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Original Research Article

Effect of Centrifugation on Motility, Sperm Capacitation and Acrosome Reaction in Soy Bean and Avocado Seed Milk Extenders of Cryopreserved Goat Spermatozoa

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Abstract

Removal of seminal plasma by centrifugation (0 centrifugation, 1 centrifugation, 2 centrifugations, 3 centrifugations) and preservation in two different tris-extenders viz., avocado seed milk (ASM) and soy bean milk (SBM) based extenders were studied for their ability to support motility, *in vitro* capacitation and acrosome reaction of spermatozoa obtained from West African Dwarf (WAD) goat bucks during cryopreservation. Semen samples collected with the aid of artificial vagina were centrifuged for one, two and three times. The centrifuged samples were diluted with the two tris-extenders each containing 20 mL of avocado seed milk and soybean milk and cryopreserved for 30 days. The results showed higher ($P < 0.05$) sperm motility ($P < 0.05$) with increased centrifugation times. Spermatozoa that were centrifuged had higher ($P < 0.05$) percentage of acrosome reaction and capacitation with increased centrifugation times compared to the control. Optimal improvement in these parameters was obtained with increased centrifugation times. The findings revealed that removal of seminal plasma by centrifugation improved sperm quality of WAD goat bucks during cryopreservation and optimum improvement was achieved consistently with 3 centrifugations.

Keywords: Centrifugation; freezing; seminal plasma; sperm viability; survival.

INTRODUCTION

Cryopreservation of goat semen is important because frozen-thawed goat semen can be utilized for artificial insemination to enhance improvement of livestock as breeders mostly use genetically superior bucks (Leboeuf et al., 2000; Martinez et al., 2007). However, cryo-damage to cells (Pegg, 2007) is a major constraint of cryopreservation because of consequent decline in sperm viability. Besides, egg yolk coagulating enzyme derived from bulbo-urethral glands in seminal plasma characterized as phospholipase A hydrolyses egg yolk lecithin to fatty acids, l isolecithin and lisolecithin, and the presence of bulbourethral secretion glycoprotein-60 causes harmful interactions between seminal plasma and egg yolk or milk (Roy, 1957; Leboeuf et al., 2000). Therefore, removal of seminal plasma can help to eliminate the toxic effect caused by interactions of bulbourethral secretion and egg yolk or milk when cryopreserved with extender from plant sources. There are discrepancies in several studies on the beneficial effect of removal of seminal plasma in goat semen. Love et al. (2005) and Kozdrowski et al. (2007) reported a beneficial effect of removing seminal plasma on the frozen semen. Purdy (2006) and Kozdrowski et al. (2007) observed that the removal of seminal plasma had advantageous effect on semen freezing and thawing quality in buck if this is done properly. The use of short-term centrifugation with a relative high g-force has been observed to cause a positive effect on sperm cryosurvival (Carvajal et al., 2004). In contrast, Tuli and Holtz (1994), Gil et al. (2000), Azeredo et al. (2001) and Peterson et al. (2007) reported that the removal of seminal plasma decreased motility in frozen-thawed spermatozoa. Furthermore, Angora buck sperm frozen with or without centrifugation showed higher percentages of subjective motility (Sariozkan et al., 2010). Moreover, species, breeds and individual variation are also critical factors because the compositions of seminal plasma and sperm membrane vary greatly between species and individuals. Daramola and Adekunle (2017) recently observed that semen washing improved sperm quality in WAD goats when cryopreserved in tris egg yolk extender. Milk based extender from plant source such as soy bean and avocado have been developed and utilized for semen cryopreservation (Saragusty et al., 2006; Fukui et al., 2008; Daramola et al., 2016).To the best of our knowledge, comparative evaluation of motility, *in vitro* capacitation and acrosome reaction of

semen centrifuged using extenders from plant sources such soy bean milk (SBM) and avocado seed milk (ASM) during cryopreservation nis not available in literature. The aim of the present study was to assess the effect of centrifugation on motility, *in vitro* capacitation and acrosome reaction of WAD goat spermatozoa cryopreserved with tris SBM and ASM extenders.

MATERIALS AND METHODS

Experimental location and animal management

The study was conducted at the Teaching and Research Farm, Federal University of Agriculture, Abeokuta, Nigeria. Five WAD bucks aged 3–4 years were used for the study. They were maintained in intensive system and fed concentrate with guinea grass (*Panicum maximum*) as supplement.

Preparation of soy bean milk and avocado seed milk

Soy bean milk and avocado seed milk were prepared using the method of soymilk preparation (Odu et al., 2012). Soy bean seeds (100 g) were washed and soaked in 500 mL distilled water and then boiled for 30 min. After boiling, the water was discarded and the whole soy bean grains were washed again and finally allowed to cool. The grains were then blended for 5 minutes and then the slurry was cooled. Soy bean milk was extracted by filtration through a clean cotton cloth, centrifuged and then boiled again for 10 minutes. The slurry was allowed again to cool and transferred into a beaker and used fresh with a tris-based extender. Avocado fruit (34 g) was washed with distilled water and split open with knife to retrieve the seed. The outer coat was removed and then transferred into boiling water for 10 min. The boiled seed was chopped and blended. After blending, 500 mL of distilled water was added to the slurry, mixed and sieved with a clean white cloth. The slurry was then transferred into a beaker and used fresh with a tris-based extender.

Semen collection, dilution and storage

Semen samples collected from 5 intact WAD bucks with the aid of artificial vagina were pooled to minimize individual differences (Bucak and Tekin, 2007). The pooled semen sample was uniformly divided into 8 parts. Six parts were washed with phosphate buffer saline (PBS) by centrifuging at 500 *g* once, twice and thrice for 5 min each in order to remove seminal plasma while the remaining 2 parts (control) were not washed. The washed samples and the control were diluted at room temperature with the two tris-extenders each containing 20 mL of avocado seed milk and soy bean milk. A two-step process consisted of 2 fractions was used for the dilution. The composition of fraction 1 solution was tris-hydroxymethyl-aminomethane (2.42 g), citric acid (1.36 g), glucose (1 g), penicillin (0.028 g), soy bean milk (20 mL) and distilled water made up 100 mL. In addition to the composition of fraction 1, 14.0% glycerol (v/v) was added to fraction 2 solution. The washed semen samples were diluted with the Fraction 1 solution. Fraction 2 solution was subsequently added at a 1:1 ratio and the sample was loaded into 0.2 mL straws and treatment replicated twice (2 straws per treatment). Thereafter, the semen samples were gradually cooled to 4 °C at a rate of 0.25 °C/min and equilibrated at 4 °C for 5 min in TYFSF Refrigerated Incubator (Model: SPX-7OB III, Hebei China). The straws were subsequently plunged into liquid nitrogen for 30 days.

Subjective microscopic evaluation of sperm motility

Subjective microscopic evaluation of spermatozoa was carried using Celestron PentaView microscope (LCD-44348 by RoHS, China). Motile spermatozoa were assessed under the microscope as described by Bearden and Fuquay (1997).

Evaluation of acrosome reaction and sperm capacitation

The method earlier described by Somanath and Gandhi (2002) was used to assess the proportion of acrosome reaction with modification as follows: Cryopreserved semen samples were thawed by plunging straws into a water bath (37 °C) for 1 min. The samples were washed with non-culture medium, and the pellets were re-suspended in culture medium. Phosphate-buffered saline (0.9% wt/vol) was subsequently added and incubated with progesterone (2.5 mg/mL) at 38.5 °C (5% CO_2 in air; 100% humidity) for 20 min to induce acrosome reaction. The proportion of spontaneous acrosome reaction was determined by adding an equal volume of phosphate-buffered saline. Spermatozoa (100 cells per slide) 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 using an upright Carl Zeiss Fluorescent Microscope (Primo Star, Germany) equipped with phase contrast and epifluorescence optics (×1000 magnification). Chlortetracycline fluorescence assay as described by Collin et al. (2000) was used to evaluate the proportion of *in vitro* capacitation of the spermatozoa.

Statistical Analysis

The study was repeated 2 times and estimations were performed for the pooled semen samples for each centrifugation consisted of two straws and each measurement was repeated four times for each parameter. The results were analyzed using a two-way analysis of variance in SAS 2000 package and Duncan

Multiple Range Test was used to separate significantly different means (P < 0.05). The model used is shown below:

$$Y_{ijk} = \mu + E_i + W_j + (AB)_{ij} + \Sigma_{ijk}$$

Where, Y_{ijkl} .....Dependent variables,
μ .....Population mean,
E_i .....effect due to i^{th} Extenders,
i .....(1, 2),
W_j .....effect due to j^{th} different centrifugation,
j .....(1, 2, 3),
EW_{ij} .....effect of ij^{th} interaction between extenders and different centrifugation,
Σ_{ijkl} .....experimental error.

RESULTS

The motility of semen centrifuged and cryopreserved with soybean milk and avocado seed milk extenders are presented in Figure 1. Higher ($P < 0.05$) sperm motilities were observed in the two different extenders as the centrifugation times increased. Spermatozoa centrifuged at 3 times had higher ($P < 0.05$) percentage motility. Motility in SBM and ASM extenders were comparable at 3 centrifugations ($P > 0.05$). SBM extenders however had higher ($P < 0.05$) motility at 1 and 2 centrifugations as well as the control compared to the ASM extenders.

Acrosome reaction of sperm centrifuged and cryopreserved in soybean milk and avocado seed milk extenders are shown in Figure 2. Spermatozoa that were centrifuged had higher ($P < 0.05$) percentage of acrosome reaction with increased centrifugation times compared to the control. Optimal ($P < 0.05$) percentage of acrosome reaction were observed at one centrifugation and three centrifugations in ASM extenders compared other centrifugation times and SBM extenders, while optimal capacitation were observed at 2 and 3 centrifugations of SBM extenders and 1, 2, and 3 centrifugations of ASM extenders compared.

Sperm capacitation of sperm centrifuged and cryopreserved in soybean milk and avocado seed milk extenders are shown in Figure 3. Spermatozoa that were centrifuged had higher ($P < 0.05$) percentage of sperm capacitation with increased centrifugation times compared to the control. Optimal ($P < 0.05$) percentage of sperm capacitation were observed at 2 and 3 centrifugations in SBM extenders and 1, 2, and 3 centrifugations in ASM extenders compared other centrifugation times and SBM extenders.

DISCUSSION

The improved sperm motility in the present study compared to the control corroborated the beneficial importance of centrifugation of spermatozoa prior to cryopreservation. The finding agreed with Leboeuf et al. (2000) that removal of seminal plasma by centrifugation of buck semen increased motility

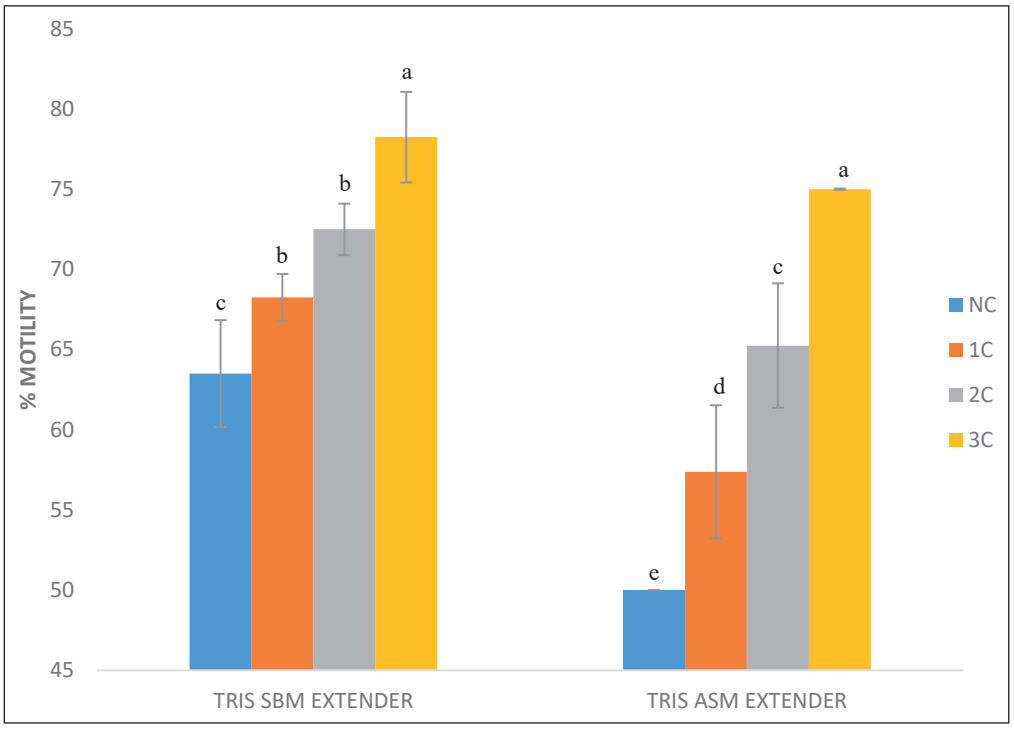

Figure 1. Motility of buck semen cryopreserved with three centrifugation protocolsin tris soy bean milk and avocado seed milk extenders. a, b, c, d, e: Mean values + SEM (n = 16) with different letters differ significantly ($P < 0.05$), SBM = Soybean milk, ASM = Avocado seed milk, NC = No centrifugation, 1C = centrifugation. 2C = centrifugations, 3C = centrifugations

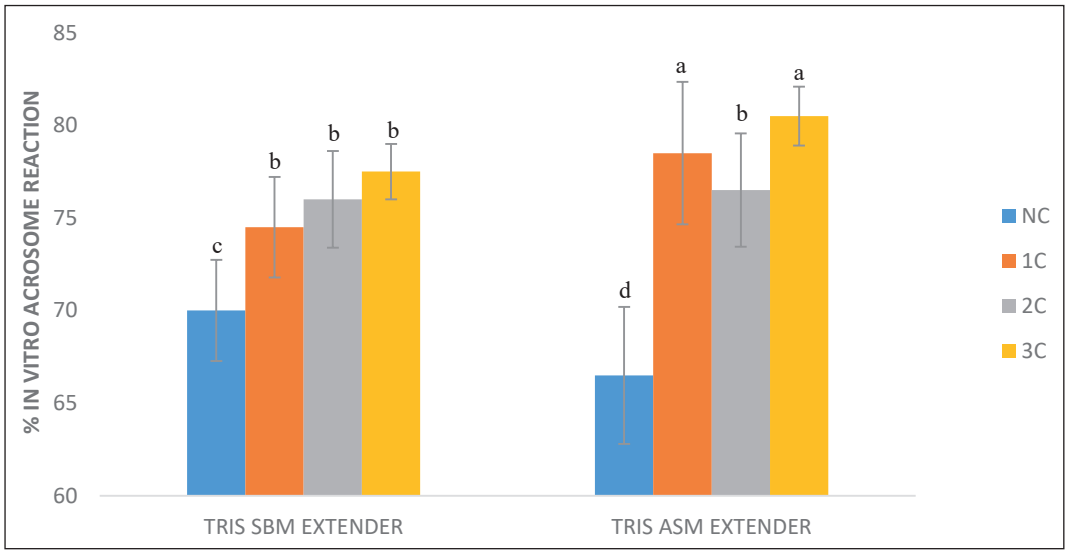

Figure 2. In vitro acrosome reaction of buck semen cryopreserved with three centrifugation protocols in tris soy bean milk and avocado seed milk extenders. a, b, c, d: Mean values + SEM (n = 16) with different letters differ significantly ($P < 0.05$), SBM = Soybean milk, ASM = Avocado seed milk, NC = No centrifugation, 1C = centrifugation. 2C = centrifugations, 3C = centrifugations

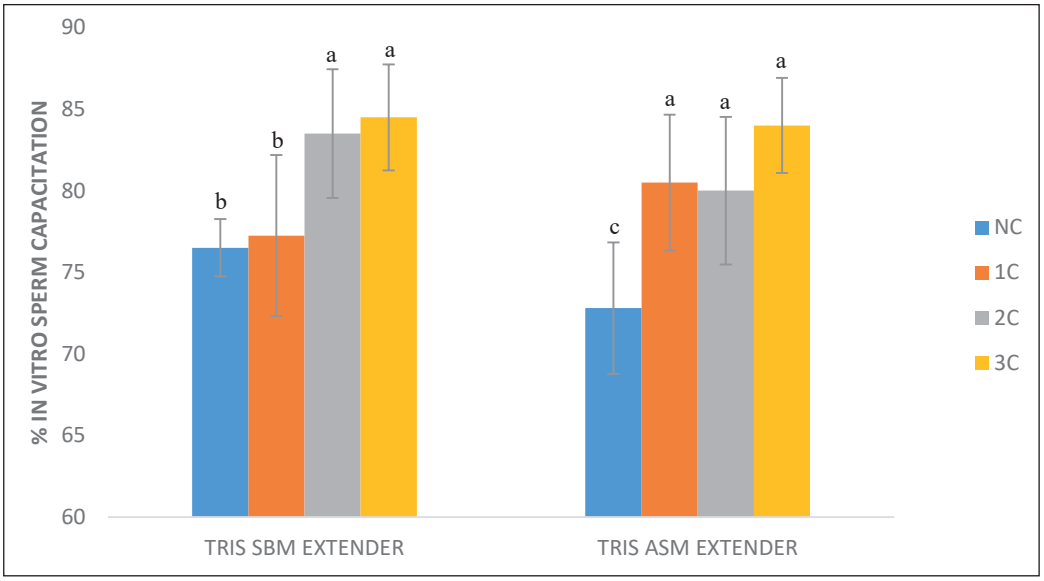

Figure 3. In vitro sperm capacitation of buck semen cryopreserved with three centrifugation protocols in tris soy bean milk and avocado seed milk extenders. a, b, c: Mean values + SEM (n = 16) with different letters differ significantly ($P < 0.05$), SBM = Soybean milk, ASM = Avocado seed milk, NC = No centrifugation, 1C = centrifugation. 2C = centrifugations, 3C = centrifugations

and the percentage of live spermatozoa during storage in egg yolk diluents (Leboeuf et al., 2000). In addition, Islam et al. (2006) and Daramola and Adekunle (2017) obtained beneficial effect of the removalof seminal plasma on the quality of goat semen during preservation in tris egg yolk extender. Some enzymes in seminal plasma, originating from bulborethral gland secretion, catalyse the hydrolysation of egg yolk lecithin and milk triglycerides of the extender, releasing sperm-toxic substances (lysolecithin and fatty acids) that lead to sperm damage (Roy, 1957; Pellicer-Rubio and Combamous, 1998). Removal of seminal plasma is usually carried out in artificial insemination programme in order to reduce its adverse effect on spermatozoa and improve sperm quality (Marti et al. 2006). The improvement in sperm quality in the present study indicated the beneficial effects of separating sperm by centrifugation from deleterious effects of coagulating enzyme such as phospholipase A in bulbourethral gland before dilution and freezing. Lebouef et al. (2000) reported that phospholipase A coagulates egg yolk extender and reduces the viability of sperm cells. In contrast, some findings indicated positive results without centrifugation (Cabrera et al.,

2005; Ari and Daşkin, 2010). Seminal plasma protects the sperm from damage by oxidative stress (Saleh et al., 2002), notwithstanding, ejaculated semen contains aging spermatozoa, defects, leukocytes and particle debris that can reduce sperm survival. Therefore, the separation of mammalian spermatozoa from seminal plasma by centrifugation is preferred and supported by several studies in order to increase motility and fertility after freeze-thaw procedure (Kozdrowski et al., 2007). In addition, different findings indicated that removal of the seminal fluid of chilled semen increases the sperm survival and motility (Kozdrowski et al., 2007).

Goat semen differs from other mammalian species due to the presence of enzymes in the seminal plasma that react with egg yolk and milk, resulting in damaged spermatozoa following production of toxic compounds (Pellicer-Rubio and Combarnous 1998). Furthermore, the lifespan of stored sperm is increased by centrifugation (Pellicer-Rubio et al., 1997). Centrifugation gave optimal sperm motility in equine as found by Waite et al. (2008) and Konyali (2012) supporting thus the results of the present study. Our results are further supported by the work of Marti et al. (2006) who showed that centrifugation yielded higher percentages of capacitated spermatozoa.

CONCLUSION

In the present study, no particular extender (ASM or SBM) was consistently superior in all the parameters, notwithstanding the results indicated that the removal of seminal plasma by centrifugation prior to dilution and cryopreservation improved sperm quality of WAD goat bucks and optimum improvement was achieved consistently with 3 washes.

Disclosure statement

The authors declare that there are no conflicts of interest.

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