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Original Research Article

Effect of sunflower lecithin on Kalahari Red goat semen during cryopreservation

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Abstract

Soybean lecithin had been used as an alternative to egg yolk in domestic animal semen extender during cryopreservation due to its characteristic phospholipid content which played a major cryoprotective role. This composition of soybean lecithin informed the replacement of soybean with sunflower lecithin (SL) in the extender for the Kalahari Red (KR) buck semen cryopreservation in this study. Effect of different levels of SL on the quality of the KR buck semen during cryopreservation using slow freezing method was evaluated. Semen samples were collected from four KR bucks of between two and two and half of age using artificial vagina, evaluated for motility and then diluted in extenders containing different levels of SL (1.5%, 3.0% and 4.5%) as experimental group and 0% SL or 20% egg yolk as control. Semen parameters including motility, acrosome integrity (AcI), membrane integrity (MI), malondialdehyde (MDA) concentration, cholesterol level and seminal arginase activity were evaluated for. The results showed that motility, acrosome integrity (AI) and membrane integrity were comparable at 0%, (22.00 ± 4.58, 82.00 ± 3.51 and 96.00 ± 2.03); 1.5%, (23.00 ± 2.08, 87.00 ± 3.79 and 89.00 ± 2.08); 3.0%, (13.00 ± 2.52, 81.33 ± 0.41 and 76.67 ± 1.20) and 4.5% (11.00 ± 4.51, 85.33 ± 9.88 and 84.00 ± 8.50), respectively, after thawing. SL at 0% had the highest ($P < 0.05$) values for MDA, cholesterol and seminal arginase activity (1.10 ± 0.008 nmol/ml, 236.35 ± 4.08 mg/dl and 0.54 ± 3.3 E-3 units/mg protein, respectively). Our data suggest that 1.5% sunflower lecithin can be used in place of soy lecithin as a substitute for egg yolk during the cryopreservation of caprine semen.

Keywords: sperm motility; acrosome integrity; membrane integrity; malondialdehyde; cholesterol; seminal arginase activity.

INTRODUCTION

The Kalahari Red (KR) goat is a meat goat breed originating from South Africa. It is a new breed in terms of recognition and the breed is becoming more popular and very important meat goat breed in South Africa, and some other neighbouring countries like Nigeria (Federal University of Agriculture, Abeokuta to be precise). The name of the Kalahari Red goats is derived from their red coat and the *Kalahari Desert*, which spans the borders of Botswana, South Africa and Namibia. Some populations of the Kalahari Red goats are also being developed in Australia, Brazil and the United States. There are limited numbers of this breed in Nigeria, however, its production can be improved upon through artificial insemination and cryopreservation. Kalahari Red goat is a very beautiful animal and has similarities in appearance with the Boer goats. It is a large breed of goat with red coloured coat. The Kalahari Red goats also have strong herding instincts for protection as well. They have long, floppy ears. They have loose skin in their neck area. They have moderately-sized, sloping horns above their ears. Their skin is fully pigmented, which allows them to forage and increase weight gains through the heat of the day. The does have full and properly attached udders and teats. The Kalahari Red goats can be used as a good crossbred for increasing hardiness and carcass size. The bucks are usually larger than the does. Average body weight of the Kalahari Red buck is about 115 kg, and that of does on is average about 75 kg.

Cryopreservation is the process where cells, tissues or any substance susceptible to damage caused by chemical reactivity or time are preserved by cooling to sub-zero temperature thereby stopping any enzymatic or chemical activity which might cause damage to the material in question. Cryopreservation of goat semen has been found to be of great necessity as cryopreserved sperm cells can be stored and used after a long period of time (Martinez et al., 2007). Extenders used for semen cryopreservation preserve sperm motility and fertility by promoting the stabilisation of the plasma membrane and providing energy substrate. An ideal extender should have nutrients as an energy source, buffer against harmful changes of pH, provide a physiological osmotic pressure and concentration of electrolytes, prevent bacterial growth and protect the cells from cold shock during the freezing and thawing processes.

Cryopreservation alters the structure and function of cells due to the formation of ice during freezing. Egg yolk is an essential component in extenders for semen cryopreservation due to its cryoprotective role. Studies have shown that egg yolk not only increases the fertilizing ability of spermatozoa at ambient temperatures but also appears to prevent sperm cell damage during cooling and freezing (Shannon and Curson, 1983; Barak et al., 1992; De Leeuw et al., 1993).

The low-density lipoproteins (LDLs) in egg yolk have been shown to play an important role in protecting sperm due to the presence of phospholipid fractions of the LDL, which form a protective film on the sperm surface (Barak et al., 1992).

However, diluting goat semen in extenders containing egg yolk can be deleterious to sperm cells due to the presence of phospholipase A, secreted by the bulbourethral glands. This phospholipase is also called Eyce (Egg yolk coagulating enzyme) or BUSgp60 bulb urethral gland secretion (LeBoeuf et al., 2000) and is responsible for the reduced viability of sperm cells that have been cooled or frozen in extenders containing egg yolk or milk, respectively (Corteell, 1981). This characteristic differentiates goat semen from other species. A viable alternative to replace the components of animal origin in extenders for freezing semen is soybean lecithin, a phospholipid that is the main component of the phosphate fraction of egg yolk and soybean (Campbell and Farrel, 2007). Salmani et al. (2014) have suggested the use of soyabean-lecithin which is a suitable plant based cryoprotectant for caprine sperms. Soya bean extracts have been widely used since the first half of the 20th century in the former Soviet Union as a component of freezing diluents for ram semen (Salamon and Maxwell, 2000). In recent decades, the use of soy lecithin has been demonstrated to be safer than egg yolk in terms of biosecurity (Bousseau et al., 1998), and it has been used for sperm cryopreservation in species such as eel (Tanaka et al., 2002), bull (Aires et al., 2003), stallion (Aurich et al., 2007), boar (Zhang et al., 2009), human (Reed et al., 2009), and particularly, ram (Gil et al., 2003; de Paz et al., 2010; Forouzanfar et al., 2010). Lecithin has been reported to have neither a cytotoxic effect (Fiume, 2001) nor a negative effect on sperm motility (Hong et al., 1986), Sunflower lecithin is a type of phospholipid found in sunflower seeds. It is a good source of phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol. Sunflower lecithin can replace soy bean lecithin as an alternative to egg yolk in mammalian semen extender during cryopreservation even though there has not been any report on the use of sunflower lecithin in preserving Kalahari Red goat semen. This study therefore aimed at determining the effect of different levels of sunflower lecithin on Kalahari Red buck semen during cryopreservation.

MATERIALS AND METHODS

Experimental animals and semen collection

Four young Kalahari red bucks of between two and two and half years of age and one matured teaser doe were used. The animals were on intensive management system and fed with concentrate and guinea grass (*Panicum maximum*). Semen samples were collected with the aid of artificial vagina. The semen samples were kept in a thermoflask at 37 °C and thereafter transported to the laboratory for evaluation of progressive sperm motility and concentration. Sperm viability and abnormality were assessed by determining the rate of live sperm using a modification of the eosin-nigrosin stain procedure as described by Chauhan and Anand (1990). A mixture of 10 µl of diluted spermatozoa and 10 µl eosin-nigrosin stains was smeared on a slide and allowed to air dry in a dust-free environment. Two hundred spermatozoa from different microscopic fields were examined under a bright-field microscope (×400 magnification), and the number of non-stained (viable) spermatozoa was counted. The sperm concentration in millions per millilitre was also determined by using a haemocytometer.

Semen dilution and freezing

Semen sample showing ≥ 70% motility were pooled. The pooled semen was diluted in a two-step process at 32 °C with tris-buffered egg yolk extender as control and lecithin based tris-extender composed of two fractions as the experimental group. Aliquots of pooled semen were diluted with the extender and the diluted semen sample were then loaded into 2 ml plastic straws (Purdy, 2006) and sealed with polyvinyl, equilibrated by cooling it to 4 °C at a rate of 0.25 °C/min in TYFSF refrigerated incubator (Model: SPX-70B 111, Hebei China). Subsequently, the straws were then placed at 4 cm above liquid nitrogen in the vaporous phase for 10 minutes before plunging them directly and

Table 1. Composition of different extenders

Extender composition	T_o	T_1	T_2	T_3
Tris (g)	2.42	2.42	2.42	2.42
Citric acid (g)	1.36	1.36	1.36	1.36
Glucose (g)	1.00	1.00	1.00	1.00
Sunflower lecithin (%)	-	1.50	3.00	4.50
Egg yolk (%)	20.00	-	-	-
Glycerol (%)	7.00	7.00	7.00	7.00
Penicillin (mg)	2.80	2.80	.028	2.80
Distilled water made up to (ml)	100	100	100	100

Table 2. Composition of sunflower lecithin

Composition	Quantity per 1 1/3 tablespoon
Phosphatidyl Choline	2.50 g (2500 mg)
Phosphatidyl Inositol	1.80 g (1800 mg)
Phosphatidyl Ethanolamine	1.10 g (1100 mg)

Source-Pack

quickly into liquid nitrogen for 7 days after which cryopreserved semen samples were evaluated for sperm viability parameters.

Thawing and sperm evaluation

The frozen semen was thawed in Clifton water bath (Model: 74178 by Nickel Electro Ltd, Weston-S-Mare Somerset, England) at 37 °C for 30 s after 7 days of cryopreservation and analysed for sperm motility, plasma membrane integrity, acrosome integrity, malondialdehyde (MDA) concentration and cholesterol content.

Sperm motility

Sperm motility was determined as described by Bearden and Fuquay (1997). 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 using different slides. Different microscopic fields were examined to observe progressively motile spermatozoa that move forward in a straight line.

Sperm plasma membrane integrity

Sperm plasma membrane integrity was determined using Hypo-osmotic swelling test (HOST) assay as described by Correa et al. (1997). Semen (10 μl) was incubated in hypo-osmotic solution (9 g fructose and 4.9 g sodium citrate/100 ml distilled water) at 37 °C for 30 minutes. Then 0.1 ml of the mixture was spread over a warm slide cover with cover slip and observed under microscope (×400 magnification). Two hundred spermatozoa were counted for their swelling characterised by coiled tail, indicating intact plasma membrane.

Acrosome integrity

Percentage of spermatozoa with intact acrosome was determined according to the method described by Ahmad et al. (2003). Fifty µl of each semen sample was added to a 500 µl formalin citrate solution (96 ml of 2.9% sodium citrate, with 4 ml of 37% formaldehyde) and mixed. A small drop of the mixture was then placed on a microscope slide and a total of 200 spermatozoa were counted in at least three different microscopic fields for each sample, using ×400 magnification. Intactness of acrosome characterised by normal apical ridge of 200 spermatozoa was assessed using microscope.

Malondialdehyde (MDA) concentration

Lipid peroxidation in the stored semen as shown by MDA concentration was measured in a thiobarbituric acid reactive substances (TBARS) according to Ohkawa et al. (1979). Then 0.37% TBA was prepared by dissolving 0.37 g TBA in 50 ml distilled water and made up to 100 ml; 15% TCA was prepared by dissolving 15 g TCA in 50 ml distilled water and made up to 100 ml and also 0.25 M HCl was prepared by measuring 0.62 ml HCl and made up to 100 ml. 0.1 ml of sperm suspension was incubated with 2 ml TBA/TCA/HCl (1:1:1) in water bath for 15 min at 95 °C. It was then placed in ice and centrifuged for 10 min at 3000 rpm. The absorbance of the clear supernatant was measured with UV spectrophotometer (SW7504 model by Surgifriend Medicals, England) at 552 nm. The concentration of MDA will be calculated as follows: MDA (µmol/L) = $A/1.55 \times 10^6$ b, where A = the absorbance of the sample, b = size of the cuvette to the nearest cm, 1.55×10^6 molar absorptivity of MDA.

Table 3. Effect of different levels of sunflower lecithin on motility, acrosome integrity and membrane integrity of KR buck semen during slow freezing method

Parameters	0% (20% EY)	1.5%	3.0%	4.5%
Motility (%)	22.00 ± 4.58	23.00 ± 2.08	13.00 ± 2.52	11.00 ± 4.51
Acrosome integrity (%)	82.00 ± 3.51	87.00 ± 3.79	81.33 ± 0.41	85.33 ± 7.88
Membrane integrity (%)	96.33 ± 2.03	89.00 ± 2.08	76.67 ± 1.20	84.00 ± 8.50

Table 4. Effect of different levels of sunflower lecithin on malondialdehyde concentration (MDA), cholesterol level and arginase activity during slow freezing method

Parameters	0%	1.5%	3.0%	4.5%
MDA (nmol/ml)	1.10± 0.0088^{a}	0.45±0.00033^{b}	0.46±0.00058^{b}	0.36±0.00033^{c}
Cholesterol (mg/dl)	236.35±4.08^{a}	17.74±0.84^{c}	22.32±0.87^{c}	91.36±0.82^{b}
Seminal arginase activity (units/mg protein)	0.54±3.3E-3^{a}	0.26±2.0E-3^{d}	0.38± 3.4E-3^{c}	0.40± 1.5E-3^{b}

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

Arginase activity

Arginase activity was carried out following the procedure of Lowry et al. (1951). Bovine Serum Albumin (BSA), 0.1 g, as standard in 10 ml water was used. One ml alkaline copper reagent (a mixture of copper sulphate reagent, sodium dodecyl sulphate solution and sodium hydroxide solution) (1:2:1) and 0.1 ml semen sample were mixed and incubated for 10 min at room temperature. After this, 4 ml Folin Ciocalteu's phenol reagent was added to the sample, mixed and incubated for 5 min at 55 °C. The absorbance of the samples was recorded at 650 nm in spectrophotometer (UV spectrophotometer, SW7504 model by Surgifriend Medicals, England). The results were expressed as units/mg protein (specific activity).

Cholesterol content

This was determined by following the procedure outlined in the manual that came with the kit namely RANDOX CHOLESTEROL STANDARD Randox Laboratory Ltd, United Kingdom.

Statistical analysis

Data obtained were subjected to one-way ANOVA and means were separated by Duncan Multiple Range Test (Duncan, 1955) using SAS 2000. The model below was used:

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

Where:
Y_{ij} .. Dependent variables
μ .... Population mean
$_i$ ..... Effect due to i^{th} level of SL inclusion, i = (0%, 1.5%, 3.0%, and 4.5%)
Σ_{ij} .. Experimental error

RESULTS

Table 3 shows the main effect of the different levels of sunflower lecithin on the viability parameters of KR buck semen during cryopreservation. There was no significant difference in the values obtained for motility, acrosome integrity and membrane integrity at 0%, 1.5%, 3.0%, and 4.5%, respectively, after thawing. The effect of sunflower lecithin on the malondialdehyde (MDA), cholesterol level and seminal arginase activity after thawing is shown in Table 4. There were significant differences ($P < 0.05$) in the values obtained for all the parameters for the control and experimental group. The control group (0%) showed significantly higher values for all the parameters than the experimental groups (1.5, 3.0 and 4.5%). The MDA level at 4.5% sunflower lecithin was significantly lower than at 1.5 and 3.0% which showed no significant difference while cholesterol level and arginase activity at 4.5% lecithin) were significantly higher than at 1.5 and 3.0%. While there was no significant difference in the cholesterol at 1.5%, the arginase activity at 3.0% was significantly higher than at 1.5%.

DISCUSSION

The use of sunflower lecithin has not been reported upon. The present study indicated decline in the percentage motility and other sperm quality parameters and agreed with previous studies on semen cryopreservation with varying semen extenders with (Salmani et al., 2014; Chelucci et al., 2014; Yotov, 2015) or without lecithin from plant origin (Ahmad et al., 2013; Farshad and Akhondzadeh, 2008; Gojen Singh et al., 2016). Studies have proven the valuable effects of soybean lecithin for cryopreservation of sperm in bull (Aires et al., 2003; Gonzales et al., 2003), ram (Forouzanfar et al., 2010; de Paz, et al., 2010) and goat (Roof et al., 2012; Salmani et al., 2014). The semen diluted in extender supplemented with 1.5% sunflower lecithin (SL) in this study was comparable with other treatments for motility and other viability parameters similar to study carried out by Salmani et al. (2014) and Yodmingkwan et al. (2016) where semen diluted in extender containing 1.5% soybean

lecithin had no significant difference from extender with egg-yolk in their motility. Similar research with soybean lecithin reported that the cryoprotective effect of soybean lecithin extender on freezing bovine sperm was similar or slightly inferior to that of the 20% egg yolk extender (Thun et al., 2002; Aires et al., 2003). There was no significant difference in the values obtained for sunflower lecithin with higher concentration unlike what was obtained in Salmani et al. (2014) and Forouzanfar et al. (2010) where there were significantly lower values obtained in soybean lecithin with higher concentration. Yotov (2015) and Gamal et al. (2016) reported a significantly higher motility for semen diluted in tris-fructose citric acid (TFC) with 1.5% soybean lecithin than with egg yolk. This difference could be as a result of higher percentage of egg yolk, glycerol or different breed of goat or location (Singh et al., 2016; Leboeuf et al., 2000) or even the source of sugar used in the extender.

The poor motility observed in the semen diluted in sunflower lecithin after thawing could be traced to the low cholesterol level produced after cryopreservation leading to cryocapacitation. This is supported by Bailey et al. (2000), when poor quality buffalo bull semen and the fertility of frozen thawed semen was attributed to damage induced by cryocapacitation during freezing and thawing. Efflux of cholesterol from sperm membrane during cryopreservation led to decrease in motility, viability and membrane integrity and the fertility of frozen-thawed semen (Singh et al., 2016; Rasul et al., 2001; Buhr et al., 1994). Muller et al. (2008) also reported that cholesterol aids in stabilising sperm membrane by regulating its fluidity during cryopreservation and the efflux or decrease in cholesterol/phospholipid ratio resulted in cryocapacitation, which is a major factor associated with reduced longevity and poor survivability of cryopreserved spermatozoa in female reproductive tract (Bailey, 2000). The role of arginase in reproduction and fertility has been reported upon. Arginase enzyme has been found in ram epididymal spermatozoa (Mendez and Martinez, 1995), prostate and other parts of the reproductive system of ram (Razmi et al., 2004), prostate, seminal plasma, testis, human sperm cells (Keskinege et al., 2001) and in different parts of the reproductive system of bulls (Razmi et al., 2005) and in the seminal plasma of goat (Turk et al., 2011). The mean seminal arginase activity recorded in all the experiment in this study for both control and experimental group were lower than that in Saneen buck (Turk et al., 2011), WAD buck supplemented with vitamin E (Daramola et al., 2016) but higher than in cat (Mahmoud et al., 2007), normal men and men of oligospermia (Elgun et al., 2000). Previous studies had reported a positive correlation between seminal arginase activity and sperm motility (Elgun et al., 2000; Eskiocak et al., 2006; Turk et al. 2011), which was also confirmed in this study except in experiment 1 where 1.5% sunflower lecithin extender recorded the significantly ($P < 0.05$) lowest seminal arginase activity. Increased arginase activity generally resulted in lower NO concentration and subsequently lead to increased sperm motility (Elgun et al., 2000). Arginase has been found to regulate the production of nitric oxide (NO) concentration (Nathan, 1997). Low concentration of nitric oxide enhanced sperm motility (Herrero et al., 2001).

Susceptibility of mammalian spermatozoa to loss of motility was connected to lipid peroxidation (LPO) (Rao et al., 1989). Oxidative stress resulted in the production of lipid peroxide due to the abundant of PUFA in sperm plasma membrane. Many studies had shown that malondialdehyde level meant to indicate LPO was increased in frozen-thawed bull semen but not in cooled semen. MDA levels do not differ in fresh and frozen semen in humans (Chatterjee and Gagnon, 2001).

The report of this study showed that extender containing SL protected the sperm membrane against lipid peroxidation as they produced significantly lower MDA compare to egg yolk extender. This was in agreement with Salmani et al. (2014). Other studies also reported that the components of extender can have effect on the frozen-thawed sperm lipid peroxidation (Bucak et al., 2008; Bucak et al., 2009) which however was not supported by Coyan et al. (2011) and Atessahin et al. (2008), who reported that lipid peroxidation in sperm was not influenced by the ingredients of cryopreservation media. It was thought that lecithin protected the sperm effectively against lipid peroxidation compare to egg yolk which can be related to the structure of egg yolk that contains more of unsaturated fatty acid susceptible to lipid peroxidation.

CONCLUSION

The findings suggest that 1.5% sunflower lecithin can be used in place of soy lecithin as an alternative to egg yolk during cryopreservation of caprine semen.

Further studies are necessary to validate the use of sunflower lecithin as semen extender in various animal species and the best level for each animal species.

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