Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst.

Effect of endometrium-derived exosomes on the development of bovine embryos

V. V. Kovpak*, O. S. Kovpak**, Y. O. Babiy*, I. O. Kharkevych*, S. S. Derkach*, S. I. Kovtun***

** National University of Life and Environmental Sciences of Ukraine, Kyiv, Ukraine*
*** Limited Liability Company "BioTexCom", Kyiv, Ukraine*
**** Institute of Animals Breeding and Genetics nd. a. M. V. Zubets of National Academy of Agrarian Science of Ukraine, Chubynske, Ukraine*

Article info

Received 14.03.2026
Received in revised form 30.04.2026
Accepted 20.05.2026

National University of Life and Environmental Sciences of Ukraine, Heroyiv Oborony st., 15, Kyiv, 03041, Ukraine.
Tel.: +38-067-935-25-70.
E-mail: vitkovpak@ukr.net

Limited Liability Company "BioTexCom", Knyazya Volodymyra Monomakha st., 2/6, Kyiv, 04107, Ukraine.
Tel.: +38-097-067-98-26.
E-mail: kovpak8887@gmail.com

Institute of Animals Breeding and Genetics nd. a. M. V. Zubets of National Academy of Agrarian Science of Ukraine, P. L. Pohrebniaka st., 1, Chubynske, Boryspil District, Kyiv Region, 08321, Ukraine.
Tel.: +38-097-667-18-35.
E-mail: kovtun_si@i.ua

Kovpak, V. V., Kovpak, O. S., Babiy, Y. O., Kharkevych, I. O., Derkach, S. S., & Kovtun, S. I. (2026). Effect of endometrium-derived exosomes on the development of bovine embryos. Regulatory Mechanisms in Biosystems, 17(3), e26051. doi:10.15421/0226051

Modern cattle reproduction is based on the implementation of *in vitro* technologies; however, the quality of such embryos often remains inferior to their *in vivo* counterparts due to the lack of paracrine signals from the maternal organism. This study evaluated the effect of exosomes isolated from an endometrial cell culture on embryo morphology, developmental dynamics, and viability. The study was based on oocyte–cumulus complexes obtained after the slaughter of cows and processed according to standard IVP protocols. Exosomes isolated by sequential ultrafiltration using a 100 kDa cut-off were added to the culture at a ratio of 1 cell equivalent per 5 embryos. To analyze efficiency, 2–4-cell embryos were randomly assigned to a control group and two experimental groups, in which exosomes were introduced at 48 hours (Exo-48h) and 96 hours (Exo-96h) of culture, respectively. This made it possible to determine the role of extracellular vesicles in compensating for the absence of the maternal environment. It was found that the addition of exosomes derived from bovine endometrium to the *in vitro* system exerted a pronounced stimulatory effect on the preimplantation development of embryos, ensuring a significant increase in the blastocyst rate regardless of the time of their supplementation. As early as on day 7 of culture (168 h), the use of exosomes improved blastulation efficiency: in the Exo-48h group, this parameter increased to 26.7 ± 0.0%, while in the Exo-96h group it reached its maximum of 27.8 ± 2.2%, which was 1.66-fold higher than in the control group (16.7 ± 1.9%). Analysis of morphological parameters revealed a significant intensification of developmental kinetics: whereas only early blastocysts (stage code 5) were present in the control group on day 7 of culture, the experimental groups showed a significant proportion of mature blastocysts (stage code 6), at 8.9–10.0% ($P < 0.05$). A specific receptive period for the paracrine effect of vesicles on embryo development was observed at the morula stage (96 h). Exosome supplementation during this period provided the highest viability parameters at the final stages of observation. On day 8 (192 h), the experimental groups showed a shift in the morphological profile towards later embryogenesis: the percentage of expanded blastocysts (stage code 7) was 2.33-fold higher than in the control group (15.6 ± 1.1% vs. 6.7 ± 1.9%). A decisive advantage of adding exosomes to the culture medium was the activation of the hatching process (stage code 8), which was completely absent under control conditions on day 8, whereas in the Exo-96h group it reached 11.1 ± 1.1%. On the final, day 9 (216 h) of observation, the prolonged effect of exosomes was confirmed by a substantial increase in implantation potential. The percentage of embryos that successfully overcame metabolic barriers and emerged beyond the zona pellucida in the Exo-96h group reached 35.5 ± 2.9%, which was twice the control value (17.8 ± 1.1%) and significantly higher than the results of early supplementation (Exo-48h — 26.7 ± 1.9%). Thus, endometrial exosomes act as effective mediators of cellular communication, enabling simulation of the maternal microenvironment, accelerating cavitation and intensifying hatching, thereby substantially improving the quality of bovine embryos produced in an *in vitro* system.

Keywords: reproductive biotechnologies; fertility; extracellular vesicles; embryonic development; cattle breeding.

Introduction

Trends in modern cattle reproduction reflect a systemic shift in priorities towards *in vitro* technologies. This transition is sustainable in nature, as evidenced by the steady dynamics of the average annual growth rate in laboratory embryo production, which stands at 12% (Viana, 2024). However, despite advances in the development of *in vitro* culture systems, the biological potential of bovine embryos produced in this way still remains substantially lower compared with their counterparts obtained *in vivo* (Rizos et al., 2002; Mazzarella et al., 2024). Thus, the artificial culture environment induces various abnormalities, including disturbances in gene expression (Lonergan et al., 2003) and the formation of so-called "stress metabolism". The latter is characterized by intensified aerobic glycolysis and excessive lactate production (Khurana & Niemann, 2000). At the cellular level, this is manifested by structural changes, in particular abnormal accumulation of lipid inclusions, disruption of intercellular junction integrity, and a reduced population of microvilli (Rizos et al., 2002). These morphofunctional disturbances result in reduced resistance to cryogenic exposure (Ferré et al., 2020) and lower transplantation efficiency (Kovpak et al., 2022; Krisher & Herrick, 2024). Given that the first week of gestation accounts for the highest percentage of gestational losses (Poliakiwski et al., 2025), optimization of embryo culture conditions with the aim of approximating them to physiological conditions is a key strategic direction for improving the efficiency of embryo transfer in cattle breeding.

Considering that the oviduct and uterus are capable of providing an ideal microenvironment at different stages of embryo development, understanding the mechanisms of this interaction is important. *In vivo*, the embryo remains in the oviduct until day 4, after which it migrates through the uterotubal junction into the uterine cavity, where it undergoes the morula stage and blastocyst formation up to day 8 (Frankenberg et al., 2016). This process is supported by secretions of the oviductal epithelium and endometrium, which provide the embryo with the necessary conditions for its growth and development (Li & Winuthayanon, 2017; Piibor et al., 2023). The key agents of embryo–maternal cross-talk are extracellular vesicles (EVs), a heterogeneous population of membrane-bound structures secreted by cells that function as mediators of paracrine and autocrine signaling (Yuana et al., 2013), transporting regulatory molecules from the maternal organism to the embryo (Raposo & Stoorvogel, 2013; Santos et al., 2025).

In complex multicellular systems, and in the reproductive tract in particular, vesicle release represents an evolutionary solution for the long-distance exchange of biomolecules, such as transmembrane receptors, genetic information in the form of mRNA and microRNA, as well as proteins, lipids, and secondary messengers (György et al., 2011; Van der Pol et al., 2012). At the same time, extracellular vesicles can ensure the elimination of excessive and/or hazardous intracellular or membrane-associated compounds (Frey & Gaipl, 2011). It should be noted that biomolecules packaged within vesicles are less susceptible to degradation, which additionally enables cargo to be delivered much more efficiently, since vesicles may be equipped with cell-type-specific adhesion receptors (Yuana et al., 2013). Based on biogenesis parameters, secretion characteristics, and size, EVs are classified into three main subpopulations: exosomes, microvesicles, and apoptotic bodies (Radchenko & Yelchaninova, 2024), while some researchers distinguish oncosomes as a separate category (Zaborowski et al., 2015). At the same time, the increasing number of publications specifically devoted to exosomes (Burke et al., 2016; Kimiz-Gebologlu & Oncel, 2022) highlights their importance in the regulation of intercellular communication and identifies them as a promising object of modern biotechnological research.

Exosomes are extracellular vesicles of endosomal origin, 40–160 nm in diameter, with an average diameter of approximately 100 nm (Kalluri & LeBleu, 2020). They were first identified in the early 1980s (Trams et al., 1981) and were initially considered to be mechanisms for the disposal of cellular waste. Recent developments have intensified the field of exosome research, and in 2017 almost 2,000 exosome-related articles were published (Bowers et al., 2020). Scientific interest in exosomes is driven by the complex intracellular mechanisms of their biogenesis, which shape their unique composition and determine their role in subsequent intercellular interactions (Mathieu et al., 2019). Bang & Thum (2012) indicate that exosomes arise within many cell types from endosomal compartments known as multivesicular bodies (MVBs). Exosome formation begins with endocytosis of the plasma membrane and the formation in the cytoplasm of a small membrane-bound vacuole and an early endosome. Components of the cell membrane, including membrane-bound signaling proteins, are captured during this process, becoming part of the endosomal membrane and, ultimately, of the exosomal membrane itself (Théry et al., 2002). The early endosome matures into an MVB through internal budding of the endosomal membrane and the formation of intraluminal vesicles (ILVs), which are nascent exosomes (Bowers et al., 2020). Fusion of the MVB with the plasma membrane results in exocytosis of ILVs into the extracellular space, where they acquire the status of exosomes (Bang & Thum, 2012). Given their small size, they have a high penetration capacity, are able to spread rapidly in the extracellular space, participate in intercellular communication through fusion with cell membranes, and influence various cellular functions (Stahl & Raposo, 2017). In the context of embryogenesis in an *in vitro* system, exosomes are considered promising tools for microenvironment modulation, as they are capable of mimicking physiological signals from the maternal organism, thereby compensating for deficits in regulatory stimuli in synthetic culture systems (Raposo & Stoorvogel, 2013; Santos et al., 2025).

Taking the above into account, it can be stated that exosomes play an important role in the regulation of early embryogenesis. Under natural conditions, the transition of the embryo from the oviduct into the uterine cavity is synchronized with a critical stage of its development – morulation (Frankenberg et al., 2016), during which endometrial exosomes act as the main carriers of maternal signals. Therefore, the addition of endometrium-derived exosomes to an *in vitro* culture system represents a strategic approach to overcoming the "*in vitro* effect". This may partially compensate for the absence of a dynamic maternal microenvironment and contribute to increasing the biological competence of bovine embryos and their capacity for further development. In view of this, the aim of the present study was to evaluate the effect of exosomes isolated from endometrial cells on the morphological characteristics and developmental dynamics of bovine embryos in an *in vitro* system.

Materials and methods

The experimental part of the study was carried out during 2025–2026 at the Educational and Scientific Laboratory "Centre for Animal Reproduction with a Sperm and Embryo Bank" of the National University of Life and Environmental Sciences of Ukraine. The source of the initial material for the study was *ex vivo* organs - uteri and ovaries – obtained from cows after commercial slaughter. The experiments were performed in accordance with the requirements of the Law of Ukraine "On the Protection of Animals from Cruelty" (Article 230 of 2006), the "General Ethical Principles for Experiments on Animals", approved by the National Congress on Bioethics, and aligned with the provisions of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 1986). The housing conditions of the cows complied with the established standards of the Order of the Ministry for Development of Economy, Trade and Agriculture of Ukraine "On Approval of the Requirements for the Welfare of Farm Animals during Keeping" (No. 224 of 2021). A positive opinion regarding the use of the experimental cows was obtained from the Local Bioethics Committee of the National University of Life and Environmental Sciences of Ukraine before the start of the experiments (Protocol No. 026 dated 10 April 2022).

Production of zygotes in the in vitro system. Oocyte maturation in the *in vitro* system. Biological material (ovaries of clinically healthy animals) was obtained at a slaughter facility and transported to the laboratory in a thermally insulated container at a temperature of 30–33 °C. The delivery time did not exceed 3 hours from the moment of collection. Under laboratory conditions, the organs were subjected to four rounds of sanitary treatment with warm (37–38 °C) Dulbecco's phosphate-buffered saline (Sigma, USA) supplemented with gentamicin sulphate at 75 μg/cm³ (Sigma, USA). Oocyte-cumulus complexes (OCCs) were recovered from antral follicles 2–8 mm in diameter in a laminar-flow cabinet by mechanical dissection of the follicular wall. The procedure was performed using oocyte and embryo manipulation medium - Wash Medium (EmbryoCloud, Spain). Morphological analysis of the OCCs was carried out using an SZ51 stereomicroscope (Olympus, Japan). Only fully competent oocytes 120–130 μm in diameter, with a compact multilayered cumulus, an intact zona pellucida, and homogeneous ooplasm without signs of vacuolization or atresia, were selected for the experiment. The selected OCCs were washed six times in Wash Medium on a heated stage (37 °C). Maturation (*in vitro* maturation, IVM) was carried out for 22–24 hours in 4-well plates (Oosafe, USA). Each well contained 300 mm³ of Maturation Medium – medium for *in vitro* maturation of bovine oocytes (EmbryoCloud, Spain) – under a layer of mineral oil (Origio, Denmark). Culture was performed at a density of 20–22 OCCs per well in a multigas incubator at 38.5 °C in a gas atmosphere containing 6% CO_2 and 5% O_2.

Preparation and capacitation of bull spermatozoa. Sperm selection and activation were performed according to a standardized protocol combining the stages of purification, separation of the motile fraction, and induction of capacitation. At the first stage, Sperm Wash Medium (EmbryoCloud, Spain) for bovine spermatozoa was used to eliminate cryoprotectant components and native plasma. A pre-thawed semen dose was added to 1 cm³ of medium at 20–25 °C, followed by centrifugation at 200 × g for 5 minutes. The pellet-washing procedure was repeated twice. The functionally active gamete fraction was isolated by the swim-up method. For this purpose, the sperm pellet after washing was carefully placed at the bottom of a tube containing 1 cm³ of Swim-up Medium (EmbryoCloud, Spain) for bovine spermatozoa. Equilibration in a thermostat for 60 minutes allowed viable cells to migrate into the upper layers of the solution, whereas atypical and immotile forms remained in the pellet. The resulting sperm suspension (supernatant) was subjected to 4-hour incubation in capacitation medium. After the activation period, the cells were centrifuged at 200 × g for 5 minutes, and the resulting pellet was resuspended in 1 cm³ of Fertilization Medium for bovine oocytes (EmbryoCloud, Spain). The final sperm concentration required for fertilization was determined using a Goryaev counting chamber.

Oocyte fertilization and embryo culture in the *in vitro* system. After completion of the maturation stage, once the readiness of the OCCs for fertilization had been verified based on the condition of the perivitelline space and cumulus, the oocytes were subjected to fertilization by *in vitro* fertilization (IVF). Gamete co-culture was performed in 4-well plates (Oosafe, USA) in Fertilization Medium for bovine oocytes (EmbryoCloud, Spain) under a layer of mineral oil (Origio, Denmark). Each well, with a volume of 300 mm³, received 5 to 10 OCCs and a motile spermatozoa fraction at a ratio of 5×10^3 spermatozoa per oocyte. Incubation lasted for 18–20 hours, after which mechanical denudation of the zygotes was performed. Further embryo development took place in 100 mm³ microdroplets of Culture Medium for bovine embryo development (EmbryoCloud, Spain). Culture was performed in groups of 5 embryos per droplet under standard conditions.

Establishment of a primary culture of bovine endometrial cells. To obtain the primary cell culture, bovine uteri collected at a slaughter facility immediately after slaughter were used. Biological material was collected from animals in the early luteal phase of the oestrous cycle, which was identified by the presence of a corpus haemorrhagicum according to the guidelines of Ireland et al. (1980). After visual confirmation of the absence of reproductive system pathologies, the organs were transported to the laboratory under isothermal conditions at a temperature of 30 °C. Under laboratory conditions, the uteri were cleaned of adjacent tissues and disinfected with a 70% ethanol solution. For cell isolation, the uterine horn ipsilateral to the ovary bearing the haemorrhagic corpus luteum was used. After a longitudinal incision along the line of mesometrial attachment, careful desquamation of the luminal layer of the endometrium was performed using sterile glass slides. The collected cellular material was transferred into prewarmed basal medium consisting of Dulbecco's modified Eagle's medium (DMEM) (Sigma, USA) supplemented with 20% foetal bovine serum (FBS) (Sigma, USA) and 10 µL/cm³ antibiotic–antimycotic solution (Sigma, USA). The viable cell suspension was seeded at a concentration of 1×10^6 cells per Petri dish (d = 35 mm, Sarstedt, Germany) (Fig. 1). The cells were cultured in a CO_2 incubator at 37 °C and 5% CO_2. The culture medium was replaced every 48 hours. On day 14 of culture, after the formation of a monolayer with 80–90% confluence, the cells were passaged at a ratio of 1:2, after prior detachment from the surface of the culture dish using trypsin/EDTA solution (Sigma, USA). Cell centrifugation was performed at a centrifugal force of 200 × g for 10 min.

Exosome isolation by ultrafiltration. The isolation of extracellular vesicles was initiated at the first passage when the endometrial cell culture reached an 80% monolayer. To prevent contamination with serum-derived exosomes, the monolayer was washed three times with phosphate-buffered saline (PBS, Sigma, USA), after which the cells were transferred to serum-free medium (DMEM supplemented with antibiotic-antimycotic solution). The conditioned medium was collected after 48 hours of incubation and subjected to repeated centrifugation using a Hettich UNIVERSAL 320R centrifuge (Germany) at 4 °C. The first two centrifugation steps – 300 × g for 10 min and 2,000 × g for 10 min – were used to remove cellular debris, while the third centrifugation step – 10,000 × g for 30 min – was used to clear the supernatant of large vesicles. The resulting supernatant was additionally filtered through a membrane with a pore diameter of 0.22 µm (Sarstedt, Germany). Exosomes were concentrated using centrifugal concentrators with a molecular weight cut-off (MWCO) of 100 kDa (Amicon Ultra-15 100,000 MWCO, Millipore Merck, Germany) at 9,000 × g for 60 min until a retentate volume of 0.5 cm³ was reached. To remove low-molecular-weight impurities, diafiltration was performed by adding 10 cm³ of PBS to the concentrate, followed by a repeated ultrafiltration cycle. The resulting exosome fraction was resuspended in 0.5 cm³ of PBS and stored at −80 °C.

Exosome dosing. To ensure biological relevance and to approximate the experimental conditions as closely as possible to physiological conditions, exosome dosing was standardized using the cell equivalent (CE) method (Al-Madhagi, 2024). One CE was defined as the total amount of exosomes secreted into the conditioned medium by 1×10^6 cells of a bovine endometrial culture over a 48-hour culture period. The use of the CE parameter made it possible to correlate the secretory activity of the cell culture with the corresponding biological response, thereby offsetting the natural variability in exosome production by cells. In the study, 1 CE was used per 5 embryos.

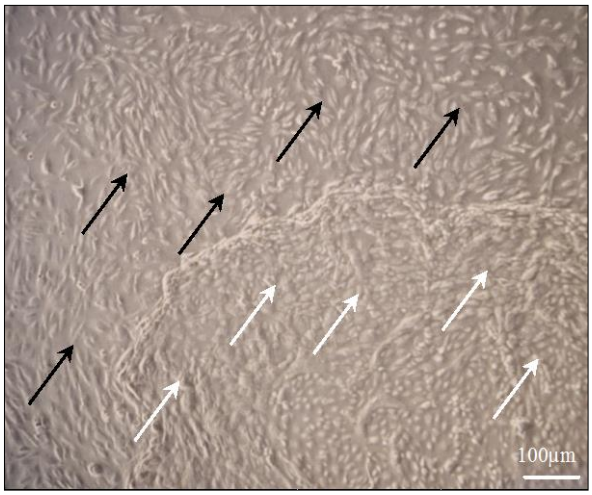

Fig. 1. Primary culture of bovine endometrial cells (day 10 of culture), phase-contrast microscopy: epithelial cells (white arrow), stromal cells (black arrow)

Group formation. To study the stage-specific effect of endometrial exosomes on embryo development in the *in vitro* system, a comparative model with different time points of their introduction was applied. At 46–48 hours after fertilisation, 2–4-cell embryos (n = 90) were randomly assigned to three experimental groups. The study was repeated three times (n = 3 for each series) to ensure the statistical significance of the results.

Group 1 (control) – culture was performed in standard Culture Medium for bovine embryo development (EmbryoCloud, Spain) without the addition of exogenous factors throughout the entire observation period.

Group 2 (Exo-48h) – the fraction of exosomes obtained from the bovine endometrial cell culture at a dose of 1 CE per 5 embryos was introduced into the culture system immediately after assessment of zygote cleavage (46–48 hours). This model was intended to simulate the prolonged effect of maternal paracrine signals from the early stages of embryogenesis.

Group 3 (Exo-96h) – exosomes at the same dose (1 CE/5 embryos) were introduced at 96 hours of embryo culture, corresponding to the morula stage, by replacing 50% of the droplet volume with fresh medium containing the appropriate amount of exosomes. This scheme models the physiological moment of embryo transition into the uterine cavity and its initial contact with the secretory profile of the endometrium.

Assessment of embryo development. The development of bovine embryos was monitored using a Nikon Eclipse Ti2-U inverted research microscope equipped with a heated stage, which made it possible to minimize thermal stress during visual analysis.

The blastocyst formation rate was assessed at 168 hours (day 7) and 192 hours (day 8) of culture. The percentage of blastocysts obtained was calculated as the ratio of formed blastocysts to the total number of 2–4-cell embryos included in the experiment, expressed as a percentage. Morphological assessment of bovine embryo quality was performed according to the classification proposed by Bó & Mapletoft (2013) and recommended by the International Embryo Technology Society (IETS). For comparative analysis, four stages of embryo development were distinguished: I – early blastocyst (stage code 5 (SC 5)): the embryo has a fluid-filled cavity, the blastocoel, the cell mass occupies 70–80% of the perivitelline space, and the overall appearance of the embryo resembles a "signet ring"; II – blastocyst (stage code 6): characterized by clear differentiation of the trophectoderm and embryoblast, the blastocoel is fully formed, and the embryo occupies almost the entire perivitelline space; III – expanded blasto-

cyst (stage code 7): the overall diameter of the embryo is increased 1.2–1.5-fold, the cell mass is closely apposed to the zona pellucida, which, in turn, is thinned by approximately one-third of its initial thickness; IV – hatched blastocyst (stage code 8): the embryos are in the process of emerging from the zona pellucida, i.e. hatching, or have already completely shed the zona pellucida (Fig. 2).

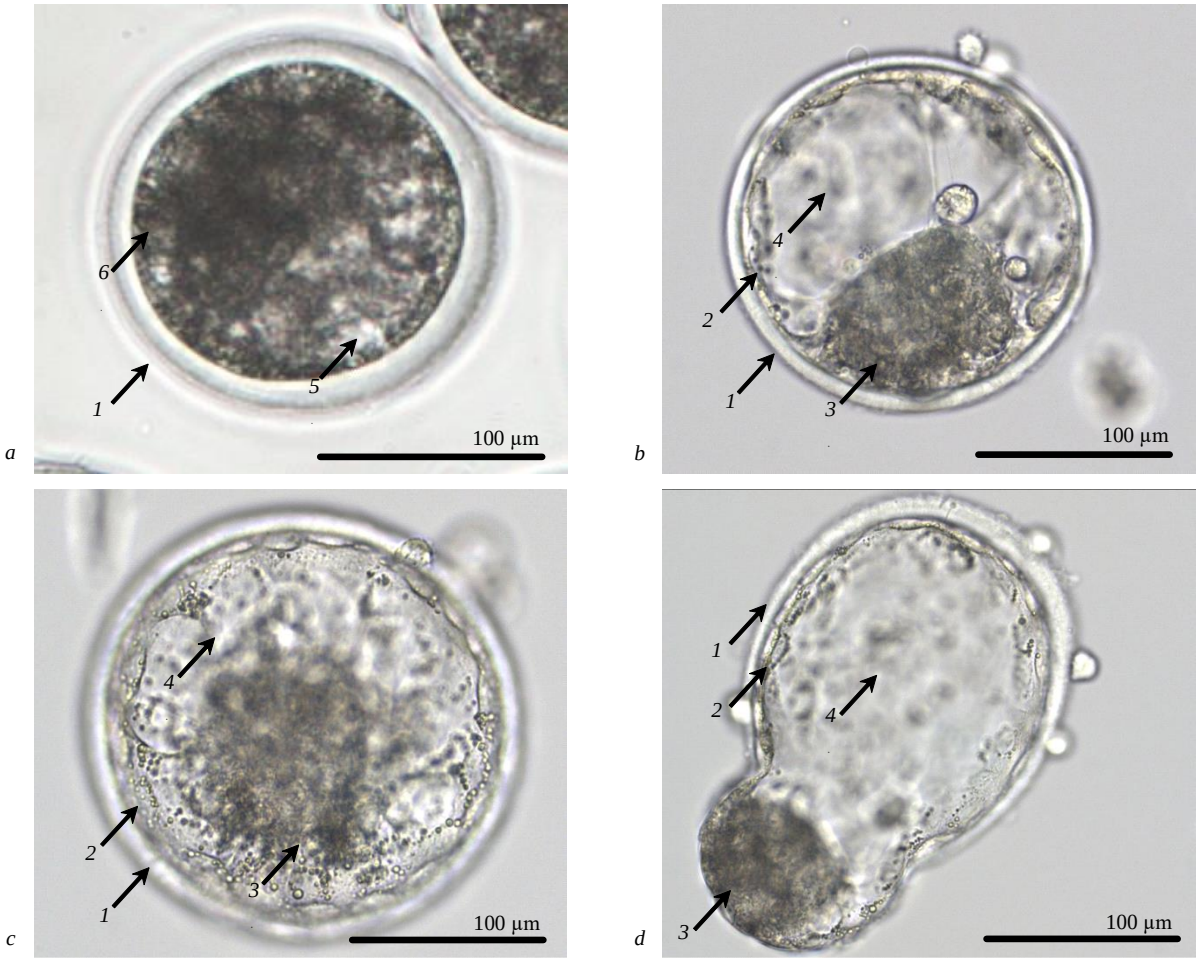

Fig. 2. Bovine embryos at different development stages (day 8), phase-contrast microscopy: early blastocyst (stage code 5) (*a*), blastocyst (stage code 6) (*b*), expanded blastocyst (stage code 7) (*c*), hatched blastocyst (stage code 8) (*d*), according to IETS standards; *1* – zona pellucida, *2* – trophoblast, *3* – inner cell mass, *4* – blastocyst cavity, *5* – initial blastocoele cavity, *6* – inner cells

Natural hatching was assessed at 216 hours of culture (day 9). Natural hatching parameters were calculated as the ratio of the number of embryos that had initiated or completed emergence from the zona pellucida to the total number of blastocysts.

Statistical analysis. The experimental data were processed using methods of variation statistics with Microsoft Excel software and the XLSTAT analytical add-in. The results obtained were presented as the arithmetic mean and its standard error (x ± SE). To assess the effect of exosomes on embryo development, one-way analysis of variance (one-way ANOVA) was applied. To identify statistically significant differences between the experimental groups at fixed time points, post hoc comparisons were performed using Tukey's HSD test. Differences were considered statistically significant at a probability level of $P < 0.05$.

Results

To assess the effect of exosomes on the preimplantation development of embryos, a series of experiments was conducted involving their addition to the culture medium at different stages of embryogenesis. Analysis of the results obtained made it possible to identify differences in the dynamics of blastocyst formation and their capacity for subsequent hatching.

It was established that on day 7 of culture (168 hours), the blastulation rate in the control group was the lowest and amounted to 16.7 ± 1.9%. The addition of exosomes to the medium significantly increased embryogenesis efficiency, regardless of the timing of their introduction ($P < 0.05$). In particular, in the Exo-48h group, the proportion of formed blastocysts increased to 26.7 ± 0.0%, which was 1.6-fold higher than in the control group. A similar trend was observed in the Exo-96h group, where the highest mean blastulation rate was recorded, at 27.8 ± 2.2%. A detailed analysis of embryo morphology on day 7 of culture additionally revealed significant differences in the rate of cavitation between the experimental groups and the control (Fig. 3).

It was found that at 168 hours of culture, the main proportion of formed blastocysts was characterized as early blastocysts (SC 5). In the control group, this parameter was 16.7 ± 1.9%, whereas in the Exo-48h and Exo-96h groups the percentage of embryos at this stage was slightly higher, at 17.8 ± 1.1%; however, no statistically significant difference compared with the control at the SC 5 stage was detected. A fundamental difference between the groups was recorded when analyzing the SC 6 stage (blastocyst). In the control group, no embryos at the SC 6 stage were detected on day 7 of culture. In the experimental groups, the proportion of embryos at this developmental stage was 8.9 ± 1.1% in the Exo-48h group and 10.0 ± 1.9% in the Exo-96h group. The difference identified between the experimental groups and the control was statistically significant ($P < 0.05$).

Further monitoring of embryogenesis on day 8 (192 hours) of culture demonstrated an increase in the differences between the groups (Fig. 4). In the control group, most embryos remained at the early stages of blastulation (SC 5 and SC 6), whereas the percentage of expanded blastocysts (SC 7) was significantly lower than in the experimental groups and amounted to only 6.7 ± 1.9%. The most substantial difference was that, under control conditions, no cases of

complete or partial hatching were recorded on day 8 of culture. By contrast, in the experimental groups, a shift in morphological stages towards late embryogenesis (SC 7 and SC 8) was observed. Thus, the percentage of expanded blastocysts (SC 7) in the groups supplemented with exosomes significantly increased to 15.6 ± 1.1% ($P < 0.05$), which was 2.3-fold higher than the control values. The results of the hatching assessment confirmed the positive effect of exosome supplementation of the culture medium on blastocyst quality. In the Exo-48h group, the percentage of blastocysts emerging beyond the zona pellucida (SC 8) was 6.7 ± 0.0%. In the Exo-96h group, the percentage of hatched blastocysts was 11.1 ± 1.1%, which was statistically higher not only than the control value ($P < 0.01$), but also than the value in the group with early exosome supplementation ($P < 0.05$).

Analysis of the results at the final stages of embryo culture in the *in vitro* system (192–216 h) confirmed the prolonged positive effect of exosomes on embryo viability and hatching capacity (Table 1). On day 8 of culture, the number of formed blastocysts in the experimental groups was significantly higher compared with the control. In the Exo-48h group, this parameter was 43.3 ± 1.9%, while in the Exo-96h group it was 46.7 ± 1.9%. At the same time, in the control group it remained at 33.3 ± 1.9% ($P < 0.05$). It should be noted that although the experimental groups demonstrated a significant advantage over the control, no statistically significant difference in the total number of embryos was detected between the experimental groups themselves on day 8.

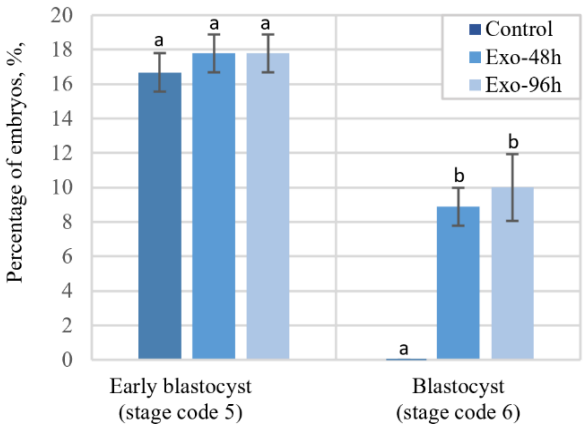

Fig. 3. Effect of exosomes on the kinetics of blastocyst development depending on the time of their introduction into the culture medium (day 7 of culture): data are presented as the arithmetic mean and its standard error (SE) based on the results of three independent experiments; statistical analysis was performed using Tukey's test; letters above the bars within the same developmental stage indicate statistically significant differences between groups ($P < 0.05$)

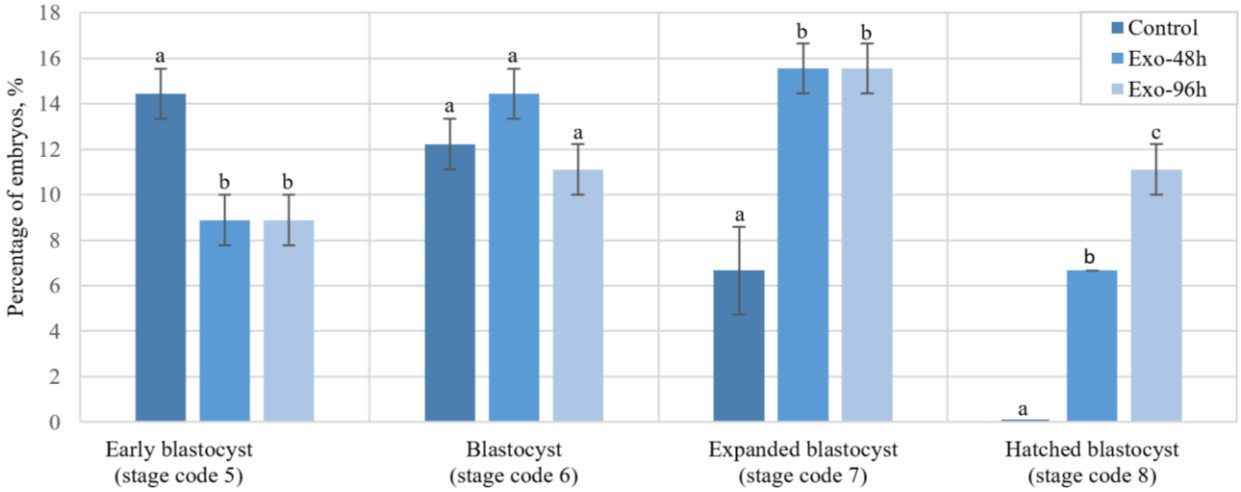

Fig. 4. Effect of exosomes on the kinetics of blastocyst development depending on the time of their introduction into the culture medium (day 8 of culture): data are presented as the arithmetic mean and its standard error (SE) based on the results of three independent experiments; statistical analysis was performed using Tukey's test; letters above the bars within the same developmental stage indicate statistically significant differences between groups ($P < 0.05$)

Table 1
Effect of exosome supplementation of the *in vitro* culture system on the development of bovine embryos (x ± SE, n = 90*)

Study groups	Blastocysts, day 8 (192 h), n (%)	Hatching, day 9 (216 h), n (%**)
Control	30 (33.3 ± 1.9)^a	16 (17.8 ± 1.1)^a
Exo-48h	39 (43.3 ± 1.9)^b	24 (26.7 ± 1.9)^b
Exo-96h	42 (46.7 ± 1.9)^b	32 (35.5 ± 2.9)^c

Note: * n = 90 – the total number of 2–4-cell embryos in each group across three replicates, with 30 embryos in each replicate; ** the hatching percentage was calculated relative to the number of 2–4-cell embryos; letters indicate significant differences between groups within a column ($P < 0.05$) according to Tukey's test.

The capacity of embryos for hatching is a decisive indicator of their implantation potential. Since 216 hours, corresponding to day 9 of embryogenesis, is regarded by most researchers as a critical time point for verifying viability and completion of preimplantation development in the *in vitro* system, this time point was selected by us as the final one for the assessment of morphological parameters. It was established that the addition of exosomes to the culture medium made it possible to substantially increase the proportion of embryos that had partially or completely emerged from the zona pellucida. In the Exo-48h group, the percentage of embryos at the SC 8 stage was 26.7 ± 1.9%, which significantly exceeded the value in the control group (17.8 ± 1.1%, $P < 0.05$). The highest efficiency was recorded in the Exo-96h group, where the proportion of hatched blastocysts was 35.5 ± 2.9%. It should be noted that this parameter was significantly higher not only compared with the control ($P < 0.01$), but also compared with the Exo-48h group ($P < 0.05$).

Discussion

The results obtained indicate that the introduction of exosomes into the *in vitro* culture system exerts a pronounced stimulatory effect on the preimplantation development of bovine embryos. Our study demonstrates, for the first time, the dynamics of the embryonic response depending on the stage of embryogenesis, revealing a specific physiological window at the morula stage (96 h), when the embryo is most receptive to external signals from exosomes isolated from the bovine endometrium. Analysis of morphological parameters confirms that exosomes not only increase the overall blastulation rate, but also

substantially accelerate cavitation, ensuring the transition of embryos to the expanded blastocyst stage. The high viability and functional competence of the embryos obtained are clearly confirmed by their capacity for hatching, which began much earlier in the experimental groups than in the control, where no cases of blastocyst emergence from the zona pellucida were recorded on day 8.

The mechanism underlying the stimulatory effect of exosomes is based on their ability to regulate critical functions of embryogenesis through multilevel signaling, encompassing the control of cell division, metabolism, and inhibition of apoptosis (Xue et al., 2024). The efficiency of such communication in the *in vitro* system is ensured by the structure of the zona pellucida. According to Vanroose et al. (2000), the pore diameter of the zona pellucida is 155–203 nm, which makes it permeable to exosomes (40–160 nm), which, after diffusion, are actively internalized by blastomeres (Pavani et al., 2018). Once in the cytoplasm, exosomes act as complex biochemical containers. Comprehensive quantitative proteomic profiling has shown that critical changes in trophectoderm cell adhesion networks – cell adhesion molecule binding, cell adhesion mediator activity, and cell–cell adhesion junctions – as well as in metabolic and gene expression networks, are mediated by endometrial exosomes, which act as protein carriers (Evans et al., 2019). In parallel with proteins, encapsulated microRNAs modulate the function of target cells by influencing embryonic morphogenesis and implantation potential (Ng et al., 2013; Balaguer et al., 2018). Thus, exosomes act as epigenetic mediators that promote the growth, division, and preparation for adhesion of embryonic cells (Chen et al., 2022; Xue et al., 2024).

The advantage we identified for exosome supplementation at 96 hours of culture, corresponding to the morula stage, has a physiological basis. In ruminants, developmental arrest in the *in vitro* system is often observed specifically on day 4 of embryo development, which corresponds to the compaction stage of 8–16 blastomeres and coincides with the time of embryo transition from the oviduct to the uterus (Graf et al., 2014). From this point onward, the embryo interacts with the maternal organism through paracrine mechanisms until the onset of implantation, which occurs approximately on day 19 of development (Maillo et al., 2016). To date, it has been demonstrated that the use of extracellular vesicles (EVs) from various sources – uterine and follicular fluids, as well as oviductal epithelium – markedly improves the quality of bovine embryos by increasing the blastulation rate and total cell number when added to *in vitro* culture systems (Fazeli & Godakumara, 2024). However, data on the effects of exosomes derived specifically from endometrial cells remain fragmented. Thus, our findings regarding the efficiency of vesicle supplementation on day 4 of embryo culture are consistent with the results of Qiao et al. (2018), who established that uterine exosomes at this stage significantly increase the blastocyst formation rate compared with other supplementation time intervals. At the same time, Perrini et al. (2018) indicate that the addition of endometrial microvesicles (100×10^6 MVs/cm) at the early stages of embryogenesis did not exert a stimulatory effect, which emphasizes the importance of synchronizing the "maternal signal" with the stage of embryo development. Aguilera et al. (2024) point to differences in the biological activity of exosomes depending on their origin. According to their data, EVs collected *in vivo* from uterine fluid more effectively stimulate an increase in blastocyst diameter and the expression of interferon tau (IFNT) compared with vesicles obtained from cells cultured in an in vitro system. It is also important to consider the donor's condition, since exosomes obtained in the presence of a pathological state in the organism may exert a suppressive effect on embryos (Wang et al., 2019; Li et al., 2023). In our study, the use of exosomes from functionally active cells obtained from healthy donors provided a rational simulation of the maternal microenvironment.

The most striking confirmation of the positive effect of exosomes was the intensification of the hatching process. This phenomenon is a critical stage that determines the ability of the preimplantation embryo to adhere and subsequently implant into the endometrium, thereby initiating the phase of placentation (Taniyama et al., 2011). It should be recalled that under control conditions, no cases of blastocyst emergence beyond the zona pellucida were recorded, indicating lower embryo competence compared with embryos in the experimental groups. It is known that three mechanisms are involved in the process of blastocyst hatching: mechanical forces acting on the zona pellucida through blastocyst expansion; weakening of the zona pellucida through enzymatic degradation; and penetration of trophoectodermal cell projections into the zona pellucida (Negrón-Pérez & Hansen, 2017). Embryo growth and development contribute to the mechanical component through thinning of the zona pellucida, which has been shown to be promoted by EVs (Chen et al., 2022). Meanwhile, embryo culture in an *in vitro* system is often accompanied by oxidative stress, which causes zona hardening and creates a mechanical barrier that prevents natural hatching (De Vos & Van Steirteghem, 2000). However, the use of exosomes helps overcome this negative phenomenon. According to studies by Zhang et al. (2022), treatment with exosomes activates cellular antioxidant defence, which not only prevents damage to embryonic structures but also maintains the elasticity of the zona pellucida.

The developmental dynamics of embryos investigated in this study indicate that the efficiency of exosomal support is closely correlated with the stage of embryogenesis at which exosome supplementation was performed. The positive effect observed with early supplementation at 48 h can be explained by the mitigation of the adverse influence of *in vitro* environmental factors during the initial stages of development. Owing to the combination of protective and regulatory functions, the addition of endometrial exosomes to the culture medium in the experimental groups enabled embryos to overcome the metabolic barriers of early embryogenesis more successfully. However, due to the irreversible degradation of exosomes under prolonged incubation conditions (Sokolova et al., 2011; Yuan et al., 2021), their subsequent effect on the hatching process was weaker compared with exosome supplementation at 96 h of culture.

At the same time, the introduction of exosomes into the culture medium at 96 h of culture, corresponding to the morula stage, demonstrated the highest efficiency. This advantage is attributable to the fact that the timing of supplementation coincides with the period of the embryo's greatest need for support to initiate cavitation and subsequent hatching. Late exosome supplementation created conditions for their maximum biological activity precisely during the period of blastocyst formation and made it possible to avoid premature degradation.

Conclusion

The conducted study confirms the pronounced stimulatory effect of endometrial exosomes on the preimplantation development of bovine embryos in an *in vitro* system, expressed as a significant increase in the blastulation rate compared with the control, regardless of the timing of vesicle supplementation. Analysis of the morphological parameters of embryos indicates a substantial acceleration of developmental kinetics and intensification of cavitation processes, as evidenced by the presence of embryos at the blastocyst stage (stage code 6) as early as day 7 of culture exclusively in the experimental groups. At the same time, the most optimal period of embryo development in the *in vitro* system was established – the morula stage, corresponding to 96 hours of culture – when embryos exhibit maximum receptivity to endometrial exosomal signals, ensuring the highest viability parameters and capacity for subsequent hatching. The decisive effect of exosomes on embryogenesis lies in their ability to initiate successful emergence of the studied blastocysts beyond the zona pellucida, which was not observed at all under control conditions on day 8, ultimately determining the high implantation potential of the embryos obtained. The data obtained indicate the high efficiency of using endometrial exosomes to simulate the maternal microenvironment, thereby enabling optimization of the preimplantation development of bovine embryos in an *in vitro* system.

This study was conducted with state funding for 2025–2027 under the project "Development of a biotechnological process for intensive reproduction of cattle and its implementation in the post-war recovery of cattle breeding", state registration number: 0125U001878.

References

Aguilera, C., Wong, Y. S., Gutierrez-Reinoso, M. A., Velásquez, A. E., Melo-Báez, B., Cabezas, J., Caamaño, D., Navarrete, F., Castro, F. O., & Rodriguez-Alvarez, L. L. (2024). Embryo-maternal communication mediated by extracellular vesicles in the early stages of embryonic development is modified by *in vitro* conditions. Theriogenology, 214, 43–56.

Al-Madhagi, H. (2024). The landscape of exosomes biogenesis to clinical applications. International Journal of Nanomedicine, 19, 3657–3675.

Balaguer, N., Moreno, I., Herrero, M., González, M., Simón, C., & Vilella, F. (2018). Heterogeneous nuclear ribonucleoprotein C_1 may control miR-30d levels in endometrial exosomes affecting early embryo implantation. Molecular Human Reproduction, 24(8), 411–425.

Bang, C., & Thum, T. (2012). Exosomes: New players in cell-cell communication. International Journal of Biochemistry and Cell Biology, 44(11), 2060–2064.

Bó, G., & Mapletoft, R. (2013) Evaluation and classification of bovine embryos. Animal Reproduction, 54, 344–348.

Bowers, E. C., Hassanin, A. A. I., & Ramos, K. S. (2020). *In vitro* models of exosome biology and toxicology: New frontiers in biomedical research. Toxicology in Vitro, 64, 104462.

Burke, J., Kolhe, R., Hunter, M., Isales, C., Hamrick, M., & Fulzele, S. (2016). Stem cell-derived exosomes: A potential alternative therapeutic agent in orthopaedics. Stem Cells International, 2016, 5802529.

Chen, K., Liang, J., Qin, T., Zhang, Y., Chen, X., & Wang, Z. (2022). The role of extracellular vesicles *in embryo* implantation. Frontiers in Endocrinology, 13, 809596.

De Vos, A., & Van Steirteghem, A. (2000). Zona hardening, zona drilling and assisted hatching: New achievements in assisted reproduction. Cells Tissues Organs, 166(2), 220–227.

Evans, J., Rai, A., Nguyen, H. P. T., Poh, Q. H., Elglass, K., Simpson, R. J., Salamonsen, L. A., & Greening, D. W. (2019). Human endometrial extracellular vesicles functionally prepare human trophectoderm model for implantation: Understanding bidirectional maternal-embryo communication. Proteomics, 19(23), e1800423.

Fazeli, A., & Godakumara, K. (2024). The evolving roles of extracellular vesicles *in embryo*-maternal communication. Communications Biology, 7(1), 754.

Ferré, L. B., Kjelland, M. E., Taiyeb, A. M., Campos-Chillon, F., & Ross, P. J. (2020). Recent progress in bovine *in vitro*-derived embryo cryotolerance: Impact of *in vitro* culture systems, advances in cryopreservation and future considerations. Reproduction in Domestic Animals, 55, 659–676.

Frankenberg, S. R., de Barros, F. R., Rossant, J., & Renfree, M. B. (2016). The mammalian blastocyst. Wiley Interdisciplinary Reviews: Developmental Biology, 5(2), 210–232.

Frey, B., & Gaipl, U. S. (2011). The immune functions of phosphatidylserine in membranes of dying cells and microvesicles. Seminars in Immunopathology, 33(5), 497–516.

Graf, A., Krebs, S., Heininen-Brown, M., Zakhartchenko, V., Blum, H., & Wolf, E. (2014). Genome activation in bovine embryos: Review of the literature and new insights from RNA sequencing experiments. Animal Reproduction Science, 149(1–2), 46–58.

György, B., Szabó, T. G., Pásztói, M., Pál, Z., Misják, P., Aradi, B., László, V., Pállinger, E., Pap, E., Kittel, A., Nagy, G., Falus, A., & Buzás, E. I. (2011). Membrane vesicles, current state-of-the-art: Emerging role of extracellular vesicles. Cellular and Molecular Life Sciences, 68(16), 2667–2688.

Ireland, J. J., Murphee, R. L., & Coulson, P. B. (1980). Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. Journal of Dairy Science, 63(1), 155–160.

Kalluri, R., & LeBleu, V. S. (2020). The biology, function, and biomedical applications of exosomes. Science, 367(6478), eaau6977.

Khurana, N. K., & Niemann, H. (2000). Energy metabolism in preimplantation bovine embryos derived *in vitro* or *in vivo*. Biology of Reproduction, 62(4), 847–856.

Kimiz-Gebologlu, I., & Oncel, S. S. (2022). Exosomes: Large-scale production, isolation, drug loading efficiency, and biodistribution and uptake. Journal of Controlled Release, 347, 533–543.

Kovpak, V., Kovpak, O., Derkach, S., Masalovych, Y., & Babii, Y. (2022). The influence of platelet concentrate on the development of cattle embryos in an *in vitro* system. Scientific Horizons, 25(9), 9–18.

Krisher, R. L., & Herrick, J. R. (2024). Bovine embryo production *in vitro*: evolution of culture media and commercial perspectives. Animal Reproduction, 21(3), e20240051.

Li, S., & Winuthayanon, W. (2017). Oviduct: Roles in fertilization and early embryo development. Journal of Endocrinology, 232(1), R1-R26.

Li, Y., Cai, L., Guo, N., Liu, C., Wang, M., Zhu, L., Li, F., Jin, L., & Sui, C. (2023). Oviductal extracellular vesicles from women with endometriosis impair embryo development. Frontiers in Endocrinology, 14, 1171778.

Lonergan, P., Rizos, D., Gutierrez-Adán, A., Moreira, P. M., Pintado, B., de la Fuente, J., & Boland, M. P. (2003). Temporal divergence in the pattern of messenger RNA expression in bovine embryos cultured from the zygote to blastocyst stage *in vitro* or *in vivo*. Biology of Reproduction, 69(4), 1424–1431.

Maillo, V., Lopera-Vasquez, R., Hamdi, M., Gutierrez-Adan, A., Lonergan, P., & Rizos, D. (2016). Maternal-embryo interaction in the bovine oviduct: Evidence from *in vivo* and *in vitro* studies. Theriogenology, 86, 443–450.

Mathieu, M., Martin-Jaular, L., Lavieu, G., & Théry, C. (2019). Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. Nature Cell Biology, 21(1), 9–17.

Mazzarella, R., Cajas, Y. N., Gonzalez Martínez, M. E., & Rizos, D. (2024). Extracellular vesicles: Emerging paradigms in bovine embryo-maternal communication. Animal Reproduction, 21(3), e20240065.

Negrón-Pérez, V. M., & Hansen, P. J. (2017). The bovine embryo hatches from the zona pellucida through either the embryonic or abembryonic pole. Journal of Assisted Reproduction and Genetics, 34(6), 725–731.

Ng, Y. H., Rome, S., Jalabert, A., Forterre, A., Singh, H., Hincks, C. L., & Salamonsen, L. A. (2013). Endometrial exosomes/microvesicles in the uterine microenvironment: A new paradigm for embryo-endometrial cross talk at implantation. PLoS One, 8(3), e58502.

Pavani, K. C., Hendrix, A., Van Den Broeck, W., Couck, L., Szymanska, K., Lin, X., De Koster, J., Van Soom, A., & Leemans, B. (2018). Isolation and characterization of functionally active extracellular vesicles from culture medium conditioned by bovine embryos *in vitro*. International Journal of Molecular Sciences, 20(1), 38.

Perrini, C., Esposti, P., Cremonesi, F., & Consiglio, A. L. (2018). Secretome derived from different cell lines in bovine embryo production *in vitro*. Reproduction, Fertility and Development, 30(4), 658–671.

Piibor, J., Waldmann, A., Dissanayake, K., Andronowska, A., Ivask, M., Prasadani, M., Kavak, A., Kodithuwakku, S., & Fazeli, A. (2023). Uterine fluid extracellular vesicles proteome is altered during the estrous cycle. Molecular and Cellular Proteomics, 22(11), 100642.

Poliakiwski, B., Smith, D., Seekford, Z., & Pohler, K. (2025). Highlighting factors contributing to pregnancy loss in beef cattle. Clinical Theriogenology, 17, 1037.

Qiao, F., Ge, H., Ma, X., Zhang, Y., Zuo, Z., Wang, M., Zhang, Y., & Wang, Y. (2018). Bovine uterus-derived exosomes improve developmental competence of somatic cell nuclear transfer embryos. Theriogenology, 114, 199–205.

Radchenko, V. V., & Yelchaninova, T. I. (2024). The role of extracellular vesicles in physiological and pathological conditions (literature review). Ukrainian Journal of Perinatology and Pediatrics, 1(97), 97–104.

Raposo, G., & Stoorvogel, W. (2013). Extracellular vesicles: Exosomes, microvesicles, and friends. Journal of Cell Biology, 200(4), 373–383.

Rizos, D., Fair, T., Papadopoulos, S., Boland, M. P., & Lonergan, P. (2002). Developmental, qualitative, and ultrastructural differences between ovine and bovine embryos produced *in vivo* or *in vitro*. Molecular Reproduction and Development, 62(3), 320–327.

Santos, G., Millán de la Blanca, M. G., Cajas, Y. N., Mazzarella, R., Cañón-Beltrán, K., Gonzalez Martínez, M. E., & Rizos, D. (2025). Deciphering embryo-maternal cross-talk: Advanced *in vitro* approaches. Animal Reproduction, 22(3), e20250059.

Sokolova, V., Ludwig, A. K., Hornung, S., Rotan, O., Horn, P. A., Epple, M., & Giebel, B. (2011). Characterisation of exosomes derived from human cells by nanoparticle tracking analysis and scanning electron microscopy. Colloids and Surfaces B: Biointerfaces, 87(1), 146–150.

Stahl, P. D., & Raposo, G. (2019). Extracellular vesicles: exosomes and microvesicles, integrators of homeostasis. Physiology, 34(3), 169–177.

Taniyama, A., Watanabe, Y., Nishino, Y., Inoue, T., Taura, Y., Takagi, M., Kubota, C., & Otoi, T. (2011). Assisted hatching of poor-quality bovine embryos increases pregnancy success rate after embryo transfer. The Journal of Reproduction and Development, 57(4), 543–546.

Théry, C., Zitvogel, L., & Amigorena, S. (2002). Exosomes: Composition, biogenesis and function. Nature Reviews Immunology, 2(8), 569–579.

Trams, E. G., Lauter, C. J., Salem Jr., N., & Heine, U. (1981). Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. Biochimica et Biophysica Acta, 645(1), 63–70.

Van der Pol, E., Böing, A. N., Harrison, P., Sturk, A., & Nieuwland, R. (2012). Classification, functions, and clinical relevance of extracellular vesicles. Pharmacological Reviews, 64(3), 676–705.

Vanroose, G., Nauwynck, H., Soom, A. V., Ysebaert, M.-T., Charlier, G., Oostveldt, P. V., & de Kruif A. (2000). Structural aspects of the zona pellucida of *in vitro*-produced bovine embryos: A scanning electron and confocal laser scanning microscopic study. Biology of Reproduction, 62, 463–469.

Viana, J. H. M. (2024). Development of the world farm animal embryo industry over the past 30 years. Theriogenology, 230, 151–156.

Wang, X., Tian, F., Chen, C., Feng, Y., Sheng, X., Guo, Y., & Ni, H. (2019). Exosome-derived uterine microRNAs isolated from cows with endometritis impede blastocyst development. Reproductive Biology, 19(2), 204–209.
Xue, Y., Zheng, H., Xiong, Y., & Li, K. (2024). Extracellular vesicles affecting embryo development *in vitro*: A potential culture medium supplement. Frontiers in Pharmacology, 15, 1366992.
Yuan, F., Li, Y. M., & Wang, Z. (2021). Preserving extracellular vesicles for biomedical applications: Consideration of storage stability before and after isolation. Drug Delivery, 28(1), 1501–1509.
Yuana, Y., Sturk, A., & Nieuwland, R. (2013). Extracellular vesicles in physiological and pathological conditions. Blood Reviews, 27(1), 31–39.
Zaborowski, M. P., Balaj, L., Breakefield, X. O., & Lai, C. P. (2015). Extracellular vesicles: Composition, biological relevance, and methods of study. Bioscience, 65(8), 783–797.
Zhang, W., Liu, R., Chen, Y., Wang, M., & Du, J. (2022). Crosstalk between oxidative stress and exosomes. Oxidative Medicine and Cellular Longevity, 2022, 3553617.