



UNIVERSITI PUTRA MALAYSIA

**COMPARATIVE EVALUATION OF BULL SEMEN QUALITY BETWEEN
ULTRA RAPID FREEZING AND SLOW FREEZING
CRYOPRESERVATION METHODS**

MUHAMMAD FARID IMRAN MUHAMMAD RAFIDI

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The logo of Universiti Putra Malaysia (UPM) is a shield-shaped emblem. It features a red and white geometric design with a central vertical element. At the top left, the letters 'UPM' are displayed in white on a red background. In the center, there is an open book with text on its pages. The entire logo is overlaid with a large, semi-transparent watermark that reads '@COPYRIGHT UPM'.

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FACULTY OF VETERINARY MEDICINE

UNIVERSITI PUTRA MALAYSIA

SERDANG, SELANGOR

2023/2024

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CRYOPRESERVATION METHODS**

MUHAMMAD FARID IMRAN MUHAMMAD RAFIDI

A project paper submitted to the
Faculty of Veterinary Medicine, Universiti Putra Malaysia
In partial fulfilment of the requirement for the
DEGREE OF DOCTOR OF VETERINARY MEDICINE
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Serdang, Selangor Darul Ehsan

DECEMBER 2023

DEDICATION

To my supervisors, family and friends.

- *Farid Imran Rafidi* -



ACKNOWLEDGEMENT

In the name of Allah, the most merciful, the most gracious, the One who gave me strength through these trying times, so that I am able to complete this project.

I extend my deepest appreciation to my supervisor, Associate Professor Dr. Nurhusien Yimer Degu, whose unwavering support, invaluable guidance, and insightful feedback have been instrumental in shaping this work. Your mentorship has been a cornerstone of my academic growth, and I am profoundly thankful for your expertise and dedication.

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LIST OF ABBREVIATION

RF	Ultra-rapid freezing
SF	Slow freezing
CASA	Computer Assisted Semen Analyzer



ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4901 - Projek.

**PENILAIAN PERBANDINGAN KUALITI SPERMA LEMBU ANTARA
KAEDAH PENYIMPANAN BEKU ULTRA PANTAS DAN
PENYIMPANAN BEKU PERLAHAN**

oleh

MUHAMMAD FARID IMRAN MUHAMMAD RAFIDI

2023

Penyelia: Professor Madya Dr. Nurhusien Yimer Degu

Penyelia bersama: Dr. Ubedullah Kaka

Penyimpanan beku sperma lembu memainkan peranan penting dalam memelihara bahan genetik untuk aplikasi pada masa depan dalam penternakan haiwan, terutamanya dalam pembiakan lembu untuk inseminasi buatan. Kajian ini mengkaji kualiti sperma lembu yang disimpan melalui dua teknik penyimpanan beku yang berbeza: kaedah penyejukan perlahan konvensional dan kaedah penyejukan ultra-pantas yang lebih baru.

Dalam eksperimen ini, sperma lembu dikutip menggunakan ejakulator elektrik dan seterusnya dicairkan dalam pencair Tris-egg yolk yang mengandungi penyeimbang Tris, fruktosa, asid sitrik, gliserol dengan kuning telur dan pencair Andromed yang boleh didapati secara komersial. Sampel sperma dibahagikan kepada dua alikot untuk penyimpanan beku. Aliquot pertama mengikuti

protokol penyejukan perlahan standard, melibatkan penyejukan berperingkat pada 5°C selama 2 jam kemudian disusun secara menegak di rak, 3-4 cm di atas permukaan gas nitrogen cecair selama 10 minit sebelum disimpan beku dalam nitrogen cecair selama 24 jam. Sebaliknya, alikot kedua mengalami penyejukan ultra-pantas, dengan fasa penyejukan cepat pada 5°C selama 30 minit, diikuti dengan penempatan segera straw ke dalam nitrogen cecair selama 24 jam. Setelah itu, straw tersebut dicairkan pada suhu 37°C selama 30 saat, dan motilitas sperma dinilai menggunakan analisis sperma bantuan komputer (CASA), manakala morfologi dan kebolehnyaawaan dinilai melalui pengecatan eosin-nigrosin.

Keputusan analisis sperma beku-cair bagi penyejukan perlahan menggunakan pencair tris-egg yolk menunjukkan peningkatan yang signifikan ($p < 0.05$) dalam kebolehnyaawaan sperma berbanding penyejukan ultra-pantas menggunakan pencair tris-egg yolk dan Andromed. Walau bagaimanapun, tidak terdapat perbezaan yang signifikan ($p > 0.05$) diperhatikan dari segi kelainan morfologi dan motiliti progresif di antara kumpulan tersebut. Kesimpulannya, penyejukan ultra-pantas mungkin bukan alternatif yang sesuai untuk penyimpanan beku sperma lembu, kerana ia tidak memberikan faedah yang ketara dari segi kebolehnyaawaan, morfologi, dan motiliti progresif.

Kata kunci: *penyejukan ultra-pantas, penyejukan perlahan, sperma lembu*

Abstract

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4901- Project.

**COMPARATIVE EVALUATION OF BULL SEMEN QUALITY
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CRYOPRESERVATION METHODS**

by

MUHAMMAD FARID IMRAN MUHAMMAD RAFIDI

2023

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Co-supervisor: Dr. Ubedullah Kaka

Semen cryopreservation plays a pivotal role in preserving genetic material for future applications in animal husbandry, notably in cattle breeding for artificial insemination. This study investigates the quality of bull semen preserved through two distinct cryopreservation techniques: the conventional slow freezing method and the comparatively novel ultra-rapid freezing method.

In this experiment, bull semen was collected using an electro ejaculator and subsequently diluted in a Tris-egg yolk extender containing Tris buffer, fructose, citric acid, glycerol with egg yolk and commercially readily available Andromed extender. The semen samples were divided into two aliquots for cryopreservation. The first aliquot followed the standard slow freezing protocol, involving gradual cooling at 5°C for 2 hours then arranged horizontally on a rack, 3-4 cm above the surface of liquid nitrogen gas for 10 minutes before being

cryopreserved in liquid nitrogen for 24 hours. Conversely, the second aliquot underwent ultra-rapid freezing, with a quick cooling phase at 5°C for 30 minutes, followed by immediate placement of straws into liquid nitrogen for 24 hours. Subsequently, the straws were thawed at 37°C for 30 seconds, and semen motility was evaluated using computer-assisted semen analysis (CASA), while morphology and viability were assessed through eosin-nigrosin staining.

The results of the frozen-thawed semen analysis for slow freezing using tris-egg yolk extender indicated a significant enhancement ($p < 0.05$) in sperm viability compared to ultra-rapid freezing using tris-egg yolk and Andromed extenders. However, there were no significant differences ($p > 0.05$) observed in terms of morphological abnormalities and progressive motility between the groups. In conclusion, ultra-rapid freezing may not be a suitable alternative for cryopreserving bull semen, as it did not yield substantial benefits in terms of viability, morphology and progressive motility.

Keywords: *ultra-rapid freezing, slow freezing, bull semen*

1.0 INTRODUCTION

Improvements in the effectiveness and sustainability of animal production for the supply of food are urgently needed as the world's population expands. In this setting, it is crucial to examine the sperm cryopreservation's historical development, consider the challenges it faces today, and comprehend the efforts being made to find solutions. This is especially important when it comes to boosting livestock fertility, with a specific focus on cattle. To increase the viability of the cattle sector, a thorough understanding of the workings of reproductive technologies and the difficulties they present is essential. Artificial Insemination (AI) stands out among these reproductive technologies as a crucial instrument for developing cattle production, enabling quicker genetic advancement and selection (Medeiros et al., 2002). Cryopreservation of male sperm is essential for increasing AI's effectiveness and success rate. It is important to recognize that existing sperm cryopreservation techniques frequently fall short in terms of effectiveness. During the freezing and thawing procedures, a sizable part of sperm sustains physiological damage, which lowers the fertility (Bailey et al., 2000).

In the realm of animal reproduction, the history of sperm cryopreservation traces its roots back to the 1600s (Sherman JK. 1964). An Italian scientist namely Lazzaro Spallanzani, made a significant advancement in artificial insemination with cooled sperm in 1784, which resulted in the birth of three puppies (Ombelet et al., 2015). Ilya Ivanovich Ivanoff, a Russian scientist,

created workable techniques for artificial insemination in farm animals nearly a century later, in 1899. However, until Phillips and Lardy's crucial discovery in 1940, there were barriers to the widespread adoption of artificial insemination. A major turning point was reached when they discovered that poultry egg yolks may protect sperm from the negative effects of cold shock during cooling and act as a cryoprotective agent (Phillips et al. 1940). In order to improve sperm preservation at low temperatures, Salisbury and colleagues later improved this method by adding Na-citrate as a buffer to egg yolk (Salisbury et al., 1940). The following significant development was made when Polge and his team discovered that glycerol has the ability to defend against freezing at both low temperatures and during the freezing process (Polge et al., 1949). The global deployment of AI underwent a revolution in the 1950s with the introduction of new extenders, packaging techniques, and procedures, particularly in the dairy industry (Walters et al., 2019). Ultra-rapid freezing procedures were conducted consecutively on human, fish, Iberian Ibex, and mouflon sperm in the years 2011, 2012, 2015, and 2017 (Boveda et al., 2018). This paper aims to compare the semen quality underwent two different types of freezing namely ultra-rapid and slow.

2.0 LITERATURE REVIEW

2.1 Bovine spermatogenesis

Spermatogenesis in bovines is a finely regulated process of germ cell multiplication and differentiation leading to the production of spermatozoa in the seminiferous tubules of the testes. The process involves the differentiation

of round spermatids into fully mature spermatozoa, and the seminiferous epithelium is composed of several generations of germ cells. In bulls, the duration of the seminiferous epithelium cycle is 13.5 days, and the total duration of spermatogenesis is 61 days, which is 4.5 times the duration of the cycle of the seminiferous epithelium. Bovines have been found to have a lower efficiency of spermatogenesis than most species examined, but higher than that of humans (Staub & Johnson, 2018). A study on the mechanism of bovine spermiogenesis based on comparative transcriptomic studies found that approximately 10 to 60 billion sperms can be obtained from bovines when semen is collected, and the molecular mechanism underlying bovine spermiogenesis has been investigated to improve the reproductive potential of cattle (Li et al., 2021). Another study demonstrated differences in the establishment of spermatogenesis in testicular tissue from bull calves, indicating that 8 weeks of postnatal life is the developmental stage in the bovine testis when elongated spermatids are first observed (Jon M. et al., 2005).

Several factors can affect bovine spermatogenesis, including environmental conditions, heat stress, disease, and genetic factors. Spermatogenesis is a highly regulated process that occurs in the seminiferous tubules of the testes and involves acrosome formation, histone replacement, and the fine regulation of gene expression (Li et al., 2021). Environmental factors such as extreme temperatures can disrupt testicular thermoregulation, interrupting and impairing spermatogenesis, leading to transitory subfertility or infertility (Capela et al., 2022). Additionally, disease, injury, and fever can decrease sperm production, while genetic factors and testicular size can also influence sperm output

potential and semen quality (Foote, 1978). Therefore, a combination of genetic and environmental factors can significantly impact bovine spermatogenesis and ultimately affect semen quality and fertility.

2.2 Methods of semen collection and quality evaluation

Several methods are used to collect bull semen, including the artificial vagina (AV) method and electroejaculation (EE). A study comparing the two methods in mithun (*Bos frontalis*) bulls found that the AV method resulted in better semen quality, including higher motility and velocity, compared to electroejaculation. However, the study also noted that electroejaculation has the potential for semen collection from free-range mithun bulls to incorporate in assisted reproductive technology procedures (Nadaf et al., 2022). Another study compared two sperm morphological evaluation methods, the eosin-nigrosin stained (ENS) method and the unstained (UNS) method, and found that both methods can be used for bull sperm morphological evaluation under field conditions, with the UNS method being a viable alternative to the ENS method due to the ease of semen smear preparation (Hanson et al., 2023). Additionally, research has shown that electroejaculated semen is at least comparable in quality to semen collected in the artificial vagina (Austin, Hupp, & Murphree, 1961). These studies demonstrate that both the AV method and electroejaculation are used for bull semen collection, and the choice of method can impact semen quality and fertility.

Bull semen quality can be evaluated using various methods, which can be broadly categorized into macroscopic and microscopic examination.

Macroscopic examination involves assessing semen characteristics such as volume, density, initial motility, sperm concentration, and total count of sperm (Filipčik et al., 2023). Microscopic examination, on the other hand, focuses on sperm morphology, motility, and other parameters like sperm membrane integrity and sperm chromatin integrity (Filipčik et al., 2023). Some of the methods used for bull semen quality evaluation include sperm morphology, sperm motility, cervical mucus penetration, zona pellucida penetration, osmotic swelling and sperm-zona binding assay. Sperm morphology is a critical component of bull semen quality evaluation, as it helps identify subfertile from fertile bulls (Hanson et al., 2023). Two common methods for sperm morphological evaluation are the eosin-nigrosin stained (ENS) method and the unstained (UNS) method (Hanson et al., 2023). Sperm motility is an important parameter in bull semen quality evaluation, and it can be assessed using various methods, such as the computer-assisted semen analyzer (CASA) system or manual observation under a microscope (Filipčik et al., 2023). Cervical Mucus Penetration is a test that evaluates the ability of sperm to penetrate cervical mucus, which is an essential step in the fertilization process (Garner, 1997). Zona Pellucida Penetration is a test that assesses the ability of sperm to penetrate the zona pellucida, the outer layer of the oocyte, which is required for fertilization (Garner, 1997). In addition, osmotic swelling is a test that measures the ability of sperm to swell in a hypo-osmotic environment, which is important for the fertilization process (Garner, 1997). Lastly, sperm-zona binding assay is a test that evaluates the binding ability of sperm to the zona pellucida, which is essential for fertilization (Kastelic, 2013)

The choice of methods for bull semen quality evaluation depends on the specific requirements of the study or application. It is essential to use a combination of tests to obtain a comprehensive assessment of semen quality, as no single diagnostic test can accurately predict fertility (Kastelic, 2013).

2.3 Semen extenders

Various extenders are utilized in cryopreserving bull semen, each presenting distinct advantages and drawbacks. Egg yolk-based extenders offer the benefit of containing cryoprotective agents that help protect the sperm during the freezing process (Rasit et al., 2019). However, they may introduce contaminants and are not universally suitable, particularly for *in vitro* fertilization or sex-sorted semen production (Anzar, Rajapaksha, & Boswall, 2019). Conversely, soybean-based extenders, commercially available as an alternative to egg yolk-based ones, present an advantage but exhibit batch-to-batch consistency issues that can lead to inconsistent fertility rates (Anzar, Rajapaksha, & Boswall, 2019). Lecithin-based extenders, another substitute for egg yolk-based extenders, have demonstrated effectiveness in preserving sperm quality. However, there are conflicting results regarding the efficacy of lecithin-based extenders, and more research is needed to determine their effectiveness (Rasit et al., 2019). Moreover, the cholesterol-cyclodextrin (CC) complex extender, devoid of egg yolk, shows promise in cryopreserving bull semen but requires more extensive research to ascertain its long-term effects on fertility (Yang et al., 2021). In summary, the choice of extender for bull semen cryopreservation depends on the specific application and the desired characteristics of the preserved semen. Egg yolk-based extenders are commonly used but may not be

suitable for all applications, while alternative extenders such as soybean-based, lecithin-based, and CC-based extenders offer potential advantages but require further research to determine their efficacy and long-term effects.

2.4 Methods of semen cryopreservation

The first method is ultra-rapid freezing methods, including vitrification, have been suggested as alternatives to slow freezing for sperm cryopreservation. This method offers a faster, simpler and cheaper freezing method (Isachenko, 2003). Hence, it has gained attention in cryopreserving wildlife sperm in field condition (Pradiee et al., 2014). By choosing animals with superior genetic features and enhancing trophy quality, common assisted reproduction techniques used in domestic animals, such as artificial insemination utilizing frozen-thawed sperm, are increasingly being used to improve wildlife management.

A study on Iberian ibexes (*Capra pyrenaica*) implemented a short equilibration duration and a permeable cryoprotectant to achieve field-ready cryopreservation of sperm (Pradiee et al., 2014). Due to its lower equipment needs, faster processing, simplicity, and cost-effectiveness, ultra-rapid cooling techniques have also shown promise in field applications, as shown by their early success in preserving human (Isachenko et al., 2011) and fish (Merino et al., 2012) sperm. This strategy has more recently been expanded to include the preservation of ejaculated sperm from Iberian ibex and European mouflon (Pradiee et al., 2015; Pradiee et al., 2017). The moderate addition of sucrose (about 100 mM) to the extender and the adoption of quick thawing rates during

sperm retrieval are essential for the successful implementation of this technology with wild ruminant sperm (Pradiee et al., 2015, 2017). Results from attempts to use glycerol's shorter pre-freezing equilibration times and ejaculated mouflon sperm's ultra-rapid cooling speeds have been mixed (Pradiee et al., 2016, 2017).

Another method is slow freezing. This method has been widely used in andrology laboratories for the cryopreservation of various species of sperm. It has been the standard method for bull sperm cryopreservation for many years. The sperm are gradually cooled over a period of 2 to 4 hours, in two or three stages, using either manual labour (Thachil and Jewett, 1981) or a programmable freezer (Serafini and Marrs, 1986). This is one method of slower freezing. The initial cooling rates, which bring the specimen from room temperature to 5°C, have been shown to be best at between 0.5 and 1°C per minute (Mahadevan and Trounson, 1984). The sample is then rapidly submerged in liquid nitrogen after being frozen at a rate of 1 to 10°C per minute as it travels from 5°C to -80°C (Thachil and Jewett, 1981; Mahadevan and Trounson, 1984; Serafini and Marrs, 1986). According to another research, seeding the specimen and keeping it at -5°C for 10 minutes may improve cryosurvival (Critser et al., 1987).

The sperm must instead be manually placed in liquid nitrogen vapor for 5 to 30 minutes before being immersed in liquid nitrogen (Sherman, 1963; Kobayashi et al., 1991; Rofeim et al., 2001). This is a quicker approach. While some studies have suggested that the slower freezing procedure is superior (Mahadevan and

Trounson, 1984), others have offered evidence in favour of faster cooling rates (Sherman, 1963).

3.0 MATERIALS & METHOD

3.1 Semen collection

Bulls were hauled through the cattle chute and secured with a head gate. As part of a physical examination, the prepuce and scrotal areas are checked for lesions. Scrotal circumference is also measured using a calliper. To prevent contaminating semen samples during the ejaculation process, hair is then cut close to the prepuce area before being cleaned with clean water and wiped till it dries.

After removing the faeces, transrectal massage of the ampullary region was performed using a clean rectal sleeve to induce ejaculation. Then, the electroejaculation probe was inserted into the rectum and controlled automatically until the semen is ejaculated. Throughout the procedure, the bull is watched for any indications of extreme pain that would call for stopping the procedure. To collect ejaculated semen, clean collecting tubes are attached to sample tubes. After ejaculating, the tube containing the semen samples will be temporarily kept in a thermos filled with pipe water that is 37°C. The sample will then be delivered right away to the lab for semen analysis.

3.2 Semen evaluation

Before the process, the semen tube was placed in a water bath set to 37°C. A drop of nett semen was placed on a glass slide and examined under a light

microscope at a 4X magnification to look at the wave pattern. Scores for wave pattern ranged from 1 to 5.

The next step was to examine the progression of motility. It was evaluated using an Androscope, mobile semen analysis unit (CASA). A drop of 3 μ l fresh semen was added to a slide that had already been heated to 37 °C and the slide was then mounted onto the Androscope. Androscope program, version 12.2, counted 5 fields on average each reading. Checking the percentage of living sperm in the semen was another step in the evaluation process. A drop of nett semen was added to 3–4 drops of Eosin–Nigrosin stain, which were then let to stand for 3 minutes on the glass slide. Next, a thin smear was applied on a glass slide, let to air dry, and then studied with a 40X objective. When observed, dead sperm should appear stained, but live sperm will look undyed. The living and dead percentages were counted and calculated using a cell counter.

Checking for semen concentration was the final step in the semen evaluation process. To obtain a 1:100 dilution, 10 μ l of fresh semen were diluted with 990 μ l of formal saline. To do a concentration count, diluted semen was placed on each chamber of haemocytometer and looked at a magnification of 40X.

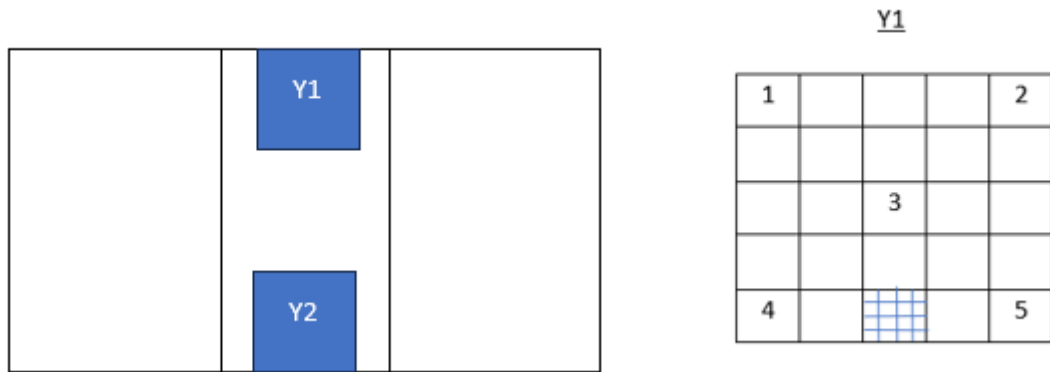


Fig. 1: Example of haemocytometer and chamber containing 25 boxes with each containing 16 small boxes

Each haemocytometer consists of Y1 and Y2 chambers, and 5 boxes containing 16 small boxes each were chosen and counted from each chamber. Semen was counted using the principle of top-left grid to avoid redundancy in counting. Total semen from Y1 and Y2 is averaged, and formula $50Y \times 10^6$ sperm/ml is used to finally obtain the concentration of sperm.

3.3 Semen cryopreservation

Fresh semen were diluted in 100ml of Tris-egg yolk extender containing 3.51 g Tris buffer, 1.25g fructose, 1.97g citric acid, 7% glycerol, and 20% egg yolk and Andromed extender. The first aliquot followed the standard slow freezing protocol, involving gradual cooling at 5°C for 2 hours. After an equilibration period of 15 min, aliquots of samples were loaded into 0.25 mL straws making each straw contain approximately 30×10^6 sperms/ml. Then arranged vertically on a rack, 3-4 cm above the surface of liquid nitrogen gas for 10 minutes before being cryopreserved in liquid nitrogen for 24 hours. Conversely, the second aliquot underwent ultra-rapid freezing, with a quick cooling phase at 5°C for 30 minutes, followed by immediate placement of straws into liquid nitrogen for 24 hours

3.4 Post thaw semen evaluation

After 24 hours, the straws were thawed at 37°C for 30 seconds, and semen motility was evaluated using Androscope (CASA), while morphology and viability were assessed through eosin-nigrosin staining.

3.5 Statistical analysis

Morphological abnormalities (%) were evaluated using One-way ANOVA while viability (%) and progressive motility (%) were evaluated using Kruskal-Wallis H Test. (IBM SPSS Statistics 27) and $p < 0.05$ was considered significant.

4.0 RESULTS

A total of eight fresh semen samples were divided in two aliquots to compare sperm recovery after the application of two cryopreservation protocols: the currently accepted slow freezing procedure and the ultra-rapid freezing protocol.

Based on the table below it shows that the fresh samples were having low volume but highly concentrated with sperm, moderate viability, low morphological abnormalities and low progressive motility of fresh semen.

Table 1: Semen parameters of fresh samples prior to cryopreservation (mean \pm SD).

Number of samples	8
Volume (ml)	3.98 \pm 2.01
Sperm concentration (10 ⁶ /ml)	2462 \pm 2686.21
Viability (%)	69.5 \pm 11.40
Morphological abnormalities (%)	21.25 \pm 10.02
Progressive motility (%)	42.42 \pm 35.80

Based on the table below it shows that Tris_SF resulted in significantly better viability than Tris_RF and Andro_RF. However, the other parameters show no significant difference between groups of cryopreservation technique.

Table 2:

Comparative results between groups after slow and ultra rapid freezing techniques (mean \pm SD).

Cryopreservation technique	Viability (%)	Morphological abnormalities (%)	Progressive motility (%)
Tris_RF	0.63 \pm 1.19 ^a	18.62 \pm 10.06	2.70 \pm 3.00
Andro_RF	3.13 \pm 8.04 ^b	15.25 \pm 7.13	2.60 \pm 2.81
Tris_SF	7.13 \pm 10.37 ^{ab}	15.99 \pm 9.08	6.20 \pm 8.09
Andro_SF	3.50 \pm 5.45	17.00 \pm 8.98	3.72 \pm 3.51

Note: Lowercase letter a indicate significant differences ($P < 0.05$) between slow freezing using tris-egg yolk and rapid freezing using tris-egg yolk. Lowercase letter b indicate significant differences ($P < 0.05$) between slow freezing using tris-egg yolk and rapid freezing using Andromed.

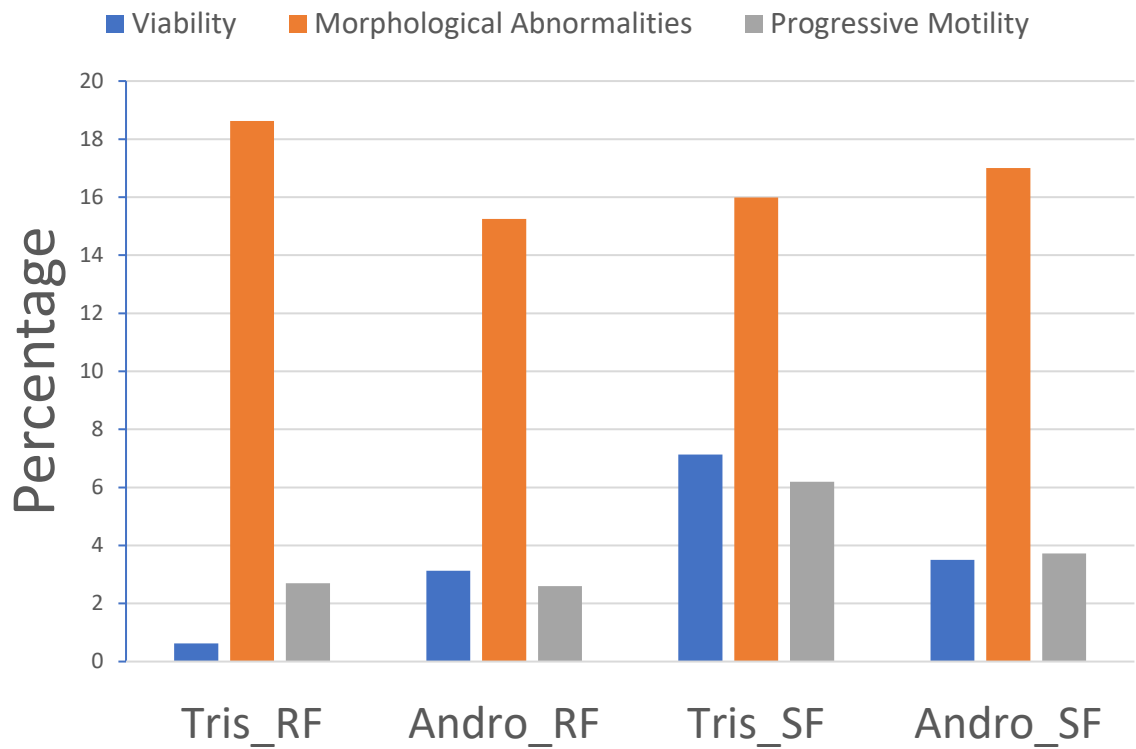


Figure 2. Comparison of post thaw sperm quality between groups after slow freezing and ultra-rapid freezing.

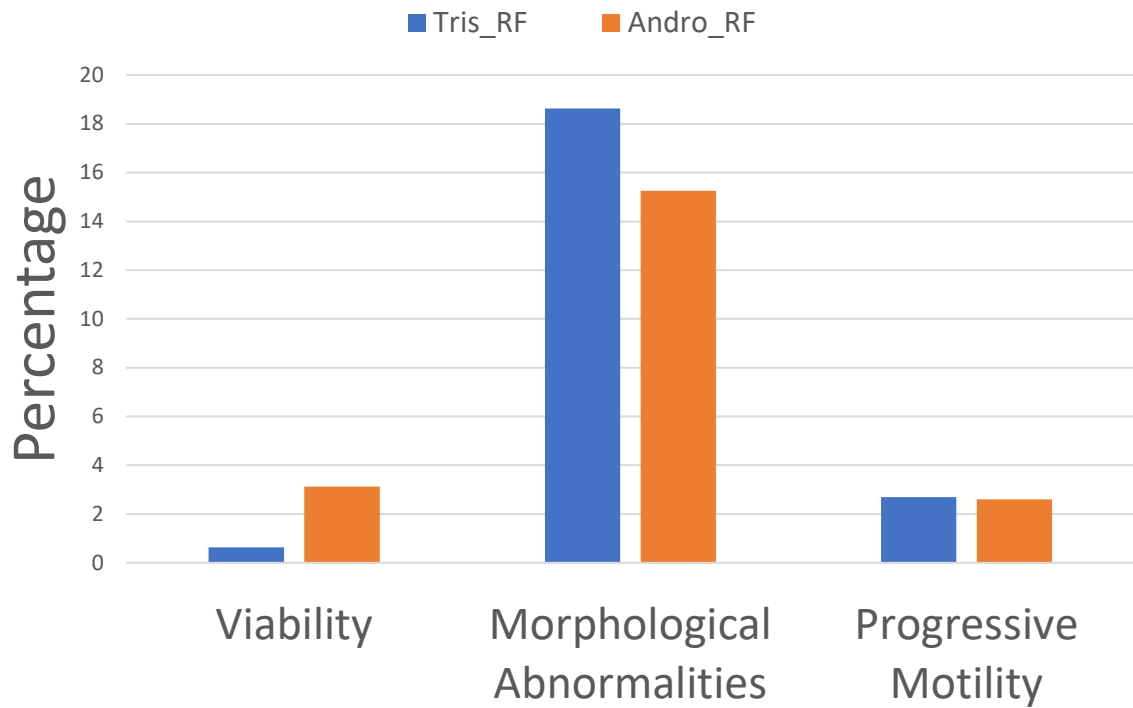


Figure 3. Comparison of post thaw sperm quality between extenders after ultra-rapid freezing.

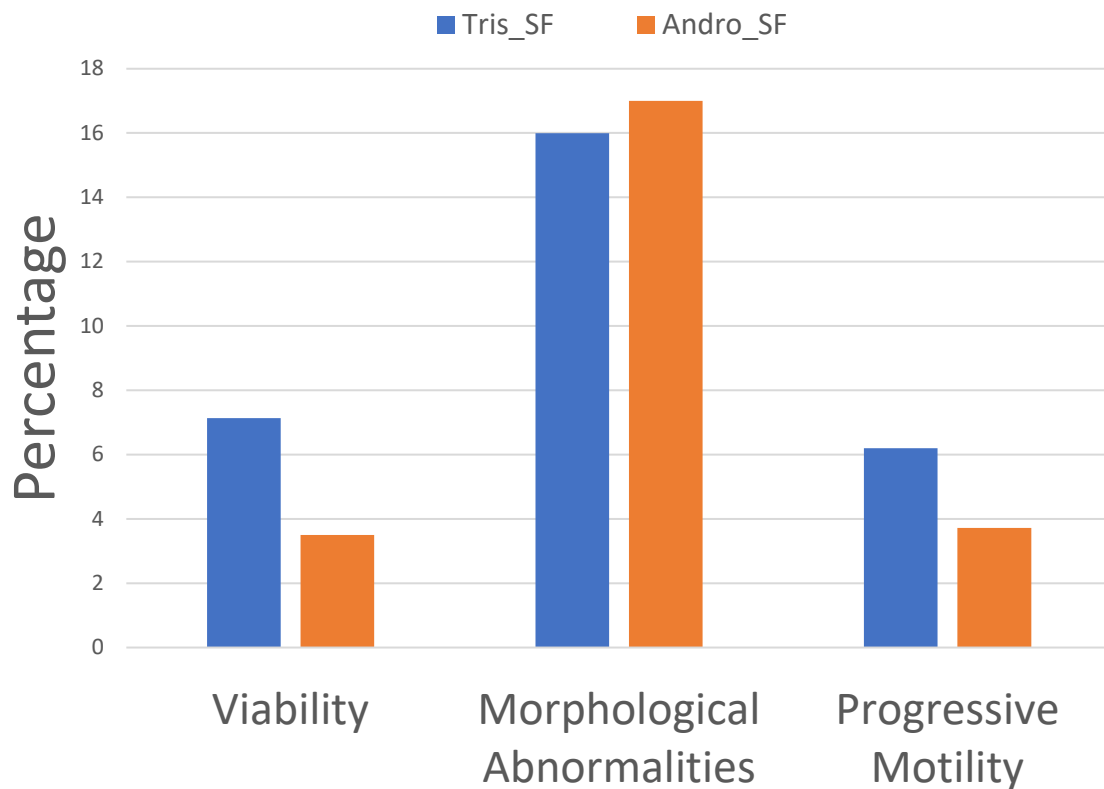


Figure 4. Comparison of post thaw sperm quality between extenders after slow freezing.

5.0 DISCUSSION

When either ultra-rapid freezing or slow freezing and egg-yolk based or Andromed extenders were used, the sperm of both of them showed reduced viability, morphological abnormalities and progressive motility compared to fresh semen parameters. The key factor of cryopreservation is highly influenced by the buffer system and concentrations of cryoprotectant used (Santiago-Moreno et al., 2006; Pradiee et al., 2017). Apart from that it also varies within species, individual within species and ejaculate within individuals (Gao et al., 1997; Thurston et al., 2002; Walters et al., 2005). These may suggest the reasons of the reduction in terms of post thaw semen quality. In addition, the other reasons are cryopreserved sperm from electroejaculation reported to have lower post thaw progressive motility compared to artificial vagina. These results agree with Jiménez-Rabadán et al., 2012. However, electroejaculation was used in this study due to the unavailability of trained bulls for the use of an artificial vagina.

For Tris-egg yolk extender, slow freezing resulted in better post thaw semen quality compared to ultra-rapid freezing. The results for ultra-rapid freezing here can be improved in the future by testing different concentrations of cryoprotectant. In this experiment, lower concentration of cryoprotectant was used mainly because ruminant sperm could not withstand the cytotoxic effect of high concentration cryoprotectant which is normally implemented in ultra-rapid freezing (Pradiee et al. 2017). Therefore, more study needs to be done

on the acceptable range for the concentration of cryoprotectant on rapid freezing in bulls.

The lower viability observed in ultra-rapid freezing, as opposed to slow freezing, can be attributed to the advantages of rapid thawing rates. It is shown that ultra-rapid freezing must implement rapid thawing rates as it prevents recrystallization within sperm cells (Isachenko et al., 2003). The formation of intercellular crystals may cause mechanical damage and highly detrimental to the cells (Luyet, 1937). However, in this experiment the thawing rates were standardized to 30 seconds for both ultra-rapid and slow freezing.

The underwhelming outcomes of slow freezing could potentially be attributed to the bulls' suboptimal semen quality. Apart from buffer system and concentrations of cryoprotectant used (Santiago-Moreno et al., 2006; Pradiee et al., 2017), the success of cryopreservation varies within species, individual within species and ejaculate within individuals (Gao et al., 1997; Thurston et al., 2002; Walters et al., 2005).

The data indicated that there were instances where the percentages of live sperm, as determined by the eosin-nigrosin stain, did not align with the percentage of progressive motility due to the fact that viability of the sperm cells was determined manually whereas the progress motility was determined automatically using Androscope (CASA). Therefore, the results for viability of the sperm cells were more likely to be observer and interpretation bias.

In addition to comparing the effects of freezing methods, this experiment also

assessed the results associated with different extenders. From the data, most of them indicate that Tris-egg yolk extender resulted in better post thaw sperm quality compared to Andromed extender. However, it is important to note that the data did not show statistical significance, suggesting that further research and analysis may be needed to draw firm conclusions on the effectiveness of this freezing technique

During the experiment, the researcher noticed the crackling of 0.25ml straws specifically for ultra-rapid freezing. This also could explain the drops in post thaw semen quality. Therefore, it is suggest to drop the semen directly into liquid nitrogen without the needs of straws for semen cryopreservation of ultra-rapid freezing (Pradiee et al,. 2017).

Apart from the lower post thaw semen quality of ultra-rapid freezing, it has its own benefits which provide a faster, simpler and cheaper freezing method (Isachenko,2003). Therefore, it is suggested to be used for field condition especially for wildlife which allowing the optimization of wild game management. Additionally, the preservation of specific species, subspecies, or ecotypes might solely rely on genome resource banking, as suggested by Santiago-Moreno and López-Sebastián in 2010.

6.0 CONCLUSION

Ultra-rapid freezing may not be a suitable alternative for cryopreserving bull semen, as it did not yield substantial benefits in terms of viability, morphology and progressive motility. Besides, the underwhelming outcomes of slow freezing itself could be explained due to the bulls' suboptimal semen quality.



7.0 REFERENCES

- Anzar M, Rajapaksha K, Boswall L (2019) Egg yolk-free cryopreservation of bull semen. PLOS ONE 14(10): e0223977. <https://doi.org/10.1371/journal.pone.0223977>
- Austin, J. W., Hupp, E. W., & Murphree, R. L. (1961). Comparison of Quality of Bull Semen Collected in the Artificial Vagina and by Electroejaculation. *Journal of Dairy Science*, 44(12), 2292-2297. [https://doi.org/10.3168/jds.S0022-0302\(61\)90057-1](https://doi.org/10.3168/jds.S0022-0302(61)90057-1)
- Bailey JL, Bilodeau JF, Cormier N. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. *J Androl.* (2000) 21:1-7.
- Capela, L., Leites, I., Romão, R., Lopes-da-Costa, L., & Pereira, R. M. L. N. (2022). Impact of Heat Stress on Bovine Sperm Quality and Competence. *Animals : an open access journal from MDPI*, 12(8), 975. <https://doi.org/10.3390/ani12080975>
- Critser, J.K., Huse-Benda, A.R., Aaker, D.V., Arneson, B.W. and Ball, G.D. (1987) Cryopreservation of human spermatozoa. I. Effects of holding procedure and seeding on motility, fertilizability, and acrosome reaction. *Fertil. Steril.*, 47, 656-663.
- E. Isachenko, V. Isachenko, J.M. Weiss, R. Kreienberg, I.I. Katkov, M. Schulz, et al. Acrosomal status and mitochondrial activity of human spermatozoa vitrified with sucrose. *Reproduction*, 136 (2008), pp. 167-173.
- E. Isachenko. Vitrification of mammalian spermatozoa in the absence of cryoprotectants: from past practical difficulties to present success. *Reprod. Biomed.*, 6 (2) (2003), pp. 191-200.
- Filipčík, R., Rečková, Z., Pešan, V., Konoval, O., & Kopec, T. (2023). Evaluation of semen parameters from Fleckvieh-Simmental bulls and the influence of age and season of collection. *Archives animal breeding*, 66(1), 113-120. <https://doi.org/10.5194/aab-66-113-2023>

- Foote R. H. (1978). Factors influencing the quantity and quality of semen harvested from bulls, rams, boars and stallions. *Journal of animal science*, 47 Suppl 2, 1–11.
- Garner, D. L. (1997). Ancillary Tests of Bull Semen Quality. *Veterinary Clinics of North America: Food Animal Practice*, 13(2), 313-330. [https://doi.org/10.1016/S0749-0720\(15\)30344-3](https://doi.org/10.1016/S0749-0720(15)30344-3)
- Hanson R., Reddick S., Thuerauf S., Webb K., Kasimanickam V., & Kasimanickam R. (2023). Comparison of bull sperm morphology evaluation methods under field conditions. *Clinical Theriogenology*, 15. <https://doi.org/10.58292/ct.v15.9425>
- Hanson R., Reddick S., Thuerauf S., Webb K., Kasimanickam V., & Kasimanickam R. (2023). Comparison of bull sperm morphology evaluation methods under field conditions. *Clinical Theriogenology*, 15. <https://doi.org/10.58292/ct.v15.9425>
- J. Pradiee, M.C. Estes, A. Lopez-Sebastián, A. Toledano-Díaz, C. Castaño, J.A. Carrizosa, et al. Successful ultra-rapid cryopreservation of wild Iberian ibex (*Capra pyrenaica*) spermatozoa. *Theriogenology*, 84 (2015), pp. 1513-1522
- J. Pradiee, M.C. Estes, C. Castaño, A. Toledano-Díaz, A. Lopez-Sebastián, R. Guerra, et al. Conventional slow freezing cryopreserves mouflon spermatozoa better than vitrification. *Andrologia*, 49 (2017), pp. 1-7
- J. Pradiee, M.C. Estes, C. Castaño, A. Toledano-Díaz, A. López-Sebastián, J. Santiago-Moreno. Cryopreservation of epididymal sperm from ibexes (*Capra pyrenaica*) using short equilibration time with glycerol. *Theriogenology*, 82 (2014), pp. 525-528
- Jiménez-Rabadán, P., et al., Effect of semen collection method (artificial vagina vs. electroejaculation), extender and centrifugation on post-thaw sperm quality of Blanca-Celtibérica buck ejaculates. *Anim.Reprod. Sci.* (2012), <http://dx.doi.org/10.1016/j.anireprosci.2012.04.005>
- John P. Kastelic, Male involvement in fertility and factors affecting semen quality in bulls, *Animal Frontiers*, Volume 3, Issue 4, October 2013, Pages 20–25, <https://doi.org/10.2527/af.2013-0029>

- Jon M. Oatley, Jerry J. Reeves, Derek J. McLean, Establishment of Spermatogenesis in Neonatal Bovine Testicular Tissue Following Ectopic Xenografting Varies with Donor Age, *Biology of Reproduction*, Volume 72, Issue 2, 1 February 2005, Pages 358–364, <https://doi.org/10.1095/biolreprod.104.030783>
- Kobayashi, T., Kaneko, S., Hara, I., Park, J.Y., Aoki, R., Ohno, T. and Nozawa, S. (1991) A simplified technique for freezing human sperm for AIH: cryosyringe/floating platform of liquid nitrogen vapor. *Arch. Androl.*, 27, 55–60.
- Lane, M., Schoolcraft, W.B. and Gardner, D.K. (1999) Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. *Fertil. Steril.*, 72, 1073–1078.
- Li, X., Duan, C., Li, R., & Wang, D. (2021). Insights into the Mechanism of Bovine Spermiogenesis Based on Comparative Transcriptomic Studies. *Animals : an open access journal from MDPI*, 11(1), 80. <https://doi.org/10.3390/ani11010080>
- Mahadevan, M. and Trounson, A.O. (1984) Effect of cooling, freezing and thawing rates and storage conditions on preservation of human spermatozoa. *Andrologia*, 16, 52–60.
- Medeiros CMO, Forell F, Oliveira ATD, Rodrigues JL. Current status of sperm cryopreservation: why isn't it better? *Theriogenology*. (2002) 57:327–44. Doi: 10.1016/S0093-691X(01)00674-4
- Nadaf, S. M., Ramesh, V., Mech, M., Haider Khan, M., Ahmed, F. A., Ponraj, P., & Mitra, A. (2022). Comparative ejaculatory response, fresh and frozen semen quality and fertility to artificial vagina vs electroejaculation method of semen collection in 22ithun (*Bos frontalis*) bulls. *Andrologia*, 54(2), e14330. <https://doi.org/10.1111/and.14330>
- O. Merino, R. Sánchez, J. Risopatrón, E. Isachenko, I.I. Katkov, E. Figueroa, et al. Cryoprotectant-free vitrification of fish (*Oncorhynchus mykiss*) spermatozoa: first report. *Andrologia*, 44 (2012), pp. 390-395
- Ombelet W, Van Robays J. Artificial insemination history: hurdles and milestones. *Facts Vis Obygn.* (2015) 7:137–43.

- Phillips PH, Lardy HA. A yolk-buffer pabulum for the preservation of bull semen. *J Dairy Sci.* (1940) 23:399–404. Doi: 10.3168/jds.S.0022-0302(40)95541-2
- Polge C, Smith AU, Parkes AS. Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature.* (1949) 164:666. Doi: 10.1038/164666a0
- R. Sánchez, J. Risopatrón, M. Schulz, J. Villegas, V. Isachenko, R. Kreinberg, et al. Canine sperm vitrification with sucrose: effect on sperm function. *Andrologia*, 43 (2011), pp. 233-241
- Rasit, U. M., Amal, S. A., Evans, H. C., Gilmore, A. A., Mustafa, H., Iis, A. R., Bambang, P., Abdullah, K., & Memili, E. (2019). Advances in Cryopreservation of Bull Sperm. *Frontiers in Veterinary Science*, 6. <https://doi.org/10.3389/fvets.2019.00268>
- Rofeim, O., Brown, T.A. and Gilbert, B.R. (2001) Effects of serial thaw–refreeze cycles on human sperm motility and viability. *Fertil. Steril.*, 75, 1242–1243.
- Salisbury GW, Fuller HK, Willett EL. Preservation of bovine spermatozoa in yolk-citrate diluent and field results from its use. *J Dairy Sci.* (1941) 24:905–10. Doi: 10.3168/jds.S0022-0302(41)95476-0
- Serafini, P. and Marrs, R.P. (1986) Computerized staged-freezing technique improves sperm survival and preserves penetration of zona-free hamster ova. *Fertil. Steril.*, 45, 854–858.
- Sherman JK. (1964) Low temperature research on spermatozoa and eggs. *Cryobiology.* 1:103–29. Doi: 10.1016/0011-2240(64)90002-1
- Sherman, J.K. (1963) Improved methods of preservation of human spermatozoa by freezing and freeze-drying. *Fertil. Steril.*, 14, 49–64.
- Staub, C., & Johnson, L. (2018). Review: Spermatogenesis in the bull. *Animal: an international journal of animal bioscience*, 12(s1), s27–s35. <https://doi.org/10.1017/S1751731118000435>

Thachil, J.V. and Jewett, M.A. (1981) Preservation techniques for human semen. *Fertil. Steril.*, 35, 546–548.

V. Isachenko, R. Maettner, A.M. Petrunkina, P. Mallmann, G. Rahimi, K. Sterzik, et al. Cryoprotectant-free vitrification of human spermatozoa in large (up to 0.5 ml) volume: a novel technology. *Clin. Lab.*, 57 (2011), pp. 643-650

Walters EM, Benson JD, Woods EJ, Critser JK. The History of Sperm Cryopreservation Importance of Sperm Cryopreservation. (2009). Available online at: www.cambridge.org (accessed February 17, 2019).

Yang, S. X., Adams, G. P., Zwiefelhofer, E. M., Rajapaksha, K., & Anzar, M. (2021). Cholesterol-cyclodextrin complex as a replacement for egg yolk in bull semen extender: sperm characteristics post-thawing and in vivo fertility. *Animal Reproduction Science*, 225, 106691. <https://doi.org/10.1016/j.anireprosci.2021.106691>