Effects of melatonin and Single Layer Centrifugation on cryosurvival of ram semen samples

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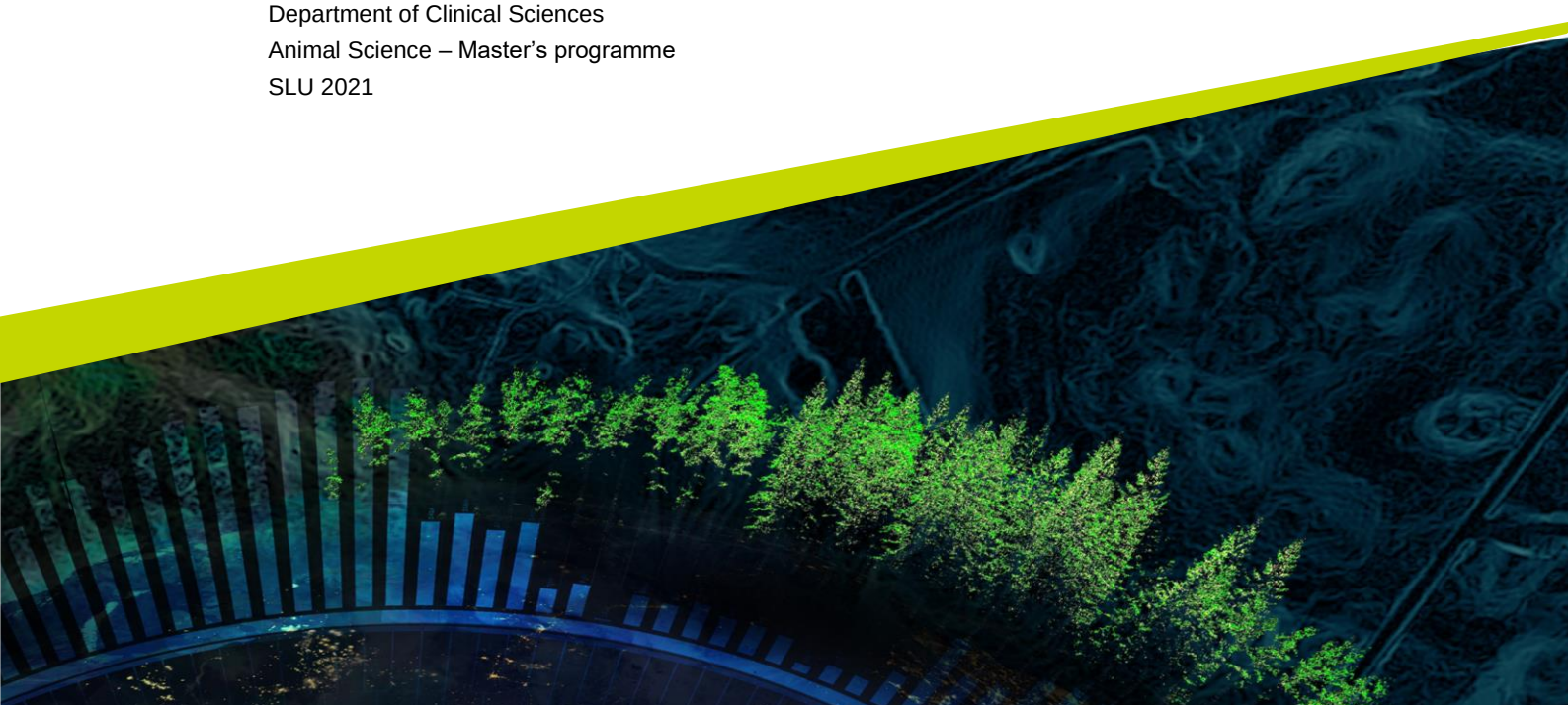

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Abstract

The composition of the ram sperm membrane renders it vulnerable to injury during cryopreservation. Production of reactive oxygen species, which are by-products of metabolism, increases during freezing and thawing, causing damage to sperm membranes and acrosomes as well as affecting mitochondrial activity. This results in a poor post-thaw yield of viable, morphologically normal spermatozoa for insemination. In the present study, we investigated the effect of melatonin addition and centrifugation through a single layer of a species-specific colloid (Ovicoll) on the quality of frozen/thawed ram semen samples.

Ejaculates from autochthonous Jezersko-Solčava rams were available for this study (n = 20). Each ejaculate was split and melatonin (1 mM) was added to the Tris-based egg-yolk-extender of one part prior to freezing. Sperm motility, viability and morphology were analysed 0, 3 and 6 hours after thawing. Membrane integrity was evaluated by HOST (hypoosmotic swelling test). Melatonin addition significantly improved sperm motility ($p < 0.05$), progressive motility ($p < 0.01$), membrane integrity ($p < 0.001$) and normal morphology ($p < 0.001$). These parameters are indicators of functional spermatozoa, suggesting that they might be able to reach and fertilize the oocyte. However, melatonin did not improve recovery rate, sperm viability and acrosome integrity. In conclusion, melatonin addition can help to protect ram spermatozoa against cryodamage, possibly by neutralisation of reactive oxygen species.

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Keywords: sheep reproduction, Jezersko-Solčava sheep breed, colloid centrifugation, melatonin

Sammanfattning

Bagge spermie membran är sårbar för skador som kan uppstå i samband med kryokonservering på grund av deras sammansättningen. Produktionen av reaktiva syrearter, som är metaboliska biprodukter, ökar vid frysning och upptining. De orsakar skada på spermiernas membran och akrosomer och påverkar även mitokondriernas aktivitet. Detta resulterar i en efter upptining minskat andel av livskraftiga, morfologiskt normala spermatozoer för insemination. I föregående studie undersöktes vilken effekt tillsättning av melatonin samt centrifugering genom ett enkelt lager av ett arts-specifikt kolloid (Ovicoll), hade på kvalitéten av frusna/upptinade bagge spermier.

För studien användes ejakulater från autochthon Jezersko-Solčava baggar (n = 20). Varje ejakulat delades upp i två delar och melatonin (1 mM) tillsättas till ett alikvot av sperma späd i Tris-baserad äggula spådningsvätska före frysning. Spermiernas motilitet, livskraft och morfologi analyserades vid 0, 3 och 6 timmar efter upptining. Membran integriteten utvärderades av HOST (hypoosmotic swelling test). Spermiekvalitet var bättre efter tillsättning av melatonin (motilitet $p < 0.05$; progressiv motilitet $p < 0.01$; membran integritet $p < 0.001$; normal morfologi $p < 0.001$). Samtliga parametrar är indikatorer för funktionella spermier, vilket indikerar att de förmodligen kan nå och befrukta oocyter. Emellertid hade melatonin ingen effekt på antal spermier efter centrifugering, spermiernas livskraft och akrosom integritet. Sammanfattningsvis, tillsättning av melatonin kan skydda bagge spermier mot kryoskada, möjligtvis genom neutralisering av reaktiva syrearter.

Nyckelord: får reproduktion, Jezersko-Solčava får ras, kolloid centrifugering, melatonin

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Abbreviations

ABTS•+	2,2`-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical cation scavenging assay
AI	artificial insemination
ALH	amplitude of lateral head displacement
AV	artificial vagina
BCF	beat cross frequency
CASA	Computer-assisted Sperm Analysis
EE	electroejaculation
F/T	frozen – thawed
GnRH	gonadotropin-releasing hormone
HOST	hypoosmotic swelling test
JS	Jezersko-Solčava sheep breed
JSR	improved Jezersko-Solčava sheep breed
kDa	kilodalton
LIN	linearity
PUFA	polyunsaturated fatty acids
ROS	reactive oxygen species
SLC	Single Layer Centrifugation
STR	straightness
VAP	average path velocity
VCL	curvilinear velocity
VSL	straight line velocity

1. Introduction

Sheep (*Ovis aries*), as small ruminants, are typically kept for meat, milk and wool production. They are tough with good adaptability and thus can graze pastures that are difficult to access. In comparison with cattle, they are smaller, hardier, easier to handle and can survive on rougher grazing. Because of their relatively short generation interval, they are often used as model animals in genetic research. The sheep population in the EU and UK in 2019 was 82.5 million (European Commission, 2020). Different breeds are available for specific purposes, such as wool type (e.g. Merino sheep breeds), flocking instincts (e.g. Romanov sheep), carcass quality (e.g. Suffolk, Charollais), milk yield (e.g. Awassi) or combination purposes. In general, they are short-day breeders, showing reproductive activity during the winter months, but some can breed throughout the year. Breeding is usually by natural mating but in some cases, assisted reproduction, particularly artificial insemination, is used instead. Pregnancy rates after artificial insemination with fresh and chilled semen doses are considered to be acceptable (Faigl *et al.,* 2012). However, with increased length of storage, there is a decrease in sperm motility, viability and fertility (Faigl *et al.,* 2012).

One of the main problems during cryopreservation is that polyunsaturated fatty acids in the membranes of ram spermatozoa are exposed to lipid peroxidation (Alvarez and Storey, 1992). Cryopreservation is detrimental to spermatozoa, increasing the production of reactive oxygen species (ROS); the latter have a detrimental effect on sperm quality and fertility. They are by-products of metabolism, but are also produced during freezing and thawing, and cause damage to sperm membranes and acrosomes as well as affecting mitochondrial activity (Ledesma *et al.,* 2014). This results in poor yield of motile, viable, and morphologically normal spermatozoa for insemination. Pregnancy rates in ewes after artificial insemination with frozen/thawed (F/T) semen samples are very low. Therefore, the uptake of AI as a breeding technique has not increased to the same extent in sheep as in cattle. Higher pregnancy rates are achieved with intracervical or laparoscopic intrauterine insemination than with vaginal deposition of F/T semen (Parkinson, 2009c). Successful insemination in seasonal ewes also depends on the season of the year and type of cycle, that is, induced oestrus in non-

breading season and natural/synchronised oestrus during the breeding season (Faigl *et al.*, 2012).

Many different approaches have been tested to improve cryopreservation and protection of spermatozoa. Based on previous findings, melatonin appears to have a role as an antioxidant and can potentially protect spermatozoa by scavenging ROS (Cebrián-Pérez *et al.*, 2014). In addition, colloid centrifugation, especially centrifugation through a single layer of a species-specific colloid, helps to improve post-thaw ram semen quality by selecting robust spermatozoa (Šterbenc *et al.*, 2019; Morrell, 2011).

2. Literature review

2.1. Jezersko-Solčava sheep breed (JS, JSR)

In Slovenia, the two most populous sheep breeds are Jezersko-Solčava (JS) and Jezersko-Solčava improved with Romanov (JSR), which are meat breeds with suitability for frequent all-year round lambing management systems (Drobnič *et al.*, 1999).

The JS is one of the autochthonous Slovenian sheep breeds. It originates from the mountain region of the Kamnik Alps and was obtained by crossbreeding the original sheep from the Jezersko-Solčava region with sheep from the regions of Bergamo and Padua in Italy. Historically, from this crossbreeding, the breed inherited its characteristic nasal bone and improved wool quality and it became capable of breeding throughout the year. The name of this breed is derived from the regions Jezersko and Solčava in Slovenia, where historically local farmers bred this sheep. It is a hornless sheep with a characteristic black patch around the eyes; with lop ears and an 'egg-shaped' head without wool. This breed is well-adapted to survive on pasture in the mountains, with year-round fertility, an average of 1.5 to 1.8 lambings per year, and with quality wool and succulent lamb-meat (Drobnič *et al.*, 1999; Feldmann *et al.*, 2005; Anon., 2010; Komprej *et al.*, 2011).

The JS breed was included in a conservation program in 1994. It is a medium-sized sheep, with white being the most usual colour, although dark brown types also exist. Shearing of the wool is done twice a year. Due to early maturity, the age of first lambing is around 13 to 17 months. The breed is described as tough with good adaptability and it is mainly distributed in regions in North Slovenia (Feldmann *et al.*, 2005).

The JSR is characterised by earlier sexual maturity than JS, good maternal instinct and year-round fertility, with three lambings in two years. The breed is mostly kept for meat production (Komprej *et al.*, 2011). The JSR is the most widely occurring sheep breed in Slovenia. Crossbreeding with Romanov started in the early eighties with the goal to improve fertility (Zagožen, 1984). This led to a year-round supply of lamb meat (Cividini *et al.*, 2012).

Year-round fertility is seen in the ability of animals to exhibit ovarian cyclicity and breeding behaviour throughout the year. This ability has been reported in some, but not all, sheep breeds (Komprej *et al.,* 2011). In general, seasonal variation of reproductive parameters is mostly obvious in ewes, but rams are also affected by this phenomenon, with decreased sperm concentration, size of testicles (volume and diameter), quality of the ejaculate and hormonal profile during the non-breeding season. However, seasonality in rams is highly influenced by genetics (Avdi *et al.,* 2004; Milczewski *et al.,* 2015).

The animals used in this study belong to the Biotechnical Faculty in Ljubljana University, and their breeding programme is described below.

2.1.1. Farm for young rams

The educational research centre, with a barn for young rams in the breeding programme (PRC) Logatec, operates within the Department of Animal Science of the Biotechnical Faculty in Ljubljana. The goal of the educational research centre farm is to improve the genetics of indigenous Slovenian sheep breeds, by testing the rams for productivity and reproduction abilities. Half of the rams are removed from the herd due to slower growth rate and another 5 to 10 % are eliminated due to other abnormalities important for vitality (correct leg conformation, backline and formation of the jaws) and reproduction ability (quality and quantity of the ejaculate). Rams with a low rating in any parameter are classified as "unsatisfactory" and are removed from the breeding programme.

Selection of lambs starts at the age of 3 to 4 months and at a body weight of around 25 to 35 kg. Selection is based on the size of the litter, growth rate from lambing to 60 ± 15 days of age, and phenotype. The lambs are selected only from certified breeders. New lambs on the farm are first settled in an isolation barn. During this period, blood samples for screening of infectious diseases are collected as part of the usual official screening scheme on farm, and at the same time they are acclimatized to the feeding regimen.

The testing period lasts for 100 days, during which the rams are weighed three times at intervals of 30 days. During testing, those with below average growth rates are removed from the herd. At the beginning and end of the testing period, blood samples are collected for screening for infectious diseases (e.g. blue-tongue). During the whole testing period, rams are fed with a specific diet and they undergo a breeding soundness examination at the end of the testing period.

After obtaining the last test results on the reproductive ability of individual rams, the evaluation committee evaluates the rams and arranges them into six groups (1A and 1B, 2A and 2B, 3A and 3B) based on expected breeding value, phenotype and reproductive ability. Rams in the first group (1A and 1B) are used for the improvement of the breed, rams rated for the second group are sold to

commercial farms with the pedigree, rams in group 3A are sold to smaller farms and those in group 3B are removed from the breeding programme. Approximately 250 animals are on test annually on the farm (Simčič, 2020). With this selection of genetically superior individuals, the farm intends to improve breed productivity, reproductive efficiency and characteristics.

2.2. Reproductive tract of sheep

2.2.1. Anatomy of the cervix and its relation to insemination

Artificial insemination with frozen semen in sheep often results in poor fertility (Kaabi *et al.,* 2006), partly due to the anatomical structure of the cervix, which obstructs the passage of the insemination pistolette (Halbert *et al.,* 1990).In addition, F/T spermatozoa show reduced ability to pass or colonize the length of the cervix and thus are lost from the reproductive tract (Smith, 2009).

The cervix is situated in the most caudal part of the uterus and is surrounded by a thick muscular-connective tissue wall. In the ewe, the organization of the inner and outer orifices of the lumen resembles a corkscrew. The length on average is about 5.5 cm (Moré, 1984). The cervix contains 4 – 7 cervical rings, and the second and third rings are usually aligned with the first one (Perry *et al.*, 2010).This disposition makes it very difficult to introduce an instrument further inside the cervix. Histologically, the superficial epithelium consists of 4 – 6 layers, which undergo cyclic changes (Moré, 1984).

In a study by Halbert *et al*. (1990), an assessment was made of the cervical anatomy in different sheep breeds using ewes in oestrus; they observed that there is individual variation in the cervix within breeds that needs to be taken into account when performing artificial insemination. They tried to assess different semen deposition sites and different routes for the insemination pipette. The best results were achieved with mid-cervical semen deposition and were more successful in the recovered tracts than in live animals (Halbert *et al*. 1990). The folds of vaginal tissue were used to classify four types of cervix: two opposing vaginal folds (type1), one vaginal fold (type 2), cluster of vaginal folds (type 3) and spiral-shaped vaginal fold (type 4). Types 2 and 3 were most frequently seen (35 % for both). However, results may not be representative due to the small population sample (Halbert *et al*. 1990).

Constriction of the transcervical passage can occur for many reasons. The occurrence of a blind space caused by vaginal folds makes identification of the cervical opening difficult, and the number, alignment, size and spacing of the rings, which vary between ewes, also contribute to the problem. There is a positive correlation between the fertility rate and depth of semen deposition (Kaabi *et al.,* 2006). Apart from the cervical folds, depth of catheter penetration

can also be affected by the age of the ewe, with increasing depth of penetration occurring in older animals (Eppleston *et al.,* 1994). The cervical rings, which are the primary physical barrier for external contamination (Perry *et al.,* 2010), limit the passage of an insemination pipette towards the uterus; the pipette can be inserted barely more than 1 cm along the cervix.

Cervical penetration can be improved by using modified pipettes or hormones to open the cervical canal. The best result is achieved with the use of a bent catheter, but its design must be based on morphological measurements (Kaabi *et al.,* 2006). To improve cervical opening, experiments in which low molecular weight hyaluronan was applied intracervically prior to insemination resulted in increased cervical penetrability by the inseminating pipette, with passage up to 2.4 cm into the cervix. Thus, in some breeds with a short cervix, the insemination pipette can traverse the entire length of the cervix. However, transcervical intrauterine insemination requires a skilled veterinarian to carry out the procedure without damaging the tissue (Perry *et al.,* 2010).

Cryoconservation causes capacitation-like changes in the spermatozoa, which reduce their ability to pass through the cervix. It results in low pregnancy rates when ewes are inseminated in the vagina or cervix (Maxwell *et al.,* 1999). Due to the anatomical construction of the cervical canal, only a limited volume of an insemination dose with relatively high concentration can be used for insemination in order to minimise semen backflow (Faigl *et al.,* 2012).

An alternative is to deposit the spermatozoa as close to the site of fertilization as possible. This procedure tends to result in higher pregnancy rates, since the spermatozoa do not have to pass the ovine cervix. To achieve suitable pregnancy rates when using F/T semen, the spermatozoa must be deposited close to the oviduct in the uterine horns, either with transcervical intrauterine insemination or with laparoscopic intrauterine insemination (Faigl *et al.,* 2012). Intrauterine insemination can results in 70% pregnancy rates, using either fresh or thawed semen. The best results are obtained when artificial insemination is performed in mid-oestrus or 12 hours after its onset (Smith, 2009).

2.2.2. Male anatomy

The function of the male reproduction organs are the production, maturation, storage and transport of spermatozoa, followed by the deposition of semen into the female reproductive tract. The testicles of most domestic livestock are located in the scrotum, in the inguinal region. In rams, the scrotum has an elongated neck. The testis consists of interstitial tissue containing Leydig cells and seminiferous tubules in which Sertoli cells and sperm-producing germinal cells are located (Parkinson, 2009c). The Sertoli cell epithelial barrier, commonly known as the blood-testis barrier, prevents immune cells carried in the blood stream from coming into contact with haploid spermatozoa in the testes. These haploid cells

would be considered as “foreign” by the immune system and therefore would be attacked if no blood-testis barrier were present (Mao *et al.,* 2018). Leydig cells are clustered around blood vessels. They are responsible for testosterone synthesis and also produce insulin 3, which is responsible for descent of the testis. In rams, testicular descent occurs in mid-gestation. At the end of the seminiferous tubules there is a transitional zone between the rete testis and spermatogenic tissue. The rete testis opens into the epididymis through efferent ducts. Rams have all the accessory sex glands: ampullae (sperm reservoir, minimal contribution to seminal plasma), prostate (its disseminated part), vesicular glands (containing citrate and fructose) and bulbourethral glands (cleaning function of the urethra) (Parkinson, 2009c).

2.3. Spermatozoa

Spermatozoa are complex cells with an inability to synthesize specific molecules and with complete dependence on the environmental conditions and extracellular medium. In their mature stage, they need to leave their parental body and function in another one, passing through many obstacles and environments, with the only purpose being to fertilize the oocyte (Cebrián-Pérez *et al.,* 2014). The discovery of the spermatozoa is attributed to Leeuwenhoek when in 1677 he described their general form and movement. Since then, with the continuous improvement of microscope technology, description and understanding of the sperm cell has improved (Fawcett, 1975).

Sperm cells are divided into several parts: a head with an acrosomal cap, neck, middle piece and tail with its subparts (Fawcett, 1975). The head includes a condensed nucleus and acrosome (Parkinson, 2009c). The shape of the nucleus is characteristic for each species (Fawcett, 1975). The mid-piece contains the mitochondria, which metabolize simple sugars, to provide energy for motility. The forward movement of spermatozoa is achieved by coordination of waves from the neck along the tail (Parkinson, 2009c).

Sperm cell production, i.e. spermatogenesis, is a complex process of changes involving a series of division (mitosis several times followed by meiosis) which transform spermatogonia to spermatozoa containing a highly organized chromatin structure. This chromatin structure protects the genome during transportation through the reproductive tracts to the site of fertilization (Oumaima *et al.,* 2018). Spermatogenesis takes place in the seminiferous tubules, in the seminiferous epithelium, where Sertoli cells provide nutritional support. After meiosis, round spermatids undergo functional and morphological changes, known as spermiogenesis, which result in the differentiation into testicle spermatozoa (Parkinson, 2009c).

After leaving the seminiferous epithelium, testicle spermatozoa lack normal motility and are immature. They gain these abilities while passing through the epididymis. Most morphological changes occur in the proximal region of the epididymis, for example changes in the plasma membrane, which help to stabilize the acrosome (Parkinson, 2009c). To be able to fertilize the oocyte, spermatozoa need to undergo capacitation in the female reproductive tract. During this process, the sperm surface membrane changes; certain lipids and proteins are segregated on its membrane, which is needed before the acrosome reaction and fertilization can occur (Barrios *et al.,* 2005). The outer acrosomal membrane fuses with the plasmalemma before the acrosome reaction. The function of acrosomal enzymes (mainly hyaluronidase and acrosin) is dispersion of the cumulus oophorus and local lysis of zona pellucida (Parkinson, 2009c).

2.3.1. Sperm membrane, seminal plasma proteins

The ram sperm plasma membrane is rich in polyunsaturated fatty acid (PUFA). This dominance of PUFA causes the spermatozoa to be highly affected by lipid peroxidation due to attack by ROS, affecting sperm motility and membrane integrity. All of these factors can lead to a decrease in fertility (Rather *et al.,* 2016).

In the ejaculate, spermatozoa are surrounded by seminal plasma, which is a complex biological mixture of several fluids, produced by the male accessory sex glands (seminal vesicles, prostate gland and bulbourethral glands) and a small volume of epididymal fluid, containing proteins and other components (Barrios *et al.,* 2005; Ledesma *et al.,* 2014). Some of the proteins help to stabilise the sperm membrane until capacitation occurs. Seminal plasma improves ram sperm viability during storage and one of its roles is to keep the spermatozoa in the decapacitated state (Ashworth *et al.,* 1994; Barrios *et al.,* 2005).

During ejaculation, seminal plasma adds volume and facilitates the passage of spermatozoa through the reproductive tracts; moreover, it also activates the spermatozoa. Components and proteins present in seminal plasma modify sperm function (mostly by stabilizing the sperm membrane and containing some decapacitating factors), affecting sperm viability and motility (Barrios *et al.,* 2005; Ledesma *et al.,* 2014).

In the study of Barrios *et al*. (2005) the focus was on seminal plasma proteins. The major ones have a relative molecular weight of 14 and 20 kilodalton, respectively. During capacitation and acrosomal reaction, these proteins are modified on the sperm membrane. Both proteins were located mostly on the acrosomal region and flagellum, so the researchers conclude that both proteins contribute to the acrosomal reaction and capacitation process, modulating motility and having a role in decapacitation.

Addition of fresh seminal plasma to freezing extenders can improve post-thaw motility and membrane integrity of the spermatozoa, especially the inclusion of low molecular weight proteins of approximately 15 – 25 kDa (Faigl *et al.*, 2012).

2.4. Electroejaculation

Semen can be collected from rams either by electroejaculation (EE) or with an artificial vagina (AV). Collection with an AV often requires a training period for the rams in the presence of an oestrous ewe (Marco-Jiménez *et al.,* 2005; Faigl *et al.,* 2012), whereas with EE, the animal must be anaesthetised and there is a question of animal welfare (Faigl *et al.,* 2012).

Semen collection from rams is relatively easy. Ejaculates from young rams in Slovenia, which are in the breeding programme, are collected by EE for a breeding soundness examination. A rectal probe with attached electrodes is placed into the ram's rectum at the pelvic brim and electrical pulses of low voltage are supplied through electrodes (Parkinson, 2009b; Abril-Sánchez *et al.*, 2019). The pulses stimulate the nerves of the hypogastric plexus, which surrounds the seminal vesicles, prostate gland and deferent ducts, resulting in semen emission. It also stimulates the pudendal nerves, resulting in contraction of the urethral muscles to induce ejaculation (Abril-Sánchez *et al.,* 2019). Many rams will ejaculate without an erection. Ejaculation should occur after four to six rhythmic stimulations of the sacral nerve plexus and the ampullae; if not, the procedure should be discontinued for several minutes (Parkinson, 2009b).

Electroejaculation is a useful procedure for obtaining semen from a large number of animals in a short time (Marco-Jiménez *et al.,* 2005; Ledesma *et al.*, 2014; Abril-Sánchez *et al.,* 2019). One advantage of EE is the possibility to collect semen throughout the year, since it overcomes the depression in sexual behaviour during the non-breeding season occurring in some seasonal sheep breeds (Abril-Sánchez *et al.,* 2019). Other advantages of EE are collection from males of wild species, males isolated from females (Abril-Sánchez *et al.,* 2019) or males with a physical injury which renders them unable to mate naturally (Ledesma *et al.,* 2014). Even though EE is an effective and easy technique, it can also be painful and stressful for the males because it can cause extreme muscular contractions. To minimise these negative effects, sedatives, hormones and anaesthetics can be used. However, anaesthesia in rams can induce a stress response as well and can also alter semen quality, and there is a need to withhold food before anaesthesia (Abril-Sánchez *et al.,* 2019).

Semen collection with an AV simulates natural mating, but does require a training period of around one to two weeks (Abril-Sánchez *et al.,* 2019). Training may take up to three months, depending on the individual (Ledesma *et*

al., 2014) and other factors, such as season, libido, willingness of the collecting rams and the breed (Malejane *et al.,* 2014).

The method of semen collection can have an effect on the quality of ejaculate and sperm parameters. The volume of the ejaculate is greater when using EE compared with AV, probably due to stronger contractions of the accessory sex glands. In rams, there is a difference in seminal plasma composition between the two collection techniques (Abril-Sánchez *et al.,* 2019). According to Robayo *et al.* (2008), sperm concentration was decreased after collecting the ejaculation by EE compared with an AV. In the study of Marco-Jiménez *et al.* (2005), a lower number of spermatozoa were found in the ejaculate after EE than after collection with an AV. However, a higher number of functional and stable spermatozoa were found in F/T ejaculates obtained by EE than by AV, and a significant effect on sperm capacitation and acrosomal integrity was also observed. One possible explanation could be the differences in seminal plasma composition. After EE there may be a higher proportion of antioxidants protecting the membrane integrity (Ledesma *et al.,* 2014).Ejaculates obtained by EE also differ in protein composition and amount compared with those collected by AV. These proteins maintain membrane stability (Ledesma *et al.,* 2014).

Due to over-stimulation of the accessory sex glands during EE, the ejaculate collected in this way has increased volume of seminal plasma compared with those collected by AV. This can result in a higher proportion of spermatozoa with an intact plasma membrane and functional mitochondria, due to seminal plasma constituents, which preserve plasma membrane integrity (Abril-Sánchez *et al.,* 2019).

The method of collection also alters the seminal plasma protein composition. Samples collected with EE have greater total protein content and a greater proportion of proteins with low molecular weight (Ledesma *et al.,* 2014) than those collected by AV; such proteins are beneficial to the sperm cells, since they improve motility and post-thaw membrane integrity (Faigl *et al.,* 2012). Ejaculates obtain by EE might contain a greater proportion of antioxidants as a result of the higher volume of seminal plasma which is responsible for protecting the spermatozoa against oxidative stress, making EE a good option for ejaculates intended for cryopreservation (Ledesma *et al.,* 2014). Ejaculates collected with EE are more resilient towards cryodamage and cold-shock than those collected with an AV (Ledesma *et al.,* 2015).

After collection, the ejaculate must be handled carefully to avoid temperature shocks (heat or cold) and to avoid contamination from the environment. The evaluation of the ejaculate occurs immediately after collection for both quantitative and qualitative parameters. The normal values for ram semen for sperm concentration are 2.0 to 5.0 * 10^9 spermatozoa/mL, volume 0.5 to 2 mL,

and motility (80 – 90 %); the proportion of morphologically normal spermatozoa should be 70 – 80 % (Faigl *et al.*, 2012; Setchell, 2014).

2.5. Artificial insemination, cryopreservation

Artificial insemination (AI) is defined as placing the spermatozoa into the reproductive tract of the female by a method other than natural mating, e.g. using special instruments (Faigl *et al.,* 2012). The procedure for AI must be simple, economical and with a high success rate. The method helps to reduce the spread of non-sexually and sexually transmitted diseases, such as foot and mouth disease, blue tongue, sheep pox or ovine brucellosis (Parkinson, 2009a); and the inclusion of variable genes into the population (Faigl *et al.,* 2012). It is an important tool for improving genetic potential and breeding progress in sheep in comparison with natural mating. Two of the major difficulties with AI are, without doubt, the preservation of the semen, either as a liquid or in the frozen state, and cervical insemination with F/T semen, because of the anatomical constitution of the ewe's cervix. Both of these factors contribute to low pregnancy rates (Rather *et al.*, 2016).

Artificial insemination is mostly done with fresh semen; due to the problem of cold shock that occurs with chilled and frozen semen (Cseh *et al.*, 2012). In sheep production, AI has not achieved the same popularity as in cattle (Smith, 2009), due to difficulties with handling and inseminating the ewes and the high cost of oestrus synchronisation in comparison with natural mating (Faigl *et al.*, 2012). However, the cost can be reduced by using vasectomised rams for the induction and detection of oestrus, and higher pregnancy rates can be attained using fresh semen instead of frozen semen (Smith, 2009). Lethal damage can be inflicted on the spermatozoa by the preservation techniques, resulting in low pregnancy rates (Faigl *et al.,* 2012). As a result, diluted semen cooled to storage temperature at+ 5 °C, should be used within 8 – 10 h of collection, since the decrease in fertility rate is 10 - 35 % for each day of storage (Cseh *et al.*, 2012).

Four types of artificial insemination techniques in ewes are available according to the site of semen deposition: vaginal insemination, cervical insemination, transcervical intrauterine insemination and intrauterine insemination by laparoscopy. The choice of technique depends on whether fresh, liquid preserved or cryopreserved semen is used. The advantage of vaginal insemination is the requirement of little skill to perform this procedure, including less time spending handling and stressing the animals (Maxwell and Hewitt, 1986). Other factors determining the choice of techniques include the degree of technical ability available and restrictions imposed by individual countries.

When using F/T insemination doses, intrauterine semen deposition is the only method with acceptable pregnancy rates (Cseh *et al.*, 2012; Faigl *et al.*, 2012).

Fertility rates from AI with F/T semen are affected by the capacity of spermatozoa to pass through the ewe's cervix. This results in a small number of spermatozoa reaching the oviducts and being available to fertilize the oocytes. Laparoscopic or trans-cervical insemination by pipette allows semen deposition directly into the uterus, thus by-passing the cervical barrier (Windsor *et al.*, 1994). With cervical insemination, the success rate with fresh semen is higher than 60 %; however, with frozen semen the conception rate is, in general, less than 40 % (Perry *et al.*, 2010).

Transcervical insemination is preferred for small ruminants (i.e. ewes, does) and involves passing the pipette into the cervix (5 – 12 mm) after locating the cervical oss using a speculum. For higher pregnancy rates, oestrus synchronisation is recommended in ewes with the use of intravaginal devices to induce oestrus. The most common route of progesterone supplementation is by intravaginal administration in the form of impregnated sponges or by using intravaginal devices (DICO, CIDR), in conjunction with equine chorionic gonadotropin. The intravaginal sponges are inserted for 9 to 19 days with equine chorionic gonadotropin administration after sponge removal or 48 h prior to removal (Wildeus, 2000). Oestrus occurs 24 to 72 h after removal of the hormonal device, and insemination should take place within a short period (Faigl *et al.*, 2012). However, transcervical insemination can cause trauma of the cervix, which may lead to induced pregnancy interruption (Faigl *et al.*, 2012). Laparoscopic insemination seems to be the most effective method of semen deposition, but is not the preferred method in Europe due to expense, welfare implications and requirement of trained personnel (Perry *et al.*, 2010).

Storage, type of extender, insemination technique, sheep breed and individual males, are factors that can affect sperm motility and viability (Faigl *et al.*, 2012). However, the success rate of artificial insemination can be improved using a higher sperm concentration in insemination doses and also by the method of insemination (Faigl *et al.*, 2012). The sperm concentration in the insemination dose depends on the insemination technique to be used; for example with intracervical insemination a concentration of 10^9 spermatozoa/mL is recommended (Parkinson, 2009a). For dilution of the semen before cryopreservation, Tris-citrate-fructose-egg yolk extenders and extenders based on saccharide complexes (Cseh *et al.*, 2012) are commonly used, with glycerol as a cryoprotectant.

Compared to fresh semen, cryopreserved semen samples can be transported over long distances and can be used for inseminating ewes when rams are not available. Cryopreservation arrests metabolic activity completely; however, it is reversible at any time. Nevertheless, cryopreservation causes a significant level of damage, and thawing can induce premature capacitation (Faigl *et al.*, 2012).

Extenders for cryopreservation should fulfil some requirements, such as adequate pH, suitable osmolality, and buffering capacity and function to protect spermatozoa against cryogenic injuries. Tris buffer together with egg yolk, fructose or glucose and citrate are well-accepted components in extenders for frozen storage of ram semen and provide suitable osmolality. The commonly used cryo-protective substance is glycerol, at an optimal concentration of 3 – 4 % (Salamon and Maxwell, 2000). Glycerol can increase cryosurvival; however, it can generate osmotic damage to spermatozoa, alter plasma membrane and interact with proteins (Farshad *et al.,* 2009). It is toxic to spermatozoa in a temperature-dependent manner. Thus, the optimal concentration depends on cooling rate, method of addition and diluent composition (Salamon and Maxwell, 2000).

The cryopreservation process and all its steps (cooling, freezing and thawing) induce serious changes in sperm function, lowering sperm fertilization ability and decreasing pregnancy rates after insemination. Cryodamage causes changes resembling premature capacitation (so-called cryocapacitation), altering the stability of the membrane and starting a cascade of events such as flagellar hyperactivation, culminating in the acrosome reaction (Ledesma *et al.,* 2015). Moreover, cryopreservation can induce DNA methylation, which is mechanism linked with epigenetic changes (Peris-Frau *et al.,* 2020). Spermatozoa which survive cryopreservation in liquid nitrogen also have to survive thawing, thus passing the critical temperature zone of – 15 ˚C to – 60 ˚C twice. Thawing must be fast enough to prevent recrystallisation of intracellular ice (Salamon and Maxwell, 2000). Using cryopreserved semen samples for insemination requires eight times more spermatozoa as fresh semen to achieve acceptable fertilization rates, due to lower sperm viability, motility and damage to the plasma membrane (Marco-Jiménez *et al.,* 2005). Cryopreservation can increase the proportion of spermatozoa with a reacted acrosome as well as increasing maturation of sperm membranes. These events may affect sperm viability and fertilizing ability. Adding antioxidants can improve storage time and quality of insemination doses, while careful timing and type of the insemination can help to increase pregnancy rates (Cseh *et al.,* 2012).

2.6. Cold shock and cryoprotectants

The greatest advantage of semen cryopreservation is the prolonged storage time that it infers, but it also has many disadvantages, such as cold shock, chemical toxicity of cryoprotectant (glycerol), oxidative stress and ice crystal formation in intracellular and extracellular spaces, as well as potential epigenetic changes. These factors can injure the spermatozoa and affect their structure and fertilizing ability (Rather *et al.,* 2016). Increasing the levels of testosterone, estradiol,

melatonin and other components in seminal plasma are reported to increase the ability of the spermatozoa to survive freezing (Cebrián-Pérez *et al.*, 2014).

Low temperatures alter sperm function. Cold shock results in sperm membrane destabilization, affecting sperm function and inducing premature capacitation. Ram spermatozoa are more affected and sensitive towards cold-shock stress than bull spermatozoa (Holt and North, 1984). Cold shock is characterized by decreased membrane integrity and viability. However, the addition of seminal plasma proteins before cooling has a positive effect on sperm survival rate. To be able to fertilize an oocyte, spermatozoa must have a functional plasma membrane; therefore, the integrity of the plasma membrane must be preserved during stressful procedures such as cryopreservation. Some of these processes can be associated with removal of seminal plasma prior to freezing (Barrios *et al.*, 2005).

Other components can be added to the extenders to increase sperm protection during cryopreservation. Higher sperm dilution rates prior to freezing provide better protection during cryopreservation, improving the post-thaw quality of ram semen (Leahy *et al.*, 2010). Glycerol in an optimal concentration of 4 – 6 % can be added, either as a two-step or one-step dilution. After addition of glycerol, the semen has to be cooled gradually (equilibration) to 5 ˚C (Faigl *et al.*, 2012). Egg yolk has a protective effect against cold shock, stabilizing ram sperm membranes during freezing and thawing (Salamon and Maxwell, 2000).

2.7. Reactive oxygen species and oxidative stress

During semen storage, there is a gradual decrease in sperm quality, such as motility, membrane integrity and fertility. One explanation for this could be attack by ROS from semen components e.g. superoxide anion radical, lipid hydro-peroxides (created by peroxidation of membrane lipids) and hydrogen peroxide (Ashrafi *et al.*, 2011). Indirect molecular damage is caused by oxygen and partially reduced species that are generated from the one-electron radical of oxygen. These reduced oxygen derivatives (i.e. ROS) and free radicals can cause damage to DNA, proteins, lipids, etc., in cells in all aerobic organisms (Manchester *et al.*, 2015).

The source of ROS production is mostly from damaged spermatozoa, spermatozoa with residual cytoplasm or from leucocytes in the ejaculate (Rather *et al.*, 2016). Sperm cells also produce ROS as a result of their metabolism. However, in some cases ROS are produced in excess, affecting sperm viability, acrosomal and DNA integrity, and mitochondrial quality (Ledesma *et al.*, 2014). Recently, excessive levels of ROS were reported to be correlated with morphological abnormalities of sperm cells in ejaculate. Those abnormal spermatozoa may produce high levels of ROS (Oumaima *et al.*, 2018).

Oxidative stress is involved in 30 – 80 % of sperm damage, due to excess production of ROS. These ROS can have both beneficial and harmful effects on spermatozoa, depending on concentration, location and duration of exposure (Oumaima *et al.,* 2018). Oxidative stress occurs where there is an imbalance between ROS production and antioxidant defence system of the cells (Casao *et al.,* 2010; Oumaima *et al.,* 2018). Seminal plasma components scavenge ROS, thereby helping to balance ROS generation and their neutralization. In ram seminal plasma there is a tendency for seasonal changes to occur in antioxidant activity. This activity can be modulated not only by the spermatozoa themselves but also by other factors, e.g. melatonin (Casao *et al.,* 2010).

Casao *et al*. (2010) showed a correlation between the level of melatonin in seminal plasma during the year with the activity of three main antioxidant enzymes (superoxide dismutase, glutathione reductase and catalase), thus supporting the role of melatonin in the antioxidant defence system. Spermatozoa as such are very sensitive to oxidative stress, due to DNA damage and apoptosis. To protect spermatozoa from oxidative stress, the accessory sex glands and epididymal epithelium secrete antioxidant enzymes and other scavengers. This activity is regulated by the circadian melatonin rhythm.

Ram spermatozoa are characterised by a high oxidative respiration capacity; mitochondrial respiration is essential for successful passage through the cervix and for retaining high fertilization capability. Mitochondrial injuries may have a significant effect on fertility rates after cervical insemination (Faigl *et al.,* 2012).

2.8. Melatonin

Melatonin is a hormone produced by the pineal gland, which controls circadian rhythm in mammals. According to Gonzalez-Arto *et al.* (2016), the synthesis of melatonin and melatonin enzymes also occurs in the testes of rams. Melatonin is produced and secreted only during distinct periods of the day. Environmental lighting (light/dark period) controls the sensitivity and production of melatonin (Klein *et al.,* 1981; Cebrián-Pérez *et al.,* 2014). Originally, it was thought that this substance is exclusively secreted from the pineal gland, but now it is known that is also synthesized in mitochondria and chloroplasts (Manchester *et al.,* 2015).

The original purpose of this molecule (to protect cells by detoxification of ROS) survives in present-day mammals. During the Great Oxygen Event (an early mass extinction), organisms which survived were those able to synthesize melatonin. Melatonin is an effective molecule in seminal plasma, which can protect cells against molecular damage, i.e. oxidative stress caused mostly by ROS (Manchester *et al.,* 2015), by direct neutralization of toxic free radicals (Casao *et al.,* 2010).

Melatonin has many different functions, such as control of lipid and sugar metabolism, defence against oxidative stress, scavenger of ROS or reactive nitrogen species, and immune regulation (Cebrián-Pérez *et al.,* 2014). Melatonin is commonly known as an antioxidant, but it can work as a pro-oxidant, for example with pro-apoptotic actions in cancer cells (Manchester *et al.,* 2015).

The function of melatonin is to reduce oxidative stress in DNA, proteins and lipids. Testing the radical scavenging ability of melatonin on the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical cation scavenging assay (ABTS•+), shows that it rapidly reduces ABTS•+ and continues to scavenge it for up to 12 minutes. Furthermore, derivatives of melatonin have a similar effect. Melatonin directly neutralizes free radicals and also removes them from cells, by metabolizing them to inactive species or by inhibiting enzymes that lead to their creation (Manchester *et al.,* 2015).

Melatonin works as the main regulator or stimulator of reproduction in photoperiodic animals, depending on whether they are long-day or short-day breeders. The level of melatonin in seminal plasma of seasonal sheep breeds shows fluctuations during the year, with peaks in the breeding season (i.e. October – November) (Casao *et al.,* 2010). Sheep belong to the group of short-day breeders, which means that increased secretion of melatonin during autumn and winter stimulates their reproduction. The main purpose of seasonal breeding is that offspring will be born during favourable conditions in spring with sufficient amount of grass for their dams to produce milk and, later on, for the lambs to graze. The synchronisation of seasonal reproductive activity and environmental conditions, as well as adjusting the gonadal activity, is through the hypothalamic-pituitary-gonadal axis with a passive signal of melatonin excretion. The information about daylight length is detected via the retina and is transformed to an endocrine signal by the pineal gland, which will increase melatonin secretion. This secretion regulates gonadotropin-releasing hormone (GnRH) production, which regulates luteinizing hormone production. Luteinizing hormone affects sperm production, sexual behaviour, sperm quality, volume and testicular weight. The decrease in semen quality during the non-breading season is due to lower melatonin concentration release, due to decreased activity of the hypothalamic-pituitary-gonadal (testicular) axis. All these hormonal changes are also reflected in the composition of seminal plasma (Malpaux *et al.,* 1996; Cebrián-Pérez *et al.,* 2014).

In a study by Casao *et al*. (2010), the minimum level of melatonin in ram seminal plasma in seasonal sheep breeds was observed during June, starting to rise again in August, reaching its maximum in October-November. This pattern of melatonin corresponds with the fact that sheep are short-day breeders. Melatonin treatment during the non-breeding season increases the level of blood testosterone. Supplementing the extenders with antioxidants seems to be

a promising method for preventing changes to spermatozoa occurring during freezing and thawing (Rather *et al.,* 2016). In previous studies, the melatonin concentration which produced the best results was 1 mM (Succu *et al.,* 2011; Ashrafi *et al.,* 2011); thus, this concentration was used in the present study.

2.9. Single Layer Centrifugation

During natural reproduction, spermatozoa pass through several selection mechanisms in the female reproductive tract that filter out defective and immature spermatozoa. It is expected that only normal spermatozoa should reach the fertilization site, the isthmic-ampullary junction (Morrell and Rodriguez-Martinez, 2010; Morrell, 2011). In assisted reproduction, sperm selection techniques used in the laboratory mimic these natural selection mechanisms and are referred to as biomimetic techniques. These sperm selection techniques can be divided into different types; sperm washing (only seminal plasma removal) or sperm selection (filtration, migration or colloid centrifugation) (Morrell and Rodriguez-Martinez, 2010). Centrifugation through a colloid e.g. Single Layer Centrifugation (SLC), mimics selection in vitro; thus it is a biomimetic technique (Morrell, 2011).

The SLC is an easy tool to improve sperm quality in F/T semen from subfertile stallions (Macías García *et al.,* 2009), improving cryosurvival and *in vitro* fertilizing capability of spermatozoa in boars (Martinez-Alborcia *et al.,* 2013), dogs (Dorado *et al.,* 2013) and bucks (Jiménez-Rabadán *et al.,* 2012). It can be used to select viable and fertile spermatozoa prior to freezing in bulls (Thys *et al.,* 2009) and camels (Malo *et al.,* 2018). In a study by Šterbenc *et al*. (2019), improvement in post-thaw ram sperm quality (motility, viability and survival rate) was shown after SLC. The SLC technique can improve sperm quality from problem stallions, increase sperm shelf-life during storage, and can improve cryopreservation, with sperm selection either prior to or after, cryopreservation, by selecting viable, motile spermatozoa with intact chromatin (Morrell, 2011). Furthermore, it can be used for removal of pathogens, thus possibly reducing antibiotic use (Morrell and Wallgren, 2011). Finally, it can be used in conservation breeding with rare breeds and endangered species, where there are reduced numbers of individuals for natural breeding (Morrell, 2011). Another advantage of SLC is that it can be scaled up, with15 mL or 150 mL of colloid being used to process 20 mL or 200 mL ejaculate, depending on the species (Morrell *et al.,* 2009).

Species-specific colloid formulations for SLC can be autoclaved to reduce potential endotoxicity and are stable in a salt solution, meaning that they can be prepared in advance (Thys *et al.,* 2009). Ovicoll (formerly known as Androcoll-

O) is a silane-coated silica colloid in a buffered salt solution optimized for ram spermatozoa (Šterbenc *et al.*, 2019); it was used in this study.

2.10. Ram semen evaluation, specification

Due to the fact that a single male animal can serve a large number of females, the impact of male subfertility on farm efficiency is high. In sheep production specifically, the problem of ram fertility is seen in decreased lambing rates or an increase in returns-to-oestrus. Routine semen evaluation tests can give useful information about ram fertility but only provide an indication of the likely fertility, since fertilization is a complex procedure affected by many variables in both males and females. Computer-assisted semen analysis (CASA) analyses sperm kinematics, providing an indication of how well the spermatozoa can migrate through the female genital tract and potentially fertilize the oocyte. The CASA can give information about different sperm characteristics, such as motion and motility, and in conjunction with various stains, can provide information about morphology and viability (Tsakmakidis, 2010).

Cervical fluid is one of the first barriers through which spermatozoa must pass on their way to the oviduct, at least in ruminants. Cervical mucus, works as a filter for spermatozoa, and only those with good kinematics are able to reach the uterine tubal junction (Robayo *et al.*, 2008).

Robayo *et al*. (2008) showed that there is a correlation between curvilinear velocity (VCL) or average path velocity (VAP) and the ability of the spermatozoa to pass through ovine cervical mucus. The ability of spermatozoa to migrate through cervical mucus depends on their hydrodynamic force. In comparison with other ruminants, ram spermatozoa move faster and follow a more linear trajectory (Robayo *et al.*, 2008). Components of seminal plasma may help spermatozoa to pass through cervical mucus (Suarez and Pacey, 2006). However, sperm fertilizing ability does not depend on a single parameter but on a combination of factors, such as the breed, the fertility of the ewes, type of oestrus (induced with exogenous hormones, or natural), the season and type of artificial insemination (Faigl *et al.*, 2012).

The common parameters measured with CASA and used in this project are: curvilinear velocity (VCL), which is an average velocity of spermatozoa over the curved path distance; amplitude of lateral head displacement (ALH), which is the mean value of side to side movement of the head; beat cross frequency (BCF),the frequency of the head crossing the average path; average path velocity (VAP),the velocity over the calculated smoothed path; straight-line velocity (VSL), the average velocity calculated from the beginning to the end of the track; straightness (STR), the proportion of straight spermatozoa calculated as

VSL/VAP * 100; and linearity (LIN), which is the proportion of linearly motile spermatozoa defined as VSL/VCL * 100 (Figures 1 and 2) (Tsakmakidis, 2010).

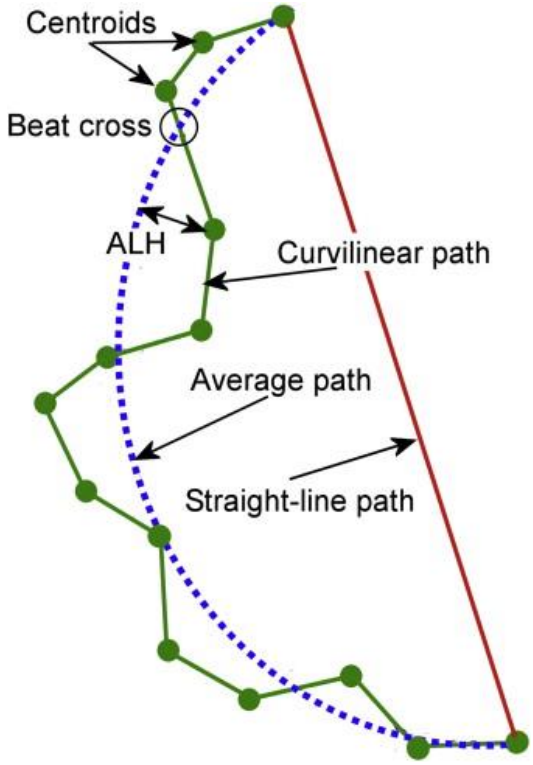

Figure 1. Diagram of CASA terminology (Amann and Waberski, 2014).
The illustration shows the path of one spermatozoon. The curvilinear path shows the actual path of the spermatozoon, with time-average velocity (VCL). Computed average path with time-average path velocity (VAP), the straight-line path is the distance between first and last position of sperm head (VSL), deviation from the average path (ALH). The beat cross is a place where the curvilinear path is crossing the average path, measured as number of crossing per second (BCF).

Figure 2. Photograph of output from CASA analysis.

3. Hypothesis and aims

Cryopreservation of ram spermatozoa is difficult due to the composition of their membranes, with the result that after thawing it is difficult to obtain a sufficient number of viable, morphologically normal spermatozoa for successful insemination.

The hypothesis is that melatonin will work as an antioxidant, with a protective role on the sperm membranes by neutralisation of reactive oxygen species, thus improving sperm cryosurvival and recovery rate after SLC. This study is based on previous findings from the study of Šterbenc *et al*. (2019).

The aims of this study were to improve the cryosurvival of ram semen samples, by addition of 1 mM of melatonin to semen extenders and selecting good quality spermatozoa by Single Layer Centrifugation after thawing.

4. Materials & Methods

4.1. Literature review

For a literature review, search engines such as Google scholar, Web of Knowledge and sites of SLU library were used, over a time span of the last twenty years. Exceptions for the time span were made mostly for literature on anatomical descriptions. The keywords were: sheep reproduction, Jezersko-Solčava sheep breed, all year round breeders, single layer centrifugation, melatonin, anatomy of the cervix in sheep, etc. Emphasis was placed on peer-reviewed articles.

4.2. Animal welfare

This study used ejaculates from young breeding rams owned by the Biotechnical Faculty of the University of Ljubljana; semen was collected by electroejaculation. The ejaculates were taken for purpose of routine andrological examination, with the remainder being used for this study. This procedure was approved by the commission for Animal Welfare of Veterinary faculty of University of Ljubljana. The Commission gave the opinion that the procedure performed on animals is in accordance with the Slovenian Animal Protection Law (Uradni list RS št. 38/2013 and 21/2018).

4.3. Preparation of solutions for two-step dilution freezing protocol and HOS analysis (see Figure 3)

4.3.1. Hypoosmotic (HOS) solution

Ingredients for HOS solution were measured using a laboratory balance (Tehtnica 200A, Železniki, Slovenia). The balance is accurate in the range of 0.05 g to 200 g with a distribution of 0.01 mg and a measurement uncertainty of 0.05 ± 0.00019 g or 199.9999 ± 0.00029 g. For 100 mL of HOS solution, 1.35 g of Fructose (D(-)-

Fructose, $C_6H_{12}O_6$, M= 180.15 g/mol, Merck, Germany) and 0.735 g of sodium citrate dihydrate (tri-sodium citrate dihydrate, $C_6H_5Na_3O_7$ x H_2O, Mr = 294.10, Merck, Germany) were weighed. Chemicals were mixed in a 100 mL volumetric flask and then diluted with the addition of distilled water to the final volume of 100 mL. The flask was marked with the date of preparation and the name of the solution. The solution was mixed for 20 minutes and a small volume was taken for osmolarity estimation. The osmolarity was measured twice with a cryoscopic osmometer (Osmomat 030, Gonotec, Germany) at a temperature of -6.9 ˚C. The calibration of the osmometer was performed with Eichlösung solution (400 mOsm/L; 12.687 g NaCl / kg H_2O, Knauer, Germany). The osmolarity of the prepared solution was 150 mOsm/L, in both measurements. Afterwards, the solution was stored at -18 ˚C until needed.

4.3.2. TRIS-based extender

All ingredients were measured using the laboratory balance described in the previous section. For the preparation of 400 mL of TRIS based extender, 12.74 g of TRIS (hydroxymethyl) aminomethane ($H_2NC(CH_2OH)_3$, M = 121.14 g/mol, Merck, Germany); 7.1448 g of citric acid monohydrate ($C_6H_8O_7$ x H_2O, 210.14 g/mol, Merck, Germany); 5.2608 g of fructose (D(-)-Fructose, $C_6H_{12}O_6$, M= 180.15 g/mol, Merck, Germany) and 10 mL of antibiotic solution were used. The antibiotic solution was prepared by adding 12 mL of bidestilled water to the antibiotic cocktail (Biladyl cocktail AB, Minitüb, Germany, with the following active units of antibiotics: *Tylosin* 60 mg, *Gentamicin* 300 mg, *Pectinomycin* 360 mg and *Lincomycin* 180 mg).

All ingredients were mixed in a 500 mL volumetric flask with the addition of distilled water to 400 mL. The solution was mixed thoroughly for 20 minutes and afterwards taken for estimation of pH and osmolarity. The osmolarity of the TRIS-based extender was 340 and 336 mOsm/L measured twice with a cryoscopic osmometer at a temperature of -6.9 ˚C, calibrated with Eichlösung solution with osmolarity 400 mOsm/L. The pH 6.96 was measured with a calibrated pH meter (MP 220, Mettler Toledo). Afterwards, the TRIS-based extender was divided into 25 mL aliquots and stored at -18 ˚C until needed. All the tubes were marked with the date of preparation and type of solution.

4.3.3. TRIS-egg yolk extender

For TRIS-egg yolk extender, egg yolk was separated from egg white. Each egg yolk was carefully rolled on filter paper to remove excess egg white. The egg yolks were collected in a tube to the desired volume of 30 mL. Afterwards, this volume of egg yolk was added to 120 mL of TRIS-based extender and mixed for 45 minutes. Then the extender was filtered through a sterile syringe filter

(Minisart® Syringe Filter, Sartorius, Germany) with a pore size of 0.45 µm. The filtered extender was divided into labelled tubes of 10 mL volume and stored at -18 ˚C until use. This extender was used to make Extender I and Extender II as follows.

4.3.4. TRIS-egg yolk extender with melatonin (Extender I melatonin) or without melatonin (Extender I control)

Extender I melatonin consisted of Tris-egg yolk extender containing 1 mM of melatonin (melatonin powder - $C_{13}H_{16}N_2O_2$, Sigma-Aldrich Chemie, Germany) dissolved in 0.1 % of dimethyl sulfoxide (DMSO; Sigma-Aldrich Chemie, Germany) and *phosphate-buffered* saline (PBS; Sigma-Aldrich Chemie, Germany). Extender I control (no melatonin addition) contained the same amount of polar solvent DMSO and PBS.

4.3.5. TRIS-egg yolk extenders with melatonin (Extender II melatonin) or without melatonin (Extender II control)

Extender II had the same composition as extender I and, in addition, contained Equex-Paste STM® (0.35 % final Equex-Paste STM® concentration; Minitüb, Germany) and 14% glycerol (Sigma-Aldrich Chemie, Germany) as cryoprotectant. Extender II melatonin contained 1mM of melatonin dissolved in 0.1 ‰ of DMSO and PBS. Extender II control with no melatonin contained the same amount of DMSO and PBS as Extender II melatonin.

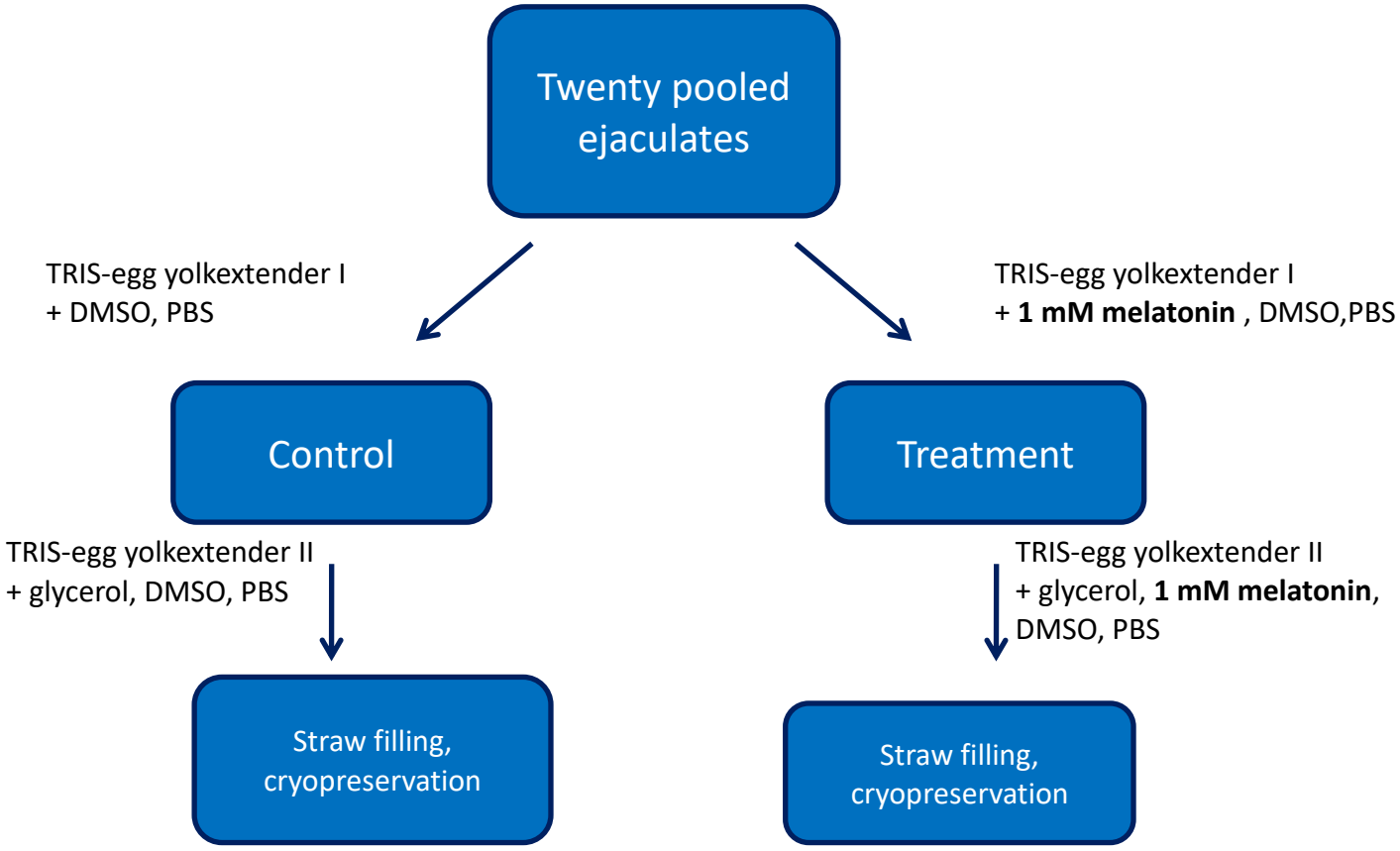

Figure 3. Diagram of process of preparation for two-step dilution protocol for sperm cryopreservation

4.4. Semen collection, processing and semen cryopreservation

Twelve rams of JS and JSR sheep breed were transported to the faculty of the Veterinary university of Ljubljana and allowed to acclimatize for two days to establish a hierarchy. After two days, semen collection was performed between routine andrological examinations.

Semen samples were collected by electroejaculation with the use of a portable electro-ejaculator (EE e320, Minitüb, Germany) with increasing electrical impulses. The electro-ejaculator consists of a battery charger with voltage of 12 – 17 V, small rectal probe and pulse controller. The rams were restrained while lying on a table in lateral recumbancy. A rectal probe was lubricated and inserted into the rectum adjacent to the accessory sex glands. Before electrical stimulation

was performed, gentle massage of the area of the accessory sex glands was implemented. The ejaculation was induced by electrical stimulation with automatically increased impulses. Ejaculates were collected into warm collecting tubes, to minimise the impact of the environmental temperature on the spermatozoa. The ejaculates were kept in a water bath at 27 °C until further processing, with a maximum of 30 minutes. The motility, concentration and volume of ejaculates were measured. To examine sperm morphology, smears of semen were prepared on glass slides. The smears were then labelled, air-dried, fixed and stained. Slides with semen were examined by optical microscopy at 1000 x magnification, under oil (Immersion oil Type N for microscopy 50cc, Nikon Japan). Semen concentration was measured with a spectrophotometer (photometer SDM6 Minitüb, Germany) and the total volume of each ejaculate was measured. The dilution rate for the spectrophotometer for ram semen was 1:500 (8 µL of semen and 4 mL of NaCl solution, 0.9 %). Before further processing, the ejaculates were pooled to avoid the individual effect of rams. Semen samples with motility less than 50 % were excluded from the experiment since it was unlikely that the spermatozoa would survive cryopreservation. In total, 20 pooled spermatozoa samples were available for this experiment.

Every pooled ejaculate was divided into two parts and diluted at room temperature with either Extender I melatonin or with Extender I control to a final concentration of 600×10^6 spermatozoa/mL. After dilution, samples were transferred to the incubator for one hour at 17 ˚C. Diluted samples were placed in a 100 mL water bath, which enabled slow cooling to + 5 °C over 2 h. After cooling to + 5 °C, extender II was added; again either with the inclusion of 1 mM of melatonin (Extender II melatonin) or without (Extender II control). Diluted semen samples were aspirated into 0.5 mL straws and sealed.

Filled straws were frozen in nitrogen vapour placed on metal ranks, 4 cm above the surface of liquid nitrogen for 45 minutes. Freezing the semen samples in nitrogen vapour took 12 minutes. Frozen samples were kept in liquid nitrogen (- 196 ˚C) in marked goblets in the liquid nitrogen tank, until thawing and analysing.

One straw from each batch was used for sperm motility evaluation. After thawing in a water bath at 37 ˚C for 17 s, samples were immediately transferred into a tube and incubated for 10 minutes in a water bath at 37 °C. Analysis was performed with a computer assisted semen analyser (Hamilton Thorne Biosciences, Version 12.3, Beverly, MA) in a counting chamber (Makler®, Sefi-medical instruments Ltd.) warmed to 37 °C. Samples in which sperm motility was less than 50 % were excluded from the experiment.

4.5. Single Layer Centrifugation (SLC)

All the media needed for the analysis were placed in the refrigerator the day before the experiment. Before starting the analyses, the media were placed in a heated block at 27 ˚C. The straws with the semen samples (melatonin and control group) were thawed in a water bath at 37 ˚C for 17 s. Three straws per semen samples were thawed, giving a final volume of 1.5 mL. Samples were put into labelled tubes and left at room temperature until the first concentration was measured.

After the first concentration measurement, the semen samples were diluted to a final concentration of 70 x 10^6 spermatozoa/mL using TRIS-based extender, and concentration was checked again. Afterwards, 4 mL of colloid (Ovicoll, SLU, Sweden) was pipetted into a 15 mL centrifuge tube and then 4.5 mL of semen sample diluted with TRIS-based extender was carefully layered on top of the colloid using an automatic pipette. Layering of the diluted semen sample was performed slowly; allowing the sample to flow down the wall of the tube, whilst holding the tube at an angle of 45˚. When layering was performed correctly, a noticeable boundary line between colloid and semen could be seen. A comparison between samples before and after centrifugation is shown in Figure 4.

The semen samples were centrifuged (Varifuge 3.0R, Heraeus Sepatech, UK) at 285 g (1100 rpm) for 20 minutes at 23 ˚C. The correct settings (i.e. *g* force, temperature, brake and time) were checked before every centrifugation. After centrifugation, visible layers could be differentiated. The supernatant and the most of the colloid were discarded into the biological trash (due to inclusion of antibiotics in the extender). The semen pellet was carefully removed, and a small amount of sample was taken for the third concentration measurement.

The semen pellet was transferred into a clean tube containing 1 mL TRIS egg yolk extender and incubated in a water bath (GFL, Germany) at 37 ˚C for 10 minutes. After incubation, smears for morphology determination were prepared, and analysis of motility, viability (Viadent®) and hypoosmotic swelling test were performed.

The samples were incubated in a water bath for a total of six hours, with all the analyses being performed three times during the incubation. First, analyses were performed at the beginning of the incubation (hour 0), again after three hours of incubation (hour 3), and lastly at the end of the incubation (hour 6). At the end of the day, any unused media and the remaining semen samples were discarded as biological material.

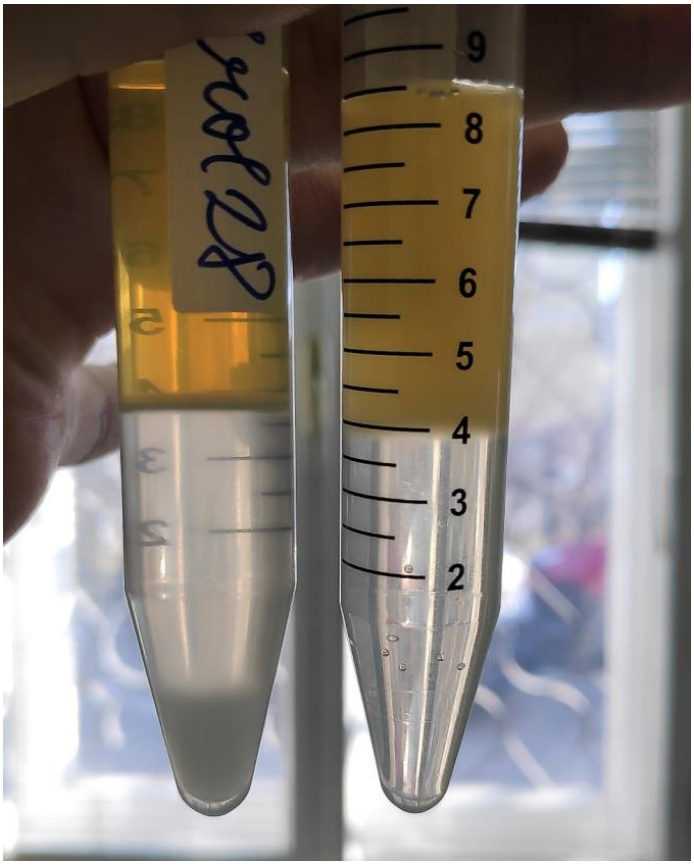

Figure 4. Comparison between samples before and after Single Layer Centrifugation. Centrifugation tube on the left is after centrifugation, with a formed pellet of selected spermatozoa on the bottom of the tube. There is also a clear line of dead and damaged spermatozoa on top of the colloid and the layer of seminal plasma with extender. Centrifugation tube on the right (before centrifugation) has a clear line between the semen sample and colloid.

4.6. Sperm analyses

4.6.1. Concentration evaluation and recovery rate estimation

For concentration estimation, a counting chamber (Neubauer improved, Brand, Germany) was used with a phase contrast microscope (Olympus BX 40, Japan) at 200 x magnification. The concentration was estimated three times for each sample during the sampling day, as described in the previous section, in duplicate.

After thawing the samples, the first concentration measurement was made, to enable dilution with TRIS-based extender to a final concentration of 70 x 10^6 spermatozoa/mL; 25 µL of the semen sample were diluted with 2.5 mL of distilled water in two tubes and mixed thoroughly. The dilution rate was 1:100. The counting chamber was filled with the diluted sample by capillary action, covered with a cover slip and left to settle for 10 minutes. Afterwards, the

spermatozoa were counted in 5 of the squares on each side of the counting chamber. The average count was used to calculate concentration using the following formula:

$$C = No.x\ dil.x\ (1\ mm3/V\ mm3)\ x\ 1000$$

Where C is the final sperm concentration (x 10^6 spermatozoa/mL), No. is the average number of counted spermatozoa, dil. is the dilution rate, Vis the volume in which the spermatozoa were counted (V = 0.0025 mm^2 x 0.1 mm x 16 x 10) and 1000 is the final volume of 1 mL. After calculating the concentration, the volumes of TRIS-base extender and semen sample were calculated to achieve the final volume of 4.5 mL needed for SLC.

After dilution, the second concentration was measured with a dilution rate 1:50 (2 x 50 µL of the semen sample were diluted with 2.5 mL distilled water). The second concentration estimation was used as a control to determine whether the dilution of samples was made properly according to the SLC protocol. The third concentration was measured after SLC and recovering the sperm pellet. The total volume of the sperm pellet was measured with an automatic pipette. The semen pellet was mixed carefully and 25 µL of the sample was diluted using 1.25 mL of distilled water in two parallel dilutions with a dilution rate 1:50. The final concentration was calculated and the recovery rate was calculated according to the following formula:

$$Yield = (number\ of\ spermatozoa\ recovered / number\ of\ spermatozoa\ in\ the\ initial\ load)\ x\ 100.$$

4.6.2. Analysis of motility and viability (Viadent®) by CASA

For viability analysis, a stain solution was prepared using 1000 µL of TRIS-based extender and Viadent stain (Hamilton Thorne Biosciences, Beverly, MA). The preparation of the Viadent stain was performed in a dark room, because of its light sensitivity. The solution was mixed until the stain had completely dissolved. Before use, the stain was stored in a dark box. Semen viability was analysed after incubation of the samples in a water bath at 37 °C for 0, 3 and 6 h, as follows: 30 µL of stain solution were incubated in a water bath at 37˚C for 5 minutes. Then 30 µL of the semen sample were added, followed by gentle mixing and incubation for an additional 5 minutes before analysis. Analysis was performed with Viadent assay® (Hamilton Thorne Biosciences, Beverly, MA) on a computer assisted sperm analyser (CASA, Hamilton Thorne Biosciences, Version 12.3, Beverly, MA) to determine viable spermatozoa.

Samples were incubated in a water bath at 37 °C for a total of six hours. During incubation, samples were taken for sperm total motility analysis, performed by CASA, at 0, 3 and 6 hours of incubation. Sperm kinematics in three randomly selected microscope fields were evaluated utilising factory CASA ram settings (frame rate 60 Hz, minimum cell size 5 pixels, VAP 75µm/s and STR

80 % for progressive cells, magnification 1.89, stage temperature 37 °C, indent fluorescence options: Viadent). For this analysis, 5 μL of the stained semen sample were loaded into a Makler® counting chamber warmed to 37 °C and covered with a cover slip.

To check that all the spermatozoa in the field were counted, a control was done using the playback function. After the analyses were finished, an Excel file was created with the motility and viability data and saved.

4.6.3. Sperm morphology

High quality semen smears are required when assessing sperm morphology. Small artefacts induced during preparation can influence the appearance of the spermatozoa.

To make a smear, a 6 μL aliquot of semen sample was placed on theslide. The resulting drop was pulled out into a smear with a second slide or a coverslip (see in Figure 5). The smear was made with minimal force to avoid breaking the sperm tails. Smears were labelled with the sample identity, treatment (melatonin or control), date, hour and the type of analysis (morphology or HOST) and were left on the heating plate for 10 minutes to air-dry. After drying, the smears were fixed and stained with Giemsa stain. The procedure was performed for time 0 (10 minutes after placing the semen samples into incubation bath), 3 and 6 h of incubation.

One litre of the fixative solution was prepared, using 125 mL of 35 % formaldehyde with 0.5 g of sodium bicarbonate ($NaHCO_3$) and 10 g of sodium chloride, with the addition of distilled water to the final volume of 1 L. For a box (containing 19 slides) of staining solution 17.1 mL of Giemsa stain (Giemsa stain, Merck, Germany) with 8.4 mL of buffer solution (disodium hydrogen phosphate/potassium dihydrogen phosphate, pH 7.00 in 20 ˚C, Merck, Germany) and 150 mL of distilled water were prepared.

Air-dried smears were placed into the glass slide rack, immersed in the fixative solution for 30 minutes and then quickly washed under running water. Afterwards, the slide rack with smears was placed into the glass staining dish with Giemsa solution for at least 16 h or overnight. Next morning, the smears were washed under running water and left to air-dry. Slides with stained smears were stored in boxes for microscopic slides before further analysis. The fixed and stained semen samples were examined by optical microscopy at 1000× magnification, under immersion oil, evaluating at least 100 spermatozoa in each sample.

Spermatozoa were classified as having normal morphology (without any visible defect) or abnormal morphology (occurrence of one or more visible defects). Spermatozoa with abnormal morphology were divided into eight groups, based on the type of defect: acrosome detached, acrosome detaching, defective

acrosome (different defects on acrosome), abnormal head (large, small, pyriform, detached, vacuolated or double heads), abnormal neck (spermatozoa with a bent, thick, thin or double-neck), abnormal mid-piece (double, bent, broken), abnormal tail (spermatozoa with bent tail, coiled tail, multiple tails, 'DAG' defect and not having the tail attached at the correct location) and cytoplasmic droplet (proximal, medial or distal droplet, according to its location on the mid-piece). Examples of these defects can be seen in Figure 6. Afterwards, the proportion of all groups was calculated and added to the Excel database.

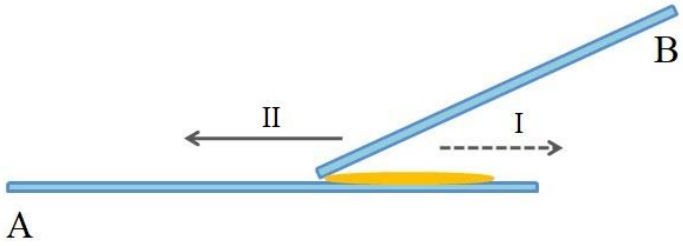

Figure 5. Schema of proper smear technique.
A: glass slide with the aliquot of semen sample. B: second slide or coverslip, positioned at 45° angle. Arrows are labelling first and second directions of movement of the second slide.

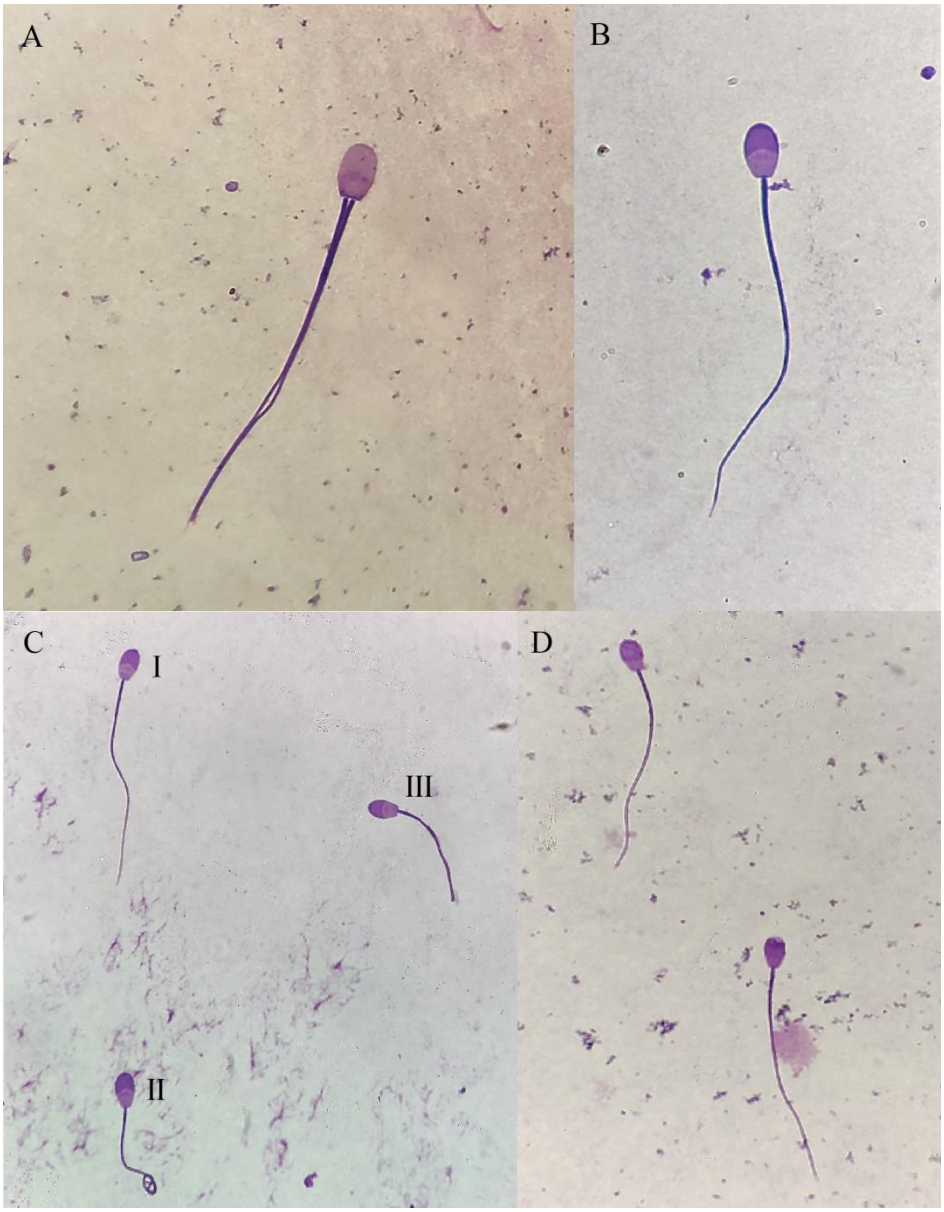

Figure 6. Morphological classification of spermatozoa.

A: spermatozoon with detached acrosome, with double neck and double tail.

B: morphologically normal spermatozoon.

C: spermatozoa from HOST classification, where I. is HOS negative and II and III HOS positive.

D: spermatozoa with an acrosomal defect and detaching acrosome.

4.6.4. Hypoosmotic swelling test (HOST)

The HOST was used as an assay to evaluate the functional activity of the sperm membrane (i.e. membrane integrity). A 500 µL aliquot of hypoosmotic solution was incubated in a water bath at 37 °C for 10 minutes. An aliquot of 50 µL of

semen sample was diluted in 500 μL of hypoosmotic solution and incubated at 37 °C for 30 minutes. After incubation, smears were made and left on a heating plate for 10 minutes to air dry. The smears were made at 0, 3 and 6 h of incubation.

Air-dried smears were fixed and stained as described in the previous section. A total of 100 spermatozoa were evaluated by phase contrast microscopy at 400 x magnification. Spermatozoa were divided into two groups: HOST positive (swollen and/or coiled tail) and HOST negative (without coiled tail). The proportions of both groups were calculated. The percentage of HOST positive spermatozoa was scored and the HOST analysis was completed after deducting the percentage of spermatozoa with a coiled tail estimated from morphology analysis.

4.7. Statistical analysis

Collected data from 20 samples were analysed, based on treatment, time and treatment*time interaction as repeated-measures data. Least squares means were adjusted by Bonferroni correction. The assumptions were checked using diagnostic plots. No apparent deviations from normality or homoscedasticity were detected and therefore there was no need to log-transform of any of the variables. A mixed model approach as implemented in the mixed procedure of the SAS (SAS Institute Inc. 2017, Version 9.4.) system was used. The fixed part of the models included treatment and hour, and their interaction. Pooled semen samples were set as a random factor. The relation between time points for each parameter (each combination of samples and treatment) were modelled using autoregressive AR (1) covariance structure. Degrees of freedom were determined according to Kenward and Roger (1997).

Statistical analysis for recovery rate was performed using ANOVA General Linear Model in Minitab (Minitab 18 Statistical Software Ltd., UK). The results are presented as arithmetic mean values.

The alpha value for this experiment was chosen to be 5 % (the experiment is confident in 95 %) and p values were compared based on the selected alpha value. The p values and F values were taken into consideration, with $p < 0.05$ values being considered as statistically significant (based on selected alpha level). However, p values in a range $0.05 < p < 0.10$ were considered to be marginally significant.

5. Results

5.1. Recovery rate of spermatozoa

The arithmetic mean values of each column of recovery rate for C group was 11.83 % and for M group 15.53 % (p = 0.147). The measured recovery rate was higher in almost every sample with the melatonin treatment, except one (Table 1). The recovery rate was low for both groups (control – C and melatonin - M), and there was no significant difference (p = 0.147) between treatments.

Table 1. *Recovery rate of spermatozoa %*

	CONTROL	MELATONIN
Sample		
1	21.45	30.15
2	6.31	8.2
3	6	8.49
4	4.88	6.45
5	4.07	6.48
6	18.04	19.39
7	7.55	8.51
8	9.94	16.7
9	5.01	6.01
10	9.53	11.92
11	9.44	11.63
12	6.07	6.54
13	9.38	26.34
14	25.32	27
15	6.96	10.88
16	15.94	9.63
17	17.07	29.16
18	13.12	15.84
19	25.38	30.91
20	15.23	20.46
Raw mean values	11.83	15.53

Comparison of the raw treatment means; p = 0.147

5.2. Motility, Viability (Viadent®) and Kinematic parameters of spermatozoa

The treatment effect was statistically significant (p = 0.0262) for motility parameter, with higher values for the melatonin group than for the control group (Table 2). The same pattern was observed for progressive motility (p = 0.0016). In both parameters, there was a highly significant decrease with time (p < 0.0001). No significant interaction was found between time and treatment.

Treatment had no significant effect on viability; however, the effect of time was highly significant (p < 0.0001). No significant interaction was found between time and treatment.

For kinematics, melatonin treatment had a positive significant effect only for BCF (p = 0.0358); for VSL, there was a trend towards significance (p = 0.0887). The time effect was highly significant for VAP, VSL, VCL, ALH and BCF. For STR, there was a trend towards significance for time (p = 0.0736) whereas LIN was not significant. No time*treatment interaction was found for any of these parameters.

Table 2. *Motility, Viability and Kinematic parameters (Least squares means ± SEM) of ram spermatozoa in the control and melatonin group at different times after thawing.*

Time (h)	**0**		**3**		**6**			
	C	**M**	**C**	**M**	**C**	**M**	**SEM**	**p**
Motility %	84.4	88.8 *	76.8	82.9 *	68.3	74.2 *	4.15	0.0262
Progressive motility %	54.8	62.6 **	47.7	54.9 **	35.0	43.7 **	4.24	0.0016
Viability %	94.2	93.5	90.4	92.6	87.9	89.3	1.39	0.3291
Kinematic parameters								
VAP µm/s	116.7	118.4	90.2	93.8	75.2	78.6	3.45	0.1567
VSL µm/s	101.4	103.9	78.6	81.8	66.9	70.4	2.92	0.0887
VCL µm/s	193.7	193.4	146.2	152.0	123.3	127.2	5.51	0.3580
ALH µm	6.5	6.4	5.9	5.7	4.7	5.1	0.32	0.9437
BCF Hz	41.9	42.4 *	39.6	40.5 *	39.1	40.7 *	0.65	0.0358
STR %	86.8	87.3	87.2	87.0	88.1	88.7	0.71	0.5996
LIN %	54.6	55.8	55.4	55.4	54.4	55.4	1.13	0.3651

C: control, M: melatonin

* indicates significant difference between treatments; * p < 0.05, ** p < 0.01, comparison between treatments (within a row) for a given time point

5.3. Hypoosmotic swelling test – HOST

Melatonin treatment had a highly significant effect on HOST ($p < 0.0001$) in comparison with control group. The sperm subpopulation with an intact membrane was higher in the melatonin group at all time points (Table 3). In contrast, membrane integrity tended to decrease with time with a trend towards significance ($p = 0.0677$). There was no significant interaction between time and treatment interaction.

Table 3. *HOS positive ram spermatozoa (Least squares means ± SEM) in the control and melatonin group at different times after thawing*

Time (h)	**0**		**3**		**6**			
	C	**M**	**C**	**M**	**C**	**M**	**SEM**	**p**
HOS positive %	64.7	70.5 **	61.7	68.9 **	62.1	67.9 **	1.59	<0.0001

C: control, M: melatonin

* indicates significant difference between treatments; ** $p < 0.01$, comparison between treatments for a given time point

5.4. Morphology of spermatozoa

For spermatozoa with normal morphology there was a highly significant effect of melatonin treatment ($p = 0.0008$); the effect of time was also highly significant ($p < 0.0001$; Table 4). No significant time*treatment interaction was found.

For abnormal spermatozoa, treatment had an effect only for tail defects ($p = 0.0013$), with fewer being seen in the melatonin group. Acrosome defects increased with time ($p < 0.0001$) and there was a trend towards significance for mid-piece defects to increase with time (p 0.0514). Tail and neck defects did not change with time, nor was there a time*treatment interaction in any of the groups.

Table 4. *Morphology of ram spermatozoa (Least squares means ± SEM) in the control and melatonin group at different times after thawing*

Time (h)	**0**		**3**		**6**			
	C	**M**	**C**	**M**	**C**	**M**	**SEM**	**p**
Morphology (%):								
Normal morphology	77.5	79.5**	70.6	73.2**	65.1	70.1**	1.68	0.0008
Acrosome detached	3.1	3.3	4.2	3.6	6.5	5.4	0.59	0.1985
Acrosome detaching	7.1	7.4	12.3	11.4	14.3	13.1	0.94	0.2864
Acrosomal abnormalities	1.9	1.8	2.8	2.6	3.9	3.4	0.65	0.4224
Tail abnormalities	7.6	5.7 **	7.2	5.3 **	6.5	5.4 **	0.77	0.0013
Head abnormalities	0.4	0.2	0.5	0.4	0.7	0.5	0.14	0.1112
Neck abnormalities	1.4	1.2	0.8	1.7	1.6	1.1	0.32	0.8739
Mid-piece abnormalities	0.9	0.9	1.4	1.6	1.4	0.9	0.29	0.6750
Cytoplasmic droplets	0.2	0.1	0.1	0.2	0.1	0.1	-	-

C: control, M: melatonin

* indicates significant difference between treatments; ** $p < 0.01$, comparison between treatments (within a row) for a given time point

6. Discussion

The aim of this study was to investigate the effect of melatonin addition together with SLC on the quality of frozen/thawed ram semen samples. In total, twenty parameters in three subgroups (CASA, HOST and morphology) were evaluated to assess the potential effect of melatonin on sperm function and morphology. Analyses were done at three-time points. Significant differences were observed in motility, membrane integrity and morphology, with better values in the melatonin group.

Previously it was shown that SLC selects motile and viable spermatozoa from defective ones, and separates them from potential pathogens and seminal plasma (Morrell, 2006). However, other studies proved the beneficial effect of seminal plasma on spermatozoa, protecting them against cold shock during cryopreservation (Barrios *et al.,* 2005; Faigl *et al.,* 2012; Ledesma *et al.,* 2014).

Another limiting factor for successful cryopreservation is the membrane composition of ram spermatozoa, which is rich in PUFA. This membrane composition will increase the risk of lipid peroxidation by ROS attack. An increased concentration of ROS leads to sperm damage, thus decreasing sperm quality (Rather *et al.,* 2016). The addition of melatonin as an antioxidant should scavenge ROS, therefore protecting the spermatozoa and improving sperm quality.

In this study, the recovery rate was very low and insufficient for artificial insemination; melatonin treatment did not have a positive effect on the recovery rate. Furthermore, concentration and total volume of the restored pellet after SLC were very low in both groups (on average 0.39 mL for control and 0.45 mL for melatonin). The values were lower than in the previous study of effects of SLC on F/T ram spermatozoa by Šterbenc *et al*. (2019), where the mean recovery rate was 21 %. The first concern was whether the colloid was overloaded with spermatozoa resulting in increased competition to pass through the interface with the colloid. The maximal recommended sperm concentration for processing fresh stallion semen is 100 x 10^6 spermatozoa/mL (Morrell and Nunes, 2018). In this experiment, the aim was to achieve a sperm concentration of 70 x 10^6 spermatozoa/mL, leaving a margin for error in case of miscalculation. Thus, the final concentration never exceeded the maximum recommended level. However,

there may be species differences in the optimal concentration because of differences in sperm morphometry.

In general, there was low quality of the ejaculates and occasionally problems arose with the semen collection. Out of the 30 pooled ejaculates, 10 were excluded due to low sperm motility (less than 50%) under the presumption that spermatozoa would not be able to survive cryopreservation (N. Šterbenc, personal communication). In the studies of Jafaroghli *et al*. (2011) and Banday *et al*. (2017), semen samples with less than 70 % motility were excluded prior to cryopreservation. In the study of Dorado *et al*. (2010) with goat semen, only semen samples with progressive motility higher than 30 % after thawing were included. Goat sperm freezability is judged by classifying as suitable or not suitable. Those classified as suitable have rapid progressive motility of 30 % or higher (Hidalgo *et al.*, 2007).

In this study, ejaculates were collected from an autochthonous Slovenian sheep breed with all-year round reproduction. The first collection was performed in the autumn and the second one at the beginning of February. However, the owners of the rams complained about the fertility of the rams during the previous year (N. Šterbenc, personal communication). The quality of the ejaculates collected from the rams at the beginning of February could have been affected by short adjustment period of the animals after transportation. If the quality of ejaculate is low prior to cryopreservation, the quality during cryopreservation and after thawing will decrease rapidly. This will result in low concentration of robust spermatozoa available for selection by single layer centrifugation, possibly explaining the low recovery rates seen in this study.

Progressive motility, with a forward movement following the typical swing of the sperm head and tail, is desirable, but other type of movements can be observed. Damaged spermatozoa will move in circles or backwards; more severe cases roll from side to side or do not move at all (Parkinson, 2009b). Motility, progressive motility and viability are crucial factors in assessing semen quality, since spermatozoa need to cross relative great distances and pass several barriers in order to reach and fertilize the oocyte. In rams, sperm motility in fresh semen varies between 80 to 90 % (Parkinson, 2009b; Faigl *et al.*, 2012).

In this study, the addition of melatonin had a positive effect on post-thaw total motility and progressive motility. Similar results were reported in the study of Succu *et al*. (2011), with the best values being for the addition of 1 mM of melatonin. In the present study, mean motility in the melatonin group after six hours of incubation were almost equivalent to the mean values of the control group after three hours of incubation. No statistical difference was seen for viability. Of the kinematic parameters, melatonin had an effect only on BCF; there was a trend towards significance for VSL to be greater in the melatonin group. Nonetheless, only VCL and VAP are thought to be correlated to the ability

of spermatozoa to pass the cervix (Robayo *et al.,* 2008), and there was no significant effect of melatonin on these parameters. Melatonin regulates several anti-oxidant enzymes, which help to reduce ROS levels, thus protecting membrane integrity and motility (Sun *et al.,* 2020).

Morphology is one of the most useful aspects of semen evaluation, since it influences sperm fertility (Šterbenc *et al.,* 2019). In ram semen, the proportion of spermatozoa with normal morphology in the fresh ejaculate varies between 70 and 80 % (Faigl *et al.,* 2012). Sperm abnormalities are categorized according to three criteria: site of the defect on the spermatozoa, site of origin in the reproductive tract during spermatogenesis, and the likely effects of the defect on fertility. There are three sites of origin: primary (defects during spermatogenesis in testes), secondary (defects formed during epididymal transit) and tertiary (after ejaculation, i.e. incorrect handling of the semen). Major defects, in relation to fertilization ability, are those of the head and acrosome, as well as the presence of proximal cytoplasmic droplets; the remainder are considered as minor defects (Parkinson, 2009b).

Melatonin treatment was associated with a positive effect on normal morphology and membrane integrity. The increase in abnormal spermatozoa over the incubation time was due to the increasing proportion of detaching acrosome and tail defects. Normal morphology in the melatonin group was above 70 % even after six hours of incubation, which is within the normal range for fresh ejaculates according to Faigl *et al*. (2012). Mean normal morphology for the melatonin group after six hours was similar to the value for the control group after three hours. The same pattern was observed for acrosomal abnormalities. Melatonin addition possibly helps to stabilize sperm cells during the freezing and thawing process, thus limiting premature capacitation or acrosomal reaction (Nur *et al.,* 2010).

Only tail defects were decreased after melatonin treatment. Defects of the tail result in immobility or reduced motility, affecting fertility (Parkinson, 2009b). During morphology evaluation of the tail, the most observed defects were coiled tails. Coiled tails can be the result of testicular degeneration (Parkinson, 2009b) or because of the presence of urine in the sample. The percentage of coiled tails in morphology was subtracted from the percentage of coiled tails in HOST, which was used to assess membrane integrity.

Melatonin treatment produced a positive effect on membrane integrity; mean membrane integrity at the end of the incubation period for the melatonin group was higher than the mean value at the beginning of incubation for the control group. Melatonin can pass the plasma membrane of the sperm cell, and thus can scavenge ROS in both intra- and extracellular spaces (Cebrián-Pérez *et al.,* 2014). Production of ROS increases during freezing and thawing, affecting plasma membrane integrity, acrosome integrity and mitochondrial activity. High levels of

melatonin can help scavenge ROS and protect mitochondria against oxidative damage, thus maintaining the structure and functional integrity of mitochondria. Melatonin as a cryoprotectant has a beneficial effect on morphology and sperm function through its ability to reduce the generation of free radicals (Sun *et al.*, 2020). It is possible that the beneficial effect of melatonin after seminal plasma removal with SLC could be due to the molecules remaining in the intracellular space and helping to protect the spermatozoa during the incubation time.

6.1. Strengths and weaknesses of the study

The strength of this study was choice of breed. Jezersko-Solčava and Improved Jezersko-Solčava sheep breeds are tough and all-year-round breeders. This means that the quality of the ejaculate should be more or less stable throughout the whole year and enables semen to be collected throughout the year. Another advantage of the study was the pooling of the ejaculates, thus limiting individual ram effects. Use of two-step dilution protocol, with the first dilution at room temperature and second dilution after gradual equilibration to 6 ˚C, and the use of TRIS-based extender containing egg yolk, are other advantages. In the study of Paulenz *et al*. (2002), the ram semen stored at 5 ˚C and diluted with TRIS-based extender, preserved the spermatozoa better than extenders based on milk or sodium citrate. The two-step dilution protocol was used according to the study of Gil *et al*. (2000). The experiment was carefully planned; the control extenders included the same amount of solvent as the melatonin treatments, thus creating equal conditions for all groups.

There are previous studies showing a positive effect of melatonin addition on cryopreserved ram semen samples, as well as the positive effect of the use of single layer colloid centrifugation on post-thaw sperm quality. The innovation of this study is in the combination of those two procedures on the quality of the ejaculate.

The main weakness of this study was the limited time, which was insufficient to allow the rams to be trained for semen collection by artificial vagina, which would help to improve welfare of the rams. Moreover, it also limited the possibilities of selecting quality rams and semen collection from the same individual ram. This would help to achieve a better quality of ejaculates prior to freezing, possibly resulting in better recovery rates, etc.

It was intended to carry out further analyses by flow cytometry, to measure mitochondrial status, membrane integrity and reactive oxygen species status objectively in an effort to understand the role of melatonin, possibly as an antioxidant. Semen collection and most of the microscopic analyses were done in Slovenia, and flow cytometry analysis should have been done in Sweden. Samples were sent to Sweden using a dry shipper but, unfortunately, the container was

damaged during transport and the straws thawed out. Despite this situation, results were available from the microscopic analysis. Transporting frozen samples always carries the risk that the samples will thaw out and be lost.

Samples were collected by electroejaculation. This is a common procedure in Slovenia for rams in the breeding programme. However, semen collection with EE may have affected the quality of the ejaculate and possibly disturbed the results. The volume of the ejaculate is greater in EE compared with AV (Abril-Sánchez *et al.,* 2019), whereas the sperm concentration is lower (Marco-Jiménez *et al.,* 2005; Robayo *et al.,* 2008). There is also evidence of a difference in seminal plasma proteins between the two collection methods (Abril-Sánchez *et al.,* 2019). However, the semen collected by EE showed an increased lifespan of morphologically normal spermatozoa, due to certain protective proteins from seminal plasma (Ledesma *et al.,* 2014). The collection technique could have positively affected the results of this study, thus intensifying the positive effect of the melatonin treatment. On the other hand, the quality of the ejaculates was low from the beginning of this study.

An interesting twist in the whole study would be to perform single layer colloid centrifugation prior to cryopreservation. The reasons for not using SLC prior to freezing were: on average very good quality of fresh ram ejaculate, the disadvantage of seminal plasma removal (some seminal plasma proteins protect spermatozoa during cryopreservation) and the time limitation for study design and pilot literature review.

In future research, it would be interesting to use different sheep breeds, preferably seasonal ones. The semen collection should be performed during the whole year since there is evidence of a fluctuation in melatonin level in the bloodstream and the ejaculate, in seasonal breeds. The semen should be collected by both methods (EE and AV) to evaluate their effect on the ejaculates and melatonin treatment. Second, the SLC would be performed either before or after freezing. For further research it would be interesting to investigate the effect of melatonin addition and SLC on DNA methylation of the spermatozoa. Last, *in vitro* fertilization trials or oocyte binding assays should also be included to evaluate the effects on fertilization rate and early embryo development, in addition to artificial insemination to evaluate the pregnancy rate, service period and natality/mortality

7. Conclusion

Melatonin addition, together with Single Layer Centrifugation, improved ($p < 0.05$) motility, progressive motility, BCF, membrane integrity and normal morphology, and decreased tail defects. The recovery rate was very low, emphasising the poor freezability of the ejaculates used.

Melatonin addition to the extenders prior to cryopreservation was fast, practical and economically viable. It can help to protect ram spermatozoa against cryodamage and attack by reactive oxygen species, and improve bad quality ejaculates. Furthermore, it can help to stabilize the insemination doses, giving a little extra time when performing artificial insemination of ewes. Insemination trials should be done to ensure that any improvement in sperm quality seen in these laboratory assays does have biological relevance.

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