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Effects of coconut (*Cocos nucifera*) water with or without egg-yolk on viability of cryopreserved buck spermatozoa

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Abstract

The effects of semen extenders containing coconut (*Cocos nucifera*) water alone or combined with egg yolk on viability of spermatozoa during cryopreservation of semen obtained from West African Dwarf (WAD) bucks were studied. Coconut water (5, 10, 15 and 20 ml/100 ml) was diluted with pooled semen samples in study I, while in study II, the pooled semen samples were diluted with egg yolk and coconut water (EYCW) at 5:10, 10:10 and 10:5 in Tris-based extenders. The control consisted of Tris-based extenders plus sodium citrate. The diluted semen samples were cryopreserved for 30 days and evaluated for sperm viability parameters. Following cryopreservation, acrosome reaction and capacitation of spermatozoa were induced *in vitro*. The results showed that motility was higher ($P < 0.05$) in coconut water extenders compared to the control and improvement in this parameter was best at 10% coconut water. More spermatozoa cryopreserved with coconut water underwent induced acrosome reaction and capacitation compared to the control and 10% coconut water had the highest values ($P < 0.05$). The results showed that motility was better preserved in EYCW5:10 and EYCW10:10 extenders ($P < 0.05$). While more spermatozoa underwent induced acrosome reaction and capacitation with coconut water and egg yolk extenders at different combinations compared to the control ($P < 0.05$), the highest values of acrosome integrity and membrane integrity were observed in EYCW5: 10 extenders. The results revealed that 10% coconut water and EYCW5:10 gave better improvement of sperm viability parameters. The findings indicated that coconut water extender improved the quality of cryopreserved spermatozoa of WAD bucks.

Keywords: acrosome reaction, freezing, semen extender, sperm quality.

Introduction

Artificial insemination with preserved semen is a viable option for genetic upgrading of West African Dwarf goats (WAD), known to possess certain valuable traits that confer adaptation to endemic trypanosomiasis challenge and hot humid tropics (Daramola and Adeloye, 2009). Cryopreservation of gametes of this breed that is in danger of extinction due to possible replacement by some breeds which do not have adaptive traits is necessary. Semen cryopreservation is a major resource for the preservation of genetic material in most domestic species and is an important tool of artificial insemination; it enhances improvement of livestock as breeders mostly use genetically superior males (Martinez *et al*., 2007). The viability of sperm at low temperatures for a long period requires dilution with an appropriate extender in order to maintain the quality of spermatozoa. Regardless of constituents of extenders however, viability of spermatozoa deteriorates during storage process at low temperatures which are possible results of lipids peroxidation or excessive production of reactive oxygen species (Peruma *et al*., 2011). Goat spermatozoa in particular are sensitive to peroxidative damage due to high content of unsaturated fatty acids in the phospholipids of plasma membrane and the relative low antioxidant capacity of goat seminal plasma (Watson, 2000). Moreover, interaction between goat seminal plasma and egg yolk or skim milk being the most common semen diluents for goat are deleterious to the sperm, a condition not observed with other mammalian seminal plasma (Pellicer-Rubio *et al*., 1997; Leboeuf *et al*., 2000). This is due to the presence of phospholipases A2, enzymes in seminal plasma that hydrolyse egg yolk lecithin in fatty acids and lysolecithin (Roy, 1957; Iritani *et al*., 1964), and react with the phosphocaseinate fraction of skimmed milk-based extenders (Pellicer-Rubio *et al*., 1997), and this could lead to production of compounds toxic to spermatozoa. A series of protection methods against reactive oxygen species have been investigated, like storage in low oxygen atmosphere or addition of antioxidants in the storage diluents (Anghel *et al*., 2010) and in some cases the results are contradicting (Maia *et al*., 2009; Sicherle *et al*., 2011). Coconut (*Cocos nucifera*) water is the clear liquid inside coconut fruits and contains essential constituents such as sugars, vitamins, minerals and amino acids (Yong *et al*., 2009; USDA National Nutrient Database, 2015) that play a vital role in aiding the antioxidant system (Evans and Halliwell, 2001). Coconut water is easy to prepare, readily available and cheap compared to egg yolk and milk. Perusals of literatures reveal no information on the cryosurvival of spermatozoa obtained from WAD buck during cryopreservation with coconut water extender. Therefore, the aim of the present study was to assess the effect of semen extenders containing coconut water alone or combined with egg yolk on viability of spermatozoa of semen obtained from WAD during cryopreservation.

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Materials and Methods

Experimental site and animals

The study was carried out at the Goat Unit of Teaching and Research Farm, Federal University of Agriculture Abeokuta, which falls within $7^0$10'N and $3^0$2'E and altitude 76 m above sea level. It lies in the South-Western part of Nigeria with a prevailing tropical climate, a mean annual rainfall of 1,037 mm and average temperature of 34.7°C. Twenty WAD bucks 2.5-3 years of were used for this study. The animals were kept under intensive management and maintained under a uniform and constant nutritional regime with concentrate feed (maize (10%), groundnut cake (3%), palm kernel cake (35%), wheat offals (50%), bone meal (2%), salt (0.2%) and premix (0.1%) supplemented with guinea grass (*Panicum maximum*).

Semen collection, dilution and cryopreservation

Semen samples were collected from six WAD bucks with the aid of an artificial vagina. A total of six semen samples (each semen sample originating from six bucks) with a minimum of 80% motility (Eriksson and Rodriguez-Martinez, 2000) were pooled to minimize individual differences (Bucak and Tekin, 2007). The pooled semen samples were diluted sequentially at the same (32°C) in a two-step process with a Tris-based extender composed of 2 fractions in 2 studies. In study I, the Fraction 1 solution contained Tris-extender consisting of Tris-hydroxymethyl-aminomethane (2.42 g), citric acid (1.36 g), glucose (1 g), penicillin (0.028 g), and distilled water made up 100 ml. Fraction 2 solution had the same composition as the Fraction 1 solution with the addition of 14.0% glycerol (v/v). Each pooled ejaculate was split into 5 equal aliquots, diluted with the Fraction 1 solution plus addition each of sodium citrate (2.9%) as control, 5, 10, 15 and 20 ml/100 ml coconut (*Cocos nucifera*) water respectively. Fraction 2 solution was subsequently added at a 1:1 ratio and the concentration of 1,435 × 10^6 sperm/ml was then determined using Photometer SDM1 (Minitud GmbH, Germany). In study II, the Fraction 1 and 2 solutions had the same composition as in study I. Each pooled ejaculate was split into 4 equal aliquots and diluted with the Fraction 1 solution plus addition each of sodium citrate (2.9%) as control, egg yolk and coconut water (EYCW) at 5:10, 10:10 and 10:5. Fraction 2 solution was subsequently added at a 1:1 ratio and the concentration of 1,658 was then determined for sperm/ml. In studies I and II, diluted semen samples were then loaded into 2 ml plastic straws (Gold Plus, Nigeria), sealed with polyvinyl, cooled to 4°C at a rate of 0.25°C/min and equilibrated at 4°C for 10 min in TYFSF Refrigerated Incubator (Model:SPX-7OB III, Hebei China). Subsequently, the straws were then placed in a rack at 4cm above liquid nitrogen in the vaporous phase for 10 min before plunging them directly and quickly into liquid nitrogen for 30 days and thereafter evaluating for sperm quality characteristics.

Progressive sperm motility

Microscopic assessment of progressive sperm motility was carried out as described by Bearden and Fuquay (1997). Briefly, cryopreserved semen samples were thawed in Clifton Water bath (Model: 74178 by Nickel Electro Ltd, Weston-S-Mare Somerset, England) at 37°C for 2 min and accessed for sperm motility using Celestron PentaView microscope (LCD-44348 by RoHS, China) at 400X magnification. A semen mount was made using 5 µl semen and the semen was placed directly on a microscope slide and covered with a cover slip. A total of 50 spermatozoa were assessed in at least five microscopic fields for progressive sperm motility and the mean of the five successive evaluations was recorded as the final motility score.

Acrosome integrity

The percentage of spermatozoa with intact acrosome was determined according to Ahmad *et al*. (2003) with some modifications. Briefly, 50 µl of each semen sample was added to a 500 µl formalin citrate solution (96 ml 2.9% sodium citrate, with 4 ml 37% formaldehyde) and mixed carefully. A small drop of the mixture was placed on a microscope slide and a total of 200 spermatozoa were counted in at least three different microscopic fields for each sample, using Celestron PentaView LCD microscope (400X magnification). Intactness of acrosome characterized by normal apical ridge of spermatozoa was examined and recorded.

Sperm membrane integrity

Hypo-osmotic swelling test (HOST) assay as described earlier (Jeyendran *et al*., 1984) was used to determine sperm membrane integrity and this was done by incubating 10 µl semen in 100 µl Hypo-osmotic solution (fructose and sodium citrate) at 37°C for 30 min, 0.1 ml of the mixture was spread over a warmed slide, covered with a cover slip and observed under Celestron PentaView LCD digital microscope (400X magnification). Two hundred spermatozoa (200) were counted for their swelling characterized by coiled tail, indicating intact plasma membrane.

Sperm abnormality

Sperm abnormality was evaluated as described by Bearden and Fuquay (1997) with the use of eosin-nigrosin smears. A thin smear of mixture of semen and eosin-nigrosin solution was drawn across the slide and dried. Abnormality of sperm cells located in the head, midpiece and tail were observed under Celestron PentaView LCD microscope (400X magnification).

In vitro acrosome reaction

Following cryopreservation, spermatozoa were thawed by plunging straws into a water bath (37°C) for 1 min and the proportion of acrosome reaction was determined as described by Tardif *et al*. (1999) with some

modifications as follows: samples of cryopreserved spermatozoa were washed with phosphate-buffered saline (PBS) that consisted of 8 g of NaCl, 0.2 g KCl, 1.44 g of Na_2HPO_4, 0.25 g of KH_2PO_4, and the pellets were re-suspended in culture medium (Calcium chloride dihydrate 265 mg/L, Magnesium chloride anhydrous 46 mg/L, Potassium chloride 200 mg/L, Sodium chloride 8000 mg/L, Sodium dihydrogen phosphate anhydrous 50 mg/L, D-Glucose 1000 mg/L). Immediately after the inclusion of 0.9% wt/vol PBS (15 µg/ml), the acrosome reaction was induced by incubating spermatozoa for 20 min with progesterone (2.5 mg/ml) at 38.5°C (5% CO_2 in air; 100% humidity). To determine the proportion of spontaneous acrosome reaction, progesterone was omitted but an equal volume of PBS was added. Spermatozoa were observed in an upright Carl Zeiss Fluorescent Microscope (Primo Star, Germany) equipped with phase contrast and epifluorescence optics, and 100 cells were counted per slide (1000X magnification). Spermatozoa with intense fluorescence over the acrosome were classified as acrosome intact (acrosome that did not react) and those with no fluorescence or a dull fluorescence along the equatorial segment as acrosome reacted.

In vitro capacitation

In vitro capacitation of the spermatozoa was evaluated using the Chlortetracycline (CTC) fluorescence assay as described by Collin *et al*. (2000). In brief, CTC (750 µm*)* was prepared in 20 mM Tris buffer containing 130 mM NaCl and 5 mM DL-cysteine (final pH 7.8). Sperm suspension (5 µl) was mixed with 5 µl of CTC solution on a warmed slide (37°C). After 30 sec, 5 µl of 0.2% glutaraldehyde in 0.5 M Tris pH 7.4 was added. Finally, 5 µl of 90% glycerol and 10% PBS (pH was adjusted to 8.6) were added to retard fluorescence fading. After adding a coverslip, the slide was examined with an upright Carl Zeiss Fluorescent Microscope (Primo Star, Germany) equipped with phase contrast and epifluorescence optics, and 100 cells were counted per slide (1000X magnification). The proportion of cryopreserved spermatozoa that exhibited pattern B according to the CTC assay was determined. Spermatozoa characterized by bright anterior head and faint fluorescence in the post-acrosomal region were classified as capacitated spermatozoa while non capacitated sperm had bright uniform fluorescence over the head.

Statistical analysis

Estimations were performed for the diluted semen samples for each treatment in repeated measurements (each measurement was repeated ten times for motility and eight times for acrosome integrity, membrane integrity, abnormality, acrosome reaction and capacitation). Data obtained were subjected to analysis of variance (ANOVA) using SAS 1999 package and results expressed as the means ± SEM. Duncan multiple range test (Duncan, 1955) was used to separate significantly different means ($P < 0.05$). The model used is shown below:

$Y_{ij} = \mu + L_{i +} \sum_{ij}$

Where,

Y_{ijk}= Dependent variables

μ = Population mean

L_i = effect due to i^{th} level of coconut water inclusion, j = (0, 5, 10, 15, 20)

$\sum_{ij}$= experimental error

Results

Spermatozoa viability of buck semen cryopreserved with coconut water

Qualitative parameters of buck semen cryopreserved with different levels of coconut water extenders are presented in Table 1. The results showed that motility, acrosome integrity, membrane integrity, acrosome reaction and capacitation were higher ($P < 0.05$) in coconut water extenders compared to the control and improvement in this parameter was consistently better at 10% coconut water. Percentage abnormality observed was however comparable among the treatments. The results (Table 1) showed that the percentage of cryopreserved spermatozoa with coconut water that underwent acrosome reaction and capacitation followed a similar pattern.

Spermatozoa viability of buck semen cryopreserved with different combinations of coconut water and egg yolk

Sperm viability parameters of buck semen cryopreserved with different combinations of coconut water and egg yolk are presented in Table 2. The results showed that motility was higher ($P < 0.05$) in coconut water and egg yolk extenders at different combinations compared to the control and the improvement in motility was more pronounced in EYCW5:10 and EYCW10: 10 extenders compared to EYCW10:5. Similarly, membrane integrity was higher ($P < 0.05$) in coconut water and egg yolk extenders at different combinations compared to the control and the improvement in membrane integrity was more pronounced in EYCW5:10 compared to other extenders. The results showed that percentage abnormality was however comparable among the treatment groups. In addition, the results showed that spermatozoa cryopreserved with coconut water and egg yolk extenders at different combinations had higher ($P < 0.05$) percentage of acrosome reaction and capacitation compared to the control.

Table 1. Mean (±SEM) spermatozoa viability of WAD buck semen cryopreserved with coconut (*Cocos nucifera*) water extender.

Parameters	Control	5%	10%	15%	20%
Motility (%)	24.5 ± 5.63^{d}	27.75 ± 6.08^{c}	35.25 ± 4.57^{a}	29.0 ± 6.07^{bc}	31.25 ± 5.47^{b}
Acrosome integrity (%)	76.0 ± 1.41^{c}	86.0 ± 2.58^{ab}	89.0 ± 1.00^{a}	84.0 ± 2.83^{ab}	90.5 ± 1.50^{a}
Membrane integrity (%)	64.0 ± 2.31^{c}	83.0 ± 2.08^{ab}	80.0 ± 1.63^{ab}	88.0 ± 1.63^{a}	76.0 ± 0.00^{b}
Abnormality (%)	1.3 ± 0.00	0.83 ± 0.22	1.2 ± 0.17	0.9 ± 0.08	1.0 ± 0.00
In vitro capacitation (%)	45.0 ± 1.91^{c}	56.0 ± 5.42^{ab}	64.0 ± 7.48^{a}	52.0 ± 2.31^{ab}	58.0 ± 3.83^{ab}
In vitro acrosome reaction (%)	39.0 ± 7.72^{c}	63.0 ± 11.12^{ab}	67.0 ± 5.00^{a}	57.0 ± 5.00^{ab}	58.0 ± 3.46^{ab}

[a,b,c ,d]Values within rows with different superscripts differ significantly ($P < 0.05$).

Table 2. Mean (±SEM) spermatozoa viability of WAD buck semen cryopreserved with different combinations of coconut (*Cocos nucifera*) water and egg yolk.

Parameters	Control	EYCW5:10	EYCW10:10	EYCW10:5
Motility (%)	24.5 ± 5.63^{c}	39.8 ± 4.16^{a}	39.8 ± 4.16^{a}	28.8 ± 2.69^{b}
Acrosome Integrity (%)	76.0 ± 1.41^{b}	79.0 ± 0.58^{a}	77.5 ± 1.26^{ab}	69.5 ± 1.50^{c}
Membrane Integrity (%)	64.0 ± 2.31^{c}	84.0 ± 1.41^{a}	73.0 ± 1.73^{b}	76.0 ± 1.41^{b}
Abnormality (%)	1.3 ± 0.00	1.25 ± 0.144	1.17 ± 0.167	0.92 ± 0.083
In vitro Capacitation (%)	45.0 ± 1.91^{c}	60.0 ± 5.89^{a}	56.0 ± 3.65^{ab}	60.0 ± 4.32^{a}
In vitro Acrosome Reaction (%)	39.0 ± 7.72^{c}	55.0 ± 5.26^{a}	52.0 ± 2.83^{a}	56.0 ± 6.32^{a}

[a,b,c]Values within rows with different superscripts differ significantly ($P < 0.05$).

Discussion

The findings indicated that inclusion of coconut water in semen extender improved the quality of cryopreserved spermatozoa of WAD bucks. The present results demonstrated that coconut water based extender provided a more adequate medium to sustain the viability of sperm cryopreserved as evidenced from its ability to maintain the sperm viability parameters better than the control. The improvement observed on sperm viability indicated that coconut water extenders contained essential constituents such as sugars, vitamins, minerals and amino acids (Yong *et al*., 2009; USDA National Nutrient Database, 2015) required for cryosurvival of spermatozoa.

The role of sugar as a source of energy, an osmolyte and a cryoprotectant for sperm survival following cryopreservation is supported in literature (Yancey, 2005; Purdy, 2006; Naing *et al*., 2010). Sugar is known to increase the osmostic potential of cells and protect the membrane from chilling-induced injury (Purdy, 2006). Goat sperm readily utilizes sugars for respiration, and these sugars also provide osmotic balance and cryoprotection (Koshimoto and Mazur, 2002; Aboagla and Terada, 2003).

In addition, the protective effect of coconut water could also be linked to its major essential amino acids which play an important role in cell membrane integrity (Sakanaba *et al*., 2004; Yong *et al*., 2009). The cryoprotective potential of amino acids in mammalian spermatozoa during freezing stemmed from their ability to form a layer on the spermatozoa surface, and positively charged molecules combined with the phosphate groups of sperm plasma membrane phospholipids has been observed (Kundu *et al*., 2001; Atessahin *et al*., 2008).

Furthermore, the improved sperm viability with coconut water indicated the ability of coconut water extenders to efficiently harness potassium contained in coconut water and spermatozoa for survival of the spermatozoa during cryopreservation. Coconut water is rich in potassium (Yong *et al*., 2009; USDA National Nutrient Database, 2015). Sperm viability is influenced by the potassium levels in storage medium and the beneficial effect of potassium on the viability of diluted spermatozoa has been demonstrated (Mansour *et al*., 2002).

Moreover, the improvement in sperm parameters observed in the present study could be linked to essential antioxidants in coconut water. In line with previous studies (Kannan and Jain, 2004; Arabi and Seidaie, 2008), low toxicity and good water solubility of antioxidants such as pyridoxine and vitamin C derived from the addition of coconut water could be attributed to the improved viability parameters in the present study. The antioxidant potential and amelioration of oxidative stress with pyridoxine and vitamin C supplementation have been reported (Shen *et al.,* 2010; Daramola e Adekunle, 2015; Daramola *et al*., 2016).

These myriads of compounds confer coconut water with the distinct biological properties necessary for protection against oxidative damage due to increased production of reactive oxygen species (or free radicals) associated with *in vitro* storage at low temperatures, especially the polyunsaturated fatty acids in the cell membrane, or to the nucleic acids in the cell nucleus (Evans and Halliwell, 2001; Peruma *et al*., 2011).

In conclusion, the improvement in motility, acrosome reacted spermatozoa as well as percentage of sperm capacitation was best at 10% coconut water. Spermatozoa cryopreserved with coconut water and egg yolk extenders at different combinations also indicated better improved motility in EYCW5:10 and EYCW10:10 extenders while acrosome integrity and membrane integrity were better improved in EYCW5:10 extenders. In all, the results revealed that 10% coconut water and EYCW5:10 gave better improvement of sperm viability parameters. The study therefore indicated the protective effects of coconut water on the

viability of cryopreserved spermatozoa of WAD bucks and its possible utilization as semen extender in cryopreservation and could be a cheap alternative for use in artificial insemination programs for this breed.

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