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Research Article

Effect of Adding Different Concentrations of Vitamin C and E to Improve Poor Semen Quality in Ram

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Abstract | Sperm quality is influenced by cryopreservation. In an egg-yolk citrate extender, the current study evaluates the impact of various vit Cand E concentrations and their fusion on inferior-quality Ram semen. In this investigation, Four Ghezel-Merinose rams, who were roughly two years old, were employed .They were kept in settings of uniform lighting, housing, and food. The farm's feeding programme was applied to the experimental animals as well. Ejaculate of the rams is divided into seven groups. For the investigation, only ejaculates with a weak motile function were chosen. Divided into four equal aliquots, each ejaculate was diluted using an Egg-Yolk-Citrate (EYC) extender penicillin, streptomycin, and distilled water to make a resulting in conc. of 4×108 sperm/ml. Six duplicates of the study were conducted for both vitamins and their fusion. The standard error of the mean (SEM) is represented as the mean ±. The effects of various treatments on motion characteristics, plasma membrane integrity, and normal acrosome morphology were evaluated using paired sample T-test. The p-value (<0.05) is considered statistically significant. After thawing, the sperm cells' motility dramatically increased with the addition of 2 mM (56.8±1.16), 4 mM (57.6±1.36), and 7 mM (62.1±1.67). The sperms viability is improved post-thawimg and at 5C after addition of anti-oxidants vitamin C. When compared to the control values, the fresh, 5C, and post-thaw sperm motility improved significantly for each concentration of vitamin E. The p-value for 2mM(0.030), 4mM(0.04), and 7mM(0.03). Based on the current findings, it is concluded that vitamin C and E are very effective antioxidants in egg-yolk citrate extender. They can also protect ram sperm during cryopreservation by reducing oxidative stress caused by thawing. Nonetheless, there aren't any appreciable variations in normal acrosome % between the treatment and control groups. This study concludes that the quality of ram semen improves on treating with ascorbic acid and tocopherol in egg-yolk citrate extender. The addition of vitamin C and Vitamin E to the sample improves the motility, viability, and membrane integrity of poor semen quality.

Keywords | Vitamin C, E, Semen, Ram, Cryopreservation, Antioxidants

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INTRODUCTION

Artificial insemination (AI) is considered as one of the main reproduction methods in animal species for improving their genetic efficiency. Semen storage which is freezing and cryopreservation are two basic ways of saving it during AI Wrathall *et al.* (2004). Nevertheless, every step in the cryopreservation process such as dilution, cooling,

freezing and thawing reduces the ability of sperms to survive for AI decreasing success rates Akhter *et al.* (2023).

The freezing of semen in rams is difficult for a number of reasons. The outer membrane of sperm cells in these animals has many fatty acids that have not reacted with hydrogen and so are easily damaged by lipid peroxidation and oxidation Zhang *et al.* (2021). Nor do ram spermatozoa contain much anti-oxidant enzymes or other protective substances in their seminal fluid. It follows that when they are frozen, more harm is done to them than would be the case with most species Zhang *et al.* (2021).

Another method could be used instead of cryopreservation which is artificial insemination using diluted fresh or chilled semen; however, this way only works for a short time Acharya *et al.* (2019). To store the seed longer and keep it good, different extenders have been developed for use on it. These agents act as buffers against things such as shock from difference in saltiness, being frozen then unfrozen, bumped around by ice crystals or exposed to too many oxygen atoms Bustani and Baiee (2021). They also supply energy in the form of ATP at an appropriate pH while keeping out germs Raheja *et al.* (2018).

Antioxidants may also help reduce oxidative damage and improve semen parameters. Many types have been tried including enzymes like superoxide dismutase found naturally within living organisms' cells themselves which neutralizes one kind called reactive oxygen species before they can attack parts such as DNA; extracts made from plants which contain large numbers of different substances some known others yet to be discovered having protective effects against various kinds of harm done to bodies including those caused by free radicals produced during reactions involving oxygen atoms; vitamins like C (ascorbic acid) E(tocopherols), olive oil and glutathione among others Kameni *et al.* (2021). They can keep sperm alive longer or make more able to swim, stop cells being broken so often, help the cap over the head stay on until it is time for fertilization and lengthen how many days after ejaculation a sample will still be good Rizkallah *et al.* (2022).

One of the most important antioxidants for male sheep sperm is vitamins C(asorbic acid) and E(tocopherols), according to Diab *et al.* (2021). This lipid-soluble antioxidant was shown by Sarangi *et al.* (2017) to be an important first defense against free radicals which give up their extra energy through hydrogen atom donation. Vitamin E also reduces lipid peroxidation when added onto semen extenders thereby preventing this compound from affecting motility negatively.

Vitamin C is a water-soluble non-enzymatic antioxidant that works well in scavenging reactive oxygen species. It can act as a radical scavenger too which means it could protect some parts of sperms including DNA while improving their swimming abilities and overall health Allai *et al.* (2018).

Therefore, we propose that if antioxidants were added specifically ascorbic acid and tocopherol into a tris-based extender it would improve poor quality ram semen. The aim of this study is therefore to determine the effects of different concentrations of ascorbic acid in combination with tocopherol individually on various parameters such as volume, concentration during preservation period using tris-based extended media for low-quality ram semen preservation; ultimately leading towards better techniques for cryopreservation artificial insemination programs in sheep breeding.

MATERIALS AND METHODS

The Consort flowchart of the study is shown in Figure 1.

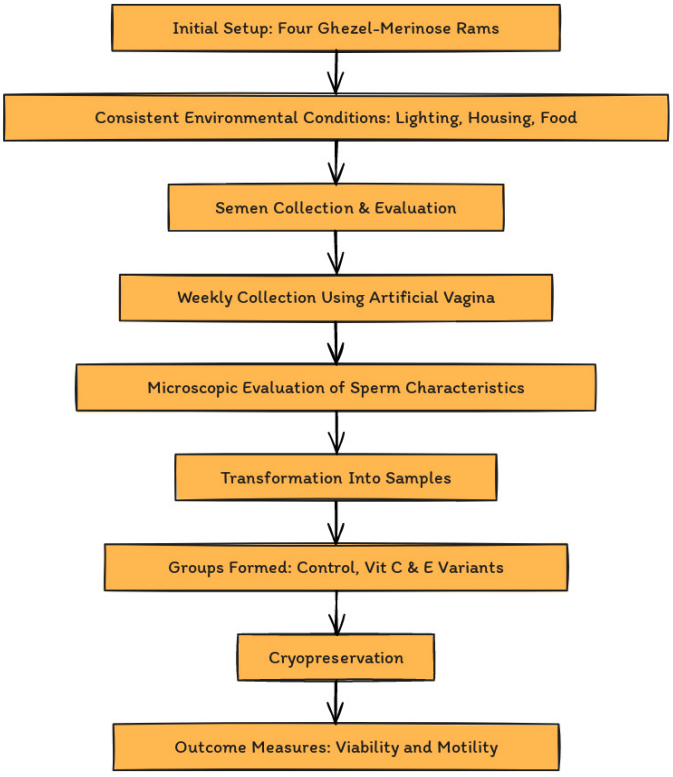

Figure 1: The Consort flowchart of the study.

Animal selection and maintenance

Four Ghezel-Merinose rams approximately two years old were selected for this study based on health screenings and reproductive history to ensure they were free from reproductive disorders. The rams were housed under uniform conditions regarding light, shelter, and feeding schedules to minimize environmental variability. They were fed a standard diet approved by the farm's feeding program and had ad libitum access to water.

Microscopic evaluation of sperm characteristics

We extracted semen once a week using an artificial vagina during the non-breeding season. We immediately submerged every ejaculate in a 37 ˚C water bath before evaluating it. We measured the volume of ejaculate by gathering it into a graduated tube. A drop or two of pure semen was put on a slide that had been warmed up at 37 ˚C without a coverslip so that the wave motion could be seen. The slide was then looked at with a phase-contrast (100×) microscope (Japan, Nikon, Eclipse, E200).

If no movement or lack of movement is seen, it is graded as 0. Spermatozoas that move slowly (<40%) are graded as 1. Some moving sperms that don't move in waves were seen and graded as 2 (30%). Some moving sperms that move slowly were seen and graded as 3 (50%). Spermatozoas that move quickly (30%) are graded as 4, and very first wave motion with moving spermatozoa (>40%) is graded as 5. One to five drops of fresh semen were placed under a coverslip under a phase contrast microscope at a magnification of around 200× to create an immersion of diluted semen. This allowed for an arbitrary estimation of the progressive motility of the sperm. We examined at least 200 spermatozoa randomly selected from at least four microscopic fields. We determined the final motility by taking the mean of four consecutive assessments. We used the hemocytometer to measure the sperm concentration. We evaluated the samples' sperm viability using the nigrosin-eosin staining method. ThThe stain was used to distinguish between viable and non-viable spermWe made the colorant solution by dissolving eosin-Y (1.67 g), nigrosin (10 g), and 2.9 g sodium citrate in distilled water (100 ml). On a warm slide, combine one drop of the semen sample with two drops of the stain, then spread the stain right away with a second slide to create the sperm suspension smears. We counted 200 cells using a phase contrast microscope at 400 magnification to determine their viability. We deemed only sperm that strictly excluded the stain as living, and deemed non-viable those that displayed partial or total purple coloring. The sperm with wave motion 1 = few sperm with weak movement were selected for the study.

Egg-yolk-citrate extender preparation

The semen was diluted using an Egg-Yolk-Citrate (EYC) extender composed of 2.9g/dl sodium citrate, 20ml/dl egg yolk, along with 1000 units/ml penicillin and 1000 µg/ml streptomycin in distilled water to achieve a final concentration of 4×10^8 sperm/ml. This extender provides nutritional support and protects against bacterial contamination during storage.

Functional integrity of the sperm plasma membrane

A 500µL solution of hypo-osmotic swelling (HOS) solution with a 75 mosm/kg concentration was mixed with 50µL of semen and left to sit in a water bath at 37 ˚C for 30 minutes. This was done to check how well the ram sperm membrane worked. We examined a small drop of incubated semen under a phase-contrast microscope at 400 magnification and counted two hundred sperm to determine if the tail was expanding or cooling. We believed that the sperm with coiled tails continued to function.

Viability of sperm

We examined the sperm viability (live-dead) using an eosin-nigrosin stain and a phase-contrast microscope (BX51, Olympus, Japan) at 400 magnification. We created the stain by combining three grams of sodium citrate dehydrate and one hundred milliliters of double-distilled water. Next, we added 1 g of eosin and 5 g of nigrosin and thoroughly mixed them with a stirrer. The idea behind this stain is that live sperm stays colorless while the dead sperm becomes stained pink by the eosin dye. Due to Nigrosin, the background is blue-black.

Transformation of semen into samples

We divided the rams' ejaculate into seven groups. We chose only ejaculates with a weak motile function for the investigation. Each ejaculate was split into four equal parts and weakened with penicillin, streptomycin, and distilled water using an Egg-Yolk-Citrate (EYC) extender. This made a solution with 4×108 sperm/ml.

Control: 1ml sperm+ Egg-yolk citrate (2.9g/dl sodium citrate+egg yolk 20ml/dl)+ 1000 units/ml pencillin + streptomycin 1000ug/ml + makeup volume to 100ml by adding distilled water.

Group 1: 1ml sperm+ Egg-yolk citrate (2.9g/dl sodium citrate+egg yolk 20ml/dl)+ 1000 units/ml pencillin + streptomycin 1000ug/ml l+ 2mM vitamin C +makeup volume to 100ml by adding distilled water.

Group 2: 1ml sperm+ Egg-yolk citrate (2.9g/dl sodium citrate+egg yolk 20ml/dl)+ 1000 units/ml+ streptomycin 1000ug/ml + 4mM vitamin C makeup volume to 100ml by adding distilled water.

Group 3: 1 ml sperm+ Egg-yolk citrate (2.9g/dl sodium citrate+egg yolk 20ml/dl)+ 1000 units/ml+ streptomycin 1000ug/ml + 7mM Vitamin C makeup volume to 100ml by adding distilled water.

Group 4:1 ml sperm+ Egg-yolk citrate (2.9g/dl sodium citrate+egg yolk 20ml/dl)+ 1000 units/ml+ streptomycin 1000ug/ml + 7mM Vitamin E makeup volume to 100ml by adding distilled water.

Group 5: 1 ml sperm+ Egg-yolk citrate (2.9g/dl sodium citrate+egg yolk 20ml/dl)+ 1000 units/ml+ streptomycin 1000ug/ml + 4mM Vitamin E makeup volume to 100ml by adding distilled water.

Group 6: 1 ml sperm+ Egg-yolk citrate (2.9g/dl sodium citrate+egg yolk 20ml/dl)+ 1000 units/ml+ streptomycin

1000ug/ml + 7mM Vitamin E makeup volume to 100ml by adding distilled water.

Frensh straws were used for the collection of sample. The straws used for the collection are 0.25ml. The aliquot was refrigerated at 5°C for 72 hours after cooling for 1.5–2 hours, in order to analyse parameters like pH, sperm viability, and progressive motility.

Semen cryopreservation

After a 4-hour equilibration period, we added semen to 0.25-ml straws at 50 °C and sealed them with powdered polyvinyl alcohol. We filled, sealed, and vacuum-frozen the straws in liquid nitrogen vapors, hovering them 2-4 cm above the liquid nitrogen for ten minutes. Finally, we submerged the straws in liquid nitrogen and placed them in a liquid nitrogen container.

Statistical analysis

Data are presented as Mean±SEM. A paired sample T-test was used to compare the mean values of vitamin C and vitamin E treatments against the control group. Significance was determined at P-values less than 0.05.

RESULTS AND DISCUSSION

Six duplicates of the study were conducted for each of vitamin C, vitamin E, and a combination of both vitamins. The mean represents the standard error of the mean (SEM). The effects of various treatments on motion characteristics, plasma membrane integrity, and normal acrosome morphology were evaluated using a paired sample T-test. The p-value (<0.05) is considered statistically significant.

The Table 1 shows the comparison of motility, viability, abnormal sperms, and membrane integrity for control, and different concentrations of vitamin C. (a superscription indicates the p-value(<0.05) is significantly different than control group).

Table 1: The comparison of motility, viability, abnormal sperms, and membrane integrity for control, and different concentrations of vitamin C.

Parameters	Semen conditions	Control	Vitamin C 2mM (Group1)	Vitamin C 4mM (Group 2)	Vitamin C 7mM (Group 3)
Sperm motility %	Fresh sperm 5 C Post-thawing	52.66±1.75 46.5±1.87 44.1 ± 1	63.1[a]±1.94 59.5±1.37 56.8 ±1.16	63.3[a] ±2.1 62.5 ±1.37 57.6 ±1.36	64.8 [a]±1.47 64.5 ±1.73 62.1 ±1.67
Live sperms%	Fresh sperm 5 C Post-thawing	71.6±1.86 59.8±3.1 55.1±1.4	70.1±1.47 59.6±1.03 72.3±1.63	70.6 ±1.83 70.6[a]±1.1.63 58.6±1.21	70.1 ±1.1 67.8[a]±1.47 71.5±1.04
Acrosomal normality	Fresh sperm 5 C Post-thawing	65.6±2.80 63.2±2.31 58.6±1.86	67.1±2.1 69.9±1.03 70.3±1.03	70.5±1.87 70.1±1.04 70.1 ±1.04	70.8±1.47 72±1.72 72.5± 1.87[a]
Intact acrosomal sperms %	Fresh sperm 5 C Post-thawing	60±1.78 58±0.894 53.6±1.36	64.3±.816 62.5±1.87 62.5±1.04	67.6±1.03[a] 66.3±1.96[a] 66±2.33[a]	72±1.78[a] 72.8±1.47[a] 74.5±1.04[a]

Table 2: The comparison of motility, viability, abnormal sperms, and membrane integrity for control, and different concentrations of vitamin E.

Parameters	Semen conditions	Control	Vitamin E 2mM Group 4	Vitamin E 4mM Group 5	Vitamin E 7mM Group 6
Sperm motility %	Fresh sperm 5 C Post-thawing	52.66±1.75 46.5±1.87 44.1 ± 1	63.16±1.94 59.51±1.37 63.33±1.21	63.3±2.1 62.5±1.37 66.33±2.16	64.8±1.47[b] 65± 1.1[b] 73.6±1.86[b]
Live sperms%	Fresh sperm 5 C Post-thawing	71.6±1.86 59.8±3.1 55.1±1.4	70.16±1.47 59.66±1.1 72.33±1.63	70.66±1.86 70.66±1.63[b] 58.66±1.21	72.83±1.47 67.83±1.47 71.5±1.1[b]
Acrosomal normality	Fresh sperm 5 C Post-thawing	65.6±2.80 63.2±2.31 58.6±1.86	65.67±2.80 63.16±2.31 58.66±1.86	67.1±1.86 69.67±1.03 70.33±1.03	70.5±1.87 70.5±104 70.5±1.04
Intact acrosomal sperms %	Fresh sperm 5 C Post-thawing	60±1.78 58±0.894 53.6±1.36	64.3333±0.86 62.5±1.87 62.5±1.04	67.66±1.03 666.33±1.96[b] 66±2.36[b]	72±1.76 72.83±1.47[b] 74.5±1.04[b]

The Table 2 shows the comparison of motility, viability, abnormal sperms, and membrane integrity for control, and different concentrations of vitamin E. (b superscription indicates the p-value(<0.05) is significantly different than control group).

Combined effect of vitamin C and E

The Table 3 shows the comparison of motility, viability, abnormal sperms, and membrane integrity for control, and different concentrations of vitamin E and C. (c superscription indicates the p-value(<0.05) is significantly different than control group).

Effect of vitamin C on sperm parameters

The impact of vitamin C, an antioxidant, on the sperm properties of frozen ram semen was assessed (Table 1 and Figure 2). When compared to the respective results, the post-thaw sperm motility improved significantly (P<0.05) in each group. The results indicate that adding vitamin C increased motility in comparison to the control group (P < 0.05), as shown in Table 1. After thawing, the sperm cells' motility dramatically increased with the addition of 2 mM (56.8±1.16), 4 mM (57.6±1.36), and 7 mM (62.1±1.67). The sperms viability is improved post-thawimg and at 5C after addition of anti-oxidants vitamin C. The acrosomal normality is not significant different than control group(p value= 0.06). The p-value 0.05 indicating suggesting that the differences are not statistically significant at the 0.05 significance level. The membrane integrity is improved by addition anti-oxidant in 4mM and 7mM (p value= 0.02).

Effect of vitamin E on sperm parameters

The impact of vitamin E, an antioxidant, on the sperm properties of frozen ram semen was assessed (Table 2 and Figure 3). When compared to the control values, the fresh, 5C, and post-thaw sperm motility improved significantly for each concentration of vitamin E. The p-value for 2mM(0.030), 4mM(0.04), and 7mM(0.03). This shows that addidtion of anti-oxidant has increased the motility of sperm post-thawing. The weak sperms selected for the study has increased mobility on addition of vitamin E. The sperm viability is improved at 4mM at 5C and 7mM of vit E post-thawing. The p-value is significantly different for 4mM(p-value= 0.032) and 7mM (p-value = 0.034). The addition of vitamin E improves the acrosomal normality at 4mM and 7mM. In the case, of membrane integrity it is improved on addition of 4mM and 7mM of vitamin E in the sperm sample.

Effect of vitamin C and E on sperm parameters:

The impact of vitamin E, an antioxidant, on the sperm properties of frozen ram semen was assessed (Table 3). When compared to the control values, the fresh, 5C, and post-thaw sperm motility improved significantly for vitamin C and E at 7mM.

The Figure 2 represent graphical distribution of Vitamin C response on semen mobility, viability, acrosomal normality, and membrane integirty. The above graph shows significant improvement of sperm mobility, viability, and membrane integrity post-thawing. However, there is no significant improvement in acrosomal normailty at 2mM, and 4mM of vitamin C concentration.

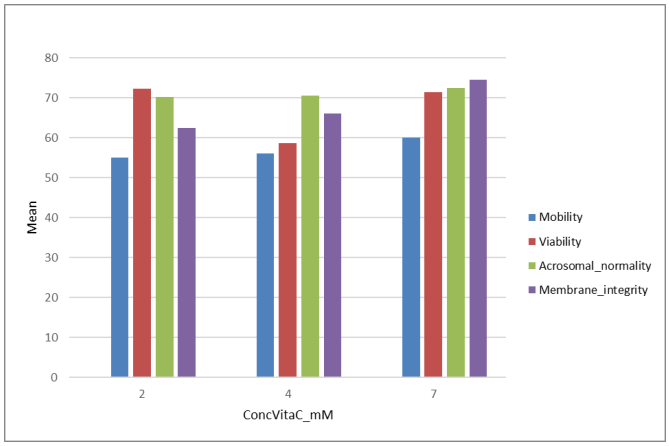

Figure 2: Graphical distribution of Vit C compared to semen characteristics.

Table 3: The comparison of motility, viability, abnormal sperms, and membrane integrity for control, and different concentrations of vitamin E and C.

Parameters	Semen Conditions	Control	Vitamin E 7mM	Vitamin C 7mM
Sperm motility %	Fresh sperm	52.66±1.75	64.8±1.47	64.8 a±1.47
	5 C	46.5±1.87	65± 1.1	64.5 ±1.73
	Post-thawing	44.1 ± 1	73.6±1.86	62.1 ±1.67
Live sperms%	Fresh sperm	71.6±1.86	72.83±1.47	70.1 ±1.1
	5 C	59.8±3.1	67.83±1.47	67.8±1.47
	Post-thawing	55.1±1.4	71.5±1.1[b]	71.5±1.04
Acrosomal normality	Fresh sperm	65.6±2.80	70.5±1.87	70.8±1.47
	5 C	63.2±2.31	70.5±104	72±1.72
	Post-thawing	58.6±1.86	70.5±1.04	72.5± 1.87a
Intact acrosomal sperms %	Fresh sperm	60±1.78	72±1.76	72±1.76
	5 C	58±0.894	72.83±1.47	72.83±1.47
	Post-thawing	53.6±1.36	74.5±1.04	74.5±1.04

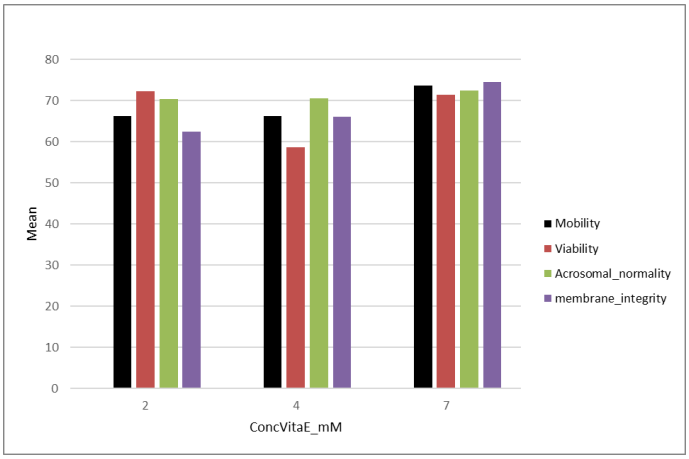

Figure 3: Impact of Vit E on selected sperm properties.

The Figure 3 represent graphical distribution of Vitamin E response on semen mobility, viability, acrosomal normality, and membrane integirty. The above graph shows significant improvement of sperm mobility, viability, and membrane integrity post-thawing. However, there is no significant improvement in acrosomal normailty at 2mM, and 4mM of vitamin C concentration.

This research study investigated how adding different amounts of the antioxidants ascorbic acid (vitamin C) and α-tocopherol (vitamin E), separately and together; to a tris-based egg yolk citrate extender affected certain quality parameters of low-quality ram semen. According to the results, this means that when these antioxidants are included especially at greater amounts like 4mM or 7mM they can greatly increase sperm motility, viability and plasma membrane integrity in comparison with controls without supplementation.

These findings align with other studies which have shown that vitamins C and E improve sperm quality across many species including rams (Akhter *et al*., 2011; Guerra *et al*., 2012; Barstow, 2018). It is believed that antioxidants protect against damage done by reactive oxygen species on spermatozoa mainly through their antioxidant properties. By scavenging ROSs and stopping lipid peroxidation, vitamin c and e can save DNA structures within cells from oxidative stress caused by free radicals which will keep them viable longer Qamar *et al*. (2023).

A study demonstrated that vitamin C and E supplementation in Tris diluent improved sperm motility and viability in cooled Awassi ram semen over five days of storage at 5°C. The antioxidants significantly decreased sperm abnormalities and acrosomal defects, suggesting their protective role against oxidative stress during storage Azawi and Hussein (2013).

As well as, vitamin E added directly to the diet was found to enhance the semen quality significantly, indicating that dietary approaches can also be beneficial for semen quality management. This finding was supported by a study on the Jing-tang Black Goat, showing improved sperm density and motility with increased dietary vitamin E (Liu *et al*., 2005).

Al-Saab and Hazem (2015) reported that subcutaneous injection of vitamin C in Awassi rams significantly increased sperm concentration and improved other semen traits, indicating a systemic effect of antioxidants when administered via injection.

Vitamins C and E together raise sperm kinematic characteristics, including curvilinear velocity Mittal *et al*. (2014). Trolox, an analogue of vitamin E, has a considerable impact on sperm's average travel velocity Minaei *et al*. (2012). Castellini *et al*. (2003) found similar outcomes using various vitamin E dosages Castellini *et al*. (2003). Hu *et al*. (2010) found that adding vitamin C to an extender dramatically increases sperm motility and straight-line velocity. Vitamins C and E, which are antioxidants, enhance sperm kinematics and guard against lipid peroxidation Akhter *et al*. (2011). Another study investigating sperm motility concluded that using a combination of Vit C and E reduces the oxidation process by improving the sperm trajectory motion Bansal and Bilaspuri (2010). Vitamins C and E, according to some previous research, can improve sperm viability and the functional integrity of the sperm plasma membrane. However, Akhter *et al*. (2023) demonstrated that antioxidants did not significantly affect the viability and integrity of the sperm plasma membrane in Kail Ram semen. These investigations disagreed with the findings of the current investigation Akhter *et al*. (2023). Adding vitamins C and E together worked better than adding each vitamin alone to protect the sperm membranes from lipid peroxidation and reactive oxygen species Lukusa (2019). The combined effects of ascorbic acid and tocopherol improved sperm parameters. Furthermore, when added separately to each vitamin, the optimal amounts of ascorbic acid and tocopherol to preserve plasma membrane integrity and sperm viability are 200 and 400 mg/l, respectively.

However, this combination seemed to work better than either one alone suggesting some kind of cooperative or additive effect between them. A recent study by Shedeed (2020) found the addition of vitamins E and C to the extender for Barki ram semen reduced oxidative stress and maintained better physical characteristics of ram spermatozoa during 48 hours of chilled storage

Country to our results, a study examining the impact of antioxidant vitamin supplementation on rams exposed to high altitude reported mixed results. While some improvements in semen characteristics were prevented

by the vitamins, there were also notable negative effects, indicating that supplementation might not consistently enhance semen quality in all environmental conditions or dosages Cofré *et al.* (2018). Another study on the supplementation of vitamins in different animal models, including rams, noted that while there were some improvements in antioxidant levels, these did not translate into significant enhancements in semen quality. This suggests that the role of vitamins may be more about maintaining baseline semen quality rather than improving it, depending on the existing health and condition of the animals involved Ozer *et al.* (2020).

Nonetheless there are limits to what we can learn from this study: only four rams were used so statistical power and generalizability may be low due to small sample size; individual differences among animals in terms of reproductive health status or age could have affected findings too; moreover it was carried out with just one breed (Ghezel-Merinose) which restricts applicability across breeds/populations.

While the treatment groups did show increased percentages of motility, viability, and membrane integrity in comparison to controls, no statistically significant differences were found between them for progressive motility or acrosomal integrity. This discovery is not entirely consistent with other research involving different species such as Merino rams and cattle (Baker *et al.*, 2021; Castellini *et al.*, 2003).

Also good to note would be that maybe more needs done on figuring out what's best – how much exactly should we use? Although it helped at higher levels like 4mM or 7mM in this investigation, previous work has recommended lower doses i.e. 200 mg/L each vitamin separately or even both together but using 400mg/L tocopherol instead of just half that amount alongside ascorbic acid (Yu *et al.*, 2019). These discrepancies illustrate further need for investigating optimal amounts by species and maybe even breeds.

Despite being limited, this research adds to the increasing number of studies that show antioxidant supplementation in semen extenders could improve the quality of stored ram sperm. By systematically evaluating different amounts of vitamins C and E, the authors were able to determine how these two antioxidants work in a dose-dependent manner, which will help guide future investigations.

To build upon and confirm these findings, it would be necessary to conduct larger scale experiments involving multiple breeds of rams while controlling for confounding variables. Moreover, fertility trials designed to measure fertilization rates and pregnancy outcomes should also be carried out so as not only translate these observations made under laboratory conditions into practical applications but also enhance reproductive efficiency within sheep breeding programs.

Additionally, researchers need to find out more about what makes vitamin C and E protect ram spermatozoa such as whether other compounds or antioxidants could be used together with them in order for this protection to happen hence come up with best formulations for preserving rams semen.

In summary, what this study did was indicate that tris-based extenders can be supplemented with antioxidants like vitamin C or E when trying to improve low-quality ram semen during storage. However there are still some areas where further investigation needs to take place so that optimal levels can be established alongside mechanisms involved thus advancing on current practices used in conserving sperms whose outcomes lead into better lamb production programs.

CONCLUSIONS AND RECOMMENDATIONS

Based on the current findings, it is concluded that vitamin C and E are very effective antioxidants in egg-yolk citrate extender. They can also protect ram sperm during cryopreservation by reducing oxidative stress caused by thawing. Nonetheless, there aren't any appreciable variations in normal acrosome % between the treatment and control groups. This study concludes that the quality of ram semen improves on treating with ascorbic acid and tocopherol in egg-yolk citrate extender. The addition of vitamin C and Vitamin E to the sample improves the motility, viability, and membrane integrity of poor semen quality.

NOVELTY STATEMENT

The addition of vitamin C and vitamin E to the poor quality semen improves the motility, viability, and membrane integrity of sperm.

AUTHOR'S CONTRIBUTION

All authors contributed equally to the manuscript.

Conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Acharya, Mohan, Burke, Joan M, Rorie, Rick W (2019). Effect of semen extender and storage temperature on motility of ram spermatozoa. Adv. Reprod. Sci., 8: 14.

Akhter S, Zubair M, Mahmood M, Andrabi, S. M. H., Hameed, N., Ahmad, E., & Saleemi, M. K. (2023). Effects of vitamins C and E in tris citric acid glucose extender on chilled semen quality of Kail ram during different storage times. Sci. Rep., 13: 18123. https://doi.org/10.1038/s41598-023-43831-2

Akhter, S., Rakha, B. A., Ansari, M. S., Andrabi, S. M. H., & Ullah, N. (2011). Storage of Nili-Ravi buffalo (*Bubalus bubalis*) semen in skim milk extender supplemented with ascorbic acid and [alpha]-tocopherol. Pak. J. Zool., 43: (**Pages?**).

Allai, L., Benmoula, A., da Silva, M. M., Nasser, B., & El Amiri, B. (2018). Supplementation of ram semen extender to improve seminal quality and fertility rate. Anim. Reprod. Sci., 192: 6-17. https://doi.org/10.1016/j.anireprosci.2018.03.019

Al-Saab, Hazem K (2015). Effect of vitamin C injection on semen quality of Awassi rams.

Azawi, Osama I, Hussein, Elias K (2013). Effect of vitamins C or E supplementation to Tris diluent on the semen quality of Awassi rams preserved at 5 C. Veterinary research forum, 2013. Faculty of Veterinary Medicine, Urmia University, Urmia, Iran, 157.

Baker, Ahmed G, Alssadi, Saram, Mohammad, Ayhan K (2021). The effect of selenium and vitamin E addition on semen quality of Awassi rams. Zanco J. Pure Appl. Sci., 33. https://doi.org/10.21271/ZJPAS.33.s1.14

Bansal AK, Bilaspuri GS (2010). Impacts of oxidative stress and antioxidants on semen functions. Vet. Med. Int., https://doi.org/10.4061/2011/686137

Barstow C (2018). Effect of Omega 3, 6 and 9 fatty acids and vitamin E on Canine semen concentration, motility, morphology and cryopreservation. Auburn University.

Bustani, Ghadeer S, Baiee FH (2021). Semen extenders: An evaluative overview of preservative mechanisms of semen and semen extenders. Vet. World, 14: 1220. https://doi.org/10.14202/vetworld.2021.1220-1233

Castellini C, Lattaioli P, Dal Bosco A, Minelli, A., & Mugnai, C. (2003). Oxidative status and semen characteristics of rabbit buck as affected by dietary vitamin E, C and n-3 fatty acids. Reprod. Nutr. Dev., 43: 91-103. https://doi.org/10.1051/rnd:2003008

Cofré E, Peralta OA, Raggi A, De Los Reyes, M., Sales, F., González-Bulnes, A., & Parraguez, V. H. (2018). Ram semen deterioration by short-term exposure to high altitude is prevented by improvement of antioxidant status. Animal, 12: 1007-1014. https://doi.org/10.1017/S1751731117002452

Diab, Hm, Ahmed, Om, Fahim, Hi, Mohamed, M. Y. (2021). Usage of some natural or synthetic compounds as antioxidant and their effects on cryopreservation and penetration of ram spermatozoa. Adv. Anim. Vet. Sci., 9: 743-753. https://doi.org/10.17582/journal.aavs/2021/9.5.743.753

Guerra, M. M. P., Câmara, D. R., Silva, E. D., & Silva, S. V. . (2012). Use of antioxidants on ram semen.

Hu JH, Tian WQ, Zhao XL, Zan, L. S., Wang, H., Li, Q. W., & Xin, Y. P. 2010. The cryoprotective effects of ascorbic acid supplementation on bovine semen quality. Anim. Reprod. Sci., 121: 72-77. https://doi.org/10.1016/j.anireprosci.2010.04.180

Kameni, Serge L, Meutchieye, Félix, Ngoula F (2021). Liquid storage of ram semen: associated damages and improvement. Open J. Anim. Sci., 11: 473-500. https://doi.org/10.4236/ojas.2021.113033

Liu H, Hongbo L, Hailing L, Hailing L, Zhihong L, Zhihong L, Z., & Shi ZhaoHong, S. Z. (2005). Effect of dietary vitamin E concentration on semen quality of goat.

Lukusa K (2019). Dietary supplementation of selenium and addition of vitamin C and E in extender to enhance semen cryopreservation and reproductive performance of Saanen goats, University of Pretoria (South Africa).

Minaei MB, Barbarestani M, Nekoonam S, Abdolvahabi, M. A., Takzare, N., Asadi, M. H., & Amidi, F. (2012). Effect of Trolox addition to cryopreservation media on human sperm motility. Iran J. Reprod. Med., 10: 99-104.

Mittal, P. K., Anand, M., Madan, A. K., Yadav, S., & Kumar, J. (2014). Antioxidative capacity of vitamin E, vitamin C and their combination in cryopreserved Bhadavari bull semen. Vet. World, 7. https://doi.org/10.14202/vetworld.2014.1127-1131

Ozer KS, Gur S, Erisir M, Kandemir, F. M., Benzer, F., Kaya, E., Sonmez, M. (2020). Influence of vitamin E and vitamin E-selenium combination on arginase activity, nitric oxide level and some spermatological properties in ram semen. Reprod. Domest. Anim., 55: 162-169. https://doi.org/10.1111/rda.13601

Qamar, Yar A, Naveed, Ilyas M, Raza, Sanan, Roy, P. K., Bang, S., & Cho, J. (2023). Role of antioxidants in fertility preservation of sperm. A narrative review. Anim. Biosci., 36: 385. https://doi.org/10.5713/ab.22.0325

Raheja, N, Choudhary, Sanjay, Grewal, Sonika. (2018). A review on semen extenders and additives used in cattle and buffalo bull semen preservation. J. Entomol. Zool. Stud., 6: 239-245.

Rizkallah, Natalie, Chambers, Caitlin G, De Graaf, Simon P, Rickard, J. P. 2022. Factors affecting the survival of ram spermatozoa during liquid storage and options for improvement. Animals, 12: 244. https://doi.org/10.3390/ani12030244

Sarangi, A., Singh, P., Virmani, M., Yadav, A. S., Sahu, S., Ajithakumar, H. M., & Rath, A. P. (2017). Effect of antioxidants supplementation on the quality of Beetal buck semen stored at 4 C. Vet. World, 10: 1184. https://doi.org/10.14202/vetworld.2017.1184-1188

Shedeed HA (2020). Evaluating the effect of adding vitamins E and C to the extender for Barki ram semen by cooling. Int. J. Environ. Agric. Biotechnol., 5. https://doi.org/10.22161/ijeab.52.10

Wrathall AE, Simmons HA, Bowles DJ, Jones, S. . (2004). Biosecurity strategies for conserving valuable livestock genetic resources. Reprod. Fertil. Dev., 16: 103-112. https://doi.org/10.1071/RD03083

Yu, X., He, S., Wang, L., Kang, M., Zhu, Y., Wang, S., & Sun, X. (2019). Effects of Vitamin C and Vitamin E on cryopreservation of Guanzhong donkey semen. Pak. J. Zool., 51: 1777-1781. https://doi.org/10.17582/journal.pjz/2019.51.5.1777.1781

Zhang, L., Sohail, T., Yongjun, L. I., Yanhu, W. A. N. G., Changjiang, C. H. U., Yunkui, F. E. N. G., & Jinliang, M. A. 2021. Effect of different extenders on the sperm quality parameters of Hu ram semen preserved at 16°C. Kafkas Üniv. Vet. Fak. Derg., 27.