

**COOLING TOLERANCE AND POST-THAW QUALITY OF BOVINE
EPIDIDYMAL SPERMATOZOA AFTER VITRIFICATION USING TRIS-
CITRATE-SUCROSE BASED EXTENDER**



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BY

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BISHOFTU, ETHIOPIA

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**A thesis submitted to the College of Veterinary Medicine and Agriculture, Addis
Ababa University in partial fulfillment of the requirements for the Degree of Master
of Veterinary Science in Veterinary Theriogenology**

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LIST OF ABBREVIATIONS

ACI	Acrosomal Integrity
AI	Artificial Insemination
ANOVA	Analysis of Variance
BSE	Bull Breeding Soundness Evaluation
BSE	Breeding Soundness Evaluation
CPA	Cryoprotectant Agents
EY	Egg Yolk
GPS	Global Positioning System
HDL	High Density Lipoprotein
HOST	Hypo-Osmotic Swelling Test
IVF	Invitro Fertilization
LDL	Low Dencity Lipoprotein
LN ₂	Liquid Nitrogen
ROS	Reactive Oxygen Species
SC	Scrotal Circumference
SFT	Society for Therigenology
TSC	Tris-Sucrose-Citrate

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ABSTRACT

Cryopreservation involves preserving living cells, such as sperm, at very low temperatures to maintain their viability for extended periods, particularly in breeding animals. An experimental investigation was conducted between December 2023 and May 2024 aimed to assess the efficacy of a specific extender media-cryopreservation combination method for evaluating the quality of epididymal sperm under field conditions. The cooling tolerance of bull epididymal spermatozoa preserved in a homemade tris-citrate-sucrose extender media was evaluated. Further, the impact of two methods of vitrification (direct droplet and straw) on post-thaw sperm quality and the effect of supplementing high (12.8%) and low (2.5%) glycerol to the base media on cryosurvival were evaluated. All physical parameters including body weight, BCS, scrotal circumference and other testicular measurements were recorded. Mean scrotal circumference, size and volume of the testis were 32.06 ± 1.2 cm; 301.80 ± 37.4 g and 334.86 ± 105.1 cm³, respectively. Pre-freeze motilities were generally higher (76.88% mass motility and 73.13% individual motility). A significant difference ($P < 0.05$) in motility was generally observed between pre-freeze and post-freeze samples. Nevertheless, a very high cooling tolerance was evident during the first 30-minute cooling period with no significant difference (70%, $p > 0.05$) in motility from prefreeze values. But a drastic 23% drop in motility was observed after 60 minutes, indicating declining cooling tolerance over time. Testicular and epididymal weight and scrotal circumference were significantly associated with improved sperm motility, suggesting that bulls with higher scores in these parameters tend to have higher sperm motility. Spermatozoa destined for vitrification showed significantly higher ($P < 0.05$) pre-freeze motility, with the head region exhibiting a higher number of morphological defects than the tail. Glycerol concentration (2.5% and 12.85) in vitrified sperm had a significant role ($P < 0.05$) in post thaw sperm parameters with higher concentrations improving cryocervival. Vitrification method also had a significant role in postthaw sperm parameters with straw vitrification in high glycerol (12.8%) having better impact in cryocervival compared to direct vitrification with lower glycerol (2.5%). In conclusion the recovery and cryopreservation of epididymal sperm from live or dead animals is a viable option in maintaining their germplasm available for future use. Further, the freezing technique is a promising method for field application particularly in evaluating preservice bulls before purchase.

Key words: Cooling tolerance, Epididymal spermatozoa, Tris citrate-sucrose extender, Vitrification

1. INTRODUCTION

Assessing the reproductive capacity of male cattle, prior to their utilization as breeding stock, would be economically prudent if feasible. Yet, accurately determining fertility through a breeding soundness assessment poses challenges in practice (Chenoweth *et al.*, 1993). Nevertheless, establishing minimum criteria for a series of accepted protocols allows for informed decisions regarding the breeding potential of the bull. An individual meeting these standards is considered to possess adequate breeding capability, and the process of conducting such a series of examinations is known as a breeding soundness evaluation (Irons *et al.*, 2007).

The evaluation of breeding soundness (BSE) is a rapid and cost-efficient method for appraising bulls prior to purchase or use. Moreover, it acts as a valuable tool in investigating cases of infertility. The use of this assessment has been linked to clear benefits, influencing herd fertility directly and indirectly by genetic connections to various other fertility traits in both sexes. The evaluation of breeding soundness is a quick and cost-effective approach for evaluating bulls before procurement or use (Chenoweth *et al.*, 1993; Irons *et al.*, 2007).

A Breeding Soundness Evaluation does not provide a guarantee regarding the high fertility of a bull or the ranking of bulls in terms of fertility. BSE's purpose includes the identification of bulls carrying undesirable heritable traits or those unlikely to achieve a high pregnancy rate within a limited breeding season (Barth, 2018). Nevertheless, BSE assesses the physical attributes of a bull essential for its mobility and athleticism in the pasture, its structural integrity, overall and reproductive development, the size and health of its testes, and the quality of its semen (Armstrong and Koziol, 2022).

Apart from assessing physical fitness, inspecting the genital tract, and measuring scrotal circumference, a significant aspect of Breeding Soundness Evaluation involves the assessment of semen quality. While categorization is based on physical examination and

the bull's capacity to meet minimal standards for testicular growth, of greater importance are the motility and morphology of sperm which necessitate evaluation before the bull commences semen production (Barth, 2018; Armstrong and Koziol, 2022).

Semen analysis during BSE is ideally conducted using samples obtained through electroejaculation. In Ethiopia, BSE in preservice bulls is a common practice post-purchase, potentially leading to the culling of bulls failing to meet breeding criteria, despite associated costs. To mitigate such losses, it is recommended that BSE be conducted at the field level, selecting only those bulls meeting the minimum semen evaluation standards. Vitrification is the preferred method for semen freezability tests in field conditions, as conventional freezing techniques often require logistical support not readily available. Vitrification offers a rapid approach to semen storage and assessment, with practical field application devoid of the need for sophisticated equipment. Both sperm motility and morphology can be effectively evaluated under field conditions. Optimal sperm cryo-survival, however, hinges on various factors such as semen responsiveness to freezing-thawing conditions, extender/cryoprotectant type, and the species/breed of the animal in question (Barbas & Mascarenhas, 2009).

However, the techniques for vitrification of bovine sperm have not yet reached full maturity and warrant further exploration (Baiee *et al.*, 2020). In light of the very big advantage particularly in the evaluation of preservice bulls, it is hypothesized that cooling tolerance and vitrification outcomes could be improved by modulating the proportion of cryoprotectant agent in the extender media.

Therefore, the major objective of this research is: To evaluate the choice for extender media-cryopreservation combination method that is applicable for field semen evaluation of preservice bulls.

Specific objectives:

- To assess the cooling tolerance of bull epididymal spermatozoa using egg yolk based homemade tris-citrate-sucrose extender media.
- To evaluate the effect of vitrification on the post-thaw quality of epididymal sperm
- To evaluate the effect of supplementation of low concentration of cryoprotectant (Glycerol) to tris-citrate-sucrose media on cryosurvival of bull spermatozoa after vitrification.
- To compare post-thaw quality of direct and straw vitrified bull spermatozoa after vitrification.

2. LITRATURE REVIEW

2.1 Breeding Soundness Evaluation

Breeding soundness is defined as the capacity of a bull to impregnate cows, while the process of breeding soundness evaluation entails assessing the reproductive capability of the male prior to mating, thereby aiding in the exclusion of sub-fertile sires from breeding schemes (Hoflack *et al.*, 2006). This is crucial as sub-fertile bulls can lead to delayed conception, extended calving seasons, diminished calf weaning weights, and heightened female culling rates (Kastelic and Thundathi, 2008). Although the most reliable indicator of fertility lies in the pregnancy rate of the females inseminated, the evaluation of breeding soundness in males provides valuable insights into the anticipated performance, thereby potentially improving the overall productivity of the herd (Chapwanya *et al.*, 2008).

While a favorable breeding soundness assessment outcome does not ensure fertility or eliminate the risk of transmitting adverse effects to herds where the bull is utilized, it does imply a diminished risk attributable to specific factors (Shiple, 1999). Bulls deemed suitable for breeding soundness demonstrated a 9% higher pregnancy rate during a breeding season in single-sire breeding herds compared to bulls with questionable breeding potential (Farin *et al.*, 1989). Bulls categorized as unsatisfactory may exhibit complete infertility, resulting in a total loss of the potential calf crop in single-sire scenarios due to various factors. Therefore, evaluating the breeding soundness of bulls before their utilization is acknowledged as an essential component of effective herd management (Irons *et al.*, 2007).

The primary aim of a bull breeding soundness evaluation is to furnish a standardized and unequivocal assessment of a bull's suitability for inclusion in a breeding program. A secondary objective may involve leveraging the data to facilitate the selection of fertility traits through genetic means. The evaluation process must adhere to principles of repeatability, reproducibility, efficiency, and safety for both the bull and the assessing clinician. Additionally, it should possess a degree of adaptability to accommodate diverse managerial needs. Within each managerial

context, there must be uniformity in data collection, interpretation, and classification of evaluation outcomes, ensuring that the results are easily comprehensible to end users. This consistency serves to uphold the credibility of the BSE, benefiting veterinarians, cattle producers, and researchers alike (Norman, 2021).

2.2.1 Components of Breeding Soundness Evaluation

Assessing a bull's potential breeding soundness involves several key components: a comprehensive physical examination, scrutiny of both external and internal genitalia through genital tract examination (which encompasses measuring scrotal circumference), and an evaluation of sperm quality (Hoflack *et al.*, 2006; Lone *et al.*, 2017).

The initial step in a bull breeding soundness evaluation involves assessing the bull's conformation, body condition, and overall physical well-being. It is imperative that the bull is adequately sized for its age, devoid of evident disease, and possesses sufficient musculature and body fat to facilitate unrestricted movement within the herd, enabling him to detect females in estrus, mount them, and successfully engage in coitus. Furthermore, the bull's feet and legs must be devoid of defects that impede mobility. Conditions such as acute or chronic laminitis, post-legged conformation, and screwclaw are among the more prevalent musculoskeletal issues that can hamper a bull's agility and athleticism. Notably, post-leg and screwclaw are potentially heritable conformation traits that can yield undesirable consequences within the herd (Chacon *et al.*, 1999; Armstrong and Koziol, 2022).

The assessment of the internal reproductive organs is typically conducted via transrectal palpation. Among the supplementary glands contributing to the composition of seminal fluid in bulls are the ductus deferens, ampullae, seminal vesicles, prostate, and bulbourethral glands (Senger, 2003). A prevalent anomaly affecting these auxiliary glands is seminal vesiculitis, necessitating meticulous examination for alterations in texture and dimensions (Alexander, 2015). The seminal vesicles, discernible as lobular structures extending laterally from the anterior aspect of the pelvic urethra, should undergo thorough scrutiny for irregularities such as marked asymmetry, presence of abscesses, adhesions, fibrotic changes, or indications of discomfort. Furthermore, attention should

be devoted to identifying the paired ampullae positioned between the seminal vesicles (Cates, 1983). Although infrequent, abnormalities in the ampullae carry significance, especially in the context of transrectal manipulation for semen retrieval. Often disregarded, the internal inguinal rings warrant scrutiny. These can be palpated transrectally approximately 15 to 20 cm ventral from the pelvic brim and 5 to 15 cm lateral from the midline. Enlarged rings may facilitate the insertion of three or more digits and are associated with a predisposition to inguinal herniation (Palmer, 2016).

The evaluation of scrotal circumference stands as an essential component within both general and reproductive assessments. This metric serves as a pivotal parameter for the appraisal of breeding soundness, primarily due to its reflection of testicular volume, which correlates closely with sperm production (Lone *et al.*, 2017). Significantly, scrotal circumference is recognized as a highly heritable characteristic and is esteemed as a reliable indicator of sperm yield (Moghaddam *et al.*, 2012). Bulls with larger testicular dimensions typically demonstrate heightened daily sperm production rates and may additionally produce female offspring who attain puberty earlier and exhibit increased ovulatory capacity during each estrus cycle (Abate *et al.*, 2023).

The measurement of scrotal circumference bears considerable importance as an indicator not only of pubertal onset but also of overall semen production, semen quality, pathological testicular conditions, and the likelihood of subfertility or infertility in general (Tesfa *et al.*, 2024). Due to its precision and straightforwardness, this measurement proves to be a valuable tool for the early selection of bulls (Lone *et al.*, 2017). Furthermore, bulls must attain minimum scrotal circumference measurements corresponding to their age in order to successfully pass a breeding soundness evaluation (Dact, 2000).

Various methods are employed for semen collection, including electroejaculation, the gloved hand technique, collection through an artificial vagina, and the epididymal gamete rescue method. Retrieving epididymal spermatozoa from deceased animals, cryopreserving them, and subsequently utilizing in vitro fertilization (IVF) are valuable techniques for preserving genetic material that would otherwise be lost. This is particularly significant for salvaging genetic material from highly productive animals or endangered species (Chaveiro *et al.*, 2015).

A fundamental aspect of sperm assessment involves the microscopic examination of motility and morphology. Motility ensures the production of an ample quantity of viable sperm by the bull, with a minimum criterion of at least 60% progressively motile spermatozoa required to pass a bull breeding soundness evaluation (BSE). Morphology assessment scrutinizes whether the bull generates properly shaped sperm capable of fertilization, with a minimum threshold of 70% normal sperm out of a total count of 100 required to pass a BSE (Dact, 2000). Assessment of sperm quantity or production is ascertained by measuring the scrotal circumference (Hopper and King, 2014).

As per the guidelines provided by the Society for Theriogenology (SFT), possessing a thorough comprehension of the minimum criteria for bull breeding soundness evaluation (BSE) is imperative for conducting a precise assessment. In order to qualify bulls as suitable prospective breeders, they must undergo a physical examination and fulfill or exceed the established minimum standards delineated within each specified category, as described in (Table 1) (Lone *et al.*, 2017).

Table 1: Minimal thresholds of scrotal circumference, sperm motility and normal sperm (Lone *et al.*, 2017)

Parameter	Threshold
Scrotal circumference	30 cm at <1.25 years of age
	31 cm at >1.25 to 1.5 years of age
	32 cm at >1.5 to 1.75 years of age
	33 cm at >1.75 to 2.0 years of age
	34 cm at >2 years of age
Percent normal spermatozoa	>70%
Sperm motility	>60% individual motility and/ or Fair gross motility

The bull breeding soundness evaluation (BSE) offers a reliable, rapid, and cost-effective means of screening and categorizing bulls based on fertility, aiming to minimize the utilization of subfertile or questionably fertile bulls. While primarily employed for naturally mating beef bulls, BSE is

also relevant for both dairy and beef bulls utilized in artificial insemination (AI) programs with cryopreserved semen (Hoflack *et al.*, 2006).

Bulls earmarked for breeding should undergo bull breeding soundness evaluation (BSE) prior to commencing semen production (Tesfa *et al.*, 2024). In Ethiopia, it's best practice to conduct BSE for young preservice breeding bulls once they've been acquired and introduced to the center. Logistically, this presents challenges because if tested bulls fail to meet the minimum criteria for breeding, they must be culled at a cost. While no established protocol currently exists, the most viable approach would likely involve conducting BSE at the field level and selecting only those bulls that meet or exceed the predetermined evaluation criteria (Hoflack *et al.*, 2006; Tesfa *et al.*, 2024).

2.2 Overview of Sperm Cryopreservation

Cryopreservation involves the utilization of extremely low temperatures to preserve living cells while maintaining their structural integrity (Baiee *et al.*, 2020). This technique is applied in both human and animal reproductive medicine to conserve fertility (Prub *et al.*, 2021). In the context of sperm, cryopreservation aims to uphold its viability and functionality at subzero temperatures, notwithstanding potential adverse effects on cell structure (Grotter *et al.*, 2019). It serves as a method for storing the genetic potential of exceptional breeding animals over extended periods, contributes to the conservation of endangered species, and facilitates the transportation and long-term storage of genetic material for subsequent use in artificial reproduction technologies (Sieme *et al.*, 2015; Sieme *et al.*, 2016; Wasilewska and Fraser, 2017).

Cryopreservation encompasses a series of sequential steps including temperature reduction, cellular dehydration, freezing, and thawing. Cooling of spermatozoa represents the fundamental and simplest aspect of the cryopreservation process, effectively lowering the metabolic rate of sperm and extending its survival. Freezing semen at -196°C is regarded as the most optimal and widely employed method for preserving the fertilizing capacity of spermatozoa (Upadhyay *et al.*, 2021).

While cryopreservation extends the storage lifespan of spermatozoa, it is linked with the occurrence of cryo-injury (Bailey *et al.*, 2000). Cryo-injury results from temperature fluctuations, induction of osmotic stress, and the formation of ice crystals, with the latter being a major determinant leading to irreversible damage by disrupting the structure and physiology of the sperm cell (Ahmed *et al.*, 2019).

Throughout the process of cryopreservation, spermatozoa experience osmotic stress resulting from changes in extracellular solute concentration following the introduction of cryoprotective agents, alongside the formation of extracellular ice during freezing and subsequent thawing. Cellular membranes demonstrate variable permeability to both cryoprotectants and water, undergoing phase transitions during both cooling and rewarming phases (Oldenhof *et al.*, 2015).

Additionally, cryopreservation compromises both the functional and structural integrity of bull spermatozoa and is associated with the generation of reactive oxygen species (ROS). Oxidative stress occurring during the freezing of mammalian sperm can lead to functional and structural impairments through ROS-mediated pathways (Baumber *et al.*, 2005). As a result, the incorporation of suitable antioxidants into semen extenders is advised to mitigate oxidative damage during the freeze-thawing process of bull spermatozoa (Ansari *et al.*, 2011). The capacity of spermatozoa to withstand cryopreservation is correlated with their ability to endure osmotic stress (Glazar *et al.*, 2009) and temperature-induced alterations in membrane structure (Oldenhof *et al.*, 2012).

Cryopreserved sperm often exhibits significant variability in survival following thawing, both among species and individuals. These differences may have a genetic basis, leading to variations in inherent cryostability. Additionally, the presence of harmful compounds in an ejaculate can diminish sperm cryostability. To address this, centrifugation processing and clean-up methods can be utilized to eliminate cell debris and harmful compounds from an ejaculate, thereby enriching samples with high-quality sperm (Sieme and Oldenhof, 2015).

Optimal sperm cryo-survival hinges on the individual sperm's ability to adapt to freezing-thawing conditions (Roca *et al.*, 2006; Fraser *et al.*, 2010; Buyukleblebici *et al.*, 2014). Furthermore, factors

such as the type of extenders, cryoprotectant, equilibration process, cooling rate, packaging method, and thawing rate, as well as the characteristics of the individual animals, breed, and species, are anticipated to influence the efficiency of cryopreservation (Upadhyay *et al.*, 2021). Spermatozoa can be cryopreserved using two methods: conventional/slow freezing cryopreservation and vitrification (Baiee *et al.*, 2020).

2.2.1 Conventional Cryopreservation

Conventional/slow freezing is the predominant technique utilized for cryopreserving spermatozoa, involving gradual cooling at rates of 1–2°C/min in steps until reaching -196°C. However, this traditional method has drawbacks primarily stemming from heterogeneous ice nucleation or uncontrolled growth of ice crystals, which disrupt sperm cells. Additionally, inadequate dissipation of latent heat, resulting in repetitive freeze-thaw cycles, can cause mechanical damage to cells (Kumar *et al.*, 2019). Intracellular ice crystal formation disrupts the cytoskeleton, while extracellular ice crystals increase solute concentration and osmolarity, leading to water loss from cells (Holt *et al.*, 2014). Equilibration time is crucial for spermatozoa to acclimate to low temperatures and facilitate the translocation of water, thereby reducing damage caused by ice nucleation during freezing-thawing (Upadhyay *et al.*, 2021).

2.2.2 Vitrification

The principle underlying vitrification involves rapidly immersing sperm into liquid nitrogen (LN₂) at a freezing temperature of -196°C. This process bypasses the formation of intracellular ice crystals, thereby minimizing damage to the cell. Unlike conventional freezing methods, vitrification results in water solidifying into an amorphous glass-like structure rather than forming ice. Achieving extremely high cooling rates (>100,000 °C/min) is essential for vitrification, and this can be accomplished using various specially designed packaging systems such as open pulled straws, Flexipet denuding pipettes, Cryotop, Cryoleaf, Cryotip, and other carrier devices. Alternatively, a direct dropping method involves suspending spermatozoa directly into liquid nitrogen. Rapid cooling rates during vitrification help prevent cell shrinkage and osmotic cell damage (Sharma and Sharma, 2020).

In addition to achieving high-speed freezing, it's crucial to ensure a rapid warming velocity during the thawing process. This facilitates the transition of water inside spermatozoa from a glassy state to a liquid state without ice crystal formation (Tao *et al.*, 2020). According to Sanchez *et al.* (2013), the temperatures reached during the devitrification process are critical for preserving morphological membrane integrity and sperm function. Various post-thaw sperm parameters, including motility, morphology, acrosomal integrity, and HOST (hypoosmotic swelling test), can be evaluated to assess the quality of vitrified spermatozoa (Tao *et al.*, 2020).

The determination of acrosomal integrity of spermatozoa through the single Giemsa's staining technique is a crucial method for assessing the fertilizing capacity of semen. This technique involves the binding of Giemsa stain to the outer acrosomal membrane but not to acrosome-reacted spermatozoa. It is valued for its simplicity, rapidity, and the ability to examine results even after many years (Chowdhury *et al.*, 2014). The hypo-osmotic swelling test (HOST) evaluates the functional integrity of the plasma membrane by assessing its ability to maintain equilibrium between the sperm cell and its environment. This test relies on the principle that under hypo-osmotic stress, there is an influx of fluid causing a normal sperm tail to coil, balloon, or swell. In contrast, a dead spermatozoon exhibits uncontrolled swelling, leading to membrane rupture and tail straightening (Gangwar *et al.*, 2018).

The approach to vitrification is more radical, aiming for the complete elimination of ice formation while mitigating toxic and osmotic changes. Vitrification is characterized by the glass-like solidification of solutions at low temperatures without the formation of ice crystals. This phenomenon is achieved by elevating the concentration of cryoprotectants and/or increasing cooling and warming rates. Additional factors that can aid in vitrification include reducing the volume of solutions and increasing hydrostatic pressure (Orief *et al.*, 2005).

The success of vitrification hinges on two critical parameters: the speed of cooling and warming rates, and the effects of dissolved substances, particularly the concentration of cryoprotectants. However, there are practical and biological limits to consider. Achieving excessively high cooling speeds is limited by practical constraints, while there is a biological threshold for the concentration of cryoprotectant that cells can tolerate during vitrification. Hence, striking a balance between

maximizing cooling rates and minimizing cryoprotectant concentration is crucial for successful vitrification (Dinnyes *et al.*, 2000).

Vitrification offers numerous advantages over conventional cryopreservation methods (Sharma *et al.*, 2015). Firstly, it eliminates the need for costly programmable freezers and significantly reduces processing time, taking approximately 10 minutes compared to 1 hour for slow freezing (Vutyavanich *et al.*, 2010). Moreover, since vitrification is performed after swim-up, it excludes seminal plasma, which may harbor pathogens (Isachenko *et al.*, 2012). Additionally, vitrification eliminates the need for any post-thaw processing. In cases where repeated cryopreservation is necessary on the same sample, vitrification has been shown to yield better post-thaw sperm parameters compared to standard slow programmable freezing methods (Vutyavanich *et al.*, 2012).

Indeed, the cryopreservation of sperm via vitrification presents a promising alternative to conventional freezing methods. By bypassing ice formation, requiring minimal equipment, and offering time efficiency and cost-effectiveness, vitrification emerges as an attractive option (He *et al.*, 2008).

2.3 Cryoprotectants and Extenders

Cryoprotectants are highly soluble substances utilized to shield biological tissue from cold shock during freezing and thawing processes. They function by altering the solute concentration in the liquid phase, displacing water from the freezing point of the solution. This mitigates water crystallization and consequently reduces cellular damage post-thawing or warming (Sharma and Sharma, 2020). Depending on their ability to traverse the cell membrane, cryoprotective agents (CPAs) can be categorized into two types: permeating/penetrating and non-permeating (Isachenko *et al.*, 2011).

Nonpenetrating and penetrating cryoprotectants serve to shield sperm cells from the physical and chemical stress induced by ice crystallization. Nonpenetrating cryoprotectants, like polymers, aid in vitrification processes, while penetrating cryoprotectants, such as sugars, assist in reducing toxicity. Although cryoprotective agents cannot prevent changes in membrane phase, they can

attenuate the rate of dehydration during freezing, thereby reducing the formation of ice crystals within the cell (Ugur *et al.*, 2019).

Penetrating or permeable cryoprotectants, including glycerol, dimethyl sulfoxide, 1–2 propanediol, ethylene glycol, propylene glycol, and methanol, are among the most popular choices. These compounds are characterized by their low molecular weight and efficiently dehydrate the cell at slow cooling speeds. On the other hand, non-penetrating cryoprotectants, such as polyvinylpyrrolidone, glucose, fructose, ficol, dextran, albumin, sorbitol, sucrose, lactose, trehalose, raffinose, and egg yolk citrate, are high molecular weight substances that are more effective when rapid cooling is employed. These non-penetrating cryoprotectants extract intracellular water using osmotic pressure differences without penetrating the cell membrane (Grotter *et al.*, 2019; Li *et al.*, 2019).

Typically, freezing media contain a combination of both types of cryoprotective agents (Benson *et al.*, 2012). Glycerol is widely used for bull sperm cryopreservation due to its ability to reduce mechanical damage to spermatozoa during the freezing process (Buyukleblebici *et al.*, 2014). Cryoprotectants are added to extenders to safeguard sperm from damage during the freezing process (Singh *et al.*, 1995). The type and quantity of cryoprotectants in semen diluent significantly influence these processes and their effects on sperm cells during freezing (ElHarairy *et al.*, 2011).

The cold shock experienced during freezing and thawing processes can significantly diminish sperm quality. The severity of injuries resulting from cold shock varies depending on the composition of extenders, the cryoprotectants employed, and the species involved (Partyka *et al.*, 2012). Various extenders, including egg yolk, have been formulated to mitigate the effects of cold shock by maximizing cooling tolerance and enhancing fertilizing ability following semen cryopreservation, thereby improving post-thaw quality (Ugur *et al.*, 2019).

Extenders play a crucial role in safeguarding sperm from cold shock, osmotic stress, and alterations in membrane fluidity and permeability, while also providing energy substrates for sperm metabolism (Batellier *et al.*, 2001; Medeiros *et al.*, 2002). A wide array of extenders have been successfully employed for bull semen cryopreservation, including citrate-sugar-based, lactose-

based, saccharose-milk-based, egg yolk-based, and various plant-based extenders. Among these, variants of Tris-egg yolk-based and milk-based extenders have emerged as universal choices for bull semen cryopreservation (Medeiros *et al.*, 2002; Barbas and Mascarenhas, 2009; Singh *et al.*, 2012).

Extenders containing 20% egg yolk are commonly utilized for cryopreserving livestock sperm from species such as cattle, buffalo, and pigs (Bathgate *et al.*, 2006). While egg yolk is recognized for its ability to prevent cell damage during cryopreservation, it contains substances within its granules, including high-density lipoproteins (HDL) and minerals, which can inhibit sperm cell respiration and reduce motility (Moussa *et al.*, 2002). However, the low-density lipoproteins (LDL) present in egg yolk serve a protective role by covering the sperm membrane during freezing and thawing processes, thus shielding sperm from damage (Amirat *et al.*, 2004).

Tris-based extenders are commonly employed for semen cryopreservation in various species, including bulls, rams, and bucks. Among these extenders, Tris with an osmolality of 375 mOsm/kg containing 2% egg yolk has been identified as particularly effective in preserving acrosomal integrity and motility after thawing. Semen frozen in Triladyl, a Tris-based diluent, has demonstrated reasonable fertility rates after Trans cervical insemination. Triladyl has shown superior in vitro results compared to extenders containing lactose-yolk and saccharose-lactose-yolk, and the addition of 2% bovine serum albumin has further enhanced its protective effect on acrosome integrity (Barbas and Mascarenhas, 2008).

2.3.1 Role of extenders in reducing cryoinjury

The function of extenders in semen preservation encompasses two primary objectives: firstly, to prolong the fertility lifespan of sperm, which can potentially extend indefinitely in the case of frozen semen; and secondly, to notably amplify the genetic contribution of individual males through artificial insemination (AI) (Layek *et al.*, 2016). To fulfill these roles proficiently, an extender must initially satisfy the basic physiological requisites of sperm. These include being iso-osmotic, possessing a near-neutral pH with an adequate buffer, and offering metabolizable substrates (Salamon and Maxwell, 2000).

For the storage of semen at low temperatures, the incorporation of cryoprotective agents is indispensable to safeguard sperm from cold shock during the gradual cooling process towards approximately 0°C, as well as from the detrimental effects of solutes and the formation of intracellular ice during freezing. Cold shock damage is believed to stem from the thermotropic phase transition of sperm membrane phospholipids, which induces a shift from a fluid to a more rigid state. This change in membrane fluidity can influence the microenvironment of various transport proteins and enzymes (Collin *et al.*, 2000). On the other hand, the formation of intracellular ice involves both thermotropic and lyotropic (dehydration-induced) phase transitions and poses an even greater threat to sperm integrity (Layek *et al.*, 2016).

The typical extenders used for freezing sperm include various components: A non-permeating cryoprotectant, which can be derived from milk or milk products, egg yolk, or yolk derivatives like low-density lipoproteins or phospholipids. A permeating cryoprotectant, most commonly glycerol. An organic buffer, typically Tris(hydroxymethyl)aminomethane (Tris). One or more sugars acting as energy substrates or osmotic regulators, such as glucose, fructose, lactose, raffinose, saccharose, or trehalose. Solute used to adjust pH and osmolarity, like sodium citrate and citric acid. Antibiotics, such as penicillin and streptomycin, to prevent bacterial contamination (Layek *et al.*, 2016).

Egg yolk-based extenders containing glycerol were among the first formulations to achieve sufficient post-thaw motility and fertility, paving the way for significant commercial success. These extenders have since become widely adopted as standard choices for cryopreserving bovine semen. However, despite the essential role of glycerol in successful sperm cryopreservation, it can also have adverse effects on sperm. These effects include inducing osmotic stress, causing alterations in membrane organization, and impacting membrane fluidity and permeability, all of which can compromise sperm function. Consequently, determining the optimal concentration of glycerol and equilibration time has been crucial in extender development to maximize its benefits while mitigating these potential side effects (Collin *et al.*, 2000).

3. MATERIALS AND METHODS

3.1 Study area

The study was conducted from December 2023 and April 2024 in Bishoftu town, situated in central Ethiopia within the East Shewa Zone of the Oromia National Regional State, approximately 44 km from the capital, Addis Ababa. According to the Bishoftu town administration report, the city comprises nine urban Kebeles and five rural Kebeles, with an estimated population of 171,227. Bishoftu town's coordinates are approximately 8.734650 latitude and 39.008533 longitude, with an elevation of 1920 meters above sea level. These coordinates correspond to 8° 44' 4.74" North and 39° 0' 30.726" East on the Global Positioning System (GPS) (Girmay *et al.*, 2022).

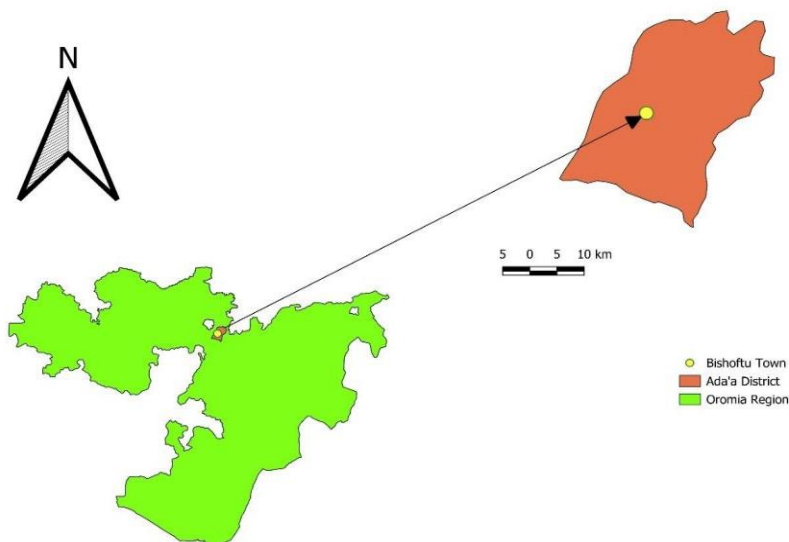


Figure 1: Geographical location of study area (Bishoftu town); *Source:* Supported by QGIS

3.2 Study animals

Bulls presented to the abattoirs and slaughtered by fulfilling all the ante-mortem inspection criteria and certain BSE records were used for sample collection. Ante mortem inspection was carried out

on each animal at rest and in motion. Records of the origin of the bulls, breed and age were recorded. The BSE during the ante-mortem inspection included evaluation of general health, scoring the bull's body condition, gait, physical activity, swelling in body parts, presence of ectoparasitism and any externally visible abnormalities. The bulls were considered as fit for slaughter after they meet the ante mortem inspection criteria.

3.3 Study Design and Sampling Method

Experimental study design was used with extender and preservation method as independent variables (extender types grouped by glycerol content, two preservation methods viz cooling at +4 degrees and vitrification). The study was conducted through collection of epididymal spermatozoa (gamete rescue) followed by dilution with specific extender and trial with preservation methods (vitrification, cooling) and post-thaw evaluation of spermatozoa.

3.4 Sample Collection

Forty four (44) (32 for cooling and 12 for vitrification) bulls presented to the abattoir were selected for sample collection and samples of fresh testes was collected from these bulls. Each testis from the individual animal was tagged and identified by code for individual animal. Then the collected testes were put in plastic bag in a chilled ice box and within 45 minutes transported to the laboratory in order to perform spermatozoa retrieval. Retrieval of spermatozoa was carried out in the laboratory by dissection of the testes and separation of the epididymis followed by spermatozoa rescue from the tail of the epididymis. Then the spermatozoa was diluted with previously prepared base media (TSC) and preliminary evaluation of motility was carried out with microscope at 10x of magnification power. Only samples with mass motility >70% were allowed to pass for the next experiment.

3.5 Preparation of Extender Media

Tris based extenders was used for the experiment. Base media containing Tris base (2.42g), citric acid (1.48g) and fructose (1.0g) dissolved in 100mL distilled water was prepared and kept at +4°C till it was used for sperm dilution. The TCS base media was portioned into the following treatments as presented in the table below (Table 2).

Table 2: Sperm cryopreservation methods with respective treatment conditions

Treatments	Method of cryopreservation
1. TCS (base media) + 20% egg yolk	Cooling at +4 °C for 30', 60' and 180'
2. TCS + 20% egg yolk + 2.5% glycerol	Direct Vitrification at -196°C
3. TCS + 20% egg yolk + 2.5% glycerol	Straw vitrification -196°C
4. TCS + 20% egg yolk + 12.8% glycerol	Direct vitrification -196°C
5. TCS + 20% egg yolk + 12.8% glycerol	Straw vitrification -196°C

3.6 Evaluation of Cooling Tolerance

The rescued spermatozoa were diluted in the base extender media (TCS) with specific amount of egg yolk were put in cryovial and went into cooling (refrigerated at +4°C). Then the portions were withdrawn from the refrigerator after 30minutes, 1hr and 3hr and thawed for 30 seconds at 37degrees for evaluation. Then the cooling tolerance was evaluated by examining the motility under microscope at 10x magnification power.

3.7 Sperm Analysis

3.7.1 Sperm Motility

The sperm motility parameter was analyzed by manual assessment method under phase-contrast microscopy. Such procedure was carried out according to the description by Le *et al.* (2019). Gross motility was observed microscopically under low power objective (10×), whereas individual

progressive motility was observed under 400× by observing and estimating the proportion of sperm moving. This was presented as percent motile. It was done by placing a 2 to 3 drop of sperm on a clean, warm microscope slide and covering with prewarmed cover slip and examined under microscope.

3.7.2 Sperm Morphology

The assessment of sperm morphology was carried out following the standard procedures described by Le *et al.* (2019). It involved putting equal volume of a well-mixed sample, 1% eosin stain and 2.9% sodium citrate on a glass slide followed by mixing eosin stain and sodium citrate at first and then mixing the sperm sample followed by smear preparation and final microscopic examination. Morphology of sperm head shape and size, sperm neck, midpiece, and tail were determined under microscopy at 40x magnification.

3.7.3 Sperm Viability

Sperm viability was determined based on the membrane integrity of the cells and can differentiate live from dead immotile sperm. The vitality parameter was assessed by the Eosin staining. This method was based on the principle that damaged plasma membrane, as seen in dead spermatozoa, allow the entry of membrane-impermeant stain, while the vital sperms exclude the dye and remain unstained. A drop of well mixed sample was mixed with an equal volume of Eosin stain and smeared on a glass slide and allowed to dry for few minutes. Two hundred cells were examined with bright optics at 40x magnification. Values were presented as live percent.

3.8 Sperm Vitrification

The principle behind the process is based on freezing of sperm by direct immersing it into liquid nitrogen which is at a freezing temperature of -196°C . This bypass the formation of intracellular ice crystals and thus the ensuing damage to the cell. In vitrification, water solidifies as an amorphous glass-like structure, and not as ice.

Sperm samples were diluted volume by volume in a small cryovial and filled in to a 100 μ L micropipette tip. Styrofoam box was prepared and filled with liquid nitrogen for vitrification and then 30 - 50 μ l of sperm were dropped via pipette at 11 cm above the level of the Styrofoam box directly into a liquid nitrogen. Then the frozen/vitrified droplets were collected using forceps and transferred in to cryovials. Finally, the cryovials with vitrified sperm pellets were stored with in liquid nitrogen for at least 24 hours. A second group of samples was prepared by filling the sperm into 0.25ml straw which is then stored into the liquid nitrogen directly (Figure 2). Each sample was withdrawn from the liquid nitrogen and thawing of vitrified balls/pellets of spermatozoa were carried out by adding 0.5ml of pe-warmed media in to cryovial. Subsequently, all pellets were carefully dispensed one by one, gently agitated, and then placed in a pre-warmed incubator at 37 degrees Celsius for 5 minutes and evaluatd for motility, AI and subjected to HOST. After being taken out of liquid nitrogen, the straws are promptly placed into prewarmed water bath set at 37 degrees Celsius for 30 seconds. They are then transferred into pre-prepared cryovials and evaluation of sperm parametrs were performed in similar manner to the droplets as outlined by Le *et al.* (2019) with brief modification.

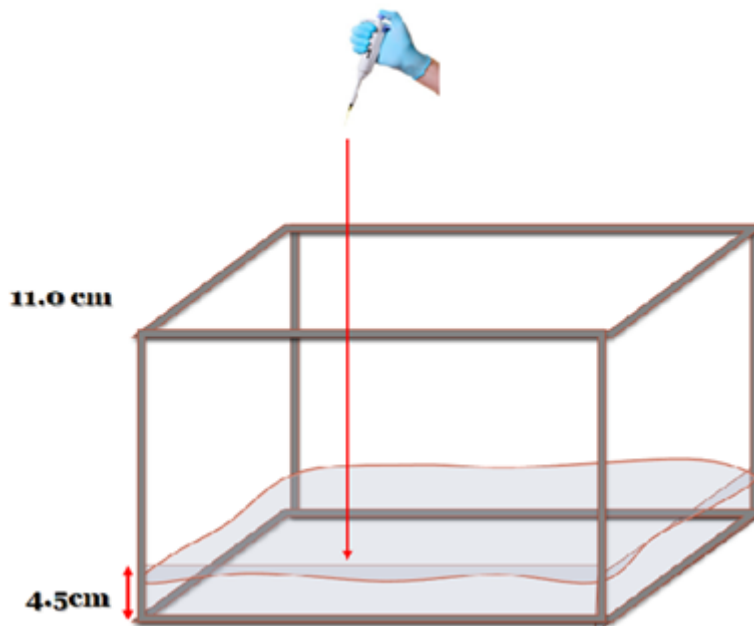


Figure 2: Process of Vitrification; *Source* (Le *et al.*, 2019)

3.8.1 Post-thaw Evaluation of Sperm

Assessment of Acrosomal Integrity Using Giemsa Staining Method

The AI (acrosomal integrity) of spermatozoa was analyzed using Giemsa staining method. The staining process was carried out by dripping sperm on an object glass. Smear preparation was made and the preparation was fixed using methanol for 10 minutes. It was stained using Giemsa stain for 1 hour and 30 minutes by immersing it in a staining jar. It was rinsed in running water and dried again. Two hundred cells were examined under a light microscope at 400x magnification. Acrosome-intact spermatozoa were characterized by purple head, while those with damaged acrosome were characterized by pale lavender head. Acrosomal status was established by counting the number of acrosome-intact spermatozoa divided by the total number of spermatozoa and multiplied by 100%, following the standard procedure described by Prihantoko *et al.* (2020).

Hypo-Osmotic Swelling Test (HOST)

The membrane integrity of spermatozoa was analyzed using hypo osmotic swelling test. The HOST solution was prepared, as recommended by Baskaran *et al.* (2020) and performed by mixing sodium citrate dihydrate (0.735 g) and d-fructose (1.0g) in 100 ml of distilled water. 1ml of hypoosmotic solution is incubated for 10 minutes and 0.1ml of the sample was mixed with the hypo-osmotic solution and incubated for 1 hour at 37°C. Following incubation, a wet preparation was subjected to examination under phase contrast optics at ×400 magnification. HOST reactivity was determined when sperm cells had either a swollen head or a coiled tail section. A total of 200 spermatozoa were evaluated in each replicate and the results was expressed as a percentage of HOST reactive sperm cells.

3.9 Data Analysis

The data collected from the laboratory investigations were recorded and coded using Microsoft Excel spread sheet software, provided by Microsoft Corporation. The SPSS software version 13 was utilized to analyse the data. Descriptive analysis was used to summarize the data, to

characterize different body and testicular parameters and to identify characteristics of epididymal spermatozoa during cooling and vitrification. The Pearson chi-square test is used to determine the significant association between prefreeze motility with scrotal circumference, total testicular weight and total epididymal weight. The One Way ANOVA was used to determine the significant difference between individual and mass motility, Prefreeze and post freeze and to determine the significant difference in sperm parameters between the vitrification methods. Level of significance was held at $P < 0.05$.

3.10 Ethical Clearance

Ethical clearance for the study was approved by and obtained from Addis Ababa University College of Veterinary Medicine and Agriculture animal research ethical review committee with an approved certificate reference number of VM/ERC/02/35/16/2024.

4. RESULT

4.1. Ante-Mortem and Physical Description of Study Animals

The bulls underwent antemortem examination while also testicular samples were collected after slaughter. The summary of description of record of antemortem findings and testicular measurements are presented in Table 3.

Table 3: Body parameters of sampled bulls recorded during antemortem and after slaughter

Parameter	N	Mean \pm SD	Minimum	Maximum
Age (Years)	32	6.06 \pm 1.1	5	9
Body weight (Kg)	32	294.03 \pm 29.9	250	380
Body condition score (on 1-9 scale)	32	5.31 \pm 0.8	4	7
Scrotal circumference (cm)	32	32.06 \pm 1.2	30	35
Total testicular weight (g)	32	301.80 \pm 37.4	236.4	397
Testicular volume (cm ³)	32	334.86 \pm 105.1	192	600
Left testis weight (g)	32	148.83 \pm 18.4	116.5	190
Left testis length (cm)	32	9.7 \pm 1.0	8	12
Left testis width (cm)	32	4.94 \pm 0.8	4	7
Left testis thickness (cm)	32	3.38 \pm 0.4	3	4
Right testis weight (g)	32	152.97 \pm 19.3	119.9	207
Right testis length (cm)	32	9.94 \pm 2.0	8	20
Right testis width (cm)	32	4.98 \pm 0.8	4	7
Right testis thickness (cm)	32	3.36 \pm 0.4	3	4
Total epididymal weight (g)	32	38.33 \pm 4.8	24.8	49.5
Left epididymis weight (g)	32	19.67 \pm 2.0	14.8	22.3
Right epididymal weight (g)	32	18.66 \pm 3.3	6.8	29.5

All animals are at slaughter age hence basically also at breeding age. The antemortem examination was essentially a Breeding soundness evaluation. There were no significant variation in body and

testicular parameters among the bulls, hence no apparent influence on variability of spermatozoa parameters among the study bulls. However, in all cases, larger scrotal circumference was significantly associated with higher prefreeze motility (OR=11.76; $p<0.05$) indicating the odds of bulls with higher SC is very high to also have a sperm that high prefreeze motility which is a sign of good quality semen. In a more similar manner, larger testicular and epididymal weights were also associated with higher sperm motility (OR= 62.93; $P<0.05$). Prefreeze motility are correlated with total testicular weight ($r=0.31$).

4.2 Assessment of Cooling Tolerance of Epididymal Spermatozoa

The average prefreeze mass motility and individual motility were 76.88% and 73.13%, respectively. There was a significant difference ($P<0.05$) in morphological abnormality by region of the sperm, with the highest affected region being the midpiece followed by the head region and tail of the sperm (Table 4).

Table 4: Summary of characteristics of epididymal spermatozoa

Parameter	N	Mean \pm SD	Minimum	Maximum
Prefreeze mass motility (%)	32	76.88 \pm 6.9	70	90
Prefreeze individual motility (%)	32	73.13 \pm 4.7	70	80
Morphology (%)	32	71.06 \pm 10.5	25	84
Head abnormality (%)	32	0.25 \pm 0.4	0	1
Midpiece abnormality (%)	32	0.34 \pm 0.5	0	1
Tail abnormality (%)	32	0.16 \pm 0.4	0	1
Live percent (%)	32	69.94 \pm 8.0	52.5	86.5

Table 5 shows that the cooling tolerance of epididymal spermatozoa at different cooling times. individual and mass motility were not significantly affected by the cooling temperature during the first 30 minutes of cooling time having 70% average motility status. But as the time increased to 60 minutes and 180 minutes, there were changes both in mass and individual motility (Table5).

Table5: Cooling tolerance at different times of cooling epididymal spermatozoa

Parameter	N	Mean \pm SD	Minimum	Maximum
Individual motility at 30 minutes (%)	32	70 \pm 7.2	50	90
Mass motility at 30 minutes (%)	32	70 \pm 7.2	50	90
Individual motility at 60 minutes (%)	32	64.38 \pm 9.1	50	70
Mass motility at 60 minutes (%)	32	56.87 \pm 8.9	50	70
Individual motility at 180 minutes (%)	32	53.13 \pm 9.0	30	70
Mass motility at 180 minutes (%)	32	42.5 \pm 11.9	30	70

There was a highly significant difference ($P < 0.001$) both in individual and mass motility of spermatozoa before and after cooling, and also during the subsequent period of chilling process (Figure 3 and 4).

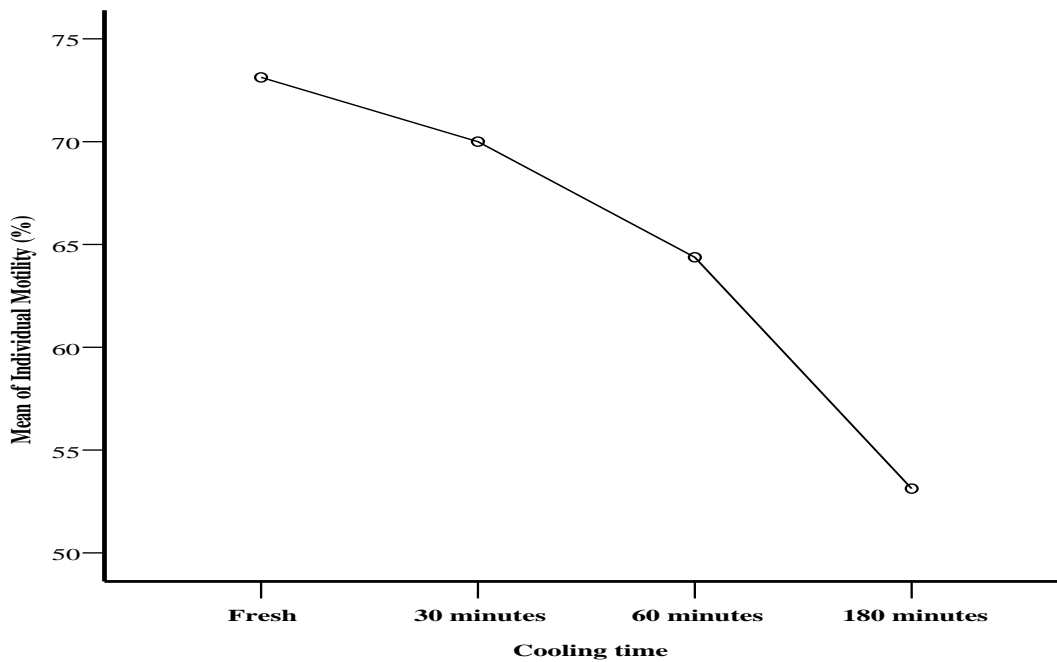


Figure 3: Mean plot of mass motility of bull epididymal spermatozoa over cooling time

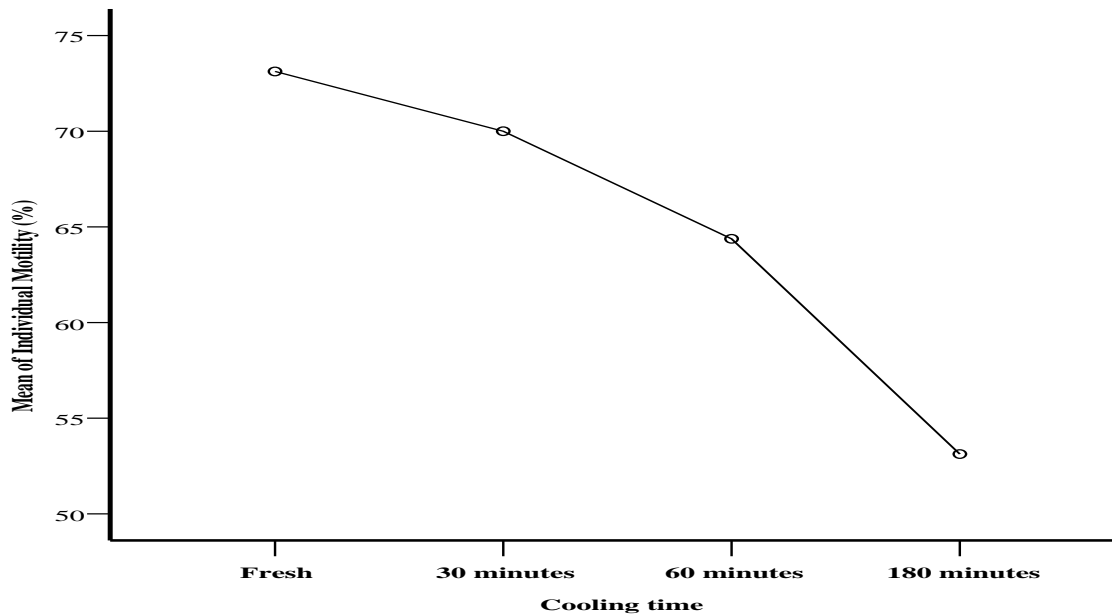


Figure 4. Mean plot of individual motility of bull epididymal spermatozoa over cooling time

The drop in Mass motility after cooling ranged from 6% to a maximum of 23% indicating the presence of high cooling tolerance during the chilling process. The drop in individual motility after cooling ranged from just 3% to a maximum of 23% indicating the presence of high cooling tolerance during the chilling process. The largest drop in motility occurred after the first hour of chilling signifying that the sperm motility is impacted as the time increased.

4.3 Assessment of Post-Thaw Quality of Direct and Straw Vitrified Bull Spermatozoa

The prefreeze mass and individual motility of spermatozoa destined for vitrification found to be significant having average motility of 76.7% and 72.7% respectively. There is a significant difference in morphological abnormality by region of the sperm, with the highest affected region being the head followed by the tail of the sperm and no abnormality detected in the midpiece region (Table 6).

Table 6: Characteristics of epididymal spermatozoa destined for vitrification

Parameters	N	Mean	SD	Min	Max
Prefreeze mass motility	12	76.7	9.8	70	90
Prefreeze individual motility	12	72.5	4.5	70	80
Morphological abnormality	12	70.6	8.7	58	82.5
Head abnormality	12	0.4	0.5	0	1
Midpiece abnormality	12	0	0	0	0
Tail abnormality	12	0.1	0.3	0	1
Live percent	12	71.7	9.3	55.5	86.5

There was a significant difference ($P < 0.05$) in individual motility between high (12.8%) and low (2.5%) concentration of glycerol with individual motility being higher with higher glycerol concentration in vitrified spermatozoa irrespective of the vitrification method. The rest of sperm parameters were not influenced by glycerol concentration (Table 7).

Table 7: Post thaw characteristics of bull epididymal spermatozoa vitrified in straw at different concentration of glycerol

Descriptives	Method of vitrifications	Glycerol concentration	N	Mean± SD	Min	Max
Mass motility	Straw	12.8%	12	18.8±5.7	10	30
		2.5%	12	14.6±5.0	10	20
Individual motility	Straw	12.8%	12	14.2±5.1	10	20
		2.5%	12	7.5±2.6	5	10
HOST reactivity	Straw	12.8%	12	30.3±4.0	25	38
		2.5%	12	27.4±5.3	20	40
AI in straw vitrified sperm	Straw	12.8%	12	30.4±4.9	22.5	38
		2.5%	12	29.6±3.4	25	35

There was a highly significant difference in sperm parameters between the vitrification methods. All parameters were higher in spermatozoa vitrified using a straw at 12.8% concentration of glycerol (Table 8).

Table 8: Post-thaw characteristics of bull epididymal spermatozoa using different vitrification methods with high concentration (12.8%) of glycerol

Parameters	Method of vitrification	N	Mean±SD	Min	Max
Mass motility	Straw	12	27.1±8.6	20	50
	Direct	12	14.6±5	10	20
Individual motility	Straw	12	22.9±6.2	10	30
	Direct	12	7.5±2.6	5	10
HOST reactivity	Straw	12	38.5±4.7	31	46
	Direct	12	27.4±5.3	20	40
AI	Straw	12	45.2±6.5	35	55
	Direct	12	29.6±3.4	25	35

There was a highly significant difference in sperm parameters between the vitrification methods. All parameters were higher in straw vitrified spermatozoa (Table 9).

Table 9: Post-thaw characteristics of bull epididymal spermatozoa using different vitrification methods with low concentration (2.5%) of glycerol

Parameters	Method of vitrification	N	Mean±SD	Min	Max
Mass motility	Straw	12	14.6±5.0	10	20
	Direct	12	7±2.5	5	10
Individual motility	Straw	12	7.5±2.6	5	10
	Direct	12	6.2±2.2	5	10
HOST reactivity	Straw	12	27.4±5.3	20	40
	Direct	12	14.7±4.5	10	25
AI	Straw	12	29.6±3.4	25	35
	Direct	12	17.2±6.6	10	31

5. DISCUSSIONS

The morphometric measurements observed in this investigation generally fall within the typical range for zebu cattle breeds. Notably, a significant distinction in scrotal circumference was noted among different weight categories, with heavier animals exhibiting a larger scrotal circumference compared to medium-sized ones, a finding consistent with prior research conducted at NAIC (Demeke, 2010). Moreover, a strong positive correlation between body weight and scrotal circumference was established, in line with findings from previous studies (Kealey, 2004; Ajitkumar *et al.*, 2011). Previous studies have also highlighted breed variations in various semen parameters. As such, certain investigations (Nasrin *et al.*, 2008) continue to advocate for Holstein Friesian breeds, suggesting superior outcomes compared to other breeds.

All samples preserved in TCS extender supplemented with egg yolk generally exhibited superior pre-freeze and post-thaw sperm parameters. The post-thaw sperm characteristics of chilled epididymal spermatozoa were notably robust, aligning with reports suggesting that epididymal sperm possess enhanced freezability and cryo-resistance irrespective of the freezing method employed (Martínez-Fresneda *et al.*, 2019). These findings are largely consistent with previous studies (Christensen *et al.*, 1999; Bearden *et al.*, 2004; Nazari *et al.*, 2020), albeit with slight discrepancies noted in the study by Bertol *et al.* (2013), which documented a decline in motility to less than 30% after 15 hours at 5 degrees Celsius. Similar to the current study, both mass and individual motility exhibited a significant decrease after 3 hours of cooling. A study by Aitor *et al.* (2021) similarly observed lower motility at times less than 2 hours, with a sharper decline over time when using a soy lecithin-based extender. Kastelic (2014) reported a reduction in total and progressive motility over time and temperature during short-term storage of Holstein bull semen. These studies employed slightly different approaches in extender media treatment, potentially contributing to variations in their findings, which could be attributed to membrane disruption resulting from structural protein disorganization, cold shock formation, disturbance of ion channels, reactive oxygen species (ROS) production, and loss of mitochondrial membrane potential associated with temperature shock (Ahmad *et al.*, 2015; Ugur *et al.*, 2019). The composition of epididymal sperm undergoes remodeling during ejaculation, leading to differences in the proteome between ejaculated and epididymal sperm, reflecting variations in freezability and

cryosurvival. While spermatozoa become more susceptible to oxidative damage during maturation in the epididymis, the epididymis provides crucial antioxidant protection, mitigating this susceptibility (Angrimani *et al.*, 2014).

The addition of antioxidants to the extender, aimed at preventing intracellular ice formation, was primarily intended to enhance sperm cryosurvival (Pipan *et al.*, 2020). Tris-based extenders supplemented with egg yolk have been extensively utilized to mitigate the effects of cold shock. The improved cooling tolerance observed in this study is likely attributed to the specific extender utilized. The cooling phase is essential to mitigate the impact of temperature fluctuations and to facilitate sperm equilibration with the diluent prior to freezing (Pereira *et al.*, 2010). Nonetheless, cooling represents a highly stressful process, leading to irreversible damage to the sperm membrane, resulting in either cell death or premature capacitation-like alterations (Garner *et al.*, 2001).

Extenders play a crucial role in safeguarding sperm from cold shock, osmotic stress, and alterations in membrane fluidity and permeability, while also providing essential energy substrates for sperm metabolism (Layek *et al.*, 2016; Aitor *et al.*, 2021). Tris egg-yolk extenders have gained prominence as universal extenders for bull semen cryopreservation (Singh *et al.*, 2012). However, over time, the quality of the extender may deteriorate due to the presence of lipids and other components vital for preserving sperm viability during storage and transportation. These components can undergo degradation, particularly when exposed to oxygen and fluctuations in temperature (Stradaioli *et al.*, 2007).

The present study uncovered a strong correlation between pre-freeze mass motility and key body parameters, including scrotal circumference, testicular weight, and epididymal weight. This suggests that bulls with larger testicular and epididymal weights and scrotal circumferences are more likely to have highly motile sperm. Importantly, this association was found to be statistically significant (p -value < 0.05), corroborating similar findings reported in studies by Kastelic *et al.* (2001), Sundararaman *et al.* (2002), and Latif *et al.* (2009).

The mean live percentage observed in our study aligns with the recommendations for a normal fertile bull as suggested by Dhimi *et al.* (1998). However, it's noteworthy that Dhimi *et al.* (1998) reported even higher values, with live sperm percentages reaching as high as 87.35% in Friesian bulls. Following cryopreservation, sperm viability undergoes a dramatic reduction due to various factors inherent to the process (Lemma, 2011). It's widely recognized that cryopreservation typically results in a 50% to 60% decrease in sperm viability (Gonzalez, 2004; Chaveiro *et al.*, 2006). As per Hafez (1993), the total abnormal sperm defect in normal fertile bull spermatozoa generally does not exceed 20%, a finding consistent with our present results.

In this study, vitrification of bull sperm generally yielded notably low outcomes in sperm parameters, although discrepancies were noted among the vitrification techniques employed. The primary challenge in sperm cryopreservation using liquid nitrogen lies in the cellular damage induced by intracellular ice formation and osmotic stress during freezing and thawing. Differences in post-thaw outcomes between conventional and vitrification methods stem from variations in freezing rates and the type and composition of cryoprotectants utilized (Ozkavukcu *et al.*, 2008; Li *et al.*, 2019). Vitrification, entailing direct immersion of sperm samples into liquid nitrogen, represents a rapid, straightforward, and cost-effective approach to sperm cryopreservation. This method of rapid freezing is presumed to mitigate damage caused by intracellular ice formation during freezing (Tao *et al.*, 2020), thus offering potential advantages over slow freezing by facilitating ease of application while minimizing cell injury (Pipan *et al.*, 2020).

The finding of a significant difference in sperm motility between high (12.8%) and low (2.5%) concentrations of glycerol, with individual motility being higher at higher glycerol concentrations, contradicts previous reports and remains subject to debate. Awad and Graham (2002) asserted that the motility of bull spermatozoa was not adversely affected by reducing glycerol concentration, and no significant differences in the percentages of motile cells were observed within a range of 2–6% glycerol. While glycerol is recognized as a permeating cryoprotectant that prevents intracellular ice formation and is commonly used at concentrations of 4-8%, it has been documented to exert toxic effects on spermatozoa, resulting in plasma membrane rupture, removal of crucial membrane proteins, and acrosomal damage, often leading to reduced fertility (Gao and Critser, 2000; Isachenko *et al.*, 2003; Awad, 2011; Gharajelar *et al.*, 2016). Glycerol

concentrations ranging from 1 to 4% have been reported to be beneficial for preserving the fertility of stallion sperm during freezing (Hoffermann *et al.*, 2011). According to Hoffermann *et al.* (2011), stallion sperm of good freezing quality can tolerate glycerol concentrations up to 3% before a decline in viability is observed. Conversely, higher post-thaw motility has been observed in epididymal sperm preserved with high glycerol concentrations, potentially indicating species-specific differences in sperm cryotolerance (Lemma, 2011).

Using higher glycerol concentrations for cryopreservation offers the advantage of preserving the structural and functional integrity of cells, thereby resulting in higher recovery rates and improved overall performance in downstream applications (Kundu *et al.*, 2000; Papa *et al.*, 2015).

In contrast to current finding, utilizing vitrification with droplets or straws did not yield significant differences in post-thaw sperm parameters (Abed-Elmdoust *et al.*, 2019). However, Díaz-Jiménez *et al.* (2018) observed in the vitrification of donkey sperm that straws exhibited greater efficiency compared to pearls. Conversely, Abed-Elmdoust *et al.* (2019) investigated Persian sturgeon (*Acipenser persicus*) fish and noted that the motility percentage of spermatozoa in both droplet-vitrified and straw-cryopreserved treatments was significantly lower than in the control group (fresh spermatozoa), with no discernible difference between the two cryopreservation techniques. Additionally, the motility duration of spermatozoa differed significantly between treatments and was lower than in the control group. While neither cryopreservation technique demonstrated superiority in terms of motility percentage, Abed-Elmdoust *et al.* (2019) concluded that the droplet vitrification technique was more effective in preserving the motility duration index of spermatozoa compared to straw cryopreservation.

In another study, Albal *et al.* (2023) observed that vitrification using mini-straws resulted in the lowest number of viable sperm post-thaw compared to vitrification using pearls. The selection of a vitrification method holds significant importance during the thawing process post-vitrification. One of the primary challenges lies in thawing droplet-vitrified pellets at 37 degrees Celsius, necessitating a prior warm-up to avoid the complete loss of motility. Conversely, thawing straw-vitrified samples is relatively easier, as the sperm is protected within the straw in a manner similar to conventionally preserved semen.

6. CONCLUSION AND RECOMMENDATIONS

Studies have shown the efficacy and potential of epididymal spermatozoa to result in fertilization *in vitro* and *in vivo* by using these spermatozoa in artificial insemination. Since sperm cells are temperature sensitive being affected by the cooling process both during temporary storage of during equilibration, it is of paramount importance to match the best extender that creates a better environment for the sperm to preserve the fertilization potential. In this regard, the finding in the present study is a promising option particularly in the attempt to improve semen quality produced at the national level. Cryopreservation by vitrification also provides the easiest and cost effective method of spermatozoa preservation and selection of potential breeding bulls at field level if appropriate extender and cryoprotectant supplementation is provided. Especially, during selection of preservice bulls at field level, as commonly practiced in Ethiopia, breeding soundness evaluation is not practical. Hence, snap freezing provides a viable option in semen quality evaluation as part of the breeding soundness test. The use of Tris-citrate-sucrose based extender showed promising results in maintaining sperm quality both after chilling and at post-thaw. A higher preefreeze motility was consequential in post cooling or freezing sperm parameters indicating the potential for improved fertility outcomes. Even though vitrification has great deal of advantage in the cryopreservation of spermatozoa, adequate care and considerations needs to be taken in the selection of cryoprotective agent in order to maximize post-thaw quality of vitrified spermatozoa. As it is shown in this study, even higher glycerol level has positive implication on the cryosurvival of vitrified spermatozoa. The study's findings underscore the importance of selecting appropriate extender media and cryopreservation methods to maintain sperm quality during the freezing and thawing processes. By evaluating parameters such as cooling rates, cryoprotectant concentrations, and post-thaw sperm characteristics, the research contributes to enhancing field evaluation of preservice bulls and the efficiency of semen preservation techniques in bovine reproductive management.

Based on the above conclusion the following recommendations are forwarded:

- Further research is essential to optimize cooling and thawing rates, as well as cryoprotectant supplementation to enhance the efficiency of bovine semen cryopreservation.
- Although sperm parameters were relatively better at higher concentration of glycerol, further experimentation has to be done on fertilizing ability of such sperm after vitrification.
- Comparative studies should be conducted to evaluate different extender formulations for preserving sperm quality post-thaw, with a focus on practical applications in semen evaluation and genetic selection of superior breeding bulls.
- Collaboration with stakeholders in the veterinary field is essential to disseminate research findings and promote the adoption of optimized sperm cryopreservation techniques for improved reproductive success in bovine species.

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8. ANNEXES

Annex 1: Field Evaluation of Some Body Parameters



Evaluation of testicle , penis and scrotal circumference in the field

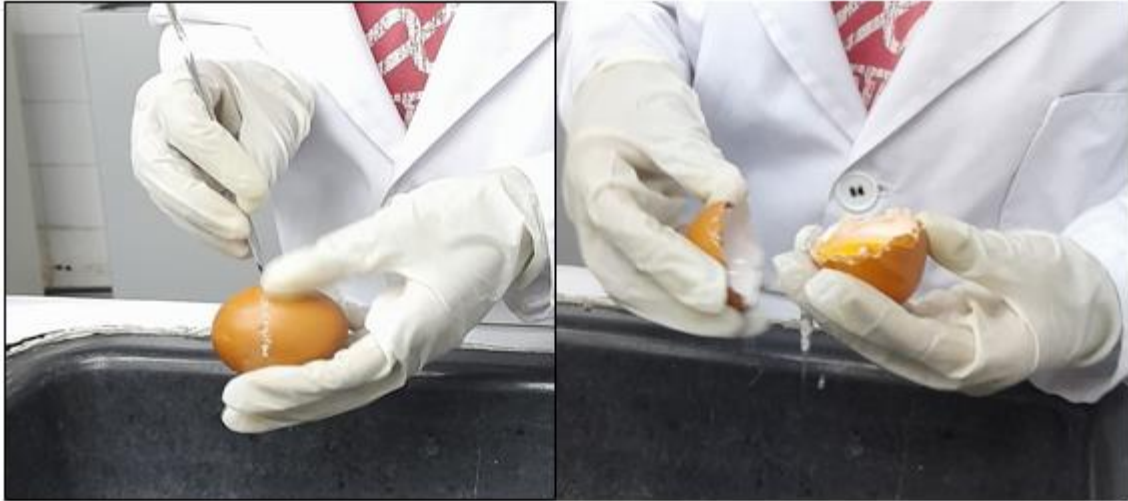
Annex 2: Pictures Captured During Extender and Treatment Preparation



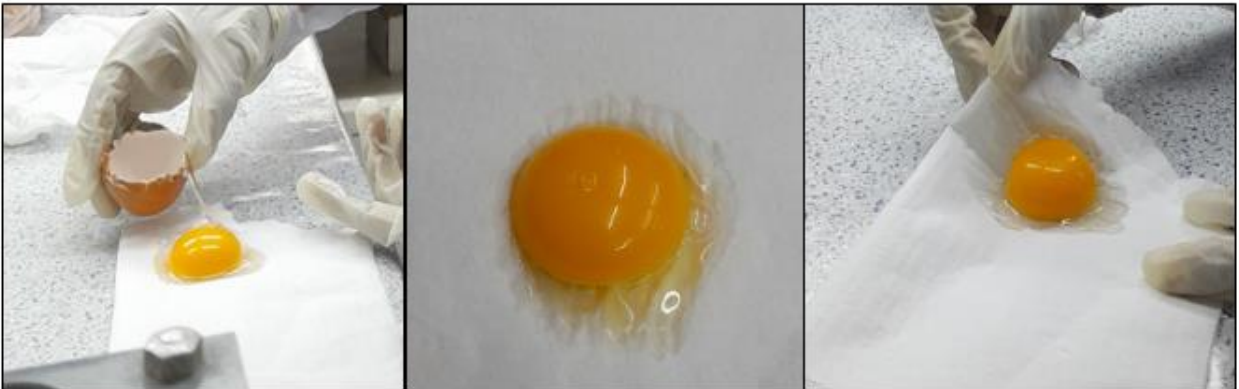
Measuring of the Base Media Using Sensitive Balance



Put the media in to flask, addition of distilled water, boiling for few minutes, cooling and storing



Preparation of fresh egg and removal of albumin



Removal of remaining albumin by placing and gently rotating the egg on paper towel



By gently piercing the outer cover, collection of the egg yolk with clean tube



Mixing the egg yolk with pre-prepared media followed by filtration on clean flask



Putting the mixture into clean tube, labling and storing

Annex 3: Sample Processing and Experimentation Pictures



Sample collection and measurement



Separation of epididymis from the testicle



Measurement of Epididymis



Tail of Epididymis



Base media



*Cutting of epididymis tail
in to slices*



*Washing of the sperm with
base media*



Collection and filtration of the spermatozoa



*Pre-freeze evaluation of
motility under microscope*



Centrifugation of spermatozoa



Sperm pellets at the bottom of the tube



Allocation of the sperm to the desired treatment groups

Annex 4: Images Captured During Vitrification of Sperm

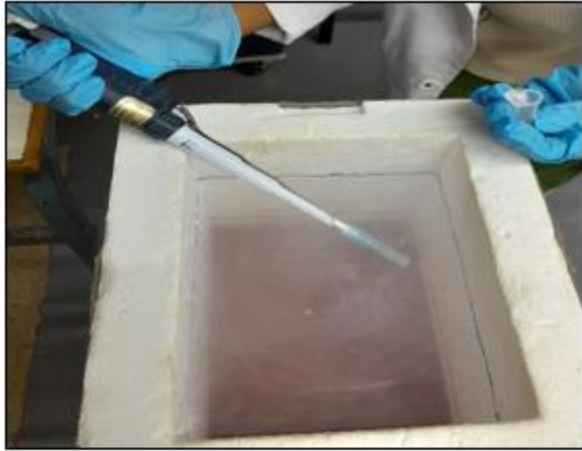


Adding Liquid Nitrogen into the Styrofoam box



Measuring 11cm above the liquid nitrogen for sperm dropping.

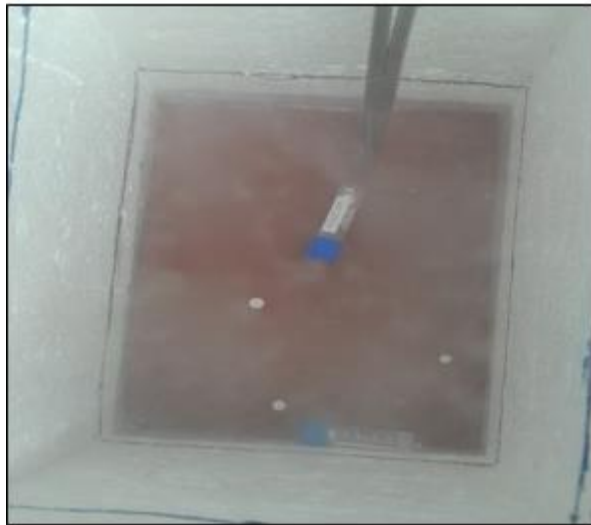
Through mixing of the sperm and treatments in the cryovial



Dropping spermatozoa directly into liquid nitrogen



Filling Straws with spermatozoa



Vitrified Ball of Spermatozoa with in liquid Nitrogen



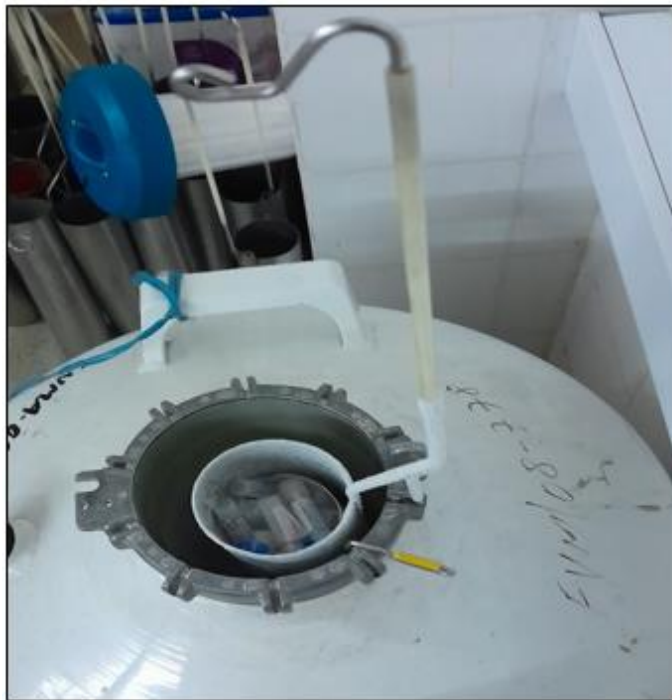
Collection of vitrified sperm into Cryovial



Cryovials filled with vitrified spermatozoa

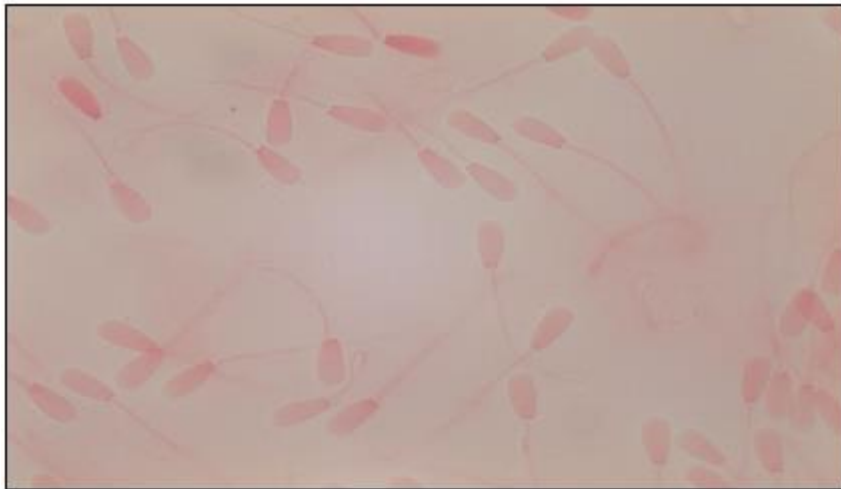


Sperm Filled straws directly dropped into liquid nitrogen



Straws and cryovials with vitrified sperm stored with in liquid nitrogen

Annex 5: Images Captured During Evaluation of Sperm Parameters



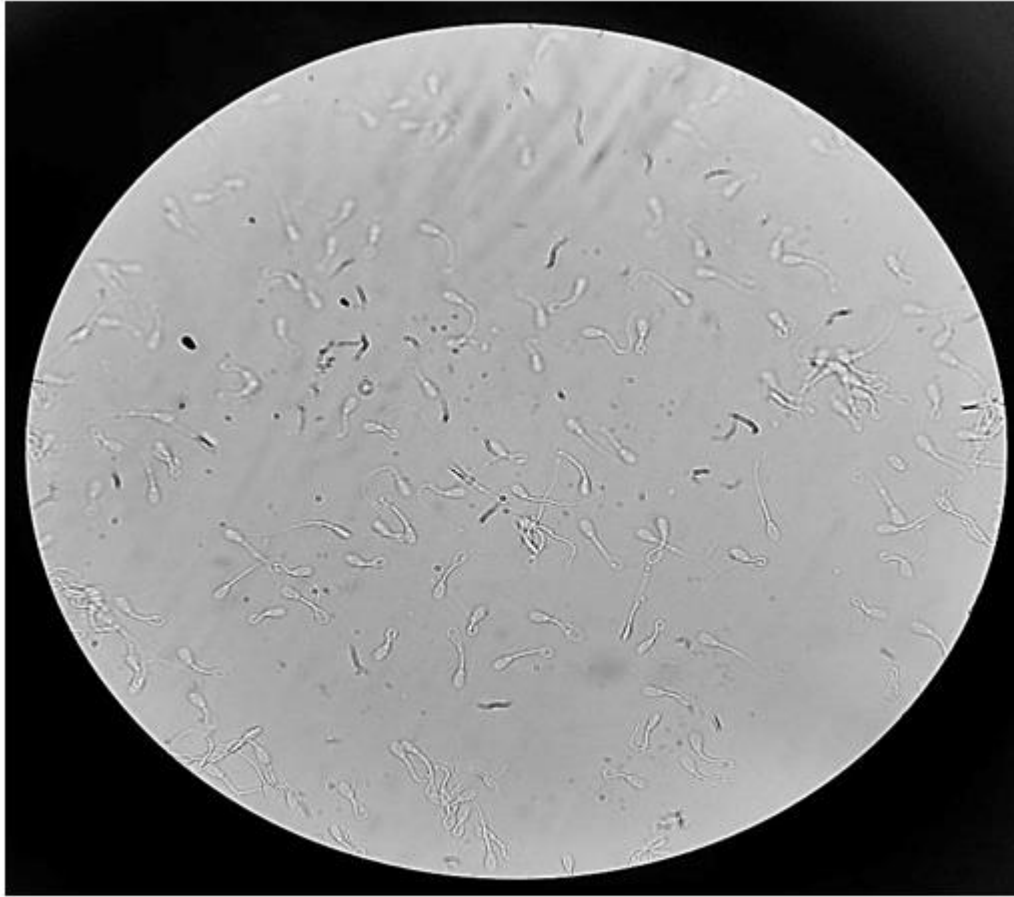
Evaluation of sperm viability by Eosin stain (pink stained head indicates viable spermatozoa)



*Black arrow indicates -
Damaged acrosome*

*Blue arrow indicates -
Intact acrosome*

Giemsa stain - for evaluation of acrosomal integrity of spermatozoa



Assessment of HOST reaction via HO solution (HOST reactive sperm indicated by tail coiling)

Annex 6: Ethical Clearance



Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/02/35/16/2024

Name of Applicant: **Bitseit Lukas (DVM, MSc student)**

Address: Department of Clinical Studies, College of Veterinary Medicine and Agriculture, Addis Ababa University

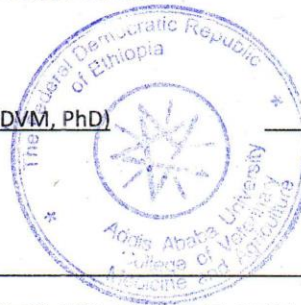
Title of the project: *cooling tolerance and post thaw quality of bovine epididymal spermatozoa after vitrification using Tris-Citrate-Sucrose based extender*

Date of application: **December, 2023**
Nature of the project: **Laboratory investigation**
Target animal species: **Cattle**
Number of animals involved: **No live animal use**
Study area: **CVMA-Bishoftu, Ethiopia**

Minutes No. and date of review: **VM/ERC/02/16/024, 26/03/2024**

The Institutional Animal Care and Use Committee of the College of Veterinary Medicine and Agriculture of the Addis Ababa University has reviewed the above research project and unanimously approved the application of **Bitseit Lukas**.

Professor Getachew Terefe (DVM, PhD)
Chairman



[Signature]
Signature

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Bishoftu, Ethiopia

Annex 7: Plagiarism Report

COOLING TOLERANCE AND POST-THAW QUALITY OF BOVINE EPIDIDYMAL SPERMATOZOA AFTER VITRIFICATION USING TRIS-CITRATE-SUCROSE BASED EXTENDER (Bistiet Lukas)

ORIGINALITY REPORT

12%	8%	9%	2%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	etd.aau.edu.et Internet Source	2%
2	S.S. Layek, T.K. Mohanty, A. Kumaresan, J.E. Parks. "Cryopreservation of bull semen: Evolution from egg yolk based to soybean based extenders", Animal Reproduction Science, 2016 Publication	1%
3	www.jirb.dormaj.com Internet Source	1%
4	www.clevelandclinic.org Internet Source	1%
5	K D Prihantoko, F Yulastuti, H Haniarti, A Kusumawati, D T Widayati, A Budiyanto. "The Acrosome Integrity Examination of Post-thawed Spermatozoa of Several Ongole Grade Bull in Indonesia Using Giemsa Staining Method", IOP Conference Series: Earth and Environmental Science, 2020	1%

