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Research Paper

The effect of adding lycopene to improve some properties of dried and frozen semen of arabian rams

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ABSTRACT

The experiment was conducted in the animal field at Karma Ali site for a time of three months, from 10/15/2023 to 1/15/2024. Four Arabian rams were purchased from local markets in Basra Governorate, aged 2.5-3 years and of similar weights. They were fed a ration consisting of a feed mixture of barley, bran, straw, and some mineral salts. Clean water was provided to them inside the pen. The rams were trained to collect semen using an artificial vagina for rams in the animal field for two weeks. The experiment included adding different concentrations of lycopene (0, 0.5, 1, 1.5) g/100 ml. The tubes were tightly closed and placed in the refrigerator. After the temperature stabilized at (5°C), they were stored in liquid nitrogen at -196°C for 10, 20, and 30 days. All the characteristics of the diluted and frozen semen were studied using (CASA). T2 (lycopene 0.5 g/100 ml) was significantly outperformed the other treatments in the percentage of individual sperm motility and live sperm in diluted and frozen semen. T2 also recorded the lowest values in the percentage of dead and deformed sperm. The 10-day freezing time significantly outperformed the other 20- and 30-day freezing time in individual sperm motility. There was no significant difference between the 10, 20, and 30-day freezing time in the percentage of live sperm. Furthermore, the 10-day freezing time showed the lowest values in dead sperm compared to the other freezing time. T2 exhibited the highest values in the straight-line velocity (VSL) and linearity of the sperm trajectory (LIN) for diluted and frozen semen compared to the other treatments. The freezing time of 10 days showed the highest values in VSL, while the freezing time of 10.20 days register the highest significant values in LIN.

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1. INTRODUCTION

It is essential that cryopreserved, frozen, and liquefied sperm remain intact in all their components, especially the acrosome, plasma membrane, and DNA. Furthermore, their individual progressive motility must be rapid and at a level that makes them effective and capable of reaching the female reproductive tract and penetrating the oocyte membranes [1]. The diluent type and composition also play an important role in maintaining the integrity of sperm during cryopreservation and freezing processes through its effect on the osmotic pressure of the sperm membrane and keeping the pH of the stored sperm within neutral limits, on the one hand. On the other hand, the diluent plays an important role in releasing enzyme antioxidants and reducing the rates of reactive oxygen species (ROS) as well as lipid peroxides (LOP) present in the seminal plasma and sperm cytoplasm, thus increasing the vitality of sperm and their ability to fertilize during artificial insemination processes [2]. Lycopene is the red, yellow, or brown pigment found in some vegetables such as tomatoes and some fruits such as apricots, watermelons, and grapefruit, but it is found in high concentrations in red-colored plants such as tomatoes [3]. Lycopene is a carotenoid derivative but does not carry vitamin A activity. It is considered a natural plant pigment produced by plants and microorganisms during photosynthesis to protect them from activity. Photosynthesis and increased photosensitivity [4].

Many researchers have also confirmed the ability of lycopene to control and prevent the formation of free radicals, reduce oxidative stress rates, and prevent the formation of nitrogen dioxide and hydroxyl radicals in the living body, whether in humans or animals [5]. In our current study, lycopene will be added to the diluted semen of Arabian rams with turmeric, egg yolk, fructose, and glycerol, and frozen in liquid nitrogen at -196°C for times of 10, 20, and 30 days to maintain and increase the rate of individual sperm motility, progressive motility, and vitality during freezing storage processes to increase the efficiency and quality of semen stored in nitrogen for longer times to achieve the highest rates of fertilization during artificial insemination in sheep.

2. RELATED WORK

Cryopreservation of ram semen is a commonly used technique in artificial insemination programs; however, it often leads to reduced sperm viability, motility, and membrane integrity due to oxidative stress. This has prompted researchers to explore various strategies to protect sperm cells during freezing and thawing processes. One such strategy involves the addition of antioxidants to semen extenders to mitigate the damaging effects of reactive oxygen species.

Natural antioxidants have shown promise in maintaining sperm quality during cryopreservation. Lycopene, a potent antioxidant found in tomatoes and other red fruits, has been highlighted for its ability to neutralize free radicals and protect cell membranes. It has been applied successfully in semen preservation for various animal species, including humans, boars, and bulls.

Its incorporation into semen extenders has led to improved motility, viability, and acrosome integrity in frozen-thawed sperm samples. Additionally, lycopene has been observed to reduce lipid peroxidation in sperm membranes, which is a key factor in cryo-damage. Although there is extensive research on the use of antioxidants in semen preservation, the specific effect of lycopene on ovine semen—particularly Arabian rams—remains limited.

Previous studies have focused mainly on synthetic antioxidants or other natural compounds, leaving a research gap regarding the use of lycopene in sheep breeds adapted to harsh environmental conditions. Arabian rams, known for their genetic and physiological uniqueness, may respond differently to such treatments. Therefore, investigating the use of lycopene in improving the cryosurvival of Arabian ram semen is both relevant and necessary.

This study aims to bridge that gap by evaluating the protective role of lycopene in the drying and freezing of semen, potentially enhancing post-thaw sperm quality and supporting more effective breeding programs.

3. METHODOLOGY

The experiment was conducted in the animal field of the College of Agriculture / University of Basra, Karma Ali site, for a time of three months, from 10/15/2023 to 1/15/2024. Four Arabian rams, aged 2.5-3 years and with similar weights, were purchased from local markets in Basra Governorate. The study included: Freezing with the addition of different concentrations of lycopene: Semen samples were collected from the four rams using artificial vaginas for rams. The semen was transferred to the laboratory using test tubes and protected from light by wrapping it with a piece of aluminum foil. The tubes were placed in a water bath at a temperature of 37°C. All special physical tests were performed (color, group and individual motility of sperm, sperm concentration, and the percentage of live, dead, and deformed sperm) using a Semen Analyzer device. The semen was then diluted with diluents. The experiment was designed to study the characteristics of Arabian ram semen diluted with levels of lycopene (0.0.5, 1, 1.5) g/100 ml, the tubes were tightly closed and placed in the refrigerator after the temperature stabilized at (5°C) and then the diluted and cooled semen samples were filled in 1 ml Eppendorf, and stored in liquid nitrogen at -196°C for 10, 20 and 30 days. In order to test the effect of freezing on the semen characteristics of rams and to study the effect of adding lycopene and the duration of freezing, various readings were taken using a computer-assisted semen analysis (CASA) device which included (motility, straight linear sperm velocity (VSL), linearity of sperm path (LIN). The percentage of live, dead and deformed sperm was also calculated using the method of [6].

Statistical Analysis

The data were statistically analyzed using [7] as a two-factor experiment. The first factor included different lycopene levels (0, 0.5, 1, and 1.5) grams, and the second factor included different freezing time, according to the following mathematical equation:

$TB_{ij}+ e_{ijk}$, $Y_{ijk}=\mu+T_i+ B_j$, Where, Y_{ijk} = the trait under study, μ = the overall mean, T_i = lycopene concentrations (0, 0.5, 1, and 1.5) grams, B_j = freezing time (30, 20, and 10) days, TB_{ij} = the interaction between lycopene concentrations and freezing storage times, and E_{ijk} = experimental error that is randomly and normally distributed with mean equal to zero and variance σ_e^2

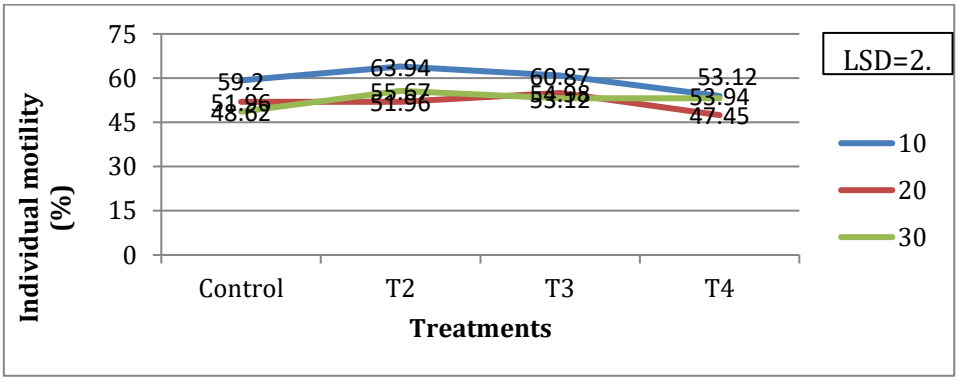
4. RESULTS AND DISCUSSION

4.1 Individual Sperm Motility

It is noted from Figure 1 that the treatment had a significant effect on the individual movement of sperm, as the T2 treatment outperformed the T3, control and T4 treatments, as the averages were 59.22, 56.32, 53.20 and 49.11%, respectively. It is also noted that the duration of freezing storage had a significant effect on the individual motility of sperm, as the 10-day time outperformed the remaining times of 20 and 30 days, with the averages being 59.49, 53.11, and 50.84%, respectively. When following up on the interaction between the treatment and the storage time, it was found that T2 at the 10-day time achieved the highest percentages significantly in the individual motility of sperm compared to the rest of the treatments and the rest of the storage times, as the individual motility was 63.94%, while the control treatment at the 30-day storage time register the lowest percentages significantly in the individual motility of sperm, as it was 48.62%. The results of the study were consistent with what was explained by [8], [9]. [10], [11], [12] in that the percentage of sperm Motility gradually and significantly decreased after freezing and thawing, and a significant change occurred during freezing time and for longer times, causing a significant decrease in motility. They demonstrated that the freezing process negatively affects individual sperm motility, and that differences in the concentrations and types of substances used in diluents are among the most important causes of sperm damage and death. Many researchers have shown that some concentrations of these substances yielded positive results, increasing sperm vitality and activity, while other concentrations had a negative and harmful effect on sperm vitality and motility. Other studies have shown that the effect of freezing on sperm motility can be mitigated by using cryoprotectants, such as glycerol and egg yolk, which can help protect sperm from membrane damage and prevent the formation of

ice crystals during freezing. They have also demonstrated that slow freezing leads to a 30-50% decrease in sperm motility compared to samples frozen by rapid freezing. A decrease in the percentage of live sperm and their motility was also observed after the samples were thawed. Rapid freezing maintains sperm motility better than slow freezing. Adding glycerol during the sperm freezing process improves sperm motility rates after thawing. The decrease in sperm vitality and motility is due to the effect of freezing and thawing, through the increased permeability of the sperm plasma membrane and the weakening of the ability of mitochondria to provide the necessary energy for it [13], [14], [15]. The results of the study were consistent with what [16], [17] found that adding levels of lycopene to diluted and frozen semen for spaced times improved individual sperm motility. They attributed this to the synergistic role played by lycopene with glycerol added during the sperm freezing process, and its antioxidant action, ridding sperm of reactive oxygen species (ROS), free radicals, and peroxides, and increasing sperm motility and activity during the thawing process.

Figure 1. The Diagram Shows the Effect of Treatments and Freezing Duration on Individual Sperm Motility (%) of Ram Semen



4.2 Percentage of Live and Dead Sperm

It is noted from Table 1 that the effect of treatment was significant (P<0.05) on live sperm, as treatment T2 significantly outperformed the control treatments, T4, and T3, as the means were 68.33, 64.19, 60.29, and 56.46%, respectively. The freezing storage time had a significant effect on live sperm, as the 10-day time significantly outperformed the 20- and 30-day storage times, as the averages were 65.49, 62.37, and 59.10%, respectively. When following up the interaction between treatment and storage time, it was found that T2, at the 10-day time, register the highest results significantly, compared to the rest of the treatments and the rest of the storage times, as the of live sperm was 72.88%, while T4, at the 30-day time, was significantly lower for live sperm, as it was 52.93%. Table 2 also shows that the treatment had a significant effect on dead sperm, as treatment T2 register the lowest significant percentages, compared to the rest of the treatments, T3, T4, and control, as the averages were 13.40, 14.41, 15.68, and 16.96%, respectively. It is noted that the freezing of 10 days had the lowest of dead sperm compared to the remaining freezing time of 20 and 30 days, as the averages were 13.87, 15.10, and 16.37%, respectively. When following up on the interaction between the treatment and the storage time, it was found that T2 at the 10-day time register the lowest of dead sperm compared to the rest of the treatments and the rest of the storage times, as it was 11.89%, while the control treatment at the 30-day time registers the highest of dead sperm, which was 18.18%. The study results are consistent with what many researchers have confirmed, that semen freezing and thawing processes lead to a decrease in the of live sperm and an increase in the of dead and deformed sperm. This is due to the morphological damage that occurs to the sperm, which is concentrated in the acrosome and sperm membranes. They also explained that the sperm freezing process increases oxidative stress, releases free oxygen species, decomposes fructose, and releases lactic acid [18], [19], [20]. Although the addition of glycerol during semen freezing with liquid nitrogen is important because it prevents the formation of ice crystals and the rupture of sperm membranes during thawing, it may be a cause of increased production of free radicals and increases sperm toxicity and membrane disruption [21]. In our current study, levels of lycopene were added, so the results were better

than the control group (without lycopene), as the second treatment had the highest significant percentage of live sperm and the lowest significant percentage of dead and deformed sperm. This may be due to the role of lycopene and its function as an antioxidant in ridding sperm of free radicals and peroxides, maintaining osmotic pressure inside the sperm, preserving its contents, and preventing the pressure inside the sperm from reaching the critical point, rupturing its membrane, and releasing its contents. Meanwhile, the higher concentrations of lycopene in the third and fourth treatments showed a significant decrease in the percentage of live sperm. This may be due to the fact that increasing the concentration of lycopene to higher levels led to sperm toxicity [22], [18], [23].

Table 1. Effect of Treatments and Freezing Duration (Months) on the Percentage of Live Sperm (%) in Ram Semen (Mean ± SE).

Storage time Treatments	10	20	30	Treatments Means
Control	66.01 ±0.22	63.98 ±0.25	62.57 ± 0.44	64.19 ± 1.50 B
T2	72.88 ± 0.17	68.13 ± 0.14	64.01 ± 0.28	68.33 ± 3.79 A
T3	63.05 ±0.16	60.93 ± 0.20	56.88 ± 0.21	60.29 ± 2.68 C
T4	60.00 ± 0.16	56.45 ± 0.61	52.93 ±0.12	56.46 ± 3.03 D
Duration Means	65.49 ± 4.93 A	62.37 ± 4.42 A	59.10 ± 4.60 AB	Interaction LSD 3.83

Means followed by different letter differ significantly at P=0.05.

Table 2. Effect of Treatments and Freezing Duration (Months) on the Percentage of Dead Sperm (%) in Ram Semen (Mean ±SE).

Storage time Treatments	10	20	30	Treatments Means
Control	15.88 ± 0.06	16.84 ±0.14	18.18 ± 0.04	16.96 ± 0.99 A
T2	11.89 ±0.28	13.55 ± 0.50	14.76 ±0.06	13.40 ± 1.26 D
T3	13.56 ±0.41	14.18 ±0.09	15.50 ± 0.36	14.41 ± 0.89 C
T4	14.17 ± 0.27	15.86 ± 0.13	17.03 ± 0.17	15.68 ±1.24 B
Duration Means	13.87 ± 1.49 C	15.10 ±1.37 B	16.37 ± 1.39 A	Interaction LSD 1.01

Means followed by different letter differ significantly at P=0.05.

4.3 Percentage of Velocity Straight Line (VSL) and Sperm Linearity (LIN)

Note from the Table 3 the treatment had a moral impact on the straight-line velocity (VSL), as the control treatment outperformed treatments T4, T3, and T2, as the averages were 32.10, 36.29, 39.49, and 42.91 micrometers/second, respectively. Freezing storage also had a significant effect on VSL, as the 30-day time was significantly superior (P<0.05) to the 20- and 10-day storage times in VSL ratios, as the averages were 37.32, 33.02, and 42.76 micrometers/second, respectively. When following up the interaction between the treatment and the storage time, it was found that T2 register the highest percentages significantly, at the 10-day time, as the VSL ratio was 47.97 micrometers/second compared to the rest of the storage times. From Table 4, it is noted that the treatment had a significant effect on the linearity of the path LIN Linearity (VCL\VSL)), as the T2 treatment outperformed the rest of the control

treatments, T3, and T4, as the averages were, as the T2 treatment was significantly superior (P<0.05) to the T3 and T4 control treatments, as the averages were 31.59, 34.91, 38.03, 41.98 respectively. It is also noted that the duration of freezing had a significant effect (P<0.05) on LIN values, as the 10-day freezing time significantly outperformed the 20- and 30-day storage times, with the averages being 33.88, 36.09, and 39.04% respectively. When the interaction between the treatment and the storage time was followed, it was found that T2 at the 10-day time register the highest values significantly compared to the rest of the treatments and the rest of the storage times, as it was 43.74%. Many researchers have shown that the types of sperm motility estimated by the sperm analysis device and according to the CASA system for frozen and thawed semen gradually differed after the thawing process.

The reason was attributed to the freezing process, which caused a decrease in the levels of protein and phospholipids in the membranes of frozen sperm [18]. In our study, the results agreed. Many researchers have found that adding lycopene improved different types of sperm motility (VSL, VAP, LIN) and reduced curved velocity and tremor. This is due to the synergistic role of lycopene with glycerol added to frozen semen in forming a foamy substance surrounding the sperm and helping prevent the formation of ice crystals, thus protecting the sperm membranes from tearing and leaking their contents [24], [25]. The results of the study were also consistent with what was found by [16], [23] that lycopene plays an important role in rearranging lipids and making them balanced with the proteins that make up the sperm membrane. This is reflected in increasing its ability and efficiency to maintain its internal contents on the one hand and raising and enhancing enzymatic antioxidants on the other. Others, and all of this is reflected in the increase in the types of sperm movement, especially progressive.

Table 3. Effect of Treatments and Freezing Storage Duration (Months) on the Straight-Line Velocity (VSL) (µm/S) for Ram Sperm (Mean ±SE).

Storage time Treatments	**10**	**20**	**30**	**Treatments Means**
Control	35.87 ± 0.31	32.13 ± 0.19	28.32 ±0.62	32.10 ± 3.24 D
T2	47.97 ±0.25	42.86 ± 0.20	37.91 ± 0.18	42.91 ± 4.29 A
T3	44.04 ±0.65	39.48 ±0.67	44.04 ±0.22	39.49 ± 3.90 B
T4	43.17 ± 0.13	34.80 ± 0.20	30.89 ± 0.35	36.29 ± 4.11 C
Duration Means	42.76 ± 5.18 A	37.32 ± 4.29 B	33.02 ± 3.82 C	Interaction LSD 3.20

Means followed by different letter differ significantly at P=0.05.

Table 4. The Effect of Treatments and Freezing Storage Duration (Months) on the Linearity of Sperm Path (LIN) (µm/S) in Rams' Sperm (Mean ± SE).

Storage time Treatments	**10**	**20**	**30**	**Treatments Means**
Control	34.62 ±0.32	32.11 ± 0.18	28.04 ±0.02	31.59 ± 2.84 D
T2	43.74 ±0.21	42.92 ± 0.10	39.29 ± 0,60	41.98 ± 2.05 A
T3	40.35 ± 0.30	37.65 ± 0.25	36.10 ± 0.13	38.03 ± 1.85 B
T4	37.46 ± 0.39	35.14 ± 0.16	32.12 ±0.18	34.91 ± 2.29 C
Duration Means	39.04 ± 3.51 A	36.09 ± 4.09 A	33.88 ± 4.37 AB	Interaction LSD 3.12

Means followed by different letter differ significantly at P=0.05.

5. CONCLUSIONS

Adding lycopene levels improved semen parameters frozen for extended cryopreservation times, including individual sperm motility (as measured by the CASA system), the percentage of live sperm, and a reduction in the percentage of dead sperm. Lycopene's ability to synergize with glycerol protects sperm membranes from rupture by preventing ice crystal formation during freezing and protecting sperm from cold shocks, in addition to its antioxidant activity in controlling the formation of free radicals and peroxides during freezing and thawing.

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Author Contributions Statement

Name of Author	C	M	So	Va	Fo	I	R	D	O	E	Vi	Su	P	Fu
Mashael Mohammed Abdulwahid	✓	✓	✓		✓	✓		✓	✓	✓	✓		✓	✓
Falah Abdulmohsen Al-Asadi	✓		✓	✓		✓	✓		✓			✓		✓

C : **C**onceptualization
M : **M**ethodology
So : **So**ftware
Va : **Va**lidation
Fo : **Fo**rmal analysis
I : **I**nvestigation
R : **R**esources
D : **D**ata Curation
O : Writing - **O**riginal Draft
E : Writing - Review & **E**diting
Vi : **Vi**sualization
Su : **Su**pervision
P : **P**roject administration
Fu : **Fu**nding acquisition

Conflict of Interest Statement

The authors declare that they have no conflict of interest regarding this research, its results, or its publication.

Informed Consent

Informed consent was obtained from the relevant authority prior to conducting the study, which involved the use of four Iraqi rams to evaluate semen characteristics after adding three levels of lycopene, cooling the semen, and freezing it for separate periods. All ethical and experimental standards were strictly followed, ensuring that the animals were cared for in accordance with international guidelines for the use of animals in scientific research. The animals underwent nutritional and veterinary care throughout the study, with all necessary measures taken to mitigate any potential problems.

Ethical Approval

Ethical approval was obtained from the University of Basra Ethics Committee to conduct this study using field animals (rams). All ethical guidelines were followed to ensure the safety and protection of the animals throughout the experiment.

Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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