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Anti-oxidative influence of butylated hydroxytoluene on chilled semen of Red Sokoto bucks and consequential conception rates in does

TK Bello[1]*, PI Rekwot[1], JO Ayo[2], AM Khumran[3] & BB Oyelowo[4]

1. *National Animal Production Research Institute, Shika, Ahmadu Bello University, Zaria, Nigeria*
2. *Department of Veterinary Physiology, Ahmadu Bello University, Zaria, Nigeria*
3. *Department of Theriogenology and Production, Ahmadu Bello University, Zaria, Nigeria*
4. *Department of Veterinary Pharmacology and Toxicology, Ahmadu Bello University, Zaria, Nigeria*

*Correspondence: Tel.: +2347030232267; E-mail: taiwokamarbello@gmail.com

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Abstract

The anti-oxidative influence of Butylated Hydroxytoluene (BHT) in three extenders on chilled semen of red Sokoto bucks and conception rates in does was evaluated. Twenty matured red Sokoto bucks were selected for this study. Two ejaculates were collected from each buck per week for a period of 4 weeks. Semen was collected from bucks using a battery-operated electro-ejaculator for semen analyses. Volume, colour, gross motility, concentration and percentage live spermatozoa were examined. A total of 10 ejaculates were further collected weekly from 10 selected bucks from the previous twenty good semen quality for 8 weeks. Semen was extended in tris egg-yolk (TEY), citrate egg-yolk (CEY) and tris-coconut water (TCW) extenders in test tubes, containing BHT antioxidant to obtain 0 (control), 0.5, 1.0, 1.5 and 2.0 mM/mL BHT. Sperm characteristics were determined at 0, 24, 48 and 72 hours of storage. Twenty multiparous does divided into 3 groups (A, B and C), of 7, 7 and 6 does respectively. These does received 250 µg cloprostenol intramuscularly, twice, 11 days apart and insemination was conducted 48 hours after. Groups A, B and C were inseminated with semen stored for 72 hours containing 1.0 mM/mL of BHT extended in TEY, 1.0 mM/mL of BHT extended in CEY and 0.5 mM/mL of BHT extended in TCW respectively. Ultrasonography was used for pregnancy diagnoses 47 days after artificial insemination. From the findings of this study, it was concluded that semen of red Sokoto bucks stored in tris egg yolk + 1.0 mM/mL BHT and chilled for 72 hours had better semen quality and conception rates.

Keywords: Butylated hydroxytoluene, Semen, Antioxidant, Extenders, Red Sokoto goats

Introduction

The population of goats in Nigeria is 78 million (FAO, 2017), with majority of these concentrated in the northern parts of Nigeria. Common breeds of indigenous breeds include; the Sokoto Red/Maradi,

West African Dwarf (WAD) goat and Sahel/desert goat- also known as West African Long-Legged goat (Fabusoro *et al*., 2007).

There is a high demand for goat and its product, it is therefore necessary to increase the number and productivity, which can be achieved through genetic improvement. Genetic improvement of goats requires the selection of superior breeding stock and the application of semen preservation and insemination techniques (Bitto & Egbunike, 2006).

In terms of genetic improvement of animals, artificial insemination is an integral process being utilized. Artificial insemination can only be possible after semen collection, semen dilution and preservation by chilling or freezing method. It has been reported that these processes cause a decline in semen quality (Allai *et al*., 2018). Preparation of suitable semen extender is an important aspect in handling and storage of semen (Purdy, 2006; Mara *et al*., 2007). The negative interactions involving phospholipids of milk and egg-yolk extenders with the bulbourethral gland secretions in semen of bucks makes the extension and preservation different from other domestic species (Purdy, 2006). This results in coagulation of sperm cells leading to death of these cells sequel to activities of egg-yolk coagulating enzyme (EYCE), which is secreted by the bulbo-urethral gland (Sen *et al*., 2015). Another protein called BUSgp60 (a 55-60-kDa monomeric glycoprotein) which is also secreted by the bulbo-urethral gland reduces vitality and motility of cooled and frozen sperm extended in milk-based extenders. Natural antioxidants present in semen protects against oxidants and its damage on spermatozoa (Saraswat *et al*., 2016). Semen dilution, however, decreases the concentrations of these natural antioxidants thereby exposing the sperm cells to increased production of reactive oxygen species (ROS) during storage causing degradation of sperm quality and fertilizing ability (Marzony *et al*., 2016). The addition of antioxidants to the semen extender seems to have the potential to augment the decrease in natural antioxidants and mitigate the negative impact of oxidative stress, as antioxidants capture free radicals and conclude the chain reaction, maintaining a redox state (Arbulu *et al*., 2021).

Butylated hydroxytoluene (BHT) is a synthetic antioxidant and an analogue of vitamin E (Patel *et al*., 2015) that has the ability to inhibit auto-oxidation reaction. Supplementation of semen extender with BHT inhibits lipid peroxidation reactions, improves sperm quality and reduces lipid peroxidation in different species (Alcay *et al*., 2016). Optimum protective levels of BHT have not been established when supplemented to semen extenders during chilling of semen from Red Sokoto bucks.

Materials and Methods

Bucks and management

Twenty (20) healthy and sexually matured Red Sokoto bucks from Small Ruminant Research Programme of National Animal Production Research Institute weighing 27.5 ± 2.5 kg and aged 12.25 ± 0.45 – 27.05 ± 1.60 months were selected for the study. The ages of the bucks were obtained from farm records. The bucks were screened for diseases and internal parasites before inclusion in to the study. All bucks were maintained under uniform management conditions using semi-intensive system of management in which they were fed with hay *ad libitum* and supplemented with commercial small ruminant concentrate at a rate of 2%/head/day.

Semen collection

Two ejaculates were collected from each buck per week for a period of four weeks giving a total of 160 ejaculates. Semen samples were collected from bucks by use of an electroejaculator (Lane Manufacturing Inc. No. 72707C). In carrying this out, the animal was adequately restrained before semen samples were collected. The probe was lubricated with petroleum jelly for easy insertion into the rectum and pushed forward slowly. A series of short electrical stimulations (10 volts) were applied intermittently for approximately 25 seconds by push of a button until erection and ejaculation are achieved. Ejaculates were placed in a waterbath at 37 ℃ before microscopic evaluations were conducted.

Semen evaluation

The gross semen characteristics that were examined included volume, colour and motility as described by Maina *et al*. (2006).

Volume and colour

The semen was collected and read from the graduated collecting tubes immediately. Semen colour was observed immediately after collection and recorded as Watery (1), Milky (2), or Yellowish/ Creamy (3).

Gross motility

Gross motility was determined by examination for wave pattern of the sperm cells movement. This was done by placing a drop of raw undiluted semen on a pre-warmed glass slide, which was cover-slipped and viewed for motility using a microscope at x 10 objective magnification. Motility was graded in percentages (Table 1).

Table 1: Grading of sperm motility

Estimated Motility	Nature of Movement
0%	No sperm movement
0-20%	Head movement only with no forward sperm progression
20-50%	Slow forward sperm progression
50-70%	Fast forward sperm progression
70-90%	Fast forward sperm progression
Above 90%	Fast wavy motion

Mandal *et al*. (2014) modified

Sperm concentration

This was determined using the haemocytometer. Semen was sucked into the red blood cell-diluting pipette up to 0.1 mark, and the volume made up to 101 mark with 10% formal saline and mixed thoroughly. A drop of the mixture was allowed to spread under the cover-slip, placed tightly on the haemocytometer after discarding few drops. The cells were allowed to settle before counting under x 40 magnification. Sperm cells were counted diagonally from top left to right bottom in 5 small squares of the improved Neubauer haemocytometer. Sperm concentration of individual buck was calculated as described by Verstegen *et al*. (2002).

Live spermatozoa

This was determined as described by Esteso *et al*. (2006). A thin smear of the semen sample was made on clean, grease-free glass slides and stained with Eosin-Nigrosin stain. The dead sperm cells were identified as those that absorbed the stain, while the live cells were those that did not absorb the stain. At least 200 sperm cells (from an average of 4 microscopic fields) were counted in a regular sequence using light microscope at x 40 magnification.

Sperm abnormalities

These were determined according to the method of Esteso *et al*. (2006). A thin smear of the semen samples were made on clean grease-free glass slides and fixed with buffered formal saline. The preparation was examined and abnormal sperm cells counted in a regular sequence using light microscope at x 100 magnification with oil immersion. A total of 400 well-spaced spermatozoa were carefully examined in each preparation and the percentage of head, mid-piece and tail sperm abnormalities were determined.

Semen collection and preparation of extenders

Ten bucks with good semen parameters were selected from the first study and a total of 10 ejaculates were collected once weekly from the 10 bucks using electro-ejaculator for a period of 8 weeks. The samples were conveyed to the laboratory in a thermoflask containing warm water kept at 37 ℃. To reconstitute the extenders, buffers were first prepared. The tris (hydroxymethyl amino-methane) and citrate (g/100 mL in distilled water) buffers were prepared to be used for tris egg-yolk (TEY), citrate egg-yolk (CEY) and triscoconut water (TCW) extender respectively. These extenders compositions are presented in Table 2. 100 mL of all extenders were prepared and placed in a beaker of warmed water (37 ^{o}C) and transported to the semen collection site. Coconut water (CW) was prepared on the day of experiment from coconut fruits. The coconut water collected from coconut fruits was first filtered through a sieve. Finally, it was centrifuged for 5 minutes at 3000 rpm and the supernatant from each tube was obtained carefully in a sterile glass bottle (Baldaniya *et al*., 2019).

Considering butylated hydroxytoluene molarity = 220, therefore 3.4 g of BHT white crystals were dissolved in 15.6 mL of absolute ethanol (99%) to make 1 molar solution. This solution was kept as stock which was then used subsequently to obtain desired concentration for every treatment. For more elaboration, 15.60 µL of the stock already contained 1 mM of BHT. This was dissolved in 2 mL of diluted semen sample to obtain 0.5 mM/mL of BHT concentration. However, the stock solution and diluted sample were not directly mixed together because the ethanol contained in the stock solution was detrimental to spermatozoa. Therefore, desired quantity of the stock was put into a clean empty cryovials first, left opened at room temperature (25 – 26 ℃) to allow the ethanol evaporate leaving the

dried BHT crystals to attach to the cryovials before 2 mL of diluted semen sample was added to the test tube, which was adequate to make up the desired concentration.

Semen samples were diluted with all extenders: tris-egg yolk, tris coconut water and citrate egg-yolk extenders in pre-warmed test tubes, containing BHT antioxidant to obtain 0 (control), 0.5, 1.0, 1.5 and 2.0 mM/mL BHT concentrations. The samples were packed into labeled Bijou bottles, placed in a padded flask and then transferred into a refrigerator at 4 ℃ for storage.

Semen evaluation

Sperm motility, morphological abnormalities and viability (% live) were measured after dilution at 0, 24, 48 and 72 hours of storage as previously described.

The results from the semen analyses provided evidence that semen containing 1.0 mM/mL of BHT extended in Tris-egg yolk, 1 mM/mL of BHT in Citrate egg yolk-extender and 0.5 mM/mL of BHT in Tris-coconut extender provided better preservative quality than other groups after chilling for 72 hours. These groups were then used for artificial insemination.

Estrus synchronisation and artificial insemination

Twenty (20) healthy does that were confirmed to have kidded before from Small Ruminant Research Programme of National Animal Production Research Institute were randomly divided into 3 groups (A, B and C), consisting of 7,7 and 6 does, respectively.

The does received 250 mcg cloprostenol each intramuscularly twice at 11 days apart. Observation for estrus commenced 72 hours after the last dose of cloprostenol and insemination was conducted on this day. Does in group A were inseminated using the transcervical method with semen containing 1.0 mM/mL of BHT extended in Tris-egg yolk, while does in group B were inseminated with semen containing 1 mM/mL of BHT extended in Citrate egg yolk-extender. Does in group C were inseminated with semen containing 0.5 mM/mL of BHT extended in Tris-coconut extender. These BHT concentrations conferred best storage qualities on sperm cells, hence their selection for insemination. All semen samples used were chilled at 4 ^{o}C for 72 hours before insemination. Semen cryo-bottles were warmed using warm water to a temperature of 37 ^{o}C before insemination was performed.

Pregnancy diagnosis using ultrasonography

A real time B-mode ultrasound scanner equipped with a 5 MHz sector probe (BMV technologies) was used for scanning 47 days after artificial insemination. Transabdominal scanning was performed after shaving the inguinal region, in front and on either side of udder of the doe. The doe was lightly restrained by one person in dorsal recumbent position. An ultrasound coupling gel was applied each time to the probe to develop good contact and to remove air between probe and animal skin. The feature determined was gestational sac. Gestational sac appeared as circular and elongated anechoic areas located cranial to the bladder at Day 30 after breeding. This was determined using the method described by Bretzlaff *et al*. (1993).

Data analyses

Data collected were expressed as means and standard deviation (± SD). Data were subjected to repeated measure analysis of variance (ANOVA). Significance of differences between treatment means was estimated using Tukey-Kramer multiple comparison test. Statistical analysis was conducted using the Graphpad Instat computer programme (Graphpad for Windows, Inc., version 8.0.2, 2019).

Results

The mean volume of semen recorded in this study ranged from 0.20 ± 0.00 to 1.83 ± 0.00 mL (Table 2). Mean semen volume above 1 mL was observed in three bucks (Table 3). Mean gross sperm motility observed in this study ranged from 36.3 ± 5.2 to 91.7 ± 1.6%. The highest sperm concentration observed in this study was 224.7 ± 17.9 x 10^6/mL, while the least was 55.0 ± 10.1 x10^6/mL. The mean values for colour ranged from 2.0 to 3.0. Seventeen of the 20 bucks had mean semen colour of 2, while 3 bucks had mean semen colour of 3 (Table 3). The age range of bucks in this study ranges from 12.25 ± 0.45 – 27.05 ± 1.60 months (Table 4). The weights also range from 20.80 ± 0.90 – 28.20 ± 2.10 kg. Semen volume was highest (1.03 ± 0.18 mL) in the oldest group of bucks. There was significant difference ($P < 0.05$) in volume observed in this group of bucks than other groups. There is no significant difference recorded for other age group of bucks in terms of general motility, sperm concentration colour and percentage live sperm cells (Table 4).

Mean gross motility decreased across all groups from 0 hours to 72 hours (Table 5). At 72 hours of chilling,

Table 2: Compositions of Tris, Citrate egg yolk and Tris coconut extender Components (in 100 mL)

Components (in 100 mL)	TEY	CEY	TCW
Tris (hydroxymethyl amino-methane) (g/100 mL)	2.9	-	2.9
Sodium citrate, dehydrate (g/100 mL)	-	2.9	-
Citric acid monohydrate (g/100 mL)	1.7	-	1.7
Fructose (g/100 mL)	1.25	-	1.25
Egg yolk (% v/v) mL	20	20	-
Coconut water (% v/v) mL	-	-	20
Penicillin 10,000 units, streptomycin 10 µg /mL	1000	1000	1000

Key: TEY- Tris egg-yolk; CEY- Citrate egg-yolk; TCW- Tris coconut-water

Table 3: Spermiogram of Red Sokoto Bucks

Buck Number	Semen Volume (ml)	Semen Colour	Sperm Motility (%)	Sperm Concentration (x 10^6/ml)	(%) Live
01	0.50 ± 0.28^{a}	2.0 ± 0.00^{a}	71.7 ± 12.4^{a}	137.3 ± 18.9^{b}	80.00 ± 3.50
02	0.80 ± 0.28^{b}	2.3 ± 0.71^{a}	86.7 ± 4.8^{a}	164.7 ± 29.2^{b}	92.50 ± 4.38
03	1.00 ± 0.60^{b}	2.0 ± 0.00^{a}	80.0 ± 16.4^{a}	150.3 ± 53.1^{b}	90.00 ± 4.72
04	0.40 ± 0.28^{a}	2.0 ± 0.00^{a}	78.3 ± 4.8^{a}	67.0 ± 24.0^{a}	87.50 ± 6.78
05	0.30 ± 0.28^{a}	2.0 ± 0.00^{a}	80.0 ± 24.6^{a}	153.7 ± 54.4^{b}	92.00 ± 6.27
06	0.60 ± 0.33^{ab}	2.0 ± 0.00^{a}	76.7 ± 18.9^{a}	84.3 ± 44.4^{a}	95.00 ± 6.92
07	0.20 ± 0.00^{a}	2.0 ± 0.00^{a}	43.3 ± 14.7^{a}	55.0 ± 10.1^{a}	80.50 ± 7.12
08	0.57 ± 0.18^{a}	2.0 ± 0.71^{a}	36.3 ± 14.7^{b}	76.7 ± 28.5^{a}	82.50 ± 4.97
09	1.83 ± 0.28^{c}	2.0 ± 0.00^{a}	73.3 ± 9.3^{b}	108.7 ± 50.1^{b}	95.00 ± 3.76
10	0.80 ± 0.38^{b}	2.0 ± 0.00^{a}	81.7 ± 20.6^{a}	160.7 ± 15.5^{b}	90.00 ± 5.76
11	1.10 ± 0.10^{b}	2.0 ± 0.00^{a}	78.0 ± 12.4^{a}	66.7 ± 8.20^{a}	82.50 ± 2.14
12	0.60 ± 0.28^{a}	2.0 ± 0.00^{a}	75.0 ± 14.1^{a}	165 ± 17.5^{c}	85.75 ± 2.96
13	0.53 ± 0.41^{a}	2.5 ± 0.82^{ab}	86.7 ± 9.3^{a}	130.7 ± 35.8^{b}	90.00 ± 3.64
14	1.07 ± 0.33^{b}	2.5 ± 0.82^{ab}	81.7 ± 20.64^{a}	162.7 ± 26.4^{b}	89.25 ± 8.06
15	0.27 ± 0.09^{a}	2.5 ± 0.82^{ab}	88.3 ± 12.4^{a}	169.3 ± 45.3^{b}	80.25 ± 6.90
16	0.47 ± 0.20^{a}	2.5 ± 0.82^{ab}	88.3 ± 12.4^{a}	161.3 ± 46.3^{b}	85.00 ± 9.89
17	0.60 ± 0.19^{ab}	2.8 ± 0.70^{ab}	85.0 ± 12.4^{a}	153 ± 33.3^{b}	85.00 ± 5.96
18	0.53 ± 0.88^{a}	3.0 ± 0.00^{b}	85.0 ± 21.4^{a}	198 ± 87.0^{b}	88.75 ± 6.20
19	0.47 ± 0.10^{a}	3.0 ± 0.00^{b}	80.0 ± 16.4^{a}	224.7 ± 50.6^{c}	95.00 ± 3.81
20	0.43 ± 0.14^{a}	3.0 ± 0.00^{b}	91.7 ± 4.5^{a}	210.3 ± 60.2^{c}	90.00 ± 5.34

^{abc} Different superscript within columns denotes significant differences (P< 0.05). Data expressed as Means ± SD, n=160

1.0 mM/mL BHT group hard the highest mean gross motility of 42.5 ± 4.9%. This value was significantly (P < 0.05) higher than values obtained for all other groups. The 2.0 mM/mL BHT group had a main gross motility of 12.50 ± 2.5% (Table 5). This value was significantly (P < 0.05) lower than values obtained for other groups. Live sperm ratio also decreased as BHT group (Table 5). At 72 hours of storage, the control group had a mean motility of 16.00 ± 2.30%, storage time increased across all groups (Table 5). The 1.5 mM/mL BHT group had the lowest live ratio (30 ± 5.8%), which was significantly (P < 0.05) lower than values obtained for all other groups. At 24 hours of storage, control group had a significantly (P < 0.05) lower sperm abnormalities (11.75 ± 1.3%) than the 2.0 mM/mL

which was significantly (P < 0.05) lower that what was obtained for all other groups except 0.5mM/mL BHT

Table 4: Age dependent spermiogram of Red Sokoto bucks

Age (months)	Weight (kg)	Scrotal Circumference (cm)	Volume (mL)	Motility (%)	Concentration ($x10^6$/mL)	Colour	% Live
12.25 ± 0.45^a	20.80 ± 0.90^a	21.90 ± 1.35^a	0.36 ± 0.1^a	77.24 ± 14.2	148.7 ± 69.8	2.4 ± 0.4	87.18 ± 6.0
20.25 ± 2.4^b	24.10 ± 1.20^b	26.20 ± 1.60^b	0.60 ± 0.08^b	73.73 ± 15.9	124.0 ± 36.8	2.16 ± 0.3	87.91 ± 5.5
27.05 ± 1.60^c	28.2 ± 2.10^b	26.80 ± 1.80^b	1.03 ± 0.5^c	77.36 ± 4.9	119.9 ± 68.7	2.17 ± 0.3	89.85 ± 5.9

^{abc} Different superscript within columns denotes significant differences ($P < 0.05$)
Data expressed as Means ± SD

Table 5: Effect of BHT on chilled semen parameters of Red Sokoto bucks stored in tris egg-yolk extender for 3 days duration

Parameter	Hours	BHT concentrations (mM/ML)				
		Control (0.0)	0.5	1.0	1.5	2.0
General Sperm Motility (%)	0	83.00 ± 6.7^w	83.00 ± 6.7^w	83.00 ± 6.7^w	83.00 ± 6.7^w	83.00 ± 6.7^w
	24	55.00 ± 8.2^x	62.50 ± 7.0^x	67.50 ± 7.0^x	55.25 ± 8.2^x	40.00 ± 5.6^x
	48	40.00 ± 0.0^y	40.00 ± 11.0^y	42.50 ± 13.8^y	55.15 ± 18.3^x	27.50 ± 5.5^y
	72	$25.00 \pm 6.1^{a,z}$	$25.00 \pm 7.9^{a,z}$	$42.55 \pm 13.8^{b,y}$	$30.70 \pm 11.5^{a,y}$	$12.50 \pm 7.0^{c,z}$
(%) Live Spermatozoa	0	90.00 ± 6.2^w	90.00 ± 6.2^w	90.00 ± 6.2^w	90.00 ± 6.2^w	90.00 ± 6.2^w
	24	75.00 ± 9.3^x	75.00 ± 8.2^x	76.00 ± 12.4^x	73.00 ± 6.5^x	71.00 ± 4.8^x
	48	70.00 ± 9.3^x	71.60 ± 4.8^y	66.00 ± 3.6^y	60.00 ± 7.3^y	65.00 ± 14.1^{xy}
	72	$55.00 \pm 7.9^{b,y}$	$58.30 \pm 12.4^{b,z}$	$60.00 \pm 8.8^{b,y}$	$30.00 \pm 16.4^{a,z}$	53.00 ± 7.5^y
Sperm Abnormalities (%)	0	8.20 ± 6.2	8.20 ± 6.2	8.20 ± 6.2	8.20 ± 6.2	8.20 ± 6.2
	24	11.75 ± 3.6^a	12.25 ± 4.2^{ab}	12.75 ± 7.9^{ab}	14.50 ± 5.9^{ab}	15.25 ± 1.4^b
	48	13.50 ± 7.9	15.75 ± 2.8	19.50 ± 6.7	22.00 ± 3.9	22.75 ± 7.0
	72	15.97 ± 8.7	15.98 ± 6.7	19.76 ± 1.9	23.86 ± 9.8	24.39 ± 5.0

^{abc} Different superscript between rows and ^{wxyz} within columns denote differences ($P < 0.05$).
Data expressed as Means ± SD n= 80

group (Table 5). At 24 hours of storage, mean general sperm motility for 0.5mM/mL BHT group (65.50 ± 5.0%) was significantly ($P < 0.05$) higher than 45.70 ± 5.5%, which was recorded for 2.0 mM/mL BHT group. At 48 hours, mean sperm motility values for 0.5mM/mL BHT was significantly ($P < 0.05$) higher than values obtained for all other groups (Table 6). At 72 hours, the value of 30.00 ± 2.5% recorded for 0.5mM/mL BHT group was significantly ($P < 0.05$) higher than other groups (Table 6). The live ratio did not differ, although they decreased as storage time increased. There was no significant differences in semen abnormalities across all groups (Table 6).

The mean general sperm motility decreased as preservation time increased. After 72 hours of chilling, the mean gross motility for 1.0 mM/mL BHT group with a value of 20.5 ± 1.5% was significantly ($P < 0.05$) higher than that recorded for other groups (Table 7). No significant differences in values were observed for other groups. Live ratio recorded revealed a decrease in life sperm cells across all groups (Table 7) as storage time increased. The 1.0 mM/mL BHT group had the highest mean value for life ratio; with a value of 61.00 ± 6.0%, but this value was not significantly different from those obtained for other groups. The values for abnormal cells recorded in this study revealed no significant differences between groups.

Semen extended in tris-coconut + 0.5 mM/mL BHT had three out of seven inseminated does confirmed pregnant with a conception rate of 42.6% (Table 7). For the group of does inseminated with semen extended in egg yolk citrate + 1.0 mM/mL BHT, three of the total number were confirmed pregnant. This group had a conception rate of 50% (Table 8). Out of the seven does inseminated with semen extended

Table 6: Effect of BHT on chilled semen parameters of Red Sokoto bucks stored in tris coconut extender for 3 days duration

Parameter	Hours	BHT concentrations (mM/ML)				
		Control (0.0)	0.5	1.0	1.5	2.0
General Sperm motility (%)	0	83.00 ± 6.7^{w}	83.00 ± 6.7^{w}	83.00 ± 6.7^{w}	83.00 ± 6.7^{w}	83.00 ± 6.7^{w}
	24	50.00 ± 18.0^{ab,x}	65.50 ± 28.2^{a,x}	50.75 ± 28.2^{ab,x}	55.92 ± 7.1^{ab,x}	45.70 ± 7.1^{b,x}
	48	30.05 ± 15.1^{b,y}	55.35 ± 20.0^{a,x}	30.00 ± 15.2^{b,y}	40.20 ± 13.1^{b,x}	30.30 ± 15.5^{b,y}
	72	5.55 ± 5.0^{c,z}	30.00 ± 14.1^{a,y}	10.20 ± 7.1^{c,z}	20.40 ± 7.1^{b,y}	10.50 ± 3.4^{c,z}
(%) Live Spermatozoa	0	90.00 ± 6.2^{w}	90.00 ± 6.2^{w}	90.00 ± 6.2^{w}	90.00 ± 6.2^{w}	90.00 ± 6.2^{w}
	24	70.50 ± 7.1^{x}	72.50 ± 7.1^{x}	77.50 ± 7.1^{x}	70.50 ± 14.0^{x}	72.50 ± 7.1^{x}
	48	65.50 ± 7.1^{xy}	70.00 ± 7.1^{x}	70.00 ± 7.1^{x}	68.00 ± 7.1^{x}	62.00 ± 7.1^{y}
	72	60.00 ± 14.1^{y}	62.50 ± 14.1^{x}	62.50 ± 14.1^{y}	65.00 ± 14.1^{x}	60.00 ± 14.1^{y}
Sperm Abnormalities (%)	0	8.20 ± 6.2^{w}	8.20 ± 6.2^{w}	8.20 ± 6.2^{w}	8.20 ± 6.2^{w}	8.20 ± 6.2^{w}
	24	12.05 ± 9.1^{xw}	13.45 ± 9.1^{x}	12.35 ± 9.1^{xw}	15.05 ± 8.7^{x}	14.15 ± 3.3^{x}
	48	14.80 ± 4.8^{x}	14.20 ± 4.8^{x}	15.50 ± 4.8^{x}	18.00 ± 6.7^{xy}	19.65 ± 9.1^{x}
	72	16.75 ± 5.3^{x}	15.05 ± 5.3^{x}	18.50 ± 5.3^{x}	20.10 ± 5.3^{y}	21.24 ± 9.8^{x}

^{abc} Different superscript between rows and ^{wxyz} within columns denotes differences (P < 0.05). Data expressed as Means ± SD n=80

Table 7: Effect of BHT on chilled semen parameters of Red Sokoto bucks in citrate egg yolk extender stored for 3 days

Parameter	Hours	BHT concentrations (mM/mL)				
		Control (0.0)	0.5	1.0	1.5	2.0
General Sperm motility (%)	0	83.00 ± 6.8^{w}	83.00 ± 6.7^{w}	83.00 ± 6.7^{w}	83.00 ± 6.7^{w}	83.00 ± 6.7^{w}
	24	63.75 ± 22.6^{x}	73.00 ± 6.7^{x}	57.50 ± 26.8^{x}	57.50 ± 29.1^{x}	57.50 ± 37.1^{x}
	48	42.50 ± 17.5^{y}	30.00 ± 23.1^{y}	30.00 ± 6.2^{y}	30.00 ± 14.5^{xy}	23.75 ± 13.7^{y}
	72	12.50 ±7.9^{b,z}	15.00 ± 4.5^{b,y}	20.50 ± 4.2^{a,z}	11.25 ± 7.3^{b,y}	8.75 ± 8.7^{b,z}
(%) Live Spermatozoa	0	90.00 ± 6.2^{w}	90.00 ± 6.2^{w}	90.00 ± 6.2^{w}	90.00 ± 6.2^{w}	90.00 ± 6.2^{w}
	24	71.67 ± 4.8^{x}	75.00 ± 1.4^{x}	76.67 ± 9.3^{x}	71.67 ± 16.9^{x}	73.33 ± 4.5^{x}
	48	60.33 ± 4.5^{y}	63.30 ± 9.3^{y}	68.21 ± 12.4^{y}	62.30 ± 4.8^{xy}	63.95 ± 9.8^{y}
	72	55.00 ± 8.2^{2}	53.00 ± 9.1^{z}	61.00 ± 16.9^{y}	58.00 ± 12.44^{y}	50.00 ± 4.2^{z}
Abnormalities (%)	0	8.20 ± 6.2^{w}	8.20 ± 6.2^{w}	8.20 ± 6.2^{w}	8.20 ± 6.2^{w}	8.20 ± 6.2^{w}
	24	10.75 ± 3.3^{w}	11.25 ± 3.3^{x}	12.05 ± 4.5^{x}	12.50 ± 7.9^{w}	13.25 ± 1.9^{x}
	48	11.50 ± 3.9^{x}	12.35 ± 5.9^{x}	15.50 ± 3.1^{y}	16.60 ± 6.2^{x}	17.75 ± 3.6^{y}
	72	12.30 ± 9.3^{x}	11.50 ± 5.9^{x}	12.70 ± 8.2^{z}	11.90 ± 6.7^{w}	15.25 ± 5.3^{xy}

^{abc} Different superscript between rows and ^{wxyz} within columns denotes differences (P < 0.05).
Data expressed as Means ± SD n=80

in tris egg yolk + 1.0 mM/mL BHT, five were confirmed pregnant. The conception rate for this group was 71.4% (Table 8). Plate I shows structures indicative of pregnancy in does which were observed during the study on day 47 of pregnancy using ultrasound. Structures seen were; conceptus (C), amniotic fluid (D) and foetal sac (E).

Table 8: Conception rates in does inseminated with different extenders supplemented with different concentrations of BHT

Group	Number inseminated of does	Pregnant does	Percentage conception
0.5 mM BHT in Tris-coconut extender	7	3	42.6%
1.0 mM BHT in Citrate eggyolk extender	6	3	50%
1.0 mM BHT in Tris egg-yolk extender	7	5	71.4%

Discussion

Spermiogram of bucks was established to aid in the selection of bucks with good semen quality. Semen colours observed were normal and varied among the bucks corroborating a previous study (Catunda *et al*., 2011). These colours are due to lipochrome pigment which is derived from the epithelium of ampulla during seminal secretion and, therefore, considered harmless to spermatozoa as they have no effect on fertility (Patel & Siddiquee 2013). The semen volume recorded in this study is similar to that reported in goats (0.78 ± 0.27 mL) by Marjuki (2011). Older bucks generally have been reported to have greater volume of ejaculate than younger bucks. This was further affirmed in this study as the three bucks with greater semen volume were matured bucks, aged above 2 years. Semen motility is an important criterion for the quality of semen and also determines the fertility in bucks (Sultana *et al*., 2013).

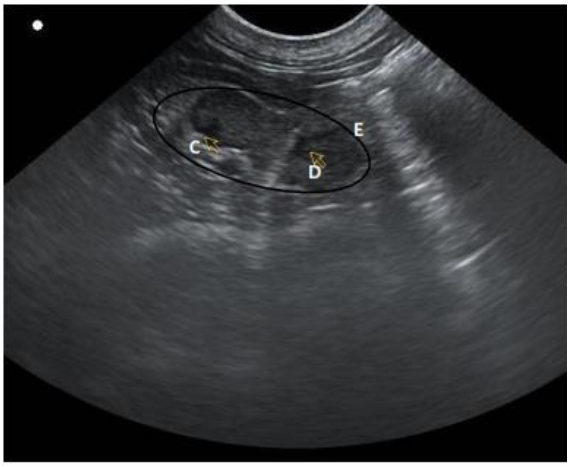

Plate I: Ultrasonograph showing conceptus (C), amniotic fluid (D) and foetal sac (E) on day 47 after artificial insemination

The average semen motility recorded in this study is similar to that reported in black Bengal bucks by Sultana *et al*. (2013), with a range of 77-81%. Semen motility is influenced by nutrition as reported by Bello *et al*. (2020a), who obtained the highest semen motility of rams fed crude protein levels of 15% as compared to those fed 10 and 20%. The semen concentration in this study is different to what was reported in Nubian goats by Elsheikh & Elhammali (2015). This difference might be due to breed difference and climatic conditions of study locations. The values for semen concentration observed in this study is also different from 437.5 ± 65.24 x 10^6/mL reported by Itodo *et al*. (2020) in red Sokoto goats. This difference could be due to the period of semen collection, as season has been reported to affected semen quality (Elsheikh & Elhammali, 2015). The live sperm in this study is similar to what was reported by Itodo *et al*. (2020) in red Sokoto goats with value of 81.00 ± 4.00%.

Beneficial effects of BHT addition on semen preservation has been confirmed and ultrastructural findings of spermatozoa also support the protective action of BHT as it reduces the damage of membrane and organelles of spermatozoa (Sharma & Singh 2019).

In this study, the effects of BHT added to citrate egg yolk (CEY), Tris- coconut and tris egg yolk (TEY) extenders on chilled caprine semen were investigated. It was observed in this study that regardless of extenders used, BHT concentration of 2.0 mM/mL had negative effects on semen characteristics. The exact mechanism by which the higher BHT concentrations negatively affected the frozen-thawed sperm characteristics remains unclear (Farshad *et al*., 2010). This finding is similar to the report by Khumran *et al*. (2017) that high concentrations of BHT (2.0 and 3.0 mM/mL), produced deteriorative effects on the sperm parameters in different extenders. The findings from this study are also similar to report by Mostafa *et al*. (2019), working on buffalo semen, where higher levels of 2.5 and 3.0 mM/mL BHT had a deteriorating effect on progressive motility, viability, morphology and acrosome as well as plasma membrane integrities compared to the control.

It is evident that greater concentrations of exogenous antioxidants in extenders have toxic effects on quality of sperm (Farshad *et al*., 2010). This might be due to

the greater concentrations of exogenous antioxidants functioning as prooxidants or may result from a redox imbalance that occurs due to interactions with existing ROS which invariably causes cellular dysfunctions (Bouayed & Bohn, 2010). Therefore, concentrations of BHT higher than optimal level might have negative effects thus distorting fertilization after insemination (Seifi-Jamadi *et al*., 2016).

The present result is similar to that of Bello *et al*. (2020b), who reported that the optimal concentration of BHT required for semen preservation vary according to the animal species and range between 0.05-2.0 mM/mL BHT. In contrast, Palomo *et al*. (2017) reported that addition of BHT at 5.0 mM improved most of the quality parameters of thawed sperm cells from rams. This variation may also be attributed to breed difference. The findings that BHT is useful at lower but detrimental at higher concentrations are consistent with the results of previous studies done on different animals. A report by Ziaullah *et al*. (2012) stated that BHT at inclusion levels of 1.0 mM/mL in extended canine semen improved post-thawed sperm quality. Higher concentrations of BHT such as 1.5 and 2.0 mM/mL in semen would have caused high fluidity within the cytoplasm of the spermatozoa beyond a critical limit, resulting in its rupture (Khumran *et al*., 2017).

Trzcinska *et al*. (2015) reported protective effects of BHT supplementation at 1 and 2 mM/mL on post-thawed boar semen quality in terms of sperm motility, viability, and acrosomal integrity. The improved sperm motility observed in this study may be attributed to the protective antioxidant effect of BHT on morphological sperm integrity, especially the integrity of the axosoma and mitochondria of the middle piece (El-Sheshtawy *et al*., 2017).

This study further corroborates the report by Ghorbani *et al*. (2015) in humans where 0.5 mM BHT resulted in both higher sperm motility and viability. In other words, BHT concentration greater than 0.5 mM results in lower sperm motility and viability which is probably due to an increased superoxide anion and H_2O_2 in sperm cells.

This study revealed that different concentrations of BHT were needed for improved preservation of sperm cells as the semen extenders were changed. The reason for the differences might be due to the differences in the constituent of each extender used. Merino *et al*. (2020) reported this, who showed that different concentrations of BHT are required for preserving semen, depending on the semen extender, the species and sperm cell membrane composition. This is further corroborated by El-Khawagah *et al*. (2020), who reported that the concentrations of butylated hydroxytoluene on buck spermatozoa varies with buck breed, and phospholipid source in the extender

A report by Mostafa *et al*. (2019) revealed that use of BHT concentrations above 2.0 mM/mL decreased sperm characteristics when added to extenders. This reduction may be attributed to the hazardous property of BHT as higher concentration of antioxidants decrease physiological level of oxidants, which is required for the normal sperm metabolism (Mostafa *et al*., 2019). These authors obtained optimal results by addition of 0.5-2.0 mM/mL BHT, depending on extender used and stage of cryopreservation. This is different from what was observed in this study, which may be due to species variation. Specifically, addition of BHT or other exogenous antioxidants has been found useful in chilled semen of various species of animals (Mostafa *et al*., 2019).

Tris-coconut extender from his study required lesser concentration (0.5mM/mL BHT) than TEY extender for optimum semen preservation after 72 hours. This result might be due to the fact that coconut water extenders contain essential constituents such as sugars, vitamins, minerals and amino acids (Yong *et al*., 2009), required for survival of spermatozoa. The findings of this study showed that, TEY extenders supplemented with 1.0 mM/mL BHT provided best preservation after 3 days. This might be because tris buffers are more stable at high temperature and other different environmental conditions than bicarbonate and sodium citrate buffers, and they preserve sperm energy by reducing fructolysis (Ubah *et al*., 2019).

Differences in sperm motility between control group and supplemented groups in this present study contraindicated the work of Khumran *et al*. (2017) in bulls, where no significant difference was observed between the treated groups and controls for general motility. This might be due to species variations differences in the semen make-up of bucks as compared to bulls.

Advancement of storage time for up to 72 hours in different extenders can significantly depress the sperm motility (EL-Seadawy *et al*., 2016). This is similar to findings in this study where sperm motility decreased across all groups as storage time increased. This further corroborates the report of Mahiddine & Kim (2021), that post-thaw parameters of cryopreserved sperm are poorer than those of fresh sperm. The sperm motility recorded in this study are similar to that reported in rams (Khalifa & Khalil

2016). The decreasing sperm motility observed was attributed to depletion of nutrients (Gibb & Aitken 2016), which inhibit fructolysis and respiration of sperm cells (Gündoğan *et al*., 2003).

In this study, beneficial effects were observed by addition of 1.0mM/mL BHT in sperm cells of red Sokoto bucks stored in tris egg yolk and citrate egg-yolk. This is in accordance with reports by Singh *et al*. (2017) in bulls, where beneficial effects were observed by addition of 1.0mM BHT which improved post thaw semen quality as evident by higher progressive motility. Bucks have the bulbo-urethral secretions which are detrimental to sperm survival. Supplementation with BHT might have mitigated the detrimental effects of the secretions, hence the reason for the increased motility observed in supplemented groups compared to the control. Khumran *et al*. (2017) further stated that BHT maintained consistently higher values of progressive motility at 0.5 mM/ mL in Bioxcell and 1.0 mM/mL in TEY and CEY than controls; hence it has potential positive effects on progressive motility. The result from this study is in disagrees with the report of Sun *et al*. (2020) in dogs, who reported 1.5 mM BHT as the optimal concentration for improving the post-thaw quality of canine spermatozoa.

In a study conducted in poultry, 0.5-1.0 mM/mL BHT had no beneficial effects on post-thaw semen parameters or fertility in chickens; instead, it decreased all the parameters (Kumar *et al*., 2019). This disagrees with the findings in this study because BHT exerts a protective effect on the sperms, which depends on its concentration in the extender, which is also species-dependent and specific (Patel *et al*., 2015). Converse to the findings in this study, the addition of BHT has been shown to have negative effects on sperm motility in Beetal goat bucks (Iqbal *et al*., 2015). Factors responsible for this effect include; breed differences, spermatozoa number per dose, application methods, thawing time and temperature, the composition of extender, and above all extent of cryo-damage itself. From this study, it was observed that a greater concentration of BHT (1.0 – 1.5 mM/mL) was required to improve the preservation of semen extended in CEY and TEY compared to that of TCW. This could be because BHT is lipid-soluble and may associate with egg yolk lipids. Therefore, scanty-free BHT may be available for permeating the sperm plasma membrane for preservation (Ziaullah *et al*., 2012).

This study detected pregnancy using ultrasound on day 47 after AI. This gives credence to a report by Karadaev (2015), who reported days 40 and 45 for accurate diagnosis of pregnancy in goats using transabdominal ultrasound. The structures seen in this study as confirmation for pregnancy were; amniotic fluid, foetal sac and conceptus, corroborating the report of Amer (2010) that criteria for positive diagnosis of pregnancy in goats include amniotic fluid, conceptus and embryonic vessicle. The foetal sac is usually not seen between days 25-30 as reported by Ali *et al*. (2020). The does inseminated with semen extended in tris egg yolk + 1 mM/mL BHT had the highest conception rate, coinciding with the highest sperm motility rate in this group after 72 hours of storage, which could have aided active transport of the sperm cells to the oviduct for fertilisation than other groups of semen.

The findings in this study are different to a report by Memon *et al*. (2011) that goats inseminated with semen extended with 2.0 mM/mL BHT had greater conception rates in boer goats than three other groups. The findings in this study are also similar to the report in pigs by Trzcinska *et al*. (2015), where the highest reproductive performance of inseminated gilts was recorded with semen cryopreserved in extender supplemented with 1.0 mM/mL BHT. However, it is worthy to note that in this study, of all the extenders selected, and none had a BHT concentration higher than 1.0 mM/mL. The difference in concentration of BHT used compared favourably with those of other authors, although the differences in breed of experimental animals, extender constituents and mode of semen storage may be contributory factors. The conception rate of 71.4% observed in the group inseminated with 1.0 mM/mL BHT in tris egg-yolk extender is higher than what was reported by Memon *et al*. (2011), who recorded a conception rate of 35.71% in goats. This might be because freeze-thawing leads to low antioxidant activity in the absence of supplementary antioxidants (Sarıozkan *et al*., 2009).

Semen from red Sokoto bucks could be extended in Tris egg yolk + 1.0 mM/mL BHT, Citrate egg yolk + 1.0 mM/ml BHT and Tris coconut water + 0.5 mM/mL BHT for better semen quality after chilling for 72 hours. In conclusion, semen from red Sokoto bucks could be extended in Tris egg yolk + 1.0 mM/mL BHT for 72 hours for good post-storage semen quality and optimum conception rate. Further studies should be conducted using a higher sample size to investigate the consequential influence of these supplemented extenders on conception rates.

Conflict of interest

The authors declare that there is no conflict of interest.

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