Comparative Efficacy of Three Semen Extenders for Preserving Bunaji and Friesian-Bunaji Cross Bull Semen during Cooled Storage

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Target Audience: local livestock farmers, Researchers, Extension Agents

ABSTRACT

Artificial insemination (AI) underpins genetic improvement and productivity in livestock, yet its success depends on semen extenders that reliably preserve sperm viability during cooled storage. This study compared the efficacy of three extenders: Tris–egg yolk (TEY), egg-yolk sodium citrate (EYS), and a novel coconut water–egg yolk blend (CWE) in preserving semen quality from indigenous Bunaji and Friesian–Bunaji crossbred bulls. The CWE formulation consisted of 68ml boiled fresh coconut water, 28ml egg yolk, 1.2g Sulphanilamide, 0.24g penicillin, 0.54g Streptomycin, 300ml distilled water. Ejaculates were collected using artificial vagina, extended at a 1:10 semen-to-extender ratio in triplicate, and stored at 4 °C. Samples were evaluated daily for six days, measuring progressive motility, viability (live/dead ratio), morphological abnormalities (coiled and bent tails) and pH stability. Two-way ANOVA with repeated measures were used to analysed data while significant differences were considered at $p < 0.05$. TEY maintained the highest motility (66.7%) and lowest proportions of dead spermatozoa (33.3%), coiled tails (0%), and bent tails (3.0%) over six days, outperforming EYS (motility 60.0%, dead 40.0%) and CWE (motility 62.5%, dead 37.5%). Crossbred bulls displayed slightly higher baseline motility (80% vs. 75%) than indigenous Bunaji. TEY was recommended for extended cooled storage in AI programmes, while CWE offers a cost-effective, locally available option for same-day inseminations in resource-limited contexts. Three ejaculates were collected on same day from each bull.

Keywords: *Semen extenders; artificial insemination; indigenous bulls; exotic bulls; sperm motility; coconut water.*

Description of the Problem

The first idea of artificial insemination A.I. is traced to the Arabs who were alleged to collect semen from the vagina of prize mares belonging to their enemies without them knowing or their consent, to breed their horses (1), while the first scientific research in AI of domestic animal was conducted by Lazzaro Spallanzani in 1784 using dog. However, the value of AI was realized for the purpose of large-scale animal breeding in Russia by Dr. Ivanovich who bred for the Calvary.

As an important genetic tool for livestock improvement, the success of AI largely depends on the quality of semen being used, as high-quality semen results in better conception rates and lower production fees. AI requires fresh semen

(to be used within 24 hours) or properly preserved semen. (2). This is because the storage of raw semen for longer periods than 24 hours causes deterioration of the semen quality, with decrease in fertilizing strength (3,4). This poses a problem for semen collected over long distances or which cannot be used within few hours of collection, as it may become useless and unfit for insemination.

To enhance AI, special chemical media known as 'semen extenders' have been discovered for preservation, extension and protection of sperm cells against various shocks during processing, storage and transportation for insemination. Semen extenders perform the same function as the seminal plasma which is secreted from the accessory reproductive organs and serves as a transport medium for spermatozoa (5,6) and also provides an environment with adequate pH for optimum metabolic activities, acting also as a buffered nutrient medium which suspends and maintains the fertility of spermatozoa. Semen extenders not only preserve and protect the viability and fertilizing ability of sperm cells, but also make up the volume of semen collected; increasing the semen volume, thus permitting more insemination per ejaculate as against the natural mating. Some known semen extenders include skimmed milk-glycerol, Lactose egg-yolk-glycerol, egg yolk citrate, Tris (hydroxymethyl), amino ethane-citric acid egg yolk glycerol, Illini Variable Temperature (IVT) extender, Cornell University extender (CUE), Tris-Coconut milk, Coconut milk-citrate, each with different preservation power and fertilization rate (7,8).

However, one of the greatest problems confronting AI practicability in Nigeria had been the difficulty in getting suitable extender for semen extension and preservation. Although, different semen extenders have been discovered, standardized and commercialized for livestock improvement, however, in Nigeria, such standard commercial extenders are not easily accessible to the local livestock farmers at an affordable rate. It is therefore needful to seek for possible alternative that can give us similar or near possible result like the commercial standard semen extender.

This study aimed to compare the efficacy of three semen extenders; Tris–egg yolk (TEY), egg-yolk sodium citrate (EYS), and a novel coconut water–egg yolk blend (CWE) for preserving semen quality from Bunaji and Friesian-Bunaji bulls during 6-day cooled storage at 4°C. Focus was mainly on the semen with no considerations on the bulls in terms of breed difference/effect, but a comparative study of the efficacy of the three extenders used on the semen samples.

MATERIALS AND METHODS

Study Area

This research was conducted at the bull stud section of the A.I. Unit of the National Animal Production Research Institute (NAPRI), Shika Zaria, and in the Animal Physiology Laboratory, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria, Kaduna, Northern Nigeria. Shika is a Guinea Savannah zone

located on Latitude 11° N and Longitude 12° E at an altitude of 640m above sea level. The average annual rainfall is 1100mm most of which falls in the month of June to October. The daily temperatures range from 14ºC to 30ºC and relative humidity of 21% during the harmattan (dry cool season) period, while during the wet season, the average temperature and humidity is 25ºC and 75% respectively (9).

Study Design

Ejaculates were collected from two different bulls (Friesian-Bunaji cross and pure Bunaji bulls); three ejaculates from each bull. The ejaculates for each bull were pooled to make up a total volume of 20ml for Friesian-Bunaji cross bull, and 18ml for pure Bunaji bull. The separately pooled ejaculates for each of the bulls were randomly assigned to the different extenders in a two by three factorial design, where the ejaculates from each bull was extended separately in the three different extenders in triplicate.

1.0 Tris-egg yolk base semen extender (TEY)
2.0 Egg yolk sodium citrate extender (EYS)
3.0 Coconut water-egg-yolk-based extender (CWE)

Bull Selection and Management

Two healthy, sexually matured bulls were selected for this research; a Friesian-Bunaji cross and a pure Bunaji breed (Table 1). The bulls were fed hay and concentrate containing maize and maize offal, cotton seed cake, wheat offal, guinea corn offal, soybean, salt, premix, bone meal, *ad libitum*. Scrotal circumference was measured in centimetre with the use of a measuring tape.

Table 1 Bulls Information

Breed	Age (Years)	BCS	Body Weight (kg)	Scrotal Circumference
Friesian -Bunaji	4	4.0	720	39
Bunaji	4	3.5	700	37

Source: NAPRI's Bulls' Record Information. Note: BCS means body condition score

Semen Collection

Semen was collected with the use of artificial vagina (AV). A teaser bull was restrained and immobilized in a secure crate or chute in standing position. The bulls from which semen was collected were led to the secure teaser while being tease around for stimulation and sexual excitement. As the bulls mounted the teaser following sexual excitement, the technician standing at about 45° by the flank of the bull with the fully assembled AV in his right hand, quickly held the prepuce of the erect penis with his left hand, and directed the penis into the AV. The semen was then collected in a graduated test tube attached at the end of the cone and placed in warm water, maintaining its temperature at 37ºC for raw semen analysis and processing. All

semen samples used were collected on the same day from bulls.

Semen Extender Preparation

Three extenders were prepared prior to semen collection and used in this study: Tris-egg yolk base semen extender (TEY), Egg yolk sodium citrate extender (EYS) and Coconut water-egg-yolk-based extender (CWE). Tris extender was prepared following the procedure by (10), egg yolk sodium citrate according NAPRI method while coconut water base extender was in accordance with the method by (11). The different extenders used are shown in the table 2 below and their respective constituents.

Table 2: Extenders Used and Constituents

Constitutes	Extenders		
	TEY	EYS	CWE
Distilled Water	100ml	100ml	100ml
Tris	3.63g		
2.9g of sodium citrate		2.9g	
1ml of streptomycin		1ml	
0.5ml of penicillin		0.5ml	0.24g
Fructose	0.5g		
Coconut Water			68ml
Egg yolk 20ml	5ml	20ml	28ml
Citric acid	1.99g		
Sulphanilamide			1.2g
Streptomycin			0.54g
Gentamycin	0.5ml		

TEY: tris egg yolk semen extender, EYS Egg yolk sodium citrate extender CWE: Coconut water-egg-yolk-based extender.

Initial Raw Semen Evaluation

The routine evaluation of fresh semen colour, volume, pH, concentration, motility, morphology, life and dead cells ratio was done. Semen colour was observed and recorded by physical eyes observation while Volume of fresh semen was recorded from the graduated mark of the semen collection tube attached at the end of the cone. All methods and procedures used for semen evaluation were followed as carried out at NAPRI. The data are as recorded in Table 3.

Semen Extension

The ejaculates from each bull were extended in the different extenders in the ratio of 1:10 (1ml of semen to 10ml of extender), stored in bijou bottles and preserved in the chilling compartment of the refrigerator at 4^{o}C, while being analysed daily for experimental period.

Ethical Statement

All procedures in this research followed the guidelines for care and use of experimental animals in Nigeria and as followed by the National Animal Production Research Institute (NAPRI) Zaria.

Limitations of the Study. The main limitations in this study were:

(1) small sample size; only one bull per breed was used in this research because of the difficulty in getting Friesian cross bulls in Nigeria

(2) lack of fertility data,

(3) 6-day storage only, since chilled preservation method was used, which only preserve semen for just few days period

(4) single storage temperature (4ºC)

Data Analysis

All data obtained from the different extenders were analysed using a two-way ANOVA with repeated measures function of JMP Genomics (Ver. 10). Tukey's HSD post-hoc test was used to compare the differences among means, while significant differences were considered at $p < 0.05$.

RESULTS

Initial Semen Evaluation

The raw semen characteristics of the Bunaji and Friesian-Bunaji cross bulls are shown in Table 3. Both breeds had cream-coloured semen. The volume of semen was slightly higher in Friesian-Bunaji bull (20ml) compared to Bunaji bull (18ml). The pH and temperature values were consistent across both groups (pH 7.0 and temperature 37°C). In terms of motility, Friesian-Bunaji bull show a modest advantage with 80% motility, compared to 75% in Bunaji bull. Sperm concentration was also higher in Friesian-Bunaji bull, with 291×10^6 sperm/ml, compared to 269×10^6 sperm/ml in Bunaji bull. Both breeds had equal percentage of live cells (75%). However, the Friesian-Bunaji bull had fewer dead cells and abnormal cells.

Table.3 Raw Semen Characteristics of Bunaji and Friesian x Bunaji bulls

Indices	Bunaji	Fresian-Bunaji
Colour	Cream	Cream
Volume (ml)	18	20
pH	07	07
Temperature (ºC)	37	37
Motility (%)	75	80
Concentration(/ml)	269 x10^6	291 x10^6
Life Cells	75	75
Dead Cells	25	20
Abnormal Cells	07	05
Normal Cells	93	95

Effect of Different Semen Extenders on Semen Characteristics of Bunaji and Friesian Cross Bulls

Table 4 shows the effect of different semen extenders on semen characteristics of pure Bunaji and Friesian cross bull.

The pH was significantly lower (P<0.05) in coconut extender (7.00) compared to egg yolk and tris which were similar (7.20). Sperm motility was highest (P<0.05) with tris extender (66.67%) with egg yolk giving the lowest motility (60.00%). The percentage of dead sperm cells was lowest (P<0.05) in tris (33.33%) and highest in egg yolk (40.00%). Coiled tail abnormalities were most prevalent in egg yolk (1.50%) (P<0.05) with none observed in tris. Bent tail abnormalities were higher (P<0.05) in egg yolk (3.50%) and coconut (3.33%) which were similar compared to tris (3.00%).

Table 4: Effect of Different Semen Extenders on Semen Characteristics of Bunaji and Friesian Cross Bulls

Parameters	Coconut	Egg yolk	Tris	SEM	P- value
pH	7.00[b]	7.20[a]	7.20[a]	8.7806e-10	<.0001*
Motility (%)	62.50[b]	60.00[c]	66.67[a]	0.48	<.0001*
Dead (%)	37.50[b]	40.00[a]	33.33[c]	0.48	<.0001*
CT (%)	0.67[b]	1.50[a]	0.00[c]	0.11	0.0002*
BT (%)	3.33b	3.50[a]	3.00c	0.10	0.0097*

[a,b,c] Means with different superscript on the same row differ significantly (P < 0.05), SEM: Standard error of mean, CT: coil tail, BT: bent tail

Effect of Time on extended Semen characteristics of Bunaji and Friesian Cross bulls

Result showing the effect of time on extended semen characteristics and of both bulls is presented in Table 5. There were significant (P<0.05) changes in all the parameters over time, with a noticeable decline in the semen fertility parameters over days, particularly after the first day.

Table 5: Effect of Time on extended Semen characteristics of Bunaji and Friesian Cross bulls

	Days							
	1	2	3	4	5	6	SEM	P value
pH	7.13[a]	7.13[a]	6.98[b]	6.70[c]	6.67d	6.67d	0.11	<0.0001
Live	63.06[a]	53.89[b]	43.06[c]	30.83[d]	17.50[e]	5.28[f]	1.37	<0.0001
Motility	63.06[a]	53.89[b]	43.06[c]	30.83[d]	17.50[e]	5.28[f]	0.49	<0.0001

[a, b,c,d,e,f] Means with different superscript on the same row differ significantly (P < 0.05), SEM: Standard error of mean

Discussion

Raw Semen

Collected semen samples from both bulls were of excellent and viable quality, with creamy colour corresponding to the findings of (12,13) for a good semen, and also in line with those of Zebu cattle reported by (14). The average volume of ejaculates from both bulls were in excellent range reported by (15) and same by (16) for Indonesian native Bali cattle; but higher compared to those reported by (17). Semen volume is such an important quality parameter because of the number of sperm cells required for optimal fertility in artificial

insemination (18). Raw semen pH was neutral for both breeds. The normal pH of good bull semen is within the range of 6.4-7.8 (19), an indication that the semen samples collected for this study are of excellent quality. Semen consists of 10% sperm cells and 90% plasma (16). (20) reported that sperm cells could be influenced by the acidity or alkalinity (pH) of the semen and further noted that acidic environment has a greater influence on sperm viability than alkaline environment.

The bulls' raw semen motilities were 80% and 75% respectively, which are far above the minimum of 30% reported by (21) needed to achieve conception. The life cells of semen samples were exactly as their respective motility. There were less than 10% abnormal sperm cells for all semen samples from each breed; similar and in alignment with the report findings of Rodriguez-Martinez, (22) who reported that a good semen should not have less than 10% abnormal cells, and is in agreement with earlier report of (23). The concentration per ml of the collected semen samples for both bulls were higher than the minimum range of 150-200 × 10^6 /ml required to achieve conception according to the findings of (13). Therefore, judging from the raw semen examination, the samples collected were highly viable and fit for artificial insemination.

Effect of Different Semen Extenders on Semen Characteristics of Bunaji and Fresian Cross Bulls

Comparing the effect of each extender on the semen characteristics, there was high significant difference ($p<0.05$) in the effect of extenders on all semen parameters at different levels. Egg Yolk Sodium Citrate and Tris Egg Yolk Extender had higher effect on the bulls' semen pH; both being at same level of effect compare to coconut water base semen extender. Whereas, the normal pH of good bull semen is within the range of 6.4-7.8 (24), and none of the extenders preserved the semen pH less than the normal range reported. Semen pH is important for spermatozoa activity as acidic pH inhibits the metabolic activity of spermatozoa, resulting to accumulation of lactic acid, causing a reduction in their motility (25,26). Sperm cells can tolerate a pH decrease to 5.5, but pH values below 5.5 are spermicidal (25**)** From the study by Aitor (27) pH at 5 °C never exceeded 6.70, whereas the pH at room temperature eventually fell to 6.22–6.28, suggesting that semen samples requiring storage for up to 24 hours should be refrigerated.

A very high significant ($p<0.05$) effect was also observed from each semen extenders' effect on semen motility, with Tris extender showing higher effect, followed by Coconut Water base extender, while Egg Yolk Sodium Citrate had the least effect and exact same is applicable with dead cells and coil tail. This result aligns with the findings of (28) Abd El-Nour *et al*. (2017) who reported that Tris extender has better buffering capacity than phosphate buffer and sodium citrate buffer solutions and has fewer toxic effects on sperm cells. On the contrary, El-Sheshtawy *et al*. (29) reported that spermatozoa diluted with coconut water and 4% glycerol even performed better than the control mixture (tris-citric acid fructose-egg yolk) in both cooled and frozen states, yields significantly better results

in semen preservation compare to other extenders. The effect of powdered coconut water on buck (male goat) semen has also been compared against Tris-diluted semen in terms of kinetic and morphological characteristics (30); and the Tris-diluted semen still managed to outdo the powdered coconut water base extender. In the same vain, (31) observed that coconut water-diluted semen couldn't out perform a commercial semen extender based on motility. The authors also noted that semen diluted with coconut water and 20% egg yolk should be used within few days for artificial insemination as its quality decreases over time due to damage in the plasma membrane of the spermatozoa. However, (32) suggested that coconut water diluted semen can still be used after five days of dilution. Both studies stored semen at 5°C.

More bent tails were recorded in Coconut water base semen extender and Egg Yolk Sodium Citrate; with both being at same level of effect on bent tail than Tris with lesser effect. Also (33) in their report stated that Extenders made from coconut water offer much potential for preserving sperm viability, morphology, motility, and integrity in both cooled and frozen sperm. As a result, coconut water can be utilized as a low-cost and natural semen extender. They further added that Coconut water preserves sperm cells alive in a liquid state until the second day of preservation and the 30-day cryopreservation period.

Effect of Time on extended Semen characteristics of Bunaji and Friesian Cross bulls

The effect of storage period on extended semen samples reveals time has an effect on semen fertility. The significant difference in semen pH declines over days similar to the findings of (27) shows even when extended and on preservation, Semen survives dilution or chilling for only a few days. The motility of spermatozoa progressively worsens also as the period of storage increases (34). Moreover, motility can also be affected by the age of the sire and environmental influences (35). However, bulls were both of good age for maximum performance and excellent spermatozoa quality. as such, the decline fertility cannot be attributed to age, but probably due to changes in pH over a prolong low storage temperature.

Conclusion

Coconut water-based extenders produced comparable outcomes when compared to other additives and diluents. No doubt, commercial extenders, on the other hand, can still produce better and higher results than coconut water extender. However, coconut water-based extender can be used as substitute in resource-limited settings requiring cost-effective alternatives, or if there aren't any commercial extenders accessible.

Extenders made from coconut water offer much potential for preserving sperm viability, morphology, motility, and integrity in both cooled and frozen sperm. As a result, coconut water can be utilized as a low-cost and natural semen extender. Coconut water preserves sperm cells alive in a liquid state until the second day of preservation and the 30-day cryopreservation period

Application

1. Considering its low cost and availability as well as its appreciable performance, I recommend coconut water as a diluent for semen extension and preservation for local livestock farmers in Nigeria who cannot afford standard or commercial extenders
2. Also, I will recommend further research findings on the efficacy of coconut water in semen preservation using deep freezing with liquid nitrogen
3. Again, there's need for further research towards harnessing the potentials of coconut water and standardizing it for semen preservation
4. I also recommend further research on the conception rate following insemination of cows with coconut water-based extender
5. Further research is also needed to investigate the performance of each breed in the different extenders to determine the preference of a particular breed in an extender.

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