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Research Article

Effects of Date Palm (*Phoenix dactylifera*) Fruit Extender on Functional Attributes of Cryopreserved Semen from West African Dwarf Goats Using Two Cryoprotocols

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Abstract

This study was carried out to determine the effects of Date Palm (*Phoenix dactylifera*) fruit extender on functional attributes of cryopreserved semen from West African dwarf goats using two cryoprotocols. Semen samples were collected with the aid of artificial vagina from four intact intensively managed bucks of ages 2 to 3 years and ejaculates were pooled for uniformity. Semen samples were thereafter diluted at 32°C with Tris date palm fruit extender (0, 5, 10, 15 and 20 ml) and subjected to slow and rapid freezing methods for 72 hours. Progressive sperm motility, livability and abnormalities were determined from cryopreserved semen samples. The experiment was laid out in a 2x5 factorial arrangement of two levels of cryoprotocol (slow and rapid freezing) and five inclusion levels of date palm fruit extender (0, 5, 10, 15, 20 ml) using a Completely Randomized Design and mean separation was done using Duncan multiple range test. Results showed that progressive sperm motility of the cryopreserved semen was higher ($P < 0.05$) in slow freezing method compared to rapid freezing (RF) method. Total abnormalities were reduced ($P < 0.05$) with slow freezing. The inclusion levels of date fruit extender did not significantly ($P > 0.05$) influence sperm functional attributes of WAD bucks. The highest ($P < 0.05$) progressive sperm motility was recorded in 10 ml inclusion of date palm fruit extender under slow freezing (SF), followed by 0 ml of slow freezing and 5 ml of rapid freezing. The study concluded that slow freezing method improved and sustained progressive sperm motility of the cryopreserved semen.

Keywords: WAD Goats; Semen; Cryopreservation; Date Palm; Slow and Rapid Freezing

Introduction

The West African Dwarf (WAD) goats possess certain valuable traits that confer adaptation to endemic trypanosomiasis challenge and hot humid tropics [1]. Artificial insemination is the most effective breeding tool that affords widespread propagation of genes carried by superior males [2]. Screening of the semen at initial stages followed by its preservation allows elimination of poor-quality semen while proper assessment of the post thaw quality of the spermatozoa can provide insight into the fertilizing capacity of the cryopreserved spermatozoa [3]. However, stored semen still encounters ultra-structural, biochemical and functional damage to the spermatozoa resulting in reduced motility, viability, fertility and impaired sperm transport. Therefore, suitable preservative methods for goat spermatozoa should be investigated.

Although semen contains antioxidants that counteract the damaging effects of lipid peroxidation and prevent excessive peroxide formation [4], the endogenous antioxidative capacity of semen may be insufficient during storage [5]. Goat spermatozoa in particular are sensitive to oxidative stress due to high content of unsaturated fatty acids in phospholipids of plasma membrane and low antioxidant capacity of goat seminal plasma [6]. Hence, goat semen requires preservation techniques that ensure adequate availability of antioxidants to sustain stability of sperm integrity.

Date palm (*Phoenix dactylifera*) has been reported to be an important fruit. It is a good source of essential nutrients [7]. Date fruit consists of 73-79% carbohydrates, 14-18% total dietary fibers, 2.5% ash, 2.1-3.0% protein [8]. Date palm fruits are good sources of natural antioxidants, containing many different antioxidant components suggests its suitability for cryopreservation. These antioxidants include carotenoids, vitamins, phenolic compounds and flavonoids and have proved to function as singlet and triplet oxygen quenchers, free radical scavengers and peroxide decomposers [9]. There are limited studies regarding freezing or cryopreservation of semen obtained from WAD goat bucks with fruit-juice like date

fruit extract rich in natural antioxidants. Therefore, this study investigates the effects of date palm fruit extender on functional attributes of cryopreserved semen from WAD bucks using two cryoprotocols.

Experimental site

The experiment was carried out at the Goat unit of Directorate of University Farms (DUFARMS), Federal University of Agriculture, Abeokuta, Nigeria located at 7°10' N and 3°2' E Google Earth., [10] with altitude 76 m above sea level. It lies within the South-Western part of Nigeria which has a prevailing tropical climate, a mean annual rainfall of 1,037 mm and an average temperature of 34.7°C. Semen evaluation was carried out in the Animal Physiology laboratory of the Department of Animal Physiology, College of Animal Science and Livestock Production, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria.

Date fruit extraction

Matured date palm fruits were purchased from a market in Abeokuta, Ogun State, Nigeria. The pulp/flesh was first removed from the seed. Date fruits of 100g weight were washed under running tap water to remove dirt and other materials. The pulp was mixed with distilled water in the ratio of 1:2 (weight/volume, pulp/water) to facilitate the blending process. Blending was done using a commercial blender (Kenwood, England). Then, the solution was filtered using a muslin cloth for the extraction of date fruit juice.

Experimental animals and management

Four WAD goats (bucks) within the ages of 2-3 years were used for this experiment. The bucks were kept separately in different pens under an intensive management system where clean water and guinea grass (*Panicum maximum*) supplemented with concentrate feed were made available to them *ad libitum*. For collection of semen, a doe was introduced into the pens which served as teaser to facilitate semen collection with the use of artificial vagina.

Semen collection, dilution and storage

Semen samples were collected from the four WAD bucks with the aid of an artificial vagina, and a doe in oestrus was introduced. Other materials used for semen collection included petroleum jelly, hot water (45 to 50°C), collection tube, micro pipette and sample bottles. Ejaculates were pooled for uniformity (to eliminate individual differences). Semen was diluted at 32°C [11,12] with a tris-based extender supplemented with date fruit juice (Tris-date fruit juice extender). After which it was subjected to two methods: slow and rapid freezing method of cryopreservation.

Experimental design

A factorial arrangement was used for the experiment which was laid out in a 2 by 5 factorial arrangement of two levels of cryo-protocol (slow and rapid freezing) and five inclusion levels of date fruit extract (0, 5, 10, 15 and 20 ml) using a Completely Randomised Design.

Protocol 1 (Slow freezing)

For this protocol, fraction 2 solution (glycerol fraction) was added subsequently to fraction 1 solution (semen fraction) (Table 1) at ratio 1:1. The pH of the extenders including that of the control was determined using a digital pH meter. Following dilution, the diluted semen samples were loaded into 2 ml plastic straws, sealed with polyvinyl and then subjected to conventional slow freezing. The straws containing the semen were cooled from 32°C to 4°C at a rate of 1°C/4 mins (0.25 ºC/min), and equilibrated at 4°C for 10 minutes in a TYFSF Refridgerated Incubator (Model: SPX-7OB III, Hubei China). Subsequently, the straws were placed in a rack at 4 cm above liquid nitrogen in the vaporous phase for about 10 min before lowering them gradually into the nitrogen tank. After 72 hours semen samples were retrieved, thawed and evaluated for sperm viability and oxidative stress parameters. Estimations were performed for the pooled semen samples in repeated measurements using different slides and each measurement was repeated five times to ensure accuracy of results.

Chemical ingredients	Fraction 1	Fraction 2
Tris-hydroxymethyl- aminomethane	2.42 g	2.42 g
Citric acid	1.35 g	1.35 g
Glucose	1 g	1 g
Penicillin	0.028 g	0.028 g
Date fruit extender levels Egg yolk	0, 5, 10, 15 and 20 ml 20 ml (control)	0, 5, 10, 15 and 20 ml 20 ml (control)
Glycerol	-	14 %
Distilled water	Added to make up 100 ml	Added to make up 100 ml

Table 1: Composition of the two fractions of Tris-date fruit extender.

Protocol 2 (Vitrification or rapid freezing)

The Vitrification procedure consisting of different cryoprotectants (Table 2) was carried out on the semen samples diluted with fraction 1 solution (Table 1) for the respective treatments as described by Srirattana., *et al*. [13], with some modifications as follows:

- The diluted semen sample (2 ml) was first exposed to 50 µl of holding medium (HM) for 10 minutes.
- Thereafter 50 µl of Vitrification solution 1 (VS-I) was added to the mixture (diluted semen sample + HM) and left for 4 minutes.
- Finally, 50 µl of Vitrification solution 2 (VS-II) was added to the mixture (diluted semen sample + HM + VS-I) and left for 1 minute.

The pH of the extenders including that of the control was determined using a digital pH metre. Following dilution, the diluted semen samples was loaded into 2 ml plastic straws, sealed with polyvinyl and subjected to Vitrification (rapid freezing). The straws containing diluted semen was placed in a canister at 4 cm above liquid nitrogen in a vapour phase for about 10 minutes in order to avoid cold shock before rapidly plunging them directly into the liquid nitrogen. After 72 hours semen samples were retrieved, thawed and evaluated for sperm viability and oxidative stress parameters. Estimations were performed for the pooled semen samples in repeated measurements using different slides and each measurement was repeated five times to ensure accuracy of results.

Cryo-protectant	Chemical composition
Holding medium (HM)	20% BSA (Bovine Serum Albumin) + PBS (Phosphate Buffer Saline) in ration 1:1
Vitrification solution 1 (VS-I)	12.5% ethylene glycol (EG) + 12.5% Dimethylesulfoxide (DMSO) + Holding medium (HM) in ratio 1:1:6
Vitrification solution 2 (VS-II)	25% ethylene glycol (EG) + 25% Dimethylesulfoxide (DMSO) + Holding medium (HM) in ratio 1:1:3

Table 2: Preparation of cryo-protectant for vitrification protocol.

Determination of sperm functional attributes

Sperm progressive motility, Sperm livability and sperm abnormalities was determined as described by Bearden and Fuquay [14].

Sperm progressive motility

The cryopreserved semen samples were thawed briefly in Clifton Water bath (Model: 74178 by Nickel Electro Ltd, Weston-S-Mare Somerset, England) at 37 ºC and assessed for sperm motility using Celestron Penta View microscope (LCD- 44348 by RoHS, China) at 400 × magnification. A 5 µL sample of semen was placed directly on a warmed microscope slide and overlaid with a 22 × 22 mm cover slip. Each semen sample was measured twice (2 slides per sample) and five microscopic fields were examined to observe progressive sperm motility (spermatozoa that move forward essentially in a straight line) by at least three observers and the mean of the five successive evaluations was recorded as the final motility score.

Sperm livability

Sperm livability was evaluated as described by Bearden and Fuquay [14] 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. The samples were observed under Celestron Penta View LCD microscope (× 400 magnification) for live and dead spermatozoa. Spermatozoa that appear white were recorded as live spermatozoa while those that pick up the stain were recorded as dead spermatozoa.

Sperm abnormalities

Sperm abnormalities were evaluated as described by Bearden and Fuquay [14] 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. The percentage of morphologically abnormal spermatozoa with defects in the head, midpiece and tail was observed under CelestronPentaView LCD microscope (× 400 magnification) and recorded.

Total abnormalities = Summation of the head abnormalities + abnormalities midpiece + tail abnormalities

Statistical analysis

Data obtained were subjected to Analysis of Variance (ANOVA) using SAS [15] and Duncan multiple range test [16] was used to separate significantly different means.

Results and Discussion

Main effect of date fruit extender inclusion on sperm functional attributes of WAD bucks

Table 3 shows the main effect of date fruit extender inclusion on sperm functional attributes of WAD bucks. There was no significant

	Parameters (%)					
Levels of inclusion (ml)	Progressive Sperm motility	Livability	Total Abnormalities	Head Abnormality	Mid piece Abnormality	Tail Abnormality
0	50.91	67.41	43.70	13.57	11.71	18.43
5	51.79	75.93	58.23	9.00	19.84	29.39
10	46.59	85.66	43.59	9.46	8.10	26.03
15	40.95	74.47	59.55	9.27	15.99	34.29
20	43.88	75.44	47.14	5.56	10.19	31.40
SEM	4.282	6.194	9.275	6.194	4.217	5.625
P-value	0.361	0.382	0.289	0.704	0.321	0.351

Table 3: Main effect of date fruit extender inclusion on sperm functional attributes of WAD bucks.

SEM = Standard error of mean.

(P > 0.05) difference in all the parameters considered on sperm functional attributes (Progressive sperm motility, Livability and Abnormalities) of WAD bucks with date fruit as semen extender.

Main effect of cryopreservation method on sperm functional attributes of WAD bucks

Table 4 shows the main effect of cryopreservation methods on sperm functional attributes of WAD bucks. The progressive sperm motility of the cryopreserved semen with slow freezing method was better (P < 0.05) than semen cryopreserved with rapid freezing method. This is attributed to the slow cooling rate that resulted in low osmotic injury to the cells which agrees with Daramola and Adekunle [17] who suggested that WAD spermatozoa were better preserved with slow freezing than rapid freezing. The livability increased (P < 0.05) with rapid freezing compared to the slow freezing cryopreservation method. Livability percentage of the spermatozoa is essential to ascertain available cells in their best functional state that could promote fertility. Rodríguez-Martínez [18] reported maximal 30% livability but livability of above 80% was observed in this study and therefore it is sufficient and viable to ensure fertilization of the does during insemination. Total abnormality of the cryopreserved semen was higher (P < 0.05) in the rapid freezing unlike in the slow freezing. In slow freezing, higher (P < 0.05) sperm head abnormality was recorded than in rapid freezing method (15.45 ± 2.432% vs 3.29 ± 2.432%). However, mid-piece abnormality was recorded with increase (P > 0.05) in rapid freezing than slow freezing. While in tail abnormality, elevated (P < 0.05) values were obtained in rapid freezing than slow freezing method. Morphological quality of spermatozoa plays crucial roles in the fertility of livestock [19]. Lower abnormalities obtained in slow freezing compared to the rapid freezing is related to the cryoprotective potential of amino acid in date fruit extender which have beneficial effect on the viability of spermatozoa under slow freezing [20].

	Parameters					
Cryoprotocols	Progressive Sperm motility	Livability	Total Abnormalities	Head Abnormality	Mid piece Abnormality	Tail Abnormality
Slow freezing	54.03^{a}	69.47^{b}	42.73^{b}	15.45^{a}	10.24	17.05^{b}
Rapid freezing	39.62^{b}	82.10^{a}	58.15^{a}	3.29^{b}	16.09	38.77^{a}
SEM	2.708	3.917	5.866	2.432	2.670	3.558
P-Value	0.001	0.034	0.042	0.002	0.136	0.008

Table 4: Main effect of cryopreservation methods on sperm functional attributes of WAD bucks.

$^{a\,b}$ Means with different superscripts in the same column differ significantly (P < 0.05).

SEM = Standard error of mean.

Interactive effect of cryopreservation methods and date fruit extender inclusion levels on sperm functional attributes in WAD bucks

Table 5 shows the interactive effect of cryopreservation methods and date fruit extender inclusions levels on sperm functional attributes of WAD bucks. There were no significant (P > 0.05) differences in cryopreservation methods and date fruit extender inclusion levels on sperm functional attributes in WAD bucks except progressive sperm motility. The highest (P < 0.05) was recorded in 10 ml inclusion of date palm fruit extender under slow freezing (SF), followed by 0 ml of slow freezing and 5 ml of rapid freezing. This is similar to the report of Daramola and Adekunle [21] who observed positive effect of pineapple and cucumber extender inclusions in the storage of WAD buck semen by improving the sperm motility. In this study, date fruit extender sustained the motility of the spermatozoa. This could be attributed to the sugar level in date fruit [22] because sugar is the source of osmolyte, cryoprotectant and energy as the main source for metabolism [23].

		Parameters					
Cryoprotocols	**Levels of inclusion (ml)**	**Progressive Sperm motility**	**Livability**	**Total Abnormalities**	**Head Abnormality**	**Mid piece Abnormality**	**Tail Abnormality**
Slow freezing	0	54.13^{a}	60.28	55.09	25.46	12.04	17.59
	5	51.27^{ab}	62.96	45.14	12.04	13.66	19.44
	10	59.92^{a}	81.76	43.38	12.87	9.77	20.79
	15	51.98^{ab}	73.49	49.19	18.54	15.77	14.88
	20	52.87^{a}	68.85	20.85	8.33	0.00	12.52
Rapid freezing	0	47.70^{abc}	74.54	32.31	1.67	11.39	19.26
	5	52.30^{ab}	88.89	71.32	5.97	26.02	39.33
	10	33.25^{cd}	89.55	43.80	6.05	6.48	31.27
	15	29.92^{d}	75.46	69.90	0.00	16.20	53.70
	20	34.91^{ab}	82.04	73.43	2.78	20.37	50.28
	SEM	3.250	3.155	5.470	2.531	2.279	4.735
	P-value	0.021	0.080	0.054	0.339	0.260	0.114

Table 5: Interactive effect of cryopreservation methods and date fruit extender on sperm functionalattributes in WAD bucks.

^{a,b,c,d} Means with different superscripts in the column differ significantly (P < 0.05).

SEM = Standard error of mean.

Conclusion

The slow freezing cryopreservation method increased progressive sperm motility and reduced total abnormalities. The main effect of date fruit extender inclusion levels did not significantly influence sperm functional attributes in WAD bucks. Date fruit extender also showed best sperm progressive motility at 10 ml inclusion with slow freezing method of cryopreservation.

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