Original Research Article

Soybean milk extender improves sperm functional and oxidative stress parameters of goat sperm during slow and rapid freezing

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Abstract

The objective of this study was to determine effects of soybean milk extender on sperm quality end points of cryopreserved goat sperm. Pooled ejaculates from West African Dwarf (WAD) goats were diluted with various amounts (0, 5, 10, 15, 20 ml) of soybean milk (SBM) in Tris-extenders, subjected to slow or rapid freezing for 30 days and subsequently thawed and evaluated. Inclusion of SBM in extenders improved sperm quality ($P < 0.05$) compared to control (egg yolk-based extender) for both slow and rapid freezing methods. Semen cryopreserved with 10 or 15% SBM extender had higher ($P < 0.05$) motility compared to other concentrations and the control using slow freezing. Although both cryoprotocols had higher ($P < 0.05$) acrosome integrity when cryopreserved with 20% SBM extender, acrosome integrity at 20% SBM was higher ($P < 0.05$) in rapid freezing compared to slow freezing. Semen cryopreserved with SBM extenders had lower ($P < 0.05$) Malondialdehyde (MDA) concentrations in 10 to 20% SBM extenders for both freezing methods, although MDA concentrations were lower ($P < 0.05$) in slow freezing compared to rapid freezing. Semen cryopreserved with SBM extenders in both protocols had fewer ($P < 0.05$) leukocytes and a higher ($P < 0.05$) acrosome reaction and sperm capacitation, whereas there was a higher ($P < 0.05$) acrosome reaction with 10 and 5% SBM extenders using slow and rapid freezing respectively. In conclusion, for goat semen cryopreserved using slow or rapid freezing protocols, SBM extenders improved functional, fertilizing and seminal oxidative stress end points.

Keywords: cryopreservation; extenders; fertility; goat; sperm viability.

INTRODUCTION

Artificial insemination of cryopreserved semen is one of the important assisted reproductive technologies for enhancing reproductive efficiency of goats (Leboeuf et al., 2000; Martinez et al., 2007). However, cryopreservation reduces sperm viability (Pegg, 2007). In addition, egg yolk, a common semen extender, limits post-freezing quality of goat semen due to its harmful interactions with bulbourethral secretions (Roy, 1957; Leboeuf et al., 2000). Alternatively, plant-derived products seem to overcome this problem. Post-thaw quality of goat sperm was improved when it was cryopreserved with tiger nut milk extender (Igbokwe et al., 2019). Soybean milk is rich in important components comparable to those in egg yolk, which is routinely used for protection of animal sperm from cold shock during cryopreservation (Campbell and Farrel, 2007; Zhang et al., 2009; USDA, 2018). Furthermore, soybean lecithin may provide more protection for sperm than egg yolk during the freeze-thawing process and also reduce the risk of introducing bacteria and mycoplasma into semen extenders (Fukui et al., 2008). In addition, although semen cryopreservation (mainly slow freezing and rapid freezing) enables long-term preservation of sperm (Sharma et al., 2015), there are conflicting reports on optimal cryopreservation methods (Choe et al., 2006; Vutyavanich et al., 2010; Tongdee et al., 2015; Daramola and Adekunle, 2016; Igbokwe et al., 2019). The objective was therefore to determine effects of varying concentrations of soybean milk extender as well as comparative effects of slow and rapid freezing protocols on sperm functional, seminal oxidative stress and fertilizing end points of WAD goat sperm.

MATERIALS AND METHODS

This study was approved by the Postgraduate Supervisory Committee of College of Animal Science

and Livestock Production (COLANIM). Animal handling and experimental procedures complied with Nigerian Institute of Animal Science (NIAS) guidelines.

Experimental location and animal management

The experiment was conducted in the Federal University of Agriculture Abeokuta, Nigeria located at 7°10′N and 3°2′E, 76 m above sea level. It lies within the South-Western part of Nigeria which has a prevailing tropical climate, mean annual rainfall of 1,238 mm and average temperature of 27.1 °C (Climate-data.org Nigeria Ogun, 2020).

Preparation of soybean milk

Preparation of soybean milk was carried in the laboratory. Soybean grains (100 g) were washed, soaked in 500 ml distilled water at 40 °C for 10 min and later boiled at 100 °C for 30 min. After boiling, grains were put in a blender for 5 min, the resulting slurry filtered through a clean cotton cloth and then boiled at 80 °C for 10 min. The suspension was cooled to 32 °C before use.

Semen dilution and storage

An artificial vagina was used to collect five semen samples (each semen sample emanated from five individual bucks) from five WAD goat bucks (between 2 and 3 years old). Ejaculates with > 80% motility were pooled, split into five equal fractions and diluted at room temperature with a Tris-based extender containing various concentrations of soybean milk. Samples were cryopreserved using slow and rapid freezing methods.

Slow freezing

Slow freezing, involving gradual temperature decreases in a stepwise manner (Mphaphathi et al., 2012) was done as described (Tarig et al., 2017). Semen samples were diluted at room temperature in a two-step process with a Tris-based extender composed of 2 fractions containing 7% glycerol. Fraction 1 solution contained tris-hydroxymethyl-aminomethane (2.42 g), citric acid (1.36 g), glucose (1 g) and penicillin (0.028 g) plus various amounts (0, 5, 10, 15 and 20 mL) of soybean milk, with distilled water added to make up 100 mL. Fraction 2 solution had the same composition as Fraction 1, plus glycerol. Egg yolk (20 mL) was used as control. Pooled fresh semen samples were split into five equal fractions and diluted with Fraction 1 solution, with Fraction 2 solution subsequently added. Following dilution, samples were loaded into 2-mL plastic straws (2 straws per treatment), sealed with polyvinyl, gradually cooled to 4 °C at 0.25 °C/min and equilibrated at 4 °C for 10 m in a TYFSF Refrigerated Incubator (Model: SPX-7OB III, Hebei China). Subsequently, straws were plunged into liquid nitrogen and stored for 30 d.

Rapid freezing

Rapid freezing or vitrification involves rapid temperature conduction with fast cooling (Mphaphathi et al., 2012), as described (Srirattana et al. 2013), with some modifications. Holding medium (HM) [phosphate-buffered saline (PBS) and 20% bovine serum albumin (BSA) (v/v) and vitrification Solution I [12.5% ethylene glycol (EG) and 12.5% dimethylsulfoxide (DMSO)] were prepared by mixing EG, DMSO and HM in ratio of 1:1:6 whereas vitrification Solution II (VS-II) consisting of 25% EG and 25% DMSO was prepared by mixing EG, DMSO and the HM in ratio of 1:1:2. Diluted samples (2 mL) were first exposed to 50 µL of HM for 10 min, then 50 µL of VS-I was added to the mixture (diluted samples + HM) and left for 4 min. Finally, 50 µL of VS-II was added to the mixture (diluted samples + HM + VS-I) and left for 1 min. Samples were loaded into 2-mL straws, replicated twice and sealed with polyvinyl and plunged directly into liquid nitrogen and stored for 30 d.

Evaluation of sperm motility

Sperm motility was determined as described (Bearden and Fuquay, 1997). Straws of semen were thawed at 37 °C and sperm motility assessed with a microscope (×400 magnification; Celestron Penta View: LCD-44348 by RoHS, China). For this, 5 µL of semen was dropped on a pre-warmed slide, then coverslipped and examined for progressively motile sperm (moved forward essentially in a straight line). Percentage progressive motility was calculated as: Number of progressive motile sperm counted / total number of sperm × 100.

Acrosome integrity

Percentage of sperm with intact acrosomes was determined (Ahmad et al. 2014). After thawing, 50 µL of semen was mixed with 500 µL formalin citrate solution. A small drop of the mixture was placed on a microscope slide and a total of 200 sperm were counted in 3 microscopic fields for each sample (× 400 magnification). Intactness of acrosome characterized by normal apical ridge was recorded. Percentage acrosome integrity was calculated as follows:

Number of sperm with normal apical ridge / total sperm counted ×100.

Membrane integrity

Hypo-osmotic swelling test assay (Zubair et al. 2013) was used to determine sperm membrane integrity. Semen (10 µL) was incubated in 100 µL of 100 mOsm/L Hypo-osmotic solution (9 g fructose plus 4.9 g sodium citrate) at 37 °C for 30 min and 0.1 mL of the mixture spread over a prewarmed slide, coverslipped and observed under a microscope (× 400 magnification). Two hundred sperm were counted and percentage of

sperm with curled tails, indicating an intact plasma membrane, was determined.

Sperm abnormalities and livability

Sperm abnormalities and livability were assessed as described (Bearden and Fuquay, 1997) using eosin-nigrosin staining. A thin smear of semen and eosin-nigrosin solution was drawn across slide and dried. Percentage of abnormal spermatozoa with defects in the head, midpiece and tail were observed under a microscope (× 400 magnification). Sperm that appeared white (unstained cells) were regarded as live sperm, whereas those that absorbed stain were regarded as dead. Sperm livability (%) was calculated as = [(Total number of live sperm)/ (Total number of sperm observed)] ×100.

Malondialdehyde (MDA) concentration

Malondialdehyde (MDA) concentrations in frozen-thawed semen were measured with thiobarbituric acid reactive substance (Yagi, 1998). Semen (0.1 mL) was mixed with 0.1 mL of 150 mM Tris-HCl (pH 7.1) and kept at 37 °C for 20 min. Subsequently, 1 mL of 10% trichloroacetic acid (TCA) and 2 mL of 0.375% thiobarbituric acid were added and the mixture kept in boiling water for 30 min. Thereafter, samples were centrifuged for 15 min at 3000 × *g*. Absorbance (532 nm) was determined with a spectrophotometer (Model SW7504, Surgifriend Medicals, England). The concentration of MDA was calculated as follows:

$$\text{Concentration of MDA (nmol/mL)} = AT - AB/1.56 \times 10^5$$

where: AT = the absorbance of the sample serum, AB = the absorbance of the blank, 1.56×10^5 molar absorptivity of MDA.

Seminal leukocytes

Peroxidase test adapted from Endtz (1974) and consistent with WHO (1992) was used to determine leukocyte counts. Peroxidase stock solution was prepared by mixing 50 mL distilled water with 50 mL 96% ethanol and 125 mg benzidine. Addition of 5 µL 30% H_2O_2 to 4 mL of peroxidase stock solution was used to prepare peroxidase working solution, with 20 µL of this solution added to 20 µL of cryopreserved semen and incubated for 5 min at room temperature. Thereafter, 20 µL of phosphate-buffered saline was added to 20 µL of peroxidase working solution and 20 µL of cryopreserved semen. Subsequently, 10 µL of the last mixture was placed on a haemocytometer and dark brown round cells were counted.

In vitro acrosome reaction

Proportion of sperm with an acrosome reaction was determined as described (Tardif et al. 1999) and subsequently modified (Daramola et al. (2016). Thawed semen was washed by centrifuging at 3000 × *g* for 5 min with phosphate-buffered saline (PBS). Pellets were re-suspended in culture medium. Immediately after inclusion of 0.9% wt/vol PBS, an acrosome reaction was induced by incubating sperm for 20 min with progesterone (2.5 mg/mL) at 38.5 °C (5% CO_2 in air; 100% humidity). Subsequently, an equal volume of PBS was added for spontaneous acrosome reaction induction. Sperm 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. Sperm with intense fluorescence over the acrosome were classified as acrosome intact, whereas those with no fluorescence or a dull fluorescence along the equatorial segment were defined as acrosome reacted.

In vitro capacitation

Chlortetracycline (CTC) fluorescence assay (Collin et al., 2000) was used to evaluate *in vitro* sperm capacitation. In brief, CTC (750 µM*)* was prepared in 20 mM Tris buffer containing 130 mM NaCl and 5 mM DL-cysteine (final pH, 7.8). Cryopreserved semen (5 µL) was added to 5 µL of CTC solution on a warmed slide (37 °C). After 30 s, 5 µL of 0.2% glutaraldehyde in 0.5 M Tris (pH 7.4) was added. Finally, 5 µL of 90% glycerol and 10% PBS (pH adjusted to 8.6) were added to retard fluorescence fading. A drop of the sample was placed on a slide, coverslipped and examined with an upright Carl Zeiss Fluorescent Microscope (Primo Star, Germany) equipped with phase contrast and epifluorescence optics. One hundred cells were counted per slide. Sperm with pattern B (bright anterior head and faint fluorescence in the post-acrosomal region) were classified as capacitated.

Statistical analyses

The experimental design was a 2 × 5 factorial arrangement; means were separated by Duncan Multiple Range Test (Duncan, 1955) using SAS 2000. The model included:

$$Y_{ijk} = \mu + C_i + L_j + (CL)_{ij} + \Sigma_{ijk}$$

Where,
Y_{ijk} = Dependent variables,
μ = Population mean,
C_i = effect due to i^{th} cryopreservation protocols, i = (1, 2,),
L_j = effect due to j^{th} level of soya milk inclusion, j = (0, 5, 10, 15, 20)
CL_{ij} = effect of ij^{th} interaction between cryopreservation protocols and levels of soya milk inclusion
Σ_{ijk} = experimental error

Table 1. Mean of sperm functional parameters of goat sperm cryopreserved with soybean milk during slow and rapid freezing

Cryoprotocols	SBM (%)	Motility (%)	ACI (%)	MEI (%)	ABN (%)	Livability (%)
Slow freezing	0	45.75 ± 2.78^{cd}	42.00 ± 1.25^{d}	44.12 ± 1.07^{d}	2.12 ± 0.35	76.25 ± 1.82^{b}
	5	50.00 ± 0.00^{bc}	50.75 ± 1.68^{c}	52.50 ± 0.82^{c}	2.00 ± 0.18	80.00 ± 2.67^{ab}
	10	57.87 ± 2.97^{a}	48.50 ± 0.62^{c}	52.25 ± 1.03^{c}	1.62 ± 0.32	82.50 ± 1.63^{ab}
	15	52.50 ± 1.99^{ab}	52.00 ± 1.19^{c}	60.25 ± 2.83^{b}	1.25 ± 0.16	85.00 ± 1.88^{a}
	20	50.75 ± 0.75^{bc}	58.00 ± 1.41^{b}	71.00 ± 1.88^{a}	1.25 ± 0.16	85.00 ± 1.88^{a}
Rapid freezing	0	41.53 ± 3.17^{d}	40.50 ± 1.18^{d}	43.62 ± 3.76^{d}	2.50 ± 0.88	78.75 ± 3.98^{ab}
	5	50.00 ± 0.00^{bc}	52.25 ± 0.88^{c}	68.50 ± 0.50^{a}	2.25 ± 0.81	81.25 ± 2.26^{ab}
	10	52.50 ± 1.63^{ab}	58.00 ± 1.19^{b}	72.00 ± 0.53^{a}	1.75 ± 0.75	86.25 ± 2.63^{a}
	15	54.12 ± 2.70^{ab}	59.75 ± 4.57^{b}	71.75 ± 1.09^{a}	1.62 ± 0.65	83.75 ± 1.82^{a}
	20	58.25 ± 3.11^{a}	69.25 ± 5.69^{a}	72.00 ± 1.73^{a}	1.37 ± 0.59	85.00 ± 1.88^{a}

^{a,b,c,d}Values within the same column with different superscripts differ ($P < 0.05$);
SBM: Soybean milk; ACI: acrosome integrity, MEI: membrane integrity, ABN: abnormality

Table 2. Mean of seminal oxidative stress parameters of goat sperm cryopreserved with soybean milk during slow and rapid freezing

Cryoprotocols	Soybean milk (%)	Malondialdehyde (nmol/mL)	Leukocyte (×10^3/ mL)
Slow freezing	0	1.25 ± 0.17^{b}	55.00 ± 0.04^{a}
	5	1.01 ±0.42^{bc}	39.38 ± 0.04^{b}
	10	0.55 ± 0.06^{c}	36.75 ± 0.03^{b}
	15	0.27 ± 0.13^{c}	35.75 ± 0.03^{b}
	20	0.67 ± 0.03^{c}	34.25 ± 0.02^{bc}
Rapid freezing	0	2.63 ± 0.11^{a}	59.62 ± 4.00^{a}
	5	2.43 ± 0.10^{a}	39.75 ± 3.84^{b}
	10	1.55 ± 0.06^{b}	39.75 ± 2.76^{b}
	15	1.29 ± 0.13^{b}	38.37 ± 3.16^{b}
	20	1.14 ± 0.04^{b}	31.00 ± 3.48^{c}

^{a,b,c}Values within the same column with different superscripts differ ($P < 0.05$).

Table 3. Mean of *in vitro* acrosome reaction and *in vitro* sperm capacitation of goat sperm cryopreserved with soybean milk during slow and rapid freezing

Cryoprotocols	Soybean milk (%)	Acrosome reaction (%)	Sperm capacitation (%)
Slow freezing	0	26.50 ± 2.71^{d}	31.5 ± 1.91^{c}
	5	50.25 ± 2.71^{b}	57.5 ± 2.82^{b}
	10	70.00 ± 3.29^{a}	71.5 ± 4.10^{a}
	15	48.50 ± 5.67^{b}	56.0 ± 4.78^{b}
	20	44.00 ± 4.47^{bc}	54.5 ± 3.41^{b}
Rapid freezing	0	34.00 ± 3.46^{c}	36.0 ± 3.62^{c}
	5	77.25 ± 3.70^{a}	51.5 ± 4.89^{b}
	10	59.50 ± 5.03^{b}	54.5 ± 4.65^{b}
	15	45.50 ± 3.11^{bc}	58.5 ± 4.37^{ab}
	20	37.50 ± 4.13^{c}	58.5 ± 7.78^{ab}

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

RESULTS

Sperm functional parameters of semen cryopreserved with varying concentrations of SBM using slow and rapid freezing are presented in Table 1. The results showed that inclusion of SBM in extenders increased ($P<0.05$) motility, acrosome and membrane integrities of sperm using slow and rapid freezing compared to control. Higher ($P<0.05$) motility was obtained in sperm cryopreserved with 10% and 20% SBM extenders using slow and rapid freezing respectively compared to other concentrations and the control. Semen cryopreserved with SBM extenders had higher ($P<0.05$) membrane integrity at all levels of SBM in rapid freezing while higher ($P<0.05$) membrane integrity was observed in semen cryopreserved with 20% SBM in slow freezing. The results showed that acrosome integrity was higher ($P<0.05$) in sperm cryopreserved with 20% SBM in rapid freezing compared to slow freezing in other concentrations and the control. Livability was slightly higher ($P<0.05$) in sperm cryopreserved with SBM extenders using slow and rapid freezing protocols.

Seminal oxidative stress parameters of semen cryopreserved with varying concentrations of SBM using slow and rapid freezing are presented in Table 2. The results showed that semen cryopreserved with SBM extenders had lower ($P<0.05$) MDA concentrations in slow freezing compared rapid freezing. However, semen cryopreserved with SBM all levels of SBM in slow freezing had lower ($P<0.05$) MDA concentrations and fewer ($P<0.05$) leukocytes compared to the control. In contrast, semen cryopreserved with SBM at 10%, 15% and 20% had lower ($P<0.05$) MDA concentrations compared to 5% and the control while lower ($P<0.05$) leukocytes was obtained in 20% SBM compared to other levels and the control in rapid freezing.

In vitro acrosome reaction and *in vitro* sperm capacitation of semen cryopreserved with varying concentrations of SBM using slow and rapid freezing are presented in Table 3. The results showed that semen cryopreserved with SBM extenders had higher ($P<0.05$) acrosome reaction compared to the control using slow or rapid freezing protocols. However, there was a higher ($P<0.05$) acrosome reaction in semen cryopreserved with 10% and 5% SBM extenders using slow and rapid freezing respectively. Similarly, the results showed that semen cryopreserved with SBM extenders had higher ($P<0.05$) sperm capacitation compared to the control using slow or rapid freezing protocols.

DISCUSSION

The SBM extender in this study sustained the quality of cryopreserved goat sperm and demonstrated its capacity to effectively preserve sperm cells. This was in agreement with previous studies that inclusion of soybean milk in semen extender improved post-thawed sperm characteristics (Papa et al., 2011; Singh et al., 2012). Protective effects of this extender could be linked to constituents of soybean that are important to promote sperm optimal survival during freezing. In that regard, soybeans contain glucose, phospholipids and vitamins (Campbell and Farrel, 2007; Zhang et al., 2009; USDA, 2018), all essential components of semen extenders. Greater progressive motility in semen diluted with SBM could have been due to glucose as source of energy in the soybean (USDA, 2018). Energy is needed to maintain sperm metabolism and to survive stress-induced dormancy caused by freezing (Gadea, 2003; Yancey, 2005). In addition, soybeans have considerable content of phospholipids and lecithin and its low viscosity (Campbell and Farrel, 2007; Zhang et al., 2009) makes it suitable for semen extender. Phospholipids reduce ice-crystal formation, stabilize the cell membrane and protect sperm from cold shock (Medeiros et al., 2002; Ezeh et al., 2014). The high percentage of livability, acrosome and membrane integrities of sperm diluted with SBM could therefore be attributed to its phospholipid content (Campbell and Farrel, 2007; Zhang et al. (2009). The present results are in agreement with previous findings that sperm motility and viability were better in soya lecithin-based extender versus egg yolk extenders (Akhtar et al., 2011; De Paz et al., 2010).

Acrosome and membrane integrities were better preserved in SBM extender; 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 integrities in soybean milk extender (Soltan et al., 2016) and soy lecithin tris-citric acid extender (Amirat et al., 2005; Stradaioli et al., 2007). Lower values for oxidative parameters indicated SBM extender 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 soy lecithin, e.g. glutathione, protected sperm viability by scavenging lipid peroxidation during freezing (Salmani et al., 2013). Intracellular MDA concentration is a stress indicator; the optimal MDA concentration obtained in slow freezing compared to rapid freezing in this study was attributed to an abrupt change in freezing temperature during rapid freezing.

Leukocytes and sperm are major sources of reactive oxygen species (ROS) during semen cryopreservation (Agarwal et al., 2003; Garrido et al., 2004). Excessive ROS, produced in large amounts by leukocytes or sperm, decrease membrane fluidity of plasma and organelle membranes and damage membrane function, ion gradients and receptor-mediated signal transduction (Sikka et al., 1996). High leukocyte concentrations, particularly activated leukocytes, are harmful to sperm functions (Henkel, 2011). The lower concentration of leukocytes after addition of SBM in this study indicated

effectiveness of antioxidative capacity of SBM in reducing defective sperm or excessive ROS produced by leukocytes or sperm.

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 SBM extender 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 SBM extenders using either slow or rapid freezing protocols. In contrast, significant differences in sperm quality parameters in slow versus rapid freezing have been reported. For example, there was a significant difference in sperm motility between slow and rapid freezing in Korean native bucks (Choe et al., 2006), superior post-thaw motility and cryo-survival in rapid versus slow freezing (Vutyavanich et al. *2010)* and improved post-thaw sperm quality parameters in slow versus rapid freezing (Daramola and Adekunle, 2016; Igbokwe et al., 2019).

CONCLUSION

Although a single concentration of SBM did not optimize every sperm end point, overall, SBM extenders improved quality of frozen-thawed goat semen in slow and rapid freezing protocols. The most desirable motility of sperm cryopreserved in slow and rapid freezing was observed with 10% and 20% SBM extenders respectively while MDA concentration was optimal in slow compared to rapid freezing.

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DISCLOSURE STATEMENT

The authors declare that there are no conflicts of interest.

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