RESEARCH ARTICLE

Sokoto Journal of Veterinary Sciences

(P-ISSN 1595-093X: E-ISSN 2315-6201)

http://dx.doi.org/10.4314/sokjvs.v20i1.1

Abba *et al.*/*Sokoto Journal of Veterinary Sciences*, **20**(1): 1 - 8.

Characteristics of fresh and frozen-thawed Uda ram semen extended with Oviplus®, egg yolk and coconut milk

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Publication History:
Received: 03-07-2021
Revised: 02-09-2021
Accepted: 07-09-2021

Abstract

Semen characteristics of Uda sheep and the effects of the egg yolk and coconut milk-based extenders on the quality of semen preserved at 4 0C and -196 0C were evaluated. Semen was collected from six Uda rams using an electro-ejaculator twice a week for ten weeks. Fresh semen samples were pooled and analyzed macroscopically and microscopically. The pooled semen was divided into 2 aliquots. One aliquot was extended in OviPlus® and egg yolk. The second aliquot was extended with OviPlus® and coconut milk. Each of the aliquots was further subdivided into 2 parts and evaluated microscopically. One part was chilled at 4 0C and evaluated for the same parameters after extension at 24, 48 and 72 hours. Second part was loaded into 0.25 ml plastic straws for cryopreservation at -196 °C and analyzed after 24 hours. The post thaw spermatozoa motility, livability and morphological abnormalities were determined at 24, 48 and 72 hours. The motility and concentration of freshly collected Uda semen were 81.7 ± 1.7 % and 3.2 ± 0.3 ×109/ml respectively. After extension, the motility decreased significantly ($P < 0.05$) from 82 % at 3 hours to 17 % at 72. The percentage live spermatozoa of the chilled semen did not differ significantly ($P > 0.05$) between the two extenders. Post thaw spermatozoa motility and livability were significantly ($P < 0.05$) reduced. There were significant differences ($P < 0.05$) between the post-thaw proportions of morphological abnormalities, between semen preserved at 4 0C and the frozen-thawed semen. In conclusion, semen motility, livability, and morphological abnormalities of Uda ram are equally preserved in coconut milk and egg yolk-based extenders at 4 0C up to 24 hours post extension. However, semen motility and livability were significantly reduced in the Uda semen earlier cryopreserved at -196 0C after thawing.

Keywords: Coconut milk, Cryopreservation, Extenders, Semen, Uda sheep

Introduction

The Uda is one of the important indigenous sheep breeds found in the Sudano-Sahelian region of West Africa including Nigeria and their characteristics have been described (Ibrahim *et al.*, 2012). Generally, Uda

sheep are managed under an extensive and nomadic system and breeding is mostly through natural mating and is among the breeds with high potential for performance and carcass characteristics (Dafur & Mbap, 2021). Semen quality plays an important role in animal breeding. It is necessary to have some parameters to predict the fertilizing ability of the semen (Rodriguez-Martinez & Barth, 2007). Sperm motility, morphology, and livability are important in breeding soundness evaluation (Evans, 1988). However, the correlation of these semen parameters with fertility is influenced by many other factors (Rodriguez-Martinez & Barth, 2007). Semen is extended to preserve its fertilizing ability during storage also increase its volume so that a single ejaculate of limited volume can be used post packaging (in form of strewing, ampoules and pellets) to inseminate a large number of animals. Components of semen extenders include glucose, fructose, tris citric acid and egg yolk which altogether provide a source of energy and protect the spermatozoa from temperature-related damage (Rather *et al.*, 2017). Semen extension with animal and plant-based extenders significantly improve sperm parameters during storage (Kasimanickam *et al.*, 2011). The extenders increase the semen volume and preserved the fertilizing capacity without affecting the semen quality thereby maintaining a suitable environment for the spermatozoa cells to survive temporarily during preservation (Vera-Munoz *et al.*, 2009).

Semen cryopreservation is an important biotechnology tool utilized to preserve the genetic trait in animals and involves semen cryopreservation through temperature reduction, cellular dehydration, freezing and thawing (Medeiros *et al.*, 2002; Jiménez-Rabadán *et al.*, 2012). For spermatozoa to survive freezing, they need to be extended in a diluent that contains cryo-protectants such as glycerol and dimethylsulfoxide (DMSO) and protect them from the deleterious consequences of freezing (Barbas & Mascarenhas, 2009). For both liquid and cryopreserved semen, previous studies have shown that fertilizing ability of stored semen is improved with antioxidants and mineral supplementation (Azawi & Hussein, 2013; Allai *et al.*, 2018).

A recent study by Oyeyemi & Olusoji (2018) had described some epididymal semen characteristics of Uda in Ibadan, Nigeria. However, there is a dearth of information on the semen characteristics of Uda rams. In addition, there is no information on freshly ejaculated, extended and cryopreserved Uda ram semen to the best of our knowledge. Thus, this study was conducted to obtain basic data on semen characteristics of Uda ram, with fresh, extended and cryopreserved, effects of semen extenders on semen characteristics as well as the effects of cryopreservation on post-thawed semen.

Materials and Methods

Study area

This study was carried out in the Department of Theriogenology, University of Maiduguri, located in the semi-arid region of Nigeria. Maiduguri is located within latitudes 10.2° N and 13.4° N and longitudes 9.8° E and 14.4° E.

Study design

A total of six matured Uda rams, aged 2-3 years, weighing between 40 to 50Kg were used for the research. The rams were kept at the Large Animal Unit of the University of Maiduguri Veterinary Teaching Hospital. The animals were made to acclimatize for two weeks while being fed groundnut hay and wheat offal. Water was provided ad libitum.

Preparation of diluents

All extenders were prepared on the day of semen collection; freshly laid eggs and mature ripe coconuts were used. The OviPlus® extender (TRIS, sugar, buffer, citric acid, antibiotics and ultrapure water) was prepared with egg yolk according to manufacturer's instruction (OviPlus® + egg yolk). Coconut milk replaced the egg yolk (OviPlus® + Coconut milk), in the coconut milk-based extender. The extenders for cryopreservation were prepared using glycerol 6% as described by Gil *et al.* (2003).

Semen collection

Semen was collected using an electro-ejaculator (Bailey® Ejaculator England), twice a week for 10 weeks. Each ram was properly restrained on lateral recumbence by two attendants; the hairs around the prepuce was clipped, washed, wiped and dried. The faeces were evacuated from the rectum using the forefinger, the electro-ejaculator probe was lubricated with KY-Jelly and was inserted gently into the rectum to a depth of 12 cm on the floor of the pelvis for stimulation. Ejaculated semen was collected into a clean graduated test tube attached to a collecting cone, the test tube was removed and placed into a water bath maintained at 37℃, and transported to the artificial insemination (AI) laboratory for macroscopic and microscopic analyses.

Pre-extended semen evaluation

Immediately after semen collection, the colour and consistency were determined using visual assessment of the semen from the test tube, while the semen volume was recorded by measuring the quantity of the semen in the test tube, using the graduated scale on the side of the collection tube calibrated in centimetre and the semen pH was determined using pH paper.

Microscopic semen evaluation

Individual progressive motility*:* The individual progressive motility were determined by adding 0.5 µl of fresh semen extended to 0.5 µl of normal saline and the mixture placed on a pre-warmed glass slide and coverslip (37℃), the slide was then observed at × 100 magnification according to the method described by Karatzas *et al.* (1997). The result was recorded in percentages.

Sperm concentration*:* Sperm concentration was determined using the haemocytometer method according to Evans & Maxwell (1987). Fresh semen was extended in formal saline at the ratio of 1:3 micro-litres. A clean warm coverslip was applied on the Neubauer counting chamber, and the extended semen was dropped on both edges of the coverslip and spermatozoa were counted on the gridded chambers under the microscope at × 40. The spermatozoa concentration was calculated according to Evans & Maxwell (1987).

Spermatozoa morphology and livability: Spermatozoa morphology and livability were determined with the aid of an eosin/nigrosin stain as described by Bjorndahl *et al.* (2003). The smear was examined using a microscope (× 100 magnification). A total of 200 spermatozoa per slide were evaluated and counted for each animal per collection. The gross structural spermatozoa abnormalities were also recorded as described by Evans & Maxwell (1987).

Analyses of extended semen

Immediately after collection, semen samples showing more than 3 + mass motility, > 75% motility, > 50% livability, < 10% morphological abnormalities and pH of about 7.0 were pooled and analyzed macroscopically and microscopically (at 37℃). The pooled semen was divided into 2 aliquots. One aliquot was extended in OviPlus® and egg yolk. The second aliquot was extended with OviPlus® and coconut milk. Each of the aliquots was further subdivided into 2 parts and evaluated for viability, individual progressive motility and morphological abnormalities. One part was chilled at 4℃ and evaluated for the same parameters immediately after extension (within 0 hours of initial collection) and at 24, 48 and 72 hours. While the second part was loaded into 0.25 ml plastic straws, chilled to 4℃ for 5 hrs, exposed to liquid nitrogen vapour at -140℃ for 10 mins and then plunged into liquid nitrogen tank for cryopreservation at -196℃ and analyzed after 24 hours.

Statistical analysis

Data were analyzed using analysis of variance (ANOVA) using GraphPad® prism software. Values of $P < 0.05$ were considered significant.

Results

The semen from the Uda ram was thick in consistency and the colour was creamy or milky. In addition, the pH was 7.0. The mean semen volume and individual progressive motility were 1.3 ± 0.1 ml and 81.7 ± 1.7 % respectively. More than 75 % of the spermatozoa were alive. Less than 9 % morphological abnormalities were recorded. The semen concentration was 3.2×10^9 spermatozoa / ml (Table 1).

The percentage individual progressive motility shown in Table 2 for coconut milk and egg yolk citrate at 0 hour, 24 hours, 48 hours, and 72 hours, showed no significant difference ($P > 0.05$) between the two extenders (coconut milk and egg yolk citrate), but were different ($P > 0.05$) with compared with the fresh semen. The spermatozoa of non extended semen were non motile when examine at 24 hours, whereas, the individual progressive motility in coconut milk and egg yolk based extenders remained motile after 24 hours, but the motility decreased over 72 hours. (Table 2).

The semen livability as shown in table 3 for semen extended with coconut milk and egg yolk citrate at 0 hour, 24 hours, 48 hours, and 72 hours showed no significance different ($P > 0.05$) with each other. However, there was significant difference in livability between extended and fresh semen. Similarly, the livability of Uda ram semen in non-extended were zero when examine at 24 hours, while the livability of semen extended in coconut milk and egg yolk based extenders remained viable after 24 hours, but the livability decreased over 72 hours (Table 3).

Table 1: Mean ± SD of semen volume, individual progressive motility, viability, morphological abnormalities and concentration of Uda ram in Maiduguri, Nigeria

Volume (ml)	Individual progressive motility (%)	Viability (%)	Morphological abnormalities (%)	Concentration ($\times10^9$/ml)
1.4	80	75	8.6	3.5
1.1	80	75	8.7	3.6
1.4	80	75	7.0	2.8
1.4	80	75	7.7	3.0
1.4	90	75	7.0	3.1
1.2	80	80	8.8	3.2
1.3 ± 0.1	81.7 ± 1.7	75.8 ± 0.8	8.0 ± 0.3	3.2 ± 0.3

Table 2: The individual progressive motility of Uda ram semen in non-extended as well as in coconut milk and egg yolk-based extenders chilled at 4℃ over 72 hours

Type of semen	Type of extension	Time from semen collection (hours)			
		0	24	48	72
Fresh		82.1±7.5^{a}	0.0^{a}	0.0^{a}	0.0^{a}
Extended	OviPlus® + Coconut milk	80.5+5.8^{a,x}	60.8+11.8^{b,y}	37.1+13.8^{c,z}	14.1+12.2^{d}
	OviPlus® + Egg yolk	81.3+5.9^{a,x}	63.8+12.4^{b,y}	39.3+14.2^{c,z}	17.3+13.2^{d}

Values with different superscripts between columns (^{a,b,c}), and within rows (^{x,y,z}) differ significantly (P< 0.05)

Table 3: Livability of Uda ram semen in non-extended and in egg yolk and coconut milk-based extender chilled at 4℃ and analyzed over 72 hours

Type of semen	Type of extension	Time from semen collection (hours)			
		0	24	38	72
Fresh		82.8±7.4^{a}	3.0±1.2^{a}	3.0±1.7^{a}	3.0±1.9^{a}
Extended	OviPlus® + Coconut milk	81.5+5.6^{a,x}	62.8+6.5^{b,y}	38.0+6.0^{c,z}	16.3+5.7^{d}
	OviPlus® + Egg yolk	81.3+5.9^{a,x}	65.5+6.4^{b,y}	40.8+6.9^{c,z}	17.3+ 8.0^{d}

Values with different superscripts between columns (^{a,b,c}), and within rows (^{x,y,z}) differ significantly (P< 0.05)

The spermatozoa morphological abnormalities for fresh pooled semen was significantly different (P < 0.05) from those of egg yolk and coconut milk-based extenders. However, no significant difference was recorded between the extenders. Similarly, the semen morphological abnormalities increased from 1 hour to 72 hours (Table 4).

The individual progressive motility of the fresh pooled semen showed a significant difference (P < 0.05) with semen extended with egg yolk and coconut milk-based extenders after cryopreservation. However, there were no significant differences (P > 0.05) between the extenders. Similarly, semen livability of fresh pooled semen showed significant differences (P < 0.05) with semen extended with egg yolk and coconut milk extenders after cryopreservation. There were no significant differences (P > 0.05) in post-thaw progressive motility between the extenders (Table 5).

Discussion

The mean semen volume found in the current study was within the range of 0.5 to 2 ml reported in ram (Hafez, 2000). However, this volume is higher than the 0.72 ± 0.2 and 1.2 ± 0.2 ml previously reported for Yankasa ram by Babashani *et al.* (2015) but similar to semen volume obtained from West African dwarf ram by Ososanya *et al.* (2014). Both authors also used electro-ejaculator for their semen collection. Using artificial vagina, Marco- Jimenez *et al.* (2005) found a mean volume of 1.2 ± 0.1 for Gurra ram in Spain. Furthermore, Azizunnesa *et al.* (2014) reported 1.2 ± 0.2 for indigenous rams in Bangladesh. This report agrees with the findings in this study on Uda ram.

The average spermatozoa concentration found in this study was is within the range previously reported in sheep by Hafez (2000). However, it is higher than the 1.7 $\times10^9$/ml to 4.7 $\times10^9$/ml reported in Yankassa sheep by Babashani *et al.* (2015). The

Table 4: The morphological spermatozoa abnormalities of fresh pooled semen and extended in egg yolk and coconut milk-based extenders 4℃

Type of semen	Type of extension	Time from semen collection (hours)			
		0	24	48	72
Fresh		3.0±1.0^{a}	3.0±1.2^{a}	3.0±1.7^{a}	3.0±1.9^{a}
Extended	OviPlus® + Coconut milk	3.3±1.3^{a,x}	3.6±0.1^{b,y}	4.3±0.1^{c,z}	4.5±0.8^{d}
	OviPlus® + Egg yolk	3.1± 0.9^{a,x}	3.5±0.5^{b,y}	4.3±0.8^{c,z}	4.6±0.8^{d}

Values with different superscripts between columns (^{a,b,c}), and within rows (^{x,y,z}) differ significantly (P< 0.05)

Table 5: The individual progressive motility, livability and morphological abnormalities of the fresh pool and post-thawed Uda semen extended with egg yolk and coconut milk-based extender observed after 24 hours.

Parameter	Fresh semen	Extended and post-thawed	
		OviPlus® + Coconut milk	OviPlus® + Egg yolk
Motility	80.0±9.7^{a}	23.7±7.6^{b}	22.7±8.9^{b}
Liveability	83.7±8.4^{a}	42.5±8.5^{b}	44.5± 10.5^{b}
Abnormalities	3.2±1.2^{a}	4.7±1.2^{b}	4.7±0.7^{b}

Different superscripts indicate significant differences within rows $^{a\ b}$ P< 0.05

values were also higher than the range of 2.0 ± 0.3 × 10^{9}/ml and 1.4 ± 0.1 × 10^{9}/ml reported in Pleven Blackhead ram semen by Yotov *et al.* (2011). However, Yotov *et al.* (2011) also reported that increased frequency of collection significantly decreased the concentration.

The average spermatozoa motility of Uda ram observed in this study is similar to 82.0 % ± 6.3 documented in Yankasa ram (Babashani *et al.*, 2015), but higher than 77.0 ± 2.2% and 77.6 ± 3.7% reported by Ghorbani *et al.* (2018) in Sanjabi rams in Iran and Marco-Jimenez *et al.* (2005) in Gurra ram in Spain, respectively. However, Azizunnesa *et al.* (2014) reported 89.0 ± 0.2 in indigenous rams in Bangladesh, which is higher than what was reported in this study. This variation is attributed to the breed of animal use for the research and management system.

Motility in the non-extended chilled semen decreased when compared with semen extended with coconut milk and egg yolk. Generally, the current study showed from the commencement to the end of preservation, there was drop in sperm motility from 82% in freshly collected semen to less than 20% at 72 hours post extension for both the extenders. This finding is in agreement with previous studies (Kasimanickam *et al.*, 2011; Rather *et al.*, 2017).

Storage temperature, irrespective of diluent, had a significant effect on both motility and viability. O'Hara *et al.* (2010) reported that the viability and fertility of fresh ram semen decreased with the passage of time and ram semen has a short fertile lifespan *in vitro*. They also found that ram semen stored at 4℃ maintained acceptable motility and viability up to 72 hours using standard skimmed milk, AndroMed®, OviPro®, and INRA 96® semen extenders. However, this study showed that coconut milk and egg yolk citrate-based extenders preserved Uda ram spermatozoa motility up to 24 hours at 4℃. Beyond 24 hours, the proportion of motile spermatozoa was low and unlikely to be fertile.

Consequently, the findings from this study suggest that the use of fresh semen shortly after collection and chilled semen at 0 hours and 24 hours post extension could be acceptable for use in artificial insemination (AI) programs in Uda rams. These findings agree with a similar report in Indian indigenous ram by Rather *et al.* (2017).

The percentage livability of spermatozoa is important in assessing semen for artificial insemination. The percentage of live spermatozoa documented in this study suggest that, it can be used successfully for artificial insemination in the ewe. Marco- Jimenez *et al.* (2005) and Ghorbani *et al.* (2018) reported semen livability of 71.9% and 71.7% in Gurra ram in Spain and Sanjabi rams in Iran, respectively.

Effect of storage on the semen characteristics in this study showed that the percentage of live spermatozoa did not differ amongst the extenders. However, the livability dropped soon after collection at 37℃ for 0-3 hours post extension for all the extended semen. The results in this study demonstrated that the inclusion of coconut milk and egg yolk citrate-based extender provided an

adequate medium to sustain the viability of Uda ram spermatozoa at least for 24 hours only.

The average spermatozoa morphological abnormalities for the rams in this study is lower than the 7.5 % previouly documented in Uda ram by Kumi-Diaka *et al.* (1985) using electro-ejaculator. Marco- Jimenez *et al.* (2005) reported 8.1 ± 1.1% in Gurra ram. However, 12.9 ± 00% and 15.8 ± 0.2% were reported by Ososanya *et al.* (2014) in West Africa dwarf rams and Babashani *et al.* (2015) in Yankasa rams respectively. Both findings were higher than what was found in this study but were all within the acceptable limit.

The proportion of abnormalities recorded for individual rams, pooled and post-thawed semen were within the acceptable values for fertile insemination as previously reported (Azizunnesa *et al.*, 2014). However, a significant difference was recorded ($P < 0.05$) in the abnormalities between semen preserved at 4℃ and the frozen-thawed at -196℃. Common abnormalities observed in this study were coiled tail, micro head, bent tail, detached tail, double head and detached head.

The mean spermatozoa concentration recorded in the current study was 3.2×10^9. This finding is lower than 3.8×10^9 previously documented in Uda ram by Kumi-Diaka *et al.* (1985). Evans & Maxwell (1987) and Hafez (2000) reported spermatozoa concentration of an adult ram to vary from 3.5 to 6.0 $\times 10^9$ spermatozoa/mls for maximum fertility. However, Gil *et al.* (2003) reported that for semen to be acceptable for artificial insemination in rams, a spermatozoa concentration of 2.5 $\times 10^9$ spermatozoa/ml is required.

Semen cryopreservation significantly reduces the quality of frozen-thawed semen (Medeiros *et al.*, 2002; Barbas & Mascarenhas, 2009). In the current study, there was a significant decline in the post-thawed spermatozoa motility and viability of Uda semen cryopreserved in liquid nitrogen at -196℃, when compared with the fresh semen. These findings showed a reduction in spermatozoa motility and spermatozoa livability after thawing. No significant difference ($P > 0.05$) was observed in individual progressive motility and livability between egg yolk and coconut milk-based extenders cryopreserved in liquid nitrogen at -196℃. Similarly, cryopreservation significantly reduced the motility and quality of frozen-thawed sperm in Lacaune and East-Friesian ram breed in Slovak Republic (Kubovicova *et al.*, 2011)

In the present study, there was a general time-related decline in semen motility, livability and increased morphological abnormalities in both coconut milk and egg yolk-based extenders. Maia *et al.* (2014) reported that ram spermatozoa produce large amounts of hydrogen peroxide which decreases post-thawed spermatozoa motility, resulting in a drastic decline in semen motility, livability and some morphological abnormalities. A decrease in temperature from room temperature (25℃) to 4℃ and gradually to -196℃ is associated with alteration of the membrane integrity, increased permeability and subsequent damage to spermatozoa (Purdy, 2006; O'Hara *et al.*, 2010). In the current study, the frozen-thawed semen significantly lost its motility and viability after storage in liquid nitrogen. This is a limitation in the study because successfully cryopreserved semen is expected to maintain acceptable post-thaw quality for artificial insemination. It is noteworthy that previous studies had established that the survival of frozen-thawed ram spermatozoa is affected by many factors, such as type of extender, cryoprotectant, packaging, freezing and thawing rates as well as the quality of spermatozoa used for freezing (Watson, 2000; Rodriguez-Martinez & Barth, 2007). Furthermore, several cryopreservation protocols were optimized through trials with different types of antioxidants and supplementation methods to reduce the ROS levels in the semen and the cell membrane damage they cause (Riesco *et al.*, 2021). Similar studies are needed to improve this coconut milk-based extension protocol using different cryoprotectants, antioxidants as well as effects of different cooling, freezing and thawing rates.

It was concluded that semen motility, livability as well as morphological abnormalities of Uda ram is equally preserved in coconut milk and egg yolk-based extenders at 4℃ up to 24 hours post extension. However, semen motility and livability were significantly reduced in the Uda semen cryopreserved at -196℃ after thawing.

Acknowledgements

We wish to acknowledge Tijjani Buba for his assistance during this study. We also acknowledge the University of Maiduguri Institution Based Research and Tertiary Education Trust Fund (Tetfund) for providing the grant used in this research.

Conflict of Interest

The authors declare that there is no conflict of interest.

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