NIGERIAN VETERINARY JOURNAL

ISSN 0331-3026

Nig. Vet. J., March 2021 Vol 42 (1): 19 – 28.

https://dx.doi.org/10.4314/nvj.v42i1.2 **ORIGINAL ARTICLE**

Effects of Coconut, Groundnut and Tigernut Milk-Based Extenders on Fresh and Chilleduda Ram Semen in Maiduguri, Nigeria

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SUMMARY

This study was conducted to determine the effects of coconut, groundnut and Tigernut milk based extenders on some characteristics of Uda ram semen in Maiduguri, Nigeria. A total of 96 semen samples were collected using electro-ejaculator from six Uda rams, twice a week, for 8 weeks. Semen was pooled and divided into 4 aliquots. One aliquot was diluted with Oviplus® solution and egg yolk (Oviplus® + egg yolk) and served as control. The other 3 aliquots were extended with coconut, groundnut and tigernut milk-based extenders respectively. The samples were chilled to 5°C and evaluated for individual motility, livability and morphologic abnormalities. These evaluations were made immediately, at 24, 48 and at 72 hours post extension. The average semen volume, individual motility, livability, morphologic abnormalities, pH and concentration of semen analyzed immediately after collection were 1.35 ± 0.1 ml, 80.9 ± 1.5 (%), 75.8 ± 2.0 (%), 8.0 ± 0.8 (%), 6.9 ± 0.1 and 3.2 ± 0.2 ($x10^9$/ml) respectively. Furthermore, it was found that coconut, ground nut and tiger nut milkbased extenders maintained good semen quality of Uda rams till 48 hours post extension when chilled at 5°C and that the tiger nut milk based extenders have better semen preservative ability than the coconut and the ground nut milk based extenders.

Keywords: Semen Extender; Tiger nut Milk; Semen quality

INTRODUCTION

Chilling is an important semen preservation protocol widely practiced in sheep breeding(Kulaksiz *et al.*, 2012).Sheep and goat spermatozoa in particular are reported to be very sensitive to peroxidative damages due to high content of unsaturated fatty acids presents in their plasma membrane and the relative low antioxidant capacity of their seminal plasma (Watson, 2000). Irreversible loss of semen quality could occurduring storage due to lipid peroxidation or excessive

production of reactive oxygen species ROS(Peruma *et al.*, 2011).

These peroxidative damages to spermatozoa could be exacerbated by microbiological contamination reported previously (Thun *et al*., 2002). To preserve semen for short or long term storage, several additives have been used to maintain the integrity of the sperm cells (Bucak *et al*., 2010, Raheja *et al*., 2018).

The search for and evaluation of alternatives to the animal-based extenders such as egg yolk and milk, by exploring extenders of plant origin has been ongoing. This became necessary because of threats of semen contamination and spread of trans-boundary diseases(Rehman *et al*., 2014).Soya-lecithin and coconut water has been found useful for preservation of semen quality and fertility (Raheja *et al*., 2018).

According to Futino*et al.*(2010), plant-based diluents, such as soya-lecithin is a good alternative to phospholipids present in egg yolk for semen preservation, because it protects the plasma membrane by refurbishing the lost phospholipid due to heat stress. In Nigeria, potential plant-based extenders include milk from coconut (*Coco nusifera*), groundnut (*Arachis hypogaea*) and Tigernut *(Cyperus esculentus L.*).

Coconut is readily available in Nigeria and coconut milk contains 35% saturated fat, 11% non-fat solid, sugar, vitamins and amino acid such as lauric acid (Raghavendra and Raghavarao, 2010). A previous study by El-Nattat*et al.*(2009a) used coconut water extender with egg yolk and different glycerol concentrationsfor cryopreservation of buffalo semen.

Tigernutis cultivated and widely available in Northern Nigeria, the milk is rich in vitamins (A, B_1, C and E), minerals, sugar and amino acid especially arginine, oleic acid and linoleic acid(Chukuma *et al*., 2010). There is no previous study that evaluated the use of Tigernut as a source of semen extender.

In addition, groundnut is also a major crop grown in the arid and semi-arid zone of Nigeria, the milk contains 44 to 56% oil, sugar, minerals, amino acids and vitamins E, K, and B (Isanga and Zhang, 2009). Amini *et al.*(2015) reported that supplementation of semen with antioxidants like oleic acid has been proven to maintain the viability and motility of liquid or cryopreserved sperm cells of rooster Govil *et al.*(1992) also stated that arginine protects spermatozoa against lipid peroxidation by increased production of nitric oxide which decreases lipid peroxidation by incapacitating free radicals. According to Kaka *et al.* (2015), addition of palmitic acid and linoleic acid to extended bull semen enhanced sperm motility and viability.

The use of coconut milk or water as component of semen extender in different species has been documented (Yong *et al*., 2009). These include buffalo semen (El-Nattat *et al*., 2009b), bovine (Lucci *et al*., 2004), caprine (Silva *et al*., 2004) and ovine (Andrade *et al.*, 2002).

Thus, there is need to evaluate the effectiveness of other readily available plant-based milk like groundnut and tiger nut milk for semen preservation. Therefore, this study was designed to determine the effects of coconut, groundnut and Tigernut milk-based extenders on some semen characteristics of Uda ram stored at 37°C and at 5°C.

MATERIALS AND METHODS

Experimental design

This research was carried out at the Andrology and Artificial Insemination Laboratory, Department of Theriogenology, University of Maiduguri. Six matured Uda rams aged 2 – 3 years weighing 40 – 45 kg were used for the study. The rams were purchased from Maiduguri livestock market and housed at the Large Animal Unit of the Veterinary Teaching Hospital University of Maiduguri. They were allowed to acclimatize

for two weeks before the commencement of the study. The rams were fed with a mixture of wheat bran and bean offal together with groundnut hay and clean water was provided *ad libitum.*

Preparation of Extenders (Coconut milk, Groundnut milk and Tigernut milk)

A commercial semen extender (Oviplus®) was used as diluent in this study. Four different extenders were prepared. Egg yolk is used to prepare the semen extender according to manufacturer's instruction. Instead of egg yolk, coconut milk, ground nut milk and tiger nut milk were used as substitutes. The extension media were always prepared fresh and stored in refrigerator a day prior to each experiment.

The preparation involved addition of Oviplus® (10%) in bi-distilled water (70%) and 20% of any of the extension media (Egg yolk, Coconut milk, Groundnut milk and Tiger nut milk). The ground nut and coconut milk were extracted using the procedure described by Onweluzo and Nwakalor(2009) and Adelodun *et al*.(2012) respectively while that of tiger nut milk was according to the procedure described by Murevanhema and Jideani (2014).

Semen collection

Semen was collected twice a week for eight weeks using an electro-ejaculator (Lane Manufacturing Inc. No. 72707C) as described by Jibril *et al.* (2011). The ejaculator probe was lubricated with KY jelly and inserted into the rectum. Penile erection was stimulated with discharge of 12 volts intermittently every 4 seconds until ejaculation according to manufacturer's instruction. The ejaculate was collected in a pre-warmed calibrated test tube. The samples were stored in a water-bath maintained at 37°C.

Semen extension, dilution ratio and post extension evaluation of Uda ram semen

Immediately after semen collection, samples were analyzed for both macroscopic (color, consistency, pH and semen volume) and microscopic changes (individual motility, concentration, livability and sperm abnormalities). Freshly collected semen were pooled and divided into four aliquots. One of the aliquots was diluted with Oviplus® + egg yolk and the remaining 3 aliquots were extended using Oviplus® + extension media (coconut milk, groundnut milk and the tiger nut milk) at ratio 1:4 respectively. Thereafter, the samples were stored at 4 ℃ in a refrigerator and were evaluated for progressive forward motility, livability and percentage abnormalities at 0, 24, 48 and 72 hours post extension. Data generated were analyzed using the GraphPad (Instat)® and presented in Mean ± Standard deviation. Analysis of Variances (ANOVA) was used to compare the means. Variables with ($p < 0.05$) were considered statistically significant.

RESULTS

The individual progressive motility of Uda ram semen evaluated immediately after collection and after storage at 4 ℃ is shown on Table I. The progressive forward motility of the spermatozoa assessed immediately after collection and extension with Oviplus® and egg yolk was 79.9 ± 1.6 %. This was significantly decreased ($P<0.05$) to 40% after the semen was cooled to 4 ℃ and stored at that temperature for 24 hours, all the plant-based extenders maintained the progressive motility significantly better ($P<0.05$) than the egg yolk-based extenders. However, 24 hours after extension, the motility remained at 65% while at 48 hours the motility was 44% with the Oviplus® and Tigernut milk extenders. By 72 hours, only 25% of the spermatozoa were progressively motile when compared to Oviplus® and egg yolk as well as other diluents (Table I). The groundnut-based milk extender showed the least motility immediately after extension (71%) compared

with all the other extenders.

TABLE I: Individual progressive motility (%) of Uda ram spermatozoa extended with various extenders and stored at 4 ℃ and evaluated at different hours post extension.

	Post Extension Storage Time (hours)			
Type of Extender	0	24	48	72
Oviplus® + Egg Yolk	$79.9 \pm 1.6^{a,w}$	$40.1 \pm 0.1^{a,x}$	$28.0 \pm 0.0^{a,y}$	$10.0 \pm 0.0^{a,z}$
Oviplus® + Coconut Milk	$75.6 \pm 7.1^{a,w}$	$60.3 \pm 10.2^{b,x}$	$41.0 \pm 12.4^{b,y}$	$20.2 \pm 8.9^{b,z}$
Oviplus® + Groundnut Milk	$71.0 \pm 11.2^{b,w}$	$59.0 \pm 13.1^{b,x}$	$35.6 \pm 11.3^{c,y}$	$15.5 \pm 6.9^{b,z}$
Oviplus® + Tigernut Milk	$78.3 \pm 6.7^{a,w}$	$65.0 \pm 7.9^{c,x}$	$44.5 \pm 16.2^{b,y}$	$25.5 \pm 9.7^{c,z}$

Values on the same column ^{a,b,c} and row ^{w,x,y,z} differs significantly at $p < 0.05$

The proportion of live spermatozoa (%) of semen extended with different extenders analyzed immediately, after storage at 4 °c and evaluated at different hours post extension is shown in Table II. Immediately after extension at 0 hours, the livability of the semen was not significantly different ($p>0.05$) between the Egg yolk, Coconut, Groundnut and Tigernut-based extenders. However, after storage at 4 °c and analyzed 24 hours post extension, semen extended Egg yolk had significantly lower livability ($p<0.05$) than the other 3 plant based extenders. At 48 hours, both the Egg yolk and Groundnut milk extended semen had significantly less livability ($p<0.05$) than the Coconut and Tigernut milk-based extenders. At 72 hours, the livability was significantly different between the extenders, with the Egg yolk extended semen having the least livability (15%) and the Tigernut milk extended semen with highest livability (31%) as shown on Table II. The livability analyzed at 24, 48 and 72 hours significantly decreased ($p<0.05$) in all of the extended semen during storage.

TABLE II: Percentage livability (%) of Uda ram semen extended with various extenders and stored at 4 ℃ and evaluated at different hours post extension

Type of Extender	Post Extension Storage Time (hours)			
	0	24	48	72
Oviplus® + Egg yolk	$73.0 \pm 8.4^{a,w}$	$51.2 \pm 2.8^{a,x}$	$33.0 \pm 0.0^{a,y}$	$15.0 \pm 0.0^{a,z}$
Oviplus® + Coconut Milk	$73.5 \pm 7.5^{a,w}$	$60.3 \pm 8.9^{b,x}$	$45.0 \pm 10.6^{b,y}$	$28.0 \pm 8.2^{b,z}$
Oviplus® + Groundnut Milk	$73.7 \pm 6.5^{a,w}$	$59.6 \pm 12.8^{b,x}$	$39.3 \pm 8.4^{c,y}$	$23.3 \pm 7.7^{c,z}$
Oviplus® + Tigernut Milk	$73.8 \pm 9.2^{a,w}$	$60.6 \pm 11.2^{b,x}$	$48.5 \pm 12.0^{b,y}$	$31.0 \pm 10.1^{b,z}$

Values on the same column ^{a,b,c} and row ^{w,x,y,z} differs significantly at $p < 0.05$

The percentage abnormalities of semen analyzed immediately after extension with Oviplus® and egg yolk was 2.8 ± 2.1 %. There were no significant differences among the extension media at 0, 24

and 48 hours post extension (Table III). However, the abnormalities increased to14.0 ± 0.0% at 72 hours in Oviplus® and egg yolk while the plant-based extenders showed no significant differences throughout the experiment. Some of the abnormalities observed included coiled tail, bent tail, double head, micro head and detached head/tail as shown in Figure 1. TABLE III: Morphologic abnormalities (%) of Uda ram semen extended with different extenders, stored at 4 ℃ and evaluated at different hours post extension

Type of Extender	**Post Extension Storage Time (hours)**			
	0	24	48	72
Oviplus® + Egg yolk	$2.8 \pm 2.1^{a,x}$	$5.1 \pm 0.3^{a,x}$	$8.0 \pm 0.1^{a,x}$	$14.0 \pm 3.1^{a,y}$
Oviplus® + Coconut Milk	$3.7 \pm 2.8^{a,x}$	$4.5 \pm 1.9^{a,x}$	$5.0 \pm 1.9^{a,x}$	$5.1 \pm 2.3^{b,x}$
Oviplus® + Groundnut Milk	$3.9 \pm 2.3^{a,x}$	$4.7 \pm 2.6^{a,x}$	$5.0 \pm 2.2^{a,x}$	$5.4 \pm 2.8^{b,y}$
Oviplus® + Tigernut Milk	$3.0 \pm 1.6^{a,x}$	$3.7 \pm 2.3^{a,x}$	$4.4 \pm 2.1^{a,x}$	$4.8 \pm 1.7^{b,y}$

Values on the same column ^{a,b,c} and row ^{x,y,z} differs significantly at $p < 0.05$

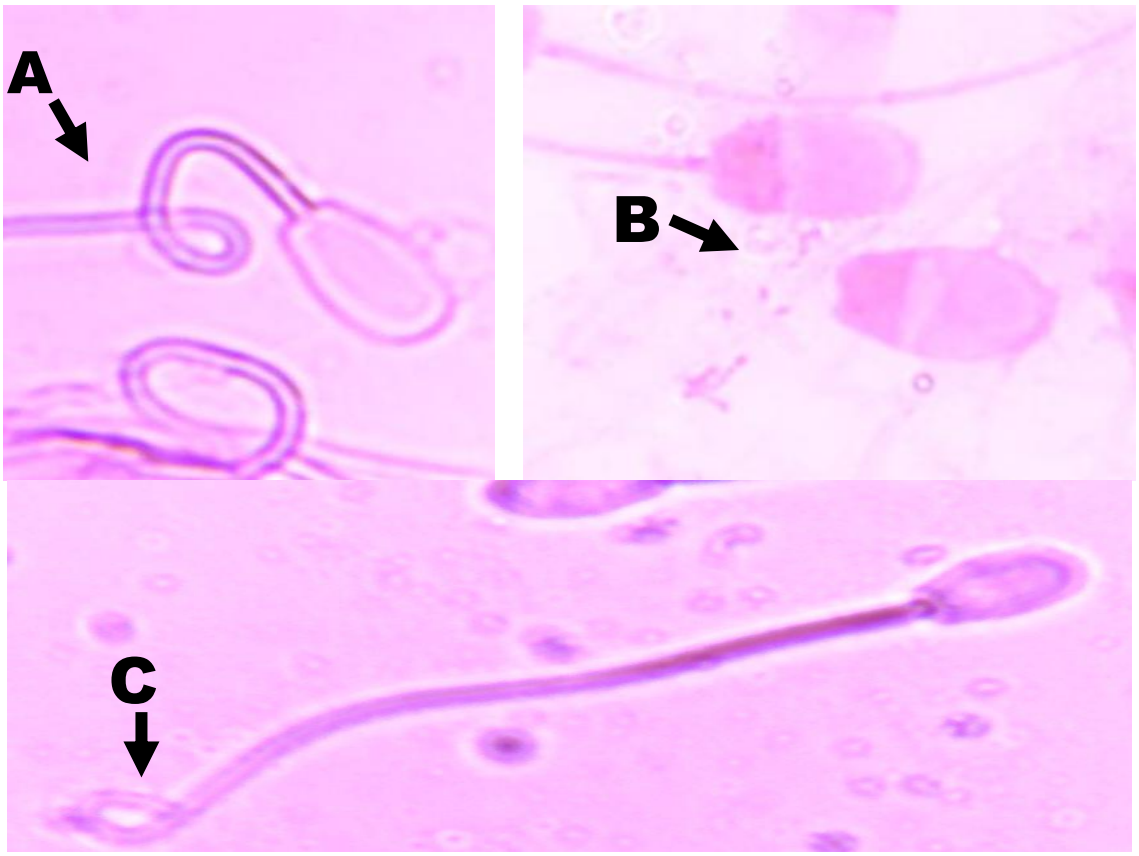

Figure 1: Some spermatozoa abnormalities observed in Uda ram semen in Maiduguri, Nigeria These include a high percentage of coiled tail, bent tail and detached tail. They include coiled tail (A), detached head (B) and coiled tail (C)

DISCUSSION

In the current study, percentage progressive motility of spermatozoa extended with Oviplus® and egg yolk and assessed immediately after collection was higher than the motility earlier reported by Oyeyemi and Olusoji (2018). This may be attributed to the different methods for semen collection used in the two studies. Oyeyemi and Olusoji (2018) flushed epididymal spermatozoa from samples obtained from the abattoir. An electro ejaculator was used to collect semen in the current study. Previous studies had shown that lower sperm motility occurred in bulls when flushing method was used compared to electro ejaculator (Silva *et al*., (2003). Muller-Scholosser (2005) also reported that the motility of epididymal spermatozoa was the first parameter to be affected by post-mortem.

The decreased motility ($P<0.05$) after storage at 4 ^{0}C for 24 hours observed in the current study was also previously reported by Kommisrud *et al*. (2002), who demonstrated that the motility of boar semen declined with days of storage, probably due to production of reactive oxygen species (ROS). According to Bilodeau *et al*. (2001), during the procedure of semen preservation, sperm cells are exposed to oxygen and visible light radiation, which will lead to formation of ROS and by extension, affect sperm motility and genomic integrity. Despite this decrease, it was observed that all the plant-based extenders maintained the progressive motility significantly better ($P<0.05$) than the egg yolk-based extenders in the current study. This may be ascribed to the considerable amount of steroids (pregnelenone and progesterone) present in egg yolk that could induce an acrosomal reaction in the sperm cells. The egg yolk serves as an excellent medium for microbial growth, as a result may lead to high risk of microbial contamination which in turn will alter the semen pH by increase production of lactic acid (Muller-Schlosser, 2005). Many researchers have reported the superiority of plant–based semen extenders giving competitive results in divergent species over egg yolk-based extenders on various semen quality assessment parameters (Stradaioli *et al*., 2004).The superior performance of plant-based extenders was illustrated with the study of Kasimanickam *et al*. (2011) who compared soy-lecithin based extender with milk containing conventional diluents and found that mitochondrial membrane potential was increased in soy-lecithin based extender. Moreover, DNA fragment index and sperm motility were also improved in soy- lecithin diluents as compared to milk-based extenders.

The groundnut milk extender showed the least motility immediately after extension in the current study. This was probably because of the high lipid content of groundnut milk (44 to 56%), which may adversely affect the semen quality (Surai and Sparks, 2001). Lipoperoxidation irreversibly abolishes the fructolytic and respiratory activity in spermatozoa, resulting in a considerable decline in their respiratory rate and motility due to decreased radical scavengers and oxidative stress (Dardmeh *et al*., 2017). However, after the initial decrease, the groundnut extenders preserved motility better than the egg yolk extenders; this may be attributed to the decrease in pH in egg yolk extenders following production of lactic acid as a result of possible bacterial contamination with storage time (Gadea, 2003).

The Tigernutmilk- based extender showed the best preservative qualities at 24 hours post extension (65%) The motility remained at 44% even at 48 hours, and this may probably be due to the abundance of oleic acid and arginine content in Tigernut milk, which is known to be an effective antioxidant and protective against lipid perioxidation (Eslami *et al*., 2016). According to Elslami *et al.* (2016), enrichment of semen with oleic acid

decreased the level of Malondialdehyde (a stable end product of lipid peroxidation used as an indirect measure of the cumulative lipid peroxidation), increased the antioxidant activity levels and finally improved the forward progressive motility of spermatozoa. Cytoprotective effects of oleic acid have been reported in somatic cells (Bucak et al., 2010a).

The significantly lower ($P<0.05$) livability of Uda spermatozoa in the Oviplus® and egg yolk extended group compared with the 3 plant-based extended groups suggests that there might be factors found in egg yolk that cause death of spermatozoa. It was reported that high level of calcium ions found in egg yolk is cytotoxic (Watson and Martin, 1976). Furthermore, Akhter *et al*.(2008) and Salmani *et al*.(2014)also reported that the composition of unsaturated fatty acid in egg yolk affect sperm metabolism resulting to lipid peroxidation, which changes the spermchromatin structure and thereby affecting the viability and fertilizing ability of spermatozoa. Findings from the current study agrees with previous reports because the livability was not significantly different ($P>0.05$) amongst the Coconut, Groundnut and Tigernut based extenders. Hence, the Tigernut extenders maintained the highest percentage livability.

With respect to morphologic abnormalities, the lowest value obtained in this study was 4.8 % in Oviplus® + Tigernut milk, this may be attributed to the antioxidants present in the plant-based extenders especially Tigernut milk. A previous study by Fraczek *et al*.(2004) suggested that antioxidants such as vitamins A, C, and E improve sperm motility and morphology.

CONCLUSION

Coconut, Groundnut and Tigernut milk-based extenders maintained semen quality of Uda ram up to 48 hours post extension at chilled (4°C) liquid storage compared to the Oviplus® + egg yolk solution. Furthermore, it was evident that Tigernut milk-based extender preserved Uda ram semen better than the Coconut and Groundnut milk=based extenders. Therefore, further studies to assess the fertility of Uda ram semen preserved under the various plant-based extenders (Coconut, Ground nut and Tigernut milk-based) should be undertaken.

ACKNOWLEDGEMENTS

We wish to acknowledge the assistance of Dr. I. M. Alkali, Dr. S. O. Asuku and Tijjani Buba for their assistance. We also acknowledge, University of Maiduguri, Institution Based Research (IBR), Tertiary Education Trust Fund (Tetfund) for the grant used in carrying out this research.

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