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

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Development of Assisted Reproductive Technologies for Ex situ *In Vitro* Gene Conservation in Sheep

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ABBREVIATIONS AND ACRONYMS

AND®	Andromed Extender
AND®200	Epididymal spermatozoa diluted with Andromed to 200×10^6 spermatozoa/ml
AND®400	Epididymal spermatozoa diluted with Andromed to 400×10^6 spermatozoa/ml
AA	Amino Acids
AGR	Animal Genetic Resources
AI	Artificial Insemination
AIPI	After Removal of Intra-vaginal Progesterone Insert
ALH	Amplitude of Lateral Head displacement
AOE	After the Onset of Estrus
AONE	After the Onset of Natural Estrus
APCAI	Air Pressure with Cervical Artificial Insemination
ARTs	Assisted Reproductive Technologies.
ASMA	Automated Sperm Morphometry Analysis
AV	Artificial Vagina
BIO®	BioXcell Extender
BIO®200	Epididymal spermatozoa diluted with BioXcell to 200×10^6 spermatozoa/ml
BIO®400	Epididymal spermatozoa diluted with BioXcell to 400×10^6 spermatozoa/ml
BCB	Brilliant Crystal Blue
BCF	Beat cross-frequency
BMC	Blanche du Massif Central
BO-IVC	Bovine *In vitro* Culture Medium
BO-IVF	Bovine *In vitro* fertilization Medium
BO-SemenPrep	Bovine Semen preparation medium
BSA	Bovine Serum Albumen
CASA	Computer-Assisted Sperm Analyzer
CE	Cauda Epididymis
CIDR	Control Internal Drug Release
CITES	Convention on International Trade in Endangered Species
COCs	Cumulus-oocyte Complexes
CR	Conception Rate
DHDTDA	Damaged Head, Damaged Tail, Damaged Acrosome

DHIT	Damaged Head, Intact Tail
DMSO	Dimethylsulphoxide
DNA	Deoxyribo Nucleic Acid
eCG	Equine chorionic gonadotropin
ED	Estrus Detection
EDC	Embryo Developmental Competence
EEJ	Electro-ejaculation
EG	Ethylene Glycol
EIGC	Ex-situ *In vitro* Gene Conservation
EPS	Epididymal Spermatozoa
ET	Embryo Transfer
EU	European Union
F	Fresh Semen
FAO	Food and Agricultural Organization
FCS	Fetal Calf Serum
FGA	Flurogestone Acetate
FTAI	Fix Time Artificial Insemination
Fr	Frozen Semen
FSH	Follicle Stimulating Hormone
g	Grams
GLY	Glycerol
GnR	Genetic Resources
GnRH	Gonadotropin releasing Hormone
HEPES	(4-(2-hydroxyethyl) Piperazine-1-ethanesulfonic acid)
HOST	Hypoosmotic Swelling Test
IBT	Intact with Bent Tail
ICSI	Intra Cytoplasmic Injection
IDD	Intact with a Distal Cytoplasmic Droplet
IHDT	Intact Head, Damaged Tail
IHITDA	Intact Head, Intact Tail, Damaged Acrosome
IHITIA	Intact Head, Intact Tail and Intact Acrosome Membrane
IN	Incision
IPD	Intact with a Proximal Cytoplasmic Droplet

ISAS	Integrated Sperm Analysis System
IU	International Units
IUCN	International Union for the Conservation of Nature and Natural Resources
IVC	*In vitro* Culture
IVEP	*In vitro* Embryo Production
IVF	*In vitro* Fertilization
IVM	*In vitro* Maturation
K-F	Kovács-Foote Staining Technique
KR	Kidding Rate
KS	Kinetic Score
LAI	Laparoscopic artificial insemination
LH	Luteinizing Hormone
LIN	Linearity
LN_2	Liquid Nitrogen
LOPU	Laparoscopic Ovum Pick-up
LOS	Large Offspring Syndrome
LR	Lambing Rate
LS	Liquid Semen
LSB	Local Sheep Breeds
MAP	Medroxyprogesterone Acetate
MOET	Multiple ovulation and embryo transfer
MOPS	3-N-morpholino propane sulfonic acid
NA	No data Available
NBL	Number of Blastocyst
NH	Number of Hatched Blastocyst
NI	No Information
NM	No Materials
NSI	Not Sufficient Information
NXB	Number of Expanded Blastocyst
OVI®	OviXcell Extender
OVI®200	Epididymal spermatozoa diluted with OviXcell to 200×10^6 spermatozoa/ml
OVI®400	Epididymal spermatozoa diluted with OviXcell to 400×10^6 spermatozoa/ml

OPU	Ovum Pick-up
PBS	Phosphate Buffered Saline
PG	Propylene Glycol
PGF2	Prostaglandin F2
PI	Propidium Iodide
PM	Progressive Motility
PVA	Polyvinyl Alcohol
REPS	Ram Epididymal Spermatozoa
RH	Relative Humidity
ROS	Reactive Oxygen Species
RT	Room Temperature
SDG	Sustainable Development Goals
SE	Standard Error of Means
SI	Sufficient Information
SL	Slicing
SOD	Superoxide Dismutase
SOF	Synthetic Oviduct Fluid
STR	Straightness
TCM	Tissue culture Media
TCN	Total Cell Number
TM	Total Motility
TW	Testicular Weight
VAP	Average Path Velocity
VCL	Curvilinear Velocity
VSL	Straight Line Velocity
WOB	Wobble

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 important 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 24-48 h 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 (KAABI et al., 2003; 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 provides nourishment and protects the sperm cells from injury during the cooling and freezing process (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 which are 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 had been explored on 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) was reported to result 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 intended 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, to 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 *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. LITERATURE REVIEW

2.1 The pros and cons of assisted reproductive technology and the need for ex-situ *in vitro* gene conservation of local sheep breeds

2.1.1 The pros and cons of assisted reproductive technology on local sheep genetic resources

The modern-day farming system employs ARTs to boost livestock productivity and reproductive performance to fulfill the enormous demand for the steady increase in the global population , with the trend dramatically increasing to 9.7 billion by 2050 **(**UN DESA, 2021). With ARTs, reducing a flock/herd size is now possible while productive performance *(meat/milk/fur production*) and quality *(less cholesterol/less intramuscular fat/lean meat)* keep increasing (DAVIS & WHITE, 2020; DALY et al., 2020). It also allows inducing estrus out-of-season in seasonal breeds of sheep, producing offspring of predetermined gender, thereby enhancing productivity and reproductive performance and consequently increasing the overall profit of the enterprise (AMIRIDIS & CSEH, 2012). With AI, a selected male can produce as many as 1.5×10^8 offspring/year (ALEXANDER et al., 2010). The ARTs allow controlling breeding in a more welfare approach (FAN-MEI et al., 2022).

Some researchers reported that using ARTs has certain drawbacks, including the negative impacts on animal health and welfare, sometimes leading to abnormal fetuses, new-borns, and even mature animals (NWOGA et al., 2022). Moreover, the ARTs and indiscriminate mating eroded valuable GnR of autochthonous livestock breeds. Similarly, the increase in selection pressure using modern ARTs resulted in the decline of genetic variability with a consequent loss of valuable adaptable genotypes among the local sheep breed (LSB) globally (TABERLET et al., 2008; SANCHAN et al., 2021). FAO's (2007) report revealed that a minimum of one livestock breed is becoming extinct every month, resulting in the extinction of 148 LSB in Europe and the Caucasus alone. North America, Europe and the Caucasus have the highest proportion of at-risk breeds globally. Still, a recent report of 2022 Global Sustainable Development Goals on LSB revealed a more remarkable paucity of information and materials globally (87.22%), in North America and Europe (92.25%), and in the Sub-Saharan African region (97.75%) (SDG, 2022). According to the (FAO, 2015) report, Africa, Asia, and Latin America are lagging in technology adoption. The

earlier mentioned regions need to step up their commitment toward gene conservation of LSB for sustainability.

A similar trend was also observed in Hungary; the 2022 effective population size data of the native Hungarian sheep breeds from Hungarian Sheep and Goat Breeders' Association (HTTPS 3) revealed that certain Hungarian sheep breeds are critically endangered (Alföldi suta racka and Sárgafejű Berke), while Cigàja and Hortobágyi Racka are at risk of extinction (*Table 1*), based on the IUCN risk status categorization (IUCN, 2012). Similarly, *in situ* gene conservation programs of the breeds exist in different national parks in Hungary: the main objective is to keep purebred animals in the colour variants *(black and white)*, and the main selection criteria are lamb weight gain, yearling weight, and phenotypic characteristics *(wool length, colour and horn appearance).* Both the *in situ* and *ex-situ* programs are supported by tenders entitled "*In situ* preservation of the genetic stock of protected native and endangered agricultural animal breeds" and "*Ex situ*, or *in vitro* preservation of the genetic stock of protected native and endangered agricultural animal breeds, and support for advisory activities preventing genetic narrowing" (HTTPS.1 and HTTPS.2). This led to the establishment of an *ex-situ in vitro* gene bank consisting of ejaculated spermatozoa of Hungarian Racka, Cikta and Cigája breeds with the cooperation of the Hungarian Sheep and Goat Breeders' Association and the National Agricultural Research and Innovation Center, Research Institute for Animal Breeding, Nutrition and Meat Science, Herceghalom between 2014-2017.

Table 1.

The effective population sizes of the native Hungarian sheep breeds as of 2022

Breeds names (Hungarian)	Number of ewes	Number of ewe-labs	Number of qualified rams
Hungarian Merino (Magyar Merinó)	10589	6116	905
Tsigai (Cigája)	3495	2816	253
Cikta (Cikta)	681	395	24
White Hortobágyi Racka (Fehér racka)	2703	1478	194
Black Hortobágyi Racka (Fekete racka)	2632	1490	146
Transylvanian Racka (Gyimesi)	1146	600	33
Polled Zackel (Alföldi Suta Racka)	33	22	0
Berke Sheep (Sárgafejű Berke)	98	109	13
Dairy Tsigai (Tejelő Cigája)	255	108	9

Source: HTTPS. 3

2.1.2 Exsitu *in vitro* gene conservation

Biodiversity refers to the variability among living organisms; terrestrial, aquatic ecosystems, and other marines with complex ecosystems that play vital roles in human existence and ensure sustainable benefits for present and future generations (KASSO & BALAKHRISHNAN, 2013). However, agricultural biodiversity is the most important and directly linked to the well-being and livelihood of human beings (KASSO & BALAKHRISHNAN, 2013). Genetic diversity refers to the within-specie variations in the functional unit of heredity (BOROKINI et al., 2010). Biodiversity conservation entails maintenance, sustainable utilization, and re-establishment of the lost and degraded biodiversity, which can be achieved by two complementary techniques: the *in-situ* and *ex-situ* gene conservation techniques. The *ex-situ* conservation involves conserving genetic diversity outside their original/natural environments *(e.g. zoo, aquarium, gene banks, among others)*, while *in-situ* conservation entails conserving them in their living habitat *(maintenance of viable population)* (MAXTED, 2013; KASSO & BALAKHRISHNAN**,** 2013). The *ex-situ* conservation technique is divided into *ex-situ in vivo* and *ex-situ in vitro* (SANCHAN et al., 2021). The *ex-situ in vitro* gene conservation (EIGC) entails cryopreserving genetic material at sub-zero temperatures (-196 ^{o}C) using liquid nitrogen

(LN_2) (MAXTED, 2013). This technique receives more attention than the former because it permits conserving GnR like live spermatozoa, oocytes, embryos, DNA, and chromosomes for an indefinite time and provides a research opportunity (KASSO & BALAKHRISHNAN**,** 2013). Recently, the Conservation Planning Specialist Group of the IUCN called for the one plan approach that simultaneously uses the two conservation techniques *(ex-situ-in-situ continuum)* to maximize gene conservation (PARAMIO & IZQUIERDO, 2016). There is a need for a better understanding of the ideal ARTs/conservation strategies that ensure the restoration of missing genotypes to the population.

Additionally, all regions/countries and animal welfare regulations should be observed while implementing gene conservation programs or transporting GnR. For example, in the EU, transporting samples of certain species requires an export or import permit by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). However, this is not the case within the EU states; otherwise, the receiving institution outside the EU must be registered exempt from a CITES permit (BOLTON et al., 2022).

Some of the drawbacks of *ex-situ* gene conservation include maintaining the organism in an artificial environment, genetic diversity deterioration, inbreeding depressions, adaptation to captivity and deleterious allele accumulation. In contrast, the significant limitations, particularly in developing countries, range from maintenance costs and personnel to unreliable electricity/power supply (KASSO & BALAKHRISHNAN, 2013). The most common ARTs used to re-establish lost genotypes back into the population by *ex-situ in vitro* gene conservation are AI, IVF/ICSI, and embryo transfers (BOLTON et al., 2022). However, other recent advanced ARTs hold greater potential *(removing invasive animal manipulation and gametes harvesting)*, including cloning, transgenesis, and somatic cell nuclear transfer, but the high cost of the techniques limits their utilization **(**BOLTON et al., 2022).

2.2 Gene conservation by semen/sperm cryopreservation and artificial insemination in sheep

Semen of different species have been successfully collected and cryopreserved in the past decades, live offspring have been produced, and breeds were regenerated back into the population by crossbreeding (SANCHAN et al., 2021). The first step is to identify the reliable and ideal semen collection method. However, different techniques commonly used in small

ruminants to collect semen spermatozoa and their alternatives for gene conservation are reviewed herein. To further increase the success of the conservation program, sample collection should be designed to target species with sufficient extant population size and genetic diversity. There is a need to understand the species' reproductive physiology better (BOLTON et al., 2022).

2.2.1 Semen collection methods

Generally, irrespective of the semen collection method, the animal should be genetically and phenotypically superior. Moreover, the selected animals need to be subjected to thorough and complete physical examinations; body condition score (3-3.5), locomotion *(no feet abnormalities)*, serving capacity and sexual vigor, size and form of testes and epididymis *(measured and compared to normal values for age, breed, and season)*, neck of the scrotum *(hard and readily palpable)*, prepuce and penis *(intact, cleaned and clipped)*, and be free from deformities *(cryptorchidism, testicular hypoplasia, spermiostasis, and varicocoel)* (SHIPLEY et al., 2007; EVANS et al., 1987). Maintaining a good plain of nutrition and ewe effects is also crucial. Stopping all stressful management practices 6-8 weeks before semen collection reduces the chance of sperm damage during the spermatogenic cycle (EVANS et al., 1987). In rams, semen collection is achieved by either AV or EEJ; simultaneously, spermatozoa can be retrieved from cauda epididymis (CE) of slaughtered, castrated, or accidentally dead elite sire for assisted reproduction or gene conservation purposes (MUJITABA et al., 2022).

2.2.1.1 Semen collection by artificial vagina

Semen collection by AV is the most preferred method by most AI practitioners but requires rams training, usually 2-3 weeks (LEBOEUF et al., 2000). It involves developing and reinforcing a male-conditioned reflex to serve a female in confined surroundings amid the operator, simultaneously for the operator to be familiar with the temperament and behaviours of the animals (EVANS et al., 1987). However, the method has specific challenges, including some rams rejecting out of estrus and dummy ewe even after training with the AV (TERRILL, 1940). It is, therefore, necessary to seek more convenient alternatives to collect semen for gene conservation.

2.2.1.2 Semen collection by electroejaculation

In rams, semen collection by EEJ involves the use of a device called an electro-ejaculator. The commonly used probes are *Bailey (Ring-type)* and *Ruakura (Longitudinal electrode-type)* (SHIPLEY et al., 2007). However, probe-type with ring electrode may stimulate irrelevant skeletal nerves and muscles inflicting more pain and stress to the animal (STAFFORD,1996). The Ruakura delivers 10-15 V output; for dried rectum, the 15 V is recommended. Moreover, lubricating the probe before inserting it is recommended (EVANS et al., 1987). It only takes 3-5 stimulations (10 to 15 s) for the ram to ejaculate. The probe is used to stimulate the organs in a rhythmic on/off sequence of 3 to 5 seconds on and 5 to 15 seconds off by applying gentle downward pressure toward the pelvic floor (SHIPLEY et al., 2007). Ejaculation was also achieved using an average value of 4 V and 90 mA within 3 m using a 3-electrode probe connected to the electro-ejaculator (ÁLVAREZ et al., 2012b). It is a simple and direct procedure with the ideal device (EVANS et al., 1987).

2.2.1.3 Spermatozoa retrieval by transrectal ultrasound-guided massage of accessory sex glands

The procedure involves exteriorizing and holding the penis using sterile gauze and observing the three accessory sex glands *(bulbourethral glands, seminal vesicles, and ampullae)* with real-time transrectal ultrasound using a 7.5 MHz linear array probe. The probe is then used to massage the ampullae vigorously back and forth to expel the spermatozoa. The bulbourethral gland is then massaged simultaneously. Firm pressure is maintained against the symphysis pubis while passing the pad of the finger in a downward and caudal direction. Simultaneously, manually massaging the penile, perineal, and pelvic parts of the urethra aids in transporting the ejaculate into the collecting funnel down to the collection tube. This procedure is repeated several times until ejaculation is achieved (SANTIAGO-MORENO et al., 2009; GUERRERO-GUTIÉRREZ et al., 2021). It is a more welfare approach to collect semen in rams than using the EEJ technique.

2.2.1.4 Spermatozoa collection from cauda epididymidis

Spermatozoa can be collected from the CE of culled, slaughtered, or accidentally dead elite sires. It is an exciting and last alternative to save the genetic diversity of the valuable

males from permanent loss in a population and is generally termed the "gametes rescue" (BOLTON et al., 2022). Gametes retrieval *post-morte*m involves collecting male genital organs or testes from a slaughterhouse/abattoir after emasculation or after the animal dies. For better quality gametes, the testicles *(>100 g)* should be maintained at refrigerated (4-8 ^{o}C) or RT (22 ^{o}C) and should be taken to the laboratory within 2 h (GANAIE et al., 2008; ALVAREZ et al., 2012). A 0.9% NaCl solution, 100 UI/mL Penicillin-G, and 100 mg/mL streptomycin sulfate may be used for the collection (ALVAREZ et al., 2012). The tunica vaginalis is removed carefully, and the epididymis is detached from the testis and rinsed with Dulbecco's Phosphate Buffered Saline at 37 ^{o}C (KAABI et al., 2003). Depending on the species involved or testicle size, the spermatozoa can be retrieved by;

a). Slicing/mincing (KAABI et al., 2003; PARRA-FORERO et al., 2015).

b). Incision methods (AHMED et al., 2019).

c). Retrograde flow/flushing via the ductus deferens *(less contamination)* (LORRAINE LEIBFRIED-RUTLEDGE et al., 1997; BERTOL, 2016) and

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

2.2.2 Sample quality assessment techniques

The success of AI categorically relies on two major factors: one of which is from males and the other from females. The male must produce and ejaculate a fertilizing sperm, the female must ovulate a viable oocyte at the right time, and its genital tract must be congenial with the spermatozoa survival (BENIA et al., 2018). In addition, up to 10-15% of rams and bucks are of unacceptable breeding quality (PEZZANITE et al., 2017). Moreover, with AI, a single ejaculate is used to inseminate/breed many females in a herd (DAVID et al., 2015). Hence, ejaculate is assessed to ascertain its quality for AI and/or predict its fertilizing ability to ensure overall AI success. An exemplary laboratory sperm quality assay should be objective, repeatable, accurate, rapid, and inexpensive (GRAHAM, 2001). We explored the key, exciting and affordable techniques for assessing the significant parameters that indicate ejaculate quality. However, some of them are subjective but promising, but for more attribution of the spermatozoa, state-of-the-art techniques like Flow cytometry (NAGY et al., 2016) or ISAS CASA system (TAPALOAGA and TAPALOAGA, 2015) can be used.

2.2.2.1 Ejaculate/spermatozoa volume

Analyzing semen quantity or ejaculate volume is paramount because ejaculate volume and concentration determine the number of semen straws/ejaculate. It will, in turn, reflect on the individual ram's genetic contributions to the offspring of future generations because volume and concentration were highly positively correlated r=0.87 as reported by BENIA et al. (2018). Or r=0.77 by GOSHME et al. (2020). Similarly, the volume and concentration have the same correlation value (r=0.96) with the number of straws in adult rams (BENIA et al., 2018). Volumetric measurements of ejaculates are achieved with a graduated collecting tube, mostly in mls. However, the acceptable volume of breeding rams' AV-collected ejaculate is ≥ 0.5 ml (EVANS et al., 1987). Ram age had no significant effects on the ejaculate volume of Ouled-Djellal rams (1.18±0.39 ml for the young ram's vs 1.29±0.23 ml for the adults). However, it is affected by seasons (higher in the Spring and Autumn and lower in Winter and Summer) (BENIA et al., 2018).

2.2.2.2 Cell concentration

Semen concentration can be determined either with the aid of a Spectrophotometer *(quick and objective)*, a Hemocytometer *(reliable but time-consuming)*, or using a Makler counting chamber *(convenient, time-consuming, and subjective)* (EVANS et al., 1987; RAI et al., 2017). For bull AI station practice, the spectrophotometer was reported to be the best, as indicated by their respective coefficients of variations: 40.40, 47.02, and 49.19 for the Spectrophotometer, Hemocytometer, and the Makler counting chamber, respectively. Furthermore, it takes an average of only 3 min to use a spectrophotometer and 10-15 min for the Hemocytometer and Makler counting chamber (RAI et al., 2017). Some factors influencing the sperm concentration of rams include age 4.01×10^9/ml in young rams vs 4.25×10^9/ml in adults, but it is not influenced by season (BENIA et al., 2018). The final sperm concentration of the insemination dose is determined by the storage method, chill or frozen (straws or pellets), while the insemination method determines the dose; $10\text{-}25\times10^6$ spermatozoa is ideal for laparoscopic intrauterine AI and $50\text{-}100\times10^6$ sperm for transcervical AI in ewes (ZHANG et al., 2021; PAULENZ et al., 2002). However, for samples to be frozen in straws, the concentration is maintained chiefly at 200×10^6 or 400×10^6 total spermatozoa per ml (ALVAREZ et al., 2012; JHA et al., 2020; SAVVULIDI et al., 2021; AKÇAY et al.,

2012). Maintaining the sperm concentration at 200×10^6/ml and double insemination by intra-cervical route within 10-12 h and 16-18 h after the onset of estrus resulted in a better non-return rate (56.5% vs 25.0%), twin born (83.3% vs 66.7%) and triplet (16.7% vs 0.0%) than 100×10^6/ml respectively (JHA et al., 2020). For chilled storage (4◦C), comparing two sperm concentrations (25×10^6/ml vs 100×10^6/ml) in Pirlak rams, using a Tris-based egg-yolk extender at RT. The 100×10^6/ml presented significantly higher motility, morphology, membrane, DNA integrity, and better oxidative stress parameters (GUNDOGAN et al., 2010).

2.2.2.3 Sperm motility

Sperm motility can be assessed subjectively by scoring the spermatozoa mass movement with a phase contrast microscope *(direct measurement)* as described by (EVANS et al., 1987). Motility can be assessed indirectly by measuring Adenosine Triphosphate, or objectively with the aid of CASA *(measures individual and overall mean sperm motility)* (AMANN & KATZ, 2004; DAVID et al., 2015). The CASA is an automated system *(hardware and software)* that can view and scan consecutive sperm images, process and analyze the data, and deliver exact, accurate, and insightful data on the individual cells and overall kinematics mean value of all the examined cells 10/04/2024 20:53:00. Motility has a strong and positive correlation with volume (0.71) and concentration (0.93) and is affected by animal age *(higher in older animals)* (BENIA et al., 2018) and genotype (GOSHME et al., 2020). But the season does not (BENIA et al., 2018; GOSHME et al., 2020). Mass motility and ram age were the significant male-factors affecting the success of AI. Mass motility is the primary predictor of fertility in sheep, and the predictability is higher when objective mass motility measurement is employed (DAVID et al., 2015).

2.2.2.4 Viability, acrosomal integrity and morphology

Sperm viability, acrosome integrity, and morphology can be assessed conveniently, although subjectively but promisingly by the Kovacs-Foote staining technique (KOVÁCS and FOOTE 1992; KÚTVÖLGYI et al. 2006). The procedure involved dropping drops of equal size (20 ml) of the viability stain (0.26% Trypan blue or 0.16% Chicago sky blue) and diluted spermatozoa sample on a well-labelled glass slide. The two drops are mixed with the

edge of another slide or by gently rocking and smeared. The slides are then parallelly attached and pulled to make two smears. The smeared slides are then air-dried near vertically at RT. The dried slides can then be fixed in a fixative for 4 min in an open staining jar. The slides on both sides are then rinsed with tap water, followed by distilled water. A Giemsa solution is then used to stain the dried fixed slides in an open staining jar at 25-40 ^{o}C for 2-4 h, depending on the semen source species. The slides are washed with tap water, differentiated in distilled water for 2 min, and air-dried in a nearly vertical position. Evaluation is achieved at ×1000 magnification for live/dead and morphological categorization as described by KÚTVÖLGYI et al. (2006). Usually, two or three hundred cells are then counted per slide and classified into the following categories: intact head, intact tail and acrosome membrane (IHITIA); intact with a proximal droplet (IPD), intact with a distal droplet (IDD), intact with bent tail (IBT), intact head, tail, damaged acrosome (IHITDA); intact head, damaged tail (IHDT); damaged head, intact tail (DHIT); and damaged head, damaged tail, damaged acrosome (DHDTDA) (KÚTVÖLGYI et al 2006).

Similarly, several techniques are available for assessing or evaluating sperm morphology, such as the ISAS CASA system (TAPALOAGA and TAPALOAGA, 2015), which help predict the fertilizing ability of spermatozoa. For sperm membrane integrity, the most used technique by researchers is the Hypoosmotic swelling test (HOST) (ZHANG et al., 2021; GUNDOGAN et al., 2010; NALLEY et al., 2013), and several staining methods are available evaluating by light (NAGY et al., 1999) or fluorescence microscopy (CHATZIMELETIOU et al., 2023) however these techniques can evaluate only spermatozoa head membrane integrity. The computer-automated sperm morphometry analysis (ASMA) evaluates the sperm head dimension or morphology (GRAVANCE et al., 1998; PERRY et al., 2021). Sperm membrane integrity is integral to sperm viability and fertilizing ability or male fertility potentials (NALLEY & ARIFIANTINI, 2013; GRAVANCE et al., 1998). With the HOST technique, the classical indicator of the sperm cells' response to hypo-osmolality is the development of bent or curled tails due to the influx of water during the re-establishment of osmotic equilibrium. This characteristic bent of the tail indicates membrane integrity (NALLEY & ARIFIANTINI, 2013).

For the ASMA technique, at least 100 cells are evaluated at ×40 objective magnification, and a sample can be analyzed objectively within 3 min. Moreover, analyzing

several spermatozoa less than recommended may lead to inaccurate results (GRAVANCE et al., 1998). The best stains for the ASMA are the Hematoxylin *(nuclear stain)* and Rose bengal stain *(acrosome stain)*, which revealed that the ASMA accurately recognized and digitized sperm cells in 95% of cases. The procedure involved placing 7 drops of thawed semen and spreading it by dragging it with the edge of another slide. The spread sample is allowed to air-dry for 1 h followed by staining, and then evaluated using the ASMA device (GRAVANCE et al., 1998). Rams with <70% normal morphological spermatozoa and <30% sperm motility are questionable and might have white blood cells in their ejaculate (PEZZANITE et al., 2017).

2.2.3 Semen processing and preservation

2.2.3.1 Semen/spermatozoa dilution

Semen processing involves the use of a semen extender to achieve semen preservation. An extender is a chemical medium for preserving, extending, and protecting sperm cells against several shocks during processing, storage, and transportation used for AI (REHMAN et al., 2013). Under the natural breeding condition, a ram can deposit up to three billion sperm cells into the ewe's vagina. Of this large number, only 100-150 million will pass through the cervix. Ultimately, only a tiny proportion will reach the fertilization site. With a semen extender, a single ram ejaculate can be extended to produce up to 20-25 semen straws, each with a concentration of at least 200 million spermatozoa, which can inseminate approximately 20 ewes (SHIPLEY et al., 2007).

Generally, ram semen is diluted to a ratio of 1:8 for straw freezing, while pellet freezing is 1:3 to 1:4 (SHIPLEY et al., 2007). Dilution is performed either in one or two steps. In the latter method, a semen sample is first diluted to half the final pre-freeze concentration, then cooled for 1.5-2 hours at 5 ^{o}C. It is then extended to the final rate with a glycerol-containing extender. In the former method, a sample is diluted straight to the targeted pre-freeze dilution rate using a glycerol-containing extender (EVANS et al., 1987). The most commonly used diluents for chilled-storage are ultra-heat-treated cow milk or Dulbecco's phosphate-buffered saline *(with or without 2% equine serum albumin or 10% FCS)* or whole milk boiled to 92-95 ^{o}C for 8-10 minutes and cooled to 30 ^{o}C before use (SHIPLEY et al., 2007). When egg-yolk-based extenders are used, a 20% egg yolk is ideal for freezing sperm regardless of the

source. Generally, for AV and EEJ-collected spermatozoa, 4% glycerol improves post-thaw sperm quality, while 8% is ideal for EPS (ÁLVAREZ et al., 2012). A better understanding of cryoprotective agents' effect on the cellular system is a precondition for improving cryopreservation procedures (CURRY & WATSON, 1994). The good extender should contain all the necessary compositions in the correct quantity and provide the spermatozoa with the desired nutrients and protection (*Table 2*).

Table 2.

Different extender compositions, types, and their functions

S/N	Extender composition	Composition types	Functions	Source
1.	Sugars	Glucose, Fructose*, Trehalose, raffinose, saccharose, and galactose	Energy supply to support sperm cell motility, increase recovery rates, thermal resistance, and acrosomal integrity of spermatozoa.	REHMAN et al. (2013); EVANS et al. (1987); GADEA (2003).
3.	Cryoprotectants	Permeating cryoprotectants • Glycerol* • Ethelene glycol* • Dimethyl sulphoxide Non-permeating cryoprotectants • Egg yolk (Low-density lipoproteins) • Milk* (Lactose and Casein). • Soy-Lecithin (Lecithin)	Prevent cryoinjury by inhibiting intracellular crystallization. Prevent intracellular crystallization, reduce seminal protein bindings, restore phospholipid lost during heating, increase acrosomal integrity, decrease damage to cell membrane lipids and improve sperm motility and viability.	BERGERON et al. (2007); REHMAN et al. (2013); BARBAS & MASCARENHAS, (2009); SMITH et al. (1979); FOULKES (1977); FUTINO et al. (2010).
4.	Buffers	Hydroxymethyl aminomethane (Tris)*, Citric acid*, Bicarbonate, Sodium phosphate, TES, MOPS, and HEPES	Maintaining media pH	REHMAN et al. (2013); GADEA (2003).
5.	Antimicrobials	Penicillin*, Streptomycin*, Polymixin B, Ceftiofur, Apramycin, Gentamycin, Kanamycin, Neomycin, linco-spectin, and tylosin.	Keep a check on the microbial contamination in extenders.	LEIN (1986); SHIN (1986).
6.	Other additional supplements	Bovine serum albumin and antioxidants {SOD, catalase, glutathione peroxidase (GPx) and cytochrome C}.	Improve motility, acrosome integrity, and post-thaw fertility rate.	HOLT (2000); CURRY & WATSON (1994); EVANS et al. (1987); FOOTE et al. (2002); MAXWELL and STOJANOV (1996).

Abbreviations: * Most commonly used/recommended as less detrimental, HEPES: (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid) MOPS: 3-N-morpholino propane sulfonic acid, SOD: Superoxide dismutase.

2.2.3.2 Sperm preservation techniques

Semen preservation techniques involve maintaining sperm's viability, morphology, integrity, and fertilizing ability for a long time until desired use (PINI et al., 2018). It is achieved by cooling (5 ºC) or freezing using LN_2 vapour (-196 ºC). Both techniques warrant successful storage and an acceptable pregnancy rate. However, the AI fertility rate in sheep relies on an interaction between the method of preservation used and the insemination method *(vaginal, cervical, and intrauterine deposition)*.

A. Chilled storage

Semen storage at low temperatures (5 ºC) significantly reduces its metabolic activity, extending its shelf-life (VISHWANAT & SHANNON, 2000). Consequently, the concentration of intracellular Na increases due to the depressed activity of the Na/K pump at 5 ºC, which is detrimental to sperm survival (VISHWANAT & SHANNON, 2000). Sperm do not naturally encounter aerobic environments. While handling and processing semen for AI, no precautions are made to prevent oxygen exposure. Oxygen removal at low temperatures improves the survival of the spermatozoa (BATELLIER et al., 2001). Similarly, when antioxidants were added to the extender, sperm motility and acrosome integrity were enhanced during refrigerated storage of bull semen. These antioxidants included superoxide dismutase (SOD), catalase, and glutathione (FOOTE et al., 2002). A Tris-glucose-egg-yolk diluent containing SOD and catalase increased the survival of ram spermatozoa kept at 5 ºC (ANEL et al., 2006). The success of using cooled stored semen depends on various compounding factors, including the storage temperature, the extender composition, the spermatozoa concentration and the volume used in AI, among others (FERNANDEZ-ABELLA et al., 2003).

B. Sperm cryopreservation

Sperm cryopreservation in rams is currently stagnant due to the rare dissemination of the technique in its field of application. This is because AI in sheep is limited to intra-uterine sperm deposition by laparoscopic due to the poor fertility observed after vaginal-cervical insemination, particularly in induced estrus (ANEL et al., 2006; CURRY, 2000). Semen freezing can be achieved in straws using liquid nitrogen (-196 ºC) or in a pellet form using

dried ice/solid carbon dioxide (-78 ºC) (EVANS et al., 1987). Semen freezing in straws can be achieved (i) manually in a Styrofoam box over liquid nitrogen vapor (static LN_2 vapor freezing) or (ii) using an automated programmable freezing machine (SAVVULIDI et al., 2021). Using a programmable freezer involves maintaining the following temperature profile: +4 °C, −10 °C (120 s), −80 °C (450 s), −120 °C (100 s), and −140 °C (180 s). After attaining the final freezing temperature at the stipulated time (−140 °C at 180 s), the semen straws are then plunged into LN2 (−196 °C) in a cryogenic container for permanent storage (MARTÍNEZ-PASTOR et al., 2006). For pellets freezing, a metal rod is used to make multiple depressions on the dried ice. A 0.1 to 0.15 ml of diluted cooled semen is then pipetted into each depression and allowed to freeze for 2 to 3 minutes (EVANS et al., 1987).

The freezing procedure for EPS is like that of AV- and EEJ-collected spermatozoa, but with slight differences: before dilution, EPS are usually washed with an isotonic buffer solution or m-PBS by single or double centrifugation at 500–600× *g* for 5–7 min or 885× *g* for 10 min, respectively. Regarding the permeating cryoprotectants, e.g., glycerol, instead of the 4.0%, which is ideal for AV- and EEJ-collected spermatozoa, the optimal level for the epididymis recovered samples is 8.0% (MUJITABA et al., 2022).

However, the techniques of semen cryopreservation offer many opportunities for artificial reproduction. It eases transporting the GnR of elite males in the forms of frozen semen and timed insemination and ensures animal GnR conservation for an indefinite period. Unfortunately, cryopreservation techniques have drawbacks, including membrane phospholipids, proteins, and sugars changes due to cryo-capacitation (PINI et al., 2018). Ram sperm membrane damage occurs mainly during freezing and thawing at -10 to -25 ºC (SALMON & MAXWELL, 2000). The major problem of concern that directly compromises post-thaw sperm fertility compared to fresh semen is the sub-lethal injury *(non-lethal biochemical or physiological changes that occur at any cryopreservation stage)* (PINI et al., 2018). It can be improved by optimizing semen handling, cell freezability, and increasing sperm population recovered cells after cryopreservation (ANEL et al., 2006).

Several factors affect the post-thaw quality of the ram semen, including the concentration of the semen at freezing (ÁLVAREZ et al., 2012), freezing system *(controlled or uncontrolled cooling rates)* (JOSHI et al., 2008), and freezing form *(using straws or in*

pellets) (SCHMEHL et al., 1986). Extra sugar *(50-100 mM of raffinose or trehalose)* in extenders improves the post-thaw ram semen quality and fertility rates following cervical insemination (JAFAROGHLI et al., 2011).

C. Alternative male gametes conservation techniques

The present-day sperm storage technique involves using LN_2 to preserve the frozen spermatozoa at sub-zero temperatures. However, it is a viable technique, though expensive, time-consuming, and challenging in certain parts of the world *(LN_2 is only sometimes available, particularly in developing countries)*. So, there is a need for an alternative way to address the earlier-mentioned challenges in the system for efficient gene conservation. The two sperm storage techniques that don't involve using LN_2 include.

a) Storage at room temperature

The success of AI rests on the quality of preserved semen. Semen storage at room temperature (RT) is simple and economical, though it only lasts for a short duration (ZHANG et al., 2021). The technique of RT sperm storage involved supplementing semen extenders with an acidic substrate *(reducing the pH)* to inhibit sperm metabolic activity and put the sperm in a reversible static state to ensure prolonged survivability of the sperm *in vitro* (HAJIHASSANI et al., 2019). In addition, sperm storage at RT requires the use of exogenous antioxidants in the semen extenders to supplement the endogenous sperm antioxidants to inhibit or delay the production and accumulation of reactive oxygen species (ROS), the high content of which is lethal to the spermatozoa *(DNA fragmentation)* (ZHANG et al., 2021). However, a low concentration of ROS has a latent role in ensuring normal fertilization (AGARWAL et al., 2006). A biological compound, Taurine (Tau)/bovine choline or taurobilin, was an effective free radical scavenging. It also has a wide range of effects: regulation of reproductive function, improved immunity, enhanced antioxidant capacity *(Amino acid peptide antioxidants in the epididymis and reproductive tract)*, slowing down cell apoptosis, and regulation of mitochondria (DE LA PUERTA et al., 2010; HUXTABLE, 1992; SARIÖZKAN et al., 2009). A 20 mM Tau supplementation maintains good sperm progressive motility (35%), plasma membrane (54%), and acrosomal integrity (86%) of Hu sheep spermatozoa stored at 15 ^{o}C for 7 days. Similarly, it presents lower ROS on day 5 than the control group (ZHANG et al., 2021).

b) Freeze-drying

Freeze-drying, also known as lyophilization, is a process that involves the direct conversion of frozen material from a solid (ice) to a vapour (gas) phase. It is an economical and friendly technique that doesn't involve using LN_2 or dry ice to store and ship frozen sperm. The sperm can be stored at RT or 4°C (GIL et al., 2014). Different sperm samples of different forms *(fresh or frozen)* or sources *(ejaculate or epididymal)* can be used for freeze-drying (GIL et al., 2014). Freeze-drying sperm samples saves storage space and costs and facilitates the transport of preserved samples. Unfortunately, freeze-dried sperm cannot be utilized for AI or IVF due to the immobility and membrane-compromised nature of the sperm. To get around those issues, intracytoplasmic sperm injection (ICSI) might be employed.

Interestingly, enhanced freeze-drying procedures ensure retaining oocyte-activating factor(s) and chromosomal integrity in rodent and mammalian species at 4 °C for up to many years and at RT for up to a year (KESKINTEPE & EROGLU, 2021). Freeze-dried mouse spermatozoa stored at 24 °C for one month maintained its chromosomal integrity; beyond this period, it decreased gradually (KANEKO & NAKAGATA, 2005). When stored at 4°C in rats for one year, it presented fewer chromosomal abnormalities than at 25 °C (HOCHI et al., 2008). This indicates that the 4°C storage condition is ideal for freeze-dried spermatozoa. A recent development in freeze-drying was the invention of a partial freeze-drying apparatus (Darya, FertileSafe, Israel). The device provides good post-thaw sperm motility of up to 46.6% and has a condensation temperature below -110 °C and a vacuum pressure of 10-100 mTorr that is obtained in less than 10 s. The lyophilization solution contains 0.26 M sorbitol and 0.165 M trehalose in egg yolk and Tris medium. A 10µl sample drop is placed on a coverslip, frozen to −10 and −25 °C and then transferred to the Darya device for 1 h drying (ARAV et al., 2018). Considering that this technique preserves sperm viability and doesn't require the use of LN_2 for storing preserved samples, it could be an answer for future assisted reproductive technologies and biobanking.

2.2.4 Thawing of semen

Semen thawing is necessary; apart from freezing injury, damage can occur at thawing if not done carefully and at an appropriate temperature. The faster the semen is frozen, the

quicker it should be thawed to obtain maximum spermatozoa recovery (EVANS et al., 1987). Instruction on the thawing procedure is given by the person or facility that froze the semen (DASCANIO & KASIMANICKAM, 2008). Two to three pellets are placed in a sterile dry test tube or Whirl Pak bag and placed in a water bath that has been preheated to 40 °C. The container is shaken or violently swirled until the pellets melt. The semen is then moved to a water bath at 30 ºC, which can be kept for up to 18 hours until insemination (SHIPLEY et al., 2007).

Thawing of straws is done similarly but should not be done at a temperature below 37 °C. Thawed straws should be wiped-dried and then de-plugged by cutting the PVA-plugged or ultrasound-sealed end of the straw using scissors before loading it into the inseminating device designed for sheep and goats (EVANS et al., 1987).

2.2.5 Insemination methods

The methods used by the AI allow the ram to fertilize many more ewes than would be feasible through normal mating. Before semen collection, the ram should undergo performance evaluations to demonstrate its superiority (COTTLE, 2010). Estrus detection (ED) is a labour-intensive daily task required by traditional AI protocols in sheep industries. Due to the elimination of ED in FTAI, the technique has now taken the role of classical AI and is a labour-effective alternative (OLIVERA-MUZANTE et al., 2011). According to MIRANDA et al. (2018), FTAI enables lambing times synchronization, the division of lambs into batches that are appropriate for satisfying market needs, an earlier start to puberty, and increased conception rates whether the estrus is visible. Progesterone-releasing devices and eCG or GnRH injection during device removal are often the fundamentals of treatment for FTAI in sheep, with a satisfactory pregnancy rate (MENCHACA & CUADRO, 2017). During the breeding season, the eCG or GnRH shortens the time between sponge removal and estrus and increases ES and ovulation efficiency (FORNAZARI et al., 2018). MENCHACA & CUADRO (2017) observed that animals receiving CIDR-eCG displayed estrus behaviours sooner than those getting CIDR-GnRH based on eCG protocol and that preovulatory LH peaks and ovulation followed these behaviours. In sheep, DE et al. (2015) found a 79.4% estrus response (374/471) and a 60.42% lambing rate (226/374), MIRANDA et al. (2018) recorded a 55.4% lambing rate, while in goats, TEKIN (2019) reported a 71.4%

estrus response and a 100.0% kidding rate. Likewise, according to REKIK et al. (2016), a 70.4% lambing rate was achieved with FGA for 14 days, and 300IU eCG was given upon sponge removal. Ovulation timing is influenced by the kind of oestrus *(natural or artificial)* and synchronization technique (COTTLE, 2010). Similarly, semen and the insemination method will impact the viability and efficiency of sperm transportation in the female reproductive tract. Both factors affect insemination time. Different AI methods, time, and recommendations in the small ruminant AI program were highlighted in *Table 3*.

Table 3.

Insemination methods, best time to inseminate and some recommendations in small ruminants

Methods	Time (Hours)	Volume (ml)	Number of progressively motile Spermatozoa	Conception/Kidding/Lambing rate (%)	Recommendations	Sources
Intravaginal/ Peri-cervical	12-18^{AONE}	0.2	$400\times10^{6\ Fr}$	NA	• Recommended during the breeding season and after estrus detection. • Fresh semen can yield good results in Does. • Extended chilled/frozen semen gives poor results. • In young ewes, inseminator should ensure depositing the semen at the anterior part of the vagina.	FAIGL et al. (2012); (PAULENZ et al., 2005); LARSEN, (2021).
	AONE	NA	$200\times10^{6\ LS.}$	78^{KR}		
Transcervical /Intracervical	55^{AIPI} or 15-17^{AONE}	0.2	$200\times10^{6\ F}$	40-70^{CR}	• Mostly used in Does. • Fresh or frozen semen can be used. • Most preferred using illumination and insemination pipette inserted to 5-12 mm depth into the doe cervix. • Cervix penetration in Does should not exceed 38 mm. • Cervix may be fully penetrated in *periparturient* ewes.	FAIGL et al. (2012); PAULENZ et al. (2005); LARSEN, (2021); TEKIN, (2019).
			$200\times10^{6\ Fr.}$	30-70^{CR}		
	AONE	NA	$200\times10^{6\ LS}$	74^{KR}		
	43-45^{AIPI}	NA	NA^{Fr}	31-32^{CR}/90-145.5^{KR}		
Laparoscopic /Intrauterine	48-65^{AIPI}	0.05	$20\text{-}40\times10^{6\ Fr.}$	60-75^{CR}	• Mostly used in Ewes. • Animals should have fasted for 12 hours. • Xylazine by IM is ideal sedative, and the ewe should be retrained in a cradle on dorsal *recumbency* raised posteriorly at angle 45^{o}. • SC injections of local anesthetic at two locations 6 cm in front of the udder and 4 cm on either side of the ventral midline. • Semen should be deposited at the middle region of the uterine horn.	FAIGL et al. (2012); JHA et al. (2020); LARSEN, (2021).
	18-22^{AOE}	0.25	$50\times10^{6\ Fr.}$	100^{LR}		

AOE: After the Onset of Estrus, AONE: After the Onset of Natural Estrus, AIPI: After Removal of Intra-vaginal Progesterone Insert, CR: Conception Rate, F: Fresh Semen, Fr: Frozen Semen, KR: Kidding Rate, LR: Lambing rate, LS: Liquid Semen LR: Lambing Rate and NA: No Data Available.
NB: Conception rates depend on the quality of semen, time of the year, ewe or doe condition, and inseminator's skill.

2.2.6 Insemination techniques to enhance the fertilizing capacity of the frozen-thawed spermatozoa

2.2.6.1 Laparoscopic artificial insemination

The Laparoscopic artificial insemination (LAI) technique is mainly practiced in small ruminants, notably sheep, to bypass its tortuous, long, and convoluted cervix with 4-7 cervical rings. It involves sperm deposition into the uterine cavity at the utero-tubal junction area, improving the thawed-semen efficiency (LIGHTFOOT & SALAMON, 1970; CSEH et al., 2012). With LAI, higher pregnancy rates can be achieved even with poor-quality semen (LIGHTFOOT & SALAMON, 1970). The LAI technique requires a low dose *(20-25 million)* of viable spermatozoa to breed an ewe as compared to 100-200 million for trans-cervical or 400 million for vaginal inseminations. Thus, with a single ram ejaculate, 50-100 ewes can be inseminated (ARAL et al., 2009; LIGHTFOOT & SALAMON, 1970). EHLINNG et al. (2006) achieved excellent lambing rates (87.5%) with EPS using the LAI technique. Therefore, using the LAI technique in sheep breeding can offer a promising result.

2.2.6.2 Air pressure with cervical artificial insemination

Using the technique of Air Pressure with Cervical Artificial Insemination (APCAI) with fresh semen in sheep also resulted in excellent pregnancy and lambing rates of 80.0% and 70.0% than cervical insemination; 46.7% and 46.7%, respectively (ARAL et al., 2009). Therefore, using the techniques of APCAI in sheep with frozen-thaw semen might improve the pregnancy and lambing rates of the poorly post-thaw spermatozoa.

2.2.6.3 *In vitro* fertilization

Post-thaw semen samples of high genetic merit animals with poor motility and kinematic parameters and/or viability due to prolonged storage should not be discarded as they can still be used for IVF. According to WILLIAMS et al. (1991), to achieve a significant penetration following a sperm and egg coincubation, a sample must have a sperm population with more than 5.0% motility and over 24.0% acrosome reaction. In boar, IKEDA et al. (2002) revealed that maintaining acrosomal integrity is more important for fertilizing ability than the motility of spermatozoa. In our laboratory, for IVF using EPS, we mainly adopt two strategies to ensure

a high fertilization rate: one at the collection and the other one at the IVF stage *(if the sample is of poor quality/motility)*;

a) At collection: we ensure that the EPS are retrieved from proximal and distal parts of the CE. This is because EPS's progressive motility and fertilizing ability increased from distal caput to distal CE (FOURNIER-DELPECH et al., 1979). Furthermore, upon sperm-eggs coincubation, proximal CE retrieved-spermatozoa present endogenous acrosome reaction faster (< 17h) with better ova penetration than the more proximal epididymis retrieved spermatozoa (22 to > 50h) (WILLIAMS et al., 1991).
b) At the IVF stage: the sample is washed twice by centrifugation, mostly at 2000 rpm for 10 min, to clean off the spermatozoa of extenders and cryoprotectants (WANI et al., 2013; AL-MEENI, 2017). In case of poor motility of the pellets, the computed/actual spermatozoa concentration is then moderated concerning its actual per cent motility at the time of the IVF. Therefore the concentration is increased to compensate for the poor motility (FINDEKLEE, 2021).

2.2.6.4 Intracytoplasmic sperm injection

Intracytoplasmic sperm injection (ICSI) is an IVF technique by which a mature sperm cell is directly injected into a matured oocyte by pricing a zona pellucida and the plasma membrane (HOCHI et al., 2008). The ICSI with spermatogonial cells, if efficiently improved, has excellent prospects, one of which is that it can shorten generation interval since the cells can be harvested from ram lamb that is as young as 2 months old. This was proved by DENG et al. (2016), which demonstrated the possibility of *in vitro* culture spermatogonial stem cells *(primordial germ cells in the testes)* collected from a 2-month-old ram lamb into haploid germ cells. Injecting *in vitro* matured sheep oocytes with the round spermatid led to successful fertilization and development of the zygotes to morulae and blastula when haploid sperm cells were used. However, the morulae rate was lower in the ICSI. Several studies have shown *in vitro* culture of spermatogonial stem cells or spermatids to haploid sperm cells in cattle, mice (ARKOUN et al., 2015) and human beings (YANG et al., 2014).

The ICSI technique in human beings is a successful procedure as it offers solutions to infertile patients. However, this is still untrue in ruminant animals (RESSAISSI et al., 2021). Several studies revealed varied reasons for the low blastocyst or success rate of ICSI in different livestock species. In bulls, HARA et al. (2011) found that the microtubule organization center

of the IVF oocytes was more functional in terms of aster formation than the ICSI-derived oocytes. Similarly, in sheep, GÓMEZ et al. (1998) and RESSAISSI et al. (2021) reported lower fertilization and blastocyst rate with ICSI compared to IVF. RESSAISSI et al. (2021) concluded that the lack of syngamy in the ICSI-derived zygotes is the major problem. Other researchers concluded that ICSI in a polar body position adversely affects fertilization rate and embryo development (NAGY et al., 1995). In contrast, GIL et al. (2014) reported that the polar body position is a reference point to pierce and inject a sperm cell to avoid damaging the metaphase plate. There is, therefore, a need to intensify research on the subject to identify the underlying problems associated with the technique to improve its success rate.

2.3 Gene conservation through embryo production and cryopreservation in sheep

Throughout a female mammal's lifespan, only a tiny portion of the oocytes in the ovaries at birth ovulate. The option of utilizing biological resources, which are generally untapped, is provided through *in vitro* maturation (IVM) (LONERGAN & FAIR, 2016). Selecting sperm for IVF is one of the first steps in embryo formation. This practice is commonly employed to select frozen-thawed spermatozoa for IVF (GORDON, 2017). Another benefit is that embryos can withstand high body temperatures owing to thermal stress better than gametes. The ET can be performed in or out of season, but the best response is achieved when donors and recipients usually are cycling (EDMONDSON et al., 2011).

2.3.1 *In vivo* embryo production

Activities for producing *in vivo* embryos in small ruminants began in the 1930s, with the first successful report coming in 1934. Since then, surgery has been used for most efforts at embryo recovery and transfer (GORDON, 2017). The procedure involves a series of steps, which include the following.

2.3.1.1 Synchronizing estrus of the donor and the recipient ewes

Exogenous hormones are utilized in small ruminants to change the physiological sequence of events associated with the estrous cycle and regulate reproduction. Understanding the physiology of ovarian follicular dynamics, the hormonal interplay at the same period in the ovaries, and the reproductive hormones interaction that drive reproduction is crucial for

efficiently utilizing this ART (RAMOS & SILVA, 2018). The estrous cycle can be manipulated to concentrate insemination and births, promote cyclicity, decrease the time between births, schedule births during a favorable time when feed is available, and employ animals that have undergone genetic improvement. Synthetic progesterone analogues were first used to study estrus synchronization/induction in the 1960s. These substances are utilized to alter the physiological estrous cycle by modifying the luteal or follicular phase (RAMOS & SILVA, 2018).

After day 5 of the cycle, PGF2 (15 mg) or Cloprostenol (125 mg) are efficacious in sheep *(estrus is day 0)*. Estrus may be synchronized by administering two dosages of prostaglandin 7-9 days apart. In cyclic or anestrous ewes, the most used drug for controlling ovulation is progestogens, impregnated intra-vaginal sponges {medroxyprogesterone (MAP) or Florogestone Acetates (FGA)} (RAMOS & SILVA, 2018). Another option is to employ a CIDR intra-vaginal plastic device impregnated with 300 mg of progesterone. It is kept in the vagina for 7 days, after which a luteolytic dosage of PGF2 is given the day before or right after the device is removed. The estrus response peaks between 72–84 hours, (ROMANO, 2021).

2.3.1.2 Superovulation of the donor animal

The foundation of multiple ovulation and embryo transfer (MOET) programs is estrus induction, synchronization, and superovulation in donor animals. Next, AI and uterine lavage are used to collect the embryos, either transferred to recipient females or preserved using cryopreservation (RAMOS & SILVA, 2018). The possibilities provided by ET are enormous. It allows exploring ways to improve embryonic development and enhance animal production and reproduction to multiply the number of offspring per dam rapidly. This method becomes a tool for genetic advancement due to the rise in the number of offspring produced per dam. It shortens the generation interval and boosts selection pressure (RAMOS & SILVA, 2018).

The *in vivo* generation of small ruminants' embryos is still variable despite recent improvements. The dissemination of this technique may be constrained by factors such as the considerable variability in superovulation response, super-stimulatory therapy, and variations in the makeup of commercially available follicle-stimulating hormone (FSH) preparations (COGNIÉ et al., 2003). Variability in the treatment response results from the ovarian response to super-ovulatory therapy being dependent on the follicular state, genetics, season, and

nutritional health of the animals. The fundamental constraint for MOET programs in small ruminant is this variation in super-ovulatory response. Superovulation therapy is ineffective in about 20 and 40% of treated females (BRASIL et al., 2016).

Moreover, one of the issues causing unsuccessful fertilization is the variability in the onset of estrus, the LH peak following hormone therapy, and the lack of synchronization at the beginning of superovulation (AMIRIDIS & CSEH**,** 2012). In order to initiate and/or synchronize the estrous cycle in small ruminants, the MOET program generally entails inserting a vaginal pessary containing progesterone analogues like FGA or MAP, or even progesterone itself, utilizing CIDR for 12 or 14 days (RAMOS & SILVA, 2018). Exogenous gonadotropin treatment starts 2 days after vaginal pessary removal to promote follicular development and produce numerous ovulations. Similar outcomes can be obtained with protocols that include vaginal progesterone pessary for 7-8 days (DRIACOURT, 2001).

2.3.1.3 Recovery of oocytes/embryos

Most embryo recovery procedures are usually carried out on day 7 after standing estrus for cryopreservation or transfer (BÓ & MAPLETOFT**,** 2013). A wide variation in the development stage and embryo quality following recovery indicates that not all the embryos are normal and it signals that the pregnancy rate might be unsatisfactory (MAPLETOFT, 1986), cited by BÓ & MAPLETOFT (2013). In small ruminants, surgical, laparoscopic, or trans-cervical techniques can be used for oocytes recovery or collection (FONSECA et al., 2016). Embryo recovery can be achieved in several ways.

i. *Surgical recovery technique:* In this case, embryos are retrieved either from the oviduct *(most efficient in super-ovulated ewes)* or the uterus (RAMON-UGALDE et al., 2008). This technique has certain drawbacks, including high equipment cost, stress to the animal, adhesions, and a decline in the number of embryos recovered in the subsequent recovery programs (FONSECA et al., 2016).
ii. *Laparoscopic ovum pick-up:* This is a minimally invasive procedure for recovering oocytes in small ruminants and doesn't affect the productive and reproductive performance of the ewe (GORDON, 2017). It leads to lesser adhesion and enables the continuous collection of oocytes (FONSECA et al., 2016).
iii. *Non-surgical embryo recovery technique:* Successful non-surgical recovery in small ruminants was first achieved in the 1980s. It is a much simpler technique and can be

performed with the sedated animal standing (FONSECA et al., 2016). It leads to fewer adhesions and produces viable offspring in goats (FONSECA et al., 2014).

2.3.2 *In vitro* embryo production

Oocyte maturation, fertilization, and embryo culture are the three significant multi-steps of *in vitro* embryo production (PUGH et al., 1991). The *in vitro* embryo production technique enables the application of technologies such as transgenesis and cloning. Ova from slaughterhouses has been a vital source for *in vitro* embryo production (SREENIVAS et al*.,* 2014). The technique improves the genetics of potential parents by combining selected gametes of elite male and female animals (PARAMIO & IZQUERDO, 2016). The significant steps involved in the *in vitro* embryo production technique are detailed below.

2.3.2.1 Cumulus oocytes complex recovery and best quality oocytes selection methods

Recovering cumulus-oocyte complexes (COCs) in small ruminants for *in vitro* embryo production with or without hormonal therapies is possible. When hormones are employed, follicular growth is encouraged, increasing follicle availability and diameter, which eases aspiration. This can increase the number of follicles aspirated by 2 to 4 each session, which improves embryo recovery, maturation, and *in vitro* production (RAMOS & SILVA, 2018). The COCs can be aspirated during laparotomy or laparoscopy, retrieved from ovaries collected from slaughterhouses, or ovaries removed during ovariectomy. The abattoir/slaughterhouses are the most readily and easily used source of COCs. Three basic methods are commonly used to retrieve the COCs from the collected ovaries: slicing, puncture, and aspiration. The first two methods give a higher number, while the last shows a higher percentage of good-quality COCs in sheep **(**WANI et al., 2000). Ovariectomy, laparotomy, and laparoscopy methods are applied in living animals. Due to its less intrusive nature and repetition in a single animal within a short time, the latter procedure is the one that is most usually employed. This method permits using genetically superior animals, prepubertal, pregnant, and those in seasonal anestrous or unsuitable for traditional reproduction (RAMOS & SILVA, 2018). Follicular aspiration hormonal treatments include follicle growth stimulation without ovulation and follicle growth induction with FSH and eCG, and they may or may not employ progestogen. It is possible to utilize the FSH or FSH: LH in many doses, a single dosage, and with or without the eCG. FSH

can quickly stimulate the initial follicular growth, and eCG can allow the follicles to continue growing until aspiration. The eCG is applied as a single injection 48 hours before aspiration, and the association with FSH can be achieved by using 80 IU of FSH with 300 IU of eCG, administered 36 hours beforehand (RAMOS & SILVA, 2018). The number and quality of oocytes retrieved are influenced by the presence or absence of corpus luteum *(higher in ovaries with no corpus luteum)* but do not affect the maturation and subsequent embryonic development. The COCs quality is the major factor affecting oocyte quality (TALUKDER et al., 2011; WIDYASTUTI et al., 2017).

The capability of oocytes to support embryonic development and maturity varies, which is termed "developmental competence" or "oocyte quality" (READER et al., 2017). The *in vitro*-derived oocytes have lower developmental competence than their *in vivo* counterparts at the maturation. Therefore, the knowledge of oocyte quality is crucial for selecting the most likely to develop (WU et al., 2007). The health and oocyte quality mainly determine the embryo developmental competence. It is crucial to evaluate the developmental and fertility potentials and ensure that the oocytes have the requisite health and quality attributes (READER et al., 2017; HOSHINO, 2018). Oocyte selection for IVF and ICSI is often based on morphological characteristics of the cumulus cells, polar body, and cytoplasm (HOSHINO, 2018; WANI et al., 2013). It is generally recognized that the oocyte is a particular and highly specialized cell that is in charge of establishing, regulating, and maintaining the embryonic genome as well as assisting in fundamental functions, including cellular homeostasis, metabolism, and cell cycle progression in the early embryo (MTANGO et al., 2008) cited in (HOSHINO, 2018). Depending on the quantity and homogeneity of cumulus cells and the oocytoplasm, WANI et al. (2013) classified COCs into three groups.

- Good oocytes: include the oocytes with several cumulus cell layers and homogeneous cytoplasm.
- Fair oocytes: are those with thin or partial layers of cumulus cells and homogeneous cytoplasm.
- Poor oocytes: comprise of the oocytes with insufficient or no cumulus cells.

They concluded that a better outcome is by using good and paired COCs. Moreover, the size of oocytes (large ≥ 2mm or small <2mm) affects the maturation, cleavage, and morula rates of sheep embryos but does not have a significant effect on the fertilization rates (WANI et al., 2013). Therefore, larger oocytes have better developmental competence than smaller ones.

2.3.2.2 *In vitro* maturation of retrieved cumulus oocytes complexes

In vitro maturation (IVM) involves the process of initiating the breakdown of the germinal vesicle (GVBD) and completion of the 1st meiotic division *(nuclear maturation)* (TALUKDER et al., 1970). The expected embryo's quality is primarily dependent on the oocyte quality. The suitable maturation medium and methods aid in achieving good MII oocytes. The immature oocytes must go through cytoplasmic and nuclear maturation to become fertile. Oocytes then extrude the first polar body and enter metaphase II, where they are ready to be fertilized (COGNIÉ et al., 2003). ZHU et al. (2018) stated that *Tissue Culture Medium-199* (TCM-199) is the most used medium for the maturation of oocytes in most of the studies conducted in different laboratories. The TCM-i serum (13 of 25 laboratories), sheep serum (5 of 25), BSA (5 of 25), and follicular fluid (1 of 25), with one laboratory omitting the use of either serum or BSA. The most typical hormone administered to the maturation medium, kept at 38.5–39 °C, 5% CO_2 for 20–24 hours, is a mixture of FSH and LH or 17-estradiol.

Finally, the developmental competence of the frozen-thawed COCs may be calculated by evaluating the proportion of apoptotic cells to normal cells after staining. WU et al. (2007) state that for double staining, the COCs must be incubated separately for 2 min at room temperature in drops of medium M2 containing 2 g/ml Hoechst 33342 and 5 g/ml propidium iodide (PI) after being subjected to BCB staining. To see the COCs under a Leica DMLB microscope, they are placed on a glass slide, crushed with a coverslip, and individually rinsed in drops of fresh M2 after incubation. A non-invasive method is to measure the COCs' enlargement 24 hours after the IVM process is finished (TALUKDER et al., 1970).

2.3.2.3 *In vitro* fertilization of the matured oocytes

Frozen-thawed spermatozoa are mostly used for IVF, but epididymal spermatozoa can also be used without detrimental effects (WANI et al., 2000). As both oocytes and sperm cells are utilized in this process, the procedure's success depends on the quality. The first step here is selecting high-quality spermatozoa, which is mainly achieved by either of the methods; swim-up or percoll gradients method (ATALLA et al., 2019), or another convenient technique. The prepared spermatozoa (1 or 2×10^6 spermatozoa/ml) and matured oocytes are co-incubated in an incubator for 16-20 hours at 39 °C and 5% CO_2 in humidified air (GARCIA-GARCIA et al., 2007). After co-incubation, the presumptive zygotes are stripped of COC and washed for IVC using a washing and equilibrated IVC medium.

2.3.2.4 *In vitro* culture of the zygotes

The culture stage of the IVEP is the longest (6-8 days) and has a significant impact on the birth weight, viability, pregnancy rate, fetal development, and embryo developmental competence (EDC) (ZHU et al., 2018). Sheep zygotes are cultured in SOF with BSA and amino acid supplements, and this mixture serves as the foundational culture medium for sheep embryos. The oxygen content is controlled at 5% (5% O_2, 5% CO_2, and 90% N_2) to lessen the likelihood of oxidation. A blastocyst is formed between days 6 and 8 of culture. The embryo culture medium is typically renewed every 48 hours or applied in a two-step culture process; in the first three days, a solution composed of SOF+AA+BSA is used to culture the embryos. They are then transferred to a new solution of SOF+AA+glucose+bovine/ovine serum or BSA on day four, where they are cultured in the medium until day eight (ZHU et al., 2018). Culturing sheep embryos in FCS-supplemented media enhances the number of cells/blastocysts. However, it significantly affects the expression of certain mRNA some imprinting genes and serum addition was observed to result in embryos with "large offspring syndrome" (LOS) after the transfer (FERNÁNDEZ-GONZALEZ et al., 2004; ZHU et al., 2018). IVF Bioscience, UK, released a complete serum-free, ready-to-use media suite for all the stages of maturation, fertilization, and culture in 2013. It comprises a synthetic serum substitute and BSA (HYTTEL et al., 2019).

2.3.3 Embryo quality assessment

The quality of embryos can be determined by examining them with a stereomicroscope or magnifying lens (×50-100 magnification). They are rated based on their physical characteristics; the integrity of the embryo's zona pellucida and the length of time post-insemination are used to determine their quality (RIZOS et al., 2002; BÓ & MAPLETOFT., 2013). Several criteria that can be used to enhance embryo development were highlighted by JONES et al. (2001). They include pre-implantation genetic testing, morphological evaluation, pronuclei expression, nucleoli orientation, ovarian/follicular vascularity, non-invasive assessment of metabolic products produced by developing embryos, and pace of blastocyst and embryo growth. BÓ & MAPLETOFT (2013) found that the stage of development with what it should be on a specific day following ovulation is the best indicator of an embryo's survivability. For evaluating EDC in humans, GEBER et al. (2001) posted two criteria which include the developmental rate *(odd number of blastomeres indicates slower division rate)* and

morphology *(thinner zonae and similar size blastomeres with little or no fragments indicate good developmental competence)*.

BÓ & MAPLETOFT (2013) classified bovine embryos using number codes based on their developmental stage (Code: 1-9) and quality (Code: 1-4).

A. Development stage evaluation code

- Code 1: Unfertilized oocytes or 1-cell embryo,
- Code 2: 2-cell embryo
- Code 3: Morula (at least 16-cells),
- Code 4: Compact morula (60-70% of the perivitelline space is occupied by the embryo mass),
- Code 5: Early blastocyst (70-80% of the perivitelline space is filled up with embryo, and the inner cell mass and the trophoblast cells are difficult to be differentiated),
- Code 6: Blastocyst (Embryo occupies a more significant percentage of the perivitelline space, blastocoel is formed, trophoblasts with a dark and compacted inner cell mass).
- Code 7: Expanded blastocyst (dramatically increased in embryo diameter and a thinner zona pellucida)
- Code 8: Hatched blastocyst (embryo is hatching out of the zona pellucida or completely hatched with a spherical and well-defined blastocoel or collapsed).
- Code 9: Expanding hatched blastocyst.

B. Quality evaluation code (Embryo morphology and integrity)

- Code 1: Excellent or good or freezable embryos (embryos have regular and sphere-shaped with uniform-sized blastomeres, color, and density. The cellular material should be 85% intact and viable embryonic mass. Intact and smooth zona pellucida, with no concave or plat surface that may cause the embryo to stick to the petri dish or straw surface).
- Code 2: Fair or transferable embryos (embryos have moderate irregular-shaped embryonic mass with fewer irregular size, color, and density of individual cells and have at least 50% intact embryonic mass).
- Code 3: Poor (significant irregular shape of the embryonic mass, size, color, and density of individual cells with 25% intact embryo mass).
- Code 4: Dead or degenerating.

2.3.4 The techniques of embryo cryopreservation

The total number of embryos produced sometimes exceeds the number required for transfer. Hence, research funding was raised to create an effective procedure for the cryopreservation of the surviving embryos (SANCHES et al., 2017). Whittingham, Leibo, and Mazur were the first to report the successful deep freezing of mouse embryos, culminating in the creation of viable young using a slow freezing technique. This opened the door for the cryopreservation of mammalian embryos in 1972 (KASAI & MUKAIDA, 2004). Cryopreservation involves:

- Suspending cells in an appropriate solution.
- Cooling them.
- Storing them in liquid nitrogen (LN_2).
- Warming them to ambient temperature.
- Reintroducing them to a physiological solution.

Cells are susceptible to a variety of damage at each stage of this process. With chilling and reheating, the development of intracellular ice causes primary injury (KASAI & MUKAIDA, 2004). Different cryoprotective agents are used to reduce cryoinjuries. Others are used to achieve cryopreservation with varying levels of success depending on their type and concentration, cryopreservation methods *(vitrification or controlled freezing)* species, oocyte source (*in vitro* or *in vivo*), the embryonic stage of development *(morula or blastocyst)*, and the protocol *(use of single or multiple permeable cryoprotectants and/or single or multiple steps removals)* adopted (MARTINEZ & MATKOVIC, 1998; (GARCIA - GARCIA et al., 2007). The *in vitro*-produced embryos present greater sensitivity to cryopreservation, a significant limitation of the cryopreservation technique (SANCHES et al., 2017). Choosing the correct technique and best quality COCs can improve the technique's success rate.

A. Cryoprotectants

Cryopreservation involves suspending cells in an appropriate solution, cooling them, storing them in LN_2, warming them to ambient temperature, and reintroducing them to a physiological solution. Cells are susceptible to various damage at each stage of this process. With chilling and reheating, the development of intracellular ice causes primary injury (BOONKUSOL et al., 2006). In addition, the cryoprotectant introduces additional injury-

causing factors, such as the agent's chemical toxicity and the cells' osmotic over-swelling after removing of the penetrated cryoprotectant. Embryos are frequently exposed to a hypertonic solution containing sucrose during removal, and in rare instances, osmotic over-shrinkage can damage the embryos (KASAI & MUKAIDA, 2004). The risk of cryoinjury can be considerably decreased by using strongly penetrating cryoprotectants. As a result, all vitrification solutions must contain a permeating agent. The following are some of the most popular penetrating cryoprotectants used to protect the intracellular organelles (KASAI & MUKAIDA, 2004).

i. Dimethylsulphoxide (DMSO)
ii. Acetamide
iii. Propylene glycol (PG)
iv. Glycerol (GLY)
v. Ethylene glycol (EG)

B. Macro molecules and small saccharides as supplementary components

In addition to permeating cryoprotectants, there are non-permeating macromolecules and small saccharides. The former makes little contribution to the osmolality of the solution. Simultaneously, the latter *(mono or disaccharides)*, smaller non-permeating molecules, have significant osmotic effects (KASAI & MUKAIDA, 2004). Including a macromolecule may aid the vitrification of a solution and would permit reducing the permeating agent's quantity needed to vitrify the solution while reducing its toxicity (KASAI**,** 2002). Others include polyvinylpyrrolidone (LEIBO & ODA, 1993) and BSA (RALL, 1987) are also used as macromolecules. Ficoll 70 offer the benefits of being less poisonous, more soluble, and viscous than other macromolecules (KASAI et al., 1990). So, the non-permeating agents are less toxic than the permeating agents.

C. Small saccharide

They are used in cryopreservation to promote dehydration and aid in vitrifying the intracellular components. It decreases the quantity of permeating cryoprotectants and their toxicity. It was discovered that adding sugar lessened a solution's toxicity considerably (KASAI et al., 1990). Moreover, adding a small saccharide will aid in preventing excessive cell swelling while removing the permeating cryoprotectant. The most prominent saccharides are glucose, galactose, trehalose, and sucrose. They are almost non-toxic when used at lower temperatures, yet they can be dangerous at higher temperatures (KASAI & MUKAIDA, 2004).

2.3.4.1 Conventional slow-rate freezing technique

Before loading the straws, the programmable freezer is first pre-cooled to begin the freezing process. After that, the straws are inserted into the freezer and chilled at a rate of -1 ºC/min to -6 ºC or -7 ºC. After that, samples are seeded to cause the permeable cryoprotective agent solution around the embryos to develop ice crystals, which furthers cellular rehydration. The temperature is then maintained there for 10 minutes. Then it is reduced at a rate of 0.3 or 0.5 ºC per minute to -30 or -35 ºC and finally, kept at -34 °C or -38°C for 2 minutes at -0.1°C/min before submerging in LN_2 (YOUNGS, 2011; MARTINEZ & MATKOVIC, 1998). The cytoplasm of the embryos and the concentrated extracellular portion in which the embryos are submerged will change into a glassy solid without forming ice when samples are cooled in LN_2 after being sufficiently dehydrated; this process is known as vitrification. As a result, vitrification in an aqueous solution refers to ice-free solidification, whereas freezing refers to ice production. Hence, since intracellular ice is lethal, embryos must be vitrified even during gradual freezing. At about -130 °C, the glass transition temperature, the cytoplasm becomes vitrified (KASAI & MUKAIDA, 2004). The slow-freezing technique has been successfully utilized to cryopreserve mammalian embryos from various species, primarily in mice, cattle, and humans. This method's drawback is that it takes a long time to chill and needs a programmable freezer to regulate the cooling pace (KASAI & MUKAIDA, 2004). The viability, pregnancy, and lambing rates of frozen-thawed sheep embryos are presented in *Table 4*.

Table 4.

Viability, Pregnancy, and lambing rates of *in vitro* and *in vivo* frozen-thawed sheep embryos

Embryo developmental stage at freezing	Cryoprotectants	Viability (%)	Pregnancy/ Lambing rate (%)	Sources
		In vitro		
Blastocyst	10% EG	30.0	-	BHAT et al. (2015)
	10% DMSO	18.6	-	
	10%EG+DMSO	20.0	-	
Blastocyst and expanded blastocyst	EG (1.5)	40.0^{H}	27.6^{P} 23^{L}	(MARTINEZ et al., 2006)
	GLY (1.4M)	20.0^{H}	-	
Morula and Blastocyst	EG (1.5M)			
		In vivo		
Morula and Hatched blastocyst	E.G. (1.5M)	72.8	73^{P}	MCGINNIS et al. (1992)
Blastocyst	EG (1.5M)	83.7	-	GARCIA - GARCIA et al. (2007)
Morula, Blastocyst, and expanded blastocyst	EG (1.5M)	54.0	-	VARAGO et al. (2014)
Compacted morula and Blastocyst	EG (1.5M)	75.4	51.3^{P}	(MARTINEZ & MATKOVIC, 1998)
	GLY (1.4M)	70.0	40.2^{P}	
Morula and Blastocyst	EG (1.5M)	45.5	62.2^{P}	(COCERO et al., 1996)
	GLY (1.4M)	27.7	47.2^{P}	

DMSO: Dimethyl sulfoxide; EG: Ethylene glycol; GLY: Glycerol; P: Pregnancy Rate, L: Lambing Rate.

2.3.4.2 Vitrification technique

The vitrification technique ensures the solidification of cells and the entire solution without forming ice crystals. Embryos vitrification is more advantageous than slow-freezing because ice-related damages are less, and embryo survival rates are higher, mainly when optimized treatment is used. Moreover, embryos can be cryopreserved quickly and easily without a programmed freezer (KASAI & MUKAIDA, 2004; SANCHES et al., 2017). Nevertheless, vitrification solutions must have a high concentration of permeating cryoprotectants, which might be detrimental to the sample due to the toxicity of the substances (KASAI & MUKAIDA, 2004).

The solution's higher cryoprotectant concentration (4-8mol/l) is the primary cause of cellular toxicity. Higher doses of cryoprotectants are used to avoid the formation of extracellular ice, which is the major drawback of this method. However, using vitrification, different embryos may now be cryopreserved with little to no viability loss (BOONKUSOL et al., 2006). Cytochalasin-b (CB) vitrified zygotes had a lower blastocyst rate (54%) than vitrified zygotes without CB (61.0%) (BOONKUSOL et al., 2006). According to KASAI & MUKAIDA (2004), the permeating agent is a necessary component, and the vitrification solutions may be divided into four types/categories.

a. Solution containing permeating agent(s),
b. A solution made up of a permeating agent(s) and macromolecule,
c. A solution composed of permeating agent(s) and a small saccharide.
d. A solution comprising the three earlier mentioned components.

A new cryopreservation protocol of Solid Surface Vitrification ensures effective cryopreservation of morula and blastocyst-stage embryos (BARANYAI et al., 2005).

2.3.5 Thawing and rehydration of cryopreserved embryos

Embryo thawing resembles semen thawing, with minor differences. The embryo-containing straws are removed from the LN_2 Dewar, kept at RT for 3-5 s (to lessen the possibility of the zona pellucida shattering), and then submerged in a 37 °C water bath for 25 to 30 seconds. Before being transferred to a recipient (surrogate) female, embryos that have been cryopreserved in GLY are submerged for 10 minutes in a 1 M solution of sucrose to remove the cryoprotective agent. Nevertheless, embryos cryopreserved in EG can be delivered straight to a recipient's uterus (YOUNGS, 2011). Embryos cryopreserved in EG can be thawed and rehydrated by exposing the frozen straws to RT for 10 s, then maintained in a water bath at 27 ^{o}C for 20 seconds. The embryos can be rehydrated sequentially in three (3) different solutions PBS+20%FCS+1%AA containing 1.0, 0.5, and 0.0M of sucrose (10 min/step) (MART´INEZ et al., 2006).

2.4 Embryo transfer in sheep

Embryo transfer (ET) aids in multiplying the population of a specific elite livestock breed in demand. It also permits easy importation and exportation of frozen embryos instead of live

animals (GORDON, 2017). The two primary embryo transfer methods to the recipients are laparoscopy and laparotomy, which result in a higher pregnancy rate (SHIN et al., 2008). In sheep, ET is mostly done surgically, which involves exteriorizing the reproductive tract, leading to surgical trauma and sometimes post-operative adhesions in the oviducts, uterus, and ovaries (ISHIDA et al., 1999). The two most challenging aspects of the embryo transfer technique are selecting the embryos with better developmental competence to ensure successful uterine implantation and the possibility of multiple pregnancies linked to the number of transplanted embryos (JONES et al., 2001). Recipients have to be deprived of feed for 48h, then suspended on a laparotomy cradle at an angle 45^{o} (SHIN et al., 2008). The laparoscopic technique is the most widely used technique for embryo transfer, and two embryos are usually transferred to the recipient's uterine horns with the ovary containing the corpus luteum (COTTLE, 2010). One exciting part of the ET technique is that the surrogate transfers her immunities to the foetuses, enhancing its ability to adapt to the local environment (WILSON, 1988).

The major limiting factors to the massive adoption of the ET technique in sheep industries are mainly related to the high cost of the procedure, lack of available pedigree and performance data, variability in superovulation protocol/treatment and complicated anatomy of the ewes reproductive tract (BARTLEWSKI, 2019).

2.5 Pregnancy diagnosis

Early and accurate detection of pregnancy provides the stockmen with several advantages ranging from re-breeding/re-inseminate the open or non-pregnant animals, grouping the animals according to their fetal numbers, thereby adjusting their nutrition, assessing the efficiency of the AI program or reproductive performance of ram, and possible diseases associated with the animals (REDDEN & PASSAVANT, 2013). Either of these methods, hormonal or non-hormonal assay can diagnose pregnancy in small ruminants. Radioimmunoassay and enzyme-linked immunosorbent assay are used for the hormonal assay to check estrone sulfate and progesterone concentrations (RAMON-UGALDE et al., 2008). Ultrasonography is used for the non-hormonal assay (REDDEN & PASSAVANT, 2013; MUNSI et al., 2019). With ultrasonography, either of these methods, the trans-rectal or the non-invasive trans-abdominal method, is used. Diagnosing the pregnancy in small ruminants as early as day 20 following natural service or AI is possible. Some practitioners are comfortable diagnosing at days 30-35, but most prefer to schedule the initial examination at day 45. At that

moment, the fetus and the placental structure are well developed. The fetal heartbeat is easy to determine, and the chances of losing the pregnancy are minimal. Most pregnancy diagnoses are made from the right-side flank to avoid image obstruction by the rumen. The probe is positioned in the pelvis dorsal to the bladder in the non-gravid uterus or early pregnancy.

Meanwhile, it is placed in the flank region or between the hind legs for late pregnancy. Counting the number of fetuses is best done on days 45-90; in this case, scanning should be done accurately, patiently, and on both sides of the plank (WIERMAN, 2019). Early pregnancy detection on a farm can improve reproductive efficiency, thereby increasing the enterprise's profit.

3. MATERIALS AND METHODS

3.1 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

3.1.1 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 August 2022.

3.1.2 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).*

3.1.3 Testicles collection

Fourteen pairs of testes from 2-5 years healthy {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).

3.1.4 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.

3.1.4.1 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 *(Fig. 1)*.

3.1.4.2 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 *(Fig. 1)*.

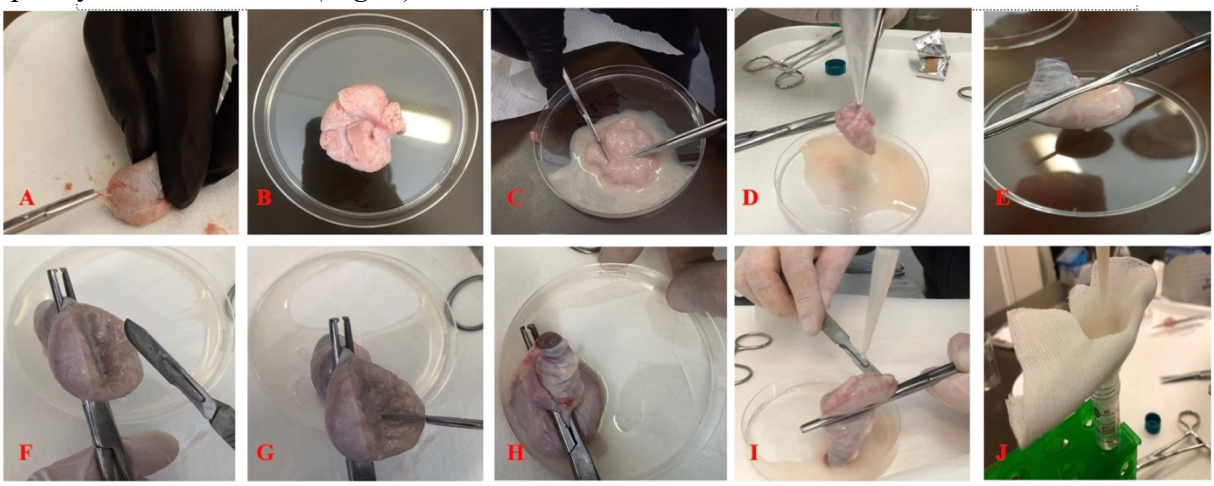


Figure 1. Epididymal sperm collection by slicing (A, B, C, D, and J) and incision (E, F, G, H, I, and J) methods. A: Striping the CE for slicing, B: Striped CE ready for slicing, C: Slicing the stripped CE, D: Rinsing the slice CE, E: Engorged CE ready for incision, F: Incising the engorged CE by deep 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).

3.1.5 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 fresh and frozen-thawed spermatozoa were assessed using a Computer-Assisted Sperm Analyzer (CASA) *(Androvision® software, Minitübe Ltd).* The samples were diluted to a concentration of $50\text{-}60\times10^6$ spermatozoa/ml 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).

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 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, while K-F-stained slides were analyzed later. The analyses, microscopical evaluations were finished later.

3.2.2 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 composition of the extenders in *Table 5.* 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 5.

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)	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%)
8	Lincomycin (15.0 mg).	Soy-lecithin (1.5 g/L)	Lincomycin hydrochloride
9	Soy-lecithin Ultrapure water	Ultrapure water (1000 ml)	Soy-lecithin Ultrapure water

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

3.2.3 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 EGERSZEGI et al. (2012) described.

3.2.4 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, 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.

3.2.5 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.

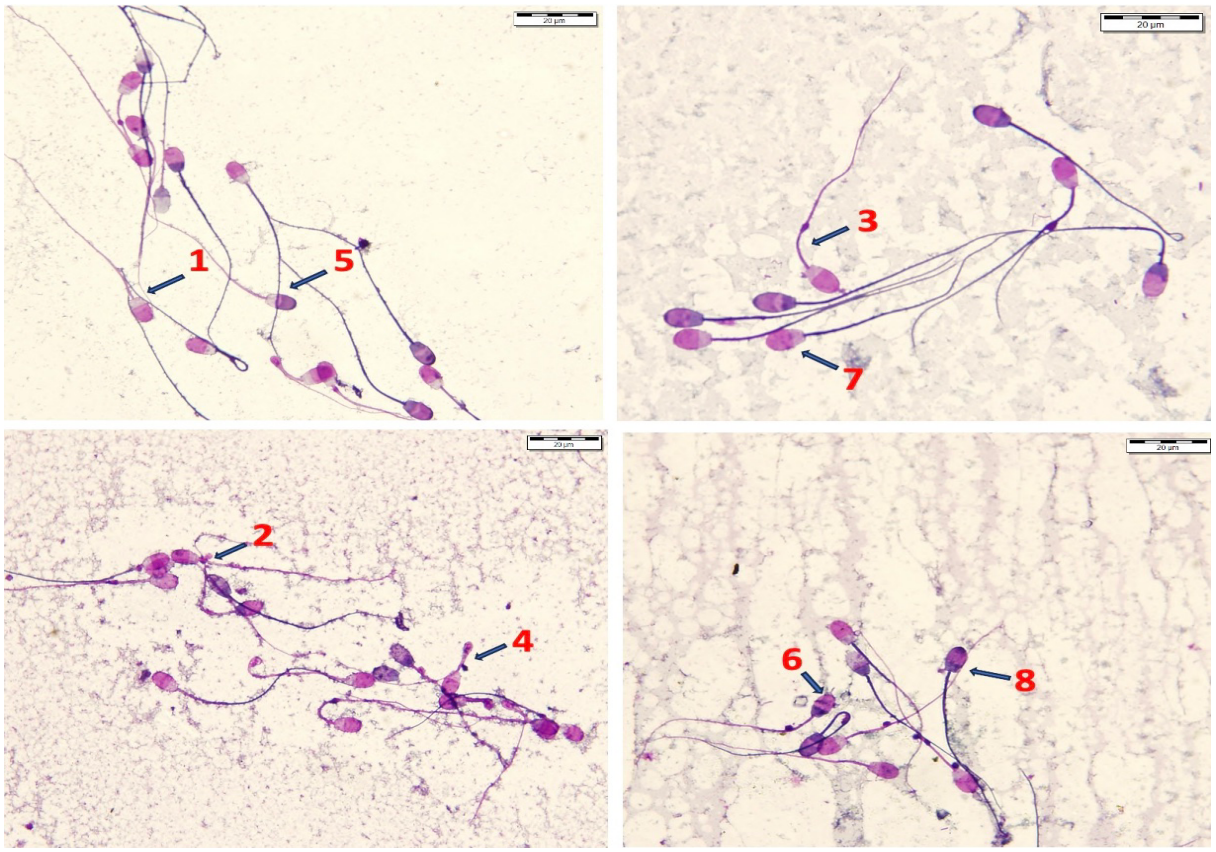
i. Standard motility and kinematic parameters assessment

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 (K-F) 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) for 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, and 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).

3.2.6 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 ^oC for 2 h)*. Freezing was done 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 ^oC for 30 s)* and assessed for standard motility and kinematic parameters. Smears were prepared for membrane integrity and morphology evaluations; *see Figure 3.*

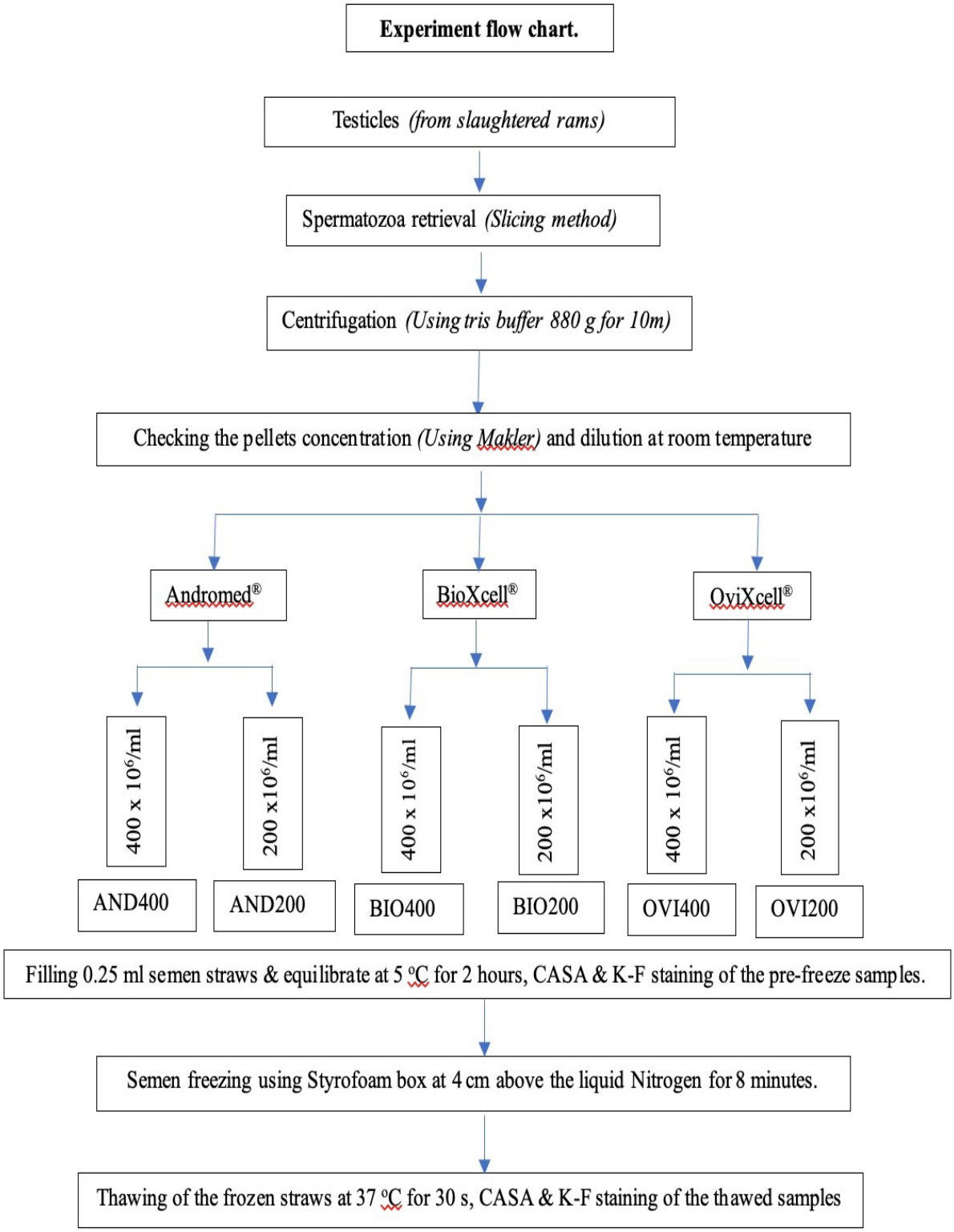


Figure 3. Flow chart of the experiment

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

3.3.1 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.

3.3.2 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 Bioscience's commercial protocol for on-site OPU.

3.3.3 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 rewashed 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 begins. They were rinsed in a 33 mm petri dish containing 2 ml of preheated (37 ºC) 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 ºC. The average number of selected COC's retrieved per ovary was calculated and presented in *Table 6*.

Table 6.

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**	

3.3.4 *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), 5% CO_2 for 22-24 h. At the end of the maturation process, the matured oocytes were then prepared for IVF.

3.3.5 *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.

3.3.6 *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 after every 24 h post-fertilization and the blastocyst rate (day 6 and 8) post-fertilization as described by WU et al. (2007).

3.3.7 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 the aid of 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 were recorded and expressed in percentage accordingly.

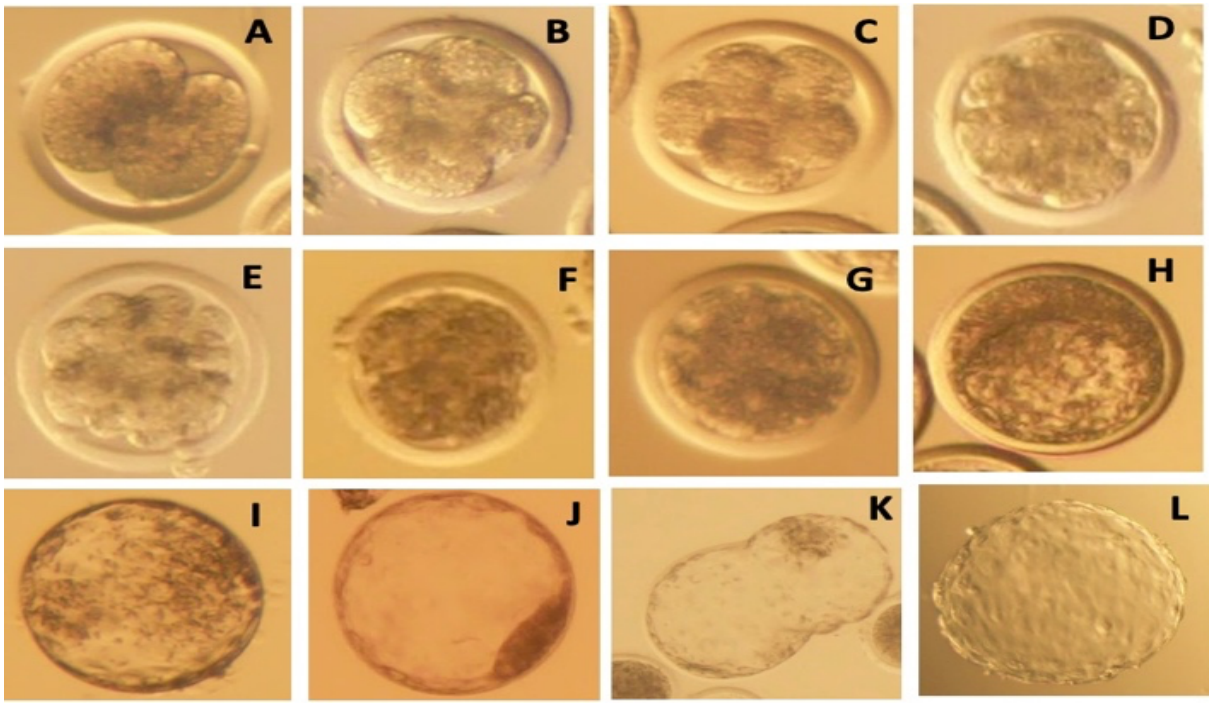


Figure 4. Developmental stages of *in vitro* produced sheep embryos. A: 2-cell, B: 4-cells, **C:** 6-to 8-cells, D: 10-to 16-cells, E: 16-to 32-cells (Early morula), F: Morula, G: Compacted morula, H: Blastocyst, I: Expanding Blastocyst, J: Expanded Blastocyst, K: Hatching Blastocyst, L: Hatched Blastocyst.

Source: (Lab work, 2022).

3.4 Data 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 student 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 per cent 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 methods mentioned above were carried out using IBM® SPSS® statistical software version 29. The embryo developmental kinetics data were analyzed with Excel and presented in simple percentages.

4. RESULTS

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

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

The efficiency of retrieving high-quality epididymal spermatozoa (EPS) is of crucial importance in the case of late processing. Spermatozoa motility and concentration are two critical factors that determine sperm function *(effective fertilization)*, with motility being the central component as it determines the fertilization potentials of an ejaculate/spermatozoa (ROBAYO et al., 2008). The purpose of the current study was to assess the effects of two EPS recovery methods *{slicing (SL) vs incision (IN)}* on fresh and frozen-thawed Merino and Hungarian Racka breed ram epididymal spermatozoa (REPS) movement characteristics and compare the fresh quality and cryo-tolerance of the two breeds.

Table 7. illustrates the effects of collection methods (SL vs 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. The weight of the testicles between the collection methods was not significantly different, signifying that any differences observed were not attributed to the variation in the testicular weight. The collection method did not significantly affect the fresh and post-thaw REPS's standard motility and kinematic parameters. However, the IN method is faster as it does not need a visceral layer of tunica vaginalis removal, making it more practical in field conditions. Nevertheless, according to our own experience, where the tunica vaginalis communis visceral layer contains bigger blood vessels, removing the tunica and slicing method is recommended to avoid extracting bloody sperm samples. In contrast, we found it quite challenging to establish a total cell number with the SL method due to wide variations in the number of cells retrieved. This is in line with the report of EHLING et al. (2006). Our findings align with that of MARTÍNEZ-PASTOR et al. (2006) that the collection method *(cuts vs flushing)* does not significantly affect the motility, total number, and post-thaw results on Iberian red deer recovered EPS. Similarly KANG et al. (2018) reported no significant ($p>0.05$) difference in the post-thaw standard motility

parameters of Hanwoo bull EPS between the flushing and mincing methods. In contrast, in rams, the IN method gave a sample with significantly ($p>0.05$) higher total motility than the mincing method (75.0 vs 59.2) (LONE et al., 2011). Our results agreed with MOGHEISEH et al., (2022) that the EPS retrieval methods have no significant effects on the post-thaw standard motility and kinematic parameters of dog EPS. There was no significant breed and collection method interaction effect on all the parameters studied under fresh and the post-thaw conditions.

Table 7.

Effects of collection methods on testicular weight, 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 (µm/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).

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

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 the effects of breed 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 8*.

The Merino breed presented significantly ($p>0.05$) higher testicular weight than the Racka breed (260.50±11.8 vs 170.82±10.1 g). This might be due to differences in testicular size/scrotal circumference or body weight between the breeds (ALLAOUI et al., 2014). Moreover, it was established that ram testicular diameter and scrotal circumferences were significantly ($p<0.01$) correlated with testicular weight (ENDALE et al., 2009). The lowest and the highest fresh TM and PM recorded in the current study (78.50±2.8 to 83.64±2.2%) and (68.93±3.3 to 73.00±3.1%) were slightly higher than that of KAABI et al. (2003), 78.4 and 64.7% retrieved 24 hrs after animal death but lower than what was reported by RAHIMIZADEH et al. (2021), 95.89 and 90.40% *(recovered few hours after the animals were slaughtered)*, respectively. This might be due to the differences in the REPS retrieval season and time after slaughtering. BERGSTEIN-GALAN et al. (2018) reported that the PM and viability of REPS declined significantly at 12 and 48 hrs after the animal death, respectively. The breed has no significant ($p>0.05$) effects on the fresh TM and PM, and this is in contrast with the result of VOZAF et al. (2022), who compared Slovak Dairy, Native Wallachian, and Improved Wallachian ram breeds. Similarly, KASIMANICKAM et al. (2007) reported that breed *(Polled Dorset vs Suffolk vs Katahdin)* affects the progressive motility of chilled-stored electro-ejaculated ram spermatozoa. The Racka breed EPS were moving straighter than EPS of the Merino breed, as indicated by their higher LIN, STR, and lower ALH values. This contrasted with KASIMANICKAM et al. (2007) results that showed that breed does not have significant effects on the kinematic parameters of chilled stored electro-ejaculated ram spermatozoa. Moreover, the spermatozoa of both breeds were rapid since a VAP > 50 μm/s were categorized as rapid-moving spermatozoa (GOOVAERTS et al., 2006).

Following thawing, all the standard motility parameters were not significantly different ($p>0.05$) between the breeds as in the fresh. The post-thaw TM of Merino 44.20±6.47% recorded was lower than that reported by ÇOYAN et al. (2011) in Merino AV collected spermatozoa; 50.9±3.1%, but our PM was higher 30.00±6.97 vs 19.6±1.1% respectively. In Racka, our TM (39.14±4.48%) and PM (27.14±3.82%) were lower than what was reported in the same breed by EGERSZEGI et al. (2012), 60.86±5.44% and 52.86±6.12% respectively. It might be due to the differences in the collection season. Our results agree with VOZAF et al. (2022) that the ram breed does not have significant effects ($p>0.05$) on post-thaw standard

motility parameters of three ram breeds, but contrasts the findings of TOHURA et al. (2019) that the breed has significant effects ($p<0.05$) on the motility of bull spermatozoa. Similarly, most of the differences in the kinematic parameters of the two breeds remain the same as in the fresh except for STR, which was significantly higher in Racka in the fresh and not significantly different between the breeds after thawing. In addition, the Racka breed presented significantly ($p<0.05$) higher LIN and BCF values in both fresh and post-thaw kinematic parameters than the Merino breed. This shows that the Racka breed EPS have better linear movement than the Merino breed EPS. Higher sperm swimming velocities determine male fertilizing ability (MALO et al., 2010), while higher BCF and lower ALH of the sperm head could facilitate the penetration of zona pellucida (GOOVAERTS et al., 2006). Similarly, LIN was reported to impact the cleavage rate following IVF positively and is a significant predictor of cleavage rate (GARCÍA-ALVAREZ et al., 2009).

Table 8.

Effects breed on testicular weight, 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]	0.0001	-	-	
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 (µm/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±07[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 superscripts within a form (fresh or post-thaw) differ significantly. Testicles number: Merino (n=14), Racka (n=14).

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

Table 9 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 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. Moreover, the Merino and Racka REPS had statistically the same cryo-tolerance ($p>0.05$). Our result contradicts ANDREEVA et al. (2017) that the breed *(Synthetic Population Bulgarian Milk vs Ile de France)* significantly affects spermatozoa cryo-tolerance. Similarly, we could not observe significant interaction effects ($p>0.05$) between the breed and the collection methods on all the parameters studied. This is because the collection methods were not significantly ($p>0.05$) different.

Table 9.

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 (μm/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).

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

4.2.1 General parameters of ram epididymal spermatozoa retrieved from different breeds

In the current study, we determined certain parameters related to the ram testicles and cauda epididymis (CE) weight and concentration of the spermatozoa retrieved from rams of three different breeds (*Table 10*). It is worth noting that many factors affecting the success of REP's cryopreservation have not been extensively studied as in AV and EE-collected ram spermatozoa (AKÇAY et al., 2012; KULAKSIZ, et al., 2012; SALAMON & MAXWELL, 2000). Among these include the pre-freeze dilution rate and the type of diluents used, particularly the readily available commercial soy-lecithin-based ones. On this note, the current study attempts to investigate the earlier-mentioned factors on the freezability of post-mortem REPS. Other researchers reported that a high dilution rate is problematic as the motile spermatozoa will stick to the glass, leaving the non-motile cells in the medium (LEAHY et al., 2010). Furthermore, the diluents/extenders were reported to affect the freezability of EPS in different species (ÁLVAREZ et al., 2012; LONE et al., 2012; MAMANI-MANGO et al., 2019). Moreover, the animal-based semen extenders were reported to contain variable compositions with high microbial contamination risk, reducing spermatozoa's post-thaw viability and acrosome integrity compared to the plant-based extenders (FERNANDES et al., 2021). Hence, it is important to identify the ideal commercially available soy-lecithin-based diluent and spermatozoa concentration for freezing REPS.

The average weight of the testes and the CE processed in this study, 157.78±22.15 and 14.25±1.38 (g), were slightly lower than what was reported by KAABI et al. (2003); 191.11±4.9 and 18.14±0.4 (g), respectively. However, our results presented higher values of standard error, which might be attributed to individual animal differences due to age and genetics, season, and breed effects.

Table 10.

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 mean**,** n=28.

4.2.2 Effects of three different commercial soy-lecithin-based semen extenders and two spermatozoa concentrations on the pre-freeze standard motility and kinematic parameters of 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 11*. It is important to note that the kinematics are important in determining spermatozoa functionality and freezing/thawing success (VAN DER HORST, 2020). Spermatozoa with high PM results in higher BCF and lower ALH (VAN DER HORST, 2020). Similarly, the VAP is preferred over PM in predicting fresh and post-thaw bull spermatozoa fertilizing potentials (NAGY et al., 2015). Moreover, the kinematic parameters show relatively high breed similarities in sheep; however, specific kinematic parameters like the VCL might vary even between individual sperm from 50 to 320 μm/s in a single field of analysis, and the spermatozoa sub-population with the highest velocities have higher cervical mucus penetration and fertilization rate (VAN DER HORST, 2020), with VCL and VAP being the only kinematic parameters that showed a significant positive correlation with cervical mucus penetration in sheep (ROBAYO et al., 2008) and litter size in pigs (HOLT, 2000). The pregnancy rate in sheep has a strong and significant positive correlation with the spermatozoa PM and VAP (r=0.62), LIN (r=0.86) and STR (r=0.55) but negatively correlated with VCL (r= -0.65), while the average litter size with LIN (r=0.87) and STR (r=0.77) (SINAPOV and YOTOV, 2023).

The current study demonstrates no significant ($p>0.05$) interaction between the extender and the spermatozoa concentrations on all the parameters. Similarly, no difference was observed among the three commercial soy-lecithin-based semen extenders, AND®, BIO® or OVI® and between the spermatozoa concentrations (200×10^6 spermatozoa/ml and 400×10^6 spermatozoa/ml on standard motility parameters of pre-freeze REPS which correspond with the results of DAŞKIN et al. (2011). The highest values of the TM and PM for the pre-freeze

samples reported in the current study were in AND®; 72.22±3.2 and 64.89±3.4% and were similar to that of KAABI et al. (2003); 78.4 and 64.7%, who retrieved the spermatozoa at 24 hrs post-mortem. We could not observe any difference between the pre-freeze spermatozoa concentrations on all the parameters.

On the pre-freeze kinematic parameters, the BCF was the only parameter significantly ($p<0.05$) different among the extenders. The REPS diluted with the BIO® and OVI® extenders were statistically the same and had significantly ($p<0.05$) higher BCF (30.18±1.1 and 29.99±1.0 Hz) than those in the AND® extender (26.80±0.8 Hz). Higher BCF value was reported to be associated with increased fertilization rates (GOOVAERTS et al., 2006). Our results on BCF correspond with that of DORADO et al. (2007) in goat buck. All other parameters were statistically the same among the extenders and between the spermatozoa concentrations.

Table11.

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

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	71.30±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.

4.2.3 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 12. 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. Under the post-thaw condition, the current study did not observe significant ($p>0.05$) interactions between the two factors in all the parameters studied. Similarly, there were no significant differences in the post-thaw standard motility parameters among the extenders and between the spermatozoa concentrations. Our result agrees with BRAGA et al. (2007) and ONDŘEJ et al. (2019), who reported no significant difference in motility (TM and PM) parameters between AND® and BIO® extenders in the post-thaw ejaculates of bull, AKÇAY et al. (2012) in rams and RASTEGARNIA et al. (2014) in buffalo. Similarly, FERNANDES et al. (2021) reported no significant difference ($p>0.05$) in the post-thaw TM (33.7 vs 41.7%) and PM (4.6 vs 5.0%) between AND® and OVI® extenders in Portuguese Merino rams AV-collected spermatozoa. However, we recorded a post-thaw TM% range of 34.89±3.9 to 38.83±3.5%, which was comparable to that reported by AHMED (2019), 20.83±5.39 to 45.00±4.47%. Our highest post-thaw TM and PM (38.83±3.5 and 31.56±3.5%) were similar to that reported by VAHEDI & EVRIGH (2018) in AV-collected ram spermatozoa; 43.13 and 32.24% but lower than that of KAABI et al. (2003) in REPS; 64.6 and 48.8%, respectively. Regarding the spermatozoa concentrations, our result was not in agreement with that of AKÇAY et al. (2012) and NASCIMENTO et al. (2008), who reported better post-thaw parameters in AV-collected ram spermatozoa frozen at 200×10^6/ml than at 400×10^6/ml. This might be due to the differences in spermatozoa source and the extenders' composition. Moreover, D'ALESSANDRO et al. (2001) and AKÇAY et al. (2012) reported that increasing the freezing concentration to 800×10^6/ml has a greater negative influence on the post-thaw quality of ram spermatozoa.

In the kinematic parameters, 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 28.72±0.9 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%). Therefore, freezing REPS in the BIO® and OVI® extenders might lead to a higher fertilization

rate than the AND® extender. Higher BCF and lower ALH of sperm heads could facilitate zona pellucida penetration (GOOVAERTS et al., 2006) and higher VAP might lead to higher cervical mucus penetration and fertilisation rates (ROBAYO et al., 2008).

The WOB parameter depicts the degree of oscillation of the sperm head/balancing (TANGA et al., 2021). The spermatozoa concentration did not affect the parameter but differed significantly between the AND® and OVI® extenders. Our results proved that the REPS frozen in the OVI® extender have more linear trajectory and progressive movement than those in the AND® extender (ISMAIL et al., 2014). Moreover, spermatozoa with higher progression tend to have higher cryo-survival and fertilization potentials (BRAVO et al., 2011). Our results on the effects of semen extenders on the WOB parameter contradict the findings of DORADO et al. (2007) in goat bucks and rabbits (DOMINGO et al., 2019) that semen extenders have no significant effect on WOB parameter. All other kinematic parameters were not significantly ($p>0.05$) different among the extenders and between the spermatozoa concentrations.

Table 12.

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	58.60±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	56.63±2.9	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	0.664	0.486	0.566	0.400	0.815	0.250
P-value Ext.*Conc.	0.723	0.946	0.648	0.855	0.978	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} differ significantly.

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

It is well established that cryopreservation decreases spermatozoa viability, functionality and fertilizing ability (BRAGA et al., 2007; D'ALESSANDRO et al., 2001; WATSON, 2000). In the current study, we used K-F viability staining technique to evaluate the REPS's head, tail, acrosome membrane integrity and morphology. Although the technique is a subjective evaluation, it is economical as it does not require a costly device and permits the investigator to see damage/abnormalities in the spermatozoa. Using this method, the head, acrosome, and tail membranes of the sperm can be assessed separately, ensuring precise determination of the lesions' location.

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 13.* Neither the extenders nor the spermatozoa concentration significantly affected the percentage of the post-thaw REPS with IHITIA. The AND® extender had significantly ($p<0.05$) lower percentage of intact REPS 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, the 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 percentage of spermatozoa with IHDT recorded in the current study (12.81±1.9 to 23.43±4.3%) was similar to that reported by NAGY et al. (1999); 20% in bulls and 5 to 25% in boars and rams and 20% in deer (NAGY et al., 2001) and 19.0% in stallion KÚTVÖLGYI et al. (2013). The 400×10^6 spermatozoa/ml spermatozoa 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%).

Based on this study, the AND® extender preserves the REPS's tail morphology better than the BIO® or OVI® extender. Spermatozoa concentrations had significant effects ($P<0.05$) only on the percentage of all intact heads of post-thaw REPS. The 400×10^6 spermatozoa/ml spermatozoa concentration was superior in preserving the REPS's head membrane integrity

better than the 200×10^6 spermatozoa/ml (45.15±5.1 vs. 37.95±3.4%). Freezing REPS with BIO® and OVI® extenders preserved the REPS's head membrane integrity better than with the AND®. The highest value of all intact heads observed in the current study was in BIO®, 45.33±3.3% and was slightly lower than what was reported by AHMED et al. (2019), 51.38±4.44% using Eosin Nigrosine staining Technique. This might be due to the differences in the extenders and the animal used. There was no significant difference ($p>0.05$) among the freezing extenders in the percentage of post-thaw REPS with DHDTDA. Our results agreed FERNANDES et al. (2021) that there was no significant difference between AND® and OVI® extenders in the per cent post-thaw abnormal spermatozoa morphology in Portuguese Merino rams.

We supposed that the percentage of all intact cells corresponds to the percentage of "live" spermatozoa with intact cell membranes and presumably actively moving spermatozoa, while cells with damaged tails and intact heads are supposed to be not moving and hence not fertile *in vivo* (NAGY et al., 1999). The percentage of all intact spermatozoa observed in the current study ranges between 19.08±2.2 and 25.03±1.5%. This agrees with the report of SALAMON & MAXWELL (2000) that only about 20-30% of post-thaw ram spermatozoa remain biologically intact. There was no significant ($p>0.05$) difference among the extenders and between the spermatozoa concentrations in terms of the per cent all intact/live spermatozoa and among the extenders.

Table 13.

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 tails (%)
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**
Concentrations (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.

4.2.5 Effects of freezing with different commercial soy-lecithin-based semen extenders on distal cytoplasmic droplets and tail defect of ram epididymal spermatozoa

The retained cytoplasmic droplets are caused by incomplete maturation in the epididymis, leading to abnormal spermatozoa morphology, impairing viability, and capacitation in boar (HENNING et al., 2021, 2022). The per cent distal droplets were also reported to increase significantly with bulls' age (MANDAL et al., 2010) and positively correlated with ROS production in men (AZIZ et al., 2004). Additionally, the presence of distal droplets had been associated with a higher percentage of ubiquitinated protein and morphological abnormality, and it also harbored 15-lipoxygenases, which are responsible for mitochondria degradation in ejaculated boar spermatozoa (KUSTER et al., 2004; FISCHER et al., 2005). However, it is a normal organelle in EPS, and the complete absence of it indicates spermatogenesis abnormality (XU et al., 2013). Similarly, maintaining the tail/flagella integrity is very important because it aids the spermatozoa's head to achieve fertilization (VAN DER HORST, 2020). Given the above-mentioned, we analyzed the effects of freezing REPS with different commercial soy-lecithin extenders on the percentage of distal cytoplasmic droplets and tail defects.

Table 14. presents the effect of freezing using different commercial soy-lecithin-based semen extenders on distal droplets and tail defects of REPS, considering that the concentrations of 200 and 400 million spermatozoa/ml were statistically the same. The data were pooled to assess the effects of freezing REPS on all distal droplets and all bent tails and the overall effects of freezing on those parameters. Significant ($P<0.05$) differences existed between the pre-freeze and the post-thaw distal droplets and bent tails in all the extenders; 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. 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. Unfortunately, we could not get a similar study to our result. The highest post-thaw per cent all distal droplets observed in the current study (AND®: 28.17±2.9%) was comparable to that reported by TURRI et al. (2014) in goat buck (27.8%) but lower than that of KAABI et al. (2003) (55.1±5.3%) in rams under similar conditions. We observed that the bent tails increased by about the same percentage as the distal droplets decreased. This means spermatozoa's moving tails suddenly get stuck and enclose the droplet (Distal

midpiece reflex or hairpin-curved). We, therefore, speculate that the decrease in the proportion of spermatozoa with distal droplets in the post-thaw samples was not due to the freezing and thawing but due to the curving of the spermatozoa tails that stuck the droplets and making the spermatozoa to be categorized into the bent tails category. This was more in the BIO and OVI extenders than the AND extender, which resulted in a higher percentage of spermatozoa with bent tails/tail defects in the former extenders than in the latter. Similarly, the overall mean proved that freezing in general significantly (P<0.05) increases the percentage of REPS with tail defects (9.29±1.3 vs 15.15±1.2%) with a significant decrease in the percentage of spermatozoa with distal droplets (all distal droplets: 32.69±2.7 vs 23.41±1.6%). Our result on the per cent distal droplets was consistent with the findings of KAABI et al. (2003).

Table 14.

Effects of freezing and three different commercial soy-lecithin-based semen extenders on the percentage of all distal droplets and all bent tails of ram epididymal spermatozoa (Mean±SE)

Extenders	Parameters					
	All distal droplets (%)		P-values Form	All bent tails (%)		P-values Form
	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 freezing effect	32.69±2.7[A]	23.41±1.6[B]	0.0001	9.30±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 within an extender[a,b] and overall[A,B] with different superscripts differ significantly.

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

Developmental kinetics indicate embryos' ability to develop into blastocysts and i mplant (DAL CANTO et al., 2012). Understanding the kinetics of the embryo pre-implantation stages will help reproductive technologists identify the ideal developmental stage to directly transfer or cryopreserve the embryos for successful pregnancies or gene conservation purposes. It is also essential because it gives a clear understanding of fertilization and different embryo developmental stages to identify the most challenging/critical stage(s) that need improvement. One of the criteria for assessing the quality of embryos *in vitro* is assessing the developmental kinetics at 24 and 30 hours and/or hatching rate. A more than 50% 2-cell embryo within 24 hrs indicates good developmental competence (FALCHI et al., 2022). In the current study, we evaluated and detailed the developmental kinetics of the sheep embryos produced from oocytes fertilized with post-thaw REPS. The overall cleavage, morula, and blastocyst rates are presented in *Table 15.*

Within 24 hours post-insemination (pi), only 25.0% of the zygotes cleaved to 2-4 cells. This is far below the indicator of good developmental competence of 50% 2-cell cleavage rate within 24-30 hrs pi as reported by FALCHI et al. (2022). The possible reasons might be that we used abattoir-sourced gametes, and it is well established that the EPS fertility rate is lower than the AV-collected spermatozoa. At 48 h pi, 26.0% reached 6-8 cells and 17% to 10-16 cells. Between 72-96 h pi, 35.0% cleaved to 16-32 cells, while 21% 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. The overall cleavage and blastocyst rate obtained in this study were below what was reported by HAJIHASSANI et al. (2019); 71.3 and 37.0, respectively. It might be that the developmental competence was hampered because we manually took the embryos from the incubator every 24 hours to assess the cleavage rate. However, the blastocyst rate was comparable to that reported by ARDESHIRNIA et al. (2017); 21.0% and KAFILZADEH et al. (2012); 22.3%. This indicates that IVEP using post-mortem/abattoir-sourced gametes has the potential to serve as an alternative to MOET and ovum pick-up in the sheep industry, and the technique might permit the rescue of valuable genes of endangered/elite dams and sire through cryo-bank.

Table 15.

Developmental stages of *in vitro* produced sheep embryos

Hours post insemination	**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**

5. CONCLUSIONS, RECOMMENDATIONS

5.1 Conclusion

Our findings revealed that the collection method does not significantly affect the fresh and post-thaw ram epididymal spermatozoa (REPS) standard motility and kinematic parameters. The incision method seems faster and more field-friendly than the slicing method. Where the tunica vaginalis communis visceral layer contains bigger blood vessels, removing the tunica and slicing method is recommended to avoid extracting bloody sperm samples. Moreover, we observed that the Hungarian Black Racka (Racka) EPS have a straighter movement than the German Mutton Merino (Merino) in fresh and post-thaw conditions. The Merino and Racka REPS have statistically the same cryo-tolerance. The results of motility parameters are diverse in literature, and it is well documented that sperm kinematics parameters are insufficient to predict semen fertilizing ability. Further investigation and more evaluation/measurements are needed for a more profound classification of REPS. There is a need to investigate whether the sides/position of the same animal's epididymis (right or left) affects the post-thaw quality of REPS.

Ram epididymal spermatozoa can behave differently than ejaculated spermatozoa during the freezing/thawing process; the membrane structure could be more unstable, so improving and optimizing the freezing technique of REPS is needed. The BIO® and OVI® extenders showed significantly higher post-thaw VAP and BCF and were superior to the AND® extender in preserving the ram epididymal spermatozoa head membrane integrity. In contrast, the AND® extender was superior in preserving the normal tail morphology of ram epididymal spermatozoa better than the BIO® and OVI® extenders. Freezing significantly decreased the percentage of spermatozoa with distal droplets and increased the percentage of ram epididymal spermatozoa with tail defects. These phenomena could be connected. All three commercial soy-lecithin-based extenders must be optimised to preserve the viability, membrane integrity and morphology of REPS better. Ram epididymal spermatozoa are suggested to freeze in 400×10^6 spermatozoa/ml concentration as it preserves its head membrane integrity better than at 200×10^6 spermatozoa/ml.

Sheep embryos with good developmental competence can be produced using abattoir-sourced oocytes and post-thaw ram epididymal spermatozoa in ivf-Bioscience Bovine media. Therefore, based on these *in vitro* studies, the cryo-preserved ram epididymal spermatozoa and abattoir-sourced/postmortem sheep oocytes can serve as a good

insurance for conserving the genetic resources of a particular sheep breed of interest through IVF. Therefore, the relevant authorities in Hungary should kindly consider the *in vitro* gene conservation technique to conserve the genetic resources of the Native Hungarian sheep breeds.

5.2 Recommendations

There is a need to investigate whether the sides/position of the same animal's epididymis (right or left) affects the post-thaw quality or characteristics of ram epididymal spermatozoa. However, where the tunica vaginalis communis visceral layer contains bigger blood vessels, the removal of the tunica and slicing method is recommended to avoid the extraction of bloody sperm samples.

All three commercial soy-lecithin-based extenders need to be optimized. Because the BIO® and OVI® extenders increase the percentage of ram epididymal spermatozoa with tail defects compared to the AND® extender. Nonetheless, the AND® extender causes more damage to the post-thaw ram epididymal spermatozoa's head than the BIO® and OVI® extenders. So, they must be optimized to preserve the viability, membrane integrity and morphology of ram epididymal spermatozoa better. There is also the need to assess the effect of these extenders on individual sperm motility and kinematic parameters and use AI to identify the best extenders among them exclusively.

Future studies should use a time-lapse camera to identify the most precise embryo developmental stages for freezing or direct transfer to the recipient ewes. Moreover, studies should compare fresh and post-thaw ram epididymal spermatozoa fertilization rates.

6. 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 membranes 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 at 400×10^6 spermatozoa/ml spermatozoa concentration preserved sperm head membrane integrity better than freezing at 200×10^6 spermatozoa/ml concentration (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.

7. PRACTICAL RESULTS

1) Both the slicing and incision methods of epididymal sperm retrieval can be used to conserve the genetic resources of endangered sheep breeds or elite sire that die 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 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 developing *in vitro*-produced sheep embryos fertilized with post-thaw ram epididymal spermatozoa.

8. SUMMARY

The local sheep breeds (LSB) are thrifty and hardier than commercial or modern. Unfortunately, interest in breeding local sheep breeds is declining each day. A report by FAO revealed that a breed of sheep is lost daily for several years, and the trend is more in Europe and the Caucasus region. Additionally, studies revealed that indiscriminate use of assisted reproductive technologies (ARTs) in the sheep industry and mating/breeding, particularly in developing countries, resulted in a global loss of local sheep genetic resources. This calls for conserving the local sheep genotypes using cheap alternative means like the epididymal spermatozoa (EPS). It was well established that viable and functional EPS could be retrieved from diseased, castrated, slaughtered or accidentally dead animals with good pregnancy outcomes. In consequence, we got prompted that many factors reported to affect the quality and cryo-tolerance of the artificial vagina and electro-ejaculated ram spermatozoa have not been well exploited in EPS despite being a cheap alternative for gene conservation. Considering the above-mentioned, we designed and conducted three experiments to achieve the following objectives.

i. Effects of ram epididymal spermatozoa (REPS) retrieval methods *(Slicing vs. Incision)* and Breed *(German Mutton Merino vs. Hungarian Black Racka)* on standard motility *(Total and Progressive Motility)* and kinematic parameters of fresh and post-thaw REPS, and compare the cryo-tolerance of the earlier-mentioned breeds' REPS.
ii. The effects of 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.
iii. Assess the developmental kinetics of *in vitro*-produced sheep embryos fertilized with post-thaw REPS.

Our findings revealed that the collection method does not significantly affect the fresh and post-thaw REPS standard motility and kinematic parameters, but the incision method is faster and more field-friendly than the slicing method. Therefore, both methods are suitable for retrieving REPS. However, where the tunica vaginalis communis visceral layer contains bigger blood vessels, the removal of the tunica and slicing method is recommended to avoid the extraction of bloody sperm samples.

Moreover, we observed that the Racka REPS have straighter movement than the Merinos in both pre-freeze and post-thaw conditions. The Merino and Racka REPS have statistically the same cryo-tolerance.

There was no significant interaction between the extender and the spermatozoa concentrations studied under both the pre-freeze and post-thaw conditions. The ram epididymal spermatozoa diluted with BioXcell® and OviXcell® presented significantly higher BCF than those diluted with the AdroMed® extender under the pre-freeze condition. Similarly, the BioXcell® and OviXcell® semen extenders were superior to AdroMed® in preserving the post-thaw ram epididymal spermatozoa VAP and BCF, acrosomal and head membranes integrity. In contrast, the Andromed® extender was superior in preserving ram epididymal spermatozoas' tail morphology. Freezing at 400×10^6 spermatozoa/ml spermatozoa concentration preserves the head membrane integrity of ram epididymal spermatozoa better than at 200×10^6 spermatozoa/ml. Therefore, REPS's ideal freezing spermatozoa concentration is 400×10^6 spermatozoa/ml. Freezing and thawing significantly increased tail defect in ram epididymal spermatozoa but reduced the percentage of spermatozoa with distal cytoplasmic droplets.

An IVF of abattoir-sourced sheep oocytes with post-thaw REPS in ivf-Bovine bioscience media resulted in a viable sheep embryo with good developmental competence: overall cleavage rate of 43.0%, a morula of 40.0%, and a blastocysts rate of 21.0%, which was comparable to those reported in the literature. This indicates that *in vitro* embryo production using post-mortem/abattoir-sourced gametes can serve as an alternative to MOET and ovum pick-up in cryo-banking of valuable local sheep breeds. It can, therefore, be used to rescue valuable genes of endangered/elite dams and sire through embryo cryo-banks. Therefore, responsible authorities need to act urgently by providing funds/grants to support the gene conservation program in Hungary because there is no ongoing conservation program now. Moreover, the breeders of the native Hungarian sheep breeders need to be introduced to the feasible techniques and draw their attention on the importance of using assisted reproductive technologies and the *ex-situ in vitro* gene conservation.

We hope these findings will be exciting and beneficial to the sheep industries and gene conservation actors as an ideal alternative to conserving local sheep genetic resources and serve as a reference material for conserving the genes of other domestic and wildlife species.

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10. 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.
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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.
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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.
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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.
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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.
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9. **Mujitaba, M. A.**, Tokár, A., Debnár, V. J., Kútvölgyi, G., Vass, N., Bodó, S.: Developmental kinetics of in vitro produced sheep embryos.
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11. **Mujitaba, M. A.**, Bodó, S., Vass, N.: The progress of electroejaculation and semen freezing in ram artificial insemination: A review.
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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.
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13. Xayalath, S., **Mujitaba, M. A.**, Ortega, A. D. S. V., Rátky, J.: A review on the trend of livestock breeds in Laos.
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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.
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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
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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.

02 February, 2024

11. STATEMENTS

STATEMENT

I wrote this thesis in the framework of the University of Debrecen Doctoral School of Animal Science for the purpose of obtaining a doctoral degree (Ph.D.) at the University of Debrecen.

Debrecen, 20.....

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PhD candidate

STATEMENT

I hereby certify that the doctoral candidate Malam Abulbashar Mujitaba has carried out his work under our supervision within the framework of the above-mentioned Doctoral School between 2019-2024. The candidate has made a decisive contribution to the results of the thesis through his/her independent creative work, and the thesis is the candidate's independent work. We recommend that the thesis be accepted.

Debrecen, 20.....

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supervisors

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