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INFLUENCE OF GLUTATHIONE ADDITION TO AN EXTENDER ON THE COOLED-STORED STALLION SPERMATOZOA

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ABSTRACT

Eighteen ejaculates were collected from three mature Arabian stallions (about 5 years of age) during breeding season (summer). Semen was collected, evaluated and extended with Tris-yolk-glucose, Milk-glucose and Non-fat Dried milk solids glucose extenders. The final extension rate was 1ml semen: 4ml extender. The extended semen was divided into 4 cervical tubes added with Glutathion (GSH) 0.0, 0.2, 0.4 and 0.8 mM/100ml‹ . The extended semen was kept at 5 ºC for up to 48 hours. The percentages of sperm motility, dead spermatozoa, abnormal spermatozoa, and acrosome damage of spermatozoa were recorded. The results showed that, the percentage of motile stallion spermatozoa extended with tris-yolk-glucose or Non-fat Dried milk solids glucose extenders significantly (P<0.05) higher than Milk-glucose extender, while the percentages of dead spermatozoa, abnormal spermatozoa and acrosome damage of spermatozoa (%) significantly (P<0.05) lower than Milk-glucose extender. Supplementation of GSH at level of 0.4 mM to the extender increased significantly (P<0.05) the percentage of motile stallion spermatozoa, while decreased the percentages of dead spermatozoa, abnormal spermatozoa and acrosome damage of spermatozoa (%) as compared with the control samples in the different extenders, during storage at 5ºC. The prolongation of storage time at 5ºC decreased significantly (P<0.05) the percentages of sperm motility and increased significantly (P<0.05) the percentages of dead spermatozoa, abnormal spermatozoa and acrosome damage of spermatozoa (%) in the different extenders or GSH concentrations and free-GSH medium (control). In conclusion, it can be recommended to use of Tris-yolk-glucose extender or Non-fat Dried milk solids-glucose extender added with 0.4 mM glutathione /100 ml for enhancement of the Arabian stallion spermatozoa, during storage at 5 ºC.

Key words: stallion, semen, extenders, glutathione.

INTRODUCTION

The horses were an important part of human life in prehistoric times. Horse domestication was a great breakthrough, bringing horsepower communications, transportation, farming and warfare. It has been estimated that only 24 % of stallions produce ejaculates that are suitable for cryopreservation and fertility for frozen semen is approximately 40 % compared to that of fresh. In stallions, motility characteristics are poorly correlated with fertility Jasko *et al*. (1992). The cryopreservation of gametes is expensive and requires sophisticated equipment. However, pregnancy rates remain low due to many variables Miller, (2008). As an arising technology, the implementation relies on the cost and feasibility, and may not be suitable to all breeding farms. Alternative procedures have been used with success to freeze semen immediately post-collection, but this requires the stallion and equipment be at the same place. The collection and shipment of cooled semen to a specialized facility makes the cryopreservation process easier, without transporting the stallion to reproduction centers Crockett *et al*. (2001). However, manipulation of equine semen during these processes reduces sperm viability and fertility due to several factors including membrane lipid peroxidation, because of its high polyunsaturated fatty acids content, making spermatozoa highly susceptible to free radicals and reactive oxygen species (ROS; Cocchia *et al*., 2011).

ROS scavengers are present in seminal plasma, with the primary ROS scavengers described in equine semen being glutathione peroxidase, superoxide dismutase, and catalase. Bozkurt *et al*. (2007) found that the stallion spermatozoa diluted in milk-based extender had greater motility and longevity than those diluted in TRIS-based and glucose-lactose extenders, Oliveira *et al*. (2014) found that the addition of 2 mM glutathione to the freezing extender increased the total motility, viability, and plasmatic membrane integrity of stallion spermatozoa de Oliveira *et al*. (2015) found also that the addition of glutathione at

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levels of 1.5 or 2.5mM were more efficient in protecting the sperm cells and yielded higher total motility after 36 hours of refrigeration. Glutathione is one of the antioxidants added to different semen specimens. It is a thiol tripeptide (ɣglutamyl cysteinyl glycine) with several biological functions found widely in the animal body, not only in somatic cells but also in gametes as well. This thiol has an important role in the ant oxidation process of endogenous and exogenous composts, as well as, in the maintenance of intracellular redox conditions. Glutathione is a natural reservoir of redox force, which can be quickly used by defend cells against oxidative stress Luberda (2005). It is synthesized from glutamate, cysteine, and glycine amino acids. Its reductive power is used to maintain thiol groups in intracellular proteins and other molecules. It acts as a cysteine physiological reservoir and is involved in the regulation of protein synthesis, cellular detoxification, and leukotriene synthesis. The protection by glutathione against oxidative damage is provided by its sulphydryl group (SH), which can be present in reduced glutathione (GSH) and oxidized glutathione (GSSG) forms. The GSH's attack against ROS is favored by the interaction with enzymes, such as glutathione reductase and glutathione peroxidase Luberda, (2005). The thiol antioxidant system is represented mainly by glutathione, the primary antioxidant in equine semen, abundant in seminal plasma. The amount of GSH in equine seminal plasma is 10 times higher than that in swine Luberda, (2005). The best result was obtained with 0.4 mM GSH level with egg yolk extender when stored up to 12 h at 25ºC in ram Solouma (2013).

Therefore, the present study aimed to study the effect of glutathione concentrations (0.0, 0.2, 0.4 and 0.8 mM/100ml) added to different extenders (Tris-yolk-glucose extender, Milk-glucose extender and Non-fat Dried milk solids glucose extender) on semen quality of the Arabian stallions, during storage at 5ºC for up to 48 hours.

MATERIALS AND METHODS

The present study was conducted in the Animal Production Department, Faculty of Agriculture, Al-Azhar University, Cairo, and Animal Production Research Institute, Agricultural Research Center, Egypt. The experimental work was carried out in Arabian stallions reared in E1-Zahra Governmental Stud, Ain-Shams Town, nearby Cairo, Egypt, during the period from March, 2015 till February, 2016. In the present study, the ejaculates of semen were collected from three healthy, intact and sexually matured (5 years of age) of Arabian stallions, during breeding season.

Semen collection:
Semen was collected once per week from each stallion by means of an Artificial Vagina (AV) using Missouri Model, while stallions mounted at mare in estrous. The AV was pre-warmed to 45-50ºC, fitted with a nylon filter coupled to a collection cup to obtain the gel-free fraction. The collected ejaculate was immediately transported to the laboratory for evaluation and processing.

Semen extension and glutathione addition:
Semen was evaluated immediately after collection then extended with the different extenders (Tris-yolk-glucose extender, Milk-glucose extender and Non-fat Dried milk solids-glucose extender). The extended semen was divided into 4 cervical tubes added with Glutathion (GSH) at levels which are Control and three treatments Respectively: 0.0, 0.2, 0.4 and 0.8 mM/100ml. The compositions of these extenders are shown in Table 1 according to Mckinnon and Voss (1993). Semen extension was carried out by adding the appropriate volume of the extender slowly to the semen. Extended semen (in tube) was kept below the level of water in water bath at 37ºC for all times to avoid fluctuations in the temperature of the extended semen. The final extension rate was 1semen: 4 extender.

Table 1: Composition of buffered yolk extenders

Extender	Ingredients	Quantity (gm/100ml distilled water)
Tris-yolk-glucose extender	*Tris aminomethan	2.40
	Glucose monohydrate	0.45
	Citric acid anhydrous	1.25
	Egg yolk (ml)	22.00
Milk-glucose extender	Dry skim milk	2.40
	Glucose monohydrate	4.90
	Sodium bicarbonate (7.5% solution)	2.00
Non-fat Dried milk solids glucose extender	Sanalac	2.40
	Glucose monohydrate	4.90
	Sodium bicarbonate (7.5% solution)	2.00

500 IU/ml penicillin + 5oo µg/ml streptomycin sulphate were added to each extender.
*Tris(hydroxymethyl) aminomethan, Aldrich Chemical Co.Ltd., Gillingham, Dorest England.
* Sanalac (instant Non-fat dry milk).

Chilling of semen at 5° C:
The test tubes containing extended semen were placed in a 500 ml beaker containing water at 30ºC with a thermometer in order to facilitate periodic checking of the water temperature during the cooling period. Another test tubes containing extended semen only (control tube) were placed in the same beaker to maintain the temperature similar to that of the semen (all the test tubes were covered with a dark plastic sheath). The beaker was placed in a refrigerator and gradually cooled till their temperature reached 5ºC throughout 1.5-2.0 hours. The cooled semen with the different extenders was kept at 5ºC for up to 0, 24 and 48 hours.

After each storage time (0, 24 and 48 hours), the percentages of sperm motility, dead spermatozoa, abnormal spermatozoa and acrosome damage of spermatozoa with the different extenders and control were determined.

Percentage of sperm motility:
Sperm motility (%) was estimated by adding one drop of gel-free semen on the dry, clean and pre-warmed (37ºC) glass slide. The drop of semen was covered by a warmed cover slip and immediately examined using high power magnification (400 x) sperm motility was estimated by observing the approximate percentage of spermatozoa moving forward motion across the field of vision with a normal vigorous swimming motion according to Salisbury *et al*. (1978).

Percentage of dead spermatozoa:
The eosin/nigrosine staining procedure was carried out according to Hackett and Macpherson (1965). The percentage of dead spermatozoa was calculated from 100 spermatozoa which were counted in each slide in different microscope fields using a hand counter.

Percentage of abnormal spermatozoa:
The morphological of abnormal spermatozoa (%) were determined by counting 100 sperm in the same smear prepared for live/dead spermatozoa ratio under oil immersion using X1000 objective of a light microscope according to Salisbary *et al*. (1978).

Percentage of acrosome damage:
Assessment of the percentage of acrosome damage of spermatozoa was done according to Watson (1975). A drop of diluted semen was smeared on a pre-warmed slide and allowed to dry in the current air. The smears were fixed by immersion in buffered formalin saline (9 ml sodium chloride + 100 ml formalin + 900 ml distilled water) for 15 minutes and then washed in running tap water for 15 to 20 minutes. The slides were dried and then immersed in buffered Giemsa stain solution for 90 minutes and after that they were rinsed briefly in distilled water and dried. A stock of Giemsa stain solution was prepared from solid Giemsa stain (Northampton, U.K. P460 D) as follows: Giemsa stain (3.8 g) was ground with absolute methanol (AR grade, 375 ml). Glycerol (AR grade, 125 ml) was added and stored at 37ºC for one week. The percentage of acrosome damage was calculated for 100 spermatozoa observed at random on each slide using oil immersion lens (x1000).

Sperm-cell concentration ($x10^6$/ml):
The spermatozoa were counted using Haemeocytomter according to Jasko *et al*. (1992).

Statistical analysis:
Data were statistically analyzed using the General Linear Model (SAS, 2008) one way analysis of variance. All statements of significant difference are based on the 0.05 or 0.01 probability levels. Duncan's New Multiple Range Test (Duncan, 1955) was used to detect differences among means. The percentage values were transformed to arcsine values before being statistically analyzed.

The following model was used:
$Yij = \mu + Ti + Eij$

Where,
Yij = Experimental observation. μ = The overall mean.
Ti = The effect of Glutathione levels, i= 0.0, 0.2, 0.4 and 0.8 mM/100ml
Eij = The errors related to individual observation.

RESULTS

1. Percentage of motile stallion spermatozoa:
Data presented in Table 2 revealed that, the percentage of motile stallion spermatozoa in tris-yolk-glucose extender and Non-fat Dried milk solids-glucose extender were significantly ($P<0.05$) higher than Milk-glucose extender. Supplementation of the extended stallion semen with glutathione up to 0.8 mM/100 ml increased significantly ($P<0.05$) the percentage of sperm motility, during storage at 5ºC for up to 48 hours in the different extenders. Among GSH supplementation levels, the highest ($P<0.05$) percentage of sperm motility was recorded with the semen containing 0.4 mM of GSH in the different extenders. While, the lowest ($P<0.05$) percentage of sperm motility was recorded with free-GSH medium (control) in the different extenders.

Table 2: Mean percentage of motile Arabian stallion spermatozoa with different extenders and glutathione levels, during storage at 5ºC for up to 48 hours.

Storage times (hours)	Tris-yolk-glucoses					Milk- glucose					Non-fat Dried milk solids-glucose				
	Glutathione level (mM/100ml)				Means	Glutathione level (mM/100ml)				Means	Glutathione level (mM/100ml)				Means
	0	0.2	0.4	0.8		0	0.2	0.4	0.8		0	0.2	0.4	0.8	
0	79.23 ±1.34	87.32 ±1.89	91.42 ±2.01	89.16 ±1.32	**86.78^{A} ±1.28**	68.68 ±1.82	75.26 ±1.59	80.71 ±1.72	76.24 ±1.65	**75.22^{A} ±1.04**	76.21 ±1.58	85.16 ±1.75	89.81 ±1.42	87.15 ±1.34	**84.58^{A} ±1.26**
24	71.82 ±1.78	80.41 ±1.82	84.79 ±1.92	82.65 ±2.11	**79.91^{B} ±1.77**	59.19 ±1.78	67.15 ±1.81	71.24 ±1.73	68.32 ±1.86	**66.47^{B} ±1.12**	68.13 ±1.34	75.24 ±1.88	83.61 ±1.74	80.29 ±1.58	**76.81^{B} ±1.18**
48	56.13 ±1.48	62.19 ±1.32	68.58 ±1.71	64.28 ±1.28	**62.79^{C} ±1.75**	43.28 ±1.16	50.33 ±1.80	54.26 ±1.33	52.37 ±1.42	**50.06^{C} ±1.48**	57.85 ±1.73	64.70 ±1.62	67.12 ±1.77	65.42 ±1.82	**63.77^{C} ±1.30**
Means	**69.06^{d} ±1.11**	**76.64^{bc} ±1.16**	**81.59^{a} ±1.98**	**78.69^{b} ±1.04**	**76.49^{a}**	**57.05^{b} ±2.16**	**64.24^{bc} ±1.82**	**68.73^{a} ±1.94**	**65.64 ±1.98^{b}**	**63.91^{b}**	**67.39^{d} ±1.08**	**75.03^{bc} ±1.89**	**80.18^{a} ±1.92**	**77.62^{b} ±1.03**	**75.05^{a}**

A-C: Means with the different superscripts in the same column, differ significantly (P<0.05).
a-d: Means with the different superscripts in the same row, differ significantly(P<0.05).

The advancement of storage time at 5˚C for up to 48 hours decreased significantly (P<0.05) the sperm motility in the different extenders or GSH levels

2. Percentage of dead spermatozoa:

Data presented in Table 3 showed significantly (P<0.05) lower in the percentage of dead spermatozoa in Tris-yolk-glucose extender and Non-fat Dried milk solids-glucose extender than Milk-glucose extender. supplementation of the extended stallion spermatozoa with GSH with 0.8 mM/100 ml decreased significantly (P<0.05) the percentage of dead spermatozoa, during storage at 5˚C for up to 48 hours in the different extenders. The highest (P<0.05) percentage of dead spermatozoa was recorded with free-semen GSH (control) and the lowest (P<0.05) value was recorded with the extended semen added with 0.4 mM GSH in the different extenders

Table 3: Mean percentage of dead Arabian stallions spermatozoa with different extenders and glutathione levels, during storage at 5ºC for up to 48 hours

Storage times (hours)	Tris-yolk-glucose					Milk- glucose					Non-fat Dried milk solids-glucose				
	Glutathione level (mM/100ml)				Means	Glutathione level (mM/100ml)				Means	Glutathione level (mM/100ml)				Means
	0	0.2	0.4	0.8		0	0.2	0.4	0.8		0	0.2	0.4	0.8	
0	19.80 ±0.87	14.48 ±0.78	6.53 ±0.91	9.72 ±0.42	**12.63^{C} ±0.10**	28.16 ±0.73	20.11 ±0.34	14.26 ±0.56	15.30 ±041	**19.45^{C} ±0.93**	21.43 ±0.84	15.34 ±0.73	**7.11 ±0.64**	9.81 ±0.43	**13.42^{C} ±0.42**
24	28.71 ±0.79	17.52 ±0.91	8.42 ±0.84	12.35 ±0.43	**16.75^{B} ±0.14**	36.12 ±0.52	25.95 ±0.74	20.16 ±0.28	23.14 ±0.34	**26.34^{B} ±1.03**	29.23 ±0.74	19.91 ±0.68	11.35 ±0.43	13.45 ±0.28	**18.48^{B} ±0.62**
48	35.05 ±0.94	22.73 ±0.87	12.42 ±0.62	18.53 ±0.48	**22.18^{A} ± 0.17**	48.92 ±1.16	32.53 ±0.94	26.83± 1.10	28.46 ±1.12	**34.18^{A} ±1.02**	38.11 ±0.82	25.92 ±0.78	14.25 ± 0.51	21.42 ±0.32	**24.92^{A} ±0.41**
Means	**27.85^{a} ±0.98**	**18.24^{b} ±0.65**	**9.12^{d} ±0.72**	**13.53^{c} ±0.81**	**17.18^{b}**	**37.73^{a} ±1.11**	**26.19^{b} ±1.32**	**20.41^{d} ±0.94**	**22.30^{c} ±0.87**	**26.65^{a}**	**29.59^{a} ±0.42**	**20.39^{b} ±0.18**	**10.90^{d} ±0.27**	**14.89^{c} ±0.42**	**18.94^{b}**

A-C: Means with the different superscripts in the same column, differ significantly (P<0.05).
a-d: Means with the different superscripts in the same row, differ significantly(P<0.05).

Regarding the effect of storage time, the advancement of storage time at 5ºC for up to 48 hours increased significantly (P<0.05) the percentage of dead stallion spermatozoa in the different extenders or GSH levels. The lowest (P<0.05) value of dead spermatozoa was recorded at zero time, while the highest (P<0.05) value was recorded at 48 hours with the different extenders

3. Percentage of abnormal spermatozoa:

Data presented in Table 4 revealed that, the percentage of abnormal stallion spermatozoa significantly (P<0.05) lower with Tris-yolk-glucose extender and Non-fat dried milk solid glucose extenders than Milk-glucose extender. Supplementation of the extended stallion semen with glutathione (GSH) up to 0.8 mM/100 ml caused decreased significantly (P<0.05) the percentage of abnormal spermatozoa during storage at 5˚C for up to 48 hours in the different extenders. The lowest (P<0.05) value of abnormal spermatozoa was recorded in the extended semen with 0.4 mM GSH and the highest (P<0.05) value was observed with semen free- GSH (control).

Table 4: Mean percentage of abnormal spermatozoa of the Arabian stallions with different extenders and glutathione levels, during storage at 5˚C for up to 48 hours

Storage times (hours)	Tris-yolk-glucose					Milk- glucose					Non-fat Dried milk solids-glucose				
	Glutathione level (mM/100ml)				Means	Glutathione level (mM/100ml)				Means	Glutathione level (mM/100ml)				Means
	0	0.2	0.4	0.8		0	0.2	0.4	0.8		0	0.2	0.4	0.8	
0	13.73 ±0.93	8.34 ±0.41	3.82 ±0.27	5.68 ±0.34	**7.89^{C} ±0.61**	23.42 ±0.28	15.25 ±0.13	9.14 ±0.14	10.44 ±0.16	**14.56^{C} ±0.19**	16.82 ±0.14	10.16 ±0.11	7.22 ±0.13	8.92 ±0.16	**10.78^{C} ± 0.16**
24	19.23 ±0.84	14.30 ±0.72	8.42 ±0.58	11.50 ±0.62	**13.36^{B} ±0.56**	30.16 ±0.64	20.91 ±0.71	13.34 ±0.65	15.17 ±0.41	**19.89^{B} ±0.22**	22.18 ±0.24	15.32 ±0.14	9.27 ±0.16	10.14 ±0.18	**14.22^{B} ±0.19**
48	26.72 ±0.56	21.43 ±0.65	12.24 ± 0.42	16.22 ±0.32	**19.15^{A} ±0.34**	37.25 ±0.41	26.24 ±0.23	18.27 ±0.11	22.40 ±0.19	**26.04^{A} ±0.22**	28.24 ±0.32	22.31 ±0.25	13.26 ±0.11	17.52 ±0.11	**20.33^{A} ±0.23**
Means	**19.89^{a} ±0.82**	**14.69^{b} ±0.61**	**8.16^{b} ±0.23**	**11.13^{c} ±0.18**	**13.46^{b}**	**30.27^{a} ±0.87**	**20.80^{b} ±0.16**	**13.58^{d} ±0.19**	**16.00^{c} ±0.21**	**20.16^{a}**	**22.41^{a} ±0.41**	**15.93^{b} ±0.16**	**9.91^{d} ±0.14**	**12.19^{c} ±0.18**	**15.11^{b}**

A-C: Means with the different superscripts in the same column, differ significantly (P<0.05).
a-d: Means with the different superscripts in the same row, differ significantly(P<0.05).

Regarding the effect of storage time, the advancement of storage time at 5ºC for up to 48 hours significantly increased (P<0.05) the percentage of abnormal stallion spermatozoa in different extenders and GSH levels (0.2, 0.4 and 0.8 mM). The lowest (P<0.05) value of abnormal spermatozoa was recorded at zero time, while the highest (P<0.05) value was recorded at 48 hours with different extender.

4. Percentage of acrosome damage of spermatozoa:

Data presented in Table 5 shows that, the percentage of acrosome damage of spermatozoa significantly (P<0.05) lower in tris-yolk-glucose extender and Non-fat dried milk solids glucose extender than Milk-glucose extender.

Supplementation of the extended stallion spermatozoa with GSH up to 0.8 mM/100 ml caused decreased significantly (P<0.05) the percentage of acrosome damage of spermatozoa, during storage at 5ºC for up to 48 hours in the different extenders.

Table 5: Mean percentage of acrosome damage of the Arabian stallions spermatozoa with the different extenders and glutathione levels, during storage at 5ºC for up to 48 hours.

Storage times (hours)	Tris-yolk-glucose					Milk- glucose					Non-fat Dried milk solids-glucose				
	Glutathione level (mM/100ml)				Means	Glutathione level (mM/100ml)				Means	Glutathione level (mM/100ml)				Means
	0	0.2	0.4	0.8		0	0.2	0.4	0.8		0	0.2	0.4	0.8	
0	9.48 ± 0.83	7.25 ± 0.76	1.12 ±0.45	2.71 ±0.53	**5.14^{C} ± 0.38**	12.53 ±0.16	9.41 ± 0.38	3.68 ±0.19	6.18 ± 0.11	**7.95^{C} ± 0.18**	8.92 ±0.12	5.12 ± 0.10	1.94 ±0.11	3.15 ± 0.16	**4.78^{C} ± 0.17**
24	15.34 ±0.46	12.16 ±0.81	2.19 ±0.11	7.87 ±0.63	**9.39^{B} ± 0.54**	19.46 ±0.43	15.27 ±0.52	6.72 ±0.71	11.51 ±0.13	**13.24^{B} ±0.26**	14.89 ±0.13	10.42 ±0.19	3.78 ±0.16	6.28 ± 0.12	**8.84^{B} ± 0.10**
48	20.22 ±0.34	16.34 ±0.61	3.72 ±0.13	9.45 ±0.12	**12.45^{A} ±0.13**	29.92 ±0.65	21.19 ±0.72	11.85 ±0.19	15.65 ±0.13	**19.90^{A} ±0.23**	22.41 ±0.21	15.33 ±0.14	5.86 ±0.13	12.36 ±0.10	**13.99^{A} ±0.11**
Means	**15.01^{a} ±0.87**	**11.94^{b} ±0.46**	**2.43^{d} ±0.14**	**6.67^{c} ±0.11**	**8.99^{b}**	**20.63^{a} ±0.23**	**15.29^{b} ±0.18**	**7.41^{d} ±0.81**	**11.44^{c} ±0.16**	**13.69^{a}**	**15.40^{a} ±0.1 2**	**10.29^{b} ±0.19**	**3.86^{d} ±0.13**	**7.26^{c} ±0.17**	**9.20^{b}**

A-C: Means with the different superscripts in the same column, differ significantly (P<0.05).
a-d: Means with the different superscripts in the same row, differ significantly(P<0.05).

The highest (P<0.05) percentage of acrosome damage of spermatozoa was recorded in the control samples and the lowest (P<0.05) percentage was recorded in the extended semen with 0.4 mM/100 ml GSH in the different extenders. The prolongation of storage time at 5ºC for up to 48 hours increased significantly (P<0.05) the percentage of acrosome damage of stallion spermatozoa in the different extenders or GSH levels. The lowest (P<0.05) percentage of acrosome damage of spermatozoa was recorded at zero time. While, the highest (P<0.05) percentage of acrosome damage of stallion spermatozoa was recorded at 48 hours in the different extenders.

DISCUSSION

Cooled equine semen is used to allow its shipment for variable periods of time. During transportation, the semen should keep the fertility and that depends on storage temperature, composition of extender and spermatic concentration per insemination dose (Heckenbichler *et al*., 2011). The cooled shipped system that we chose maintains the semen at 16 ºC for 24 h. it is low cost procedure, widely used during the breeding season and allows the shipment of semen for long distances (Melo *et al*., 2007).

Data present in Table 2 reveled that the percentage of motile stallion spermatozoa in tris-yolk-glucose was high because Tris in addition to its buffering capacity can readily diffuse into the sperm cell and serve as an intracellular buffer. Similar results fount by (Bartlett and Van Demark, 1962). These results may be due to the combination of all beneficial effects of the tris components. The addition of egg yolk which contains phospholipids and lecithin to extenders may be protect the sperm membrane against cold shock (Paulenz *et al*., 2002). Similarly, Waheed (2001) and Bozkurt *et al*. (2007) showed that tris-egg yolk extender at 5ºC was maintaining the motility of stallion spermatozoa. Moreover, Bergeron and Manjunath (2006), Bergeron *et al*. (2007), Lusignan *et al*. (2011) and El-Nady (2017) found that, the Non-fat Dried milk extender was good protection to spermatozoa because the milk contains of caseins micelles (the major proteins of milk). In stallion spermatozoa, Ball and Vo (2001) found that the addition of glutathione to semen extender prevented the loss of sperm motility by inhibition of lipid peroxidation caused by reactive oxygen species (ROS). Moreover de Oliveira *et al*. (2013), Oliveira (2014) and El-Nady (2017) reported that the addition of 2.5 mM glutathione to the freezing extender increased the total motility and the sperm viability of stallion spermatozoa. Similar trend was reported by Bustamante-Filho (2006) in stallion spermatozoa. These results may be accompanied with a precipitous fall in the rate of fructolysis (Mann and Lutwak-Mann, 1981), or may be due to change in spermatozoal morphology and coiled tail thus reducing the spermatozoal flagellum movement (El-Harairy *et al*., 2011). De Oliveira *et al*. (2015) found that the advancement of storage time at 16ºC for up to 36 hours increased significantly ($P<0.05$) the percentage of total motility of stallion spermatozoa with different levels of GSH. Moreover, cryopreservation of spermatozoa reduced motility and affects DNA integrity in humen spermatozoa (Branco *et al*., 2010) and in the Arabian stallion spermatozoa (El-Nady, 2017).

In Table 3 the low percentage of abnormal stallion spermatozoa in Tris-yolk-glucose extender may be due to the beneficial effects of tris extender allowing more protection of spermatozoa, consequently, lowering the dead spermatozoa during storage at 5ºC. El-Nady, (2017) in the Arabian stallion spermatozoa, found that, the lowest ($P<0.05$) of dead spermatozoa was recorded with glucose-yolk fructose extender, while the highest ($P<0.05$) value was recorded with Milk-glucose extender. The differences in sperm motility between GSH-treated and control samples become more evident upon increasing the storage time. De Oliveira *et al*. (2013) found that the addition of 2.5 mM glutathione to the freezing extender decreased percentage of dead stallion spermatozoa. However, Zhandi and Ghadimi (2014) and El-Nady (2017) found significantly effects of glutathione on the viability stallion spermatozoa, during storage at 5ºC. El-Nady (2017) indicated that the advancement of storage time at 5ºC for up to 48 h increased significantly ($P<0.05$) the percentage of dead cooled stallion spermatozoa added with all different concentrations of GSH. These findings may be attributed to the accumulation of lactic acid which exerts a toxic effect on sperm cell and leakage of intracellular enzymes due to the increased membrane permeability (Zeidan, 1994).

Data presented in Table 4 showed that the obtained results for abnormal spermatozoa are in agreement with those of Waheed (2001) who found that, glycine-egg yolk extender and Tris extender gave a high viability of stallion's semen than other extenders containing milk. In addition, El-Nady (2017) found that the lowest ($P<0.05$) value of the percentage of abnormal spermatozoa was recorded with tris-yolk extender, while the highest value was recorded with milk- glucose extender. Zhandi and Ghadimi (2014), Oliveira *et al*. (2014) and El-Nady (2017) mentioned that supplementation of INRA82 extender with 5 mM glutathione can preserve stallion spermatozoa during storage at 5ºC for 48 hours.

The interpretation of the obtained result concerning the abnormal sperm may be due to the increase in sperm metabolic activity, consequently, the increased lactic acid production which in turn exerts a toxic effect on sperm cell (Zeidan, 1994). The advancement of storage time of the stallion extended semen increased the percentage of abnormal spermatozoa (De Oliveira *et al*., 2013, Zhandi and Ghadimi, 2014 and De Oliveira *et al*., 2015).

The obtained results of the percentage of acrosome damage are in agreement with those of De Oliveira *et al*. (2013), El-Nady (2017) and De Oliveira *et al*. (2015) who found that, the addition of 3.5mM glutathione to the stallion semen was highly efficient for preservation of viability, acrosome integrity and membrane plasmatic integrity. These results indicated that Glutathione protect the spermatozoa from damage by inhibiting the lipid peroxidation process.

In conclusion, the Arabian stallions spermatozoa extended with Tris-yolk-fructose or Non-fat Dried

milk solids glucose extender was better than Milk-glucose extender, during storage at 5ºC. Addition of glutathione to an extender has more efficiency in maintaining the biological functions and quality of the Arabian stallion spermatozoa under refrigeration condition at 5ºC.

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تاثير اضافة الجلوتاثيون على مخفف السائل المنوى المبرد للخيول العربية

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تم في هذه الدرراسة جمع ١٨ قذفة سائل منوى من ثلاثة خيول عربية تبلغ اعمارها ٥ سنوات خلال فترة النشاط الجنسى (الصيف) وتم تقييم السائل المنوى بعد الجمع مباشرة وتخفيفه بمخففات الترس-جلوكوز ، اللبن - جلوكوز واللبن الجاف منزوع الدهن وكانت نسبة التخفيف النهائية هى ١مل سائل منوى : ٤ مل مخفف. وتم تقسيم السائل المنوى المخفف لكل مخفف لاربعة انابيب واضيف اليها الجلوتاثيون بمعدلات صفر و ٠.٢ و٠.٤ و٠.٨ ملليمول لكل ١٠٠ مللى سائل منوى مخفف وتم الحفظ على درجة حرارة ٥ درجة مئوية لمدة ٤٨ ساعة. تم تقدير كلا من النسبة المئوية لحيوية الحيوانات المنوية والحيوانات المنوية الميتة والحيوانات المنوية الشاذة والحيوانات المنوية تالفة الاكروسوم.

اوضحت النتائج زيادة النسبة المئوية لحيوية الحيوانات المنوية معنويا (على مستوى ٠.٠٥) فى السائل المنوى للخيول المخفف بمخفف الترس - جلوكوز ومخفف اللبن الجاف منزوع الدهن بينما انخفضت النسبة المئوية للحيوانات المنوية الميتة والحيوانات المنوية الشاذة والحيوانات المنوية تالفة الاكروسوم عن مخفف اللبن - جلوكوز. وقد اوضحت النتائج ان اضافة الجلوتاثيون الى المخفف ادت الى زيادة النسبة المئوية لحيوية الحيوانات المنوية للخيول معنويا (على مستوى ٠.٠٥) مع انخفاض النسبة المئوية للحيوانات المنوية الميتة والشاذة وتالفة الاكروسوم معنويا (على مستوى ٠.٠٥) مقارنة بمجموعة المقارنة وذلك فى المخففات المختلفة اثناء الحفظ على درجة حرارة ٥ درجة مئوية. كما ان التقدم فى فترة الحفظ على درجة ٥ مئوية ادى الى انخفاضا معنويا (على مستوى ٠.٠٥) فى النسبة المئوية لحيوية الحيوانات المنوية مع زيادة النسبة المئوية للحيوانات المنوية الميتة والشاذة والحيوانات المنوية تالفة الاكروسوم بدرجة معنوية (على مستوى ٠.٠٥) وذلك فى المخففات المختلفة ومع كل تركيزات الجلوتثيون. وويمن ان نستخلص من النتائج انة يمكننا ان نوصى باستخدام مخففات الترس - جلوكوز واللبن الجاف منزوع الدهن واضافة الجلوتثيون اليها بنسبة ٠.٤ ملليمول/ ١٠٠ مللى لتحسين فترة حفظ السائل المنوى للخيول العربية على درجة حرارة ٥ درجة مئوية.