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EFFECT OF EXTRACELLULAR VESICLES OF FOLLICULAR ORIGIN DURING in vitro MATURATION AND AGEING OF BOVINE OOCYTES ON EMBRYO DEVELOPMENT AFTER in vitro FERTILIZATION

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Abstract

Extracellular vesicles (EVs) isolated from ovarian follicular fluid (FF) are involved in vivo in the regulation of meiosis in female gametes. Recent studies suggested follicular EVs as potential regulators of oocyte quality capable to increase the efficiency of embryo production technologies in vitro (in vitro embryo production, IVP). In this work for the first time, we have analyzed embryo development competence of bovine oocytes after in vitro maturation (IVM) in the presence of EVs and ageing before in vitro fertilization. The aim of the study was to determine the effects of these conditions to oocyte ageing-related transformations during IVM in terms of their ability to develop blastocysts, as well as the quality of IVP embryos. The EVs were separated from FF by serial centrifugations and final ultracentrifugation at 100,000g. The isolated EV fraction contained 37.5 μg of total protein per ml of FF. Isolated vesicular fractions were analyzed using transmission electron microscopy that confirmed their enrichment in exosome-like EVs. For functional experiments, cumulus-oocyte complexes (COCs) were in vitro maturated in TC-199 medium containing 3 mg/ml of bovine serum albumin, 0.5 mM sodium pyruvate and 100 ng/ml EGF in the absence (control) or presence of EVs. Vesicles were added to IVM culture at the physiological concentration (EVs isolated from 1 ml of FF were added to 1 ml of IVM medium). After 24 hours of maturation, COCs were transferred to an ageing medium, cultured for further 12 hours, then in vitro fertilized and cultured for embryo development. At day 3 after in vitro fertilization, morphological evaluation of cleaved oocytes was carried out, and at day 7, the number of embryos developed to blastocyst stage and their quality were evaluated. The number of nuclei per embryo, calculated using the cytological method, served as an assessment of the quality of the embryo. In four independent experiments performed, the number of COCs in each group varied from 116 to 121. The cleavage rate of control oocytes was lower than that in the experimental EVs group (53.5 2.9 vs. 63.8 2.9 %, respectively, $p < 0.05$). In addition, EVs had a positive effect on embryo development up to the blastocyst stage after IVM and aging of the oocytes. In control, blastocyst rate was 17.3±1.6 %, and the presence of EVs during IVM increased this rate to 20.2±2.5 % ($p < 0.05$), whereas quality of produced embryos did not change. According to the reported data, EVs from follicular fluid added during IVM may increase the resistance of bovine oocytes to age transformations and, consequently, improve oocyte competence to embryo development after aging in vitro. Therefore, EVs can improve extracorporeal oocyte maturation and the efficiency of in vitro embryo production techniques in cattle.

Keywords: extracellular vesicles, bovine follicular fluid, bovine oocytes, in vitro maturation,

oocyte aging, embryo development

In vitro embryo production (IVP) biotechnology has broad prospects for research application and serves as the effective way to speed up genetic progress in breeding domestic animals, including cattle [1]. To date, a notable progress has been made in improving this technology, but the quality of embryos developed in vitro is still lower than in vivo [2, 3]. A limiting factors affecting the usefulness of IVP embryos is the quality of oocytes acquired during in vitro maturation (IVM) [4, 5]. However, IVM conditions still remain suboptimal and require detailing.

In standard practice, the modernization of maturation systems is aimed primarily at simulating the conditions that occur in vivo in ovarian follicles [5, 6]. A variety of molecular factors, e.g., hormones, steroids, growth factors, fatty acids, and various metabolites, are added to culture media to improve oocyte maturation [7]. Interest in thee substances is due to their presence in the follicular fluid (FF) of antral ovarian follicles, which, in turn, provides an optimal environment for oocyte growth, meiotic maturation, and acquisition of competence for future embryonic development [8]. The impact of these factors is manifested in ovarian follicles with a close bidirectional connection between the oocyte and the surrounding follicular cells [9, 10].

Recent basic research has identified secretory extracellular vesicles (EVs), including exosomes and microbubbles, as new participants in intercellular communication that are secreted by cells and can transport various regulatory factors to other cells capable of absorbing these EVs [11, 12]. In bovine ovarian follicles, EVs are present in FF [13]. They are involved in the transfer of various RNAs, proteins and lipids [14] and participate in the regulation of meiosis in the ovule and early development of the embryo [15, 16]. Such participants in intercellular communications are considered as potential regulators of oocyte quality and their competence for embryonic development under in vitro conditions and are actively studied in this aspect [17-19].

It should also be noted that the existing modern approaches to improving the efficiency of embryonic IVP technology take into account changes in the functional state of cumulus-oocyte complexes (COCs), mainly during oocyte maturation. Nevertheless, in different species, including *Bos taurus*, after the completion of the first division of meiosis in oocytes, aging processes are initiated that adversely affect the quality of mature ovules and their competence for further embryonic development [20, 21]. Under in vitro conditions, ovules isolated from different ovarian follicles and from different donor cows are heterogeneous in potential for development during in vitro maturation. In in vitro culture, some oocytes can mature, i.e., can reach the metaphase II stage of meiosis, much earlier than the period of in vitro fertilization, which leads to their earlier aging and a loss of quality compared to the rest of the population of maturing cells [22]. It is known that any delay in the oocyte fertilization can lead to low viability of embryos and to a weakening of fertility and a reduction in life expectancy of offspring if the offspring was born [20, 21, 23]. Cumulus somatic cells associated with the oocyte, which undergo apoptotic degeneration upon completion of the maturation of female gametes, can accelerate certain negative changes caused by aging [23]. The problem of rapid aging of oocytes which occurs primarily at the molecular cytoplasmic level is increasingly being addressed in the development of scientific approaches to modifying in vitro oocyte maturation systems [24-26]. However, the involvement of EVs in the regulation of aging has not yet been established.

In the present work, we for the first time investigated the competence for embryonic development in oocytes of cows (*Bos taurus*) when cultured in the presence of EVs during maturation and aging in vitro before in vitro fertilization.

The work aimed to study the influence of the tested conditions on age-related transformations in mature oocytes during IVM in terms of their further ability to develop to the blastocyst stage and also to assess the quality of the resulting embryos.

Materials and methods. In all experiments, except for specially indicated cases, reagents from Sigma-Aldrich (USA) were used.

To obtain EVs from FF, isolated post mortem cow ovaries were delivered to the laboratory on ice, freed from surrounding tissues, and repeatedly washed in sterile saline with 100 IU/ml penicillin and 50 ґg/ml streptomycin (BioPharm-Garant, Russia). The saline was cooled to +4 °C. FF was aspirated from antral follicles of 3-6 mm in diameter, 2 ml of FF were centrifuged for 15 min at 300 g and room temperature. The supernatant, free from somatic cells, was transferred into new sterile tubes. At the next stage, FF was freed from apoptotic bodies (1-5 µm in size) and large microvesicles (200-1000 nm in size) by centrifugation for 15 min at 2000 and 12000 g, respectively. EVs were isolated from purified VF by ultracentrifugation (CS 150 NX centrifuge, Hitachi, Japan) for 90 min at 100,000 g; same mode. The precipitates were pooled, diluted in 100 rl of PBS, and stored at –80 °C until use, after taking two 5 µl aliquots of from the resulting volume for EVs quantification by protein concentration and for ultrastructural analysis of the particle preparation using transmission electron microscopy (TEM).

The protein concentration was measured on a Qubit 4 Fluorometer using a Qubit Protein Assay Kit (Thermo Fisher Scientific, USA) and a Qubit protein standard with a concentration of 0.125 to 5 mg/ml.

For a morphological study, 5 µl of the EVs suspension was mixed with 5 µl of a 2% glutaraldehyde for electron microscopy (EM) (Agar Scientific, Ltd., UK) and allowed for 1 h at room temperature. Suspensions of thus fixed EVs (2 µl) were applied to nickel EM grids (Agar Scientific, Ltd., UK) coated with formvar carbon film. The samples were incubated for 60 min in a humid chamber, and the grids were washed with distilled water (3 times 10 s) by applying a 10 µl drop to the surface and water removing by touching the grid edge with the filter paper. Next, negative contrasting staining was performed with a 2% aqueous solution of uranyl acetate (Electron Microscopy Science, USA). A 10 µl drop of uranyl acetate was applied to the grid with the EVs preparation, the procedure was repeated three times with an interval of 10 s, after the removal of the last drop, the grid was dried in air. The preparations were examined using a JEOL 1011 transmission electron microscope (JEOL, Ltd., Japan) and photographed (GATAN RIO 9 camera, DigitalMicrograph3 program, Gatan, Inc., USA). Based on the TEM analysis, the presence of EVs in the samples was established and their morphology was evaluated.

For IVP experiments, isolated post mortem cow ovaries were delivered from the meat processing plant to the laboratory in warm saline and dissected as described above. Cumulus-oocyte complexes (COC) were isolated from the ovaries by dissecting the walls of the follicles with a blade, washed in TC-199 medium containing 5% fetal bovine serum (FBS), heparin (10 µg/ml) and gentamicin (50 µg/ml). The morphology of the isolated COCs was examined. For further culture, we selected rounded oocytes with homogeneous cytoplasm, a pellucid zone of uniform width, which were surrounded by several compact layers of cumulus cells.

The COCs selected by quality for in vitro maturation were cultured in 4-well plates (Biomedical, Russia) in groups (approx. 30 oocytes) in 500 µl of TS-199 medium. The medium contained 3 mg/ml of bovine serum albumin (BSA), 0.5 mM sodium pyruvate, 100 ng/ml epidermal growth factor EGF (Thermo Fisher Scientific, USA), and 50 µg/ml gentamicin in the absence (control) or

presence of EVs. Vesicular protein was added to the maturation medium at a physiological concentration (per 1 ml, the number of EVs isolated from 1 ml of FF). Drops of medium were covered with 500 μl of light mineral oil and cultured in an incubator at 38.5 °C and 5% CO_2. After 24 h according to the IVM procedure, mature COCs were transferred to the aging medium and cultured for another 10 h. For prolonged culture, a medium of the same composition was used, but without EVs.

After a period of aging, COCs were transferred to BO-IVF medium (IVF Bioscience, UK) for in vitro fertilization. Active spermatozoa obtained by the swim-up method as described previously [26] were added to the oocyte wells (final concentration 1×10^6 spermatozoa/ml). In all experiments, frozen-thawed sperm of the same bull was used for fertilization. Oocyte fertilization in vitro was performed in 4-well plates (Biomedical, Russia) in 500 μl medium drops coated with 500 μl mineral oil. Germ cells were co-cultured for 15-16 h, then the oocytes were freed from cumulus cells and adhering spermatozoa. Putative zygotes were transferred to the embryo development medium (commercial medium BO-IVC, IVF Bioscience, UK), the drops were completely covered with mineral oil and cultured at 38.5 °C in an incubator with 5% CO_2, 5% O_2 and 90% N_2. In 3 s after fertilization, the medium was replaced with a fresh medium and the fragmented zygotes was morphologically assessed; on day 7, the number of embryos that developed to the blastocyst stage was estimated.

The quality of embryos was assessed cytologically by the total number of nuclei. The 7-day-old embryos were fixed with 4% paraformaldehyde solution in sodium phosphate buffer for 60 min at room temperature. After fixation, the embryos were permeabilized for 30 min in a 0.1% sodium citrate solution containing 0.5% Triton X-100, stained for 20 min with a DAPI solution (1 μg/ml) to localize the nuclei, transferred to a dry fat-free glass, and placed in Vectashield medium (Vector Laboratories, UK). For microphotography and evaluation of preparations, a motorized microscope Axio Imager M2 (Carl Zeiss, Germany) with a fluorescent attachment and ZEN 2 pro program (Carl Zeiss, Germany) were used.

Statistical processing was performed by the ANOVA method (the SigmaStat computer program, Systat Software, Inc., USA). Experimental results are submitted as mean values (*M*) and standard errors of means (±SEM). Tukey's test was used to assess the significance of differences between the compared means. Differences were considered statistically significant at $p < 0.05$.

Results. Extracorporeal maturation is an important step in the IVP technology. Nevertheless, the competence for embryonic development of oocytes maturing in vitro still remains significantly lower vs. in vivo maturation [4, 5]. The study of the nature and mechanisms of the influence of physiological factors, primarily of natural follicular origin, in the regulation of the quality of oocytes during their maturation in vitro can contribute to solving this problem [5-7].

Previously, many authors, as well as our own studies, have shown that the EVs isolated from FF which forms the natural environment of female germ cells, in the in vitro maturation medium increases ability of oocytes for embryonic development to the blastocyst stage after fertilization [27, 28]. In addition, EVs accelerates the development of embryos [17], and improves their quality, including lower apoptosis frequency in blastomeres [27-29]. A possible mechanism for such a positive effect could be an increase in the resistance of mature eggs to age-related changes that occur in the period that precedes the activation of oocytes by sperm and reduces the competence of fertilized oocytes to embryonic development [22].

To test this assumption, in the present work, isolated post mortem oocytes of cows were cultured in IVM medium in the absence (control) or in the presence of a physiological concentration of EVs of follicular origin (experimental group of

EVs). In addition, a comparative assessment was made of the effect of the studied conditions on age-related transformations in mature oocytes during their maturation in vitro in terms of the ability to reach pre-implantation stages of development after subsequent aging and in vitro fertilization. To study the changes associated with oocyte aging, we used the model of prolonged culture [20, 21]. According to the model, COCs after the in vitro maturation for 24 h and before in vitro fertilization were additionally cultured for another 10 h both in the control and test variants. For accuracy of our experiment, COCs both during maturation and further aging were cultured in TCM-199 medium supplemented with 3 mg/ml BSA, 0.5 mM sodium pyruvate and 100 ng/ml EGF, i.e., not containing gonadotropic hormones and serum.

In the experiment, we isolated EVs from 40 ovary follicles, characterized the preparations by the vesicular protein comcentration, and confirmed the presence of EVs in the samples using TEM. The methodology for isolating EVs from bovine follicular fluid was based on differential stepped centrifugation and ultracentrifugation at 100,000 g. As practice shows [17, 19], this methodology is effective in obtaining the fraction of EVs, which are mainly exosomes ranging in size from 30 to 150 nm. The described technique was first proposed for the isolation of EVs from the oviduct fluid [30, 31] and used by us in a partial modification [19].

Thus, we isolated 37.5 μg of vesicular protein per 1 ml of FF. The total pool collected from the entire FF volume was analyzed using TEM, which confirmed the presence of exosome-type vesicles in the sample. This allows EVs to be used in functional experiments. Figure 1 shows a typical view of vesicles from FF of bovine ovaries (follicle diameter 3-6 mm). Both single and aggregated vesicles are observed.

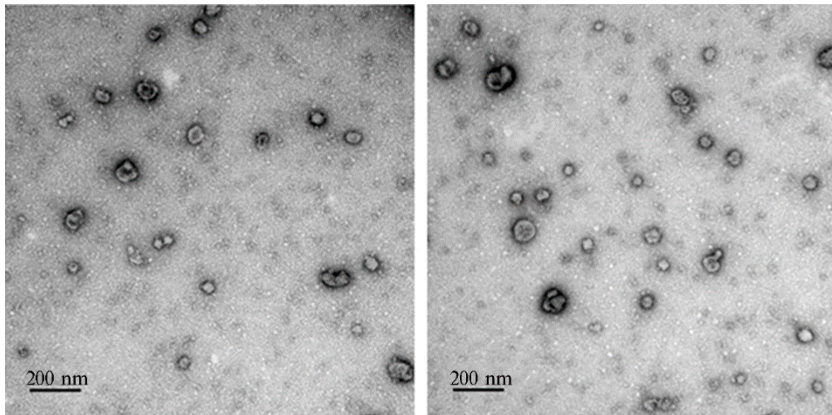

Fig. 1. Ultrastructure of extracellular vesicles (EVs) **from bovine (*Bos taurus*) ovarian follicular fluid** (transmission electron microscopy, JEOL 1011, JEOL, Ltd., Japan; GATAN RIO 9 camera, Gatan, Inc., USA; DigitalMicrograph3 program, Gatan, Inc., USA).

To evaluate the effect of EVs during the IVM period on oocyte competence for embryonic development after in vitro aging, we performed four independent experiments using vesicular protein from one isolation. Table 1 characterizes the developement of embryos when the IVP technology was applied. The proportion of fragmented zygotes (Fig. 2, A, D) on day 3 after in vitro fertilization in the control was lower vs. test cultures (53.5±2.9 vs. 63.8±2.9%, $p < 0.05$). For mature and aging in vitro oocytes, a positive effect of EVs on development to the blastocyst stage was found (Table 1, Fig. 2, B, E). In the control, the yield of blastocysts was 17.3±1.6% (see Table 1, Fig. 2, B). The presence of EVs in the maturation medium increased this indicator to 20.2±2.5% ($p < 0.05$) (see Table 1, Fig. 2, E). The trend persisted in the case of calculating this indicator from the

total number of embryos formed (41.8±1.2 vs. 32.3±1.9, $p < 0.05$).

1. Development of embryos obtained by IVP technology (in vitro production) **after maturation of cows (*Bos taurus*) oocytes in the absence** (control) **and in the presence of extracellular vesicles** (EVs) **from the follicular fluid and aging for 10 h** (*M*±SEM)

Group	Oocytes		Oocytes developed to to the blastocyst stage, %	
	total	cleaved after fertilization, %	from total	from cleaved
Control	116	53.5±2.9	17.3±1.6	32.3±1.9
Evs	121	63.8±2.9*	26.5±0.7*	41.8±1.2*

* Differences with control are statistically significant at $p < 0.05$.

2. Cytological analysis of blastocysts after in vitro fertilization of cow (*Bos taurus*) oocytes in vitro maturation in the absence (control) **and in the presence of extracellular vesicles** (EVs) **from the follicular fluid and aging for 10 hours** (*M*±SEM)

Group	Number		Average number of nuclei per blastocyst
	of experimrnts	of blastocysts	
Control	4	20	78,9±3,3
Evs	4	32	86,5±2,1

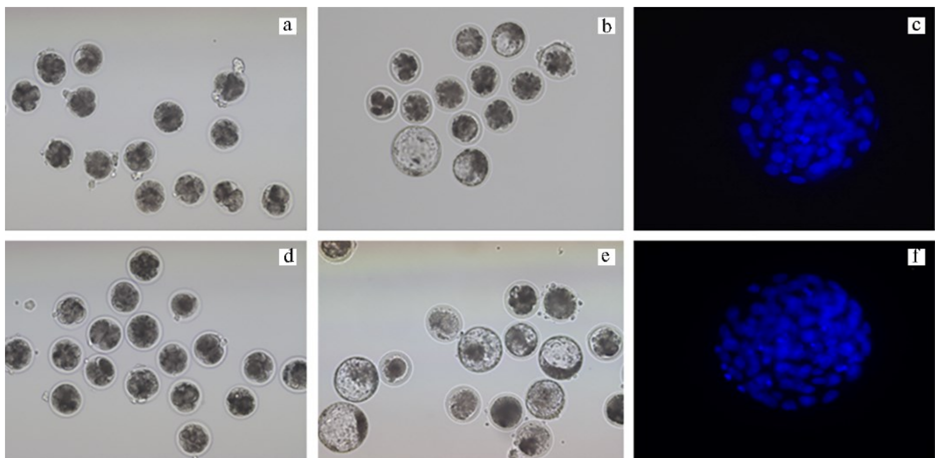

Fig. 2. Micrographs of bovine (*Bos taurus*) embryos after in vitro fertilization of oocytes matured in the absence (a-c) **or presence** (d-f) **of extracellular vesicles (EVs) from the follicular fluid and aging for 10 h:** a, d — fragmented zygotes (×100 magnification), b, e — embryos that have developed to the blastocyst stage (×100 magnification, Eclipse Ti-U microscope, Nikon, Japan); c, f — staining of nuclei in the blastocyst with DAPI (blue color; cytological preparation) (magnification ×400, microscope Axio Imager M2, Carl Zeiss, Germany).

The use of EVs did not significantly alter the quality of IVP embryos at the blastocyst stage, which was assessed by the number of nuclei on day 7 after fertilization (Table 2, see Fig. 2, C, E). According to cytological analysis, this indicator in the compared groups was the same and corresponded to the studied stage of development [32].

Oocyte aging is a complex biological process that can lead to a number of changes in the structure and function of mammalian oocytes, including DNA damage, decreased fertilization rate, disruption of mitochondrial structure, early apoptosis of oocytes, and decreased ability to develop embryos [22, 26, 33, 34]. Under in vitro conditions, changes in oocytes associated with aging can occur both during their maturation and during fertilization, which can adversely affect the development and quality of IVP embryos [25, 26, 34]. Nevertheless, despite the need to solve the problem of oocyte aging under in vitro conditions, there are very few studies aimed at finding regulators that reduce age-related transformations of oocytes. To date, the possibility of inhibition, at least partial, of the aging process when bovine oocytes are exposed to L-carnitine has been shown. The

introduction of this substance into the maturation medium reduces the level of oxidative stress and apoptosis in mature oocytes during their aging, and increases the yield of blastocysts from 20.9 (control) to 29.2% [25]. In addition, it has been found that the pituitary hormone prolactin (PRL) can specifically affect a mature oocyte and increase its resistance to aging processes, including those associated with the loss of oocyte competence for subsequent embryonic development [26]. When exposed to PRL, the proportion of oocytes that reached the blastocyst stage increased by 1.9 times compared to the control (15.2 vs. 8.2%, respectively). Here, we have demonstrated that EVs from bovine ovarian FF during maturation of oocytes can also increase their resistance to age-related changes and improve embryo development in vitro. However, unlike L-carnitine and PRL, the presence of EVs not only contributes to a similar increase in the yield of blastocysts (from 17.3 to 26.5%), but also improves the efficiency of fertilization, increasing the proportion of crushed oocytes from 53.5 to 63. 8 %.

Thus, the data obtained allow us to conclude that the use of extracellular vesicles (EVs) from the follicular fluid of cow ovaries in the in vitro maturation procedure increases the resistance of oocytes to age-related modification and, as a result, positively affects their competence for embryonic development after aging in vitro and fertilization. It is also clear that EVs when used during in vitro maturation of oocytes increase the efficiency of in vitro embryo production (IVP) biotechnology in cattle.

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