

Evaluating the effect of adding vitamins E & C to the extender for Barki ram semen by cooling

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Abstract— The present work aimed at evaluating adequacy of adding different non-enzymatic additives; i.e. vitamins E and C, in to semen diluent on physical and morphological characteristics as well as oxidative status of ram semen stored at 4°C. Ejaculates (n=80) were collected from Five sexually mature Barki rams during the period from January to April. After initial evaluation, each adequate raw ejaculate was diluted (1:10) with Tris-citrate egg yolk extender. Each diluted ejaculate was further split into seven aliquots to evaluate addition of three levels of either ascorbic acid or alpha tocopherol (0.1, 0.2 or 0.3 mM for each treatment) against control (untreated) specimens for their ability to maintain sperm viability criteria over 48 hr (T_0 and T_{48}) of chilled storage at 4 °C. The changes in seminal plasma oxidative status indices as well as enzymatic activities were also determined throughout the period of cooled storage. The results showed that addition of adequate concentrations of ascorbic acid (0.1 mM) or α -tocopherol (0.3 mM) to semen diluent can reduce ($P<0.05$) the oxidative stress and, hence, maintained ($P<0.05$) physical characteristics of ram spermatozoa compared to control throughout the 48 h period of cold storage.

Keywords— Ram semen; sperm diluent; ascorbic acid; alpha tocopherol.

I. INTRODUCTION

Generally, liquid-chilled preservation of semen has been reported to reduce the fertilizing capacity of spermatozoa along with both sperm motility and morphology over time of storage (Perumal *et al.*, 2013) as results of accumulated reactive oxygen species (ROS) which leads to the death of the sperm cells (Amidi *et al.*, 2016). The development of assisted reproductive techniques, including artificial insemination (AI) and in vitro fertilization (IVF), require improving spermatozoa physical characteristics by supplementing the diluent with a variety of antioxidant substances such as ascorbic acid (Vitamin C) and α -tocopherol (vitamin E), which were reported to play important roles as non-enzymatic antioxidant for spermatozoa (Abd El-Hamid *et al.*, 2018; Zeitoun and Al-Damegh, 2015), by protecting sperm cells from generated ROS that induce damage to sperm DNA over preservation time (Alvarez and Storey, 2005; Dandekar *et al.*, 2002). Further, both ascorbic acid and α -tocopherol were used to reduce ROS related testicular impairments in animal tissues (Acharya *et al.*, 2008), and improve the antioxidant enzymatic activity of seminal fluid (Foote *et al.*, 2002), thereby enhancing sperm motility and

viability during semen processing (Soren *et al.*, 2016). Therefore, the present study aimed to investigate the effect of supplementing semen diluent with different levels of Ascorbic acid (Vitamin C) or α -tocopherol (Vitamin E) on maintaining ram sperm traits during 48 h of chilled preservation at 4 °C, as well as determination of some antioxidant enzyme activities throughout the period of chilled storage.

II. MATERIALS AND METHODS

This study was carried out at Artificial Insemination Lab, Mariout Research Station located 34 km west of Alexandria and belonging to the Desert Research Center (DRC), Egypt.

Animal and management.

Five sexually mature Barki rams, aged from 2 - 3 years with averaged body weight of 45.0 ± 2.0 kg were used in this study from January to April. The animals were housed in closed pens throughout the experimental period. The rams were fed concentrate according to their body weight

requirements (NRC, 2007). All rams were given Egyptian clover (*Trifolium alexandrinum*) hay as a roughage ration *ad libitum*. Each animal received 1 kg /day of pelleted concentrate that contained 65% total digestible nutrients (TDN) and 14% crude protein. Fresh water was presented twice daily. Before executing the study, all rams were examined clinically and were found to be free of any disease or reproductive disorders.

Semen extender.

A Tris-citrate egg yolk extender was prepared for dilution of ram semen according to El-Bahrawy *et al.* (2004). The extender was composed of Tris buffer (0.25 Mol, 3.025 %), sodium citrate (1.67 %), and was further supplemented with egg-yolk (2.5 %) plus a mixture of Penicillin procaine (500 iu/ml) and Streptomycin (500 µg/ml) (El-Bahrawy *et al.* 2004). The extender was freshly prepared 24 hr prior to the collection sessions.

Semen collection.

A total number of 80 ejaculates were collected from the rams twice weekly using artificial vagina as previously described (El-Bahrawy *et al.*, 2004). Mean values of raw ejaculates' physical traits included in the study were volume 0.96 ± 0.06 ml, progressive motility $\geq 90\%$, pH 7.2 ± 0.1 , mass motility 4.26 ± 0.16 and sperm concentration 2374.4 ± 26.2 ($\times 10^6$ sperm/ml). Each raw ejaculate was diluted (1:10) with Tris-citrate egg yolk extender, and was further divided into two major groups. The first major group of diluted semen was then split into three aliquots, where each was supplemented with one of three levels (0.1, 0.2 or 0.3 m M) alpha tocopherol (Vit E, Sigma-Aldrich, St. Louis MO, USA). Another similar set of aliquots was supplemented with the same three levels but of ascorbic acid (vitamin, Sigma-Aldrich, St. Louis MO, USA). All six supplemented specimens were evaluated, throughout the period of the study against control (non-supplemented) group (table 1). All diluted semen groups were evaluated for physical characteristics and some biomarkers of enzyme activities during 48 hr (T_0 and T_{48}) at 4 °C of cooling period.

Table.1: Different levels of vitamins E and C supplementation within ram semen diluent.

Treatments	Experimental groups and levels of each treatment			
	Control	L ₁	L ₂	L ₃
Vitamin E	-	0.1 mM	0.2 mM	0.3 mM
Vitamin C	-	0.1 mM	0.2 mM	0.3 mM

Semen evaluation.

Total sperm motility was estimated in all diluted samples by using a phase-contrast microscope (Leica) at 40 X magnification, where an average of 5 random fields was obtained to the nearest 5%. Sperm vitality (live and dead sperm, %) were examined using the differential staining technique, where a mixture of 10 µl of semen and 5 µl of freshly-prepared eosin-nigrosin stain was smeared on a warm stage, and were examined under high power magnification (100×). Sperm abnormalities and acrosome integrity were evaluated using Romanowski's triple-stain technique (DIFF-QUICK III, Vertex, Egypt). Smears preparation and staining procedure were conducted following instructions provided by the manufacturer, and the stained smears were evaluated using a phase-contrast microscope at 100x magnification. Sperm plasma membrane integrity was determined by the hypo-osmotic swelling test (HOST) as described by Mosaferi

et al. (2005), where at least 200 sperm were evaluated at 40 X magnification.

Seminal plasma biomarkers and enzymes activity assessment.

Seminal plasma samples were obtained during the cooling period (T_0 and T_{48}) and were subjected to biomarkers and enzymes activities using commercial kits. Glutamic oxaloacetic transaminase (GOT), Glutamic pyruvic transaminase (GPT) were analyzed using colorimetric kits (Spectrum, Egypt) according to Reitman and Frankel, (1975). Alkaline phosphatase (AKP), Lipid peroxide (LP) and hydrogen peroxidase (HP) were determined using colorimetric kits (Biodiagnostic, Egypt) according to Belfield and Goldberg, 1971; Koracevic *et al.* 2001 and Aebi, 1984, respectively.

Statistical analysis.

of The 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 during the cold storage period were analyzed by the General Linear Model (GLM) procedure (SAS, 2006) using the following model:

$$Y_{ijk} = \mu + L_i + Hour_j + L_i * Hour_j + e_{ijk}$$

Y_{ijk} = observations, μ = Overall means, L_i = effect of i th Levels (i : 1-4), $Hour_j$ = effect of j th Hour (j : 1-3), $Hour * L_{ij}$ = interaction between levels and hours.

e_{ijk} = Experimental error.

The statistical significance threshold was set at 0.05 and the differences between means were detected by Duncan's multiple range test..

III. RESULTS

Effect of different levels of Vitamin E on chilled semen traits

Means values \pm SEM of physical characteristics sperm including sperm motility (%), normal sperm (%), sperm abnormalities (primary and secondary, %) acrosome integrity (%) and hypo-osmotic swelling test (HOST, %) are presented in table (2).

Overall mean of sperm motility (%) was significant higher ($P < 0.05$) in level one (L_1) and level two (L_2) of vitamin E (87.66, 92.22 ± 0.93 %, respectively) compared with both level three (L_3) (87.00 \pm 0.93%) or control (85.66 \pm 0.93%) at 48 of chilled preservation at 4°C. There was a significant interaction ($P < 0.05$) between time of preservation and levels were recorded. The higher ($P < 0.05$) value of sperm motility was recorded in all levels of vit (E) at T_0 of chilled preservation, while the low value was recorded in control (66.00 \pm 1.00 %) at T_{48} of chilled preservation at 4°C (table 2). Mean value of normal sperm (%) was significantly higher ($P < 0.05$) in (L_1) and (L_2) of

vitamin E (93.77, 92.22 ± 0.57 %, respectively) followed by L_3 (90.33 \pm 0.57%) compared with control (79.88 \pm 0.57%) at T_{48} of chilled preservation. Moreover, the higher ($P < 0.05$) value of normal sperm was recorded in all levels of vit (E) at T_0 of chilled preservation, while the low value was recorded in control (66.00 \pm 1.00 %) in T_{48} of chilled preservation at 4°C (table 2).

The results also showed that no significant was found among either levels of Vit. E and control groups in values of primary sperm abnormalities (%). However, the higher ($P < 0.05$) values of primary sperm abnormalities (%) were recorded in control and (L_1) (2.00, 2.00 ± 0.25 %, respectively) at T_{48} , while the low value was recorded in control (1.00, 1.00 ± 0.25 %) at T_0 and T_{24} throughout cooling period (T_0 - T_{48}) as illustrated in Table (2). Overall mean value of secondary sperm abnormalities (%) was significantly lower ($P < 0.05$) in L_1 (4.66 \pm 0.85%) compared with L_2 or L_3 (6.22, 8.22 ± 0.85 %) respectively, while the higher ($P < 0.05$) value was recorded in control (18.00 \pm 0.85 %). In the meantime, the lowest ($P < 0.05$) values were recorded in L_2 or L_3 compared to all treated specimens at T_0 , while the highest value was recorded in control specimens (29.33 \pm 1.48 %) in T_{48} of chilled preservation at 4°C (table 2).

Regarding sperm acrosome integrity (%), the results illustrated that both L_2 and L_1 of vitamin E increased ($P < 0.05$) (92.00, 91.55 ± 0.64 % respectively), followed by L_3 (89.00 \pm 0.64 %) and control (87.44 \pm 0.64 %) over time of storage. Furthermore, the higher ($P < 0.05$) values of acrosome integrity (%) were recorded in L_2 and L_3 , at time T_0 while the lower value was recorded in control at T_{48} of chilled preservation at 4°C. Mean value percent of hypo-osmotic swelling test (HOST, %), were increased ($P < 0.05$) in all Vit E supplemented specimens compared with control. Additionally, the higher ($P < 0.05$) values of hypo-osmotic swelling test percent (%) were recorded in L_2 and L_3 at T_0 while the lower ($P < 0.05$) value was recorded in control at T_{48} of chilled preservation at 4°C (table 2).

Table.2: Effect of adding different concentrations of vitamin E in the diluent on physical characteristics of ram semen during 48 h of coldstorage at 4°C (Mean ± SEM)

Semen characteristics	Storage time (hr)	Treatment				
		Control	Levels of vitamin E			
			L ₁	L ₂	L ₃	±SE
Sperm motility (%)	T ₀	90.00 ^c	95.66 ^a	98.66 ^a	98.66 ^a	1.61
	T ₂₄	85.00 ^d	87.33 ^b	94.20 ^b	81.66 ^c	
	T ₄₈	82.00 ^c	80.00 ^c	88.00 ^b	80.66 ^c	
Overall		85.66 ^C	87.66 ^B	92.22 ^A	87.00 ^B	0.93
Normal spermatozoa (%)	T ₀	91.00 ^b	95.33 ^a	96.66 ^a	98.00 ^a	1.00
	T ₂₄	81.00 ^e	95.33 ^a	91.33 ^b	88.00 ^c	
	T ₄₈	66.00 ^f	90.66 ^{bc}	88.66 ^{bc}	85.00 ^d	
Overall		79.88 ^C	93.77 ^A	92.22 ^A	90.33 ^B	0.57
Primary sperm abnormalities (%)	T ₀	1.00 ^b	1.33 ^{ab}	1.66 ^{ab}	1.00 ^b	0.25
	T ₂₄	1.00 ^b	1.33 ^{ab}	1.33 ^{ab}	1.66 ^{ab}	
	T ₄₈	2.00 ^a	2.00 ^a	1.66 ^{ab}	1.66 ^{ab}	
Overall		1.33	1.55	1.55	1.44	0.14
Secondary sperm abnormality (%)	T ₀	7.33 ^{de}	3.33 ^{ef}	1.66 ^f	1.00 ^f	1.48
	T ₂₄	17.33 ^b	3.33 ^{ef}	7.33 ^{de}	10.33 ^{cd}	
	T ₄₈	29.33 ^a	7.33 ^{de}	9.66 ^{cd}	13.00 ^{cd}	
Overall		18.00 ^A	4.66 ^C	6.22 ^{CB}	8.22 ^B	0.85
Acrosome integrity (%)	T ₀	94.00 ^{cd}	97.66 ^{ab}	98.00 ^a	98.00 ^a	1.10
	T ₂₄	92.00 ^{cd}	95.66 ^{ac}	90.00 ^{de}	88.00 ^e	
	T ₄₈	75.00 ^f	81.33 ^f	87.00 ^e	82.00 ^f	
Overall		87.44 ^C	91.55 ^A	92.00 ^A	89.66 ^B	0.64
HOST (%)	T ₀	73.00 ^e	87.66 ^{ab}	91.33 ^a	91.33 ^a	1.66
	T ₂₄	66.00 ^f	80.66 ^{cd}	80.66 ^{cd}	83.00 ^{bc}	
	T ₄₈	46.00 ^g	82.00 ^c	76.66 ^{de}	75.00 ^e	
Overall		61.77 ^B	83.44 ^A	82.88 ^A	83.11 ^A	0.95

^{a-f} values within the same row with different letters differ significantly ($P \leq 0.05$).

^{A, B} values within the same column with different letters differ significantly ($p \leq 0.05$).

Effect of different levels of Vitamin E on oxidative status during preservation.

Changes in seminal plasma biomarkers and enzymes activities are presented in Figure (1).

Value of glutamic oxaloacetic transaminase (GOT, IU/L), was higher ($P<0.05$) in the L₁ (270.62 ± 8.61 IU/L) at T₄₈ of chilled preservation at 4°C, while the low ($P<0.05$) values were recorded in L₂ and L₃ ($217.79, 220.40\pm8.61$ IU/L, respectively) at T₀ of chilled preservation period compared with control (239.00 ± 8.61 IU/L). The results also showed a severe decline ($P<0.05$) in glutamic pyruvic

transaminase (GPT, IU/L) level in all Vit E- supplemented specimens over storage period compared with control (Fig. 1). Value of alkaline phosphatase (AKP, IU/L), was higher ($P<0.05$) in the L₃ (398.94 ± 52.13 IU/L) at time T₄₈, while the low values were recorded in L₁ (60.69 ± 3.65 IU/L) at T₀ of preservation period. At T₄₈ value of lipid peroxide (LP, nM/mL), was lower ($P<0.05$) in L₂ (15.73 ± 3.65 nM/mL) compared with L₁, L₃ ($24.85, 26.16\pm2.63$ nM/mL, respectively) and control (24.85 ± 2.63 nM/mL). Value of hydrogen peroxidase (HP nM/mL), was higher ($P<0.05$) in the control specimens compared with differently also Vit E-supplemented specimens over storage period (Fig. 1).

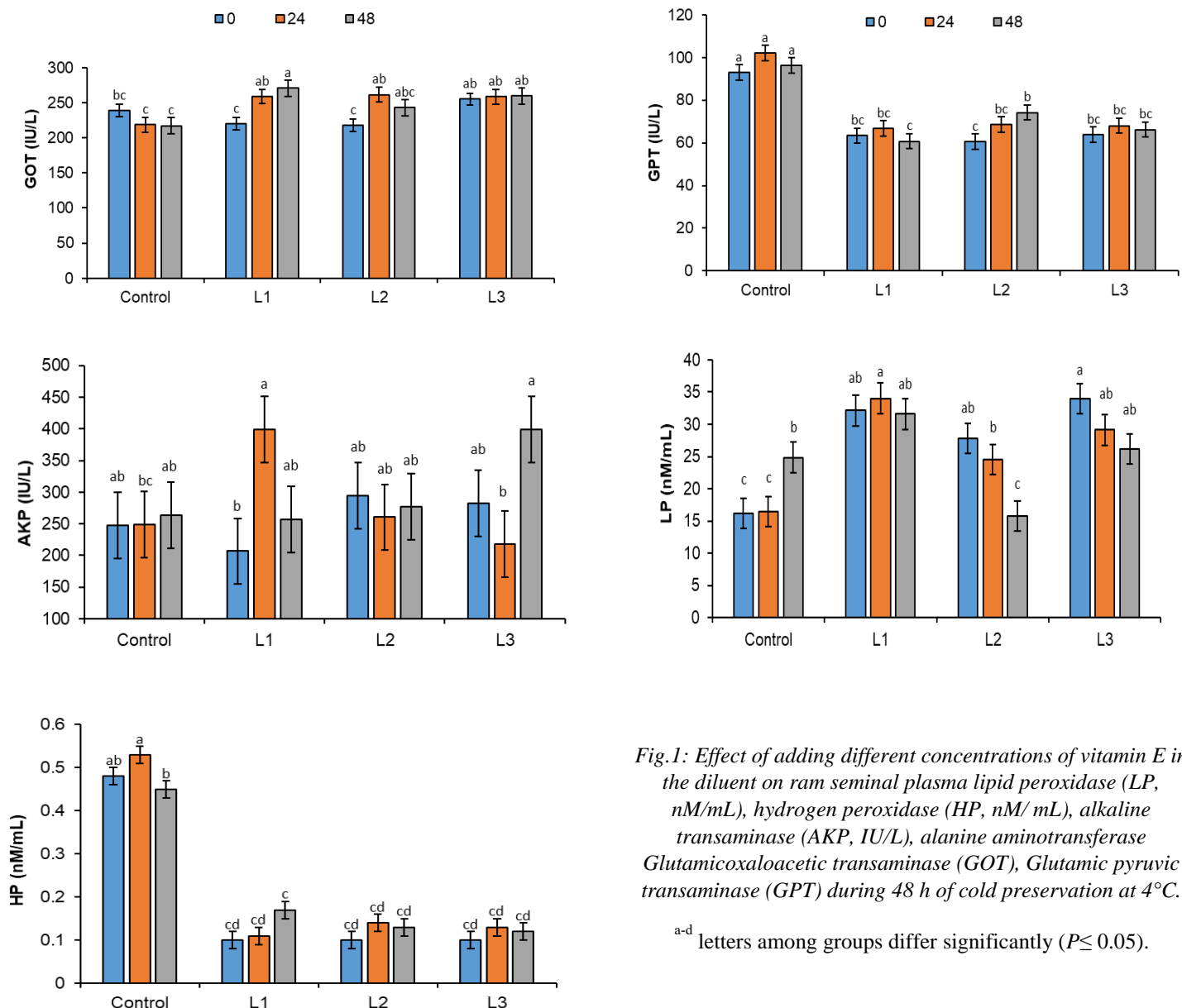


Fig.1: Effect of adding different concentrations of vitamin E in the diluent on ram seminal plasma lipid peroxidase (LP, nM/mL), hydrogen peroxidase (HP, nM/mL), alkaline transaminase (AKP, IU/L), alanine aminotransferase Glutamicoxaloacetic transaminase (GOT), Glutamic pyruvic transaminase (GPT) during 48 h of cold preservation at 4°C.

a-d letters among groups differ significantly ($P \leq 0.05$).

Table.3: Effect of adding different concentrations of vitamin C in the diluent on physical characteristics of ram semen during 48 h of cold storage at 4°C (Mean±SEM)

Semen characteristics	Storage time (h)	Treatment				
		Control	Levels of vitamin C			
			L ₁	L ₂	L ₃	±SE
Sperm motility (%)	T ₀	90.00 ^e	98.00 ^a	99.33 ^a	97.33 ^a	1.59
	T ₂₄	85.66 ^f	84.00 ^b	89.33 ^b	85.33 ^{bcd}	
	T ₄₈	82.00 ^{de}	77.66 ^c	88.00 ^{bc}	77.66 ^e	
Overall		85.66 ^C	86.55 ^B	92.22 ^A	86.77 ^B	0.93
Normal spermatozoa (%)	T ₀	91.66 ^b	98.66 ^a	97.00 ^a	98.66 ^a	1.05
	T ₂₄	81.66 ^d	91.33 ^b	91.33 ^b	88.66 ^b	
	T ₄₈	66.33 ^f	90.00 ^b	88.66 ^b	85.33 ^c	
Overall		79.88 ^C	93.33 ^A	92.33 ^A	90.88 ^B	0.61
Primary sperm abnormalities (%)	T ₀	1.00 ^{bc}	1.00 ^{bc}	1.00 ^{bc}	0.66 ^c	0.27
	T ₂₄	1.00 ^{bc}	2.00 ^a	1.33 ^{abc}	1.66 ^{ab}	
	T ₄₈	2.00 ^a	2.00 ^a	1.66 ^{ab}	1.66 ^{ab}	
Overall		1.33	1.66	1.55	1.33	0.15
Secondary sperm abnormality (%)	T ₀	7.33 ^d	1.00 ^e	2.00 ^c	1.00 ^e	1.52
	T ₂₄	17.33 ^b	6.00 ^d	7.33 ^d	9.00 ^{cd}	
	T ₄₈	29.33 ^a	8.00 ^d	9.66 ^{cd}	13.00 ^{bc}	
Overall		18.00 ^A	5.00 ^C	6.33 ^{BC}	7.77 ^B	0.88
Acrosome integrity (%)	T ₀	94.33 ^{bc}	98.66 ^a	95.33 ^{bc}	97.00 ^{ab}	1.03
	T ₂₄	92.33 ^{cd}	96.66 ^{ab}	90.66 ^{de}	87.66 ^e	
	T ₄₈	75.66 ^e	81.33 ^e	87.33 ^e	82.00 ^e	
Overall		87.44 ^B	92.22 ^A	91.11 ^A	88.88 ^B	0.59
HOST (%)	T ₀	73.33 ^e	95.33 ^a	92.00 ^{ab}	85.00 ^{cd}	1.68
	T ₂₄	66.00 ^e	80.00 ^{de}	88.66 ^{bc}	80.66 ^{de}	
	T ₄₈	46.00 ^f	82.00 ^d	76.66 ^e	73.33 ^e	
Overall		61.77 ^C	85.77 ^A	85.77 ^A	79.66 ^B	0.97

^{a-f} values within the same row with different letters differ significantly ($P \leq 0.05$).

^{A, B} values within the same column with different letters differ significantly ($P \leq 0.05$).

Effect of different levels of Vitamin C on chilled semen traits.

Means values ± SEM of sperm physical characteristics including sperm motility (%), normal sperm (%), sperm abnormalities (primary and secondary, %) acrosome integrity (%) and hypo-osmotic swelling test (HOST, %) are presented in table (3).

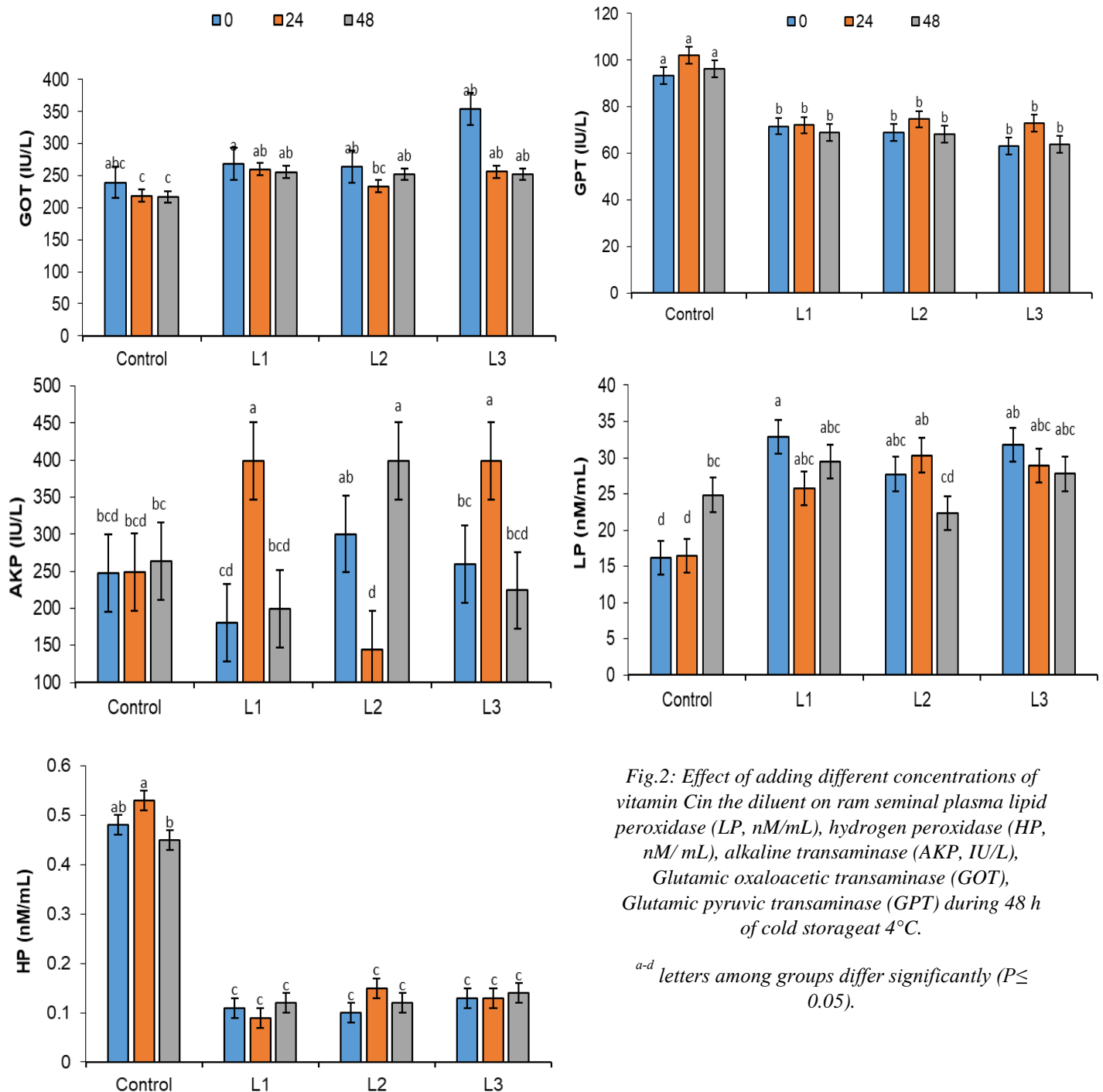


Fig.2: Effect of adding different concentrations of vitamin C in the diluent on ram seminal plasma lipid peroxidase (LP, nM/mL), hydrogen peroxidase (HP, nM/ mL), alkaline transaminase (AKP, IU/L), Glutamic oxaloacetic transaminase (GOT), Glutamic pyruvic transaminase (GPT) during 48 h of cold storage at 4°C.

^{a-d} letters among groups differ significantly ($P \leq 0.05$).

Overall mean of sperm motility percent (%) was significantly higher ($P < 0.05$) in L₂ of vitamin C (92.22 ± 0.93 %) compared with control (85.66 ± 0.93 %). Overall mean of normal sperm (%) was significantly higher ($P < 0.05$) in (L₁) and (L₂) of vitamin C (93.33 , 92.33 ± 0.61 % respectively) compared with (L₃) (90.88 ± 0.61 %) or control

(79.88 ± 0.61 %) at 48 hr of chilled preservation at 4°C. Contrarily, the lower ($P < 0.05$) percent of normal sperm was recorded in control group at T₄₈ of chilled preservation (Table 3). No significant difference was found in mean percent of primary sperm abnormalities (Table 3). There was interaction between time and treatment was recorded. The

higher ($P<0.05$) percent (%) was recorded in control and L_1 , while the lower percent was recorded in L_2 and L_3 at 48 hr of chilled preservation at 4°C (Table 3). Overall mean of secondary sperm abnormalities percent (%) was significantly higher ($P<0.05$) in control (18.00 ± 0.88 %) followed by (L_3) (7.77 ± 0.88 %) and L_2 (6.33 ± 0.88 %) compared with L_1 (5.00 ± 0.88 %) during preservation period at 4°C (Table 3). Also, the highest ($P<0.05$) percent was recorded in control group at T_{48} of chilled preservation at 4°C (Table 3).

Overall mean value of acrosome integrity percent (%) was significantly higher ($P<0.05$) in both (L_1) and (L_2) groups of vit C, while the lowest values ($P<0.05$) were recorded in control and L_3 (87.44 , 88.88 ± 1.03 %) at 48 hr of chilled preservation (Table 3). A significant ($P<0.05$) interaction was noted between time and treatment. All levels of Vit C-supplemented or control specimens over storage period at T_0 and gradual decline thought preservation period at T_{48} of chilled preservation 4°C Table (3). Mean value of hypo-osmotic swelling test (HOST, %) was significantly higher ($P<0.05$) in L_1 and L_2 (85.77 , 85.77 ± 0.97 % respectively) compared with control (61.77 ± 0.97 %). The interaction between time and levels were significant ($P<0.05$), the highest value was recorded in L_1 (95.33 ± 1.68 %) at T_0 , while the lowest ($P<0.05$) value was recorded in control (46.00 ± 1.68 %) at T_{48} of chilled preservation at 4°C (Table 3).

Effect of different levels of Vitamin C on oxidative status during preservation.

The changes in seminal plasma biomarkers and enzymes activities are presented in Figure (2).

At T_{48} of chilled preservation, value of glutamic oxaloacetic transaminase (GOT, IU/L), was higher ($P<0.05$) in all different levels of Vit C- supplemented specimens compared with control specimens. On the other hand value of glutamic pyruvic transaminase (GPT, IU/L), was increased ($P<0.05$) in the control (96.34 ± 5.19 IU/L) compared with all treated semen specimens L_1 , L_2 and L_3 (68.93 , 68.13 , 63.90 ± 5.19 IU/L), respectively (Fig. 2). In addition, value of alkaline phosphatase (AKP, IU/L), was higher ($P<0.05$) in the L_1 and L_3 at T_{48} respectively, while the low ($P<0.05$) values were recorded in L_1 at T_0 of preservation (Fig. 2). Value of lipid peroxide (LP, nM/mL), was lower ($P<0.05$) in the L_1 (22.36 ± 2.32 nM/mL) at T_{48} of preservation at 4°C , compared with semen supplemented with other levels or control (Fig. 2). At T_{48} of preservation period, value of hydrogen peroxidase (HP nM/mL), was higher ($P<0.05$) in

the control (0.45 ± 0.02 nM/mL), while the lowest ($P<0.05$) values were recorded in all levels of vit C (Fig. 2).

IV. DISCUSSION

The results of the present work showed a significant enhancement in semen viability criteria in terms of sperm motility, normal sperm, sperm abnormalities (primary and secondary), acrosome integrity and HOST-reacted spermatozoa percentages in specimens supplemented with ascorbic acid or α -tocopherol. This finding is in agreement with previous studies in different species (Abd El-Hamid *et al.*, 2018; Foote *et al.*, 2002; Yoshimoto *et al.*, 2008; Mirzoyan *et al.*, 2006), where addition of both vitamins efficiently improved physical characteristics and functional integrity of cold-stored or frozen spermatozoa. Vitamin E is one of the vitamins that cannot be synthesized by mammalian cells. Nonetheless, it has been reported to play an important role in the antioxidant defense system against ROS and LPO in the sperm cell (Almeida and Ball, 2005). Such ability was attributed to its capacity to inhibit LPO reaction in cell membranes by eliminating peroxy ($\text{ROO}\cdot$), alkoxyl ($\text{RO}\cdot$), and other lipid-derived radicals (Silva, 2006). Moreover, it has been reported responsible for reducing SOD and LP in sperm cell membrane during cooling stress (Andrabi *et al.*, 2008; Zhang *et al.*, 2001). Vitamin C, on the other hand, is considered the major non-enzymatic electron-trapping component, and plays a major role in trapping molecules in the seminal fluid (Zeitoun and Al-Damegh, 2015), thus, reducing the levels of reactive oxygen species (Fernandez-Santos *et al.*, 2009; Hu *et al.*, 2010). Additionally, it improves sperm metabolic activity and viability, as well as mitochondrial membrane potential in sperm cells (Hu *et al.*, 2010) in the present work, Vitamin C supplementation led to decrease in pH due to its strongly acidic properties (10% solution: pH 2). Similar finding has been previously reported to induce reversible or irreversible reductions in motility (Acott and Carr, 1984).

In the present study the enzyme activities of GOT in specimens supplemented with both additives increased at 48 h of preservation at 4°C alongside AKP activity. This agrees with previous observations of Zeitoun & Mona (2015), and may directly indicate sperm membrane damage (Peschet *et al.*, 2006), and increased percentages of dead and abnormal spermatozoa (Gundogan *et al.*, 2010).

Contrariwise, the results showed that values of LP, HP and GPT concentrations declined in specimens

supplemented with different levels of vit. E or C at 48 h of preservation at 4°C. This could be attributed to actions of ascorbic acid and α -tocopherol on the membrane structure in sperm cell, particularly when added to semen extenders in higher doses (AbdEl-Hamid *et al.*, 2018; Srivastava and Kumar, 2014; Gundogan *et al.*, 2010). In smaller concentrations, however, ascorbic acid through its ROS-reducing properties (Ball *et al.*, 2008) might have minimized the probable oxidative damages to the membranes, thereby preventing the enzyme leakage (Amidi *et al.*, 2016; Kheradmand *et al.*, 2006; Chatterjee and Gagnon 2001).

V. CONCLUSIONS

The results indicated that addition of adequate concentrations of ascorbic acid or α -tocopherol to semen diluent can reduce the possible damage induced by oxidative stress, hence, maintain physical characteristics of ram spermatozoa during 48 h of cold storage. Nevertheless, inclusion of the optimum levels of both vitamins combined in the diluent may further improve the fertilization potential of spermatozoa prior to application of artificial insemination (AI) and in vitro fertilization (IVF) in sheep.

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