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Developments on Improving the Technology to Obtain Large Cattle Embryos of the Desired Gender in the Republic of Kazakhstan

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ABSTRACT

Objective: Development and improvement of the technology for obtaining embryos of the desired gender and determination of the rate of maturation and fertilisation of follicular oocytes of cows in various culture systems, and investigation of the effect of micromanipulation methods on viability of zygotes and embryos, obtained through in vitro fertilisation.

Methods: In vitro method, in vivo method, hormonal stimulation method, superovulation method, transvaginal puncture of ovaries, cryopreservation method, embryo transfer.

Results: Culture system serves as the main condition for maturation and further development of embryos. Studying "in vitro" fertilisation of ova with sex-sorted semen has shown that fertilisation rate of follicular oocytes is about 75%, and the percentage of development to farther stages is 52.4% or 2.2 morphologically viable embryos per a cow-donor. 5 embryos had embryonal blocks (developmental arrests), one embryo had it at the stage of two pronuclei, and four embryos – at the stage of 4-32 blastomeres.

Conclusion: These research studies, carried out in the Republic of Kazakhstan for the first time, afforded the opportunity to apply them widely to increase the large cattle breeding stock.

Keywords
Culture Medium, Oocytes, Embryos, "in Vitro", in Vitro Fertilisation Methods.

Introduction

The aim of this study is to develop and improve the technology for obtaining embryos of the desired gender and to determine the rate of maturation and fertilisation of follicular oocytes of cows in various culture systems, as well as to investigate the effect of micromanipulation methods on viability of zygotes and embryos, obtained through in vitro fertilisation.

It is known from genetics case studies that, in sexual reproduction, the specific complex of genes that defines high-yield animal properties, is not replicated in the next generation, since it disintegrates in the course of meiosis in the parents' gametes and is combined from new haploid number of chromosomes during fertilisation process. Hence, no superior qualities of top producers are inherited by their descendants [1].

To raise the number of descendants from genetically valuable males, an artificial insemination method was developed. A genetic mother's role had remained at a low level. Hormonal stimulation and implantation of embryos, obtained from a top female producer, into the low productive specimens had facilitated its partial increase [2].

To date, current reproductive technologies, which select animals by high-quality genetically inherited traits, define the strategic direction of breeding [3].

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Now, the method of in vitro fertilisation and in vitro culturing of oocytes and embryos is a promising method to strengthen a genetic role of females, allowing for a drastic increase in the number of their descendants. The method is efficient since ova from females can be obtained all the year round and throughout an entire animal's lifetime. The problems of optimising the processes of oocytes' in vitro fertilisation and development of embryos in culture are of practical importance since the method for in vitro fertilisation of oocytes has been increasingly used. In terms of theory, these problems must be studied to develop the concepts of molecular-cellular factors, regulating the reproductive function, and to successfully implement biotechnological programs of obtaining clones and transgenic animals [4].

The principle of artificial fertilisation of agricultural livestock stems from the fact that the male ejaculate contains multimillion sperm cells, theoretically sufficient to inseminate hundreds of females. A major step towards this concept was made in late 1940s, when the team, headed by Chis Polge in Cambridge (England), had designed a technology to freeze and store animal spermia. At the same period, the methods for extracting female gametes and their manipulation were also developed [5].

The research area on improving transfer of large cattle embryos, that started to develop in Belorussia in early 90s of the previous century under the guidance of the Professor Grigoriy Fedorovich Medvedev, produced good outcomes. The methods for regulation and enhancement of reproductive capacity of cows and new veterinary preparations were devised, the methods were improved to evaluate and select stud-bulls by reproductive capacity, a method for functional preparation of stud-bulls and semen dilution technologies were developed [6].

Of scientific interest are the studies of in vitro fertilisation of Pavlova T.V. and Kazarovets T.N., they had carried out on the basis of the "BSAA Instructional farm" RUE. The performed evaluation of the results of using stud-bulls of various linear identity and selection in dairy herds indicated the direction for developing the economic traits of breeds [7].

The research conducted by Prof. Bakai A.V., Mukhtarov A.V., Mkrtchayn G.V. in the K.I. Skryabin Moscow State Academy of Veterinary Medicine and Biotechnology provide a notion of how variable the main features of the milk productivity of black-and-white cows are [8].

According to scientists, variability is among the major conditions of evolution. Genetic progress through selection is now possible only if the features of selected specimens vary. High productivity of animals depends on their individual characteristic traits, and, in this case, these traits are defined by its genetic value, and possibility – by several paratypic factors. Displaying individual characteristic traits under certain conditions specifies the variability of traits. All other factors of housing and feeding being equal, the variability of traits related to productivity is mostly defined by a genotype of animals [9].

The study results had shown that milk production of high-yield cows varies from 20 to 29%, weight fat content of milk – from 8.68 to 9.53%, protein – from 5.19 to 5.70%. Within the herd, the values of variability coefficients vary insignificantly and are almost similar. Variability in milk productivity traits states that long-term tandem selection, first aimed at selecting high-yield animals, and then maintaining quality indicators in the milk of cows, allowed first-calve heifers of various high-yield genotypes to be obtained [10].

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The latest advance in molecular biology had made it possible to integrate the molecular-genetic information into conventional selection systems and to enhance the reliability of estimating breed animals. Introduction of genetic information is now possible due to the identification of molecular-genetic markers, associated with economic traits [11].

Of interest are the studies of Singina G.N., Taradainik T.E. on optimisation of conditions to prepare sex-sorted semen of bulls for the in vitro fertilisation and evaluation of the effect of heparin in the insemination medium on embryogenesis of cows' oocytes in vitro. The data, obtained as a result of studying, show that sex-sorted semen has a lower "in vitro" fertilising capacity, than standard semen. Even though the density gradient centrifugation to obtain a fraction of active sperm cells represents a more efficient method to prepare a sex-sorted sperm to "in vitro" fertilisation, it does not produce an adequate amount of viable embryos, that the standard semen does. Hence, further refinement of certain stages of the technology for obtaining them is needed [12].

Culturing embryos constitutes an important stage of the in vitro fertilisation procedure. Its success is a decisive factor for the subsequent embryo transfer. Culturing technologies have been constantly improved, and the main attention therewith is given to nutritional media. Modern preparations are similar to the fullest in composition to the natural conditions of the maternal organism [13].

Selection of donors and recipients is among the most essential aspects in the "in vitro" technology, capable of enhancing the method efficiency. High-yield animals are selected as donors, by breed characteristics (udder and teats shape, milk flow properties, resistance, strength of bones and hooves, type and reproduction properties, and other indicators), after pedigree and offspring quality evaluation, what therefore ensures a high selection differential [14].

The process of obtaining the large cattle embryos with the use of the "in vitro" method, a state-of-the-art and advanced biotechnological method, that enables a considerable acceleration of high-yield animals reproduction process [15], comprises several stages:

- extraction of oocytes from antral ovary follicles (OPU-Ovum Pick-Up), maturation of oocytes (IVM–in vitro maturation);
- fertilisation (IVF–in vitro fertilisation);
- embryo culture (IVC – in vitro culture).

At that, ova from animals-donors can be obtained both in their lifetime through transvaginal aspiration method, and from slaughtered material (ovaries of cows from a meat-processing factory).

The use of the ovum pick-up method in the "in vitro" technology facilitates rapid offspring production of genetically valuable animals, which have reproduction failures, not related to the function of ovaries, or have no adequate superovulation response to hormonal treatment. Moreover, this technology is appropriate for obtaining viable embryos for transfer, when oocytes are picked-up from the ovaries of healthy animals, including those at various stages of pregnancy. Good quality of the obtained oocytes is a big advantage of this source [16].

The original OPU procedure requires no animal's treatment with stimulating hormonal preparations and is usually conducted twice a week, what allows for obtaining a maximum

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amount of oocytes, since the dominant follicle does not develop, if all the visible follicles are aspirated during the OPU procedure. With most selection procedures being performed once a week, the dominant follicle develops during the subsequent selection. It causes regression and degeneration of secondary follicles [17].

To date, there are different approaches to increasing the viability of oocytes in the process of their "in vitro" maturation (IVM). Of greatest interest are the attempts to model the events, happening in natural conditions. One of such approaches focuses on analysing the metabolic processes in maturating oocyte-cumulus complexes (OCC) [18].

Ova fertilisation also represents an important stage, that defines the success and efficiency of obtaining embryos employing the "in vitro" method. It is customary to use a cryopreserved semen of stud-bulls when producing embryos through the "in vitro" method in fertilisation technology [19].

When producing embryos of the desired gender through the "in vitro" method employing the in vitro fertilisation technology, over the period of experimental works a total of 94 morphologically viable sexed embryos were obtained, 46 of which were transferred to heifers-recipients, 59 embryo was frozen and placed in the embryo-bank, and 16 calves were obtained. Mean transplantability of embryos amounted to 34.7%, among them 37.9% for "in vivo" embryos and 29.4% for "in vitro" ones. The scientific research results enabled computation of the economic efficiency of the methods and finding the transplantability rate of sexed embryos in their transfer to the recipients [20].

In view of the above, it can be said that the embryos, obtained through the in vitro fertilisation, will continue to be used in the experimental studies of cell biotechnology.

The in vitro fertilisation technology is currently incorporated into the long-term breeding programs of many developed countries of the world related to breeding, improving, and preserving the existing livestock species. The extensive studies have been therefore undertaken in many countries on culturing follicular oocytes and their in vitro fertilisation and cloning the large cattle embryos.

Materials and Methods

The experimental studies were carried out employing the breeding stock of the large dairy Holstein cattle in the "Bayserke-Agro" LLC of Almaty Region of the Republic of Kazakhstan. When obtaining the material, the ovaries without visible signs of pathology were utilised, at the stage of follicular growth or with the developed corpus luteum. The study flow diagram is shown in Figure 1.

Ejaculate was obtained at the semen-collecting lot of artificial insemination in conditions of an animal facility by urethral method, using an artificial vagina of the MINITUB Company through the method, described in the manual on artificial insemination of cows [20] and GOST 32222 Reproduction means. Sperm. Methods for sampling (GOST 32222-2013) [21].

The research study was conducted in compliance with the requirements of the Bio-ethics Commission of the Federal State Budgetary Scientific Institution "Federal Centre for toxicological, radiation, and biological safety", and according to the State Standard of the Republic of Kazakhstan the "System of production development and engineering. Content and

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procedure. Patent studies" [22] and 3 years search depth.

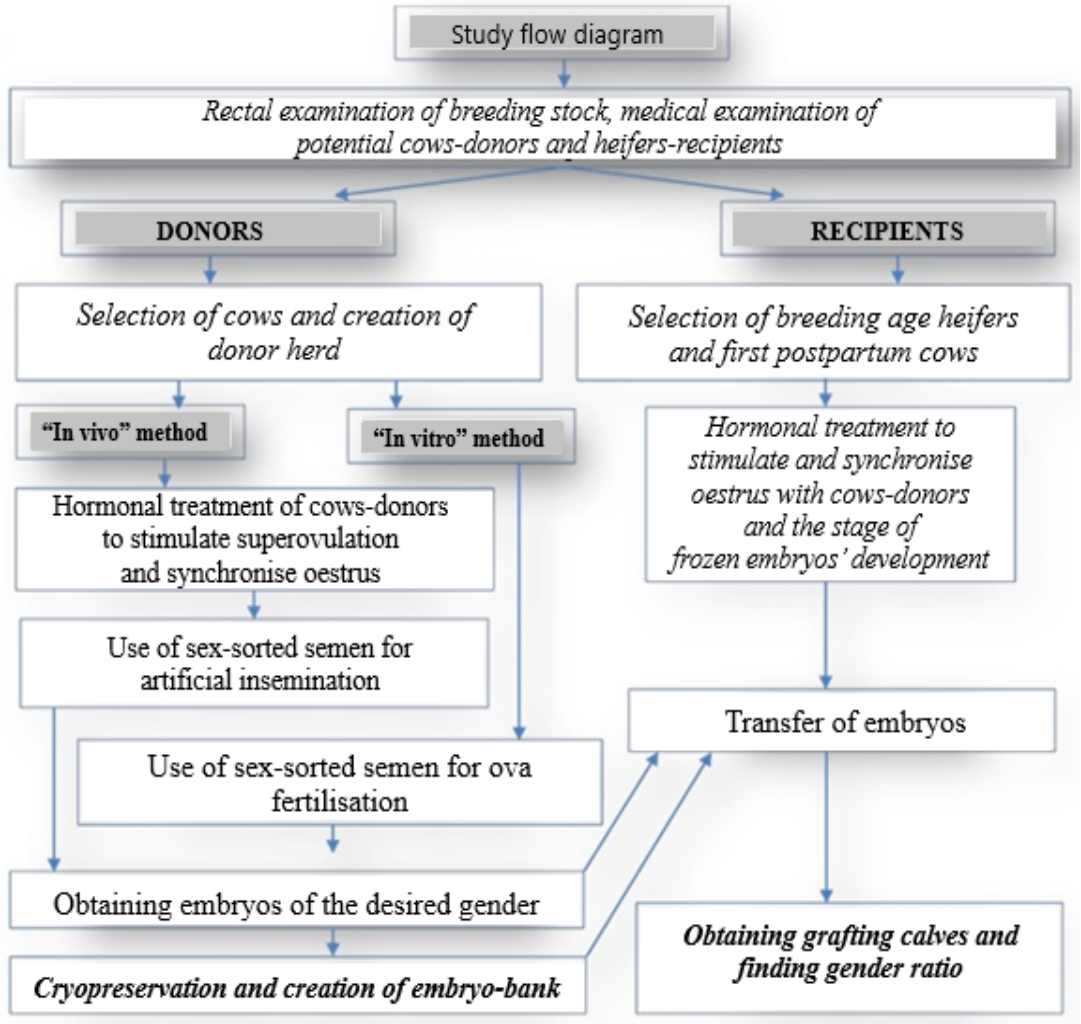

Figure 1. Diagram of conducting scientific research

The following laboratory instruments and equipment were used for the research:

- Microscopes for laboratory studies with 100-400x magnification, Axiovert 25: 03, 05, Standart 20:04, KF 2:04, Stemi 2000:06, 1000:03, analogues: microscopes of "AXIO" series (Axiovert 200, Axiovert 200M), microscope of "AXIO" series (Axioskop 40);
- Culture media to raise gametes, for performing fertilisation, sperm preparation (sperm capacitation), culturing of early embryos, culturing of late embryos (morula, blastocyst);
- A laminar box with a worktable surface heated to 37-40ºC and in-built stereo-microscope, micromanipulator with 2 injectors and 2 arms, permitted for use as appropriate;
- Cell centrifuge with up to 3000rpm variable speed (1900g);
- Solid-state thermostat (37-40ºC) to heat up test-tubes for collecting follicular fluid, the size of openings matches the external diameter of test-tubes to collect follicular fluid, permitted for use as appropriate;
- Straws for embryos, permitted for use as appropriate;
- Laboratory CO_2–incubators of model: 3211, 3548. Analogues: medical CO_2–incubator model 3111;

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- Tools for endoscopic manipulations, micropipettes for in vitro fertilisation.

As per the GOST 32277-2013 "Reproduction means. Sperm. Methods for testing physical properties and biological, biochemical, morphological analyses", a microscopic evaluation of sperm quality was performed at various stages of the research study [23].

Methodology of in Vitro Fertilisation of Oocytes

10-13 ectomised ovaries of sexually mature cows were placed in the 20ml test-tube with the PBS (Phosphate buffered saline) medium and, in a water bath at the temperature of 37ºC within an hour, they were delivered to the laboratory.

In laboratory conditions, to clean dirt, ectomised ovaries were washed 2-3 times in a warm physiological solution with antibiotics at the ratio of 50µg/ml of streptomycin and 100 U/ml of penicillin. Washed ovaries were carried over one by one to the 40 mm Petri dish, into which 1.5-2ml of the 199 medium were poured, that were previously placed in the incubator for saturation with carbon dioxide within 10-12h.

Then, beneath the laminar box, the ovary follicles were cut with a scalpel so that the follicular fluid directly pours into the medium. After aspiration of the follicle, the ovary was removed, and the dish with the medium was examined under a microscope.

It should be noted that during the ovary follicles' aspiration, OCCs (oocyte cumulus complexes) also enter the medium together with the follicular fluid. OCC is a part of the follicular epithelium, directly contacting with oocytes during folliculogenesis. Among other things, the follicular fluid contains fibrinogen causing formation of clots, and even 2-3 min after aspiration, oocytes are difficult to find and wash [24].

To overcome this problem, two options were taken:

1) Follicular fluid was examined under the microscope immediately after aspiration, and the detected oocytes were placed in a clean medium as soon as possible.
2) Follicular fluid was collected to the dishes with a heparin-containing medium, preventing gel formation.

However, the first option not always allows for finding oocytes in proper time, hence the second method was also used. To do this, the 50 IU/ml concentration of heparin was employed. 1ml of solution were added to a test-tube of the 8-10ml volume, what provides the 5-8 IU/ml final concentration of heparin in the follicular fluid.

When detecting ova, a pipettor with a glass capillary vessel was used for collecting and carrying over to a separate 4-basin dish with the medium for culturing oocytes, which was also in the incubator within 10-12h.

Prior to culturing, a quick morphological evaluation of oocytes is performed, ova with damaged pellucid zone are sorted out. Rounded-shaped ova with homogeneous cytoplasm, pellucid zone of even width, surrounded with multilayer compact follicular cells are considered suitable for studies.

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Oocytes and embryos are cultured in the incubator with 5% CO_2 in air at the temperature of 37±0.1°C and 80-90% humidity. The same conditions are met when CO_2-incubators, maintaining required temperature, humidity, and level of CO_2, are used. Prior to insemination, oocytes are assessed by morphological criteria.

In vitro capacitation of the frozen-thawed semen was performed as follows. A frozen straw with the stud-bull semen is placed in a water bath for thawing at the temperature of 37 ºC for 12-15sec. A thus defrosted semen is poured into a 15ml test-tube and poured from the top with the 199 medium to the 6ml level, then it is centrifugated within 10 min at 3000 rpm.

After centrifugating, a supernatant is poured out the test-tube, and 1ml of medium, used to culture oocytes, is poured into it; then it is placed in the incubator at the temperature of 37ºC for 30-40min. Upon the expiration of culturing, the most mobile sperm cells are in the supernatant, which is exactly applied to fertilise ova.

Prior to fertilisation, the quality of semen and the direction of sperm cells' mobility were examined under the microscope using the 10-point scale. To inseminate oocytes, the rectilinearly moving semen was used with at least 4-point mobility, i.e., at least 40% of mobile ones of the total semen amount [25].

Inseminated oocytes are placed in the CO_2 incubator at the temperature of 37ºC for 6-8h, following which the oocytes are carried over to a clean medium, also being previously placed to the incubator during 12h. 16-18h after the insemination start, ova are examined for fertilisation, i.e., for the presence of polar bodies and pronuclei – male and female. When fertilisation is detected, zygotes are carried over to a clean medium, and this procedure is further repeated every 24h. Embryos are cultured to the stage of late morula and blastocyst.

Modelling of Maturation Systems to Culture Follicular Oocytes and Embryos

To enable comparison of the processes of in vitro maturation and fertilisation of follicular oocytes and normal cleavage of embryos, the experiments were carried out to select the optimum ratio between the culture media components. Taking into account the existing literature data and own experience of testing, the systems to culture embryos and oocytes were chosen that allow for their proper and sustainable in vitro development.

To culture "in vitro" the follicular oocytes, culture systems were utilised based on the Dulbecco medium with addition of serums, 100% follicular fluid, and a ready-for-use IVF-Universal (MediCult) solution, intended to inseminate oocytes and culture embryos in vitro. The medium body contains the following components: glucose and metabolites, physiological salts, essential amino acids, vitamins, nucleotides, DNA bases, sodium bicarbonate, 50 mg/l streptomycin, 50000 IU/l penicillin, phenol red.

The medium to culture oocytes consisted of the Dulbecco medium to the extent of 90% and homologous or estrual serum to the extent of 10%. It was prepared as follows: 10ml of the Dulbecco medium were poured into the 15ml test-tube, then adding 0.1ml of serum, and the test-tube was placed in the incubator at the temperature of 37°C with 5% CO_2 for 24h, upon expiration of which the solution was employed to culture and fertilise ova.

Of great interest is the use of the follicular fluid for maturation of aspirated follicular oocytes since this medium is a natural environment for development of oocytes in the ovary follicles.

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For that purpose, for culture medium we used a 100% follicular fluid, picked up from antral follicles of the ovary of slaughtered cows.

Follicular fluid was picked up through puncturing from the Ø4-6mm ovary follicles, containing 1.0-1.5ml of fluid. After aspiration, the obtained follicular fluid was centrifugated during 5min at 2000rpm., adding heparin (Sigma) at the rate of 5U/ml. To prevent gel formation, the obtained follicular fluid was exposed to thermal treatment at 56°C during 3 min. Then the prepared medium was poured by a dosing device drop by drop 20µl each to the 40mm Petri dish. The drops were enumerated and poured from the top with paraffin oil, then placed in the incubator at the temperature of 37°C with 5% CO_2 for 24h. After a lapse of time, 2-3 selected morphologically viable oocytes, intended for culturing, were carried over to the ready-for-use FF drops [26].

Results

1. Results of Culturing Oocytes in different Culture Media

To study the nature of the effect of various culture systems on the fertilisation rate of ova, fertilisation and further development of pre-implantation embryos, the experiment was conducted in three stages employing different culture systems (Dulbecco medium with 10% homologous serum, 100% follicular fluid, IVF-Universal).

Eventually, 1243 morphologically viable oocytes, picked up from the ovary follicles of cows by the composition of nutritional media for maturation, were divided into three groups: 356 oocytes were cultured in the Dulbecco medium with 10% homologous serum, 428 and 459 oocyte - in the follicular fluid and in the IVF-Universal medium, respectively.

The oocytes therewith were classified according to three features:

1. with characteristic morphological signs of maturation;
2. without signs of maturation, i.e., without changes;
3. degenerated, i.e., with contracted or fragmented cytoplasm.

For culturing and fertilising, at least Ø140-150µm oocytes were selected, i.e., those having the size almost similar to that of the mature ovum, with a surrounding mass of follicular cells, well adjacent to the ova membrane as well.

After culturing in the “in vivo” conditions prior to insemination of oocytes with sperm cells, their maturation rate and quality were determined by morphological criteria. The results of culturing of follicular oocytes are given in Table 1.

Table 1. Results of culturing follicular oocytes

Name of medium	Dulbecco with 10% homologous serum		Follicular fluid		IVF-Universal	
	n	%	n	%	n	%
Number of oocytes	356	100	428	100	459	100
With characteristic signs of maturation	64	18	98	22.9	312	68
No signs of maturation	210	59	282	65.9	110	24
Degenerated	82	23	48	11.2	37	8

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As is seen from Table 1, maximum percentage of oocytes with signs of maturation was observed in culturing ova in the IVF-Universal medium, what was 68% or 312 of the total amount. At that, there are few, namely, only 8% of degenerated oocytes. For oocytes, cultured in the Dulbecco and FF media, typical morphological signs of maturation were seen in 18 and 22.9%, respectively. The percentage of oocytes degenerated in the Dulbecco medium was well above the rates in other media (11.2% in FF and 8% in IVF), amounting to 23% or 82 oocytes of 356. Figure 2 presents the diagram clearly showing the results.

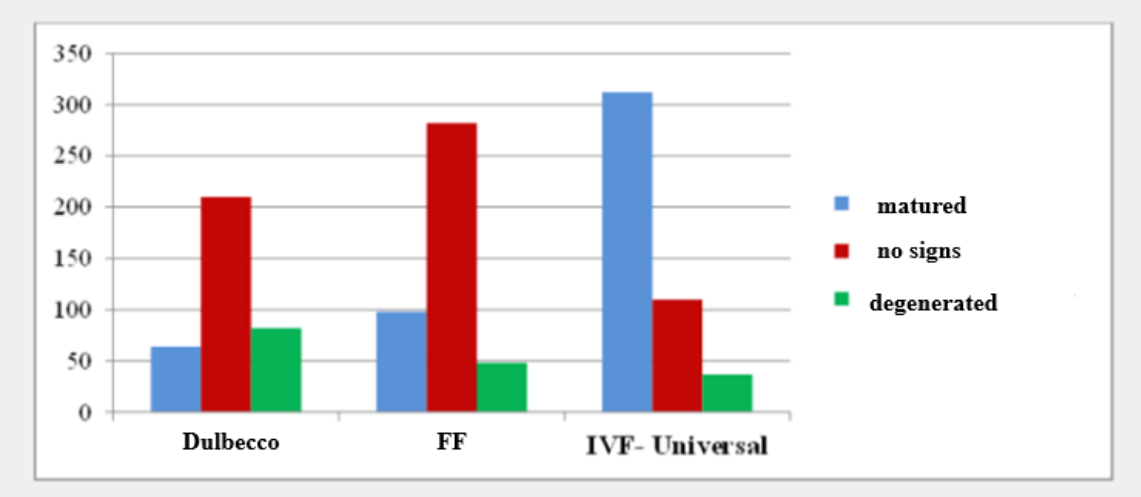

Figure 2. Maturation rate of oocytes in different culture media

The criteria, generally adopted for evaluation of ova, were applied in the morphological evaluation of the oocytes' maturation rate. And on this basis, after "in vitro" culturing, the morphological evaluation of the OCC maturation rate was conducted [27].

2. Results of Examining the Fertilisation Rate of Oocytes and Development of Embryos in the "in Vitro" Conditions

While maturing, the ovum accumulates its inner set of substances, necessary for the embryo development, and at the moment of insemination is at the stage of the II block, which is removed upon semen's entering. And, hence, the development and fertilisation of immature follicular oocytes require the conditions, corresponding to the natural ones to the maximum degree possible, i.e., to those, in which the "in vivo" oogenesis and early embryogenesis regulation mechanisms normally function. The blood serum, containing the components that facilitate survival and normal development of cells, is mostly used as a biologically active factor [28].

A problem was raised to study the effect of different culture systems on the rate of maturation, fertilisation, and further cleavage of embryos. To this effect, a Dulbecco-based medium was employed with the 10% homologous serum, 100% FF (follicular fluid) and the IVF-Universal medium, designed to perform the "in vitro" fertilisation of oocytes.

In the course of the experiment, a stepwise morphological evaluation was done, which main criteria involved unevenness of the cytoplasm state, presence of more than two pronuclei, asymmetric and fragmented division of the embryo cells.

Table 2 provides a comparative characteristic of oocytes' fertilisation and embryos' development in different culture media.

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Table 2. Comparative description of fertilisation of oocytes and development of embryos in different culture systems

Name of fluid	Dulbecco with 10% homologous serum					Follicular fluid					IVF- Universal				
	Total amount	Normal		Degenerated		Total amount	Normal		Degenerated		Total amount	Normal		Degenerated	
		n	%	n	%		n	%	n	%		n	%	n	%
Cultured and inseminated	356	274	77	82	23	428	380	88.8	48	11.2	459	422	92	37	8
Presence of pronuclei	52	41	78.8	11	21.2	125	108	86.4	17	13.6	250	224	89.6	26	10.4
2-blastomere	46	33	71.7	13	28.3	83	56	67.5	27	32.5	270	221	81.85	49	18.15
4-6-blastomere	22	19	86.36	3	13.64	48	36	75	12	25	208	196	94.23	12	5.77
16-32-blastomere	13	8	61.54	5	38.46	8	5	62.5	3	37.5	193	186	96.37	7	3.63
Morulae and blastocytes	-	-	-	-	-	-	-	-	-	-	162	158	97.53	4	2.47

As is seen from the data of Table 2, when culturing oocytes in the Dulbecco medium with 10% homologous serum, 77% of 356 oocytes remained morphologically viable, what amounts to 274 oocytes, 23% or 82 ova were sorted out due to fragmentation and degeneration of the inner cell mass. The viability rate in follicular fluid was seen in 88.8% or 380 of 428 ova, 11.2% of which were also sorted out.

The highest rates were obtained in the IVF medium, where 92% of 459 remained morphologically viable, what amounts to 422 oocytes, degeneration rate was 8% or 37 oocytes.

During subsequent culturing and insemination of oocytes with semen and observation 14-18h after, fertilisation, or the presence of two pronuclei and polar bodies was found in the Dulbecco medium in 14.6% of the total amount of oocytes or in 52 oocytes, 41 (78.8%) of which proved to be viable.

This rate in oocytes, cultured in FF, was slightly higher and amounted to 29.2% of 428 oocytes, 86.4% of which were viable. In the IVF medium, two pronuclei were found in 54.4%, what significantly exceeds the rates of both media when comparing.

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After 24-36h of culturing, fertilised oocytes cleave, and 2 blastomeres were observed under the microscope.

In the Dulbecco medium with 10% homologous serum, there were 46 embryos, or 12.9% of the total amount of fertilised cells, in 33 embryos (71.7%) of which normal cleavage of cells took place, and which has no visible signs of fragmentation, and 21.2% had pathologies, associated with fragmented and asynchronous cleavages of blastomeres.

In FF, there were 19.39% of cleaving embryos or 83 two-blastomere embryos and 67.5% of normal or 56 embryos. In IVF, the number of cleaving embryos was higher and amounted to 58.8% (270) of the number of oocytes, with 18.5% of degenerated ones.

When embryos were carried over to the clean medium and cultured to the stage of 4-6 blastomeres, 22 embryos cleaved in the Dulbecco medium. Of them 86.36% were morphologically viable, in FF - 48 or 11.2% of the number of oocytes. In the IVF-Universal medium this rate was 45.3% or 208 of 4-, 6-blastomere embryos, 94.23% or 196 embryos of which remained morphologically normal.
13 embryos cleaved to the stage of 16, 32 blastomeres in Dulbecco. In 38.46% of them, an asynchronous division of blastomeres was seen, in FF the number of embryos that continued to cleave sharply reduced and was only 1.86% of fertilised oocytes, and in the IVF medium there was almost the same amount of cleaving embryos, namely, 42%, in 96.37% of which normal cleavage occurred.

On the 6-8th day of culturing, when examined under the microscope, embryos, cultured and fertilised in the Dulbecco and FF media, stopped to develop, and their cells were contracted, whereas in the IVF-Universal medium the number of embryos at the late developmental stage was 35.3%, what amounts to 162 of all fertilised follicular oocytes.

Morphological evaluation of the fertilisation rate identified pronuclei in the Dulbecco medium in 18.9%, in FF in 32.9% and in the IVF-Universal in 59.24% of the total number of selected morphologically viable oocytes.

After 24-36h of culturing, 2 blastomeres were found in the Dulbecco medium in 16.8%, in FF in 21.8% and in IVF-Universal in 64% of oocytes, what also characterises the most reliable indicator of the fertilisation of follicular oocytes. Figure 3 demonstrates the rate of fertilisation and development of embryos among the morphologically viable oocytes.

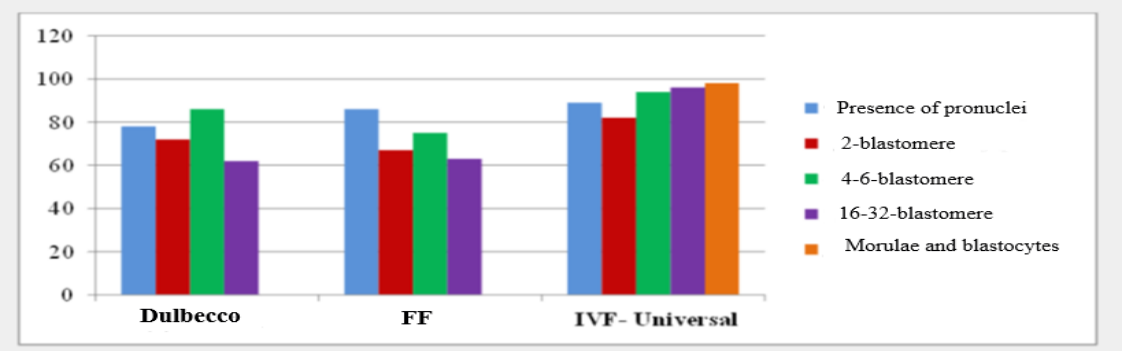

Figure 3. Rate of fertilisation and development of embryos depending on the number of morphologically viable oocytes

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During culturing, 8%, 12.6% and 49.3% of the total amount of oocytes continued to further cleave to 4, 6 blastomeres.

3. Results of Inseminating Ova with the "in Vitro Sex-sorted Semen"

15 ovaries of 9 cows were used for the research. To culture isolated follicular oocytes, standard media Universal IVF (Medium) and BlastAssist, BlastAssist System were used with addition of biologically active additives, blood serum, hormones, antibiotics. The time of culturing oocytes to the stage of metaphase II was from 30 to 40h [29].

However, the oocytes, that reached the developmental stage of metaphase II 24-27h later, are suitable for further culturing, after this period the chromosomes in the nuclei degenerate, and oocytes die. The total of 225 oocytes were cultured. After 24-36h of culturing, 167 oocytes (48.4%) reached the stage of mature ovum development.

The results of culturing and in vitro fertilisation of oocytes are given in Table 3.

Table 3. Morphological Evaluation of "in Vitro" Embryos

Stages of embryos' development	Number of embryos		Of them			
			normal		degenerated	
	n	%	n	%	n	%
Fertilised oocytes	109	48.4	80	73.3	29	26.6
2-4-8 cell	80	73.6	51	64.2	29	36.2
16-32-cell	52	47.3	30	58.2	22	42.3
Early and late morulae	30	27.5	20	67.4	10	33.3

The data of Table 3 show that after fertilisation with sex-sorted semen and culturing of 109 ova, 80 viable embryos were obtained or 73.3% of the amount of those fertilised; during further culturing 51 embryos or 64.2% reached the stage of 2, 4 and 8 cells development, 36.2% embryos stopped to further develop.

Of 51 embryos, 30 (58.2%) developed normally and reached the stage of 16-32 cells, 42.3% of embryos degenerated. After 120-168h of culturing, 30 embryos (27.5%) reached the stage of early and late morula, 20 (67.4%) embryos of them were viable.

To identify the influence of cryo- and de-preservation processes on the viability of embryos, obtained "in vitro", the studies were carried out on freezing and thawing of 20 obtained embryos, being at the early and late morula stage. Embryos were frozen in the 1.5% ethylene glycol solution.

According to the results of morphological evaluation, 12h after thawing and subsequent culturing, only 13 or 65% of 20 frozen embryos were viable, i.e., those that continued to further grow. I.e., at the rather high (73.3%) rate of "in vitro" fertilisation, 18.6% of oocytes of all the fertilised ones reached the early and late morula stage. The capacity for survival (survival rate) of early morulae, obtained "in vitro", after freezing and thawing was 65%.

Later on, a scientific research was undertaken to obtain breed sexed embryos in Holstein cows [30]. During the experimental works, the total of 36 oocytes were obtained from the ovaries of 5 cows-donors (Table 4).

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The Table 4 data show that during morphological evaluation 21 oocytes or 58.3% of all ova were selected for further culturing, and 15% were sorted out due to damaged membrane and the state of cytoplasm.

Table 4. Morphological characteristics of oocytes

Oocytes	Total amount, n	%
Total amount	36	100
Morphologically viable	21	58.3
Degenerated	15	41.7
Of them:		
with damaged pellucid zone	7	46.7
by cytoplasm state	8	53.3

Out of degenerated, 46.7% or 7 had a mechanical damage during aspiration of follicles, loss of transparency and thickened transparent shell, cytoplasm of 53.3% of ova was fragmented, dark-coloured, and contracted and had vacuoles.

There were 7.2±0.42 oocytes per a donor on an average, 4.2±0.36 of which on an average per a donor were selected during morphological evaluation for their adequacy to be further cultured and fertilised. The variability between the total amount and morphologically viable oocytes is presented in Table 5.

Table 5. Average indicators of oocytes obtained

Indicators	M±m	б	Cv %
Total amount	7.2±0.42	4.056	24.9
Morphologically viable	4.2±0.36	3.52	26.4
Degenerated	3±0.16	1.63	55.7

Variability between the total amount of oocytes and morphologically viable oocytes is 24.9% and 26.4%, respectively. There were 3±0.16 degenerated oocytes on an average with 55.7% fluctuations. The maximum and minimum values for oocytes, picked up from one ovum, were from 4 to 12.

Oocytes, picked up from the follicles, resume meiosis spontaneously and can undergo all maturation stages to metaphase II without any hormonal effects. However, this process is not equivalent to maturation, which occurs in the ovary follicles prior to ovulation. In culture conditions, only maturation of nucleus occurs with no involvement of cytoplasm. The oocyte can therefore stop to develop after fertilisation since the cytoplasm-related factors are of great importance in fertilisation.

The adequate maturation of the oocyte is required for the embryo normal fertilisation and development. Of importance is therefore a system of culturing used for the maturation [31]. No attempts to improve the culture system through modification of media composition and physical parameters of culture conditions resulted in fully solving the problem of obtaining the biologically viable embryos.

Hence, to improve the quality of early embryos in the “in vitro” conditions, embryos with such somatic cells as fibroblasts, trophoblast vesicle cells, cumulus, and epithelial cells of oviduct, were CO_2-cultured. The main attention was focused on hormonal factors and time, needed for the “in vitro” capacitation of sperm cells.

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To date, capacitation of semen from numerous types of animals is performed "ex vivo". Fresh-received or frozen-thawed semen is taken for fertilisation. "In vitro" capacitation of sperm cells is in their washing out of seminal plasm and pre-incubation prior to fertilisation, after which sperm cells acquire an increased ability to penetrate the tissues. During capacitation, the membranes of sperm cells, which acquire the ability to identify the transparent zone of ovum and receive cellular signals, are considerably re-organised, [32].

Thus, by the results of fertilising oocytes with sex-sorted semen, it was found that the fertilisation rate averages 76.2%, being characterised by the presence of female and male pronucleus 36h after insemination, when observed under the microscope. Further growth therewith is noted in 14 zygotes, what in percentage terms amounted to 66.7% of the number of original oocytes. 11 embryos or 52.4% of the total amount of morphologically viable oocytes were cultured to the late morula and early blastocyst stages. The data are given in Table 6.

It should also be noted that 2 doses or 2 mln. of mobile sperm cells, at the rate of 100 thsd. sperm cells per one ovum, were utilised for insemination, what is in compliance with the generally accepted IVF (in vitro fertilisation) standards.

Table 6. Results of fertilising follicular oocytes with sexed semen and their further development in culturing

Development stages	Total amount	Normal		Degenerated	
		n	%	n	%
Cultured and inseminated	21	21	-	-	-
Presence of pronuclei	16	15	93.7	1	6.3
2-blastomere	14	14	100	-	-
4-6-blastomere	14	13	92.8	1	7.2
16-32-blastomere	13	11	84.6	2	15.4
Morulae and blastocytes	11	11	100	-	-

5 embryos had embryonal blocks (developmental arrests), one embryo had it at the stage of two pronuclei, and four embryos – at the stage of 4-32 blastomeres. These embryos were further frozen in the 1.5% ethylene glycol solution.

To identify the fertilisation rate of aspirated oocytes and to define, how the embryos will further develop in "in vitro" culturing, transvaginal aspiration of follicles was studied. Holstein cows from the "Bayserke-Agro" LLC donor herd were used as the donors of oocytes.

Transcervical aspiration of oocytes of 4 cows was performed, applying no schemes of hormonal superovulation stimulation. Aspirated oocytes were inseminated with the sexed semen of Holstein bulls of American selection. The results of performed transcervical "Ovum pick up (OPU)" aspiration of ovaries are given in Table 7.

Table 7. Results of using the "Ovum pick up (OPU)" method to obtain embryos

Indicators	Number, pcs.	On average per donor, pcs.	
		n	%
Oocytes obtained	31	7.75±0.8	100
Morphologically viable	28	7±1.1	90.3
Ova fertilised	20	5±1.4	71.4

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Ova unfertilised	6	1.5±3.6	21.4
Degenerated embryos	4	1±4.5	14.2
Morphologically viable embryos	10	2.5±0.9	35.7

As is seen from the data of Table 7, only 31 ovum was obtained from 4 cows, of which 28 were selected for the in vitro fertilisation. Culturing and "in vitro" fertilisation enabled production of only 10 morphologically viable embryos at the stage of late morula and early blastocyst, suitable for embryo transfer. On an average, it amounted to 2.5±0.9 pcs. per a donor, 6 of which were transferred to heifer-recipients with synchronised estrous-cycle, and 4 were cryopreserved in the 1.5% ethylene glycol solution.

Discussion

1. The problems of optimising the processes of follicular oocytes' development in culture are of practical importance. In terms of theory, these problems must be studied to develop the concepts of molecular-cellular factors, regulating the processes in the ovum cytoplasm. Culture medium must be as much as possible similar by all parameters to the natural liquids, in which oocytes and embryos are in the organism during the natural course of development and fertilisation. We have therefore used several variants of culture systems, developed based on the Dulbecco medium and follicular fluid, that make it possible, according to the authors' information, to achieve the proper and sustainable in vitro development, and a ready-for-use IVF-Universal (MediCult) solution, intended to fertilise oocytes and culture embryos in vitro, as well.

Thus, it is seen from the obtained results that in culturing "in vitro" of follicular oocytes of cows, the highest maturation rates were obtained in the IVF-Universal medium, namely, 68% as compared to 18% and 22.9% in the Dulbecco and FF media, respectively. The percentage of degenerated oocytes in the IVF-Universal medium and FF is almost the same and amounts to 8% and 11.2%, respectively, when this rate in the Dulbecco medium is 23%.

2. During culturing, 8%, 12.6% and 49.3 % of the total amount of oocytes continued to further cleave to 4, 6 blastomeres. All these data demonstrate that the use of the IVF-Universal medium is far more efficient, exceeding 10 times on an average the other systems' rates in maturation and fertilisation of oocytes, and culturing embryos. It should also be noted that, in culturing embryos, an asynchronous development of embryos was seen, which, according to some authors, is typical even in natural conditions of development. Asynchronous development of embryos in culture is usually associated with heterogeneity of oocytes' maturation and subsequent differences in the time of sperm cells entry into oocytes.

It can be inferred that culture system is the major component for normal fertilisation and development of embryos. When culturing in the Dulbecco medium with 10% homologous serum and in follicular fluid, the fertilisation rate amounted to 12.9% and 19.4%, during culturing a cleavage to 16, 32 cells was 3.65% and 1.87%, respectively, after which a so-called developmental arrest occurred, i.e., the embryos stopped to further develop with their subsequent degeneration.

Alongside, the number of fertilised and cultured to the stage of 16, 32 cells in the IVF-Universal medium amounted to 58.8% and 42%, and the number of embryos cultured to the later development stages was 35.3%.

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Thus, the obtained results have demonstrated that in culturing and fertilising oocytes, the highest rates were noted in the IVF- Universal medium.

3. As a result of culturing the fertilised ova of cows, 20 (18.3%) viable embryos at the morula stage were obtained, and a high efficiency had been demonstrated during culturing the above-described culture media.

In vitro culturing of pre-implantation mammal embryos is a necessary chain in carrying out the experimental and embryological studies, as well as the embryo-technological works on cloning and obtaining transgenic animals. This method provides the basis for human infertility treatment through the in vitro fertilisation (IVF). Even though the method as such has existed for so long now, the needs of cultured embryos and optimum conditions for their "in vitro" development are not conclusively established. Nutritional media for culturing pre-implantation embryos of mammals usually comprise serum, or serum albumin. Their use leads to 50-55% maturation and fertilisation of oocytes.

However, the question on whether these protein complements are necessary, and on their role and functions, remains unclear. Up to now, not all the components of these complements have been accurately identified, and it is not always clear how their known components act on embryos.

It has been inferred from the studies that the fertilisation rate of follicular oocytes with sex-sorted semen is about 75%, and the percentage of development to farther stages is 52.4% or 2.2 morphologically viable embryos per a cow-donor.

Conclusions

For the first time in the Republic of Kazakhstan, the studies have been conducted on improvement of the methods for large cattle in vitro fertilisation and identification of the efficiency and cost-effectiveness of employing in the reproduction of large dairy cattle of embryos of the desired gender and their potential wide use in the enhancement of breeding stock in base farms.

On completion of the research works, intended to develop and improve the methods of the in vitro fertilisation in large cattle, the following results have been obtained:

1. Culture system is among the main conditions for maturation and, accordingly, further development of embryos. To explore the nature of the effect of different culture systems on the fertilisation rate of ova, fertilisation, and further development of pre-implantation embryos, the experiment was performed in three stages using different culture systems (Dulbecco medium with 10% homologous serum, 100% follicular fluid, IVF-Universal). Maximum percentage of oocytes with signs of maturation was observed in culturing ova in the IVF- Universal medium, what was 68% or 312 of the total amount.
2. Determining the fertilisation rate of oocytes and development of embryos in the "in vitro" conditions has shown that in culturing and fertilising oocytes, the highest rates were observed in the IVF-Universal medium. Morphological evaluation of the fertilisation rate enabled the detection of pronuclei in the Dulbecco medium in 18.9%, in FF in 32.9 %, and in the IVF-Universal medium in 59.24 % of the total amount of

selected morphologically viable oocytes.

3. Studying the "in vitro" fertilisation of ova with sex-sorted semen has provided the following results: the rate of inseminating follicular oocytes with sex-sorted semen is about 75%, and the percentage of development to farther stages is 52.4% or 2.2 morphologically viable embryos per a cow-donor. 5 embryos had embryonal blocks (developmental arrests), one embryo had it at the stage of two pronuclei, and four embryos – at the stage of 4-32 blastomeres.
4. To determine the fertilisation rate of aspirated oocytes and further development of embryos in "in vitro" culturing, transvaginal aspiration of follicles was studied. The results of the performed transcervical aspiration of ovaries employing the "Ovum pick up (OPU)" method are as follows: a total of 10 morphologically viable embryos at the late morula and early blastocyst stage were obtained, suitable for transfer. It amounted to 2.5±0.9pcs. per donor on an average, 6 of which were transferred to heifer-recipients with synchronised estrous-cycle, and 4 were cryopreserved.

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