Inland Norway University of Applied Sciences

Faculty of Applied Ecology, Agricultural Sciences and Biotechnology

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Master thesis

Boar sperm quality applying different preservation technologies

M.Sc. in Applied and Commercial Biotechnology

Experimental Specialization

2024

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Acknowledgement

This thesis was carried out at the Department of Biotechnology, Inland University of Applied Sciences, in the faculty of applied ecology, agricultural sciences, and biotechnology. This master thesis is part of a research Zygote project.

First, I would like to sincerely extend my greatest gratitude to my main supervisor, Professor Elisabeth Kommisrud, for her invaluable knowledge and insights. I appreciate the opportunity to study on this project and acknowledge you for your guidance, passionate supervision, encouragement, and for allowing me to learn from your experience.

I would like to express my profound thanks to Dr. Birgitte Narud for co-supervision, advice, and support, especially for your helpful feedback on reading this thesis.

I am also thankful to Teklu Tewoldebrhan Zeremichael for training lab skills, and I appreciate your assistance and support throughout the time I worked in the laboratory.

I extend my sincere thanks to Professor Jane Morrell for providing Porcicoll to be used in this master's thesis.

Also, I would like to express my gratitude to all professors and staff at INN's biotechnology department for helping to make my two years of study an amazing and unforgettable learning experience. And thanks to Norsvin for providing samples.

Finally, I would like to thank my family for standing by me and supporting me. I wish to dedicate it to my Heavenly Mom (Maman). I want to extend my most heartfelt thanks to my husband, Mojtaba, who encourages me to progress with his love and support. And my sweetheart, Melorin.

Abbreviations

AI: Artificial insemination

AID: Dead acrosome intact

AIL: Acrosome Intact Live

ALH: Amplitude of lateral head displacement

AO: Acridine orange

ARD: Dead acrosome reacted

ARL: Live acrosome reacted

BCF: Beat-cross frequency

BSA: Bovine serum albumin

BTS: Beltsville Thawing Solution

CASA: Computer-assisted sperm analysis

CPA: Cryoprotectant agents

DFI: DNA fragmentation index

DGC: Density gradient centrifugation

DMSO: Dimethyl sulfoxide

FACS: Fluorescence activated cell sorter

FITC: Fluorescein isothiocyanate

FS: Fibrous sheath

FSH: Follicle stimulating hormone

GnRH: Gonadotropin hormone-releasing hormone

HDS: High DNA stainability

LDL: Low-density lipoproteins

LH: Luteinizing hormone

LIN: Linearity

MAS: Morphologically abnormal spermatozoa

mOsm: Milliosmoles

ODFs: Outer dense fibers

PBS: Phosphate buffered saline

PI: Propidium iodide

PMT: Photomultiplier tubes

PNA: Peanut agglutinin

RLU: Relative luminescence units

ROS: Reactive oxygen species

RT: Room temperature

SCSA: Sperm chromatin structure assay

SD: Standard deviation

SLC: Single-layer centrifugation

STR: Straightness

TNE: Tris-Null-EDTA

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

UTJ: Utero-tubal junction

VAP: Velocity Average path

VCL: Velocity Curvilinear

VSL: Velocity Straight-line

WOB: Wobble

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Abstract

Commercial pig producers use artificial insemination (AI) extensively for breeding purposes. Successful outcome of AI practices is thus extremely important. Several factors important for successful fertilization, healthy embryo, and progeny development affect AI's efficiency. High-quality sperm are essential for successful fertility as AI centres use fewer spermatozoa per AI than those available by natural mating. AI centres commonly use liquid-preserved boar semen, and methods for choosing high-quality semen are important. The purpose of this thesis was to investigate potential improvements in the quality of boar semen by utilizing various techniques for semen processing and preservation. Single Layer Centrifugation (SLC) is a technique that has been developed to separate the best quality spermatozoa from semen samples. Moreover, due to the lipid composition of the plasma membrane, boar sperm is extremely susceptible to cold shock. Comparing the quality of sperm cells applying two freezing procedures was therefore included in this project.

The SLC method was used to select high-quality sperm of two breeds (Landrace and Duroc) over a period of six days storage at 18 ℃. The experiments were performed on the day of collection (Day 0) and after 3 and 6 days of storage at two different ages of the individuals included. Sperm quality evaluations included motility, viability, acrosome integrity, DNA integrity, and ATP content. The cryopreservation experiment was conducted by diluting the semen samples of Landrace and Duroc in lactose egg yolk extender and frozen in medium plastic straws by two different cryopreservation procedures: programmed freezing by IceCube and freezing in liquid nitrogen vapor. The quality of the sperm during the cooling phase (before freezing) and after thawing was evaluated.

Results of the assessment of SLC's impact on boar semen quality (both breeds) at both ages indicated that progressive motility, ATP content, viability, and acrosome integrity were mostly be enhanced in SLC samples, whereas hyperactive proportion, DFI%, and HDS% demonstrated a decline in comparison to those without SLC treatment. Comparing post-thaw samples revealed that there were some variations in male individual sperm quality; the programmed freezing demonstrated a better outcome than the freezing in liquid nitrogen vapor. For both Landrace and Duroc, the proportion of total and progressive motility as well as AIL% post-thaw IceCube samples were higher than those directly exposed to liquid nitrogen vapor. When it comes to cryopreserving boar semen, the applied programmable method showed better post-thaw sperm quality than the other method tested.

1. Background

1.1 Origins of the project

This project is a master's thesis of Inland Norway University of Applied Sciences (INN) master's degree program in Applied and Commercial Biotechnology (2021-2024), with specialization in Experimental Biotechnology.

This master project is part of a research project entitled Zygote. The regional businesses Geno SA and Norsvin SA, SpermVital AS, Klosser Innovation AS, and the academic partners Oslo University Hospital, OsloMet, SimulaMet, SINTEF, and the Swedish University of Agricultural Sciences collaborate on the project. Improving existing and developing new gamete and embryo preservation techniques is one of the Zygote project's sub-goals. Therefore, the purpose of this master's project was to explore different semen processing and preservation by applying specialized work on the quality of boar semen. By improving methods for preserving fresh and cryopreserved boar sperm, swine breeding could benefit and be improved.

2. Introduction

2.1 General structure and function of the sperm cell

The male reproductive gametes that may fertilize an ovum are known as sperm cells. These haploid cells consist of two main parts, the head, and the tail (Figure 1). The morphologies of sperm heads differ between species. For instance, rodents have sickle-shaped heads, but many species, including human sperm, have paddle-shaped heads. The sperm head is made up of a nucleus, an acrosome, and a slight amount of cytoplasm. The DNA is densely packed by nuclear proteins (protamine 1 and 2) to create a complex chromatin structure. An acrosome vesicle, which has matrix proteins and hydrolysing enzymes within, covers the proximal area of the head. Cytoplasm occupies the little gap that exists between the plasma membrane and the nucleus. Mitochondria, an axoneme, and cytoskeletal elements such as outer dense fibres (ODF) and fibrous sheaths (FS) make up the spermatozoa's tail, being the resource for the sperm cell’s motility. There are four parts that make up the tail: the neck, midpiece, principal piece, and end piece. The neck is made up of the basal plate, a redundant nuclear envelope, a few neck mitochondria, and a connecting segment with centrosomes. The proximal centriole and numerous pericentriolar matrix proteins are found in centrosomes (Toshimori, 2009). The ODFs in the midpiece of the sperm tail are surrounded by mitochondria during the last stages of spermiogenesis. Two ODFs are exchanged by the longitudinal columns of the fibrous sheaths, which are linked to one another by transverse ribs, in the principal piece of the sperm tail. The axoneme and its surrounding plasma membrane are the only components of the sperm tail's last segment (Lehti & Sironen, 2017).

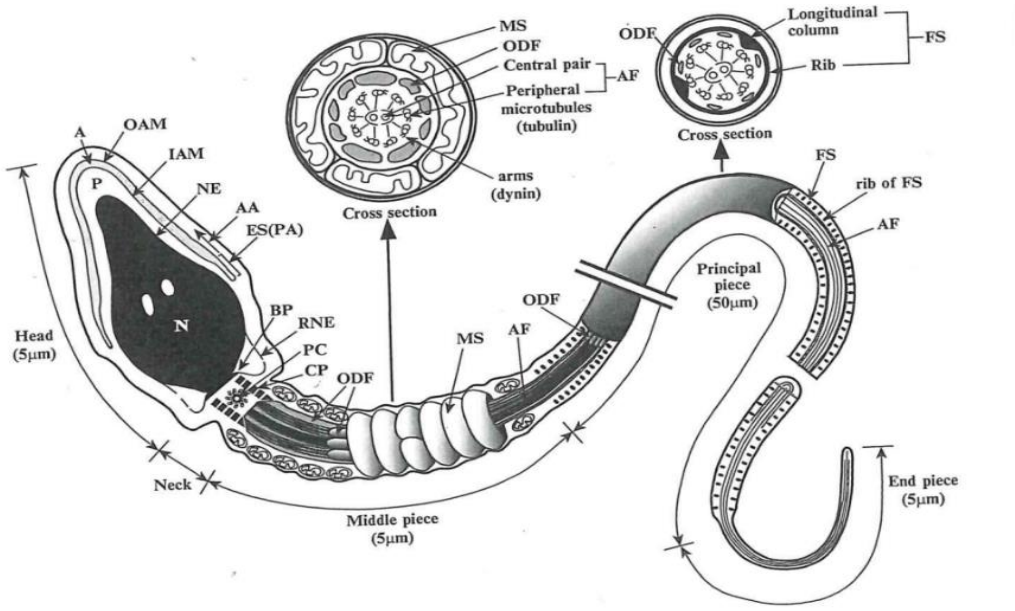

Figure 1. ***The structure of a mammalian sperm cell****. The spermatozoa consist of a head and tail. The plasma membrane completely encloses the head. A sperm cell's head is composed of two main structures: the acrosome (surrounded by the inner acrosomal membrane (IAM) and outer acrosomal membrane (OAM)) and the nucleus (surrounded by the nuclear envelope (NE)). The tail is made up of the neck, middle piece, principal piece, and end piece. The head and tail are connected by the neck, which also includes the basal body. mitochondrial sheath (MS) and the nine ODFs that surrounded the axoneme are in the middle section. Two ODFs are replaced in the principal piece of the sperm tail by the longitudinal columns of the fibrous sheaths (FS), which are joined by transverse ribs (TR). The plasma membrane surrounds the axoneme at the end of the sperm tail (Toshimori, 2009).*

2.2 Spermatogenesis

Spermatogenesis is a critical process by which males can generate reproductive germ cells. In this process, cells generated from the seminiferous tubules of the testis undergo differentiation into proper proliferation cells. Hormonal control of the spermatogenesis process includes the release of GnRH, FSH, and LH as well as gonadal steroids (testosterone and estradiol). Leydig cells release testosterone, which is then carried into Sertoli cells and altered into dihydroxytestestrone and estradiol. Blood carries testosterone and estradiol to the hypothalamus, where they have a negative feedback effect on GnRH release and, as a result, lower LH and FSH levels (Senger, 2012). The two important components of the seminiferous epithelium are the basal compartment and the adluminal compartment. Sertoli cells cover the generating population of germ cells and are connected to the basement membrane.

Spermatozoa can be developed in high quantities in testes with a high Sertoli cell number. Both FSH and testosterone have receptors in the Sertoli cell. Although Sertoli cells contain receptors for several hormones (protein and steroid), they can synthesize a range of compounds, including androgen binding protein and sulphated glycoprotein, which are important to fertility. The blood-testis barrier is composed of the Sertoli cell junctional complexes and the peritubular cells surrounding the seminiferous tubule. The blood-testis barrier is able to prevent immune cells (macrophages and lymphocytes) and immunoglobulins (antibodies)(Senger, 2012). The spermatogenesis process is categorized into three stages (Figure 2): i) proliferative phase, mitosis increases the number of spermatogonia (spermatogonial stem cells capable of self-renewing), ii) the process of meiosis starts with the primary spermatocyte, which has replicated DNA. Subsequent spermatocytes, which receive a single copy of each pair of chromosomes, subsequently reducing the number of chromosomes; iii) the round spermatid finally differentiates into the spermatozoa (Chenoweth & Lorton, 2014). The spermatozoa have completely developed morphologically and are composed of the head and tail. The head consists of highly compact chromatin and the acrosome. Protamines substitute for histones in the haploid paternal DNA, causing the chromatin to become hyper condensed in the nucleus. The acrosome is placed at the anterior of the sperm head and originates from the Golgi. The acrosome forms a large cap with membrane receptors and different proteases that are required to break down the zona pellucida and penetrate the cumulus cell layer. the cumulus cell layer. The tail (flagellum) is made up of an axoneme, a microtubule-based structure that has been preserved through evolution. The sperm's tail is essential for fertilization because it maintains sperm motility and advancement through the female genital tract (Touré, 2019).

When boars reach puberty between the ages of 5 and 6 months, every 3–4 days, waves of spermatozoa are released from Sertoli cells, and fertilization capability is developed following an additional 5-7 weeks of maturation (Flowers, 2015). At the age of 1-2 years, the boars can generate 51-150 billion sperm weekly, which results in 21-40 doses per week on average (Knox et al., 2008).

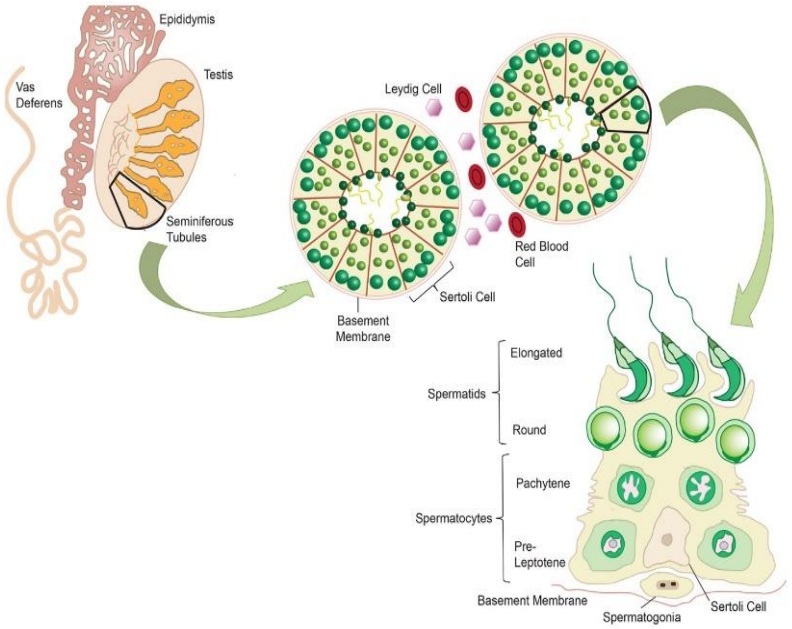

Figure 2. ***Schematic illustration of the process of spermatogenesis.*** *The diploid spermatogonium in the basal membrane of seminiferous tubules is multiplied by mitotic divisions (primary spermatocytes), followed by meiotic division where primary spermatocytes become secondary spermatocytes and finally haploid spermatids. At the final stage (differentiation), the structure of spermatids is transformed into spermatozoa (Redgrove & McLaughlin, 2014).*

2.3 Factors affecting sperm production

Sperm generation is crucial for reproduction in mammals. There are some factors that influence the number of normal sperm produced per unit of time.

Sperm production capability is significantly related to testicular size. To estimate the size of the testis, the scrotal circumference (bull) or scrotal width and length (boar and stallion) are measured. Because it is the factory of sperm production, measuring the size of the testis is an important factor that should be estimated. The greater the testicular circumference, the more sperm can be produced in each ejaculate (Senger, 2012). The length of the seminiferous tubules and the size of the testis both rise as the Sertoli cell number increases. The total number of Sertoli cells present in the testis at puberty determines the maximum amount of sperm that can be produced (between 3 and 4.5 months of age in the boar) (França et al., 2005).

After puberty, the age of the males affects sperm production. In other words, mature males have testes that are both bigger in size and more productive than young ones. In a previous study by (Knecht et al., 2017), the effect of age on boar semen quality was investigated. It

was shown that semen volume and total number of motile spermatozoa were higher in the group of 19-30 months old boars compared to the youngest boar group (8-12 months).

Production and quality of sperm are affected by environmental factors like season, temperature, humidity, and day length. The testicular temperature should be below normal body temperature to generate normal spermatogenesis. Therefore, the thermoregulatory mechanism can maintain the testicular temperature. One of the hormones that influence sperm production is LH, which could be inhibited due to heat stress (Knecht et al., 2017; Parrish et al., 2017). In a study by (Frydrychova et al., 2015), the impact of the season on semen quality parameters was determined. During different seasons, sperm motility and the percentage of morphologically abnormal spermatozoa (MAS) of boar semen were estimated. According to their findings, sperm motility was higher in the winter and spring, whereas the proportion of MAS was noticeably larger in the summer and fall. Moreover, in a project by (Peña Jr et al., 2019) was investigated how seasonal heat stress affected the DNA integrity and quality of spermatozoa taken from boars kept during the peak wet, late dry, and early dry seasons. The summer heat stress of boars kept in tropical conditions (peak wet seasons) was shown to cause sperm DNA damage and sperm concentration drops while not affecting the motility of boar spermatozoa. In addition, factors including diet, ejaculation frequency, and housing have an impact on sperm production and the quality of semen (Flowers, 2022).

2.4 Sperm cell in the female reproductive tract

After semen deposition, the sperm cells travel through the female reproductive tract towards the oviduct, to meet the egg and potentially fertilize it. The sperm cells must pass the different parts of the female reproductive tract, including the cervix, uterus, utero-tubal junction, and finally the oviduct. Sperm cells have encountered and overcame certain obstacles on their travel to the site of fertilization during the various steps. Numerous spermatozoa are lost in the process of traveling from the uterus to the oviduct; fewer than 0.01% of the spermatozoa that are inseminated reach the fertilization site. It interacts with the epithelium and the luminal fluid, which may influence sperm motility and function, without being destroyed by the immune system (Luongo et al., 2019; Miller, 2018).

The female reproductive system interacts with sperm cells in a variety of ways to get the cells to access the egg and select the optimal sperm to fertilize an egg. These interactions can be divided into two categories: physical and molecular. The physical interactions involve the

sperm's swimming reactions to fluid flows, fluid viscoelasticity, and the microarchitecture of the tract walls. Sperm surface molecules can connect with receptors in the epithelial linings of the tract as a type of molecular interaction. Effects of tract secretions on sperm, effects of seminal plasma on the tract, or interactions of sperm with immune cells of the female reproductive tract are other examples of molecular interactions (Suarez, 2016).

The architecture of cell surfaces may have an impact on how sperm swim. Fluid flows are produced by ciliary beating, smooth muscle contractions in the female reproductive tract's walls, and release of fluids into the lumen (Miller, 2018). Another physical interaction is the viscoelasticity that sperm are exposed to with viscous fluids; oviduct fluid in certain species, estrous cervical mucus, and the cumulus oophorous matrix, which is a viscoelastic network submerged in a viscous fluid. Sperm swimming velocities can be decreased by viscoelastic fluids, but they can affect sperm bending patterns and final swimming orientations (Suarez, 2016).

At the utero-tubal junction (UTJ), the sperm and epithelium are in interaction. Mammalian species differ in the structure of the UTJ. When ovulation occurs, sperm start to separate from the epithelium. They may then reattach and separate multiple times before leaving the storage area (W. V. Holt & A. Fazeli, 2016).

Increase in cytoplasmic Ca^{2+} causes sperm capacitation, while binding to the oviductal epithelium results in suppressing capacitation (Figure 3). The mechanism for inhibiting capacitation could be that oviductal catalase protects the sperm membrane from a harmful peroxidative effect by increasing the permeability of membranes to Ca^{2+}. Modification of cell surface proteins and hyperactivation occur in sperm capacitation. Sperm surface protein modifications could affect the ability to bind to oviductal receptors, and hyperactivation could force the sperm to detach from the oviductal epithelium. Ovulation-inducing hormones or signals from the preovulatory follicle may cause the oviductal epithelium to produce compounds that increase sperm capacitation and hyperactivation, resulting in sperm detachment. Furthermore, carbohydrates proteins that bind sperm to the epithelium could be changed or removed during the capacitation process (Suarez, 2008).

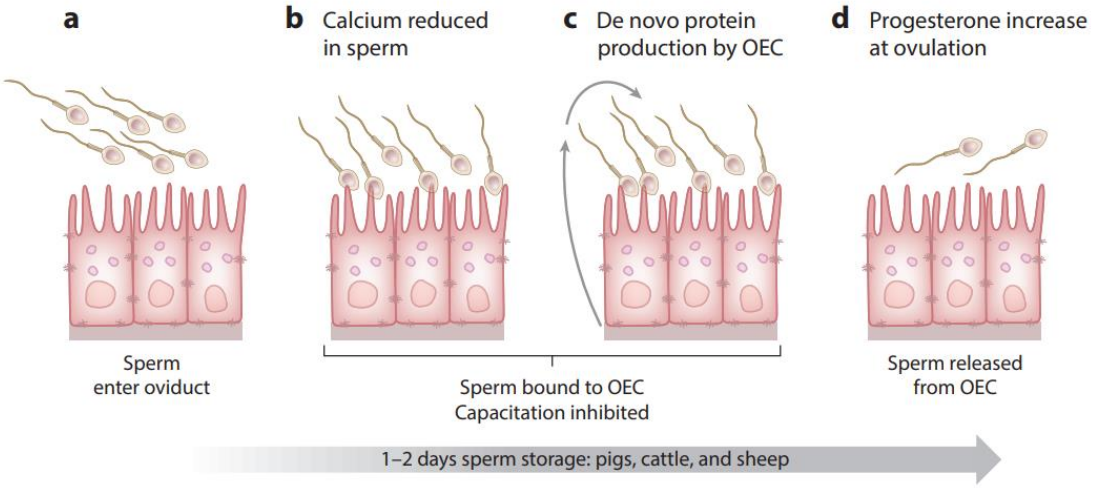

Figure 3. ***Some events that appear during sperm storage in the oviduct of domestic mammals****. a) Entering sperm in UTJ, b) Some spermatozoa attach to the surfaces of oviductal epithelial cells (OEC), decreasing in motility and cytoplasmic* Ca^{2+} *as well as inhibiting capacitation, c) The oviductal lumen secretes some proteins to protect sperm membranes and promote sperm storage, d) Increasing progesterone induces detaching sperm cells from the oviductal epithelium and resulting in sperm capacitation (William V. Holt & Alireza Fazeli, 2016).*

2.5 Artificial insemination in pig

The purpose of artificial insemination (AI) is to induce pregnancy through an *in vivo* event by delivering sperm cells (cleaned and concentrated) to the female reproductive tract. The most prevalent method of pig breeding is AI in many countries that produce pork. Currently, the usage of AI is linked to commercial production efficiency and profit. Boar semen cryopreservation causes damage to sperm cells and lower sperm viability; therefore, the AI technique is most applied with liquid-stored boar semen. The development of liquid extended semen production in the 1990s led to an improvement in the rate of genetic progress by AI. In the beginning, semen could be transported in liquid storage for up to 3 days to locations nearby the AI centre. It is now possible to keep sperm alive longer for shipment to AI facilities worldwide, due to advancements in long-term extenders (Knox, 2016). AI can be used for pigs by three methods: conventional or intracervical artificial insemination; intrauterine or post-cervical artificial insemination; and deep intrauterine insemination. For pig reproduction with liquid-stored semen, conventional AI is the most widely used method, which involves inserting a special catheter into the cervix (Figure 4). When conventional AI is carried out using frozen-thawed boar sperm, the reproductive capacity decreases significantly. During deep intrauterine insemination, the semen is inserted into the uterine horn closer to the oviduct

by using longer and more rigid catheters. Possible damage to uterine walls during the insertion of long catheters in the deep intrauterine insemination method may negatively impact the outcome of AI but reduces the amount of sperm injected by up to 20 times. However, there are some difficulties with intrauterine insemination and deep intrauterine insemination catheters entering the cervix of gilts and young sows. Since it is simpler to carry out and is less risky, producers prefer intrauterine insemination (Yeste et al., 2017).

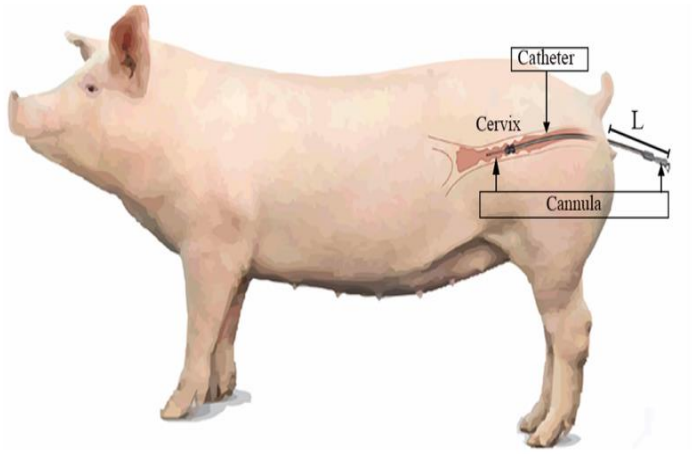

Figure 4. ***Artificial insemination (AI) in pigs.*** *The figure shows how cervical AI is performed in a gilt. This is the method of AI in pigs where the semen is deposited in deep cervical canal. L indicates the length (cm) of the instrument that extends out of the gilt during insemination (Llamas-López et al., 2019).*

2.6 Factors affecting boar semen quality during liquid storage

In Norway, AI is used widely both in pigs and cattle herds. It is critical to have consistent deliveries of higher quality semen products to compete in international markets. Farrowing rate and litter size, the two most used fertility traits in swine, are crucial production factors with significant economic impacts on pork production. Breeding expenses can be reduced because less semen is needed for reproduction (like AI) due to improved semen quality (Norsvin, 2022). It is important to keep sperm in good quality, whether in liquid/extended, or frozen conditions. Therefore, sperm must be stored in a specific medium and at certain temperatures to maintain their quality and capacity to fertilize (Pezo et al., 2019). An extender or diluent is described as an aqueous solution that contributes activity, decreases sperm metabolic function, and preserves function at low levels to maintain a suitable fertility level.

It also enables extending the volume of the ejaculate up to the required amount. The extender must contain nutrients to provide energy for the sperm metabolism and allow intrinsic motility (monosaccharides like glucose and fructose) (Gadea, 2003), protect cells from cold shock and maintain membrane integrity (bovine serum albumin) (Knox, 2016), and prevent microbial growth (antibiotics) (Bryła & Trzcińska, 2015), as well as control the pH (NaCl, KCl, sodium citrate) that due to the dilution of sperm causes a reduction in the level of certain ions that play a role in sperm viability and the osmotic pressure (bicarbonate, Tris, and HEPES) (Pezo et al., 2019).

The physicochemical characteristics of boar semen make them susceptible to cold shock. As a result, sperm samples must be stored at temperatures between 15 and 20 °C (Pezo et al., 2019). Temperatures below this range may negatively impact the viability of seminal doses. One or two steps may be used to reduce the temperature of the semen from 37 to 15-20 °C. Storage influences sperm quality, while different extenders can keep the sperm intact for a longer period. An important point to consider is that the apparent good preservation of boar sperm in liquid storage may not be associated with good reproductive success when using sperm for AI (Yeste, 2018). Several studies have been conducted to determine the response of liquid-stored sperm to bicarbonate, a capacitation inducer. In one study, as storage temperature and time increased, the specific responsiveness to capacitation conditions diminished. Cooling caused the loss of sperm quality characteristics, whereas storage time had no impact on these factors (S Schmid et al., 2013). Other factors such as increased oxidative stress, changes in sperm structure, and handling of seminal doses may have an impact on sperm quality during liquid storage. Numerous investigations were performed to improve boar semen extenders, such as adding hyaluronic acid to delay premature capacitation, different concentrations of BSA to make sperm survive up to 7 days, and reducing glutathione to increase sperm motility and membrane integrity (Yeste, 2018).

2.7 Selection of high quality sperm by single layer centrifugation (SLC)

There are several *in vitro* methods that improve the quality of sperm to achieve effective fertilization, viable embryos, and healthy offspring. Colloid centrifugation is one of the methods that isolates spermatozoa from seminal plasma and selected spermatozoa according to characteristics such as sperm motility, morphology, viability, and chromatin integrity

(Sjunnesson et al., 2013). As the highest quality spermatozoa may be denser than others, one strategy is to use density gradient centrifugation (DGC). Since the preparation of DGC is time-consuming, single-layer centrifugation (SLC), which uses just one layer of a silane-coated silica colloid, has been suggested as an effective method (Crespo-Félez et al., 2017). Therefore, SLC simplifies the DGC process and eliminates the need to prepare and layer numerous colloid preparations with various densities. During centrifugation in the SLC procedure, the motile, viable spermatozoa travel through the colloid and pellet at the bottom of the tube (Figure 5), while the seminal plasma is retained on the top of the colloid (Morrell & Wallgren, 2011). SLC is one of the colloid preparation techniques that has some positive aspects, including ready-to-use species-specific formulations, homogeneity between batches of colloid, and the capacity to scale up to process whole or large ejaculates (as is the case with the boar and the stallion). These benefits may make SLC convenient to use for commercial semen preparation (Sjunnesson et al., 2013). The first record of using SLC in boar semen was reported by using a colloid consisting of glycidoxypropyltrimethoxysilane-coated silica in a buffered salt solution (known as Androcoll^{TM-P}) (Morrell et al., 2009).

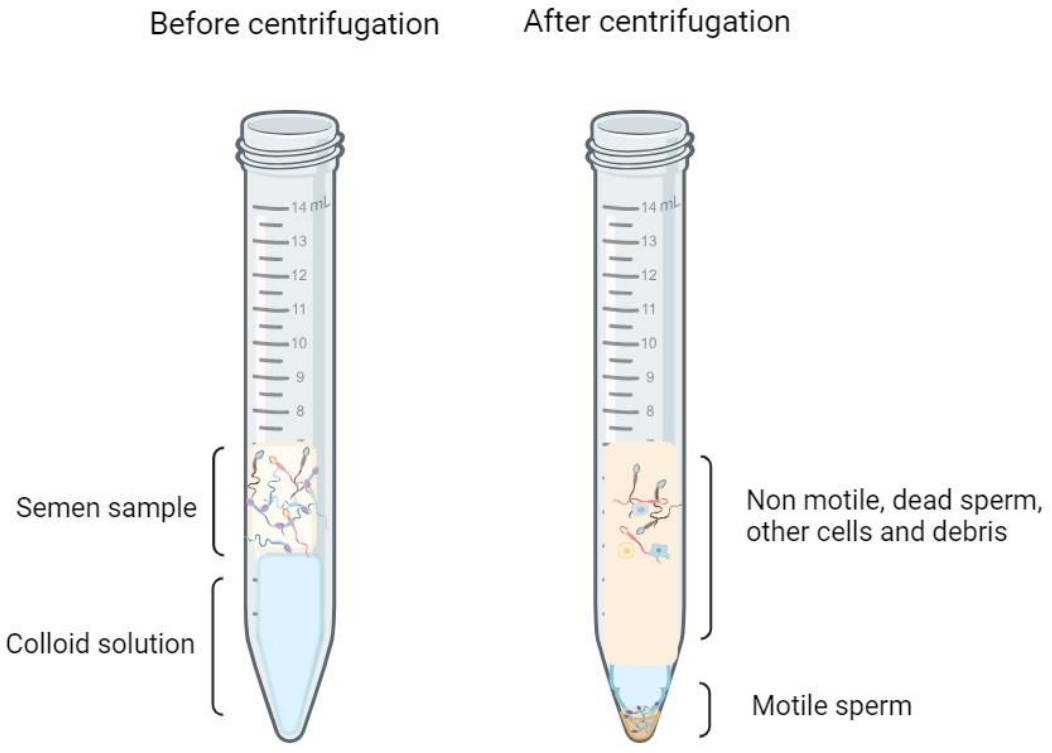

Figure 5. ***Schematic illustration of sperm selection by SLC.*** *The semen sample is loaded on top of the colloid solution. Following centrifugation, motile spermatozoa pass through the colloid and pellet at the bottom of the tube. The supernatant contains seminal plasma, semen extender, and most of the colloid, non-motile, dead sperm, and debris. The figure is created with BioRender.com by the author.*

Since liquid-stored semen rather than frozen semen is the preferred option for most AI techniques in boars, bacterial contamination occurs because of bacterial growth at normal boar semen storage temperatures (16-18 ℃). These bacteria compete with sperm for nutrients, make metabolic by-products and toxins, and may even infect the swine after insemination and lead to infertility. Removing bacteria physically from boar semen through SLC methods is suggested as an alternative technique to adding antibiotics to the extender (Martínez-Pastor et al., 2021).

2.8 Cryopreservation

Cryobiology is the study of biological material or systems at low temperatures and may be applied in e.g. theriogenology, molecular biology, engineering, and mathematics (Benson et al., 2012). Cryopreservation is the technique of preserving organelles, cells, tissues, or any other biological components in a frozen state by subjecting the samples to extremely low temperatures (Jang et al., 2017). Cryopreservation of gametes and reproductive organs may be recommended for humans, mainly children and adults with cancer, and other mammals such as endangered species (Yeste, 2016). Sperm cryopreservation is the process of freezing sperm cells and storing them for later use. The greatest effects of development in cryopreservation technology include the expansion of selective breeding in agriculture, wherein the sperm of desired males e.g., of bulls may be frozen, preserved, and transferred across local or international borders for use in AI. Other applications for the semen freezing technology include the cryopreservation of genetic resources for food fish and fishes that are used in research, the preservation of mouse, rat, and primate spermatozoa for biology and medicine research, the establishment of semen banks for the support of conservation breeding initiatives, and the preservation of regional and national agricultural breeds (Chenoweth & Lorton, 2014). The first cryopreservation experiment was recorded by Spallanzani in the 18th century when sperm cells of humans, stallions, and frogs were immobilized in snow (Großfeld et al., 2008). To develop this procedure, Polge et al. noted that glycerol acts as an effective cryoprotectant agent to protect spermatozoa against dehydration during exposure to low temperatures (Polge et al., 1949). According to these studies, AI by frozen-thawed semen produced offspring for the first time in 1951 (cattle), 1953 (humans), 1957 (pig, horse), and 1967 (sheep) (Curry, 2000).

Understanding the transportation of water via membranes and the interactions of solutes, cryoprotectants, low temperature, and membranes is important to know how cells survive during freezing and thawing treatments.

The physical changes induced by freezing a semen sample are shown in Figure 6. When the temperature is reduced, the sample's water remains liquid while active measures (seeding) are performed to include ice crystallization; at this point, the solution is known as "super cooling" (step 1). The creation of ice happens at random below the freezing point, most likely because groups of water molecules combine into clusters and start a chain reaction. A considerable amount of heat is generated during this exothermic reaction stage to raise the sample temperature (step 2). The sample temperature may remain static for a short period of time before cooling is restarted (step 3), depending on the type of sample being frozen and the cooling technology utilized. The dissolved salts and other substances become concentrated when ice begins to form during the cooling process, preventing them from forming ice (Chenoweth & Lorton, 2014).

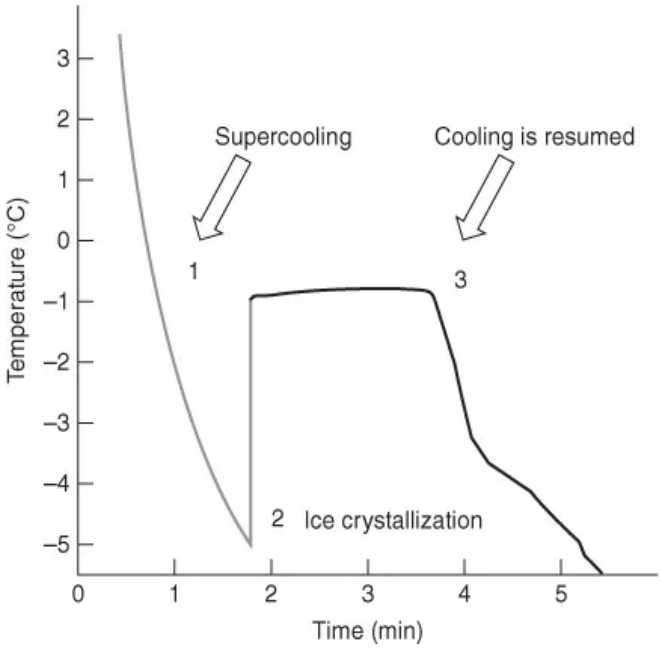

Figure 6. ***Changes in temperature during freezing of semen in plastic straws****. The temperature falls below the standard freezing point (super cooling) (1), rising quickly when the latent heat of fusion is released (2), A brief time delay of no temperature change, and then cooling is resumed (3)(Chenoweth & Lorton, 2014).*

The main drawback of the freezing-thawing method is related to the phase change of intracellular and extracellular water. The lethality of an intermediate range of temperature, between -15 ℃ and -60 ℃, is the main problem for cells that are subjected to freezing and

thawing. At -5 ℃, the extracellular medium and cells are both supercooled and not frozen. The intracellular substance is unfrozen and supercooled at temperatures between -5 and -15 ℃, but the surrounding medium starts to form ice. Water flows out of the cell due to the chemical potential of water being higher in supercooled (intracellular) than frozen state (extracellular). Cryoinjury happens when intracellular water does not entirely flow out, and ice crystals accumulate in the cytoplasm as a result. This happens when the cooling rate is very high. When the cooling rate is very low, most of the water flows out, the intracellular solutes are concentrated, and supercooling is stopped. Before reaching the temperature at which all solution contents are solidified, cells are exposed to high-solute concentrations, become dehydrated, and undergo volume shrinking of organelles and membranes (Yeste, 2016). According to "two-factor hypothesis" of (Mazur et al., 1972), lethal intracellular ice formation at high cooling rates, as well as solute/electrolyte concentration, cell dehydration, and a decrease in the unfrozen fraction in the extracellular space at low cooling rates, are the causes of cryoinjury. The ability of different cells to transfer water across the plasma membrane will affect the optimal cooling rates (Jang et al., 2017). Mazur suggested that the optimal cooling rate should be slow enough to prevent intracellular ice formation and fast enough to prevent solution effects (Mazur et al., 1972).

2.8.1 Effect of cryopreservation on sperm physiology

The sperm physiology changes during the freezing-thawing process because sperm cells are small cells with a large surface. The intracellular space's viscosity and glass transition temperature make the cells vulnerable to damage (John Morris et al., 2012). Freezing spermatozoa without using a cryoprotective agent leads to damage of the sperm's organelles because of cold shock and the induction of ice crystal formation. Reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), superoxide anions (O^{2-}), and hydroxyl radicals (OH^-) and oxidation of cellular components during this event result in membrane lipid peroxidation and destruction of cellular structure like DNA, acrosomes, and mitochondria (Hezavehei et al., 2018) (Figure *7*). Differences in spermatozoa freeze-ability have been linked to differences in plasma membrane lipid content. The boar sperm is highly vulnerable to cold shock due to the plasma membrane's abundant unsaturated phospholipid content and low concentration of cholesterol molecules. The phospholipids of the plasma membrane contribute to fluidity, and the number of sterols like cholesterol provides rigidity and stability. The boar sperm membrane transitions from a fluid to a gel phase at temps below 5 ℃ because of a low

cholesterol: phospholipid ratio and an asymmetrical distribution of cholesterol (Johnson et al., 2000; Vadnais & Althouse, 2011).

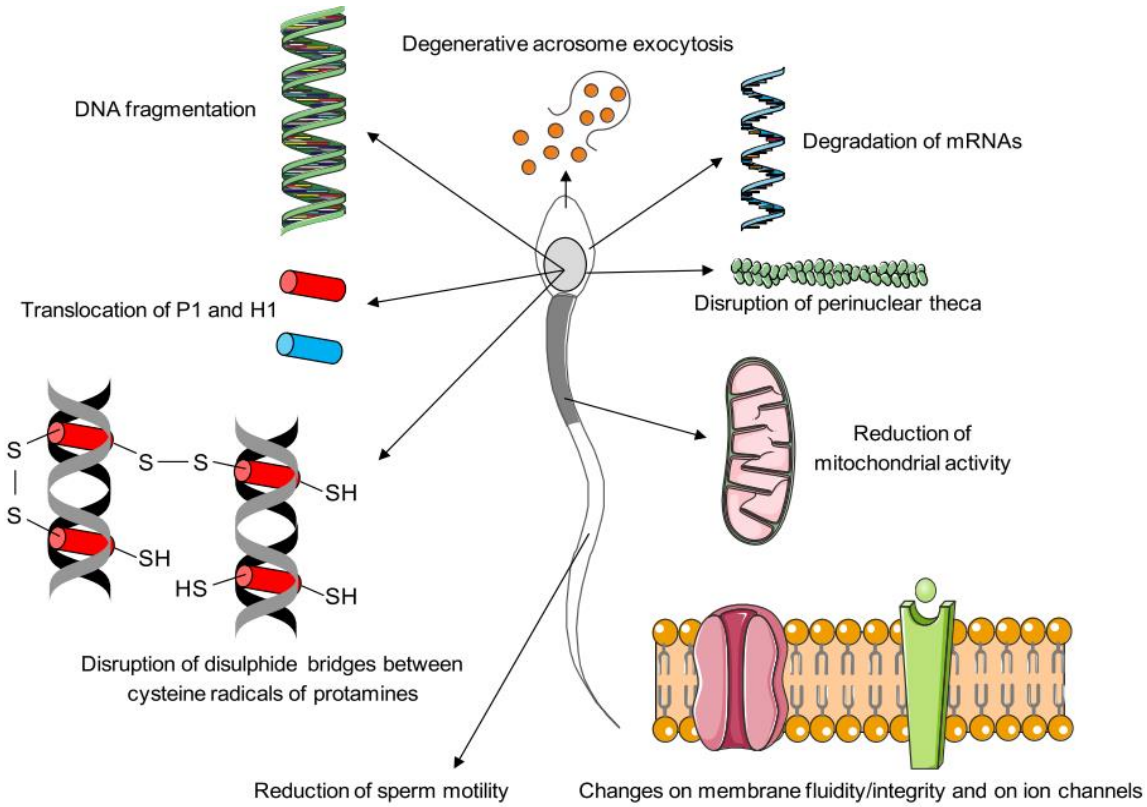

Figure 7. ***Main damages reported due to freeze-thawing procedures on boar sperm.*** *The sperm's nucleus is damaged by the cryopreservation process, which results in DNA fragmentation, translocation of protamine 1 (P1) and histone 1 (H1), and disruption of the disulfide bridges between cysteine radicals in P1. Apart from reducing mitochondrial activity and sperm motility, cryopreservation also negatively impacts acrosome integrity, plasma membrane fluidity/integrity, as well as the degradation of mRNA (Yeste, 2016).*

2.8.2 Effect of cryoprotectants in the cryopreservation process

The viability and functionality of sperm cells throughout the freezing-thawing process are significantly influenced by the composition of the medium. Under isotonic conditions, osmotic stress caused by the formation of ice crystals inside the cell causes biophysical changes in the cell as a result of excessive cell shrinkage (Cole & Meyers, 2011). Cryoprotective agents (CPAs) are applied to reduce intracellular ion concentrations by altering intracellular water content and accumulating organic electrolytes. The CPAs have an osmolarity of around 1500 mOsm (milliosmoles) while the cell has an osmolarity of 300 mOsm. In order to react to the outside pressure, the cell rejects water and absorbs CPAs to balance osmotic pressure (Sztein

et al., 2018). There are both nonpenetrating and penetration CPAs, the first ones acting outside the cells while the second ones act inside the cells.

Most of the nonpenetrating CPAs are large macromolecules, which are unable to cross the plasma membrane. This class of cryoprotectant compounds mainly includes the milk and egg yolk proteins, sugars, polyvinylpyrrolidone, hydroxyethyl starch, polyethylene glycols, and dextran. These molecules work by reducing the freezing point and raising the viscosity of the medium; as a result, they generate an extracellular protective effect and improve the efficiency of permeating CPAs (Chenoweth & Lorton, 2014). While egg yolk is a complex collection of proteins, low-density lipoproteins (LDL) act as the key protective factor. Boar sperm quality and DNA integrity are improved when the whole egg yolk component is substituted with LDL as CPAs. In studies on humans, rams, and bulls, different results have been observed when egg yolks were replaced with soy lectin. While studies in rams found a positive effect on total sperm motility, chromatin, and acrosome integrity but a reduction in mitochondrial sperm function at post-thawing, research with stallion sperm demonstrated that changing egg yolk with soy lecithin resulted in a decrease of reproductive performance (Yeste, 2016).

The solutes which can permeate to sperm cells, such as glycerol, dimethyl sulfoxide (DMSO), ethylene glycol, methanol, propylene glycol, and dimethylacetamide, are categorized as penetrating CPAs. These substitutions inhibit the lethal formation of intracellular ice and protect cells both intracellularly and extracellularly (Pezo et al., 2019). CPAs are used in cryobiology due to their positive effects at extremely low temperatures, however, their high concentrations (typically $>$1M) can cause damage to cells if added or removed incorrectly (Benson et al., 2012). The basic penetrating CPA for boar sperm cryopreservation is glycerol, with an ideal concentration between 2% and 3% (Okazaki et al., 2009; Zeng et al., 2014). Sperm cells from bulls and stallions have been shown to benefit from glutamine and dimethylformamide, respectively (Yeste, 2016).

2.9 Different methods for sperm cell quality analysis

2.9.1 CASA

Sperm motility and acrosome integrity are important for fertility and reduced sperm motility affects the fertility rate (Valverde et al., 2020). A computer-assisted sperm analysis (CASA) system is designed to offer accurate, precise, information about sperm concentration, viability, dynamics, or morphology (Lu et al., 2014). CASA has been widely used in domesticated

animal production research centres and reproductive toxicology to measure semen properties like sperm concentration and proportions of progressive motility in many animal species. In the late 1980s, advancements in the CASA technology led to great progress in analysis of sperm kinematics (Mortimer et al., 2015). The CASA technique includes estimating repeatedly taken images of a sperm suspension onto a detector array, detecting objects according to the intensity of pixels in a frame or light scatter, and collecting the data using specific software (Amann & Waberski, 2014).

A negative phase contrast microscope with a heated stage and an attached video camera are the two primary parts of a CASA system. The camera is connected to a computer that runs specialized software for various kinds of analyses (Soler et al., 2017). There are several factors that can affect the results of semen samples, such as sample temperature, time between ejaculation and examination, and the type of counting chamber. The quantity of fields examined, recording frame rate, the difference algorithm, and the type of CASA system are additional factors that can affect the CASA outcomes (Valverde et al., 2020). The CASA-Mot systems monitor the movement of a sperm head centroid, which generates a path and allows for analysis of the kinematic characteristics of the resulting track (Figure 8). Sperm motility parameters composed of VCL (m/s), calculated by adding the distance between the centroid locations of the sperm head frame by frame, divided by the amount of time that has passed; VSL (m/s), determined by adding the distance between the first and last points of the sperm track, divided by the amount of time that has passed; VAP (m/s), the average path length, is calculated by dividing the time that passed by the smoothed sperm head position in a running average, LIN (%), the level of linear progression calculated as the ratio VSL/VCL in percentage; STR (%), the ratio of VSL/VAP in percentage to measure track compactness. Two parameters that represent the characteristics of sperm wobble WOB (%), the oscillation of the actual path about the average path expressed as the ratio of VAP/VCL in percentage; ALH (m), the amplitude of the approximate sinusoidal oscillation of the sperm head around the track (which can be thought of as the maximum or the mean value along the track); and BCF (Hz), the frequency with which the sperm head crosses the average path length during acquisition (Valverde et al., 2020).

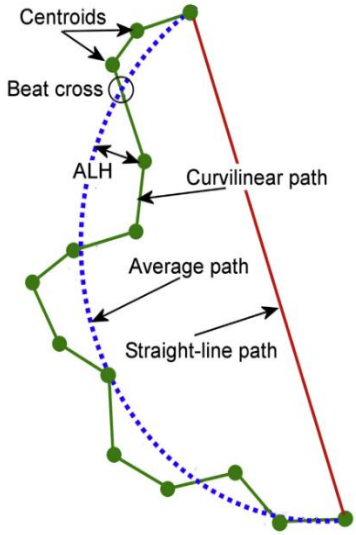

Figure 8. ***Kinematic characteristics obtained from the CASA system****. The green line shows curvilinear velocity (VCL; µm/s), the red line shows straight line velocity (VSL; µm/s) and the blue line shows average path velocity (VAP; µm/s). STR – straightness (= VSL/ VAP×100); LIN – linearity (= VSL/VCL × 100); WOB – wobble (= VAP/VCL × 100); ALH – amplitude of lateral head displacement; BCF – beat-cross frequency (Amann & Waberski, 2014).*

2.9.2 Flow cytometry

A highly sensitive and precise technique for quantifying the physical properties and fluorescence of a single particle is flow cytometry. The main advantage of flow cytometry is that it can assess a large number of particles in a short time; some systems can collect 10 to 20 parameters from each particle while running at rates of approximately 100,000 particles per second (Robinson, 2004). When single cells or particles pass through the light source (commonly a laser beam) in a direct stream of fluid, the characteristics of each individual particle (size, granularity, and fluorescence) are monitored (Figure 9). The principle of flow cytometry is based on light scattering and fluorescent emission. Light scattering correlates directly to the cell's morphology and structure; fluorescent emission is proportional to the amount of fluorescent probe attached to the cell or cellular component. The fluidic components provide the steady hydrodynamics and allow the particles to align singly in a sheath stream (Adan et al., 2017). The analysis or sorting of cells or particles is possible with the use of commercial flow cytometers. This technique could be applied to domestic animals, such as bulls, boars, rams, and rabbits, to evaluate the ratio of X- to Y-chromosome containing sperm (Johnson, 2000). Fluorescent-labelled cells may be sorted from a mixed cell population using flow cytometers called fluorescence activated cell sorters (FACS). Different properties of

fluorochrome play an important role in selecting suitable fluorescent probes for using flow cytometry in a wide range of biotechnology applications (Rieseberg et al., 2001).

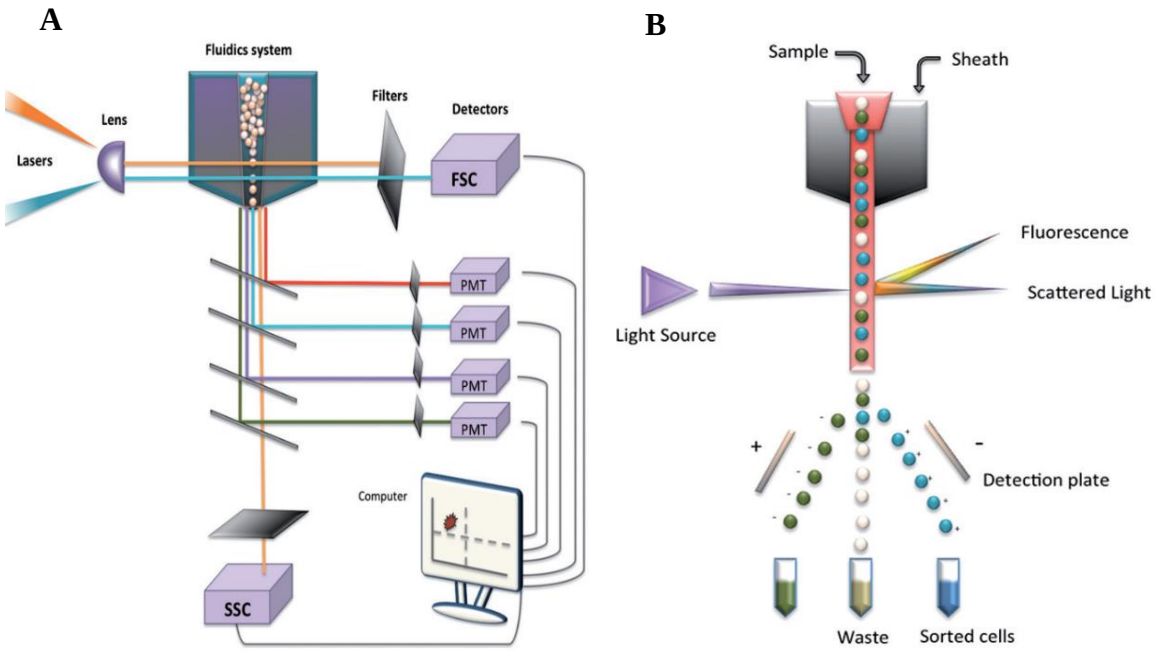

Figure 9. ***Components of a flow cytometer****. A flow cytometer consists of a fluidic system, optical system and signal detection and processing (A). Sorting flow cytometry which can be applied in sperm sexing (B) (Adan et al., 2017).*

2.9.3 Viability and acrosome integrity by flow cytometry

Fluorescent staining and flow cytometric analyses can be used to quantify sperm organelle function. Staining can be done using a single, impermeable fluorophore or a mixture of stains depending on different membrane permeabilities (Sklar, 2005). The viability of spermatozoa can be assessed using fluorescent staining: fluorescent dyes that indicate viable cells and fluorescent dyes that indicate non-viable cells. To assess sperm cell viability, most studies use membrane-impermeable probes such as propidium iodide (PI) and ethidium homodimer. PI intercalates between base pairs in double stranded DNA to bind to it. Dead cells that have leaky membranes and where PI can pass through the membrane show red fluorescent radiation (Kirchhoff & Cypionka, 2017).

The acrosome's integrity is crucial for fertility because it contains enzymes that penetrate zona pellucida. Utilizing plant lectins that have been fluorescently marked is mostly used to estimate acrosome integrity. *Arachis hypogaea* agglutinin (PNA) from peanut seed lecithin is a common lectin used. PNA attaches to galactose groups on the outer acrosomal membrane and indicates acrosome-damaged cells (Dolník et al., 2019). The PNA can be conjugated with

a fluorescent dye such as fluorescein isothiocyanate (FITC). PNA-FITC specifically attaches to the inner leaflet of the outer acrosomal membrane, and cells with damaged acrosomal membranes were estimated (S. Schmid et al., 2013).

2.9.4 Sperm chromatin structure assay (SCSA) by flow cytometry

There are several methods for evaluating the sperm chromatin structure and determining sperm DNA integrity which might influence male fertility (Evenson, 2016). The morphology, motility, or even in some cases the sperm's capacity to fertilize the egg may not be affected by DNA damage (Jonge & Barratt, 2006). However, several published studies explain the correlation that damaged DNA could limit or decrease the spermatozoon's ability to fertilize and/or negatively impact embryonic development (Middelkamp et al., 2020; Oleszczuk et al., 2016). Different methods are available to assess sperm DNA integrity and determine if sperm cells contain DNA breaks (Table 1).

Table 1. Techniques for assessment of sperm DNA integrity (Shaman & Ward, 2006).

Assay	Type of DNA break detected	Chromatin proteins removed
TUNEL	Accessible ds + ss DNA breaks	None
In situ translational	Accessible ss DNA breaks	None to few
SCSA	Non-toroidal ds + ss DNA breaks	Some histones
	(External toroidal ss + ds DNA breaks?)	(some external protamines?)
Neutral COMET	Most ds DNA breaks	ALL (histones + protamines)
Alkaline COMET	Most ds + ss DNA breaks	ALL (histones + protamines)

Sperm chromatin structure assay (SCSA) is one technique used to assess the integrity of sperm DNA. The sperm DNA damage that is commonly determined is DNA fragmentation, or DNA strand breaks (DSB) either single-strand breaks (SSB) or double-strand breaks (DSB) (Figure 10). Acridine orange (AO) is a cationic fluorescent dye that selectively binds to nucleic acids. AO interacts with double-stranded (ds) or single-stranded (ss) DNA. In SCSA technique, the DNA of sperm samples is denatured by exposure to mild acid, AO binds to DNA and colours ss (denatured, DNA single-stranded breaks can only be denatured by acid) and ds (non-denatured) DNA red and green, respectively (Agarwal et al., 2016; Champroux et al., 2016). DNA fragmentation index (DFI) is the ratio of red to total (red/red + green). The DNA is compacted by binding protamine and anchoring chromosomal DNA to the nuclear matrix. Protamines, which are connected by intermolecular disulphides, inhibit the intercalating of Acridine orange into the ds DNA (Jonge & Barratt, 2006).

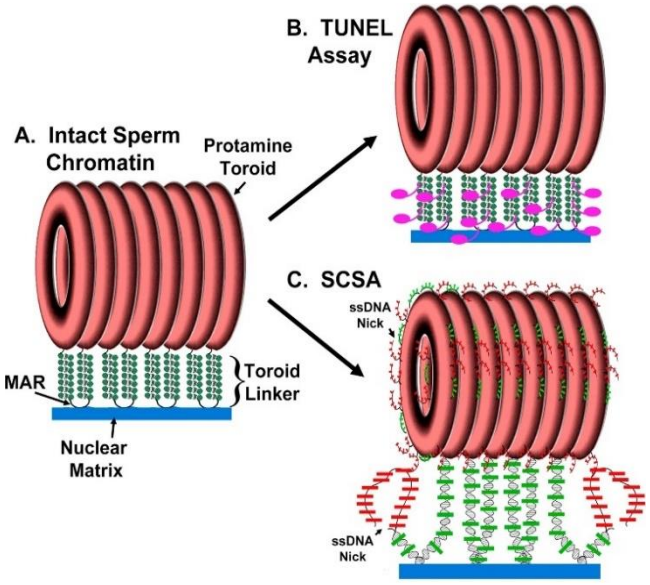

Figure 10. ***Two methods for estimating DNA breaks are TUNNEL and SCSA assays.*** *A) the intact chromatin is condensed by protamine and attached to the nuclear matrix. B) The TUNEL assay detects breaks in ds and ss DNA. C) The SCSA assay is used by Acridine Orange to measure ssDNA nicks (Evenson, 2016).*

The population of High-DNA Stainable (HDS) sperm in a semen sample represents abnormal sperm chromatin that has a high level of green fluorescence. Due to an incomplete exchange of histones for protamines, histone-complexed DNA is stained by AO at a greater level than protamine-complexed DNA. HDS populations have immature sperm morphology and are often rounder in shape because of the lack of normal condensation in HDS sperm (Jerre et al., 2019).

2.9.5 Luminescence assay

The fertilization of oocytes depends on the sperm acquiring hyperactivity, which happens during the process of capacitation. Hyperactivity is vigorous movement of the sperm tail and requires a lot of ATPs. When hyperactivity is started too early, sperm cells lose energy before they reach the oocyte for fertilization. Therefore, semen ATP content can be analysed to further assess the relationship between hyperactive motility and ATP concentrations (Tremoen et al., 2018).

To determine which cells are metabolically active, the CellTiter-Glo^{TM} Luminescent Cell Viability Assay utilizes ATP as a cofactor necessary for the luciferase reaction (Figure 11). When Mg^{2+} and ATP are present, the enzyme luciferase reacts with luciferin to create

oxyluciferin and release energy in the form of luminescence (Hannah et al., 2001). The CellTiter-Glo™ Luminescent Cell Viability Assay offers several advantages over other cell viability methods, including simplicity in addition, mixing, and measurement, sensitivity and linearity, long half-life, protection from endogenous ATPases, and minimal impact of phenol red and other additives on assay performance (Hannah et al., 2001).

Figure 11. ***The luciferase reaction in the ATP content assay.*** *Luciferase uses Mg^{2+}, ATP, and molecular oxygen to convert luciferin to oxyluciferin and light (Promega,*

2.10 Aim of the study

Boar semen for AI is currently mostly kept in liquid form, which has business drawbacks like a maximum storage time of 4 days at 16–18 °C, which further restricts long-distance shipping. Cryopreserved semen could be a preferred alternative, however, the quality of semen during cryopreservation can be impacted due to boar membrane characteristics. The primary goal of this study was to use different methods of semen processing and preservation to reveal possible enhancement of the quality of boar semen.

To obtain the primary goal, the following actions were investigated:

- Evaluation of the impact of storage conditions on the quality of semen in two Norwegian boar breeds (Landrace and Duroc)
- Selection of sperm by the single layer centrifugation (SLC) method
- Investigation of the impact of two different freezing techniques on the quality of semen after freeze-thawing

These tasks were followed by investigations of motility by CASA, sperm viability, and acrosome integrity by flow cytometry. Furthermore, the sperm DNA integrity was assessed by flow cytometry, and the ATP content of the sperm cell was measured by luminescence assay.

3. Material and Methods

3.1 Experimental plan

An overview of the experimental design on liquid boar semen is illustrated in (Figure 12). Prediluted boar semen samples were obtained, further diluted, and subjected to two sets of examinations (November 2022 and January 2023). Two distinct concentrations of the samples, 80 and 25 × 10^6 cells/mL were kept at 18 °C, over 6 days storage. The semen samples diluted to 80 mill/mL were further diluted to 25 mill/mL prior to analysis. Samples were split into two for preparation by either single-layer centrifugation (SLC) with Porcicoll or without centrifugation. Additionally, several analyses were performed to determine the impact of utilizing SLC on the properties of sperm cells for the purpose of getting high-quality sperm cells. Each semen sample was analysed in triplicate on the day of collection (Day 0), as well as after 3 and 6 days of storage at 18°C.

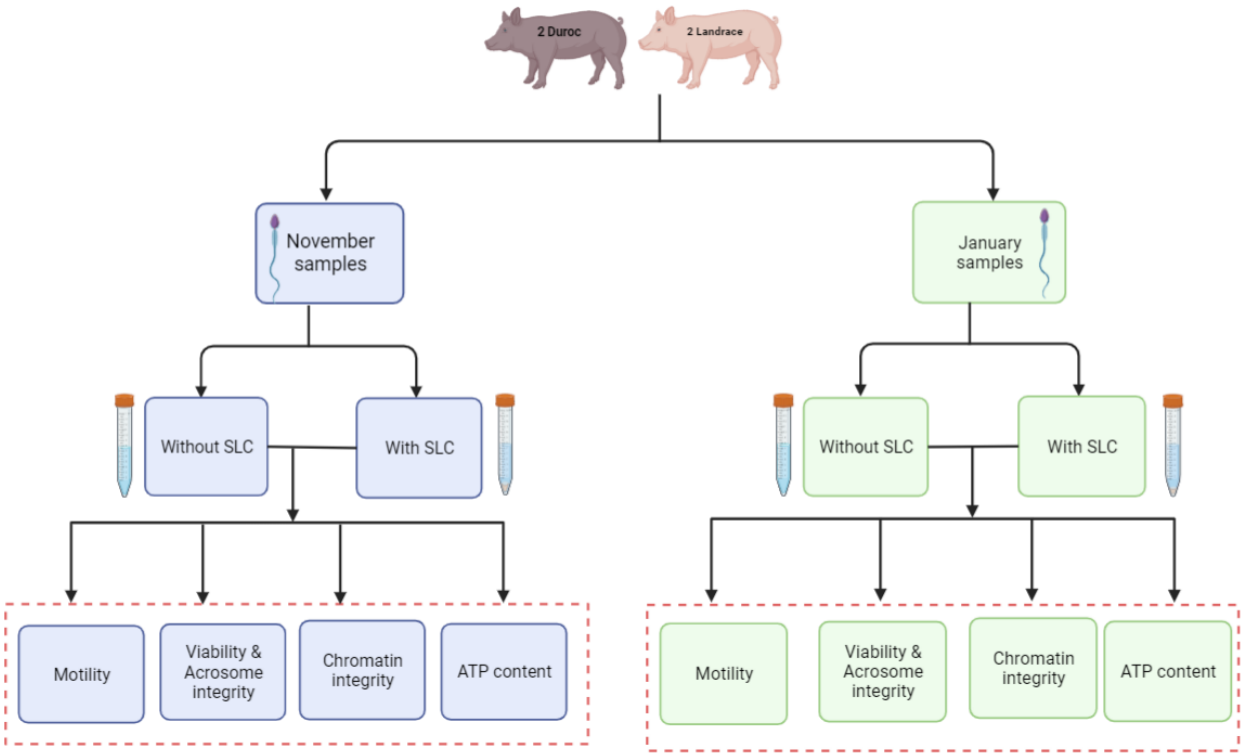

Figure 12. ***Flowchart representing the steps followed in the present study.*** *The prediluted semen of four boars (2 boars Landrace and 2 boars Duroc) was collected from Norsvin in November and January. Samples were categorized into two groups: without SLC and with SLC. Each sample (diluted to 25 × 10^6 cells/mL) was evaluated for motility by CASA, viability, acrosome integrity, and chromatin integrity by flow cytometry, as well as ATP content by luminescence assay over 6 days of storage. Without SLC: uncentrifuged semen and with SLC: centrifuged on a single layer of Porcicoll.The figure is created with BioRender.com by the author.*

Two methods were applied for cryopreservation and for comparison of the post-thaw quality of the frozen boar semen samples, as illustrated in Figure 13. Semen samples were prepared by exposing them to a lower temperature in two steps: cooling and freezing. The prepared samples were frozen using two alternative techniques: Ice Cube programming and direct freezing in liquid nitrogen vapor. The frozen straws were kept for further examination in a liquid nitrogen container. After thawing straws in a water-bath at 37 °C for 1 minute, the properties of sperm cells were also studied.

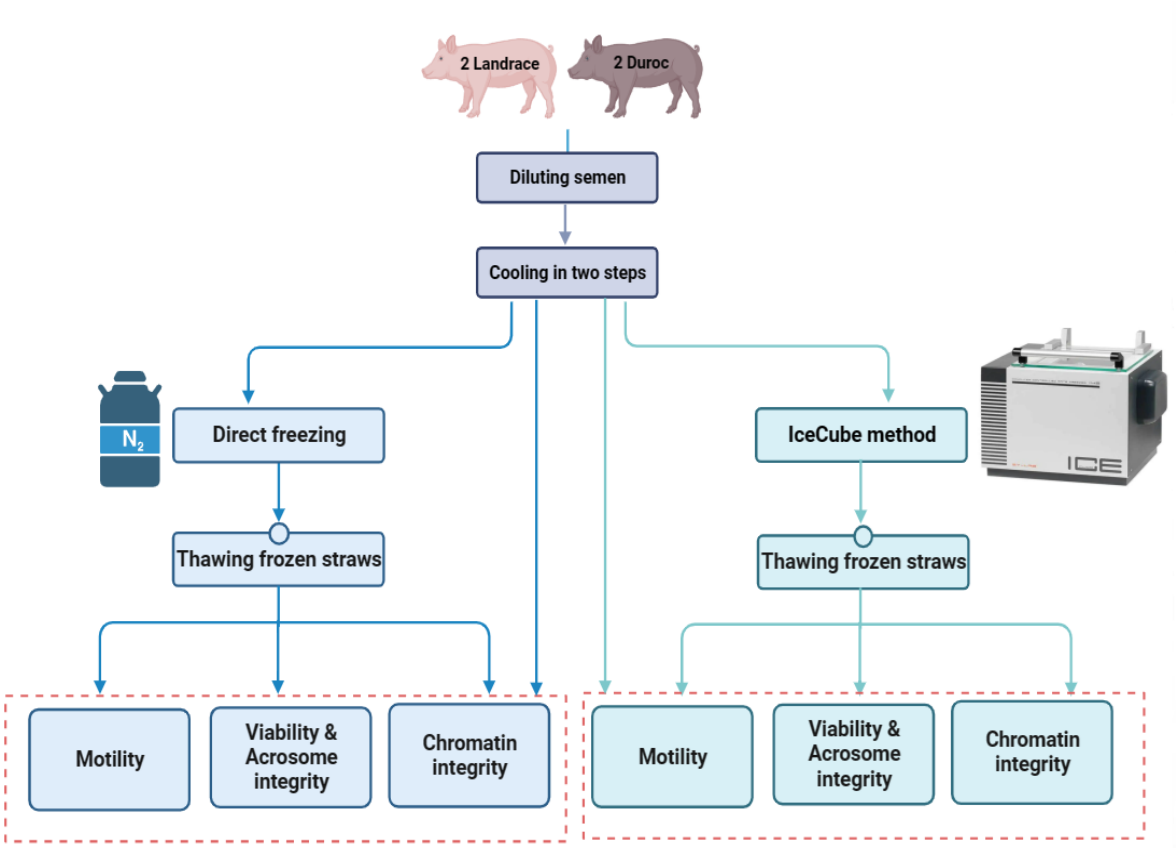

Figure 13. ***Flow diagram representing the steps followed by freezing semen samples.*** *Four boars' semen (2 Landrace and 2 Duroc) was taken from Norsvin and diluted to about 100×10^6 spermatozoa/mL. Two steps of cooling, followed by two different freezing techniques, were used to freeze the diluted semen. The thawed straws (at 37 °C, 1 minute) and the samples from the cooling phase were assessed for motility, viability and acrosome integrity, and chromatin integrity. The figure is created with BioRender.com by the author.*

3.2 Chemicals and animal material

3.2.1 Chemicals and solutions

All the chemicals utilized in the current study were from Merck in Germany, except otherwise mentioned.

Phosphate buffer saline (PBS) was prepared to be used as a sperm dilution buffer for CASA, flow cytometry and ATP content measurement analyses. The PBS solution contained the following ingredients: 137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_42H_2O$, and 1.76 mM KH_2PO_4. The pH was set to 7.4, the solution was autoclaved, and kept at 4°C. The following instructions were used to prepare the PMT buffer and acid detergent solution for SCSA experiments: 10 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4 and 0.38 M NaCl, 80 mM HCl, 0.1% (w/v) Triton X-100, pH 1.2, respectively.

3.2.2 Animal material

For this project, semen from two Norwegian Landrace and two Norwegian Duroc boars were provided by Norsvin AS (Hamar, Norway). The boars were 10-16 months old in November 2022. The boars were housed and cared for at the Norsvin artificial insemination (AI) station, in accordance with international standards and laws for keeping pigs in Norway.

3.3 Semen processing

The fresh semen samples were prepared according to Norsvin standard procedures, where raw semen was diluted (1:1) with Androstar® extender. For the experiments with cryopreservation of semen, Norsvin provided raw semen diluted (1:1) Beltsville Thawing Solution (BTS) extender.

3.3.1 Single layer centrifugation

Single layer centrifugation (SLC) was carried out using Porcicoll small, a colloid solution containing glycidoxypropyltrimethoxysilane-coated silica in a buffered salt solution (Androcoll^{TM}-P; SLC, Sweden), as described by Morrell et al. (2009). For each boar, six 15 mL falcon tubes were prepared for SLC on each analysis day. In each tube, 3 mL semen with a concentration of 80×10^6 spermatozoa/mL were layered on top of 4 mL Porcicoll pre-warmed to room temperature (RT). Following centrifugation (300 × G, 20 min, RT) by a swing-out rotor centrifuge, the supernatant (seminal plasma, semen extender and most of the colloid) was discarded with a Pasteur pipette. Approximately 2 mm colloid above the sperm pellet was left, and by using a clean pipette the pellet containing sperm cells was collected from underneath the colloid. The sperm pellet was resuspended with fresh Androstar® and diluted to 25×10^6 spermatozoa/mL. The sperm suspensions were transferred to 15 mL tubes for storage at 18 ℃. An aliquot (1 mL) of each sample was used for analysis.

3.3.2 Preparation of semen for cryopreservation

The semen was diluted to approximately 100×10^6 spermatozoa/mL using BTS extender. Diluted semen (250 mL) was cooled at 15 ℃ over 2 hours, followed at 18 ℃ for 1 hour, and a 2 mL aliquot was taken for analysis. The cooled semen was then centrifuged (10 min, 800 × G, 18 °C) and the supernatant was discarded. The pellet was resuspended at 18 °C (1:1) in a cooling extender (11% lactose with 20% egg yolk), and the sperm concentration was assessed by CASA. The sperm concentration was adjusted to 1.5×10^9 cells/mL adding more cooling extender. After 2-hours cooling to 4 °C, freezing extender (cooling extender with 12% glycerol and 1.5% Minitube Equex paste (Minitube, Tiefenbach, Germany)) was added, making the final concentrations 1×10^9 sperm/mL, 4% glycerol, and 0.5% Equex paste. Following the collection of 1 mL of diluted semen in the freezing extender for analysis, semen was frozen in medium straws (0.5 mL) (IMV Technologies, France) in accordance with the two freezing procedures.

3.3.3 Semen freezing and post-thawing

Two techniques, direct freezing in liquid nitrogen vapor, and programmed freezing by IceCube were used to freeze the straws. The programmed freezing involved putting straws in an IceCube that had been pre-programmed. The following settings were used for semen freezing, starting at 4°C with cooling rates −4, −40 and −20°C/min to −10, −120 and −150°C, respectively. Then, the straws were placed into liquid nitrogen (−196°C) to be stored. The direct freezing procedure was placing the straws 7 cm above liquid nitrogen for one minute before plunged directly in the liquid nitrogen container and stored.

The straws were thawed in a water-bath at 37 °C for 1 minute. After thawing, the semen samples were extended in commercial semen-extender TRIXcell+ (IMV Technologies, France) as a boar semen preservation medium at RT, which was then analysed by further methods (CASA, viability, acrosome integrity, and DNA integrity assessment).

3.4 Assessment of sperm motility parameters by CASA

The motility characteristics (total motility, progressive motility, and hyperactivity) were evaluated by CASA, (Sperm Class Analyzer (SCA) (Microptic; Barcelona, Spain)) which was coupled with a phase contrast microscope. The software settings were employed as follows: number of images and image rate: 30 images at 45 images/sec, cell identification: 30 μm^2 to 70 μm^2 head area, Static cells: VAP <10 μm/sec, motility criteria: based on VAP (μm/sec)

10< Slow < 25 < Medium < 50 < Rapid, Progressive motility: >70% STR, Hyperactivity: ALH>6.5 µm, VCL>80 µm/sec, LIN<65%. Each semen sample (25×10^6 spermatozoa/mL) was incubated for 10 minutes at 37°C. To ensure that the sperm cells were distributed uniformly across the chamber, 30 seconds before ending incubation, 3 µL of sample was loaded on a pre-warmed Leja 4 chamber slide (SC-100-01-02-A, Nieuw-Vennep, the Netherlands). The samples were examined in three parallels, and the average value and standard deviation of the average for each parameter were determined.

3.5 Flow cytometric analysis of sperm quality

3.5.1 Plasma membrane and acrosome integrity

Flow cytometry analysis of plasma membrane and acrosome integrity was performed using a CytoFLEX flow cytometer (Beckman Coulter, CA, USA) and results were analysed by CytExpert software (version 2.5). An unstained semen sample was used as a negative control in each run. A 488 nm laser was used as a light source for excitation. For the detection of viable sperm cells, propidium iodide (PI, LIVE/ DEAD® kit, L7011, Invitrogen) was used, which only binds to the DNA of sperm cells with damaged membranes, and therefore distinguishes between live and dead spermatozoa. The acrosome-reacted sperm cells were recognized using the lectin peanut agglutinin (PNA) from Arachis hypogea (peanut) coupled with Alexa Fluor 488 (PNA-Alexa 488, L21409, Invitrogen, UK). In addition, SYTO60 (SYTO™ 60 Red Fluorescent, S11342, Invitrogen), which is permeable to almost all cell membranes and does not act as a nuclear stain in living cells, was used as a cell marker. PI fluorescence, Alexa Fluor 488 and SYTO60 were detected in ECD (610/20 BP), FITC (525/40 BP) and PC5.5 (690/50 BP) channels, respectively. The staining solution was prepared in PBS (pH 7.4) with 2.4 mM PI, 1 mg/mL of PNA-Alexa 488 and 5mM SYTO60. The sperm samples were prepared in three parallels by transferring 30 µL semen to tubes with 970 µL of staining solution and were incubated in dark for 10 minutes at RT, before analysis. The stained samples were triggered on forward scatter for the first detection of cells, and 10,000 events were collected. The combination of forward scatter and side scatter dot plots was used to remove dead cells and debris, which have increased autofluorescence and non-specific signals. By gating for SYTO60 (a nuclear staining dye) in the PC5.5 and APC-A histograms, the sperm population was selected. The dot plots and histograms employed for the collection of data on viable sperm cells with intact acrosomes are described in Figure 14.

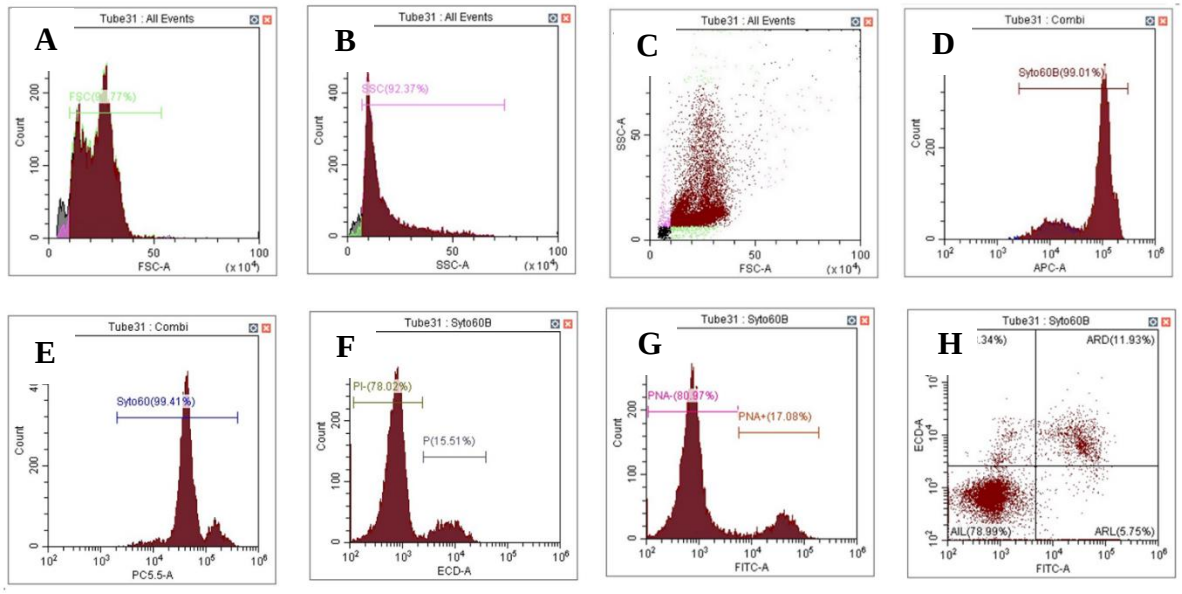

Figure 14. ***Diagrams from a flow cytometry assessment of sperm viability and acrosome integrity in a boar semen sample.*** *Semen samples were stained with propidium iodide (PI) to distinguish between live and dead sperm cells, peanut agglutinin conjugated with Alexa Fluor® 488 (PNA-Alexa488) which stains acrosome reacted sperm cells and SYTO60 which is used as a cell marker. A) Histogram from measurement of forward scatter, based on all events to inform about cell size. B) Histogram from measurement of Side Scatter, measuring granularity, all events. C) A density cytogram with SSC against FSC based on all events. D and E) represent SYTO60 as a nuclear staining dye that was gated in the APC-A and PC5.5 channels. F and G) Viability and acrosome integrity is further analysed in the gated SYTO60 population. Diagrams from this analysis are presented in ECD and FITC channels. H) the sperm cells have been gated according to the following populations: live acrosome intact (AIL), live acrosome reacted (ARL), dead acrosome intact (AID), and dead acrosome reacted (ARD).*

3.5.2 Sperm DNA integrity assays

The Sperm Chromatin Structure Assay (SCSA) was used for examining the chromatin integrity of the sperm cells, according to the procedure described previously (Evenson & Jost, 2000; Narud et al., 2020). A Cell Lab Quanta TM SC MPL flow cytometer (Beckman Coulter, Fullerton, CA, USA) was used when carrying out SCSA experiments. Flowcheck^{TM} beads (6605359, Beckman Coulter) were used to test the flow cytometer's optical alignment on each day of the experiment, and a negative control (an unstained semen sample) was included in each run. The exciting light source was a 488 nm argon laser. For a final concentration of 2 × 10^6 cells/mL in a volume of 200 µL, the semen samples were diluted in TNE buffer. Following the addition of the 400 µL detergent solution, incubation was carried out in RT for 30 seconds. Moreover, 1.2 mL of an acridine orange (AO) staining solution (6 µg/mL; A3568, Life Technologies, OR, USA) was dissolved in a buffer including 37 mM citric acid, 0.126 M Na_2HPO_4, 1.1 µM EDTA, and 0.15 M NaCl (pH 6). Following a three-minute setup mode,

data acquisition began, and 5000 events (about 200 events per second) were recorded for each sample. A 550 nm dichroic long pass mirror was used for separating the signals; a band pass filter at 525 nm detected the green fluorescence, while a long pass filter at 670 nm detected the red fluorescence. Prior to sample analysis, the flow cytometer was AO-saturated by running 1.2 mL of AO staining solution and 400 µL of acid detergent solution for 5 min. The mean green and red fluorescence signals were set to 425 ± 5 and 125 ± 5 respectively at the beginning of the examination and later after every fifth sample was evaluated in attempt to adjust the stability of the laser. Reference semen with a known DNA fragmentation index (DFI) was used in a bivariate cytogram for this procedure. The cytogram showed the FL1 (green) on the x-axis and FL3 (red) on the y-axis, both on a linear scale. FCS Express 6 Flow Cytometry Software (Denovo Software, Los Angeles, CA, USA) was applied to calculate the proportion of red (ssDNA) and green (dsDNA) fluorescent. The percent of fragmented DNA in spermatozoa (DFI, %) was determined using a histogram of the fluorescence ratio red/ (red + green). High DNA stainability (HDS, %), which refers to the spermatozoa with the strongest green fluorescent and is known as immature spermatozoa, was calculated using the bivariate cytogram. In two parallel runs, the samples were examined and for each sample, the average value and standard deviation of both DFI and HDS were calculated.

3.6 Determination of ATP content in sperm cells

Assessment of ATP concentrations in boar semen samples was measured using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA). To prepare CellTiter-Glo® Reagent, CellTiter-Glo® Substrate (lyophilized) and CellTiter-Glo® Substrate were used and stored at -20 °C until use. Prior to analysis, the semen sample was diluted to 2.5 × 10^6 cells/mL in PBS (pre-warmed at 37 °C). A white 96-well microtiter plate (NUNCTM, Denmark) was filled with 50 µL of diluted semen samples. The sample wells were then filled with 50 µL of CellTiter-Glo®Reagent (thawed and equilibrated to RT). The contents were shaken on an orbital shaker at 300 rpm (IKA® MS 3 digital, USA) to induce cell lysis. Following a 15-minute incubation at RT, the bioluminescence was assessed using a Fluostar OPTIMA plate reader (BMG LABTECH GmbH, Offenburg, Germany) and MARS data analysis software (version 1.10, BMG LABTECH, Germany). The results were given in relative luminescence units (RLU), and to convert the results to ATP values (nM), a standard curve was prepared. To prepare the standard curve, 1 µM ATP solution (disodium salt diluted in PBS) was applied at different concentrations (0–1000 nM). In each assay, control wells

contained 50 µL PBS without sperm cells (Blank) and the average of the three parallel analyses was determined in order to perform statistics.

3.7 Statistical analysis

Because of the small number of samples utilized in the present study, statistical analysis has been restricted. Three runs of the experiment were conducted to assess each parameter. The data are shown as the mean, with error bars indicating the standard deviation (SD). All statistical analyses were performed with Excel (Microsoft, USA).

4. Results

4.1 Analysis of the effects of SLC on sperm quality

4.1.1 Assessment of sperm motility parameters by CASA

To evaluate the effect of SLC on sperm motility, semen samples prepared with or without SLC were analyzed by CASA. In addition, the two breeds (Landrace and Duroc), storage time (Day 0, 3 and 6), and two timepoints (Nov and Jan samples) were compared. In general, SLC had little effect on total sperm motility. The total motility of sperm cells with and without SLC gradually decreased in both breeds (Duroc and Landrace) in the November and January samples during storage, except for the Duroc SLC samples on the day of collection (Day 0) (Figure 15). The Landrace semen displayed higher total motility than Duroc in November samples, except for the SLC samples after 6 days of storage. The total motility of the uncentrifuged Landrace samples was higher in comparison to the SLC samples, regardless of timepoints (Nov or Jan), on day 3 and 6 of storage.

For both breeds, there was an increase in progressive motility for the samples prepared with SLC, except for Landrace samples on day 6 (both ages, with SLC) (Figure 16). Over 6 days of storage, the progressive motility was higher in sperm samples collected from Landrace boars in November compared to January. Additionally, except for day 3 and 6 (Jan, with SLC), the amount of progressive sperm in Landrace samples was higher than in Duroc samples under both conditions (with SLC and without SLC).

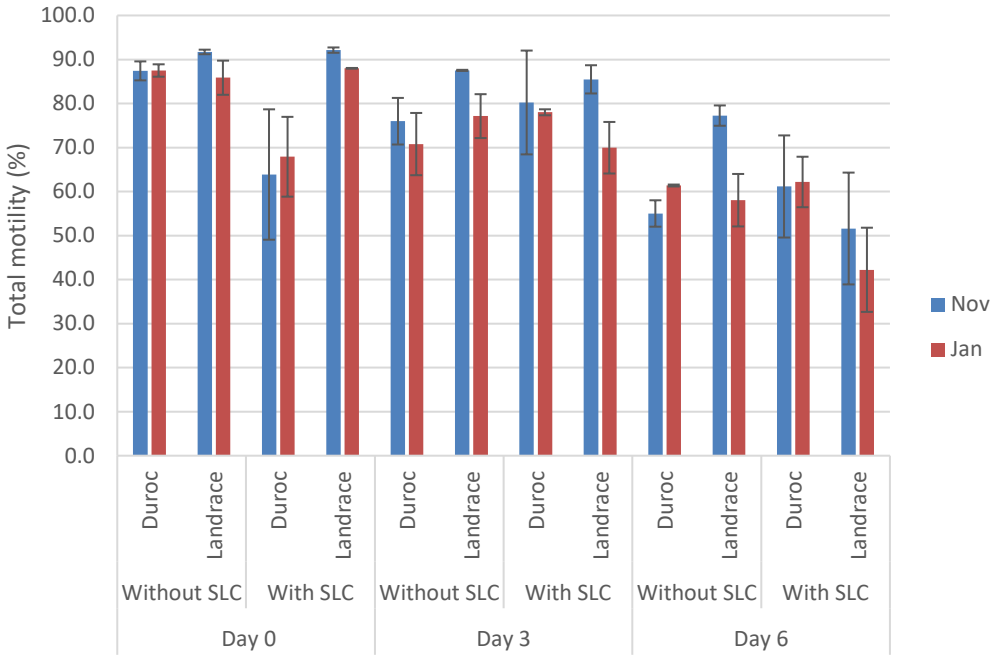

Figure 15. ***Effect of single layer centrifugation (SLC) on total motility****. The total motility was examined for 4 boars (2 Duroc and 2 Landrace) on the day of collection (Day 0) and after 3 and 6 days of storage in November (Nov) and January (Jan) samples. Without SLC: uncentrifuged sample and with SLC: centrifuged on a single layer of Porcicoll. Values are shown as mean ± SD.*

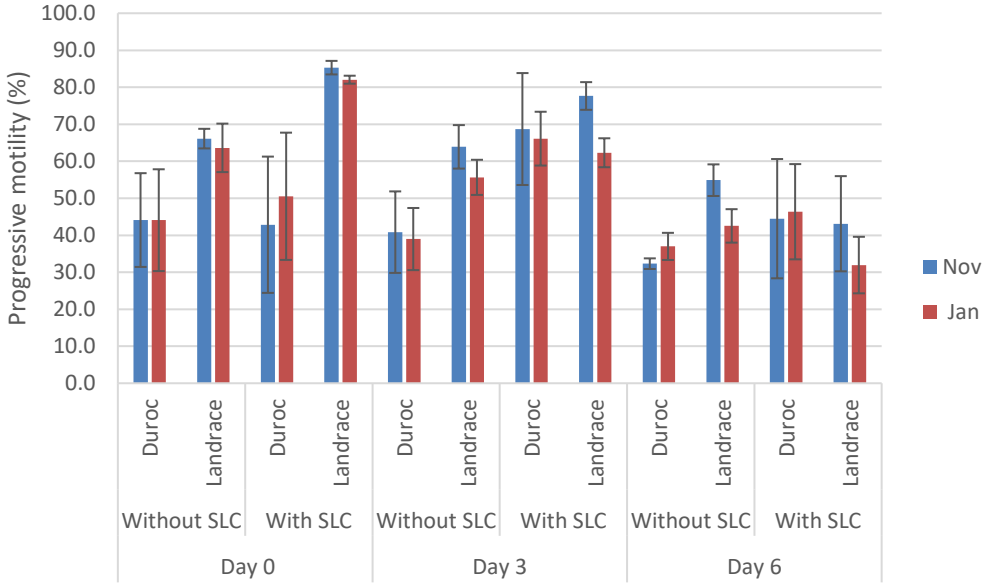

Figure 16. ***Effect of single layer centrifugation (SLC) on progressive motility.*** *The progressive motility was examined for 4 boars (2 Duroc and 2 Landrace) on the day of collection (Day 0) and after 3 and 6 days of storage in November (Nov) and January (Jan) samples. Without SLC: uncentrifuged sample and with SLC: centrifuged on a single layer of Porcicoll. Values are shown as mean ± SD.*

When SLC was applied the hyperactivity decreased dramatically in both breeds and ages (Figure 17). Compared to Landrace semen, the amount of hyperactive sperm cells was higher in Duroc samples (both with and without SLC). During the period of six days of storage, the level of hyperactivity in Duroc semen without SLC decreased in both timepoints (Nov and Jan).

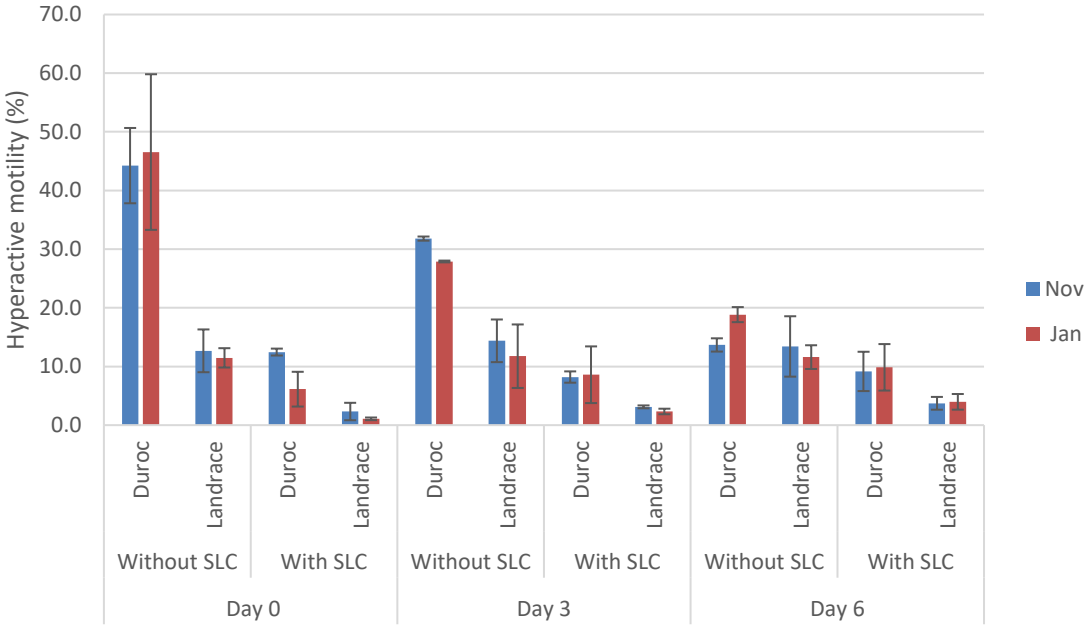

Figure 17. ***Effect of single layer centrifugation (SLC) on hyperactive motility****. The hyperactive motility was examined for 4 boars (2 Duroc and 2 Landrace) on the day of collection (Day 0) and after 3 and 6 days of storage in November (Nov) and January (Jan) samples. Without SLC: uncentrifuged sample and with SLC: centrifuged on a single layer of Porcicoll. Values are shown as mean ± SD.*

4.1.2 Examination of viability and acrosome integrity by flow cytometry

To estimate the population of acrosome intact live (AIL) cells, each sperm sample was stained with PI, PNA-Alexa 488, and SYTO60. The experiment was carried out three times, and the results (mean AIL%) are presented in Figure 18.

For both breeds and timepoints (Nov and Jan), the proportion of live sperm cells with an intact acrosome was higher in samples prepared by SLC compared to samples without SLC. Comparing the November and January samples, the percentage of AIL spermatozoa of uncentrifuged Duroc semen was almost the same. In the Duroc samples prepared by SLC, the percentage of AIL sperm seemed to be higher in the November samples compared to January

samples. For the Landrace semen samples (with and without SLC), the AIL % in November samples was higher than that of January samples over storage except for day 6 with SLC.

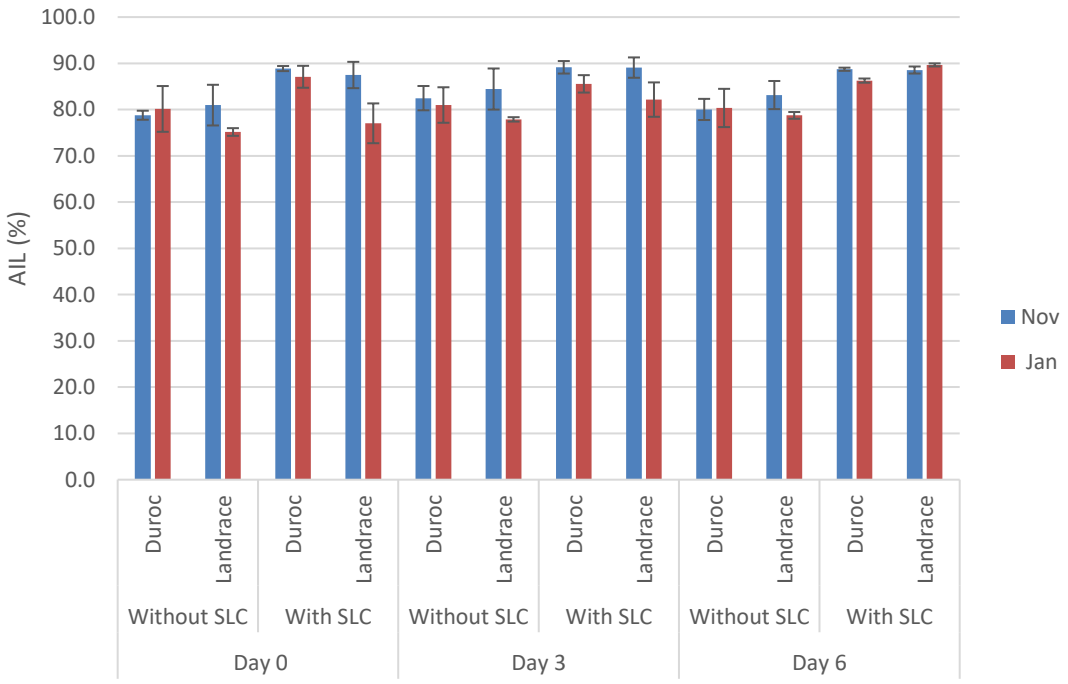

Figure 18. ***Impact of SLC on the percentage of acrosome intact live (AIL) sperm cells****. The proportion of AIL was evaluated during a 6-day storage period for samples collected from the two breeds (2 Duroc and 2 Landrace) at two timepoints (November and January). Data are presented as mean ± SD.*

4.1.3 Analysis of sperm chromatin integrity by flow cytometry

The sperm chromatin structure assay (SCSA) is valuable for evaluating DNA damage in sperm cells for both fresh and frozen-thawed samples (Evenson & Jost, 2000). DNA fragmentation index (DFI) and high-DNA stainability (HDS) were used to measure the sperm chromatin integrity (Figure 19). Uncentrifuged samples showed a higher DFI compared to samples prepared by SLC for both breeds and ages. For instance, the DFI of Landrace in January with SLC decreased 8 times compared to without SLC on Day 3 of the storage period. In January, with and without SLC, the DFI of the Landrace was higher than that of Duroc. Furthermore, Landrace semen samples in January had a higher DFI than samples in November under both conditions during the storage period—except for SLC, day 3.

In both breeds, prepared by SLC had lower HDS than samples not subjected to SLC treatment. The Duroc samples had higher HDS% than Landrace samples for both conditions and ages. The January samples for both breeds showed higher HDS than November samples.

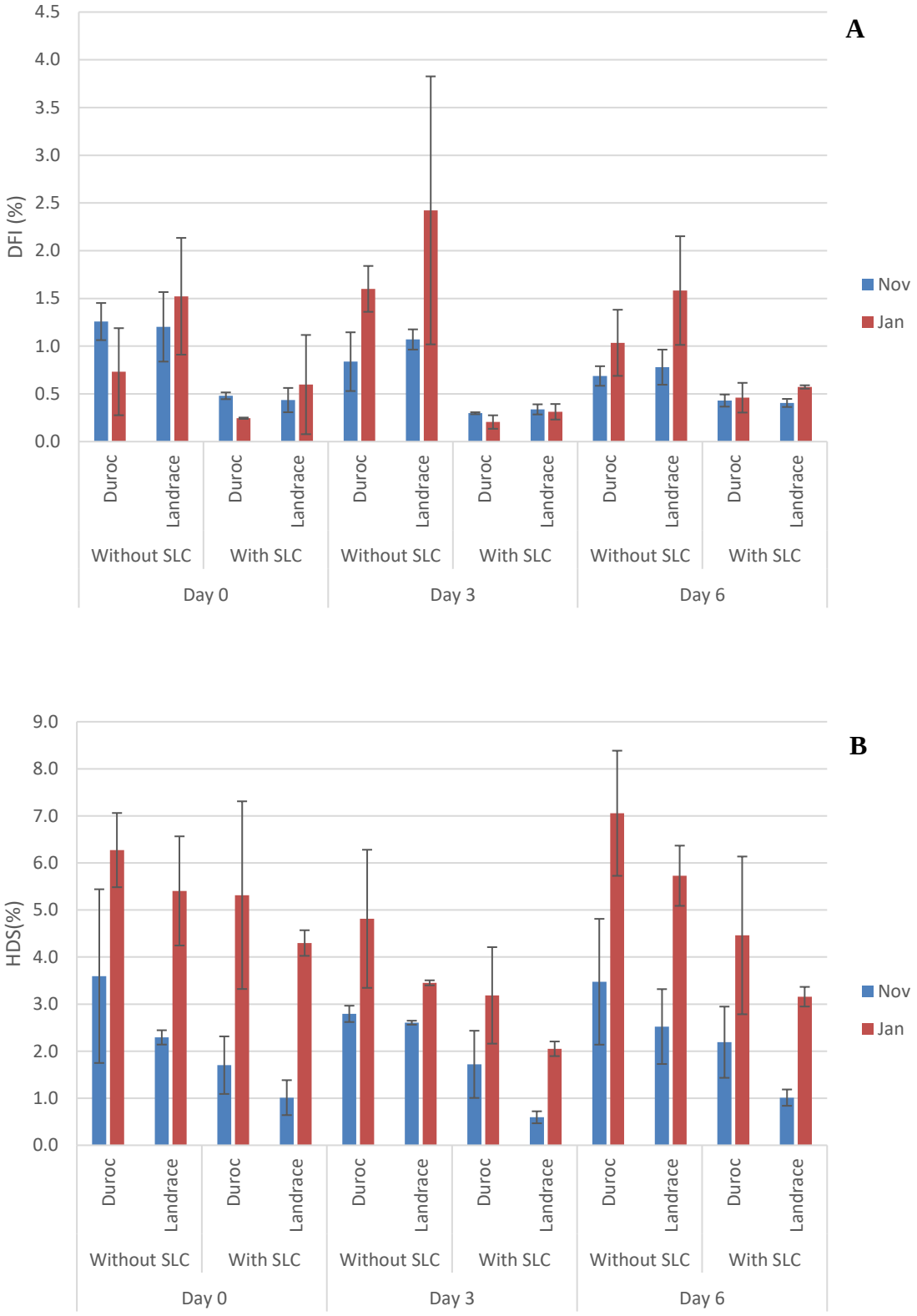

Figure 19. ***Effect of SLC on chromatin integrity.*** *The sperm quality parameters related to chromatin integrity was evaluated for 4 boars (2 Duroc and 2 Landrace) in November and January samples. A) DFI, DNA fragmentation index; B) HDS, high DNA stainability; Results are presented as mean ± SD.*

4.1.4 Evaluation of ATP content

The amount of ATP in Duroc's sperm treated with SLC was higher than in sperm without SLC at both timepoints (Figure 20). Throughout the storage period, the alternation in ATP level in the semen from Landrace was different from that of Duroc. On Days 0 and 6, the Landrace semen of November sample with SLC displayed a higher ATP content than without SLC; however, on Day 3, this character was nearly the same. The sperm ATP content of Landrace without SLC also declined in January samples. On day 0, landrace samples collected in November had a higher ATP content than corresponding Duroc samples under SLC conditions and without SLC.

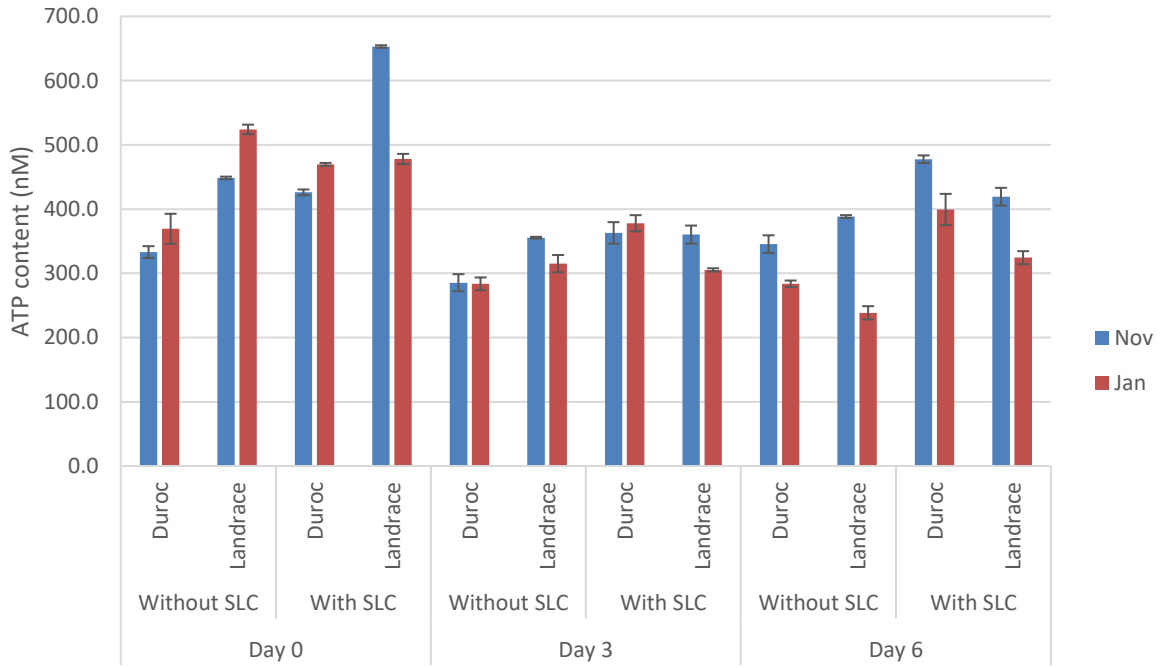

Figure 20. ***The impact of SLC treatment on ATP content (nM)****. The ATP content was examined during the storage period of 6 days, in samples collected in November and January for 4 boars (2 Duroc and 2 Landrace); Data are shown as mean ± SD.*

4.2 Comparison of two cryopreservation methods on sperm quality

4.2.1 Analysis of sperm motility parameters pre-freezing and post-thawing

Before freezing semen samples in straws, as detailed in 3.3.2 of the Materials and Methods section, samples that were collected from four boars (two Landraces and two Durocs) were cooled in two stages at 15/18 and then at 4 ℃. The data obtained from CASA for sperm motility parameter measurements are shown in Table 2. It was observed that when the temperature was reduced from 18 ℃ to 4 ℃, Landrace semen's total motility and hyperactive motility decreased. While Duroc samples showed some variation in motility parameters during the cooling phase, boar 2's motility parameters increased at 4 ℃, particularly hyperactivity, which was 2.8 times higher than at 18°C.

Table 2. ***Assessment sperm motility during cryopreservation process.*** *The motility parameters of sperm cells for 4 boars (Landrace and Duroc) evaluated before and after cryopreservation using the Direct and Ice Cube techniques. Values are presented as means ± SD.*

			18 ℃	4 ℃	Post-thaw Direct	Post-thaw Ice Cube
Total motility (%)	**Landrace**	Boar 1	90.8 ± 0.5	82.5 ± 2.1	29.11 ± 0.43	62.86 ± 2.74
		Boar 4	89.5 ± 2	85 ± 0.5	51.48 ± 6.56	62.98 ± 2.11
	Duroc	Boar 2	34.8 ± 3.9	77.2 ± 1.9	9.21 ± 2.36	19.01 ± 1.1
		Boar 3	54.6 ± 3.3	56.1 ± 0.6	4.96 ± 0.61	18.22 ± 3.34
Progressive motility (%)	**Landrace**	Boar 1	49.2 ± 1.1	54.9 ± 6.5	24.03 ± 1.53	55.05 ± 2.23
		Boar 4	56.3 ± 2.5	59.4 ± 1.5	37.29 ± 5.37	43.09 ± 1.51
	Duroc	Boar 2	12.2 ± 2.7	27.2 ± 3.1	5.91 ± 1.26	12.68 ± 1.06
		Boar 3	25 ± 0	28.8 ± 1.8	3.06 ± 0.24	14.17 ± 2.79
Hyperactivity (%)	**Landrace**	Boar 1	31.8 ± 1.3	4.8 ± 1.8	0.69 ± 0.21	2.29 ± 0.67
		Boar 4	16.9 ± 2.3	10.4 ± 0.9	0.93 ± 0.2	4.85 ± 0.64
	Duroc	Boar 2	16.2 ± 1.1	44.8 ± 3.8	0.33 ± 0.13	1.25 ± 0.08
		Boar 3	24.5 ± 3.7	15.7 ± 0.9	0.10 ± 0.18	0.68 ± 0.21

After the samples (which had been frozen using both methods of cryopreservation) were thawed, analysis revealed that the Landrace samples had higher total and progressive motility than the Duroc samples. Furthermore, when the two cryopreservation techniques are compared, the thawed semen from the IceCube technique showed a higher proportion of progressive motile sperm and total motility in both breeds than the Direct method did. For instance, in boar 1 (Landrace), the total motility of post-thaw Ice Cube was 62.9% semen; while this value was 29.1% for sample that was cryopreserved by the direct method. It was noticed that this value was 90.8% at 18°C.

4.2.2 Assessment of plasma membrane and acrosome integrity in cryopreserved samples using flow cytometry

Flow cytometry was applied to assess the viability and acrosome integrity of cryopreserved sperm cells for Landrace and Duroc boars. Thawed straws and samples of the prior cooling phase were used to evaluate acrosome intact live (AIL%) spermatozoa (Figure 21).

For both breeds, a variation between males was observed in the percentage of AIL at 4 °C compared to semen at 18 °C. After cooling to 4 °C, the percentage of AIL sperm was reduced by 3% for boar 4 (Landrace) and by 9% for boar 3 (Duroc) compared with 18 °C. When sperm were compared at 4 and 18 °C, boar 1 (Landrace) had the same AIL, but boar 2 (Duroc) had a 6% increase.

For both Landrace and Duroc boars, the percentages of live and acrosome intact sperm were reduced after thawing frozen sperm from both cryopreserved methods (Direct and Ice Cube). In comparison to sperm at 18 °C, the percentages of AIL for boar 1 (Landrace) using the Direct and Ice cube methods dropped by 40% and 19%, respectively. Higher proportions of AIL in post-thawed spermatozoa were found in Ice Cube samples as compared to post-thawed Direct samples. These results showed that for cryopreserved boar semen in both breeds, the Ice Cube method worked more effectively than Direct.

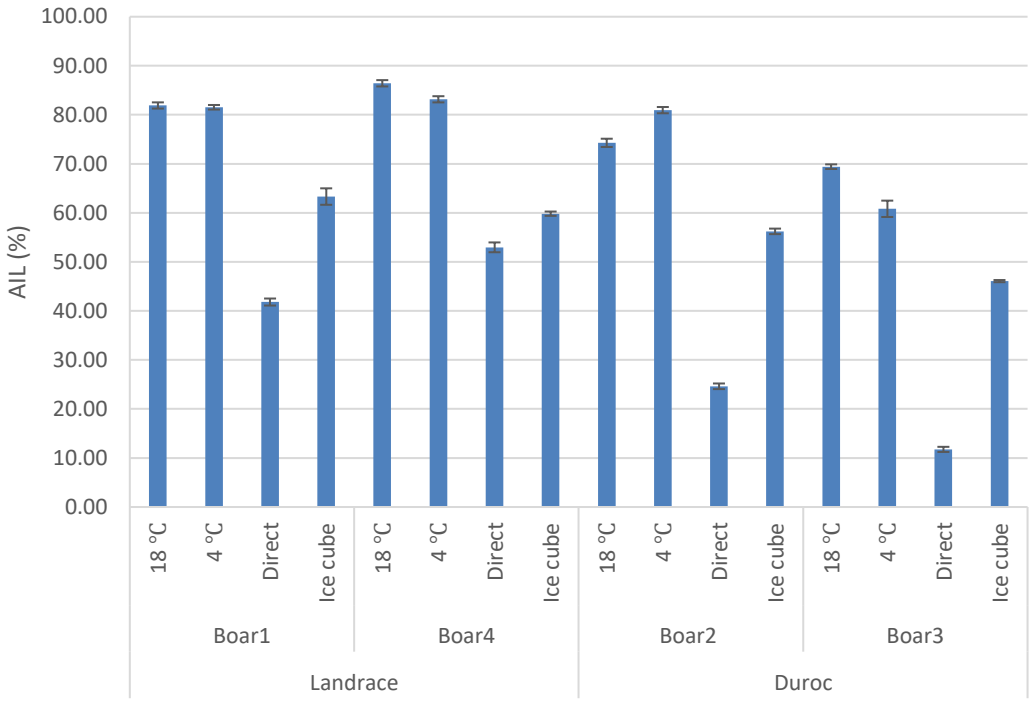

Figure 21. ***Acrosome-intact live spermatozoa (AIL%) examination during the cryopreservation process****. Analyse the samples from the prior freezing step (18 and 4 °C) as well as thawed samples (were frozen using Direct and Ice Cube methods). Boar 1 and boar 4 were Landrace and boar 2 and boar 3 were Duroc. Results are shown as mean ± SD.*

4.2.3 Analysis of sperm DNA integrity before freezing and after thawing

Since sperm DNA integrity affects reproductive outcomes, it is important to assess how cryopreservation may influence it (Lusignan et al., 2018). When the boar sperm was cryopreserved, DNA integrity was examined for integrity at 18 °C and 4 °C, as well as after using the Direct and Ice Cube methods (Figure 22).

All boars (both Landrace and Duroc) showed a decrease in the percentage of DFI and HDS when the temperature was lowered during the cooling phase prior to freezing straws. At each temperature, the Duroc semen had higher HDS compared to Landrace samples. There was no difference in the percentage of HDS (which indicates a protamine deficiency) between the two freezing methods in samples from boars 1 and 2. In contrast to the Direct method, the HDS% of frozen samples obtained through the Ice cube method was higher for boars 3 and 4. While the DFI of post-thaw Direct samples in boar 3 was lower than that of Ice Cube samples, the DFI of Landrace and boar 2 (Duroc) was approximately the same for the two cryopreserved methods.

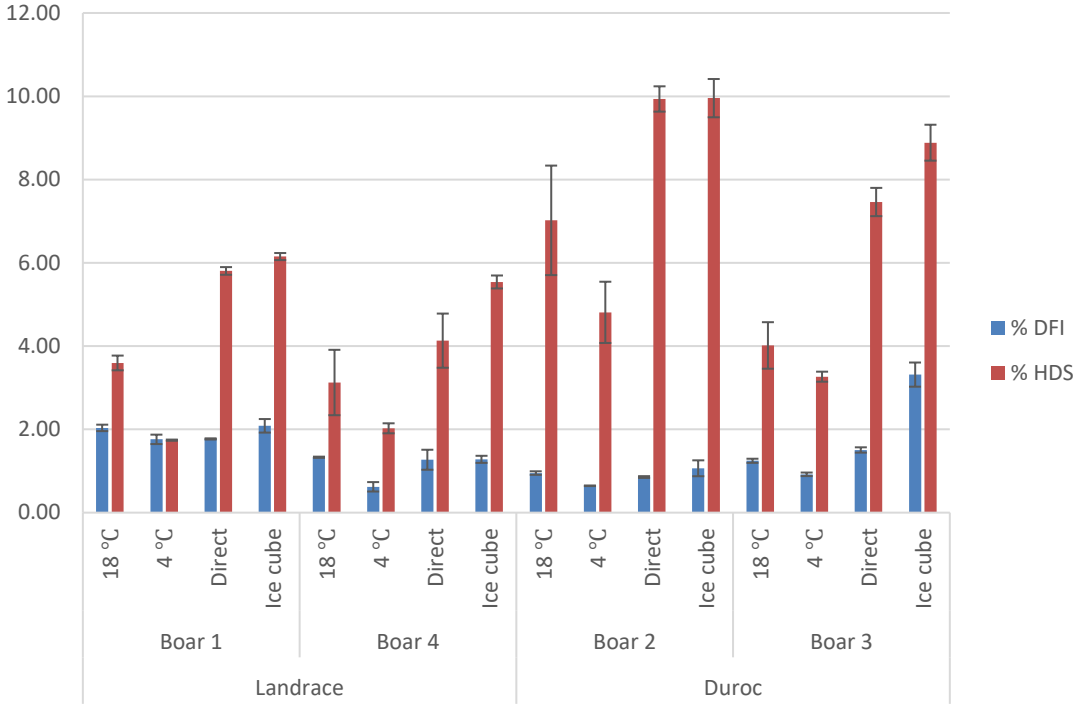

Figure 22. ***Assessment of chromatin integrity (DFI% and HDS%) during cryopreservation process****. The chromatin integrity was evaluated in semen samples collected from two Duroc and two Landrace boars, before freezing (18 and 4°C) and after cryopreservation (Direct and Ice cube). %DFI: DNA fragmentation index; HDS: high DNA stainability, data are presented as means ± S.D.*

5. Discussion

The purpose of the present study was to determine whether using different preservation methods could enhance the quality of liquid-stored boar semen as well as cryopreserved semen. This study examined the quality of semen from two Norwegian boar breeds (Landrace and Duroc) at two ages (November and January). An evaluation of the effects of the SLC method and storage on liquid-stored semen quality was performed on collection day (Day 0) and after 3- and 6-days following storage at 18 °C. Boar semen quality during the cryopreservation process, including the cooling phase (before freezing) and post-thawed samples, was assessed. The boar spermatozoa's properties, including motility, viability, acrosome integrity, DNA integrity, and ATP content, were evaluated by CASA, flow cytometry, and luminescence assay techniques, respectively.

5.1 The impacts of SLC on sperm quality

The primary objective in countries with significant use of AI and high reproductive performance is to enhance AI efficiency by producing cost-efficient, safe semen doses with high fertilizing capacity to promote the utilization high genetic value boars. Techniques for gentle semen processing, transport, storage, and methods for quality control are offered to lower the amount of sperm required for each female/year (Waberski et al., 2019). The spermatozoa (both normal and abnormal) in the ejaculate have a wide range of maturity levels (Morrell & Rodriguez-Martinez, 2016). Significant amounts of dead spermatozoa in raw semen samples negatively impact the efficiency of frozen-thawed spermatozoa and the results of *in vitro* fertilization (Roca et al., 2013). It is important to have high-quality sperm to make sure that enough spermatozoa that can fertilize reach the oocyte during AI. Due to advancements in liquid semen preservation techniques, organizations that breed pigs have established individual standards for useable semen at 60–80% for motility (Waberski et al., 2019).

In this study, the semen of the two breeds, Landrace and Duroc, that were centrifuged with Porcicoll (with SLC) and uncentrifuged semen (without SLC) demonstrated differences in the sperm motility parameters as determined by CASA. Furthermore, differences in motility parameters were observed in samples between Days 0 and 6 of semen storage for both breeds under the above conditions. Since there are studies that do not demonstrate a connection

between sperm motility and fertility (Popwell & Flowers, 2004; Xu et al., 1996), several studies have shown assessing sperm motility in extended boar semen after different storage times is an effective way to assess the quality of ejaculates and predict boar fertility (Broekhuijse et al., 2012b; Foxcroft et al., 2008; Ruiz-Sánchez et al., 2006). One of the factors necessary for sperm transportation through the female reproductive tract is the spermatozoa's progressive motility. Progressive motility has been reported to be useful for determining the quality of semen and has significant correlations with farrowing rate or litter size as fertility outcomes (Broekhuijse et al., 2012a; Kummer et al., 2013). According to Lucca et al. (2021), the progressive sperm motility at 120 h storage in Short-term extender can be used to identify less fertile boars.

The SLC technique can be used to select motile spermatozoa with normal morphology and intact membranes from a mixture of spermatozoa with different maturation levels in the ejaculate (Morrell, 2019). It can also eliminate dead spermatozoa and cellular debris from the prior stored insemination sample. Loaded semen sample on top of the colloid, is gently centrifuged. Motile spermatozoa with intact plasma and acrosomal membranes with highly condensed chromatin easily migrate through the coated silica particles (colloid) and deposit at the bottom of the tube. At the interface between the semen and the colloid, sperm with morphologically abnormal or damaged membranes, reacted acrosomes, and uncondensed chromatin are maintained (Morrell & Rodriguez-Martinez, 2016).

In the present study, progressive motility increased in the SLC-prepared samples for both breeds. This result is supported by Martinez-Alborcia et al. (2013) study where the proportion of spermatozoa with progressive motility and normal morphology after SLC by Androcoll-P was found to be higher than in control semen samples. In study by Basioura et al. (2021), SLC-treated of boar samples (by BoviPure^{TM}) showed higher percentage of progressive motility than uncentrifuged samples, and higher values of VSL and LIN compared to control.

Sperm motility hyperactivity is another important factor to consider when assessing fertility. Normally, hyperactivation takes place in the oviduct and is induced by sperm capacitation. Hyperactivated spermatozoa have the ability to detach from the oviduct and penetrate the oocyte’s zona pellucida (Schmidt & Kamp, 2004; Suarez & Ho, 2003). It is possible for sperm cells to lose energy as a result of early hyperactive motility and die before reaching the oocyte (van Son et al., 2022).

According to the present study, in both breeds, the percentage of hyperactive sperm cells dropped dramatically in samples with SLC compared to those without SLC. Generally, the proportion of hyperactive sperm cells in Duroc samples was higher than in Landrace samples.

These findings are in agreement with Tremoen et al. (2018), who reported that both Landrace and Duroc had a drop in the proportion of motile sperm cells during storage, and Duroc semen contained a significant percentage of sperm cells with hyperactive motility compared to Landrace samples.

Additionally, the study by Santiago-Moreno et al. (2017) found that using a density gradient centrifugation (DGC) on goat semen (before chilling phase of cryopreservation) can enhance sperm motility parameters. Since the ALH in hyperactive sperm increases dramatically during capacitation, the ALH remained lower in the DGC samples than in the control. As a result, selecting sperm that is non-capacitated should be simpler when employing the DGC.

In the current study, the Landrace semen samples from November had a higher percentage of total and progressive motility than the Duroc samples. In both breeds, these parameters decreased over 6 days storage. These results are supported by Stančić et al. (2011) research which demonstrate that maintaining progressive spermatozoa motility in insemination doses affected by the boar breed over the first 48 hours. Fewer semen samples with ≥65% progressive motility in Duroc compared to Swedish Landrace were found. While the amount of progressive motility declined over storage time, no significant differences were observed between the breeds after 72 hours. However, as stated by Kommisrud et al. (2002), motility and acrosome integrity are significantly affected during storage (liquid boar semen over 5 days). Sperm concentration and boar had a significant impact on motility, while boar influenced acrosome integrity. Breed had no effect on acrosome integrity or motility.

In the current study, the SLC samples had higher proportions of AIL spermatozoa. This is consistent with findings from other studies showing that SLC is effective in selecting sperm with high plasma membrane integrity in several species, including stallion (Johannisson et al., 2009), young bull (Lima-Verde et al., 2022), cat (Chatdarong et al., 2010), boar (Martinez-Alborcia et al., 2013) and goat (Jiménez-Rabadán et al., 2012).

The present AIL% results are consistent with a study by Šterbenc et al. (2019) that evaluated how the SLC of a species-specific colloid (Androcoll-O) affected the quality of spermatozoa from post-thawed rams. The study found that the AIL% was significantly higher in SLC

samples compared to control samples, while the percentage of abnormal acrosomes and morphologically abnormal spermatozoa was significantly lower in SLC samples. Moreover, a comparison was made between magnetic activated cell sorting and colloid centrifugation (methods of sperm selection) for boar semen in the study conducted by Deori et al. (2022). The results indicated that sperm quality was higher in SLC samples in contrast to magnetic activated cell sorting-selected samples, and that membrane integrity was higher in SLC samples than in controls.

According to Johannisson et al. (2009), there are two primary reasons why SLC is beneficial for maintaining the chromatin integrity of sperm. Firstly, spermatozoa that are mature and intact are selected by centrifugation at a higher density than those that are immature or damaged. Secondly, eliminating the seminal plasma (including damaged spermatozoa and leukocytes) can stop reactive oxygen species from causing additional damage to chromatin.

The mature sperm nucleus contains highly condensed chromatin that is packed so firmly because histones are replaced by protamines. Some spermatozoa may have intact membranes, normal morphology, and good motility despite having damaged chromatin. Spermatozoa with damaged chromatin can compete with intact spermatozoa to fertilize the oocyte, potentially leading to embryo failure due to limited DNA repair mechanisms (Morrell, 2019). A pervious study by Myromslien et al. (2019) has shown that the litter size of two boar breeds (Landrace and Duroc) is negatively affected by increased damaged chromatin (assessed by DFI).

The results of the current study showed that in comparison to samples prepared by SLC, uncentrifuged samples displayed a higher DFI% and lower HDS% for both breeds. Our results corroborate previous findings that chromatin integrity using SLC could improve sperm quality or better reproductive outcomes (Johannisson et al., 2009; Martinez-Alborcia et al., 2013; Morrell & Rodriguez-Martinez, 2016; Salman et al., 2023; Šterbenc et al., 2019). This is in agreement with a previous study Lacalle et al. (2023) reporting that removing bacteria contamination from boar semen by SLC caused a decrease of %HDS.

In this study, the DFI levels in Landrace boars were found to increase with age, samples of semen taken in January had a higher DFI than samples taken in November. As opposed to Myromslien et al. (2019) and Tsakmakidis et al. (2012) findings which revealed that the DFI levels for the Landrace boars (young boars) declined with increasing age.

An adequate amount of ATP is important for the spermatozoa's successful fertilization processes, including protein phosphorylation, ion transport, and capacitation. Moreover, ATP-dependent ion pumps are necessary for maintaining the integrity of the plasma membrane and the acrosomal secretory granule. The most prominent characteristic of the sperm cell is motility, which depends on ATP (Tourmente et al., 2018).

The results of the present study demonstrated that, for both breeds, the ATP level in SLC samples was mostly higher than that without SLC samples at both ages. According to a pervious study Tremoen et al. (2018), sperm cell motility characteristics in both Landrace and Duroc are correlated with ATP concentrations. The proportion of motile and progressive cells is positively correlated with higher ATP concentrations. This confirms our findings that the proportion of progressive motility as well as ATP content were higher in the samples treated with SLC. Furthermore, the Landrace semen had higher motility and ATP content compared to the Duroc samples in November.

In this study, the concentration of the samples was evaluated following SLC by Porcicoll; however, the volume underneath was not measured. According to visual observations, the volume underneath Landrace semen was higher than that of Duroc and decreased over storage. This result is consistent with Smital et al. (2004) who report that Landrace samples had a higher number of viable spermatozoa than Duroc samples. Customers of AI companies expect the best-quality semen to guarantee maximum reproductive efficiency per dose of AI purchased. The number of inseminations needed per oestrous period and the number of sperm cells per dose determine the efficiency of AI. To reduce the number of cells per AI dose, the potential for decreased field fertility should be considered (Broekhuijse et al., 2015). As a result, while AI techniques require differing numbers of sperm cells per AI dose, the total number of sperm cells in an AI dose should be above the threshold (Roca et al., 2006). Lima-Verde et al. (2022) investigated the quality of young bull semen by SLC and found that ejaculates from young bull (which are typically discarded) can be used with the SLC technique to produce sufficient quality sperm doses for AI.

5.2 Effect of the cryopreservation method on sperm quality

Frozen-thawed boar semen is not commonly used for AI in commercial pig production due to its lower fertilization capacity compared to liquid semen. Since the use of liquid-diluted semen is limited to long-distance transport and lengthy shipment times, improving cryopreservation

techniques is required (Yeste et al., 2017). Sperm quality has been demonstrated to decrease as a result of modifications during the cryopreservation procedure (Johnson et al., 2000). Mammalian sperm undergo various structural and molecular changes as a result of cryopreservation. Post-thawed semen exhibits reduced mitochondrial function, sperm motility, and changes in plasma membrane fluidity, integrity, acrosome integrity, ROS concentrations, and DNA integrity (Yánez-Ortiz et al., 2022). Understanding the variables affecting spermatozoa's survival is essential for the freezing of boar semen. These variables may be extrinsic, such as diluent composition, type, and concentration of cryoprotective agent, equilibration, freezing, and thawing procedures. Additionally, there are internal factors like the differences between ejaculates and boars and their inherent traits (Johnson et al., 2000).

Boar sperm cell cryopreservation cannot be carried out instantly due to the properties of the plasma membrane. The quality of cryopreserved boar semen is enhanced by pre-cooling steps that involve keeping the semen at 15–17 °C. This allows spermatozoa and seminal plasma components to interact for a longer period (Torres et al., 2019). The standard cryopreservation protocol has reduced cooling damage by incorporating egg yolk and adjusting the cooling rate. However, when the sample temperature is lowered from 15 to 5°C, cold injuries still happen. A minimum holding time of 1-3 hours in conventional extenders is recommended for boar sperm (Casas & Althouse, 2013). The effect of holding time (e.g., 17–5 ℃) before freezing boar semen on the quality of sperm was assessed in several studies. It was discovered that storage over time provided protection from cold shock to sperm cells (Casas & Althouse, 2013; Juarez et al., 2011; Tomás et al., 2014).

Since slow freezing is a process in which a programmable freezing machine regulates the gradual thermal equilibrium of the cells, vitrification requires high cooling rates for the purpose of solidification without ice crystal formation (Parihar et al., 2023; Pezo et al., 2019). In the present study, the Ice Cube method, a programmed freezing method, was used as slow freezing compared to the Direct method, in which samples were exposed to liquid nitrogen vapor and applied as a vitrification method. Landrace semen's total and hyperactive motility decreased, while Duroc samples showed variation in prior freezing phase. Frozen-thawed Landrace samples had higher total and progressive motility than Duroc samples. The Ice Cube technique showed a higher proportion of progressive motile sperm and total motility in both breeds. According to the AIL% analysis, the Ice Cube method performed better than the Direct method for cryopreserved boar semen in both breeds.

These findings are in agreement with observations made by Waterhouse et al. (2006). When boar sperm were cooled to 5 °C as opposed to 18 °C for both Landrace and Duroc, the percentage of live, intact acrosome sperm was found to decrease slightly. There was a noticeable decrease in the percentages of live and AIL sperm following the freezing and thawing processes.

Current observations are in compliance with results of previous the study by Petyim et al. (2007), where they compared the use of liquid nitrogen vapor and programmed freezing to assess the effects of cryodamage on human sperm characteristics. They proposed the programmed freezing method because it performed significantly better than liquid nitrogen vapor for sperm motility, vitality, and DNA integrity.

The results from the present study are consistent with previous studies by Baishya et al. (2014); Kaeoket et al. (2008) indicating that cryopreserved boar sperm in lactose-egg yolk glycerol extender obtained by Ice Cube had a higher motility percentage and viable intact acrosomes compared to the direct method. Moreover, the qualities of post-thawed semen were also influenced by the breed of boar; samples from Landraces had higher motility and AIL% than samples from Durocs. According to the study by Serrano Albal et al. (2023), the boar sperm sample's motility and viability were reduced more by the vitrification procedures than by slow freezing.

In the present study, 4% glycerol was used in the freezing extender. The prior research by Corcuera et al. (2007) examined the impact of diverse glycerol concentrations on the quality of boar sperm. It was discovered that in 4% glycerol extender (30 minutes of equilibration), frozen spermatozoa exhibited the best motility and normal acrosomes.

The assessment of chromatin integrity (DFI% and HDS%) in the current study revealed that the values indicate a reduction when semen was cooled from 18 to 4 °C. Post-thawed samples of both freezing methods showed a higher HDS% compared to the cooling phase, but differences in the percentage of DFI were observed between boars in both breeds. Fraser and Strzeżek (2005) verified that there were differences in sperm DNA resistance to the freezing-thawing process among boars. Hernández et al. (2006) stated that the cause of this individual variation in chromatin stability is unknown; defective spermatogenesis and abnormal apoptotic degeneration are associated with sperm chromatin abnormalities. Aitken (2017); Zhang et al. (2021) reported that damage sperm chromatin structure is largely caused by

reactive oxygen species. In addition, frozen-thawed spermatozoa have significantly higher levels of free radicals than fresh spermatozoa (Pini et al., 2018).

In the study by Fraser and Strzeżek (2007), radioisotope-labelled DNA was utilized to evaluate the chromatin state of frozen-thawed boar spermatozoa by a neutral comet assay. The boar semen samples in the lactose egg yolk-glycerol extenders were frozen by the Ice Cube method. They observed sperm chromatin destabilization and DNA fragmentation significantly higher in frozen-thawed semen than in fresh samples.

6. Conclusion and further perspectives

In the present study, SLC was used as a semen processing technique for Duroc and Landrace, which resulted in an improvement in the quality of boar spermatozoa. The two breeds' semen quality varied by storage conditions. Using two different freezing techniques, semen samples were evaluated. The results showed that the post-thaw quality of the samples was higher when using the programmed freezing technique than when using the other method, which directly exposed the sample to liquid nitrogen vapor.

The quality of SLC-treated semen and uncentrifuged samples was compared on the day of collection (Day 0) and after 3 and 6 days of storage. It was found that for both breeds (Duroc and Landrace), the sperm cells with SLC treatment generally had higher progressive motility, ATP content, viability and acrosome integrity while having a lower hyperactive proportion, DFI%, and HDS% than the sperm cells without SLC treatment. When day 0 and day 6 at both ages were compared, the assessment of motility characteristics showed a gradual decline in uncentrifuged samples, but the evaluation of ATP content and DNA integrity revealed variation in both breeds. It seemed that storage had no effect on the AIL% that was measured. Analysis of the quality of semen from two breeds during the cryopreservation process showed that samples displayed variation in motility parameters in the cooling phase prior to freezing. The landrace samples' total motility, hyperactivity, and AIL% decreased as the temperature was lowered from 18 to 4 ℃, but one of the Duroc semen sample's parameters increased at that temperature. Considering that the freezing methods adversely affected the semen quality in both breeds, there was a decline in motility parameters and AIL% and an increase in DNA damage (higher DFI% and HDS%). The quality of semen frozen by programmed freezing (Ice Cube) was generally higher than that of semen frozen by the direct method, according to a comparison of the results of the two freezing techniques.

Summarized, the SLC method can be used to enhance the motility characteristics, viability, acrosome integrity, and ATP content of liquid-stored semen from both Landrace and Duroc samples. The longer-term preservation applying cryopreservation of boar semen is proposed using programmed freezing instead of the direct method with liquid nitrogen vapor. For further studies on improving the quality of boar semen, conducting statistical analysis to identify the correlation between boar sperm properties and SLC-treated samples at varying storage times will be of interest. It is also suggested to measure the level of metabolites in SLC samples.

Additionally, evaluating the fertility records for both SLC samples and frozen-thawed semen is advised. This will assist in selecting high-quality semen for AI.

7. References

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