Fertility of ewe following intrauterine laparoscopic insemination with frozen-thawed semen

A M Aybazov\(^1\), N I Malmakov\(^2\), M I Selionova\(^1\), T V Mamontova\(^1\)

\(^1\) All-Russian Research Institute of Sheep and Goat Breeding – branch of the Federal State Budgetary Scientific Institution “North Caucasus Agricultural Research Center”, 15, Zootecnicheskaya St., Stavropol, 355017, Russia

\(^2\) Kazakhstan Research Institute of Animal Husbandry and Forage Production, branch of the “Research Institute of Sheep Breeding”, 51, Zhandosov Str., Alma-Ata, 050035, Kazakhstan

E-mail: velikii-1@yandex.ru

Abstract. Cryopreservation of sperm opening up possibilities for improvement of breeding work due to the rational use of the valuable animals’ genetic potential became the basis for cryobanks of biomatereal, contributed to the widespread, the exchange of gene pool. However, AI with frozen-thawed semen is not widespread in sheep as it is in other domestic species. One reason for this is the low efficiency of frozen-thawed ram semen application. The aim of research was to study the fertilizing ability of frozen-thawed semen using intrauterine laparoscopic insemination. The experiments were carried out in Kazakhstan and Russian Federation. For intrauterine insemination by laparoscopy has been used frozen in straws semen of Polypay, Suffolk (courtesy of University of Wisconsin-Madison, USA), Hampshire, Dorset, Texel, the South African Meat Merino (represented by Animal Breeding Services LTD, New Zealand), and the North Caucasian breeds. During intracervical insemination by frozen semen, the fertility of ewes was 34.4%. When intrauterine insemination using a single detection of sheep on heat, fertilization ranged from 34.7 to 43.7 %, and when using detection of sheep on heat twice a day, fertility was 68.8%. The analysis of factors that can influence the performance of laparoscopic insemination with frozen-thawed semen is presented.

1. Introduction

During the past decades, numerous studies have been intensively carried out to develop a method for the cryopreservation of semen from various types of farm animals, birds, carnivores. Searches were aimed at creating optimal models of freezing, designing various cryoprotective media, improving defrosting techniques, developing effective tests to predict the quality and biological usefulness of cryopreserved semen. With good reason it can be argued that, thanks to the achievements of primarily domestic scientists (V.K. Milovanov, I.I. Sokolovskaya, A.V. Smirnov, A.I. Lopyrin, E.M. Platov, N.A. Zheltobryukh and others), the germ cell cryopreservation technology opened up new opportunities in breeding work due to more intensive use of high-value genetic potential and creation of cryogenic storages of frozen semen (bio recourse collections) [1]. At the same time, the potential possibilities of this promising assisted reproductive method in sheep breeding are not sufficiently used or have fragmentary character. One of the reasons for this is the low efficiency of using frozen-thawed semen. It was found out the general regularity for the gametes of males in all farm animals that the cryopreservation technology reduces the number of motile sperm cells by 40...50%, leads to damage to
their individual organelles or segments, and in some cases to the death in a significant part of the cells [2]. At the same time, sperm cells damage increases as the stages of cryopreservation are performed (dilution, equilibration, freezing) [3], and the percentage of acrosome defects in frozen ram sperm is 3-3.5 times higher than in samples of freshly obtained sperm [4]. Later, using electronic microscopy, it was shown that at all stages in working with sperm; the most vulnerable organoid during freezing is the acrosome [5]. Moreover, it was found that even sperm cells with high motility can have a low fertilizing capacity due to the ultra-structural, biochemical, and functional damage [2].

It was found that the low fertilization of sheep when using frozen-thawed sperm is not fully explained by the inability of sperm cells to fertilize an egg caused by structural changes in sperm during freezing. It has been proven that by creating the most comfortable conditions for meeting and fusion of sperm cells with the egg, for example, during intrauterine insemination of sheep with frozen sperm, their fertility reached 50.7% [6]. In other experiments, even higher fertility of sheep (57.8-66.0%) was obtained [7].

Later it was proved that with intrauterine administration of frozen sperm, the fertility of sheep is 60...70%, and in some experiments it can reach 80-85% [8, 9]. Other researchers obtained more modest results when performing intrauterine insemination of sheep with cryopreserved semen (42-53%) [10]. Comparative data on the results of intrauterine insemination of sheep with natural and synchronized estrus were obtained: 48 and 54% versus 34 and 82%, respectively [11, 12]. However, they were not obtained from the results of sheep lambing, and according to the re-heat results of inseminated animals, therefore, they cannot be quite reliable.

In experiments performed on 868 sheep of the Stavropol breed laparoscopically intrauterine inseminated using frozen sperm, the fertility rate according results of lambing has made 54.5%. Differences between rams in the level of fertility ranged from 23.4 to 69.2% [13]. It is noted that the individual and pedigree features of rams can also influence the fertilized ability of cryopreserved sperm [14].

Thus, the sperm cells of the ram, despite the damage during cryopreservation and defrostation, retain a potentially high fertilizing ability.

The purpose of this work was to study the fertilizing ability of frozen-thawed sperm during its intrauterine administration by laparoscopy method using endoscopic equipment.

2. Materials and methods

In the autumn period of 2016, two experiments were conducted to study the performance of sheep intrauterine insemination with frozen and thawed sperm.

The first experiment was carried out in the Republic of Kazakhstan in October 2016 in 4 farms of the Alma Ata and Zhambyl regions on early-maturing meat semi-fine-wool sheep of the Kazakh breed at the age from 1.5 till 5 years. For their intrauterine insemination by the method of laparoscopy, sperm was used, frozen in paillettes (straws) with a volume of 0.25 ml at a concentration of 100x10^6 motile sperm cells per straw. The ram semen of Polypay and Suffolk breeds was represented by the University of Wisconsin-Madison, USA, and the ram semen of Hampshire, Dorset, Texel and South African Meat Merino (Samm) was purchased from Animal Breeding Services LTD, New Zealand.

Straws were thawed in a water bath at 38-39°C for 20 seconds, after which the motility of sperm cells was determined. Sperm was used with a mobility assessment of 4 points and more. The content of one straw within 15 minutes after thawing was used for intrauterine insemination of three sheep (0.07…0.08 ml per one sheep, respectively).

Detection of ewes on heat was carried out early in the morning once a day. Laparoscopic insemination (LI) was performed on a day of sampling according to the method proposed by Evans, Maxwell [8]. The technique of LI was as follows. Using a surgical forceps (Aesculap®, EA20), the horn of the uterus was fixed near uterine-tubal junction using endoscopic imaging, and through an incision of 1 cm on the right side of the white line it was taken out. Sperm was injected into the lumen of the uterine horn with a 1 ml syringe with an injection needle of 22G, after which it was placed into pelvic cavity. The administration of sperm into the second horn was carried out similarly.
According to the number of born lambs from experimental animals, 137-152 days after LI, insemination performance was established. 

The second experiment was conducted in the Russian Federation in October 2016 on sheep of the North Caucasian meat and wool breed at 2.5-4.5 years of age in the experimental farm of the All-Russian Research Institute for Sheep and Goat Breeding (Stavropol).

The breeding rams semen of the North Caucasian meat and wool breed was frozen in 0.2 ml paillettes on an automatic sperm freezing line (IMV, France). Before the experiment, it was stored in liquid nitrogen at -196°C in the gene pool storage of the All-Russian Research Institute for Sheep and Goat Breeding (VNIIOK). The sperm was thawed at 38...39°C for 15-20 seconds and evaluated by mobility. If 40% of sperm cells had rectilinear translational movement, intrauterine administration was performed.

Identification of sheep in the state of spontaneous heat was carried out with vasectomized teaser rams in order to ensure the purity of the experiment. At the same time, according to the timing and frequency of detecting, sheep were divided into 2 groups. In the first group, ewes in estrus status were found once a day in the morning, from 6:30 to 8:00. Selected animals on heat were inseminated intrauterinely 2-12 hours after their detection.

In the second group, the ewes on heat were chosen twice a day with an interval of 12 hours: in the morning from 6:30 to 8:00, in the evening from 18:30 to 20:00. The animals selected on heat were inseminated intrauterinely in 13-16 hours after their identification.

Intrauterine insemination was performed using the method and technique of administration described [8] with use of endoscopic equipment (Richard Wolf, Germany).

During laparoscopy, the internal genital organs (ovaries, oviducts, uterus horns and body) were visualized, and they were evaluated with detection of the presence or absence of ovulation on each ovary. For insemination in each horn of the uterus in the area of greater curvature with the help of special syringe catheter, thawed sperm was injected in a volume of 0.05 ml containing 20x10⁶ of motile sperm cells. Sheep that were lambed 145-155 days after insemination were counted.

In parallel, an intracervical insemination of sheep on spontaneous heat (n =50) with frozen sperm of the same rams was carried out. In this case, insemination was carried out twice in one heat with an interval of 8-10 hours, each time at a dose of 0.2 ml (the total insemination dose per one sheep was 0.4 ml with a content of 160x10⁶ motile sperm cells).

3. Results

The analysis of experimental data allowed us to confirm the previously revealed general biological regularity: the fertilizing ability of frozen and thawed in paillettes ram semen does not depend on the breed and place of semen obtaining and cryopreserving.

Thus, in the first experiment on intrauterine insemination of sheep, performed in Kazakhstan using the ram semen of six breeds cryopreserved in the United States and New Zealand, sheep fertility averaged 39.3% with fluctuations on farms from 36 to 43.3% in the absence of a significant difference (Table 1).

| Farm       | Inseminated sheep | Lambed sheep | Got lambs | Including |
|------------|-------------------|--------------|-----------|-----------|
|            |                   | heads | % |              | young rams | ewe lambs |
| Nadezhda   | 50                | 18   | 36.0 | 21          | 11         | 10        |
| Manzor     | 50                | 18   | 36.0 | 22          | 10         | 12        |
| Marzhan    | 30                | 13   | 43.3 | 17          | 9          | 8         |
| Koktem     | 51                | 22   | 43.1 | 28          | 15         | 13        |
| Total:     | 181               | 71   | 39.2 | 88          | 45         | 43        |

Significant differences in the ratio of young rams and ewe lambs were not observed.
The results of the second experiment in a comparative study of the fertilizing sperm ability in cervical and intrauterine insemination of sheep with frozen sperm are presented in Table 2.

**Table 2.** The results of the experiment on laparoscopic insemination of sheep with frozen sperm (Russian Federation)

| Time and frequency of sheep on heat detection | Time after detection, hours | Inseminated sheep | Of them lambed | Got lambs |
|---------------------------------------------|-----------------------------|-------------------|----------------|----------|
|                                            |                             |                   | heads          | %        | heads  | %     |
| Cervical insemination                       |                             |                   |                |          |        |       |
| 1 once a day in the morning (from 6-30 to 8-00) | 0…1                        | 50                | 17             | 34.0     | 38     | 111.8 |
| Laparoscopic insemination                   |                             |                   |                |          |        |       |
| 2 once a day in the morning (from 6-30 to 8-00) | 2-12                       | 80                | 32             | 43.7     | 40     | 125.0 |
| twice a day with an interval of 12 hours: in the morning (from 6-30 to 8-00) and in the evening (from 18-30 to 20-00) | 12-16                      | 80                | 55             | 68.8     | 72     | 130.9 |

Despite thorough preparation of ewes and selection on heat, high (4.5…5.0 points) mobility of sperm cells after thawing, good qualification of the insemination technician, during intra-cervical insemination of sheep with frozen-thawed sperm fertility was 34.0%, which is hardly acceptable result for extensive practice (Table 2). At the same time, it should be emphasized that these data are comparable with the result obtained by researchers in a large-scale experiment on intrauterine (44.89%) and cervical (31.25%) insemination with frozen sperm [15].

In the second group, where a single detection of ewes on heat and intrauterine injection of frozen-thawed sperm were used, sheep fertility was 43.7% (32 out of 80 had lambed) with a fertility of 125.0% (40 lambs from 32 sheep were got). In index of fertility, these data consistent with the result of the experiment in Kazakhstan obtained under similar methodological conditions. The significant difference in the fertility of sheep, apparently, is explained by the biological features in ewes of the North Caucasian meat and wool breed, which is known to have genetically determined high (130…140%) fertility. It is possible that this index was influenced by the natural, climatic, fodder and organizational conditions for preparing the ewes for insemination. In addition, this result is similar to the result obtained by some foreign scientists in different years in sheep with natural estrus (from 44.4 to 56.0) [16, 17, 18, and 19].

The change in the frequency of ewes on heat detection and carrying out intrauterine insemination 12-16 hours after sheep identification had a significant advantage over a single day’s morning detection and insemination 2-12 hours later. Fertility in the third group of sheep has made 68.8% (55 out of 80 sheep were lambed) with a fertility of 130.9% (72 lambs from 45 ewes were got). The difference in favor of a sheep group that was detected in the estrus state twice a day, is reliable on fertility index (P < 0.01).

4. Discussion
A scientifically based explanation of experimental data lies in the plane of knowledge about the neurohumoral regulation laws of reproductive function in sheep. It has been established that the average duration of sexual heat in ewes is from 24 to 42 hours [8, 20], ovulation from the onset of sexual heat occurs on average in 25-32 hours [8, 20], and the average biological lifetime of the egg cell, during which it is able to fertilize, is in the range from 5 to 12 hours after ovulation [8]. Finally, it was found
that the viability time of sperm cells in the horns of the uterus is 15-16 hours [21]. Based on these key parameters, as well as taking into account the method of conducting our own research, we consider it possible to make several assumptions.

When using a single detection of sheep on heat, the duration of sexual heat in animals by the time of selection can be from 0 to 24 hours. Accordingly, the use of double sheep identification with an interval of 12 hours limits the variability of the estrus duration from 0 to 12 hours. This creates the prerequisites for more accurate prediction of the probable ovulation time and, accordingly, insemination of the animals. Thus, by the time of proposed by us insemination period (12-16 hours after sheep detection) the duration of the heat by them will be from 13 to 28 hours.

We have already indicated that the average time of ovulation and of the egg cell release into the oviduct is 26-28 hours from the onset of estrus. However, the egg cell needs time to move into the lower third of the oviduct, where fertilization usually takes place. Consequently, the time we offer (12-16 hours after sheep detection) accounts for the optimal time for laparoscopic insemination, when the most favorable conditions are created for meeting male and female gametes and, respectively, ensuring high fertility of sheep.

Apparently, it is precisely this logic that can explain the low fertility of sheep during intracervical insemination with frozen sperm as compared with intrauterine administration. Despite the fact that the dose of consumed sperm was eight times higher (0.4 ml intracervical versus 0.05 ml intrauterine), its fertilizing capacity was 2 times lower. This also concerns a significantly low fertility of sheep in this group. It is logical to assume that even under the condition of more than one ovulation in a sheep, fertilization of sheep is difficult either due to the deterioration of the conditions for meeting female and male gametes, or the eggs lose their ability to fertilize. An indirect confirmation for the correctness of our calculations can serve regularity established in the experiments [22] that insemination of mares within 6 hours after ovulation provides the same degree of foalness (67.7 %), as well as insemination in the 12-hour period before ovulation (65.0 %) or directly at the time of the release of the egg from the follicle.

5. Conclusions
Based on the obtained results, it can be concluded that laparoscopic intrauterine administration of frozen thawed sperm: a) increases the effectiveness of sheep insemination compared to intracervical insemination by 2 times; b) allows you to reduce the consumption of sperm for fruitful insemination several times; c) allows you to optimize the use of expensive imported sperm from elite breeding males; d) can be used for maximum getting of descendants from rare endangered breeds and representatives of wild fauna.

References
[1] Aybazov A M, Aksenova P V, Ashurbegov K K and Kovalenko D V 2011 On the issue of the preservation of the gene pool and the biological usefulness of cryopreserved sperm Collection of scientific works of the All-Russian Research Institute for Sheep and Goat Breeding 4(1) 24–9
[2] Bagirov V A, Iolchiev B S, Volkova N A and Zinovyeva N A 2017 The influence of cryopreservation on the biological parameters of semen in Romanov and argali hybrids Agricultural Biology 52 268–73
[3] Zheltobryukh N A 1972 Violations in the sperm of rams in the process of equilibration and freezing Sheep breeding 10 33
[4] Fornusek L, Vetvickova V and Petelikova I 1981 Usinek redidel pro dlouhodobu uchovavani na akrosomy beranich spermii Veter.:Med. (CSSR) 26(4) 213–21
[5] Atroschenko M M, Kalashnikov V V, Bragina E E and Zaitsev A M 2017 Comparative study of the sperm cell ultrastructure in the epididymal, ejaculated and cryopreserved stallion sperm Agricultural Biology 52(2) 274–81
[6] Maxwell W M C 1984 Current problems and future potential of artificial insemination programmes *Reproduction in Sheep* 291–8
[7] Salamon S, Maxwell W M C and Evans G 1985 Fertility of ram semen frozen-stored for 16 years *Proc. Aust. Soc. Reprod. Biol.* 17 62
[8] Evans G and Maxwell W M C 1987 Salamon’s artificial insemination of sheep and goats (Sydney: Butterworths)
[9] Takenaka S, Fukui Y and Ono H 1985 Intrauterine insemination with frozen semen in the ewe using a laparoscope *Jpn. J. Anim. Reprod.* 31 25–7
[10] Azzarini M and Valledor F 1988 Inseminacion intrauterina o cervical con semen congelado o fresco en ovejas en celo natural *Prod Ovina* 1 1–8
[11] Reinhold G, Richter A, Schulz J and Menger H 1990 Ergebnisse der Besamung von Schafen mit gefrierkon serviertem Sperma unter Anwendung der Laparoskopie *Monatsh. Veterinaermed* 45 43–5
[12] Bustamante G, Garcia D and Sanchez L G 1990 *Evalucion de la fertilidad de semen ovino descongelado y depositado intrauterino mente por laparoscopia Memoria – III Congreso Nacionela de Produccion Ovina* (Mexico: Universidad Autonoma de Tlaxcala) pp 153–5
[13] Moroz V A, Burdukovskaya T K, Rabocheev V K, Aibazov M M, Mamytov G A, Purvis I, Maxwell W F, Osborne D, Wilson G and Moore P 1993 Results of the first stage of the Australian-Russian experiment *Sheep breeding* 3 10–4
[14] Malmakov N I, Khamzin K P, Seitpan K M and Spivakov V A 2013 Results of sheep lambing after intrauterine insemination with frozen sperm *Sci. News of Kazakhstan* 4 106–17
[15] Anel L, Kaabi M, Abroug B, Alvarez M, Anel E, Boixo J C, de la Fuente L C and de Paz P 2005 Factors influencing the success of vaginal and laparoscopic artificial insemination in churra ewes: a field assay *Theriogenology* 63 35–47
[16] Naqvi S M K, Joshi A, Das G K and Mittal J P 2001 Development and application of ovine reproductive technologies: an Indian experience *Small. Rumin. Res.* 39 199–208
[17] Anel L, de Paz P, Alvarez M, Chamorro C A, Boixo J C, Manso A, Gonzalez M, Kaabi M and Anel E 2003 Field and in vitro assay of three methods for freezing ram semen *Theriogenology* 60 1293–308
[18] Fukui Y, Kohno H, Okabe K, Katsuki S, Yoshizawa M, Togari T and Watanabe H 2010 Factors affecting the fertility of ewes after intrauterine insemination with frozen-thawed semen during the non-breeding season *J. Reprod. Dev.* 56 460–6
[19] Milovanović A, Maksimović N, Barna T, Lazarević and Delić N 2013 Laparoscopic insemination of sheep in Republic of Serbia *Biotechnology in Animal Husbandry* 29(3) 449–56
[20] Lopyrin A I 1971 *Sheep breeding biology* (Moscow: Kolos)
[21] Zheltobryukh N A and Ivkhenko V K 1976 The main reasons for the decline in the fertilizing ability of a ram *Collection of scientific works of the All-Russian Research Institute for Sheep and Goat Breeding* 38 77–84
[22] Lebedeva L F, Atroschenko M M and Burmistrova S A 2015 The main factors affecting the resultant insemination of mares with sperm, cryopreserved by Russian and foreign technologies *Agricultural Biology* 50(4) 476–85