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PRESERVATION OF RAM SEMEN EXTENDER WITH NON-TRADITIONAL INGREDIENTS IN LIQUID AND FROZEN STATE

By

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ABSTRACT

The objective of the present study aimed to define the effect of three different semen extenders (two traditional and one non-traditional) on vitality of unfrozen and frozen ram semen and its reproductive performance. To achieve this objective, six Rahmani rams (30-31 months old with live body weight of 75-80 kg) were used. Semen was collected twice a week for six weeks using artificial vagina. The semen ejaculates were extended using traditional semen diluents [Tris-egg yolk (E1) and sodium citrate-egg yolk (E2)] and non-traditional semen one as lecithin plus propolis extract dissolved in saline solution (NaCl 0.9% w/v) intravenous infusion (E3). Two unfrozen conditions (as incubation at 37ºC for up to 4 hours and stored at 5ºC for up to 4 days) and semen quality as sperm motility (%), live spermatozoa (%), normal spermatozoa (%) and intact acrosome (%) were recorded of unfrozen semen. While, through frozen semen (post-semen dilution, post- semen equilibration at 5ºC up to 180 minutes and post-thawing at 37ºC for up to 60 seconds) were recorded. In addition, reproductive performance as conception rate (%), fertility rate (%) and litters size (%) were observed with twenty-eight ewes allocated into two groups (14 ewes /group). Then, artificial insemination was performed to 1st and 2nd groups using the best either frozen-thawed semen extender in E1 or E3, respectively. Results indicated that unfrozen semen in E1, E2 and E3 showed insignificantly differs throughout the times of incubation at 37ºC and preservation at 5ºC. While, E3 was recorded better semen characteristics followed by E1 and E2. The highest ($P<0.05$) semen parameters observed with either E3 or E1 during the first hours of incubation and first day of preservation compared to E2 extender. The major frozen semen characteristics such as post-thawing motility and post-thawing intact acrosome attained significant ($P<0.05$) better quality in E3 following E1 than E2 extender. In addition, E3 showed higher reproductive performance as conception rate at 1st service (57.14%) and 2nd

service (66.67 %), fertility rate (60.00 %) and litter size (1.67) than E1 (50.00%), (57.14%), (52. 38%) and (1.09), respectively. It was concluded that ram semen can be preserved and cryopreserved in non-traditional semen extender without disruption in sperm survival through storage either liquid or frozen state.

Keywords:

Ram semen, incubation, preservation, extender, fertility rate.

INTRODUCTION

The semen extender according to present traditional invention may be contained a large number of substances commonly used in semen diluent. These include substances as hydroxymethyl aminometahne (Tris), sodium citrate, whole milk, skim milk, glycerol, egg yolk, fructose and synthetic antibiotics. Thus, the traditional invention is described in greater detail with respect to the preservation of animal semen. **Acharya *et al.* (2016)** described that decrease of ram semen characteristics extended with Tris and sodium citrate may be related to ingredients of diluent. Thus, it has been found that, surprisingly, sperm may be maintained for substantially longer periods of time without appreciable loss of motility or fertility. Non-traditional substances as lecithin, propolis extract and sodium chloride (0.9% w/v I.V.) are used as a substitute for egg yolk, synthetic antibiotics and distilled water. In this context, lecithin was demonstrated to be safer than egg yolk in terms of biosecurity and it is used for sperm preservation in ram **(Khalifa and Abdel-Hafeze, 2014)** and Billy goat **(Khalifa, 2015)**. In addition, propolis extract used in semen extenders as scavenging free radicals **(Budai *et al.*, 2014)**, natural antibiotic sources **(Khalifa *et al.*, 2016)**, antimicrobial agents **(Morrell, 2016)** and nutrient sources to spermatozoa **(El-Sheshtawy *et al.*, 2016)**. Regarding to sodium chloride solution, **Khalifa and Khalil (2016)** concluded that NaCl (0.9% w/v) achieved better conception rate than Tris extender. Generally, all extenders used for either preservation or cryopreservation storage should be the following precursors: 1) provide as an energy source; 2) buffer against harmful changes in pH; 3) be of physiologic osmotic pressure and concentration of electrolytes; 4) prevent growth of bacteria; 5) protect from cold shock during cooling and 6) have cryoprotectants to reduce the amount of freezing damage **(Vickram *et al.*, 2017).**Therefore, the aim of the present study aimed to define the effect of traditional semen extender (as Tris or sodium citrate) and non-traditional semen extender (as lecithin and propolis extract dissolved in sodium chloride solution) throughout incubation, chilling and frozen of

semen quality. Reproductive performance of post-thawing ram spermatozoa was also investigated.

MATERIAL AND METHODS

The experimental work was carried out in El-Serw Experimental Research Station belonging to Animal Production Research Institute (APRI), Ministry of Agriculture, and Egypt and in partnership with Animal Production Department, Faculty of Agriculture, Mansoura University, throughout the period from April, 2016 till March, 2017.

Experimental animals and management:

Six mature and healthy Rahmani rams achieved live body weight at 75 - 80 kg when age reached to 30 - 31 months were used in current study. They were housed in a yard with a concrete floor, roofing with asbestos and could more freely in enclosed area. All experimental rams were clinically free from external and internal parasites and had the sound history of fertility in experimental research station. The external genetalia showed normal. Rams were received 60% concentrate fed mixture in all breeding season plus fed 40% of corn silage, while rice straw was offered *ad libitum* during the experimental period. Ration requirements were represented according to **NRC (2007)**. The clean fresh water and mineral salt blocks were available freely during the experimental periods.

Semen collection and evaluation:

Seventy-two ejaculates were collected using an artificial vagina (two ejaculates weekly / up to six weeks / rams) in presence of an estrous ewe to stimulate and mating. Semen characteristics of each ejaculate / ram was evaluated individually for volume (ml), progressive motility (%), live spermatozoa (%), normal spermatozoa (%), intact acrosome (%) and sperm cell concentration$\times 10^9$ / ml. If ram ejaculates observed semen volume $\geq$ 0.8, progressive motility $\geq$75%, live sperm $\geq$85 %, normal sperm $\geq$85%, intact acrosome $\geq$85% and sperm cell concentration $\geq 2.9\times 10^9$/ml they would be pooled, extended and used in experimental procedures. Evaluation of ram spermatozoa characteristics were described by **(Salisloury *et al.*, 1978).**

Semen extension:

Semen was evaluated and pooled immediately after collection after that, semen was extended using traditional extender as Tris (E1) and sodium citrate (E2) and non-traditional extender as lecithin plus propolis extract (E3). The pooled ejaculates were divided into three parts which kept in sterile Falcon test tube under water bath adjusted at 37°C. Semen extension was

carried out by adding the appropriate volume of semen slowly to each extender type to reach extension rate at 1 ml of raw pooled semen: 6 ml / extender type / sterile Falcon test tube. The extended semen was maintained below in water both at 37°C to avoid fluctuations in the temperature until started experimental procedures.

Table (1): Compositions of the traditional and non-traditional semen extender.

Components	Traditional extender		Non- traditional extender
	E1	E2	E3
Sodium citrate (gm)	-	2.90	-
Tris (gm)	3.028	-	-
Citric acid (gm)	1.675	0.05	-
Fructose (gm)	1.250	1.250	1.250
Egg yolk (ml)	15.000	15.000	-
Lecithin (gm) [1]	-	-	3.500
Synthetic antibiotic (µl) [2]	600.000	600.000	-
Natural antibiotic (µl) [3]	-	-	600.000
Glycerol (ml)	7.000	7.000	7.000
Distilled water (ml)	100.000	100.000	-
Saline solution (ml) [4]	-	-	100.000

E1: Tris extender;E2: sodium citrate extender;E3: lecithin and propolis extender.

[1] Lecithin (from soy bean) was diglycerides and consisted of fatty acid, glycerol, phosphate group and choline.

[2] Synthetic antibiotic as Pen-Strep20/20 contained Procaine penicillin 200 mg and Dihydrostreptomycin sulphate 250mg excipient up to 1.0 ml.

[3] Natural antibiotics as propolis extract prepared by soaked 10 grams of propolis glue in 100 ml of ethanol (70%) for 24 hours. The mixture was filtrated into dark bottle and stored at 4°C until supplied to semen extender.

[4] Saline solutions (NaCl 0.9% w/v).

Preservation of unfrozen-semen:

Inclusive of either incubation time at 37°C for up to 4 hours or storage time at 5°C for up to 4 days in E1, E2 and E3 extenders. Ten replication samples / extender types / procedures were

used. Percentage of progressive sperm motility (%), live spermatozoa (%), normal spermatozoa (%) and intact acrosome of spermatozoa (%) during incubation at 37°C for 0, 1, 2 and 4 hours or storage at 5°C for 0, 1, 2 and 4 days.

Cryopreservation of frozen-semen:

Comprehensive freezing steps which consisted of equilibration period at 5°C for up to 120 -180 minutes, freezing in liquid nitrogen at -196 °C and thawing at 37°C for up to 60 seconds with E1, E2 and E3 extenders. Percentage of motility (%), live spermatozoa (%), normal spermatozoa (%) and intact acrosome of spermatozoa (%) were evaluated during post-dilution, post-equilibration and post-thawing.

Reproductive performance:

It was performed on twenty-eight mature and healthy ewes, as well as, in reproductive and productive during breeding season through September 2016. Ewes were partitioned into two groups as 1st and 2nd (n=14 each) which were inseminated using E1 and E3 extenders, respectively. The insemination was occurred with E1 compared to E3 post- thawing spermatozoa. Estrus cycle was detected with a teaser ram twice a day at 6.00 a.m. and 6.00 p.m. with 12 hours as interval period. The observation was two hours / time. Thence, if ewe in 1st or 2nd group displayed heat at 6.00 a.m. it would be inseminated with frozen-thawing straw from E1 or E3 at 6.00 p.m. of the same day and again at 6.00 a.m. in the following day, respectively. Each frozen-thawing straw of E1 or E3 included $\geq 400\times10^6$ motile sperm which placed by insemination gun on Os-cervices of ewe post speculum opened vaginal vulvas. If the ewe in 1st or 2nd groups displayed heat again after 14-19 days, it would be inseminated again. After breeding season done (34 days), the total reproductive performance was determined on the basis of pregnancy diagnosis by non-returns to heat again during breeding season. Hence, reproductive performance of either E1 or E3 analysis as following: conception rate (number of ewes conceived / number of ewes inseminated), fertility rate (number of ewes lambed / number of ewes inseminated), lambing rate as single birth (number of ewes lambing single/ number of ewes lambed), twins birth (number of ewes lambing twins/ number of ewes lambed) and litter size (number of total lambs born / number of ewes lambing).

Statistical analysis:

One-way analysis of variance (ANOVA) was carried out using IBM SPSS Statistics version 22 **(SPSS, 2013)**. When ANOVA revealed a significant treatment effect between traditional (E1 and E2) and non-traditional (E3) semen extender compositions on sperm characteristics

(%) like motility, live, normal and intact acrosome the values were compared using the Duncan's Multiple Range Test **(Duncan, 1955)** of the same SPSS program. All values are presented as mean ± standard error (SE) and differences among means were considered significant at $P < 0.05$. Conception rate results were analyzed by the Chi-square test.

RESULTS AND DISCUSSION

Preservation of unfrozen-semen:

A summary of the analysis of variance for incubated or preserved semen is presented in (Tables 2 and 3), respectively. Results showed that E1, E2 and E3 insignificantly affect semen characteristics during incubation at 37°C for up to 4 hours and storage at 5°C for up to 4 days. Semen characteristics observed better values in E3 followed by E1 than E2 extenders during preservation condition. Hence, conventional extender as Tris (E1) was usefulness as a good semen extender compared with sodium citrate (E2). These results are in agreement with those of **Paulenz *et al.* (2002)** who reported that a clear advantage of Tris based extender as an alternative of sodium citrate extender to maintain sperm motility and integrity of acrosomal membrane during storage of ram semen. Furthermore, the best sperm measurement obtained results in Tris-egg yolk extender that may be attributed to its osmolality and buffer substances compared with sodium citrate-egg yolk extender. This observation is in the line with the report of **Rakha *et al.* (2013)** who found that Tris has an osmolality at 375 mOsm/kg containing 2% egg yolk was the best in preserving acrosomal integrity and post-thawing motility. **Daramola and Adekunle, (2015)** revealed that Tris- egg yolk extender achieved the highest acrosome integrity coupled with improved acrosome reaction and capacitation. However, **Albiaty *et al.* (2016)** revealed that, the Tris and sodium citrate extenders showed superiority progressive motility (%) up to 63.87 and 61.25%, respectively. Generally, Tris-egg yolk extenders indicated less sperm damage during preservation the deleterious effect due to high density of egg yolk lipoproteins might have affected the spermatozoa lifespan **(Khumran, *et al.*, 2017)**. Results indicated that higher sperm parameters in E3 than other extenders (E1 and E2) regarding to its composition as lecithin, propolis extract and saline solution of NaCl 0.9% through preservation procedures. **Emamverdi *et al.* (2013)** established that lecithin contained major phospholipids that played an important role in building sperm cell membrane and supplies sperm freezability. Similarly, **Najafi *et al.* (2014)** recorded that extender contained lecithin as an egg yolk substitute has become available for

the preservation and cry protection of animal spermatozoa. The same results were confirmed by **Khalifa and Abdel-Hafez (2014)** who concluded that ram semen extender within lecithin at 3.5% showed a higher concentration of phospholipids which optimized to preserve the individual sperm motility, viability and intact acrosome. Moreover, **Yotov (2015)** confirmed that lecithin in extender at 1.5% (w/v) provided the best motility and viability of the chilled-stored goats' spermatozoa. Generally, **Khumran *et al.* (2017)** suggested that lecithin had a high content of low density lipoprotein, which makes it a better shock protectant and may require little synergism from an antioxidant. Moreover, the latter authors defined that density of the lipoproteins in the egg yolk used may affect sperm quality. Low density lipoproteins protect spermatozoa from damage due to cold shock while high density lipoproteins in egg yolk result in reduction of respiration and sperm motility. Propolis additive in E3 instead of synthetic antibiotics could resist bacterial contamination which improved sperm viability during incubation or storage. This is connected with the fact of **Petruska *et al.* (2014)** who reported that propolis had a wide range of biological activities including antibacterial (by damaging its cytoplasm and causing bacteriolysis), antiviral, anti-inflammatory and anti-oxidative properties. In addition, **Akandi *et al.* (2015)** revealed that when propolis added to semen basically, it could less lipid peroxidation level, an antimicrobial protective and supplied vitamins and minerals to semen extender which keep goodness sperm parameters. **Khalifa *et al.* (2016)** found that significantly mean of inhibition zone diameters in ram semen extender as control (without synthetic and natural antibiotics), using 200µl synthetic antibiotic and using 200µl of propolis glue, the circumference zone diameters were 2.37, 2.53 and 4.20mm for Gram-positive bacteria and 1.47, 1.93 and 2.20mm for Gram-negative bacteria, respectively. Regarding to replacement distilled water by saline solution (NaCl 0.9% w/v) in semen extender; it could ameliorate sperm activity in E3 during preservation procedures. This information is consistent with findings of **Hamad *et al.* (2014)** who stated that the highest value of Na^+ cation presented in saline solution plus other cations such as, K^+, Ca^{++} and Mg^{++} in the seminal plasma may play an essential role in the sperm activity and establish the osmotic balance. Finding of **Zeny (2016)** indicated that Na^+ cation may have an indication of intact sperm plasma membrane and exhibited favorable effects on the seminal quality, antioxidant balance and positive impact on all vitality of spermatozoa. On the other hand, **Khalifa and Khalil (2016)** found that ram sperm motility (%) extended with saline solution (NaCl 0.9% w/v) was recorded 81.28, 75.62 and 65.62% compared with Tris

distilled water 81.88, 76.25 and 66.25% and sodium citrate distilled water 76.88, 70.00and 61.88% during storage at 5°C for up to 1, 2 and 3 days, respectively. The prolongation of incubation at 37°C (Table 2) and storage at 5°C (Table 3) was increased sperm activity during the first hours of incubation and first day of stored conditions. Subsequently, sperm characteristics measurements declined drastically from two up to 4 hours during incubation and from 2 up to 4 day of storage. The current results, observed that E3 and E1 were the best extender followed by T2 then, the poorest sperm parameter values were obtained in sodium citrate (E2) under incubation or refrigeration conditions. From the experimental study, E3 exhibited the highest ability to sustain the viability of sperm motility. This could be attributed to capability of this extender to supply protection from cold shock (by lecithin), nutrients afford to sperm cells plus inhibit microbial growth (by propolis extract) and goodness physiologic osmotic pressure (by saline solution). Thence, the lowest in sperm parameters through advancement of preservation time could be attributed to gradual consumption of nutrients required for sperm metabolic through incubated and stored. These notions are defined by **Olurode and Ajala, (2016)** who observed that the drastic decline in sperm livability could be attributed to gradual depletion of nutrients such as potassium, sodium and plasma protein required for high metabolic demands of sperm. In addition, effect of peroxidation comes from polyunsaturated fatty acids in ram sperm cytoplasm membrane lead to lost cytoplasm membrane, decrease sperm motility and inhibit of fructolysis and respiration **(Albiaty *et al.,* 2016)**. In addition, **Acharya *et al.* (2016)** noticed that reduce of sperm characteristics be associated with waste products of reactive oxygen species (ROS) and free radical (as superoxide anion O^{-2}, hydrogen peroxide H_2O_2 and hydroxyl radical OH^-) might be involved in damaged sperm membrane.

Table (2): Semen characteristics (Mean±SE) during incubation at 37°C as affected by the different types of extenders.

Sperm characteristics	Incubation at 37°C (hours)	Extenders			Overall means
		E1	E2	E3	
Progressive motility (%)	0	85.00±0.75	82.00±1.33	86.00±0.67	84.33±0.62[a]
	1	79.50±1.17	76.00±2.45	81.50±0.76	79.00±1.00[b]
	2	73.00±1.70	69.50±2.52	75.50±1.16	72.67±1.14[c]
	4	65.00±2.23	61.00±2.87	69.00±1.63	65.00±1.42[d]
Means		75.62±4.31 [a]	72.12±4.50 [a]	78.00±3.69 [a]	75.25
Live spermatozoa (%)	0	88.10±0.97	86.90±1.42	90.50±1.03	88.50±0.71 [a]
	1	84.80±1.14	84.00±1.54	87.50±1.18	85.43±0.77 [ab]
	2	77.60±1.72	77.20±2.45	82.30±2.00	79.03±1.24 [c]
	4	71.70±2.29	71.30±2.94	77.20±2.59	73.40±1.54 [d]
Means		80.55±3.68 [a]	79.85±3.50 [a]	84.38±2.93 [a]	81.59
Normal spermatozoa (%)	0	92.40±0.88	92.00±1.13	94.00±0.83	92.80±0.56 [a]
	1	84.30±1.14	84.70±1.39	87.70±1.07	85.57±0.73 [b]
	2	74.00±1.69	75.10±1.57	79.10±1.55	76.06±0.98 [c]
	4	69.20±2.21	69.50±1.68	74.50±1.92	71.07±1.17 [d]
Means		79.98±5.20 [a]	80.32±4.99 [a]	83.82±4.35 [a]	81.37
Intact acrosome (%)	0	94.60±0.85	94.10±0.71	94.90±0.80	94.53±0.44 [a]
	1	89.30±0.80	88.50±0.95	90.10±0.90	89.30±0.51 [b]
	2	83.10±0.91	82.90±0.96	84.90±1.18	83.63±0.59 [c]
	4	76.90±1.36	76.60±1.34	79.10±1.40	77.53±0.79 [d]
Means		85.98±3.83[a]	85.52±3.72 [a]	87.25±3.39 [a]	86.25

E1: Tris extender; E2: sodium citrate extender; E3: lecithin and propolis extender.

Means bearing different superscripts within the same classification differ significantly (P<0.05).

Table (3): Semen characteristics (Mean±SE) during storage at 5°C as affected by the different types of extenders.

Sperm characteristics	Storing at 5°C (days)	Extenders			Overall means
		E1	E2	E3	
Progressive motility (%)	0	84.00±1.00	82.00±1.53	85.00±0.80	83.67±0.73 [a]
	1	73.50±1.07	73.00±2.26	76.00±0.67	74.17±0.87 [b]
	2	66.50±1.50	66.00±2.66	69.00±1.00	67.17±1.06 [c]
	4	57.00±1.70	58.00±3.09	61.00±1.01	58.67±1.22 [d]
Means		70.25±5.69 [a]	69.75±5.11 [a]	72.75±5.10 [a]	70.92
Live spermatozoa (%)	0	87.80±0.83	88.00±1.23	90.10±0.98	88.63±0.61 [a]
	1	79.00±0.84	78.10±1.57	80.40±0.97	79.16±0.68 [b]
	2	70.50±1.29	72.20±2.59	75.00±1.32	72.57±1.08 [c]
	4	63.70±1.97	64.90±3.15	69.90±1.80	66.17±1.42 [d]
Means		75.25±5.22 [a]	75.80±4.88 [a]	78.85±4.32 [a]	76.63
Normal Spermatozoa (%)	0	92.80±0.63	93.00±0.94	93.90±0.65	93.23±0.41 [a]
	1	77.20±1.21	79.00±1.33	80.70±1.19	78.96±0.74 [b]
	2	65.70±1.63	69.10±1.62	71.30±0.75	68.70±0.89 [c]
	4	59.70±1.33	62.50±1.68	64.80±1.15	62.33±0.88 [d]
Means		73.85±7.28 [a]	75.88±6.64 [a]	77.68±6.32 [a]	75.80
Intact acrosome (%)	0	94.90±0.53	94.20±0.81	95.10±0.41	94.73±0.35 [a]
	1	82.40±0.70	81.70±0.97	83.10±0.48	82.40±0.43 [b]
	2	75.30±0.73	74.80±1.27	76.50±0.81	75.53±0.55 [c]
	4	67.70±1.30	68.10±1.55	69.30±0.98	68.37±0.73 [d]
Means		80.08±5.78 [a]	79.70±5.57 [a]	81.00±5.48 [a]	80.26

E1: Tris extender; E2: sodium citrate extender;E3: lecithin and propolis extender.

Means bearing different superscripts within the same classification differ significantly ($P<0.05$).

Cryopreservation of frozen semen:

Fig.(1) shows the effect of cryopreservation procedures within E1, E2 and E3 on sperm characteristics during post-extension, post- equilibration and post-thawing. It will be observed

that semen diluted in E1 and E3 under freezing steps gave the highest ($P<0.05$) sperm measurements throughout post-extension, post- equilibration, and post-thawing compared with E2. In the current study, there were insignificant ($P>0.05$) values between semen characteristics such as normal post-diluted (NPD), normal post-thawing (NPT), intact acrosome post-diluted (IAPD) and intact acrosome post-equilibration (IAPE) at freezing steps between E1, E2 and E3. Then, the sperm values such as NPD was 92.20, 91.10 and 93.80 %, IAPD was 94.4, 93.4 and 94.7%, IAPE reached to 88.40, 86.70 and 89.20 %, NPT showed 49.60, 50.10 and 53.80 % when ram semen extended in E1, E2 and E3, respectively.

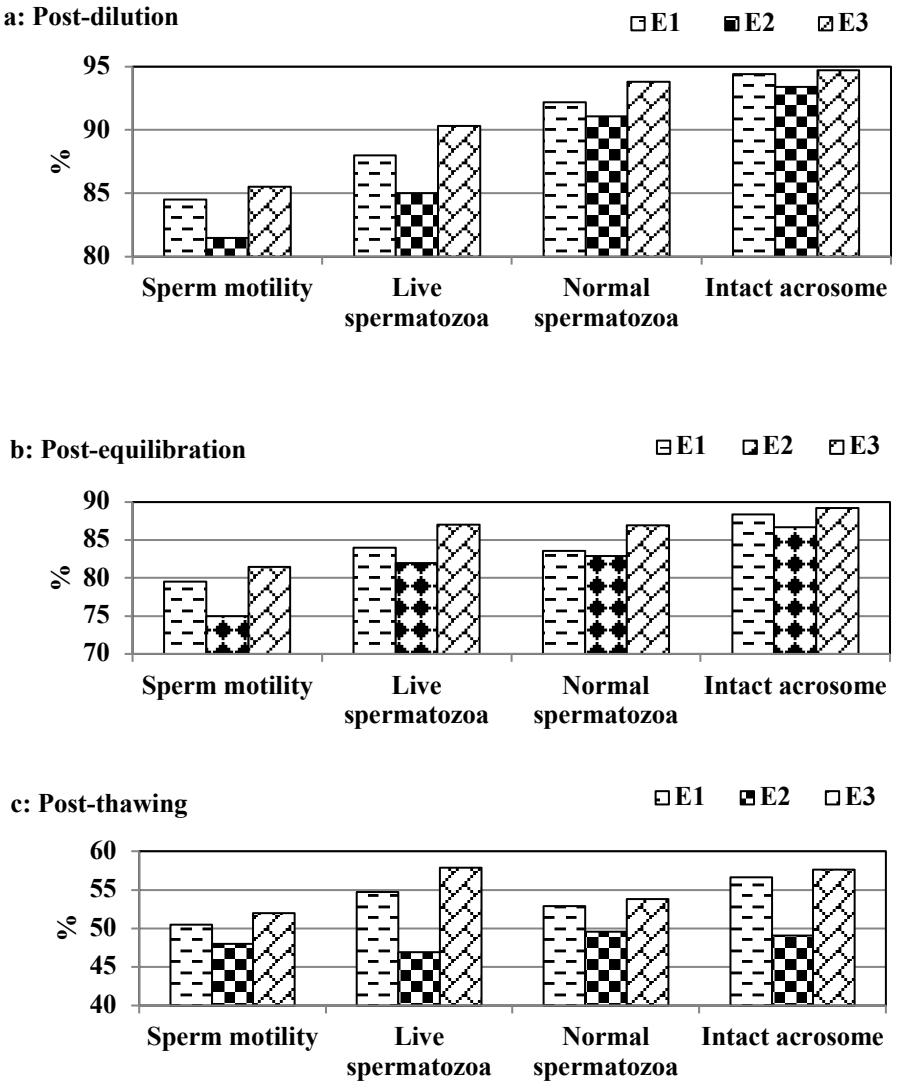

Fig. (1): Semen characteristics during cryopreservation of frozen semen as affected by the different types of extenders.

E1: Tris extender;E2: Sodium citrate extender;E3: Lecithin and propolis extender.

Thence, several authors as **Beura, *et al.* (2014), Gamal *et al.* (2016), Govindasamy *et al.* (2016) Kurmi, *et al.* (2016) and Tekin and Daşkin (2016)** reported respectively that, the supremacy of Tris (similar E1) to protect different animal's spermatozoa such as ram, bull, buffalo, goats and pigs from freezing stock dependent on osmolality and buffer in this extender compared to sodium citrate extender. Supplementation of non-traditional semen extender compositions in E3 as lecithin **(Chelucci *et al.*, 2015)**, propolis extract **(El-Battawy and Brannas, 2015)** and saline solution NaCl 0.9% w/v **(Khalifa and Khalil, 2016)** affirmed that improvement storage ability of spermatozoa without impairment in its capacity function.

Reproductive performance:

There were no significant differences among E1 and E3 in either means of conception rate (%) after 1st and 2nd inseminations or fertility rate after 1st and 2nd inseminations or litter size (Table 4). The results finding that ewes were inseminated with frozen-thawing spermatozoa diluted in E3 increased (P>0.05) reproductive performance above that obtained with frozen-thawing spermatozoa diluted in E1. The lowest of reproductive performance with E1 may be related to use conventional extender components as egg yolk and synthetic antibiotics. Actually, egg yolk is a main component in semen extender compositions for storage and freezing of most mammalian semen. Furthermore, it has been reported that addition of egg yolk reduces the progressive motility (%) and acrosomal integrity (%) and the post-thawing viability of ejaculated spermatozoa in several species, such as rams **(Khalifa and Abdel-Hafez, 2014)** and goats **(Khalifa, 2015)**. Concerning to decrease semen characteristics activity regarding to add synthetic antibiotics to extenders, **Schulze *et al.* (2016)** demonstrated antibiotics of great importance in semen extenders to ensure long life of spermatozoa and to reduce transmission of pathogens into the female tract. The use of synthetic antibiotics carries a risk of developing resistant bacterial strains in artificial insemination laboratories and their spread via artificial insemination.The highest reproductive performance in E3 may be related to use lecithin (SL) plus propolis extract dissolved in saline solution of NaCl 0.9% compared to E1. **Masoudi *et al.* (2016)** concluded that soybean lecithin (SL) in semen extender surpasses than egg yolk (EY) extender without insignificantly on sperm activity. Then, SL extender can be an efficient alternative extender to preserve ram sperm during cryopreservation procedure without adverse effects **(Khalifa, 2015)**. Based on the results, 600µl of propolis glue extract represented in E3 extender could preserve sperm

plasma membrane by improving the cells normal morphology, viability and reduce acrosome damage by preventing free radical and ROS compared with the E1 extender. These mentions agree with those carried out by **Petruska *et al.* (2014)** who reported that propolis has significantly inhibited generation of substances reverse to improve reproductive parameters such as ROS. Also, these authors defined that supplementation of propolis extract increased the sperm content of glutathione (GSH; it plays an important role in the intracellular defense against oxidative stress) after thawing and has a free radical-scavenging activity. Besides direct addition, 200µl of propolis glue extract at incubation up to 3 hours can be provided in sperm protection and improved sperm quality (as total motile spermatozoa, live, and normal) in ram semen extender **(Khalifa *et al.,* 2016)**. Saline solution used as a substitute for distilled water; it could be ameliorated reproductive performance. It is well established that the greatest Na^+ cation concentration in extender associated with high percentages of motile sperm. **Asadpour (2012)** found that Na^+ cation generally prove the osmotic balance and seminal plasma osmolality ultimately plays an important role in the activation of sperm cell for the prediction of ram fertility. The adenosine triphosphate (ATP)-generating capacity of sperm mitochondrial closely correlated with sperm motility and viability. Hence, Na^+ from extender and K^+ (presented in seminal plasma) increased metabolic of ATP in sperm cell tissues then, declined of ATP activity result in less sperm movement in female genital tract **(Zhou *et al.,* 2015)**. Actually, **Zeny, (2016)** showed that Na^+ cation has an antioxidant balance may be associated with improving sperm motility and fertilizing capacity of spermatozoa. At all events, the majority of extenders with physiological solutions NaCl 0.9% w/v I.V. Infusion (sodium Na^+ is the principal cation of the extracellular fluid and plays a large part in electrolyte disturbances and chloride Cl^- has an integral role in buffering action) based extender adequate for survival of spermatozoa and activated fertility in ram **(Khalifa and Khalil, 2016)**. With respect to lambing rate and litter size in E3. **Salem *et al.* (2013)** found that Na^+ in ewe genital mucus tract plays a higher role in vitality of sperm cell and fertility ova than other minerals. In general, **Khalifa and Khalil, (2016)** concluded that ewe inseminated saline solution as unconventional semen extender ingredients could observe single lambing rate at 81.82%, twins lambing rate at 18.18% and litter size at 1.00 compared to 90.91%, 9.09% and 0.86 in Tris as conventional extender, respectively.

Table (4): Reproductive performance of frozen-thawing ram spermatozoa diluted in with E1 or E3 semen extenders**.**

Items	E1	E3
No. of ewes inseminated at 1st service	**14**	**14**
No. of ewes conceived at 1st service	**7**	**8**
Conception rate after 1st service, (%)	**50.00±13.87[a]**	**57.14±13.73[a]**
No. of ewes inseminated at 2nd service	**7**	**6**
No. of ewes conceived at 2nd services	**4**	**4**
Conception rate after 2nd service, (%)	**57.14±20.03 [a]**	**66.67±21.08 [a]**
Total number of ewes lambing at 1st and 2nd services	**11**	**12**
Fertility rate after 1st and 2nd services, (%)	**52.38±11.17 [a]**	**60.00±11.24 [a]**
No. of ewes lambing single	**10**	**10**
Single rate, (%)	**90.91±9.09 [a]**	**83.33±11.24 [a]**
No. of ewes lambing twins	**1**	**2**
Twins rate, (%)	**9.09±9.08 [a]**	**16.67±11.23 [a]**
Litter size	**1.09±0.09 [a]**	**1.67±0.11 [a]**

E1: Traditional extender; E3: non-traditional extender

CONCLUSION

Results of the current study allowed to conclude that unfrozen and frozen-thawing ram semen in lecithin plus propolis extract which dissolved in saline solution (as non-traditional semen extender compositions) under incubation, refrigeration and frozen-thawing conditions could be maintained the highest viability of the sperm cell which resulting in ameliorated reproductive performance compared to other traditional semen extender compositions under similar condition.

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حفظ السائل المنوى للكباش المخفف بالمكونات الغير تقليدية في الحالة السائلة والمجمدة

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الملخص العربى

الغرض من هذه الدراسة إختبار تأثير ثلاثة مخففات للسائل المنوى (مخففين تقليديين واخر غيرتقليدى) على حيوية الحيوانات المنوية للكباش الغير مجمد والمجمد وكذلك الأداء التناسلى .ولتحقيق هذا الغرض أستخدم 6 كباش رحمانى عمر30-31 شهر ووزن 75- 80 كجم لتجميع السائل المنوى مرتين اسبوعيا لمدة 6 أسابيع بإستخدام المهبل الصناعي. خففت قذفات السائل المنوى بالمخففات التقليدية مثل مخفف الترس (E1) وسترات الصوديوم (E2) والغير تقليدية للسيثيسين ومستخلص البروبوليس المذاب فى محلول كلوريد الصوديوم 0,9 % المستخدم للحقن الوريدى (E3). استخدم التحضين على درجة 37مO لمدة 4 ساعات والتخزين على درجة 5مO لمدة 4 أيام وتم قياس حركة الحيوانات المنوية (%)، الحيوانات المنوية الحية (%)، الحيوانات المنوية الطبيعية (%)، الأكروسوم السليم (%)خلال التحضين والتخزين. بينما خلال التجميد تم قياس الصفات السابقة بعد تخفيف السائل المنوى، بعد الأتزان على درجة 5مO استمرت لغاية 150-180 دقيقة، بعد الإسالة على درجة 37مO لمدة 60 دقيقة. تم قياس الأداء التناسلى كمعدل حمل (%)، معدل خصوبة (%)، حجم البطن (%) بإستخدام 28 نعجة قسمت إلى مجموعتين (14 نعجة/ مجموعة) ولقحت بإستخدام المخففات E1 ، E3 على التوالى. أوضحت النتائج أن حفظ السائل المنوى الغير مجمد مع المخففات E1 ، E2 ، E3 كان غير معنوى اثناء التحضين والتخزين. بينما E3 سجل افضل قيم لصفات السائل المنوى يليه E1 مقارنة E2 و كانت أعلى قيم معنوية لصفات السائل المنوى المقاسة خلال الساعات الأولى من التحضين وخلال الأيام الأولى من التخزين لكلا من المخففات E3 ، E1 ، E2 . وصفات التجميد الأساسية كحركة الحيوانات المنوية ، الأكروسوم السليم بعد الإسالة اظهرت فروق عالية المعنوية لمخفف E3 يليه E1 مقارنة E2. والمخفف E3 أظهرارتفاع غير معنوى لمعدل الحمل بعد التلقيحة الأولى (14.57%) والتلقيحة الثانية (67.66 %) ، ومعدل الخصوبة (60.00 %) ، وحجم البطن (1.67) مقارن مع E1 (50.00%) ، (57.14%) ، (52.30 %) ، (1.09) على التوالى. وتوصى التجربة أن السائل المنوى للكباش يمكن ان يحفظ فى المخففات الغير تقليدية دون أى خلل لإعاشة الحيونات المنوية خلال التخزين في الحالة السائلة أو المجمدة .