Cryosurvival of goat spermatozoa in tris coconut milk extender supplemented with vitamin B_6

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Abstract

To maintain sperm for longer periods and cryopreserve semen, dilution with a protective extender is necessary in order to maintain fertilizing capacity of spermatozoa during in vitro storage at low temperatures. The effect of vitamin B_6 in the extender of cryopreserved semen obtained from West African Dwarf bucks was studied. Pooled semen was diluted in 100ML tris coconut milk based extender supplemented with 0, 2, 4, 6 and 8mM of vitamin B_6. The diluted semen samples were cryopreserved for 30 days and thereafter evaluated for sperm quality. Following cryopreservation, acrosome reaction and capacitation of spermatozoa were induced in vitro. The results showed higher ($P<0.05$) spermatozoa motility, acrosome integrity, membrane integrity and live sperm in extenders supplemented with vitamin B_6 compared to the control group. Motility was better sustained at all levels of vitamin B_6 inclusions, acrosome integrity and membrane integrity were better sustained in extender supplemented with 8mM inclusion levels of vitamin B_6 ($P<0.05$). Lower ($P<0.05$) concentrations of malondialdehyde (MDA) and leukocytes in extender supplemented with vitamin B6 were observed. The extenders supplemented with vitamin B_6 had the highest ($P<0.05$) values of arginase activity at 8mM inclusion. More spermatozoa cryopreserved with vitamin B_6 underwent acrosome reaction and capacitation compared to the control ($P < 0.05$) and other level of inclusion. Although a suitable supplementary level of vitamin B_6 in semen extender for one of the functional parameters of the cryopreserved buck sperm may not necessarily be the best supplementary level for the other sperm functions, the findings indicated that supplementation of semen extender with vitamin B6 improved the quality of cryopreserved spermatozoa of West African dwarf bucks.

Key words: Antioxidants, cryopreservation, pyridoxine, semen traits, WAD bucks

Cryosurvie des spermatozoïdes de boucs Nains d'Afrique de l'Ouest dans un milieu de dilution à base de lait de coco enrichi en vitamine B_6

Résumé

La cryoconservation du sperme est une technique essentielle pour la reproduction animale, permettant la conservation à long terme du matériel génétique de haute valeur. Afin de maintenir la capacité fécondante des spermatozoïdes pendant le stockage in vitro à basse température, une dilution avec un milieu de conservation protecteur s'avère nécessaire. Cette étude examine l'effet de la vitamine B6 sur la qualité des spermatozoïdes de boucs West African Dwarf cryoconservés. Le sperme collecté a été dilué dans un milieu à base de lait de coco enrichi en Tris (100 mL) et supplémenté en vitamine B_6 à différentes concentrations (0, 2, 4, 6 et 8 mM). Les échantillons dilués ont été cryoconservés pendant 30 jours avant d'être évalués pour leur qualité spermatique. La réaction acrosomique et la capacitation des spermatozoïdes ont été induites in vitro après la cryoconservation. Les résultats ont démontré une amélioration significative ($p < 0,05$) de la motilité, de l'intégrité acrosomique et membranaire des spermatozoïdes, ainsi que de la proportion de spermatozoïdes vivants dans les milieux supplémentés en vitamine B_6 par rapport au groupe témoin. La motilité spermatique était maintenue de manière optimale à toutes les *concentrations de vitamine B_6 testées. L'intégrité acrosomique et membranaire était quant à elle mieux préservée dans le milieu enrichi à 8 mM de vitamine B_6 ($p < 0,05$). De plus, les concentrations de malondialdehyde (MDA) et de leucocytes étaient significativement plus faibles ($p < 0,05$) dans les milieux supplémentés en vitamine B_6. Ces mêmes milieux ont également présenté les valeurs les plus élevées d'activité arginasique ($p < 0,05$) à la concentration de 8 mM. Enfin, un taux plus important de spermatozoïdes cryoconservés avec de la vitamine B_6 a subi une réaction acrosomique et une capacitation comparativement au groupe témoin ($p < 0,05$) et aux autres concentrations testées. En conclusion, bien qu'un niveau optimal de supplémentation en vitamine B_6 puisse varier selon les paramètres fonctionnels étudiés, cette étude démontre que l'ajout de vitamine B_6 au milieu de dilution améliore globalement la qualité des spermatozoïdes cryoconservés de boucs West African Dwarf.*

Mots-clés : Antioxydants, cryoconservation, pyridoxine, paramètres spermatiques, boucs West African Dwarf

Introduction

Semen preservation helps to keep semen over extended period of time such that it could be used for subsequent artificial insemination. However, viability of spermatozoa deteriorates at low temperatures during storage (Osama *et al.,* 2019) due to oxidative stress resulting from lipid peroxidation, which can lead to membrane damage, reduced sperm viability and lower fertility (Ahmad *et al.,* 2023). Goat spermatozoa in particular are sensitive to peroxidative damage due to the high content of unsaturated fatty acids in the phospholipids of the plasma membrane and the relative low antioxidant capacity of goat seminal plasma (Watson, 2000). Although semen contains antioxidants that balance lipid peroxidation and prevent excessive peroxide formation (Lewis *et al.,* 1997), the endogenous antioxidative capacity of semen may be insufficient during storage (Maxwell and Salamon, 1993). *In vitro* study suggested that the addition of some antioxidants to semen extender could improve the motility and survival of spermatozoa (Bilodeau *et al*. 2002). In vitro studies suggested that addition of some antioxidants to semen extenders could improve motility and survival of spermatozoa (Bilodeau *et al.,* 2002). The antioxidative properties of pyridoxine (vitamin B_6) as a scavenger for free radicals have been reported (Grimble, 1997; Kannan and Jain, 2004). Vitamin E and C have also been demonstrated as effective antioxidants in preservation of functional parameters of mammalian spermatozoa against oxidative stress (Yousef *et al.,* 2003; Ondei *et al.,* 2009). Vit B_6 (Pyridoxine) is an antioxidant (Kannan and Jain, 2004) and its protective effect on goat bucks spermatozoa during cryopreservation has been reported (Daramola *et al.,* 2015)

The antioxidative properties of pyridoxine (Vitamin B_6) as a scavenger for free radicals have been reported (Kannan and Jain, 2004). Information on protective effect of pyridoxine in extender for preserving spermatozoa of WAD bucks during cryopreservation is however is limited. The objective of this study was therefore to determine the effect of pyridoxine in semen extender on cryosurvival of spermatozoa obtained from WAD bucks.

Materials and Methods

The experiment was carried out at the Goat Unit, Directorate of University Farm (DUFARM), Federal University of Agriculture, Abeokuta which falls within 7^0 10'N and 3^0 2'E and altitude76m above sea level. It lies between South-Western part of Nigeria with a prevailing tropical climate, a mean annual rainfall of 1,037mm and average temperature of 34.7^0C.

Twenty intact WAD bucks ranged between 30 – 36 months of age and kept under semi intensive management system were used for this study. The animals were kept under intensive management and maintained under a uniform and constant nutritional regime with concentrate feed supplemented with Guinea grass (*Panicum maximum*).

Preparation of coconut milk

Mature ripe coconut was split open and the white coconut flesh was removed from the shell. Using a sterilized knife, the brownish part at the back of coconut flesh was removed. The coconut flesh was cut into bits and blended, without addition of water, in a clean electric blending machine. The finely blended flesh was wrapped in a clean sterilized white handkerchief. This was squeezed manually by hand. The extracted coconut juice was dispensed into clean sterilized centrifuge glass tubes, tightly capped, and centrifuged at 3000 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.

Semen collection, dilution and storage

Semen samples were collected from six WAD bucks that responded to semen collection with the aid of artificial vagina. Only ejaculates that reached >80% motility were pooled. The pooled semen samples were diluted at 32 ◦C in a two-step process with a Tris-based extender composed of 2 fractions. The Fraction 1 solution contained Tris-hydroxymethyl-aminomethane (2.42g), citric acid (1.36g), glucose (1g), penicillin (0.028g), Tris coconut milk (20mL) and distilled water made up 100mL as control. Fraction 2 solution had the same composition as the Fraction 1 solution with the addition of 7.0% glycerol (v/v). The pooled ejaculate was split into 5 equal aliquots, diluted with the Fraction 1 solution and supplemented each with 0, 2, 4, 6 and 8mM of vitamin B_6 in 100 mM/L diluent respectively at a final concentration of 1496×10^6 spermatozoa per millilitre and p^H of 6.99. Fraction 2 solution was subsequently added to fraction 1. Diluted semen samples were then loaded into 2 mL plastic straws, sealed with polyvinyl, cooled to 4 ºC at a rate of 0.25 ºC/min and equilibrated at 4 ºC 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 10min before plunging them directly and quickly into liquid nitrogen for 30 days and thereafter evaluated for sperm quality characteristics.

Sperm Motility

Sperm motility was determined as described by Bearden and Fuquay (1997). .

Acrosome Integrity

The percentage of spermatozoa with intact acrosomes was determined according to Ahmad *et al*. (2003).

Sperm Membrane Integrity

Hypo-osmotic swelling test (HOST) assay as described earlier (Jeyendran *et al.,* 1984) was used to determine sperm membrane integrity.

Sperm Abnormality

Sperm abnormality was evaluated as described by Bearden and Fuquay (1997).

MDA concentrations

MDA concentration as index of lipid peroxidation in the stored semen was measured in a thiobarbituric acid reactive substances (TBARS) according to Papastergiadis *et al.,* 2012.

Arginase activity

Arginase activity was carried out according to the procedure of Lowry *et al.,* (1951).

Leukocytes

Peroxidase test as recommended by WHO (1992) was used as follows: A stock solution was prepared by mixing 50 mL distilled water with 50 ml 96% ethanol plus 125 mg benzidine. The working solution was obtained by adding 5 µL 30% H_2O_2 to 4 mL of stock solution. Twenty (20) µL of working solution was mixed with 20 µL of cryopreserved semen in a small test tube. After incubation for 5 min at room temperature, 20 µL of working solution was mixed with 20 µL of phosphate-buffered saline. Then, 10 µL was placed in a haemocytometer, and peroxidase-positive cells (dark brown round cells) were counted.

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 modification as follows: Samples of cryopreserved spermatozoa were washed with non culture medium (Phosphate-Buffered Saline [PBS]), and the pellets were re-suspended in culture medium (Calcium chloride dihydrate 265mg/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 (2.5mg/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. Spermatozoa with intense fluorescence over the acrosome were classified as acrosome intact 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 CTC fluorescence assay as described by Collin *et al*. (2000).

Statistical Analysis

Data obtained were subjected to analysis of variance (ANOVA) using SAS 1999. While Duncan Multiple Range Test (Duncan, 1955) was used to separate significantly different means. The model that was used to analyse the data is stated below:

$Y_{ij} = \mu + A_i + \sum_{ij}$

Where,

Y_{ij}= Dependent variables

μ = Population mean

A_i = Effect due to i^{th} vitamin B_6 inclusion, i = 0, 5, 10, 15, 20

$\sum_{ij}$ = Experimental Error

Results

The results (Table 1) showed higher (P<0.05) sperm motility, acrosome integrity, membrane integrity and live sperm in extenders supplemented with vitamin B_6 compared to the control. Highest (P<0.05) motility was recorded at all levels of vitamin B_6 inclusion levels compared to the control. Spermatozoa cryopreserved with 8mM of vitamin B_6 had the highest (P<0.05) percentage acrosome integrity and membrane integrity compared to other inclusion levels of vitamin B_6 and the control. The results showed lower (P<0.05) percentage abnormalities at all levels of vitamin B_6 supplementation compared to the control.

Table 1: Spermatozoa viability of semen cryopreserved with different levels of vitamin B_6

Parameters	0mM	2mM	4mM	6mM	8mM
Motility (%)	39.05 ± 4.66^b	58.30 ± 2.10^a	57.50 ± 3.05^a	62.55 ± 2.23^a	58.30 ± 1.70^a
Acrosome Integrity (%)	40.00 ± 1.00^d	50.25 ± 1.31^c	57.00 ± 1.81^b	59.75 ± 1.33^b	68.75 ± 0.92^a
Membrane Integrity (%)	48.75 ± 0.65^c	68.50 ± 1.05^b	69.25 ± 0.65^b	68.50 ± 1.59^b	73.25 ± 0.53^a
Livability(%)	62.50 ± 5.59^c	75.00 ± 5.00^a	78.75 ± 3.98^a	72.50 ± 3.13^{ab}	66.25 ± 4.20^b
Abnormality (%)	4.50 ± 0.55^a	2.25 ± 0.41^b	3.00 ± 0.80^b	3.00 ± 0.94^b	3.12 ± 1.04^b

$^{a, b, c, d}$ Values within rows with different superscripts differ significantly (P<0.05)

The results in Table 2 showed lower (P<0.05) concentrations of MDA and leukocytes in extender supplemented with vitamin B_6 compared to the control. The extenders supplemented with vitamin B6 had higher (P<0.05) values of arginase activity compared to the control. However, highest (P<0.05) arginase activity were observed at 8mM of vitamin B_6 supplementation.

Table 2: MDA concentration, arginase activity and leukocyte of semen cryopreserved with different levels of vitamin B_6

Parameters	0mM	2mM	4mM	6mM	8mM
MDA Conc. (nmol/mL)	0.59 ± 0.37^a	0.16 ± 0.05^b	0.18 ± 0.09^b	0.11 ± 0.03^b	0.27 ± 0.06^b
Arginase activity (units/mg protein)	2.03 ± 0.04^d	2.48 ± 0.09^b	2.60 ± 0.14^b	2.26 ± 0.05^c	2.80 ± 0.07^a
Leukocyte (x 10^3/mL)	0.51 ± 0.04^a	0.46 ± 0.04^b	0.42 ± 0.05^b	0.44 ± 0.03^b	0.42 ± 0.04^b

$^{a, b, c, d}$ Values within rows with different superscripts differ significantly (P<0.05)

The results (Table 3) showed that the percentage of cryopreserved spermatozoa with vitamin B6 that underwent acrosome reaction and capacitation followed the same pattern. More spermatozoa cryopreserved with vitamin B_6 underwent acrosome reaction and capacitation compared to the control (P < 0.05). Highest (P<0.05) percentage of acrosome reacted spermatozoa and spermatozoa that underwent capacitation were observed at 4mM, 6mM and 8mM inclusion levels of vitamin B_6 compared to 2mM inclusion levels of vitamin B6 and the control.

Table 3: *In vitro* acrosome reaction (%) and Capacitation (%) of buck spermatozoa cryopreserved with different levels of vitamin B_6

Parameters	0mM	2mM	4mM	6mM	8mM
Acrosome reaction (%)	43.50 ± 5.45^b	43.75 ± 3.01^b	54.50 ± 6.39^a	55.00 ± 3.91^a	57.75 ± 3.59^a
Capacitation (%)	47.50 ± 2.92^b	49.50 ± 2.67^b	59.50 ± 5.50^a	62.00 ± 2.14^a	62.50 ± 2.67^a

$^{a, b,}$ Values within rows with different superscripts differ significantly (P<0.05)

Discussion

This research was designed to determine the cryosurvival effect of vitamin B_6 in Tris- coconut milk based in goat spermatozoa. In this study, our result showed that tris-coconut milk based extender supplemented with different level of Vit B_6 significantly improved motility, acrosome integrity and membrane integrity. Progressive motility was found to be significant when vitamin B_6 was used as an antioxidant source as a supplement in the extender, which could be due to the presence of antioxidant properties that shows scavenging effects against free radicals (Meister, 1992; Kannan and Jain, 2004). Vit B_6 showed significant role in the production of antioxidant enzymes that stimulates the protection system of spermatozoa against different oxidative stressors (Ehrenshaft *et al*., 1999; Bilski *et al*., 2000; Kannan and Jain, 2004). Acrosome and membrane integrities were better preserved in tris-coconut milk based extender supplemented with different level of vit B_6; this indicated the capacity of this extender to maintain sperm functional integrity. Similarly, there are several reports of better post-thaw percentage of plasma membrane integrity when semen is cryopreserved with different level of vit B_6 (Daramola *et al*., 2017; Rezaei *et al*., 2021). Lower values for oxidative parameters indicated tris-coconut milk based extender supplemented with different level of vit B_6 could reduce oxidative stress in sperm during cryopreservation. Mammalian sperm have substantial polyunsaturated fatty acids in their plasma membrane, making them prone to lipid peroxidation (Niki *et al.,* 2005; Mandal *et al.,* 2014). Antioxidant compounds in vit B_6 protected sperm viability by scavenging lipid peroxidation during cryopreservation (Skorna *et al*., 2016). Intracellular MDA concentration is a stress indicator; the optimal MDA concentration obtained tris-coconut milk based extender supplemented with different level of vit B_6 as compared to the control might be due to the antioxidant capacity of the substance in scavenging the effect of ROS. Immature and defective spermatozoa as well as contaminating leucocytes are the primary sources of reactive oxygen species in semen (Agarwal *et al*., 2003; Garrido *et al*., 2004) that lead to damaged membrane functions (Sikka *et al*., 1996). Although seminal leucocytes through their phagocytic role in eliminating defective spermatozoa is beneficial to sperm viability (Henkel, 2011), high level of leukocytes, particularly activated leukocytes have detrimental effect on sperm functions (Henkel, 2011). The low concentration of leukocytes following addition of tris-coconut milk based extender supplemented with different level of vit B_6 in this study compared to the control further indicated the antioxidative capacity of pyridoxine in reducing defective spermatozoa or excessive reactive oxygen species produced by the leukocytes or defective spermatozoa. Sperm function tests such as acrosome reaction and capacitation are better fertility predictors than traditional semen parameters (Katsuki *et al*., 2005). In this study, in vitro acrosome reaction and sperm capacitation were better with tris-coconut milk-based extender supplemented with different level of vit B_6 compared to control, indicating that this extender may enhance fertilizing ability of cryopreserved sperm. There were comparable improvements in functional, fertilizing and seminal oxidative stress end points of goat semen cryopreserved with tris-coconut milk-based extender supplemented with different level of vit B_6.

Conclusion

The addition of vitamin B_6 analogue to extender led to higher rates of integrity of the plasma membrane, motility, acrosome and mitochondrial membrane potential goat spermatozoa. Vitamin B_6 protects spermatozoa through its capacity to quench ROS accumulation and LPO during the process of preservation.

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