

# Cryosurvival of goat spermatozoa in Tris-egg yolk extender supplemented with vitamin C

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## INTRODUCTION

The WAD goats possess certain valuable traits that confer adaptation notably viability under endemic trypanosomiasis challenge, and adaptability to hot humid tropics. However, some breeds which do not have these adaptive traits are gradually replacing them to such an extent that the WAD goats are in danger of extinction. Preservation would enable producers to keep semen of this important breed, such that it could be used for Artificial Insemination over extended pe-

## SUMMARY

The effect of adding different levels of vitamin C to semen extenders on cryosurvival of spermatozoa obtained from West African Dwarf (WAD) bucks during cryopreservation was studied. Tris-egg yolk based extenders supplemented with 2, 4, 6 and 8 mM of vitamin C were diluted with pooled semen samples. The diluted semen samples were cryopreserved for 30 days and thereafter evaluated for sperm quality characteristics. Following cryopreservation, acrosome reaction and capacitation of spermatozoa were induced *in vitro*. The results showed higher ( $p < 0.05$ ) sperm motility, acrosome and membrane integrities in extenders supplemented with vitamin C compared to the control and highest values were recorded in 8 mM. Reduced concentrations of MDA were observed in extenders supplemented with 6 mM and 8 mM of vitamin C compared to other levels and the control. The extenders supplemented with vitamin C had higher ( $p < 0.05$ ) values of arginase activity compared to the control. Lower ( $p < 0.05$ ) leukocytes were observed in extenders supplemented with vitamin C compared to the control and lowest values ( $p < 0.05$ ) were observed by the addition of 8 mM. The results showed that more spermatozoa cryopreserved in extenders supplemented with 6 mM vitamin C underwent acrosome reaction and capacitation ( $p < 0.05$ ). The finding indicated that vitamin C improved sperm quality parameters. The improvement in viability parameters of cryopreserved WAD buck spermatozoa was optimum at 8 mM of vitamin C.

## Supervivencia de espermatozoides de cabra crioconservados en diluyente a base de yema de huevo-tris suplementado con vitamina C

## RESUMEN

Se estudió el efecto de suplementar a los diluyentes con diferentes niveles de vitamina C, sobre la supervivencia de espermatozoides de cabras WAD (West African Dwarf) durante la criopreservación. Para diluir una mezcla de espermatozoides, fueron usados diluyentes basados en tris-yema de huevo, suplementados con 2, 4, 6, y 8 mM de vitamina C. Las muestras de semen, diluidas fueron crioconservadas durante 30 días y a continuación se evaluaron las características de calidad del esperma. Después de la crioconservación, se indujeron *in vitro* la reacción del acrosoma y la capacitación del espermatozoide. Los resultados mostraron mayor ( $p < 0,05$ ) motilidad del esperma y superiores integridades del acrosoma y la membrana en los diluyentes con vitamina C frente al control, registrándose los valores más elevados a concentraciones de 8 mM. En los diluyentes suplementados con 6 y 8 mM de vitamina C se registraron concentraciones de MDA más reducidas que en los restantes niveles y el control. Los diluyentes suplementados con vitamina C, tienen mayores valores ( $p < 0,05$ ) de actividad de arginasa que el control. En los diluyentes suplementados con vitamina C fueron observados menos leucocitos ( $p < 0,05$ ) que en el control, correspondiendo los valores mas bajos con la adición de 8 mM. Los resultados ponen de manifiestos que mayor número de espermatozoides preservados en diluyentes suplementados con 6 mM de vitamina C presentan la reacción del acrosoma y capacitación ( $p < 0,05$ ). Los hallazgos indican que la vitamina C mejora los parámetros de calidad del esperma. La mejora de los parámetros de viabilidad espermática de cabras WAD fue optima con 8 mM de vitamina C.

riod of time. The survival of ejaculated sperm in seminal plasma alone is limited to few hours. To maintain sperm for longer periods and cryopreserve semen, dilution with a protective extender is necessary in order to maintain fertilizing capacity of spermatozoa during *in vitro* storage at low temperatures. Irrespective of the constituents of extenders however, viability of spermatozoa deteriorates at low temperatures during storage (Maxwell and Watson, 1996). Sperms are subjected to oxidative stress resulting from lipid peroxidation, which can lead to membrane damage, reduced sperm

viability and lower fertility (Donghue and Donoghue, 1997). Although semen contains antioxidants that balance lipid peroxidation and prevent excessive peroxide formation (Lewis *et al.*, 1997), the endogenous antioxidants of semen are not sufficient during storage (Maxwell and Salamon, 1993). Moreover, the levels of antioxidant decreased during the preservation process by dilution of semen with extender and excessive generation of reactive oxygen species (ROS) molecules (Andrabi, 2009; Kumar *et al.*, 2011). *In vitro* studies suggested that the addition of some antioxidants to semen extender could improve motility and survival of spermatozoa (Molinia *et al.*, 1994; Sánchez-Partida *et al.*, 1997; Bilodeau *et al.*, 2002). Vitamin C has been demonstrated as effective antioxidants (Ondei *et al.*, 2009). Perusal of literatures however revealed no information on the effect of supplementation of vitamin C as exogenous antioxidant on sperm viability of WAD buck semen during cryopreservation. Hence, the objective of the present study was to evaluate the effect of adding different levels of vitamin C to semen extenders on cryosurvival of spermatozoa obtained from WAD bucks during cryopreservation.

## MATERIAL AND METHODS

### EXPERIMENTAL SITE, ANIMALS AND MANAGEMENT

The experiment was carried out at the Goat unit of Directorate of University Farm, Federal University of Agriculture Abeokuta, Nigeria which falls within 7°10'N and 3°2'E and altitude 76 m 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°C. Six (6) WAD bucks used for this study were selected from a group of twenty (20) WAD bucks. The ages of the bucks ranged between 2.5-3 years. The animals were kept under intensive management and maintained under a uniform and constant nutritional regimen with concentrate feed supplemented with guinea grass (*Panicum maximum*).

### SEMEN COLLECTION, DILUTION AND STORAGE

Semen samples were collected from the bucks with the aid of artificial vagina. A total of six pooled semen samples (each semen sample originating from six bucks) were used. This was done for uniformity and to eliminate individual differences. Only semen samples showing >80% motility were pooled. The pooled semen samples were diluted at 32°C in a two-step process with Tris-based extenders composed of 2 fractions. The Fraction 1 solution contained Tris-hydroxymethyl-aminomethane (2.42 g), citric acid (1.36 g), glucose (1 g), penicillin (0.028 g), egg yolk (20 ml) and distilled water made up 100 ml as control. Fraction 2 solution had the same composition as the Fraction 1 solution but with the addition of 14.0% glycerol (v/v) to Fraction 2. The pooled semen samples were split into 5 equal aliquots, diluted with the Fraction 1 solution and supplemented each with 0, 2, 4, 6 and 8 mM of vitamin C respectively at a final concentration of  $618 \times 10^6$  spermatozoa/ml and pH of 6.92. Fraction 2 solution was subsequently added. Diluted semen samples were then loaded into 2 ml plastic straws, sealed with polyvinyl, cooled to 4°C

at a rate of 0.25°C/min, and equilibrated at 4°C for 10 min in TYFSF Refrigerated Incubator (Model: SPX-70B III, Hebei China). Subsequently, the straws were placed in a rack at 4 cm above liquid nitrogen in the vaporous phase for about 10 min before plunging them directly and quickly into liquid nitrogen for 30 days and thereafter evaluated for sperm quality characteristics. Estimations were performed for the pooled semen samples in repeated measurements using different slides and each measurement repeated five times.

### SPERM MOTILITY

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

### ACROSOME INTEGRITY

The percentage of spermatozoa with intact acrosomes was determined according to Ahmad *et al.*, (2003). Briefly, 50 µL of each 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 at least three different microscopic fields for each sample using Celestron PentaView LCD microscope (400× magnification). Intactness of acrosome characterized by normal apical ridge of spermatozoa was assessed.

### SPERM MEMBRANE INTEGRITY

Hypo-osmotic swelling test (HOST) assay as described by Jeyendran (*et al.*, 1984) was used to determine sperm membrane integrity. This was done by incubating 10 µL 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 (400× magnification). Two hundred spermatozoa 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 the slide and dried. The percentage of morphologically abnormal spermatozoa with defects in the head, midpiece and tail were observed under Celestron PentaView LCD microscope (400× magnification).

### MALONDIALDEHYDE CONCENTRATIONS

Malondialdehyde (MDA) concentration as index of lipid peroxidation (LPO) 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 followed by incubation in boiling water for 30 min. Thereafter, it was centrifuged for 15 min at 3000 × g inside blank tube and the absorbance was read with UV spectrophotometer (SW7504 model by Surgifriend Medicals, England) at 532 nm. The concentration of MDA was calculated as follows: The concentration of malondialdehyde MDA (nmol/ml) =  $(AT - AB) / 1.56 \times 10^5$ ; Where: AT = the absorbance of the sample serum, AB = the absorbance of the blank,  $1.56 \times 10^5$  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°C. The absorbance of the samples was recorded at 650 nm in spectrophotometer (UV spectrophotometer, SW7504 model by Surgifriend Medicals, England).

### LEUKOCYTES

Peroxidase test adapted from Endtz (1974) and as recommended by WHO (1992) was used as follows: A stock solution was prepared by mixing 50 ml distilled water with 50 ml 96% ethanol plus 125 mg benzidine. The working solution was obtained by adding 5 µL 30% H<sub>2</sub>O<sub>2</sub> to 4 ml of stock solution. Twenty (20) µL of working solution was mixed with 20 µL of thawed semen in a small test tube. After incubation for 5 min at room temperature, 20 µL of working solution was mixed with 20 µL of phosphate-buffered saline. Then, 10 µL was placed in a haemocytometer, and peroxidase-positive cells (dark brown round cells) were counted.

### IN VITRO ACROSOME REACTION

Following cryopreservation, spermatozoa were thawed (El-Shahat and Hammam, 2014) by plunging straws into a water bath (37°C) for 1 min and the proportion of acrosome reaction was determined as described by Tardif *et al.* (1999) with modification as follows: Samples of cryopreserved spermatozoa were washed with non culture medium (Phosphate-Buffered 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, D-Glucose 1000 mg/l). Immediately after the inclusion of 0.9% wt/vol PBS, the acrosome reaction was induced by

incubating spermatozoa for 20 min with progesterone (2.5 mg/ml) at 38.5°C (5% CO<sub>2</sub> 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). Sperm suspension (5 µL/L) 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 was adjusted to 8.6) were added to retard fluorescence fading. After adding a coverslip, 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 cryopreserved spermatozoa that exhibited pattern B according to the CTC assay was determined. Spermatozoa characterized by bright anterior head and faint fluorescence in the post-acrosomal region were classified as capacitated spermatozoa while non capacitated sperm had bright uniform fluorescence over the head.

### STATISTICAL ANALYSIS

Data obtained were subjected to analysis of variance (ANOVA) using SAS 1999 and Duncan Multiple Range Test (Duncan, 1955) was used to separate significantly different means. The model that was used to analyze the data is stated below:

$$Y_{ij} = \mu + A_i + L_j + \Sigma_{ij}$$

Where:

$Y_{ij}$  = Dependent variables.

$\mu$  = Population mean.

$A_i$  = Effect due to  $i^{\text{th}}$  vitamin C.

$L_j$  = Effect due to  $j^{\text{th}}$  level of inclusion ( $j = 0, 2, 4, 6, 8$ ).

$\Sigma_{ij}$  = Experimental error.

### RESULTS

The results showed higher ( $p < 0.05$ ) spermatozoa motility, acrosome and membrane integrities in extenders supplemented with vitamin C compared to the control and higher value was recorded in 8 mM (table I). Lower concentrations of MDA were observed in extenders supplemented with 6 mM and 8 mM of vitamin C compared to other levels and the control (table II). The extenders supplemented with vitamin

**Table I.** Means ( $\pm$ SEM) spermatozoa viability of semen cryopreserved with Tris egg yolk extenders supplemented with vitamin C (Medios ( $\pm$ SEM) y viabilidad de los espermatozoides del semen criopreservado con Tris diluyentes de yema de huevo enriquecido con vitamina C).

Parameters	0 mM	2 mM	4 mM	6 mM	8 mM
Motility (%)	28.20 $\pm$ 1.96 <sup>c</sup>	37.20 $\pm$ 5.74 <sup>b</sup>	38.20 $\pm$ 5.03 <sup>b</sup>	38.33 $\pm$ 4.76 <sup>b</sup>	41.40 $\pm$ 6.40 <sup>a</sup>
Acrosome Integrity (%)	40.50 $\pm$ 2.06 <sup>c</sup>	44.16 $\pm$ 2.16 <sup>b</sup>	44.00 $\pm$ 2.63 <sup>b</sup>	45.00 $\pm$ 2.16 <sup>ab</sup>	46.50 $\pm$ 2.63 <sup>a</sup>
Membrane Integrity (%)	33.00 $\pm$ 1.00 <sup>d</sup>	36.50 $\pm$ 2.06 <sup>cd</sup>	43.00 $\pm$ 4.20 <sup>bc</sup>	45.50 $\pm$ 0.96 <sup>b</sup>	54.00 $\pm$ 2.16 <sup>a</sup>
Abnormality (%)	1.17 $\pm$ 0.17	1.33 $\pm$ 0.33	1.08 $\pm$ 0.22	1.00 $\pm$ 0.14	1.00 $\pm$ 0.29

<sup>a,b,c,d</sup>= Values within rows with different superscripts differ significantly ( $p < 0.05$ ).

**Table II.** Means ( $\pm$ SEM) oxidative stress parameters of semen cryopreserved with Tris egg yolk extenders supplemented with vitamin C (Medios concentración (Medios ( $\pm$ SEM) parámetros de estrés oxidativo de semen criopreservado con Tris diluyentes de yema de huevo suplementados con vitamina C).

Parameters	0 mM	2 mM	4 mM	6 mM	8 mM
MDA concentration (nmol/ml)	0.12 $\pm$ 0.01 <sup>b</sup>	0.12 $\pm$ 0.01 <sup>b</sup>	0.13 $\pm$ 0.00 <sup>ab</sup>	0.10 $\pm$ 0.00 <sup>cd</sup>	0.11 $\pm$ 0.00 <sup>c</sup>
Arginase activity (units/mg protein)	0.57 $\pm$ 0.01 <sup>e</sup>	0.82 $\pm$ 0.01 <sup>a</sup>	0.74 $\pm$ 0.00 <sup>ab</sup>	0.70 $\pm$ 0.00 <sup>ab</sup>	0.87 $\pm$ 0.01 <sup>a</sup>
Leukocytes ( $\times 10^3$ /ml)	8.90 $\pm$ 0.21 <sup>a</sup>	7.23 $\pm$ 1.13 <sup>b</sup>	7.27 $\pm$ 0.61 <sup>b</sup>	7.50 $\pm$ 0.90 <sup>b</sup>	5.27 $\pm$ 1.22 <sup>c</sup>

<sup>a,b,c,d</sup>= Values within rows with different superscripts differ significantly ( $p < 0.05$ ).

**Table III.** Means ( $\pm$ SEM) *in vitro* acrosome reaction (%) and capacitation (%) of buck spermatozoa cryopreserved with vitamin C (Medios ( $\pm$ SEM) *in vitro*, reacción del acrosoma (%) y capacitación (%) de los espermatozoides criopreservados buck con vitamina C).

Parameters	0 mM	2 mM	4 mM	6 mM	8 mM
Acrosome reaction (%)	41.00 $\pm$ 6.81 <sup>c</sup>	40.00 $\pm$ 5.16 <sup>c</sup>	60.00 $\pm$ 0.00 <sup>b</sup>	80.00 $\pm$ 3.65 <sup>a</sup>	60.00 $\pm$ 5.89 <sup>b</sup>
Capacitation (%)	25.00 $\pm$ 1.00 <sup>d</sup>	27.00 $\pm$ 4.43 <sup>d</sup>	42.00 $\pm$ 2.58 <sup>c</sup>	75.00 $\pm$ 1.91 <sup>a</sup>	61.00 $\pm$ 1.00 <sup>b</sup>

<sup>a,b,c,d</sup>= Values within rows with different superscripts differ significantly ( $p < 0.05$ ).

C had higher ( $p < 0.05$ ) values of arginase activity compared to the control. Lower ( $p < 0.05$ ) leukocytes were observed in extenders supplemented with vitamin C compared to the control and the reduced leukocytes was more pronounced ( $p < 0.05$ ) in 8 mM (table II). The results showed that more ( $p < 0.05$ ) spermatozoa cryopreserved in extenders supplemented with 6 mM of vitamin C underwent acrosome reaction and capacitation (table III).

## DISCUSSION

The improved motility in extenders supplemented with vitamin C agreed with the findings of Reza *et al.* (2011) who reported that vitamin C supplementation in stored semen improved motility of spermatozoa. The improved motility observed in the present study could be attributed to the antioxidant potential of vitamin C (Martin *et al.*, 2002). The ability of vitamin C to enter mitochondria through facilitated glucose transporter and as water-soluble antioxidant with ability to scavenge aqueous peroxy radicals could be the possible reason for this preservative effect of vitamin C (Wainer *et al.*, 1986). The beneficial effect on motility was optimum in extender supplemented with 8 mM of vitamin C. In contrast, Shoaie and Zamiri (2008) showed that excessive amount of antioxidants caused high fluidity of plasma membrane above the desired point, making sperm more prone to acrosomal damages. However, the survival of spermatozoa in WAD buck semen in-

creased when the dosage of vitamin C added to the extenders increased. Differences in preservation protocols and constituents of extenders, the time of addition/exposure of sperm to antioxidant, concentration of antioxidants and species variations may explain, at least in part, this difference (Ashrafi *et al.*, 2011; Shoaie and Zamiri, 2008). Moreover, Thiele *et al.* (1995) reported a positive correlation between vitamin C concentration in seminal plasma and number of morphologically normal spermatozoa. The increased motility coupled with improvement in other viability parameters (acrosome and membrane integrities) agreed with Mirzoyan *et al.* (2006) that addition of vitamin C to cryopreservation medium increased motility and fertilizing ability. The findings suggested the protective role of vitamin C on cryopreserved spermatozoa in attaining fertilizing ability.

In addition, the improved acrosome and membrane integrities in extenders supplemented with vitamin C concurred with Hu *et al.* (2010) who reported protective effect of vitamin C in semen extenders. The water solubility and low toxicity of vitamin C could be responsible for the protective effect on spermatozoa observed in the present study (Asadpour *et al.*, 2011). In line with previous studies (Aurich *et al.*, 1997; Arabi and Seidaie, 2008), the higher acrosome integrity coupled with improved acrosome reaction and capacitation during cryopreservation with extenders supplemented with vitamin C compared to the control indicated the positive effect of vitamin C on cryopreserved sperma-

tozoa. Furthermore, the noticeable best improvement of viability parameters observed on cryopreserved spermatozoa at the higher level (8 mM) of inclusion indicated that vitamin C as an antioxidant might have been optimum for preserving buck sperm viability parameters, as effects of vitamin C varied with level of supplementation in the extenders; at higher concentration sperm viability parameters were maintained and best preserved in extender supplemented with 8 mM. Vitamin C seemed to act best as an antioxidant at higher level. This finding concurred with previous study that vitamin C acted as an antioxidant at high concentrations (Breininger *et al.*, 2005).

In the present study, the level of vitamin C used was optimum at higher levels (6 mM and 8 mM) for reducing MDA concentrations and indicated better protective effect of vitamin C at these levels against possible damage by lipid peroxidation (Arabi and Seidaie, 2008). Ascorbic acid is a well known non-enzymatic, water soluble natural antioxidant having capacity to reduce MDA concentrations and ROS damage by its chain breaking activity (Ho Lee, 1996; Fanaei *et al.*, 2014). The findings in this present study coupled with better sperm viability parameters suggested that addition of vitamin C as potent antioxidant to semen extenders could reduce deleterious effects of membrane LPO on cryopreserved spermatozoa (Martin *et al.*, 2002; Ondei *et al.*, 2009).

A positive correlation has been reported between sperm motility and arginase activity in seminal plasma and spermatozoa (Elgun *et al.*, 2000; Eskiocak *et al.*, 2006). The main role of arginase in testis is regulation of nitric oxide (NO) concentration (Nathan, 1997). Increased arginase activity generally results in lower NO concentration and subsequently leads to increased sperm motility (Elgun *et al.*, 2000). The direct scavenging effect of vitamin C might have enhanced arginase activity which could inhibit nitric oxide synthase activity and consequently, a decrease in NO level (Aydogdu *et al.*, 2006). This action could be the possible reason for the higher arginase activity in extenders supplemented with vitamin C in this study. Other possible reason to support this beneficial effect could be linked to activity of arginine and its derivative compounds that is known to act as a 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).

Contaminating leucocytes and immature spermatozoa are the major sources of ROS in semen (Agarwal *et al.*, 2003; Aitken *et al.*, 1992; Garrido *et al.*, 2004). Excessive ROS levels present in sperm cells' environment can either be produced in large amounts by leucocytes or the spermatozoa themselves, the process that results in decreased membrane fluidity of both plasma and organelle membranes and, consequently causes damage to membrane function, ion gradients and receptor-mediated signal transduction (Sikka *et al.*, 1996). Sequence to loss of membrane function, spermatozoa loses the ability to function properly and therefore, fertilization is impaired (Riffo and Parraga, 1996). Although contaminating leucocytes can be a critical

factor for sperm survival, the phagocytic role of leucocytes in eliminating defective spermatozoa is beneficial to sperm concentration, motility and acrosome reaction and may even stimulate sperm functions through the release of ROS (Henkel, 2011; Kaleli, *et al.*, 2000). Notwithstanding, high concentration of leucocytes, particularly activated leucocytes, are still harmful to sperm functions (Henkel, 2011). The lower concentration of leucocytes following addition of vitamin C in this study compared to the control further indicated the importance of vitamin C as antioxidant in reducing defective spermatozoa or excessive ROS produced by leucocytes or the 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 are able to undergo acrosome reaction (Arnoult *et al.*, 1996). The present *in vitro* study indicated that vitamin C was able to maintain fertilizing capacity as evidenced by better percentage of capacitation and acrosome reaction of cryopreserved spermatozoa. This is in consonant with previous report that ascorbic acid significantly increased acrosome reaction (Fanaei *et al.*, 2014).

## CONCLUSION

The findings indicated that extenders supplemented with vitamin C improved the sperm quality parameters during cryopreservation of WAD buck spermatozoa. The improvement in viability parameters (sperm motility, acrosome and membrane integrities) was optimum in extenders supplemented with 8 mM. The study resulted in reduced oxidative stress parameters in extenders supplemented with 6 mM and 8 mM (MDA), and 8 mM (seminal leukocytes) while more spermatozoa cryopreserved in extenders supplemented with 6 mM of vitamin C underwent acrosome reaction and capacitation.

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