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Coconut milk - citrate as extender for West African dwarf buck spermatozoa at room temperature

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Abstract

We studied the proportions of coconut milk and sodium citrate buffer suitable for extension of West African dwarf (WAD) buck spermatozoa at room temperature. Semen was collected from clinically healthy buck certified free of obvious andrological defects. Eight trials of semen extension were carried out using 0.1 ml of semen plus 0.5 ml buffer as individual extender. In the extenders D1 to D7, while the buffer (sodium citrate) was decreasing, the coconut milk was increasing. Statistical analyses from 5 trials showed that D2 containing 20% coconut milk and 80% citrate buffer that supported mean sperm cell motility of 52.6% was highly significant ($p = 0.018$) at 2 hours post-extension in preserving motility of extended buck semen un-refrigerated compared to both D3 (40% coconut milk and 60% citrate buffer) and D4 (50% coconut milk and 50% citrate buffer). D2 also maintained mean sperm cell motility of 45% and was highly superior ($p = 0.012$) to both D3 and D4 at 3 hours post-extension. However, in D2, there was no statistical difference ($p = 0.693$) between 2 hours and 3 hours storage time in mean motility of extended sperm cells. Similarly, there was no difference ($p = 0.106$) in mean sperm cell motility between D2 at 3 hours and D3 at 2 hours post extension. We concluded therefore, that D2 was superior to others with which it was compared; and that it preserved extended buck semen for more than 2 hours storage at room temperature.

Keywords: West African dwarf buck, spermatozoa, coconut milk, semen extension, motility

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INTRODUCTION

Artificial insemination (A.I.) is a vital labour-saving technique being used in animal production to improve livestock productivity. It was introduced by early workers like Philips and Lardy[1] and Salisbury *et al*[2]. It has been reported that there is increasing need for use of A.I. in West African Dwarf (WAD) goats[3]; this necessitates extension of semen to make A.I. economically beneficial. However, the success of A.I. is based on the ability to efficiently collect and cryopreserve spermatozoa from quality bucks (male goat) for use in inseminating does (female goats) over generations[4].

Extended buck semen can be used frozen-thawed, chilled or fresh (i.e. non-refrigerated), but when fresh semen is used, it offers better fertility and conception rate[5,6]. In addition, Blash *et al*[7] stated that the process of freezing and thawing of goat semen reduces the percentage of live sperm cells and acrosomal integrity. To meet the need of A.I., many extenders have been used. These include egg yolk- phosphate[8], skim milk[9] and orange juice[10].

Norman[11] first used coconut (*Cocos nucifera*) milk as part of extender for semen and he recorded that coconut milk had the advantage of keeping livability of fresh semen. When used fresh, buck semen extended in coconut milk was reported to give appreciable sperm cell motility and fertility with acceptable conception rate (CR) post-breeding[12]. Likewise, bulls' semen extended in coconut milk - citrate extender was reported to yield appreciable CR in cattle when used fresh[13].

Semen quality and its relationship to fertility are said to be major concern in animal production, hence accurate measurement of semen fertilizing potential is of great importance[14]. In order to assess semen quality therefore, workers in this field rely on visual and microscopic evaluation of spermatozoal progressive motility and morphology to measure sperm cell survival[15].

In Nigeria, there is a dearth of literature regarding the actual proportion of coconut milk suitable for extension of buck semen. The objectives of this study, therefore, were to determine the proportions of coconut milk and sodium citrate suitable for preserving motility and survival of WAD buck spermatozoa; and to study the duration of sustaining the motility in studied extenders at room temperature (28 ± 2^{o}C).

MATERIALS AND METHODS

Location

This study was undertaken at University of Ibadan; 6 kilometers to the north of Ibadan city, at latitude 7^{o} 26` north and latitude 3$^{o}$ 54` east, at a mean altitude of 277 meters above sea level. The annual rainfall is 1,220 mm, most of which falls between April and October, and a dry season from November to March[16].

Animals and management

Semen characteristics of two clinically healthy and fertile West African dwarf bucks were preliminarily studied during acclimatization of the bucks. The two bucks were kept in a well-illuminated pen. One was tagged "white" and the other "black". They were semi-intensively managed in clean, well-ventilated pen with a concrete floor. The bucks were about 4 years old with an average body weight of 17.5 kg, and were fed grass in the mornings and concentrate supplement in the evenings. The concentrate contained 17.25% crude protein and 212,344 kilocalories metabolizable energy per 100 kg dry feed. Each buck consumed an average of 400 g dry feed daily for about 4 weeks before and throughout the semen collection period. They were served clean fresh water *ad libitum*.

Study design

Though semen samples from the "white and "black" bucks were assessed during acclimatization, only the white buck's semen was extended as it had better semen qualities. Eight trials of semen extension were carried out. This study did not include any standard control extender other than 100% each of coconut milk and the buffer; because it was thought that for meaningful use in animal breeding, a good semen extender should be able to preserve sperm cell motility non-refrigerated for at least 2 hours, the study only compared mean spermatozoal

motility in different proportions of coconut milk-citrate extender at 2 and 3 hours after semen extension. The extender that best supported semen survival was determined by microscopic (visual) assessment of unidirectional progressive movement (motility) of sperm cells. All aseptic protocols were duly observed to minimize contamination of coconut milk, semen and diluents (extenders). Fresh, mature and ripe coconut fruit was used each time for semen extension. Animals were adequately restrained to minimize their discomforts.

Preparation of buffer

2.5% w/v trisodium citrate was prepared as described by Hafez[17]. This was kept in a 100 ml flat bottom flask in a dark cupboard until used. The pH of the buffer was determined and recorded.

Preparation of coconut milk extender

Mature ripe coconut was split open and the white coconut flesh was removed from the shell using a clean sterilized knife. The coconut flesh was cut into bits and blended, without addition of water, in a clean electric blending machine. The blending was done twice. The finely blended flesh was wrapped in a clean sterilized white handkerchief. This was squeezed using a clean orange crusher to extrude the juice. The coconut juice thereby collected was dispensed into clean sterilized centrifuge glass tubes, tightly capped, and centrifuged at 500 rpm for 20 minutes. Using a new needle and syringe, coconut milk was carefully sucked up from below the upper oily layer of the spun coconut juice.

The coconut milk thereby collected was centrifuged the second time to eliminate coconut oil and shaft. The coconut milk volume and pH were measured and recorded. Following this, requisite amounts of coconut milk and citrate buffer were dispensed into 7 differently labeled clean sterilized semen collection tubes (D1 to D7); each of these represented an extender.

The tubes were tightly capped and gently rolled to properly mix the contents and kept at 37^{o}C until used for semen extension. The pH and other parameters of each extender were measured and recorded.

Collection of semen

Using electro-ejaculation (EE) method, semen was collected as described by Oyeyemi *et al*[3]. All aseptic protocols were observed. Briefly, the EE probe was lubricated with Vaseline^{R} before insertion into the buck's rectum. With a clean warm (about 37^{o}C) sterilized funnel and semen collection tubes (bathed in warm water at 37^{o}C), semen was collected from the buck and stored at 37^{o}C in water bath until extended. Semen collection was done once a week.

Pre-extension evaluation of semen

Immediately after semen collection, a drop of semen from buck's prepuce was put on clean warm glass slide. This drop of semen was used to assess mass activity of the semen and the values recorded. Volume of collected semen was also recorded. Microscopic evaluation of semen concentration and motility was quickly done and the values recorded. Morphology of semen was also studied by staining sperm cells with Eosin-Nigrosin stain, and with Wells & Awa stain as described by Oyeyemi *et al*[3]. In each trial, prior to semen extension, volume, motility and concentration of semen were quickly assessed and the values recorded as pre-extension semen parameters.

Semen extension

Buck semen was extended as described by Ajala *et al*[18] in similar study using pawpaw juice extender. Briefly, the semen was extended by dispensing 0.1 ml semen into 0.5 ml extender (D1 to D7) previously kept at 37^{o}C. However, whenever the volume of semen collected from the buck was less than 0.7 ml, extension started from D2 to D7, to ensure that the test extenders were included. Immediate assessment of semen motility was done and recorded. Each tube of extended semen was thereafter kept away from light and left at room temperature for 6-hour period for hourly sperm cell motility assessment.

Post-extension evaluation of semen motility

This was done as described by Ajala *et al*[18] for pawpaw juice extender. Briefly, the immediate post-extension sperm cell progressive motility

was assessed and recorded as zero hour motility score in percentage (%). Subsequent motility scores were done hourly for 6 hours. Each time, the assessment of sperm cell motility always started from D2 and continued in order to D7, and lastly D1 (100% buffer).

The motility assessment was done quickly and carefully to ensure little time-lag in assessing other extended semen. The concentration of motile sperm cells of the extended semen was estimated for the most suitable extender using the average pre-extension semen volume and concentration with average post-extension motility at 2 hours storage time, and the value recorded.

Analysis of data

A descriptive analysis was performed by calculating means of sperm cell motility with results presented as mean ± standard error of mean (SEM). Using SPSS 11 software, one way ANOVA test, at 5% significant level, was used to determine whether or not there were significant differences between mean motility scores of sperm cells between the three comparable extenders at 2 and 3 hours after semen extension; and between these storage times (at room temperature) in the most superior extender.

RESULTS

The semen characteristics of the white and black bucks are as shown in Table 1 (a), (b), (c) and (d). The average motility score of sperm cells of the white buck was significantly higher ($p=0.0001$) than that of the black buck, Table 1 (a and b). Semen colour which is an indication of sperm cell concentration was consistently milky-white for the white buck while it was consistently watery for the black buck. This indicated poor sperm cell concentration of the black buck. This was supported by the mean concentration of sperm cells of white buck which was significantly higher ($p = 0.0001$) than the black buck's (Tables 1a and 1b).

However, there was no statistical difference ($p = 0.321$) between the live scores (%) of sperm cells of both bucks. The percentage of abnormal sperm cells in the white buck, in contrast to the black buck's, was less than 20% recommended by Zemjanis[19] (Tables 1c and 1d).

pH of buffer

The pH of 2.5% sodium citrate in the 8 trials ranged from 8.5 to 9.0 with an average value of 8.7 ± 0.9.

Values of coconut milk

The volumes of coconut milk obtained in the 8 trials ranged from 9 ml to 24 ml with a mean value of 20.8 ± 1.4 ml. The pH of coconut milk ranged from 5.8 to 6.1 with an average value of 6.0 ± 0.3. The relative amounts of constituents of each extender and mean pH are as shown in Table 2.

Parameters of semen prior to extension

Evaluation of the undiluted semen before extension in the 8 trials is as shown in Table 3.

Post-extension motility scores of semen

The values presented here are those of the 5 trials used in the statistical analyses for up to 3 hours post-extension (p.e.) of semen. They are shown in Table 4(a and b) for 2 and 3 hours p.e. respectively, at room temperature. In all the 8 trials, the motility scores observed dropped to virtually zero value by 4 hours p.e. The results of the 4 hours and above are not shown. The motility scores at 2 hours after extension in D2 were generally higher than in D3 and D4 in all the trials, except in trial 5. It was also observed that D3 was not different ($p = 0.444$) from D4 in preserving semen motility at 2 hours p.e.

The motility scores at 3 hours after extension followed the same pattern of decreasing motility scores from extenders D2 to D4 as observed at 2 hours p.e. (Tables 4a and 4b). No statistical difference ($p = 0.693$) was observed when mean sperm cell motility at 2 and 3 hours p.e. were compared for D2. In addition, mean motility scores of semen in D2 at 3 hours p.e. was not different ($p = 0.106$) from that in D3 at 2 hours p.e. Using 52.6% mean sperm cell motility in 0.6 ml extended semen after 2 hours, we estimated concentration of motile sperm cells in D2 as 161.5×10^6 sperm cells (i.e. 269.2 million sperm cells per ml).

Table 1a: Semen parameters of white buck during acclimatization

SEMEN CHARACTERISTICS	NUMBER OF TRIALS					
	1	2	3	4	5	MEAN ± SEM
Volume (ml)	0.5	0.5	0.6	0.3	0.3	0.44 ± 0.05
Colour	Milky white	Milky white	Milky white	Milky white	Milky white	Milky white
Mass activity (0 - 4)	4	4	4	4	4	4
Progressive Motility (%)	93	95	95	95	90	93.6 ± 0.87
Live (%)	94.20	99.40	96.20	98.12	98.30	97.2 ± 1.95
Concentration (billion sperm cells per ml)	1.77	2.58	1.84	1.15	1.49	1.77 ± 0.21

Table 1b: Semen parameters of black buck during acclimatization

SEMEN CHARACTERISTICS	NUMBER OF TRIALS					
	1	2	3	4	5	MEAN ± SEM
Volume (ml)	0.20	0.40	0.30	0.41	0.60	0.38 ± 0.06
Colour	Watery	Watery	Watery	Watery	Watery	Watery
Mass activity (0-4)	1	1	1	1	1	1
Progressive Motility (%)	50	60	45	35	35	45 ± 4.24
Live (%)	94.30	96.10	61.30	98.31	98.20	89.6 ± 6.49
Concentration (billion sperm cells per ml)	0.19	0.15	0.17	0.27	0.09	0.17 ± 0.03

Table 1c: Morphological characteristics of white buck semen in 2 trials

TRIALS	TNS	TAC	TNC	DNH	SBT	CT	A/MP	PCD	DCD	%DNH	%SBT	%CT	%A/MP	%PCD	%DCD	%TAC	%TNC
1	332	56	266	11	22	21	-	0	2	3.4	6.8	6.5	-	0.0	0.6	17.4	82.6
2	426	44	382	4	26	12	-	2	0	0.9	6.1	2.8	-	0.5	0.0	10..3	89.7
Mean	379	50	324	7.5	24	16.5	-	1	1	2.2	6.5	4.7	-	0.3	0.3	13..9	86.2

Table 1d: Morphological characteristics of black buck semen in 2 trials

TRIALS	TNS	TAC	TNC	DNH	SBT	CT	A/MP	PCD	DCD	%DNH	%SBT	PCT	%A/MP	%PCD	%DCD	%TAC	%TNC
1	32	23	9	1	7	13	-	-	-	3.12	21.9	40.6	-	-	-	71..9	28.1
2	18	12	6	5	4	3	-	-	-	27.8	22.2	16.7	-	-	-	66.7	33..3
Mean	25	17.5	7.5	3	5.5	8	-	-	-	15.5	22.1	28.7	-	-	-	69..3	30.7

TNS – Total number of sperm cells
TAC – Total abnormal cells
TNC – Total normal cells
DNH – Detached normal cells
SBT – Simple bent tail
CT – Coil tail
A/MP – Abaxially attached mid-piece
PCD – Proximal cytoplasmic droplet
DCD – Distal cytoplasmic droplet
% - Percentage

Table 2: Relative amounts of extender constituents, and mean pH of extenders

Diluent	Buffer		Coconut milk		Total volume	Semen volume	Dilution ratio	Mean pH
	ml	%	ml	%	ml	ml		
D1	0.50	100	0.00	0	0.5	0.1	1:6	8.6
D2	0.40	80	0.10	20	0.5	0.1	1:6	7.6
D3	0.30	60	0.20	40	0.5	0.1	1:6	7.1
D4	0.25	50	0.25	50	0.5	0.1	1:6	7.0
D5	0.20	40	0.30	60	0.5	0.1	1:6	7.0
D6	0.10	20	0.40	80	0.5	0.1	1:6	6.9
D7	0.00	0	0.50	100	0.5	0.1	1:6	6.4

Table 3. Pre-extension semen values in 8 trials

SEMEN	1	2	3	4	5	6	7	8	Mean ± SEM
Volume (ml)	0.70	0.80	0.50	0.20	0.45	0.40	0.60	0.80	0.56 ± 0.06
Progressive Motility (%)	90	95	95	90	90	80	90	95	90.60 ± 1.80
Billion sperm cells per ml	N.E	3.18	1.50	0.36	N.E	2.26	1.33	1.66	1.72 ± 0.35

N.E – Not estimated

Table 4a: Motility scores (%) of sperm cells at 2 hours post-extension (5 trials)

TRIALS USED	EXTENDER D2 (%)	EXTENDER D3 (%)	EXTENDER D4 (%)
1	50	25	20
2	78	35	25
5	0	0	0
6	65	30	0
8	70	8	0
MEAN ± SEM	52.6^{a} ± 12.50	19.6^{b} ± 5.98	9.0^{c} ± 4.98

Table 4b: Motility scores (%) of sperm cells at 3 hours post-extension (5 Trials)

TRIALS USED	EXTENDER D2 (%)	EXTENDER D3 (%)	EXTENDER D4 (%)
1	40	20	8
2	70	20	20
5	0	0	0
6	55	30	0
8	60	5	0
MEAN ± SEM	45^{a} ± 10.95	15^{b} ± 4.89	5.6^{c} ± 3.51

DISCUSSION

The average motility scores of WAD buck spermatozoa in 5 trials were 52.6%, 19.6% and 9.0% in D2, D3 and D4, respectively, at 2 hours post-extension (p.e.) of semen. The same parameter in the 5 trials at 3 hours p.e was 45%, 15% and 5.6%, respectively, in D2, D3 and D4. These results showed that survival of WAD buck sperm cells is inversely related to the concentration of coconut milk as the motility scores decreased from D2 to D4 at both 2 and 3 hours storage time. The reason for this observation could be that the increasing quantity of coconut milk increased the viscosity of the extenders, which thereby hindered spermatozoal progressive movement. In comparing different proportions of extenders, statistical analysis showed that after 2 hours storage of extended semen at room temperature, extender D2 preserved a mean motility score of 52.6% which was significantly ($p = 0.018$) higher than mean scores observed in D3 and D4. Also at 3 hours p.e., D2 that maintained sperm cells at average motility score of 45% was superior ($p = 0.012$) to D3 and D4. Comparison of storage time following semen extension revealed that ability of D2 at preserving sperm cell motility at 2 and 3 hours p.e. was not statistically different ($p = 0.693$). This result was suggestive of possibility of using D2 as WAD buck semen extender for duration of 3 hours at room temperature. The observation was the same when mean sperm cell motility scores at 2 and 3 hours p.e. were compared ($p = 0.609$) in D3.

These results of storage time effect on extended semen was somehow not in consonance with

report made by Dauzier[20] that fertilizing capacity of buck (and ram) semen decreased with an increase in storage time. But it was thought that a longer duration of extended semen might show a significant difference.

An astonishing observation made was that there was no difference ($p = 0.106$) in preservation of sperm cell motility when D2 at 3 hours p.e. was compared with D3 at 2 hours p.e. We suggested therefore that D3 might be useful for preserving WAD buck semen (non-refrigerated) but may be for less than 3 hours after semen extension.

The mean motility score of sperm cells at zero hour (i.e. immediate assessment post- extension) in D2 was 73% in the 5 trials (data not shown). This was higher than the pre-freezing motility score of 49% recorded by Melo and Nunes[12] for buck semen extended in coconut milk - citrate extender. This suggests that 20% coconut milk plus 80% sodium citrate buffer (i.e. D2) might be appropriate for extension of WAD buck semen intended for storage at freezing temperature; because the higher the pre-freezing motility, the higher would be the post-thawing sperm cell motility.

The 0.6 ml extended semen in D2 contained 161.5 million motile sperm cells, which may be sufficient for artificial insemination in does as Pagot[21] and Zemjanis[22] observed that 100 million sperm cells were compatible with A.I. In addition, estimation of the above motile sperm cells gave 13.5 million and 53.8 million in 0.05 ml and 0.20 ml respectively. This 0.6 ml extended semen and the motile sperm cell concentration contained in it might make a good insemination dose for does by intra-cervical route. The reason is, according to Roberts[23], workers in this field recommended 50 to 150 million motile spermatozoa in a volume of 0.05 to 0.20 ml extended semen for intra-cervical insemination, but that higher volume and numbers of spermatozoa, on the order of 10 times greater, were needed for intravaginal insemination to obtain good fertility in goat and sheep.

The ratio of semen to extender (1:6) used in this work, according to the observations, appeared suitable for sperm cell survival at 2 hours post-extension. Tewari *et al*[24] reported that a ratio slightly higher than 1:5 could still be used, though John[25] observed that buck semen viability declined with higher dilution ratio and ageing.

One factor, among others, that might be responsible for the observed drastic decline of the sperm cells motility was the non-addition of antibiotics to the extenders. This probably allowed quick depletion of sugar by the competing microbes. This probably affected trial 5 (Table 4a and 4b) in which after 1 hour of semen extension at room temperature, the motility score sharply dropped to zero value for all the extenders. In support of addition of antibiotics, Foote and Bratton[26] reported that yolk-citrate-antibiotic medium enhanced and extended the usefulness of semen. A similar study with inclusion of antibiotics is hence recommended.

It is also remarkable to report that slightly alkaline pH (7.6) of D2 made a significant difference in supporting WAD buck sperm cell motility compared to virtually neutral pH values 7.1 and 7.0 of D3 and D4 respectively. The pH value 7.6 (alkaline) of D2 was contrary to slightly acidic pH values of buck semen reported by Oyeyemi *et al*[3]. However, it was surprising that acidic extender D6 with pH similar to bucks' semen reported by Oyeyemi *et al*[3] did not give any appreciable support to motility of extended sperm cells. The same reason of high viscosity of this extender might be responsible for this observation.

Worthy of note, as well, is the observation that, though a mean motility score of 52.6% of fresh extended WAD buck semen in coconut milk at 2 hours storage period might be compatible with fertility, it remains to be proven whether it would give good reproductive performance (i.e. fertility, fecundity and prolificacy) in goats artificially inseminated. Therefore, the reproductive performance of WAD buck semen extended in 20% coconut milk plus 80% citrate buffer needs to be investigated.

This study has shown that D2 has the most suitable proportions of coconut milk and citrate buffer (20% coconut milk plus 80% citrate buffer) to support WAD buck semen motility

compared to extenders at D3 and D4. And that D2 might be suitable as buck semen extender for about 3 hours after extension of semen at room temperature.

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