integrity Research Journals

Journal of Animal Science and Veterinary Medicine
Volume 10(3), pages 291-298, June 2025
Article Number: C683F0119
ISSN: 2536-7099
https://doi.org/10.31248/JASVM2025.564
https://integrityresjournals.org/journal/JASVM

Full Length Research

Cryoprotective potential of honey in caprine semen extension

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Received 23rd April 2025; Accepted 16th June 2025

ABSTRACT: The study was carried out to determine the effect of honey as a cryoprotectant agent in caprine semen cryopreservation to improve the viability of frozen-thawed caprine semen. The semen used was collected from two healthy and proven mature bucks weighing 30 to 35 kg using an electro-ejaculator. The semen after collection was divided into five groups to form five different treatments, with the control as T1, containing only glycerol. T2, T3, T4, and T5 contained 25%, 50%, 75%, and 100% honey, respectively, and were stored in a freezer at a temperature of -18 to -20°C. After dilution and freezing, the frozen semen was assessed for livability, mass activity, morphology, plasma membrane, acrosome integrity, and pH at 24-hour intervals for four days. Each sample was thawed in a water bath at 37 °C for 60 seconds before evaluation. The effect of honey and storage day on livability, morphology, plasma membrane, acrosome integrity, and pH was significantly affected ($p>0.05$) but fell within a normal range for caprine semen viability. With increasing storage days, there was a progressive slight decrease in all the parameters measured. The result showed that including honey in cryopreservation, the diluent had no deleterious effect on the quality parameters of caprine semen. It was concluded that honey has a promising ingredient in semen extension as it is not detrimental to the quality parameters of semen after storage for up to 72 hours.

Keywords: Cryopreservation, fertilising ability, glycerol, goats, semen quality.

INTRODUCTION

Livestock, more specifically the small ruminants, are thus the key drivers for sustainable development in agriculture and allied sectors (Artabandhu, 2024). World all over, small ruminants' production, like goats, is of socio-economic importance. The goat a very useful animal is one of the most versatile domestic animals in adaptation to arid and humid, tropical, and cold, desert and mountain conditions providing people with many important products, such as meat, milk, cashmere, mohair, skins, leather, draught and pack power, and manure for crops and gardens (Aslam *et al*., 2023). In general, goats are managed in extensive and semi-extensive systems with the dual purpose of milk and meat production. Avid *et al*., 2004. Most of the flocks are local breeds, and production is concentrated over a few months; 62% of milk production is concentrated between mid-April and mid-July. In the rural areas of tropical countries like Nigeria, small ruminants are important genetic resources and play a prominent role in the sustenance of the livelihoods of impoverished families (Adamu *et al*., 2020; Agbaye and Alaba, 2024). These genetic resources of this importance need to be exploited which can be achieved through one of the assisted reproductive techniques (ARTs) such as artificial insemination (AI) associated with semen technology which allows the preserved male genetic material to be used in females that are isolated from the males (Morrell and Mayer, 2017; Santos and Silva, 2020). One of the factors in the success of AI is semen quality,

including an extender. A Semen extender is added to preserve the metabolic demands of spermatozoa, control pH changes in the extracellular environment of the spermatozoa, reduce cryogenic damage, and also control bacterial contamination (Abdul, 2018). Cryopreservation is an advanced preservation technique for tissue, cells, organelles, or other biological specimens, performed by cooling the sample to a very low temperature. It is the most common approach to improve the preservation of fertility (sperm, embryos, and oocytes) in different species that may undergo various life-threatening illnesses. It allows for the genetic screening of these cells to test the sample for diseases before use. Cheepa *et al*., 2022. Due to the cryoprotectant toxicity, there is always a need for non-toxic CPAs, as an alternative to store cells at liquid nitrogen temperature; that's why researchers recently turned back to natural materials such as honey. Honey is now relatively widely used by researchers because it is a natural component that does not require sterilization or cause considerable side effects, which makes it the most interesting natural remedy for preserving biological cells in cryopreservation (Cheepa *et al.,* 2022). Natural pure honey has numerous imperative properties besides its taste and composition. A fresh extracted honey is a viscid fluid, whose gluiness is governed by number of substances and consequently differs in its composition with especial reference to its water content (Ahmad *et al*., 2017). Honey has been successfully supplemented in semen extenders and used as a non-permeating cryoprotective agent (CPA) in the cryopreservation and vitrification of mammalian gametes (El-Sheshtawy *et al*., 2016; Shahzad *et al*., 2016; Chung *et al*., 2019; Alfoteisy *et al*., 2020). Natural honey is a mixture of 25 sugars. These sugars benefit cell viability, some of which have been successfully used in cell cryopreservation (Han and Critser, 2009). Although each sugar can work singly as a cryoprotectant, they work better synergistically when used in different combinations (Alfoteisy *et al*., 2020). Honey has a highly complex chemical and biological composition comprising various essential bioactive compounds, enzymes, amino and organic acids, acid phosphorylase, phytochemicals, carotenoid-like substances, vitamins, and minerals (Zaid *et al*., 2021). Honey has also been reported to contain various amino acids, some of which have been successfully used as non-permeant cryoprotectants for mammalian spermatozoa and oocytes (Yamada *et al*., 2011). Manyi-Loh et al. (2011) reported that honey has a wide variety of proven antioxidant components, including phenolics, peptides, vitamin C, zinc, and enzymes (e.g., catalase and glucose oxidase). In addition to the several other desirable attributes of honey, including antimicrobial properties, it shares similarities with other cryoprotectants in that it can increase extracellular osmotic activities and it does not form a crystal lattice under low temperature (Alvarez-Suarez *et al*., 2010; Alfoteisy *et al*., 2020; Balogun *et al*., 2023). Thus, the present study investigated the use of natural honey as a cryoprotective in extending caprine semen.

MATERIALS AND METHODS

Experimental site

The Experiment was carried out at the Animal Physiology Laboratory Unit, Department of Animal Science, University of Ibadan. The site is located at the latitude 7°20'N and 3°50'E, 200 m above sea level.

Experimental animal and management

Two healthy and proven mature goat bucks, 2 to 3 years of age and 30 to 35 kg, were used in the study. The animals were semi-intensively managed in clean, well-ventilated, and illuminated pens with a cemented floor.

Source of experimental honey

The honey used in this study was obtained from the Forestry Research Institute of Nigeria (FRIN), Ibadan, and was freshly diluted to the doses required using distilled water.

Experimental treatments

There was a total of five treatments in this study
T1 - Glycerol (100%) control
T2 - Glycerol + Honey (75%:25%)
T3 - Glycerol + Honey (50%:50%)
T4 - Glycerol + Honey (25%:75%)
T5 - Honey (100%)

Semen collection

The use of an electro-ejaculator accomplished the collection of buck semen. Within 2 to 3 minutes after collection, the semen was taken to the laboratory and kept in a water bath at 37 °C for evaluation.

Semen processing and preservation

Before evaluation, semen was added to the extender at a ratio of 1:25 (i.e., 0.2 mL semen was added to 5 mL of prepared extender). The semen was warmed alongside the prepared extender to 37 °C and both were mixed by gently inverting the mixture. The extended semen was frozen at -18 to – 20 ^{0}C. The extended semen samples were cooled to 5°C by placing them in a refrigerator and left to stand for I hour. At the same time, glycerol was mixed with a portion of the extender and put in the refrigerator. Straws were also placed in the refrigerator to bring them to the same temperature as the semen and glycerol extended mixture. After 1 hour, the cooled glycerol extender mixture was gradually mixed with the extended semen, and the mixture was later put into straws and left

to equilibrate for another 1 hour before placing it in the deep freezer.

Semen evaluation

Semen was evaluated *in vitro* post-freezing for the following parameters at 24-hour intervals for 3 days (0, 24, 48 and 72 hours).

Semen temperature

A digital thermometer was dipped into the semen immediately after collection, and the temperature was recorded.

Semen pH

An electronic and sensitive pH meter was used for the determination of pH.

Ejaculate colour

Semen colour was evaluated by visual observation. The consistency was scored as: 1 = Watery-cloudy, 2 = Milky, 3 = Thin creamy, 4 = Cream, and 5 Creamy grainy (Shamsuddin *et al.,* 2000). Semen was also checked for blood stains, dirt, hair or any other contaminant. Good semen should not contain all these.

Ejaculate volume

The ejaculate volume was obtained by carefully pouring the collected semen into a sterilized graduated measuring cylinder. Measurements were then obtained from the lowest level of the meniscus.

Mass activity

To evaluate the mass activity, a drop of undiluted semen was placed on a pre-warmed slide at 37 °C without a cover slip and examined under a phase contrast microscope (X100 magnification). The mass activity was scored: 0 = No motility, 1 = Few sperm with weak movement, 2 = some motile spermatozoa (20 to 40 %) without wave movement, 3 = Slow wave movement (40 to 60%) with motile spermatozoa, 4 = Rapid wave movement without whirlpool (60 to 80 %) with motile spermatozoa, 5 = Very rapid wave movement with clear whirlpool (>80%) motile spermatozoa (Avdi *et al.,* 2004).

Morphology

The eosin-nigrosin stain was used to examine the morphologically normal spermatozoa (Evans and Maxwell, 1987). For each slide, about 100 spermatozoa were counted at X400, which allows for an accurate calculation of the percentage of different sperm defects.

Livability

A drop of semen was placed on a glass slide, and a drop of eosin-nigrosin stain was added and mixed gently. It was then smeared on a slide with the edge of another clean slide, air dried, and viewed under a microscope at (X400).

Measurement of plasma membrane integrity

Measurement of Plasma Membrane Integrity The semen was mixed with the Hypo Osmotic Swelling (HOS) test solution, homogenized, and incubated for 30-45 minutes at 37°C above the warming table. The ratio used is 10-20 µl of semen with 1000 µl of HOS. The mixed sample was dropped onto the object glass and covered with a cover glass. Evaluation was carried out under a microscope with 200x magnification by counting at least 200 spermatozoa at random. Spermatozoa with intact plasma membranes are characterized by circular or bulging tails, while spermatozoa with damaged plasma membranes are characterized by straight tails (Arifiantini, 2012).

Measurement of acrosomal integrity

Integrity. The semen was mixed with 2% eosin dye in a ratio of one drop of semen to four drops of 2% eosin, then thin smear preparations were made and dried on a heating table. Observations were made under a microscope by counting 200 spermatozoa cells. Spermatozoa that still have intact acrosomal hoods are characterized by 1/2 to 2/3 of the anterior part of the head being darker than the posterior (Anwar *et al*., 2015).

Statistical analysis

Data obtained were subjected to analysis of variance (ANOVA), SAS (2003). Means were separated using the Duncan's Multiple Range Test (DMRT) of the same statistical package.

RESULTS

Pre-dilution evaluation of buck semen

Table 1 reveals the result of the initial evaluation of buck semen before freezing was done. The result indicates that the volume of the ejaculate from the buck was 1 mL with a mass activity of "4" on a scale of 1 to 5. On a percentage basis, the activity of the spermatozoa could be reported as 75 % which serves as a justification for its use in the experiment. The present normal cells were assessed as 96 % with a livability of 94 %. The colour of the ejaculate was creamy white with a pH of 6.8.

Table 1. Pre-dilution evaluation of buck semen.

Parameters	Result;s
Volume (mL)	1.0
Mass activity	++++
Normal cell (Morphology (%)	96
Live/dead (%)	94
Colour	Creamy white
pH	6.8

Table 2. Effect of honey on the quality of extended buck semen at 0 hour.

Parameters (%)	T1	T2	T3	T4	T5	SEM
Liveability	92.33^{a}	93.67^{a}	92.67^{a}	91.00^{ab}	87.33^{b}	0.40
Normal cells	87.67^{b}	92.00^{a}	90.00^{a}	84.67^{b}	75.00^{c}	0.75
pH	6.85	6.80	6.78	6.82	6.75	0.00

^{abcde} = means along the same row with the same superscripts are significantly different ($p<0.05$) from each other.

Table 3. Effect of honey on the quality of extended buck semen at 24 hours.

Parameters (%)	T1	T2	T3	T4	T5	SEM
Liveability	82.67^{a}	71.67^{b}	70.33^{b}	69.67^{b}	67.33^{b}	0.74
Normal cells (Morphology)	75.00	69.67	72.67	73.00	68.67	0.66
pH	6.51^{a}	6.42^{b}	6.34^{c}	6.25^{d}	6.18^{e}	0.00

^{abcde} = means along the same row with the same superscripts are significantly different ($p<0.05$) from each other.

Table 4. Effect of honey on spermatozoa fertilizing ability of extended buck semen at 24 hours.

Parameters (%)	T1	T2	T3	T4	T5	SEM
Acrosome Integrity	82.33^{a}	72.67^{b}	74.33^{b}	70.33^{b}	71.67^{b}	0.65
Plasma Membrane	72.67^{a}	65.00^{b}	68.00^{ab}	68.33^{ab}	67.00^{ab}	0.67

^{ab} = means along the same row with same superscripts are significantly different ($p<0.05$) from each other.

Effect of honey on the quality of extended buck semen at 0 hours

Presented in Table 2 are the mean values of livability, normal cells (morphology), and pH of extended buck semen treated with graded levels of honey at 0 hours. Similar livability mean values were observed for T1, T2, T3, and T4, while a significant reduction ($p<0.05$) was observed from T5. A significant reduction ($p<0.05$) in normal cells (morphology) was also observed from T5, when compared with T1 (control). Similar mean values were presented for pH.

Effect of honey on the quality of extended buck semen at 24 hours

Table 3 presents the mean values of livability, morphology, and pH of extended buck semen treated with graded levels of honey at 24 hours. Similar livability mean values were observed for T2, T3, T4 and T5, while T1 was significantly higher ($p>0.05$). A significant reduction ($p<0.05$) in morphology was also observed from T,5, when compared with T1 (control). There was also an observed significant difference in pH across the treatments, with T5 having the lowest mean value as compared to T1.

Effect of honey on the spermatozoa fertilizing ability of extended buck semen at 24 hours

Table 4 shows the mean values of acrosome integrity and plasma membrane of extended buck semen treated with graded levels of honey at 24 hours. Similar acrosome integrity mean values were observed for T2, T3, T4 and T5, while T1 was significantly higher ($p>0.05$). Also, similar plasma membrane mean values were observed in T2, T3, T4, and T5 with a significant increase ($p>0.05$) in T1.

Effect of honey on the quality of extended buck semen at 48 hours

Presented in Table 5 are the mean values of livability,

Table 5. Effect of honey on the quality of extended buck semen at 48 hours**.**

Parameters (%)	T1	T2	T3	T4	T5	SEM
Liveability	75.67^{a}	67.67^{b}	62.67^{bc}	62.67^{bc}	59.67^{c}	0.62
Normal cells (Morphology)	74.00^{a}	64.33^{b}	62.67^{b}	64.67^{b}	61.00^{b}	0.60
pH	6.54^{a}	6.43^{b}	6.34^{c}	6.23^{d}	6.16^{e}	0.00

^{abcde} = means along the same row with the same superscripts are significantly different (p<0 .05) from each other.

Table 6. Effect of honey on spermatozoa fertilizing ability of extended buck semen at 48 hours.

Parameter (%)	T1	T2	T3	T4	T5	SEM
Acrosome Integrity	75.00^{ab}	72.33^{ab}	72.33^{ab}	78.33^{a}	65.67^{b}	1.06
Plasma Membrane	64.33^{a}	61.67^{ab}	60.00^{ab}	60.00^{ab}	57.00^{b}	0.49

^{ab} = means along the same row with the same superscripts are significantly different (p<0.05) from each other.

Table 7. Effect of honey on the quality of extended buck semen at 72 hours.

Parameter (%)	T1	T2	T3	T4	T5	SEM
Liveability	66.33^{a}	61.33^{ab}	61.00^{ab}	61.33^{ab}	58.67^{b}	0.61
Morphology	70.67^{a}	65.33^{b}	60.33^{c}	60.33^{c}	58.67^{c}	0.36
pH	6.46^{a}	6.37^{b}	6.28^{c}	6.18^{d}	6.07^{e}	0.00

^{abcde} = means along the same row with same superscripts are significantly different (p<0.05) from each other.

Table 8. Effect of honey on spermatozoa fertilizing ability of extended buck semen at 72 hours.

Parameter (%)	T1	T2	T3	T4	T5	SEM
Acrosome Integrity	67.33^{a}	66.33^{a}	62.00^{b}	62.67^{b}	57.33^{c}	0.39
Plasma Membrane	66.67^{a}	66.33^{ab}	62.00^{c}	62.67^{bc}	57.33^{d}	0.41

^{abcd} = means along the same row with the same superscripts are significantly different (p<0.05) from each other.

morphology, and pH of extended buck semen treated with graded levels of honey at 48 hours. There was a significant difference in livability mean values across the treatments, with similarities in T2, T3, and T4, while T1 was significantly higher (p>0.05) than T5. A significant reduction (p<0.05) in morphology was also observed from T5, when compared with T1 (control). There was also an observed significant difference in pH across the treatments, with T5 having the lowest mean value as compared to T1.

Effect of honey on the spermatozoa fertilizing ability of extended buck semen at 48 hours

The mean values of acrosome integrity and plasma membrane of extended buck semen treated with graded levels of honey at 48 hours are presented in Table 6. Similar acrosome integrity mean values were observed for T1, T2, T3, and T4, while T5 was significantly lower (p>0.05). Also, similar plasma membrane mean values were observed in T1, T2, T3, and T4, with a significant reduction observed in (p< .05) in T5.

Effect of honey on the quality of extended buck semen at 72 hours

Presented in Table 7 are the mean values of livability, morphology, and pH of extended buck semen treated with graded levels of honey at 72 hours. Similar livability mean values were observed for T1, T2, T3 and T4, while T5 was significantly reduced (p<0.05). A significant reduction (p<0.05) in morphology was also observed at T5, when compared with T1 (control). There was also an observed significant difference in pH across the treatments, with T5 having the lowest mean value as compared to T1.

Table 8 shows the mean acrosomal integrity and plasma membrane values for extended buck semen treated with varying levels of honey after 72 hours. Acrosomal integrity was similar across treatments T1 to T4, but significantly lower (p<0.05) in T5. For plasma membrane integrity, T1 and T2 had similar values, T3 and T4 were also similar, while T5 showed a significant decline.

DISCUSSION

Spermatozoa are subjected to several physical and

chemical stresses during cryopreservation. Because of the harmful effects of these factors, it is inevitable to have a reduction in the quality of spermatozoa after cryopreservation (Watson 2000; Andrabi, 2007; Lemma 2011).

Livability

Significant differences in the live/death ratio at 0 hours, 24 hours, 48 hours, and 72 hours were observed. The decrease in livability in the result follows the report of Reda *et al.* (2016). However, the live-to-death ratio of the sperm cells is still in the normal range, indicating that honey contains several sugars that can provide sufficient energy substrate for spermatozoa during incubation and preservation while acting as cryo-protectant and decreasing the extent of cell injury during cryopreservation (Fukuhara and Nishikan, 1993).

Morphology

For morphological evaluation, it was observed that there was a significant difference at 0 hours. However, there was no difference observed across the treatments at 24 hours, indicating that honey has no deleterious effect on the morphology of sperm cells up to 24 hours of storage. A significant difference was observed, however, at 48 hours and 72 hours. This could be the effect of other factors such as temperature, pH, and the storage method used on the chemical properties of the natural honey. These factors, when monitored, can also result in a non-significant difference in the morphology of caprine semen up to 72 hours of storage. Ott (1978) reported that the percentage of morphological abnormalities in the semen of bucks with normal fertility should be less than 5% during the breeding season. However, a significant amount of sperm cells were normal across the treatment, which may be attributed to the vitamin content of the honey, which is reported to improve sperm morphology (Akmal *et al.,* 2006),

pH

Spermatozoa are the only cells whose activity is outside the male body, and in the inconsistent chemical milieu of sperm, seminal plasma may have profound effects on sperm quality and pH is one of the most critical factors that determine the semen quality. Spermatozoa are highly affected by pH. Mishra *et al*., 2018. Spermatozoa can metabolise glucose through glycolysis for energy supply, producing lactate. Lactate and other permeant weak acids have been shown to reduce the intracellular pH of bovine spermatozoa and many other types of cells (Alomar 2023). There was a significant difference in the pH across the treatments at 0 hours, 24 hours, 48 hours, and 72 hours. However, the pH range across the treatments at 0 hours, 24 hours, 48 hours, and 72 hours still falls within the normal range, indicating that honey has no negative effect on the pH of frozen caprine semen. The significant difference in pH and morphology across the treatment might be the result of the ultrastructural changes of the natural honey during freezing. However, the changes in pH fall within the normal range, not making the semen more acidic or too basic. Studies have shown deviated/disrupted sperm functions at high/low pH, indicating the existence of a dynamic pH regulatory system in spermatozoa (Zhou *et al*., 2015).

Plasma membrane

Plasma membrane integrity is an absolute necessity for spermatozoa. The plasma membrane serves as the first defense of cells against the external environment that can damage the cells (Prihantoko *et al*., 2020). There was a significant difference for the plasma membrane at 24 hours, 48 hours, and 72 hours across the treatment with a gradual reduction from T1 to T5 at 48 and 72 hours. This gradual reduction in post-thawed semen with the increase in storage period may be a consequence of capacitation-like changes in the plasma membrane (Green and Watson, 2001) as well as accumulated cellular injuries that arise throughout the cryopreservation process. Inappropriate ejaculate manipulation before freezing may also be the cause as suggested by Roca *et al.,* (2006).

Acrosome Integrity

Acrosome integrity is one of the determining factors of the success of fertilization. Only acrosome-intact spermatozoa can penetrate the zona pellucida and fuse with the oocyte plasma membrane (Celeghini *et al.*, 2010). There were significant differences for acrosome integrity at 24 hours, 48 hours, and 72 hours across the treatments, with a reduction as the number of hours increased. This might be a result of the metabolic reaction the sperm cell undergoes, leading to damage to the sperm cell. The differences were similar to the report of Dorado *et al*. (2007).

Conclusion

Honey can be used in caprine semen extender as it shows no negative effect on semen characteristics after storage. The use of inexpensive, readily available, and effective ingredients in semen extender with the purpose of maintaining cell membrane integrity, preserving motility, morphology, and livability as well as preventing freezing and thawing injury, may result in a significant reduction in cost and enhance the livability of extended semen.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS

The authors wish to appreciate the support of the farm staff of the teaching and research farm, University of Ibadan, Ibadan, Nigeria, and also the laboratory members of staff, Animal Physiology and Bioclimatology Unit, Department of Animal Science of the same University, for their support.

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