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BOVINE OOCYTE ABILITY TO EMBRYONIC DEVELOPMENT WHEN MATURING IN DIFFERENT TWO-PHASE CULTURE SYSTEMS

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Abstract

Existing approaches to a two-phase method of culture of cattle oocytes do not take into account their specific demands during maturation from metaphase I to metaphase II including the need for normalization of profiles of sex steroid hormones in a culture medium. The aim of the presented research was to compare the developmental competence of bovine (*Bos taurus taurus*) oocytes matured in conventional single-phase and different two-phase systems. We have studied for the first time the ovum ability to develop to the blastocyst stage and the quality of embryos produced when replacing the standard medium at the second stage of culture with a medium free of follicle-stimulating hormone (FSH) as well as by a granulosa cell culture. When using the single-phase system, cumulus-oocyte complexes (COCs) were cultured for 24 h in the medium TCM 199 containing 10 % fetal bovine serum (FBS), 10 μg/ml FSH, and 10 μg/ml luteinizing hormone (LH). In the two-phase system, oocytes matured in the same conditions for first 12 h. Then COCs were transferred to a new medium (TCM 199 containing 10% FBS or the same medium supplemented with 5 μg/ml LH) and cultured for 12 h in the presence or in the absence of granulosa cells. After 24 h of maturation, oocyte fertilization was performed. In embryos that developed to the stages of late morula and blastocyst on day 7 of culture, the degree of apoptosis and the total number of nuclei were assessed (by the TUNEL method and DAPI staining, respectively). In media collected after oocyte maturation, levels of progesterone and 17β-estradiol were determined by enzyme immunoassay. The proportions of oocytes entered the first cleavage division (57.6-68.1 %) or developed to the stages of late morula/blastocyst (16.7-20.7 %) were not associated with the culture method or medium. The transfer of oocytes after 12 h of maturation to the medium free of gonadotropic hormones resulted in 1.3-1.4-fold increase in the total number of nuclei in the late morula/blastocysts both in the absence and in the presence of granulosa cells, whereas this number decreased to the level of single-phase control on addition of LH. At the same time the proportion of apoptotic nuclei (2.3-6.9 %) did not depend on the system of oocyte maturation or the effect of LH. In the two-phase system, the level of 17β-estradiol in the maturation medium (with and without LH) was 1.3-1.4-fold lower ($p < 0.01$) as compared to the single-phase system. On the contrary, co-culture of COCs with granulosa cells led to a rise in the concentration of 17β-estradiol in the culture medium containing LH (from 418±16 to 496±26 pg/ml, $p < 0.05$). Meanwhile, no differences in the progesterone level in the culture medium of COCs were found between all the systems studied. The findings suggest that the two-phase system of maturation of bovine oocytes may be used as an alternative to the conventional IVM protocol. The transfer of oocytes after 12 h of culture to the medium free of gonadotropic hormones causes an increase in the quality of the late morula/blastocysts produced as well as leads to normalization of the 17β-estradiol profile in the culture medium at the terminal step of the ovum maturation.

Keywords: bovine oocytes, two-phase system of in vitro maturation, embryonic

The use of in vitro fertilization with embryo transplantation (IVF-ET) is considered as one of the reliable and effective methods of breeding highly productive and valuable species, as well as preserving the genetic potential of rare and endangered species [1]. Nowadays, the developed protocols for the in vitro culture and in vitro fertilization of bovine oocytes (*Bos taurus taurus*) make it possible to obtain a sufficiently high yield of morulas and blastocysts, but the potential for embryonic growth in ova that mature outside the body is still much lower than in ova in vivo [2].

In the animal body, the development of oocytes occurs in the follicles of ovaries, where they naturally and consistently acquire the capacity for nuclear maturation from the diplonema stage to metaphase II stage. The process of nuclear maturation of oocytes is accompanied by a complex of cytoplasmic transformations, encompassing changes in the organization of individual organelles and molecular transformations that are necessary for ova competence to the fertilization and subsequent embryonic growth [3]. When immature oocytes are isolated from follicles and cultured in vitro, meiosis resumes in them spontaneously and prematurely, which leads to incomplete cytoplasmic maturation and decrement of the ability to develop [4]. At the same time, environmental conditions in culture become one of the key factors determining the quality of mature oocytes [2].

At this moment, there are various approaches to the increment of the usefulness of oocytes during their in vitro maturation (IVM). The attempts to simulate events in natural conditions are the most interesting. One of such approaches focuses on the analysis of metabolic processes in maturing oocyte-cumulus complexes (OCCs) [5, 6]. Based on the analysis data, it is proposed to modify the culture medium in accordance with the energy needs of the oocytes and their metabolic cooperation with the surrounding somatic cells. Attempts in the works of other researchers have been made to use additives in the medium of paracrine factors, which are produced by oocytes and can support a certain degree of differentiation of cumulus cells [7, 8].

A differentiated approach involving the use of a two-phase culture system looks more promising, since there is a temporary inconsistency (asynchrony) between nuclear and cytoplasmic transformations in the in vitro maturation period of mammalian oocytes. The applicability of using a two-phase protocol for in vitro maturation of bovine oocytes (bovine cattle) has been actively studied in the last few years. The search for optimal methods of inhibiting the spontaneous resumption of meiosis [9, 10] is in progress. It is believed that the inhibition of meiosis can allow more oocytes to complete cytoplasmic transformations and acquire competence for development. Oocytes isolated from follicles are cultured for a while in a maturation medium with addition of meiosis regulators, mainly, inhibitors of cdk-kinases, capable of blocking the transition from diplotenes to metaphase I, and then during the absence of these substances. Nevertheless, the obtained results are quite contradictory and suggest the need to search for specific molecules for oocyte stimulation during blocking of meiosis [11-15].

It should be noted that the main cytoplasmic transformations, which ultimately determine the oocytes ability to embryonic growth, occur only at the final stage of maturation, which shows the importance of studying the specific needs of female gametes precisely in this period. According to several authors, with the in vitro maturation of bovine oocytes, as in vivo, there is an increase in the progesterone concentration and a decrease in the estrogen concentration in the culture medium. The most significant changes in the kinetics of these

processes occur 8-10 hours after the onset of IVM, which corresponds to a peri-period from metaphase I to metaphase II [16-18]. At the same time, in many conventional single-phase systems for the bovine oocytes culture that include follicle-stimulating (FSH) and luteinizing (LH) hormones or their analogs, the nature of the changes in the 17β-estradiol and progesterone ratio differs from that in vivo [19].

Therefore, it seems advisable to use a new approach to the two-phase culture. It is based on the ova maturation of bovine to the stage of metaphase I in a standard system and subsequent transfer to a new medium modified by the application of physiologically relevant substances that have a luteotropic effect. Other variant is based on granulosis cells possessing greater steroid activity than cumulus cells [20].

We have studied for the first time the ability of ova to develop to the blastocyst stage and the quality of the obtained embryos by replacing the standard medium in the second stage of culture medium free from FSH (an estrogen synthesis stimulant), as well as the culture of granulosa cells.

The aim of the presented study was to compare competence to growth in bovine oocytes that matured in the generally accepted single-phase and various two-phase systems.

Techniques. In all the experiments, except for indicated selected cases, reagents of the company Sigma-Aldrich (USA) were used.

The object of the study was oocyte-cumulus complexes (OCCs) from antrum follicles of bovine ovaries (*Bos taurus taurus*) and bulling heifers. Ovaries selected after slaughter were delivered to the laboratory in 3-5 hours at 30-35 °C and were repeatedly washed in sterile saline solution with antibiotics (penicillin 100 IU/ml, streptomycin 50 μg/ml). The OCCs were isolated by dissecting the follicle walls with a blade and were washed 3 times in TC-199 medium containing 5 % fetal bovine serum (FBS), 10 μg/ml heparin, 0.2 mM sodium pyruvate and 50 μg/ml gentamicin. Round shape oocytes with homogeneous cytoplasm and uniform width pellucid area surrounded by multilayer compact cumulus were collected for the experiments. All oocyte manipulations were performed with a stereomicroscope SMZ (Nikon, Japan) on a warm stage MATS-OZ (Tokai Hit, Japan) at 37 °C.

The OCCs were cultured in 4-well plates in groups of 30-35 pieces in 500 μl of medium at 38.5 °C in an atmosphere with 5 % CO_2 and 90 % humidity. To produce maturing oocytes, a standard single-phase (control) and two-phase culture systems were used. Using a single-phase system, the OCCs were cultured for 24 hours in a TC-199 medium containing 10 % FBS, 1 mM sodium pyruvate, 50 μg/ml gentamicin, 10 μg/ml FSH and 10 μg/ml LH. In a two-phase system, the oocytes matured under the same conditions for the first 12 hours. The OCCs were then transplanted into a new medium and cultured for a further 12 hours in the presence and absence of granulosa cells. At the second stage of two-phased culture, a TC-199 medium containing 10 % FBS, 1 mM sodium pyruvate and 50 μg/ml gentamicin (internal control) or the same medium supplemented with LH (5 μg/ml) was used.

Granulose cells were obtained by aspiration of fluid from follicles 3-5 mm in diameter and subsequent centrifugation of the material at 250 g for 10 min. After removal of the supernatant, the cells were washed twice in TS-199 medium containing 5 % FBS and 50 μg/ml gentamicin. A finite number of cells were counted in Goryaev's chamber, the proportion of living cells was determined with a 0.1 % trypan blue solution. The granulosa cells (1×10^6/ml) were pre-cultured in 4-well plates in 500 μl of TC-199 medium containing 10% FBS, 1 mM sodium pyruvate, and 50 μg/ml gentamicin. After 12 hours, 250 μl of medium was

replaced with fresh medium, then added to OCC wells. Co-culture of oocytes and granulosa cells was carried out for 12 hours.

At the end of culture, the medium was collected, frozen and stored at –20 °C. The content of progesterone and 17β-estradiol in media samples was determined by the method of enzyme immunoassay. The tests were carried out using a Uniplan (Pikon, Russia) plate-type spectrophotometer and commercial sets of NVO Immunotech reagents (Russia) according to the instructions of the proprietor companies. The sensitivity of the method was 0.4 nmol/L for progesterone and 25 pmol/l for 17β-estradiol. All analyzes were performed in duplicate, the coefficient of variation in the assay did not exceed 13 %.

Oocytes were fertilized in vitro, as described previously [21]. The OCCs matured in a single-phase or two-phase system were washed once in a Fert-TALP medium modified with 10 μg/ml heparin, 20 μM penicillamine, 10 μM hypothurine and 1 μM epinephrine, and transferred to 4-well plates (Nunc, Denmark), which contained 400 μl of the same medium, covered with an equal volume of mineral oil. Active spermatozoa obtained by the swim-up method [22] were added to the wells with mature oocytes at a final concentration of 1×10^6 spermatozoa/ml. In all experiments, frozen-thawed semen of one bull was used for oocyte fertilization. Oocytes fertilization, and embryo culture were carried out at a temperature of 38.5 °C in the atmosphere with 5 % CO_2 and 90 % humidity.

After 18-20 hours of incubation with sperm the oocytes were gently pipetted and washed in Fert-TALP medium to release cumulus cells and adherent spermatozoa. Intended zygotes were transferred to the CR1aa medium [23] and cultured for 4 days, after which the developing embryos were placed in the same medium containing 5 % FBS. On day 2 after oocytes fertilization a morphological evaluation of the divided zygotes was carried out, on day 7 the number of embryos matured to the stages of the late morula and blastocyst was determined. The evaluation was performed with a stereomicroscope SMZ (Nikon, Japan) with a ×40 magnification.

The resulting morulas and blastocysts were fixed with a 4 % solution of paraformaldehyde in sodium phosphate buffer for 60 min at the room temperature. After fixation, the embryos were permeabilized for 30 minutes in 0.1 % sodium citrate solution containing 0.5 % Triton X-100. The degree of apoptotic changes in nuclear material in embryos was determined by the TUNEL method using the In Situ Cell Death Detection Kit, fluorescein (Roche Diagnostics, Switzerland) according to the manufacturer's instructions. The embryos were then stained for 20 minutes with a DAPI solution (1 μg/ml) to localize the nuclei, transferred to dry skim glass and enclosed in Vectashield medium (Vector Laboratories, UK). Microphotography and evaluation of the preparations were performed under the motorised microscope Axio Imager.M2 (Carl Zeiss, Germany) equipped with a fluorescent attachment using the ZEN 2 pro software (Carl Zeiss, Germany). The degree of apoptosis in blastocysts was estimated by the fraction of TUNEL-positive nuclei (green color) from the total number of nuclei (blue color).

Experiments on the culture of oocytes were repeated at least 6 times. Obtained data was processed using the one-way ANOVA method and the two-way ANOVA variance analysis using SigmaStat software (Systat Software, Inc., USA). The results are presented as mean values (X) and standard error (±SEM). The significance of differences of the mean values compared was assessed using Tukey's test.

Results. Extracorporeal maturation of oocytes is an important stage in the technology of in vitro production of bovine embryos, the modeling of which can significantly improve its efficiency [7]. In the present work, along with the

standard single-phase IVM protocol, we first used the two-phase culture of bo-bovine oocytes, which assumed their maturation within the first 12 hours in a conventional system containing gonadotropic hormones, and the next 12 hours in a new medium without hormones. Besides, an attempt was made to modify the second phase by registration into the LH system and/or culture the oocytes on the monolayer of granulosa cells.

At the first stage of the work, we investigated the quantitative and qualitative characteristics of bovine oocytes during embryonic growth in vitro (Table). The ability of oocytes to enter the first cleavage division and mature to late morula/blastocyst stages (Fig. 1, A) did not depend on the method of culture. The presence of granulosa cells in the second phase of oocyte maturation did not significantly change the studied parameters, however, when LH were added to this system, there was a tendency ($p < 0.1$) to decrease the embryo yield at the advanced stages of growth in comparison with that obtained in a single-phase system.

Ability of bovine oocytes (*Bos taurus taurus*) to embryonic growth in different in vitro maturation systems (X±SEM, n = 6-7)

System (group)	GC	Number of oocytes, pcs.	Cleavage, %	Matured to late morula/blastocyst stage			
				% rate		number of nuclei	
				of oocytes	of embryos	total, pcs.	apoptopic, %
Without GC							
SPS	–	224	68.1±3.1	19.9±2.2	28.9±2.2	40.4±2.6[a]	5.4±2.2
TPS (C)	–	217	66.3±3.9	17.5±2.6	27.1±4.1	56.2±5.8[b]	2.3±0.8
TPS (LH)	–	220	66.1±4.0	16.8±2.6	25.6±3.7	41.3±3.9	5.1±0.8
In the presence of GC							
SPS	–	196	67.1±3.9	20.7±2.2	30.6±1.7	42.0±4.0[c]	6.4±1.3
TPS (C)	+	189	57.6±5.6	16.7±2.3	28.6±1.7	54.9±4.0[d]	5.1±1.1
TPS (LH)	+	195	58.2±3.1	13.9±2.6	23.8±4.0	42.4±6.9	6.9±2.7

Note. SPS — single-phase system (comparison group), TPS (C) — two-phase system, without luteinizing hormone (control), TPS (LH) — two-phase system with luteinizing hormone (5 ug/ml); GC — granulosa cells.
[a, b] $p < 0.05$, [c, d] $p < 0.05$ — the reliability of the differences between the comparison groups.

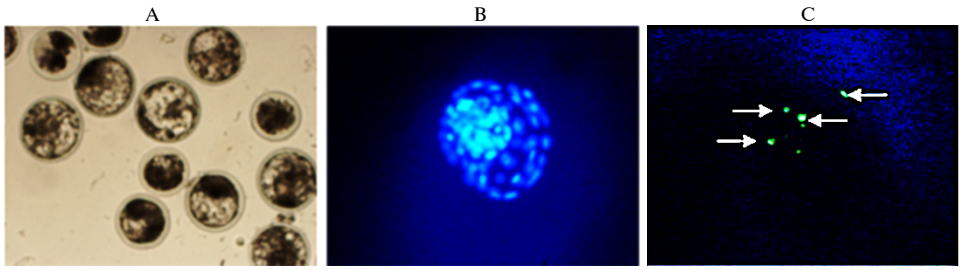

Fig. 1. Representative microphotographies of bovine embryos (*Bos taurus taurus*) on day 7 of in vitro culture and detection of apoptosis: A — morphology of morula and blastocyst (×100 magnification), B — staining of nuclei in blastocyst with DAPI (blue), cytological preparation (×200 magnification), B — staining of apoptotic nuclei in blastocyst by TUNEL method (green), cytological preparation (TUNEL-positive nuclei are marked by white arrows, ×200 magnification); microscope Axio Imager.M2, Carl Zeiss, Germany.

The quality of the embryos, which was estimated from the number of nuclei contained in the late morulas/blastocysts on day 7 after fertilization (see Fig. 1, B), was higher during the two-phase culture in the control group. The transfer of oocytes after 12 hours of maturation into a medium, gonadotropic hormones-free, led to an increase in the number of nuclei by a factor of 1.3-1.4 ($p < 0.05$), both in the absence and in the presence of granulosa cells, while adding LH. This number was reduced to the indications in a single-phase control (comparison group). At the same time, the proportion of apoptotic nuclei in the late morulas/blastocysts (see Fig. 1, C) was independent of the oocyte maturing system or the effect of LH.

As is known, the transition to a two-phase system for the culture of swine oocytes, which involves the exclusion of gonadotropic hormones in the second phase of maturing, radically changed the efficiency of the entire in vitro embryo production technology for this species of animals [24]. The data of our work are to a certain extent similar to the results observed in the maturation of swine oocytes. The absence of FSH and LH in the medium at the second stage of culture of bovine oocytes had a positive effect on the development of late morulas/blastocysts.

Next step of the work we determined the concentrations of 17β-estradiol and progesterone in culture media conditioned with maturing OCCs, depending on the culture system used, considering the importance of steroid hormones for nuclear and cytoplasmic maturation of bovine cattle oocytes [25]. At the end of the culture, the content of 17β-estradiol in the two-phase system without hormones (448±25 pg/ml) was less than in the single-phase system (583±27 pg/ml) 1.3 times ($p < 0.01$) (Fig. 2, A). Registration of LH into the two-phase system led to a more significant decrease in this indicator (up to 418±16 pg/ml, $p < 0.001$). In contrast, co-culture of OCCs with granulosa cells resulted in an increase in the concentration of 17β-estradiol in the culture medium, especially in the presence of LH (up to 496±26 pg/ml, $p < 0.05$). At the same time, we did not find any differences between all the investigated variants in the progesterone content in the culture medium of OCCs (see Fig. 2, B).

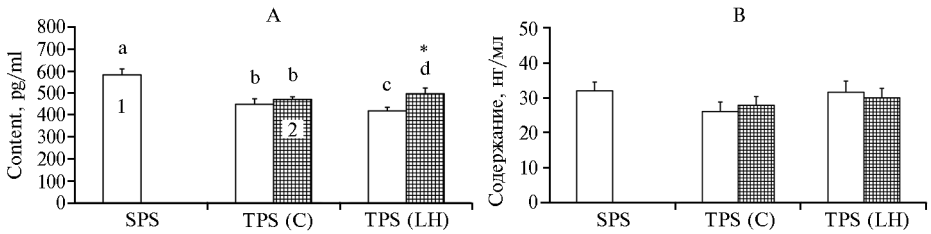

Fig. 2. The content of 17β-estradiol (A) **and progesterone** (B) **in the culture medium after in vitro maturing of bovine oocytes (*Bos taurus taurus*) in different systems:** SPS — single-phase system (comparison group), TPS (C) — two-phase system, without luteinizing hormone (control), TPS (LH) — two-phase system with luteinizing hormone (5 μg/ml); 1 — oocyte-cumulus complexes (OCCs), 2 — oocyte-cumulus complexes + granulosa cells (OCCs + GC). Vertical segments are standard errors of means (±SEM). The number of independent experiments — $n = 6$ (single-phase system), $n = 5$ (two-phase system).
$^{a, b}$ $p < 0.01$, $^{a, c}$ $p < 0.001$, $^{a, d}$ $p < 0.05$ — the reliability of the differences between the comparison groups.
* $p < 0.05$ — the reliability of the differences between OCC and OCC + GC.

The observed variations in the content of 17β-estradiol in the medium with the constant production of progesterone by the follicular cells in the second phase of oocyte maturation did not significantly affect the ability of the latter to mature to the late morula/blastocyst stage. When oocytes matured in the control two-phase system, the quality of embryos at the pre-implantation stage of growth could be associated with a decrease in the content of 17β-estradiol in the culture medium to a certain level, since the number of nuclei in them decreased both with a further decrease and with an increase in the concentration of 17β-estradiol in the presence of luteinizing hormone.

Thus, a two-phase maturation system for bovine oocytes can be used as an alternative to the conventional IVM protocol (in vitro maturation). Oocytes transfer to fresh medium without gonadotropic hormones after 12 hours of culture in a standard medium does not impair their ability to further embryonic growth and improves the quality of the obtained late morulas/blastocysts. Besides, the concentration of 17β-estradiol in the culture medium at the final stage of oocytes

maturation is reduced, which may also have some positive effect on the oocytes. Under the studied conditions, the absence of an increase in the progesterone content in the medium indicates the need to search for physiologically relevant factors that stimulate the production of progesterone in oocyte-cumulus complexes, with a view to their further application in the second phase of oocyte culture.

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