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Effect of Three Different Centrifugation on Cryosurvival of Goat Spermatozoa Supplemented with Vitamin E

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RESEARCH ARTICLE

Abstract

Three different centrifugation frequencies viz., zero time (ZTC), one time (ITW), two times (2TC) and three times (3TC) supplemented with different levels of vitamin E were studied under in vitro cryosurvival of West African Dwarf (WAD) goat spermatozoa. Semen samples were washed ITW, 2TC and 3TC and supplemented each with 2, 4, 6 and 8 mM of vitamin E in a 3x4 factorial arrangements and cryopreserved for 30 days in liquid nitrogen. Data were subjected to one-way ANOVA. Spermatozoa cryopreserved with Tris-based extender supplemented with 6 and 8 mM using 3TC and 8 mM with 2TC had highest (P<0.05) percentage motility, percentage acrosome integrity and percentage live spermatozoa while highest (P<0.05) percentage membrane integrity were observed at 4, 6 and 8 mM. Cryopreserved spermatozoa with 6 and 8 mM for 3TC had the lowest (P<0.05) percentage abnormality and highest (P<0.05) percentage spermatozoa that underwent acrosome reaction including 8 mM using 2TC. Also 4, 6 and 8 mM for 3TC and 6 and 8 mM using 2TC had the highest (P<0.05) percentage spermatozoa that underwent capacitation. The results showed lowest (P < 0.05) concentrations of Malondialdehyde MDA at 2, 4, 6 and 8 mM for 1TC, 6 and 8 mM for 2TC and ZTC; and 8 mM vitamin E for 3TC. However, the Tris extenders supplemented with 2 and 4 mM for ZTC and 4 mM for 1TC had highest (P<0.05) values for arginase activity. Cryopreservation of WAD goat buck sperm with 2TC and 3TC improved viability and fertilizing ability.

Keywords: Cryopreservation; WAD buck; seminal plasma; fertilizing ability; sperm viability.

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INTRODUCTION

Semen cryopreservation is essential for the application of reproductive techniques such as artificial insemination (AI) and in vitro fertilization (IVF) which contributes to increase production of goat and genetic selection schemes (Luo et al., 2019; Lv et al., 2019). However, cryopreservation induces ultrastructural, biochemical, and functional damages in spermatozoa due to the temperature changes resulting in decreased motility and viability. In addition, causes of reduced sperm motility are related to seminal plasma enzymes. Therefore, seminal plasma plays an important role in sperm survival during cryopreservation process (Daramola and Adekunle, 2017). The deterioration and toxic effect of the seminal plasma were observed when goat's semen was diluted with egg yolk or milk extender. Nowadays, these extenders are widely used for the frozen storage of small ruminant semen (Bustani and Baiee, 2021). The presence of enzymes (bulbourethral secretion glycoprotein-60 and egg yolk coagulating enzyme) in the seminal plasma caused the harmful interactions between seminal plasma and egg yolk or milk (Dhaher and Aziz D2021; Lv et al.,

2019). Meanwhile, bulbourethral secretion glycoprotein-60 (BUSgp60) has a triacylglycerol hydrolase activity which decreases sperm motility and movement quality by disruption of cell membrane (Anand et al., 2017). Phospholipase A2 activity of egg yolk coagulating enzyme (EYCE) catalyse the hydrolysis of egg yolk phosphatidycholine (PC) into fatty acids and lysophosphatidylcholine (LPC) and LPC has toxic effect on buck spermatozoa by acting like a detergent on biomembrane, resulting in a loss of motility, membrane integrity and consequently low fertility rate (Anand et al., 2016). Therefore, several researchers reported that the removal of seminal plasma had favourable effect on semen freezing and thawing properties in goat buck (Kozdrowski et al., 2007; Ohaneje,et al., 2021; Salama et al., 2024;). However, Tuli and Holtz (1994), Azeredo et al. (2001) and Peterson et al. (2007) observed no favourable effect of the removal of seminal plasma in buck semen cryopreservation. Removal of seminal plasma is a time-consuming process that can damage cells if it is performed improperly; however, if it is done correctly, it can be beneficial (Purdy, 2006). Therefore, some previous researches have used a variety of centrifugation solutions and concentration regimes to find out the proper method for the improvement of cryopreserved semen quality. The centrifugation regimes and centrifugation solutions used in buck semen cryopreservation were 800 x g for 15 min with Tris citric acid buffer (Tuli and Holtz, 1994), 600 × g for 10 min with Krebs-Ringer phosphate plus sodium citrate (Azeredo et al.,2001), 1200 × g for 15 min with Tris citric acid glucose (TCG) buffer (Peterson et al., 2007), 1500 × g for 10 min with TCG (Kozdrowski et al., 2007), and 1000 × g for 10 min with Ringer's latate (Sariozkan et al., 2010). In other species, several researchers have studied the effects of different centrifugation regimes. Among other, Carvajal et al. (2004) observed the influence of different centrifugation regimes (400, 800, 1600, and 2400 × g) and reported that the use of short-term centrifugation with a relative high g-force (2400 × g for 3 min) caused a positive effect on Boar sperm cryosurvival. Webb and Dean (2009) described that post thaw motility of frozen stallion sperm was not different between centrifugation treatments (700 × g for 15 min, 600 × g for 12 min, and 400 × g for 7 min). Vitamin E (α-tocopherol) functions as a peroxyl radical scavenger. It helps to maintain the integrity of long chain polyunsaturated fatty acids in the membranes of cells and thus maintain their bioactivity (Traber and Atkinson, 2007). Supplementation with endogenous antioxidant (vitamin E) could reduce the impact of oxidative stress during sperm centrifugation, storage procedures and improve extended semen quality (Michael et al., 2009). Nonetheless, studies evaluating the effects of centrifugation protocols on WAD buck semen cryopreservation are still very limited.

MATERIALS AND METHODS

Experimental Location and animal management

The study was carried out at the Goat Unit of Teaching and Research Farm, Federal University of Agriculture, Abeokuta which falls within 70 10'N and 30 2'E and altitude76m above sea level. It lies within South-Western part of Nigeria with a prevailing tropical climate, a mean annual rainfall of 1,037 mm and average temperature of 34.7 oC (Google Earth, 2022). Eight (8) intact WAD bucks aged 2.5-3 years were used for this study. The bucks were kept under an intensive management system and maintained under a uniform and constant nutritional regimen with concentrate feed supplemented with guinea grass (Panicum maximum).

Cryopreservation procedures

Semen collection, dilution and storage

The effect of three different centrifugation protocols on cryosurvival of goat spermatozoa supplemented with vitamin E obtained from WAD goat bucks was carried out in a study repeated two times. Semen samples were collected from eight WAD bucks with the aid of artificial vagina. Only ejaculates showing >80% motility were pooled to minimize individual differences as recommended Bucak and Tekin (2007). Semen samples were diluted at 32°C at 1 : 4 ratio in a buffer of the following composition: Tris-hydroxymethyl-aminomethane (2.42g), citric acid (1.36g), glucpose (1g), penicillin (0.028g), egg yolk (20 mL) and distil water was added to make it up to 100 mL (Kumar and Atreja, 2012) as control. Diluted semen samples were washed with non-culture medium (normal saline) by centrifuging at 500 rpm one time (1TC), two times (2TC) and three times (3TC) for 5 minutes in order to remove semen plasma while the control group was not washed. Following centrifugation and removal of supernatant, the sediments were then diluted at 32 ◦C in a two-step process with a Tris-based extender consisted of 2 fractions. Fraction 1 solution consisted of Tris-hydroxymethyl-aminomethane (2.42g), citric acid (1.36g), glucose (1g), penicillin (0.028g), egg yolk (20 mL) and distil water make up 100 mL as control. Fraction 2 solution had the same composition as the Fraction 1 solution with the addition of 14.0 % glycerol (v/v). The sediments were diluted with the Fraction 1 solution and supplemented each with 0 mM, 2 mM, 4 mM, 6 mM and 8 mM of vitamin E respectively. 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). Subsequently, the straws were placed in a canister at 4cm above liquid nitrogen in the vaporous phase for 10 min to avoid cold shock before plunging them

directly and quickly into liquid nitrogen. Cryopreservation was done for 30 days. Thereafter, semen samples were evaluated for sperm quality characteristics.

Evaluation

Subjective microscopic evaluation of sperm motility

Sperm motility was determined as described by Bearden and Fuquay (1997). Briefly, semen was thawed for 2 min in Clifton Water bath (Model: 74178 by Nickel Electro Ltd, Weston-S-Mare Somerset, England) at 37 °C and accessed for sperm motility using Celestron PentaView microscope (LCD-44348 by RoHS, China) at 400x magnification. A 5 µL sample of semen was placed directly on a heated microscope slide and overlaid with a 22 x 22 mm cover slip. Each semen sample was measured using different slides and ten microscopic fields were examined to observe progressively motile spermatozoa that moved forward in essentially a straight line by four observers. Mean of the ten successive evaluations was recorded as the final motility score.

Acrosome integrity

Percentage of spermatozoa with intact acrosome was determined according to Ahmad et al. (2003). Briefly, 50 µL of each thawed semen sample was added to a 500 µL 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 ten different microscopic fields for each sample. Intactness of acrosome characterized by normal apical ridge of spermatozoa was assessed using Celestron PentaView LCD microscope (400x magnification).

Sperm membrane integrity

Hypo-osmotic swelling test (HOST) assay as described by Correa et al. (1997) was used to determine sperm membrane integrity and this was done by incubating 10 µL of semen in 100 µL hypo-osmotic solution (fructose and sodium citrate) at 37 °C for 30 min and 0.1 mL of the mixture was spread over a warmed slide, covered with a cover slip and observed under Celestron PentaView LCD digital microscope (400x magnification). Two hundred spermatozoa (200) were counted for their swelling characterized by coiled tail, indicating intact plasma membrane.

Sperm abnormality

Sperm abnormality was evaluated as described 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 a slide and dried. Two hundred and forty spermatozoa were counted and morphologically abnormal spermatozoa with defects in the head, midpiece and tail were observed (400x magnification) with Celestron PentaView LCD digital microscope.

Live sperm

Sperm livability was evaluated as described by Bearden and Fuquay (1997) with the use of eosin-nigrosin stain. A thin smear of mixture of semen and eosin-nigrosin solution were drawn across the slide and dried. Semen samples were examined under a digital microscope (Celestron Penta®) LCD view at 400x magnification for live spermatozoa. Spermatozoa that appear white were recorded as live spermatozoa and those that pick up the stain were recorded as dead spermatozoa.

Malondialdehyde concentration

Malondialdehyde (MDA) concentration, an index of lipid peroxidation in the stored semen was measured in a thiobarbituric acid reactive substances (TBARS) according to Yagi (1998). For this assay, 0.1 mL of sperm suspension was incubated with 0.1 mL of 150 mM Tris-HCl (pH 7.1) for 20 min at 37 °C. Subsequently, 1 mL of 10 % trichloroacetic acid (TCA) and 2 mL of 0.375 % thiobarbituric acid were added and incubated in boiling water for 30 min. Thereafter, the mixture was centrifuged for 15 min at 3000 xg inside blank tube and the absorbance was read with UV spectrophotometer (SW7504 model by Surgifriend Medicals, England) at 532 nm. Concentration of MDA was calculated as follows: The concentration of malondialdehyde MDA (nmol/mL) = AT – AB/1.56 × 105; where AT = Absorbance of the sample serum, AB= Absorbance of the blank, 1.56 × 105 molar absorptivity of MDA.

Arginase activity

Arginase activity was carried out according to the procedure of Lowry et al. (1951). Briefly, 0.1 g bovine serum albumin (BSA) as standard in 10 mL of water was used. The tubes containing 1 mL alkaline copper reagent (a mixture of copper sulfate reagent, sodium dodecyl sulfate solution, and sodium hydroxide solution (1:2:1) and 0.1 mL thawed semen samples were mixed and incubated for 10 min at room temperature. After this, 4 mL folin Ciocalteu's phenol reagent was added to the tubes, mixed and incubated for 5 min at 55 oC. Absorbance of the

samples was recorded at 650 nm using apectrophotometer (UV spectrophotometer, SW7504 model by Surgifriend Medicals, England).

In vitro acrosome reaction

Following cryopreservation, semen were thawed by plunging straws into a water bath (37 °C) for 1 min and the acrosome reaction was determined as described by Tardif et al. (1999) with modification as follows: Samples of vitrified spermatozoa were washed with non-culture medium Phoshate Bovine Saline(PBS), and the pellets were re-suspended in culture medium (Calcium chloride dihydrate 265 mg/L, Magnesium chloride anhydrous 46 mg/L, Potassium chloride 200 mg/L, Sodium chloride 8000 mg/L, Sodium dihydrogen phosphate anhydrous 50 mg/L and D-Glucose 1000 mg/L). Immediately after the inclusion of 0.9 % wt/vol PBS acrosome reaction was induced by incubating spermatozoa for 20 min with progesterone (2.5 mg/mL) at 38.5 °C (5 % CO2 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 chlortetracycline (CTC) fluorescence assay as described by Collin et al. (2000). 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). Vitrified semen suspension (5 µL) was mixed with 5 µL of CTC solution on a warmed slide (37 °C). After 30 sec, 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. After adding a cover slip, slide was examined with an upright Carl Zeiss Fluorescent Microscope (Primo Star, Germany) equipped with phase contrast and epifluorescence optics, and 100 cells were counted per slide. The proportion of vitrified spermatozoa that exhibited pattern B according to the CTC assay was determined. Spermatozoa characterized by bright anterior head and faint fluorescence in the post-acrosome region were classified as capacitated spermatozoa while non-capacitated sperm had bright uniform fluorescence over the head.

Statistical analysis

The study was repeated 2 times and estimations were performed for the pooled semen samples for each protocol consisted of two straws in repeated measurements (each measurement repeated ten times for motility and eight times for acrosome integrity, membrane integrity, abnormality, live sperm, MDA, arginase activity, acrosome reaction and capacitation). The results analyzed using a 3 x 5 factorial arrangement in SAS (1999) package were expressed as the means +SE. Duncan Multiple Range Test (Duncan, 1955) was used to separate significantly different means ($P<0.05$). The model used is shown below:

$Yijk= \mu + Ai + Bj + (AB)\ ij + \sum ijk$

Where,

Yijkl= Dependent variables

µ = Population mean

Ai= Effect due to ithcentrifugation, i = (1, 2, 3)

Bj = Effect due to jth level of vit E inclusion, j = (0, 2, 4, 6, 8)

ABij= Effect of ijth interaction between centrifugation protocols and levels of vit E inclusion

∑ijkl= Experimental error.

RESULTS

The results (Table 1) showed higher ($P < 0.05$) sperm motility, acrosome integrity, membrane integrity and live spermatozoa in 1TC, 2TC and 3TC in Tris extenders supplemented with vitamin E compared to ZTC. Spermatozoa cryopreserved with Tris-based extender supplemented with 6 mM and 8 mM inclusion levels of vitamin E using 3TC and 8 mM inclusion levels of vitamin E using 2TC had highest ($P < 0.05$) percentage motility compared to other inclusion levels, 1TC, ZTC and the control. Motility was better sustained at 2TC and 3TC. However, the results showed highest ($P < 0.05$) percentage acrosome integrity in extender supplemented with 6 mM and 8 mM inclusion levels of vitamin E using 2TC and 3TC compared to other inclusion levels, 1TC, and ZTC. Highest ($P < 0.05$) percentage membrane integrity were observed at 4 mM, 6 mM and 8 mM inclusion levels of vitamin E using 2TC

and 3TC compared to other inclusion levels, 1TC, ZTC and the control. Membrane integrity was better sustained in extenders supplemented with vitamin E for 2TC and 3TC. However, highest (P < 0.05) percentage live spermatozoa were observed in extender supplemented with 8 mM inclusion levels of vitamin E using 2TC and 3TC compared to other inclusion levels, 1TC, ZTC and the control. However, spermatozoa cryopreserved with Tris-based extender supplemented with 6 mM and 8 mM inclusion levels of vitamin E in 3TC had lowest (P < 0.05) percentage abnormality compared to other inclusion levels, 1TC, 2TC and the control.

Table 1. Effect of different centrifugation protocols and vitamin E supplementation on viability of buck spermatozoa

		Parameter				
	Protocols(centrifugation)	**MOT (%)**	**ACI (%)**	**MI (%)**	**LIVE (%)**	**ABN (%)**
	0 Mm	32.00 ± 7.35^{i}	42.00 ± 1.15^{f}	44.00 ± 1.41^{f}	47.50 ± 2.50^{e}	4.75 ± 0.48^{a}
	2 mM	50.00 ± 0.00^{f}	54.00 ± 2.45^{d}	51.50 ± 0.96^{e}	60.00 ± 4.08^{c}	2.50 ± 0.29^{b}
ZTC	4 mM	55.00 ± 5.00^{e}	56.00 ± 2.83^{d}	62.50 ± 0.96^{d}	65.00 ± 2.89^{c}	2.50 ± 0.50^{b}
	6 mM	56.80 ± 4.16^{e}	61.50 ± 2.22^{c}	66.00 ± 0.82^{c}	70.00 ± 0.00^{c}	3.00 ± 0.00^{b}
	8 mM	65.20 ± 4.10^{c}	65.50 ± 2.06^{c}	60.50 ± 0.50^{d}	72.50 ± 2.50^{b}	2.00 ± 0.41^{c}
	0 mM	40.60 ± 2.71^{h}	45.00 ± 0.58^{f}	46.00 ± 0.82^{f}	57.50 ± 2.50^{d}	4.00 ± 0.41^{a}
	2 mM	57.40 ± 3.28^{de}	58.50 ± 2.87^{d}	60.00 ± 0.82^{d}	60.00 ± 4.08^{c}	3.00 ± 0.41^{b}
	4 mM	57.60 ± 3.31^{de}	61.00 ± 3.51^{c}	67.00 ± 0.58^{c}	70.00 ± 4.01^{b}	3.00 ± 0.41^{b}
1TC	6 mM	66.80 ± 1.83^{b}	63.00 ± 2.52^{c}	70.50 ± 0.96^{b}	67.50 ± 2.50^{bc}	2.00 ± 0.58^{c}
	8 mM	70.80 ± 1.80^{b}	67.00 ± 1.29^{b}	65.50 ± 0.50^{c}	72.50 ± 2.50^{b}	2.21± 0.25^{c}
	0 mM	45.60 ± 1.94^{g}	48.50 ± 0.96^{e}	50.50 ± 0.96^{e}	62.50 ± 2.50^{c}	3.50 ± 0.29^{ab}
	2 mM	60.60 ± 3.63^{d}	67.50 ± 0.96^{b}	65.50 ± 0.96^{c}	70.00 ± 4.08^{b}	2.50 ± 0.29^{b}
	4 mM	63.20 ± 2.99^{c}	68.50 ± 1.71^{b}	73.50 ± 0.96^{a}	72.50 ± 2.50^{b}	2.00 ± 0.41^{c}
2TC	6 mM	68.80 ± 0.73^{b}	70.50 ± 0.96^{a}	76.00 ± 0.82^{a}	67.50 ± 2.50^{bc}	1.75 ± 0.25^{d}
	8 mM	73.40 ± 1.29^{a}	70.00 ± 0.82^{a}	72.00 ± 1.41^{a}	75.00 ± 2.89^{a}	1.50 ± 0.29^{d}
	0 mM	48.80 ± 2.24^{f}	50.00 ± 0.00^{e}	51.00 ± 1.00^{e}	62.50 ± 2.50^{c}	2.75 ± 0.25^{b}
	2 mM	64.40 ± 2.59^{c}	68.50 ± 1.26^{b}	68.50 ± 0.50^{c}	70.00 ± 0.00^{b}	2.25 ± 0.48^{c}
	4 mM	66.20 ± 1.66^{b}	69.50 ± 0.96^{b}	74.50 ± 0.96^{a}	72.50 ± 2.50^{b}	1.75 ± 0.25^{d}
3TC	6 mM	71.40 ± 1.57^{a}	71.00 ± 1.73^{a}	77.50 ± 0.50^{a}	72.50 ± 2.50^{b}	1.25 ± 0.25^{e}
	8 mM	75.00 ± 1.58^{a}	73.50 ± 1.26^{a}	73.50 ± 1.71^{a}	80.00 ± 4.08^{a}	1.25 ± 0.25^{e}

Note: ^{a,b,c,d,e,f,g,h,i}Values within the same column with different superscripts differ (P < 0.05), MOT: Motility, ACI: Acrosome Integrity, MI: Membrane Integrity, ABN: Abnormality, SE: Standard Error. ZTC: Zero time centrifugation, 1TC:One time centrifugation, 2TC:Two times centrifugation, 3TC: Three times centrifugation.

The results (Table 2) showed lower (P < 0.05) concentrations of MDA in Tris egg yolk based extender supplemented with vitamin E using ZTC, 1TC, 2TC and 3TC. Lowest (P < 0.05) concentrations of MDA were observed at 2 mM, 4 mM, 6 mM and 8 mM inclusion levels of vit E for 1TC, 6 mM and 8 mM inclusion levels of vitamin E for 2TC and ZTC and 8 mM inclusion levels of vitamin E for 3TC compared to other levels of inclusions of vitamin E and their control. Reduction in MDA concentrations was more pronounced at 1TC. However, the extenders supplemented with 2 mM and 4 mM inclusion levels of vitamin E for ZTC and 4 mM inclusion levels of vitamin E for 1TC had highest (P < 0.05) values for arginase activity compared to other levels of inclusion of vitamin E for ZTC, 1TC, 2TC and 3TC.

Table 2. Effect of different centrifugation protocols and vitamin E supplementation on seminal oxidative parameters of buck spermatozoa

		Parameters	
	Protocol(centrifugation)	**MDA (nmol/mL)**	**ARG (units/ng protein)**
ZTC	0 mM	0.13 ± 0.03^{b}	1.21 ± 0.07^{k}
	2 mM	0.04 ± 0.01^{d}	3.21 ± 0.04^{a}
	4 mM	0.16 ± 0.10^{ab}	3.21 ± 0.03^{a}
	6 mM	0.01 ± 0.02^{e}	1.70 ± 0.03^{e}
	8 mM	0.01 ± 0.02^{e}	1.62 ± 0.02^{f}
1TC	0 mM	0.10 ± 0.01^{b}	1.58 ± 0.01^{gh}
	2 mM	0.02 ± 0.01^{e}	1.86 ± 0.01^{c}
	4 mM	0.01 ± 0.04^{e}	3.21 ± 0.05^{a}
	6 mM	0.02 ± 0.04^{e}	1.71 ± 0.05^{e}
	8 mM	0.02 ± 0.02^{e}	1.76 ± 0.03^{d}
2TC	0 mM	0.10 ± 0.03^{b}	1.35 ± 0.05^{j}
	2 mM	0.06 ± 0.01^{cd}	1.44 ± 0.03^{i}
	4 mM	0.06 ± 0.03^{cd}	1.89 ± 0.03^{c}
	6 mM	0.04 ± 0.01^{e}	1.57 ± 0.02^{g}
	8 mM	0.01 ± 0.01^{e}	1.63 ± 0.01^{f}
3TC	0 mM	0.18 ± 0.01^{a}	1.35 ± 0.01^{j}
	2 mM	0.07 ± 0.05^{cd}	1.70 ± 0.01^{e}
	4 mM	0.09 ± 0.01^{c}	1.46 ± 0.05^{i}
	6 mM	0.08 ± 0.01^{c}	1.62 ± 0.01^{f}
	8 mM	0.01 ± 0.01^{e}	2.11 ± 0.01^{b}

Note: ^{a,b,c,d,e,f,g,h,I,j,k} Values within the same column with different superscripts differ (P < 0.05), MDA: Malondialdehyde, ARG: Arginase activity, SE: Standard Error. ZTC: Zero time centrifugation, 1TC:One time centrifugation, 2TC:Two times centrifugation, 3TC: Three times centrifugation.

The results (Table 3) showed higher (P < 0.05) percentage of spermatozoa cryopreserved with Tris-based extenders supplemented with vitamin E using 1TC, 2TC and 3TC and ZTC that underwent acrosome reaction and capacitation. More spermatozoa (P < 0.05) cryopreserved with vitamin E using different centrifugation protocols underwent induced acrosome reaction and capacitation compared to ZTC and their control. However, Tris-based extenders supplemented with 6 mM and 8 mM of vitamin E using 3TC and 8 mM inclusion levels of vitamin E using 2TC had highest (P < 0.05) percentage of spermatozoa that underwent acrosome reaction compared to other inclusion levels of vitamin E for 2TC and 3TC, 1TC, ZTC.

Table 3. Effect of different centrifugation protocols and vitamin E supplementation on *in vitro* acrosome reaction (%) and capacitation (%) of buck spermatozoa

		Parameter	
	Protocols (centrifugation)	***In vitro* acrosome reaction (%)**	***In vitro* capacitation (%)**
ZTC	0 mM	41.00 ± 1.91^{f}	40.00±3.27^{e}

	2 mM	75.00±1.00[b]	59.00±2.52[bc]
	4 mM	53.00±3.00[d]	55.00±1.91[c]
	6 mM	59.00±1.91[cd]	57.00±2.52[c]
	8 mM	67.00±1.91[c]	56.00±2.83[c]
1TC	0 mM	48.00±3.27[e]	45.00±3.00[de]
	2 mM	69.00±4.12[b]	63.00±3.42[bc]
	4 mM	61.00±4.43[cd]	51.00±4.43[cd]
	6 mM	55.00±8.39[d]	57.00±1.91[c]
	8 mM	64.00±2.83[c]	69.00±1.91[b]
2TC	0 mM	47.00±2.52[e]	41.00±2.52[e]
	2 mM	67.50±4.50[c]	61.00±5.97[c]
	4 mM	72.50±1.26[b]	74.00±4.76[b]
	6 mM	77.00±1.91[b]	85.00±3.00[a]
	8 mM	81.00±2.52[a]	81.00±5.74[a]
3TC	0 mM	48.00±5.66[e]	49.00±1.91[d]
	2 mM	75.50±2.63[b]	74.00±1.15[b]
	4 mM	77.00±2.52[b]	81.50±3.59[a]
	6 mM	82.00±2.00[a]	86.00±3.83[a]
	8 mM	87.00±2.52[a]	86.00±4.46[a]

Note: [a,b,c,d,e,f] Values within the same column with different superscripts differ ($P < 0.05$), SE: Standard Error. ZTC: Zero time centrifugation, 1TC: One time centrifugation, 2TC: Two times centrifugation, 3TC: Three times centrifugation.

Spermatozoa cryopreserved with Tris-based extender supplemented with 4 mM, 6 mM and 8 mM inclusion levels vitamin E using 3TC and 6 mM and 8 mM using 2TC had highest ($P < 0.05$) spermatozoa that underwent capacitation compared to other inclusion levels of vitamin E for 2TC and 3TC, 1TC, and ZTC. Acrosome reaction and capacitation were better sustained in Tris-based extenders supplemented with vitamin E using 2TC and 3TC.

DISCUSSIONS

The removal of seminal plasma increased WAD buck semen motility and viability during cryopreservation process and this can be seen in the findings from Ritar and Salamon (1982), Machado and Simplicio (1992), Love et al. (2005) and Kozdrowski et al. (2007) who reported a beneficial effect of removing seminal plasma on the freezeability of semen. However, contradicts with the findings of Tuli and Holtz (1994), Gil et al. (2000), Azeredo et al. (2001) and Peterson et al. (2007) who reported that the removal of seminal plasma decreased motility in frozen-thawed spermatozoa. Following supplementation with vitamin E, WAD buck semen, with 2TC and 3TC in Tris-based extender, demonstrated higher percentages of subjective motility compared to that of that of ZTC in Tris-based extender. Some of these factors may be attributed to different processing procedures (namely, centrifugation solution, centrifugation regimes, cooling and freezing rate), and the concentration of seminal plasma remaining after centrifugation. 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 (Leboeuf et al., 2000). The absence of critical proteins, such as 20-kDa (Perez-Pe et al., 2001), 25-kDa (Lessard et al., 2000), 26-kDa (Gerena et al., 1998) caused lower recovering effect on sperm viability. Major proteins of caprine seminal plasma bind to the low-density lipoprotein fraction of hen's egg yolk leading to adhesion of these proteins to sperm membrane and prevent cryodamage (Manjunath et al., 2002). However, there was a negative correlation between one of the protein bands (16 kDa) and semen freezability due to the impaired linking of egg yolk lipoproteins to sperm membrane (Brandon et al. 1999; Zahn et al., 2006). Meanwhile, vesicular gland solutions partially inhibit the

negative effect of BUSgp-60 bulbourethral gland secretion (Nunes et al. 1982) which is called EYCE hydrolyses egg yolk phosphatidylcholine (PC) and produces lysophosphatidylcholine (LPC) which has toxic effects by acting on biomembranes of spermatozoa as a detergent. The formation and strength of detergent properties are temperature dependent (Peterson et al. 2007). Therefore, the amount of compositions formed in the seminal plasma and the strength of detrimental effect to the spermatozoa during cryopreservation process.

Acrosome and membrane integrity were higher in extender supplemented with vitamin E that were centrifuged two and three times compared with one time centrifugation and those that were not washed. According to Arnoult et al., (2012) the physiological function of BUSgp60 in goat seminal plasma could be related to the acrosome reaction because it has been shown that a high degree of similarity with lipases of the PL-RP2 subfamily, which present lipase and phospholipase activities, for participation in the energy metabolism of spermatozoa. Removal of goat SP containing BUSgp60 (called EYCE) may have decreased acrosome and membrane deformities in the semen washed two and three times groups. In addition, it may also be pointed out that centrifugation of seminal plasma positively affected acrosome and membrane integrity. In contrast to the present observation, Azerado et al. (2001) did not find any difference between centrifugation of goat semen after thawing for membrane integrity. The different results may be due to the difference in solutions used for centrifugating semen.

Lower percentage of spermatozoa with abnormality in extender supplemented with vitamin E for 2TC and 3TC observed in this study compared to 1TC and ZTC suggested that supplementing the extender with vitamin E before cryopreservation had beneficial effects on sperm morphology. It could also be linked to the fact that removal of BUSgp-60 bulbourethral gland secretion (Nunes et al., 1982) during centrifugation which hydrolyses egg yolk phosphatidylcholine (PC) and produces lysophosphatidylcholine (LPC) which has toxic effects by acting on biomembranes of spermatozoa. Moreover, the percentage spermatozoa abnormalities observed were within the range for post-thawed goat semen of Brazilian College of Animal Reproduction (Henry and Neves, 1998) in extender supplemented with vitamin E for three different centrifugation protocols and ZTC,on the other hand, the findings for higher percentage of live spermatozoa in extender supplemented with vitamin E for 2TC and 3TC observed in this study compared to 1TC and ZTC could be attributed to the removal of the protein bands (16 kDa) and semen freezeability due to the impaired linking of egg yolk lipoproteins to sperm membrane (Brandon et al., 1999; Zhan et al., 2006) and were similar with those of Ritar and Salamon, 1982 who obtained higher rates of live spermatozoa after thawing when the seminal plasma had been removed. Also, Ustuner et al. (2009) obtained similar findings with current study, and pointed out that the presence of buck seminal plasma had a detrimental effect on post-thaw motility. Hence, the solution used for seminal plasma separation may have been important.

The reduced concentrations of MDA observed in extenders following centrifugation of seminal plasma in spermatozoa supplemented with vitamin E further support the protective role of vitamin E in this study and is in consonant with previous study that vitamin E inhibits lipid peroxidation (Verma and Kanwar, 1999), indicating possible action of vitamin E in increasing antioxidant enzyme activities in the semen and thereby provide indirect protection against free radical damage in spermatozoa. Vitamin E is an efficient antioxidant that protects cells and tissues and initiates a host of receptor-mediated effects (Geva et al., 1996). The present results contributed to its protective effects against oxidative damage in spermatozoa and indicate its ability involving reduced concentration of MDA in cryopreserved WAD spermatozoa, and direct scavenging action on highly toxic hydroxyl radicals (Pähkal et al., 1998). Addition of vitamin E to cryopreservation media during freeze-thaw process decreased lipid peroxidation and apoptosis of stallion spermatozoa (Bansal and Bilaspuri, 2009), reduced injuries to ram spermatozoa (Succu et al., 2011) and decreased oxidative stress to red deer spermatozoa (Dominguez-Rebolledo et al., 2010). In this study, the removal of seminal plasma by centrifugation following the addition of vitamin E to cryopreservation media might have enhanced survival of buck spermatozoa during cryopreservation.

Positive correlation has been reported between sperm motility and arginase activity in both seminal plasma and spermatozoa (Elgun et al., 2000; Eskiocak et al., 2006). The main role of arginase in testis is the regulation of nitric oxide (NO) concentration (Nathan, 1997). Increase in arginase activity generally results in lower NO concentration and subsequently leads to increased sperm motility (Elgun et al., 2000). It is possible that besides the direct scavenging effect, the stimulatory effect of vitamin E on arginase activity may result in an inhibition of nitric oxide synthase activity and consequently, a decrease in the NO level (Aydogdu et al., 2006). This action could be the possible reason for the higher concentration of arginase activity at 1TC and ZTC in this study. Other possible reason to support this beneficial effect in this study could be linked to activity of arginine and its derivative compounds that is known to act as major reserve for adenosine triphosphate (ATP), the molecular mean of intracellular energy transfer, and as a regulatory sink for phosphate, a pivotal action in the regulation of metabolic processes (Block, 2010). This body of evidence supports the results obtained in this study and therefore indicates beneficial role of arginase activity enhanced by vitamin E for improving cryosurvival of spermatozoa.

Evaluation of acrosome reaction can be used to predict success of fertilization in artificial insemination programme. Mammalian spermatozoa undergo capacitation, a series of intracellular and membrane physicochemical changes that give spermatozoa ability to fertilize ovum (Patrat et al., 2000). Only capacitated spermatozoa have ability to undergo acrosome reaction (Arnoult et al., 1996). The present in vitro study indicated that removal of seminal plasma through centrifugation or centrifugation following supplementation of vitamin E was able to maintain fertilizing capacity as evidenced by better percentage of capacitation and acrosome reaction of cryopreserved spermatozoa. In bull spermatozoa, vitamin E administration increased plasminogen activator activity, known to be involved in sperm capacitation and acrosome reaction (O'Flaherty et al., 1999). This result was in agreement with Parrish et al. (1999) who demonstrated that vitamin E improved capacitation of bovine spermatozoa in vitro.

CONCLUSIONS

The findings of the present study indicated that cryopreservation of WAD goat buck sperm supplemented with 6 mM and 8 mM for two and three times centrifugation protocols resulted in improved viability parameters (sperm motility, acrosome integrity, membrane integrity and live sperm and reduced abnormality) and fertilizing ability parameters (acrosome reaction and sperm capacitation), and reduced MDA concentration compared to zero centrifugation protocol. This study provided the practical and beneficial effects that can be obtained by removing seminal plasma through centrifugation in WAD goat semen during cryopreservation. Semen samples obtained from WAD goat bucks would be suitable satisfactory for AI programme in three times centrifugation protocol.

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Conflicts of Interest

The manuscript does not contain clinical studies or patient data and the authors declare that they have no conflict of interest with respect to this study.

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