were common. Functional
61 analysis revealed key proteins involved in sperm-oocyte binding, fertilization and
62 embryo development, some of them lacking in *in vitro* EVs. Moreover, we showed that
63 *in vitro* produced embryos were able to internalize *in vivo* EVs during culture with a
64 functional effect in the embryo development. *In vivo* EVs increased blastocyst rate,
65 extended embryo survival over time and improved embryo quality. Our study provides
66 the first characterization of oviduct EVs, increasing our understanding of the role of
67 oviduct EVs as modulators of gamete/embryo-oviduct interactions. Moreover, our
68 results point them as promising tools to improve embryo development and survival
69 under *in vitro* conditions.

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70 INTRODUCTION

71 Successful pregnancy requires an appropriate communication between the female
72 reproductive tract and the embryo(s). Disturbance in this unique communication system
73 is associated with high rates of early pregnancy loss and it is becoming increasingly
74 evident that it also influences the developmental potential of the offspring into
75 adulthood (Baker 1998, Mahsoudi et al. 2007). Strong evidence exists with respect to
76 the signals exchange between the early embryo and the oviduct leading to an
77 appropriate embryo development and successful pregnancy (Lee et al. 2002, Alminana
78 et al. 2012, Maillo et al. 2015). Absence of these oviduct signals in ART have raised the
79 question of how much these techniques can affect the outcomes (Ostrup et al. 2011,
80 O'Neill et al. 2012). Significant gains in our understanding of the essential
81 embryotrophic components of the oviduct fluid and their interactions with the embryo
82 have been achieved in the last years (Georgiou et al. 2007, Leese et al. 2008, Aviles et
83 al. 2010, Schmaltz-Panneau et al. 2014). However, there is a need for further exploring
84 the contribution of the oviduct to the reproductive success.

85

86 Recently, exosomes and microvesicles, have been identified as essential components of
87 uterine (Ng et al. 2013, Burns et al. 2014) and oviduct fluids (Al-Dossary *et al.* 2013,
88 Lopera-Vasquez *et al.* 2017). Exosomes are 30-150 nm vesicles of endocytotic origin
89 released upon fusion of a multi-vesicular body with the cell membrane, while
90 microvesicles are 100-1000 nm in diameter and bud directly from the cell membrane
91 (Colombo et al. 2014). Both are collectively known as extracellular vesicles (EVs), and
92 are considered important tools in cell-to-cell communication (Valadi et al. 2007) by
93 transferring their molecular cargo (proteins, mRNA, miRNA) from one cell to another.
94 In the maternal tract, they have been proposed as important tools to regulate

95 gamete/embryo-maternal interactions (Al-Dossary & Martin-Deleon 2016, Burns et al.
96 2016). However, while different studies have evaluated the EVs secretion/content
97 (proteins, miRNA) produced by the uterus from *in vivo* (Ng et al. 2013, Burns et al.
98 2016) and *in vitro* origin (Greening et al. 2016, Bidarimath et al. 2017), none have
99 provided an extensive characterization of oviduct EVs content up to date. An important
100 requisite to decipher the possible role of the EVS in the embryo-oviduct dialogue. Only
101 one protein, PMCA4a, which is essential for sperm hyperactivated motility and fertility
102 have been identified in oviduct EVs (Al-Dossary et al. 2013). Despite our lack of
103 knowledge about their content, the EVs derived from *in vivo* oviduct fluid and *in vitro*
104 culture of bovine oviduct epithelial cells (BOEC), seem to improve the cryotolerance of
105 *in vitro* produced embryos (Lopera-Vasquez *et al.* 2016, Lopera-Vasquez *et al.* 2017).

106

107 Given the known differences in secretions by BOEC *in vivo* and *in vitro* (Rottmayer et
108 al. 2006), and the increasing number of studies based on EVs derived from *in vitro*
109 primary cell culture or cell lines, a comparative study of the EVs content of *in vivo* and
110 *in vitro* origin seems imperative. Thus, we aimed at: 1) deciphering the oviduct EVs
111 protein content from *in vivo* and *in vitro* origin; 2) analyzing whether embryos are able
112 to internalize oviduct EVs and 3) investigating their functional effect on embryo
113 development. For this purpose, a bovine model was used, since bovine has been
114 demonstrated to be a valuable experimental model for addressing ART-related
115 questions.

116

117 **MATERIAL AND METHODS**

118 **Collection of bovine oviduct fluid and epithelial cells (BOEC)**

119 Oviducts and ovaries were obtained from cows at local slaughterhouse (Sablé sur
120 Sarthe, France), with the permission of the direction of the slaughterhouse and the
121 agreement of local sanitary services. Oviducts and their attached ovaries were
122 transported to the laboratory at 37°C within 2-3 h after collection. For all experiments,
123 ipsilateral and contralateral oviducts from the same animal at the post-ovulatory stage of
124 the bovine estrous cycle were used. Animals showing recent ovulation sites in the
125 attached ovaries, indicating they were at postovulatory stage (1-5 days of estrus cycle),
126 were selected for EVs collection. To minimize variability, the same oviducts were used
127 for *in vivo* EVs collection by oviduct flushing than for *in vitro* EVs collection, by using
128 the conditioned media after *in vitro* BOEC primary culture. First, oviducts were
129 dissected free from surrounding tissues. Subsequently, to recover the oviduct flushing
130 the lumen of the oviduct was flushed with 500µl of sterile PBS (Sigma P4417-TAB,
131 Lyon, France). Then, BOEC were isolated by mechanical scraping of the oviduct with a
132 slide for primary *in vitro* BOEC culture as described by Van Langendonck and
133 colleagues (Van Langendonck et al. 1995). BOEC were washed three times by
134 sedimentation in 10mL of Tissue culture medium-199-Hepes (TCM-199, Sigma
135 M7528) supplemented with bovine serum albumin (BSA stock fraction V, Sigma
136 A9647) and 8µl/mL gentamycin (Sigma G1272). The resulting cellular pellet was
137 diluted 100 times in culture medium consisting in TCM-199 (Sigma M4530)
138 supplemented with 10% heat-treated foetal calf serum (FCS, Sigma F9665) and 8µl/mL
139 gentamycin before seeding. At this point, an aliquot of *in vivo* BOEC (from the day
140 (day 0) of collection) was stored at -20°C for further comparative protein analysis with
141 *in vitro* BOEC and EVs by Western blotting, while the rest of BOEC were seeded for
142 culture.

143

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144 **Bovine oviduct epithelial cell *in vitro* culture**

145 Our BOEC *in vitro* culture system has already been used to study early oviduct-embryo
146 interactions, demonstrating to be a good oviduct-like environment to support embryo
147 development *in vitro* (Cordova et al. 2014, Schmaltz-Panneau et al. 2014). BOEC were
148 cultured in 25 cm² flasks (FALCON 25cm² 353109) with TCM 199 (Sigma M4530,
149 France) supplemented with 10% Fetal Bovine Serum (Sigma F9665, France) and
150 gentamycin (Sigma G1272, 10 mg/mL) in a humidified atmosphere with 5% CO₂ at
151 38.8 °C. The medium was completely renewed at day 2. Subsequently, half of the
152 medium was replaced every two days until cells reached confluence (6-8 days). Then,
153 BOEC were washed and cultured in TCM-199 free of serum. After two days, the serum
154 free medium was completely renewed and the cells were cultured for two additional
155 days before collection of conditioned medium. BOEC viability was determined after
156 collection of conditioned media by using Live/Dead viability assay kit (LIVE/DEAD®
157 Cell Viability Assay, Life Technologies, L3224). At this point, an aliquot of *in vitro*
158 BOEC was stored at -20°C for further comparison of protein content with *in vivo* BOEC
159 (day 0) and EVs collected from them by Western blotting.

160

161 **Isolation of EVs from *in vivo* and *in vitro* origin**

162 Oviduct flushings from different animals were pooled (n=3animals per replicate; in 4
163 replicates). Conditioned media obtained from different 25cm² flasks were also pooled
164 (total of 100 mL/replicate; in 4 replicates). EVs were obtained from oviduct flushings
165 and conditioned media by serial centrifugation as described by Théry et al., (Théry et al.
166 2006). First, flushing and conditioned media were centrifuged at 300 g for 15 min,
167 followed by 12,000 g for 15 min to remove cells, blood, and cell debris and
168 ultracentrifuged twice at 100,000 g for 90 min (BECKMAN L8-M; SW41T1 rotor) to

169 pellet exosomes. The pellets were resuspended in 100 μ L of PBS and stored at -20°C for
170 further analysis.

171

172 **Transmission electron microscopy (TEM)**

173 Vesicle suspensions were diluted in PBS to attain a protein concentration of 0.6 μ g per
174 μ L. Then, 3 μ l of the sample were placed on the formvar carbon-coated grid for 5 min
175 and washed with distilled water (three times). For negative contrast the samples were
176 stained with 2% uranyl acetate for 2 min and left to dry. The micrographs were obtained
177 using TEM HITACHI HT 7700 Elexience at 80kV (with a charge-coupled device
178 camera AMT) and JEM 1011 (JEOL, Japan) equipped with a Gatan digital camera
179 driven by Digital Micrograph software (Gatan, Pleasanton, USA) at 100 kV. The
180 processing of the photos and exosome size calculation were carried out by ImageJ
181 software.

182

183 **EVs labeling, EVs-embryo co-incubation and observation**

184 *In vivo* EVs preparations from oviduct flushings (pool of three animals; 3 replicates)
185 were labeled with a lipophilic green fluorescence dye (PKH67, Sigma, France) as
186 described by Saadeldin, et al., (Saadeldin et al. 2014). PKH67 is a widely-used dye for
187 visualization of exosomes uptake by cells (Burns et al. 2014, Saadeldin et al. 2014).
188 First, a dilution of the EVs preparation was performed by mixing 25 μ L of the EVs
189 suspension in PBS with 125 μ L of diluent C (Cell mixture). In addition, 25 μ L of PBS
190 were mixed with 125 μ L of diluent C as a negative control. Then, the dye dilution was
191 prepared by adding 1 μ L dye to 250 μ L of diluent C and 125 μ L of this mixture were
192 added to EVs and Control mixtures and incubated for 5 min at room temperature (Final

193 concentration of dye is 5×10^{-6} M). To stop the labelling reaction 1 mL of free EVs-FBS
194 (previously ultracentrifuged at 100,000 g during 16h at 4°C to remove exosomes) was
195 added for 1 minute. To wash the excess of dye from EVs, the tube of EVs suspension
196 was filled with M199 media with 5% FBS (EVs-free) and twice ultracentrifuged at
197 100,000 g, 4°C, for 30 min. The final pellet was resuspended in 100µL of embryo
198 culture medium (SOF) as described below with 5% FBS (EVs-free) and used to prepare
199 the drops for embryo development.

200 *In vitro* produced embryos at the blastocyst stage (with intact zona pellucida) and
201 hatched embryos (with total or partial absence of zona pellucida) were *in vitro* cultured
202 with green-labeled EVs or control (dye-PBS) dilution for 18-20h. Prior to fixation,
203 embryos were washed twice in EVs-free medium to remove any extraneous labelled
204 vesicle not internalized. Then, embryos were fixed with 4% paraformaldehyde with
205 Saponine 0.5%, labelled with Hoechst 33342 and Actin Red Phalloidin and observed by
206 Confocal microscope (LSM780 Confocal Zeiss Observer Z1 with ZEN 2011 software).
207 For this experiment, 4 replicates were performed, with 15-20 embryos incubated with
208 green-EVs or control (dye-PBS) for each replicate.

209

210 **Proteomic analysis**

211 *Mass Spectrometry (MS) analysis*

212 EVs preparations from *in vivo* and *in vitro* origins were analyzed by SDS-PAGE
213 combined with nanoLC-MS/MS with spectral counting and extracted ion
214 chromatography (XIC) methods of quantification.

215 *Sample preparation for MS analysis*

216

217 Samples were lysed in 2% SDS pH 6.8 in Tris buffer with protease inhibitors (Sigma
218 P2714) followed by centrifugation 10 minute at 15000g. Protein concentrations in the
219 samples were determined using the Uptima BC Assay kit (Interchim, Montluçon,
220 France) according to manufacturer's instructions and using bovine serum albumin as a
221 standard. SDS-PAGE electrophoresis was carried out according to Laemmli's method
222 (Laemmli, 1970) on 10% gradient polyacrylamide gels. Reduce Laemmli buffer was
223 used for sample preparation followed by vortexing and heating in water bath at 95C° 5
224 min.

225 Forty µg of proteins from *in vivo* and *in vitro* EVs preparations were migrated
226 separately applied on 10% SDS-PAGE 8.3 cm × 7.3 cm × 1.5 mm gels (50V, 30 min)
227 (10 µg EVs preparation/replicate from 4 replicates were pooled). A brief migration was
228 performed until samples were concentrated in a single narrow band. The resulting
229 protein bands from the two pools (*in vivo* and *in vitro* EVs preparations) were stained
230 with Coomassie blue (G-250). Densitometric quantification of Coomassie blue-stained
231 protein bands was performed by transmission acquisition with an ImageScanner (GE
232 Healthcare, Orsay, France) and analyzed with TotalLab (Nonlinear Dynamics Limited,
233 Newcastle, UK) to check for the equivalent amount of protein between samples. Then,
234 each lane was cut horizontally in 3 bands for a quantitative proteomic analysis. Gel
235 slices from the two pooled samples were washed in water/acetonitrile (1:1) for 5 min
236 and in acetonitrile for 10 min. Cysteine reduction and alkylation were performed by
237 successive incubations in 10 mM dithiothreitol/50 mM NH₄HCO₃ for 30 min at 56°C
238 and 55 mM iodoacetamide/50 mM NH₄HCO₃ for 20 min at room temperature in the
239 dark. Gel slices were washed by incubation in 50 mM NH₄HCO₃/acetonitrile (1:1) for
240 10 min and by incubation in acetonitrile for 15 min. Proteins were digested overnight in
241 25 mM NH₄HCO₃ with 12.5 ng/µL trypsin (Sequencing Grade, Roche, Paris). The

242 resulting peptides were extracted from gel by successive incubations in 0.1% formic
243 acid (FA)/acetonitrile (1:1) for 10 min and in acetonitrile for 5 min. The two extracts
244 were pooled, dried, reconstituted with 30 μ l of 0.1% FA, 2% acetonitrile, and sonicated
245 for 10 min before MS analysis.

246

247 *Nano LC-MS/MS analysis*

248 Peptide mixtures were analyzed by nanoflow liquid chromatography-tandem mass
249 spectrometry (nanoLC–MS/MS). All experiments were performed on a LTQ Orbitrap
250 Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to an
251 Ultimate® 3000 RSLC Ultra High Pressure Liquid Chromatographer (Dionex,
252 Amsterdam, The Netherlands) controlled by Chromeleon Software (version 6.8 SR11;
253 Dionex, Amsterdam, The Netherlands). Five microliters of each sample was loaded on
254 trap column for desalting and separated using nano-column as previously described by
255 Labas et al., (Labas et al. 2015). The gradient consisted of 4-55% B for 120 min at 300
256 nl/min flow rate. The eluate was ionised using a Thermo Finnigan Nanospray Ion
257 Source 1 with a SilicaTip emitter of 15 μ m inner diameter (New Objective, Woburn,
258 MA, USA). Standard mass spectrometric conditions for all experiments were spray
259 voltage 1.2 kV, no sheath and auxiliary gas flow; heated capillary temperature, 275°C;
260 predictive automatic gain control (AGC) enabled, and an S-lens RF level of 60%.

261 Data were acquired using Xcalibur software (version 2.1; Thermo Fisher Scientific, San
262 Jose, CA). The instrument was operated in positive data-dependent mode. Resolution in
263 the Orbitrap was set to R = 60,000. In the scan range of m/z 300-1800, the 20 most
264 intense peptide ions with charge states ≥ 2 were sequentially isolated (isolation width, 2
265 m/z; 1 microscan) and fragmented using Collision Induced Dissociation (CID). The ion
266 selection threshold was 500 counts for MS/MS, and the maximum allowed ion

267 accumulation times were 200 ms for full scans and 50 ms for CID-MS/MS in the LTQ.
268 Target ion quantity for FT full MS was 1e6 and for MS/MS it was 1e4. The resulting
269 fragment ions were scanned at the “normal scan rate” with $q = 0.25$ activation and
270 activation time of 10 ms. Dynamic exclusion was active during 30 s with a repeat count
271 of 1. The lock mass was enabled for accurate mass measurements.
272 Polydimethylcyclsiloxane (m/z , 445.1200025, $(\text{Si}(\text{CH}_3)_2\text{O})_6$) ions were used for
273 internal recalibration of the mass spectra.

274

275 *Data processing and statistical analysis*

276 Raw data files were converted to “Mascot Generic File” (.MGF) with Proteome
277 Discoverer software (version 1.4; hermoFisher Scientific, Bremen, Germany). The
278 peptides and fragments masses obtained were matched automatically against a locally
279 maintained copy of NCBI (8 000 106 entries, download 08/07/2015). MS/MS ion
280 searches were performed using MASCOT Daemon and search engine (version 2.2.2;
281 Matrix Science, London, UK). The parameters used for database searches included
282 trypsin as a protease with two missed cleavages allowed, and carbamidomethylcysteine,
283 oxidation of methionine and N-terminal protein acetylation as variable modifications
284 and peptide charge 2 and 3+. The tolerance of the ions was set at 5 ppm for parent and
285 0.8 Da for fragment ion matches. Mascot results were incorporated into Scaffold 4
286 software (Proteome Software, Portland, USA). Peptides identifications were accepted if
287 they could be established at over 95.0% probability as specified by the Peptide Prophet
288 algorithm (Keller et al. 2002). A false discovery rate was calculated as <1% at the
289 peptide or protein level.

290 Five nanoLC-MS/MS analyses were performed for each *in vivo* and *in vitro* EVs
291 preparations. Quantifications were based on the label-free quantitative method,

292 eXtracted Ion Chromatogram peptide pattern (XIC) (Higgs et al. 2005, Wang et al.
293 2006). SIEVE version 1.3. software (Thermo Fisher Scientific) was used for XIC
294 quantification. With time-aligned chromatograms, the frame m/z and the retention time
295 (RT) were used to perform extracted ion chromatograms (XICs). The framing
296 parameters were set at 0.02 Da for the 300–1800 m/z mass range and 6 min for the RT
297 window for all MS2 data. The autodigested tryptic peptide at m/z 1082.0300 was used
298 to normalize independent samples. The algorithm determined peptide abundance
299 between 2 sample groups, frame-by-frame. A t-test was performed to characterize
300 changes between *in vivo* and *in vitro* EVs preparations. Differences were considered
301 statistically significant at p-value<0.01. Following the Proteome Discoverer version 1.3.
302 databank searches (Thermo Fisher Scientific) using the Mascot server, the .msf files
303 were integrated into SIEVE. The results were filtered with protein normalized ratios <
304 0.5 and ratio > 2, with Mascot ion scores >20. Identified frames were accepted manually
305 when peptides were validated by the Protein and Peptide Prophet Algorithms used in
306 Scaffold software.

307 MS data have been deposited to the ProteomeXchange Consortium (Vizcaino et al.
308 2014) via the PRIDE partner repository with the dataset identifier 10.6019/PXD002280.
309

310 *Data mining and Bioinformatics analysis*

311 Gene symbols and Entrez Gene IDs (bovine and putative human orthologs) were
312 mapped for all protein identifications and analyzed using the online bioinformatics tools
313 available via the biological DataBase network bioDBnet (tool db2db)
314 (<http://biodbnet.abcc.ncifcrf.gov/>;(Mudunuri et al. 2009)) and custom tools integrated in
315 a local Galaxy (Giardine et al. 2005) installation (NCBI annotation mapper,
316 Mammalian Ortholog and Annotation database, MOADb; Bick J, ETH Zurich,

317 unpublished results 2016). The background dataset for the analysis was the human
318 genome. To obtain meaningful findings of the EV proteins identified functional analysis
319 was performed using PANTHER (<http://www.pantherdb.org>; (Thomas et al. 2003)) and
320 DAVID Functional Annotation (<https://david.ncifcrf.gov>); (Huang et al. 2007)). To
321 visualize interactions among candidate EV proteins and integration of the different
322 networks the Cytoscape app ClueGO was used (<http://www.cytoscape.org/>; Cytoscape
323 3.3.0 and app ClueGO v2.2.5; (Shannon et al. 2003)).

324

325 *Western blotting*

326 Proteins were separated by SDS-PAGE (8-16% gradient polyacrylamide gels using 4 µg
327 of proteins per lane) and transferred onto nitrocellulose membranes (GE Healthcare Life
328 Sciences Whatman™) over 16h at 30v, 300A. The membranes were washed in distilled
329 water and blocked with Tris buffered saline (TBS) containing Tween 20 (0.5% (w/v)),
330 and supplemented with lyophilized low-fat milk (5% w/v) for 1h at room temperature.
331 The membranes were incubated with primary antibodies diluted in TBS-Tween
332 containing low-fat milk (1% w/v) for 2h at 37°C with gently shaking. The primary
333 antibodies used were: anti-heat shock protein 70 (HSP70; Stressgen, SPA-810); anti-78
334 kDa glucose-regulated protein (GRP78; Santa Cruz Biotechnology sc-13968); anti-heat
335 shock protein A8 (HSPA8; bioss.com, bs-5117R); anti-bovine oviduct glycoprotein
336 (OVGP; kindly donated by P.A. Mavrogianis at University of Illinois in USA); anti-
337 Myosin heavy chain 9 (MYH9 (H40), Santa Cruz Biotechnology, sc-98978); anti-
338 Cluster of Differentiation 109 (CD109, Santa Cruz Biotechnology, sc98793); and anti-
339 Lactadherin (PAS6/7, a gift from Dr. J.T. Rasmussen). After primary antibodies
340 incubation, the membranes were washed with TBS with 0.5% Tween 20 and incubated
341 overnight at 4°C under agitation with secondary antibodies. The secondary antibodies

342 used were: horseradish peroxidase (HRP)-anti-mouse (Sigma A4416) or anti-rabbit
343 (Sigma A6154). Blots were developed using a mixture of two chemiluminescence
344 substrates developing kit (GE Healthcare AmershamTH ECL SelectTH Western
345 blotting detection Reagent RPN2235 and Supersignal West Pico #34087
346 Chemiluminescent Substrate Thermo Scientific).

347

348 ***In vitro* embryo production (IVP) and EVs supplementation to *in vitro* embryo** 349 **culture**

350 Bovine embryos were produced *in vitro* as previously described by Cordova and
351 colleagues (Cordova et al. 2014). Briefly, bovine ovaries were collected at a local
352 slaughterhouse. Cumulus-oocyte complexes (COC) were aspirate, washed and
353 incubated in maturation media for 22 hours. Subsequently, COC were fertilized with
354 semen from a bull with proven fertility. Twenty hours after IVF, presumptive zygotes
355 were vortexed to remove cumulus cells and attached spermatozoa and washed into wells
356 containing 500 μ L of *in vitro* culture medium. The medium used for *in vitro* culture
357 (IVC) was synthetic oviduct fluid (SOF) medium (Holm et al., 1999) supplemented
358 with 5% fetal calf serum (FCS, MP Biomedicals, MP5418) (EVs-depleted by
359 ultracentrifugation).

360

361 Having in mind the practical application of EVs in the IVP lab, we decided to compare
362 the effect of fresh and frozen *in vivo* EVs supplementation versus control (non-
363 supplementation) in our embryo culture system. Experiments were performed in 4
364 replicates. EVs were prepared pooling oviducts from 3 animals for each replicate as
365 above mentioned. For each replicate, EVs preparations were divided in two aliquots

366 (frozen and fresh samples). Frozen samples were kept at -80 °C 3h while fresh samples
367 were kept at 4 °C until IVC media supplementation. For each replicate, EVs protein
368 concentration was measured ranging from 1.61-5.34 mg/mL and EVs supplementation
369 was added to the culture medium at a final concentration of 0.22-0.42 mg/mL. Fresh
370 and frozen EVs were diluted in IVC media and filtered (0.22µm). Then 25 µL drops of
371 IVC medium supplemented with or without EVs were prepared. Subsequently, groups
372 of 25 presumptive zygotes were cultured into these 25 µL drops of SOF medium with
373 or without EVs supplementation, overlaid with 700 µL of mineral oil. Embryo-EVs co-
374 culture was performed into 38.8 °C, 5% O₂, 5% CO₂, and 90% N₂ conditions during 9
375 days. Embryos were allocated in 3 groups according to the experimental design:
376 control, fresh EVs and frozen EVs to evaluate embryo development and quality in
377 terms of cleavage (Day 2 post IVF), blastocyst rate (Days 6, 7, 8 and 9), hatching rates
378 (Days 8 and 9) and number of cells (Day 9).

379

380 RESULTS

381 1. Differential protein profile in oviduct EVs from *in vivo* and *in vitro* origin

382 We validated the presence of EVs in both *in vivo* and *in vitro* samples by using TEM as
383 well as Western blot analysis. Transmission Electron microscopy (TEM) observations
384 confirmed the presence of EVs in bovine oviduct flushings (Figure 1A) and also in the
385 conditioned media from BOEC primary *in vitro* culture (Figure 1B). All four replicates
386 analyzed from *in vivo* and *in vitro* preparations showed a population of small-
387 extracellular vesicles (30-100nm) resembling exosomes and a population of large-
388 extracellular vesicles (>100nm-) resembling microvesicles (Figure 2A and 2 B). In the
389 literature, microvesicles ranged from (>100 up to ~1,000 nm) (Raposo & Stoorvogel

390 2013). Histograms of figure 2 showed the distribution of exosomes and microvesicles in
391 the *in vivo* (Figure 2A) and *in vitro* preparations (Figure 2B).

392

393 Western blotting for exosomal protein markers and oviduct proteins with known
394 reproductive roles were performed in EVs *in vivo* and *in vitro* preparations and their
395 cells of origin (Figure 3). EVs were positive for HSP70, a recognized exosomal protein
396 (positive control) present in 89% of exosome proteomic studies (Mathivanan et al.
397 2010, Klohonatz et al. 2016) and negative for Grp78, an endoplasmic reticulum marker
398 detected in BOEC from both origins and not in EVs (negative control) (Figure 3).
399 Moreover, *in vivo* EVs expressed oviduct glycoprotein (OVGP1), heat shock protein A8
400 (HSPA8) and myosin 9 (MYH9), while only HSPA8 and MYH9 were detected in *in*
401 *vitro* EVs. When the cells of origin were analyzed for these reproductive proteins,
402 OVGP1, MYH9 and HSPA8 were expressed in all *in vivo* samples. In contrast, MYH9
403 and HSPA8 were expressed in all *in vitro* oviduct cells whereas OVGP1 was not
404 expressed in any of the samples *in vitro*. Taken together, the Western blot (molecular)
405 data and TEM (bio-physical) showed that both *in vivo* and *in vitro* oviduct cells produce
406 EVs but with different qualitative and quantitative characteristics.

407

408 To understand the potential roles of EVs in the oviduct environment as modulators of
409 gamete/embryo-oviduct cross-talk, we performed the first proteomic analysis of oviduct
410 EVs using Mass spectrometry. This analysis allowed us to compare the *in vivo* and *in*
411 *vitro* oviduct EVs signature. Mass Spectrometry identified a total of 315 proteins, from
412 which 97 were exclusively detected in *in vivo* EVs, 47 were found only in *in vitro* and
413 175 were in common to both samples (Venn diagram, Figure 4, and Supplementary data
414 S1-S3). Moreover, Figure 4 represents the total of proteins identified in *in vivo* (270)

415 and *in vitro* (222) EVs samples. While the bar graph below shows the number of
416 proteins in common (175) or exclusive to *in vivo* or *in vitro* EVs (142). From the total
417 of 315 identified proteins, 186 were differentially expressed when *in vivo* and *in vitro*
418 EVs were compared (p-value<0.01; ratio<0.5 or ratio>2) (Supplementary data S4).

419

420 Mass Spectrometry results were confirmed by Western blot analysis on candidate
421 proteins present in EVs and associated to reproductive functions (Figure 5). Western
422 blot analysis was performed in 4 independent biological replicates for CD109, and
423 Lactadherin. While Lactadherin was expressed only in *in vitro* EVs preparations,
424 CD109 was expressed only in *in vivo* EVs preparations. These results confirmed the
425 different proteomic profile of EVs from *in vivo* and *in vitro* origin.

426

427 *2. Deciphering the EV proteomic content from in vivo and in vitro origin*

428 Mass Spectrometry data provided the first proteomic signature of oviduct EVs, and
429 identified family of proteins in their cargo characteristic of exosome protein
430 composition. The exosomal cargo included proteins involved in exosome biogenesis
431 and intracellular vesicle trafficking, including tetraspanins (CD46, CD109, CD9) and
432 Rab GTPases (RAB5C, RAB7A, RAB11B, RAB1A; ARF4). The identified tetraspanin
433 and rab family proteins were present in both *in vivo* and *in vitro* EVs. In addition, we
434 found that EVs cargo were enriched in Annexins, another class of proteins commonly
435 seen in exosomes, involved in membrane trafficking and fusion events (ANXA1,
436 ANXA2, ANXA3, ANXA4, ANXA5, ANXA7, ANXA8, ANXA11). Most of them
437 common to both EVs preparations except ANXA7 that was only expressed in *in vivo*
438 EVs. EVs were also enriched with heat-shock proteins (HSPA1A, HSP60, HSPA4,

439 HSPA5, HSPA8, HSP90AA1, HSP90AB1, HSP90B1) and other proteins related to
440 adhesion such as MFGE8/Lactadherin and integrins (ITGB1).

441

442 Besides the identification of common exosomal proteins, we performed functional
443 analysis of identified EV proteins from *in vivo* and *in vitro* origin to obtain a better
444 understanding of the role of the EV proteins in different biological processes and,
445 particularly, in gamete/embryo-oviduct communication and in supporting embryo
446 development. From the 315 proteins identified in EVs preparations, Gene ontology
447 (GO) analysis using PANTHER database revealed that a high number of these proteins
448 was involved in metabolism (24%), cellular process (20.4%), localization (10.2%),
449 developmental processes (7.7%), immune system (5.6%), response to stimulus (7.4%),
450 and in reproductive processes (1.4%) (Figure 6). Further analysis revealed that more
451 than 58% of EVs proteins involved in cellular process were associated with cell-to-cell
452 communication (Figure 6). Moreover, DAVID functional annotation clustering for EVs
453 proteins showed that clusters related to “vesicle”, “cytoplasmic vesicle”, and
454 “membrane-bounded vesicle” had the highest enrichment score (enrichment score 19.9,
455 62 proteins involved) while clusters related to fertilization were also found with a
456 relative high enrichment score (Table I). A complete list of clusters is provided in
457 Supplementary data S5.

458 A deeper analysis combining PANTHER, DAVID and GeneCards databases and the
459 literature, revealed 36 EVs proteins (11.42% from 315) involved in important
460 reproductive functions such as fertilization and embryo development (Table II).

461 Furthermore, to obtain a more integrative visualization of the differential proteins
462 identified in *in vivo* and *in vitro* EVs and their biological functions Cytoscape (app

463 ClueGO) was used. Figure 7 shows clear differential networks of functional categories
464 for *in vivo* and *in vitro* EVs proteins.

465

466 3. Demonstrating the traffic of oviduct EVs to the early embryo.

467 Here, we demonstrated that IVP embryos were able to internalize *in vivo* EVs. We
468 selected *in vivo* EVs for our experiment, since our proteomic analysis pointed out
469 important differences between *in vivo* and *in vitro* exosomes such as OVGP1 protein,
470 only present in *in vivo* EVs and involved in supporting early embryo development.

471 EVs were isolated and labeled with green fluorescent dye (PKH67), filtered ($0.22 \mu\text{m}$)
472 and co-incubated with blastocysts (with intact zona pellucida) and hatching/hatched (H)
473 blastocysts (with partial or total absence of pellucida) produced *in vitro*. Confocal
474 microscopy observations confirmed that *in vivo* EVs were internalized by blastocysts
475 (Figure 8, A-C) and H-blastocysts (Figure, D-F) and located around the nucleus. Figure
476 8 (I-J) shows that EVs were actually present in the cytoplasm and not only attached to
477 the embryo membrane. No green fluorescent EVs were observed in the negative
478 controls of embryo co-cultured with PBS-dye (G-H).

479

480 4. Oviduct EVs supplementation improved bovine *in vitro* blastocyst yield and quality

481 Having in mind the practical application of EVs in the IVP lab, we decided to compare
482 the effect of fresh and frozen *in vivo* EVs supplementation versus control (non-
483 supplementation) in our embryo culture system (4 replicates). EVs supplementation had
484 no effect on embryo cleavage (day 2) (Figure 9A) (73.58 ± 3.44 ; 76.57 ± 1.92 and
485 81.04 ± 3.64 for fresh, frozen, and control, respectively) but influenced blastocyst rates
486 over time (day 7-9) (Figure 9B). Interestingly, frozen EVs significantly improved

487 blastocyst rates at days 7 and 8 compared to fresh EVs and control (day 7: 32.99 ± 3.13 ;
488 30.18 ± 3.99 and 40.91 ± 2.61 and day 8: 34.04 ± 2.67 ; 37.92 ± 2.57 and 45.91 ± 1.12 for
489 control, fresh and frozen, respectively) (Figure 9B). Fresh EVs showed a significant
490 effect on embryo development at day 9 compared to the control (day 9: 23.78 ± 4.01 ;
491 35.2 ± 4.86 and 49.36 ± 0.64 , Figure 9). Embryo quality was measured in terms of the
492 hatching ability and number of cell/blastocyst. There were no differences on hatching
493 rates among all groups at day 8 while the addition of frozen EVs improved significantly
494 the hatching rate at day 9 (Figure 9C) from 7.5% to 26%. The total number of cells was
495 also improved by frozen EVs addition (Figure 9D). Our results showed that oviduct
496 EVs supplementation during *in vitro* embryo development improves blastocyst yield,
497 quality and extends embryo survival overtime.

498

499 **DISCUSSION**

500 Our study demonstrated that EVs are essential components of oviduct secretions from *in*
501 *vivo* and *in vitro* origins. Moreover, our results provide with the first oviduct EVs
502 signature. We found differential protein profiles between *in vivo* and *in vitro* EVs,
503 under our experimental conditions. We demonstrated that EVs from *in vivo* origin were
504 up taken by *in vitro* produced embryos and exert a functional effect by enhancing
505 embryo development and quality during *in vitro* culture.

506 TEM observations and WB from our study confirmed the presence of EVs in oviduct
507 secretions from *in vivo* and *in vitro* origin. Distribution size by TEM measurements
508 showed different populations of exosomes and microvesicles from both sources of
509 samples ranging from 30-250nm. Previous analysis performed by laboratory using
510 dynamic light scattering analysis (DLS) showed a higher abundance of bigger vesicles
511 than exosomes in EVs conditioned media when compared to oviduct fluid (Alminana C

512 2014) in contrast to current measurement by TEM. We believe that DLS results could
513 be due to artefacts, measurements of aggregates of vesicles instead of individual
514 vesicles (Muller et al. 2014). Moreover, Van del Pol et al., showed that different
515 techniques can give a different size distribution and a different concentration for the
516 same vesicle sample (van der Pol et al. 2014). Nevertheless, our results are in agreement
517 with previous studies isolating exosomes and microvesicles from bovine oviduct fluid
518 following a protocol similar to us (centrifugation at 100,000 xg) (Lopera-Vasquez *et al.*
519 2017).

520 Exosomes and microvesicles were also identified in both oviduct fluid and BOEC
521 derived conditioned media by Al-Dossary et al., (Al-Dossary et al. 2013) and Lopera-
522 Vásquez et al., (Lopera-Vasquez et al. 2016), respectively. Our study differs from those
523 in 2 important aspects: 1) our study used a wide proteomic approach to characterize
524 oviduct EVs content and decipher the role of EVs during early gamete/embryo-maternal
525 interactions; while Al-Dossary and colleagues (Al-Dossary *et al.* 2013) focused their
526 study on a specific protein approach contained in murine Oviduct EVs, PMCA4a, which
527 is essential for sperm hyperactivated motility and fertility; and 2) we compared *in vitro*
528 and *in vivo* EVs obtained from conditioned media of primary culture of BOEC and
529 oviduct fluid from same oviducts at the early post ovulatory stage (1-4 after ovulation)
530 to minimize variability. Lopera-Vásquez et al., used oviducts at the mid-luteal phase of
531 the estrous cycle (Lopera-Vasquez *et al.* 2016). Greening et al., demonstrated that
532 different hormonal environment during the estrous cycle modulate content of human
533 endometrial derived EVs (Greening et al., 2016).

534

535 Our findings suggest that BOEC *in vivo* might not secrete the same population of
536 exosomes/microvesicles than *in vitro* under our experimental conditions. It is possible

537 that the *in vitro* culture of BOEC may affect their ability to secrete and release
538 exosome/microvesicles and their content. BOEC in monolayer after several days in
539 culture are less likely to mimic the oviduct environment than BOEC *in vivo*, as reflected
540 by the loss of morphological hallmarks such as cilia and secretory granules (Rottmayer
541 et al. 2006). Differences between *in vivo* and *in vitro* exosome populations were also
542 reported by Ostman et al., (Ostman et al. 2005), when comparing exosomes secreted by
543 *in vitro* propagated tumor cells versus tumor cells grown *in vivo*. Here, we showed
544 quantitative and qualitative proteomic differences between EVs content from *in vivo*
545 and *in vitro* origin by MS analysis. Moreover, our functional analysis of the EVs
546 proteins from both sources showed that they were associated to different biological
547 functions and networks (Figure 7). Altogether, highlights the distinct functionality of *in*
548 *vivo* and *in vitro* EVs during oviduct-embryo interactions. Since EVs play a role in cell-
549 to cell communication through the transfer of their cargo, it can be expected that the
550 different proteomic oviduct EVs composition between *in vivo* and *in vitro* may exert
551 distinct functional effects on embryo(s) and gametes.

552

553 It is worthy to mention that the experimental conditions used in the present study
554 (oviduct source, oviduct collection, BOEC collection, *in vitro* cell culture system,
555 period of cell incubation and culture media, etc) might affect the *in vivo* and/or *in vitro*
556 EVs secretion/content identified here. To obtain the oviductal flushing's the conditioned
557 media from BOEC culture, we used a protocol developed in our laboratory that has
558 shown to be a good method to prepare BOEC monolayer to support embryo
559 development but also, to study the embryo-oviduct dialogue, and analysing the BOEC
560 gene expression (Schmaltz-Panneau *et al.* 2014). To avoid that the *in vivo* "debris" and
561 "apoptotic/dead cells" contained in the oviduct fluid could affect the *in vivo* EVs,

562 flushings' obtained were immediately centrifuged at 300xg, 15 min followed by
563 12000xg 15 min. Then, BOEC cells were isolated, seeded and *in vitro* cultured for 14
564 days, as in our previous studies. It is possible that EVs collected from oviducts
565 immediately post-mortem or transported under conditions different to those in our study
566 (i.e ice) could differ in EVs quality or protein abundance, despite EVs content seems
567 well-preserved. On the other hand, the use of a different culture system, with distinct
568 media, period of cell culture could also affect EVs production/content. To the best of
569 our knowledge, there is not any available study that has shown to which extend *in vitro*
570 culture can affect the EVs production and content compared to *in vivo* EVs. Even more,
571 no information exists regarding if the days of *in vitro* culture, the media or other *in vitro*
572 factors can change their content. Cell culture models are important tools for revealing
573 specific effects and mechanisms of cell populations and currently are being used for
574 many studies as a source to obtain EVs. However, it is extensively known that it is very
575 difficult to find an *in vitro* cell culture model that allows the cells to be as much as
576 possible similar to the *in vivo* ones (in regard to gene expression, secretion, EVs etc.).
577 Recently, two novel culture methods for oviductal cells based on air-liquid interphase
578 culture system (Chen *et al.* 2017) and a 3D-printing oviduct device (Ferraz *et al.* 2017)
579 have been proposed to generate oviduct fluid surrogates more similar to the *in vivo*
580 ones. Therefore, it is possible that using these novel systems, the *in vitro* content of EVs
581 could be more similar to the *in vivo* ones.

582

583 In the present study, the proteomic profiling of oviduct EVs showed an exosome
584 signature and confirmed that oviduct exosomes contain basic machinery important for
585 biogenesis, trafficking, fusion and release. However, our TEM observations also
586 revealed the presence of microvesicles. Establish methods that allow to discriminate

587 between exosomes and microvesicles is a major ongoing challenge in the field of EVs
588 (Raposo & Stoorvogel 2013). Until then, this report provides the first protein cargo
589 signature of oviduct EVs and represents the only exosome and microvesicles protein
590 resource to date.

591

592 Furthermore, oviduct EVs characterization revealed proteins with important roles in the
593 gamete/embryo-oviduct interactions, such as OVGPI, HSP90, HSPA8, HSP70,
594 Gelsolin, and Ezrin in oviduct EVs (Table II). Some of them were only identified in *in*
595 *vivo* or *in vitro* EVs (Supplementary data 1, S1 and S2). Interestingly, these proteins
596 were identified previously by Elliott and colleagues in a subset of 70kDa oviduct
597 surface proteins that bound to spermatozoa (Elliott et al. 2009). These proteins
598 enhanced *in vitro* survival of mammalian spermatozoa, particularly HSPA8 (Elliott et
599 al. 2009, Moein-Vaziri et al. 2014). The mechanism(s) by which HSPA8 or other
600 proteins are released by the oviduct epithelium and are able to enhance sperm survival
601 is still unknown. It has been suggested that heat shock proteins might be released via
602 exosomes (Campanella C 2014) or lipid raft (Pralle et al. 2000). Our results support the
603 hypothesis that exosomes/microvesicles could be one of the mechanism(s) by which
604 BOEC may release these proteins into the oviduct milieu and transport them to the
605 embryo, allowing successful gamete interactions and subsequently early embryonic
606 development.

607 Among the different reproductive proteins identified in oviduct EV under our
608 experimental conditions, we would like to highlight the presence of OVGPI, MYH9,
609 HSP90 (in its HSP90B1, HSP90AA1, HSP90AB1 forms) and lactadherin (PAS6/7 or
610 MFGE8) because of their important functions in gamete/embryo-oviduct crosstalk.
611 OVGPI is the major non-serum protein present in the oviduct fluid in different species

612 (Sutton et al. 1984, Buhi et al. 1990). It increases sperm viability and motility (Abe et
613 al. 1995); modulates sperm capacitation and fertilization (King et al. 1994) and
614 enhances development rates (Kouba et al. 2000). Furthermore, OVGPI seems to bind to
615 both gametes through the interaction of its non-glycosylated N-terminal conserved
616 region with MYH9 (Kadam et al. 2006). Our results imply that both proteins might be
617 secreted via EVs, or at least partly. Since both OVGPI and MYH9 were detected *in*
618 *vivo* EVs, while only MYH9 was expressed *in vitro* EVs. The fact that OVGPI is only
619 expressed in *in vivo* exosomes/microvesicles was not surprising since OVGPI is
620 expressed in BOEC after collection but its mRNA is strongly decreased after 7-10 days
621 of culture. Considering that OVGPI is secreted under steroid control, it is possible that
622 the lack of hormonal stimulation in our experiment could explain the absence of
623 OVGPI in the *in vitro* exosomes/microvesicles. Together with OVGPI, HSP90B1 was
624 also expressed in oviduct EVs and is associated to ZP hardening mechanism (Mondejar
625 et al. 2012). HSP90 has been shown on the surface of 25% of the live capacitated sperm
626 population that is capable of interacting with the ZP of the oocyte (Asquith et al. 2004).
627 In addition, we identified lactadherin in EVs preparations, a protein common in
628 exosomes studies and involved in ZP binding. It has been previously identified *in vitro*
629 microvesicles released by endometrial cell cultures under hormonal stimulation (Sarhan
630 et al. 2013). However, lactadherin secretion *in vitro* has been also associated to
631 unhealthy cells (Delcayre & Le Pecq 2006), despite in our *in vitro* culture system 90%
632 of BOEC were viable. It is interesting to note that exosomes are packing many proteins
633 from heat shock protein 70 and 90 families as well as other proteins involved in free
634 radicals scavenging (peroxiredoxins, thioredoxin, etc). These contents may bring to the
635 gametes and embryos some additional factors to survive in the *in vivo* and *in vitro*
636 environment.

637

638 Considering all the embryotrophic factors contained in the EVs, we evaluated whether
639 EVs could be up taken by the embryo and exert a functional effect on embryos. Our
640 results showed that oviduct EVs were internalized by the embryo being capable of
641 passing through the zona pellucida and being located around the nucleus of most
642 embryonic cells. Our results are in line with other studies showing the uptake of uterine
643 EVs by the embryo/conceptus at later stages (Vilella et al. 2015, Burns et al. 2016)
644 (Greening et al. 2016, Bidarimath et al. 2017). Burns et al. suggested that the uterine
645 EVs uptake could have an essential role in elongation of the conceptus (Burns et al.
646 2016). Vilella and colleagues (Vilella et al. 2015) demonstrated that Hsa-miR-30d,
647 contained in uterine exosomes, could induce transcriptional and functional
648 modifications in the adhesive competence of the embryo. In our study, we provide
649 strong evidence for the functional effect of oviduct EVs in supporting bovine
650 preimplantation embryo development, since EVs supplementation improved embryo
651 development and embryo quality. Our results are in part in line with Lopera-Vásquez et
652 al., (Lopera-Vasquez *et al.* 2016, Lopera-Vasquez *et al.* 2017), reporting that the use of
653 *in vitro* frozen/thawed BOEC-derived EVs improve embryo quality, by increasing the
654 number of cells and the survival of blastocyst after cryopreservation. However, these
655 authors did not report any improvement in embryo development over time or hatching
656 rates during *in vitro* culture, as our study shows. The distinct effect of EVs on embryo
657 during IVC between studies could be explained by the differences found in the protein
658 content between EVs from *in vivo* and *in vitro* origin or other EVs molecular
659 components (mRNA, miRNA). Moreover, the moment of the estrous cycle or the
660 different parts of the oviduct (Ampulla, Ithsmus) from which they were collected could
661 also have an effect (Greening *et al.* 2016, Lopera-Vasquez *et al.* 2017). Our next studies

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Alminana Brines, C., Corbin, E., Tsikis, G., Soares De Alcantara Neto, A., Labas, V., Reynaud, K., Galio, L., Uzbekov, R., Garanina, A. S., Druart, X., Mermillod, P. (2017). Oviduct extracellular vesicles protein content and their role during oviduct-embryo cross-talk. *Reproduction (Cambridge)*. 154 (4). 253-268. . DOI : 10.1530/REP-17-0054

662 will be focused in further analyzing the content of EVs at mRNA and miRNA across the
663 estrous cycle and investigating the possible epigenetic effects of EVs on
664 preimplantation embryo development.

665

666 Having in mind the practical application of EVs in optimizing IVF systems, we
667 compared the effect of fresh and frozen EVs during *in vitro* embryo development.

668 Despite both fresh and frozen EVs had a positive effect on embryo development,
669 surprisingly the use of frozen EVs showed better results for IVP. To the best of our
670 knowledge, no studies have been performed to evaluate properly the impact of the
671 freezing procedure on the EVs structure and/or content that could explain our results.

672 The information available to date in the literature is controversial with different studies
673 showing conflicting results regarding the resistance of EVs to freezing (Bosch *et al.*
674 2016). Most of studies are based on fresh or frozen samples (blood, fluid, urine) from
675 which EVs are isolated. A few studies have indicated that storing samples at -80°C do
676 not alter EVs morphology or size (Sokolova *et al.* 2011, Sarker *et al.* 2014). While
677 others have suggested that freezing may induce membrane damage and leakage of EVs
678 content in the absence of perceivable changes of size and concentration. A recent study
679 has found a significant reduction in the bi-layer membrane of frozen vesicles (-80°C)
680 when compared to fresh EVs (Teng *et al.* 2015), which could explain our results.

681 Regarding their content and functionality, studies point out that freezing seem to
682 preserve almost completely EVs associated proteins (Zhou *et al.* Kidney Int. 2006 Apr;
683 69(8): 1471–1476) and do not impair their functionality (Sokolova *et al.* 2011,
684 Jayachandran *et al.* 2012) as we have observed in our study. The alterations of EVs
685 following freezing remains a matter of debate in the field of extracellular vesicle
686 research. Although further studies are required to elucidate the impact on the freezing

687 process on EVs, our data provide clear evidence of the positive effect of fresh and
688 frozen oviduct EVs on embryo development. Therefore, our study points out that the
689 use of oviduct EVs is a good strategy to optimize *in vitro* embryo production. Further
690 studies will be conducted to evaluate whether EVs can also improve the pregnancy
691 outcomes after transferring embryos co-incubated with EVs, making them potential
692 tools for the application of other biotechnologies.

693

694 In summary, our study identified the first oviduct-derived EVs protein signature, and
695 reveals a set of proteins with important roles in gamete/embryo oviduct interactions that
696 have not been previously identified in the oviduct EVs cargo. Moreover, our results
697 highlight the differential protein cargo between *in vivo* and *in vitro* EVs. Information of
698 great importance since most of functional studies of EVs are based on EVs derived from
699 cell lines or primary culture and extrapolated to *in vivo* EVs biology and function.
700 Functionally, we demonstrated that EVs were internalized by the embryo during *in vitro*
701 culture and enhance their ability to reach the blastocyst stage, to hatch and increase their
702 survival overtime. Taken together, this study brings new insights into the contribution
703 of EVs as modulators of the oviduct-gamete/embryo crosstalk and opens up a new
704 avenue for the use of EVs to optimize ART in human and livestock species.

705

706 **CONFLICT OF INTEREST**

707 None declared.

708

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722

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945

1 **FIGURE LEGENDS**

2 **Figure 1. Transmission electron microscopy observations of bovine oviduct EVs**
3 **preparations from *in vivo* (A) and *in vitro* (B) origin.** Structures pointed by blue
4 arrows with 30-100nm size resembling to exosomes and structures pointed by red
5 arrows > 100nm size resembling to microvesicles were identified in oviduct flushing (*in*
6 *vivo*; A) and conditioned media from BOEC culture (*in vitro*; B) preparations.

7

8 **Figure 2. Histograms showing the size distribution of bovine oviduct EVs in *in vivo***
9 **and *in vitro* preparations.** Four replicates of EVs from *in vivo* (A) and *in vitro* (B)
10 preparations were analyzed using TEM and measurement of vesicles were performed.
11 Populations of exosomes (30-100nm) and microvesicles (>100nm-250) (Figure 2A and
12 2 B) were found in both EVs preparations.

13

14 **Figure 3. Western blotting characterization of bovine oviduct EVs from *in vivo* and**
15 ***in vitro* origin for known exosomal markers and oviduct proteins with known**
16 **reproductive roles.** i) Both EVs preparations were positive for HSP70, a known
17 exosomal protein marker and negative for Grp78, an endoplasmic reticulum marker
18 detected on BOEC; ii) *in vivo* EVs expressed oviduct glycoprotein (OVGP), heat shock
19 protein A8 (HSPA8) and myosin 9 (MYH9), while only HSPA8 and MYH9 were
20 detected in *in vitro* EVs; iii) When the cells of origin were analyzed for these
21 reproductive proteins, OVGP1, MYH9 and HSPA8 were expressed in all *in vivo*
22 samples, but only MYH9 and HSPA8 were expressed in *in vitro* oviduct cells.

23

24 **Figure 4. Venn diagram showing the number of bovine oviduct EVs proteins**
25 **identified exclusively in *in vivo* and *in vitro* preparations and in common.** Venn
26 illustrates that 97 proteins were exclusively detected in *in vivo* EVs, while 47 were
27 found only in *in vitro* and 175 were in common to both samples.

28

29 **Figure 5. Validation of Mass Spectrometry analysis of bovine oviduct EVs by**
30 **Western immunoblotting.** Western blot analysis confirmed MS results in the 4
31 biological replicates for CD109 and Lactadherin. CD109 was only expressed in *in vivo*
32 EVs while Lactadherin was expressed only in *in vitro* EVs.

33

34 **Figure 6. Functional analysis of bovine oviduct EVs proteins identified by Mass**
35 **Spectrometry by PANTHER database.** EVs proteins isolated from *in vivo* and *in vitro*
36 preparations were subjected to ontology and pathway analysis using PANTHER and
37 Gene ontology algorithms and subsequently classified based on their Biological
38 Process.

39

40 **Figure 7. Integrative visualization of the differential proteins identified in *in vivo***
41 **(red) and *in vitro* (green) bovine oviduct EVs and their biological functions and**
42 **protein interactions using Cytoscape app ClueGO.** Differential networks of
43 functional categories from *in vivo* (red) and *in vitro* (green) EVs proteins are shown.

44

45 **Figure 8. Uptake of *in vivo* EVs by *in vitro* produced embryos.** EVs preparations
46 from *in vivo* origin were labelled with green fluorescent dye (PKH67), filtered (0.22µm)
47 and co-incubated with blastocysts (A-C) and hatching/hatched (D-F) blastocysts.

48 Embryos co-cultured with green-labelled EVs were fixed and stained with Hoechst
49 3342 to visualize the nucleous and with Actin Red phalloidin to visualize the membrane
50 of the cells. Fluorescence (A-F) images demonstrating active uptake of green-labelled
51 Evs by embryos *in vitro* during culture. No green fluorescent exosomes/microvesicles
52 were observed in the negative controls of embryo co-cultured with PBS (G-H,
53 Controls). Detail of EVs inside the blastocyst cells, confirmed that *in vivo* EVs were not
54 only attached to the embryo membrane but were actually internalized by these cells and
55 were present in the cytoplasm (I-J). Images were obtained with 20X with an additional
56 zoom factor from 1 to 2.8X. Scale bar = 50µm.

57

58 **Figure 9. Effect of fresh and frozen *in vivo* EVs on the embryo development and**
59 **the quality of *in vitro* produced embryos.** Embryo were co-incubated with EVs for 9
60 days after IVF (4 replicates, number of initial oocytes for Control n=353; Fresh EVs
61 n=332 and Frozen EVs n=332). Cleavage (A), Blastocyst (B) and Hatched blastocyst
62 (C) rates were expressed as percentages (Mean ±SEM) and were calculated on initial
63 oocytes numbers. Number of cells /blastocyst is shown in figure section (D). Different
64 letters in the graphs represent significant differences (P<0,05). Comparison among
65 treatments in blastocyst rates are made on the same day of culture.

66

Figure 1

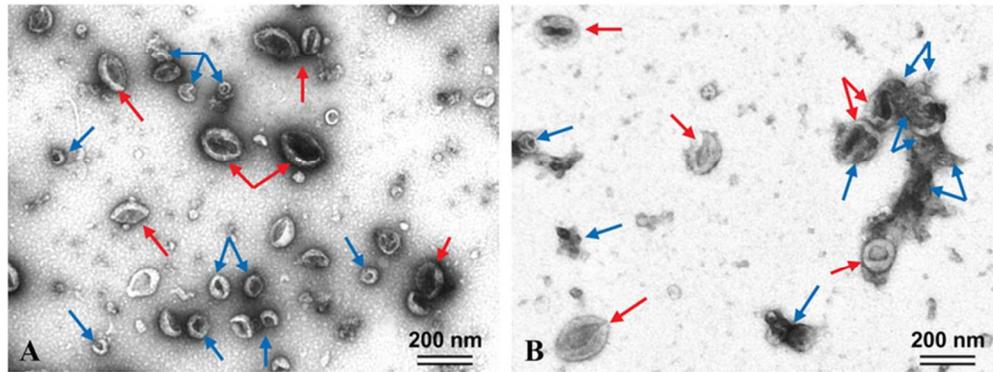


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69x29mm (300 x 300 DPI)

Figure 2

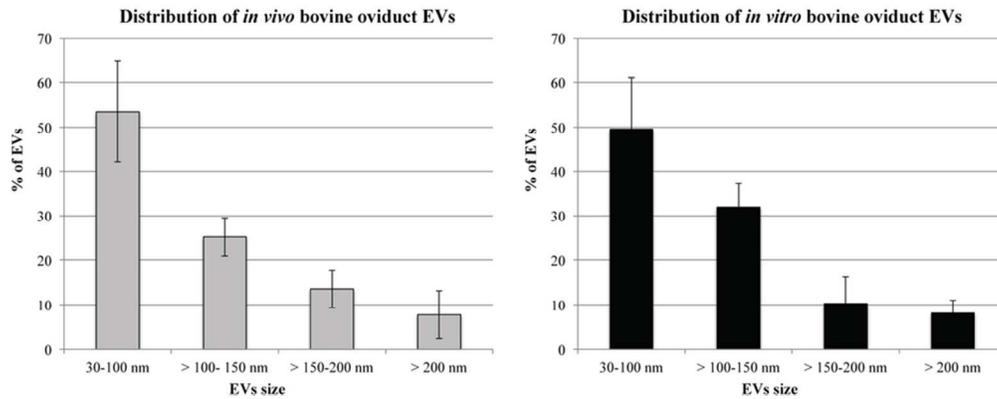


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79x34mm (300 x 300 DPI)

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Alminana Brines, C., Corbin, E., Tsikis, G., Soares De Alcantara Neto, A., Labas, V., Reynaud, K., Galio, L., Uzbekov, R., Garanina, A. S., Druart, X., Mermillod, P. (2017). Oviduct extracellular vesicles protein content and their role during oviduct-embryo cross-talk. *Reproduction (Cambridge)*, 154 (4), 253-268. DOI : 10.1530/REP-17-0054

Figure 3

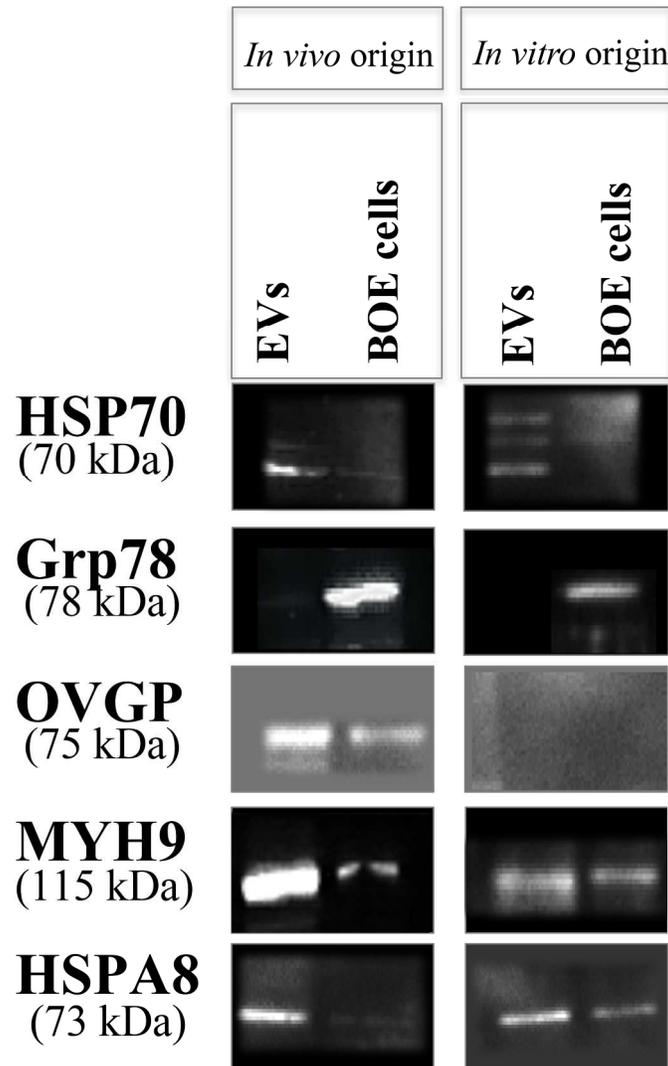


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162x292mm (300 x 300 DPI)

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Figure 4

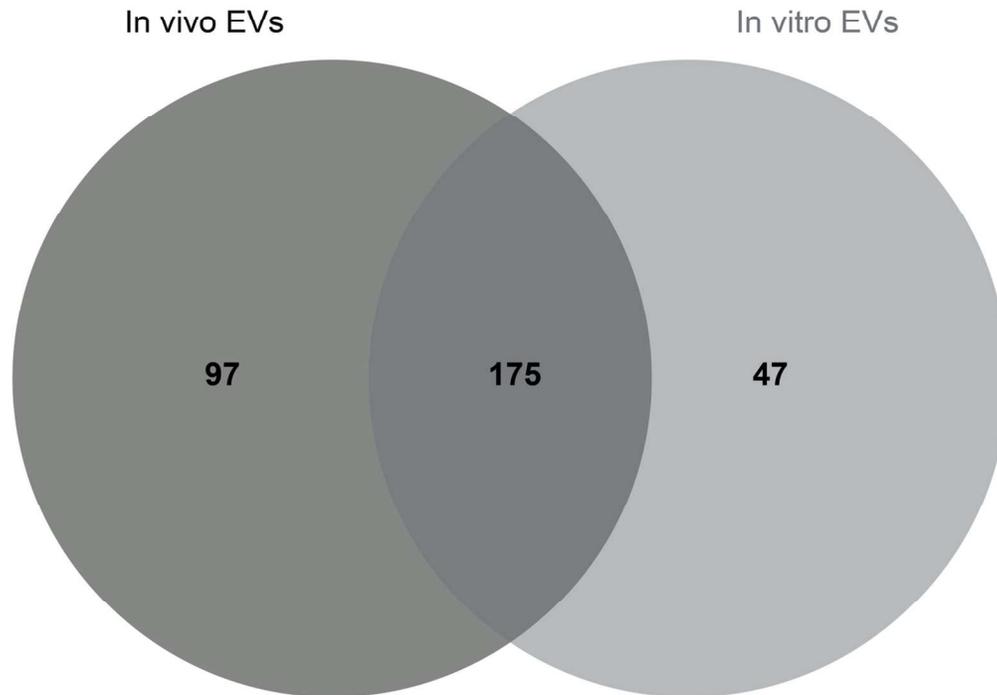


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119x93mm (300 x 300 DPI)

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Figure 5

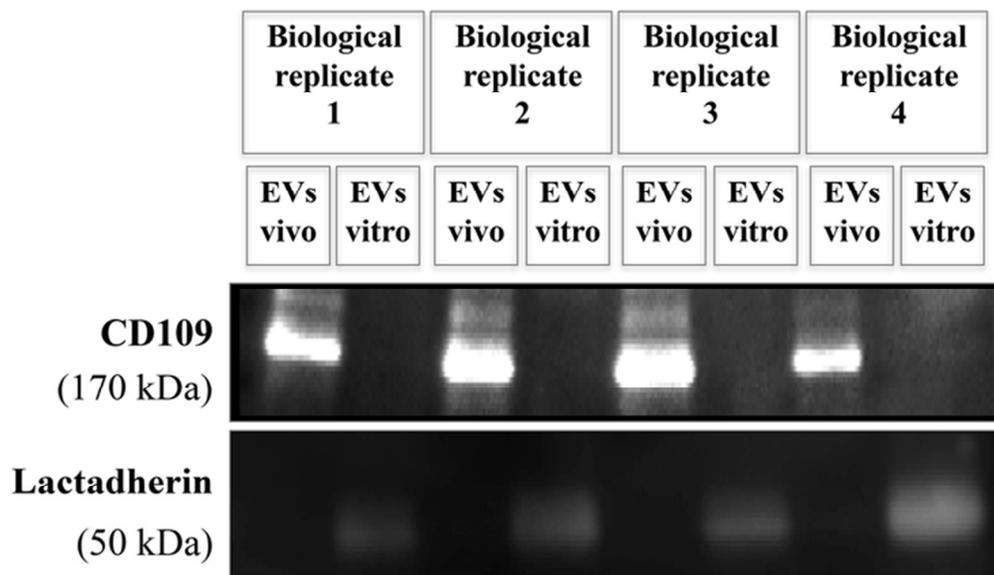


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62x42mm (300 x 300 DPI)

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Figure 6

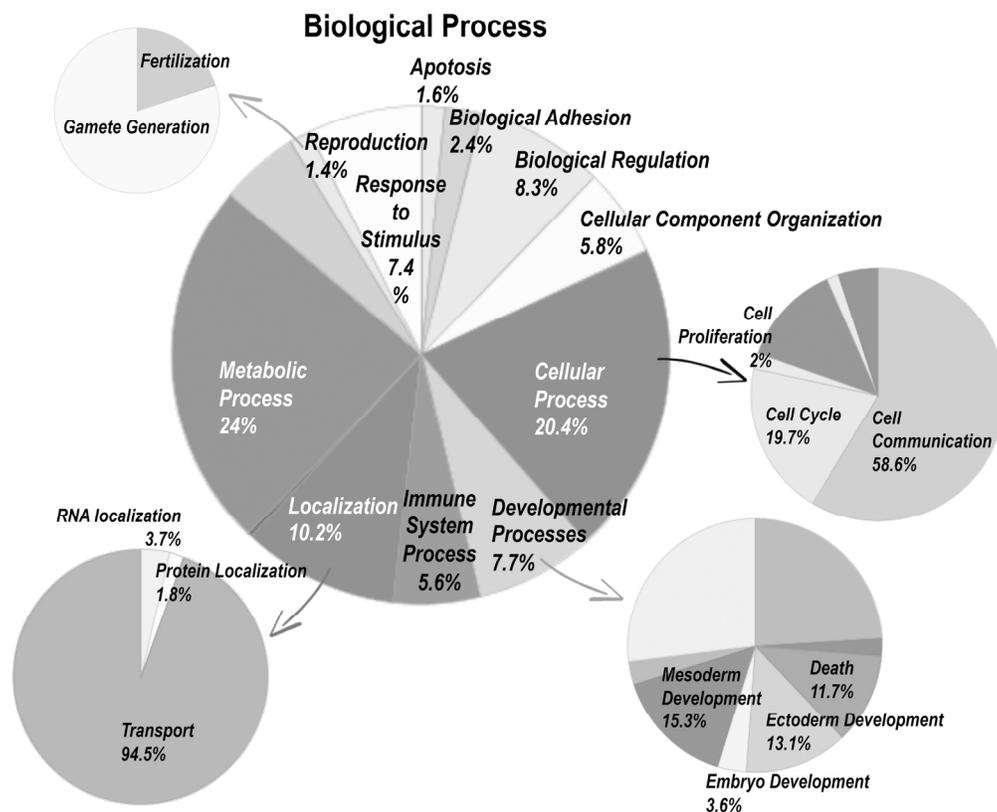


Figure 6. Functional analysis of bovine oviduct EVs proteins identified by Mass Spectrometry by PANTHER database. EVs proteins isolated from in vivo and in vitro preparations were subjected to ontology and pathway analysis using PANTHER and Gene ontology algorithms and subsequently classified based on their Biological Process.

161x144mm (300 x 300 DPI)

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Figure 8

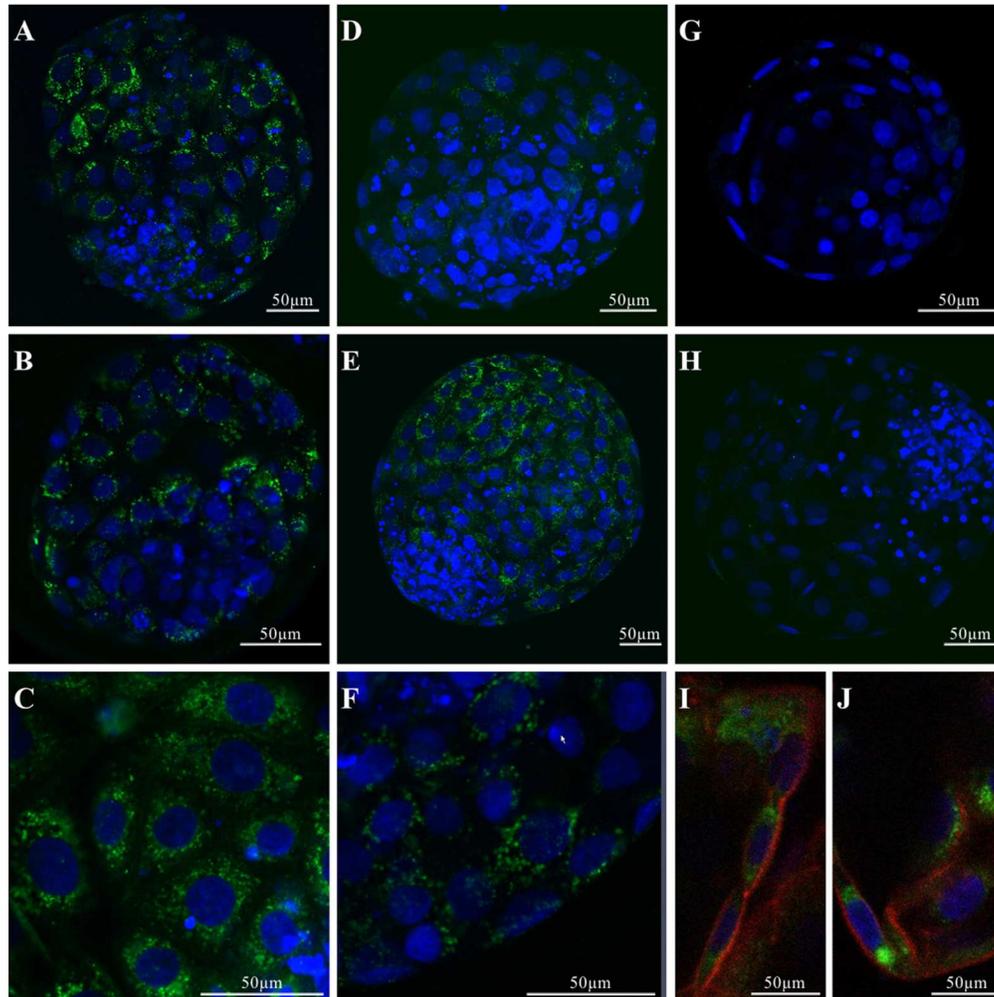


Figure 8. Uptake of in vivo EVs by in vitro produced embryos. EVs preparations from in vivo origin were labelled with green fluorescent dye (PKH67), filtered (0.22µm) and co-incubated with blastocysts (A-C) and hatching/hatched (D-F) blastocysts. Embryos co-cultured with green-labelled EVs were fixed and stained with Hoechst 3342 to visualize the nucleus and with Actin Red phalloidin to visualize the membrane of the cells. Fluorescence (A-F) images demonstrating active uptake of green-labelled EVs by embryos in vitro during culture. No green fluorescent exosomes/microvesicles were observed in the negative controls of embryo co-cultured with PBS (G-H, Controls). Detail of EVs inside the blastocyst cells, confirmed that in vivo EVs were not only attached to the embryo membrane but were actually internalized by these cells and were present in the cytoplasm (I-J). Images were obtained with 20X with an additional zoom factor from 1 to 2.8X. Scale bar = 50µm.

93x99mm (300 x 300 DPI)

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Figure 9

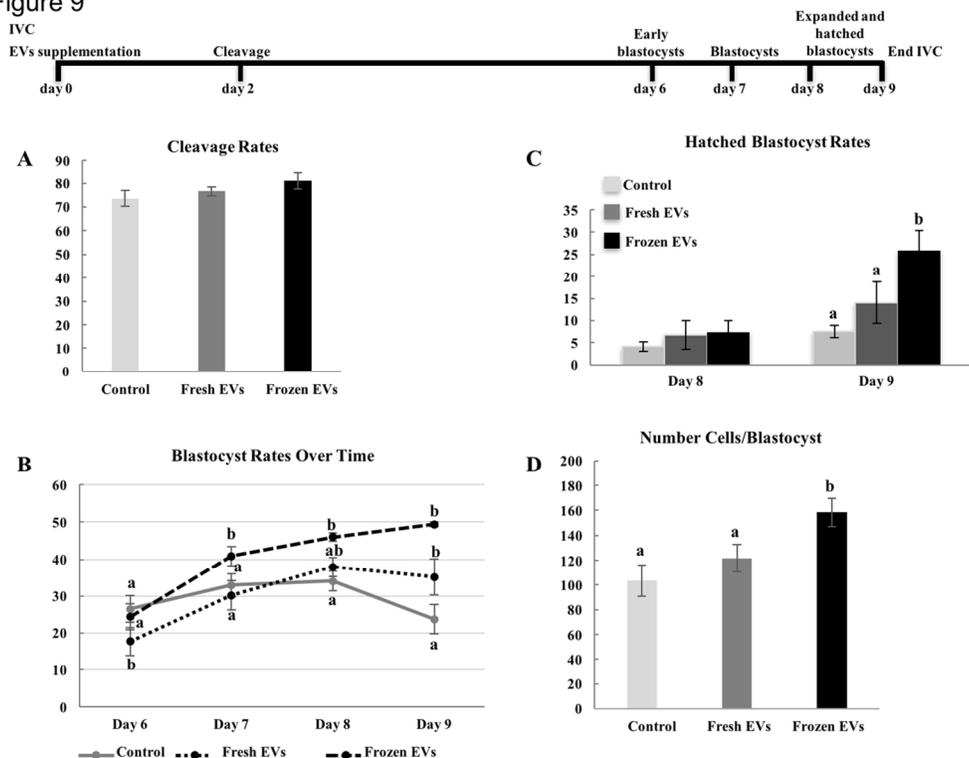


Figure 9. Effect of fresh and frozen in vivo EVs on the embryo development and the quality of in vitro produced embryos. Embryos were co-incubated with EVs for 9 days after IVF (4 replicates, number of initial oocytes for Control n=353; Fresh EVs n=332 and Frozen EVs n=332). Cleavage (A), Blastocyst (B) and Hatched blastocyst (C) rates were expressed as percentages (Mean \pm SEM) and were calculated on initial oocytes numbers. Number of cells /blastocyst is shown in figure section (D). Different letters in the graphs represent significant differences ($P < 0,05$). Comparison among treatments in blastocyst rates are made on the same day of culture.

124x98mm (300 x 300 DPI)

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1 TABLES

2 **Table I.** Selected results of DAVID functional annotation clustering for proteins
 3 identified in bovine oviduct EVs from *in vivo* and *in vitro* origin.

Representative functional terms of overrepresented annotation clusters	Enrichm. score ¹	No. Proteins
Annotation cluster for proteins identified in exosomes from <i>in vivo</i> and <i>in vitro</i> origin		
Vesicle (62, 4.3); cytoplasmic vesicle (57, 4.1); membrane-bounded vesicle (54, 4.4)	19.9	62
Ribosome (21, 17.4); translational elongation (25, 11.9); protein biosynthesis (26, 8.3)	14.3	39
Nucleotide-binding (78, 2.8); Purine ribonucleotide binding (80, 2.1)	10.9	87
Annexin (8, 34.6); Annexin 3; Annexin 4; Annexin 1; Annexin 2	8.5	10
Actin cytoskeleton organization (21, 4.5); Actin filament-based process (23,4.6)	6.8	27
ATP binding (61, 1.9); Adenyl ribonucleotide binding (61, 1.9); Purine nucleoside binding (61, 1.8);	6.6	62
Glucose catabolic process (11, 9.1); Hexose catabolic process (11, 7.7); Glycolysis (9, 9.2)	5.0	17
Vesicle lumen (9, 9.1); Cytoplasmic membrane-bounded vesicle lumen; (8, 8.5)	5.0	12
Regulation of apoptosis (36, 2.1); regulation of programmed cell death (36, 2.1); Regulation of cell death (36, 2.1)	4.6	36
Anti-apoptosis (16, 3.7); negative regulation of apoptosis (21, 2.9); negative regulation of programmed cell death (21, 2.8); negative regulation of cell death (21, 2.8)	4.3	21
Phospholipase inhibitor activity (5, 19.9); Lipase inhibitor activity (5, 15.9)	4.2	7
Myosin (8, 9.5); Myosin complex (8, 5.7)	3.9	8
Heat shock protein Hsp70 (5, 22.45); Chaperone HSP70 (4, 17.2)	3.8	5
Primary lysosome (3, 35); Specific granule (3, 20)	2.4	3
Hemostasis (10, 4.5); Regulation of body fluid levels (9, 3.7); Blood coagulation (9, 4.2); Wound healing (11, 2.8)	2.9	13
Cell migration (15, 2.6); Localization of cell (15, 2.5); Cell motility (15, 2.5)	2.7	16
Heat shock protein Hsp90 (3, 32.1)	1.7	3
Peroxiredoxin activity (4, 23.9); Response to reactive oxygen species (5, 3.2); Antioxidant activity (4, 4.07)	1.8	7
Calcium-binding region:1; low affinity (4, 13.4); Calcium-binding region:2; high affinity (4, 11.5)	2.1	4
Fertilization (5, 20.2); Single fertilization (5, 4.0)	2.2	5
Cellular ion homeostasis (16, 2.1); Chemical homeostasis (17, 1.6);	1.7	17
Actin capping (4, 12.8); Negative regulation of cytoskeleton organization (5, 4.4); Negative regulation of organelle organization (5, 2.9)	1.7	6

4 ¹geometric mean of member's p-values of the corresponding annotation cluster (in -log₁₀ scale); ²in brackets:

5 number of genes and fold enrichment of the functional term.

1 TABLES

2 **Table II.** Protein identified in bovine oviduct EVs from *in vivo* and *in vitro* origin
3 associated with reproductive roles.

Human	Protein	Reproductive	
Gene ID	Symbol	Name	Functions Source
6813	STXBP2	syntaxin binding protein 2	Gamete Generation, Fertilization PANTHER
6812	STXBP1	STXBP1 protein	Gamete Generation, Fertilization PANTHER
7348	UPK1B	uropod protein 1B	Gamete Generation PANTHER
2950	DNAH5	Dynein heavy chain 5, axonemal;DNAH5;ortholog	Gamete Generation, Fertilization PANTHER
2771	GNAI2	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 2	Gamete Generation PANTHER
23303	KIF13B	PREDICTED: kinesin family	Gamete Generation PANTHER
152007	GLIPR2	GLI pathogenesis-related 2	Gamete Generation, Fertilization PANTHER
6809	STX3	syntaxin 3	Gamete Generation, Fertilization PANTHER
3336	EMR1	EGF-like module-containing mucin-like hormone receptor-like 1;EMR1;ortholog	Gamete Generation PANTHER
2273	FHL1	Four and a half LIM domains protein 1;FHL1;ortholog	Gamete Generation PANTHER
1397	CRIP2	GLI pathogenesis-related 2	Gamete Generation PANTHER
226	ADARB1	Double-stranded RNA-specific editase 1;ADARB1;ortholog	Gamete Generation PANTHER
216	ADAM9	Disintegrin and metalloproteinase domain-containing protein 9;ADAM9;ortholog	Fertilization PANTHER
2934	GSN	gelsolin	Fertilization PANTHER
6674	SPAG1	TPA: sperm associated antigen 1	Fertilization DAVID
5016	OVGP1	oviduct glycoprotein 1	Fertilization DAVID
4179	CD46	CD46 molecule	Fertilization DAVID
928	CD9	CD9 molecule	Fertilization DAVID
4240	MFGE8	PREDICTED: lactadherin isoform X1	Fertilization DAVID
4904	YBX1	nuclease-sensitive element-binding protein 1 [Oryctolagus cuniculus]	Embryonic Development in uterus GeneCards
2288	FKBP4	FK506 binding protein 4	Embryo Implantation GeneCards
2776	GNAQ	guanine nucleotide binding protein (G protein), q polypeptide	Post-embryonic Development Literature
6194	RPS6	mCG6197 [Mus musculus]	Fertilization, Placenta development GeneCards
10521	DDX17	DEAD (Asp-Glu-Ala-Asp) box polypeptide 17, isoform CRA_h [Homo sapiens]	Embryogenesis, Spermatogenesis, Cell growth Division, Post-embryonic Development GeneCards

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11196	SEC23IP	SEC23-interacting protein [Bos taurus]	Spermatid development	GeneCards
8566	PDXK	pyridoxal kinase [Bos taurus]	Epididymis Secretory Sperm Binding Protein	GeneCards
51181	DCXR	L-xylulose reductase [Bos taurus]	Sperm surface protein	GeneCards
498	ATP5A1	Chain A, The Structure Of F1-Atpase Inhibited By Resveratrol.	Epididymis Secretory Sperm Binding Protein, EMBRYO DEVELOPMENT	GeneCards
3308	HSPA4	heat shock protein family A (Hsp70) member 4	Sperm fertilizing ability	Literature
3336	HSPE1	PREDICTED: 10 kDa heat shock protein, mitochondrial-like [Macaca mulatta]	Early Pregnancy factor	Literature
3312	HSPA8	heat shock protein family A (Hsp70) member 8	sperm fertilizing ability	Literature
7184	HSP90B1	heat shock protein 90kDa beta family member 1	Sperm fertilizing ability	Literature
3320	HSP90AA1	heat shock protein 90kDa alpha family class A member 1	Sperm fertilizing ability	Literature
3326	HSP90AB1	heat shock protein 90kDa alpha family class B member 1	Sperm fertilizing ability	Literature
302	ANXA2	annexin A2	Embryo adhesiveness to endometrium, sperm-oviduct binding	Literature
308	ANXA5	annexin A5	Formation Sperm reservoir, sperm-oviduct interaction	Literature

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