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Cryopreservation and Use of Frozen Semen in Sheep Reproduction

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Abstract

Cryopreservation of ram sperm has been in development for decades. The reason is that ram sperm is more vulnerable to any external factors than sperm of other species. Every step of the process of preserving ram sperm needs to be done carefully to avoid causing unnecessary damage. It starts with possible semen collection methods all the way through lambing after insemination with the collected and preserved semen. This process has its advantages over using natural mating, however, there are disadvantages at the same time. The selected preservation method can be influenced by the preferred insemination method, and vice versa. Substances used for dilution of semen can be combined in many ways but it has to be done carefully, not to cause harm to spermatozoa. Vaginal insemination is considered to only be successful with fresh semen, while cryopreserved semen can only show acceptable success rates when deposited directly into the uterus. The transcervical insemination technique was considered to be inappropriate with cryopreserved semen, but with newly developed devices and surgeries for this purpose, it has become a better option compared to intrauterine insemination, especially when looking at animal welfare. For insemination to be successful, many factors need to be considered on the female side whether it is management factors or synchronization methods that need to be chosen wisely.

Absztrakt

A kosok spermájának kriokonzerválása már évtizedek óta fejlődik. Ennek oka, hogy a kosok spermája érzékenyebb bármilyen külső tényezőre, mint más fajoké. Ebből adódóan a folyamat minden egyes lépését gondosan kell elvégezni a feleslegesen okozott károsodások elkerülése érdekében. Ez a folyamat a lehetséges spermagyűjtési módszerektől egészen a gyűjtött és konzervált spermával való termékenyítés utáni bárányozásig tart. Ennek az eljárásnak megvannak az előnyei a természetes pároztatással szemben, ugyanakkor megvannak a maga hátrányi is. A választott konzerválási módot befolyásolhatja az előnyben részesített termékenyítési módszer és fordítva. A sperma hígtására használt anyagok sokféleképpen kombinálhatók, de ezt nagyon óvatosan kell végezni a spermiumok károsodásának elkerülése érdekében. Míg a vaginális termékenyítés csak friss spermával való termékenyítés esetén tekinthető sikeresnek, addig a kriokonzervált spermával való termékenyítés csak akkor mutat

elfogadható sikerességi arányt, ha közvetlenül a méhbe juttatják. A transcervicalis termékenyítési technika szintén sikertelen kriokonzervált spermával, de az erre a célra újonnan fejlesztett eszközök és műtéti technikák jobb választásnak bizonyultak összehasonlítva az intrauterin termékenyítéssel különösen az állatjóllét szempontjából. A sikeres termékenyítés érdekében a nőstény oldaláról számos tényezőt kell figyelembe venni, legyenek azok menedzsment tényezők vagy szinkronizációs módszerek, melyeket körültekintően kell megválasztani.

Contents

1. Introduction

The sheep industry has a rich history which is estimated to start around 9,000 BC with their domestication. Sheep are considered to be one of the earliest animals to be domesticated by humans and the initial reason for their domestication is estimated to be their meat, milk, and skin. They were favored over other species for domestication due to their small body size, earlier puberty and maturity, higher production rates, social nature, and obedient behavior. Additionally, their products were easier to preserve compared to other similar animal products. With the growing global population and increased customer demands for livestock products, the global population of sheep is significant. Therefore, research and investment in breeding, management, nutrition, and disease control are crucial for maximizing the economic benefits of sheep production.

World distribution and population of sheep by continents – Comparison between 2006 and 2018

Continent	Sheep ($x10^6$)		World proportion (%)	
	2006	*2018*	*2006*	*2018*
Asia	416	512	40.6	43.6
Africa	244	352	23.8	30.0
Oceania	138	95	13.5	8.1
Europe	139	131	13.6	11.2
America	87	84	8.5	7.1
Total	1024	1173	100	100

Table 1- Comparison of population and distribution in 2006 and 2018 [1, 2]

Population and distribution of sheep are influenced by a range of factors, including environmental, biological, and human factors like social, economic, and cultural aspects, see Table 1. Sheep contribute significantly to meat and wool production, with Asia and Africa producing most of the sheep meat, milk production mainly occurs in the Near East and part of Europe, while wool production is prominent in countries like Australia and New Zealand. Sheep's ability to grow wool makes them economically superior in some regions, due to their ability to thrive in areas where cattle might not perform as well.

This shows that sheep production is a critical sector of agriculture, playing an important role in various aspects of human life and culture. The decline of the sheep industry could have widespread consequences, affecting both agriculture and cultural heritage [1, 2].

Statistics collected from the Food and Agriculture Organization of the United Nations (fao.org) for the year 2021 (last updated on March 21, 2023, Table 2) shows the estimated gross

production value of the meat industry worldwide, proofing the importance of maintaining the sheep industry and continue to contribute to its development.

Area	Product	Estimated value (USD x10^6)
World	Meat of sheep, fresh or chilled	67258
	Meat of sheep, fresh or chilled (indigenous)	45181
European Union (27)	Meat of sheep, fresh or chilled	2430
	Meat of sheep, fresh or chilled (indigenous)	2690

Table 2 - Estimated production value in 2021, from fao.org

Because of the importance of the sheep industry worldwide, it is important to keep the development going and artificial insemination (AI) is one of the most powerful tools for doing so. By using AI and the preservation methods available, it is easier to transfer superior semen for long distances and even across the world if needed and the selection of preferred traits needed for individual herds is easier for further development.

Objectives/Questions

The objectives of this literature review are to gather information about the different approaches of collecting semen from rams and the various methods of preserving the semen for it to be acceptable for AI. This review will also examine the different methods available for inseminating ewes, and what aspects need to be considered when choosing the appropriate insemination method, as well as ways of preparing ewes for AI, whether it is by estrous synchronization, heat detection, or what management factors need to be considered for increased chance of successful AI.

Method

When researching material to use for this review, various search engines and databases were used. CAB Abstracts, PubMed, ResearchGate, and Google Scholar were the foundation. Mainly qualitative data was selected, and the main selection criteria included keywords such as Artificial insemination, Ovis, Sheep, Ram, Semen, and Cryopreservation.

When selecting the material, I looked at how often it had been cited, what were their main references and how relevant the information was. Because of the nature of the material, papers

published before the year 2000 were not used unless cited in newer papers and included relevant information.

2. Semen collection

When starting to consider cryopreservation and/or storage of ram semen, it needs to be remembered that the semen must be collected first and then examined properly, both quantity and quality need to be examined and if everything is within normal range, the further processing of semen can be continued. The main characteristics of the semen (Table 1) must be within the normal range to be considered a good option to use for AI. Two main techniques are used for semen collection: Artificial vagina (AV) or electroejaculation (EE).

2.1.Artificial vagina

The use of AV for semen collection is the method that resembles natural mating the most but the reason it can be considered more difficult to use than EE is the fact that it usually requires training of the rams which can take up to two weeks, for them to get acquainted with the artificial vagina and for them to be able to ejaculate like in normal and natural service. However, untrained rams can often serve into an AV when an ewe has been restrained in an adjacent pen to them and is first served by an experienced ram. This can stimulate an untrained ram to serve into the AV but this does require an experienced collector who can correct the position of the AV quickly, according to the rams movements [3].

By using the AV, the libido and mating ability of the rams can be assessed along with if there is any noticeable deviation of the penis anatomy. This technique does not cause alterations in semen quality compared to natural mating [4].

The AV includes a cylinder on the outside made of strong rubber and containing a latex liner. There is a space between the outer cylinder and the liner, which is filled with warm water (45°C-48°C). The temperature is a very important stimulus for ejaculation, and if not within the optimal range, there is a risk of no ejaculation from the ram. The warm water also provides necessary pressure which resembles the turgor of the normal vagina [5]. On one end of the structure, there is a latex extension cone which contains a collecting tube for the ejaculate. It needs to be taken into account the length of the AV, the ram should ejaculate into the extension cone to prevent any possible contamination. Before using the AV, neutral lubricant should be applied around the inside of the AV, care needs to be taken not to use spermicidal substances.

An ewe is used for mounting and when the ram is led to her, he should be allowed to rub his nose against the ewe and smell her, which will cause either complete or partial erection along with pre-ejaculatory production of accessory gland secretions. When the ram is ready to mount, the collector is ready with the AV on one side of the backside of the ewe, the ram will make small thrusts first with its penis, to locate the vulva. At the time of the small thrusts, the collector grabs the sheath and deflects the penis, while guiding it into the AV. The collector cannot grab the penis itself, which is extremely sensitive and will result in unsuccessful mounting. This procedure should be done in a quiet environment, to prevent any disturbance [3].

2.2.Electroejaculate

The use of electroejaculate is a quicker method and often considered a more convenient method for semen collection because training is not a requirement before using this method.
The technique can be useful in collecting sperm from seasonal breeders outside of their normal breeding season [4].

This method consists of administering low voltage and low current electrical pulses to the rectum with a transrectal probe, which is equipped with electrodes [4].
The ram is restrained, either standing or laying on its side while the penis is held in an extruded position. A bipolar rectal electrode probe is lubricated and placed into the rectum with the electrodes facing towards the hindlegs, the probe should be located on the brim of the pelvis. With 4-6 rhythmic stimulations to the ampulla and the sacral nerve plexus, ejaculation will occur. Erection can occur if the probe is in the correct position, but some rams will ejaculate without an erection. EE is normally well tolerated, but the ram should be rested for few minutes if the procedure needs to be repeated. Repeated procedure could be necessary if there is extensive hindleg muscle stimulation or if ejaculation fails to happen after the original 4-6 stimulations. [3]

One of the disadvantages with EE is that not all rams have the same response to the electrical stimulation, some will produce semen samples with similar volume and concentration/density to a natural service or with AV, while others have obvious reduction in sperm concentration [3].
However, there have been some concerns about problems with animal welfare when using this technique. Even though it is easy to use and effective, it can be stress- and painful for the rams. To reduce the risk of negative effects of the procedure, anesthetics, sedatives and sometimes

hormones could be administered before applying the technique, but it needs to be taken into account that this could affect the quality of the semen collected. [4]

In several European countries, the use of EE is considered to be an inhumane practice and therefore is banned to use and the European Union has prohibited importation of semen is it has been collected by using EE [4].

One alternative method to EE which has been developed is Transrectal Ultrasonic-Guided Massage of the Accessory Sex Glands (TUMASG), which is better from an animal welfare point of view. It requires fewer electrical stimuli for successful ejaculation and some evidences show that the electrical pulses aren't a requirement for successful ejaculation but this alternative method does require personnel who is trained properly [4].

2.3.Comparison

When there is more than one method available, there is always the question of which one should be chosen.

The biggest difference between the methods from the labor point of view is that no training of the rams is required before using the EE. That has been the biggest reason in the past of why the EE has been the preferred method to test the quality and quantity of ram sperm production [5]. Studies have shown that between AV and EE, the composition of the ejaculated seem to be no different but that there is a difference in characteristics [6]. When using the EE technique, there are more day-to-day differences within an individual ram, in all examined characteristics; volume, sperm concentration, total sperm, and wave motion [5].

Characteristics	
Volume (ml)	1.0 (0.5 – 2.0)
Fractionated	No
Density (x10^6/ml)	2000 (1250-3000)
Motile sperm (%)	>90
Normal sperm (%)	>75

Table 3- Main characteristics of ram semen [3]

Another study showed no difference in sperm motility in fresh samples [7]. The EE method produced ejaculates of greater volume, but the sperm concentration was lower than what was obtained with AV and tended to have higher pH. This difference in concentration is due to higher production of seminal plasma when using the EE. There have been higher levels of fructose found in semen collected with EE, which is considered to be due to the consistent stimulation of the accessory glands by the electric currents, which is not the case when using the AV. However, the samples collected with the AV showed higher wave motion activity. The

collection method doesn't seem to influence the proportion of live, morphologically normal or actively progressing sperm, or affect post-thaw quality [6, 7].

Studies have shown that epididymal spermatozoa appears to be more resistant to cryopreservation, while ejaculated samples showed better quality post-thawing compared to samples from EE. EE seemed to make spermatozoa more vulnerable to cryopreservation. However, this study was done in vitro and it should be considered that even though in vitro quality of thawed epididymal samples was superior, it doesn't necessarily translate to better field fertility outcomes [8].

3. Advantages of using preserved semen

Each ram can produce enough spermatozoa for insemination of thousands of ewes. By preserving the semen, the number of offspring can be significantly increased from each ram per year. This makes genetically superior rams accessible to the sheep industry worldwide and that way help with introduction of new genetic material via international exchange of semen. Because of the possibility of international trade, superior semen can be transported internationally instead of transporting animals, preventing problems of adaptation, such as lack of resistance to local diseases.

The number of rams needed in individual farms can be significantly reduced and therefore lowering the cost housing, feeding, and labor for each farm [9, 10].

Preserved semen can eliminate problems and/or contagious diseases that can appear when mixing animals from different flocks [11]. It can also lead to effective improvement genetically of the national sheep population due to the possibilities of applying genetic material of high value to even small flocks [10].

Using preserved semen reduces the risk of venereal diseases and because of extensive examinations of semen before processing and mandatory testing, the risk of pathogens being transmitted with semen reduces significantly. The pathogens causing the biggest threats are Brucella melitensis, Brucella abortus and Brucella ovis [9].

4. Disadvantages of using preserved semen

Detecting estrous in ewes can easily be the most difficult and problematic part of all AI programs, ewes don't normally show signs of estrous unless in the presence of a male and

therefore an infertile ram is necessary to detect ewes in estrous. Another possibility is to administer synchronization drugs to each ewe and that way determine the timing of estrous [9]. Fertility, regarding both rates of pregnancy and litter size, is adversely affected when using artificial insemination, especially when frozen-thawed semen is used. There have been intensive laboratory studies on how to keep ram semen viable after freezing and thawing, but despite them all, the process of freezing and thawing still reduces the viability of spermatozoa significantly and therefore isn't capable of good fertilization rates [10].

It can occur that a small number of especially selected blood lines in combination with increasing use of AI, makes the possibility of inbreeding greater and increases the risk of decline in genetic variation [9, 11].

Cervical insemination of ewes with frozen-thawed semen requires large amount of viable spermatozoa being applied in the cervix and because of the processed semen, the spermatozoa have impaired motility, causing failure in reaching the site of fertilization [10].

Considering all the technology around the process and the insemination, it all has its cost and that is no different when preserving and using ram semen. The costs include the collection and the assessment of semen, the processing, freezing and storing of the semen, delivery of AI, labor costs, and cost for drugs used for synchronization of ewes [10].

There is always a possibility that a genetic fault could be transmitted from a superior ram if it is a recessive trait, this is especially a possibility if the recessive trait is present at a low rate in the general population, meaning that many individuals need to be bred before the condition appears in a homozygous progeny, good example of this is poor hind leg/foot conformation [9].

It can happen that there are bacterial pathogens in the semen, usually obtained from contamination of the ejaculate, that are not parts of the routine testing of the samples, which could be resistant to the antibiotics that are present in the extenders used or can even avoid them by forming biofilms [11].

5. Preservation methods

Nowadays, only two methods for preservation seem to reach acceptable results in fertility when using AI in sheep. These methods are cooling down to 15°C and freezing for laparoscopic AI. However, it needs to be considered the interaction between the preservation method and the

insemination technique. Because of the complex female anatomy of the cervix, there are problems that hold back how widespread the usage of frozen-thawed semen has become [12].

5.1.Fresh semen

Fresh semen indicates it being used immediately after it has been collected, most often after dilution [11]. Not too much material has been published about using fresh semen in sheep reproduction. However, one study showed that using AI with fresh semen, either diluted with egg yolk citrate or a commercial soybean lecithin-based extender called AndroMed, can be an effective method. It can also be a practical method for increasing lamb production and enhancing the genetic quality of sheep during the non-breeding season, especially in certain regions. In the study, pregnancy rates after vaginal AI during the non-breeding season were found to be approx. 32.6-39.3% depending on experimental groups. These rates were within range which previous studies had reported for fresh diluted semen. The same study compared the already mentioned diluents (egg yolk citrate and AndroMed), showing that the reproductive parameters observed with AndroMed were slightly higher but not significantly higher than those observed with the egg yolk citrate. Furthermore, it may be a useful technique to expose ewes to rams 3 hours after AI to increase the immediate reproductive performances of the sheep. However, it comes with a drawback – The biological father of the newborn lambs becomes uncertain [13].

5.2.Chilled semen

Chilled semen is the preservation of extended semen in a liquid state. Main methods of this preservation technique are storage at reduced temperature, either 0-5°C or 10-15°C, and another method is to store the semen at ambient temperature by inactivating spermatozoa, which is reversible. By storing extended semen at a reduced temperature, we are extending the life of the spermatozoa by slowing down their metabolism while inhibiting bacterial growth. It is required to inhibit bacterial growth because normally bacteria grow by consuming the nutrients available in semen extenders and therefore decreasing the available resources for the spermatozoa. If the bacteria manages to survive in the beginning but dies in the stored semen, they could release endotoxins which are toxic to spermatozoa [11].

Various changes occur to the spermatozoa when stored in liquid state. These changes include a drop in motility, integrity of morphology and their fertility. With longer storage, the

more the changes are of decreased quality, regardless of dilution or its rate, temperature, or condition of the storage. Furthermore, there is a reduction in the spermatozoa capability of survival in the female reproductive tract and there are higher risks of embryonic loss [14]. Studies have shown that storing ram semen at 5°C is more favorable than at 15°C, as it preserves sperm motility and viability over time, a high proportion of sperm retains its ability to penetrate artificial mucus for up to 72 hours of storage and storing semen at 5°C for 72 hours post-collection does not reduce its ability to fertilize oocytes in vitro, that is if cold shock is prevented in the cooling process. Cold shock can be prevented by adding lipids as extenders, such as egg yolk or soybeans. Lipids have been found to protect sperm against cold shock. Storing semen at 15°C causes more pronounced detrimental effects to spermatozoa and longer storage has negative effect on sperm motility and morphology, potentially due to the accumulation of toxic metabolic byproducts. The same study showed that prolonged storage of semen is associated with a linear decrease in pregnancy rates in ewes after AI and it is advised to perform cervical AI within 24 hours to achieve higher pregnancy rates. The choice of diluent does not show a significant effect on semen quality when stored at 5°C. Chlortetracycline treatment has shown potential to improve post-thaw sperm motility and plasma membrane integrity. [15–18].

One research [18] showed that UHT skim milk supplemented with 5% egg yolk, either alone or with 2% glycerol, showed promise in preserving ram semen at 5°C for up to 48 hours. While sperm quality declined after 48 hours, this timeframe was considered suitable for practical use in AI procedures.

5.2.1. Fertility after Liquid Storage

There are researches that show that it is possible to obtain acceptable pregnancy rate when using liquid ram semen which has been stored for 12 hours at 5°C when deposited at the cervix by doing the insemination after proper estrous detection [19].

It is known that there is a gradual decline in motility and morphological integrity of spermatozoa after being stored in liquid storage, leading to a rapid decline in fertility. However, as stated previously, the duration of the storage is the most important factor when it comes to successful usage. Lowered motility of spermatozoa is mainly a problem due to the anatomy of the ewe cervical anatomy and the complex process of reaching the site of fertilization, which includes muscle contractions and ciliary action. It also needs to be taken into consideration the limited survival of spermatozoa in the oviduct. If spermatozoa have aged in storage, the risk of

embryonic mortality increases. Embryonic mortality is the death of fertilized ova and embryos up to the implantation stage. Aging of spermatozoa is known to affect embryonic survival, potentially leading to abnormal embryo development and high embryonic mortality rates [14].

5.3. Cryopreserved semen

Cryopreserved semen, or frozen semen, is the method that in an optimal environment, semen can be stored indefinitely without losing qualities that are needed for AI. Cryopreservation involves temperature reduction, cellular dehydration, freezing and thawing. The main point of cryopreservation is to slow down or stop the cellular metabolic rate of spermatozoa while being frozen in liquid nitrogen at -196°C and after frozen storage, that they survive, and functionality is restored after thawing. This makes it possible to keep spermatozoa from superior donors for unlimited periods of time and opens the possibility of preserving genetic pools along with making it possible to use sperm as efficiently as possible with AI. The semen collected is mixed with solutions for protection, which usually contain lipoproteins, sugars and some cryoprotectants, like glycerol. By adding the solutions, the membrane integrity of the spermatozoa is reserved during the process of cooling, freezing, and thawing. During the procedure, the spermatozoa will always suffer structural, biochemical, and functional damage, resulting in reduced motility and fertilizing ability. This damage is believed to be caused by ice formation, both extracellular and intracellular. Before the solution is frozen, it induces the efflux of intracellular water via ice crystallization, cell contraction and there is also a possibility of ion influx. Cryopreservation can also lead to the release of certain enzymes and loss of important substances in sperm [11, 20, 21].

Formation of reactive oxygen species (ROS) can also be induced by cryopreservation, which can impair fertility [21]

The cryopreservation of ram sperm presents challenges due to its sensitivity to freezing and thawing processes. Various attempts have been made to improve post-thaw sperm viability using additives, antioxidants, and modifications to cryoprotectant composition and cryopreservation processes. These attempts have yielded varying degrees of success, with ram sperm motility after thawing ranging from 34% - 61% in different studies [22].

While a relatively high proportion (40-60%) of ram spermatozoa retain their motility after freeze-thawing, only about 20-30% remain biologically undamaged. Spermatozoa may be motile but damaged, making it uncertain whether such cells can effectively fertilize an egg.

Motility and structural integrity of spermatozoa are affected differently, and it's unclear whether these changes occur simultaneously or at different stages of the freeze-thaw process. Spermatozoa that are weakened but not mortally injured during freeze-thawing, and those that are aged after thawing and insemination in the female reproductive tract, may initiate non-viable embryos that perish at an early stage. Ram spermatozoa generally experience more severe ultrastructural damage compared to bull spermatozoa. Motility is better preserved than the morphological integrity of spermatozoa after both slow and fast freezing. [16].

5.3.1. Freezing process

The goal of freezing semen is to slowly decrease the temperature from +5°C to -196°C, this process is necessary to try to prevent any damage to the sperm cells.

When the process of freezing is starting, the semen that has been selected needs to be extended with selected diluent and cooled gradually down to 5°C. This is done to try to extend the lifespan and decrease the metabolic activity of the spermatozoa. The dilution is done according to specific ratios for the selected diluent to make sure that the sperm concentration in each insemination dose is appropriate to warrant high fertility after insemination. Care needs to be taken not to have too concentrated semen because that would cause a decrease in the spermatozoa metabolic activity.

For the best results, the cooling process needs to be gradual and slow, to keep the spermatozoa functional. If done too fast from 30°C down to 0°C, there will be sperm damage, also called the "cold shock". This needs to be prevented from happening and that is done by slowly cooling the semen down to 5°C over 1.5-2 hours, by adding the diluents in stages, mix them, and keep the whole mixture around body temperature. The cold shock has been linked to lipid phase transition effects. Such effects can contribute to poor control of intracellular calcium concentrations at low temperatures. This would explain why calcium-chelating agents are included in semen diluents. Evidence suggests that the plasma membrane undergoes structural rearrangements involving lipids and proteins during thawing, influenced by temperature and solute interactions during freezing.

When the temperature goes under 5°C and until it reaches -10°C, the water inside the spermatozoa freezes and increases the risk of ice crystal formation. The aim is cell dehydration, which is regulated by the freezing rate and the goal is for that to happen as fast as possible.

The cooling process must be slow enough to allow for adequate cellular dehydration but fast enough to make sure that the remaining intracellular fluid freezes. By this slow cooling, water manages to escape the cells by osmosis and that way prevents intracellular ice formation which kills the spermatozoa. The cooling rate which has been considered optimal, that is from body temperature down to 5°C, is -10°C per hour and to prevent cold shock as much as possible, adding cryoprotective agents such as egg yolk or milk has shown good results. The semen extender plays a crucial role in determining the optimal cooling rate and packaging for preserving sperm [20, 21, 23, 24].

Cryopreservation methods for gametes, such as sperm, involve either slow freezing or vitrification. Slow freezing uses low concentrations of cryoprotectants, which can cause chemical toxicity and osmotic shock. Vitrification is a rapid method but is typically not used for sperm due to slow heat transfer in sperm cells, which can lead to solution effects or crystallization. [21]

The recommended procedure is to transfer semen to liquid nitrogen (LN2) for storage at a rate of -15°C/min from +5°C to -100°C. Quick freezing rates of 15-60°C/min have shown a reasonable survival rate for sperm cells, with an estimated effective cooling rate of around 20°C/min or more for ram sperm. This slow freezing technique helps maintain cell hydration and promoting sperm cell survival [20, 25]. Cooling rates vary between researchers, one study [26] claims that a cooling rate of -30°C/min is recommended for cryopreservation, while other researchers [27] suggest a range of cooling rate of 15-60°C/min, claiming it leads to better post-thawing results.

The dilution ratio has been found to significantly affect the survivability of ram sperm after freezing and thawing. Sperm diluted at a ratio of 1:2 showed higher total motility and progressive movement compared to a 1:1 dilution ratio at various time points post-thaw. However, there is no standard dilution rate available and early researches used dilution rates ranging from 11-26 fold, while today 2-5 fold dilution rates are more commonly used. These studies suggest that the dilution ratio plays a crucial role in preserving the quality of frozen-thawed ram semen [16, 28, 29].

A study was done on Malpura and Bharat Merino ram spermatozoa to investigate the impact of the initial freezing temperature on the survival and acrosomal integrity. Using 3 different temperatures initially (-25°C, -75°C, -125°C) and analyzing the samples after thawing

during a 4-hour incubation at 37°C. The results showed that the sperm motility was highest in samples frozen at -125°C, with 80.3% of motile cells after thawing and 63.7% during post-thaw incubation. Samples frozen at lower temperatures exhibited slightly lower motility (73.9-75.9%). Acrosomal integrity was also found to be significantly better in samples frozen at -125°C compared to the lower temperatures. These results suggest that an initial freezing temperature at -125°C provides the best cryopreserving ability for ram spermatozoa [30].

5.3.2. Thawing process

Cryopreservation of ram sperm is challenging due to low post-thaw sperm survival, especially when compared to other species such as bulls and stallions. The variability in sperm response to cryopreservation exists among individuals within the same species, which can be attributed to differences in sperm membrane permeability to water and cryoprotectants. Ram sperm has the highest permeability, making it more vulnerable to cryopreservation-induced damage [22].

Thawing is the process of reversing freezing, where a solid phase turns into a liquid. It significantly impacts sperm cell survival during cryopreservation. Sperm quality is negatively affected during both freezing and thawing due to exposure to critical temperature ranges twice: Once during cooling to -196°C and again during thawing. The thawing rate depends on whether the cooling rate was fast enough to induce intracellular freezing or slow enough to dehydrate the cells. Ram semen is typically thawed at temperatures between 38-42°C for 30 seconds. However, thawing at higher temperatures (60-75%) can yield similar post-thaw motility, acrosome integrity, and sperm fertility. Thawing at a lower temperature is practical for using frozen-thawed ram semen under farm conditions. Typically, approx. 40-50% of spermatozoa die after freezing and thawing, and even though frozen-thawed ram semen may contain a significant proportion of motile cells (40-60%), only around 20-30% of them are physiologically active. Furthermore, it needs to be noted that individual ejaculates of ram sperm should have >40% forward-moving spermatozoa after thawing and >30% after 5-6 hours of incubation to be considered acceptable for insemination. This shows us that the thawing phase is equally crucial for sperm survival as the freezing phase [16, 20, 21].

5.4.Extenders and diluents

When preparing semen for cryopreservation, the semen requires specific diluents for its extension. When picking extenders, they must have adequate pH, appropriate buffering capacity

and osmolality suitable to protect the spermatozoa from cryogenic injury, which is reached by reducing the physical and chemical stress that induce the freezing and the thawing process [31].

Semen diluents are used to increase ejaculate volume and maintain sperm fertility for extended periods. They should provide nutrients, protect against temperature-related damage, maintain pH stability, inhibit bacterial growth, and preserve sperm cells. Ideal diluents have high water solubility, low solubility in other solvents, low salt and buffer concentrations, minimal temperature effects, stable cation interactions, high ionic strengths, and chemical stability. The composition of the diluent significantly affects sperm quality and longevity [20].

Semen extenders typically consist of components like a penetrating cryoprotectant (such as glycerol, ethylene or propylene glycol, and dimethyl sulfoxide), a buffer (such as Tris to maintain the pH), saccharides (such as glucose, lactose and raffinose), salts (sodium citrate or citric acid), and antibiotics (such as penicillin and streptomycin). Penetrating cryoprotectants enhance sperm survival during cryopreservation by altering membrane properties, reducing intracellular ice formation, and increasing membrane fluidity at lower temperatures. In contrast, non-permeating cryoprotectants work externally by lowering the freezing temperature of the medium and reducing extracellular ice formation without crossing the cell membrane [31].

5.4.1. Glycerol

The primary cryoprotective agent for preserving spermatozoa is glycerol, despite the investigation of numerous alternatives. The exact mechanisms behind glycerol's superior cryoprotective properties compared to other agents remain uncertain. Glycerol's cryoprotective effects are most pronounced at higher concentrations but must be balanced and controlled against its toxicity which occurs at high levels [21, 32].

Semen extenders containing glycerol can be added to semen in two ways: one-step or two-step methods. In the one-step method, the extender is added all at once after semen collection, while in the two-step method, a portion of the extender (without glycerol) is added after collection, and the rest (with glycerol) is added after cooling but before freezing. Adding glycerol at 5°C is better for sperm survival than at 30°C. Stepwise dilution reduces sperm stress and using cryoprotectants in stages improves sperm survival compared to adding them all at once.

Glycerol is commonly used for ram sperm cryopreservation. It lowers the freezing point of the solution, reducing ice formation, and dehydrates cells through an osmotic mechanism. However, as mentioned previously, glycerol can be toxic to sperm and therefore limit its use.

The ideal concentration of glycerol is 4-7%, with concentrations above 6% harming sperm survival [20].

The timing and temperature of glycerol addition can impact sperm quality. Some studies suggest adding glycerol after cooling, while others recommend different protocols [31].

Research was done in 2002 to investigate the effects of glycerol on ram sperm preservation with two experiments. The results from that research suggested that adding glycerol did not impact the quality of fresh or cryopreserved ram sperm in vitro significantly. However, the study noted that the impact of glycerol on fertility in vivo, particularly within the female genital tract, differs [33]. However, other studies claim that adding glycerol at 5°C provides better sperm quality post-thaw compared to adding it at 15°C, and membrane intactness was significantly higher at 5°C. This same study investigated the effect of an extender called Bioexcell1, which contains glycerol concentration of 6.4%. This extender showed a general better sperm quality parameters, compared to 3.2% glycerol and lower glycerol concentration appeared to result in more stressful treatment and reduced membrane integrity [34].

There are researchers that have investigated if there are differences in extender resistance depending on the source of the semen from rams. Their results suggest that if samples are ejaculated or EE method is used, a glycerol concentration of 4% is suitable for cryopreservation, while 8% glycerol is more effective for epididymal sperm samples, this could possibly be due to greater resistance to glycerol's toxic effects [8].

5.4.2. Egg yolk

The presence of egg yolk in extenders has been suggested to provide protection against cold shock and oxidative stress during liquid storage, maintains motility, and prevents acrosomal enzyme loss [20, 35]. Low-density lipoproteins in egg yolk help maintain sperm membrane integrity [31].

During cryopreservation, it is considered to be the egg yolks lipid component that shields the sperm plasma membrane and acrosome from thermal damage [20].

Studies have shown that both fresh egg yolks and lyophilized egg yolks can be successfully used for cryopreservation. Lyophilization is a freezing and dehydration process which is commonly used for preservation of biological content to maintain stability and viability for longer periods of time. The process is done at <30°C, causing minimal thermal damage of

protein. Therefore, egg yolk extenders could be stored for longer and even used later for semen cryopreservation [36].

Research has shown that a diluent containing 7% glycerol and 20% egg yolk offers better protection to sperm compared to milk-based extenders when assessing sperm motility, pH levels, and viability. Glycerol and egg yolk were highlighted as crucial components in the protective capacity of the diluent [37].

Various research has been done to investigate how successful egg yolk is, in extenders and also in combination with milk extenders. One research came to the conclusion that increasing the egg yolk concentration above 5% in milk extenders does not significantly improve sperm characteristics after thawing, except for membrane intactness, which improved with 10% egg yolk content [34].

5.4.3. Milk diluents

Whole milk or skim milk has often been used as an extender for ram semen. The protein component of milk is recognized for its ability to stabilize or counteract pH fluctuations, protecting sperm during dilution, and acting as a chelating agent. The milk should be heated before using it for semen dilution to inactivate lactenin, which is toxic to sperm. In the past, milk has often been compared to synthetic diluents and that comparison shows that milk is considered more favorable for insemination over synthetic diluents. Skim milk has been found to be better for storage at 15°C and addition of antibiotics showed improvement in fertility when using milk diluents, with fertility rates around 72% [14].

5.4.4. Sugars

Different sugars have been tested for their ability to preserve sperm motility during storage. Some sugars provide an energy source, while others maintain osmotic pressure [14].

It has been demonstrated that hypertonic extenders containing sugars can enhance post-thaw sperm characteristics such as motility, viability, and reduced sperm abnormalities, including acrosomal and membrane integrity. The mechanism behind the cryoprotective effect of sugars is not entirely understood, but it is believed that they may help stabilize the sperm membrane, reduce intracellular ice formation, and prevent lipid phase transitions during freezing. Incorporating sugar concentrations ranging from 50-100 mM into ram semen freezing diluents can be effective for improving semen quality and fertility outcomes. There is research

that suggests that higher concentrations of sugars (70-100 mM) tend to yield better results in terms of sperm quality after freezing and thawing. In particular, 100 mM trehalose or raffinose shows the most significant improvements in sperm parameters, both in vitro and in terms of fertility rates after cervical insemination in ewes [38]. The sugars that are used for semen diluents can be categorized into several groups. Citrate-sugar-based, lactose-based, saccharose-based and raffinose-based. Citrate-sugar-based diluents include different types of sugar, such as fructose and glucose. These hypertonic citrate-sugar diluents were commonly used for ram semen but became less common after the late 1960s. Lactose-based diluents were used as the main component in the past but resulted from poor to moderate lambing rates. Saccharose was used in synthetic diluents to protect acrosome integrity. Synthetic antioxidants, such as Vitamin E, and chelating agents were added to inhibit lipid peroxidation and various combinations were tested to improve cryoprotection [16, 21].

5.4.5. Caffeine

One study investigated the effects of different doses of caffeine on the physical characteristics of ram sperm during a 48-hour chilled preservation period. The results showed that caffeine supplementation had a positive impact on various semen characteristics, depending on the applied dose. Low and medium doses of caffeine improved several sperm characteristics, including normal sperm count, cell membrane integrity, and reduced primary abnormalities after 48 hours of chilled preservation. These findings are consistent with previous studies in other animal species, such as bulls, and they suggest that caffeine enhances sperm function by increasing intracellular calcium levels, promoting hyperactivation of sperm, and stimulate production of molecules which have positive effects on sperm motility and integrity. However, high and very high doses of caffeine were associated with decreased sperm motility, potentially due to increased enzyme leakage and oxidative stress [39].

5.4.6. Antioxidants

Extenders used for semen preservation at 23°C are supplemented with exogenous antioxidants to protect spermatozoa from oxidative stress. Temperature plays a crucial role in the effectiveness of antioxidants, with some antioxidants preserving sperm motility better at lower temperatures. However, the benefits of antioxidants depend on their dosage and the temperature of storage. Experiments with catalase as an exogenous antioxidant showed that it improved

acrosome integrity and total motility in ram sperm at specific concentrations, but higher doses were cytotoxic. L-carnitine and acetyl-L-carnitine have shown promise in preserving sperm motility at higher temperatures, but their effects can vary depending on concentration. Pyruvate was found to maintain metabolic pathways and improve motility during storage. It also reduces lipid peroxidation and acts as an antioxidant. Melatonin, known for its role in circadian rhythms, also acts as a mitochondrial-targeted antioxidant, preventing lipid peroxidation by scavenging free radicals. Its effectiveness depends on the dosage and the type of ROS present. Research into the use of these antioxidants, particularly at 23°C, is essential for developing effective semen extenders that can preserve sperm quality during liquid storage [24].

5.4.7. Others

Soybean lecithin is a safer alternative then egg yolk, with lower microbial contamination risk. It has been used in extenders, with positive effects on sperm motility and viability [31].

Tris and Tes-based extenders were found to be superior to skim milk-based extenders in preserving sperm quality. Additionally, the protective effects of the Tes-based extender were like those of the Tris-based extender. This study supports the idea that Tris and Tes-based extenders, particularly when supplemented with egg yolk, are more effective in preserving the quality of ram spermatozoa during liquid storage [35].

Study was done to assess the use of a synthetic extender called AndroMed, which does not contain egg yolk, for intrauterine AI with frozen-thawed ram semen in Japanese sheep. AndroMed is a Tris-based buffer containing phospholipids, citric acid, sugar, antioxidants, and glycerol and has been used successfully for cryopreservation of semen in other species. The study compared the fertility results of using AndroMed with those of traditional extenders containing egg yolk or bovine serum albumin (BSA). The results showed that AndroMed performed similarly to the extenders containing egg yolk or BSA, with lambing rates ranging from 55.2-64.5%. The study highlighted the advantages of using a synthetic extender like AndroMed, as it reduces the risk of contamination with bacteria and mycoplasma that can occur with egg yolk-based extenders. Additionally, using a completely defined, synthetic extender provides a more hygienic option for semen preservation. While laparoscopic AI has been the most effective method for AI with frozen-thawed semen, this study suggests that cervical or vaginal AI with semen frozen using AndroMed could be a practical and effective alternative [40]. Another study, which used Suffolk and Suffolk crossbred ewes during the non-breeding

season, explored different AI methods with fresh-diluted and frozen-thawed semen and using AndroMed. This study showed that intrauterine AI on a fixed-time basis with AndroMed resulted in higher fertilization rates (62.5%) compared to cervical AI with fresh-diluted semen (44.4%) [41].

Antibiotics are necessary when preserving semen, penicillin and streptomycin are most used, to prevent any bacterial growth during storage.

6. Insemination techniques

There are different methods available for inseminating ewes. Each method has its advantages and disadvantages which need to be taken into account when choosing which method is the best for each occasion. The selection criteria can include the preservation method used on the semen and what method can be used according to available equipment.

When using hormonal synchronization, it is considered optimal insemination time between 48-58 hours after the synchronization [16].

6.1.Vaginal insemination

The basis of vaginal insemination is depositing semen into the cranial part of vagina, without trying to locate the cervix. This method is the simplest method for insemination because it needs minimal requirements regarding technical knowledge and handling accommodations for the ewe since the cervix doesn't need to be located. Because of this, a very high number of spermatozoa is needed in each insemination to attempt successful migration through the female genital tract. The best chance of fertilization with vaginal insemination is after estrous detection during the natural breeding season and the ideal timing for the insemination is before ovulation which happens approx. 12-18 hours after the onset of estrous. Fertility has only been on an acceptable level when fresh or chilled semen, at 15°C, has been used. Studies have shown that the highest conception rates are reached when the insemination is done twice per day after detecting estrous [9, 10, 12].

6.2.Cervical insemination

With cervical insemination, the cervix is located with a speculum and a light source. This method seems to have the best results when the hindquarters of the ewe are elevated. The cervical structure in the ewe is what makes this method difficult to use. The structure is complex, tortuous and it doesn't dilate during estrous, making it difficult to deposit the semen into the

cervix. Generally only the first fold of the cervix can be reached to deposit the insemination dose or up to 2 cm. The conception rates are still better than in vaginal insemination, making this method relatively easy and cheap, but the conception rate also correlates with how deep into the cervix the semen is deposited to. The conception rate has acceptable success with this method if insemination happens 15-17 hours after estrous has been detected [9, 10].

6.3.Transcervical insemination

This method is more recent than the other methods and with it, we try to achieve intrauterine insemination but through the cervix. To achieve this, the cervix is fixed by grasping it with forceps, and making a way for insemination instrument to get as far as possible through the cervix. This can cause trauma both with the forceps and with the instrument going through the cervix, resulting in the possibility of difficulties with conception in the next estrous [9, 10, 42].

Although there is some natural cervical relaxation during estrous, studies have shown that exogenous cervical dilatators (e.g. oxytocin, estradiol, FSH) in sheep could increase relaxation of the cervix in estrous before ovulation and that way increase the chances of successful transcervical insemination [42, 43].

6.3.1. Surgery for cervical dilatation

An interesting approach to help with successful transcervical insemination has recently come to light, to prevent the trauma that can come with that procedure when passing the insemination catheter through the cervix, and hopefully decrease the prevalence of laparoscopic AI. This is described as a surgical approach to overcome the challenging cervical anatomy. Surgical ablation or incision of the cervical folds is done to allow the passage of an insemination catheter and appropriate semen deposition into the uterus. This surgery should be performed within 24 hours post-partum when the cervix is more relaxed and easily manipulated. The cervix undergoes changes in the peri-partum period, including relaxation of smooth muscle layers and softening of connective tissue, making it suitable for surgery. These changes limit the potential for trauma and fibrosis during the procedure. This proposed surgical approach is considered safe, from an animal welfare point of view, and does not compromise the animal's health. It still maintains the cervix's role as a barrier protecting the endometrium and conceptus from pathogens. In the study, they found that ewes that underwent surgical procedures, such as incisions of cervical folds, achieved high pregnancy and lambing rates, similar to those achieved

with laparoscopic AI. These rates were significantly higher than those reported for transcervical AI using frozen-thawed semen in previous studies. One more advantage of using surgical procedure is that it is considered “Once in a Lifetime” interventions, as they can be performed on an ewe without the need for repeated surgeries [44].

6.3.2. Devices to help with transcervical insemination

A new catheter called DARIO (Dispositivo Anti Reflujo para Inseminación Ovina in Spanish, marketed by Consorcio Mercantil de Huesca, S.L.) was designed for AI in Rasa Aragonesa ewes. This catheter has a conical shape, a soft tip with central and lateral holes for semen deposition, and a hemispherical body that blocks the cervical opening, reducing retrograde flow. It fits the cervix well and reduces visible injuries to the cervix. It is made of a flexible polymer and can be attached to the standard AI catheter. Field tests were conducted between 2013-2016, involving 662 ewes inseminated with DARIO and 637 with a traditional catheter. The AI procedure using DARIO was similar to traditional catheters, with a focus on minimizing retrograde flow. The results showed that DARIO significantly increased fertility and fecundity rates compared to traditional catheters. Fertility rate was defined as the percentage of ewes lambing after AI, fecundity rate as the average number of lambs born per lambing ewe, making it a promising tool for AI. [45].

Another device has been developed and tested to see if it would affect semen quality, fertilization rates, and pregnancy rates. The catheter tested was made of semi-flexible stainless steel, and was 17.5 cm long with a 17-gauge size. It had a 4 mm brass bulb at the end. This catheter design was similar to one used for embryo transfer. It was long enough to reach the front part of the uterine horn, making it more effective. Its flexibility allowed it to pass through the cervix smoothly without blocking the semen’s path. The catheter had an internal diameter of 1.07 mm and an external diameter of 1.47 mm. The brass bulb at the tip helped guide it through the cervix’s rings and allowed technicians to monitor its progress using ultrasound. A stainless-steel fitting securely attached the catheter to an AI gun, ensuring no semen loss. The AI gun was designed to work with 1 cm^3 semen straws, compensating for the catheter’s void volume. This design allowed for easy manipulation through the cervix and into a uterine horn. Several experiments were conducted to evaluate the catheter’s effectiveness and they found that it didn’t affect semen quality, sperm numbers, fertilization rates, or pregnancy rates. This

suggests that using this catheter for AI in sheep doesn't interfere with fertility if cervical manipulation is minimized [46].

6.4.Intrauterine insemination

Direct intrauterine insemination is done laparoscopically. The method was originally developed to try to avoid going through the cervix and that way increase the conception rates while using AI with frozen-thawed semen. The biggest advantage of this method is the small number of spermatozoa in each dose that is needed compared to the other methods. The ewes need to be sedated and restrained properly. The procedure is performed close to the udder after local anesthetics have been administered. It is recommended to inflate the abdomen to avoid damaging any possible structures during the procedure. The uterus is located, and a small stab wound is created where a simple pipette can be introduced into the uterine lumen and the semen can be deposited straight into the uterus. There are some disadvantages to this method, it is an invasive technique, it requires a veterinarian to carry out the procedure, and in terms of labor and equipment, it is very expensive. There are also concerns about how acceptable laparoscopic and transcervical insemination methods are, on the grounds of animal welfare [9, 10].

6.5. Conception rates

The success of achieving conception in ewes is influenced by various factors such as the method and timing of insemination, whether the ewes are in synchronized or spontaneous estrous, and whether fresh or frozen semen is used (See Table 4). Furthermore, the management and overall health of the ewes, along with the meticulous management of the AI program, play a crucial role in determining the conception rates [9].

Using vaginal route for AI has shown highly variable success rates but it has been proven that using this technique is not suitable to use with frozen semen [10]. There has been more success in conception rates with the cervical technique. This method has shown good results when using fresh or chilled semen but if using frozen-thawed semen, the success rate is unacceptably low. A higher success rate is during the ewe's breeding season compared to non-breeding seasons. Studies have shown that 80-90% conception rates were achieved with this technique on Hungarian sheep farms, when using fresh semen [9, 10].

Site of insemination	Semen type	Number of motile sperm ($x10^6$)	Lambing rate (%)
Intravaginal	Fresh	400	20-60
	Frozen		5-20
Intracervical Synchronized estrous Spontaneous estrous	Fresh	200	40-80
	Frozen		25-40
	Frozen		30-60
Intrauterine	Fresh	20	70-95
	Frozen		40-80
Transcervical	Fresh	200	40-80
	Frozen		30-70

***Table** 4 - Typical lambing rates of different AI routes [8]*

The transcervical route has shown acceptable conception rates. However, without using the previous mentioned surgical approach for easier AI, it can cause trauma to the ewe's cervix and compromise their ability to conceive naturally afterwards [10].

As seen in Table 4, intrauterine insemination has shown good conception rates and it has been reported that a pregnancy rate of 71.4% has been achieved with this technique in Israel [9, 10].

7. Preparation of ewes

There are many factors that need to be considered before AI can take place and many of them need to be taken care of by management before insemination should take place.

Ewes are what is called "short day breeders", meaning that their regular and spontaneous estrous cycles happen when daylight decreases or is short. This occurs from early fall until late winter. Due to the short period of time within the year, many farmers decide to synchronize their flocks either to have them ovulate at the same time during breeding season or to induce ovulation during non-breeding seasons to increase the number of lambs per year per ewe and therefore, increase the production at the farm.

7.1. Synchronization methods

Synchronization of ewes can be done either with administering exogenous hormones or by using methods which don't require exogenous hormones but can induce return of estrous cycle by mimicking normal conditions for their usual estrous cycle.

7.1.1. With hormones

There have been many different hormonal treatments developed up till now to manipulate the sexual cycle of sheep. When choosing the appropriate treatment, it needs to be considered if it is the breeding season or if it's a non-breeding season. Depending on the season, different hormones should be used. For example, during breeding season, prostaglandins are used because the ewes already have functional corpus luteum in the ovaries, while during non-breeding season, other hormones are most commonly used for estrous stimulation [47].

Progestogens

Any changes in concentrations of progesterone are a crucial part of the process that results in ovulation. Progesterone and its synthetic analogues simulate the function of the corpus luteum and therefore they suppress the normal cyclic activity by blocking the release of gonadotropins. When the administered progestogen concentration decreases and/or is non-existent anymore, this blockage disappears and normal cycle activity returns [48].

Progestogen treatment is widely used for synchronization in ewes, either on their own or in combination with other hormones, and they can be used either to induce estrous during non-breeding season or for synchronization for already cyclic ewes during normal breeding season, but during non-breeding season, it is crucial to use Equine Chorionic Gonadotropin (eCG) when the sponges are removed [48].

Most common route used in ewes is with impregnated sponges intravaginally and the recommended usage is a 14-day treatment program, which will result in return to estrous in 2 days after the progesterone administration is stopped. It has been recorded that inducing ovulation in ewes can cause inadequate formation of corpus luteum, which results in either premature regression or limited progesterone production. With limited progesterone production, there is less stimulation of the endometrial glands for their secretion, which in normal levels promotes pre-implantation of embryos. This lack of stimulation has been considered as a predisposing factor for early embryonic death [48–50].

Prostaglandins

The time interval between estrous is controlled by the corpus luteum and its life span. This means that if the corpus luteum is destroyed or removed, estrous is induced, this process is called luteolysis. Luteolysis can be induced by using exogenous prostaglandins (PGF2a) as an

intramuscular injection, which will also interfere in the normal cyclic activity. In normal conditions, PGF2a is produced by the endometrial glands as a response to stimulation caused by oxytocin to cause luteolysis when the luteal phase ends if there is no pregnancy. After ovulation, the developing corpus luteum is usually refractory for 3-5 days and responsive to exogenous PGF2a for 9 days, if injected into responsive ewes, estrous will be induced approximately 36-46 hours later but the occurrence of ovulation depends on the dominant follicle present and its stage of development. It must be noted that when the process of natural luteolysis has started, exogenous PGF2a will have no effect, and due to the fact that PGF2a are abortion-inducing drug, it cannot be used in animals if there is any chance of pregnancy and this should be confirmed if it is not known if pregnant or not, before using PGF2a [48, 49].

If estrous is being induced during anestrous, it is not enough to use PGF2a alone because there is no luteal tissue to work with during that time. However, a research has shown that using two PGF2a injections with 10 days apart, in combination with Gonadotropin-Releasing Hormone (GnRH) before the first PGF2a injection, leads to higher lambing rate in ewes in non-breeding season [49].

Melatonin

Melatonin is effective in regulating the reproductive cycle of sheep because they respond to short days. It can be supplemented either orally or through subcutaneous implants. Before inserting the implant, ewes should be separated from males for at least 7 days, and after implant insertion, they should remain separated for 30-40 days. After the separation period, rams are introduced, even though peak mating activity normally occurs around 60 days after the implantation. By supplementing melatonin, the breeding season can be accelerated by 2-3 months, without affecting fertility. Melatonin implants can also stimulate reproductive behavior in rams, but higher doses are required compared to the ewes. However, melatonin should not be used in ewes that have never lambed before [48].

Gonadotropin-Releasing Hormone (GnRH) and derivatives

GnRH is responsible for regulating the release of anterior pituitary hormones, which play a crucial part in reproductive processes. GnRH has however been less commonly used in sheep, despite its effectiveness in stimulating ovulation and delaying luteolysis.

Using eCG alone to induce estrous in seasonally anestrous ewes has not proven to be very successful. Administering progesterone to ewes before the injection of eCG can result in synchronized estrous and ovulation, improving the success with estrous induction. Stimulating an early return to cyclical reproductive activity in lactating ewes, especially those that are lactating heavily, has proven to be difficult [48].

7.1.2. Without hormones

Hormones are not the only way to manipulate the normal cycle of ewes, other methods include altering the light exposure and exposing the ewes to a fertile ram to induce estrous.

Light

The seasonal reproductive behavior in these animals suggests the presence of an endogenous circannual rhythm, which plays a key role in regulating seasonal reproductive changes. This rhythm is synchronized by changes in photoperiod. To control the cyclicity of reproduction in sheep and goats, artificial alterations of photoperiod have been used. However, sheep require decreasing daylight to stimulate reproductive activity. Creating artificially short days during midsummer can be challenging and costly due to the need for daylight-proof housing. An alternative approach is to stimulate reproductive activity by exposing animals to artificially long days and then returning them to the natural shorter day length. For example, exposing ewes to 22 hours of "daylight" for at least one month late in pregnancy or at parturition, followed by a return to natural day length, can stimulate reproductive activity. This treatment results in ewes having a fertile estrous approx. 3 months after lambing, eliminating the need for daylight-proof housing [48].

The "Ram Effect"

Introducing a ram to a group of ewes can trigger the female's reproductive cycle, even if they are not in their usual breeding season. This phenomenon, known as the "Ram Effect", has been extensively studied in goats and sheep. It occurs in response to various signals from the male, including scent of sex pheromones and behavior. Exposure to these male signals leads to the secretion of a hormone called Luteinizing Hormone (LH), which then triggers ovulation and the return of regular ovarian activity. However, it's important to note that this doesn't result in perfect synchronization of the ewe's reproductive cycles [48]. Interestingly, these pheromones from a new male can even override the inhibitory effect of Progesterone, except during the

midluteal phase. When you expose anestrous ewes to these male sex pheromones, it can induce ovulation in about 46-50% of them without any other treatment. However, the estrous behavior that usually accompanies ovulation is silent, and the luteal phase following ovulation is shorter in these ewes. In ewes, prior exposure to progestogen is necessary for normal estrous behavior and luteal function after ovulation. Researchers have found that more out-of-season ewes show estrous behavior when they receive progestogen treatment along with exposure to novel male sex pheromones (approximately 77%) compared to just being exposed to a ram. Using progestogens along with exposure to male sex pheromones not only increases the chances of inducing normal estrous behavior and ovulation but also results in pregnancy in about 50% of the females that are mated. Therefore, it's common to use progestogens and male sex pheromones together to improve reproductive performance in ewes that are out of their normal breeding season [49].

7.2.Management factors

There are many factors taking part in successful AI and often, farmers handle the majority of it, especially when it comes to the factors affecting ewes. Without managing these correctly, the chance of successful AI decreases.

Body condition

Body Condition Score (BCS) of ewes significantly influences their estrous activity. Ewes with very low BCS (1.5) and those with very high BCS (3.5-4) are less likely to exhibit estrous. Ewes with BCS of 3 have the highest conception rate during estrous and ewes with very low or very high BCS levels can have difficulties conceiving, leading to longer lambing intervals. Ewes on either side of the moderate BCS have increased risk of aborting, suggesting that ewes with moderate BCS (2-3.5) have better metabolic well-being [51]. Well-nourished ewes with good nutrient intake respond more effectively to the breeding season, showing an increase in ovulation rate. Flushing, which involves providing additional nutrients before mating, leads to a rapid increase in the ovulation rate. In regions with unpredictable food availability, improving the nutrition of ewes through flushing or higher body weights at mating has been shown to enhance lambing and twinning rates. Body nutritional condition is considered crucial for fertility, even more so than body weight [52].

Ram nutrition

Several studies have shown that the diet of rams can influence the size of their testes and sperm production. A balanced diet appears to be important for optimal reproductive function in rams. Specific components of the diet, such as Vitamin E, have been suggested to have a positive effect on increasing both the quality and quantity of semen produced by rams [52].

Age

Young and maiden ewes typically have lower fertility compared to adult ewes, especially after cervical AI. This declined fertility is likely due to impaired sperm transport and lower mucus production in the cervical canals during estrous. Numerous studies have reported a decrease in AI success as ewe age increases. This decline in fertility is estimated to be around 2-3% per year of age for various sheep breeds. Some studies have observed a significant drop in lambing rates with increasing age. The detrimental effect of increasing ewe age on fertility may be attributed to higher risks of reproductive disorders and decreased ovulation rates, leading to lower-quality ovulated oocytes compared to younger ewes. The optimal age for cervical AI appears to be around 2-5 years old, with the highest fertility rates observed in ewes aged between 2-4.5 years. Beyond this age range, fertility tends to decline significantly. Younger and older ewes are often recommended for natural mating rather than AI, as their fertility tends to be higher in such circumstances [52].

Lambing-AI interval

It is well established that ewes require a resting period after lambing to allow for uterine involution, the process of the uterus returning to its normal size and condition. In high-production systems where there is a need to maximize reproductive rates, there may be a tendency to reduce the time between lambing and AI. This can have a negative impact on fertility. Studies have shown that reducing the interval between lambing and AI to less than 40-50 days can lead to a significant decrease in fertility. This decrease in fertility may occur even with natural mating. Most experts recommend not inseminating ewes any sooner than 50 days after lambing. This allows an adequate resting period for uterine involution and supports better fertility outcomes in the future [52].

7.3.Heat detection

There are not many useful methods to detect heat in ewes. They rarely exhibit signs of heat without ram present and therefore it is necessary to involve rams in the process. Teaser rams

are considered the best way to detect estrous because they are incapable of impregnating ewes, but they still show normal copulation behavior. The best candidates for teaser animals are young virgin rams but sexually mature. The ideal teaser animal should be disease-free, show a high level of libido, be small enough for immature ewes but large enough for mature ewes. The teaser ram should be friendly and not show any aggressive tendencies, and it is always helpful if they have a distinct color to differentiate them from the herd. For a ram to become a teaser ram, the flow of semen needs to be blocked and most often vasectomy or epididymectomy are used to ensure that the flow is blocked.

Intact rams can also be used for heat detection, but it is important to ensure that they can't impregnate the ewes. Aprons can be used, and they are also helpful in the way that colored chalk can be added onto the apron to mark the ewes in heat when the ram jumps on them, but the apron blocks successful penetration at the same time.

Results

With increased importance of food production in the world due to growing global population, it is also important to maintain good lambing rates within the sheep production industry. Each ram that is used for semen collection and sperm preservation needs to be thoroughly screened for infectious diseases or hereditary faults, and the sperm needs to be thoroughly examined for proper parameters which are important to achieve successful AI. The processes of freezing and thawing of the semen need to be done properly and carefully for acceptable quality and quantity of sperm for each insemination dose and cold shock should be prevented. Diluents and extenders have to be chosen according to specific criteria and availability, but they must always have adequate pH, appropriate buffering capacity and osmolality suitable to protect the spermatozoa from cryogenic injury. They should include penetrating cryoprotectant, a buffer, saccharides, salts, and antibiotics.

Ewes need to be prepared appropriately and farm management need to take care of their body condition, it needs to be moderate if the intention is to achieve successful AI.

Conclusions

Most of the research material regarding semen preservation and AI in sheep reproduction all aim to find the most suitable way to increase the lambing rate while getting the most out of each insemination dose. However, due to the vulnerability of ram sperm to factors happening outside of the ram's body, it can be expensive to try to achieve successful AI. The cost can either be purely monetarily, or the animal's welfare can also be involved. With the constant development of the industry, it is possible to find a suitable way to achieve acceptable success rates with AI. Due to the development, the new devices that have been invented for transcervical insemination, give the hope of it being possible to achieve successful rates with frozen-thawed semen without the need of surgery. However, the newly found surgery on the cervical folds also shows that with one surgery that doesn't need to be performed for each insemination, frozen-thawed semen can be used without affecting animal welfare.

Even with increased development in preserving semen, it needs to be noted that many factors for successful AI lies in the hands of the farm management, with proper nutrition, heat detection, resting periods between lambing and estrous synchronization or other methods used for preparing ewes for AI.

Summary

Ram semen preservation has been proven to be more difficult than semen preservation in other species because of the spermatozoa vulnerability to factors outside of the ram's body. This causes the success rate of AI to be lower when compared to other species. Even with the complications around the preservation, there are methods available to preserve semen and inseminate ewes successfully. With the constant development of technology around food producing industries, more options become available every year, and with research and studies becoming more available worldwide, it enhances the chance of success. Not only are rams' spermatozoa more vulnerable in the whole process, the ewe's female reproductive tract, specifically the cervix, is complex and can become a barrier for sperm migration into the uterus where fertilization takes place. Without overcoming these hurdles, AI won't become successful.

There are multiple methods of inseminating ewes, each method with advantages and disadvantages. Vaginal insemination can only be successful with fresh semen, and frozen-thawed semen needs to be deposited directly into the uterus to be successful. Frozen-thawed semen has undergone enough damage to spermatozoa, resulting in unsuccessful migration through the cervix into the uterus to fertilize eggs.

To increase the chances of successful insemination, farm management can ensure proper preparation of ewes, such as maintain moderate body condition, ensure proper nutrition, synchronize estrous if needed, and depending on season (breeding season / non-breeding season) which medical protocols need to be considered.

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Thesis progress report for veterinary students

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Thesis title: Cryopreservation and use of frozen semen in sheep reproduction

Consultation – 1st semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2021	April	15	Initial consultation	
2.	2021	Oct	20	Progress report	
3.	2022	Oct	10	Progress report	
4.	2023	Feb	23	Progress report	
5.	2023	April	11	Progress report	

Grade achieved at the end of the first semester: 5

Consultation – 2nd semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2023	May	20	Progress report	
2.	2023	June	15	Progress report	
3.	2023	July	2	Progress report	
4.	2023	Sept	10	Progress report	

5.	2023	October	20	Draft sent for approval	

Grade achieved at the end of the second semester: 5

The thesis meets the requirements of the Study and Examination Rules of the University and the Guide to Thesis Writing.

I accept the thesis and found suitable to defence,

.....

signature of the supervisor

Signature of the student: .....

Signature of the secretary of the department: .....

Date of handing the thesis in 15 November, 2023

I hereby confirm that I am familiar with the content of the thesis entitled

CRYOPRESERVATION AND USE OF FROZEN SEMEN IN SHEEP REPRODUCTION

..... written by SIGRIÐUR AUÐNA GUÐGEIRSDÓTTIR HJARÐAR

(student name) which I deem suitable for submission and defence.

Date: Budapest, 04. day 11. month 2023 year

Horváth András

..... Supervisor name and signature

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