An-Najah National University

Faculty of Graduate Studies

INCORPORATING DIFFERENT LEVELS OF ADENOSINE WITH TRIS- EGG YOLK AND ANDROMED® DILUENTS USED IN FREEZING SEMEN OF RAMS

By

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This Thesis is Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Animal Production, Faculty of Graduate Studies, An-Najah National University, Nablus - Palestine.

2025

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Dedication

To my mother, who taught me the meaning of struggle and perseverance, and has always been a model of sacrifice and giving. To my beloved wife, who supported me with all her love and provided me with strength and patience throughout this journey. You have always been my pillar and companion at every step. And to my sweet children, whose innocence and smiles eased the hardships of the days, making all efforts worthwhile. And to my supervisor, who never hesitated to provide guidance and advice, and who exemplified dedication and commitment. I deeply appreciate his constant support and valuable guidance, which had a significant impact on the completion of this work.

Acknowledgements

First and foremost, I would like to thank my wonderful supervisor, Dr. Jihad Abdullah, thank you for your faith in me, your trust, exceptional mentorship and continuous support throughout my research.

I am also thankful to Dr. Hatem Attalla from the Department of Veterinary Medicine at the Faculty of Agriculture and Veterinary Medicine/An-Najah University for his valuable contribution and assistance at the beginning of my research journey. His guidance and advice were pivotal in clarifying many concepts and determining the correct direction for my work.

To JICA agency, with all my gratitude to you, for giving me the opportunity to join in CEPAD training program.

To Artificial Insemination Center BBIB-Singosari (Indonesia), staff, thank you for your training and assistance in completing this work.

To my colleagues at the Beit Qad Agricultural Station and Palestinian Center for livestock Improvement, no words can describe my feelings of gratitude and appreciation for you, as you helped me overcome all obstacles to achieve this accomplishment.

Declaration

I, the undersigned, declare that I submitted the thesis entitled:

INCORPORATING DIFFERENT LEVELS OF ADENOSINE WITH TRIS- EGG YOLK AND ANDROMED® DILUENTS USED IN FREEZING SEMEN OF RAMS

I declare that the work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's Name: **Mousa Khaleel**

Signature: 

Date: **30/01/2025**

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INCORPORATING DIFFERENT LEVELS OF ADENOSINE WITH TRIS- EGG YOLK AND ANDROMED® DILUENTS USED IN FREEZING SEMEN OF RAMS

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Abstract

Background: In ovine reproduction, cryopreservation techniques of ram semen are particularly important in genetic improvement programs, as they accelerate the rate of transmission of superior genetic material to low productive flocks in the population, enable the storage of valuable germplasm for a long time, and eliminate geographical restrictions in artificial insemination. However, cryo-freezing techniques of ovine sperm still face difficulties limited their opportunity of success. Ram spermatozoa are particularly prone to thermal and oxidative stress during freezing process and thawing. Adenosine is omnipresent intrinsic molecule composed of adenine linked to a ribose sugar by β-glycosidic bond, it has been demonstrated to ameliorate energy metabolism and antioxidant defense system, yet its potential use in the cryo-freezing of ovine semen is still undiscovered.

Aims: This study aims to evaluate the capability of adenosine at different concentrations (0%, 0.5%, 0.75%, and 1%) to protect ram sperm functions and structure during the freezing process. Furthermore, the study compared the cryo-potency and cryo-efficacy of Andromed® and Tris-egg yolk diluents in maintaining sperm quality.

Methods: Semen was collected every five days over a one-month period from six mature Assaf rams aged 3 to 4 years and diluted with commercial Andromed® and conventional Tris-egg yolk fortified with different levels of adenosine. Semen quality parameters including motility, viability, thermal resistance, membrane integrity and morphology were evaluated after 30 minutes and 3 hours of dilution, and post-thawing. The data were subjected to analysis of variance (ANOVA) with comparison of means using Bonferroni test.

Results: Adenosine, particularly at 0.75%, significantly improved ram semen parameters across all stages ($P < 0.05$). Moreover, sperm quality parameters were significantly lower ($P < 0.05$) in samples extended with Tris-egg yolk than those extended in Andromed®.

Conclusion: Incorporating adenosine with semen extenders improves ovine semen quality parameters during cryopreservation. Additionally, Andromed® appear to be a promising freezing extender to replace Tris-egg yolk extender.

Keywords: Adenosine, Cryopreservation, Ovine semen, Tris-egg yolk, Andromed®.

Chapter One

Introduction

In breeding industry, cryopreservation of ovine sperm is a major technique in genetic improvement programs, it paves the way for large scale artificial insemination (AI) projects, accelerates the genetic progress via transmission of superior genetic material in order to improve productivity of sheep flocks in the population, eliminates the vast distance restrictions, and allows storage of valuable germplasm over a prolonged period, ensuring its availability even after the ram's death (Saha et al., 2022; Ntemka et al., 2018).

Apart from these benefits, ovine sperm faces significant challenges to survive during cryopreservation , which they limits the widespread application of frozen semen in AI projects (Byrne et al., 2000; Curry, 2000; Salamon & Maxwell, 1995). Over the past few years, the accumulation of human knowledge at the cellular level has contributed to understanding the nature of spermatozoa, complexities of freezing process and possibility of a successful sperm cryopreservation ,especially with ovine (Saha et al., 2022).

Ovine spermatozoa are more sensitive to thermal and oxidative stress during freezing-thawing process compared to bovine and other species (Gillan et al., 2004; Salamon & Maxwell, 2000); this sensitivity is mainly imputed to the greater concentrations of polyunsaturated fatty acids (PUFAs) in the sperm cell membrane, which are particularly susceptible to temperature change and osmotic imbalances during cryopreservation (Carro et al., 2022; Allai et al., 2018; Mandal et al., 2014; Khan & Ijaz, 2008; Salamon & Maxwell, 1995). Temperature inconstancy, combined with high unsaturated/saturated fatty acid ratio and absence of antioxidants in ovine spermatozoa, contributed to lipid peroxidation in cell membrane; consequently, induce ultrastructural damage , impair of motility, and decrease sperm viability as well as fertilization capacity (Saha et al., 2022; Fang et al., 2016; Maxwell & Watson, 1996). Moreover, the handling and diluting of semen through freezing- thawing cycle induce both physical and chemical damage of sperm cell, which led finally to rupture of spermatozoa cell membrane, organelles damage, and contraction of sperms (L. Zhang et al., 2024; Maxwell & Watson, 1996; Maxwell & Salamon, 1993).

Researchers have tried to counteract these limitations and difficulties in order to enhance the cryo-survival and minimize the cryoinjuries of ovine spermatozoa, through investigation of different procedures and methods, such as using varied freezing techniques (Saha et al., 2022; Mishra et al., 2018), altering freezing rates (Fang et al., 2016; Byrne et al., 2000), comparing the efficacy of different semen extenders (Bustani & Baiee, 2021; Gogol et al., 2019; Rekha et al., 2016), manipulating in semen extender ingredients (Abdel-Khalek et al., 2018), and adding antioxidants , amino acids and antibiotics to cryoprotective solution (Allai et al., 2018; Hussain et al., 2018).

Adenosine is a natural organic compound found both inside and outside the cells (Della Latta et al., 2013). It consist of a purine nucleobase known as adenine, and D-ribofuranose molecule linked by β-glycosidic bond (Layland et al., 2014; Della Latta et al., 2013).

Adenosine performs pivotal role in sperm cell mechanisms and functions. It serves as an essential components in adenosine monophosphate (AMP) and adenosine triphosphate (ATP) synthesis (Layland et al., 2014). In spermatology, adenosine stimulates spermatozoa to start and amplify the flagellar movement (Zhu et al., 2018; Romac et al., 1994; Fraser & Duncan, 1993). This enhancement in sperm motility was focused the researchers' attention to understand the mechanism of its occurrence. Previous investigations have verified the presence of A_2 adenosine receptors in sperm cell membrane (Fénichel et al., 1996; Fraser & Duncan, 1993). The interaction between adenosine and these receptors on the surface of spermatozoa cell activates adenosine cyclase, which amplifies intracellular signaling pathways. This in turn promote the synthesis of cyclic adenosine monophosphate (Wendlandt et al., 2023; Fraser & Duncan, 1993). Cyclic adenosine monophosphate (cAMP) contributes to regulate and control sperm functions via activating the pathway of protein kinase A (Balbach et al., 2018; Branham et al., 2006), which it plays a vital role in sperm capacitation and sperm hyperactivation (Balbach et al., 2018; Liguori et al., 2004).

Besides that, adenosine act as stimulating agent to antioxidants defense system; it helps to lower cellular oxidative damage and maintain the integrity of spermatozoa membrane via induction the enzymatic antioxidants, like glutathione peroxidase (Y. Zhang et al., 2005; Maggirwar et al., 1994). However, its concentration must be carefully controlled,

as excessive levels may have inhibitory or pathological effects by desensitizing receptors, disrupting metabolic processes, or organs damage (Borea et al., 2017; Burnstock & Knight, 2004).

Many studies in humans, mice, and others have been done to evaluate the effect of cAMP and ATP as an additive on semen quality (Jannatifar et al., 2020; Rodríguez-Miranda et al., 2008; Edwards et al., 2007), and the effect of adding adenosine to fresh and cooled semen media (Bezerra et al., 2019; Vahidi et al., 2011), but the effect of adenosine on frozen semen has not been determined. Understanding its influence in this context could provide valuable insights into improving reproduction technologies, particularly in ovine.

1.1 The general objective

The general objective of this study is to evaluate the effect of adding different concentrations of adenosine (9-β-D-Ribofuranosyladenine) to Tris-egg yolk and Andromed® diluents on post-thawed ram sperm parameters.

1.2 The specific objectives

The study was conducted to:

- Evaluate the action of adding (0%, 0.5%,0.75% and 1%) of adenosine on Assaf ram semen parameters (motility, thermal resistance, viability, membrane integrity, and morphology) through different freezing stages (after 30 minutes of dilution, after 3 hours of dilution, and post-thawing).
- Determine the optimal concentration of adenosine in semen diluents
- Assess the freezing efficiency of Andromed® and Tris-egg yolk diluents in maintaining Assaf ram semen quality across different stages (after 30 minutes of dilution, after 3 hours of dilution, and post-thawing).

1.3 Literature Review

1.3.1 Artificial Insemination and its Benefits

Several techniques were used to speed up the rate of genetic improvement. One of these techniques is AI, which plays a vital role in selection and breeding management. In assisted reproduction technology (ART), AI is consecutive process that begins with defining of breeding objectives, developing of breeding structure, selecting sires and targeted flocks, managing rams' and ewes' nutrition, along with monitoring health status,

and finally inseminating the ewes with good quality semen by qualified technician (Haile et al., 2020; Cueto, 2019).

With AI techniques, sheep breeders have the opportunity to inseminate their ewes with semen from rams with high genetic merit, which maximizes the outcomes of breeding programs (Alvares et al., 2015; Ballesteros Hernández et al., 2015). Besides that, artificial insemination decreases the risk of sexually transmitted infections through testing the males before introducing them into breeding and genetic improvement programs (Ballesteros Hernández et al., 2015; Shipley et al., 2007). Moreover, once semen is collected, it undergoes thorough analysis to verify is pathogens-free, and treated with antibiotics, which in turn also enhances diseases control(Shipley et al., 2007). Additionally, AI increases the economic efficacy and flexibility of breeding schemes through eliminating the need of transporting the elite rams from place to another and maximizing the benefits from them (Cueto, 2019). Overall, AI technique helps to improve productivity, profitability, and sustainable farming practices.

However, the application of AI technique in ovine, using fresh semen is widespread and common, while using of frozen semen is still limited, this may be due to the complexity of cryopreservation process, the difficulty to obtain high-quality frozen semen, and the obstacle to achieving a satisfactory conception rate after trans-cervical insemination, which subsequently limits expansion (Cueto, 2019).

1.3.2 Cryopreservation of ovine semen and challenges

It is an axiom in ART that semen cryopreservation is particularly important, as previously stated, it speeds up the rate of transmission of superior genetic material in population and enables preservation the germplasm of elite males for a long period of time (Cueto, 2019).

Cryopreservation revolution began in 1949, when the protective ability of glycerol in preserving sperm during freezing process was first discovered (Lonergan, 2018). Since that time the studies and research have continued to investigate the efficacy of different cryofreezing extenders, and various cryoprotectant agents on the quality of frozen semen, with attention on bovine semen at the beginning ; this is for two main reasons , which are the high cryotolerance capacity of bovine sperm, and to meet the demand in global dairy industry for long-term storage of elite bulls semen (Lonergan, 2018; Walters et al., 2009).

However, cryopreservation of ovine sperm has progressed slowly over the years, and this is directly attributed to high sensitivity of ovine spermatozoa to cold shock , oxidative and osmotic stress during freezing process, which ultimately results in unacceptable outcomes (Allai et al., 2018; Salamon & Maxwell, 1995). This sensitivity is primarily due to the unique lipid and fatty acid profile of ovine spermatozoa (Carro et al., 2022) , as well as the lack of antioxidants in ovine sperm cell (Salamon & Maxwell, 1995). Phospholipid peroxidation, particularly of PUFAs (arachidonic (AA) and docosahexaenoic (DHA) fatty acids) occur through freezing-thawing cycle, due to oxidative stress and rapid temperature change (Salamon & Maxwell, 1995). Oxidative and thermal stress stimulate the formation and releasing of oxidants like reactive oxygen species (ROS), which in turn mainly attack PUFAs within the cell membrane, and result in lipid damage (Ayala et al., 2014).

By the time, many efforts have been made to achieve success in cryopreservation of ovine sperm. Researchers found that freezing protocol using a two-step accelerating controlled cooling rate with freezing rate of 60 °C/min from −10° C minimizes cryogenic injuries to ram spermatozoa and significantly improves post-thaw sperm quality parameters (Galarza et al., 2019). Moreover, other workers reported that using automated freezing technique resulted in better post-thaw sperm quality, when compared to traditional technique (Yánez-Ortiz et al., 2022). In contrast, some research indicated that there were no significant differences between automatic and traditional method (Yánez-Ortiz et al., 2022). In addition, many studies have been conducted to investigate the effect of ultrafast vitrification on spermatozoa quality, this technique may minimize the toxicity of cryoprotectants, and maintain the integrity of sperm cell; however, inacceptable sperm quality was resulted after using it with ram semen, which determines the use of this technology in ovine AI programs (Yánez-Ortiz et al., 2022).

Additionally, comprehensive studies on the protective effect of diluents component led to understand the best medium for ram sperm survival after freezing. It has been found that lactose and fructose enhanced spermatozoa functions better than sorbitol (Chanapiwat et al., 2012), whereas other researchers found that raffinose and saccharose were better than lactose, fructose and glucose in stabilizing and maintaining membrane protein-lipid complex of sperm cell, and the combination of two type of sugars was more efficient, than when implemented single sugar alone (Salamon & Maxwell, 1995).

Furthermore, the inclusion of vitamins such as alpha-tocopherol, cobalamin, and ascorbic acid, enzymes like oxidized glutathione, and superoxide dismutase (SOD), amino acids like cystine, HCL, l-glutamine, and l-proline, hormones like testosterone, and insulin, and other substance such as strawberry, green tea, honey, and fish oil to semen diluents improved the kinetic and quality parameters of spermatozoa by neutralizing ROS, quenching free radicals and stabilizing the PUFAs within the cell membrane (Bustani & Baiee, 2021; Hussain et al., 2018).

Recently, efforts to replace animal origin components such as egg yolk with synthetic alternatives or plant origin components like soybean lecithin and trehalose in cryopreservation extenders, aim to improve standardization and reduce variability as well as minimize the possibility of contamination and enhance the biosecurity procedures (El-Shereif et al., 2022; El-Sisy et al., 2016).

Regardless of these efforts and advancements, reduced motility and viability of ovine of frozen semen still hinder widespread application of ram frozen semen in large AI programs (Alvarez et al., 2019). On the other hand, the ewe's complex cervical anatomy represent an additional challenge to the wide usage of frozen ram semen, as it is impossible to apply intrauterine insemination, expect by using laparoscopic surgical technique, which is very expensive and there are concerns in its use related to the animal's welfare (Alvarez et al., 2019; Allai et al., 2018). Further confirmations that ram frozen semen is unsatisfactory for wide application in the field is that the conception and pregnancy results, which are still low compared to fresh and cooled semen (Cueto, 2019).

1.3.3 Semen diluents

Seminal plasma offers relatively minimal stability and protection to spermatozoa cell during cooling and freezing process, so it is important to use diluents in order to provide maximum protection for sperm, maintain sperm viability for a long time, and also expand the volume of semen ejaculates (Salamon & Maxwell, 1995). In cryopreservation of ovine semen, it is necessary to choose the appropriate diluent formula to maintain sperm viability, and membrane integrity at reduced temperature.

Early semen diluent was water then it was replaced with basic saline solutions (NaCl) , which maintained sperm activity for a long time (Bootwalla & Miles, 1992). In the 1940_s, the role of egg yolk in semen diluent was discovered, which represented a qualitative leap

in preserving bull semen (Lonergan, 2018; Walters et al., 2009; Bootwalla & Miles, 1992). Additionally, glycerol function as penetrating cryoprotectant, it was discovered in 1949 by chance, which later led to made of more effective and useful semen extenders (Lonergan, 2018; Bootwalla & Miles, 1992).

Over the years, the accumulation of knowledge has contributed to introducing more complex and efficient formulations containing buffering agents, energy sources, cryoprotectants and other substances like antibiotics and antioxidants (Bustani & Baiee, 2021; Bootwalla & Miles, 1992).

Cryoprotectants play an important role in preserving spermatozoal membrane integrity; these cryoprotectants are classified as penetrating and non-penetrating protective agents (Bustani & Baiee, 2021; P. H. Purdy, 2006). The introduction of penetrating cryoprotectants such as glycerol (Foote, 2002), ethylene glycol (Holt, 2000), and dimethyl sulfoxide (Jones, 1973) has significantly improved sperm quality by preventing intracellular ice formation and minimizing osmotic stress during freezing and thawing (Santiani et al., 2005; Holt, 2000). Non-penetrating cryoprotectants from animal origin such as egg yolk and skim milk and from plant origin such as soybean lecithin provide phospholipids and lipoproteins that protect sperm plasma membrane from cold shock and oxidative damage during cooling, freezing and post thawing (Bustani & Baiee, 2021; Raheja et al., 2018).

Buffer is a one of the major ingredients of semen diluents, it assists to maintain the ideal pH level for sperm vitality during temperature reduction (Salamon & Maxwell, 2000; Leelasiri et al., 1995). However, the information available about the pH behavior during cryopreservation is incomplete due to difficulty in testing pH after freezing (Leelasiri et al., 1995). Whatever, semen diluents shall contain buffering agents such Tris (Tris(hydroxy-methyl) aminomethane), sodium citrate, phosphate, or zwitterion buffers like TES free acid, HEPES, and PIPES (Salamon & Maxwell, 2000; Leelasiri et al., 1995). Previous research investigated the effect of these buffers on post -thaw survival of spermatozoa, and generally it result in good motility and fertility of frozen sperm after thawing (Leelasiri et al., 1995).

Diluents should also contain sugars as an energy source and cryoprotectants to maintain metabolism and functions of sperm cell (Bustani & Baiee, 2021; Leelasiri et al., 1995).

By the meaning of energy, fructose, glucose, and mannose were used in semen extenders (Leelasiri et al., 1995), fructose is considered the perfect sugar in term of preserving the functional integrity of plasma membrane, and motility (Bustani & Baiee, 2021). However , none of the other sugars have been shown to supply energy for spermatozoa (Maxwell & Salamon, 1993).

Since there is no antioxidant in ram semen and freezing process stimulates ROS formation and releasing (Salamon & Maxwell, 1995), researchers added a wide varieties of antioxidants to semen diluents, including vitamins such as ascorbic acid and α-Tocopherol, enzymes such as glutathione reductase and SOD, in addition to some minerals like selenium and zinc to act as protective and motility enhancing agents (Bustani & Baiee, 2021; Hussain et al., 2018).

Additionally, researchers supplemented semen extenders with antibiotics to inhibit and control microbial growth during cooling and freezing process (Bustani & Baiee, 2021; Hussain et al., 2018; Leelasiri et al., 1995). Various antibiotics like ceftiofur, gentamycin, and penicillin or combinations of Penicillin-streptomycin, and penicillin-neomycin have been routinely added to semen diluents (Bustani & Baiee, 2021; Raheja et al., 2018).

Furthermore, amino acids, fatty acids, and other additives were added to semen diluents in order to minimize DNA fragmentation, protect plasma membrane integrity, and enhance the post-thaw viability, and fertility of spermatozoa (Allai et al., 2018; Hussain et al., 2018). Amino acids such as cystine, glutamine, and methionine act as scavengers, and protect sperms from lipid peroxidation , which finally led to improve post-thaw sperm quality (Allai et al., 2018). Fatty acids such as oleic acid and docosahexaenoic acid were added to semen extenders, particularly ram semen extenders to overcome the vulnerability of ovine spermatozoa to cold shock and lipid peroxidation (Allai et al., 2018; Raheja et al., 2018). Another additives such as hormones, Coenzyme Q10, royal jelly, butylated hydroxytoluene, astaxanthin, coconut oil, honey, fish oil, and more have been added to semen diluents in the context of continuous efforts to improve the quality of frozen semen (Bustani & Baiee, 2021; Allai et al., 2018; Raheja et al., 2018).

In the same context, several researchers have evaluated the efficiency of different semen extenders. Previous research revealed that plant protein based extenders could provide a similar cryoprotective effects to common animal protein based extenders such as Tris-

egg yolk and milk extenders (Ntemka et al., 2018). This conclusion was supported by other research revealed that soybean lecithin-based diluents like Andromed® appear to be a good and safer substitutes to egg yolk-based diluents like Tris- egg yolk for cryopreservation of ram and bull semen (Bustani & Baiee, 2021; Murphy et al., 2018; Baharum et al., 2017). Interestingly, researchers also found that using lipolyzed egg yolk diluent during freezing of ram semen achieved similar results to fresh egg yolk diluent, suggesting that lipolyzed egg yolk diluent can be efficiently used to freeze ovine semen (Alcay et al., 2015).

Overall, these efforts underscore the continuous advancements and foreground the need for further research to determine the efficiency of numerus diluents used in ovine semen cryopreservation in order to improve ovine frozen-thawed sperm quality.

1.3.4 The Role of Adenosine in sperm functions

Adenosine(9-β-D-Ribofuranosyladenine) is a purine nucleoside composed of adenine attached to a ribose sugar (Layland et al., 2014). In mammals cells, adenosine initiates its function through its interaction with four subtype receptors belonged to G-protein-coupled receptors family, which is A1, A2A, A2B, and A3 (Sheth et al., 2014).

In the scope of adenosine role in molecular and cellular biology, adenosine is known to regulate energy transmission via its interactions with adenosine diphosphate (ADP) and ATP (Layland et al., 2014). It plays an energetic role in the regulation of cellular homeostasis, and modulation in the release of neurotransmitters (Cunha, 2001). Furthermore, adenosine plays a dynamic role in a wide range of cell tissues, it modulates the mechanism of T-cell proliferation, and cytokines secretion in immune cells (Sheth et al., 2014). Interestingly, adenosine also act as catalyst to various cellular enzymes classified as antioxidants, via activation of A3 receptor (Maggirwar et al., 1994).

Previous litterateurs have investigated the role of adenosine and its receptors in reproductive physiology. Adenosine plays a vital regulatory role in spermatogenesis, sperm maturation and capacitation as well as acrosome reaction (Bellezza & Minelli, 2017). Furthermore, adenosine participates in spermatozoa functions via interaction with A2A receptors in sperm cell (X. Zhang & Ma, 2024). Recently, it has been proven that there are A2A receptors within the plasma membrane in sperm flagellum region (Chen et al., 2024). The interaction between adenosine and this receptor regulates the calcium

influx, and stimulates an enzyme called adenosine cyclase, which subsequently leads to increase intracellular cAMP production and modulate dynamin adenosine triphosphatases (ATPases) activity (X. Zhang & Ma, 2024; Fraser & Duncan, 1993). Previous research have proved the role of cAMP in regulating spermatozoa functions. The synthesis and accumulation of cAMP has been linked to enhance sperm capacitation (Balbach et al., 2018; Fraser, 1990), motility (Chen et al., 2024; Jannatifar et al., 2020; Balbach et al., 2018; Vahidi et al., 2011; Romac et al., 1994), and fertilizing ability (Chen et al., 2024; Fraser, 1990, 1981).

However, the addition of adenosine to animal semen cryopreservation diluents has not been investigated yet. Our study may provide new insight into its potential use and benefits in the field, particularly in ovine sperm cryopreservation.

Chapter Two

Methodology

2.1 Location and time

The experiment was conducted at Beit-Qad Agricultural Station. (32.47104506213847, 35.35696929549924), which is affiliated with the National Agriculture Research Center (NARC) in Palestine from April 2024 to July 2024. 2.2 Animals

2.2.1 Breed

Assaf sheep breed was be selected for experimentation. It currently one of the most prevalent sheep breeds in Palestine, comprising about 36.8% of the total sheep population in the country (PCBS, 2021) .The Assaf sheep breed is a dual-purpose breed (meat and milk) was developed by crossing local Awassi breed (fatty tailed sheep) with East Frisian sheep breed (Known for its high milk production).

Assaf sheep are medium sized breed, very hardy animals and adapted in semi-extensive and extensive management system. Moreover, the ewes produce approximately 197 litters of milk to 5 months of lactation and have three lambing per two years with 1.35 twining rate (Ahmed & Abdallah, 2012).

2.2.2 Rams

Six mature Assaf rams aged 3 to 4 years and weighing 90-110 kg, exhibiting a good health, and have body condition score 3 to 3.5(on a scale of 1 to 5)(Maurya et al., 2010) were selected for this study.

The rams were trained to collect semen with the aid of two ewes treated with estradiol cypionate.

During the entire study duration, all rams were maintained under controlled conditions and received a balanced diet formulated to optimize reproductive performance. The daily ration consisted of 2.0 to 2.3 kg of concentrated feed, containing 250-280 g of crude protein (CP), 6-7 g of calcium (Ca), 3.5-4 g of phosphorus (P), 2-2.5 g of magnesium (Mg), and 3-4 g of sodium (Na) (National Research Council (U.S.), 2007). Additionally, a straw–hay mix was provided ad libitum to ensure proper fiber intake and maintain

rumen health. Moreover, they were included in the health and vaccination program at the station (Soest, 1994).

Figure 1

Assaf Ram in Beit-Qad Agricultural Station

2.2.3 Ewes

Two Assaf ewes aged between two to three years old, with weights ranging from 55 to 60 kilograms were selected to serve as teasers for training rams on the artificial vagina, and collect semen.

Ewes treated with 1.2 mg of Estradiol cypionate (E.C.P.® *zoetis*) by intramuscular route, once weekly for 3 weeks, this kept them in heat for 5 weeks during the training period.

2.3 Laboratory environment

The laboratory at Beit Qad Station was designed to meet precise biosecurity standards, ensuring the production of high-quality ovine semen while minimizing contamination risks to the lowest possible level. To maintain a controlled environment, the facility was

divided into designated sections, including changing rooms, clean areas (dilution room, filling and sealing room and storage room), and other operational sections.

Before starting laboratory work, strict hygiene protocols were followed to uphold cleanliness and safety. After the staff entered, the laboratory door was kept closed to prevent contamination from external environments. Staff were required to wash and disinfect their hands with 70% alcohol before entering the workspace. Proper laboratory attire, including a white lab coat and designated laboratory slippers, was mandatory to ensure biosecurity. all surfaces and equipment regularly disinfected and sterilized before and after each work session, along with weekly fumigation to enhance sterilization. Essential tools, such as the artificial vagina and glassware were sterilized by autoclaving according to guideline to eliminate potential pathogens. Unnecessary items were avoided in the laboratory and there was a strict rule against eating, drinking, and smoking inside the laboratory. In addition, movement of staff within clen areas was controlled to prevent cross-contamination (NDDB, 2015).

2.4 Preparation of semen diluents

The primary goal of using diluents is to extend the lifespan of sperm and decrease their metabolic activity.

Sugars provide essential nutrients to maintain sperm motility and viability, buffers protect sperm from environmental stress by sustaining pH stability and osmotic balance, cryoprotectants protect sperm cells from cold shock, prevent ice formation and minimize osmotic stress while antibiotics prevent bacterial contamination during storage and handling.

Semen dilution is conducted using specified ratios with appropriate diluents to ensure that the volume of semen utilized for insemination contains an adequate amount of sperm per dosage (Rasul et al., 2000).

2.4.1 Preparation of Tris-egg yolk diluent

Tris-egg yolk extender was prepared by a qualified laboratory technician following a two-step protocol.

In the first step, a Tris buffer solution was formulated by dissolving 2.42 g of Tris(hydroxy-methyl)aminomethane Sigma Chemical Co., St. Louis, USA), 1.28 g of citric acid monohydrate (Sigma-Aldrich, St. Louis, MO, USA) , and 2.16 g of D-fructose (Merck, Darmstadt, Germany) in 100 ml of sterile water (B. Braun Melsungen AG, 2024) (Nalley & Arifiantini, 2011).

The second step involved the preparation of the freezing extender by combining 74% of the Tris buffer with 6% glycerol (Merck, Darmstadt, Germany), 20% (v/v) fresh egg yolk, and 500 IU/ml of penicillin G and streptomycin sulfate (Merck, Darmstadt, Germany) (Nalley & Arifiantini, 2011).

The extender was prepared 36 hours before the collection of semen and placed in a refrigerator at 4°C.

2.4.2 Preparation of Andromed® diluent

To prepare 100 ml of Andromed® diluent, the manufacturer's guideline was followed. 20 ml of Andromed® concentrated media (Minitube, Germany, 2024) was warmed to + 35°C then mix it with 80 ml of sterile water (B. Braun, Melsungen AG, 2024) also maintained at +35°C by using a magnetic stirrer (Guohua Laboratory Instrument Group, China).

2.5 Preparation of adenosine solution

Adenosine powder was weighed using an analytical balance (Mettler-Toledo, Columbus, United States). Then, it was dissolved in sterile water (B. Braun) and stirred at 37°C for 15 minutes by a magnetic stirrer (Guohua) to achieve homogeneity.

2.6 Incorporating adenosine with diluents

Adenosine solution was added to the Tris-egg yolk and Andromed® diluents at the designated concentrations (0%, 0.5%, 0.75%, and 1% V/V).

The mixture was gently stirred at 37°C for 5 minutes to ensure uniform distribution before semen dilution.

Figure 2

Preparation of Diluents in the Lab

2.7 Semen collection

2.7.1 Preparation of artificial vagina

Artificial vagina (AV) is a tool employed in animal breeding centers to facilitate collection of semen from a variety of species, particularly from bovines, equines and ovine.

AV utilizes both thermal and mechanical stimulation to induce ejaculation from males. It is composed of outer rubber cylinder with valve, inner liner made of soft rubber(latex), receiving cone, collection vial and insulation bags (Shukla, 2011).

Before using of AV (Minitube, Germany, n.d.), the space between rubber cylinder and inner liner was filled and pressurized with warmed water (42 °C to 44°C) from the valve, the inner surface of rubber liner (3cm) was lubricated with sterile jelly (Repro jelly, Minitube, Germany). After that, the graduated collection vial was attached to the end of the receiving cone (Shukla, 2011) .

2.7.2 Collection process

Semen collection was conducted early in the morning (7:30 AM) every five days over a one-month period, yielding six ejaculates from each ram.

Rams and ewe were brought to semen collection area 30 minutes before start of semen collection, the ewe was tied in collection pen, then each ram was given stimulation by giving 3 false mounts and each false mount followed by 2 minutes restraint.

After stimulation, AV was used to collect semen from the rams (Saha et al., 2022; Shukla, 2011).

After collection of each semen sample, it was placed in 4.1L water bath (Water bath with cover and 6 cylindrical inserts, Minitube, Germany) at 37°C for further analysis.

Figure 1

Prepared AV

2.8 Fresh semen evaluation

A wide variety of assays have been established and developed for evaluating sperm quality.

The purpose of fresh semen evaluation is to determine whether the quality parameters meet the minimum requirements established for the sample in this research.

2.8.1 Gross appearance of ram's ejaculate

Immediately after semen collection, each sample underwent a comprehensive macroscopic evaluation, assessing the following key parameters: volume (ml), color (categorized as normal or abnormal), and pH (classified as in range or out of range).

The volume of each ejaculate was precisely measured and recorded. The ejaculates of this study had to be at least 0.5 ml as the normal physiological range established between 0.5–2 ml (Shukla, 2011).

Additionally, the color and appearance of the semen ejaculate were assessed as a preliminary indicator of sperm concentration and possible contamination, with normal variations ranging from milky-white to thick creamy (Shukla, 2011). Any ejaculate that showed an abnormal color or appearance was not used in this study.

The pH of fresh semen ejaculate was assessed by pH meter (HAM238100, Hamilton , USA), with normal range of 6.7-6.8 (Bar-Sagie et al., 1981). The ejaculates with pH level more than 6.9 were not used in the study.

2.8.2 Assessing of sperm concentration

The concentration of each semen ejaculate was checked using a spectrophotometer (Photometer SDM1, Minitube, Germany).

The spectrophotometer quantifies the light absorbed by semen sample, higher sperm counts leading to more light absorption and by creating a standard curve relating absorbance to sperm numbers, it become possible to efficiently, quickly and precisely determine the sperm concentration.

The procedure consists of two steps. First, 2.5 ml of sodium citrate buffer are placed in colorimeter tube with 0.1 ml of fresh semen and mixed gently. Then, the cuvette placed in SMD1 spectrophotometer and the results saved (Shukla, 2011).

The concentration had to be $\geq 2500 \times 10^6$ for the ejaculates to be used in the experiment.

2.8.3 Eye motility evaluation of fresh semen

Sperm become motile cells following their passage through the epididymis. Sperm motility is essential during fertilization. Therefore, the evaluation of motility parameters has special importance in assessing semen quality.

When assessing motility, sperms are typically categorized as progressive motile, non-progressive motile, or immotile (Shukla, 2011).

Sperm motility of fresh semen ejaculates was subjectively evaluated through direct observation under a phase-contrast microscope with a magnification 400× (Motic ba310e, Motic group, China).

At least 75% progressive motility was required for the ejaculate to be used in the experiment.

2.8.4 Morphology evaluation of fresh semen

Assessing ram sperm morphology is fundamental for evaluating reproductive efficiency, as structural abnormalities can significantly impact fertilization potential. Defects in the head, midpiece, or tail may compromise motility, hinder capacitation, and lower fertility rates (Erdem et al., 2016).

In this study, 10 µl of fresh semen sample was added to micro tubes containing 100µl of buffered formol saline. Then, 5 µl of this mixture was placed on a slide and covered with a glass cover slip, left for one minute to allow the drop to spread smoothly (Shukla, 2011).

At least 200 sperm were counted at magnification 1000 × under a phase contrast microscope (Motic ba310e).

The semen ejaculates with less than 10% abnormality were pooled.

2.9 Pooling of semen

Pooling of semen ejaculates in experiments helps minimize the variability (Ram effect) and ensures more consistent and representative results. It also reduces time and cost without compromising the statistical power.

After initial evaluation, all accepted semen ejaculates were pooled together in 20 ml sterile plastic culture tubes. Immediately after pooling, 150 µl was taken and equally divided into five tubes for evaluation. Note that, each pooled or diluted sample was evaluated three times to increase confidence in the results and reduce random errors.

2.10 Evaluation procedure after pooling

2.10.1 Motility evaluation

Sperm motility is a critical parameter in reproductive technology**,** fundamentally influencing the ability of sperms to navigate through the female reproductive tract, facilitating the interaction with and fertilization of the oocyte (Suarez & Pacey, 2006).

As in the previous steps, the sperm were categorized as progressive motile, non-progressive motile or immotile. Total motility, an additional measure was used in this study and refers to all sperms showing movement regardless of whether the movement is progressive or not (Shukla, 2011).

Firstly, Eye motility (%) was assessed under a phase contrast microscope (Motic ba310e) with a magnification400 ×. Next, Sperm motility assessment using Computer-Assisted Sperm Analysis (CASA, Minitube, Germany): total sperm motility (%), progressive motility (%), and average path velocity(µm/s) were evaluated by CASA using AndroVision® software with customized slides (Lija, IMV Group, Netherland) (Palacín et al., 2013).

Figure 2

Motility Assessment by CASA

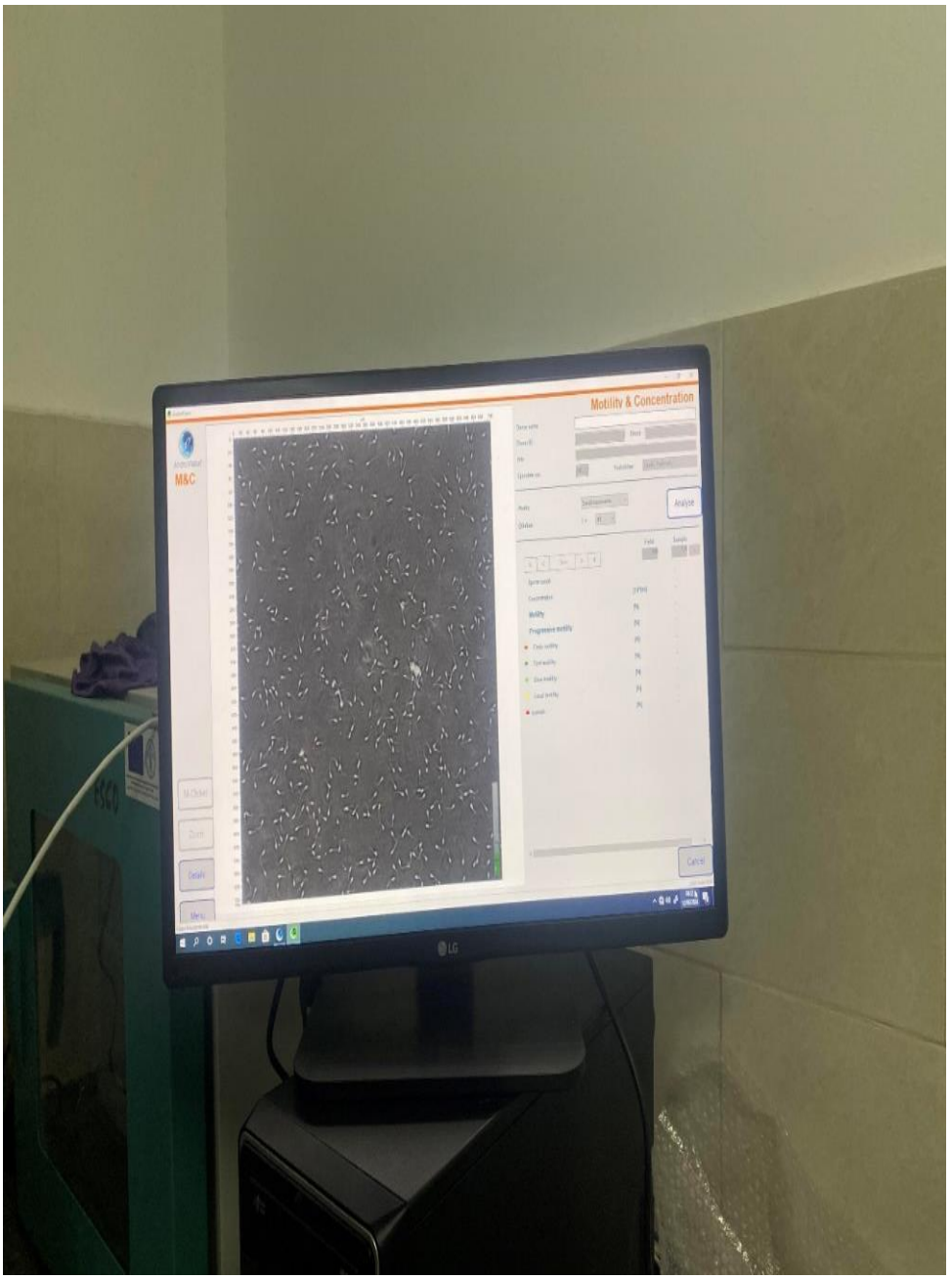
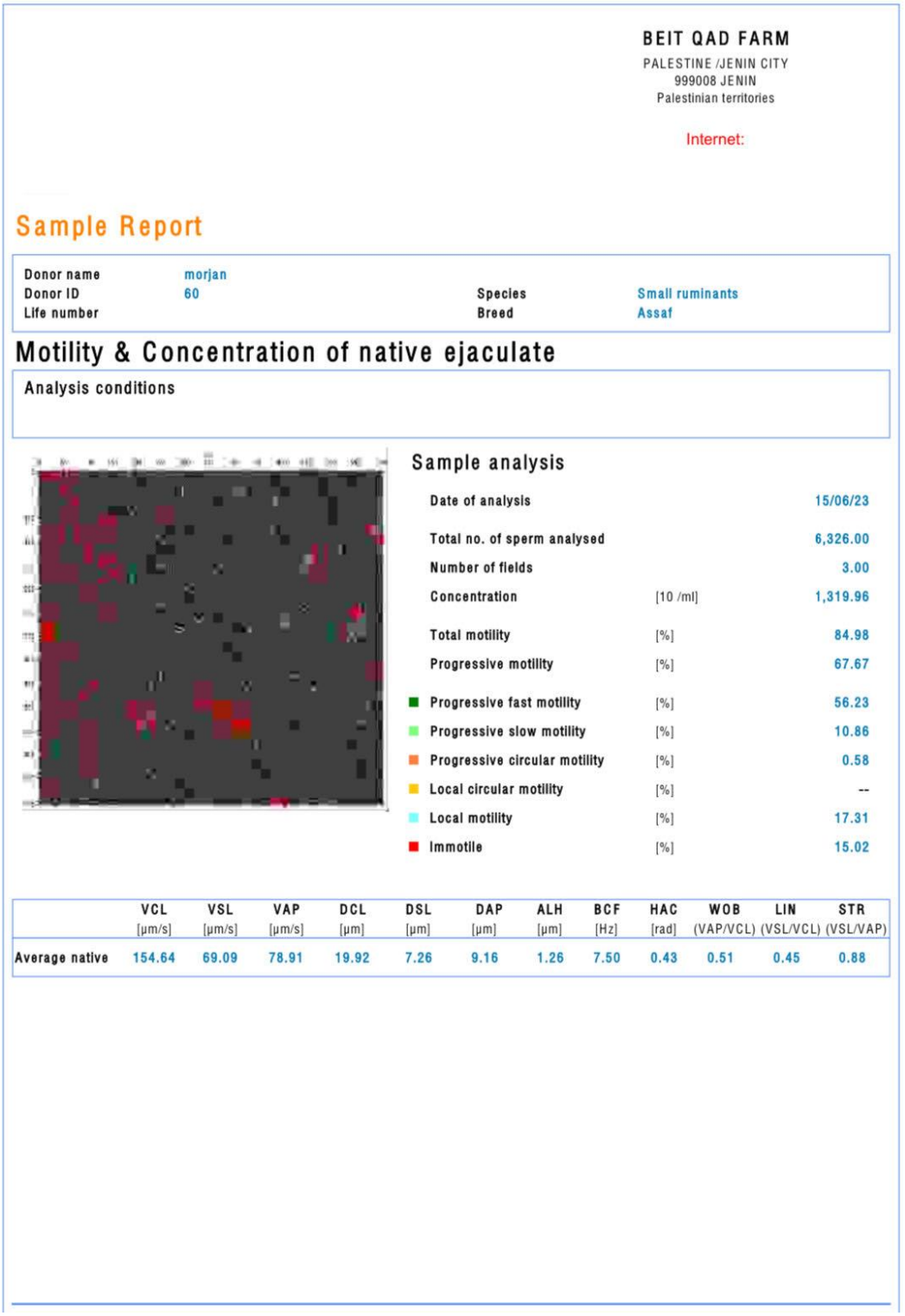


Figure 3

CASA Report during the Experiment, which includes Total and Progressive Motility, Average Path Velocity and Other Parameters



BEIT QAD FARM
PALESTINE /JENIN CITY
999008 JENIN
Palestinian territories

Internet:

Sample Report

Donor name	morjan		
Donor ID	60	Species	Small ruminants
Life number		Breed	Assaf

Motility & Concentration of native ejaculate

Analysis conditions

Sample analysis

Date of analysis		15/06/23
Total no. of sperm analysed		6,326.00
Number of fields		3.00
Concentration	[10 /ml]	1,319.96
Total motility	[%]	84.98
Progressive motility	[%]	67.67
Progressive fast motility	[%]	56.23
Progressive slow motility	[%]	10.86
Progressive circular motility	[%]	0.58
Local circular motility	[%]	--
Local motility	[%]	17.31
Immotile	[%]	15.02

	VCL [µm/s]	VSL [µm/s]	VAP [µm/s]	DCL [µm]	DSL [µm]	DAP [µm]	ALH [µm]	BCF [Hz]	HAC [rad]	WOB (VAP/VCL)	LIN (VSL/VCL)	STR (VSL/VAP)
Average native	154.64	69.09	78.91	19.92	7.26	9.16	1.26	7.50	0.43	0.51	0.45	0.88

2.10.2 Thermal stress test

In spermology, thermal stress test (TST) or thermo-resistance test is a crucial test for evaluating sperm resilience and viability after exposure to a high temperature environment (Bacinoglu et al., 2008). It predicts the longevity and fertilizing capability of sperm in the female reproductive tract (Fiser et al., 1991).

Directly after pooling, 30µl of pooled semen sample was incubated at 46°C in water bath (Minitube) for 15 minutes. Therefore, the total motility (%) and progressive motility (%) were evaluated by CASA (Minitube) using AndroVision® software (Bacinoglu et al., 2008).

2.10.3 Viability Test

Live/dead sperm viability test is an important quality test in determining sperm fertility via evaluating the permeability of sperm's plasma membrane (Shukla, 2011).

In this study, live/dead sperm percentage was estimated through using Eosin-Nigrosine staining technique by following Hancock procedure (Shukla, 2011).

Briefly, 30 g of nigrosine (Nigrosine water soluble, Sigma-Aldrich, Germany) was dissolved in 300 ml of sterilized water (B Braun) by heating and stirring over a digital hotplate stirrer (YR02937, Kalstein, France). Then, 5 g of eosin (Eosin Y disodium salt, Sigma-Aldrich, Germany) was dissolved in the prepared nigrosine solution by the same method. Finally, stain mixture was filtrated and stored at 4°C in glass vacuum bottle.

Sperm smear was prepared by mixing a drop of pooled semen with two drops of stain on the end of a warm slide. Afterwards, the mixture was left to sit for 2 minutes. Then, the mixture was spread immediately with the aid of a second slide. The smear was dried by keeping it on a laboratory heating plate (Warming plate with integrated control unit, Minitube, Germany) at 36.8°C (Shukla, 2011).

A total of 200 spermatozoa were examined under a light microscope (Bx53, Olympus, Japan) using 100× oil immersion objective.

Sperms that were partially or completely stained with purple were considered non-viable; only sperms showing strict exclusion of stain were considered viable (Shukla, 2011). The results were recorded in excel data sheet for statistical analysis.

2.10.4 Morphology evaluation

A normal mature spermatozoon consists of a flattened head that contains the nucleus, a neck that contains the centrioles, and a flagellum that contains the axoneme (Shukla, 2011). The reason for the evaluation of sperm abnormality is its negative effect on viability and motility. So, morphology assessment is a fundamental test in fertility evaluation.

As in the previous steps ,10 µl of pooled semen sample was added to micro tubes containing 100µl of buffered formol saline. From this mixture, 5 µl was placed on a microscope slide, covered with a glass coverslip, and examined under a phase-contrast microscope (Motic ba310e) at 1000× magnification.

A minimum of 200 sperm cells were counted during the observation. Spermatozoa were classified as having normal or abnormal morphology. Then, spermatozoa defects were classified as acrosome, head, mid-piece, and tail defects (Özturkler et al., 2001). The results were recorded in excel data sheet for statistical analysis.

2.10.5 Functional integrity evaluation

The integrity of spermatozoa plasma membrane is crucial for maintaining viability. Hypo osmotic swelling test (HOST) was used in the experiment to evaluate the functional integrity of the spermatozoal membrane.

Briefly, 20 µl of pooled semen sample was diluted in 350 µl sodium sulphate–fructose solution (75 mOsm) and incubated in 41 °C water bath (Minitube) for 45 minutes, then it fixed with 50 µl formalin-buffered saline. At that point,10 µl of prepared samples was used for evaluation. Two-hundred cells were evaluated under a phase-contrast microscope (Motic ba310e) at 400× magnification. Sperms were categorized based on their response to the hypo-osmotic environment into two distinct groups: reacted sperm, characterized by a visibly coiled tail, indicating an intact and functional plasma membrane, and non-reacted sperm, which displayed no visible tail coiling, suggesting compromised membrane integrity (Ramu & Jeyendran, 2013; Drevius & Eriksson, 1966).

The percentage of HOST-positive sperm was then calculated using the following formula:

$$\%\text{HOST} - \text{postive sperm} = \left(\frac{\text{Number of reacted sperm}}{\text{Total sperm counted}}\right) \times 100 \qquad 1$$

2.11 Semen dilution and packaging

Each pooled semen sample was divided equally into eight aliquots, with each aliquot was gradually diluted using one of the adenosine-supplemented diluents at concentrations of 0%, 0.5%, 0.75%, and 1% (V/V) at 37°C. Semen to diluent ratio was set at 1:4, ensuring consistency in sperm concentration across all treatments (Salamon & Visser, 1973; Lightfoot & Salamon, 1970).

The diluted samples were incubated in a beaker containing 40 ml of water at room temperature for five minutes, before being transferred to a cooling castle (Minitube). Subsequently, they were immediately filled and sealed in 0.25 ml plastic straws (Minitube) using the MPP Uno (MPP Uno, automatic filling and sealing machine for straw, Minitube). At the same time straws were labeled automatically by LEIBINGER JET3up inkjet printer (Minitube).

After 30 minutes, 150 µl of each treatment was taken, and equally divided into five tubes for evaluation as previously described (the same evaluation steps used after pooling).

Figure 4

Diluted Semen Samples in Cooling Castle Machine

2.12 Equilibration period and freezing process

The equilibration and cooling process is an essential step in ram semen cryopreservation, allowing gradual adaptation of sperm cells to low temperatures and cryoprotective agents before freezing process. In our experiment, straws were maintained for three hours (one and a half hour of cooling and one and a half hour of holding) inside the cooling castle (Minitube) at 5°C (Leelasiri et al., 1995).

After the equilibration period, 150 µl of each treatment was taken and equally divided into five tubes for evaluation as previously described (the same evaluation steps used after pooling). Then, all straws were transferred to IceCube14S, automatic freezer chamber (SY-LAB, Minitube) where they were frozen in five minutes with the following curve: five °C to minus ten °C at five °C/minute; minus ten °C to minus one hundred thirty°C at sixty °C/minute (Purdy, 2017). All frozen straws were stored at -196°C in liquid nitrogen container (Vozaf et al., 2021).

Figure 5

The MPP Uno, Automatic Filling and Sealing Machine for Semen Straw Preservation

Source: Minitube 2025

Figure 8

The IceCube14S, Automatic Freezer Chamber for Semen and Embryo Cryopreservation

Source: Minitube 2025

Figure 6

Semen Straws After Filling and Sealing in Beit-Qad Lab

2.13 Thawing and sperm analysis

Twelve days after storing straws in the liquid nitrogen container, three frozen straws were randomly selected for each treatment and thawed in a water bath (Minitube) at 37°C for 30 seconds (Purdy, 2017).

After thawing, the straws from each treatment were mixed gently. Then, 150µl was taken and divided into five tubes equally for final evaluation as the same previous steps after pooling and cooling.

2.14 Data collection

For each ejaculate, detailed data were recorded, including ram ID, date of collection, and initial evaluation. Furthermore, Semen quality parameters were recorded immediately after evaluation at each stage.

Additionally, freezing details such as freezing time, number of processed straws, straws information and specific treatment conditions were also documented. Finally, all collected data were compiled into an excel sheet for subsequent statistical analysis.

2.15 Statistical Analysis

The experimental design was a factorial treatment design with four levels of adenosine and two types of diluents arranged in a randomized complete block design (RCBD), where the collection session considered as a block. In the analysis model, blocks were treated as random effect while adenosine level and diluent type were considered as fixed effects.

The effect of adenosine levels and types of extenders were tested according to the following statistical model:

$$Y_{ijk} = \mu + B_k + \alpha_i + \beta_j + (\alpha \times \beta)_{ij} + e_{ijk}, \quad 2$$

where Y_{ijk} is the quantitative response variable, μ is the overall mean, B_k is the random effect of the k^{th} block (collection session), α_i is the fixed effect of i^{th} adenosine level, β_j is the fixed effect of the j^{th} extender type; $(\alpha\times\beta)_{ij}$ is the interaction effect, and e_{ijk} is the random error.

The data were analysed using mixed model procedures of IBM SPSS Statistics version 25.

Before analyzing the data, the variables were subjected to test of normality using Shapiro-wilk test, and the distribution was normal for all response variables.

The analysis of variance (ANOVA) was used for analysis of the results. To compare treatment means, the Bonferroni test was utilized.

2.16 Ethical considerations

Currently, there are no local regulations governing the animal research and experiments. Therefore, we followed the principles outlined by (National Research Council, 2011) in the guide for the care and use of laboratory animals.

Team members were informed about this study, its objectives, work procedures, evaluation methods, and potential risks. Furthermore, the farm manger, workers, and semen collectors received special instructions and training course on animal rights and welfare. Moreover, the animals were housed in modern, well planned and designed barns at Beit-Qad station.

In addition, biosecurity procedures, and animals' health status were monitored daily by qualified veterinarian. The animals also were introduced in the station's diseases prevention program, and given medical care and appropriate treatment in the case of illness.

Finally, all procedures and activities carried out in this study involving team members and study animals were designed to minimize distress and discomfort.

Chapter Three

Results

3.1 The effect of adenosine and diluents on motility parameters

Motility parameters obtained by Eye and CASA for Assaf rams during the three different freezing stages were presented in Tables 1 and 2. Adenosine and diluent had a significant impact ($P < 0.05$) on all motility parameters: Eye Motility (EM), Total Motility (TM), Progressive Motility (PM), and Average Path Velocity (VAP).

Table 1 presented the effect of adenosine levels on motility parameters. After 30 minutes of dilution, as adenosine level increased, there was an overall improvement in motility parameters. Adenosine at 0.75% resulted in the highest EM%, TM%, PM%, and VAP ($P < 0.05$), demonstrating its positive effect on sperm motility during the early dilution phase.

After equilibrium (after 3 hours of dilution), a similar trend was observed, where increasing adenosine level up to 0.75% improved all motility parameters significantly ($P < 0.05$) compared to the control (0% adenosine) and the other adenosine levels. This suggested that adenosine, particularly at 0.75%, provided stability to sperm function during this phase, possibly by enhancing energy metabolism and membrane integrity.

Post-thawing, adenosine positively influenced motility, with 0.75% adenosine yielding the highest improvements in EM%, TM%, and PM%, along with a notable increase in VAP ($P < 0.05$). The motility values were significantly ($P < 0.05$) higher at 0.75% adenosine than those of the control (0% adenosine), which exhibited the lowest motility parameters ($P < 0.05$) and other adenosine levels. These findings confirmed that adenosine, especially at 0.75%, played a crucial role in preserving the ram sperm motility throughout cryopreservation.

Table 2 presented the effect of diluent type on motility parameters. The results indicated that diluent type significantly ($P < 0.05$) influenced sperm motility parameters at all stages of the freezing process, with Andromed® consistently outperforming Tris-egg yolk in maintaining higher motility values. After 30 minutes of dilution, sperm extended in Andromed® exhibited superior motility, showing significantly higher total and

progressive motility, along with higher average path velocity ($P < 0.05$) compared to Tris-egg yolk. This suggested that Andromed® provided better initial support for sperm function.

Following equilibration stage (after 3 hours of dilution), Andromed® continued to maintain higher motility parameters ($P < 0.05$) than Tris-egg yolk, demonstrating its ability to protect sperm cells from osmotic and thermal stress during the cooling process. While motility declined at this stage in both extenders, sperm preserved in Andromed® retained a significantly better movement profile, indicating its enhanced protective properties.

Post-thawing, as expected, a notable decline in motility was observed in both extenders due to cryodamage. However, sperm samples diluted with Andromed® still exhibited significantly higher motility and velocity ($P < 0.05$) compared to those with Tris-egg yolk, confirming its superior ability to maintain sperm functionality after freezing and thawing.

Additionally, the lack of a significant interaction across all stages was observed ($P > 0.05$); this suggested that the relationship between adenosine and motility parameters was consistent across different diluents.

Overall, the use of Andromed® diluent instead of Tris-egg yolk extender and the addition of adenosine to extenders, especially at 0.75%, enhanced ram sperm motility across all stages, with statistically significant differences noted ($P < 0.05$).

Table 1

Effect of Adenosine Level% on Motility Parameters

Adenosine leve	EM%	CASA Parameters		
		TM%	PM%	VAP (µm/s)
After 30 minutes of dilution				
0%	67.86[1,]	72.29[d]	62.93[d]	113.66[d]
0.5%	70.98[c]	76.14[c]	65.85[c]	118.27[c]
0.75%	74.36[a]	78.73[a]	68.93[a]	121.05[a]
1%	71.89[b]	77.26[b]	67.00[b]	119.70[b]
SEM	0.50	0.36	0.66	0.46
After 3hours of dilution				
0%	61.31[d]	64.76[d]	53.21[d]	105.03[d]
0.5%	66.01[c]	69.95[c]	58.92[c]	113.71[c]
0.75%	70.49[a]	74.61[a]	64.59[a]	117.75[a]
1%	67.94[b]	71.89[b]	60.93[b]	115.62[b]
SEM	0.65	0.58	0.46	0.88
Post thawing				
0%	36.85[d]	39.98[d]	28.82[d]	82.34[d]
0.5%	41.58[c]	45.55[c]	36.48[c]	88.62[c]
0.75%	51.15[a]	57.77[a]	45.87[a]	93.64[a]
1%	44.18[b]	48.48[b]	39.11[b]	89.55[b]
SEM	0.54	0.56	0.63	0.96

EM: eye motility; CASA: computer-assisted sperm analysis; TM: total motility; PM: progressive motility; VAP**:** average path velocity; µm/s: micrometers per second. Data were reported as mean and standard error of the mean (SEM). [1] Values with different superscripts in the same column and stage are significantly different ($P < 0.05$).

Table 2

Effect of Diluent Type on Motility Parameters

Diluent	EM%	CASA Parameters		
		TM%	PM%	VAP (µm/s)
After 30 minutes of dilution				
Andromed®	72.21[1, a]	77.63[a]	67.96[a]	118.91[a]
Tris-egg yol	70.23[b]	74.58[b]	64.40[b]	117.44[b]
SEM	0.47	0.31	0.64	0.41
After 3hours of dilution				
Andromed®	67.42[a]	71.63[a]	61.21[a]	113.91[a]
Tris- egg yo	65.45[b]	66.99[b]	52.61[b]	112.15[b]
SEM	0.61	0.53	0.39	0.71
Post thawing				
Andromed®	44.93[a]	49.30[a]	38.91[a]	89.73[a]
Tris-egg yol	40.95[b]	44.59[b]	33.58[b]	80.35[b]
SEM	0.39	0.42	0.54	0.84

EM: eye motility; CASA: computer-assisted sperm analysis; TM: total motility; PM: progressive motility; VAP**:** average path velocity; µm/s: micrometers per second. Data were reported as mean and standard error of the mean (SEM).**[1]**Values with different superscripts in the same column and stage are significantly different ($P < 0.05$).

3.2 The effect of adenosine and diluents on sperm thermal resistance

The effect of adenosine level and diluent type on TM% and PM% obtained by CASA after TST during three different freezing stages was presented in Tables 3, 4, and Figure 10. Both adenosine and diluent had significant effects on TM% and PM% after TST in all stages.

Table 3 showed that adenosine levels significantly influenced sperm thermal resistance across all freezing stages ($P < 0.05$). After 30 minutes of dilution, motility parameters improved as adenosine concentration increased. Although all adenosine-incorporated samples exhibited higher motility ($P < 0.05$) than the control (0% adenosine), and 0.75% adenosine resulted in the highest values ($P < 0.05$), suggesting its potential stabilizing effect on sperm motility and longevity under thermal stress conditions.

After equilibrium (after 3 hours of dilution), the same trend persisted, with 0.75% adenosine showing the most significant improvement ($P < 0.05$) in maintaining both total and progressive motility. This indicated that adenosine, particularly at 0.75% concentration played a crucial role in protecting ram sperm cells from thermal stress and maintaining spermatozoa longevity.

Post-thawing, a sharp decline in motility was observed due to cryodamage. However, 0.75% adenosine still demonstrated the highest thermal resistance, with significantly better motility ($P < 0.05$) than the control (0% adenosine) and other adenosine levels. These findings confirmed that adenosine, particularly at 0.75%, enhanced sperm resilience against thermal stress and cryoinjury, making it a valuable additive for improving sperm survival during cryopreservation.

The results presented in Table 4 provided a comparative assessment of Andromed® and Tris-egg yolk diluents on sperm motility parameters (TM% and PM%) after samples were subjected to thermal stress at different stages of the freezing process. Subsequent to 30 minutes of dilution, semen samples diluted with Andromed® extender showed higher total and progressive motility ($P < 0.05$) compared to semen sample diluted with Tris-egg yolk extender. This suggests that Andromed® diluent may be a better choice for maintaining sperm activity and motility during this early stage.

After equilibrium stage, sperm motility still significantly higher ($P < 0.05$) in semen samples diluted with Andromed® compared to semen samples diluted with Tris-egg yolk extender.

Post-thawing, sperm motility after TST was remarkably dropped due to cryogenic damage and lipid peroxidation. However, sperm samples extended in Andromed® retained significantly higher motility ($P < 0.05$) values compared to those in Tris-egg yolk, confirming its greater ability to mitigate cryoinjury and maintain post-thaw functionality of ram frozen sperm. These findings highlighted the protective role of Andromed® in minimizing the detrimental effects of thermal stress and cryoinjury.

The interaction between adenosine level and diluent type significantly influenced progressive motility (PM%) after 30 minutes of dilution and thawing stages ($P < 0.05$), whereas no significant differences were observed in TM% across all stages ($P > 0.05$),

and PM% after the equilibrium stage ($P > 0.05$). Due to this selective significance, the interaction effect was specifically presented for PM% after thermal stress in the post-dilution and thawing stages in Figure 10.

After 30 minutes of dilution, sperm motility exhibited a general improvement with increasing adenosine concentration in both diluents, but Andromed® consistently maintained higher progressive motility values ($P < 0.05$) compared to Tris-egg yolk. The highest significant progressive motility after thermal practices ($P < 0.05$) was observed in Andromed® supplemented with 0.75% adenosine. The significant interaction at this early stage, indicating that the response to adenosine varied between diluents, and suggesting that Andromed® supplemented with 0.75% adenosine may provide the most effective protection against thermal stress.

Post-thawing, the interaction effect was also significant ($P < 0.05$), and sperm progressive motility after thermal practises was significantly higher ($P < 0.05$) when semen samples diluted in Andromed®. Just like after the dilution stage, Andromed® supplemented with 0.75% adenosine result in highest significant progressive motility after thermal exposure ($P < 0.05$). However, these results revealed that the intensity of adenosine's effect on post-thaw progressive motility after thermal practices was dependent on the diluent type used, and Andromed® supplemented with 0.75% adenosine was the best choice to minimize the detrimental effects of thermal stress. Overall, these findings proved that adenosine played a dynamic role in maintaining sperm motility under thermal condition, and its protective capacity was enhanced when incorporated into Andromed® diluent.

In general, we can conclude that incorporating adenosine up to 0.75% with Andromed® and Tris-egg yok semen diluents improved thermal resistance of ovine spermatozoa, and Andromed® was more efficient in protecting the vital functions of sperm when were exposed to thermal practices compared to Tris-egg yolk.

Table 3

Effect of Adenosine Level% on Sperm Thermal Resistance

Adenosine level	TM%	PM%
After 30 minutes of dilution		
0%	60.36[1, d]	34.20[d]
0.5%	64.78[c]	38.36[c]
0.75%	68.68[a]	43.03[a]
1%	66.33[b]	40.00[b]
SEM	0.49	1.01
After 3hours of dilution		
0%	53.05[d]	26.53[d]
0.5%	58.82[c]	33.52[c]
0.75%	64.09[a]	40.94[a]
1%	60.27[b]	35.67[b]
SEM	0.89	0.54
Post-thawing		
0%	10.15[d]	4.56[d]
0.5%	16.11[c]	8.80[C]
0.75%	26.06[a]	16.92[a]
1%	20.61[b]	12.37[b]
SEM	1.15	0.89

TM: total motility; PM: progressive motility. Data were reported as mean and standard error of the mean (SEM). [1]Values with different superscripts in the same column and stage are significantly different ($P<0.05$).

Table 4

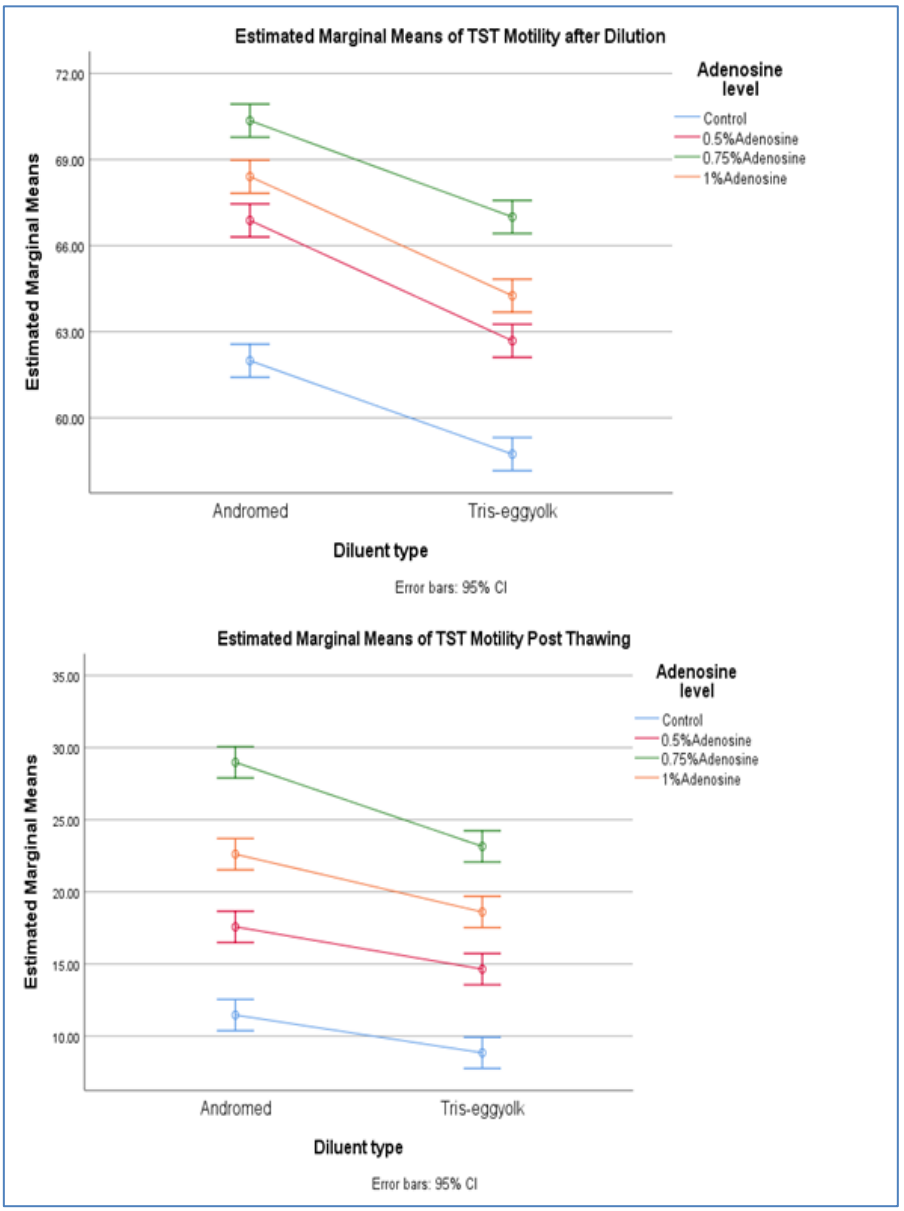
Effect of Diluent Type on Sperm Thermal Resistance

Diluent	TM%	PM%
	After 30 minutes of dilution	
Andromed®	66.91[1, a]	41.84[a]
Tris-egg yolk	63.17[b]	35.96[b]
SEM	0.47	0.99
	After 3 hours of dilution	
Andromed®	60.86[a]	36.54[a]
Tris- egg yolk	55.26[b]	31.63[b]
SEM	0.86	0.47
	Post thawing	
Andromed®	20.75[a]	13.12[a]
Tris-egg yolk	16.31[b]	9.25[b]
SEM	1.16	0.86

TM: total motility; PM: progressive motility. Data were reported as mean and standard error of the mean (SEM). [1]Values with different superscripts in the same column and stage are significantly different ($P<0.05$).

Figure 7

Effect of Diluent × Adenosine Level Interaction on Progressive Motility After Thermal Stress Test



3.3 The effect of adenosine and diluents on live sperm percentage

Tables 5 and 6 presented the effect of different adenosine levels and diluent type on sperm viability, measured as a percentage of live sperm across three key stages during the freezing process: after 30 minutes of dilution, after equilibrium (after 3 hours of dilution), and post-thawing. Adenosine and diluent had significant impacts ($P < 0.05$) on live sperm percentage in all stages.

Table 5 illustrated the effect of adenosine on live sperm percentage. After 30 minutes of dilution, no meaningful differences in live sperm percentage were found between the control(0%adenosine), 0.5% adenosine, and 1% adenosine ($P > 0.05$), but 0.75% adenosine significantly ($P < 0.05$) enhanced the live sperm percentage compared to the control and other adenosine levels, suggesting that this concentration provided the most effective early stabilization of ram sperm cells.

After equilibrium (after 3 hours of dilution), a more pronounced effect of adenosine was observed. Sperm viability significantly improved ($P < 0.05$), when adenosine was added to semen diluents. The 0.75% adenosine showed the highest percentage of live sperm ($P < 0.05$) compared to the control and other adenosine levels. This indicated that adenosine, specifically at 0.75% concentration played a protective role during this stage.

Post-thawing, adenosine at all its different levels significantly ($P < 0.05$) and positively affected the live sperm percentage, especially at 0.75%, which showed the best cryoprotective benefits ($P < 0.05$). This indicated that adenosine, particularly at 0.75% concentration boosted the viability of post-thawed ovine sperm, and minimized the negative effect of cryopreservation process. Thus, these results suggested that incorporating adenosine with ovine cryopreservation diluents, especially at 0.75% concentration had beneficial effects on sperm viability and vitality during freezing and thawing.

Table 6 illustrated the effect of diluent type (Andromed®/Tris-egg yolk) on the percentage of live sperm. Subsequent to dilution, semen samples diluted with Andromed® showed a higher percentage of live sperm ($P < 0.05$) compared to samples diluted with Tris-egg yolk. This superabundance of live sperm in samples diluted with Andromed®, suggested its potential superiority and ability to protect sperm membrane during this initial stage.

Post equilibrium (after 3 hours of dilution), the percentage of live sperm remained higher in samples diluted with Andromed® ($P < 0.05$) compared to samples diluted with Tris-egg yolk. This is another confirmation of the exceptional capability of Andromed® to enhance sperm survival during cryofreezing process.

Post-thawing, sperm viability stayed significantly higher ($P < 0.05$) in semen samples diluted with Andromed®.This conclusively confirmed the superiority of Andromed® over

Tris-egg yolk in the ability to maintain ovine sperm viability and vitality, through enhancing their survival potential.

These findings revealed that the use Andromed® in cryopreservation ovine semen has shown a high capability to maintain sperm viability, and it may be a better alternative to Tris-egg yolk diluent.

Despite the independent benefits of adenosine and diluent type on sperm viability, their interaction effect was not statistically significant ($P > 0.05$), implying that the two factors independently affected the live sperm percentage with no observable synergistic or antagonistic effects when combined.

Table 5

Effect of Adenosine Level% on Live Sperm Percentage

Adenosine level	Live %
After 30 minutes of dilution	
0%	82.76[1, b]
0.5%	82.78[b]
0.75%	84.31[a]
1%	83.15[b]
SEM	0.42
After 3 hours of dilution	
0%	73.01[d]
0.5%	74.62[c]
0.75%	76.59[a]
1%	75.21[b]
SEM	0.37
Post thawing	
0%	52.57[d]
0.5%	56.98[c]
0.75%	60.96[a]
1%	58.32[b]
SEM	0.89

Live%: live sperm percentage. Data were reported as mean and standard error of the mean (SEM). [1] Values with different superscripts in the same column and stage are significantly different ($P<0.05$).

Table 6

Effect of Diluent Type on Live Sperm Percentage

Diluent	Live%
After 30 minutes of dilution	
Andromed®	84.63[1, a]
Tris-egg yolk	82.36[b]
SEM	0.43
After 3 hours of dilution	
Andromed®	75.49[a]
Tris- egg yolk	74.23[b]
SEM	0.37
Post thawing	
Andromed®	59.16[a]
Tris-egg yolk	55.06[b]
SEM	0.86

Live%: live sperm percentage. Data were reported as mean and standard error of the mean (SEM). [1] Values with different superscripts in the same column and stage are significantly different (P<0.05).

3.4 The effect of adenosine and diluents on functional integrity of spermatozoal membrane

The integrity of the sperm membrane, a crucial factor in post-thaw sperm functionality, was influenced by both diluent and adenosine significantly ($P < 0.05$) across all stages, while their interaction did not have a significant effect ($P > 0.05$).

In Table 7, the results displayed the effect of different levels of adenosine on membrane integrity at different freezing stages.

After 30 minutes of dilution, increasing adenosine levels in the extender showed a slight improvement in intact sperm percentage, but significant ($P < 0.05$), suggesting a possible stabilizing effect during this early stage.

After equilibrium (after 3 hours of dilution), membrane integrity improved with increasing adenosine levels ($P < 0.05$), especially at 0.75%, which showed the highest intact sperm percentage ($P < 0.05$). This increases highlighted adenosine's ability to enhance membrane resilience and resistance against osmotic and thermal stress, particularly at 0.75% concentration.

Post-thawing, higher adenosine levels (0.75% and 1%) resulted in better membrane integrity ($P < 0.05$) compared to the control and 0.5% adenosine. Further confirmation that higher adenosine concentrations, particularly 0.75% concentration played a vital role in protecting spermatozoal membrane integrity.

Overall, these findings implied that adding adenosine to extenders, particularly at 0.75% provided a protective effect via enhancing membrane stability and resistance against cryodamage during the freezing-thawing cycle.

Similarly, diluent choice appears to had a significant effect ($P < 0.05$) on sperm membrane integrity in this study. The results in Table 8 focused on the effect of Andromed® and Tris-egg yolk in maintaining spermatozoal membrane integrity during the freezing process.

After 30 minutes of dilution, Andromed® showed a higher ($P < 0.05$) intact sperm membrane percentage compared to Tris-egg yolk, indicating its exceptional role in stabilizing the spermatozoa membrane at this initial stage.

After equilibrium (after 3 hours of dilution), Andromed® outperformed Tris-egg yolk ($P < 0.05$), showing greater capability to preserve sperm membrane integrity.

Post-thawing, Andromed® maintained significantly higher membrane integrity than Tris-egg yolk ($P < 0.05$), further confirming its superior protective effect and capacity.

On the whole, these results suggested that Andromed® extender was more effective at protecting spermatozoal membrane integrity and provided better osmotic balance across all stages.

Despite the significant effect of both diluents and adenosine, the interaction was not statistically significant ($P > 0.05$), which confirms that there is no synergistic or antagonistic effect.

Table 7

Effect of Adenosine Level% on Sperm Membrane Integrity

Adenosine level	Host positive (intact%)
After 30 minutes of dilution	
0%	77.44[1, d]
0.5%	78.02[c]
0.75%	79.59[a]
1%	78.58[b]
SEM	0.427
After 3 hours of dilution	
0%	50.56[d]
0.5%	53.09[c]
0.75%	58.18[a]
1%	54.57[b]
SEM	0.924
Post thawing	
0%	37.05[d]
0.5%	41.25[c]
0.75%	48.20[a]
1%	42.95[b]
SEM	0.824

Host positive (intact%): percentage of reacted sperm. Data were reported as mean and standard error of the mean (SEM). [1]Values with different superscripts in the same column and stage are significantly different ($P<0.05$).

Table 8

Effect of Diluent type on Sperm Membrane Integrity

Diluent	Host positive (intact%)
After 30 minutes of dilution	
Andromed®	79.86[1, a]
Tris-egg yolk	76.80[b]
SEM	0.42
After 3 hours of dilution	
Andromed®	56.12[a]
Tris- egg yolk	52.23[b]
SEM	0.90
Post thawing	
Andromed®	44.38[a]
Tris-egg yolk	39.35[b]
SEM	0.80

Host positive (intact%): percentage of reacted sperm. Data were reported as mean and standard error of the mean (SEM). [1] Values with different superscripts in the same column and stage are significantly different (P<0.05).

3.5 The effect of adenosine and diluents on sperm abnormalities

Table 9 illustrated the effect of varying adenosine levels on sperm abnormalities at various stages of the freezing process.

After 30 minutes of dilution, no major differences were observed among different adenosine levels (0, 0.5, 0.75, and 1%) as the percentage of acrosome, head, mid-piece, tail defects and total abnormal sperm remained relatively consistent across all adenosine levels ($P > 0.05$). This suggested that, adenosine did not significantly influence sperm structure ($P > 0.05$), within this early stage.

Following the equilibrium stage (after 3 hours of dilution), variations in sperm abnormalities became more apparent. The control group (0% adenosine) exhibited the highest percentage of total abnormal sperm and the percentage of all sperm morphological defect types ($P < 0.05$). In contrast, Adenosine-incorporated samples exhibited a significant decrease in the percentage of total abnormal sperm and all types of abnormalities ($P < 0.05$). Among all levels, 0.75% adenosine exhibited the most significant improvements, particularly in head and mid-piece regions ($P > 0.05$),

suggesting that supplementation the extenders with adenosine at 0.75% played an essential role in protecting sperm structure and had a defensive and preservative effect during cooling and equilibration. However, at 1% adenosine, a slight increase, but significant ($P > 0.05$) in the total sperm abnormalities percentage was noted compared to 0.75% adenosine, suggesting that excessive adenosine supplementation might have led to metabolic imbalances, osmotic stress, or toxicity.

Post-thawing, the differences between the adenosine levels became more evident and the beneficial effects of adenosine supplementation became more pronounced. The highest percentages of total abnormal sperm and all types of structural abnormalities were observed in the control group ($P < 0.05$), underscoring the detrimental effects of the freeze-thaw process on sperm morphology. On the other hand, all adenosine-incorporated sperm samples showed a progressive decline in in total abnormal sperm percentage and all types of structural abnormalities percentages ($P < 0.05$), highlighting adenosine's cryoprotective potential in minimizing ram sperm morphological damage caused by freezing and thawing. Among all levels, the most substantial reduction in abnormalities was observed at 0.75% ($P < 0.05$) with the greatest improvements recorded in the head and mid-piece regions ($P > 0.05$), further confirming its efficacy in preserving ovine sperm nuclear integrity and ATP production during cryopreservation.

Overall, adenosine demonstrated a capacity to reduce sperm abnormalities while maintaining the structural integrity of ovine sperm, particularly after equilibrium and post-thawing stage, with the 0.75% concentration showing the most consistent improvements against all defect categories, principally head and mid-piece defects.

Table 10 illustrated the effects of diluent type on sperm abnormalities. The evaluation was conducted as we mentioned before at three different stages during the cryopreservation process: after 30 minutes of dilution, after equilibrium (after 3 hours of dilution), and post-thawing.

After 30 minutes of dilution, total abnormal sperm percentage and the percentages of all categories of sperm abnormalities were lowered in sperm samples diluted with Andromed® than those diluted with Tris-egg yolk ($P < 0.05$), suggesting that the choice of diluent had an immediate impact on sperm morphology during this initial stage and Andromed® had greater capacity to preserve sperm membrane integrity and morphology.

After equilibrium (after 3 hours of dilution), the trend remained consistent and the protective effect of Andromed® became more evident and visible. Andromed® continued to show a lower total abnormal sperm percentage and lower percentages of all sperm defect categories compared to Tris-egg yolk ($P < 0.05$), except for acrosome defect, which remained very close in both diluents ($P > 0.05$). This indicated that Andromed® better preserved sperm structural integrity during the cooling and equilibration phase.

Post-thawing, sperm abnormalities increased in both diluents, as expected, due to the cryoinjury. However, Andromed® still maintained a significantly lower percentage of abnormal sperm ($P < 0.05$), and significantly lower percentage of head, mid-piece, and tail defects compared to Tris-egg yolk ($P < 0.05$), while the percentage of acrosome defect remained very close in both diluents as in the previous stage ($P > 0.05$). These results were clear evidence of Andromed®'s superiority in preserving ovine sperm morphology, even after thawing.

These findings indicated that Andromed® was more effective in preserving stability of sperm cell structure during cryopreservation, making it the preferred choice over Tri-egg yolk for maintaining sperm quality.

Despite this, the interaction between diluents and adenosine was not statistically significant ($P > 0.05$) in influencing sperm abnormalities at any stage of the freezing process. Consequently, the observed reduction in sperm abnormalities were primarily associated with the individual beneficial effects of adenosine and type of diluent used rather than an interactive enhancement between the two factors.

Table 9

Effect of Adenosine Level% on Acrosome, Head, Mid-piece, Tail Defects and Total sperm abnormalities Percentage

Adenosine level	Acrosome	Head	Mid-piece	Tail	Total
After 30 minutes of dilution					
0%	4.09	0.31	1.62	6.36	12.35
0.5%	4.12	0.30	1.60	6.31	12.32
0.75%	4.14	0.31	1.59	6.28	12.30
1%	4.15	0.30	1.57	6.29	12.29
SEM	0.37	0.07	0.12	0.52	0.92
After 3 hours of dilution					
0%	$4.44^{1, a}$	0.66^{a}	2.92^{a}	7.81^{a}	15.83^{a}
0.5%	4.18^{b}	0.44^{b}	2.80^{b}	7.52^{b}	14.90^{b}
0.75%	3.84^{c}	0.28^{c}	2.75^{b}	7.34^{cd}	14.18^{c}
1%	4.07^{b}	0.48^{b}	2.80^{b}	7.40^{bc}	14.75^{b}
SEM	0.23	0.17	0.23	0.20	0.50
Post-thawing					
0%	5.96^{a}	0.85^{a}	3.07^{a}	10.56^{a}	20.40^{a}
0.5%	5.48^{b}	0.70^{b}	2.97^{ab}	10.01^{b}	19.15^{b}
0.75%	5.11^{d}	0.45^{c}	2.87^{b}	9.60^{c}	18.01^{d}
1%	5.30^{c}	0.68^{b}	2.90^{b}	9.90^{b}	18.78^{c}
SEM	0.39	0.15	0.24	0.28	0.58

Total: total abnormal sperm percentage. Data were reported as mean and standard error of the mean (SEM).[1]Values with different superscripts in the same column and stage are significantly different ($P<0.05$).

Table 10

Effect of Diluent Type on Acrosome, Head, Mid-piece, Tail Defects and Total sperm abnormalities Percentage

Diluent	Acrosome	Head	Mid-piece	Tail	Total
After 30 minutes of dilution					
Andromed®	3.91[1, b]	0.23[b]	1.53[b]	6.01[b]	11.55[b]
Tris-egg yolk	4.32[a]	0.39[a]	1.67[a]	6.60[a]	12.98[a]
SEM	0.34	0. 09	0.17	0.46	0.87
After 3 hours of dilution					
Andromed®	4.14	0.40[b]	2.73[b]	7.45[b]	14.70[b]
Tris-egg yolk	4.12	0.52[a]	2.90[a]	7.58[a]	15.22[a]
SEM	0.22	0.12	0.21	0.13	0.48
Post-thawing					
Andromed®	5.43	0.61[b]	2.89[b]	9.15[b]	18.03[b]
Tris-egg yolk	5.50	0.76[a]	3.12[a]	10.22[a]	19.55[a]
SEM	0.37	0.15	0.23	0.25	0.77

Total: total abnormal sperm percentage. Data were reported as mean and standard error of the mean
(SEM).[1]Values with different superscripts in the same column and stage are significantly different ($P<0.05$).

Chapter Four

Discussion

4.1 Overview of study findings

This study investigated the impact of incorporating different levels of adenosine (0%, 0.5%, 0.75%, and 1%) with two widely used diluents (Andromed® and Tris-egg yolk) on ovine sperm quality throughout the freezing process, particularly on post-thaw quality of Assaf ram semen.

Our results demonstrated that adenosine supplementation, particularly at 0.75%, significantly enhanced sperm motility, viability, membrane integrity, resistance to thermal stress, and reduced sperm abnormalities. Furthermore, Andromed® consistently outperformed Tris-egg yolk in preserving ovine sperm quality parameters across all freezing stages. These findings underscore the potential of adenosine as a valuable additive to semen extenders and highlight the superior protective effects of synthetic egg yolk-free extenders (Andromed®).

4.2 Adenosine and sperm motility

With the development of microscopes and CASA systems, sperm motility appeared as a fundamental approach for the evaluation of male fertilization potential and reproductive capacity (Waberski et al., 2022). In ART, sperm motility is a basic assessment to make the decision in selection programs, as motility immediately influences the sperm's capability to navigate and migrate properly in the female reproductive tract and fertilize the ovum (Waberski et al., 2022).

However, our study revealed that there is a strong positive correlation between adenosine and sperm motility during cryopreservation of ovine semen. Incorporating adenosine with ovine semen extenders, particularly at 075% significantly enhanced ($P < 0.05$) motility parameters including total motility, progressive motility, and average path velocity. These findings are consistent with previous research emphasizing the role of adenosine in sperm motility via its interaction with A2A receptors within the membrane in sperm tail (X. Zhang & Ma, 2024; Vahidi et al., 2011), these receptors play a regulatory role in marinating sperm functions (Chen et al., 2024). The activation of these receptors

motivates cAMP production (Fraser, 1990) and triggers dynamin ATPases activity, leading to increased mechanical energy production in sperm flagellum (Caroppo, 2021; Romac et al., 1994), thereby enhances the flagellum's oscillation, and promotes sperm to start moving (Chen et al., 2024).

Additionally, our results are in agreement with previous studies validated the presence of cAMP-activated protein kinase (AMPK) in the head and midpiece region of spermatozoa cell (Zhu et al., 2018). Higher adenosine level induce the cAMP production and accumulation, which in turn lead to activating AMPK (Zhu et al., 2018). Once AMPK is activated, its play a vital role in marinating cellular energy balance (Zhu et al., 2018; Liguori et al., 2004), and modulating spermatozoa motility via altering lactate, and ATP level (Zhu et al., 2018; Hurtado de Llera et al., 2012).

Moreover, similar results were obtained when adenosine was added to infertile men semen (Vahidi et al., 2011), and Andromed® extender used for storage of ram semen in liquid state (Bezerra et al., 2019), indicating the coherence of scientific evidence on the role of adenosine in enhancing sperm motility.

4.3 Diluent type and sperm motility

Successful semen cryopreservation can be achieved when suitable diluents is used along with appropriate cooling and freezing procedure (Frim & Mazur, 1983). In the present study, the choice between Andromed® and Tris-egg yolk for cryopreservation of Assaf ram semen was investigated.

The results revealed that Andromed® diluents preserved and improved sperm motility parameters better than Tris-egg yolk diluents ($P < 0.05$) during the cryopreservation process.

The superiority of Andrormed® in maintaining sperm motility may be because it contains soybean lecithin instead of egg yolk. Egg yolk contain a small spherical structures globules called yolk globules, these globules contribute to an increase in the viscosity of the medium, as in Tris-egg yolk diluent, and when spermatozoa are suspended in a high viscosity medium their ability to move freely and efficiently can be compromised (Aires et al., 2003). Thus, it may be lead to impair sperm motility when the diluent used contain egg yolk (Vishwanath & Shannon, 2000). The possibility of bacterial contamination

associated with the use of Tris-egg yolk may also be another reason for Andromed® superiority (Bustani & Baiee, 2021).

However, Our results are harmonized with previous literatures, which documented that soybean lecithin-based extenders, such as synthetic Andromed®, demonstrate elevated or at least similar capability to egg yolk-based extenders, such as Tris-egg yolk, in maintaining and upgrading functional motility of ovine (El-Azzazi & Yaseen, 2016; Depaz et al., 2010; Forouzanfar et al., 2010) and bovine (Sawitri et al., 2021; Miguel-Jimenez et al., 2020; Baharum et al., 2017) spermatozoa.

4.4 Adenosine and sperm viability

In our study, the term of sperm viability referred to live sperm percentage in semen samples. Sperm viability is an important factor in assessing semen quality, as it significantly and positively correlated to sperm motility and fertilizing capacity (Shukla, 2011).

Incorporating adenosine with semen diluents, particularly at 0.75% concentration significantly improved sperm viability ($P < 0.05$). As we mentioned earlier, adenosine play a vital role in cellular homeostasis and metabolism, alongside its stimulating behavior to antioxidant defense system, and ATPases. These functions of adenosine may have contributed to improve sperm membrane stability, and reduce lipid peroxidation, consequently lead to prevent premature apoptosis, and maintain live sperm percentage.

The results of our research are consistent with previous studies on thawed human semen (Huang et al., 2003), frozen-thawed boar semen (Funahashi & Nagai, 2001), and cooled ram semen (Bezerra et al., 2019).

4.5 Diluent type and sperm viability

In present study, we observed that the live sperm percentage was significantly higher ($P < 0.05$) in sperm samples diluted with Andromed® compared to sperm samples diluted with Tris-egg yolk across all stages during the cryofreezing process.

As previously stated, Andromed® contain soybean lecithin as a replacement for egg yolk. Soybean lecithin and egg yolk have the similar key components, and they are phosphatidylcholine, palmitic acid, oleic acid, and stearic acid (Bustani & Baiee, 2021).

These components have properties that enable it to prevent cold and freeze shock, providing stability to spermatozoa membrane during cooling and freezing process (Bustani & Baiee, 2021).

However, Andromed® has features that may be the reason for its superiority over Tris-egg yolk diluents. It is egg yolk-free extender, and does not contain any component from an animal protein source (Baharum et al., 2017). Furthermore, it contains a unique combination of tylosin, gentamicin, spectinomycin, and lincomycin, which are microbial protein synthesis inhibitors(Santos & Silva, 2020). These features contributes to reduce the potential risk of microbials contamination and consequently, improve the stability of sperm cell structure and sperm motility, reduce the occurrence of sperm agglutination, and protect lipids in the spermatozoa membrane from peroxidation, which finally lead to enhance sperm viability (Santos & Silva, 2020).

In the light of this, our findings are in agreement with previous research that emphasized the excellent capability of Andromed® in maintaining the quality and viability of sperm (Sawitri et al., 2021; Lydia et al., 2012; Aires et al., 2003).

However, some researchers reported that no significant differences between soybean lectin based extender and egg yolk based extender in preserving sperm viability (Bustani & Baiee, 2021; Gogol et al., 2019), while others found that egg yolk based extender was more effective in preserving sperm viability during freezing and thawing procedures (A Swelum et al., 2019). This difference in results between studies may due to species-specific factors, different biosecurity procedures, different freezing protocols, and variations in egg source and quality (Anzar et al., 2019).

4.6 Adenosine and thermal stress resistance

As previously mentioned, TST is an important test in assigning the degree of deterioration in semen quality after freezing and thawing (Fiser et al., 1991). It estimates the spermatozoa resilience, longevity and fertilizing capacity in the female reproductive tract (Fiser et al., 1991).

Within this research, the incorporation of adenosine with semen diluents was significantly improved ($P < 0.05$) the sperm resistance and the 0.75% adenosine concentration showed the best cryoprotective effect ($P < 0.05$).

These results highlight the close and positive association between adenosine and sperm longevity, confirming previous research findings on the effective role of adenosine in regulating spermatozoa function (X. Zhang & Ma, 2024), and stimulating its fertilizing ability (Fraser, 1990).

4.7 Diluent type and thermal stress resistance

During the comparison of Andromed® and Tris-egg yolk, semen samples diluted with Andromed® showed better thermal resistance ($P < 0.05$) compared to those diluted with Tris-egg yolk.

As is well known, Andromed® is soy bean lecithin-based extender (Baharum et al., 2017), and soy bean lecithin has lower viscosity compared to egg yolk (Li et al., 2015). This is mainly because egg yolk lecithin mainly contains a higher concentration of phosphatidylcholine than soybean lecithin (Li et al., 2015). Although phosphatidylcholine is important component to preserve sperm during cooling and freezing, but the high viscosity it causes as in Tris-egg yolk extender leads to negative results such as the inability of the sperm to breathe and move properly (Salmani et al., 2013; Crespilho et al., 2012). This in turn may be linked to Andromed® appearing more efficient in enhancing sperm's ability to resist thermal stress.

After all, our observations align with previous reports in the field, which indicated that soy bean lecithin-based extenders like Andromed® maintain similar or superior sperm quality compared to egg yolk-based extender (Sawitri et al., 2021; Baharum et al., 2017; Aires et al., 2003).

4.8 Adenosine and functional integrity of spermatozoal membrane

The spermatozoal plasma membrane plays a crucial role in maintaining homeostasis (Anzar et al., 2019), defending against extracellular agents, and facilitating sperm capacitation and interaction with oocytes (Tapia et al., 2012). Its structural integrity is essential for optimal sperm function and fertility (Ramu & Jeyendran, 2013).

Our study found that adenosine, particularly at 0.75%, significantly improved sperm membrane integrity ($P < 0.05$), as reflected in HOST results. This is likely due to adenosine's role in reducing lipid peroxidation (Correia & Vale, 2024), regulating lipid homeostasis (Koupenova & Ravid, 2013) and other functions as we mentioned earlier.

Concerning the effect of adenosine on integrity of spermatozoal membrane, no study has been conducted.

4.9 Diluent type and integrity of sperm membrane

The study revealed that Andromed® consistently outperformed ($P < 0.05$) Tris-egg yolk in preserving spermatozoa membrane integrity across all freezing stages.

The superior performance of Andromed® in maintaining sperm membrane integrity maybe related to the various reasons we discussed earlier. The protective properties and low viscosity of soy bean lecithin (Li et al., 2015),and its antioxidant capacity (El-Azzazi & Yaseen, 2016) are closely related to the efficiency of Andromed® as it is the main ingredient . Furthermore, when egg yolk-based extender is used, ovine sperm become more susceptible to phospholipids peroxidation due to the greater concentrations of PUFAs in the plasma membrane (Carro et al., 2022), and the lack of antioxidants in sperm cell of ovine (Salamon & Maxwell, 1995). In addition, lipid particles in egg yolk-based extenders may have an adverse impact by forming a physical barrier around spermatozoa, potentially disrupting their natural trajectory (Crespilho et al., 2012). Moreover, the reduced percentage of intact sperm in Tris-egg yolk compared to Andromed® in our study may be related microbial aspects.

However, our results are in agreement with previous studies who reported that soy bean lecithin-based diluents are efficient in protecting the spermatozoa membrane (El-Azzazi & Yaseen, 2016; Chelucci et al., 2015; Crespilho et al., 2012; Amirat et al., 2005).

4.10 Adenosine and sperm morphology

In terms of morphology, sperm cells consist of three main parts: head, midpiece, and tail.

The head of sperm is usually oval in shape and contains the nucleus with DNA as well as an acrosome, which holds the enzymes (Shukla, 2011).Sperms with defects in the head, such as those that are elongated, detached, pyriform, too small, oversized, round head or double heads, may have difficulty piercing the egg's outer layer (Zona pellucida) and are more susceptible to DNA damage (Moskovtsev et al., 2009).

The mid-piece (body), which contains mitochondria organelles that are responsible for producing energy and centrioles (Shukla, 2011). Sperm with mid-piece abnormalities,

such as asymmetrical insertion of the midpiece into the head, sharply bent, thick or thin midpiece, may result in energy deficiencies, affecting motility (Shukla, 2011).

The tail part (flagellum) consists of two pieces (the principle and end pieces); both pieces are necessary for sperm motility (Shukla, 2011). Tail defects such as a short, bent, or coiled tail can cause erratic movement or an inability to swim effectively. However, prior studies have established a strong correlation between cryopreservation and damage in structural morphology of sperm cells (Shukla, 2011) .

Our study revealed that adenosine supplementation significantly reduced ($P < 0.05$) sperm abnormalities, particularly in head and mid-piece regions, with 0.75% adenosine showing the most significant effect ($P < 0.05$). This suggests that adenosine may play a protective role in preserving sperm structural integrity through its regulatory mechanisms in cell homeostasis, and its other important functions that we mentioned previously (X. Zhang & Ma, 2024; Bellezza & Minelli, 2017; Fraser & Duncan, 1993) .

No studies have been conducted to evaluate the impact of adenosine as an additive to semen cryofreezing diluents on the morphological abnormalities of animal sperm. The findings of this study can be regarded as the first assessment.

4.11 Diluent type and sperm morphology

Andromed® resulted in a lower percentage of sperm abnormalities ($P < 0.05$) compared to Tris-egg yolk. This suggested that Andromed® may be a good and better alternative to Tris-egg yolk extender in future cryopreservation of ram semen protocol.

Many factors that contribute to the improvement in stability and integrity of sperm structure when diluting semen samples with Andromed®, have been mentioned previously. Several studies supported our findings which that soybean lecithin- based extenders like Andromed® provide exceptional protection for sperm through freezing process (El-Azzazi & Yaseen, 2016; Aires et al., 2003).

4.12 Strength points and limitations of study

4.12.1 Strengths

1. Innovative approach to cryopreservation One of the key strengths of this thesis is the novel incorporation of adenosine into ram semen extenders. This innovation presents new opportunities for enhancing cryopreservation strategies in ovine frozen semen, potentially improving post-thaw sperm quality and function.
2. Comprehensive sperm quality assessment .The research thoroughly evaluated sperm quality using multiple critical tests, including motility, viability, and morphology, at three essential stages of cryopreservation:

- After 30 minutes of dilution
- After 3 hours of dilution
- Post-thawing

This multi-stage analysis ensured the reliability and validity of the results, offering a deeper understanding of the changes that occur in the sperm and its function throughout the cryopreservation process.

3. Thermal stress test. The inclusion of TST further strengthened in our study. This test provided essential information into sperm resistance, longevity, and potential fertility.
4. Comparison of diluents .The study also compared the effectiveness of Andromed® and Tris-egg yolk diluents, providing valuable data for selecting the most suitable diluent for freezing Assaf sheep semen.
5. Adherence to ethical standards .Strict ethical guidelines were followed to ensure the well-being and appropriate care of the study's animals. This commitment to ethical research practices represents an additional strength of the study.

4.12.2 Limitations

Regardless of the contribution of this study to the development of frozen semen diluents for ovine, it has several limitations that should be acknowledged:

1. Limited sample size. The study was conducted on only six rams, which may restrict the generalizability of the findings. A larger sample size would strengthen the conclusions and improve statistical power.
2. Short experimental duration. The short duration of the study did not allow for an assessment of the long-term viability of cryopreserved sperm across different seasons.

Seasonal variations may influence sperm quality, and future research should consider extended observation periods.

3. Focus on a single Breed. The study exclusively examined the sperm from Assaf breed, which may limit the applicability of the findings to other ovine breeds or species. Further investigations involving multiple breeds would enhance the broader relevance of the results.
4. Variability in Egg Yolk Quality. The inconsistency in egg yolk quality, and the possibility of microbial contamination presents a potential challenge, as it may affect the reproducibility of results associated with the Tris-egg yolk extender. Buy eggs from a single source may contribute to minimize the variation in egg yolk quality and conducting tests to ensure that egg yolk is free from microbial contamination in future research could help mitigate this variability.

4.13 Contribution of findings to existing knowledge

The findings from our study on adenosine's role in ram semen cryopreservation significantly enhanced our understanding in several key areas. The research demonstrates that adenosine, a molecule known for its role in cellular energy metabolism, enhances sperm membrane integrity, viability, thermal resistance, and motility during the freezing and thawing process., deepening our knowledge of its biological effects on ovine sperm.

The study also focused on the challenges and determinants facing the successful ovine semen cryopreservation, as ovine sperm is particularly susceptible to thermal stress and oxidative damage, primarily due to the high content of PUFAs in its membrane and the lack of antioxidant content, underscoring the need for species-specific semen extenders formulation and different cryopreservation protocols to enhance sperm quality in rams and similar livestock.

Furthermore, the incorporation of adenosine into semen diluents showed potential for enhancing diluent quality, which is particularly relevant for breeding stations and semen banks. Improved diluents could lead to higher post-thaw sperm viability and fertility rates, making this finding highly valuable for artificial insemination programs.

Moreover, this study opens the door for future research into the optimal concentrations of adenosine for each species, the adenosine pathway and function in sperm cell when it used as additive to cryofreezing media.

While this study primarily focused on ovine sperm and two types of diluents, it contributes to the broader understanding of adenosine's role in reproductive science. The findings may have implications for cryopreservation strategies in other species, including humans and bovines.

4.14 Recommendations and conclusion

4.14.1 Recommendations

- We recommend incorporating adenosine at a concentration of 0.75% with ovine semen diluents.
- Andromed® is preferable to the Tris-egg yolk extender for use in ovine sperm banks and breeding stations.
- Future research should include larger sample sizes and diverse species to improve the generalizability of results.
- To build on the results we found, future studies should also explore the effect of combining adenosine with other antioxidants in semen diluents to determine the optimal formulation for improving frozen semen quality, particularly in ovine.

4.14.2 Conclusion

We evaluated the effects of incorporating varying adenosine concentrations (0, 0.5, 0.75, and 1%) in Tris-egg yolk and Andromed® diluents on the frozen semen quality of Assaf rams. Adenosine supplementation improved all quality parameters during freezing and thawing, with the 0.75% concentration yielding the most significant enhancements in motility, viability, membrane integrity, thermal resistance, and normal morphology. Additionally, our study indicated that Andromed® was more efficient than Tris-egg yolk in maintaining the frozen sperm quality of ovine. However, the interaction between adenosine and diluents was not significant in most cases, suggesting that adenosine's effects on semen quality are consistent across different diluents. Further research is necessary to fully elucidate the role of adenosine in spermatozoa physiology and optimize cryopreservation protocols for improved reproductive success in ovine and other species.

List of Abbreviations

Abbreviation	Meaning
AI	Artificial Insemination
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ART	Assisted reproduction technology
NARC	National agricultural research center
cAMP	Cyclic adenosine monophosphate
PUFAs	Polyunsaturated fatty acids
ATP	Adenosine triphosphate
AV	Artificial vagina
AP	After pooling
SOD	Superoxide dismutase
HOST	Hypo osmotic swelling test
RCBD	Randomized Complete Block Design
ANOVA	Analysis of variance
EM	Eye motility
TM	Total motility
PM	Progressive motility
VAP	Average path velocity
TST	Thermal stress test
Live%	A percentage of live sperm
CASA	Computer assisted sperm analysis
CP	Crude Protein
Mg	Magnesium
P	Phosphor
Ca	Calcium
Na	Sodium

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Appendices

Appendix A

Initial Evaluation of Ram's Ejaculates

CS No.	Ram ID	Volume(ml)	Clarity	Color	Conc(10^9/ml)	Eye Motility (%)
1	46	1.92	Clear	Creamy	4.98	85
1	54	1.55	Clear	Creamy	5.12	77
1	52	1.52	Clear	Creamy	5.07	78
1	53	1.33	Clear	Creamy	4.96	85
1	48	1.12	Clear	Thin Creamy	4.15	75
1	60	1.85	Clear	Creamy	5.31	85
2	46	2.00	Clear	Creamy	5.11	85
2	54	1.42	Clear	Creamy	5.06	82
2	52	1.50	Clear	Creamy	4.98	78
2	60	1.93	Clear	Creamy	5.16	85
2	53	1.57	Clear	Creamy	4.97	78
2	48	1.38	Clear	Thin Creamy	3.22	76
3	60	1.96	Clear	Creamy	5.35	85
3	48	1.26	Clear	Creamy	4.47	80
3	46	1.85	Clear	Creamy	5.19	80
3	52	1.48	Clear	Creamy	4.88	75
3	54	1.52	Clear	Creamy	4.96	75
3	53	1.44	Clear	Creamy	4.87	76
4	48	1.23	Clear	Thin Creamy	3.66	75
4	60	1.96	Clear	Creamy	5.22	85
4	46	1.98	Clear	Creamy	5.05	85
4	52	1.58	Clear	Creamy	4.92	82
4	53	1.47	Clear	Creamy	4.86	81
4	54	1.45	Clear	Creamy	5.02	82
5	53	1.55	Clear	Creamy	4.83	85
5	54	1.72	Clear	Creamy	5.24	83
5	60	2.05	Clear	Creamy	5.16	86
5	48	1.33	Clear	Thin Creamy	3.50	75
5	46	1.98	Clear	Creamy	5.13	86
5	52	1.62	Clear	Creamy	4.90	84
6	60	1.97	Clear	Creamy	5.26	80
6	46	2.11	Clear	Creamy	5.11	81
6	48	1.43	Clear	Creamy	4.55	75
6	53	1.58	Clear	Creamy	4.88	80
6	54	1.82	Clear	Creamy	4.94	82
6	52	1.56	Clear	Creamy	4.97	85

Appendix B

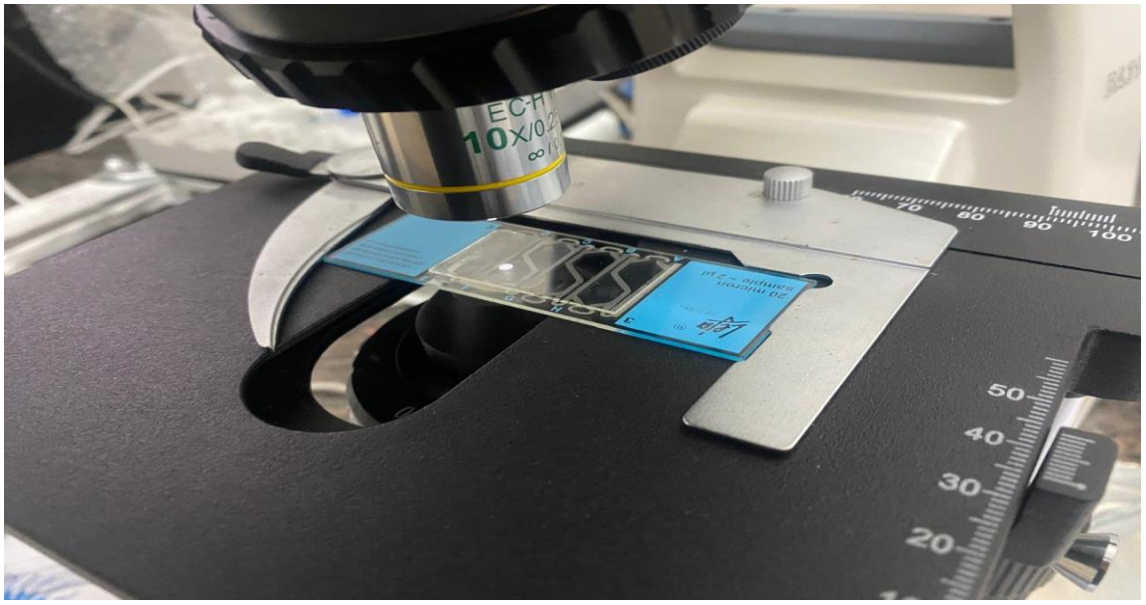
Effect of Diluent × Adenosine Level Interaction on Progressive Motility After Thermal Stress Test

Diluent	Adenosine level	PM%
	After 30 minutes of dilution	
	0%	35.90[1, d]
	0.5%	40.97[c]
Andromed®	0.75%	47.11[a]
	1%	43.38[b]
	0%	32.51[g]
	0.5%	35.76[f]
Tris-egg yolk	0.75%	38.95[e]
	1%	36.63[f]
SEM		1.04
	Post-Thawing	
	0%	5.77[d]
	0.5%	9.84[c]
Andromed®	0.75%	18.97[a]
	1%	13.90[b]
	0%	3.94[h]
	0.5%	7.77[g]
Tris-egg yolk	0.75%	14.47[e]
	1%	10.84[f]
SEM		0.95

PM: progressive motility. Data were reported as mean and standard error of the mean (SEM).[1] Values with different superscripts in the same column and stage are significantly different ($P < 0.05$).

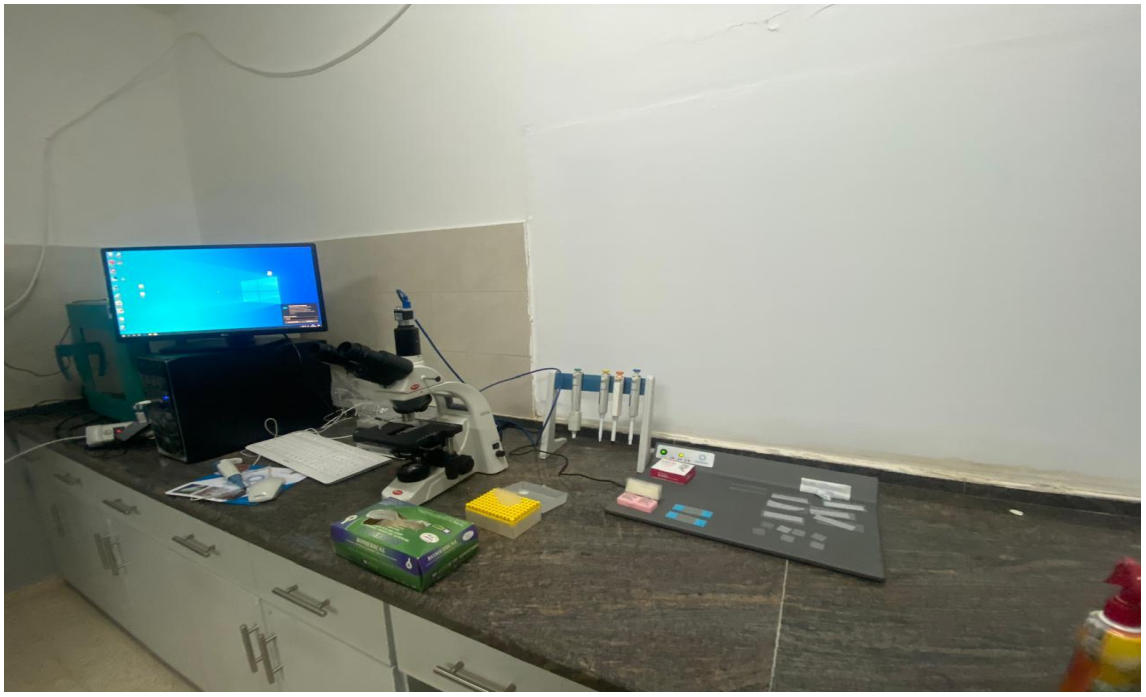
Appendix C

Leja Slides Which was Used in the Experiment



Appendix D

The Picture Show the CASA Device, Motic ba310e Microscope, and Heating Plate



Appendix E

The Picture show samples Reception Area

جـامعـــة النجاح الوطنية

كليـــة الدراســـات العليـــا

دمج تراكيز مختلفة من مركب الأدينوسين مع محاليل التمديد تريس–صفار البيض والأندروميد المستخدمة في تجميد السائل المنوي للكباش

إعداد

موسى خليل

إشراف

د. جهاد عبد الله

قدمت هذه الرسالة استكمالا لمتطلبات الحصول على درجة الماجستير في الانتاج الحيواني، من كلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس– فلسطين.

2025

دمج تراكيز مختلفة من مركب الأدينوسين مع محاليل التمديد تريس–صفار البيض والأندروميد المستخدمة في تجميد السائل المنوي للكباش

إعداد

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اشراف

د. جهاد عبد الله

الملخص

الخلفية: في علم تكاثر الأغنام، تكتسب تقنيات حفظ السائل المنوي للكباش بالتجميد أهمية خاصة في برامج التحسين الوراثي، حيث تساهم في تسريع نقل الموروثات الجينية المتفوقة إلى القطعان ذات الإنتاجية المنخفضة، وتُمكّن من الحفظ طويل الأمد على الموارد الوراثية القيّمة، كما تحدّ من القيود الجغرافية في برامج التلقيح الاصطناعي. ومع ذلك، لا تزال تقنيات تجميد الحيوانات المنوية للضأن تواجه صعوبات تحد من إمكانية نجاحها. حيث إن الحيوانات المنوية للكباش أكثر عرضة للإجهاد الحراري والتأكسدي أثناء التجميد والإذابة. الأدينوسين هو جزيء جوهري موجود في كل مكان ويتكون من الأدنين المرتبط بسكر الريبوز بواسطة رابطة جليكوسيدية. وقد ثبت أنه يحسن استقلاب الطاقة والدفاعات المضادة للأكسدة؛ إلا أن تطبيقه المحتمل في حفظ السائل المنوي للضأن بالتجميد لا يزال غير مكتشف.

الأهداف: تهدف هذه الدراسة لتقييم القدرة الواقية للأدينوسين عند تراكيز مختلفة (0%,0.5%,0.75%,1%) في حماية وظائف الحيوانات المنوية للكباش وشكلها خلال عملية التجميد. بالإضافة إلى ذلك، يقارن البحث الكفاءة والفعالية التبريدية لكل من الممدد التجاري أندروميد والممدد التقليدي تريس–صفار البيض في الحفاظ على جودة الحيوانات المنوية.

الأساليب: تم جمع السائل المنوي من ست كباش عساف تتراوح اعمارها بين 3 –4 سنوات كل خمسة أيام لمدة شهر، وتم تمديد السائل المنوي باستخدام ممددات الأندروميد وتريس–صفار البيض المدعمة بالأدينوسين. تم تقييم عينات السائل المنوي من حيث الحركة، والحيوية، والمقاومة للحرارة، وسلامة الغشاء والشكل بعد 30 دقيقة و3 ساعات من التخفيف وبعد الإذابة. خضعت البيانات لتحليل التباين (ANOVA) مع اختبار بونفورني لتقييم تأثيرات تركيز الأدينوسين ونوع المدد.

النتائج: أدى استخدام الأدينوسين، خاصة بتركيز 0.75 %, إلى تحسين جودة الحيوانات المنوية بشكل ملحوظ في جميع مراحل التجميد ($P < 0.05$). علاوة على ذلك، تفوق الممدد أندروميد على المدد تريس–صفار البيض في الحفاظ على جودة الحيوانات المنوية المجمدة ($P < 0.05$).

الخاتمة: أدى إدراج الأدينوسين في ممددات السائل المنوي إلى تحسين جودة الحيوانات المنوية للضأن خلال التجميد. كما أثبت الممدد الأندروميد أنه بديل واعد لممدد تريس–صفار البيض في تجميد السائل المنوي للأغنام.

الكلمات المفتاحية: الأدينوسين، التجميد، السائل المنوي للضأن، تريس–صفار البيض، الأندروميد