Effect of Ergothioneine Supplementation in Tris Extender on the Quality of Cooled Awassi Ram Semen

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Abstract

The aim of this research is to evaluate the effects of mixing tris diluent with ergothioneine on the properties of the semen collected by Awassi Rams. This investigation was carried out by the College of Agricultural Engineering Sciences, which comprises the Department of Animal Production at the University of Baghdad and the Department of Animal Science at the University of Sulaimani. In this technique, semen samples were collected from eight rams that were between the ages of two and three years old. Once semen was obtained from each animal by means of an artificial vagina, the semen was then diluted with Ergothioneine at varying concentrations. Ergothioneine was added to the semen extender at concentrations of 0, 5, 10, and 15 mM, and at varying time intervals (0, 24, 48, and 72 hours following the dilution). Cooling preservation at 5 °C demonstrated that supplementation with 5 mM ergothioneine significantly improved sperm motility and plasma membrane integrity in Awassi rams compared with higher concentrations and the control group ($P < 0.05$), particularly during the initial phase of storage. However, extended preservation up to 72 h was associated with increased DNA fragmentation, especially at 15 mM, while ergothioneine treatments overall reduced malondialdehyde levels, indicating a mitigation of oxidative stress.

Keywords: Antioxidant capacity, Sperm viability, Oxidative stress, Semen preservation.

Introduction

The cooling preservation of Awassi ram semen at 5 °C is associated with a progressive decline in sperm quality, including reduced motility, decreased plasma membrane integrity, increased DNA fragmentation, and elevated oxidative stress due to reactive oxygen species accumulation. Although Tris extender is widely used, its ability to protect sperm cells during extended storage remains limited, prompting investigation into effective antioxidant supplements such as ergothioneine and determination of their optimal concentration to improve semen quality during cooling preservation.

The Awassi sheep are one of the most commercially significant breeds in Iraq for the production of skin, milk, and meat. [1]. Sheep are vital to Iraq's agriculture, and their reproduction and improved

ISSN 2072-3857

productivity are critical to population growth and profitability [2]. The principal challenge facing agricultural operations in Iraq was reduced sheep production, driven by genetic factors, disease transmission, and inadequate management [3]. The reproductive behaviour of rams is influenced by the season in some breeds and areas [4]. Ergothioneine (EGT) is a naturally occurring sulfur-containing amino acid derivative primarily synthesized by fungi and some bacteria, and in animals, it comes from dietary sources. Recognized as one of the most efficient antioxidants, it has a unique thiol/thione structure that enables it to scavenge ROS and protect cellular macromolecules from oxidation. In ram semen, Ergothioneine (EGT), an essential low-molecular thiol, occurs naturally in several plants and animals. It is a distinctive antioxidant that scavenges singlet oxygen and hydroxyl/peroxyl radicals [5]. [6] report increased EGT levels across several tissue systems. The components include the liver, kidneys, ocular structures, erythrocytes, and seminal fluid. Ergothioneine in semen extenders has been shown to maintain semen DNA integrity, notably in some studies, thereby safeguarding semen against oxidized and peroxidized fructolysis agents and to improve post-thaw motility and DNA quality in ram semen [7]. Cooling, freezing, and thawing of semen inside the genitourinary organs lead to membrane instability and diminish the efficacy of artificial insemination [8]. The aim of this study was to evaluate the effect of different concentrations of ergothioneine supplementation in a Tris-based extender on the quality of cooled semen of Awassi rams. Specifically, the study investigated its impact on sperm motility, plasma membrane integrity, DNA fragmentation, and oxidative stress markers during storage at 5 °C over different preservation periods.

Material and Methods

This research was carried out by two universities: the College of Agricultural Engineering Sciences, comprising the Department of Animal Production at the University of Baghdad, and the Department of Animal Science at the University of Sulaimani. In this technique, semen samples were collected from eight rams that were between the ages of two and three years old. [9] used artificial vaginas constructed for sheep and goats to collect these samples. Afterward, the semen samples were divided into four treatments, each diluted with a different ergothioneine concentration: 0, 5, 10, or 15 mM. Ergothioneine used in this study was obtained from Sigma-Aldrich (St. Louis, MO, USA) with analytical-grade purity [2]. Following dilution of the semen sample with an equivalent volume of Tris solution, the Tris extender consisted of Tris (3.63 g), citric acid (1.99 g), fructose (0.5 g), egg yolk (20%), and antibiotics dissolved in distilled water to a final volume of 100 ml., the sample was processed according to the techniques outlined by [9]. After cooling, the samples are sent to the Institute of Higher Diagnosis of Infertility, where they are maintained at 5 degrees Celsius for 0, 24, 48, and 72 hours. The following procedures should be carried out in accordance with [10]: evaluate the motility of the individual; evaluate the integrity of the plasma membrane in accordance with [11]; examine the DNA that has been damaged in accordance with [12]; and measure the concentration of malondialdehyde in accordance with [13].

All information gathered in Excel was used in the statistical analysis. Calculations were carried out using the criteria for each treatment. Data were analyzed using a two-way

ISSN 2072-3857

ANOVA with ergothioneine concentration and storage time as fixed factors. SAS programs [14], and Duncan's multiple range test [15] was used to compare means at ($P < 0.05$). It was determined that these variations may be considered significant at the 5% level.

Results and Discussion

Figure 1. It was shown that cooling preservation at 5 Cº did not significantly affect semen motility at time 0 for control parameters or for 10 and 15 mM ergothioneine (74.2 percent, 75.4 percent, and 73.6 percent, respectively). While a significant difference ($P < 0.05$) with 5 mM (77 percent). When comparing the control group with the 15 mM group at 24, 48, and 72 hours, no discernible differences were observed. There were three different percentages that were associated with the control group: 62% and 61.4%, 42.4% and 43%, and 52.4% and 53.4%, respectively. Figure 1 demonstrates that the portion of individual motility that reached 77% occurred at 0 hours, which was the greatest possible value.

The incorporation of ergothioneine into the Tris solution significantly improved plasma membrane integrity in Awassi rams throughout the first phase of preservation. The 5 mM ergothioneine treatment showed significantly ($P < 0.05$) higher plasma membrane integrity than the 10 mM and 15 mM concentrations, with success rates of 81.6% and 80.2%, respectively. The control achieved a higher success rate of 79.2%. As shown in Figure 2, 72-hour cooling preservation at 5 degrees Celsius for the same feature showed a considerable advantage at a 5 mM concentration (53.6 %).

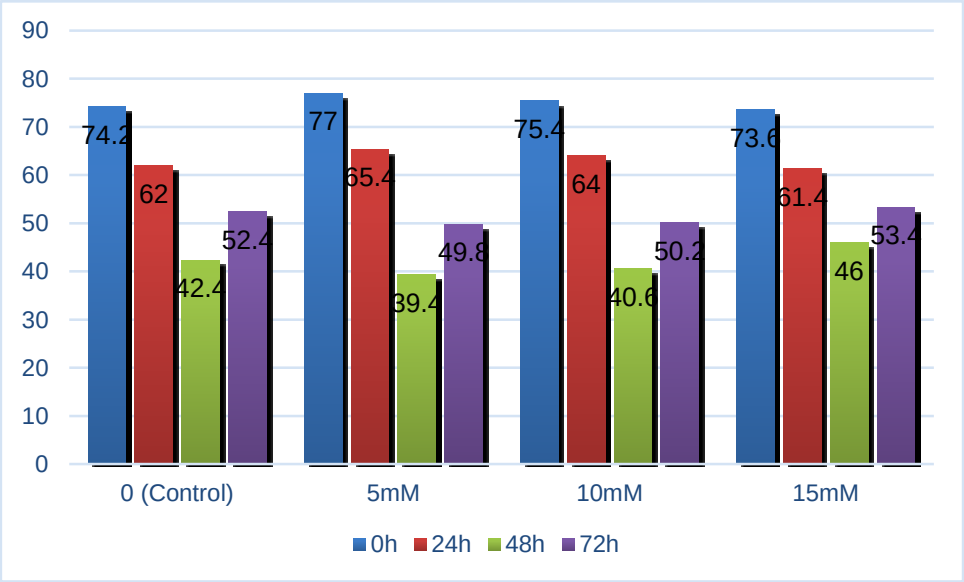
Regarding the proportion of DNA fragmentation during the first hour of chilling preservation, there were no

significant differences between the control treatment and the 15 mM Ergothioneine concentration (8% and 8.33%, respectively). The amount of DNA fragmentation was at its maximum at 15 mM preservation after 72 hours of cooling preservation, at 13.33% (Fig. 3).

The evaluation of malonaldehyde content in the seminal plasma of Awassi rams revealed a baseline concentration of 0.58 µmol/10^9 semen and a maximum of 2.467 µmol/10^9 semen. Awassi rams were used for the measurement. The Awassi ram showed a notable reduction in malondialdehyde concentration (in µmol/10^^{9} semen) after 72 hours of cooling preservation at 5°C. This contrasted with the control group, which had the lowest malondialdehyde concentration, measured at 2.476 µmol/10^^{9} semen. The decreases in this regard were reported across a wide range of therapies.

ISSN 2072-3857

Fig. 1.



Impact of concentration of Ergothioneine on the individual motility of semen during various periods (0, 24, 48, and 72 hours)

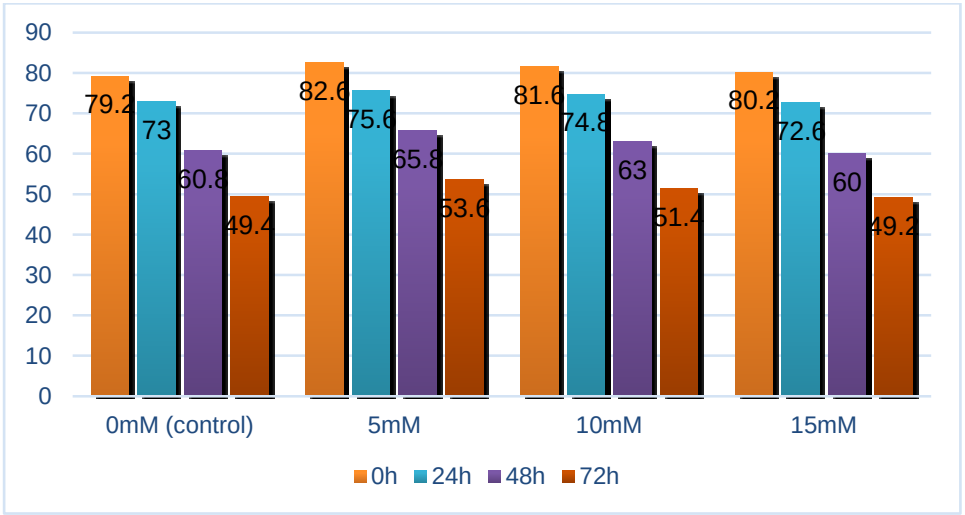


Fig. 2. Impact of concentration of Ergothioneine on the plasma membrane of semen during various periods (0, 24, 48, and 72 hours)

ISSN 2072-3857

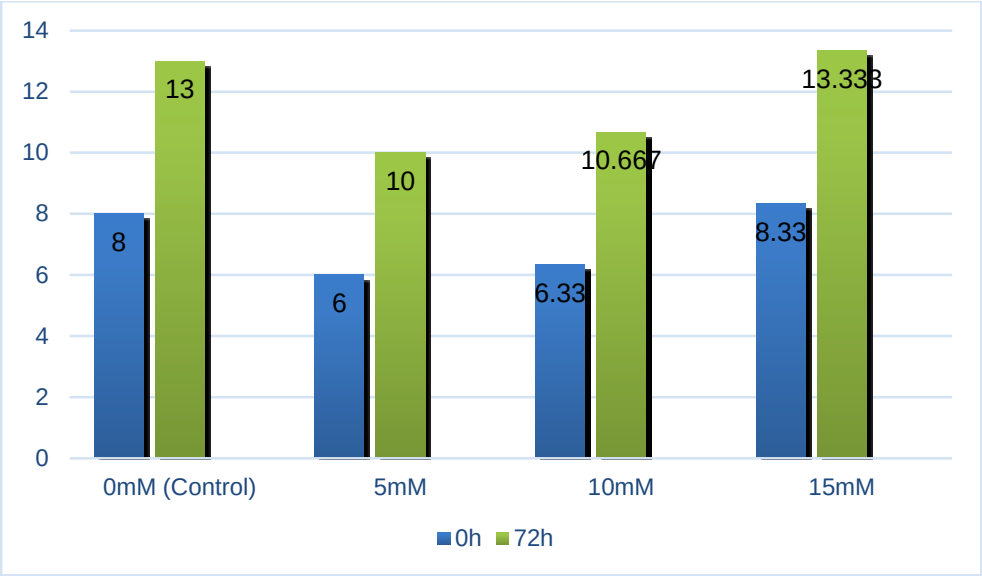


Fig. 3. Impact of concentration of Ergothioneine on the DNA fragmentation of semen during various periods (0, 72 hours)

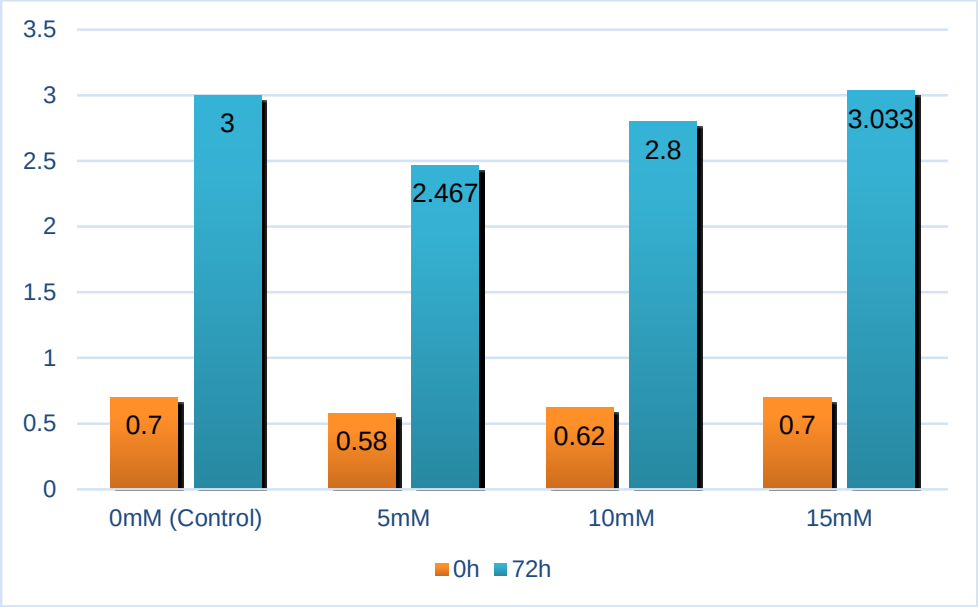


Fig. 4. Impact of concentration of Ergothioneine on the malondialdehyde of semen during various periods (0, 72 hours)

The present study was designed to evaluate the effect of ergothioneine supplementation in a Tris-based extender on the quality of cooled Awassi ram semen during storage at 5 °C. The findings indicate that ergothioneine plays a significant role in preserving semen quality,

ISSN 2072-3857

particularly during the early stages of cooling preservation. Oxidative stress has been shown to peroxidize the lipids of the semen plasma membrane and fragment DNA, contributing toward semen damage during semen processing and cryopreservation. Polyunsaturated fatty acids predominate the semen membrane, and their oxidative attack is specific. Ergothioneine neutralizes ROS, including superoxide anions, hydroxyl radicals, and hydrogen peroxide, thereby reducing lipid peroxidation and maintaining membrane integrity and semen functionality [16]. The antioxidant properties of ergothioneine are mainly due to its potential to donate electrons and quench free radicals without taking a pro-oxidant status. It also chelates transition metals (e.g. iron and copper), limiting the formation of highly reactive hydroxyl radicals through the Fenton reaction [17]. Moreover, ergothioneine is also integrated into reproductive tissues and seminal plasma via the organic cation transporter OCTN1, thereby allowing localized antioxidant protection. Semen extenders that have shown improvements in semen motility, membrane integrity, and post-thaw semen quality, including by minimizing free radical exposure during freezing and thawing, include supplementation with ergothioneine in ram semen [18]. In addition, ergothioneine might safeguard DNA in semen from oxidative fragmentation and promote chromatin integrity, thereby supporting successful fertilization and embryo development [7]. The fundamental goal of semen preservation is to sustain semen functioning for a long amount of time by cryopreservation [19]. This is accomplished by either fully halting metabolic processes or by lowering metabolic activity through chilling. Semen is preserved from the time it is collected until it is needed for fertilization. This allows for the successful completion of the process. According to [20], oxidative damage is a significant factor contributing to unsuccessful fertilization. All of the components that comprise a live creature, including proteins, nucleic acids, lipids, and carbohydrates, are susceptible to being impacted by oxidative stress. The 5 mM concentration showed the highest percentage of individual movement during the 24-hour cryopreservation phase. Compared with the 10 mmol concentration (65.4 ± 0.67%), this percentage did not differ significantly from 64.0 ± 0.44%. [21] added ergothioneine to frozen rams' semen and found no significant positive or negative effects. [22, 23] found that preservation procedures reduced semen metabolic activity by releasing enzymes and ions. Repeated cycles of freezing and thawing lead to a loss of semen characteristics. According to [24, 25], tests conducted on goats and bull semen showed improved

ISSN 2072-3857

performance. This result is in line with the conclusions of those studies. Oxidative stress is linked to the presence of antioxidants in the body. [26, 27] report that reactive oxygen species generated during the freeze-thaw cycle may adversely affect semen motility and reduce mitochondrial energy production. The results of using Pomegranate Juice as a Tris Diluent on Some Semen Characteristics of Awassi Rams showed that a 5% concentration gave the best performance for the studied traits during the preservation [28].The results of the current experiment indicate that the injection of ergothioneine at low doses improved semen motility, plasma membrane integrity, DNA fragmentation, and malondialdehyde levels. Both of these improvements were seen.

Conclusion

Based on the data presented in this study, adding a low level of Ergothioneine to the Tris diluent improved the stability of the parameters at 0, 24, 48, and 72 hours. This suggests that Ergothioneine may act as a stabilizing agent, enhancing the stability of biological specimens.

Acknowledgment: Both the Animal Science Department at the University of Sulaymaniyah and the Department of Animal Production at the University of Baghdad were very kind in allowing the experiment to be conducted at their respective facilities and labs. The authors would like to express their heartfelt gratitude to both departments.

References

1. Azawi, O. and M. Ismaeel, *Effects of seasons on some semen parameters and bacterial contamination of Awassi ram semen.* Reproduction in domestic animals, 2012. **47**(3): p. 403-406.
2. Salamon, S. and W.M.C. Maxwell, *Storage of ram semen.* Animal reproduction science, 2000. **62**(1-3): p. 77-111.
3. Yimer, N., et al., *Comparison of cryopreservative effect of different levels of omega-3 egg-yolk in citrate extender on the quality of goat spermatozoa.* Pakistan Veterinary Journal, 2014. **34**(3).
4. Karagiannidis, A., et al., *Seasonal variation in semen characteristics of Chios and Friesian rams in Greece.* Small ruminant research, 2000. **37**(1-2): p. 125-130.
5. Fang, Y. and R. Zhong, *Effects of oxidative stress on spermatozoa and male infertility*. 2019: IntechOpen.
6. Ari, U., et al., *Effect of L-(+)-Ergothioneine (EGT) on freezability of ram semen.* Int

J Anim Vet Adv, 2012. **4**(6): p. 378-383.
7. Coyan, K., et al., *Ergothioneine attenuates the DNA damage of post-thawed Merino ram sperm.* Small Ruminant Research, 2012. **106**(2-3): p. 165-167.
8. Marti, E., et al., *Seasonal variations in antioxidant enzyme activity in ram seminal plasma.* Theriogenology, 2007. **67**(9): p. 1446-1454.
9. Evans, G. and W.C. Maxwell, *Salamons' artificial insemination of sheep and goats*. 1987.
10. Haboby, A., et al., *Effect of vitamin E and selenium on semen quality, sexual activity, and some blood parameters of Awassi Rams.* J Agric Invest, 2004. **2**: p. 58-62.
11. Jeyendran, R., et al., *Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics.* Reproduction, 1984. **70**(1): p. 219-228.
12. Tejada, R.I., et al., *A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence.* Fertility and sterility, 1984. **42**(1): p. 87-91.
13. Kumaresan, A., M. Ansari, and A. Garg, *Modulation of post-thaw sperm functions with oviductal proteins in buffaloes.* Animal reproduction science, 2005. **90**(1-2): p. 73-84.
14. Edition, F., *SAS® 9.3 In-Database Products.* 2012.
15. Duncan, D.B., *Multiple range and multiple F tests.* biometrics, 1955. **11**(1): p. 1-42.
16. Cheah, I.K. and B. Halliwell, *Ergothioneine; antioxidant potential, physiological function and role in disease.* Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 2012. **1822**(5): p. 784-793.
17. Aruoma, O.I., et al., *Antioxidant action of ergothioneine: assessment of its ability to scavenge peroxynitrite.* Biochemical and biophysical research communications, 1997. **231**(2): p. 389-391.
18. Najafi, A., et al., *Different concentrations of cysteamine and ergothioneine improve microscopic and oxidative parameters in ram semen frozen with a soybean lecithin extender.* Cryobiology, 2014. **69**(1): p. 68-73.
19. Bucak, M.N., et al., *The effect of antioxidants on post-thawed Angora goat (Capra hircus ancryrensis) sperm parameters, lipid peroxidation and antioxidant activities.* Small ruminant research, 2010. **89**(1): p. 24-30.

ISSN 2072-3857

20. Agarwal, A., R.A. Saleh, and M.A. Bedaiwy, *Role of reactive oxygen species in the pathophysiology of human reproduction.* Fertility and sterility, 2003. **79**(4): p. 829-843.
21. YILDIZ, S., et al., *The effects of L-ergothioneine, N-acetylcystein and cystein on freezing of ram semen.* KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ, 2015. **21**(1).
22. Curry, M.R., *Cryopreservation of semen from domestic livestock.* Reviews of reproduction, 2000. **5**(1): p. 46-52.
23. Morris, G.J., et al., *Freezing injury: the special case of the sperm cell.* Cryobiology, 2012. **64**(2): p. 71-80.
24. Ibrahim, S.F., et al., *A study of the antioxidant effect of alpha lipoic acids on sperm quality.* Clinics, 2008. **63**(4): p. 545-550.
25. Tuncer, P.B., et al., *The effect of cysteine and glutathione on sperm and oxidative stress parameters of post-thawed bull semen.* Cryobiology, 2010. **61**(3): p. 303-307.
26. Twigg, J., et al., *Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants.* Human reproduction (Oxford, England), 1998. **13**(6): p. 1429-1436.
27. Kao, S.-H., et al., *Increase of oxidative stress in human sperm with lower motility.* Fertility and sterility, 2008. **89**(5): p. 1183-1190.
28. Zhina, A., E. Muhsin, and S. Shawkat, *Effect of Adding Pomegranate Juice to Tris Diluent on Some Semen Characteristics of Awassi Rams.* Indian Journal of Ecology, 2021. **48**(17): p. 340-343.

ISSN 2072-3857