AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1206-1213
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Cell cultures

UDC 636.2:591.39

doi: 10.15389/agrobiology.2019.6.1206eng
doi: 10.15389/agrobiology.2019.6.1206rus

FINAL MATURATION OF BOVINE OOCYTES IN A FERT-TALP MEDIUM INCREASED THEIR QUALITY AND COMPETENCE TO IN VITRO EMBRYO DEVELOPMENT

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The authors declare no conflict of interests
Acknowledgements:
Supported by grant from Russian Foundation for Basic Research (project No. 17-29-08035) and the Ministry of Science and Higher Education of the Russian Federation
Received September 17, 2019

Abstract

In vitro maturation (IVM) of the oocytes is an important step for in vitro embryo production (IVP). In vitro culture during maturation decreases oocyte quality and therefore, IVM conditions need to be improved. Routinely, IVM of bovine oocytes is performed using one-step medium, the TC-199 complemented with fetal bovine serum (FBS) and gonadotropins. However, the asynchrony of oocyte nuclear and cytoplasm maturation processes may require the differential hormonal environment during IVM. In the present work, for the first time we have compared the efficiency of the two-step IVM protocols, which include the first step in standard conditions and final maturation in hormone-free mediums TC-199 or Fert-TALP; the last one is a routine medium for in vitro fertilization (IVF). The objective was to study the effects of two-step IVM protocols to chromosome modifications and apoptotic events in mature oocytes and a quality of in vitro produced embryos. Oocyte-cumulus cells complexes (OCC) were in vitro matured during 16 h in medium TC-199 complemented with 10 % FBS, 10 μg/ml follicle-stimulating hormone and 10 μg/ml luteinizing hormone, and then transferred to either medium TC-199 without gonadotropins (System 1) or Fert-TALP medium (System 2) for additional 8 h of IVM. After final IVM, nuclear state and apoptosis were checked in a part of the mature oocytes. IVF was performed on remaining OCC to analyze in vitro embryo development. No difference was observed in nuclear maturation rates between the systems 1 and 2 after 24 h IVM: 83.3 % and 84.7 % of the oocytes reached metaphase-II. Apoptosis rate were significantly lower ($p < 0.05$) in the oocytes matured in System 2 (11.7±0.7 %) compared to System 1 (19.4±1.1 %). In addition, blastocyst rate after 7 days of in vitro embryo development was significantly higher ($p < 0.05$) in System 2 (30.0±2.9 %), than in System 1(17.4±0.4 %). Oocyte cleavage rate, cell number and apoptosis rate in the blastocysts were similar in both IVM systems. In conclusion, two-step IVM system using final 8-hour maturation in Fert-TALP medium is more advantageous compared to a system including hormone-free TC-199 medium at the end of IVM. Fert-TALP IVM system decreased apoptosis rate in the oocytes and increased their competence to in vitro embryo development after IVF.

Keywords: bovine oocytes, in vitro maturation, maturation media, apoptosis, embryo development

In bovine cattle, reproductive cellular technologies, including in vitro embryo production (IVP), have extensive scope of application in scientific research, as well as in supporting reproduction, maintaining genetic diversity and breeding of animals possessing the preset properties [1-3]. By now, there has been a significant advancement in development of IVP technology; the usefulness of embryos developed in vitro, however, is still considerably lower than of those developed in vivo. Moreover, many issues relating to the vitality of the new breed are still unsolved [4, 5].

Standard IVP technology involves several phases, the first of which is in vitro ovum maturation. In immature oocytes, in response to extraction from a follicle and placement in culture medium, meiosis restarts and nuclear transfor-

mations from diplotene stage to metaphase II stage (MII) commence. Oocytes also undergo various structural and molecular transformations at cytoplasm level (cytoplasmic maturation) which are necessary to prepare an oocyte for fertilization and further embryonic development [6]. As is commonly known, when the oocytes restart meiosis in vitro, the same processes of nucleus maturation as in oocytes in vivo occur in them, while the level of cytoplasm maturity remains insufficient, affecting the quality of ova and, after their fertilization, the quality of IVP embryos. The attempts to resolve that issue, despite persistent effort of the researches, have still not yielded a desirable result. The conditions in which oocytes mature remain suboptimal and require further simulations [4, 7, 8].

Most times, one-step culture in TC-199 medium supplemented with fetal bovine serum (FBS) and gonadotropic hormones is used for in vitro maturation of cow oocytes. It was demonstrated that in such conditions, the average of 80% of immature oocytes reach metaphase II of the second meiotic division [9] but only 15-40% develop to the blastocyst stage [10, 11]. Low production of embryos may be caused by a combination of factors. First, one-step culture omits the temporal mismatch (asynchrony) between nuclear and cytoplasmic transformations. Second, TC-199 is a complex medium developed for culturing somatic cells, and although the serum contained in it serves as a source of growth factors, amino acids and endotoxins [12], which is important for maturity level, it is a variable component that can be the cause of reduction in the number of IVP embryos [13].

Earlier, for the first time we demonstrated that two-step culture of cow oocytes, when the standard system is used at the first step and is followed by oocytes being moved to a fresh medium free of gonadotropic hormones, can increase the number of nuclei in embryos at the blastocyst stage [14]. Such improvement could be caused by corresponding cytoplasmic transformations occurring during the final stage of maturing, on which the oocytes' capacity for further embryonic development ultimately depends. We have assumed that two-step culture in media differing in composition at the initial and final stages of oocyte maturation may be an alternative (differentiated) approach in the methodology of in vitro embryo production, and that further research may be required to improve its efficacy [14].

In this paper we have for the first time assessed the expediency of use during the second maturation stage of Fert-TALP [15], which, unlike TC-199, is a less complex serum-free solution. In addition, Fert-TALP medium is further used to fertilize the mature ova, thus its use during the period of maturing may reduce the stress arising in oocytes upon forced change in culture conditions when transitioning from maturing stage to in vitro fertilization.

The purpose of this paper is studying the state of chromosomes and degree of apoptotic generation in oocytes maturing in two different two-step culture systems and the assessment of effect of these conditions on development and quality of IVP embryos.

Techniques. In all experiments, except for the expressly stated cases, the Sigma-Aldrich (USA) reagents were used.

Cow ovaries (*Bos taurus taurus*) collected after slaughtering, were delivered to the laboratory within 3-5 hours at 30-35 °C, were freed of adjacent tissue and washed multiple times in a sterile physiological solution containing antibiotics (100 MU/ml penicillin, 50 μg/ml streptomycin). Oocyte-cumulus cells complexes (OCC) were isolated from the ovaries by dissecting the follicle walls with a blade, flushed 3 times in TC-199 medium containing 5% of fetal bovine serum (FBS), heparin (10 μg/ml), sodium pyruvate (0.2 mM) and gentamicin (50 μg/ml), and morphologically studied. For further culture, orbed oocytes with

homogenous cytoplasm, regular-width pellucid zone surrounded by multilayer compact cumulus were selected. All oocyte manipulations were performed under SMZ stereomicroscope (Nikon, Japan) at 37 °C.

After selection, OCC were cultured in groups, 20-30 each, in 500 μl medium at 38.5 °C in the atmosphere containing 5% CO_2 at 90% humidity. To produce mature oocytes, a two-step system was used. For the first 16 hours, OCC were cultured in TC-199 medium containing 10% FBS, sodium pyruvate (1 mM), gentamicin (50 μg/ml), follicle-stimulating hormones (FSH) (10 μg/ml) and luteinizing hormones (LH) (10 μg/ml) (standard maturation medium), whereafter they were moved to a fresh medium for another 8 hours. During the second maturation stage, TC-199 containing 10% FBS, sodium pyruvate (1 mM) and gentamicin (50 μg/ml), or a medium for further fertilizing Fert-TALP [15] that contained 114 mM NaCl, 3.2 mM KCl, 0.4 mM NaH_2PO_4, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25 mM $NaHCO_3$, 10 mM HEPES, 10 mM sodium lactate, 0.25 mM sodium pyruvate, bovine serum albumin (BSA, 6 mg/ml) and gentamicin (50 μg/ml).

After 24 hours of maturing, a portion of oocytes was freed from cumulus cells, fixed and used for cytological analysis of maturity and apoptosis as per the protocol described [16]. Another portion was moved to Fert-TALP medium supplemented with BSA (6 mg/ml), heparin (10 μg/ml), penicillamine (20 μM), hypotaurine (10 μM) and epinephrine (1 μM) for in vitro fertilization and assessment of capacity for further embryonic development.

Then, 1.5 hours prior to fertilization of oocytes, the straws containing frozen semen were unfrozen, active spermatozoa were produced by "swim-up" method in Sperm-TALP medium [17] containing sodium pyruvate (1mM) and BSA (6 mg/ml). For this purpose, the contents of straws were underlaid, in 220 μl portions, to the 1.8 ml test tubes (Nunc, Denmark) with 1 ml of Sperm-TALP medium and were incubated (a MCO-18AIC, Sanyo, Japan) for 50 minutes. In the end of incubation, 750 μl of the upper layer was collected, diluted with fresh medium and centrifuged at 300 g for 10 minutes. The resulting pellet containing motile sperm were added to the fertilization medium containing OCC (spermatozoa concentration: 1.5×10^6/ml).

Gamets were incubated jointly for 18-20 hours, whereafter the prospective zygotes were moved to the embryo-development medium [14] and cultured for 7 days. On day 2 after fertilization of oocytes cleaved zygotes were morphologically analyzed, and in the end of culturing the number of embryos that have developed to the stage of blastocyst was counted.

The resulting blastocysts were fixed with 4% paraformaldehyde solution and subjected to permeabilization in Triton X-100 solution. The extent of apoptotic changes of nuclear material in embryos was determined by TUNEL method using the In Situ Cell Death Detection Kit, fluorescein (Roche Diagnostics, Switzerland) in accordance with the instructions of the manufacturer. The embryos were then stained with DAPI solution (1 μg/ml) for localizing the nuclei, and transferred to the glass slide for analysis. Microphotography and evaluation of preparations was carried out under Axio Imager.M2 fluorescent microscope equipped with 65 HE filter (for TUNEL, excitation at λ = 445-470 nm) and 49 (for DAPI, excitation at λ = 365 nm) using digital camera Axiocam 506 and ZEN 2 pro application (Carl Zeiss, Germany). The apoptosis rate in the embryos was evaluated by the TUNEL-positive nuclei percentage of the total number of nuclei.

In statistical processing, one-way analysis of variance was applied (SigmaStat application by Systat Software, Inc., USA). The results are given as means (M) and standard errors of the means (±SEM). To evaluate the significance of

differences between the compared means, Tukey's test was used

Results. The medium surrounding oocytes in vitro critically affect the quality of ova, which makes these conditions a subject of targeting. In the paper presented, in order to induce the maturation of cow oocytes, instead of standard one-step IVM protocol we used two-step oocyte cultivation that implied their maturation for the first 16 hours in TC-199 medium containing 10% FBS and gonadotropic hormones and for the next 8 hours either in TC-199 free of hormones (system 1) or in Fert-TALP medium [15] (system 2). The time of changing the medium of 16 hours was selected on the basis of previous data on the initial shows of deterioration of oocyte quality occurring by that period of their in vitro maturing [18].

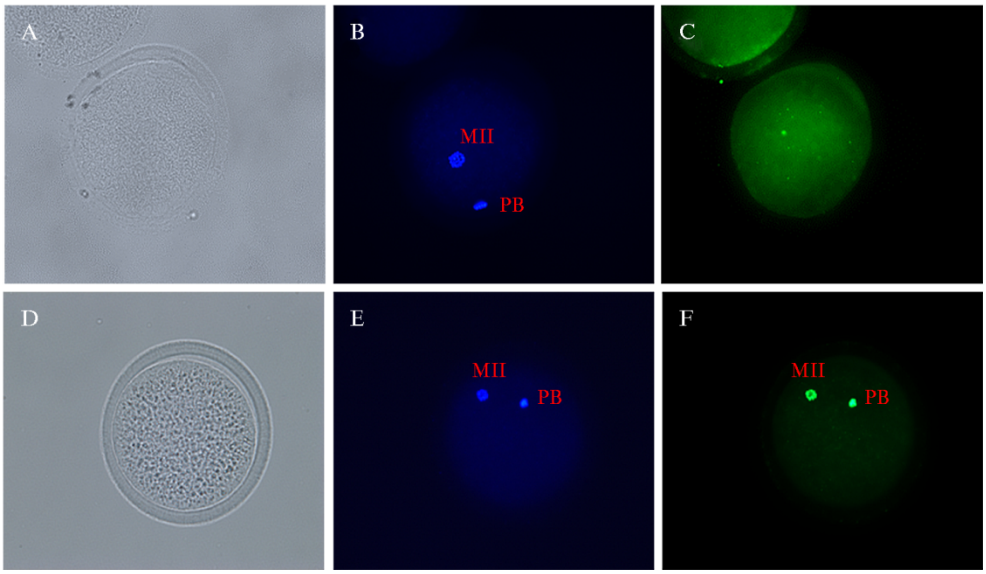

Fig. 1. Microphotographs of cytological preparations of mature cow oocytes (*Bos taurus taurus*) after 24 hrs of in vitro maturing: A, D — oocyte morphology, B, E — staining nuclear material using DAPI (blue color), MII — chromosomes at the stage of metaphase of the second meiotic division, PB — polar body; C, F — staining nuclear material using TUNEL method, upper section (C) — oocytes without any shows of apoptosis, lower section (F) — with shows of apoptosis (TUNEL-positive MII chromosomes and PB are stain green). ×400 zoom, fluorescent microscope Axio Imager.M2 equipped with 65 HE filter (for TUNEL, excitation at λ = 445-470 nm) and 49 (for DAPI, excitation at λ = 365 nm) with color digital camera Axiocam 506 (Carl Zeiss, Germany).

1. State of chromosomes and frequency of apoptotic degeneration in cow oocytes (*Bos taurus taurus*) after maturing for 8 hours in different culture systems (*M*±SEM)

Test group (system)	Count		Percentage	
	experiments	total oocytes	oocytes at MII stage	MII oocytes with shows of apoptosis
System 1	4	85	83.3±5.6	19.4±1.1
System 2	4	83	84.7±2.4	11.7±0.7*

Note. MII — metaphase II; system 1: oocyte maturing for the first 16 hours in TC-199 medium containing 10 % fetal bovine serum (FBS) and follicle-stimulating hormones (FSH) and luteinizing hormones (LH), and for the next 8 hours in TC-199 medium free of hormones; system 2: oocyte maturing for 16 hours in conditions similar to those of system 1 and for the next 8 hours in Fert-TALP fertilization medium free of heparin, hypotaurine and epinephrine.

* Differences between the compared groups are statistically significant at $p < 0.05$.

Cytological analysis did not reveal any impact of the culture system on completion of nuclear maturation. Percentage of oocytes at MII meiosis stage (Fig. 1, B-E) after 24 hours of maturing (16 + 8 hrs) was similar in both groups and made 83.3 and 84.7 % (Table 1). At the same time, the percentage of oocytes with the shows of apoptosis (see Fig. 1, F) was lower (11.7±0.7 %) when

maturing in Fert-TALP medium, while for those cultured in TC-199 containing FBS this value increased by 7.7% ($p < 0.05$) (Table 1).

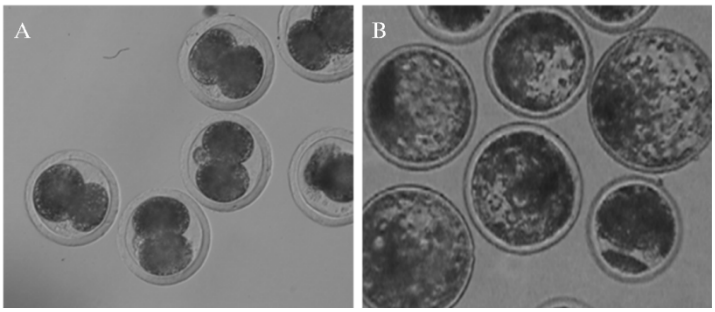

Fig. 2. Microphotographs of cow oocytes (*Bos taurus taurus*) cleaved after in vitro fertilization and of embryos developed to the blastocyst stage (B) (×200 zoom, Eclipse Ti-U microscope by Nikon, Japan).

Capacity of mature oocytes for development after in vitro fertilization that serves a criterion of the level of cytoplasmic maturity was evaluated by the oocytes' capability to the first cleavage division (Fig. 2, A) and reach blastocyst stage (see Fig. 2, B) (Table 2).

2. Cow oocyte (*Bos taurus taurus*) embryonic developmental capability in different systems for in vitro maturing (*M*±SEM)

Test group (system)	Count		Oocyte cleavage rate, %	Development to blastocyst stage, %	
	experiments	total oocytes		of number of oocytes	of number of embryos
System 1	5	150	65.4±1.1	17.4±0.4	26.6±0.9
System 2	5	123	71.7±1.7	30.0±2.9*	42.1±4.9**

N o t e. For experiment design (media and systems) see Table 1.
*, ** Differences between the compared groups are statistically significant at $p < 0.05$ and $p < 0.01$.

The percentage of cleaved oocytes determined on the 2nd day of cultivating did not vary between the experimental groups and made 65.4±1.1 and 71.7±1.7 % for system 1 and 2, respectively. Nevertheless, the impact of conditions of the second step of oocyte maturing (8 hours) on their development to blastocyst stage was found. In case of transfer of OCC after 16 hours of cultivating to TC-199 medium, the production of blastocysts was 17.4±0.4 %. Use during the relevant period of cultivation of Fert-TALP medium increased this value up to 30.0±2.9 % ($p < 0.05$) (see Table 2).

Use of two compared protocols of two-step oocyte culture did not significantly change the quality of IVP embryos that were assessed by the nuclei count on day 7 after fertilization (Fig. 3, C), however when culturing during the second stage of maturing in Fert-TALP medium, the tendency towards increase of this value was observed (Table 3). The percentage of embryonic nuclei with shows of apoptosis (see Fig. 3, C) did not also change between experimental groups and made 5.1±0.9 and 4.6±0.4 % for system 1 and system 2 respectively (see Table 3).

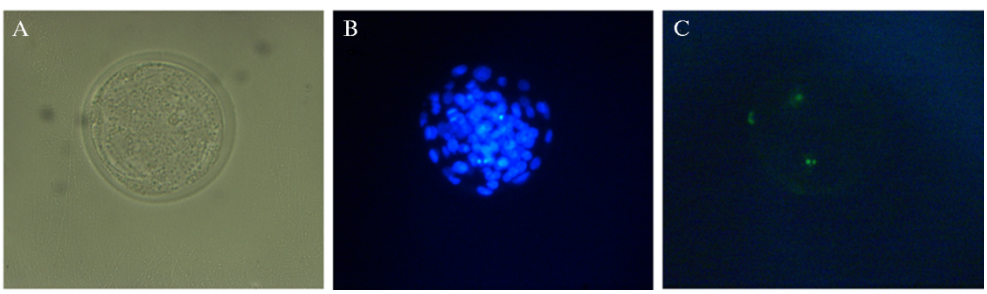

Fig. 3. Microphotographs of cytological preparations of bovine embryos (*Bos taurus taurus*) on day 7 of culture: A — blastocyst morphology; B — staining blastocyst nuclei using DAPI (blue color); C — staining apoptotic nuclei in blastocyst using TUNEL method (TUNEL-positive nuclei are stained green). ×200 zoom, Axio Imager.M2 fluorescent microscope by Carl Zeiss, Germany.

3. Quality of IVP (in vitro embryo production) **embryos after 8 hours maturing of cow oocytes (*Bos taurus taurus*) in different culture system followed by in vitro fertilization** (*M*±SEM)

Maturing medium (system)	Experiments	Total blastocyst count	Blastocysts nuclei count	
			total	apoptotic, %
TCM + 10 % FBS (1)	5	26	62.9±5,2	5,1±0,9
Fert-TALP (2)	5	37	72,2±5,1	4,6±0,4

N o t e. For experiment design (media and systems) see Table 1.

The expediency of application of two-step bovine IVM protocol has been actively studied over the past few years. In order to resolve the problem of asynchrony between nuclear and cytoplasmic maturation, OCC extracted from follicles are for some time cultured in maturation medium supplemented by meiosis inhibitors, and then without them [19]. However, cytoplasmic transformations, on which the oocytes developmental capability ultimately depends, occurs not on the initial but on the final stage of IVM, which demonstrates the relevancy of research of the specific needs of female gametes during this very period [4].

Therefore, this work for the first time compares two-step IVM protocols that include cow oocyte maturation during the first step in the standard medium and maturation in one of two hormone-free media (TC-199 and Fert-TALP) during the final step. Although TC-199 medium containing FBS is commonly used in the majority of one-step IVM protocols and is capable of sustaining the high ova capability for further development [15], in our two-step system during the final maturation stage it turned out to be less effective in terms of embryo production than Fert-TALP medium. Moreover, its use in the similar conditions has adversely affected the apoptotic degradation in ova. The cause of such effect could be not only the fact that TC-199 medium, unlike Fert-TALP, is a complex solution, but also the fact that it is blood serum instead of BSA that is used there as a source of protein. It is believed that adding the serum results in uncertain and changing conditions of cell cultivation [11, 13, 20, 21]. In addition, it is shown that adding the serum to the embryo cultivation medium negatively affects their development at early cleavage stages and alters the ultrastructure and the nature of gene expression during the later stages of in vitro embryo development [22-24].

Thus, in two-step in vitro maturation (IVM), culturing oocytes during the second-step maturation (8 hrs) in Fert-TALP medium is more preferable than in TC-199 medium containing fetal bovine serum. Such conditions improve the quality of mature oocytes and their embryonic developmental capability after in vitro fertilization.

REFERENCES

1. Lonergan P., Fair T. In vitro-produced bovine embryos: dealing with the warts. *Theriogenology*, 2008, 69: 17-22 (doi: 10.1016/j.theriogenology.2007.09.007).
2. Galli C., Duchi R., Crotti G., Turini P., Ponderato N., Colleoni S., Lagutina I., Lazzari G. Bovine embryo technologies. *Theriogenology*, 2003, 59(2): 599-616 (doi: 10.1016/S0093-691X(02)01243-8).
3. Ponsart S., Le Bourhis D., Knijn H., Fritz S., Guyader-Joly C., Ottern T., Lacaze S., Charreaux F., Schibler L., Dupassieux D., Mullaart E. Reproductive technologies and genomic selection in dairy cattle. *Reproduction, Fertility and Development*, 2013, 26(1): 12-21 (doi: 10.1071/RD13328).
4. Blanco M.R., Demyda Peyrõs S., Moreno M.M., Genero E. Developmental competence of in vivo and in vitro matured oocytes: a review. *Biotechnology and Molecular Biology Review*, 2011, 6: 155-165.
5. Nagano M. Acquisition of developmental competence and in vitro growth culture of bovine oocytes. *Journal of Reproduction and Development*, 2019, 65(3): 195-201 (doi: 10.1262/jrd.2019-022).
6. Hyttel P., Xu K.P., Smith S., Greve T. Ultrastructure of in-vitro oocyte maturation in cattle. *J. Reprod. Fertil.*, 1986, 78: 615-625 (doi: 10.1530/jrf.0.0780615).
7. Abd El-Aziz A.H., Mahrous U.E., Kamel S.Z., Sabek A.A. Factors influencing in vitro production of bovine embryos: a review. *Asian Journal of Animal and Veterinary Advances*, 2016, 11: 737-756 (doi: 10.3923/ajava.2016.737.756).
8. Niemann H., Wrenzycki C. Alterations of expression of developmentally important genes in preimplantation bovine embryos by in vitro culture conditions: implications for subsequent de-

velopment. *Theriogenology*, 2000, 53: 21-34 (doi: 10.1016/s0093-691x(99)00237-x).
9. Dominko T., First N.L. Timing of meiotic progression in bovine oocytes and its effect on early embryo development. *Molecular Reproduction and Development*, 1997, 47: 456-467 (doi: 10.1002/(SICI)1098-2795(199708)47:4<456::AID-MRD13>3.0.CO;2-U).
10. Ward F., Enright B., Rizos D., Boland M., Lonergan P. Optimization of in vitro bovine embryo production: effect of duration of maturation, length of gamete co-incubation, sperm concentration and sire. *Theriogenology*, 2002, 57(8): 2105-2117 (doi: 10.1016/s0093-691x(02)00696-9).
11. Russell D.F., Baqir S., Bordignon J., Betts D.H. The impact of oocyte maturation media on early bovine embryonic development. *Molecular Reproduction and Development*, 2006, 73(10): 1255-1270 (doi: 10.1002/mrd.20553).
12. McKiernan S.H., Bavister B.D. Different lots of bovine serum albumin inhibit or stimulate in vitro development of hamster embryos. *In Vitro Cell Dev. Biol. — Animal*, 1992, 28A: 154-156 (doi: 10.1007/bf02631084).
13. Sagirkaya H., Misirlioglu M., Kaya A., First N.L., Parrish J.J., Memili E. Developmental potential of bovine oocytes cultured in different maturation and culture conditions. *Animal Reproduction Science*, 2007, 101: 225-240 (doi: 10.1016/j.anireprosci.2006.09.016).
14. Singina G.N., Lebedeva I.Yu., Shedova E.N., Taradajnic T.E., Mityashova O.S., Tsyndrina E.V., Danch S.S. Bovine oocyte ability to embryonic development when maturing in different two-phase culture systems. *Agricultural Biology* [*Sel'skokhozzyaistvannaya Biologiya*], 2017, 52(4): 776-784 (doi: 10.15389/agrobiology.2017.4.776eng).
15. Parrish J.J. Bovine in vitro fertilization: in vitro oocyte maturation and sperm capacitation with heparin. *Theriogenology*, 2014, 81(1): 67-73 (doi: 10.1016/j.theriogenology.2013.08.005).
16. Lebedeva I.Yu., Singina G.N., Shedova E.N., Lopukhov A.V., Mityashova O.S. Resistence of bovine oocytes to age-realated changes after exposure to luteotropic factors during the second phase of culture till the metaphase II stage. *Agricultural Biology* [*Sel'skokhozzyaistvannaya Biologiya*], 2018, 53(6): 1202-1211 (doi: 10.15389/agrobiology.2018.6.1202eng).
17. Singina G.N., Bagirov V.A., Danch S.S., Taradainik T.E., Dotsev A.V., Zinovieva N.A. In vitro production of interspecies hybrid embryos of cattle (*Bos taurus*) and wisent (*Bison bonasus*). *Agricultural Biology* [*Sel'skokhozzyaistvannaya Biologiya*], 2016, 51(6): 824-829 (doi:10.15389/agrobiology.2016.6.824eng).
18. Lebedeva I.Yu., Singina G.N., Lopukhov A.V., Zinovieva N.A. Dynamics of morphofunctional changes in aging bovine ova during prolonged culture in vitro. *Cell and Tissue Biology*, 2014, 8(3): 258-266 (doi: 10.1134/S1990519X14030080).
19. Guemra S., da Silva Santo E., Zanin R., Monzani P.S., Sovernigo T.C., Ohashi O.M., Verde Leal C.L., Adona P.R. Effect of temporary meiosis block during prematuration of bovine cumulus-oocyte complexes on pregnancy rates in a commercial setting for in vitro embryo production. *Theriogenology*, 2014, 81(7): 982-987 (doi: 10.1016/j.theriogenology.2014.01.026).
20. Salhab M., Tosca L., Cabau C., Papillier P., Perreau C., Dupont J, Mermillod P., Uzbekova S. Kinetics of gene expression and signaling in bovine cumulus cells throughout IVM in different mediums in relation to oocyte developmental competence, cumulus apoptosis and progesterone secretion. *Theriogenology*, 2011, 75(1): 90-104 (doi: 10.1016/j.theriogenology.2010.07.014).
21. Mastromonaco G.F., Semple E., Robert C., Rho G.J., Betts D.H., King W.A. Different culture media requirements of IVF and nuclear transfer bovine embryos. *Reproduction in Domestic Animals*, 2004, 39: 462-467 (doi: 10.1111/j.1439-0531.2004.00548.x).
22. Lonergan P., Kearney-Flynn M.O., Boland M.P. Effect of protein supplementation and presence of an antioxidant on the development of bovine zygotes in synthetic oviduct fluid medium under high or low oxygen tension. *Theriogenology*, 1999, 51: 1565-1576 (doi: 10.1016/s0093-691x(99)00099-0).
23. Abe H., Yamashita S., Itoh T., Satoh T., Hoshi H. Ultrastructure of bovine embryos developed from in vitro-matured and fertilized oocytes: comparative morphological evaluation of embryos cultured either in serum-free medium or in serum-supplemented medium. *Molecular Reproduction and Development*, 1999, 53: 325-335 (doi: 10.1002/(SICI)1098-2795(199907)53:3<325::AID-MRD8>3.0.CO;2-T).
24. Wrenzycki C., Herrmann D., Carnwath J.W., Niemann H. Alterations in the relative abundance of gene transcripts in preimplantation bovine embryos cultured in medium supplemented with either serum or PVA. *Molecular Reproduction and Development*, 1999, 53: 8-18 (doi: 10.1002/(SICI)1098-2795(199905)53:1<8::AID-MRD2>3.0.CO;2-K).