

Effects of pyridoxine in combination with different antioxidants on viability and oxidative stress parameters of cryopreserved goat buck semen

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SUMMARY

The effects of pyridoxine alone or combined with different antioxidants on viability and oxidative stress parameters of cryopreserved sperm of goat bucks were studied. Pooled semen samples diluted with tris-egg yolk extenders were supplemented in the Study I: with 0, 2, 4, 6 and 8 mM of pyridoxine; in the study II with 2 mM of pyridoxine (P) each combined with different concentrations (0, 2, 4, 6 and 8 mM) of vitamin E (PE), vitamin C (PC) and melatonin (PM); and in study III: with 2 mM of pyridoxine each supplemented with different combinations of vitamin C, vitamin E and melatonin (PCEM) each at different concentrations (0, 2, 4, 6 and 8 mM). The results showed higher ($p < 0.05$) sperm motility in extenders supplemented with 4 mM and 6 mM pyridoxine, or its combination in PM4 and PM6 followed by PC4, PC6 and PE4; and PCEM6 extenders. Extenders supplemented with pyridoxine alone or its combination at all levels of vitamins E, PM2, PCEM2, PCEM4 and PCEM8 had higher ($p < 0.05$) acrosome integrity. Extenders supplemented with pyridoxine alone or in combination with all levels of vitamins E and PC2, PCEM2 and PCEM8 had higher ($p < 0.05$) membrane integrity. Sperm abnormal and seminal leukocytes reduced ($p < 0.05$) at 4 mM, 6 mM and 8 mM of pyridoxine or in combination with other antioxidants. Extenders supplemented with pyridoxine alone or combinations with antioxidants (PCEM4, PCEM6 and PCEM8) had reduced ($p < 0.05$) malondialdehyde (MDA) concentrations. The findings indicated that extenders supplemented with pyridoxine alone or in combination with other antioxidants improved viability and reduced oxidative stress parameters of cryopreserved sperm of goat bucks.

Efectos de la piridoxina en combinación con diferentes antioxidantes sobre parámetros de viabilidad y estrés oxidativo del semen caprino criopreservado

RESUMEN

Se estudiaron los efectos de la piridoxina sola, o en combinación con diferentes antioxidantes, sobre los parámetros de viabilidad y estrés oxidativo de los espermatozoides caprinos criopreservados. Muestras combinadas de semen, diluidas con diluyente yema de huevo-tris se complementaron, en el estudio I con 0, 2, 4, 6 y 8 mM de piridoxina; en el estudio II con 2 mM de piridoxina (P), en combinación con diferentes concentraciones (0, 2, 4, 6 y 8 mM) de vitamina E (PE), vitamina C (PC) o melatonina (PM); y en el estudio III con 2 mM de piridoxina suplementada con combinaciones de vitamina C, vitamina E y melatonina (PCEM) a diferentes concentraciones (0, 2, 4, 6 y 8 mM). Los resultados mostraron mayor ($p < 0,05$) motilidad del esperma con diluyentes suplementados con 4 y 6 mM de piridoxina, o su combinación en PM4 y PM6, seguido de CP4, PC6 y PE4; y PCEM6. Los diluyentes suplementados solo con piridoxina o su combinación, en todos los niveles con vitamina E. PM2, PCEM2, PCEM4 y PCEM8 mostraron mayor ($p < 0,05$) integridad del acrosoma. Los diluyentes de piridoxina solo, o en combinación con todos los niveles de la vitamina E y con PC2, PCEM2 y PCEM8 determinaron mayor integridad de la membrana ($p < 0,05$). La cantidad de espermatozoides anormales y leucocitos seminales se redujo ($p < 0,05$) con 4 mM, 6 mM y 8 mM de piridoxina o en combinación con otros antioxidantes. Los diluyentes suplementados con piridoxina solo, o con combinaciones de antioxidantes (PCEM4, PCEM6 y PCEM8) redujeron ($p < 0,05$) las concentraciones de malondialdehído (MDA). Los resultados indican que los diluyentes suplementados con piridoxina sola o en combinación con otros antioxidantes mejoran la viabilidad y reducen los parámetros de estrés oxidativo de los espermatozoides caprinos criopreservados.

ADDITIONAL KEYWORDS

Artificial insemination.
Extenders.
Freezing.
Sperm.

PALABRAS CLAVE ADICIONALES

Inseminación artificial.
Diluyentes.
Congelación.
Esperma.

INFORMACIÓN

Cronología del artículo.
Recibido/Received: 25.01.2016
Aceptado/Accepted: 20.07.2016
On-line: 15.01.2017
Correspondencia a los autores/Contact e-mail:
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INTRODUCTION

West African Dwarf (WAD) goats (*Capra hircus*) are endowed with some useful qualities that help to adapt to hot humid tropical conditions (Daramola and Adeloje, 2009). Preservation of gametes from the breed is important for artificial insemination programme in order to sustain the adaptable qualities of this breed. Maintaining sperm viability requires dilution with a protective extender that can sustain fertilizing capacity of spermatozoa during storage at low temperatures. However, sperm quality still reduces during freezing (Maxwell and Watson, 1996) due to susceptibility of goat spermatozoa to lipid peroxidation or excessive production of reactive oxygen species and depleted antioxidants of seminal plasma (Beconi *et al.*, 1993; Watson, 2000).

Studies have shown the effects of antioxidants on sperm viability and in some cases the results are inconclusive or contradictory (Maia *et al.*, 2009; Anghel *et al.*, 2010; Sicherle *et al.*, 2011). Pyridoxine (vitamin B6) is known to possess antioxidative property (Kannan and Jain, 2004). Several findings have supported the role of vitamin E, C and melatonin as effective antioxidants for cell protection against oxidative stress induced by free radicals (Tan *et al.*, 1993; Pähkla *et al.*, 1998; Yousef *et al.*, 2003; Ondei *et al.*, 2009).

Although, the protective effect of pyridoxine has recently been reported (Daramola *et al.*, 2015), information about the ideal combination of pyridoxine with different antioxidants for improving sperm viability of cryopreserved goat bucks is however not available yet in literature. The objective of the present study was therefore to determine the ideal combination of pyridoxine with vitamin E, vitamin C and melatonin on sperm viability parameters (motility, acrosome integrity, membrane integrity, and sperm abnormality) and oxidative stress parameters (malondialdehyde concentrations and leukocytes) of cryopreserved semen obtained from WAD goat bucks.

MATERIALS AND METHODS

The study was investigated at the Goat Unit of Teaching and Research Farm, Federal University of Agriculture, Abeokuta. The area lies between South-Western part of Nigeria with a prevailing tropical cli-

mate, a mean annual rainfall of 1,037mm and average temperature of 34.7°C. and falls within 7° 10'N and 3° 2'E and altitude 76m above sea level. Six (6) WAD goat bucks used for this study were 2.5 to 3 years old. The animals were kept under an intensive management system and maintained under a uniform nutritional regimen with concentrate feed supplemented with guinea grass (*Panicum maximum*).

SEMEN COLLECTION, DILUTION AND STORAGE

Semen samples showing > 80 % motility were collected from 6 WAD goat bucks with the aid of an artificial vagina and pooled. The pooled semen samples were diluted at room temperature in a two-step process with Tris-based extenders composed of 2 fractions in 3 studies.

STUDY I

Fraction I solution contained Tris-extender consisting of Tris-hydroxymethyl-aminomethane (2.42 g), citric acid (1.36 g), glucose (1 g), penicillin (0.028 g), egg yolk (20 mL) and distilled water made up 100 mL. Each pooled ejaculate was divided into 5 equal parts, diluted with Fraction I solution plus addition of different levels of pyridoxine (Superdrug Stores plc, Admail 838, Croydon, U.K) as follows:

- Tris-extender + 2 mM pyridoxine
- Tris-extender + 4 mM pyridoxine
- Tris-extender + 6 mM pyridoxine
- Tris-extender + 8 mM pyridoxine
- Tris-extender (without pyridoxine) as Control

Fraction I and II solutions had similar composition but with addition of 14.0% glycerol (v/v) to Fraction II. Equivalent volume of Fraction II solution (Fraction I and Fraction II at ratio 1:1) was thereafter added.

STUDY II

Fraction I and II solutions had similar composition as in study I but with addition of 14.0% glycerol (v/v) to Fraction II. Each pooled ejaculate was divided into 13 equal parts, diluted with Fraction I solution plus addition of pyridoxine and other antioxidants as follows:

- Tris-extender + 2 mM pyridoxine + 2 mM vitamin E (PE2)

Table I. Means sperm viability parameters, malondialdehyde concentration and leukocytes of WAD goat buck semen cryopreserved with different levels of pyridoxine (Parámetros medios de viabilidad del esperma, concentración de malondialdehído y leucocitos de semen caprino (WAD) criopreservado con diferentes niveles de piridoxina).

Extenders	MOT (%)	ACI (%)	MEI (%)	ABN (%)	MDA (nmol/mL)	LEU (x10 ³ /mL)
Control	44.33 ^d	77.00 ^b	77.50 ^b	1.25 ^a	0.11 ^a	0.46 ^a
2 mM	55.67 ^c	82.50 ^a	88.00 ^a	1.08 ^a	0.02 ^c	0.45 ^a
4 mM	58.33 ^{ab}	86.00 ^a	89.50 ^a	0.92 ^b	0.09 ^b	0.32 ^b
6 mM	61.33 ^a	82.50 ^a	91.50 ^a	0.83 ^b	0.08 ^b	0.29 ^b
8 mM	55.67 ^c	85.50 ^a	90.50 ^a	0.83 ^b	0.04 ^{bc}	0.44 ^b
SEM	6.71	1.19	1.11	0.14	0.02	0.06

a, b, c, d Values within columns with different superscripts differ (p<0.05); MOT = Motility, ACI = Acrosome Integrity, MEI = Membrane Integrity, ABN = Abnormality, LEU = Leukocytes, SEM = Standard Error Mean

Tris-extender + 2 mM pyridoxine + 4 mM vitamin E (PE4)

Tris-extender + 2 mM pyridoxine + 6 mM vitamin E (PE6)

Tris-extender + 2 mM pyridoxine + 8 mM vitamin E (PE8)

Tris-extender + 2 mM pyridoxine + 2 mM vitamin C (PC2)

Tris-extender + 2 mM pyridoxine + 4 mM vitamin C (PC4)

Tris-extender + 2 mM pyridoxine + 6 mM vitamin C (PC6)

Tris-extender + 2 mM pyridoxine + 8 mM vitamin C (PC8)

Tris-extender + 2 mM pyridoxine + 2 mM melatonin (PM2)

Tris-extender + 2 mM pyridoxine + 4 mM melatonin (PM4)

Tris-extender + 2 mM pyridoxine + 6 mM melatonin (PM6)

Tris-extender + 2 mM pyridoxine + 8 mM melatonin (PM8)

Tris-extender only without antioxidant as Control

Equal volume of Fraction II solution was subsequently added.

STUDY III

Each pooled ejaculate was divided into 4 equal parts, diluted with the Fraction I solution plus addition of pyridoxine plus different combinations of other antioxidants as follows:

Tris-extender + 2 mM pyridoxine + 2 mM each of vitamin C, E and melatonin (PCEM2)

Tris-extender + 2 mM pyridoxine + 4 mM each of vitamin C, E and melatonin (PCEM4)

Tris-extender + 2 mM pyridoxine + 6 mM each of vitamin C, E and melatonin (PCEM6)

Tris-extender + 2 mM pyridoxine + 6 mM each of vitamin C, E and melatonin [PCEM8]

Tris-extender without antioxidant as Control

Equal volume of Fraction II solution was subsequently added.

In studies I, II and III, diluted semen samples were loaded into 2 mL plastic straws, sealed with polyvinyl, cooled to 4 °C at a rate of 0.25 °C /min and equilibrated at 4 °C for 10 min in Refrigerated Incubator (TYFSF SPX-70B III, Hebei, China). Subsequently, the straws were placed in a rack at 4 cm above liquid nitrogen in the vaporous phase for 10 min before plunging them directly into liquid nitrogen for 96 h and thereafter evaluated for sperm quality and oxidative parameters.

SUBJECTIVE EVALUATION OF SPERM MOTILITY

Progressive sperm motility was evaluated in line with procedure described by Bearden and Fuquay (1997). Semen was thawed for 2 min in Water bath (74178-Clifton Water Bath, Nickel Electro Ltd, Weston-S-Mare Somerset, England) at 37 °C and assessed for sperm motility using Celestron PentaView digital microscope (LCD-44348 by RoHS, China) at 400x magnification. sample of semen (5 µL) was placed directly on a warmed microscope slide and overlaid with a cover slip (22 x 22 mm). For each sample, five microscopic fields were examined to observe progressively motile spermatozoa that moved forward in basically a straight

Table II. Means sperm viability parameters, malondialdehyde concentration and leukocytes of WAD goat buck semen cryopreserved with 2 mM pyridoxine in combination with different levels of vitamin E, C and melatonin (Parámetros medios de viabilidad del esperma, concentración de malondialdehído y leucocitos de semen caprino (WAD) criopreservado con 2 mM de piridoxina en combinación con diferentes niveles de vitamina E, C y melatonina).

Extenders	MOT (%)	ACI (%)	MEI (%)	ABN (%)	MDA (nmol/ mL)	LEU (x 10 ⁹ / mL)
Control	44.33 ^e	77.00 ^c	77.50 ^c	1.25	0.38 ^a	0.46 ^a
PE2	56.80 ^d	86.00 ^{ab}	93.00 ^a	1.00	0.35 ^b	0.49 ^a
PE4	74.80 ^b	85.50 ^{ab}	92.00 ^a	0.75	0.22 ^e	0.25 ^b
PE6	66.80 ^c	89.50 ^a	92.00 ^a	0.67	0.26 ^d	0.28 ^b
PE8	68.80 ^c	92.00 ^a	94.50 ^a	0.42	0.25 ^d	0.27 ^b
PC2	58.60 ^d	77.80 ^c	88.50 ^a	0.75	0.30 ^c	0.45 ^a
PC4	77.20 ^b	78.50 ^c	81.50 ^b	1.00	0.21 ^e	0.35 ^{ab}
PC6	77.00 ^b	78.50 ^c	86.50 ^{ab}	0.92	0.23 ^{de}	0.31 ^b
PC8	58.40 ^d	76.50 ^c	84.00 ^b	0.75	0.24 ^{de}	0.40 ^{ab}
PM2	53.40 ^d	84.00 ^{ab}	84.00 ^b	0.75	0.36 ^b	0.42 ^{ab}
PM4	89.20 ^a	73.50 ^c	78.50 ^c	0.92	0.17 ^f	0.47 ^a
PM6	80.60 ^a	77.50 ^c	83.00 ^b	0.75	0.25 ^d	0.30 ^b
PM8	64.40 ^c	74.50 ^c	80.00 ^b	0.83	0.17 ^f	0.17 ^c
SEM	4.39	1.25	1.64	0.11	7.69	0.07

^{a, b, c, d, e, f} Values within columns with different superscripts differ (p<0.05); MOT = Motility, ACI = Acrosome Integrity, MEI = Membrane Integrity, ABN = Abnormality, LEU = Leukocytes, SEM = Standard Error Mean

line by three observers. The mean of the five successive observations was recorded as the final motility score.

ACROSOME INTEGRITY

The percentage of spermatozoa with intact acrosomes was determined according to Ahmad *et al.* (2003). Semen sample (50 μ) was added to a 500 μ L formalin citrate solution (96 mL 2.9 % sodium citrate, with 4 mL 37 % formaldehyde) and mixed carefully. A small drop of the mixture was placed on a microscope slide and a total of 200 spermatozoa were counted in at least three different microscopic fields for each sample, using Celestron PentaView LCD digital microscope (400x magnification). Spermatozoa that showed normal apical ridge in acrosome region were assessed as intact acrosomes.

SPERM MEMBRANE INTEGRITY

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

SPERM ABNORMALITY

Sperm morphological abnormalities were evaluated in line with procedure by Bearden and Fuquay (1997) with the use of eosin-nigrosin smears. A thin smear of mixture of semen and eosin-nigrosin solution was drawn across the slide and dried. The percentage of morphologically abnormal spermatozoa with defects in the head, midpiece and tail were observed under Celestron PentaView LCD digital microscope (400x magnification).

MALONDIALDEHYDE (MDA) CONCENTRATION

Malondialdehyde concentration in the stored semen was determined in a thiobarbituric acid reactive substance (TBARS) as described by Papastergiadis *et al.* (2012). Sperm suspension (0.1 mL) was incubated with 0.1 mL of 150 mM Tris-HCl (pH 7.1) for 20 min at 37 °C. Afterward, 1 mL of 10 % trichloroacetic acid (TCA) and 2 mL of 0.375 % thiobarbituric acid was added and then incubated in boiling water for 30 min. Thereafter, it was centrifuged for 15 min at 3000 g and the absorbance was read with UV spectrophotometer (SW7504 model by Surgifriend Medicals, England) at 532 nm. The concentration of MDA was calculated as follows: The concentration of MDA (nmol/mL) = AT - AB/1.56 $\times 10^5$; Where: AT = the absorbance of the semen sample, AB = the absorbance of the blank, 1.56 $\times 10^5$ molar absorptivity of MDA.

SEMINAL LEUKOCYTES

Seminal leukocytes were counted using peroxidase test adapted from Endtz (1974) and as recommended by WHO (1992). Mixture of distilled water (50 mL), 50

mL 96 % ethanol and 125 mg benzidine was prepared as stock solution. A working solution was obtained by adding 5 μ L 30 % H₂O₂ to 4 mL of stock solution. The working solution (20 μ L) was mixed with cryopreserved semen (20 μ L) in a small test tube and incubated for 5 min at room temperature. Subsequently 20 μ L of working solution was mixed with 20 μ L of phosphate-buffered saline. Then, 10 μ L was placed in a haemocytometer, and peroxidase-positive cells (dark brown round cells) were counted.

STATISTICAL ANALYSIS

Data obtained were subjected to analysis of variance (ANOVA) using SAS 1999 (in a 3 \times 5 factorial arrangement in study II and III), while Duncan Multiple Range Test (Duncan, 1955) was used to separate significantly different means.

The models used are shown below.

Study I

$$Y_{ij} = \mu + L_i + \Sigma_{ij}$$

Where,

Y_{ijk} = Dependent variables

μ = Population mean

L_i = effect due to i^{th} level of pyridoxine inclusion, $i = (0, 2, 4, 6, 8)$

Σ_{ij} = experimental error

Study II and Study III

$$Y_{ijk} = \mu + A_i + B_j + (AB)_{ij} + \Sigma_{ijk}$$

Where,

Y_{ijkl} = Dependent variables

μ = Population mean

A_i = Effect due to i^{th} combination of antioxidants $i = (1, 2, 3)$,

B_j = Effect due to j^{th} level of antioxidants inclusion, $j = (0, 2, 4, 6, 8)$

AB_{ij} = Effect of ij^{th} interaction between combination of antioxidants and levels of antioxidants inclusion

Σ_{ijkl} = Experimental error

RESULTS

Sperm viability parameters of WAD goat buck semen cryopreserved with extenders supplemented with different levels of pyridoxine are shown in **Table I**. The results showed higher ($p < 0.05$) sperm motility, acrosome integrity and membrane integrity in extenders supplemented with pyridoxine compared to the control. Spermatozoa cryopreserved with 4 mM and 6 mM of pyridoxine had the highest percentage motility ($p < 0.05$). However, acrosome integrity and membrane integrity were comparable among the different levels of pyridoxine. Reduced percentage of abnormal spermatozoa and seminal leukocytes were more pronounced ($p < 0.05$) in extenders supplemented with 4 mM, 6 mM and 8 mM. Reduced concentration of MDA

($p < 0.05$) was recorded in extenders supplemented with pyridoxine compared to the control.

Table II shows sperm viability parameters of WAD goat buck semen cryopreserved with extenders supplemented with 2 mM pyridoxine in combination with different levels of other antioxidants. The results showed higher ($p < 0.05$) sperm motility in extenders supplemented with pyridoxine in combination with other antioxidants compared to the control. Optimal motility was observed in PM4 and PM6 extenders followed by PC4, PC6 and PE4. Higher acrosome integrity was observed in extenders supplemented with pyridoxine in combination with all levels of vitamins E and PM2 compared to other treatments and the control. Higher membrane integrity was observed in extenders supplemented with pyridoxine in combination with other antioxidants compared to the control except PM4. Abnormal sperm cells were comparable among the different extenders. Reduced MDA concentrations were observed among the different antioxidants in combinations with pyridoxine compared to the control. Seminal leukocytes were comparable among the extenders except PM8 that had the lowest values.

Sperm viability parameters of WAD goat buck semen cryopreserved with extenders supplemented with 2 mM pyridoxine in combination with different antioxidants at different levels each are presented in **Table III**. The results showed higher ($p < 0.05$) sperm motility in extenders supplemented with pyridoxine in combination with other antioxidants compared to the control. Highest ($p < 0.05$) motility was observed in PCEM6 and followed by PCEM8. Semen extenders supplemented with PCEM2, PCEM4 and PCEM8 had the highest acrosome integrity. The results showed higher ($p < 0.05$) membrane integrity in extenders supplemented with pyridoxine in combination with other antioxidants compared to the control and the highest values were obtained in extenders supplemented with PCEM2 and PCEM8. Reduced ($p < 0.05$) percentage of abnormal sperm cells and seminal leukocytes were observed in extenders supplemented with pyridoxine in combination with other antioxidants compared to the control. Reduced MDA concentrations were obtained in extenders supplemented with PCEM4, PCEM6 and PCEM8 compared to PCEM2 and the control.

DISCUSSION

The supplementation of pyridoxine alone in the present study improved the percentage of sperm motility, acrosome and membrane integrities. The improvement in these parameters coupled with reduced MDA concentration in extenders supplemented with pyridoxine revealed the beneficial impact of pyridoxine on sperm viability and this could be attributed to its potential antioxidant property. Pyridoxine has been reported to have antioxidant properties (Kannan and Jain, 2004). Pyridoxine as a phenolic compound has been reported to function as an antioxidant (Ehrenschaft *et al.*, 1999; Bilski *et al.*, 2000; Kannan and Jain, 2004). In addition, pyridoxine as a cofactor of glutathione peroxidase could destroy hydrogen peroxide, thereby serving as a modulator of oxidative stress (Meister, 1992; Kannan and Jain, 2004).

Antioxidants are generally accepted and satisfactory to be important in cell functions (Pierre *et al.*, 1994; Kazez *et al.*, 2000; Lee *et al.*, 2001). Cryopreserved cells are subjected to stress that leads to membrane changes. These changes during freeze-thawing process obstruct the transbilayer phospholipids asymmetry of mammalian sperm and damage to plasma membrane increases susceptibility to lipid peroxidation due to high production of reactive oxygen species (Anghel *et al.*, 2010). These harmful effects eventually lead to impairment of sperm motility and functional membrane integrity (Aitken, 1984; Alvarez and Storey, 1989; Aitken *et al.*, 1993). The present study agreed with recent finding by Daramola *et al.* (2015) that goat buck extender supplemented with pyridoxine improved sperm motility, capacitation and acrosome reaction during cryopreservation and this could be due to its capacity to enter to mitochondria as water-soluble antioxidant (Mahfouz *et al.*, 2009). Sperm motility is the most commonly affected characteristics during cryopreservation and the main cause of reduced fertility after freezing/thawing (Donnelly *et al.*, 2001). It has been suggested that motility, membrane integrity and mitochondrial functions are similarly affected by cryopreservation (Henry *et al.*, 1993), an indication of partial dependence of sperm motility on mitochondrial function. Moreover, mitochondria in sperm cells encase axosome, connect with dense fibers in mid-piece and

Table III. Means sperm viability parameters, malondialdehyde concentration and leukocytes of WAD goat buck semen cryopreserved with 2 Mm pyridoxine in combination with vitamins C, E and melatonin each at different levels (Parámetros medios de viabilidad del esperma, concentración de malondialdehído y leucocitos de semen caprino (WAD) criopreservado con 2 mM de piridoxina en combinación con diferentes niveles de vitaminas C y E y melatonina).

Extenders	MOT (%)	ACI (%)	MEI (%)	ABN (%)	MDA (nmol/ mL)	LEU ($\times 10^3$ / mL)
Control	29.60 ^d	76.00 ^c	64.00 ^c	1.30 ^a	0.29 ^a	0.54 ^a
PCEM2	56.80 ^c	79.00 ^a	93.50 ^a	0.83 ^b	0.32 ^a	0.40 ^b
PCEM4	53.40 ^c	77.50 ^{ab}	81.00 ^b	0.92 ^b	0.22 ^b	0.25 ^c
PCEM6	80.40 ^a	72.50 ^d	84.50 ^b	0.58 ^b	0.22 ^b	0.25 ^c
PCEM8	72.40 ^b	79.50 ^a	89.00 ^a	0.67 ^b	0.21 ^b	0.35 ^{bc}
SEM	3.56	1.27	1.56	0.08	0.01	0.06

^{a, b, c, d.} Values within columns with different superscripts differ ($p < 0.05$); MOT = Motility, ACI = Acrosome Integrity, MEI = Membrane Integrity, ABN = Abnormality, LEU = Leukocytes, SEM = Standard Error Mean

produce adenosine triphosphate (Perumal, *et al.*, 2013) and high level of reactive oxygen species could result in damaged axoneme and mitochondria in the sperm (Aitken and Clarkson, 1987). The study therefore indicated that pyridoxine alone or in combination with other antioxidants such as vitamins C and E as well as melatonin could maintain sperm viability parameters as evidenced by improved percentage of motility, acrosome integrity, membrane integrity, acrosome reaction and reduced MDA concentrations of cryopreserved spermatozoa. The results further supported the role of pyridoxine as antioxidant in maintaining sperm parameters during cryopreservation and agreed with Daramola *et al.* (2016) that these antioxidants are compatible in improving sperm viability.

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 (Kaleli, *et al.*, 2000; Henkel, 2011), high level of leucocytes, particularly activated leukocytes have detrimental effect on sperm functions (Henkel, 2011). The low concentration of leukocytes following addition of pyridoxine alone or in combinations with other antioxidants 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.

Melatonin as an antioxidant has been shown to maintain and preserve mitochondria through different mechanisms (Martin *et al.*, 2000). The ability of melatonin as a potent cyclic AMP (cAMP) stimulator (Yung *et al.*, 1995) could account for its capacity to trigger sperm motility through its direct action on axoneme of sperm tail (Lindemann, 1978) or indirectly through its action on cell membrane as secondary messenger (Garbers and Kopf, 1980). The results in the present study agreed with Kaya *et al.* (2001) who observed that melatonin administration to rams *in vivo* improved post-thawed sperm viability.

Vitamin E is similar to melatonin in membranes due to its ability to directly suppress free radicals (Sinclair, 2000; Ball *et al.*, 2001; Yousef *et al.*, 2003). The results were in consonant with previous study that supplementation of some antioxidants (vitamins A, C or E) to semen extenders improved motility, viability, morphology and fertilizing ability of stored roosters' semen (El-Nasry *et al.*, 2004). The role of vitamin C in the restoration of physiological composition of polyunsaturated fatty acids in cell membrane has been reported and therefore important in sperm functions (Lewis *et al.*, 1997). This result indicated that these antioxidants play important role in reducing damage associated with excessive reactive oxygen species production during cryopreservation (Aitken and Clarkson, 1987). The improved sperm viability parameters could also be due to appropriate incorporation of these antioxidants in the extenders.

CONCLUSION

The improvement in motility was best in 4 mM, 6 mM, PM4, PM6 and PCEM6 when the extenders were supplemented with pyridoxine alone or in combinations with different antioxidants. A suitable supplementary level of pyridoxine alone or in combinations with other antioxidants was not necessarily the best for all sperm parameters, however the findings indicated that supplementation of semen extenders with pyridoxine alone or in combinations with other antioxidants improved sperm viability and reduced oxidative stress parameters.

ACKNOWLEDGEMENTS

The animals and chemicals used in this study were funded by Federal University of Agriculture Abeokuta, Nigeria through the Directorate of Grant Management of the under the grant number FUNAAB-DGM/01-2012. The Authors are also grateful to the Laboratory Technologists of the Department of Animal Physiology, Federal University of Agriculture Abeokuta for their technical assistance.

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