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Effects of liquid storage of buck semen at refrigeration temperatures on sperm viability and fertility to develop ready to use goat semen diluent

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ABSTRACT

It is generally acknowledged that lower pH throughout liquid storage can decrease sperm motility and energy production, which may be advantageous for maintaining sperm viability during cryopreservation. Hence, the purpose of this study was to see how pH affected sperm viability, motility, membrane integrity, and oxidative changes after 5 days of storage in chemically specified extenders at refrigerator temperatures to formulate a ready to use goat semen diluent. Ejaculates were extended with tris-citric acid-fructose diluent with 20% or without egg yolk and stored at 5°C for 5 days. Sperm motility, live count, acrosome integrity, hypo-osmotic swelling positive spermatozoa, pH, and malondialdehyde all substantially differed significantly ($P < 0.05$) during different days of liquid storage. The pH of diluted semen decreased considerably ($P < 0.05$) from its original pH of 6.46 on 0 day to pH 6.06 on the 5th day of liquid storage at refrigeration temperature. Diluted semen at refrigeration temperature having 20% egg yolk had significantly higher ($P < 0.05$) seminal characteristics compared to liquid semen without egg yolk. The liquid storage of semen quality became worse day by day due to a drop in pH and egg yolks acted as a cryoprotectant against the harmful effects of chilling. Hence, 20% egg yolk may be used to enhance the keeping quality of diluted buck semen at refrigeration temperature for up to 5 days. Based on this result, we formulated a ready to use goat semen diluent for liquid semen storage at room temperature and refrigeration temperature.

1. INTRODUCTION

Liquid semen storage is a good alternative for artificial insemination (AI) compared to frozen semen [1,2]. A limited improvement has been achieved in liquid semen viability and fertilizing potential [3]. Seminal plasma with milk or egg yolk had a detrimental impact on the longevity of buck sperm cells [4]. There are few studies on the liquid conservation of buck semen in extending solution with specific chemical combinations that do not contain milk or egg yolk [5]. Lower pH decreases the metabolism and motility of sperm, which is a favorable sign that the spermatozoa would remain alive during preservation [6]. Buck semen was best kept in liquid form at a pH of 6.04; however, either pH 6.61 or pH 5.54 dramatically reduced sperm viability [7]. Our findings and those of previous research [6] show that maintaining high sperm motility during semen storage depends on the extender's starting pH. Monitoring the pH of the semen extender at various points during storage is crucial for optimizing the liquid semen conservation process. This outcome will be helpful when the freezing of semen is carried overnight to a laboratory. This is crucial for gene bank operations as well, as semen samples are often gathered in one place and sent to a central facility for processing and storage. AI is a powerful technique utilized in various mammals for breed improvement. Initially, its aim was to enhancing the number of insemination doses from a single ejaculate but its success was hindered by the lack of a suitable dilution medium [8]. It is extensively used in cattle and buffalos but less in other mammals such as sheep, horses, deer, and goats. AI plays a significant role in improving feasibility and enhancing economic efficiency. Fresh or well-preserved semen is required for effective AI, with approximately 95% of all, AI utilizing preserved semen [9]. Therefore, the preservation medium is crucial to maintaining semen quality. Proper preservation involves chilling or cryopreservation, with reduced temperature to induce sperm inactivity while stored in liquid form. The widely accepted cooling temperature for maintaining semen quality in goats is between 4°C and 5°C, which helps in decrease sperm metabolism. Chilled semen has advantages of higher fertility rates compared to cryopreserved semen. This results in reduced insemination doses, lowering storage expenses, and making AI more accessible to stakeholder. In addition, chilled semen has an extended lifespan within the female reproductive tract and exhibits higher fertilization rates than frozen semen. Developing suitable dilution mediums for preserving

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goat semen during chilling or cryopreservation is essential to further optimize and propagation of AI techniques [10].

This study aimed to determine the impact of pH on sperm viability, motility, membrane integrity, and oxidative alterations after storage in chemically specified extenders at refrigeration temperatures for 5 days.

2. MATERIALS AND METHODS

Except where otherwise noted, all chemicals were purchased from Sigma compounds Co. in St. Louis, Missouri, USA. All experimental methods used during the investigation adhered to institutional animal care and use committee guidelines.

2.1. Extender Medium Preparation

The diluent for buck semen is formulated with the following: 3.604 g of tris, 1.902 g of citric acid, 1 g of fructose, 100 mg of streptomycin, 60 mg of penicillin, and 100 ml of triple distilled water. The pH of the solution was adjusted to 6.8. This sperm medium contains a basic extender with hen fresh egg yolk 20% (v/v) [11].

2.2. Semen Collection and Evaluation

Semen was collected from breeding bucks (N-12) that were kept under semi-intensive management. Semen samples were taken biweekly after being stimulated in the presence of an estrus doe using an artificial vagina. Ejaculates volume, color, consistency, and mass activity were evaluated. Ejaculates from the same buck that had a mass motility of +4 or higher were pooled and split into five equal parts. Tris diluents with 20% (v/v) egg yolk and without egg yolk were used for semen dilution. After dilution and 24 h interval of liquid semen storage at refrigeration temperatures (5°C) for 5 days, various parameters including progressive sperm motility, sperm viability, hypo-osmotic swelling (HOS) integrity, acrosome integrity, pH, and malondialdehyde (MDA) levels were measured.

2.3. Motility Assessment

Sperm motility was observed by placing diluted semen (10µl) onto a clean slide, free of grease, and warmed to a temperature of 38°C at a magnification of 400X under a phase contrast microscope. The average values of two different specialists were taken into consideration while calculating the progressive sperm motility [12].

2.4. Sperm Viability Assessment

The live/dead spermatozoa were determined using the Eosin-Nigrosine stain [3]. Sperm cells that were alive before staining did not take any color and were seen as white transparent under a microscope.

2.5. Acrosome Integrity Assessment

Giemsa stain was employed to evaluate the spermatozoa's acrosome functionality. On a spotless, free of grease slide, a small smear of diluted semen was created and allowed to air dry. Then, the spotless slide was placed in Hancock's fixative and heated to 38°C for one hr. The slides were cleaned under flowing water and allowed to air dry. Then, the slides were submerged in Giemsa stain for 2 h at 38°C (6 ml Giemsa stain, 4 ml Sorenson buffer, and 90 ml triple-distilled water). The slides were stained, then cleaned with flowing water, and allowed to air dry. The acrosome morphology on the plates was investigated using an oil immersion microscope objective. We counted at least 200 sperms and computed the proportion of acrosome changes [13].

2.6. Functional Membrane Integrity Assessment

The HOS test was employed to evaluate the spermatozoa's membrane integrity [14]. Hypo-osmotic solution (150 mOS) and diluted semen (10: l) were combined in a microtube and incubated at 38°C for 2 h. The semen drop was put on a glass slide after incubation, put coverslip over the drop and seen under 400X. Along with the total number of spermatozoa measured, strong coiling was described when the sperm linear tail turned into heavily coiling [15].

2.7. MDA Estimation

Thiobarbituric acid (TBA) was used to measure the generation of MDA, as described by Zanganeh [16], with a few minor adjustments to sperm concentration and incubation period. The supernatant was extracted after the buck semen sample had been centrifuged at 7000 rpm at a 20mm radius for 5 min and washed in PBS/Tris buffer. The spermatozoa pellet was subsequently re-dissolved in Phosphate-Buffered Saline (PBS, pH = 7.2). On adding 2 mL of TBA-Trichloroacetic acid (TCA) reagent (15%, w/v TCA; 0.375%, w/v TBA; and 0.25 N HCl) to 1 ml of spermatozoa suspension, the amount of lipid peroxide in the spermatozoa was determined. The mixture was subjected to a 45-min treatment in a hot water bath. The resultant suspension was centrifuged at 7000 rpm at a 20 mm radius for 5 min after cooling. The leftover supernatant was separated out, and a ultraviolet spectrophotometer was used to detect the absorbance at 535 nm. According to the particular absorbance coefficient (1.56×10^5/mol cm^3), the MDA concentration was calculated.

$$\text{MDA}\ (\mu\text{mol / ml}) = \frac{\text{OD} \times 10^6 \times \text{total volume}\ (3\ \text{ml})}{1.56 \times 10^5 \times \text{test volume}\ (1\ \text{ml})}$$

2.8. Statistical Analysis

The Statistical Package for the Social Sciences ver. 16.0 (IBM®, USA) was used to conduct the statistical analysis. The percent of sperm motility, live, acrosome intact, HOS +ve sperm, and MDA were all conditional factors in the factorial model. The alteration in pH was an independent variable. To determine the significant difference between various parameters at $P < 0.05$, a *post hoc* test Duncan LSD T3 was performed. To give distinct superscripts to variables based on significant differences ($P < 0.05$) observed among different variables, a homogeneity test was also carried out.

3. RESULTS

The percentages of motile, viable, hypo-osmotic swollen sperm, acrosome integrity, MDA, and pH for each day were averaged and shown in Figures 1 and 2. In fresh semen (0 day), the percentages of progressively motile spermatozoa, live spermatozoa, hypo-osmotic swollen spermatozoa, and acrosome integrity were 82.86 ± 1.01, 85.32 ± 0.99, 77.13 ± 1.69, and 79.60 ± 0.65, respectively. The sperm grade considerably ($P < 0.05$) declined day by day at refrigeration temperature. Sperm motility, live sperm count, acrosome integrity, HOS +ve spermatozoa, and MDA substantially differed significantly ($P < 0.05$) at different day intervals due to a decrease in pH levels of diluted semen. On the 5th day of liquid storage at refrigeration temperature, the pH of diluted semen decreased considerably ($P < 0.05$) from its original pH of 6.46 to 6.06, and seminal characteristics were below the permitted range for AI [Figure 2].

Throughout the entire incubation period of 5 days, there was a significant ($P < 0.05$) decrease observed in the total motility of

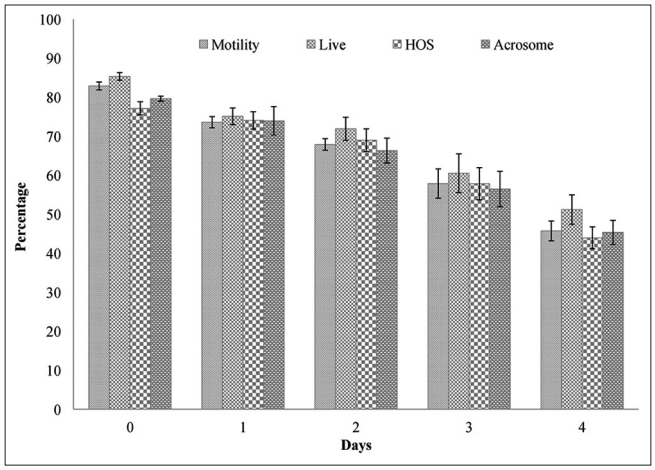

Figure 1: Effect of keeping duration at refrigeration temperature on semen qualities.

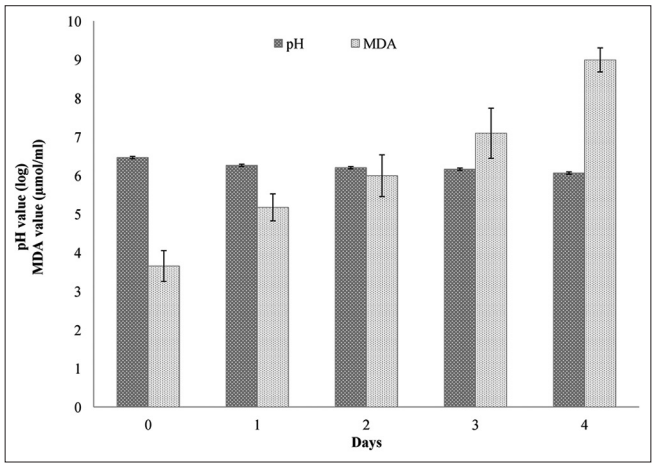

Figure 2: Effect of keeping duration on pH and malondialdehyde at refrigeration temperature.

sperm in semen diluent having 20% Hen`s egg yolk. In a time-dependent manner, the presence of an acidic environment resulted in a significant reduction in the percentage of total motile spermatozoa. In addition, the acidic environment (pH = 6.06) also decreased the viability and membrane intactness of sperm [Figure 1]. Sperm HOS rate was assessed, and the findings revealed a significant decrease in HOS rate at pH = 6.02 (43.93 ± 2.81%, $P < 0.05$) when using a semen diluent containing 20% egg yolk medium, as compared to pH 6.46 (82.86 ± 1.01%). Moreover, a correlation between the HOS rate and sperm movement was examined at 24-h intervals over a span of 5 days, considering various pH levels. A direct association was observed regarding the total motility of sperm, indicating a positive correlation. Similar findings were noted regarding the relationship between the HOS rate and sperm viability, demonstrating a positive correlation.

The lipid peroxidation index MDA concentration was 3.65 ± 0.40 µM in fresh semen. The concentration of MDA (µM) exhibited a significant daily increase ($P < 0.05$) throughout the study, but on the 5th day, it significantly increased ($P < 0.05$) 8.99 ± 0.31 [Figure 2]. Lipid peroxidation increased over a span of time compared to the day of collection to the 5th day of storage at freezing temperature.

4. DISCUSSION

The goat sperm progressive motility decreased significantly ($P < 0.05$) at an incubation period of 5 days in semen diluent having 20% Hen's egg yolk. The acidic environment increases significantly according to the time elapsed and decreases the percentage of total progressive motile spermatozoa. The chemically rich condition can exert a significant impact on sperm quality and sperm motility and is a crucial element for assessing the quality of semen and its capacity to fertilize. Sperm mobility can be directly impacted by the pH of the semen [17]. Since pH modifies sperm's metabolic rate and motility, it also changes their vitality [18]. These findings showed that sperm motility may be directly impacted by the pH of the semen. One of the main sperm fertilization factors is pH [19]. These observations support our findings that a low pH of the semen is linked to reduced sperm motility and male infertility.

The HOS test was utilized to evaluate the integrity of the sperm membrane. We found that in a neutral environment, there were large proportions of live sperm. The HOS test was shown by Ranjan [11] to have a substantial positive correlation with sperm motility. Our findings, which are in line with the findings of this study, also revealed a substantial positive link between HOS and sperm motility, indicating that an aberrant pH of 6.06 shortens sperm life and damages the cell membrane.

Sperm motility was altered by the pH of the semen, suggesting that sperm activity may potentially be influenced by the pH of the vaginal milieu. Following ejaculation, sperm travel through the female reproductive way along with seminal plasma, making them susceptible to different chemicals present in both the semen and the female reproductive system. These chemicals can directly impact sperm movement and metabolic activity, thereby influencing the entire way of capacitation and fertilization [20]. Supplementing Triladyl® with 10% (v/v) chicken egg yolk enhances the cryopreserved *in vitro* and *in vivo* quality of Beetal buck spermatozoa when compared to a tris-citric acid extender containing 20% (v/v) egg yolk [21]. Sperm motility, kinematics, plasma membrane, acrosome, mitochondria and DNA integrities, as well as *in vivo* fertility (59.72% after single freezing versus 52.9% after double freezing; p>.05), remain acceptable following the double freezing protocol, although they are slightly lower than after a single freeze cycle in Beetal bucks [22]. Changes in pH levels occur not just in semen but also in the vagina during infections such as trichomoniasis, bacterial vaginosis, and cytolytic vaginosis. These alterations can have an impact on sperm motility and capacitation, potentially leading to infertility [23].

Previous research has established that mammalian spermatozoa exhibit intricate mechanisms for controlling intracellular pH. The role of the H^+ ion in controlling sperm functional characteristics has been made clear by the discovery of the proton-gated Hv1 channel in mammalian spermatozoa [24]. Sperm pH within cells is precisely regulated by H^+ fluxing, which also collaborates with other ions to control pH. The existence of the HCO_3 system provides more insight into the intricate process of pH control [25]. The exterior pH of sperm with its intricate regulation system may have a direct impact on the intracellular pH of sperm. Studies have also demonstrated that sperm intracellular pH affects ionic regulation along the sperm membrane and that intracellular pH displays a linear connection with external pH [26]. The sperm motility was linearly reduced in the present study when the pH was reduced from 6.46 to 6.06, resulting in pH levels contributing to regulate sperm motility. Sperm movement and pH level at storage

showed a substantial association. In addition, whereas storage with a gradually decreasing extender pH decreased sperm motility, storage with an initial extender pH increased from 6.04 to 6.25 or storage with an artificially stable pH-enhanced sperm quality [26].

Dialyzed epididymal plasma and bicarbonate were used to enhance the intracellular pH of goat semen together with changes in the extracellular pH, which started the caput-epididymal sperm's forward motility [27]. Understanding pH and its impact on spermatozoa function can aid in the development of approaches to comprehend and address the fundamental reasons for pH-related infertility. The discovery of appropriate agents to restore pH during freezing and thawing will be aided by a comprehension of the role of pH in the control of spermatozoa functionalities.

When diluter was refilled at 48 h intervals during buck semen storage as opposed to when it was not, sperm motility, functional membrane, and acrosome intactness were all considerably greater [17]. The current findings showed that sperm motility was enhanced by yolk coating by raising sperm resistance to pH decrease at liquid semen preservation. Egg yolk is frequently used in semen extenders to defend spermatozoa from cold shock when undergoing the freeze-thaw procedure. Many writers have suggested that the low-density proteins (LDL) found in the egg yolk may be substantially liable for sperm preservation, even if the meticulous method by which the yolk defends spermatozoa is not entirely apparent. LDL may cling to cell membranes throughout the thawing process, protecting sperm membranes [28]. Yolk may possibly have antioxidant benefits during freezing and liquid nitrogen preservation [29]. As an outcome, the additional function that the present findings have given to the yolk boosts sperm tolerance to pH variations during semen preservation.

Lipid peroxidation increased with time compared to the day of collection to the 5th day of storage at refrigeration temperature. This showed that the generation of reactive oxygen species is more due to an increase in dead sperm percent in semen samples.

We had developed CIRG Dyl a Tris-based semen diluter which maintain the functional qualities of post-chilled goat semen. It serves as a medium for preserving sperm, extending its viability for fertilization for a period of up to 3 days. It possesses the ability to uphold and safeguard sperm metabolic activities, manage the medium's pH, regulate bacterial transmission and contamination, as well as minimize cryogenic damage. It maintains the pH at 6.8–7.2, provides energy, antibiotics to prevent contamination, and anti-freezing shock. These characteristics facilitate sperm storage, transportation, and its application in assisted reproductive techniques such as AI, *in vitro* fertilization, intracytoplasmic sperm injection, and various research endeavours. This can be stored at room temperature and should be used within 2 months from the date of manufacture.

5. CONCLUSION

The research indicates a strong relationship between a gradual decrease in pH levels and a corresponding decline in sperm motility over an extended period of storing buck semen at refrigeration temperatures. Interestingly, the inclusion of egg yolk in semen samples has shown substantial improvements in seminal qualities compared to samples without egg yolk. In response to these findings, a ready-to-use goat semen dilutor called CIRG Dyl has been developed. This dilutor is designed to preserve diluted semen for up to 24 h, and fortifying it with 20% egg yolk has demonstrated even more promising results. With this addition, the quality of storage for the diluted semen can be extended up to 5 days at refrigeration temperatures. This breakthrough has substantial implications for liquid semen AI technology in goats, offering a more efficient and effective method for preserving semen quality over an extended period.

6. FUTURE PROSPECTIVES

6.1. Enhanced Reproductive Efficiency

The use of CIRG Dyl fortified with egg yolk can potentially revolutionize goat breeding programs by allowing for the extended storage of high-quality semen. This advancement may lead to improved reproductive efficiency by enabling breeders to use semen from superior bucks over a wider geographical area and time frame.

6.2. Cost-Effective Breeding Programs

Longer preservation times and improved semen quality could contribute to more cost-effective breeding programs. Breeders can potentially reduce the need for constant transportation of live bucks for mating, as well as the associated costs, by utilizing stored semen from high-quality donors.

6.3. Research and Development

Further research could focus on refining the composition of dilutors and fortification methods beyond egg yolk to optimize semen preservation. This might involve exploring alternative additives or compounds that could further enhance sperm viability and longevity.

6.4. AI Adoption and Training

As liquid semen AI technology advances, there might be a need for training and dissemination of knowledge among goat breeders regarding proper AI techniques to solve the problem of quality buck shortage. Education and training programs could be developed to ensure the correct use of this technology for maximum efficiency and success.

Overall, the integration of CIRG Dyl with egg yolk fortification presents a promising future for liquid semen AI technology in goats, offering numerous opportunities to enhance breeding practices, genetic diversity, and reproductive efficiency in goat farming.

7. AUTHOR CONTRIBUTIONS

MK and RR designed and conducted the study. MK collected the data and prepared the manuscript. MK is involved in field sampling, studies and interpretation, sample maintenance, testing and assay standardization. RR and AB did the data analysis, manuscript drafting and reviewing. The manuscript was reviewed by all authors.

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

The study protocol was approved by the Institutional Animal Ethics Committe, ICAR-CIRG, Makhdoom, Mathura, UP [Project Mentioned at Sr. No. 08, Date: 20/02/2021].

11. DATA AVAILABILITY

All data generated and analyzed are included in this research article.

12. PUBLISHER'S NOTE

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13. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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