Microsurgical separation of deconserved embryos

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Abstract. At the current level of development of cell engineering methods in cattle breeding, the accelerated production of monozygotic twins is acquiring important scientific and economic significance. This will make it possible to determine the effectiveness of breeding methods in assessing genetic progress in a population over a number of generations. The method of embryonic cloning by microsurgical separation of embryos is close to solving this problem. Of particular interest is the long-term preservation of demi-embryos to obtain genetic analogues. But the possibilities of preserving demi-embryos at low temperatures are limited, since existing methods are multi-stage and extremely difficult to implement in practice. The search for applied methods of microsurgery in combination with long-term preservation of demi-embryos is an urgent task. Early studies have reported micromanipulation of frozen-thawed embryos. In 1984, in experiments by S. Willadsen & R. Godke, after microsurgery of deconserved embryos, the survival rate was 54.2%. Data on survival rate are not provided. No other data was found, and therefore we conducted studies to determine the influence of the processes of cryo- and deconservation, and dissection of embryos on the survival and ability of demi-embryos to develop totipotently after transplantation. The research was carried out in 2022 at the Regional Research and Production Center for the Reproduction of Farm Animals of Kalmyk State University named after B.B. Gorodovikov. The efficiency of dissection of intact embryos was 86.1% - after separation of 61 embryos, 105 suitable demi-embryos were obtained out of 122 possible, which was 5.7% higher than the same figure for dividing deconserved embryos - 80.4% (37 suitable demi-embryos out of 46 possible after separation 23 embryos). The survival rate of demi-embryos as the ability to nidate in the recipient's uterus in intact and depreserved demi-embryos differed within acceptable limits - 52.2% when transplanting 1 intact demi-embryo (12 pregnant out of 23 recipients) and 65.9% when transplanting 2 demi-embryos (27 pregnant from 41). Similar indicators for transplantation of deconserved demi-embryos were respectively: 42.8 (3 pregnant recipients out of 7 transplanted) and 60.0% (9 pregnant recipients out of 15).

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1 Introduction

Increasing the yield of calves when producing twins using biotechnological methods is an important reserve for increasing milk and meat production. At the same time, monozygotic twins are of particular interest from a scientific and practical point of view [4, 7]. The possibility of creating large groups of monozygotic twins in a short time will make it possible to determine with high accuracy the effectiveness of the selection methods used based on the assessment of genetic progress in the population over a number of generations [1-2, 4, 7].

Currently, a new direction has emerged in biotechnology - the artificial production of identical twins using microsurgical methods of embryonic cloning [3-4, 6, 9]. The cows obtained after transplantation of parts of the embryos can be assessed for their own productivity and the best ones can be selected as mothers of bulls. An urgent task is the possibility of long-term storage of separated embryos, since genetic copies of valuable animals with long-term preservation can be used at any time to obtain genetic analogues [4-5, 7, 13].

Numerous studies in this direction are being carried out all over the world and the results obtained indicate the possibility of deep freezing of embryo halves under certain conditions [8-12]. However, today the possibilities for long-term preservation of demi-embryos at low temperatures are limited. The existing technique is a multi-stage process, difficult to implement in production conditions - when freezing half-embryos after separation, a prerequisite for preserving their viability is packaging in agar cylinders, and during the depreservation process, removal and removal of the cryoprotectant [9-10].

In studies by a group of American scientists, halves of 5-6-day-old morulae were placed in an agar cylinder and cultured for 24-48 hours in sheep oviducts [8, 11]. Compacted half-embryos extracted from the oviducts were frozen using 10% glycerol in an accelerated mode. After thawing and removal of the cryoprotectant, 70% of the halves were viable. Pregnancy after surgical transplantation of 18 halves was 61.2%. As a result of the experiment, 11 live calves were obtained, including 8 monozygotic twins. The technique used is difficult to implement, but is most effective compared to other methods [7, 10, 14].

According to L. Piccard et al. (1984) when freezing half-embryos, the use of an agar cylinder is mandatory. The authors compared the results of culturing frozen-thawed halves of embryos in an agar cylinder and without it. In the first case, 70% of the halves remained viable, in the second case the results were unacceptable - only 6%.

In the experiments of S. Willadsen & R. Godke (1984), part of the separated embryos was packed into the zone pellucidus and placed in agar, and part of the halves were left outside the zone pellucidum. After freezing using glycerol as a cryoprophylactic, the half-embryos were thawed, the glycerol was removed, and they were cultured for 36 hours. In this experiment, only 26.6% of the halves without the zona pellucida remained viable. The same authors conducted an experiment on the separation of embryos after freezing and thawing. The level of safety of demi-embryos after cultivation was twice as high – 54.2%. The results of the experiment indicate the possibility of micromanipulation on previously frozen and then thawed embryos, but no data are provided on the ability of the resulting demi-embryos to nidate in the recipient's uterus.

We did not find additional data in this area of research, and therefore experiments were conducted to determine the influence of the processes of cryo- and deconservation and dissection of embryos on the survival and ability of demi-embryos to nidate after transplantation to recipients.

The purpose of the research was to study the influence of cryo- and deconservation processes on the possibility of embryo dissection, the safety and ability of demi-embryos for nidation and totipotent development after transplantation.

In connection with this goal, the following tasks were identified:

- Study the possibility of microsurgical dissection of frozen embryos after deconservation.
- Determine the viability and ability for nidation of demi-embryos obtained by dissection of deconserved and intact embryos in a comparative aspect.

2 Materials and methods

The studies were conducted in 2022-23. at the Regional Research and Production Center for the Reproduction of Farm Animals of Kalmyk State University named after B.B. Gorodovikov.

The material for conducting experimental studies on microsurgical separation was freshly obtained and frozen cattle embryos. Embryos were obtained from Kalmyk beef breed of cows using in vivo technology. Generally accepted methods, materials and equipment were used in the procedures for poliovulation stimulation, elution, quality assessment and embryo freezing.

In the experiment on the separation of deconserved embryos, high-quality embryos at the morula stage were selected, frozen on a ZEM-4 program freezer after 3-stage equilibration in glycerol solutions increasing to 10% concentration in a cooling mode from 20°C to –36 °C at a rate of 1.0 to 0.3°C/min with spontaneous seeding at 6°C. Thawing was carried out in two stages: 10 sec in air and 25 sec in a water bath at 25°C. The cryoprotector was removed in 3 steps in glycerol solutions of decreasing concentration with the addition of 0.3 M sucrose and subsequent washing in two PBS solutions. For microsurgical separation, 23 morulae were selected, which, after thawing and removal of glycerol, retained the integrity of the zona pellucida and embryonic complex, morphologically assessed as “excellent” and “good”.

Microsurgical dissection of fresh and deconserved embryos was performed using a mobile micromanipulator f. Bachofer (Germany) with one instrument - a microscalpel, which was controlled using a joystick. The second instrument, a microsuction cup for embryo fixation, was not used. This was due to the fact that after thawing, the embryos were washed from a culture medium containing blood serum, which has antistatic properties and prevents the zona pellucida from adhering to the plastic of the Petri dish. The effect of adhesion of an embryo washed from serum to plastic has been successfully used in various manipulations with embryos, including their separation. Embryo dissection was carried out as follows. 3 drops of Dulbecco's medium without blood serum were placed in a Petri dish. To wash the embryo from the culture medium, it was transferred to the first drop, then to the second. When transferred to the third drop, the embryo, washed from the serum, under the influence of electrostatic potential, firmly adheres to the bottom of the cup and, when dissected, does not slip out from under the microscalpel blade. The embryonic complex of the morula was divided strictly in half with a microscalpel in a vertical plane directly through the zone pellucida.

The resulting halves of embryos without the zona pellucida were transferred to a culture medium containing 20% calf blood serum, in which they were kept for 60 minutes. During this time, viable half-embryos, in the process of compaction, restore the ability to nidate in the recipient's uterus. If separation is unsuccessful, the compaction process is disrupted, which is accompanied by degeneration of half-embryos.

Demi-embryos obtained by dissection of freshly obtained and deconserved embryos were transplanted into recipient heifers with a sexual cycle of 7 days, corresponding to the age of the embryos. One recipient was transplanted with 1 or 2 demi-embryos into the uterine horn ipsilateral to the ovary with a functioning corpus luteum.

3 Results

As a result of experiments on dissection of freshly obtained or intact embryos, 61 embryos were divided. As a result of separation, 116 symmetrical half-embryos were obtained (Table 1). The asymmetric division, critical for the blastomere complex, had 6 halves. 116 morphologically suitable half-embryos out of the maximum possible 122 (95.1%) were put into cultivation. After 60 minutes, in 105 halves out of 116 (90.5%), the process of compaction of the embryonic complex to a spherical state was noted, which indicated the preservation of their viability. Thus, the number of suitable demi-embryos after dissection of intact embryos was 86.1% (105 out of 122 possible).

Table 1. Efficiency of microsurgical dissection of deconserved embryos.

State of the embryos	Separated embryos - possible halves	Morphologically suitable halves, n – %	Compact after cultivation n – %	Compact from the number of possible, n-%
Freshly received	61 – 122	116 – 95.1	105 – 90.5	105 – 86.1
Decanned	23 – 46	42 – 91.3	37 – 88.1	37 – 80.4

When dividing embryos using a similar technique after deconservation, the following results were obtained. A total of 23 suitable morulae were separated. As a result, 42 morphologically suitable half-embryos were obtained out of 46 possible (91.3%), of which, after cultivation for 60 minutes, the beginning of compaction of the cell complex was noted in 37 halves - 88.1% of the number placed for cultivation, or 80.4% viable from the number of possible (37 out of 46).

Thus, the efficiency of embryo dissection after deconservation was inferior to the same indicator after separation of intact embryos by 5.7% - 80.4 versus 86.1%, respectively.

The resulting demi-embryos were transplanted into recipient heifers of breeding age, taking into account the sexual cycle synchronized with the age of the embryos (7 days). Transplantations were performed of 1 and 2 demi-embryos per recipient into the uterine horn, ipsilateral to the ovary with the corpus luteum (Table 2). The recipients' pregnancy was determined by rectal examination 2.5 months after transplantation.

Table 2. Results of transplantation of demi-embryos obtained by separating freshly obtained and deconserved embryos.

Indicators	State of demi-embryos	
	freshly received	decanned
Transfers of 1 demi-embryo	23	7
Pregnant recipients (n – %)	12 – 52.2	3 – 42.8
Transfers of 2 demi-embryos	41	15
Pregnant recipients (n – %)	27 – 65.8	9 – 60.0

The efficiency of transplantation of 1 demi-embryo from the separation of intact embryos was 52.2%, which was 9.4% higher than the same indicator when transplanting demi-embryos from the dissection of deconserved embryos - 52.2 versus 42.8%, respectively. After transplantation of 2 separated intact demi-embryos into 41 recipients, pregnancy was established in 27 animals (65.8%), while from transplantation into 15 recipients of separated deconserved embryos in 9 (60.0%), or 5.8% lower.

4 Discussion

The results of the studies show that the method of separating embryos after deconservation can be an alternative to cryopreservation of separated embryos. Pregnancy rates from 42.8% to 60.0% when transplanting 1 or 2 depreserved demi-embryos are quite high indicators, comparable in effectiveness to transplantation of freshly obtained demi-embryos - 52.2 and 65.8%, respectively. It should be noted that the results obtained are in accordance with the known factor of reducing the level of engraftment by up to 10% when transferring frozen-thawed embryos compared to freshly obtained ones.

5 Scientific novelty

At the current level of development of cell engineering methods in cattle breeding, the accelerated production of monozygotic twins is acquiring important scientific and economic significance. This will make it possible to determine the effectiveness of breeding methods in assessing genetic progress in a population over a number of generations. The method of embryonic cloning through microsurgical separation of embryos allows us to solve this problem. In this case, of particular interest is the long-term preservation of demi-embryos to obtain genetic analogues. But the possibilities of preserving demi-embryos at low temperatures are limited, since existing methods are multi-stage and extremely difficult to implement in practice. The search for applied methods of microsurgery in combination with long-term preservation of demi-embryos is an urgent task.

Early studies have reported micromanipulation of frozen-thawed embryos. Thus, in 1984, in the experiments of S. Willadsen & R. Godke, after microsurgical separation of deconserved embryos, the level of safety was 54.2%. We did not find any other data, and therefore studies were conducted to determine the influence of the processes of cryopreservation, deconservation and dissection of embryos on the survival and ability of demiembryos to develop totipotently after transplantation.

The research was carried out in 2022 at the Regional Research and Production Center for the Reproduction of Farm Animals of Kalmyk State University named after B.B. Gorodovikov. The results obtained showed that the efficiency of dissection of deconserved and intact embryos was close in value - 80.4 versus 86.1% preservation, respectively. The survival rate of demi-embryos after transplantation, as the ability for nidation and totipotent development in the recipient's uterus, in intact and depreserved demi-embryos differed within acceptable limits - when transplanting 1 demi-embryo into recipients - 52.2 versus 42.8%, respectively, and 65.8 versus 60.0 % when transplanting 2 demi-embryos.

6 Conclusion

The result of the conducted studies should be considered that the effectiveness of transplantation of demi-embryos obtained by microsurgical dissection of deconserved and freshly obtained embryos is within the extent of the acceptable difference between the transplantation of intact (not divided) frozen-thawed and freshly obtained embryos. Therefore, the method of embryonic cloning under consideration can be successfully used in the reproduction of cattle for the accelerated production of monozygotic twins.

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References

1. V.Yu. Babenkov, N.V. Chimidova, A.I. Khakhlinov, A.V. Ubushieva, V.I. Manzhiev, The role of reproductive biotechnologies in the reproduction and preservation of the gene pool of rare and endangered cattle breeds, Livestock and feed production, **106**, **1**, 67-76 (2023)
2. V.Yu. Babenkov, A.I. Khakhlinov, R.D. Sangadzhiev, E.Yu. Makarova, O.S. Mergulchiev, Biotechnological aspects of accelerated reproduction of the Kalmyk meat breed, Bulletin of the Peoples' Friendship University of Russia. Series: Agronomy and animal husbandry, **18**, **1**, 71-79 (2023)
3. V.Yu. Babenkov, Obtaining twins of cattle using biotechnological methods, Uch. zap. Vitebsk State acad. vet. Medicine, **36**, **1**, 126-127 (2000)
4. L.V. Golubets, Innovative technologies in breeding and selection of pedigree livestock: monograph (GGAU, Grodno, 2019)
5. B.P. Zavertyaev, Biotechnology in reproduction and selection of cattle (Leningrad, 1989)
6. N.G. Kletsko, V.F. Volynchuk, Methodological approaches to cloning mammalian embryos, Bull. scientific works of VIZH, **65**, 22-23 (1982)
7. L.K. Ernst, N.I. Sergeev, Transplantation of embryos of farm animals (Agropromizdat, Moscow, 1989)
8. H. Lehn-Jensen, W. Raii, Cryonicroscopie observations of cattle embryos during freezing and thawing, Theryogenology, **10**, **2**, 263-277 (1983)
9. J.P. Ozil, Y. Heyman, J.P. Renard, Production of monozugotic twins in cows by micromanipulation and cervical transfer, Soc. Study of Fertility (Cambridge) Abstracts, 10 (1981)
10. S.M. Willadsen, H. Lehn-Jensen, C. G. Fehilly, R. Newcomb, The production of monozygotic twins of preselected parentage by micromanipulation of non-surgically collected cow embryos, Theriogenology, **15**, 23-29 (1981)
11. S.M. Willadsen, C. Polge, Attempts to produce monozygotic quadruplets in cattle by blastomemre separation, Vet. Rec., **100**, 211-213 (1981)
12. O. Dochi, Direct transfer of frozen-thawed bovine embryos and its application in cattle reproduction management, J Reprod Dev., **65**, **5**, 389-396 (2019)
13. B. Rodríguez-Alonso, J.M. Sánchez, M. Hamdi, M. McDonald, V. Havlicek, U. Besenfelder, P. Lonergan, D. Rizos, Asynchrony between the early embryo and the reproductive tract affects subsequent embryo development in cattle, Reprod Fertil Dev., **32**, **6**, 564-571 (2020)
14. P.C. Dos Santos-Neto, F. Cuadro, M. Souza-Neves, M. Crispo, A. Menchaca, P.C. Dos Santos-Neto, Refinements in embryo manipulation applied to CRISPR technology in livestock, Theriogenology, **208**, 142-148 (2023)