



UNIVERSITI PUTRA MALAYSIA

**EVALUATION OF GANODERMA LUCIDUM AS AN ANTIOXIDANT
SUPPLEMENT IN GOAT SEMEN CRYOPRESERVATION**

AMINAH SAUDAH MAZIDAH BINTI ZULKEFLI

**Ip
FPV 2023 9**

**EVALUATION OF GANODERMA LUCIDUM AS AN ANTIOXIDANT
SUPPLEMENT IN GOAT SEMEN CRYOPRESERVATION**

AMINAH SAUDAH MAZIDAH BINTI ZULKEFLI

A project paper submitted to the
Faculty Of Veterinary Medicine, Universiti Putra Malaysia

In partial fulfilment of the requirement for the
DEGREE OF DOCTOR VETERINARY MEDICINE

Universiti Putra Malaysia (UPM)

Serdang, Selangor Darul Ehsan

DECEMBER 2023

CERTIFICATION

It is hereby certified that we have read this project paper entitled “Evaluation of Ganoderma Lucidum as An Antioxidant Supplement In Goat Semen Cryopreservation”, by Aminah Saudah Mazidah binti Zulkefli and in our opinion, it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD4999 Final Year Project.

ASSOC. PROF DR NURHUSIEN YIMER DEGU
DVM (ADDIS ABABA UNIVERSITY), PhD (UPM)

Associate Professor
Faculty of Veterinary Medicine,
Universiti Putra Malaysia
(Supervisor)

DR. MARK HIEW WEN HAN
DVM (UPM), PhD (PURDUE UNIVERSITY)

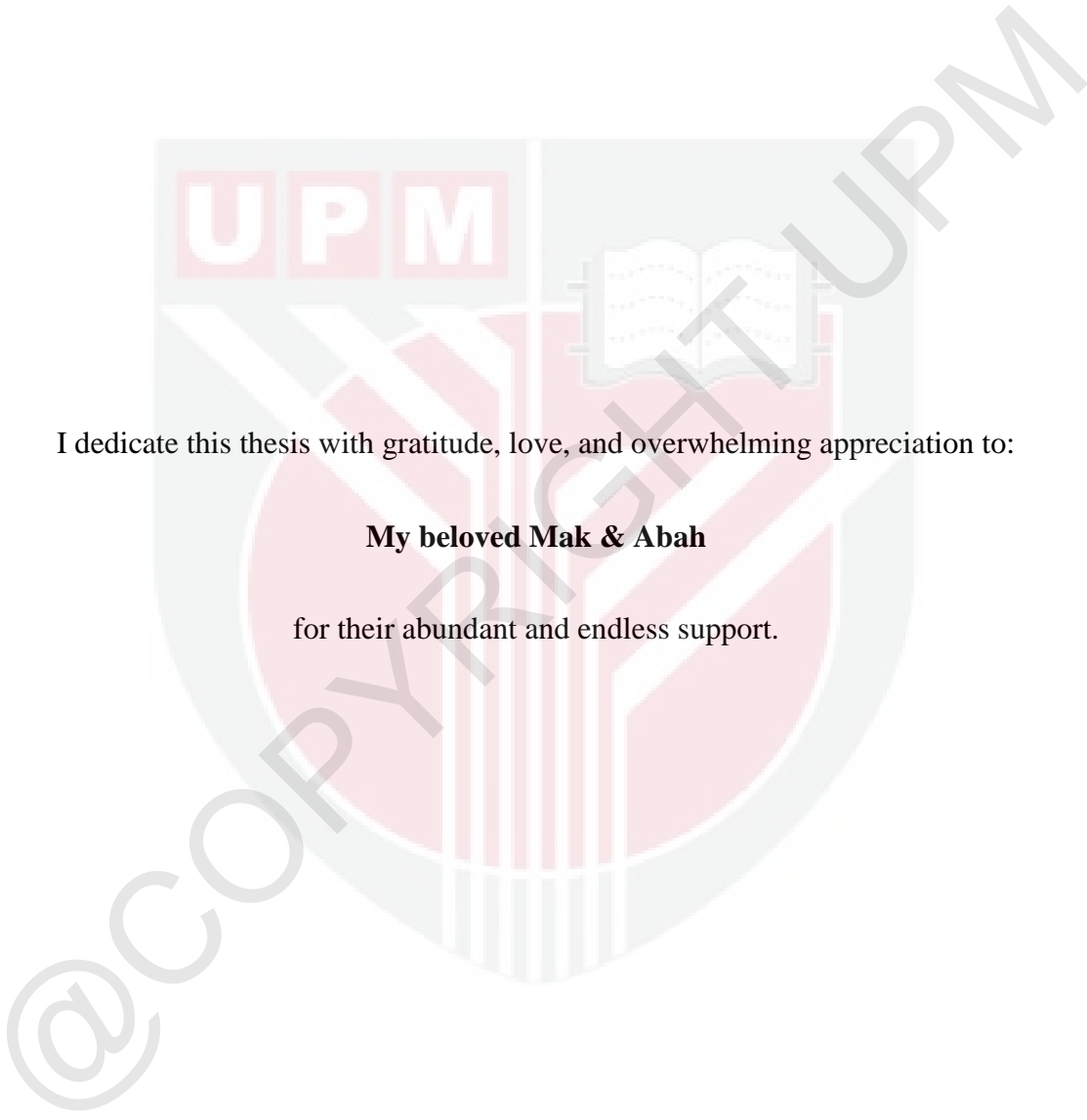
Senior Lecturer
Faculty of Veterinary Medicine,
Universiti Putra Malaysia
(Co-Supervisor)

DEDICATION

I dedicate this thesis with gratitude, love, and overwhelming appreciation to:

My beloved Mak & Abah

for their abundant and endless support.



ACKNOWLEDGEMENT

First and foremost, I would like to express my utmost gratitude to Allah S.W.T for granting me patience, perseverance and keeping myself sane throughout this FYP period which is quite challenging at times. He is the one who granted me every chance I am here up to this moment. Alhamdulillah.

To my parents and siblings, thank you so much for the support by mentally and physically being there for me when I needed them the most to gain strength, and also for the financial support too. May He bless all of you and give you a better rizq each day. Ameen.

To my supervisor who lent me this amazing idea for me to experience, it was really great to have this project as my first stage of experience in research field. I owed him a lot for the guidance and insights to make this project happened. Thank you to Farid, my FYP partner as well, for helping me throughout the process.

To my friends, especially my roommate, Kam. Thank you for lending me your shoulders to cry on. Thank you for the advices, validations and reassurance that I would love to hear from. Hope things are easier for you too.

To everyone else that directly and indirectly involved in conducting this project; Mr Tijjani, Dr Mark, Dr Halimatun, En Fahmi, Farhan and fellow staffs of Faculty of Agriculture and Faculty of Veterinary Medicine, without all of your help and guidance, I would not be able to carry out this project smoothly. Only God can repay their great help to me.

TABLE OF CONTENTS

TITLE	I
CERTIFICATION	II
DEDICATION.....	III
ACKNOWLEDGEMENT.....	IV
TABLE OF CONTENTS	V
LIST OF FIGURES	VI
LIST OF TABLES	VII
LIST OF ABBREVIATIONS	VIII
ABSTRAK	IX
ABSTRACT.....	XI
1.0 INTRODUCTION.....	1
1.1 Hypothesis.....	2
2.0 LITERATURE REVIEW	3
2.1 Ganoderma lucidum (GL).....	2
2.2 Semen Collection and Cryopreservation	2
3.0 MATERIALS AND METHODS	7
3.1 Semen Extender and Extract Preparation.....	7
3.2 Animals Selection and Semen Collection	7
3.3 Semen Processing and Microscopic Analysis	8
3.3.1 Sperm mass and individual motility.....	9
3.3.2 Sperm concentration.....	10
3.3.3 Sperm viability and general morphological abnormality.....	10
3.4 Semen Chilling and Cryopreservation	11
3.5 Malondialdehyde (MDA) And Superoxide Dismutase (SOD) Assessment	11
3.6 Statistical Analysis	11
4.0 RESULTS	13
5.0 DISCUSSION	22
6.0 CONCLUSION	26
7.0 RECOMMENDATIONS.....	27
REFERENCES.....	28

LIST OF FIGURES

- Figure 1 Rates of sperm mass motility with different GL concentrations
- Figure 2 Rates of individual progressive motility of sperm with different GL concentrations
- Figure 3 There is no significant difference ($p>0.05$) among all extender groups toward individual progressive motility.
- Figure 4 Rates of live sperm cells with different GL concentrations
- Figure 5 There is no significant difference ($p>0.05$) among all extender groups toward viable sperm.
- Figure 6 Rates of dead sperm cells with different GL concentrations
- Figure 7 There is no significant difference ($p>0.05$) among all extender groups toward viable sperm.
- Figure 8 Rates of sperm's general morphological abnormalities
- Figure 9 There is no significant difference ($p>0.05$) among all extender groups toward general morphological abnormalities sperm parameter

LIST OF TABLES

- Table 1 Motility scale of sperm
- Table 2 Results of Superoxide dismutase (SOD) assay
- Table 3 Comparison of SOD concentration between the extenders



LIST OF ABBREVIATIONS

GL	Ganoderma lucidum
%	Percentage
IPM	Individual Progressive Motility



ABSTRAK**PENILAIAN TERHADAP *GANODERMA LUCIDUM* SEBAGAI ANTIOKSIDAN
TAMBAHAN DALAM PENGKRIOPRESERVASI AIR MANI KAMBING****By****Aminah Saudah Mazidah binti Zulkefli****2023****Penyelia: Prof Madya Dr Nurhusien Yimer Degu**

Penggunaan antioksidan semula jadi telah menarik perhatian yang besar sebagai strategi berpotensi untuk meningkatkan kualiti dan kebolehjangkaan spermatozoa yang dibekukan disebabkan oleh fungsi untuk melindungi spermatozoa daripada kecederaan oksidatif ketika proses kriopreservasi. *Ganoderma lucidum* (GL), sejenis cendawan perubatan yang terkenal dengan sifat antioksidannya berpotensi untuk meningkatkan kejayaan pengawetan air mani dalam pelbagai spesies haiwan. Kajian ini bertujuan untuk meneroka potensinya sebagai tambahan antioksidan dalam mengekalkan kualiti spermatozoa kambing selepas pembekuan dengan menilai kesan penggunaannya terhadap kebolehjangkaan dan gerakan spermatozoa selepas proses pembekuan. Sampel air mani telah dikumpulkan tanpa penggabungan daripada jumlah tiga ekor kambing (*Capra hircus*) jantan berusia di antara tiga hingga lima tahun dari Ladang 15 UPM di mana sepuluh ejakulasi berjaya diperolehi. Ejakulasi dikumpulkan menggunakan vagina tiruan (VT) dengan kehadiran seekor kambing betina berumur tiga tahun. Air mani tulen dianalisis bagi kualitinya, dan hanya sampel air mani yang mempunyai sekurang-kurangnya 70% motiliti am yang diproses lebih lanjut dan dicairkan ke dalam penyebet telur Tris yang mengandungi tiga kepekatan ekstrak GL yang berbeza. Keputusan data dianalisis menggunakan ujian ANOVA dua hala dan Kruskal-Wallis H, yang menunjukkan bahawa sperma yang telah dibekukan dengan 0%, 0.5% dan 1.0% *G. lucidum* dalam penyebet tidak menunjukkan perbezaan yang ketara ($p > 0.05$) terhadap parameter kualiti

sperma termasuk pergerakan progresif individu (%), kebolehjangkaan (%) dan kelainan morfologi (%) selepas pencairan. Data analisa Superoksida dismutase (SOD) tidak menunjukkan ketara bagi walau dengan penambahan GL sebagai antioksida. Kesimpulannya, penambahan GL dalam penyebat belum dapat dibuktikan sebagai pilihan antioksidan yang baik untuk pengawetan air mani kambing. Walau bagaimanapun, penyelidikan lanjut terhadap fungsi *G. lucidum* dalam mengekalkan kualiti sperma selepas pencairan patut dijalankan.

Kata kunci: *Ganoderma lucidum*, cendawan Reishi, cendawan Lingzhi, antioksida, kambing (*Capra hircus*), pengkriopreservasi air mani

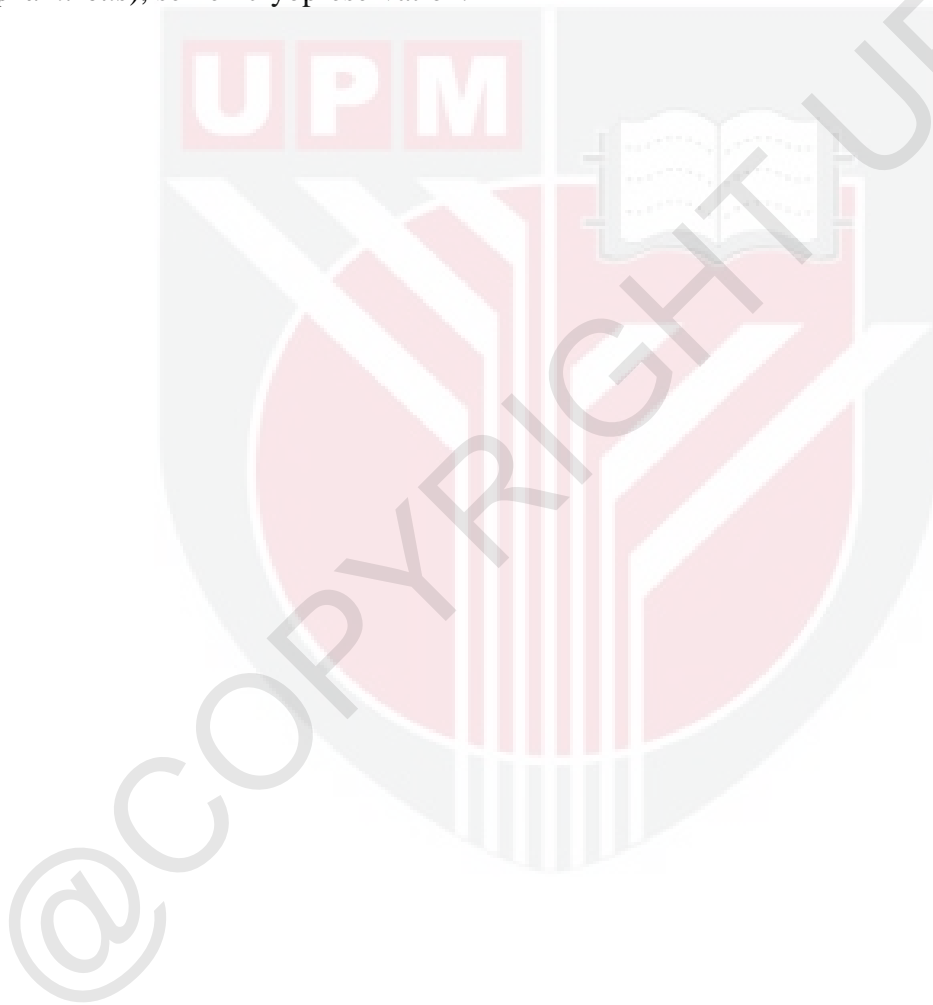


ABSTRACT**EVALUATION OF *GANODERMA LUCIDUM* AS AN ANTIOXIDANT
SUPPLEMENT IN GOAT SEMEN CRYOPRESERVATION****By****Aminah Saudah Mazidah binti Zulkefli****2023****Supervisor: Assoc. Prof Dr Nurhusien Yimer Degu**

The application of natural antioxidants has gained considerable attention as a potential strategy to enhance the quality and viability of cryopreserved spermatozoa due to its function in protecting the spermatozoa from oxidative injury caused by freezing and thawing. *Ganoderma lucidum* (GL), a medicinal mushroom with well-documented antioxidant properties, has emerged as a promising candidate for improving the success of semen cryopreservation in various animal species. This study explores the potential of GL as an antioxidant supplement in preserving the quality of buck spermatozoa post-cryopreservation through evaluating the impact of it on sperm viability and motility. Semen samples were collected without pooling from a total of three individual bucks with ages ranging from three to five years at Ladang 15 UPM until ten ejaculates were achieved. The ejaculates were collected using artificial vagina (AV) with the presence of a three years old teaser doe. The neat semen was analysed for its quality, and only semen samples with at least 70% general motility were further processed and being diluted into Tris-egg yolk extenders that contain three different concentrations of GL extracts. The data results were analysed using two-way ANOVA and Kruskal-Wallis H test, showing that sperm that were cryopreserved with 0%, 0.5% and 1.0% GL in semen extender has no significant difference ($p > 0.05$) toward sperm quality parameters including individual progressive movement (%), viability (%) and morphological abnormality (%) at post-thaw.

Superoxide dismutase (SOD) data analysis also showed that it is not statistically significant despite having GL as antioxidant supplement. In conclusion, supplementation of GL in the extender is yet to be proven as an excellent choice of antioxidant for goat semen cryopreservation. However, further research against the functions of GL in preserving the sperm quality at post-thaw should be conducted.

Keywords: *Ganoderma lucidum*, Reishi mushroom, Lingzhi mushroom, antioxidant, goat (*Capra hircus*), semen cryopreservation.



1.0 INTRODUCTION

Semen cryopreservation is a technique used to store sperm samples for future use. The capacity to cryopreserve goat sperm is difficult, but it has received attention because of the prospective benefits, such as the ability to store and transport sperm for artificial insemination and genetic enhancement projects. Cryopreservation involves freezing the sperm at low temperatures, which might degrade the quality of the sperm sample, resulting in decreased motility and viability. As a result, optimizing the cryopreservation method is critical to minimize damage while maintaining sperm viability and motility (Purdy, 2006; Lv et al., 2019).

The use of suitable extenders and cryoprotectants as well as addition of antioxidants can aid in the protection of sperm cells during the freezing process. Several studies have explored the effects of antioxidant supplementation on the quality of cryopreserved goat semen, with a focus on improving sperm motility, viability, and morphology. Given the susceptibility of goat sperm to oxidative stress during cryopreservation, the potential benefits of antioxidant supplementation in preserving sperm quality are of great significance for goat breeding and artificial insemination programs in the future (Hassan et al., 2021; Zhang et al., 2022).

Incorporation of antioxidants have been shown to possess protective properties against oxidative stress that compromise sperm function during the cryopreservation process. According to Hassan et al. (2021), the use of plant extracts or specific antioxidant compounds in semen extenders has demonstrated promising results in maintaining sperm quality. Li et al. (2022) has mentioned about strong antioxidative properties of *G. lucidum* in their studies, suggesting that there is a potential in improving sperm quality in rats. However, there is limited research on the use of *G. lucidum* as an antioxidant supplement in extenders for goat semen cryopreservation although there have been several studies on the use of natural products as antioxidants in semen cryopreservation of different livestock species.

The main objective of current study is to evaluate the potential of *G. lucidum* as an antioxidant supplement for goat semen cryopreservation although there are limited studies on the antioxidative properties toward post-thaw sperm quality. Hence, the purpose of this study is to bridge the knowledge gap pertaining to the limited understanding regarding the roles of *G. lucidum* as an antioxidant supplement and its impact on post-thaw sperm quality.

1.1 Hypothesis

Null hypothesis (H₀): The addition of *Ganoderma lucidum* as supplement during semen cryopreservation has no significant effect on the post-thaw quality of buck spermatozoa.

Alternative hypothesis (H_A): The addition of *Ganoderma lucidum* as supplement during semen cryopreservation has significant effect on the post-thaw quality of buck spermatozoa.

2.0 LITERATURE REVIEW

2.1 *Ganoderma lucidum* (GL)

Ganoderma lucidum (Lingzhi or Reishi), a traditional medicinal mushroom, historically valued in Chinese medicine for fostering well-being and extending lifespan, has been utilized in Japan and Korea for preventing and treating diverse ailments (Watchel-Galor, 2011) in human traditional medicine. Revered as "God's herb" in ancient China, it was thought to prolong life, invigorate the spirit, and maintain vitality (Oke et al., 2022). In these recent years, *G. lucidum* has gained attention due to its potential health benefits, including its antioxidant properties. Renowned for its immunomodulatory, antioxidative, and antimicrobial attributes, this mushroom serves as a widely favoured supplement for humans, both healthy individuals and cancer patients in conjunction with standard treatments (Watchel-Galor, 2011).

The mushroom consists of bioactive substances that contribute to its therapeutic properties, including triterpenes, polysaccharides, and other active ingredients (Li et al., 2022). It has been discussed by Büyük et al. (2022) and Li et al. (2022) that *G. lucidum* antioxidative qualities may contribute to its capability to improve reproductive function, by alleviating oxidative stress as it may exert negative impact on sperm quality and function. The addition of *G. lucidum* has recorded a good performance in enhancing sperm motility rate in rats which plays an important factor in male fertility as suggested by Büyük et al. (2022). In their studies, they investigated the effects of GL towards testicle tissue, spermatogenic series cells and sperm motility in mice by administering 2 ml physiologic serum with different dosage of 500 mg/kg, 2500 mg/kg and 5000 mg/kg GL extract orally. In another study by Ali & Ghazal (2013), similar

method was also applied where three groups of rabbits were fed with diet supplemented with GL at different dosage of 0g/ton, 200g/ton and 400g/ton diet. In that study, both treated groups recorded improvement in terms of libido and sperm quality parameters but 200g/ton was more an ideal dosage for mature buck. It also has been proven that triterpenoids in *G. lucidum* have improved the histomorphology and function of tests in middle-aged male mice by reducing oxidative stress and cellular death (Li et al., 2022).

2.2 Semen collection and cryopreservation in goats

In current study, the method of semen collection used for bucks is by artificial vagina (AV). It is the most preferred method due to its positive impact on the quality of the semen collected. There are several studies have compared semen collection techniques and found that the AV method yields better semen quality than the other methods, for instance, the use of an electro-ejaculator (EE) or palpation. It is supported by Bopape et al. (2015) where the study conducted a comparison between AV and EE for semen collection in bucks, they found that the AV was more suitable to collect semen from the species, particularly in indigenous bucks. This is because the AV yielded better semen quality as evidenced by parameters such as sperm concentration, motility and pH (Memon, 1986). Moreover, post-thaw sperm quality demonstrates significant improvement when collected via artificial vagina, as indicated by elevated percentages across all sperm parameters (Jiménez-Rabadán et al., 2016; Lukusa et al, 2020). Due to the superiority of the AV method of collection from goats, it has been further supported by researches on other species such as drake semen collection and the implementation of egg yolk as semen extender (Kasai et al., 2001).

In semen cryopreservation, superoxide dismutase (SOD) is an essential antioxidant enzyme that plays a crucial role in protecting spermatozoa from oxidative stress, thereby influencing sperm quality and fertility. In the context of goat semen cryopreservation, the evaluation of SOD levels and its impact on sperm quality parameters is of significant importance. SOD is a class of metalloenzymes that catalyse the dismutation of superoxide radicals into oxygen and hydrogen peroxide, thus serving as a primary defence against oxidative stress (Mara et al., 2007). It protects them from the damaging effects of reactive oxygen species (ROS) and preserving their functional and structural integrity. Therefore, the addition of antioxidants to the cryopreservation medium holds promise in mitigating the adverse effects of oxidative stress on sperm quality and improving post-thaw sperm parameters in goats (Dorado et al., 2010). While malondialdehyde (MDA) is a by-product of lipid peroxidation, a chemical process that occurs when polyunsaturated fatty acids in cell membranes are oxidized by reactive oxygen species (ROS). It is used as an indicator of a biomarker of oxidative stress and membrane damage in various biological samples, including semen, as suggested by Gangwar et al. (2016). Therefore, the evaluation of MDA levels in goat semen cryopreservation can provide valuable understandings into the extent of oxidative stress and its potential impact on sperm quality parameters.

A number of studies has been conducted to investigate the SOD and MDA activities due to supplementation of antioxidants in semen cryopreservation. For instance, Sharafi et al. (2018) has discussed the effects of honey and *Nigella sativa* oil as natural antioxidants. It resulted an improvement of fresh and post-thaw sperm quality and survival rates where it significantly reduces MDA activity and increases SOD activity. Another study also used natural antioxidants from ginger and echinacea extracts to observe their effects toward the quality and fertility potential of frozen-

thawed sperm in rams (Merati & Farshad, 2020). It has also recorded a significant reduced MDA levels and increased SOD activity. Meanwhile, a study by Sobeh et al. (2021) investigated the effects of a polyphenol-rich extract from *Entada spiralis* on the quality of cryopreserved goat semen. The study found that the addition of the polyphenol-rich extract to semen extenders improved post-thaw sperm quality by reducing MDA levels and increasing SOD activity. The authors suggested that the polyphenol-rich extract could be used as a natural antioxidant supplement in semen extenders for goat semen cryopreservation.

Hence, in current study, it aims to observe and evaluate the effects of *G. lucidum* as antioxidant whether it could improve the sperm quality parameters by reducing the MDA levels and increasing SOD activities.

3.0 MATERIALS AND METHODS

3.1 Semen Extender and Extract Preparation

Tris-egg yolk extender were prepared by mixing well 3.8g Tris, 2.1g citric acid and 20% chicken egg yolk in water to make 100ml extender solution. 1g D-fructose and 6.8% glycerol were added after the solution is adequately mixed. In preparation of the extract, *Ganoderma lucidum* (GL) powder is obtained from human's Ganoderma health supplement capsules (Gano Excel Sdn Bhd, Malaysia). To prepare the stock solution, 1g of GL powder from the capsule was mixed with 100ml of deionized water. The mixture was then soaked for three hours at room temperature to be fully dissolved and was centrifuged at 1000rpm for 30 minutes. The supernatants were filtered using a 0.45 microns membrane filter to filter out any sediments according to the methods described by Ali & Mervat (2013). There were two extenders that were added with GL extract, which T1 (0.5% GL) and T2 (1.0% GL), while another extender without addition of the extract (0% GL) was used as a control. In the preparation of T1, 0.5ml of the extract was taken from the stock solution to be included into the 100ml of Tris-egg yolk extender, making 0.5% GL extract in the extender. Similar technique was also applied to prepare 1.0% GL extract. All extender groups were maintained for their pH of 6.8 to 7.0.

3.2 Animals Selection and Semen Collection

Semen samples were collected without pooling from a total of three individual bucks from different breed with ages ranging from three to five years at Ladang 15 UPM until ten ejaculates were achieved. All of these bucks were previously trained for AV. Apart from being healthy, bucks showing normal and active behaviour were selected. AV is

the most commonly used technique of collecting semen for goats as the procedure is painless, convenient, resembling natural mating process and not stress-inducing. The ejaculates were collected using artificial vagina (AV) with the presence of a four years old doe as a dummy. The goats were all fed with fresh fodder napier grass twice per day and were given drink from tap water sources daily, and the pens were cleaned once daily in the morning. The bucks and doe were observed for any abnormality in terms of behaviour and temperament. The AV was set up by assembling the cylinder with a rubber lining attached at the opening. A temperature-controlled thermos was filled with warm water approximately around body temperature (37°C) before being poured into the AV. After complete assemble, lubricant (K-Y* Lubricating Jelly, Johnson & Johnson Medical, United States) was administered to the inner liner's open end in the artificial vagina, while a 15ml Eppendorf conical tube was inserted into a clear conical plastic attached at the other end of AV for semen collection. The tube and the conical plastic sheet were ensured to be dry and there was no debris or water that came out of the AV. The collected ejaculates were stored inside the 15ml Eppendorf conical tube before being transported to the lab for further processing. Prior to collection, the teaser doe was tied and secured to the designated crush to allow mounting of the buck and gives better ejaculate. After multiple false mount attempts, the person with the AV directed the penis to the AV when the buck shows sign of ejaculating. Collected semen in the test tube was protected in a prewarmed thermal pouch to avoid direct heat and sunlight.

3.3 Semen Processing and Microscopic Analysis

After collection, the semen was evaluated for its volume, colour, pH, general motility before further processing. Samples showing at least 70% general motility were further

processed and assessed for other several parameters, such as sperm concentration, individual progressive movement, viability and general morphological abnormality before being diluted into the extenders for chilling.

3.3.1 Sperm mass and individual motility

Mass motility of spermatozoa was assessed by placing one drop of neat semen on a warm slide to observe the wave motion using a sperm motility grading scale as indicated by David et al. (2015) and Mandal et al. (2014). The scale range was standardised as grade 0 (no motion) to grade 5 (fast distinct swirl). To assess individual progressive movement of the sperm, 10 μ l of net semen was diluted with 90 μ l of control group tris extender with a coverslip placed on the slide and was observed under 40x objective under the microscope. The motility was recorded and evaluated using the scale provided by David et al. (2015) in Table 1 below.

Table 1 Motility scale of sperm.

Grade	Characteristics
0	No swirl – nil or sporadic oscillation of individual sperm
1	No swirl – generalized oscillation of individual sperm only
2	Very slow distinct swirl
3	Slow distinct swirl
4	Moderately fast distinct swirl
5	Fast distinct swirl

3.3.2 Sperm concentration

A specialized counting chamber like the improved Neubauer haemocytometer was utilized for determining sperm concentration (Mortimer, 1994; Centola, 2011; Bjordahl, 2010; WHO, 2010; Agarwal et al., 2016a; Centola, 2018). In this study, 10 μ l of neat semen was diluted in 990 μ l of distilled water at a ratio of 1:1000. Subsequently, the diluted semen was loaded into both chambers using a micropipette to aid in settling. The loaded haemocytometer was then placed under a microscope at 40x objective to count the sperm heads within the subdivided smaller squares at the four edges and center of the counting chamber. Sperms that fall within the grid squares were counted. The sperm were counted from top left grid to top right, moving down toward the centre and bottom left to right in order to avoid redundant counting. Total sperm from first and second chamber was averaged and the final concentration of sperm were calculated using $50Y \times 10^6$ sperm/ml formula.

3.3.3 Sperm viability and general morphological abnormality

After semen collection, the sample were maintained in constant room temperature to preserve the sperm viability. Percentage of live and dead sperm cells was assessed by mixing 10 μ l of fresh semen with 20 μ l of Eosin-Nigrosin staining on a clean glass slide and let sit for around 2 to 3 minutes for the stain to evenly absorb with the semen. A thin smear was made on the slide and air dried, then live and dead sperm cells were observed under 40x and recorded by counting 200 sperm cells. Live sperm cells appear white or unstained, while dead sperm cells bind to the Eosin and stained pink. Apart from sperm viability, percentage of morphologically abnormal sperm head (small, giant, and

detached), midpiece (deformed and irregular shape) and tail (coiled, absent and bent) were also recorded.

3.4 Semen Chilling and Cryopreservation

The diluted semen was divided equally for each extender groups (0%, 0.5% and 1.0% GL) and was packed into 0.25ml straws and sealed. The packed straws were chilled under 4°C to 5°C for 3 hour and then were prepared for cryopreservation by arranging the straws on the rack 3 to 4cm above the liquid nitrogen vapour for 10 minutes. For storage, the straws were plunged into the liquid nitrogen inside the tank under -196°C. Post-thaw evaluation took place after five days by placing the straws inside a waterbath containing 37°C water for few seconds, then assessing the sperm quality parameters such as motility, viability and morphological abnormalities.

3.5 Malondialdehyde (MDA) And Superoxide Dismutase (SOD) Analysis

Straws that were harvested from the cryotank were kept inside the freezer under temperature of -20°C. The samples were centrifuged at 1500rpm for 10 minutes (Hoa et al., 2022) to obtain the supernatant and were further processed according to protocols of respective goat malondialdehyde (MDA) and superoxide dismutase (SOD) ELISA kits provided by ELK Biotechnology (ELK Biotechnology CO., Ltd, Denver, USA).

3.6 Statistical analysis

The data related to post-thaw sperm quality parameters such as sperm motility, live/dead sperm, and morphological abnormalities were collected and analysed using Two-way ANOVA and Kruskal-Wallis H tests via IBM SPSS Statistic version 27. A

significance level of 0.05 ($p < 0.05$) was utilized to evaluate the effects of different *G. lucidum* concentrations in the extender toward post-thaw semen quality parameters.



4.0 RESULTS

Each sperm quality parameters for all ten samples were evaluated using Two-Way ANOVA and Kruskal-Wallis H Test, comparing the effects of each extender with different concentrations of *G. lucidum* towards the sperm at post-thaw.

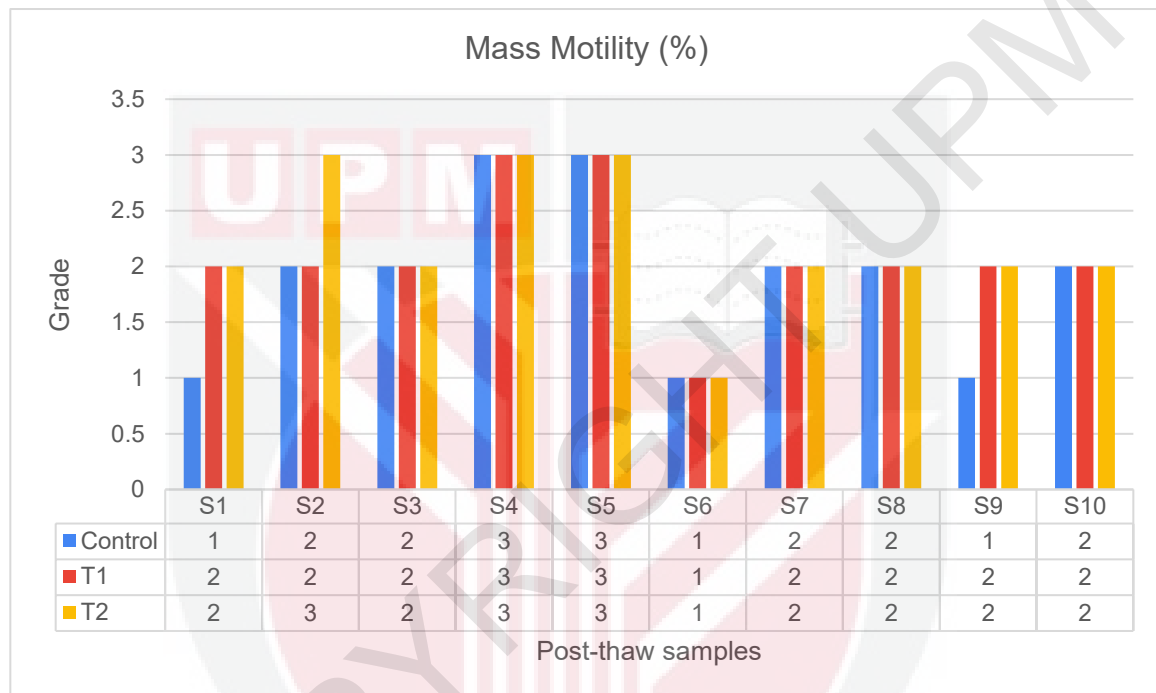


Figure 1 Rates of sperm mass motility with different GL concentrations

Based on chart shown in Figure 1, the highest grade of mass motility has been recorded by is grade 3 of motility. In general, both extenders containing 0.5% GL (T1) and 1.0% GL (T2) showed similar grades of mass motility of the sperm in most samples at post-thaw.

Data from Figure 2 shows that there is a difference in terms of effects of *G. lucidum* concentration, where T2 (1.0% GL) causes the sperm individual progressive motility rates to be the highest in first few samples but T1 (0.5% GL) was more predominant in the last few samples. However, none of these extenders; control (0% GL), T1 (0.5% GL), and T2 (1.0% GL) were significant ($p>0.05$) toward the sperm quality parameter of individual progressive movement.

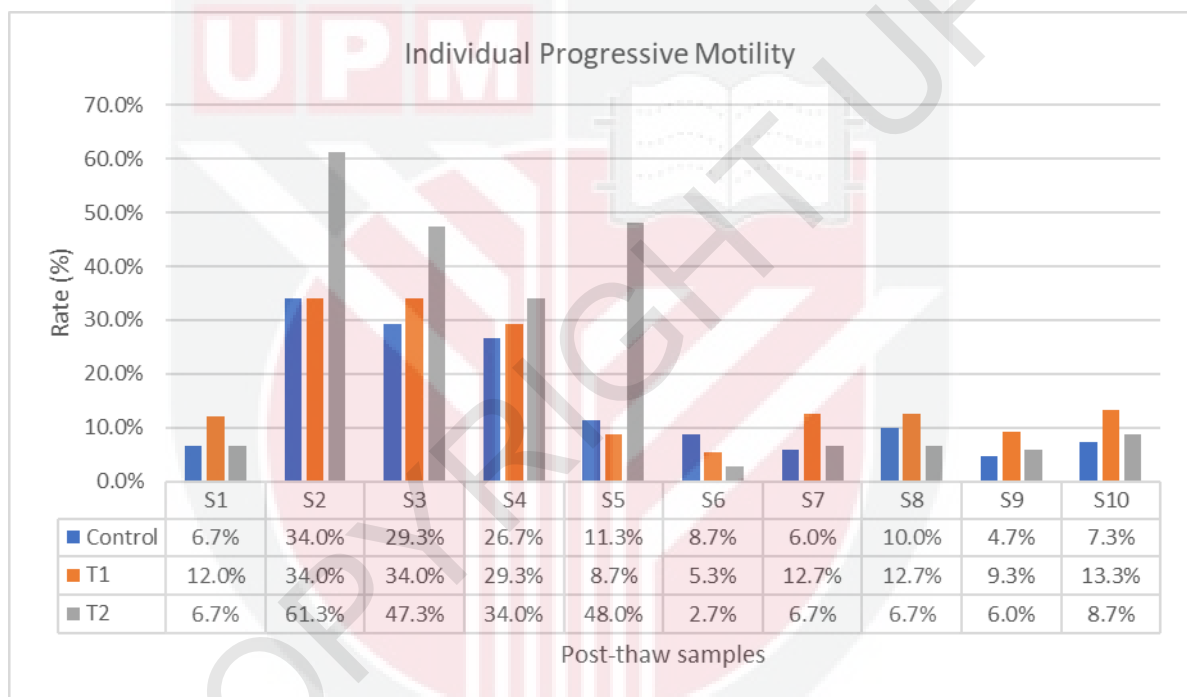


Figure 2 Rates of individual progressive motility of sperm with different GL concentrations

There is also a comparison between the three extender groups where samples from T1 extender containing 0.5% GL contributes to highest individual progressive motility rate despite there is no significant difference between all of the treatment groups.

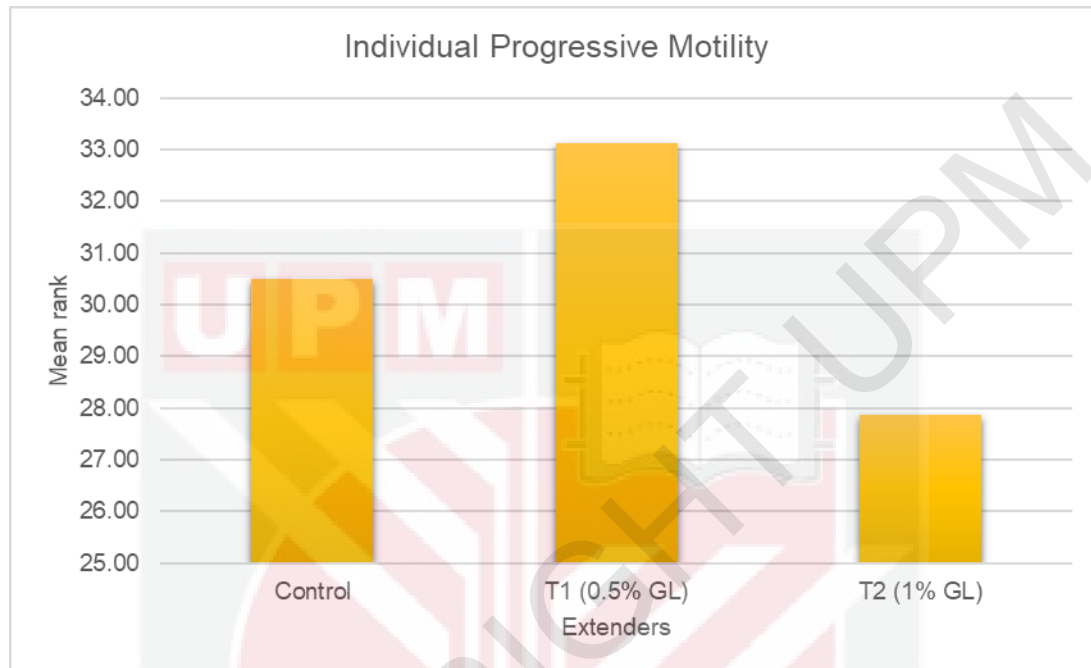


Figure 3 There is no significant difference ($p>0.05$) among all extender groups toward individual progressive motility.

Figure 4 to Figure 7 showed the effects of different concentration of *G. lucidum* against the viability of the sperm. As shown in Figure 4, rate of live sperm recorded by control group extender (0% GL) was 86.0%, while both T1 and T2 with respective concentration of 0.5% and 1.0% GL recorded 88.0% rates of live sperm cells. Followed by chart in Figure 5, there is no significant difference ($p>0.05$) for all three different extenders toward the sperm parameter. Apart from that, Figure 6 showed that 21.0% dead sperm rate was the highest for control group (0% GL), followed by 22.0% for T1 (0.5% GL) and 27.5% for T2 (1.0% GL). However, all these three extender groups are not statistically significant ($p>0.05$) in improving the rate of sperm viability despite T1 (0.5% GL) shows the highest percentage.

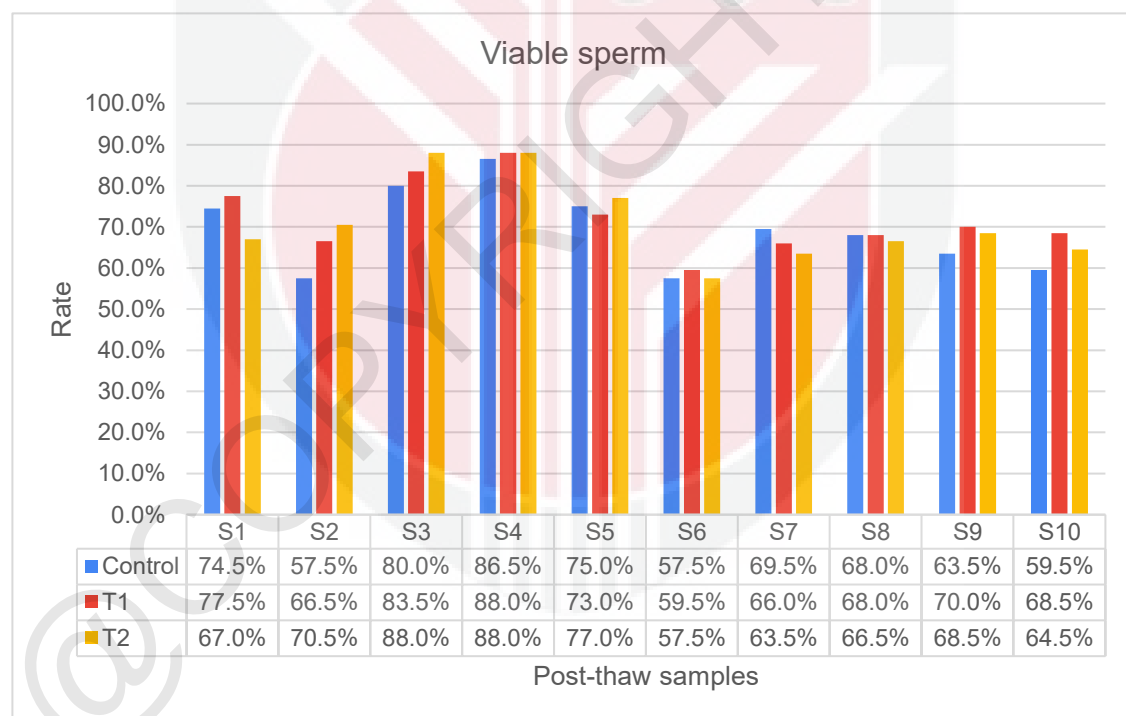


Figure 4 Rates of live sperm cells with different GL concentrations

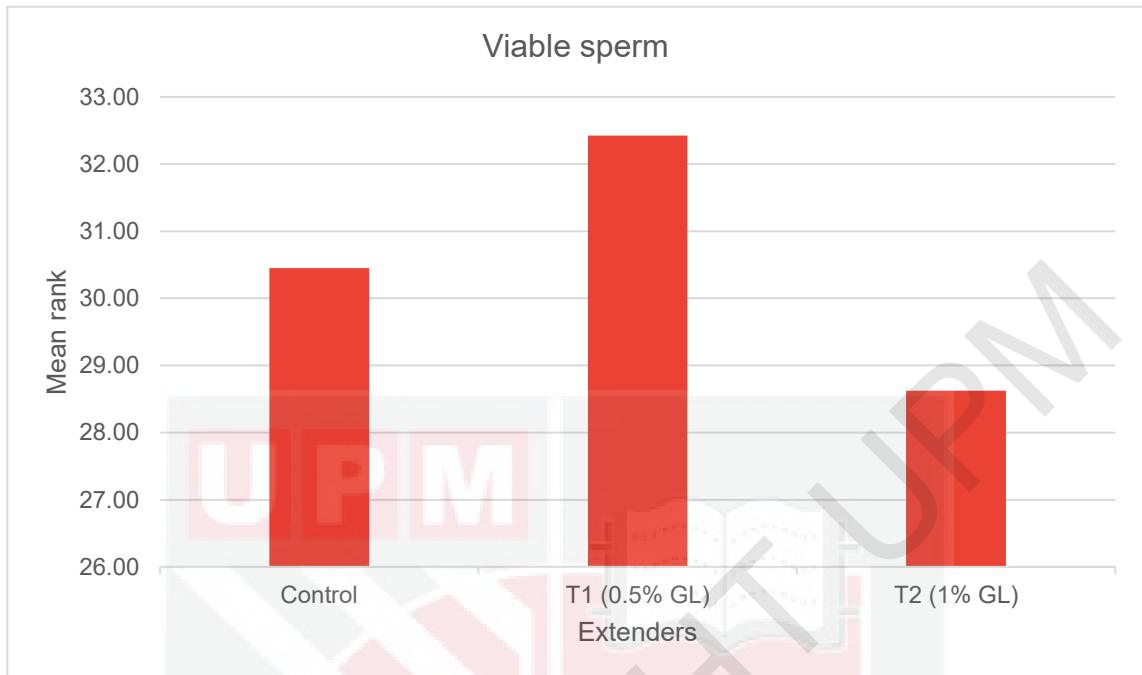


Figure 5 There is no significant difference ($p>0.05$) among all extender groups toward viable sperm.

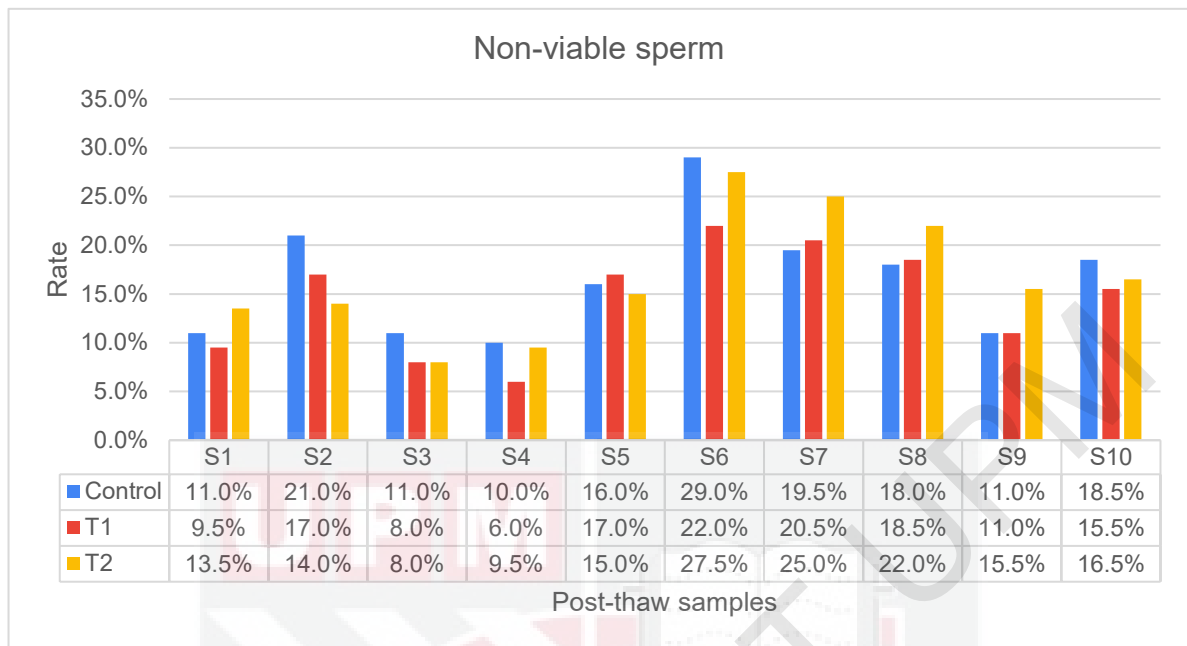


Figure 6 Rates of dead sperm cells with different GL concentration

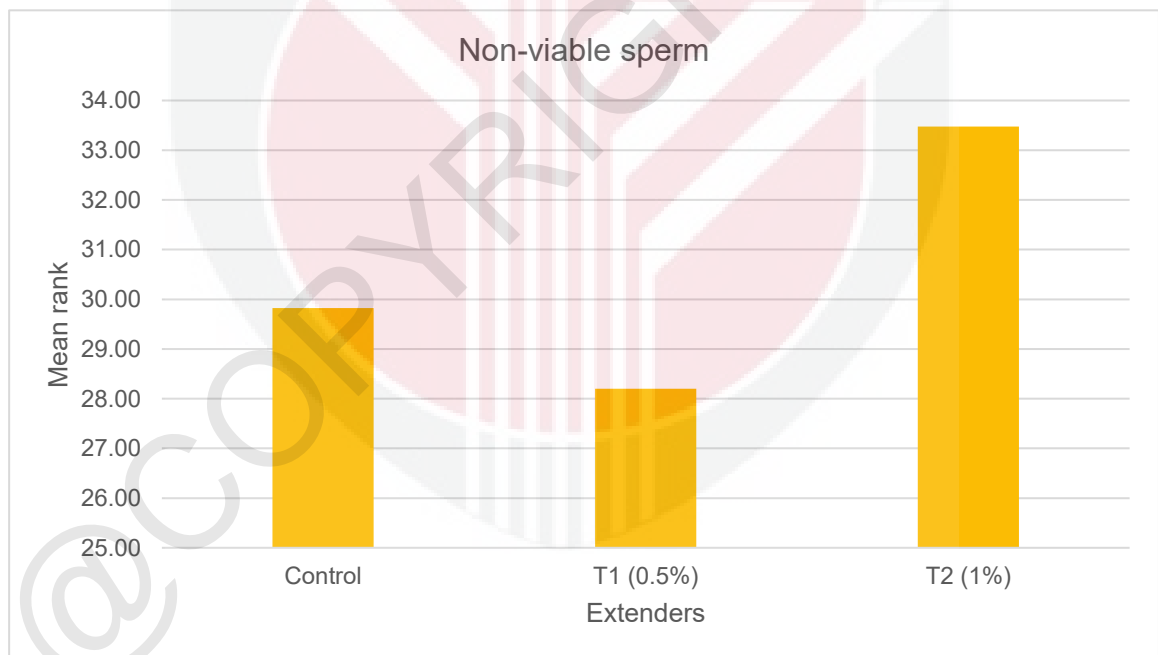


Figure 7 There is no significant difference ($p > 0.05$) among all extender groups toward viable sperm.

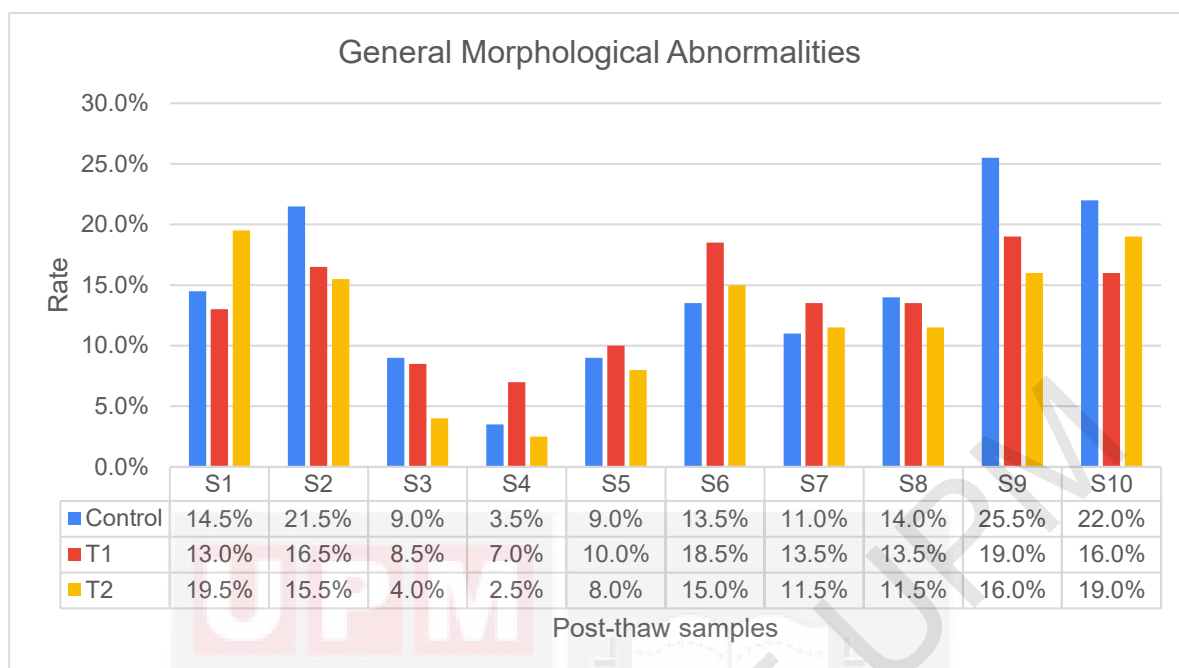


Figure 8 Rates of sperm's general morphological abnormalities.

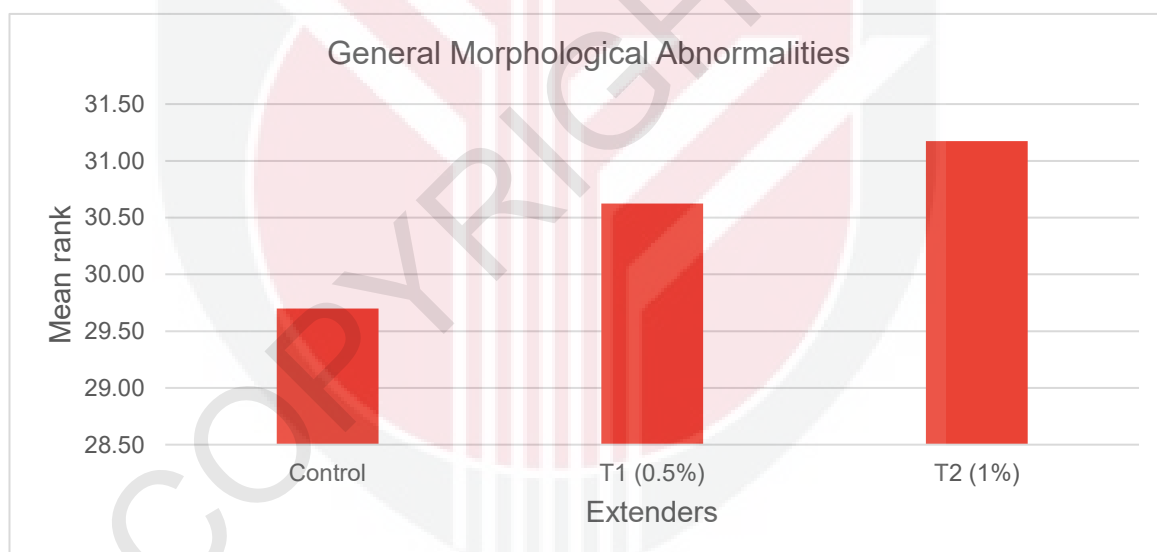


Figure 9 There is no significant difference ($p > 0.05$) among all extender groups toward general morphological abnormalities sperm parameter.

The highest rate of general morphologically abnormal sperm recorded is 25.5%, followed by 19.5% and 19.0% from control (0% GL), T2 (1.0% GL) and T1 (0.5% GL) respectively. However, there was no significant difference ($p > 0.05$) among all extender groups toward the sperm parameter.

Based on Table 2, referring to the optical density (OD) values of samples from T1 (0.5% GL) and T2 (1.0% GL) is lower compared to the control (0%GL). It depicts that the samples from control extender does not sufficiently contain SOD concentration as there was no incorporation of GL to the extender. Comparing to both T1 and T2, the OD values were indicative of higher SOD concentrations in the samples due to the supplementation of the antioxidant in the extenders. To justify the SOD assay results in relation to the previous data, it is concluded that the extenders with GL, T1 and T2 could have provided antioxidative functions and roles in improving the post-thaw sperm quality parameters. Meanwhile Table 3 depicts the comparison of the superoxide dismutase (SOD) among the three extender groups, where the data from all of the extenders were not statistically significant as the p-values were more than 0.05. Hence, in this study, it is not proven that the supplementation of GL could improve post-thaw sperm quality parameters.

Apart from that, the attempt of assessing malondialdehyde (MDA) levels was previously done, however, due to unknown errors in the assay procedure and time constraint of redoing the procedure, the data was not included in the report of this study.

Table 2 Results of Superoxide dismutase (SOD) assay

	Control	T1	T2	Calculated concentration	Std. deviation (OD)
S1	0.403	0.328	0.367	0.483	0.031
S2	0.552	0.13	0.221	0.37	0.181
S3	0.36	0.28	0.149	0.305	0.087
S4	0.466	0.266	0.273	0.429	0.093
S5	0.622	0.192	0.171	0.417	0.208
S6	0.301	0.387	0.11	0.31	0.116
S7	0.375	0.25	0.267	0.363	0.055
S8	0.201	0.187	0.198	0.191	0.006
S9	0.73	0.15	0.147	0.441	0.274
S10	0.446	0.129	0.104	0.243	0.156

Table 3 Comparison of SOD concentration between the extenders

Extenders	Mean±SEM	p-value
Control	0.2007±0.126	p>0.05
T1	0.2213±0.313	p>0.05
T2	0.4542 ±0.297	p>0.05

5.0 DISCUSSION

The use of antioxidants to improve post-thaw sperm quality parameters such as mass motility, individual progressive motility, viability sperm rates, and general morphological abnormalities has sparked interest in recent years. However, the lack of a statistically significant improvement in sperm quality parameters post-thaw, such as mass motility, individual progressive motility, live and dead sperm rates, and general morphological abnormalities, despite the use of antioxidants, has been a topic of interest in reproductive field. There were findings of several research that have been inconclusive, with some demonstrating favourable effects and others demonstrating no significant correlation between antioxidant supplementation and better sperm quality parameters (Saraswat et al., 2012; Ahmadi et al., 2016; Sadaghiani et al., 2020). For example, a study by Andrabi et al. (2011) was conducted to assess the significant improvement of sperm quality parameters, where they investigated the effect of antioxidants on post-thaw goat sperm characteristics and discovered that adding antioxidant during cryopreservation of goat sperm in Tris extender had boosted thawed spermatozoa motility and decreased lipid peroxidation.

While the forementioned study found that antioxidants improved sperm motility, it did not find a statistically significant improvement in other sperm quality criteria such as individual progressive motility, living and dead sperm rates, or general morphological defects. Similarly, Bucak et al. (2009) studied the influence of antioxidants on microscopic semen characteristics, lipid peroxidation, and antioxidant activity in cryopreserved Angora goat semen. According to the findings of this study, antioxidants had a good effect on some sperm quality metrics and oxidative stress markers. The statistical significance of these effects on post-thaw sperm quality metrics such as mass motility, individual progressive motility, live and dead sperm rates, and general morphological abnormalities, on the other hand, has not been consistently observed.

In addition, there were multiple factors contributing to the statistically insignificant data in current study. Despite incorporating *G. lucidum* as antioxidant supplement in the extenders, it is not sufficiently proven that the antioxidative properties significantly improve the sperm quality parameters. Based on current study, it is might due to the type and concentration of antioxidants, the extender composition, individual buck differences, as well as semen processing time and condition.

Type and concentration of antioxidants can affect post-thaw sperm quality due to their role in regulating oxidative stress and redox balance in sperm cells. According to Michael et al. (2007), antioxidant molecules are critical to minimise the impact of oxidative stress during the freeze-thaw process, therefore improving semen quality post-thawing. The effectiveness of antioxidants in preserving the quality of sperm is dose-dependent, and the concentration of antioxidants used in cryopreservation media has been shown to influence post-thaw sperm motility, viability, and morphology (Qamar et al., 2023). However, in this study, the dosage or concentration of *G. lucidum* extract might not be the ideal concentration for the antioxidant properties to be properly utilised as it has not been used in semen extenders yet for the time being. Therefore, there is no enough evidence to support the antioxidative roles of *G. lucidum* supplementation in the extenders to provide better protection against oxidative stress and their mechanism is not fully investigated.

Based on current study, the sperm quality parameters were not significantly improved although *G. lucidum* has been included as antioxidant supplement in the extender might also due to the extender's composition. For instance, in this study, the extender used was Tris-egg yolk extender. According to Nuti (2016), the egg yolk is detrimental to goat sperm due to the toxic effect of lysolecithins and fatty acids resulting from the hydrolysis of lecithins by phosphotidase, an enzyme produced by the bulbo-urethral glands of the buck. This process catalyses the production of toxic compounds that are harmful to goat spermatozoa. This is

where the antioxidants should play role to mitigate the oxidative stress impact toward the sperm quality during cryopreservation. However, due to the insufficient protective effects of *G. lucidum* in current study, the post-thaw sperm quality could not be well-preserved although glycerol has been added to the extender as a cryoprotectant.

Individual differences are also believed to be some of the factors affecting the post thaw sperm quality (Karagiannidis et al., 2000; Hahn et al., 2019). Several studies have looked into the influence of individual buck differences on post-thaw sperm quality in cryopreserved goat sperm. Additionally, Jiménez-Rabadán et al. (2016) suggested that the method of sperm collection had a detrimental effect on post-thaw sperm quality in bucks but not in rams, showing individual differences between the two species. On top of that, the semen collection time and temperature from each buck could also affect sperm quality. For instance, a study by Hahn et al. (2019) that looked at the impact of different handling temperatures and assessment time points following sperm collection on buck sperm quality discovered substantial influences on raw sperm viability, motility, and semen pH, showing the importance of individual buck variability in sperm quality. The study analysed that the raw goat semen can remain at room temperature for up to 10 minutes without affecting its overall quality (Hahn et al., 2019). Therefore, it is recommended that semen samples should be evaluated within 10 minutes of collection for consistent results. However, in this study, it was possibly due to some technical errors during the semen processing such as fluctuations of surrounding temperatures and duration of semen processing where it took much longer time and caused delay, hence affecting the sperm quality as well.

In this current study, the data analysis from SOD assay showed were not statistically significant. In addition to that, despite the potential benefits of *G. lucidum* as antioxidant supplements, the importance of its impact on goat sperm quality indicators has not always been confirmed. There were several researches previously conducted have investigated the

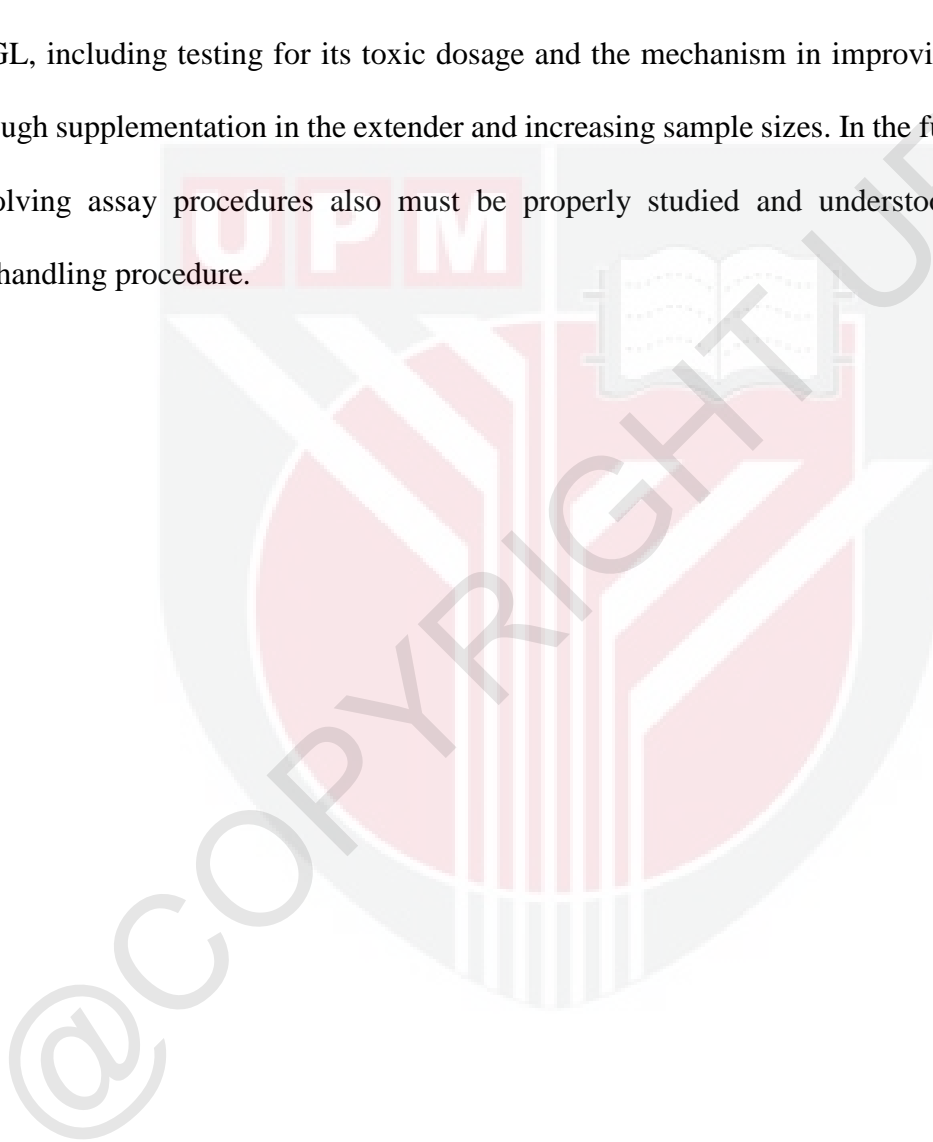
correlation between antioxidant supplementation, SOD activity, and sperm quality measures. For instance, Aitken and Clarkson (2017) investigated the relationship between sperm quality parameters and antioxidant enzyme activity, particularly superoxide dismutase (SOD), in human sperm. The researchers discovered there is no link between sperm motility and seminal SOD activity. This research suggested that the activity of SOD, an important antioxidant enzyme, did not have a significant association with sperm motility which is a crucial indicator of sperm quality in the context of human sperm. Gharagozloo and Aitken (2020) claimed that poor sperm quality caused by oxidative stress and lipid peroxidation may be influenced by the activity of reactive oxygen species (ROS). While the study did not directly examine the effect of antioxidant supplementation and SOD, it does provide insights into the complicated relationship between oxidative stress, lipid peroxidation, and sperm quality, emphasizing the multiple nature of factors impacting sperm function. However, when current study was conducted, due to the incomplete data analysis from malondialdehyde (MDA) assay, it could not properly support the forementioned justifications on the idea of the goat sperm quality parameters did not significantly improved despite being supplemented with antioxidant.

6.0 CONCLUSION

In conclusion, it is apparent that the sperm quality parameters such as individual progressive motility, viability and general morphological abnormalities did not significantly improve despite having *G. lucidum* as antioxidant supplementation in the semen extenders. It is not yet sufficiently proven that the choice of antioxidant was ideal or optimum in terms of dosage and concentration to fully utilise the antioxidative functions and roles of the *G. lucidum*, as both T1 (0.5% GL) and T2 (1.0% GL) extenders did not show significant differences. Hence, in this study, there is no significant improvement observed in post-thaw sperm quality parameters and null hypothesis is accepted.

7.0 RECOMMENDATIONS

Further comparative and investigative studies regarding the effectiveness of *G. lucidum* as antioxidant supplement in goat semen cryopreservation is highly recommended to be conducted in the future to observe and evaluate the correct dosage and concentration of the GL in the extender. Preliminary testing is advisable for determining optimum and suitable dosage of GL, including testing for its toxic dosage and the mechanism in improving sperm quality through supplementation in the extender and increasing sample sizes. In the future studies, any involving assay procedures also must be properly studied and understood to avoid any mishandling procedure.



REFERENCES

1. Ahmadi, S., Bashiri, R., Ghadiri-Anari, A., & Nadjarzadeh, A. (2016). Antioxidant supplements and semen parameters: An evidence-based review. *International journal of reproductive biomedicine*, 14(12), 729.
2. Ali, W. A. H., & Ghazal, M. N. (2013). In vivo and in vitro studies on the effect of Ganoderma on rabbit reproductivity, semen preservation and artificial insemination. *Journal of Animal and Poultry Production*, 4(12), 715-731.
3. Ahmad, Z., Ali, L., Ahmed, H., Shah, S. A. H., & Andrabi, S. M. H. (2020). Superoxide dismutase in extender improves the in vitro quality and in vivo fertility of cryopreserved water buffalo (*Bubalus bubalis*) spermatozoa. *Cryoletters*, 41(4), 194-201.
4. Ansari, M. S., Rakha, B. A., Ullah, N., Andrabi, S. M. H., & Akhter, S. (2011). Glutathione addition in tris-citric egg yolk extender improves the quality of cooled buffalo (*Bubalus bubalis*) bull semen. *Pakistan Journal of Zoology*, 43(1).
5. Büyük, B., Demirci, T., Demir, N., & Türkön, H. (2021). Can ganoderma lucidum be an alternative nutritional supplement for enhancing sperm motility rate?. *Izmir Democracy University Health Sciences Journal*, 4(1), 47-61.
6. Bucak, M. N., Sarıözkan, S., Tuncer, P. B., Ulutaş, P. A., & Akçadağ, H. İ. (2009). Effect of antioxidants on microscopic semen parameters, lipid peroxidation and antioxidant activities in Angora goat semen following cryopreservation. *Small ruminant research*, 81(2-3), 90-95.
7. Centola, G. M. (2018). Laboratory Evaluation of Bulk Seminal Parameters.
8. Chauhan, M. S., & Anand, S. R. (1990). Effect of egg yolk lipids on the freezing of goat semen. *Theriogenology*, 34(5), 1003-1013.
9. David, I., Kohnke, P., Lagriffoul, G., Praud, O., Plouarboué, F., Degond, P., & Druart, X. (2015). Mass sperm motility is associated with fertility in sheep. *Animal reproduction science*, 161, 75-81.
10. Dorado, J., Rodríguez, I., & Hidalgo, M. (2007). Cryopreservation of goat spermatozoa: Comparison of two freezing extenders based on post-thaw sperm quality and fertility rates after artificial insemination. *Theriogenology*, 68(2), 168-177.
11. Ghajari, G., Chakraborty, A., Moosavi, S. A., & Saremi, M. (2022). The Protective Effect of Ganoderma Lucidum in Mice-Exposed to Sertraline. *Personalized Medicine Journal*, 7(26), 6-12.

12. Gangwar, C., Kharche, S. D., Kumar, S., & Jindal, S. K. (2016). Cryopreservation of goat semen: status and prospects. *Indian Journal of Small Ruminants (The)*, 22(1), 1-10.
13. Hahn, K., Failing, K., & Wehrend, A. (2019). Effect of temperature and time after collection on buck sperm quality. *BMC veterinary research*, 15, 1-7.
14. Hassan, S. A., Khalil, W. A., Hassan, M. A., Yousif, A. I., Sabry, O. M., Wink, M., & Sobeh, M. (2021). Antioxidant and antiapoptotic effects of a *turraea fischeri* leaf extract on cryopreserved goat sperm. *Animals*, 11(10), 2840.
15. Karagiannidis, A., Varsakeli, S., & Karatzas, G. (2000). Characteristics and seasonal variations in the semen of Alpine, Saanen and Damascus goat bucks born and raised in Greece. *Theriogenology*, 53(6), 1285-1293.
16. Kasai, K., Izumo, A., Inaba, T., & Sawada, T. (2001). Efficiency of artificial vagina method in semen collection from Osaka Drakes. *Journal of Applied poultry research*, 10(3), 206-210.
17. Mandal, S., Chattopadhyay, S., & Batabyal, S. (2014). Comparative Evaluation of Fresh and Frozen Semen Quality of Jersey (Exotic) and Gir (indigenous) Bulls. *Environment and Ecology*, 32(3), 878-880.
18. Li, Y., Liang, W., Han, Y., Zhao, W., Wang, S., & Qin, C. (2022). Triterpenoids and Polysaccharides from *Ganoderma lucidum* Improve the Histomorphology and Function of Testes in Middle-Aged Male Mice by Alleviating Oxidative Stress and Cellular Apoptosis. *Nutrients*, 14(22), 4733.
19. Lin, Z. B., & Zhang, H. N. (2004). Anti-tumor and immunoregulatory activities of *Ganoderma lucidum* and its possible mechanisms. *Acta Pharmacologica Sinica*, 25(11), 1387-1395
20. Lv, C., Wu, G., Hong, Q., & Quan, G. (2019). Spermatozoa cryopreservation: State of art and future in small ruminants. *Biopreservation and biobanking*, 17(2), 171-182.
21. Mara, L., Dattena, M., Pilichi, S., Sanna, D., Branca, A., & Cappai, P. (2007). Effect of different diluents on goat semen fertility. *Animal reproduction science*, 102(1-2), 152-157.
22. Memon, M. A., Bretzlaff, K. N., & Ott, R. S. (1986). Comparison of semen collection techniques in goats. *Theriogenology*, 26(6), 823-827.
23. Merati, Z., & Farshad, A. (2020). Ginger and echinacea extracts improve the quality and fertility potential of frozen-thawed ram epididymal spermatozoa. *Cryobiology*, 92, 138-145.

24. Michael, A., Alexopoulos, C., Pontiki, E., Hadjipavlou-Litina, D., Saratsis, P., & Boscus, C. (2007). Effect of antioxidant supplementation on semen quality and reactive oxygen species of frozen-thawed canine spermatozoa. *Theriogenology*, 68(2), 204-212.
25. Nuti, L. C. (2016). Goat semen collection and processing. *Cornell Univ*, 40, 184-185.
26. Natarajan, S., & Remick, D. G. (2008). The ELISA Standard Save: Calculation of sample concentrations in assays with a failed standard curve. *Journal of immunological methods*, 336(2), 242-245.
27. Oke, M. A., Afolabi, F. J., Oyeleke, O. O., Kilani, T. A., Adeosun, A. R., Olanbiwoninu, A. A., & Adebayo, E. A. (2022). Ganoderma lucidum: Unutilized natural medicine and promising future solution to emerging diseases in Africa. *Frontiers in pharmacology*, 2876.
28. Parepalli, Y., Chavali, M., Pamanji, S. R., & Singh, M. (2021). Ganoderma lucidum: extraction and characterization of polysaccharides, yields and their bioapplications. *Algerian Journal of Research and Technology (AJRT)*, 5(1), 35-48.
29. Purdy, P. H. (2006). A review on goat sperm cryopreservation. *Small ruminant research*, 63(3), 215-225.
30. Qamar, A. Y., Naveed, M. I., Raza, S., Fang, X., Roy, P. K., Bang, S., ... & Cho, J. (2023). Role of antioxidants in fertility preservation of sperm—A narrative review. *Animal bioscience*, 36(3), 385.
31. Turri, F., Madeddu, M., Gliozzi, T. M., Gandini, G., & Pizzi, F. (2014). Effect of testicle postmortem storage on goat frozen-thawed epididymal sperm quality as a tool to improve genebanking in local breeds. *Animal*, 8(3), 440-447.
32. Tijjani, H. U., Faisal, A., & Asmad, K. (2014). Effect of different glycerol concentrations on sperm quality after preservation of Malaysian indigenous cockerel semen. *Savannah Journal of Agriculture*, 9(1), 56-67.
33. Wachtel-Galor, S., Yuen, J., Buswell, J. A., & Benzie, I. F. (2012). Ganoderma lucidum (Lingzhi or Reishi): a medicinal mushroom.
34. Sadaghiani, S., Fallahi, S., Heshmati, H., Teshnizi, S. H., Chaijan, H. A., Ebrahimi, F. F. A., ... & Alizadeh, F. (2020). Effect of antioxidant supplements on sperm parameters in infertile male smokers: a single-blinded clinical trial. *AIMS Public Health*, 7(1), 92.
35. Saraswat, S., Jindal, S. K., Priyadharsini, R., Ramachandran, N., Yadav, S., Rout, P. K., ... & Goel, A. K. (2012). The effect of antioxidants supplementation to cryopreservation protocol on seminal attributes and sperm membrane characteristics in Sirohi goat. *J. Phys. Pharm. Adv*, 2, 49-58.

36. Sharma, A., & Sood, P. (2020). Caprine semen cryopreservation and the factors affecting it: an overview. *Veterinary Sciences: Research and Reviews*, 6(1), 46-57.
37. Shahverdi, A., Rastegarnia, A., & Topraggaleh, T. R. (2014). Effect of extender and equilibration time on post thaw motility and chromatin structure of buffalo bull (*Bubalus bubalis*) spermatozoa. *Cell Journal (Yakhteh)*, 16(3), 279.
38. Sharafi, M., Borghei-Rad, S. M., Hezavehei, M., Shahverdi, A., & Benson, J. D. (2022). Cryopreservation of Semen in Domestic Animals: A Review of Current Challenges, Applications, and Prospective Strategies. *Animals*, 12(23), 3271.
39. Sobeh, M., Hassan, S. A., Hassan, M. A., Khalil, W. A., Abdelfattah, M. A., Wink, M., & Yasri, A. (2020). A polyphenol-rich extract from *Entada abyssinica* reduces oxidative damage in cryopreserved ram semen. *Frontiers in Veterinary Science*, 7, 604477.
40. Tang, W., Liu, J. W., Zhao, W. M., Wei, D. Z., Zhong, J. J., & Ganoderma, L. (2007). Two novel antioxidants from *Ganoderma lucidum*. *Journal of Agricultural and Food Chemistry*, 55(4), 1331-1337.
41. Xia, Q., Zhao, Q., Zhu, H., Cao, Y., Yang, K., Sun, P., & Cai, M. (2022). Physicochemical characteristics of *Ganoderma lucidum* oligosaccharide and its regulatory effect on intestinal flora in vitro fermentation. *Food Chemistry: X*, 15, 100421.
42. Zhang, W., Min, L., Li, Y., Lang, Y., Hoque, S. M., Adetunji, A. O., & Zhu, Z. (2022). Beneficial Effect of Proline Supplementation on Goat Spermatozoa Quality during Cryopreservation. *Animals*, 12(19), 2626.
43. Zhu, M. D., Ren, X. X., Hu, Z. H., Tang, W., & Zhao, M. W. (2008). A new antioxidant from *Ganoderma lucidum*. *Chemistry & Biodiversity*, 5(2), 269-273