



Effects of storage temperature and extension media on motility of caprine spermatozoa

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Abstract

The survivability of caprine spermatozoa was compared in semen extenders prepared from the conventional egg-yolk citrate and goat-milk citrate. Motility was comparable in both sets of extenders during the first 24 hours post-extension when extended ejaculates were stored at either room temperature (28 °C) or refrigerator temperature (5 °C). Motility in goat milk citrate extenders at 5 °C was significantly higher ($p < 0.05$) than that stored at 28 °C and in egg-yolk citrate extenders at both investigated temperature ($p < 0.05$) 48 hours post extension. Beyond 72 hours of storage, motility in both extenders and at both temperature was less than 40%. The results showed that goat milk in part can replace egg-yolk as a medium for semen extension. Therefore, an extender with concentration of 10-20 % heated goat milk, 70-80% sodium citrate and 10% egg-yolk could be used to store goat semen at 5 °C for 2-3 days.

Keywords: Egg-yolk citrate, Goat-milk citrate, Motility, Semen extension, Spermatozoa

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Introduction

The importance of goats in livestock economy of people living in subsistence agriculture in the humid zone of West Africa has made them the species of choice for peasant farmer and for supply of protein and other products in the sub-region (Bitto & Egbunike, 2012). The West African Dwarf (WAD) breed of goat; the most popular genotype in Nigeria (which supplies excellent quality meat, milk, skin and other products), has been adjudged one of the most prolific in the world, with a remarkably high reproductive potential (Wilson, 1989; Gall *et al.*, 1992). Despite these excellence characteristics, WAD goat is the lowest in meat and milk production in Africa (Steel, 1996). This is largely due to low improvement in its reproductive potentials. Selective breeding to develop and improve this animal require the use of modern reproductive biotechnology such as Artificial Insemination (AI). AI may be regarded as first generation assisted reproductive technology that is the most widely used and the one that has made the most significant contribution to genetic improvement worldwide (Evans & Maxwell, 1987; Chemineau *et al.*, 1991, Leboeuf *et al.*, 2000). The

increase need for use of AI in WAD goats has been reported (Oyeyemi *et al.*, 2000). The advantage of AI over natural mating is in its increase number of offspring per sire, allowance of spatial and temporal dissociation between collection of spermatozoa and fertilization, limitation of disease spread and decrease offspring generation interval (Leboeuf *et al.*, 2000). The success of AI is based on the ability to efficiently collect, evaluate and cryopreserve semen from quality bucks for use in inseminating does over generation (Ngoula *et al.*, 2012). In order to maximize the economic benefit of AI, there is need for extension of the semen. Extended buck semen can be used frozen-thawed, chilled or fresh but when fresh semen is used, it offers better fertility and conception rate (Langford *et al.*, 1979, Hackett & Wolynetz, 1981). Several extenders have been formulated and used in order to meet the need of AI including egg-yolk phosphate (Phillips & Lardy, 1940) skim milk (Almquist & Wickersham, 1962) orange juice (Bonadonna *et al.*, 1962) coconut milk-citrate (Sule *et al.*, 2007), snail mucus-egg yolk extender (Ajadi *et al.*, 2012).

However, majority of these extenders are not very suitable media for semen extension and storage (Bitto *et al.*, 2007). Egg yolk and skim milk in semen extender exert a protective effect against cold shock upon chilling or freezing of spermatozoa (Watson, 1981; Colas, 1984). Milk as semen extender has also been found to have detrimental effect on sperm cells (Purdy, 2006). This is due to presence of bulbourethral gland secretion (BUSgp60) in semen which reacted with the glycoprotein in milk. BUSgp60 lipase activity is strongly increased by milk. It hydrolyses triglyceride in skim milk and produce fatty acids (Oleic acid). The oleic acid released, from triolein by BUSgp60 exert a strong deteriorating effect on goat spermatozoa (Pellicer-Rubio *et al.*, 1997). Heat (>50°C) has been identified as an important factor that denature protein. Denaturing the glycoprotein in the milk could increase the survivability of spermatozoa in milk. Therefore, this work is carried out to compare the effect of egg yolk-citrate and heated goat milk on sperm motility at two different storage temperature in an attempt to develop a suitable extender that would be optimum for use in biotechnology in a developing nation like Nigeria.

Materials and Methods

Experimental animal

A sexually mature West African Dwarf buck aged about 2½ years weighing 16.0 kg which was purchased from the local market was used for this study. It was housed in experimental goat pens of the Department of Veterinary Surgery and Reproduction, Faculty of Veterinary Medicine, University of Ibadan. The animal was fed with concentrates and fresh elephant grass and supplied with clean water *ad-libitum*. In addition, it was acclimatized for the experiment two weeks prior to the commencement of the study during which it was dewormed with ivermectin (Ivomec®,

Hoescht, Germany) at a dose rate of 1 mg/100kg body weight. At the time of purchase, the buck was examined physically for breeding soundness.

Preparation of buffer (2.9% Sodium citrate)

2.9g of Tris-Sodium Citrate was dissolved in sterile distilled water in a volumetric flask up to 100 ml mark to make 2.9% solution of Tris-Sodium Citrate (Na₃C₆H₅O₇·2H₂O). The solution was kept overnight in a dark cupboard.

Preparation of diluents

- Fresh goat milk was obtained from lactating does in the goat research unit of the Department of Veterinary Surgery and Reproduction, University of Ibadan. The milk obtained was heated at 95°C for about 10 minutes. After cooling to room temperature, it was centrifuged at 4,000 revolutions per minute for 5 minutes. The cream was separated and discarded.
- Egg-yolk: Fresh eggs were disinfected with 70% ethyl alcohol. They were then cracked and poured in to the egg-yolk separator which separated the albumen from the egg-yolk. The egg-yolk was then centrifuged at about 4,000 revolutions per minute for 5 minutes. Composition of the extenders (A and B) - see Tables 1 and 2.

Semen collection and evaluation

(a) Semen collection

Semen was collected by the Electroejaculator (EE) method as described by Oyeyemi *et al.* (2000). Semen was collected twice weekly (Mondays and Thursdays) between the hours of 08.00 and 10.00 a.m. A total of ten collections were made during this study; five each for goat milk citrate extender (A) and egg-yolk citrate extender (B).

Table 1: Extender- A (Goat-Milk citrate extender)

Diluents	Goat milk (%)	Sodium citrate (%)	Egg-yolk (%)	Total volume (%)
I	0	90	10	100
II	5	85	10	100
III	10	80	10	100
IV	15	75	10	100
V	20	70	10	100

Table 2: Extender B (Egg-yolk citrate extender)

Diluents	Goat milk (%)	Sodium citrate (%)	Egg-yolk (%)	Total volume (%)
I	10	90	0	100
II	10	85	5	100
III	10	80	10	100
IV	10	75	15	100
V	10	70	20	100

The EE probe was lubricated and connected to the rheostat which was equally connected to the mains supply. The EE probe was then introduced into the rectum of the WAD buck which has been properly restrained. The rheostat was operated to deliver variable voltages by rotating the knob clockwise and anti-clockwise to increase and decrease the voltage over a period of time until the buck ejaculated. The ejaculate was collected into a clean sterile graduated collecting tube which carries a clean sterile glass funnel. The collecting tube was contained in a protector kept warm at 37°C by warm water.

(b) Semen evaluation

Freshly collected semen samples were evaluated before extension for colour, volume, mass activity, sperm progressive motility, sperm concentration, live/dead ratio and sperm morphology by standard laboratory methods (Bitto *et al.*, 2007), while sperm progressive motility was evaluated post-extension using standard laboratory method as previously mentioned. The volume of ejaculate collected was read directly from graduated collection tube and subsequently recorded. Mass activity was done qualitatively while progressive motility was determined and recorded as percentages (Noakes *et al.*, 2001). Sperm concentration was determined using the improved Neubauer haemocytometer (Coffin, 1953; Bearden & Fuquay, 1997). Morphological examination of the spermatozoa was carried out and abnormalities of the sperm cells located in the acrosome, head, mid-piece and tail were observed under a microscope and recorded.

Direct smear of semen with eosin-nigrosin stain was done for each ejaculate by touching a spreader slide to a drop of a mixture of the stain and fresh semen (5:1) and then drawing the mixture across the surface of a pre-warmed clean glass slide at 45° angle. 0.2 ml of the fresh semen was transferred to a sample bottle containing 1 ml of buffered formal saline at 37°C. This was later used to study some morphological abnormalities of the spermatozoa, using phase-contrast microscope (Hancock, 1957; Sekoni, 1992; Sekoni, 1993). Wet preparations under small cover slips were examined in a regular sequence at a magnification of x1000 for abnormal acrosomes, detached heads, proximal and distal cytoplasmic droplets, abnormal mid-piece and sperm tail abnormalities. A total of 400 well-spaced spermatozoa was carefully examined and counted in each preparation by scanning. Eosin-nigrosin stained smears were used for the study of head abnormalities by systemically counting four hundred spermatozoa at a magnification of x1000 under oil immersion.

Semen extension

Semen was extended by dispensing equal volumes of 0.05ml of the ejaculate into bottles containing about 8.0ml of the different extenders. A set was stored at room temperature and the other set was slowly cooled to 5°C and stored in the refrigerator. Daily monitoring of percentage motility for the set of different dilutions were carried out and recorded. Prior to extension, 0.05 ml of antibiotic (Pen-strep®) was added to each of the dilutions.

Statistical analysis

All data were recorded as mean \pm standard deviation and were subjected to Pearson's correlation analysis and tested using the 2-tailed student *t*-test.

Results

Pre-extension values for spermogram of the WAD buck is as shown in Table 3. Spermatozoa motility decreases progressively with time in both types of extenders and in all diluents as depicted (Figures 1-4). Spermatozoa survived for a longer period in extenders stored at 5°C than those stored at room temperature as depicted by the relatively high motility values shown (Figures 1 and 3). It was also observed that spermatozoa stored in goat-milk citrate extender had higher average motility after 48 hours of storage than the egg-yolk citrate extended spermatozoa (Figures 1 and 2). When stored at room temperature, higher spermatozoa motility values were recorded for goat-milk-citrate extender than the egg-yolk-citrate extender (Figure 2). Diluent – AIII maintained spermatozoa with relatively higher motility and for longer period (Figures 1 and 2). This is followed closely by Diluent – AV for the goat-milk-citrate extender (Figures 1 and 2). It was also observed that Diluent – AI on extension showed presented highly reduced spermatozoa motility (Figures 1 and 2). Similarly, Diluent – BV maintained spermatozoa with relatively higher motility values for longer period (Fig, 3 and 4) and is closely followed by Diluent – BIII for the egg-yolk-citrate extender (Figures 3 and 4). Also, it was observed that poorest motility was shown by Diluent –BI (Figures 3 and 4), however, the spermatozoa motility value was poorer in Diluent – BI than Diluent – AI (Figures 1-4). Within days, the spermatozoa motility tends to increase down the Table as concentration of the diluents increases for both extenders as shown in (Figures 1 – 4). Spermatozoa stored at 5°C survived for about 96 hours (4 days) while those stored at the room temperature (28°C) can survive for 72 hours (about 3 days) as shown (Figures 1 – 4). At about 72 hours, there tends to be significant difference

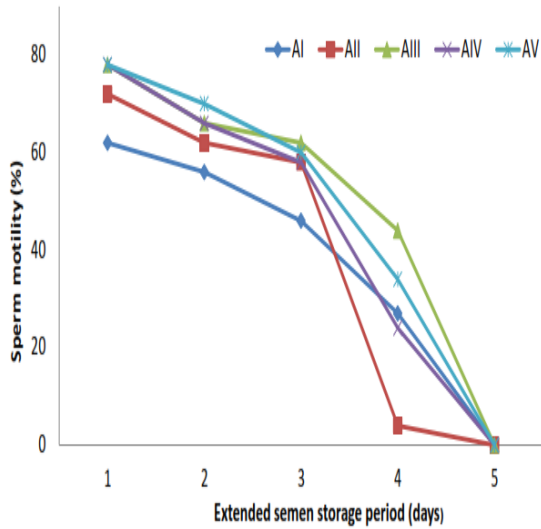


Figure 1: Percentage motility of sperm cells in extender-A stored in refrigerator at 5°C

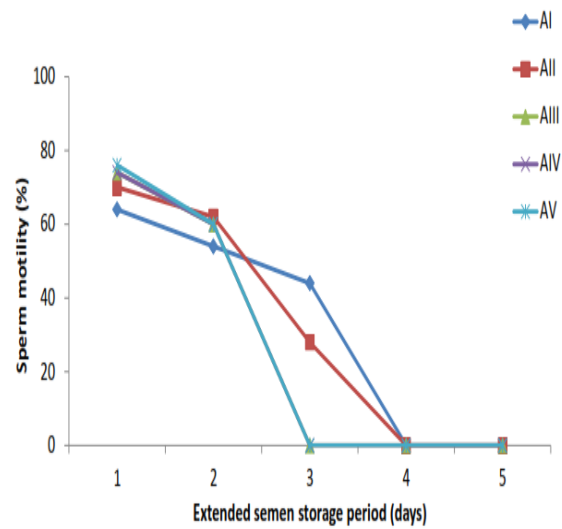


Figure 2: Percentage motility of sperm cells in extender-A stored at room temperature

Key

- AI= Diluent containing 0% Goat Milk + 90% Sodium citrate + 10% Egg yolk
- AII= Diluent containing 5% Goat Milk + 85% Sodium citrate + 10% Egg yolk
- AIII= Diluent containing 10% Goat Milk + 80% Sodium citrate + 10% Egg yolk
- AIV= Diluent containing 15% Goat Milk + 75% Sodium citrate + 10% Egg yolk
- AV= Diluent containing 20% Goat Milk + 70% Sodium citrate + 10% Egg yolk

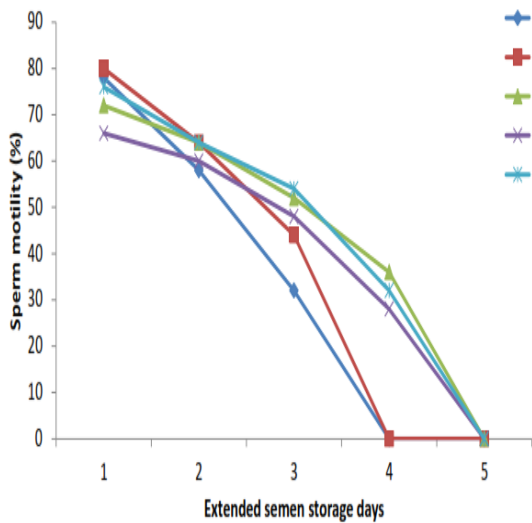


Figure 3: Percentage motility of sperm cells in extender-B stored in refrigerator at 5°C

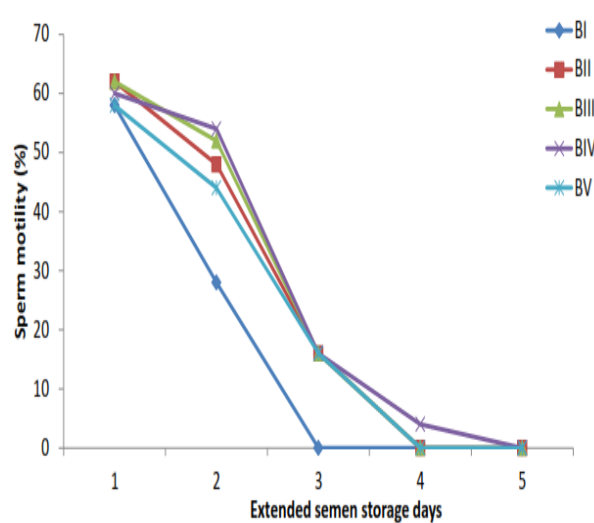


Figure 4: Percentage motility of sperm cells in extender-B stored at room temperature

Key

- BI = Diluent containing 10% Goat Milk + 90% Sodium citrate + 0% Egg yolk
- BII = Diluent containing 10% Goat Milk + 85% Sodium citrate + 5% Egg yolk
- BIII = Diluent containing 10% Goat Milk + 80% Sodium citrate + 10% Egg yolk
- BIV = Diluent containing 10% Goat Milk + 75% Sodium citrate + 15% Egg yolk
- BV = Diluent containing 10% Goat Milk + 70% Sodium citrate + 20% Egg yolk

Table 3: Pre-extension Spermogram of the WAD Buck (Mean±SEM)

Trials	Volume (ml)	Mass activity	Motility (%)	Live/dead ratio (%)	Concentration (x10 ⁹ spermcells/ml)	Morphology (%)	
						Normal	Abnormal
A (5)	0.75±0.06	3.0±0.30	88.0±2.00	94.8±1.99	2.304±0.04	94.45±0.57	5.55±0.57
B(5)	0.67±0.03	4.0±0.00	86.0±2.45	89.8±3.40	2.38±0.01	93.25±1.15	6.75±1.15

($P < 0.05$) in spermatozoa motility values of Diluents – AII, AIII, AIV and AV stored at 5°C and room temperature (Figure 1) while significant difference ($P < 0.05$) for Diluent – AI stored at both investigated temperature was only noticeable at about 96 hours post storage. Similarly, at about 48 hours diluents BI and BII stored at both 5°C and 28°C showed significant difference in spermatozoa motility (Figures 3 and 4). Difference in significance ($P < 0.05$) of spermatozoa motility for diluents – BIII, BIV and VB was only noticeable at about 72 hours of storage at both investigated temperature (Figures 3 and 4.)

Discussion

The pre-dilution semen characteristics were within the normal values reported for this breed (Udeh & Oghenesode, 2011, Ajala *et al.*, 2012, Sule *et al.*, 2007 and Ajadi *et al.*, 2012). In addition, the pre-extension gross and subjective motility were scored to be +3 and 88% respectively. This is related to earlier report for WAD +3,90% Ajadi *et al.*, 2012, +4,90% Ngoula *et al.*, 2012, +4,93% Sule *et al.*, 2007, +3, 92% Udeh & Oghenesode, 2011, +3,92.5 Ajala *et al.* (2012).

Spermatozoa motility depreciates progressively with time in both types of extenders and in all dilution ratios. This result is in line with Peterson *et al.* (2007) that the percentage motile spermatozoa in buck semen stored in liquid form for 72h, progressively decline overtime irrespective of whether storage occurred at 4 or 18°C. The drastic decline in sperm motility of stored liquid semen could be attributed to gradual depletion of nutrients such as potassium, sodium and plasma protein required for high metabolic demands of sperm transport through the female genital tract.

The refrigerator temperature (5°C) had significantly ($p < 0.01$) higher motility values compared to room temperature (25°C) in all the trials. This could be attributed to the fact that refrigerator temperature help to reduce metabolic process in stored liquid semen which resulted in the utilization of nutrients such as fructose by the sperm cells (Aboagle & Terada, 2003). This observation is in line with the report of Bayemi *et al.* (2010) and Udeh & Oghenesode, (2011) that refrigerator temperature recorded highest viability of stored bull and goat semen, respectively. However addition of antibiotics could have prolonged the survival of spermatozoa at room temperature in line with report of Foote & Bratton (1950) which indicated that extender containing antibiotic enhanced and prolong the usefulness of the semen.

Although sodium citrate has been reported to be good extender (Adeyemo *et al.*, 2007, Udeh & Oghenesode, 2011), preserving sperm motility up

to 24hours. The addition of either egg-yolk or goat milk or both prolonged the sperm viability in the extender beyond 24 hours due to composition of the added components.

The spermatozoa stored in goat-milk citrate extender had higher average motility than egg yolk-citrate extender. This report is in line with Yotov *et al.* (2011) who observed that bull semen extended in milk without antibiotic had fertility result higher than those obtained with yolk citrate extender. However, skim milk extender cannot preserve the semen for AI for more than 24hours (Ngoula *et al.*, 2012) as done by the boiled goat milk. This was attributed to presence of 60 kilodalton glycoprotein with triglyceride lipase activity in the bulbourethral secretion which reacts with the skim milk to produce oleic acid from triolein with resultant deteriorating effect on the spermatozoa (Pellicer-Rubio *et al.*, 1997). However the effectiveness of boiled milk could be due to destruction of the reactant in the goat milk. It could also be due to hydrolysis of lactose to glucose and galactose in boiled milk which provides an exogenous source of energy for the spermatozoa. Other factors such as changes in the protein and a decrease in the ionic calcium and change in interfacial tension may be involved (Bergeron *et al.*, 2007).

The poor preservation ability of egg-yolk citrate extender used in this study could be due to the presence of egg-yolk coagulating enzyme (EYCE) in the seminal plasma of semen which destroys the spermatozoa (Kozdrowski *et al.*, 2007, Noakes *et al.*, 2001).

This study also revealed that goat-milk extended semen when stored at 5°C (refrigerator), the spermatozoa motility can be maintained for up to a period of 96 hour (4 days) after ejaculation. However for good conception rate the semen must be used within 2-3 days in line with Peterson *et al.* (2007) who reported that sperm motility decline progressively overtime in semen whether the semen is stored at 4 or 18°C.

In conclusion, the result of this study suggested that boiled goat-milk can comparably support the survival of caprine spermatozoa. Liquid semen stored in goat-milk-citrate extender under refrigerator condition maintained the highest viability of sperm cell compared to other storage condition. Also semen extended in goat-milk-citrate can be stored conveniently for up to a period of two days at room temperature making this medium suitable for field condition.

It is therefore recommended that for better AI and fertility trials, extender with concentration point of 10-20% goat-milk (heated), 70-80% sodium citrate and about 10% egg-yolk could be used and stored semen should be inseminated within 2-3 days.

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