Effect of bovine oviductal extracellular vesicles on embryo development and quality *in vitro*

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Abstract

The aim of this study was to evaluate the effect of extracellular vesicles (EV) from oviductal fluid (OF), either from the ampulla or isthmus, on the development and quality of *in vitro*-cultured bovine embryos. Zygotes were cultured in synthetic oviduct fluid (SOF + 3 mg/mL BSA) without calf serum (C– group), in the presence of 3×10^5 EV/mL from ampullary or isthmic OF at either 1×10^4 g (10K) or 1×10^5 g (100K), and compared with SOF + 5% FCS (C+ group). OF-EV size and concentration were assessed by electron microscopy and nanotracking analysis system. Embryo development was recorded on Days 7–9, and blastocyst quality was assessed through cryotolerance and gene expression analysis. Lower blastocyst yield was observed on Day 7 in the C– and OF-EV groups (12.0–14.3%) compared with C+ (20.6%); however, these differences were compensated at Days 8 and 9 (Day 9: 28.5–30.8%). Importantly, the survival rate of blastocysts produced with isthmic 100K OF-EV was higher than that of C+ and C– group at 72 h after vitrification and warming (80.1 vs 34.5 and 50.5% respectively, $P < 0.05$). In terms of gene expression, blastocysts produced in the presence of 100K isthmic OF-EV upregulated the water channel *AQP3* and *DNMT3A* and *SNRPN* transcripts compared with the C+, with the expression in C– being intermediate. The lipid receptor *LDLR* was downregulated in C+ compared with all other groups. In conclusion, the addition of oviductal fluid extracellular vesicles from isthmus, to *in vitro* culture of bovine embryos in the absence of serum improves the development and quality of the embryos produced.

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Introduction

Despite considerable improvements in assisted reproductive technologies in the last several decades, conditions of *in vitro* embryo production are far from the physiological (Lonergan & Fair 2008). The *in vitro* deficiencies are reflected in lower embryo developmental rates and quality of the blastocysts produced when compared to their *in vivo* counterparts (Lonergan 2007), evidenced by lower cryotolerance (Rizos *et al.* 2008), altered inner cell mass/trophectoderm cell ratio (Plourde *et al.* 2012), altered gene expression patterns (Niemann & Wrenzycki 2000) and lower pregnancy rates of transferred embryos (Pontes *et al.* 2009). Biological complements added to culture media, such as serum and serum albumin, improve embryo developmental rates. However, it has been shown that serum exhibits a biphasic effect, increasing the number of transferable embryos but reducing their quality reflected in short- (Rizos *et al.* 2003) and/or long-term effects such as large offspring syndrome (Lazzari *et al.* 2002).

The oviduct is a dynamic organ where fertilization and early embryo development takes place. Regulatory mechanisms modulate the fluidic milieu composition to allow sperm capacitation, transport and fertilization of the mature oocyte in the ampulla and early embryonic development in the isthmus (Leese *et al.* 2007, Rodriguez-Martinez 2007). It has been shown that the oviductal environment can support embryonic growth up to the blastocyst stage and produce better-quality embryos across a wide range of species after trans-species transfer (Rizos *et al.* 2010). Moreover, the use *in vitro* of oviductal epithelial cells (OEC) allows the study of mechanisms involved in sperm storage in the oviduct (Abe & Hoshi 1997) and the embryotrophic effects on early embryo development and quality (Cordova *et al.* 2014). Thus, the use of *in vitro* models can provide evidence on physiological embryo–maternal interactions, mechanisms that are difficult to study *in vivo*.

The OF is generated by transudation from plasma into the oviductal lumen supplemented with secreted substances from OEC (Menezo & Guerin 1997).

Secretions present in the OF affect oocyte and sperm function (Killian 2011, Mondéjar *et al.* 2013) and comprise proteins, such as glycoproteins and lactoferrin, involved in gamete interaction (Ghersevich *et al.* 2015), oviductin, osteopontins and the complement protein C3 important for early embryo development (Tse *et al.* 2008), and oviductal glycoprotein 1 (OVGP1) important for sperm–zona pellucida binding (Coy *et al.* 2012). The use of pure bovine OF during *in vitro* maturation of porcine oocytes prior to fertilization improves embryo development and quality, protecting them against adverse impacts of mtDNA transcription/replication and apoptosis induced by the culture environment (Lloyd *et al.* 2009). In contrast in cattle, OF treatment of matured oocytes had no effect on fertilization parameters, cleavage rate, blastocyst yield or morphology. However, several gene transcripts related to embryo quality were upregulated in blastocysts (Cebrian-Serrano *et al.* 2013). When bovine zygotes were cultured *in vitro* with low concentrations of OF (1.25% and 0.625%), it was demonstrated that OF had a positive effect on the development and quality of the produced blastocysts (Lopera-Vasquez *et al.* 2015).

Extracellular vesicles (EVs) play an important role in intercellular communication and the regulation of physiological and pathological processes (for review, see Yanez-Mo *et al.* 2015). They contain and may transfer different bioactive molecules (proteins, mRNAs, miRNAs (Simons & Raposo 2009, Silveira *et al.* 2012) and lipids (Raposo & Stoorvogel 2013)). EV nomenclature commonly relates to EV size and origin, and includes exosomes (30–200 nm) of endosomal origin and microvesicles (MV) (100–1000 nm), those EV that bud from the plasma membrane. These major vesicle subtypes are commonly enriched by differential centrifugation (Théry *et al.* 2006). Smaller vesicles are typically isolated by sedimentation at 100,000–200,000 *g* (100–200 K) (Théry *et al.* 2009), whereas MVs are typically reported to be enriched at 10,000–20,000 *g* (10–20 K) (Witwer *et al.* 2013).

EVs have been identified *in vivo* in all body fluids studied including amniotic fluid, urine and blood (Simpson *et al.* 2008). Regarding reproduction, EVs have been reported in follicular fluid (Silveira *et al.* 2012), uterine fluid (Burns *et al.* 2014) endometrial environment (Ng *et al.* 2013) and seminal plasma (Piehl *et al.* 2013). Saadeldin and coworkers (Saadeldin *et al.* 2014) showed that the addition of exosomes isolated from the conditioned medium of parthenogenetic embryos increased the developmental competence of cloned embryos. Recently, we provided evidence that EV derived from media conditioned by bovine oviduct epithelial cells (BOEC) improves blastocyst quality and induces cryoprotection in *in vitro* culture to the same extent as classical co-culture with fresh BOEC monolayers (Lopera-Vásquez *et al.* 2016).

Thus, the aim of the present study was to isolate EV from bovine OF and evaluate their effect on early embryonic development and quality of the blastocysts *in vitro*. This may contribute to the improvement of assisted reproduction technologies in mammals and humans as well as providing new insights on early embryo–maternal communication.

Materials and methods

Unless otherwise stated, all chemicals were purchased from Sigma Aldrich Química S.A.

Extracellular vesicle isolation

Ipsilateral oviducts from slaughtered heifers at the early or mid-luteal phase of the estrous cycle (based on corpus luteum morphology) were transported to the laboratory on ice. All oviducts were washed twice in PBS, and trimmed free of tissue on a cooled surface. The ampulla and isthmus were separated through the ampullary–isthmic junction, identified where the oviduct diameter first exhibited a marked reduction in size. Each oviductal region (ampulla and isthmus) was washed with 5 mL of PBS (4°C). After a first centrifugation for BOEC elimination (400 *g*), EV were pelleted under two sequential centrifugal (*g*) forces: (i) 10,000 *g* (10 K) and (ii) 100,000 *g* (100 K) following the procedures of Théry and coworkers (Théry *et al.* 2006) with minor modifications. Briefly, after BOEC removal, the OF was centrifuged at 10 K for 60 min at 4°C. Then, the supernatant was removed and centrifuged again at 100 K for 60 min at 4°C and the resultant pellet was recovered (Avanti J30i, Beckman Coulter). The EV recovered at 10 K and 100 K *g*-forces were washed in PBS and pelleted again under the previous conditions.

EV characterization

Nanoparticle tracking analysis (NTA)

A representative part (100 µL) of the resultant pellet (~400 µL obtained vesicles) was used to determine the size and number of EV by NTA with Nanosight LM10 and NTA 2.3 Software (Nanosight, Wiltshire, UK). EV concentration was standardized, and samples were frozen for embryo culture.

Transmission electron microscopy (EM)

For negative staining of exosomes, ionized carbon and collodion-coated copper EM grids were floated on a sample drop, washed and stained with 2% uranyl acetate (in double-distilled water) for 1 min and visualized in a JEM-1010 (JEOL, Tokyo, Japan) transmission EM.

Western blot

EV preparations were lysed in non-reducing Laemmli loading buffer and resolved in a 4–25% gradient SDS-PAGE gel (Biorad). Proteins were transferred to a PVDF membrane (Biorad), blocked with 10% skimmed milk and incubated

with the following primary antibodies: anti-CD 9mAb VJ1/20, anti-ERM 90:3pAb and anti-TSG101 mab (Abcam) (Lopera-Vásquez *et al.* 2016) followed by peroxidase-coupled secondary antibodies and revealed detected by chemiluminescence with an ImageQuant LAS4000 biomolecular imager (GE LifeSciences).

In vitro embryo production

Oocyte collection and in vitro maturation

Immature cumulus oocyte complexes (COCs) were obtained by aspirating follicles (2–8 mm) from the ovaries of postpubertal heifers and cows collected at slaughter. Class 1 and 2 COCs were matured for 24 h in 500 µL of maturation media (TCM 199 supplemented with 10% (v/v) fetal calf serum (FCS) and 10 ng/mL epidermal growth factor) in a four-well dish, in groups of 50 COCs per well at 38.5°C under an atmosphere of 5% CO₂ in air, with maximum humidity.

Sperm preparation and in vitro fertilization

Frozen semen from a previously tested Asturian Valley bull (ASEAVA, Asturias, Spain) was thawed at 37°C in a water bath for 1 min and centrifuged for 10 min at 280g through a gradient of 1 mL of 40% and 1 mL of 80% Bovipure according to the manufacturer's specification (Nidacon Laboratories AB, Gothenborg, Sweden). The sperm pellet was isolated and washed in 3 mL of Boviwash (Nidacon) by centrifugation at 280g for 5 min. The pellet was re-suspended in the remaining 300 µL of Boviwash. Sperm concentration was determined and adjusted to a final concentration of 1 × 10⁶ sperm/mL for IVF. Gametes were co-incubated for 18–22 h in 500 µL of fertilization media (Tyrode's medium with 25 mM bicarbonate, 22 mM Na lactate, 1 mM Na-pyruvate, and 6 mg/mL fatty acid-free BSA supplemented with 10 mg/mL heparin sodium salt, Calbiochem) in a four-well dish, in groups of 50 COCs per

well under an atmosphere of 5% CO₂ in air, with maximum humidity at 38.5°C.

In vitro culture of presumptive zygotes

At approximately 20 h after insemination (p.i.), presumptive zygotes were denuded of cumulus cells by vortex and cultured in 500 µL of Synthetic Oviduct Fluid, (SOF) (Holm *et al.* 1999) – (with 4.2 mM sodium lactate, 0.73 mM sodium pyruvate, 30 µL/mL BME amino acids, 10 µL/mL MEM amino acids, 1 µg/mL phenol-red) with or without OF-EV (see 'Experimental design' section for clarification) in a four-well dish, covered with mineral oil, in groups of 50 per well, at 38.5°C under an atmosphere of 5% CO₂, 5% O₂ and 90% N₂. When stated, SOF was supplemented with 3 mg/mL BSA (A9647 – Sigma) or 5% FCS (F2442 – Sigma).

Assessment of embryo development and quality

Embryo development

Cleavage rate was recorded at Day 2 (48 h p.i.), and cumulative blastocyst yield was recorded at Days 7, 8 and 9 p.i. under a stereomicroscope.

Embryo quality

Blastocyst vitrification

The ability of the blastocyst to withstand cryopreservation was used as a quality indicator. Days 7 and 8 blastocysts were vitrified in holding medium (HM) (TCM199 supplemented with 20% (v/v) FCS) and cryoprotectants, following the procedures of Rizos and coworkers (Rizos *et al.* 2002), in a two-step protocol using the Cryloop device (Hampton Research, Aliso Viejo, CA, USA). First step: HM with 7.5% ethylene glycol, 7.5% dimethyl sulfoxide and second-step final solution: HM with 16.5% ethylene glycol, 16.5% dimethyl sulfoxide and

Table 1 Details of primers used for qRT-PCR.

Gene		Primer sequence (5'–3')	Fragment size (bp)	Gene bank accession no.
H2A.Z	Forward	AGGACGACTAGCCATGGACGTGTG	212	NM_016750
	Reverse	CCACCACCAGCAATTGTAGCCTTG		
AQP3	Forward	CGGTGGTTTCTCACCATCA	299	NM_001079794.1
	Reverse	CAGAGGGGTAGGTGGCAAAG		
AQP11	Forward	GCTTCGTATCCACCTGCTGT	102	NM_001110069.1
	Reverse	TGCAGTGAAAGTGCCAAAGT		
ATP1A1	Forward	GCGGACACGACAGAGAATCA	158	NM_001076798.1
	Reverse	GAGGCATCACCCGCTACAG		
LDLR	Forward	CAAAACCCCGATCATCCCCA	194	NM_001166530
	Reverse	TCGACCCTGAAGTGGAAACG		
LDHA	Forward	TTCTTAAGGAAGAATATGTC	310	NM_174099.2
	Reverse	TTCACGTACGCTGGACCAA		
DNMT3A	Forward	CTGGTGCTGAAGGACTTGGGC	318	AY271299
	Reverse	CAGAAGAAGGGGCGGTTCATC		
IGF2R	Forward	GCTGCGGTGTGCCAAGTGAAAAAG	201	NM_174352.2
	Reverse	AGCCCCTCTGCCGTTGTACCT		
GRB10	Forward	GAAGACGGGACAAGCAAAGT	291	XM_010803961.1
	Reverse	CTGGCACCACTGAACCATCTG		
SNRPN	Forward	AACAGCACGTACCAGAGGTG	144	NM_001079797.1
	Reverse	ATGCCTGTCTCAGTTCTGCC		

0.5 M sucrose. The blastocysts were warmed in two steps in HM with 0.25 M and 0.15 M sucrose and then cultured in 25 µL droplets of SOF with 5% FCS. Survival was defined as re-expansion of the blastocoel and its maintenance for 24, 48 and 72 h.

Gene expression analysis

Poly(A) RNA was extracted from five pools of 10 blastocysts from each experimental group using the Dynabeads mRNA Direct Extraction Kit (Dyna Beads, Oslo, Norway) with minor modifications (Bermejo-Álvarez *et al.* 2008). Immediately after extraction, the RT reaction was carried out following the manufacturer's instructions (Bioline, Ecogen, Madrid, Spain) using poly(T) primer, random primers and MMLV reverse transcriptase enzyme. The quantification of all mRNA transcripts was carried out by qPCR with two repetitions for all genes of interest. qPCR was performed by adding a 2 µL aliquot of each cDNA sample to the PCR mix containing the specific primers. All primers were designed using Primer-BLAST software to span exon–exon boundaries when possible (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Primer sequences and the approximate sizes of the amplified fragments of all transcripts are shown in Table 1. For quantification, qPCR was performed as described previously (Bermejo-Alvarez *et al.* 2010); PCR conditions were tested to achieve efficiencies close to 1. The comparative cycle threshold (CT) method was used to quantify expression levels. Values were normalized to the endogenous control, *H2AFZ*. 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 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 *H2AFZ* CT value for each sample from each gene CT value of the 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 $2^{-\Delta\Delta CT}$.

Experimental design

The developmental capacity of bovine zygotes and the quality of the produced blastocysts cultured *in vitro* with EV from

Figure 1 Experimental design.

Figure 2 Electron microscope images and NTA analyses of OF-EV isolated at 10 K (A and C) and 100 K (B and D) g-forces. Western blot analysis of OF-EV with EV markers (E).

OF of the ampulla (A) and the isthmus (I) from early luteal phase obtained at 10,000 g (10 K) and 100,000 g (100 K) was assessed (Fig. 1). At approximately 20 h after insemination, presumptive zygotes were cultured with frozen/thawed

Table 2 Concentration OF-EV isolated from OF at different cycle phases at different g-forces (10–100 K).

Luteal phase	Centrifugal force (g)	n	Concentration*
Early (Day 1–4)	10 K	5	2.8 ± 1.2 ^a
	100 K	5	9.28 ± 4.1 ^b
Medium (Day 4–8)	10 K	5	1.25 ± 0.3 ^a
	100 K	5	3.98 ± 0.5 ^a

n: Number of oviductal samples used in nanoparticle analysis system (Nanosight). K: Represents 1 × 10³. *EV particles⁸/mL ± s.d. ^{a,b,c}Values in the same column with different superscripts differ significantly (P < 0.05).

Table 3 Concentration OF-EV isolated from the ampulla and the isthmus at different g-forces (10–100K).

Centrifugal force (g)	n	Concentration*
Ampulla		
10K	3	9.0 ± 3.4 ^{ab}
100K	3	10.5 ± 3.1 ^b
Isthmus		
10K	3	3.6 ± 2.5 ^{ac}
100K	3	7.5 ± 0.2 ^{ab}

n: Number of oviductal samples used in nanoparticle analysis system (Nanosight). K: Represents 1×10^3 .

*EV Particles⁸/mL ± s.d. ^{a,b,c}Values in the same column with different superscripts differ significantly ($P < 0.05$).

OF-EV from the ampulla and isthmus isolated at 10K and 100K and diluted in SOF supplemented with 3 mg/mL BSA (C–) under adjusted concentration of 3×10^5 EV/mL. A group of SOF supplemented with 5% fetal calf serum was included (C+). Embryo 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 h up to 72 h after warming or frozen in LN₂ in groups of 10 and stored at –80°C for gene expression analysis. A total of 11 replicates were carried out.

Statistical analysis

Results of EV concentration, cleavage rate, blastocyst yield, survival after vitrification/warming and relative mRNA abundance for candidate genes were analyzed using one-way ANOVA ($P < 0.05$). All analyses were performed with the SigmaStat software package (Jandel Scientific, San Rafael, CA, USA).

Results

Oviductal fluid contains extracellular vesicles

Extracellular vesicles were enriched from OF by ultracentrifugation at two different centrifugal forces (10K and 100K). Transmission electron microscopy revealed a larger (with some vesicles larger than 500 nm) and more scarce vesicle population in the 10K pellet, whereas the 100K pellet was mainly composed

of smaller vesicles of less than 200 nm of diameter (Fig. 2A and B respectively). Nanosight analyses confirmed a broader size distribution in the 10K pellet, encompassing vesicles larger than 500 nm, whereas the 100K pellet revealed a more homogeneous and smaller distribution (Fig. 2C and D respectively). These vesicles expressed some of the classical markers described for exosomes (ERM, TSG101 and tetraspanin CD9 proteins) (Lopera-Vásquez *et al.* 2016) (Fig. 2E). Regarding vesicle number, the 100K pellet contained more vesicles than the 10K pellet, this difference being more evident in the early phase of the estrous cycle (Table 2). Because the early embryo is still in the oviduct during the early luteal phase, we thereafter focused on this early phase isolating OF from either the isthmus or the ampulla section of the oviduct for the present experiment. These analyses revealed that in the ampulla, vesicles were more or less equally divided in the 10K and the 100K pellet, whereas the isthmus had a higher concentration of smaller vesicles mainly recovered in the 100K pellet (Table 3).

Extracellular vesicles isolated from isthmus OF have a positive effect on the quality of in vitro-produced bovine embryos

Addition of OF-derived EV to *in vitro* embryo culture resulted in no differences in terms of cleavage rates (range: 88.0–89.6%) and blastocysts yield at Day 8 (range: 24.6–26.9%) and Day 9 (range: 28.5–30.8%), as shown in Table 4. As previously reported, the blastocyst yield at Day 7 in the C+ group was significantly higher (20.6%) compared with the rest of the groups (range: 12.0–14.3%) ($P < 0.05$).

Interestingly, the survival rate at 24 h after vitrification/warming of blastocysts produced with 100K OF-EV from isthmus was significantly higher than C+, C– and ampulla-10K groups (91.3% vs 48.3%; 71.0%; 62.2% respectively, $P < 0.05$). At 48 and 72 h, these differences were increased with a survival rate of isthmus-100K group of 80–89%. The survival rate of the blastocysts from isthmus-10K group was also significantly higher than that in C+ at all time points (Table 5).

Table 4 Effect of *in vitro* embryo culture with OF-EV (ampulla–isthmus) isolated at different g-forces (10–100K × g) on development *in vitro*.

Centrifugal force (g)	n	Cleavage*	Blastocyst yield*		
			Day 7	Day 8	Day 9
Control (C+)	490	432 (88.5 ± 2.2)	99 (20.6 ± 2.1) ^a	129 (26.9 ± 1.9)	143 (29.6 ± 2.3)
Control (C–)	566	498 (88.1 ± 1.3)	75 (13.4 ± 1.8) ^b	139 (24.6 ± 1.7)	168 (29.7 ± 2.5)
Ampulla					
10K	514	460 (89.6 ± 1.3)	62 (12.0 ± 1.6) ^b	133 (26.0 ± 2.1)	152 (29.7 ± 2.1)
100K	462	406 (88.0 ± 1.1)	63 (13.8 ± 1.9) ^b	116 (25.3 ± 2.4)	131 (28.5 ± 2.2)
Isthmus					
10K	557	494 (88.6 ± 1.5)	75 (13.7 ± 1.1) ^b	135 (24.6 ± 2.2)	164 (29.6 ± 1.9)
100K	549	488 (89.1 ± 1.2)	68 (14.3 ± 3.0) ^b	126 (26.1 ± 4.1)	151 (30.8 ± 4.5)

K: Represents 1×10^3 .

*Results presented as n (% ± s.e.) where n represents total number of presumptive zygotes placed in culture. ^{a,b}Values in the same column with different superscripts differ significantly ($P < 0.05$).

Table 5 Survival rate after vitrification and warming of D7–8 blastocyst cultured with OF-EV (ampulla–isthmus) isolated at different *g*-forces (10–100K×*g*).

Centrifugal force (<i>g</i>)		<i>n</i>	Blastocyst survival after vitrification/warming*			
			4 h	24 h	48 h	72 h
Control (C+)		80	65 (80.4±5.2)	43 (48.3±9.1) ^a	37 (41.3±7.0) ^a	31 (34.5±8.0) ^a
Control (C–)		105	92 (87.6±4.5)	77 (71.0±6.6) ^{ab}	64 (58.0±7.7) ^{ab}	56 (50.5±8.1) ^{ab}
Ampulla	10K	88	78 (85.9±4.6)	61 (62.2±10.4) ^{ab}	52 (50.8±11.5) ^{ab}	48 (46.3±12.2) ^{ab}
	100K	64	59 (89.9±3.6)	50 (73.5±7.4) ^{abc}	49 (71.9±7.7) ^{bc}	45 (66.8±7.6) ^{bc}
Isthmus	10K	119	114 (96.6±1.8)	100 (83.6±4.7) ^{bc}	93 (78.6±3.3) ^{bc}	86 (70.5±4.4) ^{bc}
	100K	97	93 (96.7±1.6)	87 (91.3±3.8) ^c	84 (89.3±4.3) ^c	79 (80.1±3.9) ^c

n: Total number of presumptive zygotes placed in culture. K: Represents 1×10³.

*Data are presented as *n* (%±s.e.). ^{a,b,c}Values in the same column with different superscripts differ significantly (*P*<0.05).

The expression levels of three genes related with membrane trafficking (*AQP3*, *AQP11* and *ATP1A1*), two genes related to metabolism (*LDLR* and *LDHA*) and four genes involved in epigenetics (*DNMT3A*, *IGF2R*, *GRB10* and *SNRPN*) were determined in blastocysts cultured in the presence (C+) or absence (C–) of FCS or supplemented with EV from OF from the isthmus enriched either at 10K (Isthmus 10K) or 100K (Isthmus 100K) (Figs 3 and 4). The addition of 100K isthmus EV induced a significant upregulation of the water channel *AQP3*, *DNMT3A* and *SNRPN* genes compared with the group supplemented with serum (C+), whereas blastocysts cultured with BSA (C–) showed intermediate but not significantly different levels. The lipid receptor *LDLR* was significantly downregulated in C+ compared with the rest of the groups. The remaining genes analyzed displayed no differences among groups.

Discussion

In this study, we report that the *in vitro* culture of bovine embryos with OF-EV from the isthmus, in the absence of serum improves the quality of the produced embryos. We found that embryo development was not affected by

the presence of OF-EV, but the quality of the produced embryos in terms of cryotolerance and the expression of genes related to metabolism and epigenetics were improved. To our knowledge, this is the first attempt at isolating and characterizing EV from the ampullary and isthmus OF and evaluating their effect during *in vitro* culture.

The first stages of early bovine embryo development occur in the oviduct, where the embryo spends around 4 days (Hackett *et al.* 1993). The OF comprises the secretions of BOEC and the transudation into the oviduct lumen of blood plasma (Ellington 1991). To date, the effect of bovine OF *in vitro* has been evidenced in porcine- and bovine-matured oocytes in terms of ZP modulation and embryo quality (Coy *et al.* 2008, Lloyd *et al.* 2009, Cebrian-Serrano *et al.* 2013); and during *in vitro* culture of bovine zygotes with a positive effect on embryo development and quality (Lopera-Vasquez *et al.* 2015).

The EVs are membrane vesicles secreted by most cell types that are nowadays considered mechanisms of intercellular communication (Yanez-Mo *et al.* 2015). Different EV populations associate different morphological characteristics and RNA profiles (Lötvall *et al.* 2014). The analyses by NTA of the size

Figure 3 Relative mRNA transcription in bovine *in vitro* blastocysts (D7–8 p.i.) cultured with OF-EV (isthmus) isolated at different *g*-forces (10–100K) of genes related with membrane trafficking (*AQP3*, *AQP11*, *ATP1A1*) and metabolism (*LDLR* and *LDHA*). Data are relative to mean of internal gene H2AFZ. Data presented are mean±s.e. ^{a,b}Different superscripts indicate significant differences for each gene (*P*<0.05).

Figure 4 Relative mRNA transcription in bovine *in vitro* blastocysts (D7–8 p.i.) cultured with OF-EV (isthmus) isolated at different *g*-forces (10–100K) of genes related with epigenetics (*DNMT3A*, *IGF2R*, *GRB10* and *SNRPN*). Data are relative to mean of internal gene H2AFZ. Data presented are mean±s.e. ^{a,b}Different superscripts indicate significant differences for each gene (*P*<0.05).

and concentration of EV from maternal reproductive tract show that they are heterogeneous. Human follicular fluid EV are polydisperse, including MV and exosome-sized EV, with an average of ≈ 220 nm and a mean concentration of $\approx 2.7 \times 10^6$ EV/mL (Tannetta *et al.* 2014). In ovine uterine fluid, EVs showed a mean size of ≈ 148 nm in a $\approx 200 \times 10^9$ EV/mL (Burns *et al.* 2014). Our samples also presented polydispersed populations. NTA analyses revealed a broadly distributed population in the 10K pellet and a more narrow and smaller-sized distribution in the 100K pellet, even though the mean size of both samples was not significantly different irrespective the *g* force used for isolation. Moreover, the concentration of small vesicles was greatly increased in the early phase of the cycle, and predominant at the isthmus, whereas in the ampulla, vesicles were more or less equally divided in the 10K and the 100K pellet. This may be explained by the fact that the isthmus is the part of the oviduct where the embryo develops *in vivo*, up to ≥ 16 cell stage, before entering the uterus and that the vesicles are responsible for the interaction between the oviduct and the early embryo.

The role of EV present in the reproductive tract is a recent field of research. Silveira and coworkers (Silveira *et al.* 2012) isolated MV and exosomes of equine ovarian follicular fluid and evidenced the presence of proteins and miRNAs that were present in the follicular cells, suggesting EVs play an important role in cell-to-cell communication within the mammalian ovarian follicle (Silveira *et al.* 2012). Soheli and coworkers (Soheli *et al.* 2013) demonstrated a miRNA transport mediated by exosome and non-exosome structures in bovine follicular microenvironment, associating the extracellular miRNA with the growth status of the oocyte (Soheli *et al.* 2013). Using an *in vitro* model, Ng and coworkers (Ng *et al.* 2013) identified and examined the presence and role of EV of the uterine cavity. The miRNA present in the uterine EV allowed a bioinformatic identification of pathways possibly influenced after the EV uptake by trophectoderm or endometrial epithelium at the time of implantation or after sperm transfer during the transit across the uterus (Ng *et al.* 2013). Burns and coworkers (Burns *et al.* 2014) evidenced the presence of EV in the uterine fluid of pregnant and cyclic ewes. The differences identified in the molecular contents determined by the pregnancy status suggested different EV sources (endometrium conceptus), supporting the idea that EV in the uterine fluid plays a relevant role in the establishment and maintenance of pregnancy (Burns *et al.* 2014). The same group clearly demonstrated the function of EVs from conceptus trophectoderm and uterine epithelia in conceptus elongation and conceptus–endometrial interactions during early pregnancy in sheep (Burns *et al.* 2016). Al-Dossary and coworkers (Al-Dossary *et al.* 2013) revealed the expression and secretion via oviductal exosomes of plasma membrane

Ca^{2+} -ATPase 4a (PMCA4a– Ca^{2+} homeostasis) in the female reproductive tissues and luminal fluids during estrous, and their uptake by sperm with a possible role in sperm viability during storage in the oviduct, capacitation and acrosome reaction (Al-Dossary *et al.* 2013).

In spite of the recent efforts made to elucidate the function of EVs during reproductive events, the role of bovine OF-EV in early embryo development is still unknown. Our results show for the first time that EV can be isolated from the isthmus of the bovine oviduct at the early luteal phase and can be used successfully during *in vitro* culture with developmental rates similar to serum-containing media. In a similar *in vitro* system, we recently demonstrated a positive effect of EV secreted by BOEC monolayer on the developmental capacity of bovine zygotes and the quality of the produced embryos *in vitro* (Lopera-Vásquez *et al.* 2016). Data from this study, in which we assessed different EV doses (as low as 7.5×10^4), showed no significant differences, indicating that we may be probably working at saturating conditions in this *in vitro* conditions. In addition, we could also demonstrate that some of the deleterious effects of serum were reduced by the presence of EVs extracted from BOEC cultures *in vitro* (Lopera-Vásquez *et al.* 2016). Here, the quality of the blastocysts cultured with isthmus-100K OF-EV was higher than that of blastocysts produced in semi-defined media with serum or BSA in terms of cryotolerance. This effect may be possibly due to cell–cell communication mechanisms related to the dialog between the oviduct and the early embryo. Thus, it is not surprising that the positive effect is mostly observed with the small vesicle population recovered from the isthmus, the part of the oviduct where *in vivo* the early embryo develop up to ≥ 16 cell stage. Interestingly, the number of small vesicles greatly increases in the early phase of the oestrous cycle, when most likely an oviduct–embryo interaction takes place. Thus, the OF complexity is associated with the dynamic nature of oviductal epithelial cell populations at different stages of estrous cycle (Yániz *et al.* 2000). This is in agreement with a recent study from our group (Maillo *et al.* 2016) that identified 2287 genes differentially expressed between ampulla and isthmus in pregnant animals after characterization of the bovine oviductal transcriptome on Day 3 between cyclic and pregnant heifers.

A plausible molecular explanation for the increased survival rates after vitrification in the isthmic OF-EV is the upregulation of the water channel AQP3. However, the difference with the C– group was not significant, suggesting an additional role of serum in the regulation of this gene. In a previous study, we also detected a significant upregulation of AQP3 in blastocysts cultured in the presence of oviductal fluid, which also exhibited higher cryotolerance than those cultured in the presence of serum (Lopera-Vasquez *et al.* 2015). Aquaporins selectively conduct water molecules allowing the

rapid movement of water through the membrane and thereby facilitating survival after freezing and thawing. In particular, the artificial expression of *AQP3* in mouse oocytes (Edashige *et al.* 2003) improved survival after cryopreservation and *AQP3* has been proposed as a major water and cryoprotectant transporter in bovine morulae (Jin *et al.* 2011). In contrast, two other genes related with membrane trafficking, another aquaporin (*AQP11*) and the Na/K-ATPase α -1-subunit (*ATP1A1*), essential for blastocyst expansion in mice (Barcroft *et al.* 2004), did not differ between groups. We also analyzed the expression of a lipid receptor (*LDLR*) and a glycolytic enzyme (*LDHA*) that has been related with anaerobic glycolysis in bovine cumulus cells (Bermejo-Alvarez *et al.* 2010). *LDHA* did not display significant differences between groups, but *LDLR* was significantly downregulated in the presence of serum compared with the other groups. This result is in agreement with our previous findings in bovine embryos (Lopera-Vasquez *et al.* 2015) and may be the consequence of higher amount of lipids in the serum supplemented media. In this perspective, *LDLR* has been reported to be downregulated in embryos derived from obese mice, and it was proposed as a regulator of lipid uptake in blastocysts (Bermejo-Alvarez *et al.* 2012).

Finally, the *de novo* DNA methyltransferase *DNMT3A* and the imprinted gene *SNRPN* were downregulated in the group supplemented with serum compared with isthmus 100K OF-EV. However, the imprinted gene *IGF2R* and the putatively imprinted gene *GRB10* did not reveal significant differences, although did show a similar trend. Altered expression patterns of imprinted genes may be indicative of imprinting disorders. Imprinting disorders caused by artificial reproductive techniques are known to cause phenotypic alterations in the offspring such as the large offspring syndrome (Young *et al.* 2001). In particular, abnormal *SNRPN* imprinting causes Prader-Willi syndrome in humans (Özcelik *et al.* 1992). Similar to humans, bovine *SNRPN* is a paternally expressed imprinted gene (Lucifero *et al.* 2006), and its expression is positively linked with methylation in bovine. Thus, a reduced expression of *DNMT3A* may have caused a reduction in methylation and expression of *SNRPN*, similar to the one observed in bovine placenta derived from IVF embryos compared with AI (Suzuki *et al.* 2009).

In conclusion, bovine OF-EV can be isolated from the isthmus by ultracentrifugation at 100 K and used in *in vitro* embryo culture with a positive effect on embryo quality, reflected in cryotolerance. Regarding gene expression patterns, significant differences of important genes related with metabolism, epigenetics and water channel traffic were observed compared with the group cultured in the presence of serum. The presence of isthmus-100K OF-EV also slightly increased the abundance of some of these genes compared with the no-serum control. These results provide evidence of the essential association

of the oviductal environment and developing embryo confirming an early embryo–maternal dialog. Further studies of OF-EV characterization will elucidate possible mechanisms of communication between the mother and the embryo mediated by the EV.

Declaration of interest

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

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