Extracellular vesicles from oviductal and uterine fluids supplementation in sequential in vitro culture improves bovine embryo quality

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Research Article

Keywords: exosomes, miRNAs, embryo development, cryopreservation, lipid metabolism, oviduct, uterus, cattle.

Posted Date: April 21st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1553800/v1

Extracellular vesicles from oviductal and uterine fluids supplementation in sequential in vitro culture improves bovine embryo quality

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Abstract

Background: In vitro production of bovine embryos is a well-established technology, but the in vitro culture (IVC) system still warrants improvements, especially regarding embryo quality. This study aimed to evaluate the effect of extracellular vesicles (EVs) isolated from oviductal (OF) and uterine fluid (UF) in sequential IVC on the development and quality of bovine embryos. Zygotes were cultured in SOF supplemented with either BSA or EVs-depleted fetal calf serum (dFCS) in the presence (BSA-EV and dFCS-EV) or absence of EVs from OF (D1 to D4) and UF (D5 to D8), mimicking in vivo conditions. EVs from oviducts (early luteal phase) and uterine horns (mid-luteal phase) from slaughtered heifers were isolated by Size Exclusion Chromatography. Blastocyst rate was recorded on Days 7-8 and their quality was assessed based on lipid contents, mitochondrial activity and total cell numbers, as well as survival rate after vitrification. Relative mRNA abundance for lipid metabolism-related transcripts and levels of active hormone-sensitive lipase (pHSL) proteins were also determined. Additionally, the expression levels of 383 miRNA in OF- and UF-EVs were assessed by pRT-PCR.

Results: Blastocyst yield was lower ($p<0.05$) in BSA groups compared to dFCS groups. Survival rates after vitrification/warming were improved in dFCS-EVs compared to dFCS ($p<0.05$). EVs increased ($p<0.05$) blastocysts total cell number in dFCS-EV and BSA-EV compared to dFCS and BSA groups, while lipid content was decreased and mitochondrial activity increased in dFCS-EV ($p<0.05$). Lipid metabolism transcripts were affected by EVs (*PPARGC1B* and *ACACA*, $p<0.05$) and protein source in the medium (*CD36, PLIN2* and *ATGL*, $p<0.05$). Levels of pHSL were lower in dFCS ($p<0.05$). Twenty miRNA were differentially expressed between OF- and UF-EVs and only bta-miR-148b was increased in OF-EVs ($p<0.05$).

Conclusions: Mimicking physiological conditions using EVs from OF and UF in sequential IVC does not affect embryo development but improves blastocyst quality regarding survival rate after vitrification/warming, total cell number, lipid content, mitochondrial activity and relative changes in expression of lipid metabolism transcripts and lipase activation. Besides, EVs miRNA contents may contribute to the observed effects.

Keywords: exosomes, miRNAs, embryo development, cryopreservation, lipid metabolism, oviduct, uterus, cattle.

Introduction

In vitro production (IVP) of bovine embryos is applied to animal breeding programs to enhance reproductive efficiency and genetic gain, and has been widely used in many countries for commercial and research purposes [1]. However, there is still the need for improvements, particularly regarding embryo quality in comparison to in vivo derived (IVD) embryos [2–4]. This lower quality is observed in terms of their reduced cryotolerance [5], altered gene expression [3,6], metabolism [7,8], morphology [9] and developmental kinetics [10]. Also, lower pregnancy rates are observed [11], which can be related to long-lasting effects of in vitro culture (IVC) resulting in various failures in post-transfer development up to and after calving [12]. IVP embryos when exposed to in vivo conditions improve quality, which is further increased with longer times of exposure [13]. Besides, modifications to the post-fertilization IVC conditions can improve blastocyst quality [5]. Therefore, improvements to the IVC system are still necessary to obtain IVP embryos of better quality for higher pregnancy rates and healthy offspring births, the ultimate outcome for efficient IVP systems.

Among the reasons for lower quality and cryotolerance, is the higher lipid contents of IVP embryos compared to IVD [2,14]. When lipid contents are reduced by different means, improved cryotolerance has been reported [15–18]. One of the factors implicated with higher lipid accumulation in IVP embryos has been the use of serum during IVC [19–22]. Fetal calf serum (FCS) is a common supplement in IVC. Although it has been related to reduced embryonic quality, it paradoxically stimulates embryo development [20]. Serum-free embryo culture is possible, but an altered expression of lipid metabolism genes has been reported. Therefore, such conditions still need to be optimized [23].

Amidst strategies used to improve embryo development in vitro, are efforts to mimic the physiological conditions observed in vivo. Preimplantation embryo development in vivo initiates within the oviduct, where the embryo will remain for about four days, and then will enter the uterus [24]. During this period, the embryo will develop mostly dependent on nutrients present in oviductal (OF) and uterine fluid (UF), until implantation around Day 20 [25]. The importance of components supplied by the female reproductive tract to the developing embryo, especially during early development, has been highlighted (reviewed by [26,27]). Previous studies have shown that the use of medium conditioned by bovine oviduct epithelial cells (BOECs) [28] or supplemented with low concentrations of OF in IVC medium [29] improve quality and cryotolerance of IVP embryos. Less is known relative to the use of UF in vitro. However, embryo quality was also enhanced when the IVC medium was supplemented with a low concentration of OF during the initial four days, followed by a sequential culture with a low concentration of UF up to the blastocyst stage until Day 9 [30]. Extracellular vesicles (EVs) are present in both fluid types, and may be involved, at least in part, in the positive effects reported when these fluids are added during IVC.

EVs are membranous vesicles secreted by many cell types and identified in several body fluids, containing diverse substances (proteins, lipids, metabolites and nucleic acids, including miRNAs), and are considered a cell signalling system, involved in various physiological and pathological processes (reviewed by [31,32]). EVs can be classified according to their size in two types: the small EVs (smaller than 200 nm) and the large EVs (bigger than 200 nm) [33]. In the female reproductive system, EVs have been found in several species (reviewed by [34,35]), including fluids from bovine oviduct [36,37] and uterus [38,39]. Supplementation of IVC medium with EVs isolated either from BOECs conditioned medium [28] or from OF [36,37], showed positive effects on embryo development and quality. EVs isolated from bovine UF also favoured the development and quality of somatic cell nuclear transfer bovine embryos [39]. Additionally, EVs from BOECs conditioned medium had positive effects on embryo quality in terms of cryotolerance, even in the presence of serum [28]. This improvement could be related to lower lipid contents in such embryos, but this parameter was not assessed in this study. Interestingly, Almiñana et al. [37] recently detected the lipogenesis enzyme fatty acid synthase (FASN) among mRNAs in EVs from bovine OF, besides other mRNAs and microRNAs (miRNAs, small non-coding RNAs acting in post-transcriptional regulation, [40]) related with lipid metabolism, which could potentially affect lipid metabolism in embryos exposed to theses EVs.

In the present study, we have investigated the effects of EVs from OF and UF, when supplemented to IVC medium in a sequential culture system. We hypothesized that this supplementation could improve embryo development and quality, and affect the lipid contents and expression of lipid metabolism-related genes in IVP embryos. The miRNA contents of these EVs were investigated as well. New knowledge in this field could

provide the basis for the development of better culture systems, which could bring improvements to the quality of in vitro produced embryos.

Materials and Methods

Experimental design

The developmental capacity of bovine zygotes and the quality of the produced blastocysts cultured in vitro with EVs from OF and UF, mimicking the physiological preimplantation environment in vivo was assessed. At approximately 20 h after insemination, presumptive zygotes were cultured in 4 groups: (i) BSA: SOF with 3 mg/mL BSA; (ii) dFCS: SOF with 5% dFCS; (iii) BSA-EV: SOF with 3 mg/mL BSA supplemented with 3×10^5 EVs/mL from OF (D1 to D4) and 3×10^5 EVs/mL from UF (D4 to D9); and (iv) dFCS-EV: SOF with 5 % dFCS supplemented with 3×10^5 EVs/mL from OF (D1 to D4) and 3×10^5 EVs/mL from UF (D4 to D9). BSA and dFCS groups underwent media renewal on Day 4 (Figure 1). Blastocyst development was assessed on Days 7, 8 and 9. To assess blastocyst quality a representative number of Days 7–8 blastocysts from each group were either vitrified/warmed, and survival rate was recorded every 24 hours up to 72 hours after warming or fixed and stained for mitochondrial activity, lipid content and total cell number analysis. In addition, Day 7–8 blastocysts were frozen in liquid nitrogen in groups of 10 and stored at -80 ^{o}C for gene expression or western blot for protein analyses. miRNA contents in EVs were analysed as well.

Materials

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA).

Isolation and characterization of small Extracellular Vesicles (EVs)

Isolation and characterization of EVs from bovine oviduct and uterine fluids analysis was performed according to Cañón-Beltrán et al. [41], with minor modifications.

Oviductal and uterine flushing for EVs isolation

Five oviducts and five uteri from slaughtered heifers were selected according to the stage of the corpus luteum, and transported to the laboratory on ice. Only oviducts corresponding to Stage 1 (from Day 1 to Day 4 of the estrous cycle) and uteri to Stage 2 (from Day 5 to Day 10; [42]) ipsilateral to the corpus luteum were used. Each oviduct and the corresponding uterine horn was trimmed free of associated tissues and washed in cold Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS^{-}). Then, each oviduct was flushed with 1 mL cold PBS^{-} from ampulla to isthmus while each uterine horn was flushed with 2.2 mL cold PBS^{-} towards the uterine tubal junction. During all processes the temperature was maintained at 4 ºC. Each oviduct and uterine flush sample was recovered and centrifuged once at 300 g for 7 min to remove cells, then the supernatant from each sample was centrifuged again at 10,000 g for 30 min at 4 ºC to remove cellular debris [43]. The resulting supernatants were each separately passed through a 0.22 µm filter and stored at 4 ºC until EVs isolation the next day.

Small extracellular vesicle isolation by Size Exclusion Chromatography

OF-EVs and UF-EVs were isolated by size exclusion chromatography (SEC), according to manufacturer's instructions (Pure EVs®, HBM-PEV; Hansa BioMed Life Sciences). Briefly, columns were firstly washed with 30 mL PBS^{-} and then each OF ($\simeq$1 mL) or UF ($\simeq$2 mL) sample was loaded onto the top of a SEC column, and when the sample was completely within the column, 11 mL PBS^{-} were loaded. Immediately, the first 3.0 mL were discarded and the following 2.0 mL of EVs-rich fractions were collected. Next, the 2.0 mL samples containing EVs were submitted to ultracentrifugation (UC) at

100,000 g for 1 h at 4 ºC using an Optima L-90K ultracentrifuge (Beckman Coulter, Fullerton, CA, USA) and a swinging-bucket rotor (SW 41 Ti). Supernatants were then discarded and each pellet was suspended in 100 µL cold PBS. Thirty microliters were taken from each of the five EVs suspensions of OF and UF and pooled, from which part was frozen at -20 ºC to be used in IVC (100 µL EVs suspension from OF and 100 µL from UF) and 5 µL were used for characterization by nanoparticle tracking analysis (NTA) and 5 µL for transmission electron microscopy (TEM). From the remaining volume of the original five EVs samples (Stage 1 OF and Stage 2 UF), 70 µL were used for miRNA analysis. Another ten samples of EVs from Stage 1 OF and from Stage 2 UF were collected and isolated in the same way and their EVs suspensions were entirely used for western blot analysis.

EVs characterization

Nanoparticle tracking analysis (NTA)

Analysis of absolute size distribution of EVs was performed using a NanoSight LM-10 system equipped with a CCD video camera and particle-tracking software NTA 3.1 Build 3.1.45 (NanoSight Ltd., Minton Park, UK). With NTA, particles are automatically tracked and sized based on Brownian motion and the diffusion coefficient. After isolation, 5 µL of EVs solution were diluted in 95 µL of filtered PBS^-. The NTA measurement conditions were detection threshold 2 to 3**,** camera level 13, temperature 22 ºC and measurement time 60 s. Three recordings were performed for each sample. After EVs concentration of each sample was determined, the samples were diluted to a standardized concentration (3 x 10^5 particles/mL, [28,36]), aliquoted and stored frozen at -20 ºC for later use in embryo culture experiments.

Transmission electron microscopy (TEM)

Five microliters of each EVs suspension were diluted in 45 µL. Next, the 25 µL of EVs preparations were allowed to adsorb onto Formvar/carbon-coated 200 mesh copper grids (Agar Scientific, Essex, UK) for 1 min at room temperature. Grids were then washed twice with distilled water. For negative staining, grids were transferred to a 50 µL droplet of 2% uranyl acetate for 20 seconds, followed by blotting the excess liquid. Grids were air-dried and TEM visualizations were performed at the National Center for Electron Microscopy of the Universidad Complutense de Madrid (ICTS-CNME-UCM) using a JEOL JEM1010 (100kV) transmission electron microscope (Jeol Ltd., Tokyo, Japan) equipped with a Megaview II CCD camera integrated into iTEM Olympus Soft Imaging Solutions software (Olympus, Tokyo, Japan).

Western blot for EV marker proteins

EVs preparations were lysed in 1× RIPA buffer (Cell Signaling Technology, 9806S), supplemented with 1× protease, phosphatase Inhibitor Cocktail (Roche, Basel, Switzerland). Protein concentrations in the EVs samples were determined by the Bicinchoninic acid assay (BCA) (Micro BCA Protein Assay Kit; 23325). A total of 35 µg of protein per sample was suspended in Laemmli loading buffer, then loaded and separated in a 4–12% gradient SDS-PAGE polyacrylamide gel. Proteins were transferred onto nitrocellulose membranes (GE Healthcare Life Sciences Whatman™). The membranes were washed in distilled water and blocked with 3% bovine serum albumin (BSA) in PBS-T (PBS+0.1% Tween-20) for 30 min at room temperature. The membranes were incubated with primary antibodies diluted in PBS-T supplemented with 3% BSA overnight at 4 °C with gentle shaking. Three primary antibodies were used as EVs biomarkers and one as negative control: anti-tetraspanin cell surface protein CD9 antigen (1:1000, anti-CD9 mAb, 13403, Cell Signaling Technology, D3H4P, Danvers, MA, USA), anti-heat shock protein 70 (1:1000, anti-HSP70 mAb,

C92F3A-5, Enzo Life Sciences, NY, USA); anti-ALG-2-interacting protein X protein (1:1000, anti-ALIX mAB, sc-53540, Santa Cruz Biotechnology, CA, USA). As negative control, anti-Calnexin (1:1000, anti-CANX mAb sc-23954, Santa Cruz Biotechnology, CA, USA) was used to indicate the absence of cell contamination in EVs samples. After primary antibodies incubation, the membranes were washed with PBS-T and incubated for 2 hours under agitation with secondary antibodies. The following horseradish peroxidase (HRP) conjugated secondary antibodies were used: goat anti-rabbit IgG-HRP (1:5000, 7074S, Cell Signaling Technology, Danvers, MA, USA) or horse anti-mouse IgG-HRP (1:5000, 7076S, Cell Signaling Technology, Danvers, MA, USA). The membranes were again washed three times in PBS-T for 5 min and incubated for 1 min in the Immobilon Forte Western HRP substrate (#WBLUF0100, Millipore, Burlington, MA, USA) and revealed by chemiluminescence with an Image Quant LAS500 biomolecular imager (GE Healthcare Life Sciences, USA, 29005063). The blood, pancreas, lungs and ureters tissues of mice were used as positive controls for the proteins CD9, HSP70, ALIX and CANX where they are respectively highly expressed.

EVs miRNA contents analysis

EVs RNA extraction

Total RNA (including small RNAs) was extracted (n=3 per group) using the miRNeasy Mini Kit (217004, Qiagen, Venlo & Limburg, the Netherlands) according to manufacturer's instructions. RNA concentration and quality were evaluated by NanoDrop™ (Thermo Scientific) and samples with the highest RNA amount were used for analysis.

miRNAs analysis

241 Analysis of miRNA contents was performed as described by Da Silveira et al. [44], with
242 minimal modifications. Briefly, reverse transcription was conducted on the total RNA
243 (120 ng/sample) using the miScript PCR System (Qiagen, Venlo & Limburg, the
244 Netherlands) according to the manufacturer's instructions. Briefly, total RNA, including
245 small RNA fraction, were incubated with 5x miScript Hiflex Buffer, 10x miScript
246 Nuclei mix, RNAse free water, and miScript reverse transcriptase at 37 ºC for 60 min,
247 followed by 5 min at 95 ºC. The relative abundance levels of 383 mature miRNAs were
248 established via quantitative real-time (qRT-PCR), using the miSCRIPT II RT kit
249 (Qiagen, Venlo & Limburg, the Netherlands), according to manufacturer´s instructions.
250 Reactions were prepared in 6 µL master mix containing 2x QuantiTect SYBER Green
251 PCR Master Mix (Qiagen), 10x miScript Universal Primer, miRNA specific forward
252 primers and 0.024 µL of 1:4 diluted cDNA. qRT–PCR was conducted in a 384-well
253 plate using the Quantstudio 6 System (Applied Biosystems). The PCR cycling
254 conditions were 95 °C for 15 min, 45 cycles of 94 °C for 10 s, 55 °C for 30 s, and 70 °C
255 for 30 s followed by a melting curve analysis to confirm the specificity of cDNA
256 amplification. To quantify miRNAs expression level in EVs isolated from OF and UF,
257 raw cycle threshold (Ct) values were normalized to the geometric mean of bta-miR-99b,
258 Hm/Ms/Rt T1 snRNA and RNT43 snoRNA, as internal controls. OF and UF-EVs
259 samples were analysed in triplicate, and only miRNAs with a Ct value less than 37, and
260 detected in at least two of the three samples in each group, were considered to be
261 present.

262 **In vitro embryo production**

263 **EVs depletion of FCS for use in IVC experiments**

264 To produce foetal calf serum (FCS) depleted from its EVs (dFCS), heat-inactivated FCS
265 was ultra-centrifuged at 100,000 for 18 hours at 4 ºC using an Optima-L-100XP

Beckman Coulter ultracentrifuge. Under a laminal hood the supernatant (dFCS) was collected, aliquoted and stored at -20 ºC for later use in embryo culture. The same batch was used in the entire experiment.

Oocyte collection and in vitro maturation

Immature cumulus-oocyte complexes (COCs) were obtained by aspirating follicles (2–8 mm) from the ovaries of mature heifers and cows collected from local abattoirs. COCs were selected and matured in four-well dishes (Nunc, Roskilde, Denmark) in 500 µL TCM-199 medium supplemented with 10% (v/v) FCS and 10 ng/mL epidermal growth factor (EGF), in groups of 50 COCs per well, for 24 hours at 38.5 °C and under an atmosphere of 5% CO_2 in air with maximum humidity.

Sperm preparation and in vitro fertilization (IVF)

IVF was performed as described previously [36]. Briefly, frozen semen straws (0.25 mL) from an Asturian Valley bull previously tested for IVF were thawed at 37 ºC in a water bath for 1 min and sperm cells were selected on a gradient of Bovipure (Nidacon Laboratories AB, Göthenborg, Sweden). Sperm concentration was determined and adjusted to a final concentration of $1x10^6$ sperm cells/mL. Gametes were coincubated for 18-22 hours in 500 µL fertilization medium (Tyrode's medium with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate and 6 mg/mL fatty acid-free BSA) supplemented with 10 µg/mL heparin sodium salt (Calbiochem, San Diego, CA, USA) in a four-well dish, in groups of 50 COCs per well, under an atmosphere of 5% CO_2 with maximum humidity at 38.5 °C.

In vitro culture of presumptive zygotes

At approximately 18-22 hours post-insemination (hpi), presumptive zygotes were denuded of cumulus cells by vortexing for 3 min and then cultured in groups of 20-25 zygotes in 25 µL droplets of culture medium (synthetic oviductal fluid, (SOF); [45] with 4.2 mM sodium lactate, 0.73 mM sodium pyruvate, 30 µL/mL basal medium eagle (BME) amino acids, 10 µL/mL minimum essential medium (MEM) amino acids and 1 µg/mL phenol-red). Embryos were cultured in medium that was supplemented with 3 mg/mL BSA or 5% dFCS in the presence (BSA-EV and dFCS-EV) or absence (BSA and dFCS) of 3×10^5 EVs/mL from OF (D1 to D4) and UF (D5 to D9), under mineral oil at 38.5 ºC and an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. BSA was included as an additional comparison group as it has been shown to improve embryo quality and cryotolerance [30,36].

Assessment of embryo EVs uptake, development and quality

EVs uptake by embryos

EVs isolated from OF and UF were labelled with a lipophilic green fluorescent dye (PKH67, Sigma, USA) as described by Almiñana et al. [37] with some modifications. Firstly, 25 µL of the EVs suspension in PBS were mixed with 125 µL of diluent C (Cell mixture) and for the negative control, 25 µL of PBS^- without EVs were also mixed with 125 µL of diluent C. Next, the dye was diluted in diluent C (1:250), and each sample (EVs and controls) was then placed into 125 µL of the dye mix and incubated for 5 min at room temperature (final concentration of dye is 5×10^{-6}M). To stop the labelling reaction 250 µL per sample of 1% BSA in PBS^- was added for 1min. The samples (dye-EVs and dye-PBS^- negative control) were washed four times (three 15 min centrifugations at 2,000 g and one for 20 min) with 9 mL PBS^- in Amicon Vivaspin® filters (Sartrius Stedim, Germany). The samples (100 µL) were then filtered with a 0.22

µm filter and diluted 1:1 with SOF medium (2x) without phenol red and with BSA 3 mg/mL (final concentration), and the embryos were cultured in this medium. The labelled EVs from OF and the PBS^- negative control (labelled but without EVs) were used in the culture of D3 8-cell embryos for 5 hours, and the labelled EVs from UF and the PBS^- negative control (labelled but without EVs) were used in the culture of D8 blastocysts also for 5 hours. After the culture period, embryos were then collected and washed twice in PBS to remove any labelled vesicles that were not internalized, followed by fixation in 4% paraformaldehyde (PF) during 30 minutes, washing, and labelling with Hoechst 33342. Finally, embryos (10-14 per group) were placed in a droplet of ProLong Diamond Antifade Mountant on a glass slide, covered with a coverslip, and then analysed under a multispectral confocal microscope (Leica TCS-SP8 STED 3X) using the 63X objective.

Embryo development

Cleavage rate was recorded on Day 2 (48 hpi) and cumulative blastocyst yield was recorded on Days 7, 8 and 9 pi.

Embryo quality

Blastocyst vitrification

The ability of the blastocyst to withstand cryopreservation was used as an indicator of quality. Day 7–8 blastocysts from each treatment (N=67, 52, 86 and 64 for BSA, BSA-EV, dFCS and dFCS-EV, respectively) were vitrified in holding medium (HM), consisting of TCM 199 supplemented with 20% (v/v) FCS, and cryoprotectants as described previously [2] in a two-step protocol using the Cryoloop device (Hampton Research, Aliso Viejo, CA, USA). In the first step, HM was supplemented with 7.5% ethylene glycol and 7.5% dimethyl sulfoxide; in the second step, HM was supplemented

with 16.5% ethylene glycol, 16.5% dimethyl sulfoxide and 0.5 M sucrose. Blastocysts were warmed in two steps in HM with 0.25 and 0.15 M sucrose and then cultured in 25-mL droplets of SOF with 5% FCS. Survival was defined as re-expansion of the blastocoel and its maintenance for 72 hours after warming.

Mitochondrial activity measurement, lipid content quantification and total cell number in blastocysts

For mitochondrial activity, blastocysts (D7-8) from each treatment (N=29, 29, 33 and 27 for BSA, BSA-EV, dFCS and dFCS-EV, respectively) were first suspended in 100 µL PBS without calcium and magnesium supplemented with 0.1% polyvinylpyrrolidone (PVP). Next, blastocysts were equilibrated for 15 minutes in culture medium supplemented with 5% FCS and then incubated for 30 minutes at 38.5 °C in 400 nM/mL MitoTracker DeepRed (Molecular Probes, Eugene, USA); blastocysts were then fixed in 4% PF for 30 min at room temperature. For lipid content analysis (N=28, 26, 25 and 29 for BSA, BSA-EV, dFCS and dFCS-EV, respectively), the blastocysts were fixed in PF and then permeabilized with 0.1% saponin for 30 min and stained for 1 hour with 20 µg/mL Bodipy 493/503. For total cell number the blastocysts (N=57, 55, 58 and 56 for BSA, BSA-EV, dFCS and dFCS-EV, respectively) were stained with Hoëchst 33342 (10 µg/mL) for 30 min, after being stained either for mitochondria or for lipids. After each stain the blastocysts were washed in PBS+0.1% PVP three times for 5 minutes each. Finally, blastocysts were mounted in 3.8 µL mounting medium (ProLong Gold, Thermo Fisher Scientific) between a coverslip and a glass slide and sealed with nail polish.

Slides were examined using a laser-scanning confocal microscope (Leica TCS SP2) equipped with an argon laser excited at 488 nm and whose emission spectrum is 500-537 nm for visualization of lipid droplets. For mitochondria, excitation and emission

were set at 543 nm and 580– 650 nm, respectively. The format, laser, gain and offset were kept constant for every sample.

For the assessment of mitochondrial activity, the fluorescence signal intensity (pixels) was quantified. Serial sections of 5 µm were made for each blastocyst and a maximum projection was accomplished for each one. Images obtained were evaluated using the ImageJ program (NIH; http://rsb.info.nih.gov/ij/). After selection using the freehand selection tool, each blastocyst was measured to determine its area and its integrated density (IntDen), which corresponds to pixel intensity. In addition, the background fluorescence of an area outside the blastocyst was measured. Fluorescence intensity in each blastocyst was determined using the following formula: Relative fluorescence = IntDen - (area of selected blastocyst x mean fluorescence of background readings). Fluorescence intensities are expressed in arbitrary units (a.u.) [46]

The lipid quantity in blastocysts was obtained by analysis of the total area of lipids in each embryo. We captured three images of each blastocyst: one in the middle of the blastocyst (the image with the largest diameter) and the other two in the middle of the resulting halves. We used a 63X objective at a resolution of 1024 x 1024 and images were analysed using the 'nucleus counter' tool, set to detect, distinguish and quantify droplet areas with the ImageJ software (NIH, USA) and expressed as the area of lipid droplets relative to the total area of the blastocyst without its cavity (μm^2). For blastocysts, lipid quantity was corrected by area, to account for varying blastocyst sizes. After verification of a significant correlation ($r^2 = 0.84$ and $P < 0.0001$ by Pearson's correlation test) between lipid quantity of three sections in 30 blastocysts (10 per group) we chose the section with the largest area per embryo to be analysed [22]. The number of cells per blastocyst was determined by counting the Hoechst-stained cells under an

385 epifluorescence microscope (Nikon 141731) equipped with a fluorescent lamp (Nikon
386 HB-10104AF) and UV-1 filter.

387 **Gene expression analysis**

388 Gene expression analysis was performed using Day 7–8 blastocysts (pools of 10 in three
389 replicates per treatment). All samples were washed in PBS, snap-frozen in LN_2 and
390 stored at -80 ºC until mRNA extraction analyses. Poly(A) RNA was extracted using the
391 Dynabeads mRNA Direct Extraction Kit (Ambion; Thermo Fisher Scientific) with
392 minor modifications [47]. Immediately after poly(A) RNA extraction, reverse
393 transcription (RT) was performed using a MMLV Reverse Transcriptase 1st-Strand
394 cDNA Synthesis Kit according to the manufacturer's instructions (Epicentre
395 Technologies). Poly(T) random primers and Moloney murine leukaemia virus (MMLV)
396 high-performance reverse transcriptase enzyme were used in a total volume of 40 µL to
397 prime the RT reaction and to produce cDNA. Tubes were heated to 70 ºC for 5 min to
398 denature the secondary RNA structure and the RT mix was then completed by adding
399 50 units of reverse transcriptase. Samples were incubated at 25 ºC for 10 min, to help
400 the annealing of random primers, followed by incubation at 37 ºC for 60 min, to allow
401 the RT of RNA, and finally at 85 ºC for 5 min to denature the enzyme. All mRNA
402 transcripts were quantified in duplicate using a Rotorgene 6000 Real Time Cycler
403 (Corbett Research). RT–quantitative polymerase chain reaction (qPCR) was performed
404 by adding 2 µL aliquot of each cDNA sample (~60 ng µL-1) to the PCR mix (GoTaq
405 qPCR Master Mix, Promega) containing the specific primers to amplify transcripts for
406 the genes (Additional file 1: Table S1). The selection of genes to be evaluated was
407 carried out considering the expression of key genes in lipid metabolism. All primers
408 were designed using Primer-BLAST software

(http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to span exon–exon boundaries when possible. For quantification, RT-qPCR was performed as described previously [48]. The PCR conditions were tested to achieve efficiencies close to 1. Relative expression levels were quantified by the comparative cycle threshold (CT) method [49]. Values were normalized using two housekeeping (HK) genes: H2AZ1 and ACTB (Additional file 1: Table S1). Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above the background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to a doubling of the amplified PCR product. According to the comparative CT method, the ΔCT value was determined by subtracting the mean CT value of the two HK genes from the CT value of the gene of interest in the same sample. The calculation of ΔΔCT involved using the highest treatment ΔCT value (i.e. the treatment with the lowest target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Fold-changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT}$.

Protein analysis in blastocysts

Protein analysis was performed using Day 7–8 blastocysts (pools of 20 in 3 replicates per treatment). Embryo samples were lysed in RIPA buffer supplemented with 1× protease, phosphatase Inhibitor Cocktail. Proteins were resolved in SDS-PAGE (4-12% acrylamide gel loading 45 µL of total protein per well) and transferred to a nitrocellulose membrane (Amersham™ Protran™ 0.45 NC), blocked with 3% BSA in PBS-T and incubated overnight at 4 ºC with the following primary antibodies: HSL rabbit (1:1000 in 3% BSA + 0.1% PBS-T, #4107S, Cell Signaling Technology, Boston, MA, USA), pHSL rabbit (phospho-HSL, 1:1000 in 3% BSA + 0.1% PBS-T, #4139S, Cell Signaling Technology, Boston, MA, USA). The membranes were washed three

434 times in 1X TBS-T for 5 min and incubated for 2 hours at room temperature with a goat
435 anti-rabbit IgG-HRP (1:5000 in 1% BSA + 0.1% PBS-T, #7074, Cell Signaling
436 Technology, Danvers, MA, USA). The membranes were again washed three times in
437 1X TBS-T for 5 min and incubated for 5 min in Enhanced Chemiluminescence kit
438 (RPN2109, ECL™, Amersham GE Healthcare, Buckinghamshire, UK) and detected by
439 an ImageQuant LAS 500 chemiluminescence CCD camera (GE Healthcare Life
440 Sciences, USA, 29005063).

441 The monoclonal anti-β-actin−peroxidase antibody produced in mouse (Merk,
442 Darmstadt, Germany, A3854) was used as the loading control. For this purpose, after
443 the chemiluminescent detection of HSL and pHSL, membranes were stripped and then
444 wash extensively in TBS-T and repeating the blocking step, the membrane is re-probed
445 with an anti-actin antibody. In all cases, intensities of protein bands [(optical density
446 (OD)] were quantified by ImageJ software and normalized relative to the abundance of
447 actin in each lane and phosphorylation level was expressed as phosphorylated
448 pHSL/HSL. The ratio of the OD of the protein concerned (HSL/pHSL) in relation to
449 actin is presented in the form of bar charts [41].

450 **Statistical analysis**

451 Cleavage and blastocyst rates, and vitrification data were analysed by *Chi*-square test.
452 Number of cells per blastocyst, relative mRNA abundance and protein level
453 (pHSL/HSL) were normally distributed with homogeneous variance, so one-way
454 analysis of variance (ANOVA) followed by Tukey´s test was performed to evaluate the
455 significance of differences between groups. For mitochondrial activity and lipid content,
456 data were analysed by Kruskal-Wallis followed by Dunn´s pairwise multiple
457 comparisons test. The correlation analysis for lipid quantification in blastocyst was

458 determined by linear regression r^2 test and significance of the correlation by F test.
459 Values were considered significantly different at $P<0.05$. Unless otherwise indicated,
460 data are presented as the mean ± s.e.m. All analyses were made with the SigmaStat
461 software package (Jandel Scientific, San Rafael, CA). For miRNA, data were displayed
462 as the mean ± SD of replicate samples. Statistical analyses were performed using SAS
463 9.3 Software (SAS Institute). A Student's t-test was used to assess statistical
464 differences, and normality was verified using a Shapiro–Wilk test, where a statistical
465 difference of 5% was considered to be significant.

466 # Results

467 **OF and UF EVs characterization**

468 **NTA**

469 This analysis was performed to characterize the size and concentration of particles
470 present in the pools of the five samples isolated from OF and UF. NTA showed that
471 concentration in OF was 2.97×10^{10} particles/mL, while the mean size was 177.5 nm
472 and modal size 137.2 nm. In UF concentration was 7.98×10^{10} particles/mL and mean
473 and modal sizes were 216.5 and 151.2, respectively. Concentration/Size distribution of
474 EVs from oviductal and uterine samples, which were used for IVC experiments, are
475 shown in Figure 2A.

476 **TEM**

477 Figure 2B shows representative images from TEM analysis for the presence and
478 morphology of EVs in oviductal and uterine samples. The structures observed are
479 compatible with EVs, confirming NTA data and their presence in the samples analysed.

480 **Western Blotting**

Marker proteins for EVs CD9, HSP70 and ALIX were detected in OF and UF samples (Figure 2C). The negative control CANX was not detected in any samples, except for its positive control tissue (urethra). These observations further confirm the presence of EVs in the samples analysed and the absence of cell contamination.

In vitro culture

EVs uptake

To confirm that EVs added to the media were taken up by embryos, therefore, would be capable of affecting their development and/or quality, 8-cell embryos (Day 3) and blastocysts (Day 8) were cultured for 5 hours with fluorescently labelled EVs from OF and UF, respectively. As observed in Figure 3, uptake of both types of EVs was confirmed with their presence inside the blastomeres.

Embryo development

As shown in Table 1, cleavage was not affected by treatments ($P>0.05$) and the mean rate was 89.9%. Blastocyst rate on Day 7 of culture, however, showed that groups treated with BSA, with or without EVs (16.2 and 14.1 %, respectively, $P>0.05$), were lower ($P<0.05$) than dFCS groups, with or without EVs (30.5 and 31.1%, respectively, $P>0.05$). On D8, BSA and BSA-EV (31.0 and 26.2%, respectively) still had lower ($P<0,05$) blastocyst rates than dFCS and dFCS-EV (40.7 and 39.8 %, respectively, $P>0.05$), and BSA-EVs had lower development than BSA ($P<0.05$). The same pattern was seen on D9, where BSA and BSA-EV (34.6 and 29.2%, respectively), were lower ($P<0.05$) than dFCS and dFCS-EV (41.7 and 41.4%, respectively, $P>0.05$) and BSA-EV was lower than BSA ($P<0.05$).

Table 1. Cleavage (D2) and cumulative blastocyst development rates (D7-9) after in vitro culture with BSA or dFCS supplemented or not with OF-EVs (D1-4) and UF-EVs (D5-8) (28 replicates).

Treatment	IVC	Cleaved	blastocysts		
		D2	D7	D8	D9
	n	n	n	n	n
		(% ± SEM)	(% ± SEM)	(% ± SEM)	(% ± SEM)
BSA	1584	1427	248	484	548
		(89.5±1.0)	(16.2±1.5)[a]	(31.0±1.9)[a]	(34.6±1.7)[a]
BSA-EV	1853	1677	261	496	555
		(89.7±1.1)	(14.1±1.7)[a]	(26.2±2.0)[b]	(29.2±2.1)[b]
dFCS	1594	1435	476	648	659
		(89.5±0.9)	(30.5±2.0)[b]	(40.7±2.5)[c]	(41.7±2.4)[c]
dFCS-EV	1473	1337	460	590	615
		(90.7±0.9)	(31.1±2.5)[b]	(39.8±2.7)[c]	(41.4±2.8)[c]

506 [a,b,c] Values with different superscripts within a column are significantly different (P<0.05).

507

508 **Embryo quality**

509 **Vitrification**

510 As a parameter to assess the quality of embryos produced in the different treatments, the
511 survival rate after vitrification and warming of blastocysts cultured up to 72 hours in
512 SOF with 5% dFCS was measured (Table 2). Survival rate at 72 hours was highest for
513 dFCS-EV (87.8%, P<0.05) compared to its respective control (dFCS = 69.2%)
514 demonstrating that the presence of EVs improved blastocyst quality. The other groups
515 were similar among themselves with high survival at this time point ranging from 67.1
516 to 73.1% (P>0.05).

517 **Table 2**. Survival rates after vitrification and warming of Day 7-8 blastocysts cultured
518 with BSA or dFCS supplemented or not with OF-EVs (D1-4) and UF-EVs (D5-8).

Treatment	IVC	4h	24h	48h	72h
	n	n	n	n	n

		(% ± SEM)	(% ± SEM)	(% ± SEM)	(% ± SEM)
BSA	67	64	56	51	49
		(95.4±2.8)	(78.5±6.6)	(68.6±8.9)	(67.1±8.1)[a]
BSA-EV	52	48	43	41	39
		(93.9±6.1)	(84.2±8.7)	(79.2±9.8)	(73.1±10.7)[a]
dFCS	86	80	75	67	61
		(93.4±2.6)	(86.6±4.0)	(75.8±5.7)	(69.2±5.8)[a]
dFCS-EV	64	63	60	57	54
		(97.9±2.1)	(93.3±4.1)	(91.2±4.3)	(87.8±5.7)[b]

519 [a,b] Values with different superscripts within a column are significantly different (P<0.05).

520 **Total cell number, mitochondrial activity and lipid contents**

521 Results for total cell numbers in Figure 4 show that although there was no variation in
522 terms of blastocyst production by adding EVs to culture medium, the total nuclei in
523 blastocysts showed differences among treatments.

524 Both groups with EVs did not differ between themselves (BSA-EV = 137.1 ± 3.2 and
525 dFCS-EV = 143.5 ± 3.6, P>0.05), but had increased cell numbers (P<0.05) compared to
526 their respective controls (128.8 ± 2.5 and 124.6 ± 3.5 for BSA and dFCS, respectively),
527 which were also not different (P>0.05). Therefore, the presence of EVs during embryo
528 cultured showed a positive effect, irrespective of the type of protein supplementation
529 used.

530 Regarding mitochondrial activity (Figure 5), EVs supplementation did not affect
531 embryos relative to its respective control for BSA-EV in comparison with BSA,
532 (P>0.05), but for dFCS-EV there was an increase in mitochondrial activity compared to
533 all other groups (P<0.05).

534 Finally, regarding lipid contents (Figure 6), the group cultured with dFCS associated
535 with EVs showed reduction (dFCS-EV = 0.277 ± 0.03 lipid droplet area in μm^2,

P<0.05) compared to its control (dFCS=0.466 ± 0.02 μm^2) and to BSA-EV (0.377 ± 0.02 μm^2) and was lower but not different from BSA (0.374 ± 0.02 μm^2). Thus, in embryos cultured with dFCS, the presence of EVs caused a reduction in lipid contents, while in those cultured with BSA, the addition of EVs had no effect.

Gene expression

As seen in Figure 7, analyses have shown that there was variation in transcripts abundance regarding the presence of EVs, where *PPARGC1B* increased and *ACACA* reduced (P<0.05) in embryos cultured with the vesicles in both media. Regarding *CD36*, *PLIN2* and *PNPLA2*, however, there was an effect by EVs, but it depended on the type of protein supplementation. EVs caused reduction in transcript abundance for *CD36* in BSA-EV compared to its control BSA (P<0.05), while for dFCS-EV there was an increase in this transcript in comparison to its control dFCS (P<0.05). There was a reduction for *PLIN2*, but only in dFCS-EV compared to dFCS (P<0.05), and the same was observed for *PNPLA2*, but in BSA-EV relative to BSA (P<0.05).

Lipolysis proteins

Levels for active (phosphorylated) and total HSL (rate-limiting enzyme for lipolysis) were determined by western blotting in D7-8 blastocysts cultured in medium with BSA, BSA-EV, dFCS and dFCS-EV. As observed in Figure 8, results showed that HSL and pHSL were detected in all groups and that HSL phosphorylation level was significantly lower (P<0.05) in dFCS group compared with all groups.

miRNA in EVs from OF and UF

miRNA contents of EVs in OF and UF were also analysed, and 333 mature miRNAs were detected, from which the twenty most abundant in both fluids are listed in Additional file 2: Table S2. Most of them are common to EVs from both fluids, and the

top four are the same (bta-miR-615, bta-miR-323, bta-miR-494 and bta-miR-631) and in a similar order of enrichment, with little variation. Only two were exclusive to OF (bta-miR-493 and bta-let-7a-5p) and two to UF (bta-miR-200c and bta-miR-1225-3p).

When the abundance of miRNAs between the two EVs origins was compared, 20 were shown to be significantly differentially expressed ($P<0.05$), and of these, 19 were more abundant in EVs from UF, while only one was more abundant in EVs from OF (bta-miR-148b, Figure 9).

Discussion

In the present study, we have used a sequential in vitro culture system, by supplementing embryo culture medium with EVs isolated from OF obtained at early luteal phase (Stage 1) and used during the first 4 days in culture, followed by use of EVs isolated from uteri obtained at mid-luteal phase (Stage 2) for the remaining days (up to Day 9). This system intends to mimic the sequence of events undergone by the embryo during its early development in vivo. Besides, EVs have been shown to participate in the communication between embryo and oviduct [37] and uterus [50], and OF and UF as well as EVs isolated from OF and BOECs conditioned medium showed improvements to embryo development and/or quality [29,30,36]. The main findings were that although embryo development was not increased, embryo quality was improved in those cultured with EVs and that lipid metabolism-related genes, as well as an active lipase protein, are affected by their supplementation. Also, the protein source in the medium may influence the functional effects of EVs on bovine embryos produced in vitro.

EVs characterization

583 Small EVs isolated from OF and UF were characterised by NTA, TEM and WB. The
584 results have shown the expected features for EVs by the three methods, i.e., size,
585 morphology and presence of protein markers. The morphology is the typical cup-shaped
586 seen in TEM as previously described for the bovine oviduct [36,51] and uterus [38,39].
587 The marker proteins for EVs CD9, HSP70 and ALIX [33] were also identified in
588 previous studies in EVs from oviduct (HSP70, [51] and CD9, [36]) and uterus samples
589 (HSP70, [38], CD9, [39]), similar to our observations. Few studies have analysed EVs
590 from bovine oviducts and concentrations found by Almiñana et al. [51] for fluid from
591 Stage 1 oviducts were lower (about 5 x 10^8 to 1.0 x 10^9 particles/mL) than the observed
592 in the present study (~3 x 10^{10} particles/mL). A previous study in our laboratory also
593 with Stage 1 oviducts observed EVs smaller than 200 nm and concentration of 7.5 to
594 10.5 x 10^8 /mL [36]. The sizes of EVs were not different compared to the other
595 aforementioned studies (<200 nm), but concentrations were higher in ours. These
596 differences are probably due to distinct isolation methods, which can affect particle
597 concentration, purity and size [52].

598 Regarding EVs in bovine UF there is little information available. Only one study also
599 evaluated EVs from Stage 2 uteri and observed that size was mostly between 50-150 nm
600 measured by TEM, but NTA was not performed and concentration was not assessed
601 [39]. In another study using EVs isolated from bovine uteri at different days of
602 pregnancy (17 to 22 days), Kusama et al. [38] also reported sizes of 50-150 nm
603 determined by TEM and 109 to 137 nm by NTA. Particle concentrations were in the
604 order of 10^6 particles/mL, so they were also higher in our study (10^{10} particles/mL), as
605 were sizes (~200 nm), and such differences are probably due to the isolation method, as
606 mentioned above. In a recent study from our group we found concentration and size of

607 EVs from OF and UF (30 to 200 nm and 3.4 and 6.0 x10^{10} particles/mL, respectively),
608 throughout the estrous cycle [53], similar to the current study.

609 **EVs uptake**

610 Blastocysts cultured with labelled EVs from OF and UF showed presence of fluorescent
611 spots within cytoplasm of blastomeres, confirming that embryos are able to internalize
612 EVs added to culture medium from both origins and at stages of development analysed
613 (from OF in D4 embryos and from UF in D7 blastocysts). Similar observations were
614 also reported in studies using labelled EVs from OF, UF, FF or derived from medium
615 conditioned by cultured embryos [37,50,54,55]. Besides, pores in bovine embryos zona
616 pellucida are of about 200 nm [56], which matches the size of EVs isolated from the
617 fluids used in embryo culture. Therefore, as EVs are taken up by embryos, their cargo,
618 which includes proteins, mRNA, miRNA, among other molecules [57], can affect
619 embryo functions during in vitro culture, and may contribute, at least in part, to the
620 effects seen in the present study. One curious observation was that some of the effects
621 of EVs appeared to affected by the type of protein supplementation present in the
622 embryo culture media (BSA or dFCS), and in most cases was observed when dFCS was
623 the protein source (more details in following sections). The reasons for this are unclear,
624 but could be related with serum somehow affecting the process of EVs uptake or of a
625 subpopulation of the vesicles. Studies on effects of culture conditions are mostly
626 focussed on EVs secretion, and quantitative analysis and characterization of factors
627 affecting EVs uptake are not well defined, as also separating EVs subpopulations [58–
628 60]. However, a recent study using HeLa and A431 cell lines, showed that uptake may
629 be increased in presence of serum [61], so differences in uptake efficiency could impact
630 target cell response and this could be a possibility also in embryos. This level of
631 analysis was not the aim of this experiment and the method used (fluorescent lipophilic

dye staining) would not be appropriate for such study [62], but this would be an interesting point to consider in future studies.

In vitro embryo development

EVs supplementation during embryo culture in vitro did not improve embryo development, as blastocyst rates were similar in dFCS and dFCS-EV groups. Surprisingly, however, development was reduced when EVs were added to BSA containing medium. When culturing embryos with complete FCS and EVs isolated from BOECs conditioned medium, lack of effect of EVs have also been reported [28]. In contrast, positive effect of EVs on embryo development has been observed by Qiao et al. [39] when using EVs only from UF in medium with BSA from D4 and by Almiñana et al. [37] using EVs only from OF in medium with dFCS. Differences in culture conditions (only OF or only UF EVs *vs* sequential use of OF and UF-EVs), type of embryo (cloned embryos in [39]), EVs isolation method and amount of EVs supplemented to culture medium, could account for the diverging results between studies. Regarding EVs in medium with BSA, Lopera-Vasquez et al. [36] and Banliat et al. [63] did not report any effect of EVs, contrary to our observations, but they both used different isolation methods. No other studies have directly compared EVs addition in culture media with different protein supplementation, so the reasons for diverging effects of EVs when using BSA or dFCS are unknown, but as mentioned earlier, serum could affect EVs uptake, as seen in cell lines [61], which could lead to different effects on cells.

Embryo quality

Vitrification

When assessing embryo quality by blastocyst survival rates at 72 hours after vitrification and warming showed that addition of EVs had no effects in medium with BSA, as BSA-EV did not differ from its respective control (73 x 67% for BSA-EV and BSA, respectively). However, the addition of EVs to dFCS group increased survival (87.8 vs 69.2%, respectively), indicating a positive effect of EVs in presence of serum. The supplementation of EVs in embryo culture medium and effects on cryotolerance were reported in only two previous studies. Lopera-Vasquez et al. [28] observed that using EVs isolated from BOECs conditioned-medium, but in medium devoid of any protein source, had higher survival rates compared to complete serum (50 x 20%). This effect, however, could be due to the exclusion of serum, as this has been shown to increase embryo cryotolerance (56 x 26%, Lopera-Vasquez et al. [29]). Also, the use of either BSA or dFCS alone improved cryotolerance [28–30,36], and no additional effects of OF, OF+UF or EVs from BOECs were reported. However, a positive effect of oviduct EVs has been described when they were isolated from the isthmus of the oviduct (80 x 51%), suggesting that EVs may differ depending on the region from which they were isolated [36]. The characterization of EVs from different regions would be interesting to bring new information to better understand their different effects on aspects of embryo development and quality. Nevertheless, in the present study, EVs were isolated from the whole oviduct and used in sequence with EVs from the uterus, so conditions are different, but a positive effect was also observed. Besides, this was the same group showing lower lipids, suggesting this could be related to better cryotolerance as previously reported [15–18].

Total cell numbers

Blastocyst quality was also assessed by other parameters including total cell numbers, mitochondrial activity and lipid content. Our results using EVs from OF and UF in

680 sequential culture corroborate previous studies of a positive effect on cell numbers.
681 Indeed, increased cell numbers in embryos were also reported when using EVs isolated
682 from BOECs-conditioned medium with BSA [28], from UF in medium with BSA from
683 D4 to D8 [39], and also from OF in medium with dFCS [37]. EVs isolated from
684 medium conditioned by cultured embryos also show this same effect [55,64].

685 Such effect would be due to a stimulus to cell proliferation as reported by Hung et al.
686 [65], who observed a proliferative effect of EVs from follicular fluid on bovine
687 granulosa cells in vitro. A similar effect could be caused by EVs from OF and UF on
688 embryo cell division. When analysing the transcriptome of EVs from OF, Almiñana et
689 al. [51] reported the presence of transcripts for genes related to cell cycle regulation,
690 which could potentially be transferred by EVs uptaken by embryos, stimulating cell
691 division, therefore increasing embryo cell numbers. Such possibility seems plausible, as
692 horizontal transfer of mRNAs and proteins between bovine granulosa cells were shown
693 to occur through EVs, and phenotypical changes in cells uptaking EVs, including
694 increased proliferation, have also been reported [66]. It would be interesting to further
695 investigate and confirm the transfer, via EVs from OF and/or UF, of molecules
696 regulating the cell cycle in embryos exposed to such vesicles.

697 **Mitochondrial activity**

698 Regarding mitochondrial activity in blastocysts, only dFCS-EV embryos showed
699 increased activity. These results suggest that EVs are capable of stimulating embryo
700 mitochondrial activity, but this effect can be influenced by the protein source in the
701 culture medium, as for embryos cultured with BSA, the presence of EVs had no effect.
702 Coincidently, the dFCS-EV group that had increased mitochondrial activity also had the
703 lowest lipid contents, therefore this increased activity may go along with higher lipid
704 consumption for β-oxidation in mitochondria of treated embryos. Prastowo et al. [67]

have reported a close relationship between lipid contents and mitochondrial function. Bovine granulosa cells exposed to EVs showed an increased in mitochondrial activity [66], therefore, there was an effect from EVs from OF and/or UF on embryonic mitochondrial activity.

Lipid contents

When analysing lipid contents in blastocysts cultured with EVs in a medium with different protein sources, we observed that only the dFCS-EV group had lower contents, suggesting effects of EVs when embryos are cultured with serum, but not with BSA. Substitution of serum for BSA in embryo culture medium has been shown to decrease lipids [68], and apparently the addition of EVs did not result in an additional decrease. On the other hand, embryos cultured with serum usually show increased lipid contents [20]. In the present study, we have used dFCS, which has been shown to improve embryo quality and cryotolerance [28], as also observed with the use of BSA [30], suggesting a possible reduction in lipids.

On the other hand, the supplementation of EVs was capable of causing a significant reduction in embryo lipids compared to dFCS alone, which was not different from BSA groups. The mechanism by which EVs would cause such reduction is presently unknown but is probably related to EVs cargo, as it can affect functions in target cells [66]. Almiñana et al. [51] have detected several transcripts for lipid metabolism enzymes and miRNAs that affect them. Therefore, EVs could affect embryo lipid metabolism through modifications on mRNA and/or protein expression. As described later, we have observed changes in transcript abundance for several genes related to lipid metabolism, reinforcing this possibility.

Taken together, the observations for total cell number, mitochondrial activity and lipid content indicate that EVs improve parameters related to embryo quality, and also, that

such effect can be influenced by the protein source supplemented to the embryo culture medium. To the best of our knowledge, this is the first study to indicate the effects of EVs on mitochondrial activity and lipid metabolism in bovine embryos.

Expression of lipid-metabolism genes

The presence of EVs affected five of the analysed transcripts (*PPARGC1B*, *ACACA*, *CD36*, *PLIN2* and *PNPLA2*) and the patterns of expression were varied. Most transcripts were decreased (*PARGC1B, PNPLA2*, *PLIN2* and *CD36*) and two were increased (*ACACA* and *CD36*). The most common outcome of miRNAs on mRNA levels is a decrease by degradation of the targeted transcript [69], however, increased transcription mays also be induced by miRNAs [70,71]. On the other hand, increased transcripts in target cells may be a result or direct transfer of mRNA, and this could be the case for *ACACA* and *CD36*, as their transcripts were identified in OF-EVs [51]. However, miRNAs that can target *ACACA* [51] were also identified in this study. mRNAs for *PPARGC1B*, *PNPLA2* and *PLIN2* were also detected in OF-EVs by Almiñana et al. [51], but they were decreased in embryos in this study, as were for *LDLR* and *FASN* that were not affected, so transfer of mRNAs would not explain this outcome. Regulation of target cells functions by EVs is quite a complex topic and yet undergoing expansion of knowledge. Bauersachs et al. [72] have analysed mRNA and miRNAs contents reported as present in OF-EVs and compared with the transcriptome of embryos cultured with such EVs, and concluded they do regulate expression in embryos and that such regulation may be carried out by different mechanisms including increased delivery of mRNAs, translation of delivered mRNAs into proteins that then modulate expression or by miRNAs that downregulate mRNAs or modify expression in other ways. Whether miRNAs and/or mRNAs in EVs used in this study are influencing these transcripts cannot be discriminated, and warrant further studies.

Another observation regarding effects of EVs, was that some transcripts were affected differently by EVs depending on the protein source present in culture medium. *PNPLA2* and *PLIN2*, both decreased, but only when EVs were added to medium containing BSA or dFCS, respectively. Also, CD36 showed an inverse response, where EVs lead to upregulation when in medium with dFCS, and upregulation with BSA. Reasons for this outcome are unknown, but could be partly related with the presence of serum, as some differences in other results in this study appeared to be influenced mostly by serum. BSA does not contain EVs [55], but serum, although depleted of its EVs, still has some left, as the depletion process (ultracentrifugation) does not result in their complete removal [73,74]. Thus, some EVs still present in dFCS in association with those added to the culture medium (from OF and UF in this study), could lead to a different effect than when adding to medium containing BSA, which would have only the added EVs from the fluids. Also, as mentioned before, if serum can somehow affect uptake of EVs, as observed in other cells [61], this would in turn also affect cargo delivery and the function of target cell. More studies are needed to understand the interactions between EVs, protein source and their effects on embryonic gene expression.

Taken together, EVs were shown to affect transcripts levels of lipid-related genes, involved in different aspects of lipid metabolism as lipogenesis (*PPARGC1B* and *ACACA*), lipolysis (*PNPLA2*), lipid uptake (*CD36*) and accumulation (*PLIN2*), so EVs from OF and/or UF may influence lipid metabolism of embryos. Also, type of protein source in culture medium may interfere in the effect of these EVs in vitro.

Lipid metabolism proteins

HSL is an essential enzyme in lipolysis and is activated when phosphorylated [75]. Confirming its presence at mRNA level, HSL protein was also detected, as was its

779 phosphorylated (active) form (pHSL) in all groups, showing that the enzyme is also
780 functional in bovine embryos. This protein had only been detected previously in bovine
781 oocytes and cumulus cells [76,77]. This is the first confirmation of its protein in
782 embryos, indicating its certain participation in embryonic lipid metabolism, as the
783 activated form was also present. HSL phosphorylation level was lower in dFCS,
784 suggesting this groups would have lower lipolysis rates, and this could be related to the
785 higher amount of lipids found in this group. The presence of EVs in the medium with
786 dFCS had a positive effect on the stimulation of lipolysis and could counteract the effect
787 of serum suggesting an important role in embryonic lipid metabolism. Recently, Zhuan
788 et al. [78] have shown in porcine oocytes that stimulation of HSL activity improves
789 oocyte maturation and embryo development, while its inhibition resulted in increased
790 lipids in oocytes, associated with lower mitochondrial activity. This is in accordance
791 with our observation that lower active HSL corresponded with higher lipid amounts and
792 lower mitochondrial activity in dFCS blastocysts compared to those to which EVs were
793 added to culture with dFCS.

794 Interestingly, transcript levels for HSL did not vary with any treatment, further
795 supporting the idea that transcript levels do not correlate to lipases activities [79], and
796 that detection of the active form of the enzyme can be a better indicator of effects on
797 lipid metabolism. The mechanism by which EVs could affect activity of HSL is
798 unknown, but EVs have been shown to participate in the regulation of cell metabolism
799 by multiple pathways [80].

800 **miRNA in EVs used for IVC**

801 As EVs may carry different types of cargo and among them there are miRNAs, which
802 are small molecules known to modulate target-cell function through effects on the

803 expression of transcripts and proteins [69–71]. Therefore, we also looked at their
804 content in the EVs used in our culture system. From the twenty most abundant miRNAs
805 in EVs from OF and UF, bta-miR-200b was also detected by Almiñana et al. [51] in
806 EVs from bovine OF samples. When comparing miRNA contents in EVs from OF and
807 UF, two of the differentially expressed (bta-miR-151-3p and bta-miR-24-3p), were also
808 reported in EVs from OF in the same study, in agreement with present observations.
809 These two miRNAs are involved in the control of expression of *FASN* and *ACACA* [51],
810 which are genes related with lipid metabolism, and both transcripts were detected in
811 embryos in this study, but only *ACACA* was influenced by the EVs supplementation in
812 the culture medium. EVs increased transcripts for *ACACA* possibly due to one or both
813 of these miRNAs, as these molecules may also induce transcription [70,71]. And
814 although *FASN* transcripts were not affected, it is possible that miRNAs interfered with
815 protein levels. This possibility, however, remains to be addressed in future studies.

816 Some of the miRNA detected in EVs from OF and UF samples, such as bta-miR-181,
817 bta-let-7a, bta-let-7b, bta-miR29a, bta-miR-151 and bta-miR-494 have been related with
818 implantation in mice [81,82], sheep [83] and humans [84]. From the 20 differentially
819 expressed, almost all of them were more abundant in EVs from UF, suggesting that
820 these miRNAs could be involved with the arrival of the embryo to the uterus. The only
821 miRNA that was lower in EVs from UF compared to those from OF (bta-miR-148b)
822 was reported to be related to the suppression of proliferation, migration, and invasion in
823 human tumour cells [85], suggesting that its reduction in the uterus would allow for an
824 increase in these cell activities, necessary for embryo implantation in the uterus. This
825 miRNA was also related with the action of interferon-τ produced by embryonic
826 trophoblastic cells, which lowers the expression of this miRNA to reduce the
827 inflammatory response in bovine endometrial epithelial cells [86]. This observation

828 suggests that the reduction of bta-miR-148b in the uterus compared to the oviduct,
829 could be related with favouring the implantation process, but more studies are necessary
830 to address this assumption. As nearly all differentially expressed miRNAs were
831 upregulated in the uterine EVs, this is probably a reflection of the highly complex
832 function of the uterus in regulating embryo development and the process of
833 implantation and placental development. Most studies on uterine EVs and miRNAs in
834 bovine have addressed the peri-implantation period (D16-22, [38,87–90]) and have
835 shown the importance of embryo-maternal communication through EVs during this
836 period [91]. However, the cross-talk between the embryo and uterine epithelium is quite
837 intense also at early stages of development (D5-8 as in our model; [92]), and EVs and
838 miRNAs may probably participate in this communication. Interestingly, one of the
839 upregulated miRNAs in UF-EVs was bta-miR-155, which was shown to be more
840 expressed in in vivo produced embryos between D7-9 of development compared to
841 those in vitro [93]. This miRNA could be indicative of better quality of embryos and
842 maybe transferred or induced by uterine EVs.

843 As there are no studies comparing EVs from OF and UF and especially regarding the
844 miRNAs in EVs from bovine UF, the roles of these miRNAs for early embryo
845 development remain to be investigated. Even though miRNAs in EVs can regulate
846 functions in target-cell [94], it is also possible, that at least part of effects reported in
847 this study, could be due to the other constituents of their cargo. Bauersachs et al. [72]
848 reported that both mRNA and miRNA in OF EVs regulate the transcriptome of embryos
849 cultured with them, and by varied mechanisms. Lipidome in EVs is less studied [95],
850 but recently Banliat et al. [63] compared the lipidome in OF EVs and of embryos
851 exposed to these EVs and observed changes in embryonic lipids profile. Therefore, it

must be considered that other cargoes (mRNA, proteins, bioactive lipids, among others), may also be involved in effects caused by EVs during IVC.

Conclusions

Although EVs do not affect embryo yield, they improve quality parameters including cell number, and when in medium with dFCS, they reduced lipid contents and increased mitochondrial activity and post-vitrification survival; such observations are possibly given, at least in part, by effects on expression of lipid metabolism related genes, modulating their expression by mRNAs, miRNAs and/or proteins contained in these EVs, besides an increase in the active form of HSL, suggesting activated lipolysis. Further studies are needed to understand which cargo may be affecting which cellular functions and how they may be leading to the observed results, something quite challenging as it is still difficult to isolate different types of EVs, which carry different components within.

Abbreviations

IVC: In vitro culture, IVP: In vitro production, EVs: extracellular vesicles, OF: Oviductal fluid, UF: uterine fluid, dFCS: EVs-depleted fetal calf serum, FCS: Fetal calf serum, BSA: bovine serum albumin, miRNAs: microRNAs, NTA: nanoparticle tracking analysis, TEM: transmission electron microscopy, *LDLR*: low density lipoprotein receptor, *ACACA:* acetyl-CoA carboxylase alpha, previous name ACC, *FASN:* fatty acid synthase, alias FAS, *PLIN2:* perilipin 2, *PNPLA2:* patatin like phospholipase domain containing 2, alias ATGL adipose triglyceride lipase, *PPARGC1B*: PPARG coactivator 1 beta, *FAPBP3*: fatty acid binding protein 3, LIPE: lipase E, hormone sensitive type, alias HSL hormone sensitive lipase.

Supplementary Information

Additional file 1: Table S1. Details of primers used for reverse transcription–quantitative polymerase chain reaction.

Additional file 2: Table S2. Top 20 miRNA in EVs from OF and UF

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation, to any qualified researcher. In addition, all generated or analysed data derived from this study are included in this published article and its Supplementary data files

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

This work was supported by the Spanish Ministry of Science and Innovation (PID2019-111641RB-I00 to DR, and RTI2018-093548-B-I00 to AGA); São Paulo Research Foundation, Brazil (FAPESP; #2017/20339-3 and CNPq-Brazil 304276/2018-9 to CLVL; #2019/04981-2 to RM; 2014/22887-0 and 2015/21829-9 to JCDS); Minciencias-Colombia Postdoctoral Fellowship (848-2019) to KC-B; Secretaria de

898 Educación Superior, Ciencia y Tecnología e Innovación (SENESCYT-Ecuador) to
899 YNC.

900 **Authors' contributions**

901 CLVL and DR supervised the project and designed the experiments. CLVL, KC-B,
902 YNC, MH, AY, MGMB, PBB, EG, RM and JCS performed the experiments. CLVL,
903 KC-B, YNC, MH, EG, RM, JCS, AGA and DR analyzed the experiments and provided
904 technical support and discussions. CLVL, KC-B, YNC, MH and DR wrote the
905 manuscript. All the authors reviewed the manuscript.

906 **Acknowledgments**

907 Special thanks are extended to the: slaughterhouses (Transformación Ganadera de
908 Leganés SA; Matadero de Madrid Norte, San Agustín de Guadalix; and Carnica
909 Colmenar SC, in Madrid, Spain) for providing access to the biological material
910 (ovaries) used in the present study and to the Spanish Association of Breeders of
911 Selected Cattle of the Asturian Valley Breed (ASEAVA) for providing the semen
912 used in the present study. We thank from the Department of Animal Biotechnology,
913 CSIC-INIA, Dr. Covadonga Alonso for the use the confocal microscope and Dr. Rafael
914 Blasco Lozano and Dr. María del Mar Lorenzo Gilsanz for the use of the
915 ultracentrifuge; Dr. Maria Jesus Cocero and Mrs. Pilar Marigorta from the Department
916 of Animal Reproduction, CSIC-INIA for the use of the epifluorescence microscope.

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Figure Legends

Figure 1. Experimental design.

A EVs isolation and characterization. Oviducts (n=5) and uterine horns (n=5), ipsilateral to a Stage 1 (oviduct) or Stage 2 (uterus) corpus luteum, were flushed with PBS (1 and 2 mL, respectively), and after centrifugation to remove cells and cellular debris, samples were filtered (0.22 µm) and EVs isolated by Size Exclusion Chromatography. EVs samples were concentrated by ultracentrifugation and pellets resuspended in 100 µL PBS. Thirty microliters from each of the five EVs suspensions from oviduct (150 µL) and uterus (150 µL) were pooled and from this, 5 µL were used for EVs characterization by nanoparticle tracking analysis (NTA) and 5 µL for transmission electron microscopy (TEM), and the rest was frozen at -20 ºC and used in IVC. The remaining volume of the original five EVs samples (70 µL) was used for miRNA analysis. To have enough protein amount (35 µg) for EV biomarkers to be detectable by

1253 western blot, a pool of 10 oviducts (Stage 1) and uterine horns (Stage 2) flushings was
1254 obtained and prepared the same way. **B** Embryo culture in vitro. At approximately 20 h
1255 after insemination, presumptive zygotes were cultured in 4 groups: BSA: SOF with
1256 0.3% BSA (3 mg/mL w/v); dFCS: SOF with 5% dFCS; BSA-EV: SOF with 0.3% BSA
1257 supplemented with 3×10^5 EVs/mL from OF (D1 to D4) and 3×10^5 EVs/mL from UF
1258 (D4 to D9); and dFCS-EV: SOF with 5 % dFCS supplemented with 3×10^5 EVs/mL
1259 from OF (D1 to D4) and 3×10^5 EVs/mL from UF (D4 to D9). BSA and dFCS groups
1260 underwent media renewal at 96 hpi (Day 4). Blastocyst development was assessed on
1261 Days 7, 8 and 9. A representative number of Days 7–8 blastocysts from each group was
1262 assessed for quality either by vitrification/warming (survival rate at 24, 48 and 72
1263 hours) or fixed and stained for total cell number, mitochondrial activity and lipid
1264 content and analysis. In addition, Day 7–8 blastocysts were frozen in liquid nitrogen in
1265 groups of 10 and stored at -80 ^{o}C for gene expression or western blot for protein
1266 analyses. **C** miRNA contents in EVs. Total RNA in oviducts and uteri EVs samples
1267 (n=3) was extracted with a miRNeasy mini kit, reversed transcribed with the miScript
1268 PCR System and relative miRNA levels determined by qRT-PCR.
1269 **Figure 2.** OF and UF-EVs characterization.
1270 **A** Averaged finite track length adjustment Concentration/Size graphs for NTA of
1271 particles in fluid isolated from oviducts (left) and uterine horns (right). **B** Representative
1272 images from electron microscopy analyses of OF (left) and UF (right) samples. Some
1273 EVs and their sizes are indicated in the images. **C** Representative images of membranes
1274 treated for detection of EVs marker-proteins (CD9, HSP70 and ALIX) and EVs negative
1275 protein (CANX) in samples isolated from oviduct (OF-EVs) and uterus (UF-EVs). PC =
1276 positive controls (CD9 = blood, HSP70 = pancreas, ALIX = lung, CANX = urethra). MW

= molecular weight marker. Numbers indicate the MW. Expected MW: CD9 = 22, 24 and 35 kDa, HSP70 = ~70 kDa, ALIX = 90 kDa and CANX = 90 kDa.

Figure 3. Representative image of embryo on Day 3 of IVC with EVs from OF and UF. **A** Representative image of embryo on Day 3 of culture in vitro with EVs from OF and **C** on Day 8 with EVs from UF. Nuclei in blue indicate cells (blastomeres) and EVs labelled with PHK67 (green). **B** Negative control for OF in D3 embryo and **D** for UF in D8 embryo. 63 X.

Figure 4. Total cell number in blastocysts IVC.

Total cell number in blastocysts cultured in vitro with BSA or dFCS supplemented or not with OF-EVs (D1-4) and UF-EVs (D5-8) (BSA=57, BSA-EV=55, dFCS=58, dFCS-EV=56). Data are the means±s.e.m. Different letters differ show statistically significant differences between groups ($P<0.05$).

Figure 5. Representative fluorescence images of mitochondrial activity in bovine blastocysts.

A Representative fluorescence images of mitochondrial activity in bovine blastocysts cultured in vitro in medium with BSA, BSA-EV, dFCS and dFCS-EV . Images captured on 63X objective. **B** Quantification of mitochondria fluorescence intensity in arbitrary unites (au) in blastocysts cultured in vitro with BSA or dFCS supplemented or not with OF-EVs (D1-4) and UF-EVs (D5-8). (BSA=29, BSA-EV =29, dFCS=33, dFCS-EV =27). Data are the means±s.e.m. * Indicates the group differs significantly from the others ($P<0.05$).

Figure 6. Representative fluorescence images of lipid droplets in bovine blastocysts.

A Representative fluorescence images of lipid droplets in bovine blastocysts cultured in vitro in medium with BSA, BSA-EV, dFCS and dFCS-EV. Images captured on 63X objective. **B** Quantification of total area of lipids in D7-8 blastocysts cultured in vitro with

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1305 **Figure 7.** Relative mRNA abundance of lipid metabolism-related genes in blastocysts.
1306 Relative mRNA abundance of lipid metabolism-related genes in blastocysts cultured in
1307 vitro with BSA or dFCS only or supplemented with OF-s (D1-4) and UF-EVs (D5-8)
1308 (BSA, BSA-EV, dFCS and dFCS-EV). Bars represent the relative abundance of the
1309 transcripts analysed and normalized to *H2AFZ* and *ACTB* as housekeeping genes. The
1310 experimental groups are represented by columns. Data are the means±s.e.m. Different
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1312 **Figure 8.** pHSL phosphorylation levels in bovine blastocysts.
1313 Effect of in vitro culture medium with BSA or dFCS supplemented or not with OF-EVs
1314 (D1-4) followed by UF-EVs (D5-8) on pHSL phosphorylation levels in bovine
1315 blastocysts. **A** pHSL phosphorylation level. Expression levels were normalized to the
1316 abundance of endogenous control actin. Phosphorylation level was expressed as
1317 pHSL/HSL. Data are expressed as means±s.e.m. *Indicates the group differs significantly
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1319 lipolysis protein, total HSL and phosphorylated p-HSL, and ACTB.
1320 **Figure 9**. Relative abundance of miRNAs in EVs from OF and UF in bovine embryos.
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Figures

Figure 1

Experimental design. A EVs isolation and characterization. Oviducts (n=5) and uterine horns (n=5), ipsilateral to a Stage 1 (oviduct) or Stage 2 (uterus) corpus luteum, were flushed with PBS (1 and 2 mL, respectively), and after centrifugation to remove cells and cellular debris, samples were filtered (0.22 µm) and EVs isolated by Size Exclusion Chromatography. EVs samples were concentrated by ultracentrifugation and pellets resuspended in 100 µL PBS. Thirty microliters from each of the five EVs suspensions from oviduct (150 µL) and uterus (150 µL) were pooled and from this, 5 µL were used for EVs characterization by nanoparticle tracking analysis (NTA) and 5 µL for transmission electron microscopy (TEM), and the rest was frozen at -20 oC and used in IVC. The remaining volume of the original five EVs samples (70 µL) was used for miRNA analysis. To have enough protein amount (35 µg) for EV biomarkers to be detectable by western blot, a pool of 10 oviducts (Stage 1) and uterine horns (Stage 2) flushings was obtained and prepared the same way. B Embryo culture in vitro. At approximately 20 h after insemination, presumptive zygotes were cultured in 4 groups: BSA: SOF with 0.3% BSA (3 mg/mL w/v); dFCS: SOF with 5% dFCS; BSA-EV: SOF with 0.3% BSA supplemented with 3×105 EVs/mL from OF (D1 to D4) and 3×105 EVs/mL from UF (D4 to D9); and dFCS-EV: SOF with 5 % dFCS supplemented with 3×105 EVs/mL from OF (D1 to D4) and 3×105 EVs/mL from UF (D4 to D9). BSA and dFCS groups underwent media renewal at 96 hpi (Day 4). Blastocyst development was assessed on Days 7, 8 and 9. A representative number of Days 7–8 blastocysts from each group was assessed for quality either by vitrification/warming (survival rate at 24, 48 and 72 hours) or fixed and stained for total cell number, mitochondrial activity and lipid content and analysis. In addition, Day 7–8 blastocysts were frozen in liquid nitrogen in groups of 10 and stored at -80 oC for gene expression or western blot for protein analyses. C miRNA contents in EVs. Total RNA in oviducts and uteri EVs samples (n=3) was extracted with a miRNeasy mini kit, reversed transcribed with the miScript PCR System and relative miRNA levels determined by qRT-PCR.

Figure 2

OF and UF-EVs characterization.

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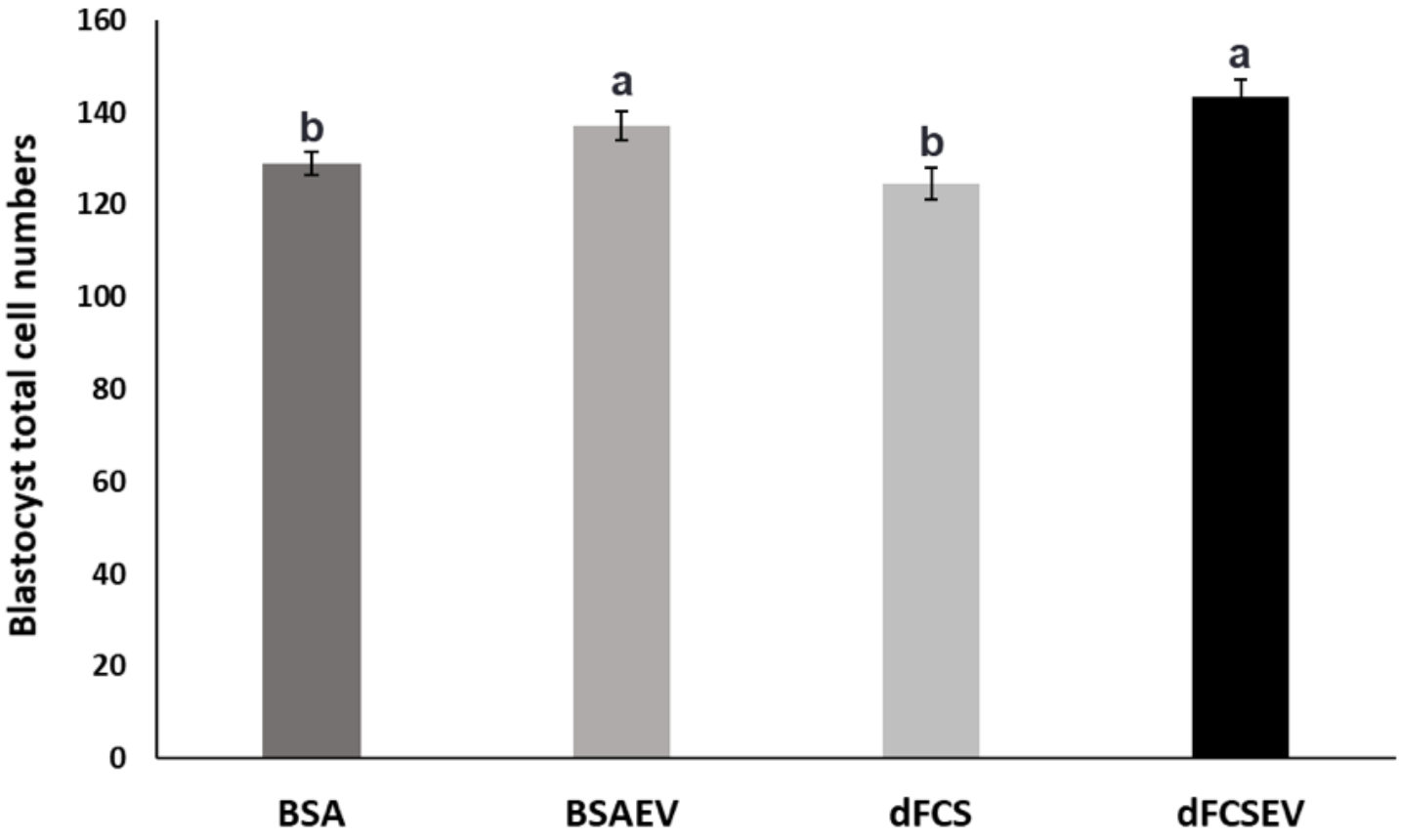

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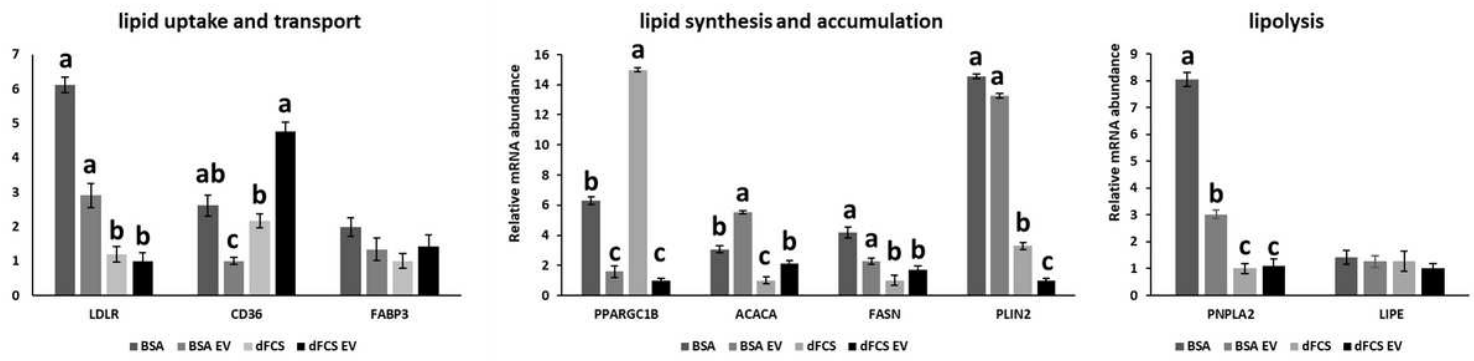

Figure 7

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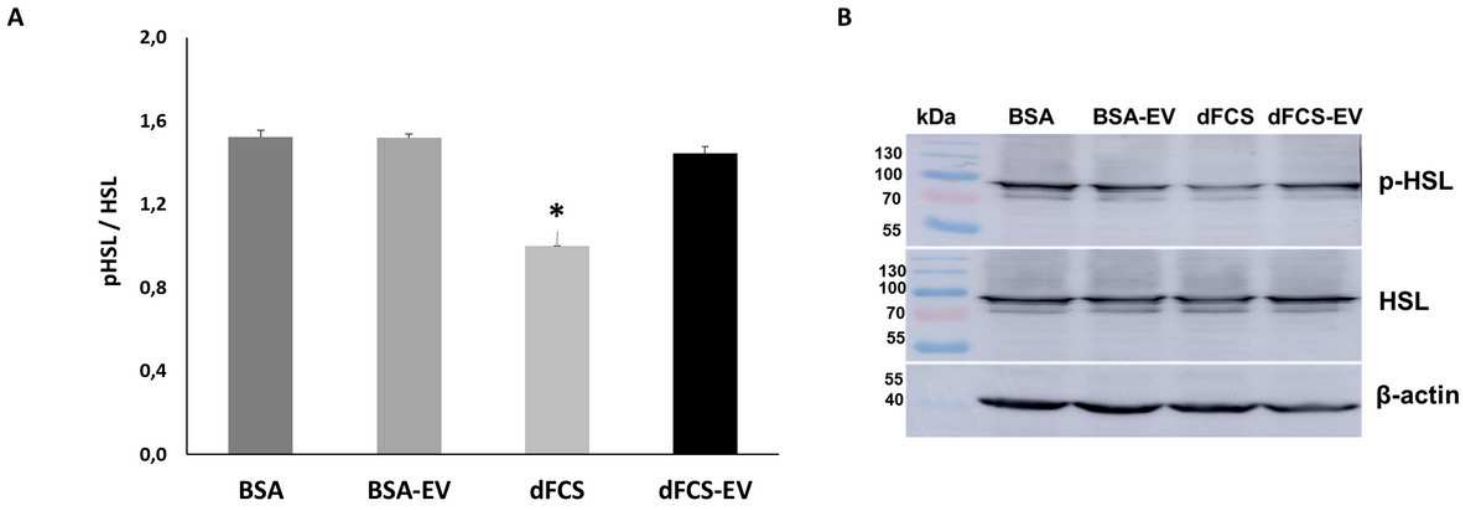

Figure 8

pHSL phosphorylation levels in bovine blastocysts.

Effect of in vitro culture medium with BSA or dFCS supplemented or not with OF-EVs (D1-4) followed by UF-EVs (D5-8) on pHSL phosphorylation levels in bovine blastocysts. A pHSL phosphorylation level. Expression levels were normalized to the abundance of endogenous control actin. Phosphorylation level was expressed as pHSL/HSL. Data are expressed as means±s.e.m. *Indicates the group differs significantly from the others (P<0.05). B Representative images of membranes treated for detection of lipolysis protein, total HSL and phosphorylated p-HSL, and ACTB.

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Relative abundance of miRNAs in EVs from OF and UF in bovine embryos.

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Supplementary Files

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