Article:
Dissanayake, K., Nõmm, M., Lättekivi, F. et al. (2020) Individually cultured bovine embryos produce extracellular vesicles that have the potential to be used as non-invasive embryo quality markers. Theriogenology, 149. pp. 104-116. ISSN: 0093-691X

https://doi.org/10.1016/j.theriogenology.2020.03.008

Theriogenology 149 (2020) 104–116

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Individually cultured bovine embryos produce extracellular vesicles that have the potential to be used as non-invasive embryo quality markers

Keerthie Dissanayake [a, c], Monika Nõmm [b], Freddy Lättekivi [a], Yosra Ressaissi [a, d], Kasun Godakumara [a], Arina Lavrits [a], Getnet Midekessa [a], Janeli Viil [a], Rikke Bæk [e], Malene Møller Jørgensen [e, f], Sourav Bhattacharjee [g], Aneta Andronowska [h], Andres Salumets [i, j, k, l], Ülle Jaakma [b], Alireza Fazeli [a, m, *]

[a] Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia
[b] Chair of Animal Breeding and Biotechnology, Estonian University of Life Sciences, Tartu, Estonia
[c] Department of Anatomy, Faculty of Medicine, University of Peradeniya, Sri Lanka
[d] Faculty of Veterinary Medicine, University of Teramo, Italy
[e] Department of Clinical Immunology, Aalborg University Hospital, Denmark
[f] Department of Clinical Medicine, Aalborg University, Denmark
[g] School of Veterinary Medicine, University College Dublin (UCD), Belfield, Dublin 4, Ireland
[h] Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Poland
[i] Competence Centre on Health Technologies, Tartu, Estonia
[j] Institute of Genomics, University of Tartu, Tartu, Estonia
[k] Department of Obstetrics and Gynecology, University of Helsinki and Helsinki University Hospital, Helsinki, Finland
[l] Department of Obstetrics and Gynecology, Institute of Clinical Medicine, University of Tartu, Tartu, Estonia
[m] Department of Oncology and Metabolism, University of Sheffield, United Kingdom

ARTICLE INFO

Article history:
Received 8 February 2020
Received in revised form
6 March 2020
Accepted 8 March 2020
Available online 4 April 2020

Keywords:
Extracellular vesicles
Nanoparticles
Embryo
Bovine

ABSTRACT

Extracellular vesicles (EVs) are membrane-bound biological nanoparticles (NPs) and have gained wide attention as potential biomarkers. We aimed to isolate and characterize EVs from media conditioned by individually cultured preimplantation bovine embryos and to assess their relationship with embryo quality. Presumptive zygotes were cultured individually in 60 μl droplets of culture media, and 50 μl of media were collected from the droplets either on day 2, 5 or 8 post-fertilization. After sampling, the embryo cultures were continued in the remaining media until day 8, and the embryo development was evaluated at day 2 (cleavage), day 5 (morula stage) and day 8 (blastocyst stage). EVs were isolated using qEVsingle® columns and characterized. Based on EV Array, EVs isolated from embryo conditioned media were strongly positive for EV-markers CD9 and CD81 and weakly positive for CD63 and Alix among others. They had a cup-like shape typical to EVs as analyzed by transmission electron microscopy and spherical shape in scanning electron microscopy, and hence regarded as EVs. However, the NPs isolated from control media were negative for EV markers. Based on nanoparticle tracking analysis, at day 2, the mean concentration of EVs isolated from media conditioned by embryos that degenerated after cleaving (8.25×10^8/ml) was higher compared to that of embryos that prospectively developed to blastocysts (5.86×10^8/ml, $p < 0.05$). Moreover, at day 8, the concentration of EVs isolated from media conditioned by degenerating embryos (7.17×10^8/ml) was higher compared to that of blastocysts (5.68×10^8/ml, $p < 0.05$). Furthermore, at day 8, the mean diameter of EVs isolated from media conditioned by degenerating embryos (153.7 nm) was smaller than EVs from media conditioned by blastocysts (163.5 nm, $p < 0.05$). In conclusion, individually cultured preimplantation bovine embryos secrete EVs in the culture media and their concentration and size are influenced by embryo quality and may indicate their prospective development potential.

* Corresponding author. Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia.
E-mail address: alireza.fazeli@ut.ee (A. Fazeli).

https://doi.org/10.1016/j.theriogenology.2020.03.008

1. Introduction

Nanomaterials, including nanoparticles (NPs), are conventionally defined as particles with a size of ≤100 nm in at least one dimension [1], and thus NPs with its conventional spherical shape are typically ≤100 nm in all dimensions. However, NPs vary based on many factors including source, size, and composition. Both synthetic and biological NPs have a wide range of applications in medicine [2]. Extracellular vesicles (EVs) are membrane-bound biological NPs that are secreted both *in vivo* and *in vitro* by many cell types under physiological and pathological conditions [3–5], while they are found in all biological fluids, such as blood, milk, synovial fluid, and also in conditioned cell culture media [6,7]. Their molecular cargo primarily includes proteins, nucleic acids (including messenger and non-coding RNA) and lipids [8]. The compartmentalization of the molecular cargo within EVs protect the labile cargo from inactivation and further degradation in the extracellular environment [9]. Secreted EVs are internalized by the target cells and thus, the molecular cargo of the EVs alters the activity and phenotype of the recipient cells and can also induce (epi) genetic modifications [10]. Moreover, EVs are gaining wider attention as potential biomarkers [11] and therapeutic DDSs (drug delivery systems) [12].

The production of embryos *in vitro* has multiple applications, such as using assisted reproductive technologies (ART) to treat human subfertility, animal breeding, and research in the preimplantation embryonic development [13,14]. Despite recent advances in embryo culturing protocols, the quality of embryos produced *in vitro* is lower compared to their *in vivo* counterparts [15]. Compared to individual embryo culture, higher blastocyst formation rates and better quality have also been reported when embryos were cultured in groups [16] or with other somatic cells, such as the oviductal epithelial cells [17]. This could be due to the release of various autocrine and paracrine factors by these cells, which support the development of embryos in groups rather than in individual culture.

Traditionally, the method of choice to evaluate the embryonic quality has been the microscopic evaluation of embryo morphology. The field has further been revolutionized with the introduction of time-lapse microscopic systems enabling continuous monitoring of the embryos [18]. However, ART based live birth rates have yet not lived up to the expectations despite the technological advances in the field [19]. The biggest hurdle to overcome is to develop a reliable method to evaluate the quality of the generated embryos in order to select the best candidate for transfer. As a result, research focusing on the identification of a non-invasive biomarker of embryonic quality has emerged as an active field. By now, different non-invasive *in vitro* methods based on the analysis of conditioned embryo culture media, within the areas of metabolomics, proteomics, and small non-coding RNA, have been tested [20]. However, so far, no biomarker clearly stands out as an early, consistent and sensitive way to predict the embryonic developmental or transfer success.

It has been shown that *in vitro* produced preimplantation embryos of several mammalian species, including bovine [21], porcine [22], murine [23] and humans [24] secrete EVs. Giacomini et al. (2017) demonstrated that the EVs secreted by human embryos were uptaken by the primary human endometrial epithelial and stromal cells. Pavani et al. (2018) further illustrated that when EVs isolated from the bovine embryo conditioned media were supplemented to the embryo culture systems, they were internalized by the embryos; thus, providing evidence of embryo-embryo interactions via EVs [25]. Both these findings support the role of EVs in intercellular communications in mammalian preimplantation embryogenesis.

Melisho et al. (2017) reported the release of EVs by individually cultured bovine blastocysts produced by *in vitro* fertilization (IVF) and parthenogenetic activation (PA), during the days 7–9 of *in vitro* development. They found out that the concentration of vesicles, released between day 7–9 of development, was higher in IVF blastocysts with arrested development between day 9–11 of *in vitro* culture compared to competent PA blastocysts [21]. This indicated that the quality of embryos influences the release of EVs during embryonic development. Moreover, the same group recently investigated the release of EVs from individually cultured embryos during blastulation [26]. As these studies evaluated the EVs derived from the bovine embryos at a later stage, i.e., blastocyst stage, of preimplantation embryo development, it is still unknown if EVs are released at earlier stages of bovine embryonic development.

Cell culture media, including embryo culture media, can contain EVs and other NPs deriving mainly from the supplemented serum or serum derivatives, such as bovine serum albumin (BSA). Therefore, the depletion of these EVs from culture media is recommended when used for EV research [27]. However, even under EV depleted culture media conditions, it is difficult to quantify the exact proportion of remaining EVs out of all NPs due to the limitations of the technology. Also, the effects of EV depletion on the viability of individually cultured zygotes are yet to be verified. Adding to this, Pavani et al. (2018) cultured bovine embryos in groups using culture media supplemented with EV-depleted BSA (by ultracentrifugation) and regular BSA [25]. Although the blastocyst rates were unchanged, a significant impairment of the embryo quality, as measured by the total and inner cell mass cell numbers of the blastocysts, grown with EV depleted media, was noted indicating that some of the vital factors derived from the BSA might have been lost during the ultracentrifugation procedure. Nevertheless, the effects of EV depletion of BSA on individual embryonic culture systems remain to be established.

EVs in the culture media conditioned by embryos may vary depending on the developmental stage and the quality of the embryos. These differences could be apparent in terms of the quantity or the size of the EVs secreted, or in terms of the molecular cargo, such as proteins and nucleic acids. This way, these EVs could serve as a potential biomarker of embryonic quality and their prospective development. As these EVs can reflect the functional and physiological status of the developing embryos, they could potentially complement the morphology-based assessment of the embryo quality through a non-invasive embryo quality assessment. Therefore, the aims of the current study were firstly, to study the effects of depletion of EVs from the culture media on the developmental potential of individually cultured bovine embryos and secondly, to isolate and characterize EVs from culture media conditioned by *in vitro* produced and individually cultured bovine embryos, based on their quality, developmental stage, and prospective development.

2. Materials and methods

2.1. In vitro embryo production (IVP)

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich/Merck (Germany or USA). Bovine embryos were produced as previously described by Nõmm et al. (2019) with modifications of the embryo culture system [28]. Abattoir-derived cattle ovaries (*Bos taurus*) were transported to the laboratory in 0.9% sterile NaCl solution within 4 h after the sacrifice at ~32–37 °C and washed twice in a freshly prepared 0.9% NaCl solution.

Cumulus-oocyte complexes (COCs) were aspirated from follicles with a diameter of 2–8 mm using a vacuum pump (Minitüb GmbH, Germany). Quality code 1 COCs [29] were washed and matured in groups of 50 in 500 μl of IVM-medium (supplemented with 0.8% fatty acid-free BSA fraction V in 4-well plates (Nunc, Roskilde, Denmark). The COCs were incubated at 38.5 °C with 5% CO_2 in humidified air for 22–24 h.

Frozen-thawed semen was used to fertilize the matured COCs. Washed spermatozoa were diluted to the final concentration of 1×10^6 motile sperms per ml. The COCs and sperms were co-incubated in groups of 50 in 500 μl of Fert-TALP media in 4-well plates at 38.5°C with 5% CO_2 in humidified air for 18–20 h.

Cumulus cells were removed from the presumptive zygotes by vortexing, and the denuded embryos were transferred individually into 60 μl droplets of modified Synthetic Oviduct Fluid with amino acids and myo-inositol (SOFaaci) containing 0.8% BSA under mineral oil [28], for single embryo culture, which allowed sampling at different time points. The presumptive zygotes were cultured at 38.5 °C, 5% CO_2 and 90% N_2 with humidified air for eight days. Embryos were morphologically evaluated at day 2, 5 and 8 post-fertilization, and the developmental stages and embryo quality were recorded [29]. The three distinct development stages were: cleavage, morula and blastocyst stage.

2.2. Collection, storage, and categorization of embryo conditioned media

Conditioned media samples (50 μl) were collected at day 2 (cleavage stage), day 5 (morula stage), and day 8 (blastocyst stage) post-fertilization from different batches of individually cultured bovine embryos. After collecting the conditioned media, the embryos at day 2 and 5 were continuously cultured in the remaining 10 μl culture media droplet until day 8. The collected conditioned and control media were stored at −80 °C until further experiments.

Based on the morphological evaluation of the embryos on days 2, 5 and 8 post-fertilization, the collected conditioned media samples were categorized, and the samples relevant to the study were defined as follows.

Conditioned media collected at day 2: media conditioned by embryos cleaved by day 2 and subsequently developed to blastocysts by day 8 (hereafter referred as “Day 2 good quality embryo media”), and media conditioned by embryos that cleaved by day 2 but subsequently degenerated (hereafter referred as “Day 2 bad quality embryo media”).

Conditioned media collected at day 5: media conditioned by embryos that developed to morula by day 5 and subsequently developed to blastocysts by day 8 (hereafter referred as “Day 5 good quality embryo media”), and media conditioned by embryos developed to morula by day 5 but subsequently degenerated (hereafter referred as “Day 5 bad quality embryo media”).

Conditioned media collected at day 8: media conditioned by embryos that developed to blastocysts by day 8 (hereafter referred as “Day 8 good quality embryo media”), and media conditioned by embryos developed to morula by day 5 but subsequently degenerated (hereafter referred as “Day 8 bad quality embryo media”).

In parallel, culture media samples were incubated for 2, 5 and 8 days, without embryos and labeled as “Day 2 control”, “Day 5 control” and “Day 8 control,” respectively.

2.3. Benchmarking qEVsingle® size exclusion chromatography columns

Commercial size exclusion chromatography columns (qEVsingle/70 nm by Izon Sciences, UK, product code SP2), specially designed to isolate EVs, were used for isolation of EVs from embryo conditioned media and controls. Initially, a column was benchmarked using RPMI-1640 media supplemented with 10% FBS to verify the performance of the qEV columns and the fractions which contained the EVs and the ones that contained proteins. In brief, 2 ml of complete media (RPMI-1640 media supplemented with 10% FBS), which were known to contain EVs due to the supplementation of FBS, was subjected to sequential centrifugation at 400g for 10 min and at 2000 g for 10 min to remove any larger particles. Subsequently, the media was concentrated up to 150 μl using Amicon® Ultra-2 10 K centrifugal filters (Merck Millipore Ltd, Ireland, catalog number UFC201024) at 3200 g for 40 min. The concentrated sample was used for EV purification using a qEVsingle column, while each eluted fraction (200 μl) was collected separately. The concentration of the EVs in each fraction was measured using ZetaView® nanoparticle tracking analyzer (PMX 120 by Particle Metrix GmbH, Inning am Ammersee, Germany). Similarly, the protein concentrations in each of the eluted fractions were measured using the Modified Lowry Protein assay kit (Thermo Scientific, U.S.A, catalog number-23240) according to the manufacturer’s protocol.

2.4. Isolation of EVs from the embryo conditioned media and controls

Even though embryos are unlikely to introduce dead cells or larger particles such as apoptotic bodies to the conditioned media due to the *zona pellucida* (ZP), sequential centrifugation was carried out to remove such potential cells or bigger particles as recommended as such particles could clog the qEVsingle® columns, and affect the purification process. Dulbecco’s phosphate-buffered saline (DPBS, Sigma® Life Science, UK, catalog number D8537) was filtered using 0.2 μm Ministart® syringe filters and was used freshly for sample dilution and EV isolation. Firstly, the culture media samples stored at −80 °C, were thawed on ice, while 45 μl of media were diluted in sterile-filtered DPBS to a final volume of 150 μl. These samples were then subjected to double centrifugation steps. Initially, the diluted samples were centrifuged at 400 g for 10 min at 4 °C to remove any dead cells and debris, while 145 μl from the supernatant was transferred to another fresh tube. The collected supernatants were centrifuged at 2000 g for 10 min to remove any apoptotic bodies. After centrifugation, 140 μl of the supernatant was transferred to another new tube and kept in ice until the isolation of EVs.

A standard protocol recommended by the Izon Science Ltd. was followed during the whole process of EV-isolation using qEVsingle size exclusion columns. Briefly, the columns were mounted vertically in a holder and were equilibrated by running through 10 ml of fresh filtered (0.2 μm) elution buffer (DPBS). Then, the sample (140 μl of media) was added to the top of the column, and fraction (200 μl) collection was initiated immediately. When the sample leveled with the upper column filter, the column was topped up with the elution buffer. The first five fractions (total of 1000 μl), which was the void volume, were collected together and discarded. Fractions 6–9 (total of 800 μl) were collected and pooled as EVs elute in these fractions should there be any in the sample. The size and the concentrations of EVs in the pooled fractions were then determined using nanoparticle tracking analyzer-ZetaView®.

2.5. Nanoparticle tracking analysis (NTA)

The measurement of the concentration and size profile of NPs of the samples were carried out using NP tracking analyzer-Zetaview®. Zetaview® was calibrated using 100 nm particle size standards (Applied Microspheres BV, Netherlands. Catalogue no. 10100). Before sample measurements, the concentration of the

filtered DPBS was measured to confirm the purity (i.e. minimum amounts of NPs), of the DPBS used. The NP concentration and the size profiles of the samples were measured in scatter mode under the following settings: sensitivity: 75, shutter: 100, frame rate: 30 fps and number of cycles: 3. Each biological sample was measured in triplicates. In between measurements, the measurement cell of the instrument was thoroughly washed with Milli-Q® water and DPBS before the injection of the next sample in order to minimize the inter-sample contamination.

2.6. High throughput multiplexed phenotyping of EVs (EV Array)

Characterization of the EVs, based on the EV markers, was carried out using EV Array [30]. IVC media conditioned by individually cultured day 5 bovine embryos that developed to morula (n = 40, 2 ml when pooled) and IVC media incubated till day 5 without embryos as control (n = 40, 2 ml when pooled) were used. Each sample was subjected to sequential centrifugation, as previously described, to deplete potential larger particles. From both embryo conditioned and the control media, 100 μl fractions were transferred to two separate tubes and labeled as media before EV isolation (sample and control). The rest of the conditioned and the control samples were subjected to EV isolation using qEVsingle columns. Fractions 6–9 were collected, pooled and concentrated to a final volume of 100 μl using Amicon® ultra-2 10 K centrifugal filters at 3200 g for 30 min at 4 °C. All samples were stored at −80 °C till used for EV protein profiling by EV Array.

Microarray slides were produced for the EV Array using a sciFLEXARRAYER S12 (Scienion AG, DE). Shortly, antibodies were printed on epoxy-coated slides (SCHOTT Nexterion, Germany) with a coated PDC3 size 60 (Scienion AG). Positive and negative controls were biotinylated human IgG (100 mg/ml) and PBS with 5% glycerol, respectively. One anti-bovine antibody (CD63, clone CC25, BioRad, CA, USA) together with a total of 21 anti-human antibodies, were used for production of the EV Array and listed in the following with the corresponding clone, if available: EGFR (Antibodies-online.com, Germany); Hsp 90 (IGF1), p53 (pAb 240), Flotillin-1, TSG101 (Abcam); CD63 (AbD serotec, UK); CD9 and CD81 (Ancell, MN, USA); Alix (3A9, Biolegend, CA, USA); HLA-G (87G, Novus Biologicals, CO, USA); Annexin V, Cathepsin D, Tspan8 (458811), CD82 (423524), CD151 (210127), Hsp 70 (242707), and LAMP-1 (R&D Systems, MN, USA); EpCam (0.N.277), GRP78 (N-20) and AKAP (C-20, Santa Cruz Biotechnologies, TX, USA). Antibodies were diluted in PBS with 5% glycerol and printed in triplicates at 200 mg/ml.

The EV Array was performed as described by Jørgensen et al. (2013), with modifications. In short, the microarray slides were initially blocked (50 mM ethanolamine, 100 mM Tris, 0.1% SDS, pH 9.0) prior to incubation with 40 μl sample diluted in wash-buffer (PBS/0.05% Tween®20). The incubation was performed in Multi-Well Hybridization Cassettes (ArrayIt Corporation) at RT for 2 h followed by overnight incubation at 4 °C. Biotinylated anti-bovine antibody against CD9 (Antibodies-Online.com, DE) was diluted 1:1500 and used to detect retained EVs using Cy5-labeled streptavidin (Life Technologies, MA, USA) diluted 1:1500. Scanning and spot detection was performed as previously described [31].

2.7. Transmission electron microscopy (TEM)

Embryo culture media conditioned by *in vitro* cultured single bovine embryos that developed to morula stage were collected at day 5 and pooled together (n = 60, 3 ml). As the control, embryo culture media (3 ml) incubated for 5 days without embryos were used. The pooled conditioned media and control media were subjected to sequential centrifugation at 400 g for 10 min and 2000 g for 10 min to remove any existing larger particles. Subsequently, the supernatants were concentrated to 150 μl using Amicon® Ultra-15 10 K centrifugal filters (Merck Millipore Ltd. Ireland, catalog number UFC901024) by centrifuging at 3200 g for 45 min. From the concentrated media, EVs were isolated using qEVsingle columns, as described previously. Fractions 6–9 were collected, pooled and subsequently, concentrated using Amicon® ultra-2 10 K centrifugal filters by centrifuging at 3200 g for 80 min to a final volume of 60 μl. A droplet of the purified EV samples from the conditioned media and control were placed on formvar/carbon-coated 200 mesh grids (Agar Scientific, Stansted, UK) and allowed to adsorb for 20 min. Next, samples were fixed in Karnovsky fixative (2% paraformaldehyde and 1% glutaraldehyde; Sigma-Aldrich, Germany; Polysciences, USA, respectively) before being contrasted in uranyl oxalate [mixture of 4% uranyl acetate (Polysciences, Warrington, USA) and 0,15 M oxalic acid (Sigma-Aldrich, Schnelldorf, Germany)] and embedded in a mixture of methylcellulose (Sigma-Aldrich, Schnelldorf, Germany) and uranyl acetate (Polysciences, Warrington, USA). Samples were observed with a JEM 1400 transmission electron microscope (JEOL Ltd. Tokyo, Japan) at 80 kV, and digital images were acquired with a numeric camera (Morada TEM CCD camera, Olympus, Germany).

2.8. Scanning electron microscopy (SEM)

Embryo culture media conditioned by *in vitro* cultured single bovine embryos that developed to the morula stage were collected on day 5 and pooled together (n = 30, 1.5 ml). The pooled conditioned media were then subjected to sequential centrifugation at 400 g for 10 min and 2000 g for 10 min to remove any existing dead cells or larger particles. Subsequently, the media was concentrated to 150 μl using Amicon® Ultra-2 10 K centrifugal filters by centrifuging at 3200 g for 30 min at 4 °C. From the concentrated media, EVs were isolated using qEVsingle columns, as described previously. Fraction 6–9 were collected together and subsequently concentrated using Amicon® ultra-2 10 K centrifugal filters (3200 g for 60 min) to a final volume of 60 μl. The concentrated sample was frozen at −80 °C and transferred to a scanning electron microscopic (SEM) facility. The sample was thawed and a drop of the isolated EV sample was left on an aluminum foil for overnight drying and was imaged the following day in a Hitachi S-4300 SEM microscope after sputter coating the samples with gold.

2.9. Statistical analysis

Log-rank test was used to compare the survival distributions of embryos in EV depleted and regular IVC media culture settings. Obtained p-values were corrected for multiple testing with Bonferroni correction. Linear mixed models (LMM) fit via residual maximum likelihood (REML) approach were used to test for statistically significant differences in the concentration, and the average size of NPs observed in the three experimental groups and developmental stages. The LMMs were fit being nested for the three developmental stages with experimental replicate (or batch) as the mixed effect to test for differences between experimental groups in their respective developmental stage. P-values were obtained from t-tests on the resulting estimated marginal means (EMMs) and were further subjected to Tukey adjustments. Statistical analysis was conducted in R [32] using packages lme4 [33] and emmeans [34]. The means of the 3 technical measurement replicates for each biological sample were used in these tests. In order to compare nanoparticle concentrations in specific size ranges (bins), the nanoparticle concentrations in each size bin were normalized beforehand by dividing by the sum concentration of all size bins for the given sample. This resulted in normalized concentration values, i.e. fraction of total concentration in each size bin for the given sample.

2.10. Experimental design

Two experiments were carried out to accomplish the objectives. In the first experiment, the effects of EV-depletion on individual and grouped embryonic development was assessed. During the preparation of IVC media, EVs in BSA were depleted by ultrafiltration. BSA was ultrafiltered, using Amicon® Ultra-15 10 K centrifugal filters, by centrifuging at 3600*g* for an hour under sterile conditions. The flow-through of the filtration was used when supplementing BSA to the culture media. Such prepared EV-depleted IVC media was used to culture embryos individually and in groups for 8 days. As a control, embryos were cultured individually in regular IVC media (without EV-depletion) for 8 days.

In the second experiment, presumptive zygotes produced *in vitro* were cultured individually in regular IVC media to isolate and quantify the EVs from the conditioned media based on the development stage and prospective embryonic development. Presumptive zygotes were individually cultured in IVC media under mineral oil for 8 days, and their development was assessed at day 2 (cleavage stage), day 5 (morula stage) and day 8 (blastocyst stage) post-fertilization. Fifty μl of media, conditioned by embryos, were collected at day 2 ($n = 35$), day 5 ($n = 35$) and day 8 ($n = 35$) post-fertilization and stored at −80 °C. After collecting the conditioned media, the embryos were cultured in the remaining 10 μl of media until day 8. The collected conditioned media samples were categorized based on embryonic development up to day 8. This experiment was carried out in three replicates using oocytes collected on three different days. Similarly, all embryos were cultured individually to characterize the EVs isolated from conditioned media by EV Array, nanoparticle tracking analysis, transmission and scanning electron microscopy.

3. Results

3.1. Experiment 1: evaluation of individual and grouped embryo development in EV-depleted and regular IVC media

In this experiment, presumptive zygotes were cultured both individually and in groups in EV-depleted media and individually in regular IVC media to assess the impact of EV depletion on individual and grouped embryonic cultures. The results showed that although 54.5% of embryos reached the morula stage, only 3% reached the blastocyst stage during the individual embryo culture in EV-depleted IVC media (Table 1). In contrast, 39.3% of the individually cultured embryos in regular IVC media reached the blastocyst stage. The overall survival of individually cultured embryos was found to be significantly poorer ($p = 0.013$, log-rank test, Bonferroni correction) when cultured in the EV-depleted media compared to regular IVC media. Interestingly, the survival of embryos in the EV-depleted media improved significantly ($p = 0.014$, log-rank test, Bonferroni correction) when cultured in a group compared to single embryo cultures. Embryos cultured in groups in EV-depleted medium were able to develop to the blastocyst stage at a rate similar to individual embryos in regular IVC media.

3.2. Experiment 2: individual embryo culture for isolation of EVs and their characterization

3.2.1. Individual culture of bovine embryos

Presumptive zygotes were cultured individually in 60 μl of regular IVC media under mineral oil until day 8 post-fertilization. Subsequent to the collection of 50 μl of conditioned media at day 2 or day 5, those embryos were allowed to culture in the remaining 10 μl media till day 8. For each time point, a total of 105 presumptive zygotes were cultured individually (35 zygotes for each of the three replicates). Embryo development was assessed based on morphological parameters.

In the day 2 group, 25% of the zygotes had developed into blastocysts, and 27% had cleaved by day 2, but had subsequently degenerated (Table 2). In the day 5 group, 28% of the zygotes had developed into blastocysts, and 17% had degenerated after developing into a morula by day 5. In the case of embryos at the time-point of day 8 post-fertilization, while 23% of the zygotes had developed to blastocysts, 21% of the zygotes had degenerated after developing into a morula by day 5. Therefore, the blastocyst rate did not seem to be affected by the collection of 50 μl of media before day 8.

3.2.2. Benchmarking qEVsingle size exclusion chromatography columns

Before isolating the EVs from embryo conditioned media, a qEVsingle® column was benchmarked using cell culture media known to contain EVs. Benchmarking confirmed the clear separation of the EVs from soluble proteins in the culture media (Fig. 1) as the result of size exclusion chromatography. Based on this observation, it was decided to collect fractions 6–9 to isolate the EVs from embryo-conditioned media and controls.

3.2.3. Characterization of the EVs isolated from culture media conditioned by individually cultured bovine embryos

EV samples isolated from culture media conditioned by individually cultured bovine embryos were characterized by EV Array, NTA, TEM, and SEM.

The EV Array technology was used to phenotype EVs using EV markers. Twenty-two different antibodies against EV-, surface- or surface-associated markers were used to capture EVs onto a microarray (EV Array). Detection of the captured EVs was performed using an anti-bovine CD9 antibody. The EV Array experiment showed that NPs isolated from media conditioned by embryos were strongly positive for CD9 and CD81. Weak signals were also detected for CD63 (using both anti-human and anti-bovine antibodies) together with CD82, p53, Alix and HLA G (Fig. 2A and B). In contrast, particles isolated from control culture media (incubated for 5 days without embryos) were only very weakly positive for some of the markers such as CD9, CD81, CD82, p53, Alix and HLA G and therefore the presence of EVs in these samples cannot be confirmed. Moreover, the enrichment of EVs, as shown by the signal intensity for EV markers, following EV isolation by qEVsingle is evident when comparing sample I and sample II. Similarly, a weak increase in the fluorescent signal intensity was observed for control media samples when comparing the sample III and IV.

Fig. 3 illustrates the average size distributions of EVs isolated from embryo conditioned media and NPs isolated from control media of day 2, 5, and 8 samples. Most of the particles were distributed within the 30–330 nm size range.

Culture media conditioned by 60 individually cultured bovine embryos that developed to morula by day 5 (day 5 media) were pooled and the isolated EVs were used for TEM based characterization (Fig. 4A). TEM imaging visualized EVs and most of these particles were in the size range of 50–150 nm (supplementary materials, S1). Similarly, culture media conditioned by 30 individually cultured bovine embryos that developed to morula by day 5 (day 5 media) were used for SEM-based characterization and confirmed the spherical shape of the EVs (Fig. 4B).

3.2.4. Mean concentrations and sizes of EVs are associated with embryo quality

When presenting and comparing the mean concentrations and diameters of particles that were measured using NTA, particles

Table 1
Individual and group culture of embryos in EV-depleted and regular IVC media.

Individual and group culture of embryos in EV-depleted and regular IVC media.

	Individual embryos cultured in regular IVC media	Individual embryos cultured in EV-depleted IVC media	Group culture of embryos in EV-depleted IVC media
	n (%)	n (%)	n (%)
Oocytes	33 (100)	33 (100)	63 (100)
Zygotes	31(93.9)	27 (81.8)	53 (84.1)
Morula	20 (60.6)	18 (54.5)	37(58.7)
Blastocysts	13 (39.3)	1 (3.0)	25 (39.6)
p-value		0.013*	0.014**

Data are represented as numbers (n) or percentages (%). IVC- *in vitro* culture. IVC media consisted of modified synthetic oviductal fluid with amino acids, and myo-inositol (SOFaaci) supplemented with 0.8% BSA. For EV depletion, the dissolved BSA used to supplement IVC media was ultrafiltered using Amicon®- 15 10k filters by centrifuging at 3200 g. * p-value resulting from Log-rank test for the overall survival of individually cultured embryos in EV-depleted IVC media compared to regular IVC media. ** p-value resulting from Log-rank test for the overall survival of group cultured embryos in EV-depleted IVC media compared to individually cultured embryos in EV-depleted IVC media.

Data are represented as numbers (n) or percentages (%). IVC- *in vitro* culture. IVC media consisted of modified synthetic oviductal fluid with amino acids, and myo-inositol (SOFaaci) supplemented with 0.8% BSA. For EV depletion, the dissolved BSA used to supplement IVC media was ultrafiltered using Amicon®- 15 10 k filters by centrifuging at 3200 g * p-value resulting from Log-rank test for the overall survival of individually cultured embryos in EV-depleted IVC media compared to regular IVC media. ** p-value resulting from Log-rank test for the overall survival of group cultured embryos in EV-depleted IVC media compared to individually cultured embryos in EV-depleted IVC media.

isolated from embryo conditioned media were denoted as EVs and particles isolated from control media were denoted as NPs. This is because particles isolated from embryo conditioned media were proven to contain EVs and particles isolated from control media were not (based on the results of the EV Array).

At day 2, the mean concentration of EVs isolated from day 2 bad quality embryo media (8.25×10^8/ml) was 1.41-fold higher compared to that of day 2 good quality embryo media (5.86×10^8/ml, $p < 0.05$) (Fig. 5A and B). Moreover, at day 2, the mean concentration of the NPs isolated from the control media (8.65×10^8/ml) was as high as the mean EV concentration of day 2 bad quality embryo media. In contrast, at day 5, there was no difference in the mean EV concentrations between day 5 good quality and day 5 bad quality embryo media. However, at day 8, the mean concentration of EVs isolated from day 8 bad quality embryo media (7.17×10^8/ml) was 1.26-fold higher compared to that of day 8 good quality embryo media (5.68×10^8/ml, $p < 0.05$) (Fig. 5A and C). Furthermore, a gradual drop in the NP concentrations in the control media (8.65×10^8/ml, 6.47×10^8/ml, and 5.37×10^8/ml at day 2, day 5 and day 8 respectively) was observed as the incubation duration prolonged.

The size profiles of EVs isolated from embryo conditioned media and NPs isolated from control media were further analyzed to determine if there were differences between groups in specific size ranges. Particles in size ranges of 61–90 nm, 91–120 nm, and 121–150 nm were compared as this is the size range in which the NTA results can be expected to be most accurate. Furthermore, this size range corresponds to the expected size range of exosomes. At day 2, statistically significant differences were noted between good quality embryo media and control media concerning 61–90 nm and 91–120 nm sized particles (Fig. 6B and C and between good quality embryo media and bad quality embryo media with regards to 91-120 nm sized particles (Fig. 6C). Moreover, at day 8, differences were noted between good and bad quality embryo media in terms of 61–90 nm and 91–120 nm sized particles (supplementary materials, S2). However, on day 5, no differences were observed between any of the groups based on any of the size ranges analyzed (supplementary materials, S3).

Interestingly, significant differences in the mean diameters of the EVs isolated from the media conditioned by individually cultured bovine embryos and NPs isolated from controls were observed at day 2 and day 8 (Fig. 7),). At day 2, the mean (±CI) diameter of EVs isolated from good quality and bad quality embryo conditioned media were 166 ± 9 nm and 159 ± 8.8 nm, respectively and were higher compared to the mean concentration of NPs isolated from the control media which was 150 ± 9 nm ($p < 0.05$). In

Table 2
Morphological assessment of embryo development.

Timepoint of media collection	Day 2	Day 5	Day 8
Presumptive zygotes (n)	35	35	35
Not cleaved by day 2 (%)	26.66 ± 2.05	23.80 ± 3.10	24.76 ± 3.11
Cleaved by day 2, but subsequently degenerated (%)	26.66 ± 4.11	31.42 ± 3.56	31.42 ± 4.85
Developed to morula by day 5, but subsequently degenerated (%)	21.90 ± 1.55	17.14 ± 1.34	20.95 ± 3.39
Developed to blastocysts by day 8 (%)	24.76 ± 2.05	27.61 ± 2.80	22.85 ± 4.85

Data are represented as counts (n) or mean ± SEM of percentage. At each time point, conditioned media were collected from 105 samples (n = 35, triplicates for each time point of media collection). Embryo quality was assessed based on the morphological parameters.

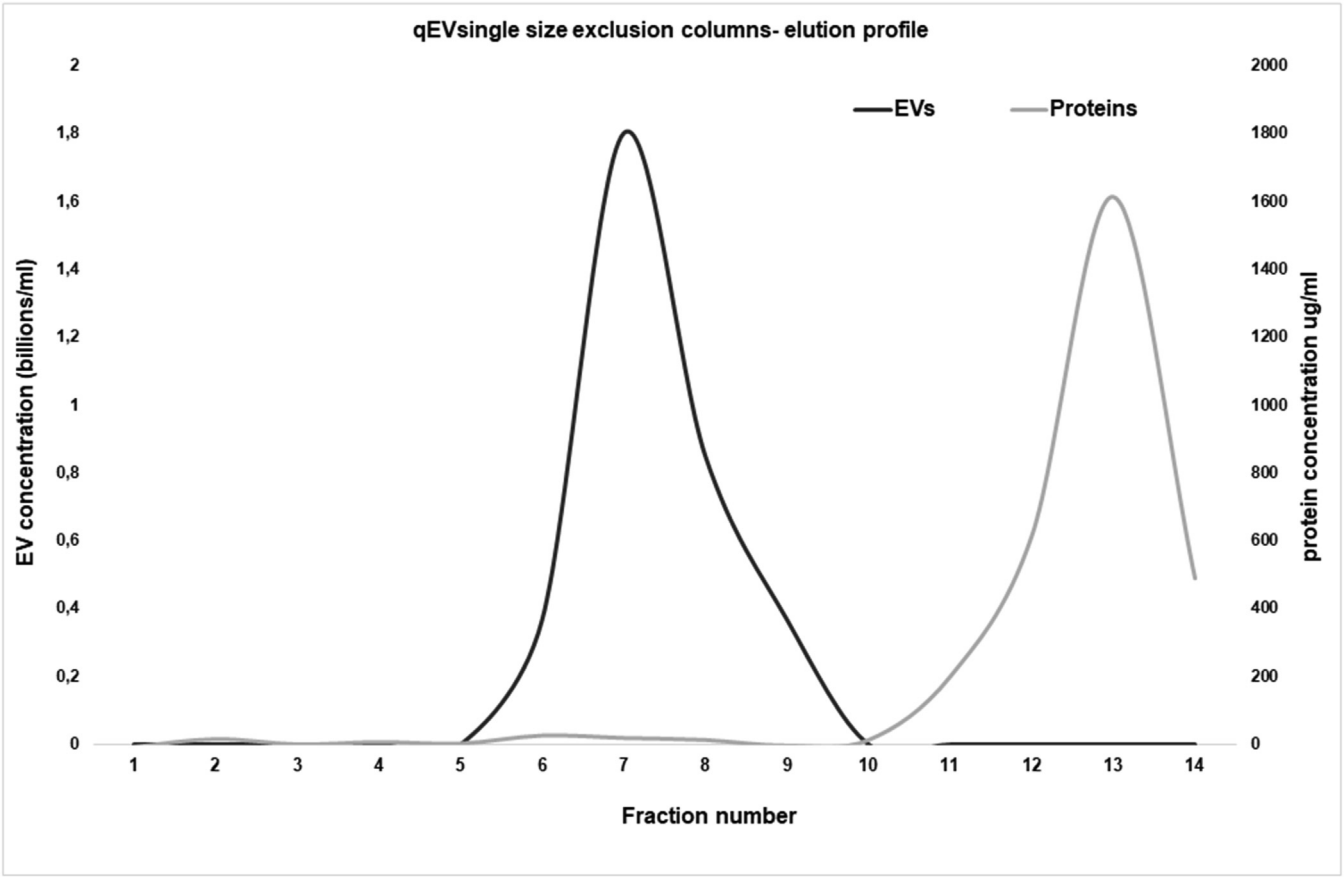

Fig. 1. Elution profile of qEVsingle size exclusion chromatography columns. The elution profile of the column was tested using RPMI-1640 supplemented with 10% FBS. Fractions 7–8 were enriched with EVs, while fractions 6 and 9 had detectable quantities of EVs. Proteins were only detectable after the 10th fraction and peaked at fraction 13. Each fraction is 200 μl.

contrast, on day 5, there were no differences in the mean diameters of EVs isolated from the good quality and bad quality embryo media. However, at day 8, the mean (±CI) diameter of EVs isolated from good quality embryo media (163 ± 7 nm) was higher compared to that of bad quality embryo media (154 ± 11 nm) ($p < 0.05$). Moreover, a gradual increase in the mean diameters of the NPs isolated from control media, as the incubation duration prolonged, was observed.

4. Discussion

Isolation of EVs from culture media conditioned by single bovine embryos is challenging due to the low sample volume and the limitations of the isolation and detection technologies. In this study, primarily, we attempted to isolate and characterize the EVs in the media conditioned by single bovine embryos along the entire preimplantation development i.e. 2 cell, morula and blastocyst stages. However, Melisho et al.(2017) isolated EVs from individually cultured bovine blastocysts, which were released between day 7–9 of *in vitro* development [21]. Moreover, those embryos had been cultured in groups till day 7 to select the blastocysts to culture them individually from day seven onwards. Moreover, recently, the same group further investigated the release of EVs from individually cultured bovine embryos during blastulation [26].

According to the guidelines of the International Society for Extracellular Vesicles (ISEV), depletion of EVs from culture media is expected to be carried out when such media are used for cell culture in EV-based research [27]. It has been previously shown that EV-depletion has an impact on the development of embryos in terms of quality [25]. As a part of this study, we carried out *in vitro* production of the embryos, both individually and in groups, in EV-depleted media to assess their development. Impaired blastocyst formation rate in EV-depleted embryo culture media (3%) compared to individual embryo culture in regular IVC media (39.3%) indicated that EV-depleted IVC media is lacking some vital elements, needed for embryos to develop to blastocysts. Due to the process of EV depletion, the media may have lost other vital factors, along with EVs, if there is any, from the media that are crucial for embryo growth. In contrast, when the embryos were cultured in groups within EV-depleted conditions, the blastocyst rate was not affected. This indicates that in the group culture systems, despite the culture media is EV-depleted, the embryo-derived EVs and other paracrine molecules would help to promote the development of embryos. Indeed, improved embryonic development had been demonstrated when *in vitro* produced (IVP) embryos were cultured in groups in many mammalian species [35,36]. Therefore, EV-depleted IVC media were not used in the subsequent individual embryo culture experiments due to: (i) the poor individual embryonic development under EV-depleted conditions; (ii) claims of the previous studies that BSA used to supplement culture media do not carry EVs [25]. Therefore, IVC media supplemented with regular BSA was used for the subsequent single embryo culture experiments.

For isolation of EVs from media, qEVsingle/70 nm SEC columns were used. These columns were designed for isolating EVs from small sample volumes of up to 150 μl and have an optimal recovery

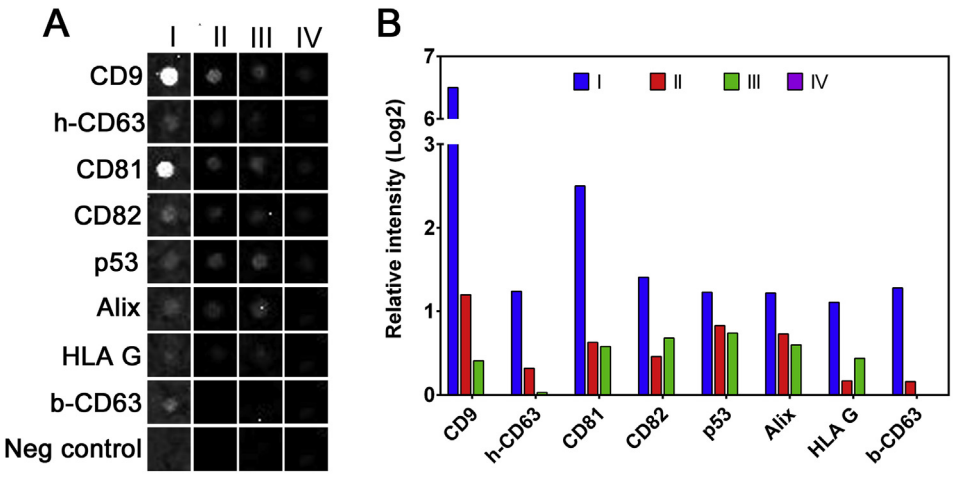

Fig. 2. Phenotyping of the EV population in bovine embryo conditioned media. EVs in the embryo conditioned media and controls were profiled using an EV Array printed with different capturing antibodies. For embryo conditioned media, culture media collected from 40 individually cultured embryos that developed to morula by day 5 were pooled (2 ml). For control media, culture media incubated for five days without embryos were pooled (2 ml). From each group, 100 μl were aliquoted and used as media before EV isolation (sample II and IV of figure B). From the remaining media samples, EV isolation was carried out, concentrated to 100 μl each (sample I and III of figure B). **(A)** Microarray spots obtained by fluorescent detection with anti-bovine CD9 antibody. Positive signals were only detected from the visualized spots. (**B)** Histogram of the log 2 transformed fluorescence intensities relative to the negative control spot. **Sample I**: EVs isolated from media conditioned by individually cultured bovine embryos for five days, **Sample II:** Culture media conditioned by individually cultured bovine embryos (before SEC), **Sample III:** NPs isolated from culture media incubated for 5 days without embryos, **Sample IV:** Culture media incubated for 5 days without embryos (before SEC).

range of 70–1000 nm. They can separate nanoparticulate materials within this size range from proteins and other particles in the media based on size differences. The benchmarking of the qEV-single SEC columns enabled to identify the fractions containing EVs while separating them from the proteins and other smaller molecules in the samples. We were able to isolate and detect nanoparticles in embryo conditioned media and the control media samples in all types of samples incubated up to days 2, 5 and 8.

The characterization of EVs isolated from embryo conditioned media was carried out using EV Array, NTA, TEM, and SEM. EV Array technology was used to identify the EVs using antibodies against EV-, surface- or surface-associated markers. The major advantage of this technology over other available methods to detect EV proteins is that it requires only a very limited quantity of samples [31]. The test was strongly positivity for CD9 and CD81 and less strongly positive for CD63 together with CD82, p53, Alix and HLA G. This confirmed the secretion of EVs to the culture media by individually cultured bovine embryos as early as day 5 of *in vitro* culture. However, in terms of NPs isolated from day 5 control media, only very weak signals were observed for some of the markers. It indicates that despite control media have higher levels of NPs, as measured by NTA, they do not respond to EV Array antibodies. Considering the high sensitivity of the EV Array, it is possible to conclude that there are no EVs in the control media. This finding corroborates with the findings of Pavani et al. (2018) who demonstrated that BSA lyophilized powder used for supplementing

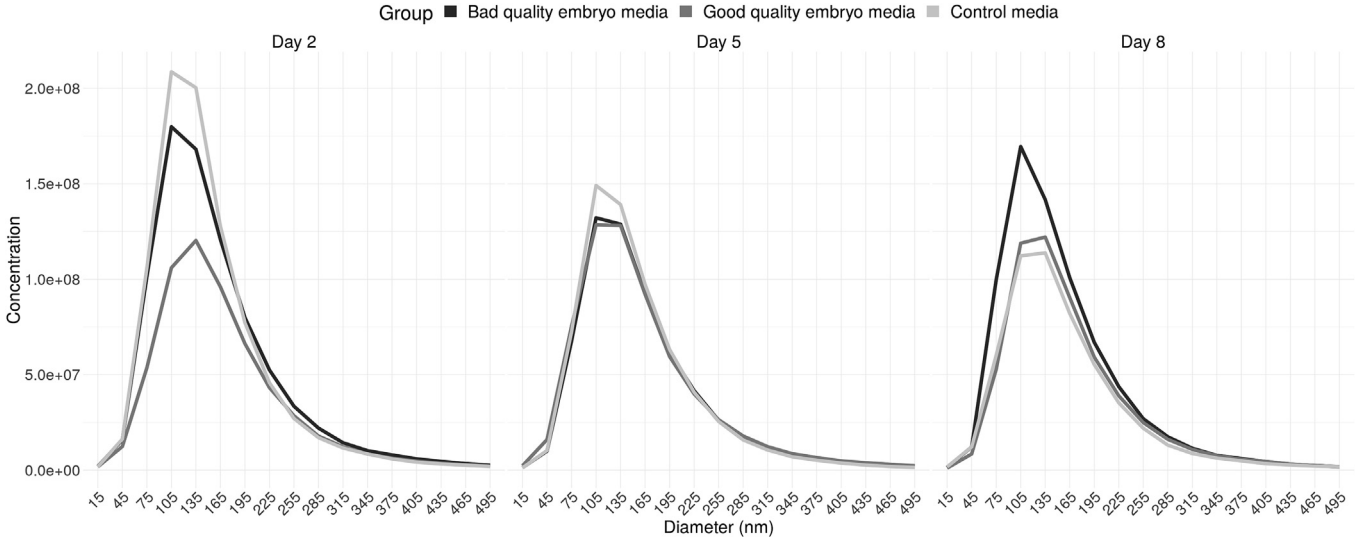

Fig. 3. Nanoparticle Tracking Analysis based characterization of average size profiles of EVs isolated from day 2, day 5, and day 8 embryo conditioned media and NPs isolated from control media samples. "Day 2 good quality embryo media" (n = 23); "Day 2 bad quality embryo media" (n = 22); "Day 2 control" (n = 15); "Day 5 good quality embryo media" (n = 25); "Day 5 bad quality embryo media" (n = 19); "Day 5 control" (n = 15); "Day 8 good quality embryo media" (n = 24); "Day 8 bad quality embryo media" (n = 20); "Day 8 control" (n = 15). Concentrations are expressed as particles/ml.

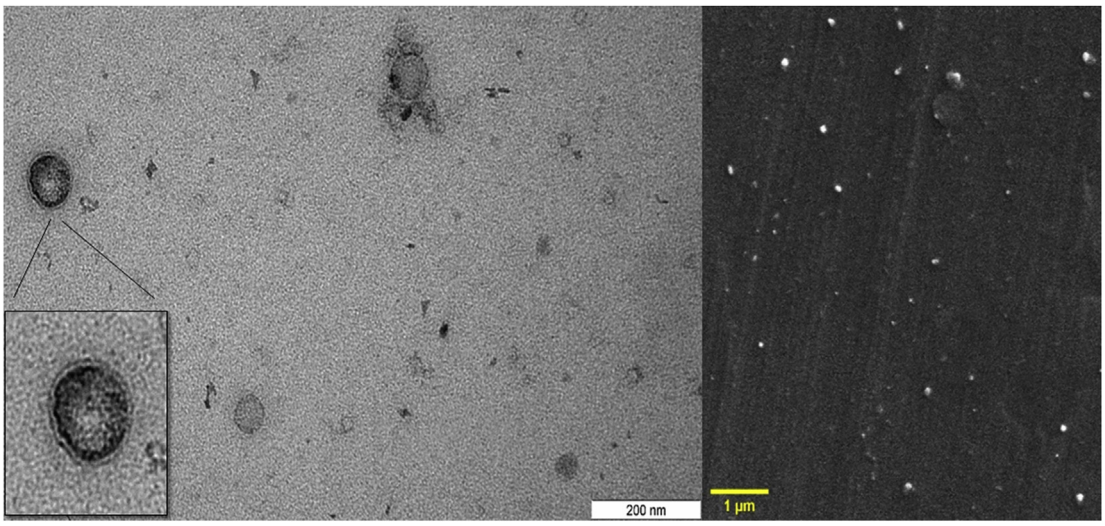

Fig. 4. Electron microscopy-based characterization of EVs. (A) TEM imaging of EVs isolated from a pool of individually cultured bovine embryo (n = 60) conditioned media developed to morula by day 5. The majority of EVs were in a size range of 50–150 nm. The magnified part of the image zoomed in to an individual EV exhibiting cup-like shape typical to EVs, that is associated with sample processing. The Scale bar is 200 nm. **(B)** SEM imaging of EVs isolated from a pool of individually cultured bovine embryo (n = 60) conditioned media developed to morula by day 5. White dots in the dark background indicate EVs which are heterogeneous in terms of their size. Scale bar is 1 µm.

IVC media did not contain EVs [25]. To characterize the BSA derived NPs, they carried out western blot assays and immunogold staining

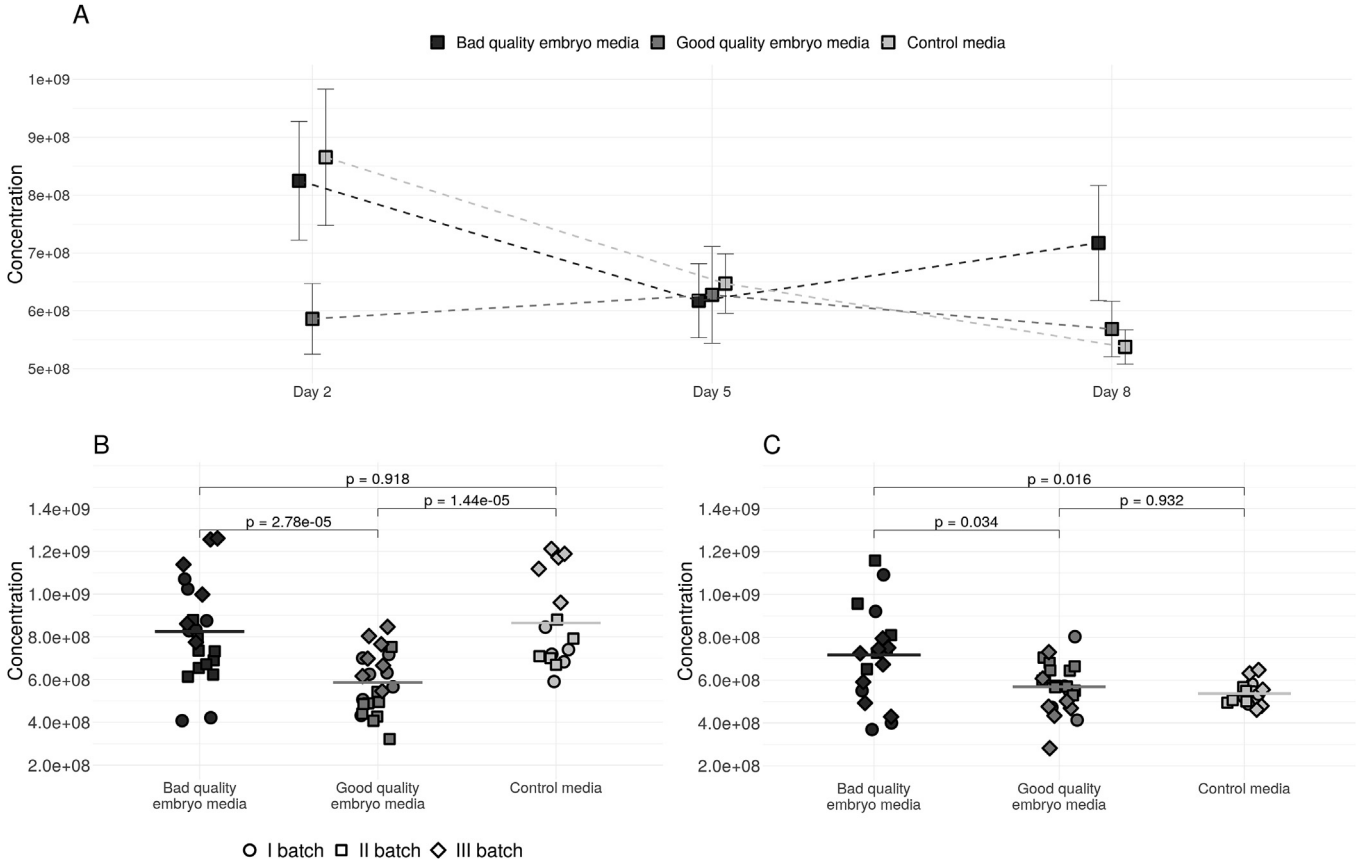

Fig. 5. Changes in the concentrations of EVs (per ml) isolated from culture media conditioned by individually cultured bovine embryos and the NPs (per ml) isolated from control media. (**A)** Mean concentrations of EVs isolated from media conditioned by individually cultured bovine embryos at day 2, 5, and 8 post-fertilization and the NPs isolated from control media. (**B–C)** Comparison of the concentrations of EVs isolated from media conditioned by individually cultured bovine embryos at day 2 and day 8, respectively. "Day 2 good quality embryo media" (n = 23); "Day 2 bad quality embryo media" (n = 22); "Day 2 control" (n = 15); "Day 5 good quality embryo media" (n = 25); "Day 5 bad quality embryo media" (n = 19); "Day 5 control" (n = 15); "Day 8 good quality embryo media" (n = 24); "Day 8 bad quality embryo media" (n = 20); "Day 8 control" (n = 15). Different shapes in **B** and **C** indicate the measurements of the samples from 3 replicates and each such shape represents the mean of the three measurements of each biological sample. Differences were considered to be statistically significant if $p < 0.05$. Error bars are 95% confidence intervals.

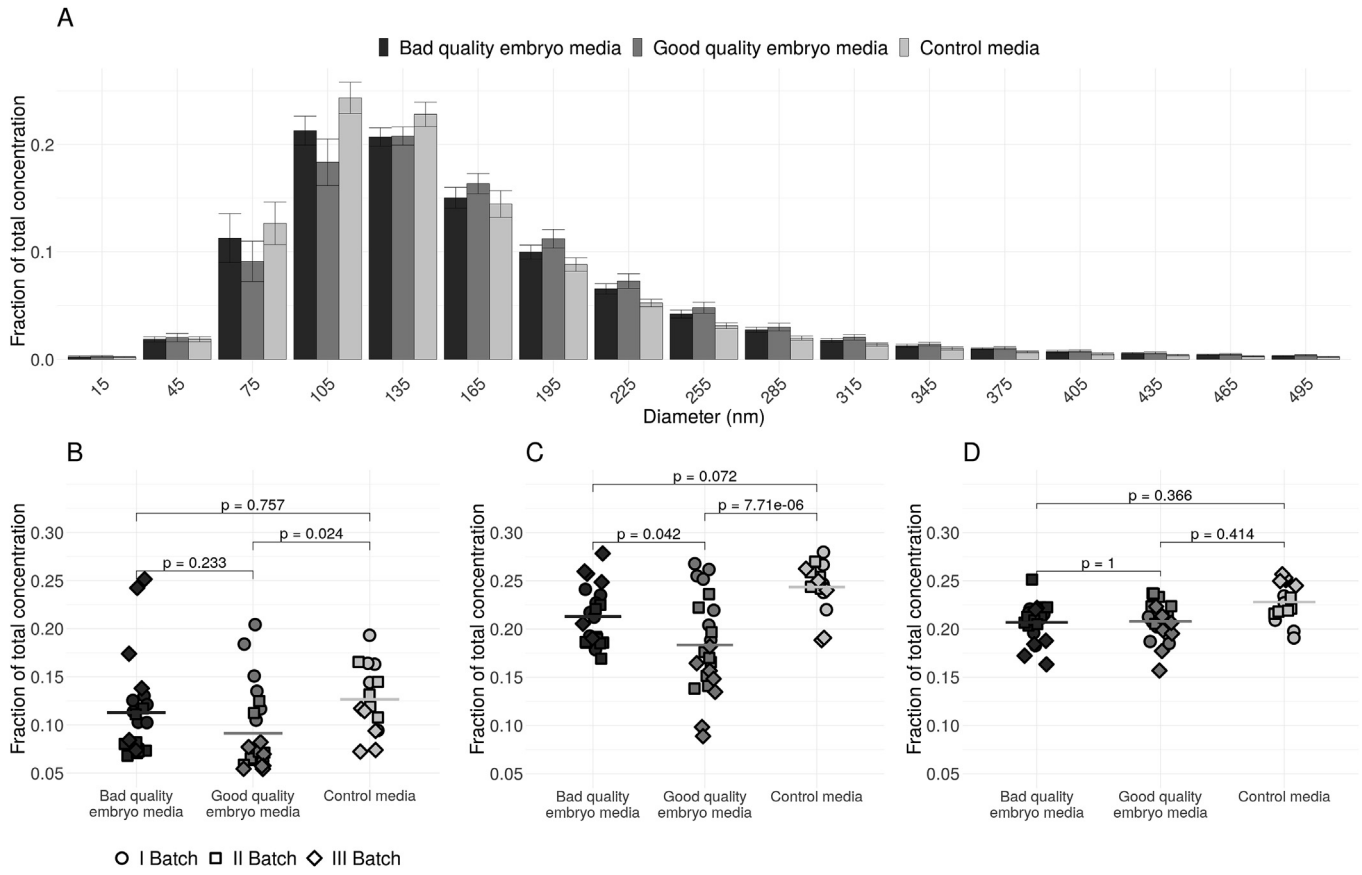

Fig. 6. Comparison of the concentrations of EVs (per ml) isolated from day 2 embryo conditioned media and the NPs (per ml) isolated from day 2 controls, based on size ranges. (**A**) The overall distribution of the particles isolated from day 2 good quality embryo media (n = 23), Day 2 bad quality embryo media (n = 22) and day 2 controls (n = 15). Data are presented as fractions of the total concentration. (B, C, D) Comparison of the concentrations of EVs/NPs isolated from day 2 embryo conditioned media and controls, based on size ranges: B; 61–90 nm, C; 91–120 nm, D; 121–150 nm. Different shapes in B, C, and D, indicate the measurement of the samples from three replicates and each such shape represents the mean of the three measurements of each biological sample. Differences between groups were considered to be statistically significant if $p < 0.05$. Error bars are 95% confidence intervals.

of these particles and concluded that BSA derived NPs were unlikely to include EVs [25]. Stolk et al. (2015) isolated EV-like NPs from BSA ('sham' BSA EVs) and tested them in comparison to the mesenchymal stem cell (MSC)-derived EVs [37]. They reported that such 'sham' BSA EVs were different from the MSC-derived EVs in terms of surface protein markers. Furthermore, the EV Array used in our study demonstrated the enrichment of EVs in samples subsequent to purification with qEVsingle columns and their concentration with Amicon® Ultra-2 10 K centrifugal filters.

NTA based characterization of the EVs isolated from day 2, 5 and, 8 embryo conditioned media showed that most of the particles are distributed within the 30–300 nm size range. Similarly, the control media also had NPs most abundantly in the same size range indicating the non-specificity of qEVsingle SEC columns for EVs. With regards to the sample measurement using NTA, in the scatter mode, it tracks nanoparticles irrespective of whether they are EVs or not. Therefore, NTA-ZetaView® can not specifically identify NPs as EVs. TEM imaging showed EVs in size range of 50–150 nm. However, EVs were not abundant in these images, which is possibly due to the low concentration of EVs in embryo conditioned media or loss during processing. The SEM also visualized nearly round-shaped EVs isolated from embryo conditioned media.

Biological analyses are evolving towards single-cell technologies, such as single-cell genomics, that provide a clearer understanding of complex biological processes at a single-cell level. Preimplantation embryos, such as morula, are composed of a limited number of pluripotent cells. Therefore, studying single embryos warrants single cell-based technologies for better results. The ISEV has proposed Minimal Information for Studies of Extracellular Vesicles ("MISEV") in 2014 and updated in 2018 [27]. As most of the EV studies are based on EVs deriving from bulk tissues and cell cultures with higher cell quantities, achieving such requirements is not much of an issue for those studies. In contrast, when dealing with single cells or a limited number of cells such as preimplantation embryos, this is a very challenging task due to the scarcity of the materials and the limitations of the EV-isolation and detection technologies.

Considering the results of SEM, TEM, and the EV Array analysis, we conclude that the NPs present in the culture media conditioned by embryos in the current investigation is indeed EVs. Hence, it is justified to use the term EVs for NPs purified from bovine embryo conditioned culture media. Such proof of evidence was not present for the NPs isolated from the control media; hence they were considered as NPs.

It was observed that the mean EV concentration of the culture media conditioned by day 2 good quality embryos were lower compared to day 2 bad quality embryos, considering the concentration of NPs in day 2 controls as the baseline/background. At day 2, the embryos of both these groups were morphologically similar though their subsequent development was different. Therefore, the concentration of EVs isolated from day 2 conditioned media foreshadowed the prospective development of the embryos, as those

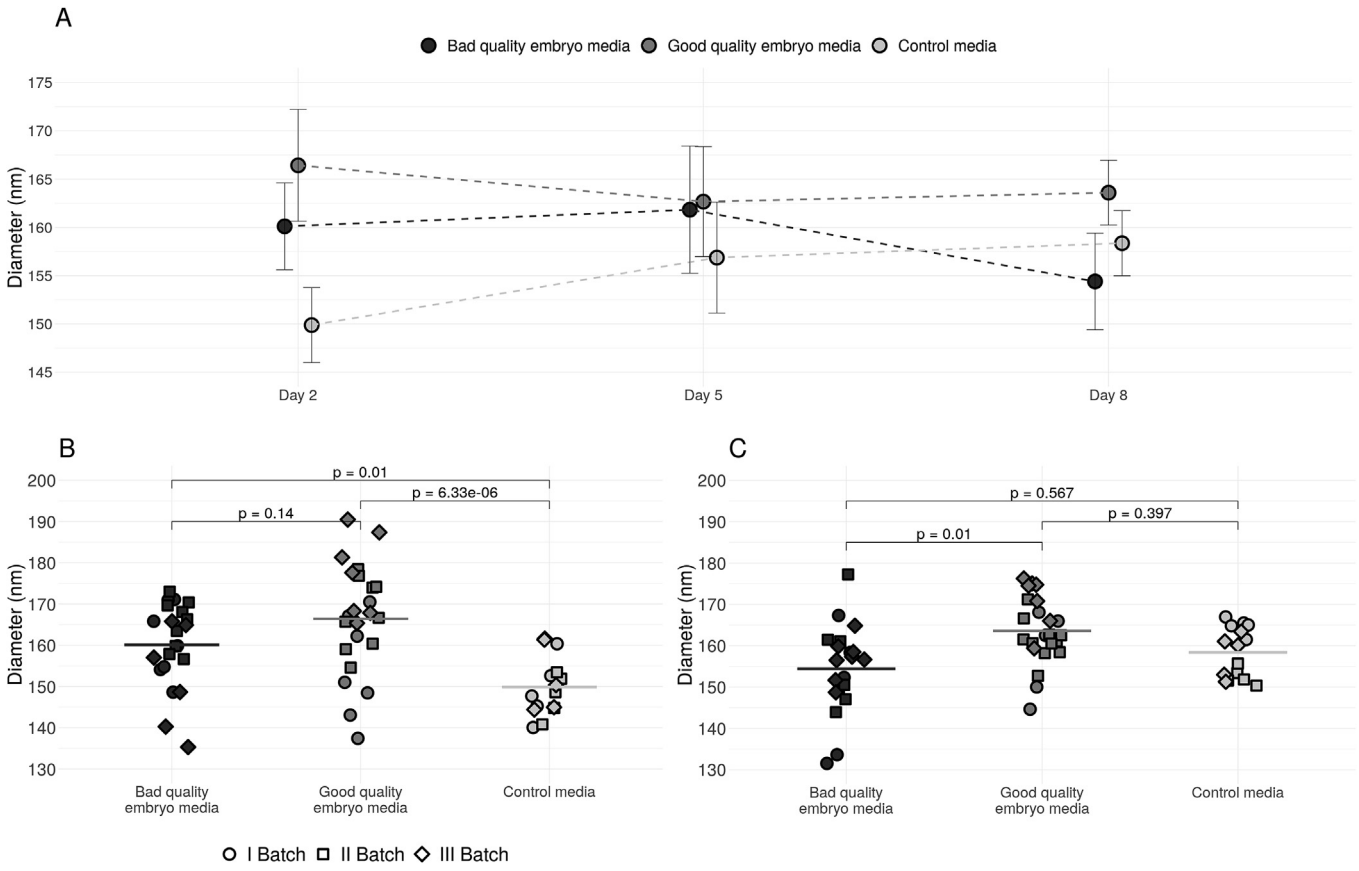

Fig. 7. Mean diameters of EVs isolated from culture media conditioned by individually cultured bovine embryos and the NPs isolated from control media. **(A)** Mean diameters of EVs isolated from media conditioned by individually cultured bovine embryos at day 2, 5, and 8 post-fertilization and NPs isolated from controls. "Day 2 good quality embryo media" (n = 23); "Day 2 bad quality embryo media" (n = 22); "Day 2 control" (n = 15); "Day 5 good quality embryo media" (n = 25); "Day 5 bad quality embryo media" (n = 19); "Day 5 control" (n = 15); "Day 8 good quality embryo media" (n = 24); "Day 8 bad quality embryo media" (n = 20); "Day 8 control" (n = 15). **(B and C)** Statistical comparison of the mean diameters of EVs isolated from media conditioned by individually cultured bovine embryos at day 2 and day 8, respectively. Different shapes in B and C indicate the measurement of the samples from 3 replicates and each such shape represents the mean of the three measurements of each biological sample. Differences between groups were considered to be statistically significant if $p < 0.05$. Error bars are 95% confidence intervals.

embryos producing fewer EVs were more likely to develop to blastocysts. Moreover, it is possible that while developing embryos release EVs to the culture media, they uptake other NPs existing in the culture media. Similarly, they may reuptake the released EVs. Therefore, we propose the hypothesis of a possible dynamic exchange of NPs and EVs between the embryo and its surrounding microenvironment, resulting in the net decrease of concentration of particles in the embryo conditioned media.

The media conditioned by embryos that degenerated after reaching the morula stage (Day 8 bad quality embryo media) had a higher mean EV concentration compared to the embryos that developed to blastocysts by day 8 (Day 8 good quality embryo media). It is possible that when the embryos are degenerating, they release more EVs to the media. This observation corroborates with a recent study by Melisho et al. (2019), who reported that non-viable early blastocysts derived EVs were higher in concentration compared to viable early blastocyst derived EVs [26]. In contrast, the current study has investigated three morphological levels of preimplantation embryo development i.e. 2 cell stage, morula and blastocyst. A previous study by the same researchers showed that the concentration of vesicles released by IVF blastocysts with arrested development is higher compared to competent PA blastocysts [21]. Contrary to day 2 and day 8, mean concentrations of EVs isolated from day 5 good quality and bad quality embryo media were not different. Subsequent analysis of particle concentrations based on size ranges demonstrated differences in the concentrations of EVs between good quality and bad quality embryo media in day 2 and day 8 samples. This observation further proves the physical differences in EVs isolated from good quality and bad quality embryo media.

In general, the sizes of most of the EVs isolated from embryo conditioned media ranged between 30 and 300 nm. This size range corroborates with previous studies which reported bovine embryo-derived EVs are in the similar size range [21,25]. The average diameters of EVs isolated from day 2 good quality and bad quality embryo conditioned media were higher compared to the NPs in day 2 control media. Moreover, the difference of the average diameters of EVs isolated from day 8 good quality embryo media and day 8 bad quality embryo media were statistically significant, with bigger size demonstrated for good quality embryo-derived EVs in our study. However, the study by Melisho et al. (2017) did not observe a difference in EV sizes released by competent and non-competent blastocysts [21]. This could be due to the differences in the EV isolation methods and NTA methods used in the 2 studies.

The limiting factor for the exchange of EVs between the embryo and its environment (including the culture media), compared to the somatic cells, is the presence of *zona pellucida* (ZP). The ZP is an outer covering of mammalian oocytes and early embryos consisting of highly modified glycoproteins [38]. Its porosity and permeability have been thoroughly studied [39,40]. The ultrastructure of the ZP

of *in vivo* and *in vitro* created zygotes are different, and *in vitro* zygotes were found to have higher pore density [41]. An ultrastructural study of the ZP in bovine embryos showed that the average number (per 5000 μm^2) and average diameter of the outer pores in ZP varied between different early embryonic development stages with mean outer pore diameters of zygotes and morula being ca 223 nm and 155 nm, respectively [42]. The average diameters of the EVs isolated from all the 3 stages of embryos were of similar sizes and supported the possibility of permeation by most of the NPs, including EVs, across the ZP. However, the permeability of molecules across the ZP is determined not only by their size but also by their biochemical and physicochemical properties. Turner and Horobin (2004) demonstrated this in mouse embryos using colored probes and could predict the passage of lipid and lipid-containing molecules [40]. However, this model could not predict the permeability of ZP to proteins and nucleic acids. Therefore, pore sizes and the permeability of the ZP would decide the size and type of NPs, including EVs, that would pass in or out of the embryos across the ZP.

The NTA-based quantification of the EVs in the conditioned media, in combination with morphological grading of embryos, can be used to develop a scoring system that would grade the embryos for uterine transfer. Such kind of combined analysis would enhance embryo grading process and would make it more trustworthy compared to simple morphology-based embryo grading, and thus, would assist embryologists in selecting the best embryos for transfer. In a recent study by Melisho et al. (2019), a novel model has been constructed to identify viable embryos using a combination of EV characteristics and blastocyst morphokinetics [26]. This kind of combined non-invasive embryo scoring system would enhance the outcome of IVF treatment in the future. In that regard, the presence of regular embryo culture media, rather than EV-depleted media, would be more supportive as it provides an optimum environment for the embryo culture. Moreover, studying the surface characteristics of these particles, such as surface proteome, zeta potential, and the molecular cargo, such as nucleic acids and proteins, would provide more information regarding the usability of EVs as a possible biomarker of embryo developmental capacity.

In conclusion, the depletion of EVs from the culture media had a negative impact on the individual embryo culture, as was evidenced by a drop in the blastocyst formation rate in EV-depleted media. Individually cultured preimplantation bovine embryos secrete EVs to the culture media. The concentrations of nanoparticles in the embryo conditioned media are modulated based on the developmental stage of the embryo and embryo quality, and thus, may indicate embryo's further potential for development. Further advancement of the current technologies is needed to enable profiling EVs deriving from single cells such as zygotes and 2 cell staged embryos. This would provide more useful information about the early preimplantation embryo quality and lead to identifying novel biomarkers that would be decisive in selecting embryos for transferring to the uterus.

Author contributions

M.N., K.D, and A.F and UJ developed the concept; K.D., M.N., and A.F. designed the experiments; M.N. developed and optimized individual embryo culture systems and produced embryos *in vitro*, Y.R. produced embryos for TEM and EV Array-based characterization; K.D. and A.L. experimented for the optimum EV purification system for single embryos; K.D. carried out EV purification from embryo conditioned media and NTA analysis, and sample preparation for EV characterization by EV Array, TEM and SEM; K.G. and G.B. contributed for EV purification and NTA analysis; M.M.J. and R.B. performed EV Array and provided written materials in methods and results; A.A. performed TEM and provided written materials in methods and results; S.B. performed SEM and provided written materials in methods and results; F.L. and K.D. carried out data analysis, K.D. wrote the initial draft of the manuscript with conceptual contributions from A.F., M.N., K.G., J.V. and G.B.; and all the authors participated in discussing the initial draft and agreed to the final manuscript; A.F., U.J., and A.S. supervised.

Declaration of competing interest

The authors have no conflicts of interest.

CRediT authorship contribution statement

Keerthie Dissanayake: Conceptualization, Methodology, Validation, Investigation, Data curation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Monika Nõmm:** Conceptualization, Methodology, Investigation, Writing - review & editing. **Freddy Lättekivi:** Formal analysis, Data curation, Writing - original draft, Visualization, Writing - review & editing. **Yosra Ressaissi:** Investigation, Writing - review & editing. **Kasun Godakumara:** Investigation, Writing - review & editing. **Arina Lavrits:** Validation, Writing - review & editing. **Getnet Midekessa:** Investigation, Writing - review & editing. **Janeli Viil:** Validation, Writing - review & editing. **Rikke Bæk:** Investigation, Writing - review & editing. **Malene Møller Jørgensen:** Investigation, Writing - review & editing. **Sourav Bhattacharjee:** Investigation, Writing - review & editing. **Aneta Andronowska:** Investigation, Writing - review & editing. **Andres Salumets:** Writing - review & editing, Supervision, Resources, Funding acquisition. **Ülle Jaakma:** Methodology, Project administration, Resources, Writing - review & editing, Supervision, Funding acquisition. **Alireza Fazeli:** Conceptualization, Methodology, Project administration, Writing - original draft, Resources, Writing - review & editing, Supervision, Funding acquisition.

Acknowledgments

This work was supported by the European Union's Horizon 2020 research and innovation program under grant agreement No 668989 (Project TRANSGENO), the Estonian Ministry of Education and Research (grant IUT34-16); Enterprise Estonia (grant EU48695); the European Commission Horizon 2020 research and innovation program under grant agreement 692065 (project WIDENLIFE) and European Commission Horizon 2020 research and innovation program under grant agreement 692299 (Project SEARMET); Rep-Eat-H2020-MSCA-COFUND713714.

Authors thank CellFit-European network of excellence (COST Action CA16119) for facilitating interaction and networking between the authors, Particle Metrix, Germany for their valuable advice regarding nanoparticle tracking analysis, and Annika Häling for her support during the laboratory work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2020.03.008.

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