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CHANGE OF CULTURE MEDIUM POSITIVELY INFLUENCES THE DEVELOPMENT AND QUALITY OF in vitro CATTLE EMBRYOS

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Abstract

Although the practice of producing in vitro embryos (IVP) has become routine in the world, the quality of IVP embryos is still lower than that of in vivo embryos, and the conditions for obtaining in vitro embryos still need to be specified. This original research article presents data on the development and quality parameters of IVP embryos in cattle (*Bos taurus*), depending on the volume of in vitro culture medium (IVC) and its refreshment protocols. Post mortem bovine oocytes were cultured in a maturation medium, fertilized in vitro with frozen-thawed sperm, and transferred to 500 or 100 μl of BO-IVC medium (IVF Bioscience, UK) for embryonic development. The effects of not refreshing the IVC medium during the 8-day embryonic development period (NMR), half-refreshed medium (HRM) and completely refreshed medium (CRM) were compared. For HRM or CRM, after 3 days of culture, half of the initial 500/100 μl volume was replaced with fresh medium or embryos were transferred to 500/100 μl fresh medium. On day 8 of NMR, HRM, and CRM culture, the number of embryos developed to the blastocyst stage (BL) was evaluated, and the BL quality was assessed as per the total number of nuclei and the apoptotic nuclei counted cytologically. The 8-day-old blastocysts were also cultured for 2 days to the stage of hatched BL (HBL). CRM has been shown to improve embryo production regardless of the volume of the IVC medium when compared to NMR culture. Blastocyst formation increased from 23.0±1.5 and 25.8±0.8 % for 500 μl and 100 μl, respectively, to 45.7±4.8 and 52.1±4.9 % ($p < 0.01$), while apoptosis decreased from 6.53±0.88 and 6.47±0.66 to 3.60±0.12 and 3.50±0.29 % ($p < 0.05$ and $p < 0.01$). The yield of hatched blastocysts increased from 14.9±1.5 and 11.6±3.3 to 25.2±3.9 and 40.8±3.2 % ($p < 0.05$ and $p < 0.001$) from the total number of fertilized oocytes. In a 100-μl volume, the number of nuclei in BL also increased from 175.8±13.5 to 224.3±6.7 ($p < 0.05$). Similarly, the HRM culture was more favorable for embryo development compared to the NMR culture. As in the CRM culture, the blastocyst formation increased up to 44.9±0.7 and 44.1±5.0 % ($p < 0.01$ and $p < 0.01$ 0.05), and the HBL yield increased up to 26.2±3.2 and 27.7±1.4 % ($p < 0.05$). In a 100-μl volume, the number of nuclei in BL increased up to 230.4±8.4, ($p < 0.05$) while the proportion of apoptotic nuclei decreased to 3.10±0.17 ($p < 0.01$). No significant differences were found between CRM and HRM cultures for all parameters studied. However, with CRM and a 100-μl volume, the rates of BL and HBL were the highest. Thus, in cattle, CRM and HRM cultures, as compared to NRM without medium change, have a positive effect on the development and quality of IVP embryos. The noted effect obviously depends on the volume of the IVC medium. For BO-IVC medium with a volume of 500 μl, both manipulations to refresh the medium are effective, for a volume of 100 μl, CRM is the best.

Keywords: in vitro embryonic development, culture medium, IVP, IVC, cattle

The development and practical application of assisted reproductive technologies for in vitro embryo production (IVP) and embryo transplantation into recipient animals makes it possible to increase the rate of genetic progress in the selection of commercial breeds and the efficiency of programs for the conservation of small and gene pool breeds [1]. In vitro embryos suitable for transplantation develop from oocytes matured and fertilized under artificial conditions. Oocytes

can be obtained both in vivo (most often by transvaginal follicle puncture) [2] and post mortem [3]. In both cases, the goal is to get more offspring from better and more unique mothers.

Every year, the use of in vitro embryos is becoming more and more common worldwide, however, in terms of quality, IVP embryos are still inferior to in vivo embryos [1, 4]. IVP embryos are less resistant to freezing [4], and their viability after transplantation to recipients is lower than in embryos in vivo [3, 5].

An important procedure in IVP technology on which embryo quality depends is culturing oocytes after fertilization to the pre-implantation stages of development. In cattle, fertilized oocytes are cultured for approx. 7 days. The derived embryos that reached the blastocyst stage (BL) are either frozen or transplanted into recipient animals. The conditions for in vitro embryonic development critically affect both quantitative (the proportion of embryos at the BL stage) and qualitative (BL viability) BL parameters which are indicators of the IVP effectiveness [3].

For the development of cattle embryos in vitro, nutrient media self-prepared in the laboratory and simple in composition are most often used [6]. They should include the SOF medium [7] which is a synthetic analogue of the oviduct fluid, as well as the CR1aa medium [8]. In order to improve embryonic development, along with bovine serum albumin (BSA), at the final stage of culture, it is recommended to add fetal bovine serum (FBS) to both solutions, since FBS enables the embryos overcome the block at the morula stage, increases the release of BL and improves their quality [9, 10].

With the increasing commercialization of IVP technology over the past decade, there has been a need to develop off-the-shelf commercial in vitro embryo culture (IVC) media. The peculiarity of these media is that they provide a high yield of high-quality embryos of pre-implantation stages of development in the absence of FBS, which can be a source of pathogens and complicate the exchange of embryonic materials. In addition, the use of such media simplifies the procedure for obtaining embryos and makes it possible to standardize the conditions for their culturing [1, 6].

There is ample evidence in the literature that the media in which embryos are cultured ultimately determine their developmental potential and quality [11-13]. However, the modes of incubation, in particular the conditions for renewal of the medium, can improve this effects. Changing the culture medium is a common practice during the IVC procedure that positively influenses the development of the embryos, providing them with the necessary nutrients and removing toxic metabolites, such as ammonia and oxygen free radicals accumulated in the culture medium [14-16]. During in vitro culture, embryos secrete into the medium paracrine factors, such as epidermal growth factor, platelet activating factor, insulin-like growth factor, as well as messenger RNA and microRNA, which play an important role in the regulation of embryonic development [17-19]. These molecules, according to recent studies, are packed in extracellular vesicles (EVs) that can be secreted or taken up by the embryos [20, 21]. Changing the culture medium during the IVC procedure removes these important factors as well as EVs, and, therefore, can reduce the potential for in vitro embryonic development.

In cattle, IVC medium is usually replaced every 2-3 days of culture [3, 15, 22]. However, there are many reports about the dubious need for such a procedure and the possibility to reach high rates of development and quality of IVP embryos without changing or with a partial change of medium [16, 23, 24]. The last two approaches have become particularly popular due to the use of incubators with a low oxygen atmosphere [25]. That is, at the stage of maturation and fertilization, oocytes are cultured at the natural O_2 level (20%), and from the stage of the

putative zygote, at a lower air concentration of O_2. As a result, the production of reactive oxygen species and oxidative stress in the cells decrease. There is an opinion that, despite many years of experiments and routine work on obtaining IVP embryos, optimal mode of changing the IVC medium for both cattle [16] and other animals [26] has not yet been established. This is especially true for recently appeared new commercial solutions for which information is either limited or not available.

In our work, the embryonic development of mature and in vitro fertilized oocytes occurred in the commercial BO-IVC medium (IVF Bioscience, UK) chosen due to its high efficiency in IVP of embryos in cattle [27, 28]. The communication we present is the first to demonstrate the influence of the studied conditions on the development of in vitro fertilized bovine oocytes to the blastocyst stage. In addition, we assessed the quality of derived blastocysts in terms of the number of nuclei, the frequency of apoptosis, and viability.

The aim of the study was to study the influence of the conditions of the BO-IVC change on the quantitative and qualitative characteristics of embryos during their development in vitro. The possible dependence of such an effect on the volume of the culture medium was also evaluated.

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

To produce embryos, isolated post mortem cow ovaries were delivered to the laboratory, freed from adjacent tissues, and repeatedly washed in sterile saline containing penicillin (100 IU/ml) and streptomycin (50 μg/ml) (BioPharmGarant, Russia). Cumulus-oocyte complexes (COCs) were dissected with a blade from the ovaries and separated from the walls of the follicles. COCs were washed 3 times in TC-199 medium containing HEPES (25 mM), Na-pyruvate (0.5 mM), PBS (5%), heparin (10 гg/ml) and gentamicin (50 μg/ml). During washing, the morphology of the COCs was examined to select oocytes for further culture according to common criteria [29].

For maturation, COCs were incubated for 24 h in TS-199 medium containing HEPES (25 mM), Na-pyruvate (0.5 mM), follicle-stimulating and luteoinizing hormones (10 μg/ml), epidermal growth factor (10 ng/ml) (Thermo Fisher Scientific, USA), FBS (10%) and gentamicin (50 μg/ml).

After maturation, COCs were subjected to in vitro fertilization (IVF). They were washed once in BO-IVF medium (IVF Bioscience, UK) and placed in drops of the same medium 30 min before contact with spermatozoa. Oocytes were fertilized using a fraction of active spermatozoa obtained by the swim-up method [30]. The straw with frozen sperm was thawed, the content of the straw was transferred to the bottom of test tubes containing 1 ml of Sperm-TALP medium [31] and placed in an incubator (MCO-18AIC, Sanyo, Japan) for 50 min. After incubation, the 750 μl upper layer from the tube was transferred to another tube containing Sperm-TALP medium and centrifuged at 300 g for 7 min (a centrifuge 3-30KS, Sigma, Germany). The sediment of motile spermatozoa was added to the BO-IVF medium (IVF Bioscience, Great Britain) containing previously transferred mature COCs, the final concentration of spermatozoa was 1.5×10^6/ml. Germ cells were co-cultured for 15-16 h. Then in Fert-TALP medium the oocytes were carefully released from cumulus cells and adhering spermatozoa and placed in the medium for embryonic development.

Embryos were cultured in 4-well plates (Nunc, Denmark) in 500 or 100 μl drops of BO-IVC medium (IVF Bioscience, UK) under a layer of mineral oil in an incubator (MCO-MCO-50M-PE, Sanyo, Japan) at 38.5 °C and a gaseous atmosphere containing 6% CO_2, 5% O_2 and 89% N_2. Three variants of incubation were compared, i.e., without medium replacement (NMR), with completely

refreshed mediume (CRM), and with half-refreshed medium (HRM). For NMR, during the entire period (8 days) the embryos developed without refreshing culture medium. For CRM, after 3 days of IVC, the embryos were transferred for further development into drops of fresh medium. For HRM, after 3 days of incubation, half of the initial volume of media (500 or 100 μl) was removed and replaced with an equivalent volume of fresh media. In all variants, after 3 days of IVC, the proportion of fragmented zygotes was assessed morphologically, on day 8 of culture, the embryos that developed to the BL stage was counted, and their viability and quality were estimated.

For quality analysis, a portion of 8-day-old BLs was fixed for 60 min with a 4% solution of paraformaldehyde. After fixation, the embryos were permeabilized for 30 min in a 0.1% sodium citrate solution containing 0.5% Triton X-100. For detection of apoptotic nuclei by the TUNEL method, the In Situ Cell Death Detection Kit, fluorescein (Roche Diagnostics, Switzerland) was used. Nuclei were stained for 20 min in a DAPI solution (1 μg/ml), transferred to a glass slide with an adhesive Superfrost Plus coating (Thermo Fisher Scientific, USA), and placed in Vectashield medium (Vector Laboratories, UK). Cytological preparations were viewed and photographed (an Axio Imager M2 fluorescent microscope equipped with a filter for TUNEL, excitation at 445-470 nm, and DAPI, excitation at 365 nm; an Axiocam 506 digital camera with ZEN 2 pro software, Carl Zeiss, Germany). The total number of nuclei in the embryos and the number of TUNEL-positive nuclei were counted.

To assess the viability, the second portion of the 8-day-old BLs were cultured for 2 days in a BO-IVC medium containing 5% PBS until the hatching stage. The hatched BLs (HBLs) at the beginning and at the end of culturing were counted.

Statistical processing was performed by the ANOVA method using the SigmaStat program (Systat Software, Inc., USA). The experimental results are presented as mean values (*M*) and their standard errors (±SEM). Tukey's test was applied to assess the significance of differences between the compared means.

Results. The in vitro matured oocytes used to produce embryos had an equal competence for further development. In the two culture options used, the results of cleavage on day 3 after in vitro fertilization did not differ significantly, and the proportion of oocytes that cleaved after fertilization ranged from 67.6 to 74.0% (Table 1).

The experiment revealed the influence of the IVC medium replacement mode on the development of in vitro fertilized oocytes to the BL stage (Fig. 1, A-B, Table 1). For 500 μl BO-IVC drops without changing the medium for 8 days, the BL yield was the lowest and amounted to 23.0±1.5%. Partial or complete change of the medium after 3 days of embryo growth equally increased the indicator 1.9-fold ($p < 0.01$). With a decrease in the drop volume to 100 μl, the influence of medium replacement on the development of zygotes up to the BL stage did not change. The described effects remained unchahged when the percentage of development to BLs was calculated from the number of fragmented zygotes, that is, from the total number of embryos obtained after fertilization.

1. Development of in vitro fertilized cow (*Bos taurus*) oocytes to the blastocyst stage depending on the mode of IVC (in vitro culture) **medium replacement** ($n = 6$, *M*±SEM, day 8 of culture)

Option	Oocyte number	Oocytes cleaved after fertilization, %	Development to the blastocyst stage, %	
			of total oocytes	of cleaved oocytes
500 μl drops				
No refreshed medium	148	67.6±1.9	23.0±1.5	34.1±2.4
Completely refreshed medium	166	69.9±2.6	45.7±4.8**	65.1±4.8***
Half-refreshed medium	138	74.0±2.9	44.9±0.7**	58.5±1.6**

		100 μl drops		
No refreshed medium	68	68.4±3.4	25.8±0.8	37.9±2.8
Completely refreshed medium	88	72.5±4.5	52.1±4.9**	71.5±2.3***
Half-refreshed medium	94	72.2±2.3	44.1±5.0*	60.9±5.7**

*, **, *** Differences vs. no refreshed medium are statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

2. Cytological analysis of bovine (*Bos taurus*) blastocysts (day 8) **depending on the mode of IVC** (in vitro culture) **medium replacement**

Option	Number of experiments	Total number of blastocysts	Number of nuclei per blastocyst, *M*±SEM	
			total	apoptotic, %
		500 μl drops		
No refreshed medium	3	17	216,9±9,8	6,53±0,88
Completely refreshed medium	3	38	180,4±9,2a	3,60±0,12*
Half-refreshed medium	3	31	198,3±15,4	4,30±0,46
		100 μl drops		
No refreshed medium	3	9	175,8±13,5	6,47±0,66
Completely refreshed medium	3	23	224,3±6,7*b	3,50±0,29**
Half-refreshed medium	3	21	230,4±8,4*	3,10±0,17**

*, ** Differences vs. no refreshed medium are statistically significant at $p < 0.05$ and $p < 0.01$.
ab Differences between mean values for the same mode of medium refreshment labeled with different letters are statistically significant at $p < 0.05$.

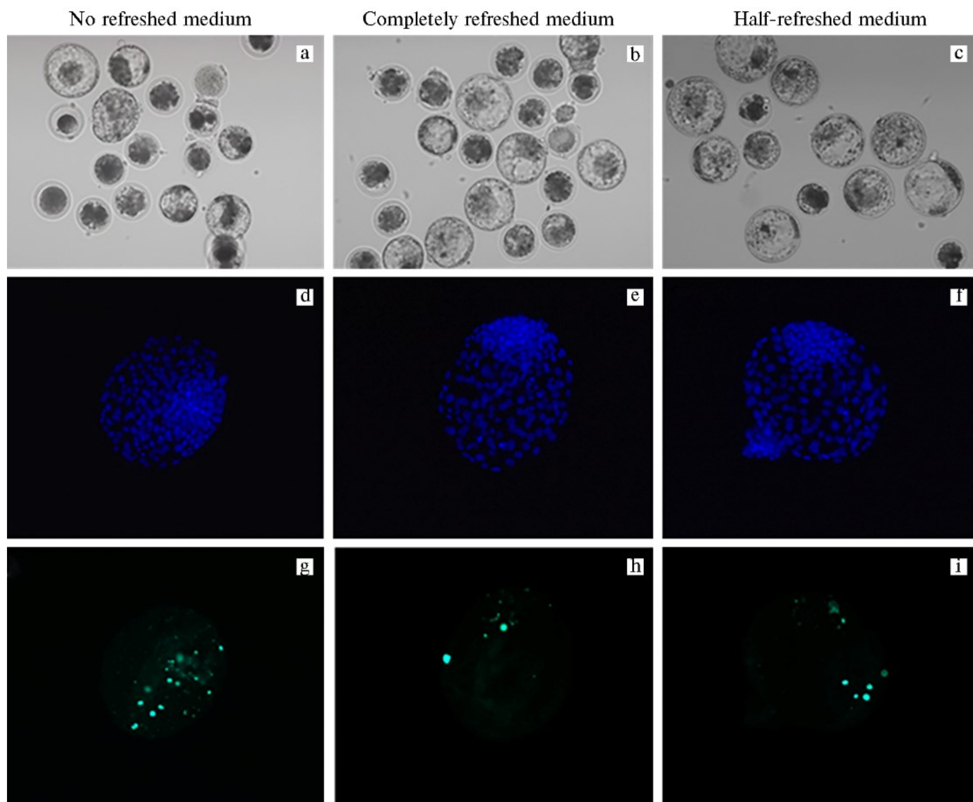

Fig. 1. Micrographs of bovine (*Bos taurus*) embryos after 8 days of in vitro culture under different modes of IVC (in vitro culture) **medium replacement:** a-c — embryos that have developed to the blastocyst stage (magnification 100×, microscope Eclipse Ti-U, Nikon, Japan); d-f — staining of nuclei in the blastocyst with DAPI (blue color; cytological preparation), g-i — staining of apoptotic nuclei in the blastocyst by TUNEL method (green color; cytological preparation) (magnification ×400, microscope Axio Imager M2, Carl Zeiss , Germany).

Cytological analysis testified to a rather high quality of the embryos that developed in vitro under the compared modes for the renewal of the IVC medium (Table 2). With a high average number of nuclei in BLs on day 8 (see Fig. 1, D-

F), no significant differences occurred between the modes if the embryos developed in 500 μl drops. On the contrary, with a decrease in the volume of IVC medium to 100 μl, CRM led to an increase in the analyzed parameter compared to NRM ($p < 0.05$), and also compared to CRM at 500 μl drops ($p < 0.05$) .

The tested modes (NRM, HRM, and CRM) also affect the incidence of apoptotic degeneration in BL. NRM mode + 100 μl drops negatively affected the proportion of apoptotic nuclei in BL (see Fig. 1, G), significantly increasing this indicator compared to both the CRM (Fig. 1, H) and HRM (see Fig. 1, I) ($p < 0.01$). As the volume of the IVC medium increased, the effect became less pronounced. With CRM, the analyzed parameter decreased compared to NRM ($p < 0.05$), but not to HRM. With a partial replasmant of the medium, we did not find significant differences, although in embryos there was a tendency to a decrease in the proportion of nuclei with apoptosis.

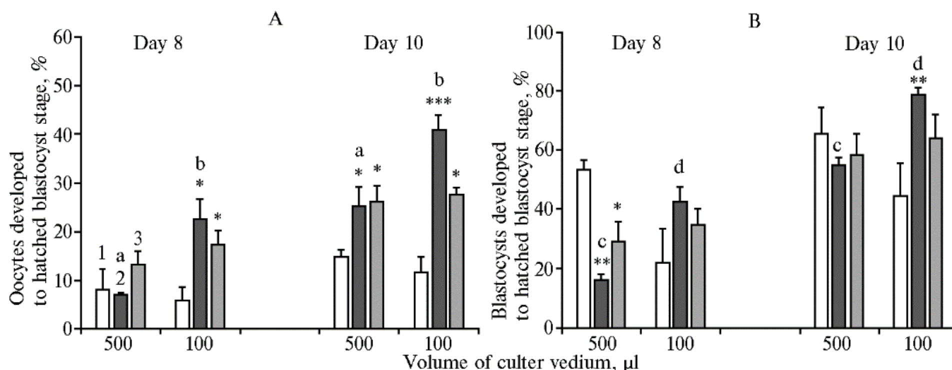

Fig. 2. Viability of bovine (*Bos taurus*) blastocysts after 8 and 10 days of in vitro culture under different modes of IVC (in vitro culture) **medium replacement:** 1, 2 and 3 — no refreshed medium, completely refreshed medium and half-refreshed medium, respectively (n = 3, M±SEM).
*, **, *** Differences vs. no refreshed medium are statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$.
ab, cd Differences between the means for one mode of medium replacement on day 8 and day 10 marked with different letters, are statistically significant at $p < 0.05$ and $p < 0.01$

It was found that the proportion of IVF oocytes that reached the HBL stage on day 10 of IVC, which serves as a criterion for the viability of IVP embryos, was higher in the CRM and HRV groups than in the NRM group (Fig. 2, A). In the latter case, this indicator for 500 and 100 μl drops did not differ and amounted to 14.9±1.5 and 11.6±3.3%, respectively. CRM and HRM cultures in 500 μl IVC medium drops increased the proportion of IVF oocytes that reached the HBL stage to 25.2±3.9 and 26.2±3.2% ($p < 0.05$), in 100 μl drops up to 40.8±3.2 ($p < 0.001$) and 27.7±1.4% ($p < 0.05$), respectively. That is, under the CRM mode, a decrease in the volume of IVC medium from 500 to 100 μl increased the viability of the embryos ($p < 0.05$), and the viability index here reached a level that was the highest among the tested cultures. It should be noted that when the proportion of IVF oocytes at the HBL stage was assessed on day 8 of IVC, a positive effect in CRM and HRM cultures vs. NRM occurred only in 100 μl drops ($p < 0.05$). In addition, in CRM culture, the viability index, as on day 10, was higher compared to that in 500 μl drops ($p < 0.05$).

The viability of embryos was additionally assessed by the proportion of HBLs to the total number of blastocysts (see Fig. 2, B). If culturing embryos for 10 days in 500 μl drops, the proportion of HBLs did not differ significantly between the modes of medium change and amounted to 65.6±8.7, 54.6±2.9 and 58.5±7.1% for the groups of NRM, CRM and HRV, respectively. On the contrary, with a decrease in the volume of the culture medium, this indicator was higher in CRM group than in NRM group (78.7±2.4 vs. 44.4±11.1%, $p < 0.01$), and

significantly ($p < 0.05$) exceeded the values in the same group for 500 μl drops. On day 8 in 500 μl drops, the HBL percentage of the total blastocyst number was higher in NRM group vs. CRM and HRM groups, 53.3±3.3 vs. 16.2±2.0% ($p < 0.01$) and 29.4±6.3% ($p < 0.05$). In 100 μl drops, the HBL percentage was the lowest among the compared modes, 22.2±11.1, 42 .6±4.9 and 35.0±5.0% for NRM, CRM and HRM, respectively.

Currently, the IVC media used in the IVP technology can provide a high yield of embryos suitable for freezing and transplantation into recipient animals. Nevertheless, the in vitro conditions that ensure the development of the embryo still require detailing [3, 6]. In particular, it remains unclear whether there is a need for an IVC medium refreshing procedure recommended every 48-72 h during embryo culture. On the one hand, the replacement of the medium provides the embryos with nutrients and removes harmful metabolites [14-16], on the other hand, this procedure leads to a stressful change in the microenvironment of the embryos and the removal of embryotropic factors necessary for embryo development [15, 16].

In the present work, embryos were cultured in 500 and 100 μl drops of commercial BO-IVC medium and assumed the embryo development to the blastocyst stage either without changing the culture medium or with complete or partial (50%) medium change. Our findings showed that the CRM mode vs. the NRM mode after 3 days of IVC provided a significant increase in BL yield (see Table 1), a decrease in the frequency of apoptosis (see Table 2), and an increase in the BL viability assessed as percentage of the total number of fertilized oocytes (see Fig. 1, A). These parameres did not depend on the IVC medium volume (500 or 100 μl drops), while the number of nuclei in BLs increased in 100 μl drops (see Table 2). A partial change of culture medium (HRM) was also more efficient in several indicators vs. culture without a change of medium. The blastocyst formation frequency (see Table 1) and viability (see Fig. 1, A) were higher similar to CRM group, and in 100 μl drops, an increase in the number of nuclei and a decrease in apoptotic nuclei in BLs (see Table 2) occurred. We did not find any significant differences between CRM and HRM in all the studied parameters; nevertheless, for CRM and 100 μl culture, there were the highest rates of BL development and the HBL number. In general, these results indicate a negative impact of the NRM culture on the development and quality of IVP embryos.

Our data are partly consistent with a recent study on rabbits, in which HRM was more effective in terms of BL development and quality (the number of nuclei) than in vitro culture without medium change. In addition, HRM and CRM modes similarly reduced the rate of apoptosis in embryos [26]. In the work of these authors, as in our study, the medium was changed once after 3 days of culturing. Nevertheless, in experiments on cattle, which are not so numerous, on the contrary, the CRM mode compared to NRM mode led to a decrease in the number of BL and HBL [23] and to deterioration in the quality and viability of embryos [16]. There are also reports of no differences between these cultures [24]. The fact that in these studies bovine embryos were cultured in SOF medium that was changed every 48 h IVC and the results of our own studies draw us to two assumptions. Firstly, such a frequent medium change can cause stress and CRM can negatively affect the development and quality of embryos [32], and, secondly, there is a need for detailed parameters of SOF medium refreshing.

Thus, complete (CRM) and partial (HRM) culture medium renewal positively affect the development and quality of in vitro produced (IVP) bovine embryos as compared to in vitro cultures without medium change (NRM). CRM provides significantly increased blastocyst production from 23.0±1.5 and 25.8±0.8% to 45.7±4.8 and 52.1±4.9% ($p < 0.01$) for 500 and 100 μl drops of the culture

medium. In addition, the apoptosis rate in embryos decreases from 6.53±0.88 and 6.47±0.66% to 3.60±0.12 and 3.50±0.29% ($p < 0.05$ and $p < 0.01$). In CRM culures, the yield of hatched blastocysts (HBLs) from the total number of fertilized oocytes increases from 14.9±1.5 and 11.6±3.3% to 25.2±3.9 and 40.8±3.2% ($p < 0.05$ and $p < 0.001$). All these changes occur regardless of the culture medium volume (data are presented for 500 and 100 μl, respectively). For 100 μl under CRM mode, the number of nuclei per blastocyst also increases (from 175.8±13.5 to 224.3±6.7, $p < 0.05$). For HRM mode, the frequency of blastocyst formation increaseы (up to 44.9±0.7 and 44.1±5.0%, $p < 0.01$ and $p < 0.05$), as does the proportion of HBL (up to 26.2±3.2 and 27.7±1.4%, $p < 0.05$) (data are presented for 500 and 100 μl, respectively). For 100 μl culture medium under HRM mode, the number of nuclei per blastocyst increases (up to 230.4±8.4, $p < 0.05$), while the proportion of apoptotic nuclei decreases (to 3.10±0.17%, $p < 0.01$). The revealed effects obviously depend on the BO-IVC medium (IVF Bioscience, UK) volume in IVC (in vitro culture). For 500 μl, both modes of medium renewal are effective, while for 100 μl, the CRM mode ensures the best results. The CRM mode and 100 μl culture medium provide the the highest development rates of blastocysts and the number of HBLs. Therefore, BO-IVC medium (IVF Bioscience, UK), if refreshed after 3 days of in vitro culture, can be recommended for in vitro production of cattle embryos.

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