



Research Article

Cryosurvival of Ram Spermatozoa after Supplementing the Diluent with L-Ascorbic Acid or α -Tocopherol

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ABSTRACT

Typically, cryopreservation has detrimental effects on physical and morphometric properties of spermatozoa and, hence, on sperm fertilizing capacity. In the current investigation we evaluated the efficiency of supplementing ram semen diluent either with L-Ascorbic acid or α -Tocopherol on ameliorating cryopreservation-induced oxidative stress. Ejaculates ($n=15$) were collected from 5 adult Barki rams, 3 ejaculates each, by an artificial vagina during January, 2017. After evaluation, ejaculates of each collection session were pooled, diluted (1:10) with glycerolized Tris-citric acid egg yolk and were further split into 7 aliquots corresponding to the following groups: control (untreated), vitamin C (0.1, 0.2 or 0.3 mM) and vitamin E (0.1, 0.2 or 0.3 mM) (T_0). Thereafter, all specimens were equilibrated for 5 hr at 4°C before being processed for cryopreservation. Post-thaw physical and morphological sperm properties were determined by CASA. The results showed that both low vitamin C (Vit- C_{LD}) and high vitamin E (Vit- E_{HD}) levels in preservation medium improved ($P<0.05$) post-thaw physical and morphological properties of spermatozoa, thus, they were considered the optimum levels of each individual supplement. Furthermore, specimens supplemented with Vit- E_{HD} recorded the highest ($P<0.05$) values of post-thaw motility, viability, normal spermatozoa and sperm functional integrity compared to both control and Vit- C_{LD} supplemented groups. Contrariwise, the later groups recorded the highest ($P<0.05$) percent of secondary sperm abnormalities compared to that of Vit- E_{HD} specimens. These results elucidate that supplementing the diluent with 0.3 mM α -Tocopherol is most appropriate to increase ram sperm cryosurvival *in vitro*, which would be beneficial for maximizing utilization of cryopreserved ram semen for AI and IVF schemes.

Key words: L-Ascorbic acid; α -Tocopherol; Oxidative stress; Cryopreservation; Ram semen

INTRODUCTION

Application of artificial insemination (AI) and embryo transfer (ET) in small ruminants is, to date, still lacking although it is substantially required to develop breeds of genetic merit (Baldassarre and Karatzas, 2004). Therefore, developing sperm processing procedures to maintain sperm fertilization potential in these species became in focus of recent research.

The full potential of semen utilization, particularly for commercial application, depends on using cryopreserved doses (Ashrafi *et al.*, 2013). However, cryopreservation has detrimental effects on physical and morphometric properties of spermatozoa and, consequently, on sperm fertilizing capacity (Munsi *et al.*, 2007; Peña *et al.*, 2009). Ram spermatozoa, in particular, are highly vulnerable to the oxidative damage and subsequent accumulation of reactive oxygen species (ROS) occurring during

cryopreservation of semen doses (Bilodeau *et al.*, 2002; Alvarez and Storey, 2005). The seminal fluid encompasses a natural defense system against ROS damage via enzymatic and non-enzymatic antioxidants (Fejercakova *et al.*, 2013). However, dilution of semen prior to processing divests sperm cells from the protective effects of these naturally-existed antioxidants (Mostafa *et al.*, 2009).

Vitamin C (ascorbic acid) and vitamin E (α -tocopherol) are considered the major non-enzymatic electron trapping molecules in semen (Zeitoun and Al-Damegh, 2015). Ascorbic acid has been reported to play a role in protecting sperm from generated ROS (Dandekar *et al.*, 2002), and in maintaining the genetic integrity of sperm cells by preventing oxidative damage to sperm DNA (Alvarez and Storey, 2005). Further, both ascorbic acid and α -tocopherol were used to reduce oxidative stress-related testicular impairments in animal tissues

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(Acharya *et al.*, 2008), as well to improve the antioxidant enzymatic activity of seminal fluid (Foote *et al.*, 2002), thereby protecting sperm motility and viability during semen processing (Soren *et al.*, 2016). Nevertheless, the protective effects of both vitamins when added to the diluent either separately or combined on post-thaw sperm traits varied considerably among previous studies due to specie differences and levels incorporated in the diluent.

In this regard, the present experiment was implemented to evaluate efficiency of supplementing the diluent either with ascorbic acid or α -tocopherol on ameliorating oxidative stress and enhancing cryopreservation capacity of ram spermatozoa.

MATERIALS AND METHODS

Animals and management

Five adult Barki rams aged 36 - 48 months and an average body weight of 45.0 ± 2.0 kg belonging to the Artificial Insemination Lab., Mariout Research Station, Desert Research Center, Egypt were used during January, 2017. The rams were housed in a fenced stockyard throughout the period of the study, and were allowed to graze daily from 0800 to 1400 h. Thereafter, they were fed a concentrate mixture according to their protein and energy requirements (NRC, 2007), and Egyptian clover, *Trifolium alexandrinum*, hay was provided *ad libitum*. Fresh water was presented once daily after returning from the pasture. Before executing the experiment, all rams were clinically examined and were found free of disease or reproductive disorders.

Preparation of cryopreservation diluent

A glycerolized Tris-citric acid egg yolk extender was prepared for cryostorage of ram spermatozoa as previously described (Rateb, 2018). Briefly, the base extender consisted of 3.63% Tris buffer (w/v), 1.99% citric acid (w/v), 0.5% glucose (w/v) and antibiotics; i.e. 500 iu/mL Penicillin procaine and 500 μ g/mL Streptomycin and was further supplemented with 40% egg-yolk (v/v). Soon after preparation, the diluent was clarified from egg yolk granules by centrifugation at 2400 g for 15 min and aspiration of the clear supernatant. The clarified base diluent was prepared 24 hr prior to each collection session and was stored at 4°C until use.

Semen collection

Ejaculates (n=15) were collected from the 5 rams, 3 ejaculates each, throughout the period of study. Semen collection was performed twice weekly at 0700 hr by an artificial vagina. Ejaculates contaminated with urine or exhibited strange color or odors were disposed. Soon after collection, each raw ejaculate was transported to the laboratory, and was further evaluated for sperm physical and morphometric properties. Only ejaculates recorded >4.0 mass motility score (5=highly motile; 0=immotile) and >90% progressive motility (David *et al.*, 2015) were further pooled and processed.

Experimental design

All good quality ejaculates were pooled and diluted (1:10) with glycerolized (2% glycerol, v/v) diluent. The

diluted specimens were further split into 7 aliquots using a split sample technique. The first aliquot served as control (untreated), whereas the other 6 aliquots were supplemented either with 0.1, 0.2 or 0.3 mM vitamin C (L-Ascorbic acid, Sigma-Aldrich, St. Louis MO, USA; Cat. no. A92902), or the same 3 previous concentrations but of vitamin E (α -Tocopherol, Sigma-Aldrich, St. Louis MO, USA; Cat. no. 258204). The supplemented diluents were added to the pooled ejaculates corresponding to the 7 groups under investigation (portion-A, T₀). Soon after, the specimens were equilibrated for 3 hr at 4°C. Afterwards, a second aliquot of chilled-glycerolized diluent (portion-B, T₃) was added to portion-A to reach a final concentration of 4% glycerol in medium. All specimens were then equilibrated for another 2 hr at 4°C (T₅) before being packed in 0.5 mm French straws using a mini-tube filling and sealing machine (Model 133, Mini-tube, Germany). The straws were placed in a mini-tube biological freezer and were exposed to nitrogen vapor (-80°C) for 10 min before being immersed in liquid nitrogen. The frozen straws were stored under liquid nitrogen surface (-196°C) until post-thaw physical and morphological traits were evaluated.

Semen evaluation

Sperm motility (%) was evaluated by a phase-contrast microscope (Leica) at 400 x magnification. Viability was assessed by mixing and smearing 10 μ L of semen and 5 μ L of freshly-prepared eosin-nigrosin stain on a warm stage, and smeared slides were examined at 1000x magnification. Romanowski's triple-stain method (DIFF-QUICK III, Vertex, Egypt) was used to evaluate primary and secondary sperm abnormalities, as well as acrosomal integrity. Smears preparation and staining processes were conducted according to the manufacturer's instructions, and stained smears were evaluated by a phase-contrast microscope at 1000x magnification. Integrity of sperm plasma membranes were determined by the hypo-osmotic swelling (HOS) test (Mosaferi *et al.*, 2005), where at least 200 sperm were evaluated at 400 x magnification.

Statistical procedure

The data was checked by Shapiro-Wilk's test and were found fitting the normal distribution. Repeated measures analysis of variance (ANOVA) was used to compare changes in physical and morphometric sperm properties among different levels within each treatment (vitamins C and E), as well among control and the optimum concentration of each treatment. The statistical significance threshold was set at 5% and the differences between means were detected by Duncan's post-hoc test. The data were analyzed by IBM-SPSS statistics program for windows (IBM-SPSS, 2013). The data are expressed as means \pm standard error (SEM).

RESULTS

Effect of L-Ascorbic acid (vitamin C) supplementation on cryosurvival of spermatozoa

The results showed that all sperm physical and morphological properties were affected ($P < 0.05$) by freezing/thawing cycle except for sperm primary

abnormalities (Fig. 1). The highest ($P<0.05$) percentages of post-thaw motility, viability and normal spermatozoa were observed in low vitamin C level (Vit-C_{LD})-supplemented group, whereas the lowest ($P<0.05$) were recorded in both control and specimens supplemented with high level of vitamin C (Vit-C_{HD}) (Fig. 1). No significant difference was observed among different vitamin C levels in medium and percent of primary abnormalities. However, both control and Vit-C_{HD} groups recorded the highest ($P<0.05$) percent of secondary abnormalities, while the lowest was observed in Vit-C_{LD} group (Fig. 1). Meanwhile, the percent of intact acrosome was not influenced ($P<0.05$) by level of vitamin C supplementation in the diluent, though the control group recorded the lowest ($P<0.05$) values compared to all supplemented specimens (Fig. 1). Furthermore, the highest ($P<0.05$) percent of intact sperm cell membranes were observed in Vit-C_{LD} and Vit-C_{LD} groups, whereas the lowest ($P<0.05$) were recorded in both control and Vit-C_{HD} groups (Fig. 1).

Protective effect of α -Tocopherol (vitamin E) inclusion in sperm diluent

Similar to that of vitamin C supplementation, the results also showed that freezing/thawing cycle affected ($P<0.05$) all sperm physical and morphological properties, but not sperm primary abnormalities, in all groups (Table 1). Regarding the effect of vitamin E level in diluent, the result showed that specimens supplemented with medium (Vit-E_{MD}) or high (Vit-E_{HD}) level of vitamin E recorded the highest ($P<0.05$) values of post-thaw motility, viability and normal spermatozoa, while the lowest ($P<0.05$) was recorded in the control group (Table 1). Although the percent of primary abnormalities were not affected among control and treated groups, the values of secondary abnormalities were affected ($P<0.05$) by level of vitamin E supplementation in medium. In this respect, the lowest ($P<0.05$) values were recorded in both Vit-E_{MD} and Vit-E_{HD} groups, while the highest ($P<0.05$) values were observed in control (Table 1). With respect to sperm functional integrity, the results showed that Vit-E_{HD} group recorded the highest ($P<0.05$) values of intact

acrosome and sperm cell membrane integrity post-thaw, whereas the lowest ($P<0.05$) values were observed in control (Table 1).

Determining best adjunct to enhance ram sperm cryopreservation aptitude

From the previous results, it can be inferred that both low vitamin C (Vit-C_{LD}) and high vitamin E (Vit-E_{HD}) levels in medium markedly ($P<0.05$) improved post-thaw physical and morphological properties of spermatozoa. Thus, these 2 levels were considered the optimum levels of each individual supplement and, hence, they were further evaluated for sperm cryosurvival against control to determine the most adequate adjunct.

In this regard, the results clarified that specimens supplemented with Vit-E_{HD} recorded the highest ($P<0.05$) values of post-thaw motility, viability, normal spermatozoa and sperm functional integrity criteria compared to both control and Vit-C_{LD} supplemented groups (Fig. 2). Contrariwise, the later groups recorded the highest ($P<0.05$) percent of secondary sperm abnormalities, whereas the lowest ($P<0.05$) was observed in Vit-E_{HD} group (Fig. 2).

DISCUSSION

Typically, procedures related with cryopreservation produce cold shock on spermatozoa due to excessive formation of free oxygen radicals (Bucak *et al.*, 2009). Since mammalian sperm plasma membranes contain high concentrations of polyunsaturated fatty acids, they are highly susceptible to free radicals-induced peroxidative damage particularly following cryopreservation (Lenzi *et al.*, 2002). Consequently, inhibition of post-thaw sperm motility through the depletion of ATP (Armstrong *et al.*, 1999), and loss of both sperm viability (Wongtawan *et al.*, 2006) and membrane functional integrity (Ashrafi *et al.*, 2013) have been observed. Therefore, presence of natural antioxidants in semen specimens prior to freezing has been reported to increase the proportion of motile spermatozoa of higher fertilization potential post thaw (Breininger *et al.*, 2005).

Table 1: Effect of different levels of vitamin E enclosure in the diluent on physical characteristics of cryopreserved ram sperm (mean \pm SEM).

Parameter	Observation time	Treatment			
		Control	Vit-E _{LD}	Vit-E _{MD}	Vit-E _{HD}
Motility (%)	T ₀	85.0 \pm 1.6 ^A	93.0 \pm 1.2 ^A	93.0 \pm 1.2 ^A	91.0 \pm 1.9 ^A
	Post-thaw	54.0 \pm 1.2 ^{c,B}	58.0 \pm 1.2 ^{b,B}	77.0 \pm 1.2 ^{a,B}	78.0 \pm 1.2 ^{a,B}
Viability (%)	T ₀	82.8 \pm 1.6 ^A	85.2 \pm 1.4 ^A	89.6 \pm 2.5 ^A	89.2 \pm 2.2 ^A
	Post-thaw	66.2 \pm 0.7 ^{c,B}	59.0 \pm 1.0 ^{b,B}	72.0 \pm 1.2 ^{a,B}	74.0 \pm 1.0 ^{a,B}
Normal sperm (%)	T ₀	85.8 \pm 1.2 ^A	79.4 \pm 1.2 ^A	85.6 \pm 2.3	86.0 \pm 1.9
	Post-thaw	43.6 \pm 1.4 ^{c,B}	64.8 \pm 2.1 ^{b,B}	81.0 \pm 2.6 ^a	84.6 \pm 2.0 ^a
Primary abnormalities (%)	T ₀	2.0 \pm 0.4	2.4 \pm 0.4	1.4 \pm 0.2	1.8 \pm 0.3
	Post-thaw	1.8 \pm 0.4	1.6 \pm 0.2	1.8 \pm 0.2	1.4 \pm 0.2
Secondary abnormalities (%)	T ₀	12.2 \pm 0.9 ^B	18.2 \pm 1.2 ^B	13.0 \pm 2.4	12.2 \pm 2.2
	Post-thaw	54.6 \pm 1.4 ^{a,A}	33.6 \pm 2.5 ^{a,A}	17.2 \pm 2.0 ^b	14.0 \pm 2.0 ^b
Intact acrosome (%)	T ₀	88.0 \pm 1.7 ^A	76.2 \pm 1.5 ^A	82.6 \pm 2.3 ^A	85.6 \pm 1.1
	Post-thaw	42.8 \pm 2.3 ^{d,B}	59.0 \pm 2.4 ^{c,B}	76.0 \pm 1.7 ^{b,B}	82.4 \pm 1.1 ^a
Intact cell membrane (%)	T ₀	85.4 \pm 1.6 ^A	73.8 \pm 1.2 ^A	78.4 \pm 1.6	79.0 \pm 1.0
	Post-thaw	39.4 \pm 2.9 ^{c,B}	53.0 \pm 2.0 ^{b,B}	73.4 \pm 1.4 ^a	78.8 \pm 1.7 ^a

^{a-d} letters among groups in the same row differ significantly ($P<0.05$); ^{A,B} letters in the same column within each parameter differ significantly ($P < 0.05$); **Vit-E_{LD}, MD, HD**, the diluent was supplemented with 0.1, 0.2 or 0.3 mM vitamin E, respectively; **T₀**, immediately after dilution; **Post-thaw**, immediately after thawing cryopreserved straws.

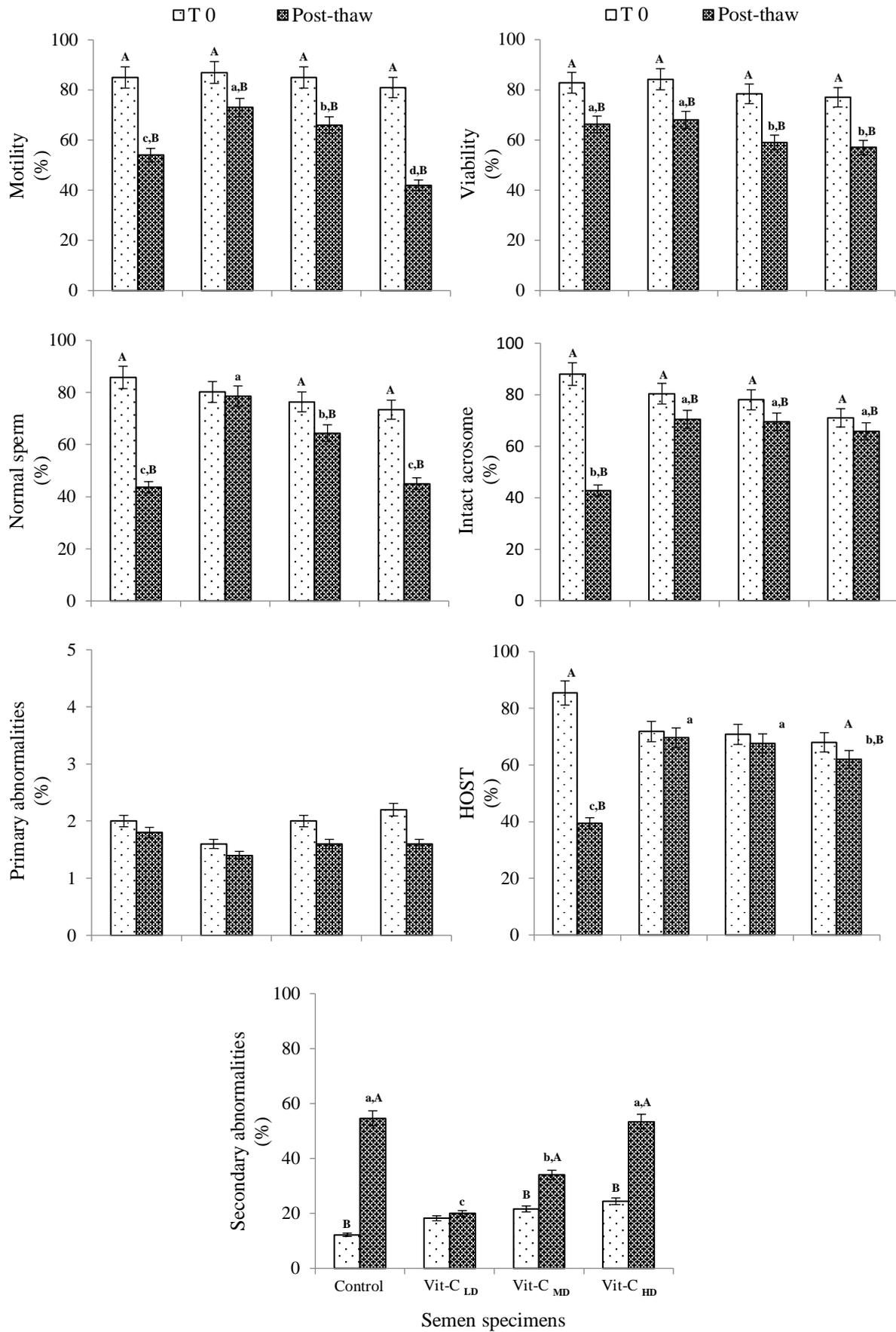


Fig. 1: Post-thaw physical and morphological properties of ram spermatozoa after supplementing the diluent with different levels of ascorbic acid (vitamin C) (mean \pm SEM). ^{A,B} letters within each treatment differ significantly ($P < 0.05$). ^{a-d} letters among groups differ significantly ($P < 0.05$). Vit-C _{LD, MD, HD} : Sperm diluent was supplemented with 0.1, 0.2 or 0.3 mM ascorbic acid, respectively.

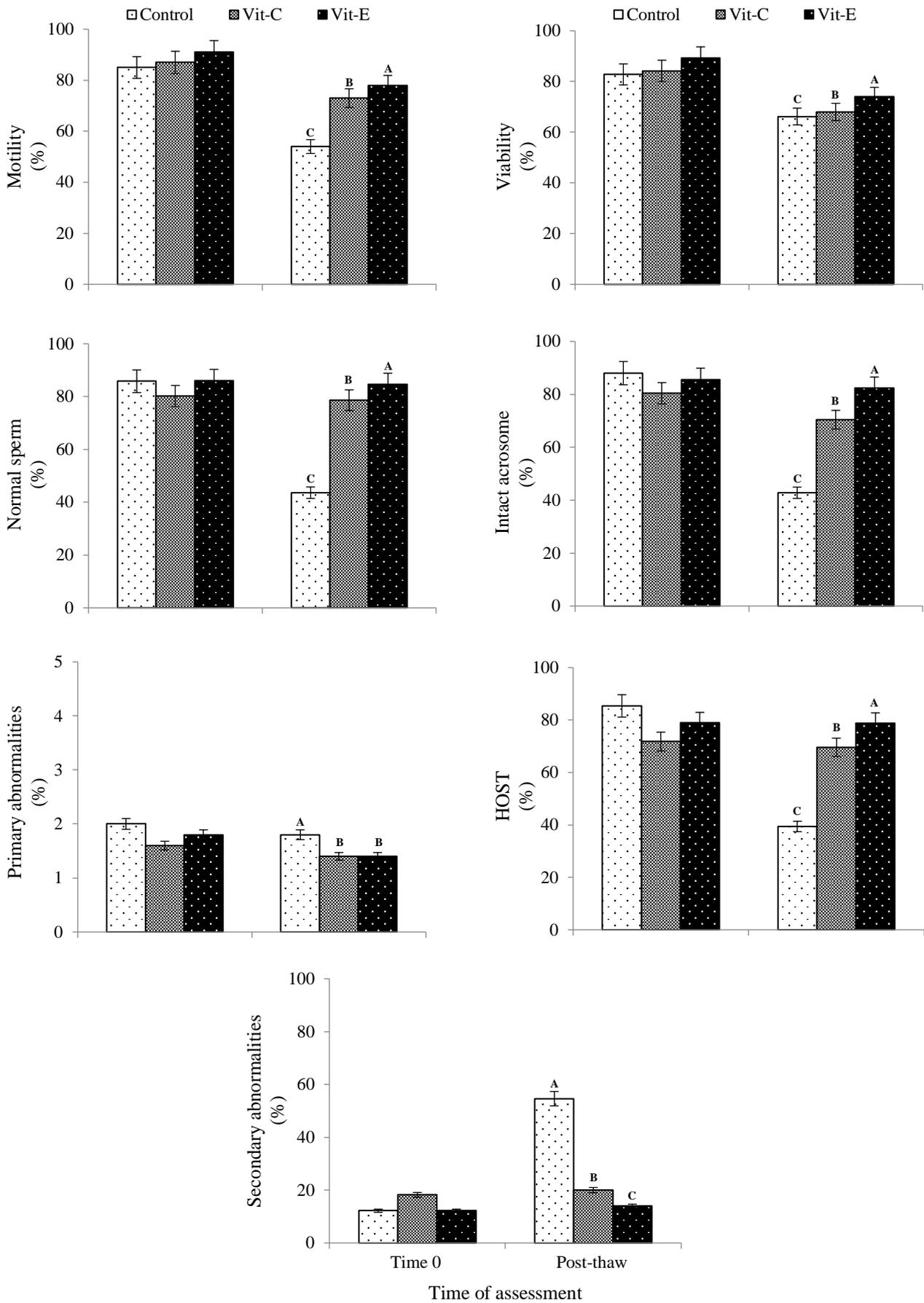


Fig. 2: Effect of supplementing sperm diluent with vitamin C or vitamin E on post-thaw evaluation of ram spermatozoa (mean \pm SEM). ^{A-C} letters among groups differ significantly (P<0.05). Vit-C, diluent was supplemented with 0.1 mM vitamin C; Vit-E, diluent was supplemented with 0.3 mM vitamin E.

In the present results, cryopreservation of ram spermatozoa in control specimens was attributed with loss of motility and increased levels of altered acrosome and sperm cell membranes post-thawing. Nonetheless, it was clearly evident that supplementing the diluent either with ascorbic acid or α -tocopherol had profound effects on cryopreservation potential of spermatozoa in a dose-dependent manner.

In this regard, supplementing the diluent with low ascorbic acid level (0.1 mM) was associated with highest post-thaw sperm traits. This finding is in agreement with earlier reports of other workers in the topic, where inclusion of ascorbic acid in the diluent at high concentrations was detrimental to sperm motility of frozen-thawed spermatozoa in different species (Aurich *et al.*, 1997; Sanchez-Partida *et al.*, 1997; Foote *et al.*, 2002;). This was suggested to be attributed to possible decrease in pH caused by the strongly acidic (10% solution: pH 2) ascorbic acid, since such low pH has been reported earlier to induce reversible or irreversible reductions in motility (Acott and Carr, 1984). On the other hand, supplementing mammalian semen diluent with adequate levels of vitamin C at time of extension has been reported to increase sperm progressive motility (Azawi and Hussein, 2013), and enhance sperm cytological parameters (Mirzoyan *et al.*, 2006) and acrosomal integrity (Yoshimoto *et al.*, 2008). It also improved sperm metabolic activity and viability as well as mitochondrial membrane potential and sperm cell membrane integrity (Hu *et al.*, 2010). Therefore, ascorbic acid was considered a very efficient antioxidant that can be used to reduce the oxidative stress provoked by thawing of spermatozoa (Fernandez-Santos *et al.*, 2009; Hu *et al.*, 2010).

Most animals can synthesize ascorbic acid from glucose via the glucuronic acid pathway (Lewis *et al.*, 1997). Ascorbic acid was found in the epididymal fluid of several species including rams and was regarded the major water-soluble antioxidant found in both blood and seminal plasma (Surai *et al.*, 2001). The seminal plasma contains 10 times higher ascorbic acid concentration than that of blood plasma; thus, it has been reported to contribute to about 65% of seminal plasma total antioxidant capacity (Yousef *et al.*, 2003; Nowaczewski *et al.*, 2005;). *In vivo* studies indicated that ascorbic acid is required as a cofactor for at least eight enzymes (Halliwell and Gutteridge, 1999). The role of vitamin C in ameliorating the adverse effects of reactive oxygen radicals has been thoroughly elucidated (Padayatty *et al.*, 2003). Presence of ascorbic acid in extracellular fluids assists in various mechanisms involved in protecting cells against oxidative stress by numerous disruptive free radical processes including LPO. The antioxidant capability of ascorbic acid was ascribed to its capacity to react with free radicals particularly those with a peroxy group (Carr and Frei, 2002). In the present results, the improvement in semen quality by addition of vitamin C to ram semen diluent is more likely related to an inhibition of lipid peroxidation of the sperm DNA and plasma membrane as was reported by Barati *et al.* (2011). Furthermore, vitamin C scavenges superoxide anions and singlet oxygen thereby protecting the lipoproteins from detectable peroxidative damage (Silva, 2006).

The results also showed that inclusion of 0.3 mM α -tocopherol in ram sperm diluent improved post-thaw sperm physical and cytological traits. A higher vitamin E concentration may act as an oxidation stimulator rather than an antioxidant (Breininger *et al.*, 2005). Therefore, further increase in α -tocopherol level supplementation in semen diluents (3.5 mM or more) had deleterious effects on sperm motility (Batool *et al.*, 2012). Contrariwise, our results are in line with those reported previously (Jeong *et al.*, 2009).

A-tocopherol, vitamin E, comprises a group of chain-breaking lipid-soluble antioxidants; i.e. tocopherols and tocotrienols, which defend the organism against oxidative stress (Buetter, 1992). Vitamin E was regarded the primary component of the antioxidant system of spermatozoa and one of the major membrane protectants against ROS and LPO (Almeida and Ball, 2005). Unlike ascorbic acid, mammalian cells cannot synthesize vitamin E. Therefore, once membrane tocopherol is consumed during the period of oxidative stress exposure, cellular lipids are subjected to peroxidation which can result in toxic damage (Zhang *et al.*, 2001). The protective effect of vitamin E noted in the present study can be attributed to its antioxidant capability as previously reported by Sen *et al.* (2004). Such protective effect has been related to capacity of vitamin E to inhibit LPO reaction in cell membranes by eliminating peroxy (ROO•), alkoxy (RO•), and other lipid-derived radicals (Silva, 2006). It also works against the superoxide anions and reduces the lipid peroxidation of sperm cell membrane during freeze-thawing stress. Thus, higher percent of post-thaw live spermatozoa with intact acrosome and plasma membrane integrity, in extenders containing α -tocopherol acetate, has been observed (Andrabi *et al.*, 2008).

Furthermore, in consonance with a previous finding in humans (Askari *et al.*, 1994), our results clearly elucidated that although both vitamins efficiently mitigated the deleterious effects of cryopreservation-induced oxidative damage, α -tocopherol was more potent than ascorbic acid in protecting spermatozoa.

Conclusions

On the basis of the present results, it could be concluded that although cryopreservation reduced the motility and functional integrity of ram spermatozoa, addition of adequate concentrations of ascorbic acid or α -tocopherol to semen diluent can reduce the oxidative stress occurring during the freezing/thawing cycle thereby protecting spermatozoa and enhance their post-thaw physical and cytological traits. Nevertheless, inclusion of the optimum levels of both vitamins combined in the diluent may improve cryopreservation capacity and fertilization potential of spermatozoa even further. Yet, this needs to be addressed in future studies.

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