AGRICULTURAL BIOLOGY,
2023, V. 58, Iss. 6, pp. 1100-1111
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 636.2:591.39

doi: 10.15389/agrobiology.2023.6.1100eng
doi: 10.15389/agrobiology.2023.6.1100rus

PRESENCE OF FOLLICULAR FLUID EXTRACELLULAR VESICLES DURING in vitro MATURATION OF DONOR COW (*Bos taurus)* OOCYTES INCREASES THEIR ABILITY TO in vitro EMBRYO DEVELOPMENT

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by the Russian Science Foundation, project No. 19-16-00115-П
Final revision received November 03, 2023
Accepted November 30, 2023

Abstract

In vitro embryo production (IVEP) technology from the oocytes isolated by transvaginal follicle puncture (ovum-pickup, OPU) is widely used to generate a number of descendants from the most valuable donor cows and becomes a routine in cattle breeding programs to replicate and conserve precious genotypes. To increase the efficiency of OPU/IVEP-technology, in this work for the first time, bovine OPU-oocytes were matured in the presence of extracellular vesicles (EVs) from follicular fluid (FF), and the ability of treated oocytes to IVEP after in vitro fertilization was investigated. The aim was to study the effects of FF EVs on oocyte maturation and capacity to develop up to blastocyst of OPU-oocytes, as well as to resistance of such blastocysts to freezing. EVs were obtained from FF by differential centrifugations followed by ultracentrifugation at 100,000 g. The preparations were analyzed by transmission electron microscopy that confirmed the presence of exosome-size EVs. Oocyte donors were sexually mature Yaroslavl breed heifers (n = 6) following natural cycle. OPU was performed twice a week. The isolated oocytes were in vitro matured in TC-199 medium, supplemented by fetal bull serum (10 %), follicle stimulating and luteinizing (10 μg/ml) hormones, epidermal growth factor (10 ng/ml) in absence (control) or presence of EVs (experiment). Vesicular preparations were added to the in vitro maturation (IVM) medium in the physiological concentration (EVs isolated from 1 ml of FF per 1 ml of medium). After 24 hours of IVM, the oocytes were subjected to in-vitro fertilization and in vitro embryo culture. At day 3 after fertilization, oocyte morphology was checked, and at day 7 of culture, a number of embryos developed to blastocyst (Bl) was determined. The resulting Bl were frozen and stored for some time at –196 °C. Then the blastocysts were thawed and in vitro cultured until hatching to determine their viability. A total of 10 independent experiments were performed, 57 and 56 oocytes were analyzed in control and treatment, respectively. No impact of experimental conditions on nuclear maturation rate was evidenced. The mature oocytes were similar in control and EVs-treated groups and accounted for 90.4±5.6 % and 94.3±3.1 %, respectively. In addition, the presence of EVs during IVM did not change oocyte cleavage rate after vitro fertilization, 78.6±7.3 % and 86.7±4.9 % in control and EV-treated groups, respectively. However, beneficial effect of EVs on blastocyst rate was found. In control, 26.6±5.8 % of OPU oocytes developed to Bl. EVs added during IVM increased blastocyst rate to 41.2±3.2 % ($p < 0.05$). EVs also tended to increase cryoresistance of resulting blastocysts. In control, blastocyst hatching rate after thawing and short-term culture was 29.1±8.8 % and increased to 53.3±9.2% in the EVs group. Thus, addition of EVs from cow follicular fluid during IVM culture increases oocyte quality and, consequently, their competence to embryo development after in vitro fertilization. Therefore, using of follicular fluid EVs during in

vitro maturation of OPU oocytes can improve the efficiency of OPU/IVEP technologies in cattle.

Keywords: extracellular vesicles, bovine follicular fluid, bovine oocytes, in vitro maturation, oocyte aging, embryo development

Biotechnologies aimed at obtaining embryos from living donors and their transplantation into recipient animals are widely used in livestock breeding to increase the number of offspring from the best mothers in order to more fully realize their genetic potential in generations and accelerate genetic progress in cattle breeding [1, 2].

Embryos are obtained either in vivo using Multiple Ovulation and Embryo Transfer (MOET) technology or in vitro. In MOET, the essence of the technique is to induce superovulation in donor cows through hormonal treatment followed by artificial insemination, embryo flushing and transplantation of fresh or frozen-thawed embryos into recipients [1]. The technology for obtaining embryos in vitro (in vitro embryo production, IVEP), in turn, includes the isolation of female germ cells (oocytes), their maturation and fertilization in vitro, followed by in vitro culture of the resulting zygotes to embryonic stages suitable for freezing and transplantation [2]. Oocytes are recovered from the ovarian follicles of living cows and heifers most often by transvaginal follicle puncture (ovum pick up, OPU). Unlike MOET, OPU can be performed repeatedly and over relatively long periods of time [3, 4]. In addition, to isolate oocytes from follicles, it is not necessary to perform hormonal stimulation of the ovaries, and therefore, the procedure can be repeated at shorter intervals, (usually twice or once a week, compared to obtaining embryos in vivo, no more than once every 2 months. According to statistics of recent years, the number of in vivo embryos used in practice for transplantation into recipients is approximately at the same level [5, 6], and those obtained in vitro (in vitro production, IVP) continue to increase with an average annual growth rate of 12% [7]. This is due, first of all, to the fact that IVEP technology, which involves the use of donor OPU oocytes (OPU/IVEP technology), has now become an alternative to the traditional program for obtaining embryos in vivo [6] and is increasingly used for commercial purposes in many countries [7, 8].

An important condition for the practical use of OPU/IVEP technology remains its efficiency, which, undoubtedly, when compared in retrospect, has increased significantly in recent years, but is nevertheless inferior in a number of MOET indicators. First of all, we are talking about a decrease in the quality of embryos obtained using OPU oocytes and the resistance of OPU/IVEP embryos to freezing, as well as a decrease in their viability after transplantation compared to those obtained in vivo [9-11]. Continuing research on optimizing the stages of OPU/IVEP technology and identifying factors affecting its effectiveness will solve this problem.

As is known, the effectiveness of OPU/IVEP technology is primarily determined by the quality of oocytes before the in vitro fertilization procedure, which they acquire during in vitro maturation (IVM) [12, 13]. However, IVM conditions still remain suboptimal and require improvement.

In routine practices of in vitro bovine reproductive biotechnologies, maturation of bovine oocytes is carried out in optimized media containing, at a minimum, growth factors such as EGF and bovine fetal serum [14], and commercial serum-free media are also used. When developing serum-free media which were initially less effective, various components were used (hormones, amino acids, antioxidants, fatty acids, vitamins, metal ions and various biological preparations). When added, these components could have a positive effect on the cytoplasmic maturation of the oocytes and increase in vitro the number and quality of blastocysts capable of developing into a viable fetus [15, 16]. However, the quality of

OPU oocytes maturing in vitro, usually in small groups, is usually lower than in culture with a large number of cumulus-oocyte complexes (COCs), usually from 25 to 50 in 0.5 ml of medium. OPU oocytes obtained from some cows are often surrounded by fewer cumulus cells (CCs) which can reduce the results of in vitro maturation, primarily cytoplasmic. The addition of 5% follicular fluid (FF) to the IVM medium, which is the optimal medium for oocyte maturation, increases their competence to embryonic development in vitro, especially in the culture of individual oocytes [17]. In addition to the hormonal and steroid components of FF, secretion products of follicular cells and plasma derivatives are contained in extracellular vesicles (EVs), in particular in nanovesicles with a diameter of 30-150 nm called exosomes. Such EVs contain various regulatory factors involved in the molecular dialogue of the oocyte with follicular cells, and primarily with cumulus cells. EVs concentrated in follicular fluid are taken up by target cells via intercellular connections and transzonal projections between the oocyte and the surrounding CCs [18, 19]. Bovine FF EVs contain various proteins [20], lipids [21] and nucleic acids, including microRNAs that regulate gene expression in target cells, in particular in the oocyte [22, 23]. These components are necessary for the development of the oocyte and the formation of its competence to embryonic development. FF vesicular factors are involved in the regulation of signaling pathways that control the development of the follicle and oocyte in it, serve as mediators of cell responses to hormonal and environmental stress, and influence oocyte maturation. In vitro, EVs from FFs enhance the proliferation of follicular granulosa cells, increase their steroid synthesis, cumulus expansion, reduce apoptosis in CCs and oocytes, and influence the activation of various signaling pathways in CCs [22]. FF EVs, when added to IVM medium, improve IVEP efficiency and embryo quality in vitro in cows [24, 25] and positively influence the development and survival of embryos under thermal stress in cows [25]. Although the mechanisms of these effects are far from fully understood, they involve regulation by microRNAs of the functions of specific proteins and lipids that mediate molecular signals in oocytes and CCs and thus influence the oocyte maturation.

In the presented work, we for the first time studied the effect of FF EVs added to the maturation medium on the competence of bovine OPU oocytes to embryonic development in vitro.

The purpose of the work was to study the effect of cow FF EVs on the maturation of OPU oocytes during IVM and on their ability to develop in vitro to the blastocyst stage. The influence of the tested conditions on the resistance of the resulting blastocysts to freezing was also assessed.

Materials and methods. Reagents from Sigma-Aldrich (USA) were used in the work except for specially indicated cases.

To isolate EVs, cow ovaries obtained from a meat processing plant were transported to the laboratory on ice. Ovaries were freed from surrounding tissues and washed in a sterile physiological solution which was pre-cooled to +4 °C and contained antibiotics (100 IU/ml penicillin and 50 μg/ml streptomycin; BioPharmGarant, Russia). To obtain EVs, FFs were aspirated from antral follicles of 3-6 mm diameter and centrifuged for 15 min at +20 °C and 300 g to remove somatic cells. All subsequent centrifugations were also carried out at +20 °C. The supernatant was re-centrifuged for 15 min at 2000 g to remove apoptotic bodies 1-5 μm in size. A third centrifugation (15 min at 12,000 g) was carried out to remove large microvesicles measuring 200-1000 nm. After this preliminary purification, the EVs were pelleted by ultracentrifugation for 90 min at 100,000 g (CS 150 NX centrifuge, Hitachi, Japan). The supernatant was diluted with sterile phosphate-buffered

saline (PBS, pH 7.4) and re-centrifuged for 90 min at 100,000 g for more complete isolation of EVs. The pellets from the two ultracentrifugations were combined and dissolved in PBS. Two 5 μl aliquots were taken from the obtained samples. One of the aliquots was used to determine the number of EVs by protein concentration on a Qubit™ 4 Fluorometer using a Qubit Protein Assay Kit (Thermo Fisher Scientific, USA) and a Qubit protein standard 0.125 to 5 mg/ml. The second aliquot was used for ultrastructural analysis of the particle preparation using transmission electron microscopy (TEM), for which it was mixed with an equal volume of 2% glutaraldehyde (electron microscopy quality, EM) in PBS (Agar Scientific, Ltd., UK) and fixed for 1 h at room temperature. 2 μl of fixed EVs samples were applied to nickel EM grids (Agar Scientific, Ltd., UK) coated with carbon-coated Formvar film and incubated for 60 min in a humid chamber. The grids with EVs deposited on the film surface were washed with distilled water to remove PBS salts. To do this, a drop of 10 μl was applied to the film surface 3 times; after 10 s, it was removed by touching the filter paper with the edge of the mesh. Negative contrast with a 2% aqueous solution of uranyl acetate (Electron Microscopy Science, USA) was carried out in a similar way (3 times for 10 s), removing a drop of the solution by touching the edge of the filter paper mesh. After removing the last drop, the mesh was air dried. Ultrastructural studies were performed usinga JEOL 1011 transmission electron microscope (JEOL, Ltd., Japan). The samples were photographed with a GATAN RIO 9 digital camera using the DigitalMicrograph3 program (Gatan, Inc., USA). Based on the electron microscopy analysis, the EVs in the obtained samples were detected and their morphology was assessed.

The concentration of EVs was measured (a ZetaView nanoparticle analysis apparatus, Particle-Metrix, Germany), calibrated with control 100 nm particles. EVs preparations from cow FFs were diluted in a ratio from 1:1000 to 1:5000 in sterile PBS (0.1 μm filter) and examined in laser (λ = 488 nm) measurement mode using 1 ml of the diluted preparation. The concentration was calculated (specialized program ZetaView, version 8.05.14 SP7).

When obtaining IVEP embryos using OPU technology, oocyte donors were sexually mature, clinically healthy Yaroslavl heifers (*Bos taurus*) (n = 6) aged 3.2-3.5 years. The donors' diets were balanced in terms of energy, nutrients and biologically active substances in accordance with the animals' needs. OPU was performed 2 times a week with an interval of 3 or 4 days.

Transvaginal puncture of the follicles was performed using an OPU system, which included a Versana Active ultrasound scanner with a convection broadband probe (frequency 5 MHz), a probe holder (GE HealthCare, USA), and a vacuum pump (Mini-tube, Germany). Animals were individually fixed in a pen and epidural anesthesia was performed with 2% novocaine. Visible follicles were aspirated with a needle connected by silicone tubing to a 50-ml centrifuge tube. Dulbecco modified medium added with the of 10% fetal bovine serum (FBS) (Biolot, Russia) and heparin (18 IU/ml) was used as aspiration medium. Aspirates from each donor (1 OPU session) were filtered individually, washed with PBS supplemented with 1% PBS (Biolot, Russia). Oocytes were looked for under a stereomicroscope (Nikon, Japan) and transferred to Petri dishes with TC-199 medium, containing 5% FBS and 50 μg/ml gentamicin (TC-199M).

The total pool of oocytes collected from each donor was washed three times in TC-199M and divided according to morphological criteria into three quality categories, good, medium and poor. Oocytes with homogeneous cytoplasm surrounded by more than one layer of compact cumulus were assigned to good quality group; oocytes with homogeneous or moderately heterogeneous cytoplasm, with one layer of cumulus cells (CC) or partially surrounded by CC were assigned

to medium quality group. Oocytes with heterogeneous cytoplasm with signs of granulation or lysis, naked cells, and mature cumulus-oocyte complexes (COCs) were classified as poor. Only oocytes of good and medium quality were considered suitable for further work.

Selected oocytes of good and medium quality were pooled and cultured in groups (4-8 oocytes) in 200 μl drops of medium applied to the bottom of a well of a 4-well plate (Biomedical, Russia) and completely covered with mineral oil. As a control, TC-199 with FBS (10%), sodium pyruvate (0.5 mM), follicle-stimulating hormone (10 μg/ml), luteonizing hormone (10 μg/ml), epidermal growth factor (20 ng/ml) and gentamicin (50 μg/ml) was used as an IVM medium. In the experiment, EVs were added to this medium in a physiological concentration, that is, vesicular protein isolated from 1 ml of follicular fluid was added to 1 ml of IVM medium. Oocytes were cultured at 38.5 °C and 5% CO_2 (an incubator MCO-18AIC, Sanyo, Japan). After 24 h maturation, the oocytes were subjected to in vitro fertilization as described previously [26].

For in vitro fertilization, straws with frozen sperm of a Yaroslavl bull were thawed, and active sperm were obtained by the swim-up method using the Sperm-TALP medium [27] as described [28]. The isolated spermatozoa were added to the fertilization medium with the COCs previously transferred, so that the sperm concentration was 1.5×10^6/ml.

Germ cells were co-cultured for 10-11 h, and then the oocytes were separated from CC and adherent sperm and assessed morphologically. The percentage of maturation was fetermined as the proportion of oocytes with polar bodies (PB) to the total number of oocytes. Purified fertilized oocytes were transferred to 100 μl drops of BO-IVC medium (IVF Bioscience, UK) in the wells of a 4-well plate (Nunc, Denmark) for embryo development (an incubator (MCO-50M-PE, Sanyo, Japan, 38.5 °C and 5% CO_2, 5% O_2 and 90% N_2). Three days after fertilization, the medium was replaed, and the cleaved zygotes were morphologically assessed. On day 7 of incubation, the number of embryos that had developed to the blastocyst stage was counted.

The resulting blastocysts were frozen using an automated freezer CL-8800 (Cryologic, Australia) in a 1.5 M ethylene glycol solution (IVM Technologies, France), and then stored in liquid nitrogen vapor for at least 1 month. To assess viability, embryos were thawed and cultured in BO-IVC medium (IVF Bioscience, UK) supplemented with 5% FBS for 3 days until the hatching stage.

Cleavage, development to the blastocysts and hatched blastocysts were evaluated under a stereomicroscope (Nikon, Japan). In some cases, IVEP embryos were photographed (an Eclipse Ti-U microscope, Nikon, Japan).

Statistical processing of the data was performed using one-way analysis of variance in the SigmaStat program (Systat Software, Inc., USA). Data are expressed as means (*M*) and standard errors of the means (±SEM). The significance of the differences in the compared means was assessed using the Tukey test (p not more than 0.05).

Results. Preparations enriched with extracellular nanovesicles were obtained by successive centrifugations of FF from antral follicles measuring 3-6 mm. Isolation was completed by ultracentrifugation at 100,000 g and washing of excess protein with PBS. Such preparations, diluted in phosphate buffer, contained EVs similar in size to exosomes [29, 30], as well as clusters of lipoproteins (Fig. 1, A). In preparations, the total protein content was 31.18±4.4 μg/ml of FF. The EVs concentration of per 1 ml of FF was $2.4\text{-}4.5\times10^{12}$ with an average particle size of 132.4-135.9 nm (see Fig. 1, B).

A total of 321 ultrasound-visible follicles were aspirated in 34 OPU sessions, and 156 oocytes of varying quality were isolated from these follicles. After

morphological assessment, oocytes of good and moderate quality were cultured for maturation in either control IVM medium or medium containing EVs. Table 1 shows that the groups had comparable indicators both for the OPU effectiveness and in the quality of oocytes used for research. The first parameter was assessed by the number of aspirated follicles per OPU session and by the proportion of retrieved oocytes from the number of aspirated follicles. The second parameter, was assessed by the proportion of oocytes of good and average quality suitable for cultivation from the total number of isolated oocytes.

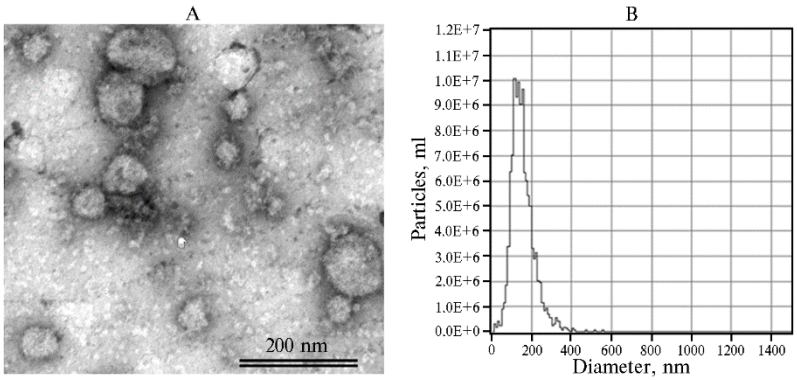

Fig. 1. Analysis of extracellular vesicles (EVs) **from the follicular fluid** (FF) **of the antral follicles of cows:** A — preparation of EVs (transmission electron microscopy, JEOL 1011, JEOL, Ltd., Japan); B — graph of size (diameter) distribution and concentration of particles in a diluted preparation of EVs from the FF of cow ovaries (Laboratory of Cell Biology and Electron Microscopy, Faculty of Medicine, University of Tours, 2022).

1. Isolation and quality assessment of OPU oocytes from Yaroslavl heifers (*Bos taurus*) in the control and test (EVs) **groups** (Ernst Federal Research Center — VIZh, Moscow Province, 2023)

Parameter	Control	EVs
Number of OPU sessions, *N*	18	16
Aspirated follicles, *n*	164	157
Number of aspirated follicles per OPU session, *n*	9.11	9.81
Total number of isolated oocytes, *n*	80	76
Proportion of oocyte recovery from the number of aspirated follicles, %	55.7±7.2	53.3±7.3
Proportion of good quality oocytes from the total number of isolated oocytes, %	33.3±7.3	32.5±8.7
Proportion of oocytes of average quality from the total number of isolated oocytes, %	39.6±5.2	46.7±8.8
Proportion of oocytes suitable for cultivation from the total number of isolated ones, %	72.9±4.3	79.3±5.5

N o t e. In vitro maturation mediumwithout or with extracellular vesicles from the follicular fluid of cows mean control and treatment (EVs) groups, respectively.

Table 2 shows the results of maturation and embryonic development of OPU oocytes.

2. Competence to embryonic development after in vitro fertilization of OPU oocytes of Yaroslavl heifers (*Bos taurus*) as influenced by extracellular vesicles (EVs) **from the follicular fluid in the maturation medium** (Ernst Federal Research Center — VIZh, Moscow Province, 2023)

Parameter	Control	EVs
Tepetitions, *N*	10	10
Number of oocytes, *n*	57	56
Proportion of mature oocytes, %	90.4±5.6	94.3±3.1
Proportion of fragmented oocytes, %	78.6±7.3	86.7±4.9
Proportion of oocytes that have developed to the blastocyst stage, %	26.6±5.8	41.2±3.2*

N o t e. Control means medium for in votro maturation IVM.
* Differences with control are statistically significant at $p < 0.05$.

The EVs in the IVM medium did not affect the nuclear maturation of OPU oocytes. The percentage of maturation as proportion of oocytes with PB was equally high, more than 90%, in both groups. We also did not reveal the effect of

vesicles on the ability of oocytes to enter the first cleavage division (Fig. 2, A). The proportion of cleaved oocytes after in vitro fertilization was approximately 70%. Nevertheless, a positive effect of EVs from FF on the development of mature and in vitro fertilized oocytes to the blastocyst stage was found (see Fig. 2, B). When culturing OPU oocytes in the control IVM medium, the blastocyst yield was 26.6±5.8%. EVs added to the IVM medium increased the blastocyst yield 1.5-fold ($p < 0.05$), up to 1.37 blastocysts per OPU session vs. 0.77 blastocysts per OPU session in the control.

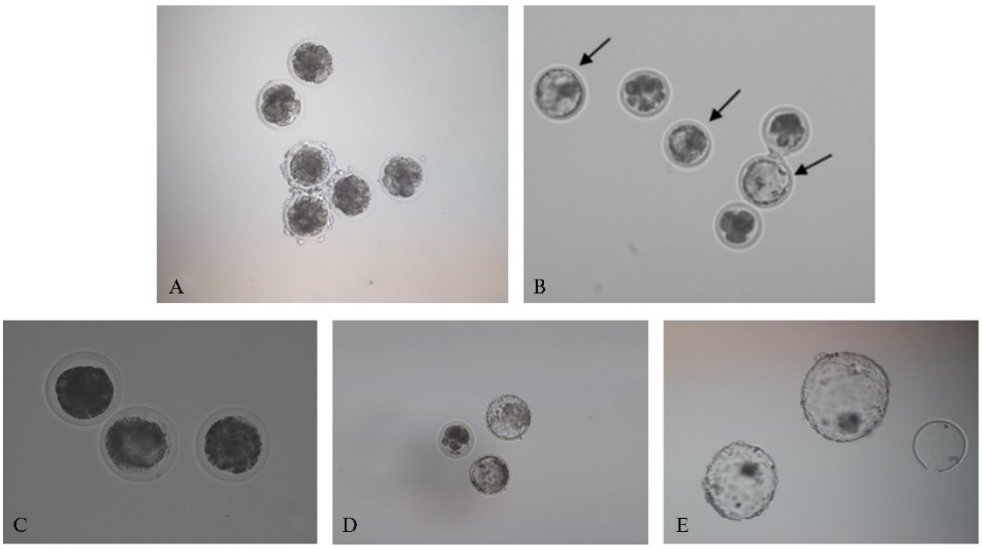

Fig. 2. Embryos of Yaroslavl cattle (*Bos taurus*) developed after in vitro fertilization of OPU oocytes: A — cleaved oocytes; B — embryos that have developed to the blastocyst stage (Bl, marked by arrows); C — frozen Bl right after the defrosting procedure (magnification ×200); D — thawed BL after 24 h culture; E — thawed Bl that reached the hatching stage after 3 days of culture. Microscope Eclipse Ti-U, Nikon, Japan (magnification ×100 except as indicated for B) (Ernst Federal Research Center — VIZh, Moscow Province, 2023).

3. The influence of extracellular vesicles (EVs) **from follicular fluid in the maturation medium of OPU oocytes of Yaroslavl heifers (*Bos taurus*) on the viability of frozen-thawed blastocysts after short-term incubation in vitro** (Ernst Federal Research Center — VIZh, Moscow Province, 2023)

Group	Number		Hatched blastocyst, %
	repetitions, *N*	blastocysts, *n*	
Control (in vitro maturation medium IVM)	10	14	29.1±8.8
EVs (IVM + EVs)	10	22	53.3±9.2*

* Differences with control at $p = 0.081$.

We also determined the long-term effects of FF EVs in the oocyte maturation medium using a test for the freezing resistance of developing blastocysts. When studying the survival of blastocysts after the freezing-thawing procedure, a positive effect of vesicle preparations on maturing oocytes was discovered (Table 3). Exposure to EVs during the maturation of OPU oocytes was responsible for an increase, although not significant, in the proportion of hatched blastocysts (see Fig. 2, E) which likely resulted from the increase in their viability and/or developmental potential before freezing.

Extracellular vesicles are membrane-coated secretory granules that have been found in all types of biological fluids [18], including fluid from female ovarian follicles, which is the natural environment of oocytes during their development in vivo. It has been shown that EVs are involved in intercellular communications within the follicle, since they are secreted by cells and are able to transfer their

contents to other cells due to the ability of the latter to absorb them [18, 19]. In this regard, EVs are being actively studied, including in cows, as potential regulators of oocyte quality and competence to embryonic development in vitro [24, 25, 31-33].

To date, it has been shown that EVs added to IVM medium for post mortem bovine oocytes increases the yield of blastocysts from 26% (control) to 37% [32], and improves the quality of embryos [24]. In addition, it has been found that EVs can improve performance of mature oocytes, increasing their resistance to age-related transformations [34] and protecting from stress [25].

In the present work, we used cow oocytes isolated from the ovarian follicles of individual donors using the OPU method. To our knowledge, this is the first study in which OPU oocytes were cultured to mature in the absence (control) or presence of EVs, then fertilized in vitro and cultured to the blastocyst stage. Moreover, we investigated both the effect of EVs on the maturation and development of OPU oocytes to the blastocyst stages and the resistance of these blastocysts to freezing and thawing. EVs from the FFs of antral follicles (diameter 3-6 mm), according to the generally accepted classification [29, 30], were exosomes in size (see Fig. 1, A, B). Their presence in the maturation medium did not affect the nuclear maturation of OPU oocytes and their ability to enter the first cleavage division after fertilization (see Table 2). However, we have shown that EVs from bovine ovarian fluid, when added to the medium during oocyte maturation, can improve embryo development in vitro. When exposed to EVs, the proportion of oocytes that reached the BL stage increased compared to the control from 26 to 42%, which was even slightly higher than other researcher reported for post mortem cow oocytes [32]. In addition, the presence of EVs in the IVM medium may have a long-term effect, as there was a tendency for the viability of the resulting BLs to increase after the freeze-thaw procedure. In our opinion, the statistical uncertainty in determining the influence of EVs in the IVM medium on the cryostability of Bl may be due to the fetal bovine serum in the medium. The negative effects of some PBS components have previously been reported in similar studies on EVs isolated from media conditioned by oviductal epithelial cells [35].

Thus, the use of extracellular vesicles EVs from the follicular fluid (FF) of bovine ovaries in the in vitro maturation (IVM) procedure improves the quality of oocytes and, theretore, their competence to embryonic development after in vitro fertilization. Preparations enriched with extracellular vesicles from cow FFs can be used during extracorporeal oocyte maturation to more effectively produce high-quality cattle embryos using OPU/IVEP technology.

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