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Effect of Some Alternative Components of Egg Yolk in Tris-Extender on Sperm Characteristics of Ram Semen Frozen with Two Methods of Packaging Semen

Abdel-Khalek, A. E.[1]; W. A. Khalil [1]; B. E. El-Saidy[2]and A. I. A. Yousif [2]

[1]Department of Animal Production, Faculty of Agriculture, Mansoura University, Mansoura 35516, Egypt

[2]Animal Production Research Institute, Agriculture Research Center, Dokki, Giza, Egypt

ABSTRACT

This study aimed to evaluate the effect of alternative components of egg yolk in Tris extender and semen packaging methods during cryopreservation on sperm characteristics and antioxidant system in seminal plasma of frozen-thawed semen of Finnish Landrace rams. Semen was collected from 5 sexually matured Finnish rams (50-70 kg LBW and 2-4 years old) by artificial vagina once weekly for 7 weeks. Only semen with mass motility of ≥70% was pooled and diluted with Tris-citric extender containing 15% egg yolk (Tris-EY) or 1% soybean lecithin (Tris-SBL) or 2 mM butylated hydroxytoluene (Tris-BHT). Semen was extended at a rate of 1:5 (semen/extender) with three extender types. After semen extension, semen was placed for cooling in the refrigerator (5^{o}C) for 4 hours as equilibration period and packaged in 0.25 ml French straws or 0.25 ml pellets in liquid nitrogen. Semen was thawed at 37°C for 30 s. Semen was evaluated after dilution, equilibration and thawing, for progressive motility, livability and abnormality of spermatozoa and curled tail spermatozoa responded to a solution of osmolarity of 75 mOsm for 30 min. The concentration of total antioxidants (TAC), malondialdehyde (MDA**)** and lactic dehydrogenase (LDH) activity in post-thawed seminal plasma were determined. Results showed that sperm characteristics, including percentages of progressive motility, livability, abnormality and curled tail in post-diluted, post-equilibrated or post-thawed semen were not affected significantly by the type of extender. Progressive motility and curled tail percentages in post-thawed semen were higher ($P<0.001$; $P<0.05$) in straws than in pellets. Livability and abnormality percentages were insignificantly better at straws than in pellets. The recovery rate of motility and livability was higher at straws than in pellets. All sperm characteristics indicated insignificant effect of interaction between type of extender and semen packaging method. TAC was higher ($P<0.05$), while MDA concentration was lower ($P<0.05$) in Tris-SBL and Tris-BHT than in Tris-EY. The activity of LDH was insignificantly the highest in Tris-BHT than in other extenders. The TAC, MDA concentration and LDH activity in post-thawed semen were not affected significantly by semen packaging method. Effects of interaction between type of extender and semen packaging method on each of TAC, MDA concentration and LDH activity were not significant. The current study concluded the successful usage of Tris-SBL or Tris-BHT in comparing with the possible disadvantages of using egg yolk in Tris-based extender of ram semen.

Keywords: *Ram semen, lecithin, butylated hydroxytoluene, sperm function, antioxidant capacity.*

INTRODUCTION

Chilled-frozen semen is the most potent technique for rapid genetic improvement in domestic animals using artificial insemination (AI) (Ax *et al*., 2000). In mammalian spermatozoa, high concentrations of polyunsaturated fatty acids (PUSFA) in the plasma membrane and lack of antioxidant enzymes in spermatozoa (Ziaullah *et al*., 2012) leads PUSFA to volatile to reactive oxygen species (ROS), induced a subsequent sperm functions loss and peroxidative damage in mammalian spermatozoa (Nair *et al*., 2006). Therefore, mammalian sperm preservation is a complex process, including several factors in order to obtain good semen quality (Ferdinand *et al*., 2014). Cryoprotectants, extenders, cooling rates, thawing rates and semen packaging method are major factors affecting cryopreservation success (Cotter *et al*., 2005; Andrabi, 2007; Clulow *et al*., 2008). Successful storage and quality of semen may vary depending on the extender used (Salamon and Maxwell, 2000). Extender composition aids with the stabilization of cells during the freezing-thawing process (Nur *et al*., 2010).

Egg yolk is considered to be an excellent cryoprotectant for the sperm cryopreservation at various concentrations in different species (Demianowicz and Strezek, 1996). The accurate mechanism of egg yolk which aids to protect sperm during freezing process is unknown. However, (Moussa *et al*., 2002) has suggested that the low-density lipoproteins (LDL) of egg yolk could protect spermatozoa against cold shock and improve sperm motility. Also, LDL could protect sperm membranes by involving to cell membranes during the freezing-thawing process (Graham and Foot, 1987). Furthermore, the presence of substances in the yolk that prevents the gaseous exchange of sperm or decreases sperm motility has accelerated the demand to replace whole egg yolk with the cryoprotective fraction alone (Pace and Graham, 1974; Watson and Martin, 1975; Haidl and Schill, 1994).

Egg yolk and skim milk are the most widely used as a non-penetrating cryoprotectants for preserving goat spermatozoa (Purdy, 2006) and ram, bull, equine, boar and human semen because it can protect the membrane damage as a result of its large content of lecithin (Rehman *et al*., 2014). Egg yolk, seminal plasma and milk proteins as animal principle ingredients increase the risk of microbial contamination of semen storage media (Wrathall *et al*., 2008). Soybean lecithin (SBL) extender as a lipid/lipoprotein source can be a substitute component of the animal's origin ingredients (Vidal *et al*., 2013) as skimmed milk (Papa *et al*., 2011) or conventional extenders that include egg yolk (Zhang *et al*., 2009) in semen extenders for semen cryopreservation. Also, butylated hydroxytoluene (BHT) has been tested as a cryoprotectant potentiality and optimal inclusion level in canine semen (Sahashi *et al*., 2011). The BHT has been used for minimizing

cryoinjury in cryopreserving semen of rams (Watson and Anderson, 1983), bovine bulls (Shoae and Zamiri, 2008), goats (Naijian *et al*., 2013) and human (Merino *et al*., 2015).

On the other hand, freezing method semen choice depends on individual preferences and needs. Variable effects have been occurred by semen packaging (straw or pellets) on sperm motility after the freezing-thawing process in ram (Awad, 1989) and rabbits (Daader and Zeidan, 2008).

Therefore, this study aimed to evaluate the effect of alternative components of egg yolk (SBL or BHT) in Tris-extender and semen packaging methods (straws or pellets) during cryopreservation on sperm characteristics and antioxidant system in seminal plasma of frozen-thawed Finnish rams semen.

MATERIALS AND METHODS

The current study was carried out at Animal Production Research Station, Sakha, Kafrelsheikh Governorate, located in the northern part of Nile Delta (latitude 31° 15'N and longitude 31° 45'E), belonging to Animal Production Research Institute (APRI), Agricultural Research Center, Ministry of Agriculture, Egypt, in cooperation with Physiology and Biotechnology Laboratory, Department of Animal Production, Faculty of Agriculture, Mansoura University, Egypt, during the period from August, 2016 until February, 2017.

Animals:

A total of 5 sexually matured Finnish Landrace rams (50-70 kg LBW) aged (2-4 years), adaptability to semen collection by artificial vagina were selected for semen collection. All experimental animals were raised under the same environmental conditions in the experimental farm and kept under the semi-open shaded yard.

Feeding requirements were calculated according to the recommendations of APRI, Ministry of Agriculture, Egypt. Each ram was fed concentrate feed mixture (CFM) at a level of 1.250 kg (14% CP) plus 1.250 kg berseem hay during August-November or 5 kg Egyptian fresh berseem (*Trifolium alexandrinum*) during December-February, with free access to trace mineralized salt lick blocks and drinking water all time.

Semen collection:

Semen was collected from 5 Finnish rams once weekly for 7 weeks before feeding at 7.0 a.m. to 8.0 a.m. by using the conventional artificial vaginal method (35 ejaculates). Semen ejaculates were transferred immediately after collection to the laboratory and then placed in water bath at 37°C. Only semen with mass motility of ≥70% was pooled and divided into three extender types and two methods of semen packaging (straws and pellets).

Semen extender preparation:

Tris-citric-egg yolk extender (Tris-EY) containing 3.025 g Tris (Sigma Chemical Co., St. Louis, MO, USA), 1.66 g citric acid monohydrate (Sigma), 1.25 g glucose (Sigma, Aldrich), 15% fresh egg yolk, 5% glycerol, 100 IU/ml penicillin and 100 µg/ml streptomycin was used as a control extender. Egg yolk (15%) in Tris-EY was replaced by 1% soybean lecithin (L-a-phosphatidyl choline, LAB: product number MC041) in the 2nd extender (Tris-SBL) or 2 mM butylated hydroxytoluene (BHT) in the 3rd extender (Tris-BHT).

Extenders were gently shaken and warmed up to 37°C in a water bath before use. Osmolarity and pH were assessed before addition of cryoprotectants and was adjusted to the osmolarity of 300 mOsmol and pH value of 7.3.

Semen freezing and thawing

Immediately, tubes containing the extended semen was gently shaken and placed in a water bath containing warm water (37°C). Pooled semen was diluted at a ratio of 1:5 (semen/extender) with three extender types. After semen extension, semen was placed for cooling in the refrigerator (5°C) for 4 hours as equilibration period.

Equilibrated semen was frozen by two methods of packaging, the first was 0.25 ml French straws (IMV, L'Agile France) and the 2nd was 0.25 ml pellets. The straws were then plunged into liquid nitrogen at -196°C, while pellets were made according to Awad (1989).

At least three straws and three pellets from each extender type of pooled ejaculates (n=7) were thawed in a water bath at 37°C for 30 s to evaluate sperm characteristics in post-thawed semen.

Semen evaluation:

Progressive motility, livability and abnormality of spermatozoa were assessed, and curled tail spermatozoa responded to a solution of osmolarity of 75 mOsml in term hypo-osmotic swelling test (HOS-t) for 30 min was also assessed by adding 0.1 ml of semen sample in a tube contains 0.9 ml of HOS media. These parameters were evaluated immediately in post-diluted, post-equilibrated and post-thawed semen.

Antioxidants analysis:

Concentration of total antioxidants (Koracevic *et al*. 2001), malondialdehyde (MDA, Ohkawa *et al*., 1979) and lactic dehydrogenase (LDH) activity (Bais and Philcox, 1994) were determined in post-thawed seminal plasma using commercial kits (Biodiagnostic, Egypt) and spectrophotometer (SPECTRO UV-VIS AUTO, UV-2602, Labomed, USA).

Statistical analysis:

The General Linear Model procedures of (SAS 2004), GLM analysis of variance (ANOVA, two ways design) were used for statistically analyzing data to determine the effect of extender type, semen packaging method and their interaction on sperm characteristics and antioxidant system in semen of Finnish rams. The Differences among treatment means were tested according to Duncan multiple range tests (Duncan 1955).

RESULTS

Sperm characteristics:

Effect of type of extender:

Sperm characteristics, including percentages of progressive motility, livability, abnormality and curled tail (membrane integrity in response to HOS-t) either in post-diluted, post-equilibrated or post-thawed Finnish ram semen were not affected significantly by the type of extender (Table 1).

Table 1. Sperm characteristics in post-diluted, post-equilibrated and post-thawed semen of Finnish rams as affected by the extender type.

	Sperm characteristics (%)			
Type of Extender	**Progressive sperm motility**	**Sperm livability**	**Sperm abnormality**	**Curled tail spermatozoa**
Post-diluted semen:				
Tris-EY	77.10±1.81	69.80±1.68	12.00±0.65	68.80±3.38
Tris-SBL	75.70±2.00	67.40±0.97	12.80±1.67	67.10±3.97
Tris-BHT	75.00±2.15	65.40±0.90	13.40±1.13	65.40±4.03
P-value	0.75	0.06	0.24	0.26
Post-equilibrated semen:				
Tris-EY	69.28±2.0	64.28±1.0	14.00±1.2	62.14±0.9
Tris-SBL	68.57±2.1	62.85±0.9	15.57±1.4	62.85±1.1
Tris-BHT	67.85±1.5	62.00±0.6	15.28±1.4	61.14±1.5
P-value	0.86	0.19	0.67	0.59
Post-thawed semen:				
Tris-EY	45.35±1.33	46.28±1.40	34.4±1.10	46.64±1.75
Tris-SBL	45.00±1.28	45.64±1.46	35.5±1.10	46.78±1.38
Tris-BHT	43.57±1.10	44.92±1.34	36.4±1.12	46.57±1.34
P-value	0.57	0.70	0.46	0.99

Despite the observed insignificant differences among different types of extenders used in this study on sperm characteristics during different semen processes. Tris-EY showed a lower rate of decrease in motility, livability and curled tail of spermatozoa and lower increase in sperm abnormality after dilution as compared to Tris-SBL and Tris-BHT. However, both Tris-SBL and Tris-BHT showed an opposite trend of Tris-EY after equilibration. Yet, all types of extenders exhibited similar trend of change in all sperm characteristics in frozen/thawed semen (Table 2)

These effects reflected nearly similarity in using soybean lecithin (SBL) or butylated hydroxytoluene (BHT) as an alternative to egg yolk in the Tris-based extender, regardless semen packaging method.

Table 2. Change rate (%) in sperm characteristics after dilution, equilibration and thawing of Finnish ram as affected by the extender type.

	Sperm characteristics (%)			
Extender Type	**Motility**	**Livability**	**Abnormality**	**Curled tail**
Fresh semen	78.5	75.4	8.8	74.0
After dilution:				
Tris-EY	-1.78	-7.42	36.36	-7.02
Tris-SBL	-3.50	-10.61	45.45	-9.32
Tris-BHT	-4.45	-13.26	52.27	-11.62
After equilibration:				
Tris-EY	-10.14	-7.90	16.66	-9.68
Tris-SBL	-9.41	-6.75	21.56	-6.33
Tris-BHT	-9.53	-5.19	14.72	-6.51
After freezing/thawing:				
Tris-EY	-34.54	-28.00	145.71	-24.94
Tris-SBL	-34.37	-27.38	180.00	-25.56
Tris-BHT	-35.78	-27.54	138.21	-23.83

Effect of semen packaging method:

Sperm characteristics, including percentages of progressive motility and curled tail in post-thawed Finnish ram semen, were higher significantly ($P<0.001$; $P<0.05$) in semen of straws than that in pellets form. Also, sperm characteristics, including percentages of livability and abnormality tended to be better in semen of straw than in pelleted semen, but the differences were not significant (Table 3).

When the effect of semen packaging methods was expressed in term of recovery rate, also packaging semen in straws showed a higher recovery rate of sperm motility and livability than those packaged in pellets form (Table 3).

Such result showed that freezing ram semen in straws is better than in pelleted form, regardless type of extender.

Table 3. Sperm characteristics in post-thawed semen of Finnish rams as affected by semen packaging method.

Item	Sperm characteristics (%)			
	Motility	**Livability**	**Abnormality**	**Curled tail**
Semen packaging method:				
Straw	45.47±1.08[a]	48.71±1.09	35.8±1.01	48.57±0.89[a]
Pellet	43.80±0.91[b]	42.52±0.66	35.1±0.79	44.76±1.33[b]
P-value	0.260	0.0001***	0.640	0.030*
PE semen[(1)]	68.56	63.04	-	-
Recovery rate:				
Straw	66.32	77.26	-	-
Pellet	63.88	67.44	-	-

*** Significant differences at P<0.05. *** Significant differences at P<0.001. PE: Post-equilibrated**

Effect of interaction between extender types and semen packaging method:

Analysis of variance of all sperm characteristics studied indicated insignificant effect of interaction between type of extender and semen packaging method on all characteristics, reflecting better sperm characteristics of semen packing in straws than in pellets form with all types of extenders (Table 4).

Table 4 .Effect of interaction between extender types and semen packaging method on sperm characteristics in post-thawed Finnish ram semen.

Type of Extender	Packaging Method	Sperm characteristics (%)			
		Motility	Livability	Abnormality	Curled tail
Tris-EY	Straw	45.71±2.02	49.00±2.10	34.30±1.63	48.57±1.68
	Pellet	45.00±1.89	43.57±1.25	34.60±1.60	44.71±3.04
Tris-SBL	Straw	45.71±2.02	48.85±2.20	35.40±1.82	48.71±1.55
	Pellet	44.28±1.70	42.42±0.99	35.60±1.39	44.85±2.17
Tris-BHT	Straw	45.00±1.89	48.28±1.63	37.60±1.85	48.42±1.65
	Pellet	42.14±1.01	41.57±1.19	35.30±1.27	44.71±1.99
P-Value		0.83	0.91	0.67	0.99

Antioxidant capacity and enzyme activity in post-thawed seminal plasma:

Effect of extender type:

Antioxidant system, in terms of total antioxidants capacity (TAC) and malondialdehyde (MDA), were affected significantly (P<0.05) by type of extender. Results showed that TAC was significantly (P<0.05) higher, while MDA concentration was significantly (P<0.05) lower in Tris-SBL and Tris-BHT than in Tris-EY extender. However, lactic dehydrogenase (LDH) activity was insignificantly the highest in Tris-BHT than in other extenders (Table 5). These results indicated that using SBL or BHT in Tris-extender as an alternative to egg yolk had beneficial effects in reducing lipid oxidation or peroxidation in the cell membrane of spermatozoa during freezing in liquid nitrogen.

Table 5. Total antioxidants capacity, malondialdehyde concentration and lactic dehydrogenase activity in seminal plasma of post-thawed Finnish ram semen as affected by the extender type.

Extender Type	Total antioxidants capacity (Mm/l)	Malondialdhyde (nmol/ml)	Lactic dehydrogenase (U/ml)
Tris-EY	1.87±0.14^{b}	1.53±0.18^{a}	357.97±61.55
Tris-SBL	2.18±0.11^{ab}	0.92±0.04^{b}	304.91±44.07
Tris-BHT	2.40±0.15^{a}	0.56±0.02^{b}	446.57±29.27
P-value	0.03 *	0.0005***	0.13

*** Significant differences at P<0.05. *** Significant differences at P<0.001.**

Effect of semen packaging method:

Antioxidant system, in terms of TAC, MDA concentration and LDH activity in post-thawed Finnish ram semen were not affected with semen packaging method, indicating similar sperm characteristics of ram semen frozen in straws or in pelleted form (Table 6).

Table 6. Total antioxidants capacity, malondialdehyde and lactic dehydrogenase activity in seminal plasma of post-thawed Finnish ram semen as affected by semen packaging method.

Semen packaging method	Total antioxidants capacity (Mm/l)	Malondialdhyde (nmol/ml)	Lactic dehydrogenase (U/ml)
Straw	2.02±0.09	1.00±0.18	415.84±48.64
Pellet	2.27±0.15	1.01±0.15	323.80±26.52
P-value	0.10	0.97	0.11

Effect of the interaction between extender type and semen packaging method:

Effects of interaction between type of extender and semen packaging method on each of TAC, MDA concentration and LDH activity were not significant, reflecting better antioxidant defence system in semen packing in straws than in pellets form with all types of extenders (Table 7).

Table 7. Effect of the interaction between extender type and semen packaging method on total antioxidants capacity, malondialdehyde and lactic Dehydrogenase activity in seminal plasma of post-thawed Finnish ram semen.

Extender Type	Packaging Method	Total Antioxidants Capacity (Mm/l)	Malondialdhyde (nmol/ml)	Lactic dehydrogenase (U/ml)
Tris-EY	Straw	1.76±0.12	1.50±0.39	418.24±121.64
	Pellet	1.97±0.27	1.56±0.14	297.71±22.70
Tris-SBL	Straw	2.18±0.14	0.97±0.07	338.19±80.05
	Pellet	2.17±0.21	0.87±0.06	271.63±46.85
Tris-BHT	Straw	2.12±0.10	0.54±0.04	491.09±36.50
	Pellet	2.67±0.16	0.59±0.03	402.05±31.12
P-value		0.32	0.87	0.91

DISCUSSION

The current study aimed to compare the effectiveness of soybean lecithin (SBL) or butylated hydroxytoluene (BHT) as an alternative component in the Tris-based extender, on sperm characteristics, including progressive motility, livability, abnormality and curled tail spermatozoa in post-diluted, post-equilibrated and post-thawed semen. It is of interest to

note that each of sperm motility and livability percentage in the present study had a positive relationship with the percentage of curled tail spermatozoa as affected by the type of extender or semen packaging methods. In the same way, Gil *et al*. (2003) and Salmani *et al*. (2014) reported that functional membrane integrity of spermatozoa has a positive effect and direct relationship with sperm motility. During cryopreservation, equilibration period was important for protecting sperm motility and membrane integrity (Leite *et al*., 2010). It is worth noting that each of Tris-SBL or Tris-BHT showed a lower rate of reduction in motility, livability and response to HOS-t and a lower rate of increase in abnormality of spermatozoa than Tris-EY after equilibration. Similar results were obtained by Khalifa and Abdel-Hafez (2014), who indicated that Tris-citric acid extender supplemented with 3.5 g soybean lecithin had higher progressive motility and viability than Tris-citric acid extender supplemented with 15% egg yolk in post-equilibrated semen. This improvement may be attributed to that the observed reduction rate of sperm characteristics during refrigeration was attributed to changes in the pH of extension, osmolarity and growth of bacteria (Futino *et al.,* 2010) in Tris-SBL or Tris-BHT as compared to Tris-EY. Also, Shahverdi *et al*. (2014) observed significant interactions between equilibration time and extenders for sperm motility and membrane integrity. Freezing-thawing process negatively influenced sperm motility and livability, abnormality and membrane integrity. After freezing/thawing, ram semen had a nearly similar rate of change in all types of extenders. Similarly, El-Badry *et al*. (2014) found that addition of 15% egg yolk to an extender resulted in the highest sperm post-thaw motility, viability index, sperm membranes, acrosomal and DNA integrities. Depending on egg yolk which contains cholesterol, phospholipids, and low-density lipoprotein, it prevents the formation of ice crystal, thus protects sperm plasma membrane integrity against cold shock (Hu *et al.,* 2010).

The present results indicated nearly similar effects of different types of extenders (Tris-EY, Tris-SBL or Tris-BHT) on all sperm characteristics studied in frozen semen of Finnish rams. These findings are in agreement with previous studies on ram and goat semen cryopreservation which reported no differences between egg yolk and lecithin based extenders in viability, motility (Salmani *et al*., 2014) and functional membrane integrity of spermatozoa (Emamverdi *et al*., 2013). Similar results were reported on cryopreservation of ram (Forouzanfar *et al*. (2010) and goat (Roof *et al*. 2012) semen. Recently, Üstüner *et al*. (2014) indicated insignificant differences in the percentage of progressive motility in frozen-thawed ram semen diluted by extenders containing 1% soybean lecithin or 20% egg yolk. Also, Emamverdi *et al*. (2013) found insignificant differences in percentages of total motility and total abnormality in post-thawed semen diluted with Tris-SBL or Tris-EY extender. However, the addition of BHT in canine semen led to a significant improvement in semen quality (Neagu *et al*., 2010). This similarity may be due to that most semen extenders contain egg yolk and skim milk as sources of lipoprotein (Moussa *et al*., 2002), soybean contains high contents of low-density lipoproteins and egg yolk contains lecithin (Forouzanfar *et al*., 2010) that protects sperm cells against cold shock and other damage. SBL may play a more protective role for spermatozoa than egg yolk during the freezing-thawing process, and therefore reduces the risk of bacterial and mycoplasma into freezing extenders (Fukui *et al*., 2008). However, Zhang *et al*., (2009) reported that the boar sperm motion characteristics, plasma membrane integrity and acrosome integrity were superior in SBL-extender comparing with egg yolk extender. This is because of the provided poor protection by egg yolk for boar spermatozoa during cryopreservation unlike for bovine sperm (Benson *et al*., 1967; Bathgate *et al*., 2006).

Although both Tris-SBL and Tris-BHT extenders had similarity with Tris-EY, they reflected significant improvement in the antioxidant system of semen, in terms of increasing total antioxidant capacity and decreasing MDA concentration as well as increasing LDH activity in seminal plasma of post-thawed semen. This means a high level of lipid peroxidation (LPO) its end products (MDA) in Tris-EY extender than in the other extenders. Similar results were reported by Chelucci *et al*. (2015). Overall, egg yolk contains high levels of unsaturated fatty acids susceptible to LPO. Regardless of the source (polyunsaturated fatty acids from sperm cell membranes, from extenders or both), LPO which induced by ROS disrupts sperm motility and impairs all the sperm functions which are dependent on the integrity of plasma membrane, including sperm-oocyte fusion and ability to undergo acrosomal exocytose (Bansal *et al*., 2011). SBL as a substitute to egg yolk in semen extender may have played a protective role during freezing because of low viscosity, improvement of the sperm membrane function and rearrangements phospholipids of the sperm cells membrane (Fukui *et al*., 2008). Furthermore, the effective antioxidant components in soy lecithin as glutathione may protect the sperm viability through scavenging the LPO and prevention of MDA formation during freezing process (Salmani *et al*., 2014). Also, SBL extender recovered motility, plasma membrane and acrosome integrity, maintain semen quality, improve the freezability, apoptosis status and mitochondrial activity after thawing ram spermatozoa (Emamverdi *et al*., 2013; Singh *et al*., 2013). Molecular features revealed that sperm functionality in egg yolk extender was affected, as evidenced by lower DNA integrity, and higher lipid peroxidation in comparison with spermatozoa cryopreserved in soybean lecithin extender (Chelucci *et al*., 2015). As showed in this study, Tris-BHT extender caused higher activity of LDH than in Tris-EY and Tris-SBL, respectively. The increase in activity of LDH enzyme was in accordance with the findings of Jones (1997), who reported that reduced LDH activity in seminal plasma indicated disturbed sperm function and metabolism. These findings may indicate higher microbial contamination of semen diluted with Tris-EY than that with Tris-SBL or Tris-BHT, being the best for the later extender.

Concerning the effect of semen packaging method, the present results indicated the superiority of straws as a better method for semen packaging as compared to pelleted form, regardless type of extender, in all sperm characteristics studied in ram semen after dilution, equilibration and thawing. Kowalczyk and Lukaszewicz (2015) found a

higher reduction in sperm motility of frozen semen in pellets than in straws. The obtained better post-thawed motility and plasma membrane integrity of ram spermatozoa in straws than in pelleted form proved in our study were also reported in goats (Bezerra *et al*., 2012), boar (Dai *et al*., 2009). Also, Hunton *et al*. (1987) indicated that semen frozen in straws was more motile than pelleted semen, but both applied cryopreservation methods (pellets and straws) caused a decrease in the percentage of sperm livability. Pickett and Berndtson (1974) reported that superior sperm viability, greater storage efficiency, and higher conception rates resulting from storage in plastic straws have led to rapid increases in the use of this packaging technique. During cryopreservation, the plasma membrane integrity disruption caused by disarrangement of lipids within the membrane may encourage further cellular damage and then lead to sperm death (Chelucci *et al*., 2015). In addition, straws have been packaged with more hygienic semen, also the use of vials or straws readily allows the accurate identification of samples. Although pellets have the advantage of allowing a rapid drop in temperature to be achieved, they are not suited for easy identification after freezing. In addition, the re-use of the carbon dioxide block or metal plate carries the potential risk of cross-contamination with spermatozoa from the previous freezing. On the other hand, the use of straws considerably reduces the risk of cross-contamination during cryopreservation (Bezerra *et al*., 2012). In general, the present results did not reflect the significant effect of semen packaging method on total antioxidant capacity, MDA concentration or LDH activity.

The successful usage of Tris-SBL or Tris-BHT comparing with the possible disadvantages of using egg yolk, including its potential to be a cause of allergic reactions, the risk of bacterial contamination and its variable effect on semen, which may allow the save using SBL or BHT as an alternative of egg yolk in Tris-based extender of ram semen.

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تأثير استخدام بعض المكونات البديلة لصفار البيض فى مخفف الترس على خصائص الحيوانات المنوية فى السائل المنوى للكباش المجمد بطريقتين لتعبئة السائل المنوى

عبد الخالق السيد عبدالخالق[١] ، وائل أحمد خليل[١] ، بدير السيد الصعيدى[٢] و أحمد إبراهيم على يوسف[٢]

[١]قسم انتاج الحيوان ، كلية الزراعة ، جامعة المنصورة

[٢]معهد بحوث الانتاج الحيوانى ، مركز البحوث الزراعية، وزارة الزراعة

الهدف من هذه الدراسة هو تقييم تأثير المكونات البديلة لصفار البيض في مخفف ترس وطرق تعبئة السائل المنوي أثناء الحفظ بالتجميد على صفات السائل المنوى ونظام مضادات الأكسدة في البلازما المنوية للسائل المنوى للكباش الفنلندى بعد التجميد والإسالة. تم جمع السائل المنوي من ٥ كباش فنلندي ناضجة وزنها حوالى (٥٠-٧٠ كجم) و عمر (٢-٤ سنوات) بإستخدام المهبل الصناعى مرة واحدة أسبوعيا لمدة ٧ أسابيع.أخذت عينات السائل المنوى ذات حيوية لاتقل عن ٧٠%. تم جمع السائل المنوى ثم تخفيفه بإستخدام مخففات (١٥% صفار البيض مع الترس – حمض الستريك) ،(١٪ ليسيثين فول الصويا مع الترس – حمض الستريك) أو(٢ ملي مول من البيوتيلاتيد هيدروكسى تولوين مع التريس – حمض الستريك). تم تخفيف السائل المنوي بمعدل ١:٥ (السائل المنوي / المخفف) بإستخدام المخففات الثلاثة. بعدعملية التخفيف تم وضع السائل المنوي للتبريد في الثلاجة على درجة (٥ْم) لمدة ٤ ساعات كفترة موازنة، تم تعبئة السائل المنوى المخفف في قصيبات (٠,٢٥ مل) أو فى شكل حبيبات (٠,٢٥ مل)، ثم تم الحفظ بوضعها في النيتروجين السائل. تم إسالة السائل المنوي عند ٣٧ درجة مئوية لمدة ٣٠ ثانية. تم تقييم صفات السائل المنوي بعد التخفيف وبعد الموازانة وبعد التجميد والإسالة من خلال الحركة التقدمية، الحيوية، الحيوانات المنوية الشاذة وإلتواء ذيل الحيوانات المنوية كإستجابة لمحلول منخفض الإسموزية (٧٥ مللى اوزمول) لمدة ٣٠ دقيقة . تم قياس تركيز مضادات الأكسدة وتركيز المالون داي ألدهيد ونشاط إنزيم لاكتيك دهيدروجينيز في البلازما المنوية بعد الإسالة. وقد أظهرت النتائج أن صفات السائل المنوي، بما في ذلك النسب المئوية للحركة التقدمية والحيوية والشواذ والذيل الملتوى بعد التخفيف وما بعد الموازنة وكذلك بعد الإسالة لم تتأثر بشكل كبير بنوع المخفف. كانت النسبه المئويه للحركة التقدمية والتواء الذيل بعد الإسالة في السائل المنوي المعبأ في قصيبات أعلى من المعبأ فى حبيبات. وكانت نسب الحيوية والشواذ أفضل بشكل ملحوظ في السائل المنوي المعبأ في قصيبات من المعبأ فى حبيبات. وكان معدل إسترجاع حركة وحيوية الحيوانات المنوية في السائل المنوي المعبأ في قصيبات أعلى من المعبأ فى حبيبات. كما أظهرت جميع صفات السائل المنوى تأثير غير معنوي للتفاعل بين نوع المخفف وطريقة تعبئة السائل المنوي. كانت نسبة مضادات الأكسدة أعلى فى حين كان تركيز المالون داي ألدهيد أقل عند إستخدام مخفف ليثيسين فول الصويا ومخفف البيوتيلاتيد هيدروكسى تولين من مخفف صفار البيض. وكان نشاط إنزيم لاكتيك دهيدروجينيز أعلى بكثير في مخفف البيوتيلاتيد هيدروكسى تولوين مما كان عليه في المخففات الأخرى. لم يكن هناك تأثير لطريقة تعبئة السائل المنوى على تركيزمضادات الأكسدة والمالون داي ألدهيد ونشاط إنزيم لاكتيك دهيدروجينيز في السائل المنوي بعد التجميد والإسالة. لم يكن هناك تأثيرمعنوي للتفاعل بين نوع المخفف وطريقة تعبئة السائل المنوي على كل من تركيزمضادات الأكسدة و تركيز المالون داي ألدهيد ونشاط انزيم لاكتيك دهيدروجينيز. وقد خلصت هذه الدراسة إلى نجاح إستخدام مخفف ليثيسين فول الصويا ومخفف البيوتيلاتيد هيدروكسى تولوين بالمقارنة بالتأثير السلبى المحتمل عند إستخدام صفار البيض في مخفف الترس لتخفيف السائل المنوي للكباش.