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FROZEN-THAWED BULL SPERMATOZOA QUALITY AND FERTILITY RATE ADDED WITH SELENO-ZINC METHIONINE

1-EFFECT OF ZINC METHIONINE AND SELENO- METHIONINE ON FREEZABILITY AND FERTILITY OF BULL SEMEN.

By

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ABSTRACT

The present study aimed to study the effect of different levels of some antioxidants on post-diluted (PDSM) post- equilibrated (PESM) and post - thaw (PTSM) sperm motility. Also, the effect of these antioxidants on the livability%, acrosomal integrity%, levels of some enzymes as well as on the conception rate was investigated. Semen was collected from five mature Friesian bulls. Good semen ejaculates with at least 70% motility were pooled then split semen fraction was used for experiment. Three levels (0.5, 1.0 and 1.5mM) of each of the antioxidants, zinc methionine (Z-Met.) and seleno- methionine (Sel-Met.) were tested. Six semen fractions were diluted with Tris diluents enriched with the above levels of the antioxidants, the seventh fraction act as a control. The diluted semen was cooled, equilibrated and packaged in 0.25ml straws and freezed on the surface of liquid nitrogen. After at least 48 hours' storage, straws were thawed in water bath at 38 - 40 ºC for 30 - 60 seconds. Results indicated that, the all levels of the two antioxidants used were exerted a beneficial effect on the all parameters studied with the best PDSM, PESM, PTSM, high mortality and livability %, lower acrosomal damage, least enzymatic leakage and fructose level and the highest conception rate were observed when extended semen was enriched with (1.5 mM) zinc methionine.

Keywords:

Anti-oxidant, seleno, zinc methionine, semen, freezing, Friesian bulls.

INTRODUCTION

Semen cryopreservation is an important section of artificial insemination programs **(Watson, 2000)**, it allows preservation of semen fertility for a long time. Sperm cells have a high

content of polyunsaturated fatty acids (PUFA) in their membranes and they are exposed to aerobic condition during processing before freezing and a little endogenous antioxidant to protect them against reactive oxygen species (ROS) that may be present **(Foote *et al*., 2002).** The processes of cooling, freezing and thawing produce physical and chemical stress on sperm membrane that reduces sperm viability and their fertilizing ability. The cold shock of spermatozoa is associated with oxidative stress induced by reactive oxygen species generation. The process of freezing has been resulted in a significant reduction in endogenous antioxidant content in bovine **(Bilodeau *et al.,* 2000)** and buffalo **(Gabr, 2009)** semen accompanied by changes in sperm composition, organization of sperm membrane and in turn a marked reduction in sperm viability. Some attempts have been made to preserve sperm parameters, especially sperm motility by adding zinc, copper, selenium, vitamins, antioxidants, amino acids and coenzymes to the semen before freezing **(Foote *et al.,* 2002**; **Shiva Shankar Reddy *et al*., 2010 and Dorostkar *et al.,* 2012).** Zinc is essential elements for a multitude of body functions, including the acids balance **(Hahn and Baker, 1993),** DNA and nutrients metabolism **(Banerjee, 1988),** immunity protection **(Gross *et al.,* 1979),** fertility **(Apgar and Traves, 1979)** and many of other physiological processes. Also, the importance of zinc as co-factors for superoxidate dismutase activity **(Keen and Graham, 1989),** and glutathione peroxidase (GSHPx) system that regulates extra-and intracellular peroxidases **(Burk and Hill, 1993).** Till today, more than 200 zinc dependent enzymes and hormones have been identified in all the main biochemical path ways **(Smith and Akinbamizo, 2000).** Zinc influences the process of spermatogenesis **(Wong *et al.,* 2002),** controls sperm motility **(Wroblewski *et al.,* 2003)** and stabilizes sperm membrane **(Kendall *et al.,* 2000).** Selenium (Se) is physiologically important, because it acting as an integral component of the enzyme glutathione peroxidase **(Erskine,1993)**. This element is a component of selenoproteins (including glutathione peroxidase, iodothyronine deiodinase, and selenoprotein P and W and thioredoxin reductase) which plays an important structural and enzymatic functions **(Flohe *et al.,* 2000)**. Selenium is an inactivator of toxic heavy metals, causes apoptosis in tumer cells **(Behne *et al.,* 1996),** prevents or delays the cell aging by protecting the mitochondrial membranes **(Wesołowski and Ulewicz , 2000),** and protects the cell lipids from damaging effects of reactive oxygen species **(Musik *et al.,* 2003).** Selenium is present in the mide-piece of spermatozoa and is associated with Cys-rich protein of the mitochondrial sheath **(Kleene *et al.,* 1990).** A deficiency of Se causes changes in mid-

piece architecture leading to breakage of head and tail of spermatozoa and impaired of sperm motility **(Maiorino *et al.,* 2006).**Selenium level in blood is positively correlated with acrosomal integrity **(Bertelsmann *et al.,* 2007)**. Therefore, the present study aimed to investigate the effect of two different anti-oxidants on post- thaw sperm motility, recovery rate (freezability), acrosomal damage, enzymatic activity and fertility of frozen-thawed bull semen.

MATERIAL AND METHODS

The present study was carried out at El-Gemmizah Experimental Station, El-Gharbiya Governorate, belonging to the Animal Production Research Institute, Agricultural Research Center.

Animals and management system:

Five sexually mature Friesian bulls weighted 490-550 kg and aged 2.5 - 3.5 years old were used in the present study. All bulls were in healthy condition and clinically free of external and internal parasites with a sound history in the herd. Palpation of external genetalia showed that, they were typically normal. Feeding according to the live body weight (LBW) as recommended by Animal Production Research Institute (APRI) for adult Friesian bull requirements. Bulls were fed individually at 8.00 a.m. and 3.00 p.m., while fresh water and mineral blocks were available all the day times and housed individually under semi-open sheds.

Semen collection:

Semen was collected from all bulls by means of an artificial vagina twice a week for 12 weeks. Two successive ejaculates were obtained from each bull during collection day. Ejaculates with ≥ 70% sperm motility were pooled and used for different treatments.

Semen extension and treatment:

Semen was evaluated immediately after collection, extended with Tris-yolk fructose (TYF) (3.028 g tris amino methane, 1.675g citric acid anhydrous, 1.25g fructose, 7% glycerol, 20 ml egg-yolk, 500 I.U penicillin and 500 mg streptomycin added to 100 ml distilled water according to **Salisbury *et al.*(1978)**. Extended semen was divided into 7 aliquots as shown in table, 1. Six aliquots were supplemented with two types of antioxidants on three levels including zinc- methionine (0.5, 1.0 and 1.5 mM) and seleno-methionine (0.5, 1.0 and 1.5 mM) and one aliquots without antioxidants (control). Semen was extended with different

types and levels of antioxidants at a rate of 1 semen: 10 extenders (v. /v.) at 37ºc and cooled at 5ºC for 4 hours as equilibration period as described by **El-Harairy *et al.* (2010)**. Then, the cooled semen was packed in straws (0.25:0.50 ml) and freezed in liquid nitrogen (-196 ºC) as described by **Salisbury *et al.* (1978)**. Frozen-thawed semen samples were centrifuged at 1000 g for 15 minutes. The supernatant was collected and kept at -20 ºC till analysis.

Semen evaluation:

Percentages of post-diluted, equilibrated and post - thawed sperm motility, livability and normality were determined according to **Salisbury *et al.* (1978)**. Acrosomal damage of spermatozoawas estimated using Giemsa stain as described by **Watson(1975)**. In post-thawed semen, aspartate-aminotransferase (AST), alanine-aminotransferase (ALT), acid phosphatase (ACP)and alkaline phosphatase (ALP) enzymes were determined colorimetrically according to **Reitman and Frankel (1957),** using commercial Kits (Stanbio Kit, Texas, USA). Fructose concentration was measured according to **Barakat and EL-Sawaf (1964).**

Conception rate:

Fertility trial was done on one hundred and five normal cyclic Friesian cows (in privet farms) which divided into seven groups (six treatments and one control, each including 15 cows). In all treatment groups, cows were artificially inseminated with frozen-thawed semen extended with TYF extender supplemented with 2 types of antioxidants, zinc-methionine (Z-Met.) and seleno-methionine (Sel-Met.) with three levels for each (0.5, 1.0 and 1.5 mM), respectively. Each cow was artificially inseminated twice a day within10-12h interval with straw (0.25ml) of the thawed-frozen semen containing $20x10^6$ motile spermatozoa, by recto-vaginal insemination technique **(Salisbury *et al.*, 1978)**. Pregnancy diagnosis was performed by rectal palpation 60 days after insemination.

Table (1): Composition of Tris-yolk fructose extender (TYF), containing different type and levels of anti-oxidants used in bull semen dilution.

Ingredients	Type of extender						
	G1	G2	G3	G4	G5	G6	G7
Tris (g)	3.028	3.028	3.028	3.028	3.028	3.028	3.028
Fructose (g)	1.25	1.25	1.25	1.25	1.25	1.25	1.25
Citric acid (g)	1.675	1.675	1.675	1.675	1.675	1.675	1.675
Egg yolk ml	20	20	20	20	20	20	20
Glycerol, %	7	7	7	7	7	7	7
Penicillin (IU/ml)	500	500	500	500	500	500	500
Streptomycin (mg/ml)	500	500	500	500	500	500	500
Distilled water/ml up to	100	100	100	100	100	100	100
Zinc-methionine(mM)	0.0	0.5	1.0	1.5	0.0	0.0	0.0
Seleno-methionine(mM)	0.0	0.0	0.0	0.0	0.5	1.0	1.5

G_1 = without antioxidants (control). G_2=supplemented with zinc- methionine at a level with 0.5mM. $G_{3=}$ supplemented with zinc- methionine at a level with 1.0 mM**.** $G_{4=}$ supplemented with zinc- methionine at a level with 1.5mM. $G_{5=}$ supplemented with seleno-methionine at a level with 0.5mM. $G_{6=}$ supplemented with seleno-methionine at a level with 1.0 mM. $G_{7=}$ supplemented with seleno-methionine at a level with 1.5mM**.**

Statistical analysis:

Statistical analysis of data was carried out according to **Snedecor and Cochran (1982)** using General model Program of **SAS (1990).** Percentage values were transformed to arcsines before being analyzed. Duncan Multiple Range Test, **Duncan (1955)** was used to test the significance of differences among means at a level of $P < 0.05$. Pergnancy results were analyzed by Chi-square test.

RESULTS AND DISCUSSION

Sperm motility (%):

Results in (Table 2) showed that, the percentage of post-diluted sperm motility (PDSM) was significantly ($P<0.05$) higher with all antioxidants groups than the control group (G1). Among antioxidant groups, PDSM was significantly ($P<0.05$), higher with G4 and G6, while the lowest ($P<0.05$) value with G7 group. However, differences between G2, G3, G5 and G7

groups on PDSM were insignificant. Concerning equilibration period, all antioxidants supplementation significantly (P<0.05) increased the percentage of post-equilibrated sperm motility (PESM) as compared to G1, except for G7, which did not differ from that of the G1. Among antioxidant groups, PESM was significantly (P<0.05), higher in G4 group, followed by G3, G6, G5 and G2 groups respectively. While the lowest (P<0.05) value was in G7 group. However, differences among G2, G3, G5 and G6 groups or between G4, G3 and G6 groups were insignificant (Table 2).

Table (2): Post-diluted, equilibrated and thawed sperm motility (%) of Friesian bulls supplemented with different types and levels of antioxidants.

Antioxidant type (mM)		Sperm motility (%)		
		Post-dilution	Post-equilibration	Post-thawing
Control (G1)		73.92±1.78^{c}	65.25±1.61^{c}	41.67±2.31^{d}
Zinc-methionine (Z-Met.)	0.5 mM (G2)	77.75±1.90^{b}	72.83±1.75^{b}	54.58±1.89^{bc}
	1.0 mM (G3)	80.25±1.67^{b}	76.33±1.26^{ab}	57.92±1.78^{ab}
	1.5 mM (G4)	83.75±1.21^{a}	78.83±1.28^{a}	63.08±1.46^{a}
Seleno-methionine (Sel-Met.)	0.5 mM (G5)	79.08±1.45^{b}	73.25±1.51^{b}	50.75±1.49^{c}
	1.0 mM (G6)	83.25±1.72^{a}	74.75±1.72^{ab}	59.50±1.44^{ab}
	1.5 mM (G7)	75.42±1.75^{b}	67.08±1.42^{c}	44.08±1.95^{d}

$^{a, b, c, d;}$ **Means denoted within the same column with different superscripts are significantly different at P<0.05.**

With regard to frozen-thawed semen, the percentage of post- thawed sperm motility (PTSM) was significantly (P<0.05) decreased with G1 and G7 groups as compared to the other treated groups. However, differences between G4, G3, and G6 groups or between G2, G3 and G6 groups on PTSM were insignificant (Table 2). Generally, the highest (P<0.05) and the lowest (P<0.05) value of PDSM, PESM and PTSM were recorded with G4 and G1 groups, respectively. Such results indicated that beneficial effect of all antioxidants supplementation on motility of cryopreserved spermatozoa during all semen process, especially with (1.5 mM) Z-Met. Supplementation in G4. In agreement with the best results obtained in this study, percentage of sperm motility, livability, normality and fertility rate are reduced by sperm processing and cryopreservation involved in semen preservation in artificial insemination

programs **(Wathes *et al.*, 2007)**, while the antioxidant defense capacity of the semen is decreased **(Bilodeau *et al.*, 2000)**. During freezing, the production of ROS **(Bilodeau *et al.*, 2000 and Ball *et al.*, 2001)** can induce changes in membrane function and structure of spermatozoa. The detrimental effects of freezing could be blocked, at least in part, by the addition of exogenous antioxidant, since the cell employs the endogenous antioxidant and thioredoxin system to reverse oxidative stress. In addition, an alteration in antioxidant defense system may occur **(Bilodeau *et al.*, 2000),** including decrease in intracellular antioxidants content **(Gadea *et al.*,2004)**. During the processing of semen, supplementation of antioxidants was exerted beneficial effect on bull **(Foote *et al.*, 2002 and Chatterjee *et al.*, 2001)** and buffalo **(Gabr, 2009 and Shiva Shankar Reddy *et al.*, 2010)** spermatozoa.

Sperm livability (%):

Results presented in (Table 3) revealed that, the percentage of post-diluted sperm livability (PDSL) was insignificant. However, it tended to be higher with all antioxidant groups than the control one (G1). Concerning the equilibration period, the percentage of post-equilibrated sperm livability (PESL) was significantly ($P<0.05$) higher with all treatment groups than the control group (G1). Among the treated groups, it was significantly ($P<0.05$) higher with G4 than other treated groups. However, the differences effect between G2, G3, G5, G6 and G7 groups on PESL was insignificant (Table 3).

Table (3): Post-diluted, equilibrated and thawed sperm livability (%) of Friesian bulls supplemented with different types and levels of antioxidants.

Antioxidant type (mM)		Sperm livability (%)		
		Post-dilution	Post-equilibration	Post-thawing
Control (G1)		79.50±1.75	63.25±1.58^{c}	47.17±1.65^{d}
Zinc-methionine (Z-Met.)	0.5mM (G2)	81.25±1.63	68.83±1.54^{b}	56.92±1.32b^{c}
	1.0 mM (G3)	82.33±1.33	71.83±1.29^{b}	59.83±1.09^{b}
	1.5 mM (G4)	84.00±1.17	76.92±1.11^{a}	64.75±1.12^{a}
Seleno-methionine (Sel-Met.)	0.5 mM (G5)	82.83±1.45	70.67±1.52^{b}	58.00±1.30b^{c}
	1.0 mM (G6)	82.67±1.39	70.17±1.30^{b}	60.42±1.08^{b}
	1.5mM (G7)	81.17±1.57	68.67±1.08^{b}	55.33±1.54^{c}

$^{a, b, c, d}$; Means denoted within the same column with different superscripts are significantly different at $P<0.05$.

With regard to post- thawing semen, the percentage of post-thawed sperm livability (PTSL) was significantly ($p<0.05$) increased with all antioxidants supplementation as compared to control (Table 3). Among antioxidants supplementation, PTSL was significantly ($p<0.05$), being the highest value with G4, moderate with G2 and G5, while the lowest value was observed with G4 group. However, the differences effect between G2, G3, G5 and G6 groups or between G2, G5 and G7 groups on PTSL was insignificant (Table 3). Generally, the highest ($P<0.05$) value of the percentage of PDSL, PESL and PTSL was recorded with G4 group, while the lowest ($P<0.05$) value was observed with G1 group. Such results indicated that beneficial effect of all antioxidants supplementation on livability of cryopreserved spermatozoa with Z-Met. At level of 1.5 mM in group (G4). These results are in agreement with those reported by **(Alavi-Shoushtari *et al.*, 2009)** in buffalo who showed that a significant correlation between zinc content of seminal plasma and sperm parameters including motility, viability and normality. Moreover, zinc ions may be positively correlated with semen quality. **El-Hawary, (2010)** found beneficial effect of zinc plus vitamin E treatment on quality of fresh and cryopreserved semen. Also, **El-Sheltawi *et al.* (1999)** concluded that *in vitro* and / or *in vivo* supplementation of zinc increased motility and livability of spermatozoa and plasma membrane integrity. **Sikka (2001)** indicated that zinc as a co-factor of copper/zinc superoxide dismutase (Cu/Zn SOD), played a major role in the protection of spermatozoa against peroxidative damages of reactive oxygen species. **Joanna Rogalska *et al.* (2009)** concluded that organic zinc supplementation has shown better results in comparison to double dose of inorganic zinc supplementation. **Singh *et al.* (2000)** reported that dietary supplementation with methionine produced a beneficial effect on semen quality and freezability in buffalo-bulls and rabbit bucks, respectively. Moreover, our results are compatible with those of **Khalifa (2001) and Coyan *et al.* (2010)** who recorded that *in vitro* supplementation of ram and buffalo semen extenders with methionine resulted in pronounced enhancement in post-thaw sperm motility and plasma membrane integrity. Better motility, livability, normality and acrosomal integrity after equilibration and freezing of semen observed in this study could be attributed to increased antioxidant capacity of zinc ions, as reported by **Sikka (2001) and Kamran Dorostkar *et al.* (2014).**

Sperm normality (%):

Data presented in (Table 4) showed that, all antioxidant groups significantly ($P<0.05$) increased the percentage of post-diluted sperm normality (PDSN) as compared to the control

group (G1). With regard to equilibration period, all antioxidant groups significantly (P<0.05) increased the percentage of sperm livability (PESN) as compared the control group (G1). In frozen-thawed semen, the percentage of post-thawed sperm normality (PTSN) was significantly (P<0.05) higher with all antioxidant groups as compared to the control group.

Table (4): Post-diluted, equilibrated and thawed sperm normality (%) of Friesian bulls supplemented with different types and levels of antioxidants.

Antioxidant type (mM)		Sperm normality (%)		
		Post-dilution	Post-equilibration	Post-thawing
Control (G1)		81.00±0.92^{e}	73.83±1.14^{e}	52.42±2.05^{e}
Zinc-methionine (Z-Met.)	0.5 mM (G2)	88.17±0.55^{b}	80.75±0.83^{cd}	72.50±0.94b^{c}
	1.0 mM (G3)	88.42±0.48^{b}	84.17±0.79^{b}	74.83±0.94^{a}
	1.5 mM (G4)	90.50±0.34^{a}	87.08±0.57^{a}	77.42±0.87^{a}
Seleno-methionine (Sel-Met.)	0.5 mM (G5)	85.33±0.71c^{d}	79.33±0.64^{d}	69.83±1.26^{cd}
	1.0 mM (G6)	87.00±0.71b^{c}	81.75±0.85^{c}	72.08±1.14^{bc}
	1.5 mM (G7)	83.42±0.75^{d}	78.58±0.58^{d}	66.58±1.23^{d}

$^{a, b, c, d e;}$ **Means denoted within the same column with different superscripts are significantly different at P<0.05.**

Among antioxidant groups, PTSN was significantly (P<0.05) higher with G4, and G3, groups than other antioxidant groups, moderate with G2 and G6, while the lowest value was recorded with G7. However, the differences between G2, G5 and G6 groups or between G5 and G7 groups on PTSN was insignificant (Table 4). Generally, the highest (P<0.05) value of the percentage of PDSN, PESN and PTSN was recorded with group G4, while the lowest (P<0.05) value was observed with the control group (G1). Such results indicated that beneficial effect of all antioxidants supplementation on normality of cryopreserved spermatozoa, especially with a level (1.5 mM) of Z-Met. These findings are in agreement with those of **Alabi *et al.* (1985)** who revealed that *in vitro* incubation of ram spermatozoa with Seleno-methionine significantly (P<0.05)improved sperm motility, livability and oxygen consumption. **Khalifa, (2001)** indicated that *in vitro* supplementation of buffalo semen extenders with methionine resulted in pronounced enhancement in post-thaw buffalo sperm motility, viability and plasma membrane integrity besides a clear reduction in the post-thaw sperm abnormalities. The protective effect of seleno-methionine and zinc-methionine on

sperm functional and structural characteristics when included in the pre-freeze preparation may be attributed to that seleno-methionine and zinc-methionine penetrated the cell membrane easily, enhancing intracellular glutathione and superoxide dismutase enzymes, which plays a major role in the protection of spermatozoa against peroxidative damages of ROS **(Sikka, 2001 and Dorostkar *et al.*, 2012)**.

Acrosomal damage:

As presented in (Table 5) results revealed that, the percentage of acrosomal damage post-diluted (ADPD) of spermatozoa was significantly (P<0.05) lower with all treated groups than the control group (G1). Among treated groups, ADPD was significantly (P<0.05), being the lowest value with G4 group and the highest value with G7 group. However, the differences between G2, G3, G4 and G6 or between groups G2, G3, G5, G6 and G7 groups were insignificant. Concerning equilibration period, antioxidants supplementation significantly (P<0.05) decreased the percentage of acrosomal damage post- equilibration (ADPE) of spermatozoa as compared to G1. In frozen-thawed semen, the percentage of acrosomal damage post-dilution (ADPD) of spermatozoa was significantly (P<0.05) lower with all antioxidant supplementation as compared to control.

Table (5): Percentage of acrosomal damage (%) during post-diluted, equilibrated and thawed of Friesian bulls' spermatozoa supplemented with different types and levels of antioxidants.

Antioxidant type (mM)		Acrosomal damage (%)		
		Post-dilution	Post-equilibration	Post-thawing
Control (G1)		12.58±0.65^{a}	19.92±0.77^{a}	26.67±1.02^{a}
Zinc-methionine (Z-Met.)	0.5 mM (G2)	9.33±0.45b^{c}	15.08±0.54^{c}	17.33±0.51b^{c}
	1.0 mM (G3)	8.75±0.43b^{c}	13.17±0.56^{d}	15.83±0.55^{c}
	1.5 mM (G4)	7.83±0.47^{c}	10.50±0.50^{e}	13.33±0.48^{d}
Seleno-methionine (Sel-Met.)	0.5 mM (G5)	9.67±0.57^{b}	16.50±0.53b^{c}	18.58±0.50^{b}
	1.0 mM (G6)	8.92±0.51b^{c}	15.83±0.58b^{c}	17.42±0.48b^{c}
	1.5 mM (G7)	9.92±0.50^{b}	17.08±0.51^{b}	19.08±0.57^{b}

$^{a, b, c, d, e}$; Means denoted within the same column with different superscripts are significantly different at P<0.05.

Among antioxidant groups, ADPT was significantly ($p<0.05$), being the lowest value with G4 group, and the highest value with G7 group. However, the differences between G2, G5, G6 and G7 or between G2, G3 and G6 were insignificant (Table 5). Generally, the lowest ($P<0.05$) values of ADPD, ADPE and ADPT were recorded with G4 group, while the highest ($P<0.05$) values were recorded with the control (G1). Such results indicated that beneficial effect of all antioxidants supplementation on acrosomal damage of cryopreserved spermatozoa during all semen process, especially with (1.5 mM) Z-Met. Supplementation in G4. **El-Battawy and Rowida Riad (2011)** indicated that, the addition of 1.0 and 2.0 mM methionine to extended rabbit semen induced remarkable physiological effects on semen quality and improved sperm motility, viability, DNA and membrane integrity during conservation for 3 - days-long period at 5 ºC, and freezability. In buffalo, **Kamran Dorostkar *et al.* (2014)** showed that, the addition of zinc to the extender gave a higher protection of sperm protection of sperm progressive motility, viability, membrane integrity and DNA stability through the process of dilution, equilibration and freez-thawing and caused a higher total antioxidant capacity (TAC) as compared to the control group. Furthermore, they found that, *in vitro* supplementation of buffalo semen extenders with zinc resulted in pronounced enhancement in post-equilibration and thawed sperm motility, viability membrane integrity and DNA stability. Regarding the addition of seleno-methionine to the semen extender, the current study showed that seleno-methionine significantly ($P<0.05$) increased of motility, livability and normality and decreased percentage of acrosomal damage of spermatozoa through the process of dilution, equilibration and freez-thawing. **Kamran Dorostkar *et al.* (2014)** showed that *in vitro* supplementation of zinc was significantly ($P<0.05$) higher protection of the plasma membrane of buffalo spermatozoa and lower of sperm DNA damage through the process of dilution, equilibration and freeze-thawing than the control group. **Pankaj Kumar *et al.* (2014)** showed the beneficial effect of supplementation of zinc and selenium on ant oxidative status of seminal plasma which may provide better protection to spermatozoa from oxidative damage.

Enzymatic activity (u/10^9 spermatozoa) and fructose concentration (mg/100ml) in Post-thawed:

The activities of transaminases, (AST and ALT) and phosphatases (ACP and ALP) enzymes in post-thawed bull semen (Table 6) were significantly ($P<0.05$) lower in all treated groups

than the control group. There were significant differences in the activity of AST, ALT, ACP and ALP enzymes in post-thawed bull semen between treatment groups.

Table (6): Enzymatic activity (u/10^9 spermatozoa) and fructose concentration (mg/100ml) in post-thawed in Friesian bull semen as affected by different types and levels of antioxidants.

Antioxidant type (mM)		Enzymatic activity (u/109 spermatozoa)				Fructose (mg/100ml)
		AST	ALT	ACP	ALP	
Control (G1)		39.00±3.41^{a}	28.75±2.69^{a}	330.00±16.45^{a}	163.75±8.98^{a}	308.35±3.78^{a}
Zinc-methionine (Z-Met.)	0.5 mM (G2)	21.50±1.19^{cd}	15.25±1.49^{c}	164.00±9.84^{bc}	77.00±7.03^{cd}	254.07±15.60^{bc}
	1.0 mM (G3)	19.25±1.49^{de}	13.75±1.49^{c}	149.25±9.51^{c}	66.75±8.92^{d}	243.60±15.98^{cd}
	1.5 mM (G4)	16.00±1.30^{e}	9.25±0.85d	116.25±7.88^{d}	55.25±7.76^{d}	208.65±10.04^{d}
Seleno-methionine (Sel-Met.)	0.5m M (G5)	26.50±1.32^{bc}	19.25±1.75^{bc}	150.25±7.08^{bc}	94.00±8.50^{bc}	261.40±11.02^{bc}
	1.0m M (G6)	23.75±1.50^{bc}	16.50±1.55^{bc}	171.75±8.42^{bc}	81.75±7.59^{cd}	229.85±15.17^{cd}
	1.5 mM (G7)	28.50±1.44^{b}	21.75±2.01^{b}	189.75±8.35^{b}	109.75±10.19^{b}	290.41±5.44^{ab}

$^{a, b, c, d, e}$; Means denoted within the same column with different superscripts are significantly different at P<0.05.

Generally, the lowest and the highest (P<0.05) values of AST, ALT, ACP and ALP enzymes in in post-thawed bull semen were recorded with G4 and G1 groups, respectively (Table 6). **El-Harairy *et al.* (2010)** mentioned to storage of bull semen at low temperature caused structural damage as a result of cold shock. The changes involved damage to plasma membrane over the acrosome and outer acrosomal membrane and damage to the plasma membrane of the middle piece. These changes are followed by decreased in the proportion of spermatozoa with intact acrosome and increase the amount of enzymes released from the intracellular to extracellular medium. The present results are in agreement with those obtained by **El-Sheltawi *et al.* (1999)** who found that *in vivo* and /or *in vitro* supplementation of zinc proved to protected the plasma membrane of buffalo spermatozoa during freezing and thawing as indicated from the minimum rate of AST, ALT, ACP and ALP enzymes released to extracellular medium. The obtained results regard to activity of AST and ACP enzymes in post-thawed bull semen were almost higher than the corresponding values of ALT and ALP

activity as estimated by several authors in seminal plasma **(El-harairy *et al*., 2010)** in Friesian, **(Gabr, 2009 ; El-Hawary, 2010)** in buffalo. **Taha *et al*. (2000)** confirmed quality semen was characterized by lower activity of AST and ALT enzymes in barki and awassi rams. **Kumar *et al.* (2006)** revealed that zinc supplementation may induce the membrane stabilizing action of zinc that prevent leakage of enzymes, proteins and other vital components of spermatozoa in order to extend the functional life of spermatozoa. Present results showed that fructose content of frozen-thawed semen decreased with all antioxidant groups as compared to G1 and G7 groups, being the highest value with G1 and the lowest value with G4. **Marai *et al*. (1998)** who indicated that, the increase in percentage of motility of spermatozoa during equilibration and post-thawing increased, sperm metabolic activity and, consequently, increased the amount of fructose consumed, as well as, fructolysis.

Conception rate:

Results in (Table 7) indicated greatly higher conception rate (93.33%) of cows inseminated with the extended semen supplemented with (1.5 mM) Z-Met. than the other groups. In addition, the conception rates of cows inseminated with thawed semen to which were added three levels of antioxidants were significantly (P<0.05) higher than the control group (G1). Moreover, the percentage of conception rate in cows inseminated with semen supplemented with zinc methionine was significantly (P<0.05) higher than those enriched with seleno- methionine, especially at levels of 1.0 and 1.5 mM.

Table (7): Conception rate (%) of cows artificially inseminated with thawed semen.

Items	Experimental groups						
	G1	G2	G3	G4	G5	G6	G7
Number of inseminated cows	15	15	15	15	15	15	15
Number of conceived cows	8	11	12	14	10	11	10
Conception rate %	8/15 53.33^{e}	11/15 73.33^{c}	12/15 80.00^{b}	14/15 93.33^{a}	10/15 66.67^{d}	11/15 73.33^{c}	10/15 66.67^{d}

$^{a, b, c, d, e}$; Means denoted within the same column with different superscripts are significantly different at P<0.05.

The results of current study indicated that,addition of zinc-methionine and seleno- methionine at levels of (0.5, 1.0, and 1.5 mM) respectively, to the extended bull semen improved the sperm motility, livability and normality while at level of (1.5 mM) reduced the acrosomal

damage of spermatozoa as compared to the control group especially when zinc-methionine was supplemented. Similar results were reported by **El-Harairy *et al*. (2010)**, **Gabr (2009) and El-Hawary (2010).** In conclusion, addition of antioxidant zinc or seleno-methionine to Tris-yolk fructose extender (TYF) improved semen quality of bulls. Also, addition of zinc methionine at a level of (1.5 mM) during cryopreservation of bull semen showed the highest sperm motility, recovery rate and intact acrosomes in post-thawed semen with lowest enzymatic activity and fructose concentrations, and highest conception rate of Friesian cows.

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