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APPLICATION OF MODERN BIOTECHNOLOGICAL AND GENETIC METHODS IN THE SYSTEM OF PRESERVING THE GENE POOL OF THE UKRAINIAN BROWN DAIRY BREED

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Abstract

The authors investigated the possibility of using modern biotechnological methods for obtaining in vitro embryos from females of the local breed with the desired complex genotype by the A2A2/BB beta- and kappa-casein, cryopreservation and long-term storage in the system of preserving the gene pool. During the first stage of work, 27 oocytes–cumulus complexes (OCCs) were obtained from the ovaries of heifers, of which 66.7% were selected as suitable for in vitro cultivation, and from the ovaries of cows - 69.2% of oocytes–cumulus complexes (45 of the 65 obtained oocytes–cumulus complexes) were suitable for further cultivation. Our cytogenetic analysis of heifer egg cells showed that 33.3% (9 out of 27 cultured oocytes–cumulus complexes) of cells were at the metaphase II stage of meiosis, and 66.7% had chromosomal disorders. The second stage involved the fertilization of egg cells matured outside the body. During this stage, it was found that the level of zygote formation on average reached 23.8%, and further cultivation also provided in vitro fragmentation of embryos at the level of 22.2% (10 embryos from 63 inseminated eggs). It was found that the overall level of embryo formation after fertilization of egg cells of cows matured outside the body was 33.3%. For the conservation and rational use of breeding (genetic) resources of cattle at the cellular level, it is necessary to create cryobanks of gametes for long-term storage in order to further sell them for reproduction.

Key words**:** *gene pool, oocytes–cumulus complex, embryo, semen dose*

INTRODUCTION

There are still many local breeds of farm animals in the world, despite the fact that only a few specialized and highly productive breeds dominate the commercial sector. Unfortunately, state-of-the art and advanced technologies and effective breeding programs further reduce the competitiveness of local breeds. As a result, highly productive breeds are increasingly replacing local breeds, which leads to a decrease in the population of local breeds and even the threat of their extinction, and this places scientists before the challenge of developing measures to preserve genetic diversity [7; 8; 14]. This, in turn, requires scientific institutions, higher educational institutions of agricultural profile and veterinary laboratories to develop measures to study and identify valuable adaptive and productive traits of native animals [13].

Recently, dairy cattle breeding has increased the requirements for the quality characteristics of milk. According to the results of research, scientists have found that cow milk usually contains two main types of beta-casein – A1 and A2 [6, 9]. It is proven that researchers have found a possible link between milk consumption and certain diseases, such as type 1 diabetes, cardiovascular disease, Sudden Infant Death Syndrome, schizophrenia and autism, gastrointestinal diseases, prostate cancer, and other diseases [1].

It is shown that cattle of local breeds are characterized by a higher frequency of the desired A2A2 genotype compared to specialized dairy breeds [3, 4].

Successful conservation of breeds should take into consideration the biological, industrial, and cultural aspects that affect them. To be successful, all three aspects should be considered [12, 2].

Conservation and development strategies based on quantified strengths and weaknesses should be developed to preserve native breeds. Conservation of local breeds should include improving their genetic potential and managing breeds for future use. Effective management of local cattle resources includes identification, characterization, evaluation, documentation, and storage. To create regional banks of genetic resources, it is necessary to involve breeders, communities, public organizations and other stakeholders in conservation programs. As a rule, one of the three main conservation strategies for farm animal breeds is followed. The first two are in situ and ex situ in vivo preservation of live offspring. The third strategy involves preserving biological material (embryos, semen, etc.) in cryobanks.

Cryopreservation plays an auxiliary role in the conservation and improvement of breeds.

Within the existing local breed management system, the strategy should simultaneously ensure both the breed preservation and improvement. Restoration of the structure can provide two thousand semen doses from 15-30 stud bulls and 300 embryos with the same number of males and females.

Within the existing local breed management system, the strategy should combine genetic improvement and conservation. To restore the cattle population, it is necessary to preserve about 2,000 doses of frozen semen each from 15-30 bulls, and 300 embryos with the same number of males and females.

It is possible to ensure biological diversity by preserving other biomaterials (blood, tissues, stem cells). It is believed that if it is not possible to preserve the breed *in situ*, another *ex situ in vivo* conservation strategy should be used[13].

Sumy Region, located in the North-Eastern Ukraine, is almost the only region of Ukraine where local brown cattle, represented by the Lebedyn and Ukrainian Brown dairy breeds, are stored. The main breeding area of these breeds is Sumy and Okhtyrka Districts of Sumy Region.

The aim of the research was to develop a scheme for preserving the Brown cattle in the North-Eastern Region of Ukraine using biotechnological and genetic methods.

MATERIALS AND METHODS

The research was conducted on the regional breeding farms for breeding the Brown cattle, in the Sumy State-Owned Breeding Center. The object of the study was follicular oocytes obtained from the ovaries of animals at Nadiia Experimental Agricultural Farm State-Owned Enterprise of the Institute of Agriculture of the North-East of the National Academy of Agrarian Sciences of Ukraine located in. Donors of oocytes–cumulus complexes (OCCs) were three heifers and five cows of the Ukrainian Brown dairy breed. The ovaries were delivered to the reproduction biotechnology laboratory of M. V. Zubets Institute of Animal Breeding and Genetics of the National Academy of Agrarian Sciences from the slaughterhouse of Nadiia EAF of NAAS 6 hours after the animals were slaughtered in a thermos with sterile saline solution (0.9% NaCl) at a temperature of +32–38°C. Oocytes were obtained by cutting visible antral follicles with a blade, after which they were washed with the Dulbecco's medium (Sigma-Aldrich D 5773) with 1 U/mL of heparin (Biochemi) and 40 U/mL of gentamicin, caught with a Pasteur pipette and washed three-four times in the medium 199 (Sigma-Aldrich M 2520), which contained 10% pre-inactivated fetal calf serum (Sigma F 7524), and evaluated by morphological features through the microscope (MBS-9 USSR).

Oocytes with homogeneous fine-grained ooplasm, intact transparent shell, dense or partially loosened cumulus were used for cultivation. Maturation of OCC outside the body was carried out in quadrilateral plates

(Costar) for 24 hours at a temperature of +38.5°C and 5% CO_2 content in an incubator, in medium 199 with 20% pre-inactivated estrus serum of cows, 2.0 mm of sodium pyruvate, 2.92 mm of calcium lactate, 40 mcg/ml of gentamicin. After maturation outside the body, the egg cells were fertilized *in vitro*. Cryopreserved ejaculated germ cells of a stud bull were used for fertilization of Final 1008 of the Lebedyn breed (blood capacity Lebedyn40.6/Swiss59.4). Semen capacitation was performed with heparin (100 U/ml) according to the methodology of Parrish J.J. et al. [10]. Separation of germ cells from seminal plasma and diluent was carried out by the swim-up method.

Before insemination, oocytes matured outside the body were partially released from the surrounding cumulus cells by mechanical means (pipetting through a smaller diameter pipette). Co-incubation of egg cells and germ cells was carried out in a thermostat at a temperature of +38.5°C and 5% CO_2 content in the air, in drops of Fert.-TALP medium. After 18 hours of co-incubation, the zygotes were washed from adhering germ cells and transferred to a CDM medium for further cultivation. The zygote culture medium was supplemented with 20% fetal calf serum. After further 96-hour cultivation, the embryos underwent morphological evaluation, and the embryos not corresponding to the stage of development were selected for cytogenetic analysis.

Genotyping was carried out for cattle of the Ukrainian Brown dairy breed of 7 stud bulls (Sumy State-Owned Breeding Center) and 8 cows and heifer kept on the farm of Nadiia Experimental Agricultural Farm State-Owned Enterprise of the NAAS Institute of Agriculture of the North-East. Genotyping was carried using the appropriate method [3; 5].

RESULTS AND DISCUSSIONS

The current state of the Brown cattle in the North-Eastern Region of Ukraine is characterized primarily by a rapid decline in the number of cows (Fig. 1) and the complete absence of live stud bulls in the breeding centers of Ukraine.

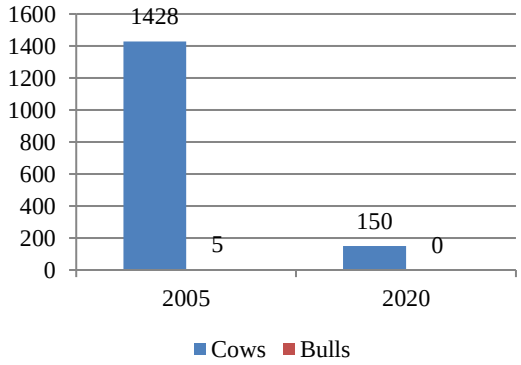

Fig. 1. Population of the experimental breed cattle in breeding farms and centers, heads
Source: Own research.

Only small reserves of semen of bulls crossbred with the Swiss breed (intermediate genotypes during the creation of the breed (Table 1)) give hope for its preservation.

Table 1. Reserves of stud bull semen in the Sumy State-Owned Breeding Center

Line	Stud bull	Conditional blood relationship, %*	Semen reserves, thousand doses
Elehanta 148551	Final 1008 A2A2/BB	L40.6/S59.4	4.5
	Murat 79 A1A1/AA	L12.5/S87,5	2.0
Minus 370	Parom 2075 A1A1/AB	L75/S25	1.5
Bravyi 1510	Rohiz 5002 A1A2/AA	L75/S25	11.0
Balkon 1799	Zaichyk 17000 A1A2/BB	L75/S25	8.9
Suprime 124652	Zalp 17505 A1A1AB	L75/S25	6.2
Balkon 1799	Chystyi 17035 A1A2/AA	L62.5/S37.5	5.8

* blood capacity:- Lebedyn; S –Swiss

Source: Own research.

That is why, as scientists note [11], cryopreserved semen of farm animals, which retains a high ability to fertilize for 25 or more years, is important for the preservation of local domestic breeds of cattle.

Brown cattle are a promising target for conservation and breeding, given their high proportion of preferred beta- and kappa-genotypes. Based on the results of our

research, it was found that stud bulls whose semen is stored in the Sumy Breeding Center have a sufficient level of frequency of the desired A2 and B alleles (beta- and kappa-casein, respectively) (Fig. 2).

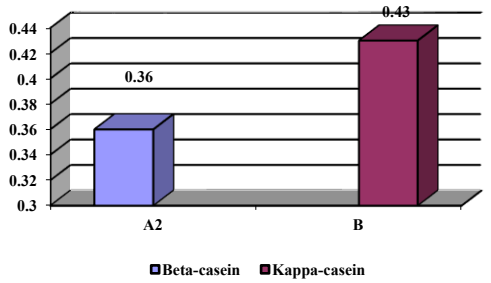

Fig. 2. Frequency of desired beta- and kappa-casein alleles in stud bulls
Source: Own research.

Their use makes it possible to obtain cattle with the desired genotype by A2A2 beta-casein and BB kappa-casein.

However, today the limited number of animals in breeding farms does not ensure their rapid reproduction. We have developed and begun implementing a scheme for organizing the use of biotechnological measures for the conservation of these livestock. According to this scheme, we have studied the possibility of using modern biotechnological methods for obtaining *in vitro* embryos with the desired genotype from heifers and cows of the local breed, cryopreservation and long-term storage in the system of preserving the gene pool of autochthonous breeds. We carried out a complex of works on the organization of obtaining, cultivating and fertilizing eggs cells of heifers and cows matured outside the body, and obtained embryos for further cryopreservation and long-term storage. Experimental Agricultural has the status of a breeding reproducer for breeding cattle (Certificate No. 9289). According to the site class determination data, it was recognized that heifers No. 8012882133, 8012882205, 8011870917 and cows No.No. 5900471930, 5900153371, 5900153352, 5900324430, 8011871058 are animals of the of the studied breed. According to genetic studies, the genotype of these animals by beta- and kappa-casein is A2A2/BB, which makes them particularly valuable in terms of creating micro-populations of dairy cattle with unique qualities. This, in turn, creates prerequisites for the preservation of the local breed. That is why the reproductive material of these animals has been used for biotechnological studies in the system of preserving the gene pool at the cellular level (Table 2).

Table 2. Number of cows and heifers received by OCC

Experiment variants	Number of OCC received				
	total	unsuitable for cultivation		suitable for cultivation	
	n	n	% ±m	n	% ±m
Heifers (n=3)	27	9	33.3[a] ±9.0	18	66.7[a] ±9.0
Cows (n=5)	65	20	30.8[a] ±5.7	45	69.2[a] ±5.7

Source: Own research.

Thus, we obtained 27 OOCs from the ovaries of heifers, of which, according to morphological assessment, 9 (33.3%) were unsuitable for cultivation, and 18 (66.7%) were selected as suitable for cultivation *in vitro*. 65 OCCs were obtained from cows, of which 45 (69.2%) were suitable for further cultivation, and 20 (30.8%) were assessed as unsuitable for biotechnological manipulation.

The next stage of our work was the cultivation of the resulting OCCs, fertilization of egg cells with cryopreserved ejaculated germ cells of stud bulls, and the production of embryos. From the selected oocytes of heifers n=18, from cows, respectively, n=45 were put for cultivation, cultured for 24 hours and fertilized *in vitro*. To obtain embryos *in vitro* with high genetic potential, egg cells were fertilized with cryopreserved ejaculated germ cells of the stud bull Final 1008, whose semen has been stored in the cryobank for more than 30 years, and its complex genotype is A2A2/BB. The use of semen from this stud bull will ensure the production of embryos with the desired A2A2/BB genotype. After defrosting, germ cells showed activity at the level of 65.0%.

As a result of fertilization of egg cells matured outside the body, the level of zygote formation on average reached 20.0% (Table

3). Further cultivation also resulted in the *in vitro* fragmentation at 20.0%.

Table 3. Results of *in vitro* fertilization of egg cells of heifers and cows

Experiment variants	Number of cells to be fertilized *in vitro*	Number of embryos in stages of							
		2 cells		3-4 cells		5-8 cells		9-16 cells	
		n	% ±m	n	% ±m	n	% ±m	n	% ±m
Heifers (n=3)	18	0	0.0^{a}	0	0.0^{a}	0	0.0^{a}	0	0.0^{c}
Cows (n=5)	45	15	33.3^{b} ±7.0	10	22.2^{b} ±6.2	10	22.2^{b} ±6.2	5	11.1^{d} ±4.7
Total	63	15	23.8^{b} ±5.4	10	15.8^{b} ±4.6	10	15.8^{b} ±4.6	5	7.9^{d} ±3.4

Notes: c : d P<0.05; a : b P<0.001, Student's t-test. In this Table, various superscripts indicate the likely difference between the indicators
Source: Own research.

It should be noted that in the case of *in vitro* fertilization of heifers' egg cells after 96 hours of cultivation, the development of embryos outside the body did not occur. According to the results of cytogenetic analysis, 6 cells (33.3%) out of 18 were at the stage of metaphase II of meiosis, and 12 out of 18 (66.7%) had chromosomal disorders.

When studying the formation of embryos after fertilization of mature cow eggs outside the body, the formation of 15 2-cell embryos was observed after 24 hours of cultivation (33.3%). During the next 72 hours of cultivation, embryo fragmentation was observed in 22.2% of cells. After 96 hours of outside the body cultivation of fertilized egg cells obtained from the ovaries of cows, embryo fragmentation was observed in 11.1% of embryos.

The overall level of embryo formation after fertilization of egg cells matured outside the body cows was 33.3% (15 embryos out of 45 fertilized egg cells). It should be noted that the total insemination index of cows is 4.0, which can explain the low yield of embryos outside the body.

The resulting embryos at the early morula stage were frozen by vitrification.

Only embryos of good and excellent quality were used for freezing. Cryopreservation was carried out by immersing straws and embryos in liquid nitrogen using balancing and vitrification solutions. Before freezing, the embryos with the lowest amount of culture medium were transferred to a balancing solution containing 10% glycerol (Sigma G 2025), 20% propanediol (Sigma-Aldrich 16033), and 20% fetal calf serum in Dulbecco's phosphate buffered saline. Cell balancing was carried out for 10 minutes at room temperature. The embryos were then transferred for 30 seconds to a vitrification solution (25% glycerin, 25% propanediol, 20% fetal calf serum in Dulbecco's phosphate buffered saline), pre-cooled to +4°C, placed in 0.25 ml of plastic straw (ART.NR: 13407/3010 Minitube) (Fig. 3) and immersed in liquid nitrogen.

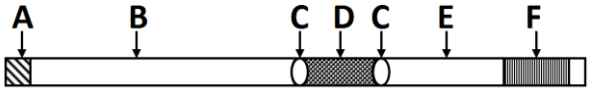

Fig. 3 Scheme of embryo filling in straw for cryopreservation.
A – plug, B – 0.75 m sucrose solution, C – air, D – vitrification solution with the biological object, E – vitrification solution, F – polymer plug
Source: Own research.

It is worth noting that cows had a capacity of 5,500 kg of milk per year with a fat content of 4.25% and protein content of 3.32%, so embryos have a high genetic potential.

Thus, when using complex breeding, biotechnological and genetic methods for obtaining embryos in vitro, it was found that the delivery of ovaries within 6 hours after the slaughter of animals (with the required 1.5-2 hours) affected the level of formation of embryos at the preimplantation stages of development, which was only 11.1%. The use of modern biotechnological methods can provide the essential amount of biological material from animals with high genetic potential and the desired complex genotype by beta- and kappa-casein and more efficiently use such material in the system of preserving the gene pool of breeds based on obtaining embryos in vitro. Thus, our proposed scheme for preserving the Brown dairy breeds using biotechnological and genetic methods includes:

- continuous monitoring of the quality of semen products of stud bulls in the gene pool storage;
- determination of animal genotype by beta- and kappa-casein;
- selection of animals (in case of their culling by age, level of productivity and state of health, the exception is animals with diseases of the reproductive organs);
- obtaining biomaterial (ovaries) and transporting them to the biotechnological laboratory in a set time;
- obtaining an OCC;
- cultivation of obtained OCCs;
- fertilization of egg cells with germ cells of a stud bull;
- embryo obtaining
- cryopreservation and storage of embryos in liquid nitrogen;
- use of embryos to reproduce the population of the local breed.

CONCLUSIONS

Measures have been developed to arrange the use of the modern biotechnological method of cultivation and fertilization of oocytes matured outside the body to obtain *in vitro* embryos cattle with the desired complex genotype while preserving the gene pool of autochthonous breeds.

For the conservation and rational use of breeding (genetic) resources of cattle at the cellular level, it is expedient to create cryobanks of gametes for long-term storage in order to further sell them for reproduction. The results of the research have revealed the need for a deeper study of the biological processes occurring in the animal body, taking into account individual, age and other characteristics, while preserving autochthonous breeds.

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