Extracellular vesicles secreted during blastulation show viability of bovine embryos

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Abstract

Extracellular vesicles (EVs) secreted by blastocysts may be clinically relevant, as indicator of embryo viability on *in vitro* fertilization. We tested if the characteristics of EVs secreted during blastulation are related to embryo viability. Morulae were individually cultured in SOF media depleted of EVs until day 7.5 post IVF. Viable embryos were determined by a system of extended *in vitro* culture of bovine embryos until day 11 (post-hatching development). Afterward, a retrospective classification of blastocyst and culture media was performed based on blastulation time (early blastulation (EB) or late blastulation (LB)) and post-hatching development at day 11 (viable (V) or non-viable embryo (NV)). A total of 254 blastocysts and their culture media were classified in four groups (V-EB, NV-EB, V-LB, NV-LB). Group V-EB had a larger blastocyst diameter (170.8 μm), higher proportion of good-quality blastocysts (77%) and larger mean size of population of EVs (122.9 nm), although the highest concentration of EVs (5.75×10^9 particles/mL) were in group NV-EB. Furthermore, small RNA sequencing detected two biotypes, miRNA (86–91%) and snoRNA (9–14%), with a total of 182 and 32 respectively. In differential expression analysis of miRNAs between V versus NV blastocysts, there were 12 miRNAs upregulated and 15 miRNAs downregulated. Binary logistic regression was used to construct a non-invasive novel model to select viable embryos, based on a combination of variables of blastocyst morphokinetics and EVs characteristics, the ROC-AUC was 0.853. We concluded that characteristics of EVs secreted during blastulation vary depending on embryo quality.

Reproduction (2019) **158** 477–492

Introduction

There is increasing evidence of the important roles of secreted extracellular vesicles (EVs) in cell-cell communication; those vesicles can be identified in various biological fluids, including blood and urine, as well as in media from *in vitro*-cultured cells (van der Pol *et al.* 2012, Tkach and Thery 2016). Among their biological roles, EVs participate in embryo-maternal crosstalk (Saadeldin *et al.* 2014, Lopera-Vasquez *et al.* 2016, 2018, Almiñana *et al.* 2017, Kurian and Modi, 2019). It has been shown that EVs secreted by the oviduct or the endometrium can modulate the developmental potential of *in vitro*-produced embryos (Lopera-Vasquez *et al.* 2017, Lv *et al.* 2018, Wang *et al.* 2019). Other reports have identified EVs secreted by embryos, potentially having a role in paracrine communication between cells during early embryonic development (Saadeldin *et al.* 2014, Mellisho *et al.* 2017, Qu *et al.* 2017). However, in the embryo, presence of the zona pellucida (ZP) may modulate secretion of EVs. However, there is scarce information about characteristics of EVs secreted during early embryonic development and how

their characteristics may be affected by embryo quality (Ferreira *et al.*, 2013) and developmental stage (Mellisho *et al.* 2017, Lopera-Vasquez *et al.* 2018).

Determining biological characteristics of EVs, including variations in their morphology, size, concentration, composition, cellular source and biogenesis, requires use of a combination of technologies (Colombo *et al.* 2014). EVs contain various types of molecules such as proteins, lipids, mRNA and non-coding RNAs (Braicu *et al.* 2015). It was recently reported that miRNAs are selectively secreted by the developing human embryos into the culture medium and may be biomarkers of embryonic development (Kropp *et al.* 2014, Rosenbluth *et al.* 2014). Also, in this year, Battaglia *et al.* (2019) demonstrated the presence of miRNA in exosomes from blastocoel fluid. In addition, Rosenbluth *et al.* (2014) identified miR-191 as highly expressed, in human embryos with chromosomal alterations, with potential as a biomarker for genetic preimplantation diagnosis.

Besides, relationship between embryo competence and developmental kinetics have been reported for early cleavage (Kaith *et al.* 2015, Milazzotto *et al.* 2016), early blastulation (Shapiro *et al.* 2008, Muthukumar *et al.*

2013), and early hatching (Utsunomiya *et al.* 2004). Thus, morpho kinetic parameters may be helpful in improving classical embryo selection (Meseguer *et al.* 2011, Kaith *et al.* 2015, Milewski *et al.* 2016). Early division favors transcript abundance at all stages and increases blastocyst production (Gutiérrez-Adán *et al.* 2004), whereas early blastulation has been related to better synchrony of embryo development (Hoelker *et al.* 2006, Shapiro *et al.* 2008). Blastocyst formation is an essential event in preimplantation embryo development during which many molecular and morphological changes occur (Watson and Barcroft 2001, Madan *et al.* 2005, Rekik *et al.* 2011). Between compact morula and blastocyst stages, the first cell lineage differentiation occurs, forming the inner cell mass (ICM) and trophoctoderm (Rossant and Tam 2009, Maître 2017).

A critical step in early embryonic development occurs while embryos transit through the oviduct and reach the uterus. During this interval, there is bidirectional communication with the maternal environment to promote development and survival (Zumoffen *et al.* 2013, Maillo *et al.* 2016). This communication is mediated by autocrine, paracrine and endocrine signaling (O'Neill 2008). During *in vitro* culture, effects of co-culture systems on embryonic development reflect existence of embryo tropic factors secreted into the culture medium (Watson *et al.* 1999, Killian 2004). Several molecules crucial to cellular processes occurring during embryogenesis and embryo communication with its environment have been identified in embryo culture media, including amino acids, fatty acids, metabolites (Seli *et al.* 2007), proteins (Cortezzi *et al.* 2011) and miRNAs (Cortezzi *et al.* 2011, Kropp *et al.* 2014).

We tested the hypothesis that during blastulation of bovine embryos, morphokinetics and the biological attributes of their secreted EVs (e.g. size, concentration, and small RNA) relating to on embryo viability. Our objectives were to (a) evaluate the differences in blastocyst morphokinetics and the size, concentration and small RNA cargo of EVs secreted during blastulation related to viable and non-viable embryos and (b) establish the potential use of these attributes as a novel predictive model for embryo viability.

Materials and methods

All experiments were approved by the Ethics Committee of the Faculty of Veterinary Sciences, University of Concepcion (permit no. CBE-17-2017).

Experimental design

Embryos were cultured in groups (~25 zygotes per well) until day 5 (D5) of development after *in vitro* fertilization (morulae stage). At D5, morulae were selected and individually cultured (80 µL) in synthetic oviduct fluid media depleted of EVs (SOFdepleted) until D7.5. From D5 to D7.5, embryos were

visually monitored for blastocoel formation as an indicator of embryonic development kinetics. This allowed to classify blastocysts as early (day 6.5; experimental group D6.5) or late (day 7.5; experimental group D7.5) blastulating embryos. At day 7.5, morphological characteristics of the blastocysts were determined; embryo quality, developmental stage and blastocyst diameter, using criteria described in the IETS manual (Stringfellow and Givens 2010). Also, at day 7.5, culture media from individual embryos were collected and identified as matched with the corresponding embryo. D7.5 blastocysts were transferred individually to fresh SOFdepleted (140 µL) until day 11 (D11) to assess their post hatching developmental (viability) according to growth performance and diameter (Fig. 1).

Experimental groups: according to *in vitro* post hatching development

At D11, blastocysts were classified into three categories according to their post hatching: (1) viable (V), size >270 µm, quality 1 and 2; (2) less viable (LV), size >160 µm and <270 µm, quality 3; and (3) non-viable (NV), size <160 µm, quality 4 or degenerated. For further binary logistic regression analysis, we consider only the two outermost groups (V and NV) due to the significant differences existing in their morphological development of the blastocysts at day 11. Embryo culture media from less viable blastocyst were excluded from the analysis to avoid unwanted bias (Fig. 1).

The culture media collected from individual embryos at D7.5 were retroactively assigned to one of the following experimental groups according to the embryo score of D11:

- G1: viable with early blastulation (V-EB); (*n* = 73)
- G2: non-viable with early blastulation (NV-EB); (*n* = 68)
- G3: viable with late blastulation (V-LB); (*n* = 61)
- G4: non-viable with late blastulation (NV-LB); (*n* = 52):

In vitro embryo production

Ovaries from beef cattle were collected at an abattoir house Frigosur Ltda, Chillan, VIII Region – Bio-Bio located 8 km (15-min drive) from the laboratory, following standard procedures described by Rodríguez *et al.* (2008). The cumulus-oocyte complexes (COCs) were *in vitro* matured (IVM) in four-well dishes (25–30 COCs per 500 µL well) in maturation media for 20–22 h as described (Velasquez *et al.* 2016). Oocytes were fertilized using frozen-thawed commercial semen of a beef bull (Semex, Madison, WI, USA) previously used in other *in vitro* fertilization procedures with good blastocyst production and following standard protocols used in our laboratory (Velasquez *et al.* 2016). After 18–22 h of IVF, presumptive zygotes were placed in 0.5 mL of TCM-Hepes + 10% of fetal bovine serum, cumulus cells were mechanically denuded by 4-min vortexing. Thereafter, they were washed three times before culture.

Presumptive zygotes were *in vitro* cultured (IVC) in groups in four-well plates (25–30 zygotes per 500 µL well) in SOF supplemented with 0.37 mM trisodium citrate, 2.77 mM myo-inositol, essential and non-essential amino acids (final concentration 1×), gentamycin (50 µg/mL), 3 mg/mL essentially

Figure 1 Schematic illustration of experimental design. Morulae (day 5 post IVF) were collected and individually cultured in SOFdepleted up to day 7.5. Embryos at blastocyst stage were determined at day 6.5 (early blastulation) and day 7.5. At day 7.5, all blastocysts were evaluated and the following were determined: blastocyst stage (BS), blastocyst quality (BQ) and blastocyst diameter (BD). All blastocysts were transferred to fresh SOFdepleted and individually cultured up to day 11. At day 11, blastocysts were classified according to their post-hatching viability (C: viable, NC: non-viable).

fatty acid-free BSA, 2% fetal bovine serum (FBS) and 10 ng/mL EGF in a balanced gas atmosphere consisting of 5% CO₂, 5% O₂ and 90% N₂, 100% humidity at 39°C.

On day 5 post IVF, morulae were selected (stage 3 – early morula and stage 4 – morula) using criteria described in IETS manual (Stringfellow and Givens 2010). Morulae were washed three times in SOFdepleted and placed in a 96-well plate (80 µL per well) for individual culture until day 7.5. During this period, embryos were monitored, and the formation of blastocoel was verified (EB at day 6.5 and LB at day 7.5).

On day 7.5, morphology characteristics of the blastocysts were measured; embryo quality and developmental stage and blastocyst diameter, using criteria described in IETS manual (Stringfellow and Givens 2010). Embryo quality was graded based on morphological integrity of embryos (number of cells, degree of fragmentation and regularity, scale from 1 to 4). Codes were defined as follows (Bó and Mapletoft 2013): quality 1 (excellent or good), quality 2 (fair), quality 3 (poor) and quality 4 (dead or degenerating). Embryo diameter was measured using the 5-Mpx Micrometrics digital camera software (Arquimed, Santiago, Chile). On day 7.5, embryo culture media was collected individually and identified with the matching embryo and stored individually at –80°C until analysis. The blastocysts of day 7.5 were transferred to SOFdepleted (140 µL) until day 11 to assess their *in vitro* post hatching developmental viability according to their growth performance and diameter at day 11 (Brandão *et al.* 2004, Fischer-Brown *et al.* 2004, Mellisho *et al.* 2017). On day 11, morphology characteristics of the blastocysts were measured – embryo quality and developmental stage and blastocyst diameter. Individual embryo culture was carried out at 39°C under gas mixture (CO₂, 5%; O₂, 5% and N₂, 90%).

Isolation of EVs from culture media

The technique of EVs isolation from individual embryo culture media was previously described by Mellisho *et al.* (2017). Five hundred microliters of PBS, previously filtered through a 0.22 µm syringe filter, were added to culture media collected from individual embryos (~80 µL) and centrifuged at 700×g for 10 min (to eliminate cellular debris). The supernatant was subjected to a second centrifugation at 2500×g for 20 min (to eliminate apoptotic bodies). Finally, the recovered supernatant (approximately 500 µL) containing the EVs (microvesicles and exosomes) was analyzed by NTA to determine the concentration and size distribution of nanoparticles in culture media from individual preimplantation embryos.

Nanoparticle tracking analysis (NTA)

Isolated EVs from individual embryo culture media were analyzed by NTA on a NanoSight NS300 (Malvern Instruments Ltd, UK) equipped with a sCMOS camera. Size distribution and concentration of nanoparticles in liquid suspension was measured. At least 60 particles per frame were measured. Negative controls (PBS and SOFdepleted) had less than seven particles per frame. Sample was injected in continuous sample flow with a syringe pump into the sample chamber at room temperature (RT). Each sample was measured in triplicate with the same camera settings: acquisition time of 60 s and detection threshold setting of 8. The detection threshold was similar in all samples. For this analysis, a 488 nm laser beam was applied to the diluted suspension of vesicles. Data were captured and analyzed using NTA analytical software, version 3.2 Dev Build 3.2.16. Graphical analysis showed particle size distribution of the nanoparticles in the groups and concentration was reported as particles per milliliter.

Transmission electron microscopy analysis

Transmission electron microscopy was used to identify EVs. The EVs suspended pellets were deposited on formvar-carbon-coated copper grids for whole-mount preparations and subjected to transmission electron microscope (TEM) as described (Mellisho *et al.* 2017). Two grids were prepared and washed and fixed in 1% glutaraldehyde in 0.1 M PBS, and then were contrasted in uranyl-oxalate solution (pH 7.0) for 5 min and methyl cellulose-UA on ice for 10 min. A total of 20 fields and images were processed using ImageJ 1.47t software. Grids were visualized on a Zeiss EM 900 TEM (Zeiss, Germany) operated at 80–90 kV.

Analysis of EV markers

EVs cannot be used directly in flow cytometry characterization, instead they were bound to latex beads as described (Mellisho *et al.* 2017). Briefly, EVs (35 μ L or 4×10^8 particles/mL) were incubated with 4 μ m aldehyde/sulfate latex beads (0.125 μ L or 1.25×10^5 particles/mL) (Life Technologies) in a 100 μ L final volume of PBS and incubated overnight at 4°C. Next, 22 μ L of 1 M glycine/PBS (100 mM final concentration) was added and mixed gently (to block unbound sites of latex beads) and kept for 45 min at RT. The MVs/beads complexes were washed twice with 1 mL of PBS/0.5% bovine serum albumin by centrifugation at $1500 \times g$ for 3 min at RT. The MVs/beads complexes were incubated with primary FITC-conjugated anti-human antibodies against CD63 (Abcam, catalog no. 18235, clone MEM-259) or CD9 (Abcam, catalog no. 34162, clone MM2/57) for 1 h at RT. A negative control antibody reaction was performed using latex beads alone incubated with anti-CD63, anti-CD9, anti-CD81 and anti-CD40L for 1 h at RT. The labeled MVs/beads complex were pelleted and washed twice as above with 1 mL of PBS/0.5% bovine serum albumin, and finally 100 μ L pellets were resuspended in 400 μ L of focusing fluid and subjected to flow cytometry using Attune™ NxT Flow Cytometer (Life Technologies, Inc). EVs isolated from bovine follicular fluid was used as positive control.

Exosome RNA isolation and quantification

EVs isolated from individual embryo culture media and analyzed by NTA were further processed for small RNA sequencing. For this, three biological replicates and four technical replicates were used yielding 12 samples (G1.1, G1.2, G1.3; G2.1, G2.2, G2.3; G3.1, G3.2, G3.3; G4.1, G4.2, G4.3). Each replicate was the pool of EVs from ~20 individual embryo culture media. The EVs from each pool were concentrated (~250 μ L) using Amicon Ultra Centrifugal Filters (Amicon® Ultra-15, 100 KDa) to maximize RNA extraction and library preparation. A new filter device (ultracel centrifugal filter device with a 100K NMWL $2500 \times g$) was used for each replica. Immediately, sample pellets were frozen at -80°C pending isolation of RNA. Samples ($n=12$) were sent on dry ice (-80°C) for small RNA isolation, quantitation, library preparation, and sequencing using NextSeq 500/550 Mid Output Kit v2 high-throughput sequencing platform (Norgen Biotek Corp, Thorold, ON, Canada). Total RNA was extracted

from EVs secreted by bovine blastocysts using plasma/serum RNA Purification Kit (#55000, Norgen Biotek), the final elution volume was 15 μ L. For RNA quantification, 1 μ L of RNA was used in a 20 μ L RiboGreen assay (Norgen's in-house developed modification). miR-21 and 5S rRNA by RT-qPCR was used as quality control of RNA. One microliter of RNA was used in 5 μ L TruScript RT reaction using miR-21 stem-loop RT primer or 5S rRNA Reverse Primer. Entire RT reaction was used in 20 μ L of qPCR reaction using miR-21 or 5S rRNA primers (Supplementary Table 1, see section on [supplementary data](#) given at the end of this article).

Small RNA sequencing analysis

Small RNA isolation, quantitation, quality control, library preparation, sequencing (10M reads per sample) and bioinformatics analysis were outsourced to Norgen Biotek. Small RNA populations were prepared for sequencing using a Multiplex Small RNA Library Prep Set for Illumina following the manufacturer's protocol. The initial step of small RNA-Seq analysis was to evaluate sequence read quality and removal of adapter sequences (Truseq 3': TGGGAATTCTCGGGTGCCAAGG). Reference or database sequences used were bostau8 (Genome; <https://genome.ucsc.edu/>), miRBase version 21 (for miRNAs; release date: 2014-06-1; <https://mirbase.org>), Ensembl (for snRNA, snoRNA and rRNA; *Bos taurus* Genome assembly: UMD3.1; <https://www.ensembl.org>) and gtrNadb (for tRNAs; <https://gtrnadb.ucsc.edu>), RNadb (piRNAs; <https://matticklab.com/rnadb>).

sRNA detection (adapter removal, quality control, mapping, counting) was performed using Oasis (V2.0; <https://oasis.dzne.de/>) (Capece *et al.* 2015) with the input reads FASTQ files. For differential expression analysis, the module (Oasis) used as input count files that were output from the 'small RNA detection' module. The differential expression analysis powered by DESeq2 (Love *et al.* 2014) facilitated comparison of miRNAs expression between viable versus non-viable groups.

Prediction of novel miRNA

Prediction of novel miRNAs was performed with miRDeep2 software (Oasis Platform) in 'sRNA Detection' module reads FASTQ files, annotates known sRNAs, and novel miRNA. Novel predicted miRNAs were saved in Oasis Platform, small RNA database (Dweep *et al.*, 2011).

Target prediction and gene ontology analysis

The target genes of 11 miRNAs upregulated were predicted using the algorithms in miRWalk database version 3.0 (<http://mirwalk.umm.uni-heidelberg.de/>) according to established parameters (Dweep *et al.* 2011). The novel upregulated miRNA was not included in this analysis. For prediction of miRNA targets by miRWalk, filter set was used to identify the 3'UTR and retained only the cross-database predicted targets (Using miRanda, miRDB, miRWalk and TargetScan database). Embryo viability-associated target genes of the miRNAs upregulated present in EVs formed the basis of miRNA-mRNA interaction network that was visualized using mirWalk graphics available at <http://mirwalk.umm.uni-heidelberg.de/graph/>. Then, all

targets were classified according to their function in several ways (gene ontology (GO)): molecular function, biological process and cellular components. Functional annotation of the miRNA target genes was carried out by PANTHER classification system, version 13.1 (Mi et al. 2013). The official gene symbols (example: ENSBTAT00000023657) of miRNA target genes were uploaded in tab-delimited text format to PANTHER available at <http://www.pantherdb.org/>.

Statistical analysis

Size, concentration and miRNA cargo of EVs according to embryo viability

Measurable variables of EVs (mean size, mode size and concentration) of each experimental group (G1, G2, G3, G4) were analyzed for normality of distribution by using Kolmogorov–Smirnov test and then compared, with a non-parametric *U* Mann–Whitney test. Analysis of identified miRNA cargo used data from the filtration cut-off values of five counts per million (CPM5) based on EdgeR algorithm. Data were compared to the 791 mature cattle miRNAs curated in miRBase, version 21 (*Bos taurus* for miRNAs; release date: 2014-06-1; <https://mirbase.org>). Selection of differentially expressed miRNAs was based on $\log_2$ FC ($\log_2$ -fold change) of more than 1.5 (upregulated) or less than -1.5 (downregulated), and with an FDR (false discovery rate) of ≤ 0.05 .

Clustering analysis to discriminate viable of non-viable embryo

Principal Component Analysis (PCA) was used along with multi-dimensional dataset and cluster analysis (CA) to detect differences among samples of viable and non-viable embryo, applying the same approach previously adopted for dataset (Penkova 2017). In this work, a multivariable dataset of blastocyst morphokinetics (stage of development, quality and diameter of blastocyst at day 7.5), EVs size (mean, mode), concentration and counts of miRNA and snoRNA (CPM > 5) were analyzed by PCA and CA among groups of viability and blastulation (G1, G2, G3 and G4). Statistical analysis was performed with SAS system, version 8 for windows (SAS Institute Inc., 1999).

Predictive models of embryo

The predictive model of embryo viability proposed here was based on binary logistic regression to describe the dichotomous-dependent variable of the blastocyst (viable=1 and non-viable=0). A set of independent variables (morphokinetics of blastocysts and characteristic of EVs) were analyzed with multiple regression. Logistic regression generated the coefficients and its standard errors and significance level of an equation to calculate a probability, where values between 0.5 and 1 predict embryo viability. To verify the predictive power of the algorithm, the following indicators were used: analysis of the receiver-operating characteristic (ROC) with determination of area under the curve (AUC), percentage correct predictions, and Omnibus tests. Statistical significance was determined at the $P < 0.05$ level. A rough guide for classifying the accuracy

of a predictive model is 0.90–1 = excellent, 0.80–0.90 = good, 0.70–0.80 = fair, 0.60–0.70 = poor, 0.50–0.60 = fail. Statistical analysis was performed with the program IBM SPSS Statistics, version 19 (IBM).

Results

Blastocyst morphokinetics according to embryo viability

A total 4665 COCs of quality 1 and 2 were used for *in vitro* fertilization, producing 1371 good-quality morulae in group culture (efficiency 29.4%). Morulae were further cultured individually in EVs-depleted media, up to the blastocyst stage. There were 230 blastocysts at day 6.5 (early blastulation, 16.8%) and 327 blastocysts at day 7.5 (LB; 23.9%). In all, 40.6% of morulae developed to blastocyst. At day 7.5, all blastocysts independent of the timing of reaching that state were collected and kept in extended individual culture up to day 11. At day 11, 254 embryos were selected and based on their developmental performance were divided into four groups: V-EB, NV-EB, V-LB, NV-LB. Non-hatched blastocysts were discarded. Diameter at day 7.5 was larger in V-EB (170.8 μ m; $p < 0.05$) (Fig. 2; Supplementary Table 2). No differences in diameter were observed in day 7.5 blastocysts from groups NV-EB and V-LB; however, blastocysts from NV-LB were the smallest embryos at that stage (146.5 μ m; $P < 0.05$) (Fig. 2; Supplementary Table 2). In addition,

Figure 2 Blastocysts growth from day 6.5 up to day 11. (A) Viable embryos despite their time of blastulation showed a lineal growth from day 7.5 up to 11, compared to non-viable embryos. Different letters within the same developmental day (6.5, 7.5 or 11) indicate statistical differences ($P < 0.05$). (B) Representative pictures from blastocyst morphology (days 6.5 and 7.5) during blastulation time and post-hatching development (day 11).

V-EB group was the only one with hatched blastocysts at day 7.5, and it had the highest percentage of best quality blastocysts at day 7.5 (77% quality 1 blastocysts and 10% in hatching stage (Supplementary Table 2)). Conversely, embryos from NV-LB group showed delayed development; 96.1% of the blastocysts at day 7.5 were at early stage and 37% of embryos were classified as quality levels 3 and 4 (Supplementary Table 2).

Size, concentration and small RNA cargo of EVs according to embryo viability

The NTA analysis characterized populations of EVs in terms of size and concentration. The EVs analyzed were secreted during the transition from morulae to

blastocyst (day 5–7.5; Fig. 3A). Filtered PBS solution and SOF-depleted media used for embryo culture were included as negative control: these solutions had very low concentration of particles that did not influence characterization of EVs populations secreted by individual embryos (Fig. 3B). Isolated EVs were visualized by TEM (Fig. 3C) and confirmed as EVs by immunoidentification of surface markers CD9, CD81, CD63 and CD40L (Fig. 3D).

Population of EVs showed significant differences among groups of embryos, with the highest mean size corresponding to EVs from V-EB embryos (122.90 nm; $P < 0.05$) (Fig. 3A and B; Supplementary Table 3). However, there were not significant differences between viable and non-viable embryos with LB. Regarding

Figure 3 Measurements of the size and concentration of EVs secreted from bovine blastocyst. (A) Size and concentration of EVs populations measured with Nanoparticle tracking analysis (NS300). In A, Y-axis indicates concentration (10^8 particles $\times$ mL), while X-axis indicates size distribution (nm). Depleted SOF media and filtered PBS were included as negative controls. (B) Descriptive statistical values (mean, mode, concentration) of EVs populations and comparison measures between viable versus non-viable embryo groups. Different letters within EVs characteristic mean statistical differences with $P < 0.05$. (C) Representative pictures from transmission electron micrographs showing morphology of EVs secreted by bovine blastocysts and (D) flow cytometry analysis of EVs markers (CD9, CD63, CD81, CD40L).

EVs concentration, culture media from NV-EB had the highest concentration of EVs (5.75×10^9 particles/mL) (Fig. 3A and Supplementary Table 3).

High-throughput sequencing was used to determine small RNA content in EVs derived from preimplantation bovine embryos during blastulation. Overall 14.9 million raw reads (ranging from 9 to 18 million) were achieved. After bioinformatics processing, 0.24–3.03% were mapped as unique reads. The most abundant biotypes of

small RNA were miRNA (86–91%) and snoRNA (9–14%) (Fig. 4A). Bioinformatics analysis identified a total of 182 miRNAs; 161 were known miRNAs and 21 putative novel miRNAs (p-bta-miR-) (Table 1). For this identification, the cut-off was set at five counts in EVs from embryo culture media. These newly identified miRNAs have apparently not been reported in other species. Further, miRNAs/snoRNAs counts were compared between viable and non-viable embryos. Eight miRNAs (bta-miR-1, bta-miR-

Figure 4 Distribution of small RNA species in EVs secreted by preimplantation bovine embryo during blastulation (from day 5 up to day 7.5 of embryo development). (A) Distribution of small non-coding RNAs obtained from EVs from blastocyst according to their viability. (B and C) Venn diagram depicting counts of miRNAs and snoRNAs (CPM > 5) found in EVs from viable (blue/green circle) and non-viable embryos (red/black circle). (D) Heatmap profile of miRNA counts (CPM > 5) from EVs secreted by bovine blastocysts. Global color scale shows upregulated (green) and downregulated (red) miRNA expression levels.

Table 1 Identification of a novel miRNAs (CPM ≥ 5 per sample) of EVs from embryo culture media.

Mature candidate ID*	Mature sequence	Length	Chromosome	Mature start	Mature end	Mature strand
p-bta-miR-101	AGGGUCAGGUAAGGGUCUCCACU	23	chr5	55981753	55981775	+
p-bta-miR-110	UUGUCCUGGUCGCGGCCUCUGC	23	chr7	5779012	5779034	+
p-bta-miR-119	GGUUAGCCAGGACACAGCGGAG	22	chr4	115606058	115606079	-
p-bta-miR-124	UUUCACCCGGUCUCUUUGGCUC	22	chr4	5633784	5633805	+
p-bta-miR-129	CCGAGCGCGGACCUCGCGGCCU	23	chr24	50939210	50939232	+
p-bta-miR-134	UGUGGUCUGUGUUCUGUAAGA	21	chr1	75157296	75157316	+
p-bta-miR-14	UGGCACACGACUGGCGGUGGC	22	chr26	37549333	37549354	-
p-bta-miR-20	GCACGGGUGGUUCAGUGGUA	20	chr2	41564663	41564682	+
p-bta-miR-24	UCCAGGAUAUGUGCGUGUAACU	22	chr9	24769364	24769385	-
p-bta-miR-26	UGCACACCCGUGGUCACUCCU	22	chr23	28502263	28502284	-
p-bta-miR-28	AUCGCGACGGGUAUCUCU	18	chr11	11314991	11315008	-
p-bta-miR-31	AAAACCUCACGAACUCUUUGG	22	chr27	15045875	15045896	+
p-bta-miR-34	UUAGGAUUGACAGAUUGA	18	chr10	34366159	34366176	+
p-bta-miR-35	CCGGGCCUGGGCCGAGCCCCA	21	chr15	36372562	36372582	-
p-bta-miR-39	UCGAGUGCUGAAGUGACCUUCU	22	chr21	7226582	7226603	+
p-bta-miR-47	GGACAGUGUCAGUUGGGCA	19	chrX	113743090	113743108	-
p-bta-miR-58	UAAGACCUCACAUGCCUUGCAGC	23	chr19	41310531	41310553	+
p-bta-miR-61	CGCGCUGCAUGCCGGGAGGCU	21	chr13	63816904	63816924	-
p-bta-miR-69	UCCUCAGUAGCUCAGUGGUA	22	chr12	82691418	82691439	+
p-bta-miR-71	UAGAGCAGACGACUCAGAUACCG	23	chr20	5593899	5593921	+
p-bta-miR-72	UUCGGGCGGCGCGGGUCCCGG	22	chr10	9674464	9674485	-
p-bta-miR-85	CGCCCUUCUGUACUCGCGCCG	22	chr11	107268646	107268667	-

*Nomenclature oasis, predicted.

125b, bta-miR-184, bta-miR-2368-3p, bta-miR-2417, bta-miR-2889, p-bta-miR-124, p-bta-miR-71) were exclusively detected in media from viable embryos (including EB and LB) (Fig. 4B). Conversely, there were 24 exclusive miRNAs in media from non-viable embryos (Fig. 4B).

A total of 32 snoRNAs identified (Fig. 4C), of which two (SNORD110, SNORD81) were exclusively present in media from viable embryos (including early and LB), whereas media from non-viable embryos had three exclusive snoRNAs (SCARNA24, SNORD97, SNORD48). Heatmap (Fig. 4D) illustrated that viable and non-viable embryos had a differential miRNA expression pattern (FDR < 0.05).

For differential expression analysis, data were filtered by $\log_2$ ratio > 1.35 or < -1.35, P value adjusted (FDR) < 0.05 and P < 0.05. Based on expression level analysis of each miRNA, 27 of the 182 identified miRNAs were confirmed to exhibit significantly differential expression levels in viable versus non-viable embryos, despite blastulation time (Fig. 5 and Supplementary Table 4). Among these miRNAs, 12 miRNAs had higher expression levels in viable embryos (Fig. 5A), whereas the remaining 15 miRNAs were downregulated in these embryos (Fig. 5B). The miRNAs deregulated the most, according to the $\log_2$ fold change (FC) of expression were bta-miR-2284ab (FC = 4.6), bta-miR-124a and bta-miR-124b (FC = -3.5) (Supplementary Table 4). Five of the 12 miRNAs with a higher expression level were exclusively identified in viable embryos (bta-miR-1, bta-miR-184, bta-miR-2368-3p, bta-miR-2889, p-bta-miR-71). When miRNAs from embryos that reached blastulation at different time (early or late) were compared considering

the same criteria (FDR < 0.05 and P < 0.05), no differentially expressed miRNAs were found despite embryo viability. Among the 32 snoRNAs identified, one (SNORD20 ENSBTAG00000043297) was upregulated (FDR < 0.05) in EVs of viable embryos compared to non-viable embryos (Supplementary Table 5).

Potential target genes of 11 known miRNA upregulated (Fig. 5C) and 12 known miRNAs downregulated (Fig. 5D) in viable embryos were identified using miRWalk 3.0 database, yielding 9890 and 14307 target genes, respectively, with miRNA potential binding sites in their 3'UTR respectively. Putative novel miRNAs ($n = 4$; p-bta-miR-14, p-bta-miR-26, p-bta-miR-61, p-bta-miR-71) were not included in this analysis, as no target genes are known at present. In upregulated, three miRNAs can potentially regulate 58% of target genes: bta-miR-2449 (2477 genes), bta-miR-320a (1947 genes) and bta-miR-7863 (1246 genes) (Fig. 5C). Therefore, the interaction network between miRNA and target genes is common in cellular mechanisms (example: predicted target genes and upregulated miRNAs were used to construct the interaction network using the MirWalk graphic software (Fig. 5E)). All targets were classified according to their function in two categories of GO: molecular function (Fig. 5F) and biological process (Fig. 5G) using PANTHER classification system. In GO annotation for molecular function, most target genes (84%) were related to binding (GO:0005488), catalytic activity (GO:0003824) and transporter activity (GO:0005215). In GO annotation for biological process, most target genes (69%) were related to binding cellular process (GO:0009987), metabolic process (GO:0008152), biological regulation (GO:0065007)

Figure 5 Differential miRNA expression profiles in EVs and the target gene analysis. From 27 miRNAs, 12 are upregulated in viable embryos (A) and 15 are downregulated in these embryos (B). ** indicated statistical significance with corrected FDR < 0.01; * indicated statistical significance with corrected FDR < 0.05. (C and D) Prediction of potential target genes for 11 known miRNAs upregulated (C) and 12 known miRNAs downregulated (D) using MirWalk 3.0 database. (E) Interaction network among target genes of 11 miRNAs upregulated (bta-miR-2284ab, bta-miR-2449, bta-miR-2368-3p, bta-miR-184, bta-miR-7863, bta-miR-2889, bta-miR-1, bta-miR-320a, bta-miR-1246, bta-miR-10b, bta-miR-10a): Orange nodes indicate a target gene; Blue nodes indicate a miRNA with upregulated expression. (F) Nine significantly enriched GO for molecular function were achieved using the target genes of upregulated miRNAs and (G) twelve significantly enriched GO for biological process were achieved using the target genes of upregulated miRNAs.

Figure 6 Clustering analysis of blastocyst morphokinetics and EVs properties identifies viable preimplantation embryos. PCA plot and clustering analysis for four groups according to developmental viability and blastulation time (V-EB, NV-EB V-LB and NV-LB): (A) clustering according to blastocysts morphokinetics data, (B) clustering according to EVs characteristic (size and concentration), (C) clustering combining variables from blastocyst morphokinetics and EVs characteristic (size and concentration), (D) clustering according to counts of miRNA and (E) snoRNA (RNA) cargo of EVs from embryo culture media. EB, early blastulation; LB, late blastulation; NV, non-viable; V, viable.

and response to stimulus (GO:0050896). Gene ontology analysis for target genes of downregulated miRNAs had similar results (data not shown).

Clustering analysis of blastocyst morphokinetics and EVs characteristic

A multivariate data analysis using PCA and CA was performed including different variables (Fig. 6). Blastocysts morphokinetics data (stage, quality and diameter of blastocyst at day 7.5) allowed identification of two groups, discriminating according to moment of blastulation (EB vs LB), but not according to embryo viability (V vs NV) (Fig. 6A). EVs characteristics (mean size, mode size and concentration) allowed to discriminate between blastocyst from V-EB and NV-EB but not between embryos at LB (Fig. 6B). However, when blastocyst morphokinetics data and EVs characteristic are combined in the same analysis, there is a better discrimination of viable and non-viable embryos from early blastulation group (Fig. 6C). Counts of miRNA and snoRNA (CPM > 5) were performed in three replicates per group, with a marked discrimination among the four groups for both embryo viability and blastulation time (V-EB, NV-EB, V-LB and NV-LB) (Fig. 6D and E).

Non-invasive model to predict embryo viability

Binary logistic regression was used to construct non-invasive models to predict embryo viability, based on the values of seven variables, including blastocyst morphokinetics characteristics (blastulation time (BT), stage (BS), quality (BQ) and diameter (BD) of blastocyst at day 7.5) and EVs characteristic (mean size (ME), mode of size (MO) and particle concentration (CO)), measured in 254 embryos. In Model-1, all independent variables were analyzed; the most significant predictors associated with embryo viability identified for this model were blastocyst stage, blastocyst quality, blastocyst diameter, mean size of EVs and concentration of EVs ($P < 0.05$) (Table 2). Mode size of EVs and BT were not significant in Model-1.

Model-2 included most significant variables from Model-1 (Supplementary Tables 6 and 7), whereas

Model-3 included highly significant variables ($P < 0.011$). In Model-4 and 5, EVs characteristic were excluded; Model-4 considered all variables from the embryo, whereas Model-5 excluded BT (Table 2). Based on statistical test of predictive power, Model-1 and 2 achieved a ROC-AUC of 0.853 (95% CI, 0.806–0.900; $P < 0.001$) and correct predictions of 77.2 and 77.6% respectively (Table 2, Supplementary Table 6 and Supplementary Fig. 1). Model-3 with only three variables had an ROC-AUC similar to Model-1 and 2, but a lower percent of correct prediction (Table 2). Conversely, the analysis of predictive power for Models-4 and 5, achieved an ROC-AUC of 0.714 (95% CI, 0.651–0.777; $P < 0.001$) and 0.724 (95% CI, 0.662–0.786; $P < 0.001$) and correct predictions of 65.4 and 66.9% respectively, indicating the value of EVs characteristics to predict embryo viability (Table 2 and Supplementary Fig. 1).

The logistic function (logit) for Model-2:

$$Y = \ln[p/(1-p)] = -2.280 + (-0.868)*BS \\ + (-0.470)*BQ + (0.047)*BD \\ + (0.027)*ME + (-0.051)*CO$$

The estimated probability is:

$$p = \text{Exp } Y / (1 + \text{Exp } Y)$$

Where:

ln is the natural logarithm, log exp, where $\exp = 2.71828...$

$\ln[p/(1-p)]$ is the log odds ratio, or 'logit'

$p/(1-p)$ is the 'odds ratio'

p is the probability that the event Y occurs, $p(Y=1)$

If $p > 0.5$, predict embryo viability

If $p < 0.5$, predict embryo non-viability

Discussion

Recently we and others reported secretion of EVs by bovine, porcine and human embryos (Ferreira et al. 2013, Saadeldin et al. 2014, Mellisho et al. 2017, Battaglia et al. 2019). EVs are recognized as carriers

Table 2 Predictive non-invasive models of embryo viability with the use of binary logistic regression.

Variables	Model-1 (full variables)	Model-2 (significant variables)	Model-3 (min. significant variables)	Model-4 (blastocyst morphokinetics)	Model-5 (blastocyst morphology)
Constant	-1.662	-2.280	-6.386	-0.819	-0.585
Blastocyst kinetics					
Blastulation time (early = 1, late = 0)	-0.622	–	–	-0.472	–
Blastocyst morphology					
Day 7.5; blastocyst stage	-0.874	-0.868	–	-0.672	-0.760
Day 7.5; blastocyst quality	-0.509	-0.470	–	-0.530	-0.477
Day 7.5; blastocyst diameter	0.048	0.047	0.036	0.039	0.039
EVs characteristics					
Mean of EVs	0.046	0.027	0.025	–	–
Mode of EVs	-0.034	–	–	–	–
Concentration of EVs	-0.041	-0.051	-0.053	–	–
Power of predictive algorithm					
Accuracy of model (ROC-AUC)	0.853	0.853	0.834	0.724	0.714
AUC: 0.80–0.90, model good					
Percent correct predictions (bigger, better the model)	77.2%	77.6%	72.4%	65.4%	66.9%
Omnibus tests of coefficients and model $P < 0.01$	0.001	0.001	0.001	0.001	0.001

Note: Values in the columns corresponded to coefficients of the variable in the logistic function of each model. Below the logistic function of Model-2 is represented. This model had significant variables and the highest percentage of correct predictions.

of proteins, lipids, and nucleic acids and can transfer this cargo to recipient cells, participating in cell-to-cell communication (Raposo and Stoorvogel 2013, Kowal *et al.* 2014, Battaglia *et al.*, 2019). Although EVs have not been as intensively studied during preimplantation embryo development as in other cell types, it seems that EVs characteristics (mean size, mode of size and particle concentration, as well as their cargo) can be associated with embryo stage and quality embryos (Ferreira *et al.* 2013, Saadeldin *et al.* 2014, Mellisho *et al.* 2017). In fact, we reported that parthenogenetic and IVF embryos secreted dissimilar populations of EVs (Mellisho *et al.* 2017). In the same research, we also observed a high variability in characteristics of EVs secreted by non-viable blastocyst, whereas viable embryos had less polydispersity of EVs (Mellisho *et al.* 2017). Skog *et al.* (2008) and Sharma *et al.* (2011) mentioned that polydispersity can be indicative of the origin of the vesicles; for example, tumor-derived EVs had greater polydispersity than those from healthy cell lines.

Based on that information, in this work, we investigated the potential of characterization of EVs as a non-invasive marker of bovine embryo viability. Using the described experimental design and the adjustment of protocols to isolate and characterize EVs, it was possible to derive information from individual embryos. EVs population from viable and non-viable embryos showed high variability in diameter (range 10–300 nm) and concentration, what is not observed in EVs-depleted media used as negative control for NTA analyses. This diameter range is in concordance to previous report for bovine (Mellisho *et al.* 2017), human (Ferreira *et al.* 2013) and porcine (Saadeldin *et al.* 2014) embryos. However, PCA and cluster analysis allowed discriminated viable embryos with early blastulation group, but not at LB.

Perhaps this was due to a difference in EVs secretion depending on embryo developmental kinetic.

In bovine embryos, there is a peri-implantation period when growth and many molecular and morphological changes occur despite a lack of direct contact with the maternal endometria (Graf *et al.* 2014). Therefore, bovine embryos maintained in a culture system after blastocyst stage to evaluate post hatching development are a convenient model to study embryo viability (Brandão *et al.* 2004, Velasquez *et al.* 2016, 2017). To accomplish this, conventional embryo culture system was modified; first embryos were cultured in groups until morula stage to promote development, whereas thereafter, morulae were cultured individually in EVs-depleted media. This facilitated the assessment of development performance of individual embryos and avoid contamination of EVs from FBS (Kropp *et al.* 2014, Kropp and Khatib 2015, Mellisho *et al.* 2017). However, under this experimental design, embryos were exposed to non-optimal culture conditions, consequently, the blastocyst rate was reduced from above 30% in routine laboratory group culture (unpublished) to ~12%. Most embryos blastulated early at day 6.5, this favored greater blastocyst diameter at day 7.5 (average = 170.8 µm) and post-hatching development. Also 32% of early blastulated embryos were later classified as viable (V-EB), whereas 16% of late blastulated embryos were viable (V-LB).

Prospective use of morphokinetics to improve selection by using early (Wong *et al.* 2010, Motato *et al.* 2016) or late development parameters have been reported (Campbell *et al.* 2013). In the present study, both morphokinetic and morphology variables of blastocyst at day 7.5 were significantly related to embryo viability. The prediction power of both models

(Model-4 and Model-5) achieved an ROC-AUC of 0.714 and 0.72, respectively, being statistically considered as 'fair' models to select viable embryo. Similar results were reported using models based on time-lapse embryo monitoring, achieving an ROC-AUC of 0.7 (Swain 2013), 0.602 (Motato *et al.* 2016), 0.70 (Milewski *et al.* 2015) and 0.71 (Milewski *et al.* 2016), whereas a model that considers blastocyst morphology, achieved an ROC-AUC of 0.56 for implantation prediction (Motato *et al.* 2016).

Limitations of embryo viable prediction based on embryo morphology and/or morphokinetics led researchers to pursue more accurate approaches. During early development, embryos interact with the environment. *In vitro*, embryos consume substrates and oxygen from the culture system while secreting proteins and other molecules, thus changing characteristics of the culture media (Ménézo *et al.* 2013). The secretome of the oocyte/embryo likely reflects their biochemical or epigenetic status (Katz-Jaffe and Gardner 2008). This information has been used to determine an association between embryo metabolomics or secretion and their developmental competence, in order to design non-invasive models for embryo scoring. For instance, in human embryos, Seli *et al.* (2011) and Dominguez *et al.* (2015) proposed a strategy combining time-lapse and proteome or metabolomic analysis in spent culture media. In this species, extreme values for ROC analysis were published when comparing metabolomic (NIR spectroscopy, AUC=0.92) versus morphology score (AUC=0.55) for pregnancy prediction (Li *et al.* 2015). However, data were not consistent between laboratories and the method was not considered useful in daily practice (Vergouw *et al.* 2014). In this work, we proposed a novel combined predictive model of embryo viability based on variables of morphokinetics of blastocysts and characteristic of EVs that achieved ROC-AUC of 0.853 for Model-1 and Model-2. Statistically it was considered as 'good' model to select viable embryos. Model-1 included all variables evaluated, whereas Model-2 excluded BT without decreasing ROC-AUC value and the percent of correct predictions. The second model was useful from a practical point perspective, as it avoided disturbing embryo culture during blastulation. Certainly, incorporating EVs properties increases accuracy of embryo selection, as judged from comparisons of ROC-AUC values of good models versus Model-4 and Model-5. These data supported the possibility to improve embryo selection using conventional morphological criteria of the blastocyst combined to characteristics of EVs obtained by NTA. It was corroborated that NTA is very sensitive to analyze EVs population of individual embryos, it is easy and fast and thus can be performed very shortly after sample collection, allowing for appropriate and timely decisions about embryo fate. Besides, data are quantitative and easy to interpret.

Attempts to relate certain characteristics of spent culture media with bovine embryo viability have been published, but so far with non-convincing results, and the predictive value of proposed systems is still controversial and unfeasible (Reviewed by Rødgaard *et al.* 2015, Capalbo *et al.* 2016). That EVs contain molecules with regulatory potential emphasizes their biological importance, we analyzed the miRNA cargo of EVs from embryo culture media using small RNA sequencing, identifying 161 known miRNAs and 32 snoRNAs in EVs. Clustering analysis of miRNA/snoRNAs also allowed to discriminate viable embryos from both EB and LB groups. Moreover, differential miRNA expression patterns were observed between viable and non-viable embryos. However, when EB and LB were compared, no miRNAs were significantly differentially expressed despite embryo viability. In some of the compared miRNAs, there were *P* values <0.05, but none of them met the FDR criterion set by us. Therefore these miRNAs were not considered for the analysis of their biological significance. Seemingly, blastulation (early or late) is the milestone after which there are no relevant changes in miRNA expression pattern in our experimental conditions. In addition, when BT was incorporated as a variable into the proposed model, it did not improve the percentage of positive prediction and thus it was excluded from the selected model (model-2).

We paid attention to miRNAs differentially expressed between viable and non-viable embryos. It has been proposed that miRNAs are selectively secreted into the culture medium by developing human embryos making them potential biomarkers of embryonic viability (Kropp *et al.* 2014, Rosenbluth *et al.* 2014, Kropp and Khatib 2015, Lin *et al.* 2019). In this work, 5 of the 12 upregulated miRNAs were identified solely in EVs secreted by viable embryos, of these, bta-miR-2284ab was upregulated the most. In the endometrium of goats, miR-2284ab is upregulated during the receptive phase (Song *et al.* 2015) as well as in cumulus cells of bovine oocytes that developed to blastocysts (Uhde *et al.* 2017).

Other miRNAs deregulated in our findings have been reported to participate in different reproductive events. The expression of bta-miR-1 is also higher in EVs from viable embryos. This miRNA acts in different functions including cell proliferation, cell cycle, apoptosis, cell migration and invasion (Li *et al.* 2012, Osaka *et al.* 2014). Members of miR-1 family regulate the balance between differentiation and proliferation by interacting with multiple targets in different cell types (Chen *et al.* 2006). In lung cancer and carcinoma cells, miR-1 suppresses cell proliferation by inducing arrest in the G0/G1 phase (Fujji *et al.* 2019). During embryo development, miR-1 induces angiogenesis in zebrafish (Lin *et al.* 2013), while in human and mouse miR-1 participates in the differentiation of skeletal and cardiac muscles (Chen *et al.* 2006). In the literature search we did not find any other report of miR-1 expressed in early embryos or in

EVs secreted by preimplantation embryos. In this research we did not find a relation between embryo viability and the high expression of miR-1. Others reported that insulin-like growth factor 1 (IGF-1) is a target of miR-1 (Elia *et al.* 2009, Li *et al.* 2012, Hu *et al.* 2013). IGF-1 and 2 and their respective binding proteins (IGFBPs) are expressed in the endometrium of cattle (Wathes *et al.* 1998), but the concentration of IGF-1 decreases from day 0 to 18 in cattle carrying an embryo (Mense *et al.* 2018). IGF-1 concentration increases again between days 42 and 60 in pregnant cows suffering embryonic/fetal mortality (Mense *et al.* 2018). The presence of miR-1 in EVs from viable embryos here could potentially lead to diminish the expression of IGF-1 in endometrium. Alternatively, viable embryos may take rid of unwanted miRNAs, like miR-1, to avoid cell arrest mediated by this or other miRNAs.

Recently we found bta-miR-1 to be downregulated in EVs secreted from day 3 to morula by embryos that developed to blastocysts, compared with those that became blocked and did not develop further (unpublished data). It is tempting to propose that there would be a kinetic of release of miRNA potentially harmful for the embryo. It has been reported that levels of miRNA in cells do not necessarily correlate with the level in EVs derived from them; some miRNAs are abundant in EVs while rare in cells and conversely (Maida *et al.* 2016). In line with this, bta-miR-184 was found expressed only in EVs from viable embryos here. However, in human and mice embryos, miR-184 suppresses cell proliferation and induces apoptosis in trophoblastic cells; higher expression is related to embryo reabsorption (Zhang *et al.* 2019).

In this work, it was found bta-miR-10b upregulated in EVs from viable embryos. This is in contrast to Lin *et al.* (2019) who found that the expression of miR-10b was higher in culture medium from slow and arrested preimplantation bovine embryos. However, this contradictory result might be due to different experimental designs; Lin *et al.* (2019) collected culture media from one-cell to blastocyst, while we collected media from morula to blastocyst and only from those embryos that reached blastocyst stage.

In our work bta-miR-200c among several other miRNAs were significantly downregulated in EVs from viable embryos. The expression of miR-200 family members is low in the receptive uterus in humans and mouse while is upregulated in infertile female (Liu *et al.* 2016, Zheng *et al.* 2017). miR-200c inhibits uterine epithelial cells receptivity and embryo implantation in mouse (Zheng *et al.* 2017). The low expression of miR-200c in EVs from viable embryos in this work would favor endometrium receptivity. Interestingly, miR-200c is activated by the pluripotency transcription factors OCT4 and SOX2 to induce or maintain cell pluripotency

(Wang *et al.* 2013). In this sense, it would be a 'necessary' miRNA for a developing embryo and consequently would be conserved (i.e., not secreted). Previously, we found bta-miR-200c upregulated in more competent bovine embryos produced *in vitro*, compared to cloned embryos (Castro *et al.* 2010). Also, Kim *et al.* (2019) reported the upregulation of miR-200c-3p in mouse embryo outgrowths compared with non-outgrowth blastocysts. In line with this, here bta-miR-26 was found downregulated in EVs from viable embryos, while Castro *et al.* (2010) reported it was upregulated in more viable IVF embryos compared to clones.

Considering the facts discussed, it seems that EVs secretion plays a role in regulating the expression pattern of miRNAs in bovine blastocysts what is also related to embryo viability. Therefore, miRNA cargo of EVs could be evaluated in order to select more competent embryos. Regardless, validation analysis of the most prominent miRNAs is needed to include them in prediction models of embryo viability.

Exosomes are derived from major sorting hubs of endosomal trafficking; therefore, they are at the center of a signaling network that connects external stimuli with trophoblast cells intrinsic features. Differences in composition between EVs of viable and non-viable embryos may be due to the existence of regulated sorting mechanisms determining the phenotype and functional properties of these vesicles. Also, EV-associated miRNAs can travel long distances and become messages, regulating different biological process that may have an extraordinary impact on early embryo-maternal crosstalk (de Candia *et al.* 2016).

In conclusion, heterogeneous populations of EVs were secreted by individual embryos in an embryo viability-dependent manner. Furthermore, secreted miRNA and snoRNA were differently packaged into distinct populations of EVs related to embryo viability. Also, our binary logistic regression models (Model-1 and Model-2) effectively discriminated between viable and non-viable embryos, using blastocyst morphokinetics variables and EVs morphological parameters. It will be worth to define how miRNA cargo can collaborate by adding valuable predictive features to improve overall performance of pregnancy.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-19-0233>.

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.

Funding

This work was partially supported by FONDECYT 1170310 from the Ministry of Education of Chile. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author contribution statement

E A M, L R-A: Conceptualization, E A M: Data curation, E A M, M A B, L R-A: Formal analysis, L R-A: Funding acquisition, E A M, L R-A: Investigation, E A M, A E V, L R-A: Methodology, L R-A: Project administration, L R-A, F O C: Resources, E A M, J C, M A B: Software, L R-A: Supervision, L R-A, F O C: Validation, E A M, L R-A: Visualization, E A M, L R-A: Writing (original draft preparation), E A M, L R-A, A E V, J C, M A B, F O C: Writing (review and editing) and all authors read, edited and approved the final manuscript.

Acknowledgements

The authors express their appreciation to Victor Gallardo Alvarez for his collaboration in retrieving ovaries from the abattoir. They are grateful to Dr John P Kastelic (Professor, Head Department of Production Animal Health, University of Calgary) for his contribution reviewing this manuscript.

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Received 27 May 2019

First decision 6 August 2019

Revised manuscript received 27 August 2019

Accepted 1 October 2019