Theses of Doctoral (PhD) Dissertation

DEVELOPMENT OF ASSISTED REPRODUCTIVE TECHNOLOGIES FOR EX SITU *IN VITRO* GENE CONSERVATION IN SHEEP

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1. INTRODUCTION

Sheep play an essential role in providing meat, milk, and wool to human livelihood and, besides, being a ready source of income in developing countries, particularly to rural dwellers (ESTEVEZ-MORENO et al., 2019). The present geometric increase in the human population necessitated using assisted reproductive technologies (ARTs) to improve livestock genetic gain, productivity, and reproductive performance (AMIRIDIS & CSEH, 2012). However, the increase in selection pressure using ARTs and poor or lack of proper identification and conservation strategies resulted in a drastic loss of valuable local sheep genotypes globally and changed the sex ratio, which is more in the developing countries and Europe (TABERLET et al., 2008). The International Union for the Conservation of Nature and Natural Resources (IUCN) reported that a species with an effective population size below 50, 250, or 1000 is considered Critically Endangered, Endangered, or Vulnerable, respectively (IUCN, 2012).

Moreover, the 2022 Global Sustainable Development Goals reports on the number/proportion of local sheep breeds (LSB) genetic resources (GnR) revealed a huge global and continental dearth of information and materials; Globally: 87.22%, Europe and North America: 79.64% and Sub-Saharan Africa: 97.75% (SDG, 2022). There is, therefore, no sufficient data on the conservation program of such important genotypes. Furthermore, BOLTON et al. (2022) reported that the current extinction rate is 1000-10000-fold greater than the natural rate, which is a devastating threat to our survival. It, therefore, calls for the need to stage-manage by devoting extra effort to recognize and conserve LSB globally. The *ex-situ* conservation involves conserving biological diversity outside their natural living habitats, while *in-situ* conservation entails conserving them in their living habitat (MAXTED, 2013). *Ex-situ in vitro* gene conservation (EIGC) entails cryopreserving genetic material at sub-zero temperatures (-196 ^{o}C) using liquid nitrogen (MAXTED, 2013). It is a vital insurance policy that enables the indefinite conservation of animal genetic resources (AGR), followed by the use of ART to regenerate a genotype of interest in the population (BOLTON et al., 2022). Techniques like AI, IVF or ICSI, oocytes and embryo cryopreservation are among the major techniques used to achieve EIGC of endangered species (HOWARD et al., 2016; BRISKI & SALAMONE, 2022).

The *in vitro* embryo cryopreservation technique is a challenging sector of sheep embryo biotechnology. The progress in the sector has been relatively slow in the past few years (ZHU et al., 2018). The results are still inconsistent, unpredictable, and variable, presenting an essential limitation to its commercial application (PARAMIO & IZQUERDO, 2016). The

technique is still inefficient in sheep, with approximately 70-90% of prophase I oocytes maturing to metaphase II stage, 50-80% cleaved to at least 2-cells at 24-48h post insemination, and barely 20-50% of the prophase I oocytes attain to the blastocyst stage, on day 7-8 after fertilization (ZHU et al., 2018). More studies are needed in the field to improve the overall efficiency of ARTs and EIGC.

The AI technique involved the manual deposition of spermatozoa into the receptive female reproductive tract, accelerating the rate of genetic progress in a selection (MEDEIROS et al., 2002). Semen collection is considered the preliminary step in the AI program and is achieved in small ruminants, usually using either an artificial vagina (AV) or electroejaculation (EEJ) (LEDESMA et al., 2015). For gene conservation or ARTs studies, an excellent alternative is to retrieve the spermatozoa from the cauda epididymis (CE) of slaughtered, castrated, or accidentally dead elite sires (MUJITABA et al., 2022). They can be retrieved differently depending on the species involved or epididymal size. The most used epididymal spermatozoa (EPS) retrieval methods include;

a). Slicing/mincing (PARRA-FORERO et al., 2015)

b). Incision methods (KARJA et al., 2010; AHMED, 2019).

c). Retrograde flushing via ductus deferens *(less contamination)* (BERTOL, 2016) and

d). Floatation *(in species with tiny testicles)* (BERTOL, 2016).

Good pregnancy rates have been reported using EPS in sheep: 87.5% and 58.5% (EHLING et al., 2006; RICKARD et al., 2014), goats: 61.2% (OCAMPO et al., 2021), stallions: 27.8% and 64.0% (HEISE et al., 2010; MIRÓ et al., 2020), and 75.0% in red deer (SOLER et al., 2003). Therefore, it can be used to conserve the genetic resources (GnR) of elite sires.

A semen extender is a solution/diluent that nourishes and protects the sperm cells from injury during cooling and freezing (REHMAN et al., 2013). It is one of the factors that affect the fertility of cervical insemination (ALVAREZ et al., 2012b). It has been reported by some researchers to influence the quality of the post-thaw EPS in sheep greatly (ALVAREZ et al., 2012a; LONE et al., 2012) and alpaca (MAMANI-MANGO et al., 2019). Extenders can be conventional *(tris-citric egg yolk)* or commercially prepared and classified based on their origin/composition. They include egg yolk-based (Biladyl®, Botu-Bov® BullXcell®, and Triladyl®), milk-based (INRA96®), soy lecithin-based (Andromed®, Bioxcell®, Biociphos Plus®, Botu-Bov®-soy lecithin, and OviXcell®), and protein-free (OptiXcell®) (KHATUN et al., 2021; AIRES et al., 2003; RASTEGARNIA et al., 2014; ABDUSSAMAD et al. 2016; MURPHY et al., 2017). Different studies were conducted on the effects of commercially prepared semen extenders on the freezeability of spermatozoa of bull (ABDUSSAMAD et al.,

2016; BRAGA et al., 2007) (AIRES et al., 2003; CRESPILHO et al., 2012), buffalo (MEENA et al., 2010); goat buck (JIMÉNEZ-RABADÁN et al., 2012); and rams (GIL et al., 2003; FUKUI et al., 2008; KULAKSIZ et al., 2012).

In recent years, researchers highlighted the problems associated with egg yolk-based extenders, including microbial contamination risk and the wide variability of its components, reduced post-thaw viability, and sperm acrosomal integrity due to endotoxin production (AKÇAY et al., 2012; KULAKSIZ et al., 2012). Alternatively, a plant-based cold shock protector, soy-lecithin, can be used. Several studies have been conducted on different semen extender's effects on the freezability of AV-collected ram spermatozoa (ARI et al., 2011; BOHLOOLI et al., 2012; FERNANDES et al., 2021). However, this wasn't the case with REPS; little had been done, particularly on the soy-lecithin-based commercially available semen extenders (ÁLVAREZ et al., 2012; LONE et al., 2012). Most of the studies conducted on the REPS were related to the effects of collection methods (LONE et al., 2011), handling/storage conditions or transportation temperature (KAABI et al., 2003; TAMAYO-CANUL et al., 2011; LONE et al., 2012), washing (AHMED et al., 2016), and egg yolk-based extenders (ÁLVAREZ et al., 2012; LONE et al., 2012; AHMED et al., 2019) on its post-thaw quality characteristics. Little has been explored about the ideal commercial soy-lecithin-based semen extenders and spermatozoa concentration for freezing REPS.

Dilution rate or sperm freezing concentration is another exciting factor worth investigating on REPS freezeability. Some researchers have reported it to affect the quality/success of AV-collected cryopreserved spermatozoa in sheep (AKÇAY et al., 2012; D'ALESSANDRO et al., 2001; LEAHY et al., 2010). The lower concentration (200×10^6 spermatozoa/ml) resulted in better post-thaw quality parameters than the higher doses (400×10^6 or 800×10^6 spermatozoa/ml) (AKÇAY et al., 2012; NASCIMENTO et al., 2008). However, extreme dilution was found to affect the membrane integrity of ram spermatozoa negatively and cause a capacitation-like changes (MAXWELL & JOHNSON, 1999) and cryopreservation has an additive effect damaging the cells (LEAHY et al., 2010). Moreover, for a successful artificial insemination (AI) program, the technique employed in depositing spermatozoa into the receptive female reproductive tract determines the dilution rate (D'ALESSANDRO et al., 2001). To the best of our knowledge, there are fewer studies on the ideal spermatozoa concentration of REPS that leads to less detrimental effects on its post-thaw quality.

Hence, the current study intends to assess the effects of EPS collection methods, ram breed, three (3) different commercial soy-lecithin-based semen extenders, and two spermatozoa concentrations on the freezability of REPS. Finally, it will study the developmental kinetics of

in vitro-produced sheep embryos fertilized with post-thaw REPS to enhance the conservation of local sheep breed GnR.

1.1. Research Aims

The study aimed to assess the effect of four different factors (EPS collection methods, ram breed, commercially available soy-lecithin-based semen extenders, and two spermatozoa concentrations) on the quality of pre-freeze and post-thaw REPS and to finally assess the developmental kinetics of sheep *in vitro* produced sheep embryos fertilized with the post-thaw REPS using ivf-Bioscience bovine media.

1.2. Specific objectives

The study attempts to assess the effects of;

1. epididymal sperm collection methods *(Slicing vs Incision)* and ram breed *(German Mutton Merino and Hungarian Black Racka)* on the motility and kinematic parameters and compare the cryo-tolerance of the earlier-mentioned breeds' REPS.
2. three (3) different commercial soy-lecithin-based semen extenders (AdroMed®, BioXcell®, and OviXcell®) and two spermatozoa concentrations (200×10^6/ml vs 400×10^6/ml) on freezability of REPS.
3. the developmental kinetics of *in vitro*-produced sheep embryos fertilized with post-thaw REPS.

2. MATERIALS AND METHODS

2.1 Effect of epididymal sperm collection methods *(Slicing vs Incision)* and ram breed on the motility and kinematic parameters of fresh and post-thaw ram epididymal spermatozoa.

2.1.1 Study location and duration

The experiments were conducted at the Department of Precision Livestock Farming and Animal Biotechnics spermatology laboratory, Institute of Animal Sciences, Kaposvár Campus, Hungarian University of Agriculture and Life Sciences, Herceghalom, Hungary, between February 2022 and March 2023.

2.1.2 Media, reagents, and materials

Andromed® semen extender *(one-step, 200 ml, Minitube, Tiefenbach, Germany),* 0.25 ml transparent semen straws (*Minitube, Tiefenbach, Germany*) and PBS tablets *(Gibco, Lot:2565974)* were used for the experiment. The extender was reconstituted according to the manufacturer's guidelines, filled into sterilized 10 ml centrifuge tubes, and stored at frozen condition until required. All other plastic wares were purchased from Falcon® *(Corning, Inc., USA).*

2.1.3 Testicles collection

Fourteen pairs of testes from 2-5 years old healthy {(free from reproductive disorders and with the required European Union Animal Health Certificate (2016 EC regulations, article 144)} German Mutton Merino (Merino) /7 rams/ weighing 80-100 kg and Hungarian Racka Black variants (Racka) /7 rams/ weighing 55-60 kg were collected during the non-breeding season from a slaughterhouse in Hungary. They were transported to the laboratory in an icepack (4-5 ^{o}C) in the scrotum within 2-3 h, stored overnight in a refrigerator (4-5 ^{o}C) to simulate field conditions, and processed the following day as described by EGERSZEGI et al. (2012).

2.1.4 Epididymal sperm collection methods

After removing the scrotal sac and lamina parietalis of tunica vaginalis, the testis with epididymis was weighed using a digital weighing scale. Each epididymis was carefully separated, and spermatozoa of both cauda epididymides (CE) of the same ram were retrieved randomly by either the *slicing* or *incising* method.

2.1.4.1 The slicing method

The visceral layer of tunica vaginalis covering the CE is carefully removed to avoid blood contamination. The stripped CE was washed with PBS solution *(prepared in the lab using PBS tablets: Gibco, Lot:2565974)*, then cut out and sliced with a scalpel in a Petri dish containing 3 ml of Andromed® semen extender. The sliced CE was allowed in the extender at room temperature for 10 minutes to enhance spermatozoa collection. It was then rinsed with 2 ml of the semen extender and filtered with gauze sheets *(Figure. 1)*.

2.1.4.2 The incision method

We adopted the AHMED et al. (2019) procedure with little modifications. We used an Andromed® semen extender to retrieve spermatozoa, and a hemostatic forceps was used to engorge the CE to facilitate emptying the spermatozoa. After that, a single deep longitudinal incision at the ventral part of the CE with fewer blood vessels and 3-4 parallel incisions in the inner part was made using a sterile scalpel. It was then pressed against the bottom of the petri dish containing 3 ml of Andromed® semen extender to aid in emptying the spermatozoa and then rinsed with 2 ml of the same extender. Finally, a sterile gauze was used to sieve the epididymal tissue debris (*Figure* 1).

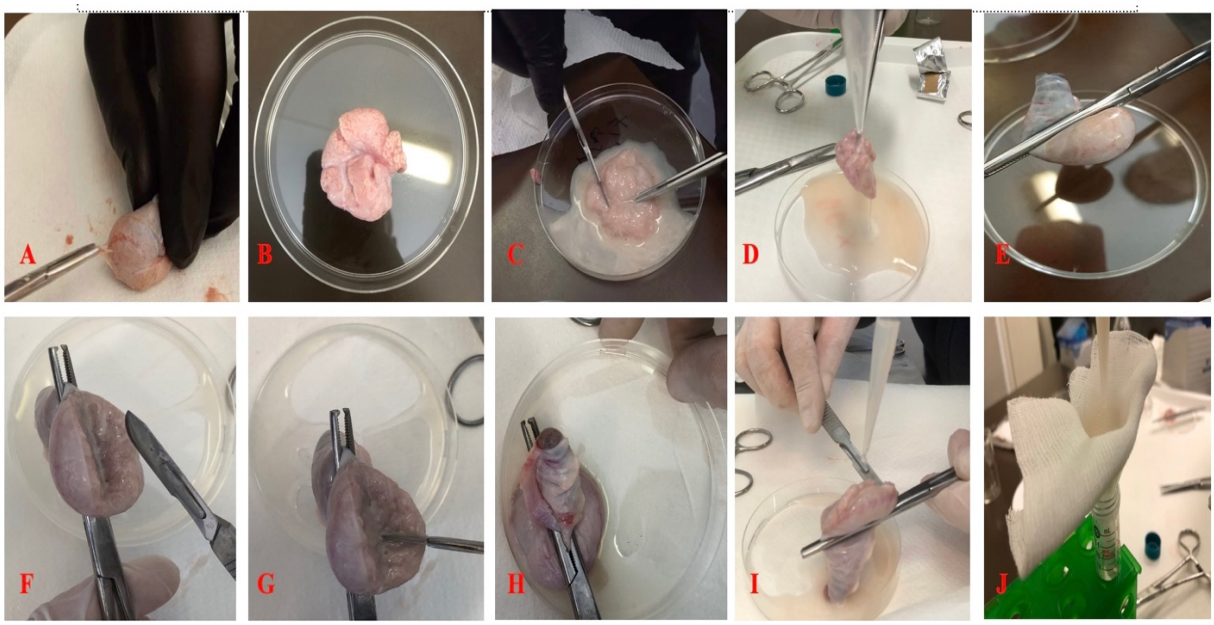


Figure 1. Epididymal sperm collection by slicing (A, B, C, D, and J) and incision (E, F, G, H, I, and J) methods. A: Striping the CE for slicing, B: Striped CE ready for slicing, C: Slicing the stripped CE, D: Rinsing the slice CE, E: Engorged CE ready for incision, F: Incising the engorged CE by deep vertical incision, G: Small horizontal incisions, H: Pressing the incised CE against the bottom of the Petri dish to aid in emptying the CE spermatozoa, I: Rinsing the incised CE, H: Filtering the retrieved CE spermatozoa.

Source: Lab work (2022).

2.1.5 Epididymal sperm dilution, equilibration, freezing and motility assessment

Sperm concentration was assessed with a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) using a phase contrast microscope at 200× magnification. Upon establishing the concentration, each sample was extended using an Andromed® at room temperature to a final concentration of 200×10^6 spermatozoa/ml and manually filled and sealed using PVA into well-labelled 0.25 ml French Mini straws.

The filled and sealed straws were equilibrated in a refrigerator (5 °C for 2 h), and freezing was done manually in a Styrofoam box at 4 cm above the liquid nitrogen (LN_2) for 8 min. Finally, the frozen straws were plunged into the LN_2 for permanent storage. After about 2 weeks, the frozen samples were thawed *(37 °C for 30 s).* The motility and kinetic parameters of the pre-freeze and frozen-thawed spermatozoa were assessed using a Computer-Assisted Sperm Analyzer (CASA) *(Androvision® software, Minitübe Ltd).* The samples were diluted to 50-60×10^6 spermatozoa/ml concentration using the same extender. At least 10 random fields per sample or a total of 500 spermatozoa were analyzed for standard motility {Total motility (TM%), Progressive motility (PM%)}, and Kinematic parameters: Curvilinear velocity (VCL, μm/s), Average path velocity (VAP, μm/s), Straight line velocity (VSL μm/s), Linearity (LIN = VSL/VCL×100%), Straightness (STR = VSL/VAP×100%), Beat cross frequency (BCF, Hz), Wobble (WOB = VAP/VCL×100%) and Amplitude of lateral head displacement (ALH, μm), as described by GOOVAERTS et al. (2006) and KANG et al. (2018).

2.2 Effects of three different commercial plant-based semen extenders and two spermatozoa concentrations on freezeability of ram epididymal spermatozoa

2.2.1 Study location and duration

The experiment was conducted at the Department of Precision Livestock Farming and Animal Biotechnics spermatology laboratory, Institute of Animal Sciences, Kaposvár Campus, Hungarian University of Agriculture and Life Sciences, Herceghalom, Hungary, between November 2022 and March 2023. The analyses and microscopical evaluations were finished later.

2.2.2 Media, reagents, and materials

Three (3) different commercial soy-lecithin-based semen extenders, AdroMed® (AND®) *(13503/1200 CSS one-step, 200 ml)*, BioXcell® (BIO®) *(016218 Easy to use, 250 ml)*, and OviXcell® (OVI®) *(020997 ready-to-use extender, 100 ml)* were purchased from Minitube

Ltd (Tiefenbach, Germany), and IMV technologies, (L'Aigle France), see the extenders composition in *Table 1.* The AND® and BIO® extenders were reconstituted according to the manufacturer's guidelines, filled into sterilized 10 ml centrifuge tubes, and stored at frozen condition until required. All other plastic wares were purchased from Falcons® *(Corning Inc., USA)*, while 0.25 ml transparent semen straws were purchased from IMV Technologies, L'Aigle, France.

Table 1.

Compositions of the three commercial soy-lecithin-based semen extenders

S/N	Andromed (100ml)	BioXcell (100ml)	OviXcell (100ml)
1	Phospholipids	Glycine (0.2 g/L)	Amino-acid
2	TRIS	Tris (2.3 g/L)	Salts
3	Citric acid	Monohydrate citric acid (2.5 g/L) Sodium Citrate (6.2 g/L) Potassium chloride (0.8 g/L)	Buffers and salt
4	Sugars	Fructose (1.2 g/L) Monohydrate lactose (0.8 g/L) Anhydrous glucose (0.5 g/L) Hydrate of calcium lactate (0.7 g/L)	Sugars
5	Antioxidants	Taurine (0.005 g/L)	Taurine
6	Glycerol (6.7%)	Glycerol (7.0%/40.2 g/L)	Glycerol
7	Tylosin (5.0 mg) Gentamycin (28.6 mg) Spectinomycin (30.0 mg) Lincomycin (15.0 mg).	Tylosin tartarate (0.33 g/L) Gentamycin sulphate (0.24 g/L) Spectinomycin, Lincospectin 100 (0.385 g/L)	Tylosin tartarate Gentamycin Spectinomycin sulfate (<0.2%) Lincomycin hydrochloride
8	Soy-lecithin	Soy-lecithin (1.5 g/L)	Soy-lecithin
9	Ultrapure water	Ultrapure water (1000 ml)	Ultrapure water

Sources: Extenders leaflets; (PENITENTE-FILHO et al., 2017).

2.2.3 Testicles collection

Nine (9) pairs of intact testes were collected during and outside the breeding season from nine adult healthy rams {(free from reproductive disorders and with the required European Union Animal Health Certificate (2016 EC regulations, article 144)} of different breeds: Merino (4), Racka (3), and Dorper (2) from a slaughterhouse in Hungary between November 2022 and March 2023. They were transported to the laboratory in a cold box within 2 h and processed within 24 hours to simulate field conditions as described by EGERSZEGI, et al. (2012).

2.2.4 Epididymal sperm collection

The testes were weighed using a digital weighing scale, and the spermatozoa were retrieved by slicing as in the first experiment with little modifications. The stripped CE was washed with PBS solution, then sliced with a scalpel in a Petri dish containing 3 ml of Tris-citric acid fructose buffer solution {Tris (Hydroxyl methylamino methane): 3.028 g, Citric acid

monohydrate: 1.70 g, Fructose: 1.25 g, and distilled water add to 100 ml)} as described by AHMED et al. (2016). The sliced CE was allowed in the Tris buffer solution for 10 minutes to enhance spermatozoa collection, rinsed with 2 ml of the Tris buffer and filtered with gauze sheets. The final volume was recorded. The Tris buffer solution was added to each sample from each CE, making an equal volume of 10 ml and centrifuged at 880 g for 10 minutes instead of 885 g for 10 minutes, as reported by AHMED et al. (2016). Finally, the supernatant was removed, and the pellets from each ram with a good mass motility score of 4-5 were mixed.

2.2.5 Sample quality assessment

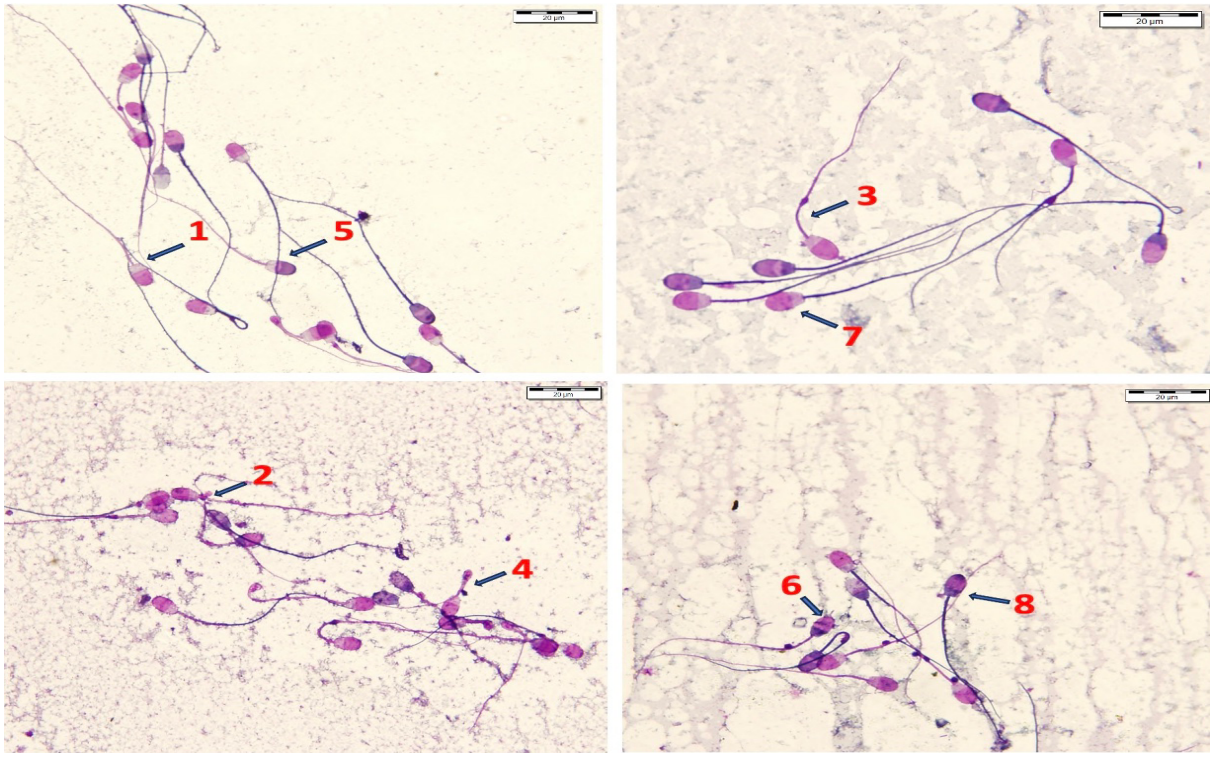
Spermatozoa concentration, standard motility and kinematic parameters were determined as described earlier. Spermatozoa viability, membrane integrity and morphological assessment were achieved using the Kovacs-Foote staining technique (K-F).

i. Standard motility and kinematic parameters assessment

Standard motility and kinematic parameters were assessed as described in experiment one.

ii. Viability and morphology assessment

Acrosome, head and tail membrane integrity and morphology and acrosome, head and tail membrane integrity of spermatozoa were evaluated by a modified Kovács-Foote staining method using 0.16% Chicago sky blue 6B (Sigma-Aldrich, St. Louis, MO, C-8679) viability stain, neutral red (Sigma N 2880), formaldehyde fixation and Giemsa solution (Sigma GS-500) acrosome staining as described by KOVÁCS & FOOTE (1992) and KÚTVÖLGYI et al. (2006). Slides were evaluated using an oil immersion objective with bright field microscopy at ×1000 magnification with a yellow filter for better live/dead differentiation, as described by KÚTVÖLGYI et al. (2006). A total of three hundred (300) cells were counted on each slide and classified into eight categories: Intact head, intact tail, acrosome membrane, normal morphology (IHITIA), Intact with a proximal cytoplasmic droplet (IPD), Intact with a distal cytoplasmic droplet (IDD), Intact with a tail defect (bent, broken, hairpin curved, coiled tail) (IBT), Intact head, tail, damaged acrosome (IHITDA), Damaged head with intact tail (DHIT), Intact head with damaged tail (IHDT) and Damaged head, damaged tail, damaged acrosome (DHDTDA), as described by KÚTVÖLGYI et al. (2006). Different spermatozoa categories are shown in *Figure 2*. In addition, all distal cytoplasmic droplets and all bent, hairpin-curved tails were counted regardless of intact or damaged membranes, per cent, all intact (IHITIA+IPD+IDD+IBT), all intact heads (IHITIA+IPD+IDD+IBT+IHITDA+IHDT) and all intact tails (IHITIA+IPD+IDD+IBT+IHITDA+DHIT) were also calculated. Values obtained for each category were presented in percentages.



1: IHITIA, 2: IPD, 3: IDD, 4: IBT, 5: IHITDA, 6: DHIT, 7: IHDT 8: DHDTDA
Intact head, intact tail, and acrosome membrane (Intact: IHITIA), Intact with a proximal droplet (IPD), Intact with distal droplets (IDD), Intact with a bent tail (IBT), Intact head, tail, damaged acrosome (IHITDA), Damaged head with intact tail (DHIT), Intact head with damaged tail (IHDT) and Damaged head, damaged tail, damaged acrosome (DHDTDA).
Figure 2. The different post-thaw ram epididymal spermatozoa categories (Evaluated using a modified Kovacs-Foote staining technique, magnification ×1000, using a light microscope with an oil immersion objective).
Source: Lab work (2023).

2.2.6 Sample dilution, equilibration, freezing and thawing

Samples were checked for concentration with a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) using a phase contrast microscope at ×200 magnification. Part of the sample was taken and divided into three aliquots, and each of the aliquots was diluted with one of the commercial semen extenders to a concentration of 400×10^6 spermatozoa/ml at room temperature to give AND®400, BIO®400, and OVI®400. Part of each extended sample was aliquoted again and further diluted with the corresponding extender to a final concentration of 200×10^6 spermatozoa/ml giving, AND®200, BIO®200, and OVI®200. The extended samples were manually filled and sealed using a Polyvinyl alcohol (PVA) into well-labelled and colour-coded French Mini straws.

Freezing of REPS was conducted in a similar way as the conventional AV-collected spermatozoa freezing. The filled and sealed straws were equilibrated in a refrigerator (5 °C for 2 h). Freezing was done manually in a Styrofoam box at 4 cm above the liquid nitrogen (LN_2) for 8 min. Finally, the frozen straws were plunged into the LN_2 for permanent storage. After about 2 weeks, the frozen samples were thawed *(37 °C for 30 s)* and assessed for standard

motility and kinematic parameters and smears were prepared for membrane integrity and morphology evaluation, see *Figure 3*.

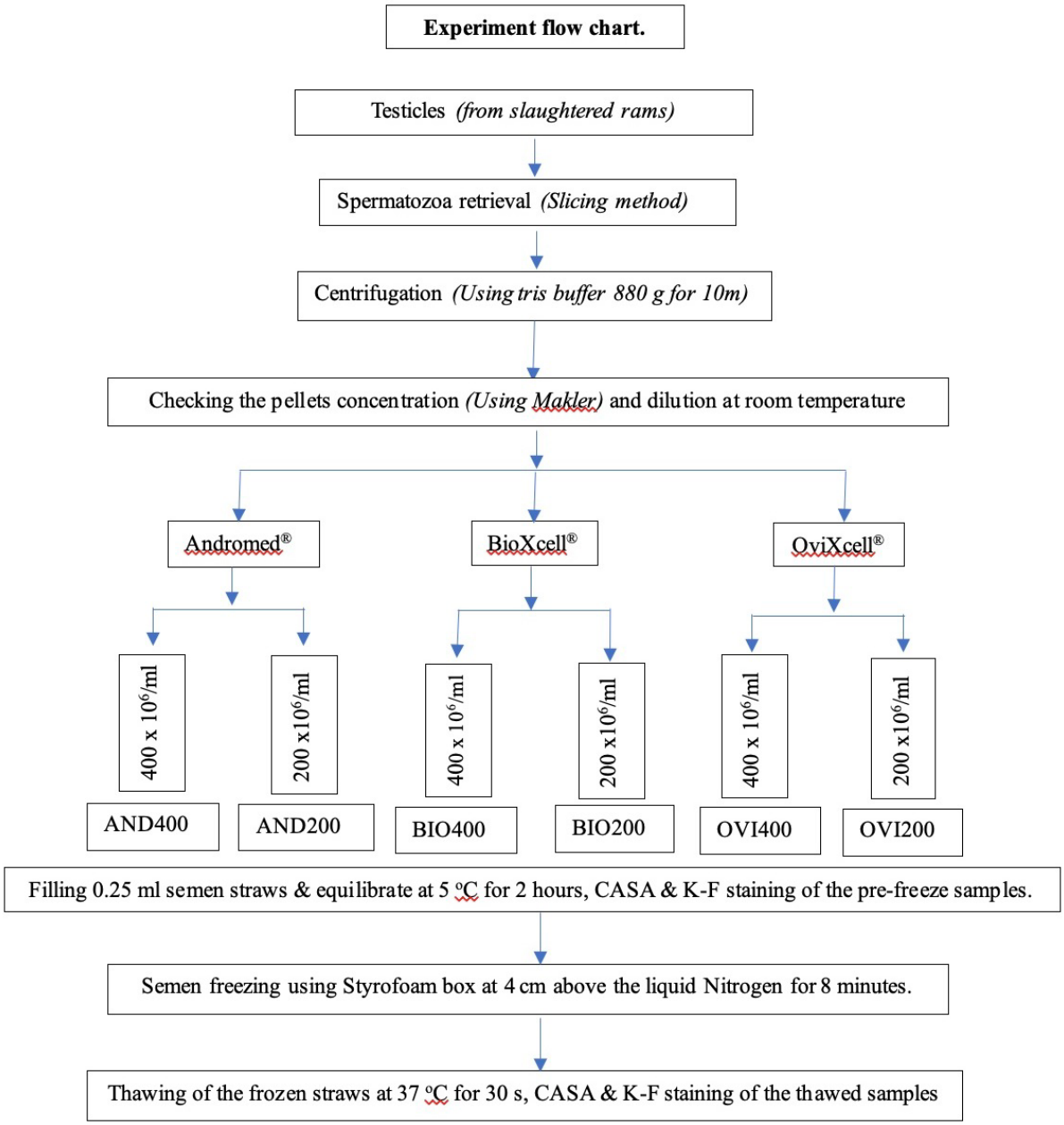


Figure 3. Flow chart of the experiment.

2.3 Effects of using frozen-thawed ram epididymal spermatozoa on the developmental kinetics of *in vitro*-produced sheep embryos.

2.3.1 Study location and duration

The experiment was conducted at the Department of Precision Livestock Farming and Animal Biotechnics Embryology Laboratory, Institute of Animal Sciences, Kaposvár Campus, Hungarian University of Agriculture and Life Sciences, Herceghalom, Hungary, between February 2022 and July 2022.

2.3.2 Materials and Media

Materials and media used for this experiment include a laminar flow hood, incubator, centrifugal machine, stereomicroscope, benchtop heating block, pipette sets, 4-Well plate (4-WP), sterile filter, syringe, injection needle, eye dropper/Pasteur pipette burner, racks, Petri dishes, capillary tubes, 70% ethanol, Phosphate-Buffered Saline (PBS), Fetal Calf Serum (FCS), antibiotics, sterile surgical blade, sterile hand globes, ovum pick up (OPU) medium, washing medium (Wash), bovine *in vitro* maturation medium (BO-IVM), bovine *in vitro* culture medium (BO-IVC), Bovine semen preparation medium (BO-SemenPrep), oil, bovine *in vitro* fertilization medium (BO-IVF). The media were purchased from ivf Bioscience (United Kingdom), and *in vitro* embryo production procedures were conducted according to the manual guide of ivf Biosciences' commercial protocol for on-site OPU.

2.3.3 Sourcing of ovaries and cumulus-oocyte complexes (COC-s) retrieval

Five (5) programs consisting of 216 cumulus-oocyte complexes (COC's) were conducted. Ovaries were sourced from the slaughterhouse. Upon collection, the excised ovaries were rinsed using a PBS solution, maintained at 37 ^{o}C using an incubator and transported to the laboratory within 2-3 h of collection. Extra tissues were trimmed using scissors, and the ovaries were washed again in a beaker containing pre-warmed PBS solution. The ovaries were then transferred into a 60 mm petri dish containing a sterile filtered Dulbecco's phosphate-buffered saline (PBS) solution supplemented with 10% FBS (PBS+10% FBS) maintained at 37 ^{o}C using a benchtop heating block. The COC's were retrieved by the aspiration and slicing methods with the aid of a syringe pitted with 16 G needle and a surgical sterile scalpel handle blade. The retrieved COC's were then held in PBS+10% FBS at 37 ^{o}C for a short period before the maturation process began. They were rinsed in a 33 mm petri dish containing 2 ml of preheated (37 ^{o}C) washing medium, and only grade A and B COC's were selected for maturation. The selected ones were rinsed in CO_2-equilibrated BO-IVM media maintained at 37 ^{o}C. The average number of selected COC's retrieved per ovary was calculated and presented in *Table 2*

Table 2.

Average number of cumulus oocytes complex retrieved per ovary

Programs	Ovary number	Cumulus oocyte complex
M01	8	30
M02	7	43
M03	7	45
M04	11	51
M05	13	47
Total	**46**	**216**
Average number of COC's retrieved/ovary	**4.7**	

2.3.4 *In vitro* maturation of COC's

The COC's were washed in a pre-heated washing medium and matured in a group (25-50 oocytes/group with a ratio of 1 oocyte/2-5 µl medium) using 4WP containing 500 µl of CO_2 equilibrated BO-IVM. They were then incubated in an incubator maintained at 38.5°C in humidified air (21% O_2) and 5% CO_2 for 22-24 h. At the end of the maturation process, the matured oocytes were then prepared for IVF.

2.3.5 *In vitro* fertilization

Frozen-thawed REPS were washed by double centrifugation at 300 g for 5 m using BO-SemenPrep media. Sperm concentration was determined using a Makler counting chamber, and the required volume was calculated according to Bovine ivf commercial protocol for on-site OPU. Matured COC's with expanded layers of cumulus cells and uniform cytoplasm were graded and selected for the IVF described by WANI et al. (2013). The matured COC's and the prepared REPS pellets were co-incubated for 18 h in an overnight equilibrated 50 µl bovine *in vitro* fertilization medium droplet (1.0×10^5 motile spermatozoa/drop) with an oil overlay with the incubator set as described earlier.

2.3.6 *In vitro* culture

Presumptive zygotes were denuded of cumulus cells mechanically with the aid of a denudation pipette and cultured in an overnight CO_2 equilibrated 50 µl drop of oil overlayed bovine *in vitro* BO-IVC medium with the incubator set at 38.5 ^oC, 5.6% CO_2, 6.0% O_2 in a humidified air (98% RH). The resulting embryos' developmental competence was assessed by checking the cleavage rate every 24 h post-fertilization and the blastocyst rate (days 6 and 8) post-fertilization as described by WU et al. (2007).

2.3.7 Assessing the developmental competence of the *in vitro*-produced sheep embryos.

Development competence/cleavage was assessed every 24 h post-fertilization, and embryo pictures were taken daily with a microscope-pitted camera to evaluate the developmental kinetics until day 7 (Figure. 4). The proportion of zygotes that developed and cleaved to the blastocysts was recorded and expressed in percentage accordingly.

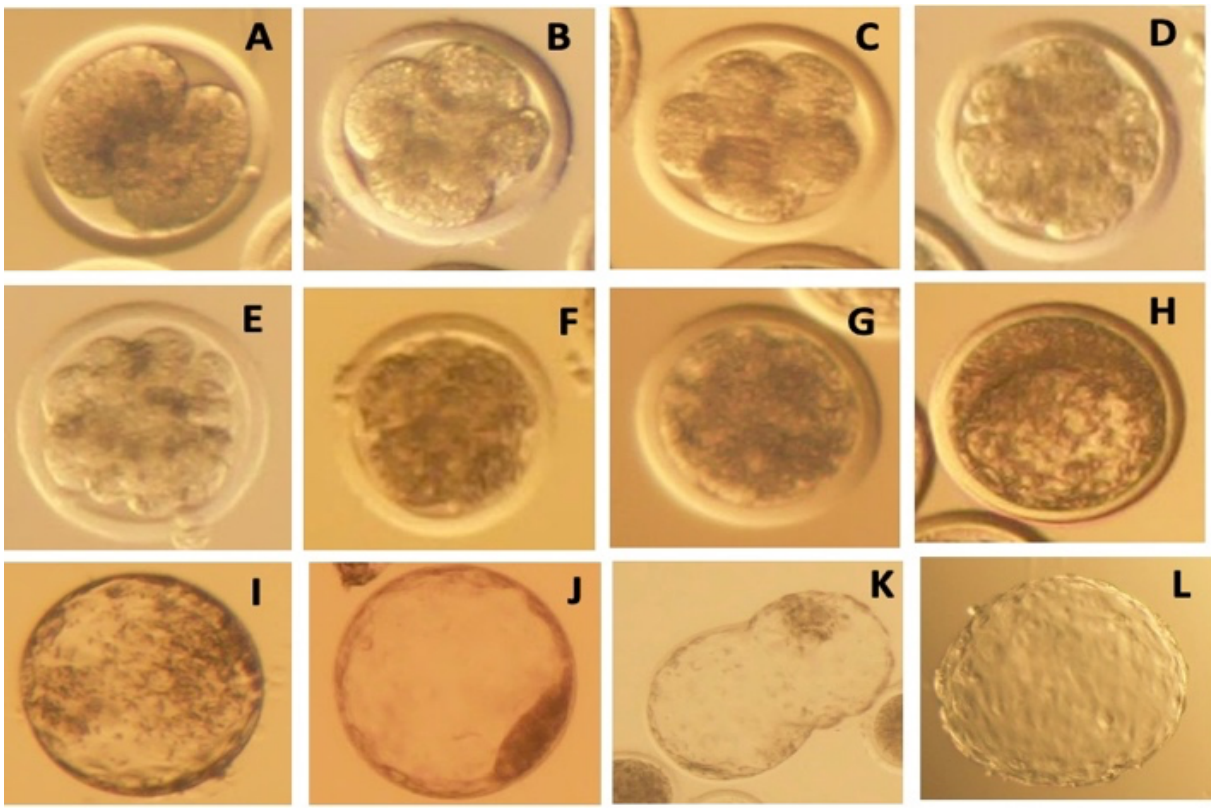


Figure 4. Developmental stages of *in vitro* produced sheep embryos. A: 2-cell, B: 4-cells, C: 6-to 8-cells, D: 10-to 16-cells, E: 16-to 32-cells (Early morula), F: Morula, G: Compacted morula, H: Blastocyst, I: Expanding Blastocyst, J: Expanded Blastocyst, K: Hatching Blastocyst, L: Hatched Blastocyst.

The developmental kinetics of the embryos were calculated using ivf-BioScicence formulae. Total Blastocyst (T) was calculated using NBL + NXB + NH - - - - equation 1. Where: T= Total blastocyst; K= Developmental kinetics; NBL= Number of blastocysts; NXB= Number of expanded blastocysts; NH=Number of hatched blastocysts.

Source: Lab work (2022).

2.4 Statistical analysis

Data from fresh and frozen-thawed samples were collected and recorded. The data were tested for normality using Shapiro-Wilk and were normally distributed. A two-way Analysis of Variance was used to check the effects of collection methods and breed *(Pooled the slicing and*

incision methods data) under the two conditions *(Fresh and Post-thaw)* separately, with the level of the significant set at $p<0.05$.

The model for the experiment was $Y_j = a+bx_1+cx_2+ x_1*x_2$

Where:
Y = dependant variable (e.g TM)
a = intercept
x_1 = Breed (Merino or Racka)
x_2 = Method (SL or IN).

Effects of freezing (fresh vs. post-thaw) within the breed were analyzed using a paired t-test. In contrast, the cryo-tolerance of the two breeds was calculated using the following formulae CT= Values after thawing/Values before freezing×100% as described by Ehling et al. (2006). The differences between breeds (Merino vs. Racka) were analyzed using an independent sample t-test, and the significance was checked using a two-tail test. Results were presented as means±standard error of means (SE).

For the extender experiment, we did not consider the breed effect because of the wide variations between them, and the sole aim was to identify the ideal concentration, extender, and their most suitable interactions for freezing REPS regardless of the breed to enhance gene conservation. The data were tested for normality using Shapiro-Wilk, and normality was achieved using a Two-step transformation method. Pre-freeze, post-thaw, and Kovacs-Foote-stained REPS data were collected, recorded, and analyzed for descriptive statistics. A general linear model using two-way Analysis of Variance was used to analyze the extender and sperm concentration *(400×10^6 vs 200×10^6)* on the standard motility, kinematic parameters, and viability and morphological parameters with the level of the significant set at $p<0.05$. Means were separated using the Tukey post hoc test. The effects of freezing using different commercial soy-lecithin-based semen extenders and the overall effects of freezing and thawing on the percent all distal droplets and all bent tails categories were analyzed using a paired sample T-test, and the significance differences were checked using a two-tail test. Results were presented as means ± standard error of means (SE). The abovementioned methods were carried out using IBM® SPSS® statistical software version 29. The embryo developmental kinetics data were analyzed with Excel and presented in simple percentages.

3. RESULTS

3.1 Effect of epididymal sperm collection methods and breed on the motility and kinematic parameters of fresh and post-thaw ram epididymal spermatozoa

3.1.1 Effects of collection methods on the fresh and post-thaw ram epididymal sperm standard motility and kinematic parameters

Table 3. illustrates the effects of collection methods {Slicing (SL) vs Inscision (IN)} on fresh and post-thaw REPS. Testicular weight does not differ between the collection methods (SL vs IN; $p>0.05$). Similarly, the collection method did not have significant ($p>0.05$) effects on standard motility and all the kinematic parameters in the fresh and post-thaw REPS. There was no significant ($p>0.05$) breed and collection method interaction effect on all the parameters studied under fresh and post-thaw conditions.

Table 3.

Effects of collection methods on testicular weight and standard motility, and kinematic parameters of fresh and post-thaw ram epididymal spermatozoa (Mean±SE)

Parameters	Fresh		*p*-value	Post-thaw		*p*-value
	Collection methods			Collection methods		
	Slicing	Incision		Slicing	Incision	
TW (g)	213.45±16.9	217.82±16.3	0.789	-	-	-
TM (%)	82.07±2.5	80.07±2.8	0.598	45.83±5.4	36.67±4.9	0.324
PM (%)	73.00±2.9	68.93±3.4	0.376	31.58±5.2	25.08±4.9	0.504
VCL (μm/s)	132.61±7.8	129.09±9.8	0.781	102.73±5.2	106.01±5.9	0.776
VAP (μm/s)	66.24±3.3	63.39±4.1	0.591	50.41±2.4	51.33±2.9	0.880
VSL (m/s)	47.04±2.3	44.70±3.2	0.572	38.89±1.9	39.72±2.7	0.845
LIN (%)	35.57±1.2	34.29±1.0	0.368	37.42±0.8	36.67±0.8	0.587
STR (%)	70.86±1.6	69.43±1.4	0.483	76.50±1.0	76.42±1.4	0.913
WOB	49.93±0.6	48.86±1.1	0.389	44.75±3.7	48.08±0.4	0.463
BCF (Hz)	26.19±0.6	25.60±0.6	0.451	25.93±0.7	25.92±0.96	0.907
ALH (μm)	4.86±0.3	4.7±0.3	0.655	3.85±0.2	3.83±0.15	0.811

ALH: Amplitude of the lateral head displacement, BCF: Beat cross frequency, LIN: Linearity, PM: Progressive motility, SE: Standard error of mean; STR: Straightness; TM: Total motility, TW: Testicular weight, VAP: Average path velocity; VCL; Curvilinear velocity; VSL: Straight line velocity, WOB: Wobble.
Testicles Number: Slicing (n=14), Incision (n=14).

3.1.2 Effects of breed on testicular weight and fresh and post-thaw ram epididymal spermatozoa motility and kinematic parameters

Considering that there was no significant difference ($p>0.05$) between the collection methods on all the parameters, the data were pooled and analyzed to determine breed effects on testicular weight (TW; g), total (TM; %) and progressive motility (PM; %) and kinematic parameters of fresh and post-thaw REPS. The results were summarized and presented in *Table 4.* The Merino breed had significantly ($p<0.05$) higher TW than the Racka breed. In contrast, the Racka breed presented significantly ($p<0.05$) better specific kinematic parameters (LIN, STR, BCF and ALH) than the Merino breed. All other parameters were not significantly different ($p>0.05$) between the breeds.

The breed has no significant ($p>0.05$) effects on the post-thaw standard motility parameters and kinematic parameters except for LIN and BCF, which were significantly ($p<0.01$; $p<0.05$) higher in the Racka breed.

Table 4.

Effects breed on testicular weight and standard motility, and kinematic parameters of fresh and post-thaw ram epididymal spermatozoa (Means±SE)

Parameters	Fresh		*p*-values	Post-thaw		*p*-values
	Breed			Breed		
	Merino	Racka		Merino	Racka	
TW (g)	260.50±11.8[a]	170.82±10.1[b]	0.001	-	-	
TM (%)	83.64±2.2	78.50±2.8	0.182	44.20±6.5	39.14±4.5	0.498
PM (%)	73.00±3.1	68.93±3.3	0.376	30.00±6.9	27.14±3.8	0.702
VCL (μm/s)	140.13±8.5	121.58±8.6	0.152	104.76±4.7	104.09±5.9	0.936
VAP (μm/s)	68.39±3.3	61.24±3.8	0.186	50.05±2.0	51.45±2.9	0.728
VSL (m/s)	46.78±2.3	44.96±3.2	0.659	37.75±1.7	40.41±2.6	0.456
LIN (%)	33.2±1.0[b]	36.6±0.9[a]	0.022	35.40±0.7[b]	38.21±0.7[a]	0.008
STR (%)	67.9±1.6[b]	72.43±1.1[a]	0.032	74.90±1.3	77.57±1.1	0.127
WOB (%)	48.43±0.9	50.36±0.8	0.127	47.30±0.4	45.79±3.2	0.698
BCF (Hz)	24.86±0.5[b]	26.92±0.6[a]	0.013	24.43±0.7[b]	26.99±0.7[a]	0.027
ALH (μm)	5.22±0.2[b]	4.35±0.3[a]	0.022	4.10±0.2	3.66±0.1	0.064

ALH: Amplitude of the lateral head displacement; BCF: Beat cross frequency; LIN: Linearity, PM: Progressive motility, SE: Standard error of mean; STR: Straightness; TM: Total motility, TW: Testicular weight, VAP: Average path velocity; VCL; Curvilinear velocity; VSL: Straight line velocity, WOB: Wobble.
Means in the same row with different superscript within a form (fresh or post-thaw) differ significantly.
Testicles number: Merino (n=14), Racka (n=14).

3.1.3 Comparison of cryo-tolerance of Merino and Racka ram epididymal spermatozoa

Table 5 presents the effects of freezing on standard motility and kinematic parameters and compares the cryo-tolerance of Merino and Racka REPS. Freezing and thawing significantly ($p<0.05$) reduced the values of TM, PM, VCL, VAP, and ALH but significantly ($p>0.05$) increased the STR, with the WOB and BCF being the only parameters not significantly ($p>0.05$) affected in both breeds. The VSL of Merino significantly ($P<0.05$) declined following freezing and thawing, while Racka's did not. The Racka and the Merino breeds REPS have statistically the same cryo-tolerance ($p>0.05$).

Table 5.

Comparison of the cryotolerance of Merino and Racka breeds ram epididymal spermatozoa (Mean±SE)

Parameters	Merino		*p*-values	Black Racka		*p*-values	Cryo-tolerance		
	Fresh	Post-thaw		Fresh	Post-thaw		Merino CT (%)	Racka CT (%)	*p*-values
TM (%)	83.64±2.2[a]	44.20±6.5[b]	0.0001	78.50±2.8[a]	39.14±4.5[b]	0.0001	52.30±6.9	49.91±5.3	0.783
PM (%)	73.00±3.1[a]	30.00±6.9[b]	0.0001	68.93±3.3[a]	27.14±3.8[b]	0.0001	39.42±8.7	39.67±5.3	0.980
VCL(µm/s)	140.13±8.5[a]	104.76±4.7[b]	0.003	121.58±8.6[a]	104.09±5.9[b]	0.020	76.66±5.2	88.44±5.2	0.133
VAP(µm/s)	68.39±3.3[a]	50.05±2.0[b]	0.0001	61.24±3.8[a]	51.45±2.9[b]	0.004	73.60±4.5	85.75±4.2	0.067
VSL (m/s)	46.78±2.3[a]	37.75±1.7[b]	0.012	44.96±3.2	40.41±2.6	0.088	82.29±5.7	92.39±5.4	0.220
LIN (%)	33.2±1.0	35.40±0.7	0.247	36.6±0.9	38.21±0.7	0.074	108.76±6.1	104.82±2.3	0.505
STR (%)	67.9±1.6[a]	74.90±1.3[b]	0.039	72.43±1.1[a]	77.57±1.1[b]	0.001	113.22±5.5	107.33±1.8	0.261
WOB (%)	48.43±0.9	47.30±0.4	0.598	50.36±0.8	45.79±3.2	0.165	96.22±0.9	90.97±6.3	0.498
BCF (Hz)	24.86±0.5	24.43±0.7	0.740	26.92±0.6	26.99±0.7	0.915	100.45±2.7	100.49±2.5	0.792
ALH (µm)	5.22±0.2[a]	4.10±0.16[b]	0.005	4.35±0.3[a]	3.66±0.1[b]	0.001	79.13±4.9	86.12±3.4	0.241

ALH: Amplitude of the lateral head displacement; BCF: Beat cross frequency; CT: Cryo-tolerance, LIN: Linearity, PM: Progressive motility, SE: Standard error of mean; STR: Straightness; TM: Total motility, VAP: Average path velocity; VCL; Curvilinear velocity; VSL: Straight line velocity, WOB: Wobble.
Means in the same row with different superscripts within a breed[a,b] (Merino or Racka) differ significantly.
Testicles number: Merino (n=14), Racka (n=14)

3.2 Effects of three different commercial soy-lecithin-based semen extenders and two spermatozoa concentrations on freezeability of ram epididymal spermatozoa

3.2.1 General parameters of ram epididymal spermatozoa retrieved from different breeds

In the current study, we determined specific parameters related to the ram testicles and cauda epididymal (CE) weight and concentration of the spermatozoa retrieved from rams of three different breeds (*Table 6*).

Table 6.

General parameters of the ram epididymal spermatozoa retrieved from different breeds

Parameters	Range	Mean±SE
Testicular weight (g)	113.07-308.09	157.78±22.15
Cauda epididymal weight (g)	7.89-20.39	14.25±1.38
Spermatozoa concentration (10^6/ml)	5800-14240	9061.44±845.53

SE: Standard Error of the means, n=28.

3.2.2 Effects of three different commercial soy-lecithin-based semen extenders and two spermatozoa concentrations on standard motility and kinematic parameters of pre-freeze ram epididymal spermatozoa

The effects of three different commercial soy-lecithin-based semen extenders and two spermatozoa concentrations on pre-freeze REPS were presented in *Table 7.* There was no significant ($p>0.05$) interaction between the extender and the spermatozoa concentration on all the parameters studied, so we presented the main treatment effect. Similarly, the standard motility and all the kinematic parameters showed no significant ($p>0.05$) difference among the extenders and between the spermatozoa concentrations except for BCF. The BIO® and OVI® extenders had significantly ($p<0.05$) higher BCF (30.18±1.1 and 29.99±1.0 Hz) than the AND® extender (26.80±0.8 Hz).

Table 7.

Effects of three different commercial soy-lecithin-based semen extenders and two spermatozoa concentration on standard motility and kinematic parameters of pre-freeze ram epididymal spermatozoa

Extenders	Standard motility and kinematic parameters (Mean±SE)									
	TM (%)	PM (%)	VCL (μm/s)	VAP (μm/s)	VSL (μm/s)	LIN (%)	STR (%)	BCF (Hz)	WOB (%)	ALH (μm)
Adromed®	72.22±3.2	64.89±3.4	163.94±5.8	76.85±2.3	54.05±2.4	32.83±1.3	70.00±2.2	26.80±0.8[a]	46.61±0.5	5.55±0.2
BioXcell®	69.00±3.8	62.44±4.0	168.11±3.9	82.21±2.3	60.64±3.1	35.50±1.6	72.83±2.4	30.18±1.1[b]	48.44±0.6	5.21±0.2
OviXcell®	67.61±3.7	60.78±3.9	169.06±3.2	83.16±2.2	62.00±3.4	35.94±1.5	73.22±2.2	29.99±1.0[b]	48.56±0.7	5.27±0.1
P-value	0.633	0.727	0.863	0.336	0.215	0.267	0.463	0.020	0.080	0.695
Concentrations (10^6/ml)										
200	67.85±3.3	61.26±3.4	167.48±3.7	81.14±1.9	59.80±2.5	35.26±1.2	72.85±1.9	29.24±0.9	48.04±0.6	5.25±0.2
400	71.37±2.5	64.15±2.8	166.59±3.6	80.34±1.9	58.00±2.5	34.56±1.2	71.19±1.9	28.71±0.8	47.70±0.5	5.42±0.2
P-value	0.170	0.231	0.556	0.379	0.302	0.808	0.584	0.834	0.985	0.181
P-value Ext.*Conc.	0.619	0.643	0.852	0.744	0.659	0.887	0.840	0.854	0.946	0.712

ALH: Amplitude of the Lateral Head displacement; BCF: Beat Cross Frequency; Ext*Conc.: Extender*Concentration interaction effects, LIN, Linearity of movement; PM: Progressive motility, SE: Standard Error of mean; STR: Straightness; TM: Total motility, VAP: Average pathway velocity, VCL: Curvilinear velocity, VSL: Straight line velocity, n=9.
Means in the same column with different superscripts [a,b] differ significantly.

3.2.3 Effects of three different commercial soy-lecithin-based extenders and two spermatozoa concentrations on standard motility and kinematic parameters of post-thaw ram epididymal spermatozoa

Table 8 presents the effects of the three commercial soy-lecithin-based semen extenders and two spermatozoa concentrations on REPS's post-thaw standard motility and kinematic parameters. Like the pre-freeze condition, there was no significant ($p>0.05$) interaction between the extender and the spermatozoa concentration on all the parameters, so we presented the main effect of the extenders and the spermatozoa concentrations. The standard motility parameters of the post-thaw REPS were also not significantly ($p>0.05$) different among the extenders and between the spermatozoa concentrations. The BIO® and OVI® extenders had statistically the same VAP (77.78±3.2 and 80.48±3.1 μm/s) and BCF (32.81±1.1 and 32.46±1.0 Hz) and were significantly higher ($p<0.05$) than the AND® extender (67.72±3.5 μm/s and 28.27±1.0 Hz). Moreover, the OVI had significantly higher ($P<0.05$) per cent WOB than the AND® extender (50.56±0.8 vs 47.67±0.7%), while BIO® and OVI® were statistically the same (49.56±0.9 vs 50.56±0.8%). All other kinematic parameters were statistically the same ($p>0.05$) among the extenders and between the spermatozoa concentrations.

Table 8.

Effects of three different commercial soy-lecithin-based semen extenders and two spermatozoa concentrations on standard motility and kinematic parameters of post-thaw ram epididymal spermatozoa

Extenders	**Standard motility and kinematic parameters (Mean±SE)**									
	TM (%)	PM (%)	VCL (μm/s)	VAP (μm/s)	VSL (μm/s)	LIN (%)	STR (%)	BCF (Hz)	WOB (%)	ALH (μm)
Andromed®	34.89±3.9	27.11±3.4	139.55±6.3	67.72±3.5^{a}	50.58±3.3	35.72±1.4	74.06±2.3	28.72±0.9^{a}	47.67±0.7^{a}	4.41±0.2
BioXcell®	38.83±3.5	31.50±3.1	156.72±5.0	77.78±3.2^{b}	58.96±3.9	37.11±1.8	74.28±2.5	32.81±1.1^{b}	49.56±0.9^{ab}	4.42±0.2
OviXcell®	37.61±3.7	31.56±3.5	157.39±5.4	80.48±3.1^{b}	61.46±3.9	38.33±1.7	75.00±2.4	32.46±1.0^{b}	50.56±0.8^{b}	4.55±0.2
P-Value	0.893	0.509	0.191	0.024	0.154	0.554	0.816	0.012	0.044	0.849
Concentrations (10^6/ml)										
200	34.33±2.3	27.33±2.2	150.40±5.3	75.43±3.2	57.92±3.4	37.74±1.4	75.41±1.9	31.83±0.9	49.37±0.8	4.33±0.1
400	39.89±3.5	32.78±3.1	152.04±4.3	75.22±2.4	56.07±2.8	36.37±1.3	73.48±1.9	30.83±0.8	49.15±0.6	4.58±0.2
P-value	0.170	0.249	0.878	0.957	0.534	0.486	0.566	0.400	0.815	0.250
P-value Ext.*Conc.	0.723	0.946	0.648	0.855	0.913	0.976	0.959	0.827	0.882	0.927

ALH: Amplitude of the Lateral Head displacement; BCF: Beat Cross Frequency; Ext.*Conc.: Extender*Concentration interaction effects, LIN, Linearity of movement; PM: Progressive motility, SE: Standard Error of mean; STR: Straightness; TM: Total motility, VAP: Average pathway velocity, VCL: Curvilinear velocity, VSL: Straight line velocity, n=9.
Means in the same column with different superscripts^{a,b} differs significantly.

3.2.4 Effects of three different soy-lecithin-based commercial semen extenders and two spermatozoa concentrations on the post-thaw viability and morphological characteristics of ram epididymal spermatozoa

The effects of different soy-lecithin-based commercial semen extenders and the two spermatozoa concentrations on the post-thaw viability and morphological characteristics of REPS were presented in *Table 9.* There was no significant ($p>0.05$) interaction between the extender and the spermatozoa concentration. Similarly, neither the extender nor the spermatozoa concentration significantly affects the percentage of the post-thaw REPS with IHITIA. The AND® extender had significantly ($p<0.05$) lower percentage of REPS with intact with bent tails, all intact heads, and all bent tails categories (2.56±0.6, 34.64±3.2 and 9.74±1.4%) than the BIO® (8.14±1.5, 45.33±3.3, and18.33±2.4%) and OVI® (7.19±1.3, 44.68±2.9, and 17.39±1.7%) extenders, respectively. In contrast, BIO® and OVI® extenders had significantly ($p<0.05$) lower categories of REPS with damaged head intact tail (2.91±0.7 and 2.53±0.4%) than the AND® (6.31±1.1%). The 400×10^6 spermatozoa/ml concentration resulted in a significantly ($p<0.05$) higher percentage of all intact head categories than the 200×10^6 spermatozoa/ml (45.15±5.1 vs 37.95±3.4%).

Table 9.

Effects of three different commercial soy-lecithin-based semen extenders and two spermatozoa concentrations on viability and morphological parameters of post-thaw ram epididymal spermatozoa

Extenders	Viability and morphometric parameters (Mean±SE)												
	IHITIA (%)	IPD (%)	IDD (%)	IBT (%)	IHITDA (%)	DHIT (%)	IHDT (%)	DHDTDA (%)	All intact (%)	All intact head (%)	All intact tail (%)	All distal droplets (%)	All bent tials (%)
Andromed®	5.92±1.2	0.87±0.3	9.72±1.4	2.56±0.6^{a}	0.04±0.0	6.31±1.1^{a}	15.52±1.8	59.06±3.4	19.08±2.2	34.64±3.2^{a}	25.42±2.9	28.44±2.9	9.74±1.4^{a}
BioXcell®	6.55±1.1	0.91±0.2	9.44±1.4	8.14±1.5^{b}	0.03±0.0	2.91±0.7^{b}	20.27±2.5	51.73±3.3	25.03±1.5	45.33±3.3^{b}	27.97±1.9	21.89±2.7	18.33±2.4^{b}
OviXcell®	7.46±1.3	0.68±0.2	9.33±1.5	7.19±1.3^{b}	0.02±0.0	2.53±0.4^{b}	20.00±1.8	52.79±3.0	24.66±2.4	44.68±2.9^{b}	27.21±2.6	20.33±2.4	17.39±1.7^{b}
P-Value	**0.658**	**0.736**	**0.981**	**0.005**	**0.843**	**0.017**	**0.193**	**0.242**	**0.094**	**0.030**	**0.771**	**0.100**	**0.003**
Concentration (10^6/ml)													
200	5.43±1.3	0.70±0.3	9.03±2.1	6.39±1.9	0.04±0.0	4.83±0.8	16.36±2.2	57.23±4.1	21.55±2.8	37.95±3.4^{A}	26.41±3.7	24.15±4.1	16.81±2.9
400	7.86±1.9	0.94±0.3	9.96±2.1	5.53±1.1	0.02±0.0	3.00±0.9	20.83±4.0	51.83±5.1	24.29±2.9	45.15±5.1^{B}	27.32±3.4	22.96±3.7	13.49±2.1
P-Value	**0.083**	**0.339**	**0.587**	**0.542**	**0.648**	**0.115**	**0.064**	**0.160**	**0.267**	**0.049**	**0.760**	**0.713**	**0.128**
P-Value Ext.*Conc.	**0.634**	**0.968**	**0.946**	**0.978**	**0.361**	**0.351**	**0.692**	**0.918**	**0.750**	**0.724**	**0.984**	**0.833**	**0.790**

DHDTDA: damaged head, damaged tail, damaged acrosome, DHIT: Damaged head with intact tail, Ext.Conc.: Extender*Concentration interaction effect, IBT: Intact with a bent tail, IHDT: Intact head with damaged tail, IHITDA: Intact head, tail, damaged acrosome, IHITIA: Intact head, intact tail, and acrosome membrane (Intact); IPD: Intact with proximal droplet, SE: Standard error of the means.

Stained with a modified Kovacs-Foote staining technique, three hundred cells were evaluated and categorized per slide using a bright field microscope with an oil immersion objective at ×1000 magnification, n=9.

Means in the same column with different superscripts among extenders^{a,b} and between spermatozoa concentrations ^{A,B} differ significantly.

3.2.5 Effect of freezing with different commercial soy-lecithin-based semen extenders on all distal droplets and tail defect of ram epididymal spermatozoa

Table 10 presents the effects of freezing REPS with commercial semen extenders on all REPS distal droplets and tail defects. Considering that the 200 and 400 million spermatozoa/ml concentrations were statistically the same. The data were pooled to assess the effects of freezing equilibrated REPS on all distal droplets and all bent tails and the overall effects of freezing. Significant ($P<0.05$) differences existed between the equilibrated and the post-thaw distal droplets and bent tails in all the extenders and the overall means; AND: 38.51±4.8 vs. 28.17±2.9% and 5.52±1.3 vs. 9.74±1.4%, BIO: 32.92±5.5 vs. 21.72±2.8 and 11.24±2.7 vs 18.33±2.4%, OVI: 26.62±3.6 vs 20.33±2.5% and 11.31±2.4 vs. 11.31±2.4 vs. 17.39±1.7%, and the overall means: 32.69±2.7 vs. 23.41±1.6% and 9.29±1.3 vs 15.15±1.2% for all distal droplets and all bent tails, respectively.

Table 10.

Effects of freezing and thawing with different commercial soy-lecithin-based semen extenders on distal droplets and tail defects of ram epididymal spermatozoa (Mean±SE)

	All distal droplets		P-values	All bent tails		P-values
Extenders	Pre-freeze	Post-thaw		Pre-freeze	Post-thaw	
AND	38.51±4.8[a]	28.17±2.9[b]	0.002	5.52±1.3[a]	9.74±1.4[b]	0.003
BIO	32.92±5.5[a]	21.72±2.8[b]	0.009	11.24±2.7[a]	18.33±2.4[b]	0.003
OVI	26.62±3.6[a]	20.33±2.5[b]	0.032	11.31±2.4[a]	17.39±1.7[b]	0.002
Overall	32.69±2.7[a]	23.41±1.6[b]	0.0001	9.29±1.3[a]	15.15±1.2[b]	0.0001

AND: Andromed extender, BIO: BioXcell extender, OVI: OviXcell extender, SE: Standard error of the means, *n*=9.

Stained with a modified Kovacs-Foote staining technique, three hundred cells were evaluated and categorized per slide using a bright field microscope with an oil immersion objective at ×1000 magnification.

Means in the same row with different superscripts differ significantly.

3.3 The developmental kinetics of *in vitro*-produced sheep embryos fertilized with post-thaw ram epididymal spermatozoa

In the current study, we evaluated and detailed the developmental kinetics of sheep embryos from oocytes fertilized with post-thaw REPS. The overall cleavage, morula, and blastocyst rates are presented in *Table 11.*

Within 24 hours post-insemination (pi), only 25.0% of the zygotes cleaved to 2-4 cells. At 48 h pi, 26.0% reached 6-8 cells and 17.0% to 10-16 cells. Between 72-96 h pi, 35.0% cleaved to 16-32 cells, while 21.0% reached the morula stage. At 120 and 144 h pi, the blastocyst rates were 6.0% and 10.0%, respectively. Finally, at 168 h pi, the overall cleavage, morula, and blastocyst rates were 43.0%, 40.0 and 21.0%, respectively.

Table 11.

Developmental stages of *in vitro*-produced sheep embryos

Hours post insemination (Hours)	**Developmental stages**	**Cleavage/number of oocytes**	**Percentage**
24	2-4 cells	55/216	25.0
48	6-8 cells	57/216	26.0
72	10-16 cells	37/216	17.0
	16-32 cells	76/216	35.0
96	16-32 cells	76/216	35.0
	Morula	45/216	21.0
120	Morula	86/216	40.0
	Blastocysts	13/216	6.0
144	Blastocysts	22/216	10.0
	Expanding blastocysts	20/216	9.0
	Expanded blastocysts	13/216	6.0
168	Blastocysts	12/216	5.6
	Expanding blastocysts	6/216	2.8
	Expanded blastocysts	13/216	6.0
	Hatching blastocysts	9/216	4.2
	Hatched blastocysts	10/216	4.6
	Overall cleavage	**93/216**	**43.0**
	Overall morula	**86/216**	**40.0**
	Overall blastocysts	**45/216**	**21.0**

4. NEW SCIENTIFIC RESULTS

The results of this PhD dissertation revealed that:

1) Ram epididymal spermatozoa can be retrieved by either slicing or incision method without adversely affecting its fresh and post-thaw standard motility.
(Fresh TM: 82.07±2.5 vs 80.07±2.8%, PM: 73.00±2.9 vs 68.93±3.4% and Post-thaw TM: 45.83±5.4 vs 36.67±4.9%, PM: 31.58±5.2 vs 25.08±4.9%) and kinematic parameters (Fresh VSL: 47.04±2.3 vs 44.70±3.2 μm/s, VAP: 66.24±3.3 vs 63.39±4.1 μm/s and Post-thaw: VSL: 38.89±1.9 vs 39.72±2.7 μm/s, VAP: 50.41±2.4 vs 51.33±2.9 μm/s), for slicing vs incision methods respectively.

2) In the current study, the Hungarian Black Racka breed had epididymal spermatozoa with straighter and more linear movement under both fresh (STR: 72.43±1.1 vs 67.9±1.6% and LIN: 36.6±0.9 vs 33.2±1.0%) and post-thaw (LIN: 38.21±0.7 vs 35.40±0.7%) conditions than the German Mutton Merino breed.

3) Based on this finding, the German Mutton Merino and Hungarian Black Racka epididymal spermatozoa had the same cryo-tolerance (TM: 52.30±6.9 vs 49.91±5.3%, PM: 39.42±8.7 vs 39.67±5.3) and (VCL: 76.66±5.2 vs 88.44±5.2%, VAP: 73.60±4.5 vs 85.75±4.2%, and VSL: 82.29±5.7 vs 92.39±5.4%).

4) The BioXcell® and OviXcell® extenders were superior to Andromed® extender in preserving the post-thaw ram epididymal spermatozoa specific kinematic parameters (VAP: 77.72±3.2 and 80.48±3.1 vs 67.72±3.5 μm/s and BCF: 32.81±1.1 and 32.46±1.0 vs 28.72±0.9 Hz), and head membrane integrity (all intact head: 45.33±3.3 and 44.68±2.9 vs 34.64±3.2%), respectively. In contrast, the Andromed® extender was superior to the BioXcell® and OviXcell® extenders in preserving ram epididymal spermatozoa tail morphology (Intact with tail defect: 2.56±0.6 vs 8.14±1.5 and 7.19±1.3% and all bent tails: 9.74±1.4 vs 18.33±2.4 and 17.39±1.7%).

5) Freezing and thawing significantly decreased the proportion of ram epididymal spermatozoa with distal cytoplasmic droplets (Pre-freeze vs. Post-thaw: AND: 38.51±4.8 vs 28.17±2.9, BIO: 32.92±5.5 vs 21.72±2.8, and OVI: 26.62±3.6 vs 20.33±2.5%), while tail defect increased significantly (AND: 5.52±1.3 vs 9.74±1.4, BIO: 11.24±2.7 vs 18.33±2.4, and OVI: 11.31±2.4 vs 17.39±1.7%). Moreover, the cryopreservation in 400×10^6 spermatozoa/ml spermatozoa concentration preserved

sperm head membrane integrity better than freezing at 200×10^6 spermatozoa/ml (All intact head: 45.15±5.1 vs 37.95±3.4%).

6) Viable embryos with good developmental competence (Overall cleavage, Morula and Blastocyst rates: 43.0, 40.0, and 21.0%) can be produced *in vitro* using post-thaw ram epididymal spermatozoa in ivf-Bioscience bovine media.

5. PRACTICAL RESULTS

1) Both the slicing and incision methods of epididymal sperm retrieval can be used to conserve the genetic resources of endangered sheep breed or elite sire that dies suddenly.
2) The incision method is more field-friendly than the slicing method because it is faster.
3) Freezing ram epididymal spermatozoa in BioXcell® and OviXcell® extenders at 400×10^6 spermatozoa/ml concentration preserves its' VAP, BCF, and head membrane integrity better than in Andromed® extender.
4) The ivf-Bioscience bovine media can be used for sheep *in vitro* embryo production, because it supports the development of *in vitro*-produced sheep embryos fertilized with post-thaw ram epididymal spermatozoa.

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7. PUBLICATIONS IN THE FIELD OF RESEARCH

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Subject: PhD Publication List

Candidate: Malam Abulbashar Mujitaba
Doctoral School: Doctoral School of Animal Husbandry
MTMT ID: 10073737

List of publications related to the dissertation

Foreign language scientific articles in Hungarian journals (4)

1. **Mujitaba, M. A.**, Kútvölgyi, G., Egerszegi, I., Vass, N., Bodó, S.: In vitro conservation of gametes: the way forward to conserve the genetic resources of autochthonous sheep breeds.
   Anim Welfare Etol Tartástechn. 19 (1), 118-125, 2023. ISSN: 1786-8440.
   DOI: http://dx.doi.org/https://doi.org/10.17205/SZIE.AWETH.2023.1.118

2. **Mujitaba, M. A.**, Kútvölgyi, G., Debnár, V. J., Tokár, A., Posta, J., Bodó, S., Vass, N.: The impact of retrieval method and breed on the motility and kinematic parameters of fresh and post-thaw ram epididymal spermatozoa.
   Acta Vet. Hung. 71 (3-4), 210-218, 2023. ISSN: 0236-6290.
   DOI: http://dx.doi.org/https://doi.org/10.1556/004.2023.00945
   IF: 0.9 (2022)

3. **Mujitaba, M. A.**, Vass, N., Bodó, S.: The recent state of cryopreservation techniques for ex-situ gene conservation and breeding purposes in small ruminants: A review.
   Agrártud. Közl. 2020 (1), 81-87, 2020. ISSN: 1587-1282.
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4. **Mujitaba, M. A.**, Vass, N., Bodó, S., Angyal, E.: The recent state of embryo production techniques in sheep breeding - A review.
   Állatteny. takarm. 69 (4), 429-443, 2020. ISSN: 0230-1814.

Foreign language scientific articles in international journals (1)

5. **Mujitaba, M. A.**, Egerszegi, I., Kútvölgyi, G., Nagy, S., Vass, N., Bodó, S.: Alternative Opportunities to Collect Semen and Sperm Cells for Ex Situ In Vitro Gene Conservation in Sheep.
   Agriculture-Basel. 12 (12), 1-17, 2022. EISSN: 2077-0472.
   DOI: http://dx.doi.org/10.3390/ agriculture12122001
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Hungarian conference proceedings (1)

6. Tokár, A., **Mujitaba, M. A.**, Debnár, V. J., Vass, N., Kútvölgyi, G., Nagy, S. T., Bodó, S.: Juh embriók in vitro létrehozása.
In: XXVIII. Ifjúsági Tudományos Fórum : Konferenciakötet. Szerk.: Bene Szabolcs, Magyar Agrár- és Élettudományi Egyetem Georgikon Campus, Keszthely, 83-88, 2022. ISBN: 9786156338075

Foreign language abstracts (5)

7. **Mujitaba, M. A.**, Radnainé Szentpáli, J., Debnár, V. J., Tokár, A., Vass, N., Bodó, S., Kútvölgyi, G.: The influence of different commercial plant-based semen extender and pre-freeze dilution rates on freezability of ram epididymal spermatozoa.
In: 31st International Congress of the Hungarian Association for Buiatrics, Hungarian Association for Buiatrics, Budapest, 189-190, 2023. ISBN: 9786158141338

8. **Mujitaba, M. A.**, Tokár, A., Debnár, V. J., Vass, N., Bodó, S., Kútvölgyi, G.: Collection and freezing post-mortem epididymal sperm cells for in vitro fertilization in sheep.
In: XXV. Tavaszi Szél Konferencia 2022 : Absztraktkötet. Szerk.: Molnár Dániel, Molnár Dóra, Doktoranduszok Országos Szövetsége, Budapest, 726-727, 2022. ISBN: 9786158205481

9. **Mujitaba, M. A.**, Tokár, A., Debnár, V. J., Kútvölgyi, G., Vass, N., Bodó, S.: Developmental kinetics of in vitro produced sheep embryos.
In: "FIBOK 2022" : Fiatal Biotechnológusok V. Országos Konferenciája = 5th National Conference of Young Biotechnologists : Program és angol nyelvű összefoglalók = Program and abstracts. Szerk.: Bánfalvi Zsófia, Gócza Elen, Olasz Ferenc, Pál Magda, Posta Katalin, Várallyay Éva, MATE Genetika és Biotechnológia Intézet, Gödöllő, 111-111, 2022. ISBN: 9789632699998

10. Tokár, A., **Mujitaba, M. A.**, Debnár, V. J., Kútvölgyi, G., Nagy, S. T., Bodó, S.: In vitro fertilization with ram epididymal sperm.
In: "FIBOK 2022" : Fiatal Biotechnológusok V. Országos Konferenciája = 5th National Conference of Young Biotechnologists : Program és angol nyelvű összefoglalók = Program and abstracts. Szerk.: Bánfalvi Zsófia, Gócza Elen, Olasz Ferenc, Pál Magda, Posta Katalin, Várallyay Éva, MATE Genetika és Biotechnológia Intézet, Gödöllő, 122-122, 2022. ISBN: 9789632699998

11. **Mujitaba, M. A.**, Bodó, S., Vass, N.: The progress of electroejaculation and semen freezing in ram artificial insemination: A review.
In: XXIV. Tavaszi Szél Konferencia 2021 : Absztraktkötet. Szerk.: Molnár Dániel, Molnár Dóra, Doktoranduszok Országos Szövetsége, Budapest, 106, 2021. ISBN: 9786155586996

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List of other publications

Foreign language scientific articles in Hungarian journals (3)

12. Xayalath, S., **Mujitaba, M. A.**, Ortega, A. D. S. V., Rátky, J.: Opportunities and challenges for pig production in Vientiane Capital, Laos: a review.
Rev. Agric. Rural Dev. 11 (1-2), 3-8, 2022. ISSN: 2063-4803.
DOI: http://dx.doi.org/10.14232/rard.2022.1-2.3-8

13. Xayalath, S., **Mujitaba, M. A.**, Ortega, A. D. S. V., Rátky, J.: A review on the trend of livestock breeds in Laos.
Acta Agrar. Debr. 2021 (1), 227-237, 2021. ISSN: 1587-1282.
DOI: http://dx.doi.org/10.34101/ACTAAGRAR/1/9047

14. Ortega, A. D. S. V., **Mujitaba, M. A.**, Xayalath, S., Gutierrez, W., Soriano, A. C., Szabó, C.: Perspectives of the livestock sector in the Philippines: A review.
Acta Agrar. Debr. 2021 (1), 175-188, 2021. ISSN: 2416-1640.
DOI: http://dx.doi.org/10.34101/actaagrar/1/9101

Foreign language scientific articles in international journals (2)

15. Xayalath, S., **Mujitaba, M. A.**, Ortega, A. D. S. V., Khangembam, R., Novotniné Dankó, G., Rátky, J.: Effects of birth weight on puberty and the reproductive performance of crossbred Moo Lath x Duroc gilts = A születési súly hatása az ivarérésre és a szaporodásbiológiai teljesítményre keresztezett Moo Lath x Duroc kocasüldőknél.
J. Cent. Eur. Agric. 24 (2), 303-310, 2023. EISSN: 1332-9049.
DOI: http://dx.doi.org/10.5513/JCEA01/24.2.3811
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16. **Mujitaba, M. A.**, Bodó, S., Vass, N., Abdullahi, U.: A review on the effect of endocrine disruptors on reproductive performance of small ruminant.
Nat. Res. Sustain. Develop. 10 (2), 145-160, 2020. ISSN: 2066-6276.
DOI: http://dx.doi.org/10.31924/nrsd.v10i2.051

Total IF of journals (all publications): 5,2
Total IF of journals (publications related to the dissertation): 4,5

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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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