AGRICULTURAL BIOLOGY,
2023, V. 58, Iss. 6, pp. 1088-1099
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

Assisted reproductive technologies

UDC 636.3:591.391.1:57.085.23

doi: 10.15389/agrobiology.2023.6.1088eng
doi: 10.15389/agrobiology.2023.6.1088rus

THE RESULTS OF PRODUCTION AND TRANSPLANTATION OF IVEP EMBRYOS IN SHEEP (*Ovis aries*)

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by the Ministry of Science and Higher Education of the Russian Federation
Final revision received July 19, 2023
Accepted August 03, 2023

Abstract

In vitro embryo production (IVEP) in sheep is necessary to develop because of its use in breeding and conservation of valuable animals and possible creation of new genotypes by genomic editing. In the present work for the first time in national practice, full-fledged ovine embryos were produced in vitro and live lambs were born after their transplantation to recipient ewes. The aim of this work was to model the main steps of IVEP technology in this species, and to evaluate its efficiency in vitro and in vivo. Female germ cells were obtained post mortem from the ovaries of sexually mature ewes and sheep of various breeds and ages after slaughtering. Cumulus-oocyte complexes (COCs) (n = 1028) were retrieved by dissecting of visible follicles and only high-quality COCs (n = 620) were cultured for 24 h, 25-35 COCs per 500 ml of TC-199 medium supplemented by 10 % fetal calf serum, 10 μg/ml of FSH and 10 μg/ml LH, 10 ng/ml of epidermal growth factor. A part of mature oocytes (n = 96) was used for cytological analysis of nuclear maturation rate, and other oocytes (n = 524) were transferred to BO-IVF medium (IVF Bioscience, UK) for in vitro fertilization. The granules of Katadin breed ram frozen semen were thawed and treated by "swim-up" method in Sperm-TALP medium (G.N. Singina, 2019). Mature oocytes were co-cultured with ram sperm in BO-IVF for 15-16 hours and then were transferred to BO-IVS medium (IVF Bioscience, UK) for in vitro embryo development. At day 2 of culture, cleavage rate was evaluated and a part of cleaved embryos was transplanted to recipient animals; at day 7, development to blastocyst occurred. Two-day embryos were transplanted synchronously to cycling Romanov breed ewes (n = 6) by endoscopic surgical method (V.A. Lukanina et al., 2023) using two-port laparoscopy under local anesthesia. After 35-42 days of transplantation, recipient ewes were examined for pregnancy, and fetus development was monitored until live lamb birth. According to cytological analysis, oocyte nuclear maturation rate was 77.1 % (74/96), 316 out of 524 mature and fertilized oocytes were cleaved (60.3 %), and 92 cleaved embryos were transplanted to recipient animals. Remaining early embryos (n = 224) continued in vitro development and 34.8 % reached blastocyst stage. According to ultrasound diagnostics after embryo transplantation, pregnancy rate was 50 % (3/6), and 33.3 % (2/6) transplantations resulted in live offspring. Thus, reported data demonstrated efficiency of IVEP technology in sheep: produced embryos were full-fledged and capable to develop to viable offspring. There is a good reason to believe that proposed technology of in vitro embryo production and transplantation to recipient ewes can be applied to reproduction technologies and gene editing in ovine.

Keywords: *Ovis arie*, domestic sheep, oocytes, in vitro maturation, in vitro fertilization, embryos, IVEP, transplantation

Assisted reproductive technology (ART) in husbandry uses mature and in vitro fertilized female gametes (oocytes) of domestic animals to produce embryos outside the body and their transplantation to recipients. The ART improvement

and practice may provide preservation and replication of valuable genotypes and the creation of new genotypes by genomic editing [1, 2]. According to statistics, in vitro embryo production (IVEP) from farm animals in the world increases every year [3]. The leader remains cattle with approximately 50% of such embryos out of the total number produced, and a significant part of them is intended for commercial use [4].

In small ruminants, particularly sheep, IVEP has so far had limited commercial use compared to cattle, although interest in IVEP technology is also growing [5]. In sheep, this approach is an alternative to multiple ovulation and transfer embryo production (MOET) technology. Its applicability in breeding is limited by the high cost and the unpredictability of the result [1, 5] because of the dependence on the reproductive status of the donor [6], significantly fluctuated superovulatory response to hormonal stimulation [7], insemination efficiency [8], and early luteal regression [6]. In addition, both embryo retrieval and transplantation require surgical manipulations [5, 6]. Recently, a serious impetus is the increased interest in creation of modified animals using a simple and effective CRISPR/Cas9 based genome editing system [9, 10]. Its application most often involves introduction into the cytoplasm of oocytes fertilized in vitro [11, 12]. Finally, for sheep as a farm animal, there is an easy and affordable way to retrieve oocytes from the ovaries post mortem. This expands the use of IVEP as a model in reproductive biology, including improving assisted reproductive technologies that are used to solve some human fertility problems and to conserve endangered animal species [13].

IVEP includes several main stages. These are extraction of female germ cells (post mortem or from living donors) from ovarian follicles, culture of isolated oocytes for in vitro maturation (IVM), in vitro fertilization (IVF) of mature oocytes using fresh or frozen semen, as well as IVF oocyte culture to produce embryos of different developmental stages (early or blastocyst) [13]. The first fact of IVEP for sheep embryos was reported in Cambridge (UK) in 1986 [14]. Due to the work of scientists from different countries, individual stages of IVEP were improved and, as evidenced by a number of reviews [2, 5, 13], this provides a significant increase of the efficiency of the method, but still its indicators, with rare exceptions, are inferior to those of cattle [2, 5]. Therefore, there is a need to continue such experiments. The overall performance of IVEP in terms of the number of embryos and their development to the blastocyst stage ranges from 18 to 89% and from 7 to 79%, respectively. However, there are significant differences between experiments due to particular protocols, the germ cell origin, the age, physiological status and genetic background of donors [2, 5, 13].

In domestic practice, the need to develop IVEP technology in sheep is especially acute. We have not found a single work that contains information about obtaining viable embryos from ewe oocytes that have matured and been fertilized outside the body.

Here, we report for the first time the IVEP production of high-quality sheep embryos after transplantation of which live offspring were born.

The aim of our study was to develop IVEP technology in sheep and evaluate its effectiveness in vitro and in vivo.

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

Obtaining IVEP embryos. The ovaries of sexually mature ewes of different breeds and ages were collected post mortem at a meat processing plant and delivered to the laboratory within 2-4 h, where they were freed from excess tissue and washed several times in a physiological solution containing 100 IU/ml penicillin and 100 μg/ml streptomycin (OOO BioPharmGarant, Russia). To isolate cumulus-oocyte complexes (COCs), the ovaries were alternately placed in a

Petri dish (OOO Biomedical, Russia) with a diameter of 100 mm with TS-199 medium containing 2% fetal bovine serum (FBS), 10 μg/ml heparin and 50 μg/ml gentamicin (TS-199M) and fixed using surgical tweezers. The walls of the visible ovarian follicles were dissected with a blade held in surgical tweezers or a scalpel. After dissecting a batch of 5-7 ovaries, the Petri dish was examined under an SMZ stereomicroscope (Nikon, Japan) and the COCs were selected and transferred into fresh TS-199M medium. After completing a similar treatment for all ovaries delivered to the laboratory, the total pool of collected COCs was washed 3 more times. During the last washing, suitable for culture COCs of oocytes with homogeneous cytoplasm surrounded by at least one layer of cumulus cells (CC) were selected [15].

To mature in vitro, COCs were cultured in TC-199 medium containing HEPES (25 mM), Na-pyruvate (0.5 mM), follicle-stimulating and luteinizing hormones (10 μg/ml), epidermal growth factor (10 ng/ml) (Thermo Fisher Scientific, USA), FBS (10%) and gentamicin (50 μg/ml). Incubation was carried out in 4-well plates (OOO Biomedical, Russia) in groups of 25-35 COCs in 500 μl drops of medium. After 24 h of in vitro maturation (IVM), part of the COCs was used for cytological analysis of nuclear maturation [16], the remaining part was transferred to BO-IVF medium (IVF Bioscience, UK) for in vitro fertilization (IVF).

Oocytes were fertilized using the active sperm fraction obtained by the swim-up method according to a previously described protocol with minor modifications [16]. Granules of frozen sperm of a Katahdin ram were thawed in Sperm-TALP medium [17], 200 μl were transferred to the bottom of tubes containing 1 ml of same medium, and incubated for 1 h. After incubation, the top 700 μl layer was transferred to another tube with Sperm-TALP medium and centrifuged. The resulting sediment, containing motile sperm, was added to the BO-IVF medium with previously matured COCs placed there, the sperm concentration was 1×10^6/ml of fertilization medium. Oocytes and spermatozoa were incubated together for 15-16 h, then female germ cells were carefully released in fresh drops of BO-IVF from CC, dead and adherent spermatozoa, and transferred to embryo development medium.

Embryos were cultured in drops of BO-IVC medium under a layer of mineral oil in an Embryovisor plate incubator (Westtrade LTD, Russia) at 38.5 °C and a three gas mixture of 5% CO_2, 5% O_2 and 90% N_2. After 2 days of culture, a morphological assessment of the fertilized oocyte cleavage into blastomeres was carried out and a part of early embryos, having two or more blastomeres, was selected for transplantation into recipients. The remaining portion was transferred to fresh BO-IVC medium and cultured for another 5 days under similar conditions until the blastocyst stage to assess viability in vitro. Morphological assessment of embryo development was carried out according to common criteria [18].

The isolated COCs and the embryos that derived after in vitro fertilization were photographed using an Eclipse Ti-U inverted microscope (Nikon, Japan).

E m b r y o t r a n s p l a n t a t i o n. The embryos were transplanted into sexually mature Romanov ewes aged 17-18 months, the animals had previously gone into natural heat (the physiological yard of the Ernst Research Center — VIZh, October 2022). The number of estrus was not taken into account before use. Recipient ewes were preliminarily stimulated to estrus by intramuscular injection of 125 μg of cloprestenol (Bioveta, Czech Republic) 13 and 2 days before the expected estrus [19]. Animals with signs of estrus were detected using a sample ram. On day 2 after heat detection, 2 day IVEP embryos were endosurgically transplanted (n = 6) using two-port laparoscopy under local anesthesia, as described previously [19].

Embryos were transferred into the uterine lumen (ipsilateral to the ovary

containing at least one functional corpus luteum, CL) using a 10 mm (outer diameter) capillary pipette connected to a 1 ml syringe. The capillary was filled as follows: 25-30 mm medium for embryo transfer, 2-3 mm air, 30-40 mm medium containing embryos, 2-3 mm air, 25-30 mm medium. The medium used was TS-199 containing HEPES (25 mM), gentamicin (50 μg/ml) and 10% FBS. The number of corpora lutea per recipient averaged 2.2, varying from 1 to 4.

35-42 days after embryo transfer, recipient ewes were examined for pregnancy. The development of fetuses was monitored until the birth of live offspring. Diagnosis in both cases was carried out using a portable ultrasound scanner Draminski 4Vet Slim (DRAMIŃSKI S.A., Poland), equipped with a convex sensor with the main frequency 2-8 MHz. Lambs born were examined for general health, identified by sex, and weighed.

The reliability of the origin of the surrogate lambs was assessed using microsatellite markers (short tandem repeats, STR) [20]. Mothers and offspring were studied for 11 STRs comprising 2 multiplex panels, the INRA005, INRA23, MAF65, McM527, SPS113, INRA063 (panel 1), and HSC, MAF214, OarAE129, OarCP49, OarFCB11 (panel 2). Genomic DNA of lambs and recipient ewes was isolated from ear tissue using the DNA-Extran kit (ZAO Synthol, Russia) and the perchlorate method. Amplification was run in a 15 μl final volume in PCR buffer containing 1.5 mM $MgCl_2$, 200 μM dNTP, 0.5 mM primer mixture, 1 unit Taq polymerase (JSC Dialat Ltd, Russia) and 10-100 ng of genomic DNA. Conditions were as follows: initial denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 20 s; 63 °C (panel 1) and 55 °C (panel 2) for 1 min; 72 °C for 1 min; final elongation at 72 °C for 10 min. Amplification fragments were identified (an ABI Prism 3130xl genetic analyzer, Applied Biosystems, USA). Primary STR data were processed using the GeneMapper 4 program (Applied Biosystems, USA). The probability of exclusion of relationship between animals was assessed using the GenAlEx 6.5 program [20]. The calculated parameters were PI (probability of genotype matching), PX1 (exclusion of one parent in a pair), PX2 (exclusion of relationship when one parent is known, but the other genotype is not available), PX3 (exclusion of the relationship of the alleged parent couple).

Results. A total of 174 post mortem ovaries were delivered to the laboratory (Fig. 1, A), from which we isolated 1028 COCs, on average 5.9 per ovary. Based on the morphological assessment, 620 COCs (60.3%) were suitable for in vitro maturation, on average 3.56 per ovary (see Fig. 1, B) and cultured in IVM medium.

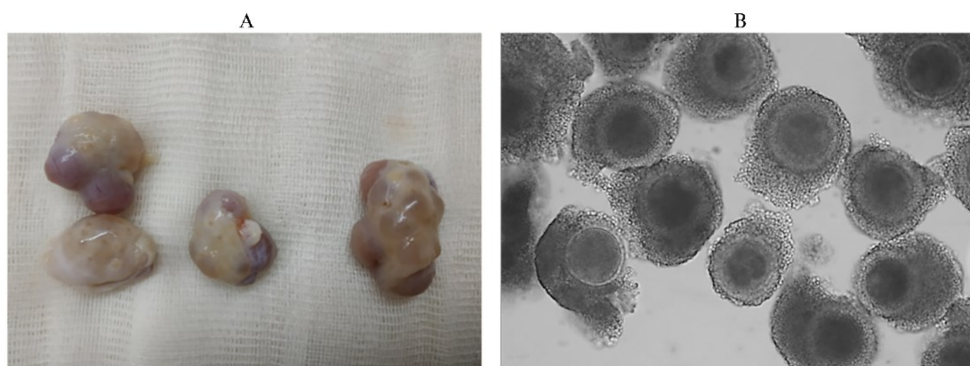

Fig. 1. Ewe ovaries used to isolate oocytes (A)**, and a micrograph of cumulus-oocyte complexes isolated for cultivation** (B)**.** A microphotography was made on an inverted microscope Eclipse Ti-U (Nikon, Japan), magnification ×100 (Ernst Federal Research Center — VIZh, Moscow Province, 2022).

After 24 h of incubation, 96 COCs were selected for cytological assessment

of nuclear maturation in oocytes, and 524 COCs were subjected to in vitro fertilization followed by assessment of the in vitro development to blastocysts (7 day culture). In addition, the viability of some 2 day embryos after transplantation into recipient animals was studied.

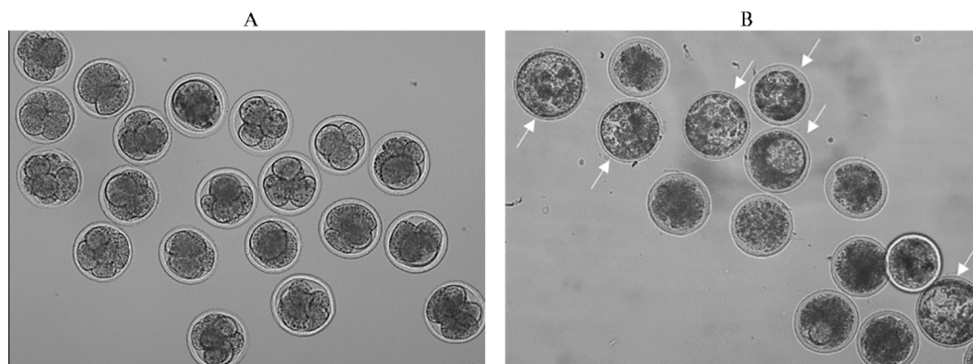

Fig. 2. Microphotographs of embryos developed from ewe oocytes matured and fertilized in vitro: A — day 2 cleaved embryos; B — 7 day embryos, including those that have reached the blastocyst stage (indicated by an arrow; an inverted microscope Eclipse Ti-U, Nikon, Japan, magnification ×100) (Ernst Federal Research Center — VIZh, Moscow Province, 2022).

Cytological analysis confirmed that the proportion of maturation, that is, the percentage of oocytes at metaphase II of the meiotic division from the initial number of cultured COCs, was acceptable and averaged 77.1% (74 out of 96). Of the 524 mature and fertilized oocytes, 316 oocytes (60.3%, Fig. 2, A) passed the first cleavage division; 92 cleaved embryos were transplanted. Of the rest 224 cleaved embryos that continued to develop in vitro, 78, or 34.8%, reached the blastocyst stage (see Fig. 2, B) In general, the percentage of blastocyst development vs. the number of COCs set for maturation was 21.0%.

1. The effectiveness of IVEP embryo transplantation to recipient Romanov ewes (Ernst Federal Research Center — VIZh, Moscow Province, **2023)**

Recipient number	Transplanted emvryos, *n*	Ultrasound examination results	Lambs born, *n*	
			total	alive
062	14	No	0	0
195	14	Yes	3	2
5750	19	No	0	0
039	13	No	0	0
005	15	Yes	0	0
26	17	Yes	1	1

N o t e. The pregnancy (yes/no) was assessed by an ultrasound examination on days 35-42 after embryo transfer.

Table 1 shows the results of transplantation of in vitro obtained embryos. Ultrasound diagnostics confirmed that after embryo transplantation, pregnancy occurred in 3 out of 6 recipients (50%) (Fig. 3, A). The proportion of transplants that resulted in the birth of live offspring (see Fig. 3, B) was 33.3% (2 out of 6). One ewe (recipient No. 195) naturally gave birth to three lambs, two females and one male, two of which (a male and a female) were alive and healthy (see Fig. 3, B), and one lamb was born dead, but full-term. The second ewe (recipient No. 26) similarly gave birth to one healthy ram. In the first case, the viability of the transplanted embryos was 21.4% (3 out of 14), in the second 5.9% (1 out of 17). In recipient No. 195, the duration of the gestation period from the date of embryo transfer was 147 days, the average weight of the born lambs was 2.97 kg, in recipient No. 26 it was 145 days and 3.0 kg, respectively.

To control for origin, we determined microsatellite profiles of animals (Table 2). This method is generally accepted for confirming/excluding kinship in a mother—offspring pair [20].

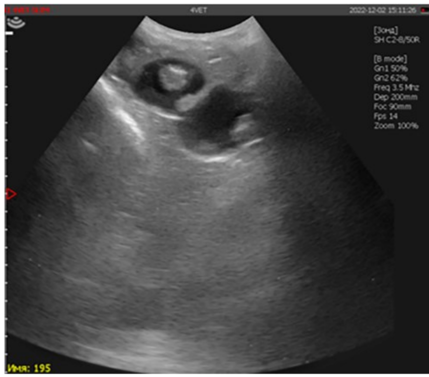

Fig. 3. **In vivo development of IVEP sheep embryos after transplantation:** on the left, ultrasound diagnostics (ultrasound scanner Draminski 4Vet Slim, DRAMI SKI S.A., Poland), 42 days after embryo transplantation, recipient No. 195; on the right, ewe No. 195 with two newborn lambs 3 h after lambing (March 17, 2023, Ernst Federal Research Center — VIZh, Moscow Province).

2. Microsatellite profiles of recipient Romanov ewes and surrogate lambs and STR panel indicators (Ernst Federal Research Center — VIZh, Moscow Province, 2023)

STR	Family 1			Family 2	
	M195	O1	O2	M26	O
INRA005	131/131	129/131	129/131	131/131	147/149[a]
SPS113	140/152	140/148	148/148[a]	140/148	140/152
McM527	169/169	169/171	173/175[a]	169/183	169/177
INRA23	201/205	205/223	203/203[a]	205/221	203/203[a]
MAF65	125/125	131/137[a]	131/135[a]	135/135	125/125[a]
INRA063	181/181	181/187	189/205[a]	173/183	177/181[a]
HSC	276/286	276/282	276/286	286/286	272/276[a]
OarCP49	106/108	94/102[a]	96/100[a]	100/106	98/106
OarAE129	147/149	147/149	139/139[a]	149/149	147/147[a]
MAF214	190/200	188/192[a]	190/192	192/192	190/190[a]
OarFCB11	131/135	123/135	127/139[a]	131/139	131/145
Parameter	**Family 1**			**Family 2**	
PI	8.58×10^{-10}			1.07×10^{-7}	
PX1	99.92			98.96	
PX2	98.31			92.25	
PX3	99.999			99.928	

N o t e. Family 1 — mother (M) recipient No. 195, offspring (O) 1 and 2, family 2 — mother (M) recipient No. 26, offspring (O); the letter ([a]) indicates microsatellite alleles in the offspring profile that do not coincide with the maternal.

The 11 STR based assessment of the origin of the surrogate lambs born by recipient No. 195 revealed the probability of genotype matching (PI) with a lower threshold of 8.58×10^{-10}. The accuracy of confirming the origin for two (PX1) and one (PX2) parents was 99.92 and 98.31%, respectively, the accuracy of excluding parents (PX3) was 99.99%. A comparison of allelic profiles in female and male offspring demonstrated their discrepancy with the surrogate mother at 3 and 8 microsatellite loci, respectively, and between each other at 6 loci, which excludes any probability of consanguinity. The lack of relationship with the surrogate mother (PX3) was also confirmed in the offspring born by recipient No. 26 for 7 microsatellite loci with an exclusion accuracy of 99.93%. A combined analysis for two surrogate families showed the accuracy of excluding consanguinity for two parents (PX3) to 99.9999%.

In sheep, due to the high cost and high invasiveness of the procedure for obtaining female germ cells from live animals, oocytes are usually removed from the ovaries post mortem [2], which involves either dissection of the follicle walls or aspiration of their contents [21]. The latter approach is considered simpler, but it does not allow full use of the generative potential of the original biological

material, and also, according to some reports, negatively affects the quality of the isolated COCs [22]. Careful dissection of the ovarian follicle walls with a cutting instrument increases the number of COCs recovered and better preserves their structure [5]. Using this technique, we recovered an average of 5.9 COCs from one ovary, of which 60% were suitable for obtaining embryos in vitro. Other researchers have reported similar results when choosing this method [5, 21, 22].

Oocytes isolated from follicles, in order to become suitable for fertilization, must mature in vitro, that is, must reach the metaphase II of the meiotic division. They must undergo the necessary cytoplasmic transformations [4]. It is well known that in vitro maturation is a key step in obtaining good quality oocytes, determining their competence for embryonic development. In sheep, a recent analytical review showed that 70-90% of oocytes mature in vitro [2]. In this case, we are talking about both donor (obtained from living animals) and post mortem oocytes. However, there are studies that report lower values of this parameter [23, 24] (Table 3). In our study, the maturation percentage of oocytes isolated from follicles post mortem is comparable to higher values and amounted to 77%.

3. The effectiveness of the in vitro production embryos from ewe oocytes isolated post mortem (according to the literature)

Country	Breed	Semen	IVM, %	Embryo development, %			Reference
				of total number of mature oocytes	up to the blastocyst stage		
					1	2	
Australia	Merino	Frozen	93.1	73.7	41.8	56.7	[15]
Brazil	No data	Frozen	88.0	68.0	33.8	48.9	[25]
Great Britain	Texel[б]	Frozen	63.2	89.7	40.4	45.1	[23]
Iran	No data	Frozen	92.0	63.3	20.0	31.7	[26]
Iran	Lori-Bakhtiari	Fresh	No data	81.6	31.7	38.7	[27]
Iran	Sajabi[б]	Fresh	No data	66.2	20.6	31.0	[28]
Spain	Rasa Aragonesa[б]	Fresh	No data	74.3	30.0	40.0	[29]
Spain	No data	Frozen	No data	60.4	4.0	6.7	[30]
Italy	Sarda sheep	Fresh	No data	80.0	30.0	38.0	[31]
Italy	Sarda sheep[б]	Frozen	92.0	74.5	22.4	59.2	[32]
Italy	Sarda sheep	Fresh	73.2	58.6	11.6	19.8	[33]
China	No data	Fresh	48.4	72.3	29.9	41.4	[24]
Mexico	Rideau Arcott[a]	Fresh	д/о	76.5	21.3	27.7	[34]
Poland	No data	Frozen	д/о	57.9	21.9	36.7	[35]
Portugal	Merino	Fresh	87	41.9	15.6	37.4	[36]
		Frozen		45.1	19.5	42.8	
Saudi Arabia	Naimi	Fresh	No data	29.9	7.1	23.5	[37]
		Frozen		18.8	1.3	7.0	
Saudi Arabia	Najdi	Frozen	No data	35.0	5.9	17.2	[37]
				18.98	0.7	4.1	
Uruguay	No data	Frozen	No data	69.8	35.2	50.5	[38]

Т щ е е. IVM — in vitro maturation; 1 — percentage of the number of mature oocytes, 2 — percentage of the number of cleaved embryos; [a] and [b] — information about the breed for sperm or oocytes, respectively.

The source of sperm for in vitro fertilization of mature oocytes can be either fresh or frozen semen (see Table 3). Here, we use the latter. This is more practical, in addition, there are few reportws about a decrease in the fertility of cryopreserved vs. native semen in vitro [30, 37]. Active sperm are obtained either by the swim-up method or by density gradient centrifugation, e.g., in Percoll. The question of which techniques are more effective in case of sheep semen remains open [5], so we preferred the swim-up method which we use for IVEP in cattle [16].

For the success of IVF, it is important that the germ cell co-incubation environment is capable of providing sperm with ideal conditions for their penetration into the oocytes. Most laboratories use synthetic oviduct fluid, supplemented with the necessary capacitating agents [1]. We set out to simplify and make this IVEP step more reproducible, so commercial BO-IVF medium (IVF Bioscience, UK) was used for co-incubation of oocytes and sperm. The proportion

of oocytes that proceeded to cleavage which is a criterion for assessing the frequency of fertilization. In our work, this estimate was 60.3%, being comparable with the values established by other authors [26, 30, 33, 35] (see Table 3), and in some cases exceeding these values [36, 37].

In our study, to determine the competence of fertilized oocytes to embryonic development, they were either cultured for 7 days until the blastocyst stage, or transplanted into the uterine horn of recipient ewe after 2 days of incubation and cleavage. The yield of embryos at the blastocyst stage was 21.0% from the COCs subjected to maturation, and 34.8% from the number of cleaved oocytes. If compare these values with those presented in Table 3, we can see that in general they are consistent with the results of other authors [26, 32, 35, 26], but there are also more successful [15, 23, 25] and less successful [30, 37] works. The discrepancies can be explained by peculiar protocols used, differences in the quality of COCs, and the influence of the physiological state and genetic background of germ cell donors [2, 5, 13].

In our study, after transplantation of IVEP embryos, pregnant ewes accounted for 50% (3/6), and 33.3% (2/6) of transplantations resulted in the birth of lambs. The offspring (three lambs, two rams and one ewe) are the first in domestic practice; currently the animals are healthy and their development is being monitored.

Given the proportion of oocyrw cleaved after fertilization to the Bl stage in vitro (34.8%), the described positive effect of group embryo transplantation [5] and the multiple pregnancy of the Romanov ewes, we transplanted from 13 to 19 2 day embryos per recipient. Approximately that number of embryos has been reported by other authors [39, 40].

Thus, the results we obtained under in vitro maturation of sheep oocytes (77%), in vitro fertilization (60%) and in vitro development of embryos (34%) indicate the compliance of the IVEP technology we used with world analogues. The obtained embryos could develop into viable offspring. There is every reason to believe that the proposed IVEP technology with embryo transplantation into recipient animals can be practiced during reproduction and genome editing in sheep.

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