j.Egypt.net.med.Assoc 78, no 4. 825 – 843 (2018)

EFFECT OF SYNTHETIC AND NATURAL ANTIOXIDANTS ON PRESERVATION AND FERTILITY OF RAM SPERMATOZOA

By

Khalifa, E. I. [1], Mohamed, M. Y.[1], Mahdy, T.M.M.[1] and Khalil, W. A. [2]

[1]Animal Production Research Institute (APRI), Ministry of Agriculture, Dokki, Giza, Egypt.

[2] Animal Production Departments, Faculty of Agriculture, Mansoura University.

Correspondence author: xyezz@yahoo.com.

ABSTRACT

The current experiment was conducted to evaluate the effect of synthetic (vitamin E and glutathione) and natural (rosemary extract) antioxidants on ram sperm characteristics during three sessions (equilibration, frozen-thawing and fertility rate) using three mature Rahmani rams. Semen collection was performed twice weekly for four weeks by artificial vagina. In equilibration and frozen-thawing sessions ejaculates were pooled split into four aliquots. The1stpart(P1) was controlwithout any antioxidant type.However,thevitamin E, glutathione and rosemary extract were added in each concentration 50 mg/ml of each to another three portions P2, P3 and P4, respectively. After that, straws were stored at 5 ℃ as equilibration period and frozen at -196 ℃ by using liquid nitrogen. Sperm characteristics as progressive motility,viability, normality of spermatozoa,integrity of acrosome and concentration of lipid peroxidation (by assay malondialdehyde acid, MDA) were investigated post-equilibration and in frozen-thawing conditions. In addition, conception rate was calculated within frozen-thawing of P1, P2, P3 and P4 using forty ewes as well as in reproductive and productive performance. The results showed that sperm characteristics and sperm recovery rate were higher ($P<0.05$), but concentration of MDA was lower ($P<0.05$) in P2, P3 and P4 than P1 extenders post-equilibration and thawing conditions. Furthermore, there were different values among P1, P2, P3 and P4 extenders in conception rate of ewes; it achieved 37.50, 53.33, 53.33 and 57.14%, respectively. The findings of this study indicated that extenders supplemented with rosemary extract (as natural antioxidant) could be ameliorated sperm characteristics, pregnancy rate and reduced oxidative stress parameters corresponding to vitaminEand glutathione(as synthetic antioxidant)during cryopreservationofram spermatozoa.

Keywords:

Natural and synthetic antioxidants, cryopreservation, ram spermatozoa characteristics, fertility rate.

INTRODUCTION

Various strategies have been used to improve semen conservation process which including the usage of non-penetrated cryoprotectants (as sucrose), dissolving of extender ingredients (as saline solution), altering the cooling materials (as soybean lecithin), antibiotics (as propolis) and the most importantly is supplementing semen dilutes media with various antioxidants. Thus, **Souza *et al.* (2017)** confirmed that the antioxidants are the essential defence factors against oxidative stress produced by free radicals during semen keeping especially lipid peroxidation. The lipid composition in sperm plasma membrane makes this structure sensitive to oxidative damage because of a higher polyunsaturated/saturated fatty acids ratio in ram spermatozoa than other species **(Akalin *et al*., 2015)**. Actually, the addition of antioxidants to semen diluents could be extended storage time, improve motility, reduce the degree of cellular damage, protected acrosomal membrane and increase the viability and fertilization capacity of spermatozoa.Among substances with recognised antioxidants effectiveness were natural or synthetic to be added to extenders. In this context, vitamin E (synthetic non-enzymatic antioxidant) is one of the most important lipid-soluble primary defense antioxidants and has principal role as an antioxidant. Hence, **Soltanpour *et al.* (2014)** reported that supplementation 5mM of vitamin E has significantly improved semen quality parameters during liquid storage time at 5ºC for up to 72 hours. Moreover, **Zeitoun and Al-Damegh (2015)** concluded that 5 IU of vitamin E/ml supplemented to ram semen extender during chilled storage could enhance sperm survival and reduced free radicals formation. In addition, **Mahnaz *et al.* (2016)** indicated that supplementation 2mM of vitamin E as antioxidant is recommended to facilitate the enhancement of ram sperm cryopreservation techniques. On the other hand, **Motemani *et al.* (2017)** demonstrated that concentrations of vitamin E (α-tocopherol) at 4.8 mM can be efficient for preservation of bull spermatozoa in freezing status and conquer reactive oxygen species (ROS) accumulation. **Nikolovski *et al.* (2014)** accentuated that glutathione prevents the damage of important cellular components developed by reactive oxygen species such as free radicals and peroxides. However, **Sarlós *et al.* (2002)** found that the ram ejaculate contains somewhat lower amounts of glutathione

peroxidase, but the concentration of this enzyme markedly decreases when the semen is diluted. Furthermore, **Solouma (2013)** stated that glutathione at levels of 0.4 mM could preserve ram sperm motility. **Zeitoun and Al-Damegh (2015)** reported that the addition of glutathione up to 2 mM observed enhanced progressive motility, live sperm, and abnormal sperm and reduced lipid peroxidation in stored ram semen extender at 5°C for up to 96 hours. Some studies were observed that many herbal medicinal plants have great antioxidant potential. In this regarded, rosemary extract have been tested for development of the natural antioxidant formulations in the areas of medicine and nutrition. **Tabassomi and Alavi-Shoushtari (2013)** demonstrated that spermatozoa are highly sensitive to ROS which induced damage while, strong expression of Cu/Zn has been considered the primary antioxidant defense in cells , ROS at minimum levels has been reported to have a variety of physiological roles in sperm capacitation and antimicrobial defense. Moreover, **Motlagh *et al.* (2014)** revealed that rosemary aqueous extract may acts as an appropriate antioxidant against cryopreservation-mediated decrease in sperm viability and motility after freeze-thawing cycles. **Daghigh-Kia *et al.* (2014)** indicated that improvement of post-thawing motility of frozen bull spermatozoa when either 5 or 10g of rosemary leaves were used. **Gad and Sayd (2015)** recommended that rosemary extract contained the highest concentration of phenolic substances obtained from the leaves. Thus, these contains are a potent antioxidant having inhibition effect of superoxide anion production, as well as lipid peroxidation and free radical scavenging activities **(Oliveira *et al.*, 2017)**.

Much work has been done by the addition of natural and synthetic antioxidants to ram semen extenders, but information on their use in native breed as Rahmani rams' spermatozoa preservation and fecundity are not supported. Therefore, the present study was undertaken to investigate the comparison efficacy between adding synthetic antioxidant (vitamins E and glutathione) and natural antioxidant (rosemary extract) in diluent to evaluate ram sperm quality (pre or post- frozen) and fertility.

MATERIAL AND METHODS

This experimental study was carried out from March to July 2017 at El-Serw Research Station belonging to Animal Production Research Institute (APRI), Agriculture Research Center, Ministry of Agriculture, and Egypt.

Feeding animals and semen collection:

Three mature Rahmani rams 3.0 to 3.6 years of age and an average of 75.0 kg body weight of proven fertility were used in this study. All rams were housed individually in pens on semi-slatted floors fed with a diet according to the recommendations of the National Research Council **(NRC, 2007)**. The based ration offered on 60:40 ratio of concentrate feed mixture to forage (as maize silage), respectively. Rice straws *ad libitum* and fresh water had free access through the experimental periods. The semen samples were collected twice weekly for four weeks by artificial vagina. Sperm samples from each ram were analysed separately to take in consideration the variability of animals. From each male two ejaculates were collected at a period of 10-15 min and then the samples were pooled and designed to experimental work.

Sperm processing

Immediately after collection, semen samples were pooled to eliminate individual differences and plunged into a water bath maintained at 37 ℃ prior to evaluation. The pooled semen samples were investigated for progressive motility, viability, normality of spermatozoa, sperm concentration and integrity acrosome. Semen samples that showed either more than 80% viability and motility or 90% normality of spermatozoa and integrity acrosome or sperm concentration 2.9×10^9 were selected for this experiment. After primary observation, semen samples were split into four aliquots and different types of antioxidants diluted at a 1:4 (semen: diluents). The 1st part (P1) was control (free of antioxidant type). However, 2nd part (P2), 3rd part (P3) and 4th part (P4) were supplemented with 50 mg/ml of each antioxidant types as vitamin E, glutathione and rosemary extract, respectively. In general, ingredients of Tris semen extender and antioxidant levels are defined in (Table 1).

Table (1): Ingredients of Tris semen extenders and antioxidant levels.

Ingredients	Semen extender types			
	P1	P2	P3	P4
Tris (gm)	2.442	2.442	2.442	2.442
Sodium citrate (gm)	0.145	0.145	0.145	0.145
Citric acid(gm)	1.340	1.340	1.340	1.340
Fructose (gm)	0.750	0.750	0.750	0.750
Egg yolk (ml)	15.000	15.000	15.000	15.000
*Vitamin E (mg)	-	50.000	-	-
**Glutathione (mg)	-	-	50.000	-
***Rosemary extract (mg)	-	-	-	50.000
Penicillin (IU)	1000.000	1000.000	1000.000	1000.000
Streptomycin (mg)	200.000	200.00	200.000	200.000
Glycerol (ml)	5.000	5.000	5.000	5.000
Distilled water added up to	100ml	100ml	100ml	100ml

P1: semen diluent without antioxidant; P2: semen diluent with vitamin E; P3: semen diluent with glutathione; **P4: semen diluent with rosemary extract**.

* Vitamin E: solution 50 mg/ml in ampoule 1ml (Sigma. Co.) according to **(João *et al.*, 2018)**.

** Glutathione: 50 mg / ml (from glutathione vital contain 600 mg glutathione powder dissolved in 4 ml of solution) according to **(Wang and Dong 2017)**.

*** Rosemary extract was extracted by 5 g of fresh leaves rosemary was added to 100 ml of boiling distilled water maintained for 10 min. Once the rosemary extract had cooled up to 25°C and then filtered to remove the leaves before use according to **(Daghigh-Kia *et al.*, 2014)**.

Evaluation of microscopic sperm parameters:

Sperm cells concentration

The sperm concentration was evaluated by means of a hemocytometer as $n\times10^9$/ml.

Progressive motility:

The sperm progressive motility was determinate subjectively by preparing a wet mount of diluted semen by placing one drop of semen under cover slip and recorded the proportion of spermatozoa moving progressively straight forward at higher magnification (400×) of the microscope. At least 200 spermatozoa, selected randomly from five microscopic fields, were examined. The mean of five successive estimations was recorded as the final progressive motility.

Livability spermatozoa %:

Sperm viability of the semen samples were assessed by means of the eosin- nigrosin staining. The sperm suspension smear was prepared by mixing a drop of the semen sample with 2 drops of the stain on a warm slide and spreading the stain with a second slide immediately. The viability was determinate by counting 200 cells under the light microscope (400×). Sperm showing partial or complete purple color was considered dead and only sperm showing strict exclusion of the stain were considered alive.

Morphologically normal spermatozoa%:

A thin smear of mixture of semen and eosin-nigrosin solution was drawn across the slide and dried. The percentage of morphologically normal spermatozoa without defects in the head, mid piece and tail were count (100 sperm) and observed under light microscope at magnification (400×).

Integrity acrosome %:

Semen sample (50 µL) was added to a 500 µL formalin citrate solution (96 mL 2.9 % sodium citrate, with 4 mL 37 % formaldehyde) and mixed carefully. A small drop of the mixture was placed on a microscope slide and a total of 200 spermatozoa were counted in five different microscopic fields for each sample using magnification phase contrast microscope (400×). Spermatozoa that showed normal apical ridge in acrosome region were assessed as intact acrosomes.

Biochemical assay as lipid peroxidation (LPO) concentration:

After equilibration (pre-frozen) and thawing (post-frozen) the LPO concentration was measured. The level of LPO was estimated by measuring the level of malondialdehyde acid (MDA) using commercial kit LPO-586 (Oxis Research, Burlingame, CA, US) with sensitivity at 0.5µM and 0.5 to 4.0 µM as range curve.

Experimental design:

Effects of antioxidant addition on semen diluents during cooling and post- equilibration

Diluting sperm with P1, P2, P3 and P4 were kept in the fridge and reached to 5^{o}C the qualitative parameters such as progressive motility, live sperm, normal sperm, integrity acrosome and LPO concentration were checked after 3 hours of equilibration period.

Effects of addition antioxidant on quality of frozen-thawing semen extenders:

Semen samples were filled in 0.50 ml straws and sealed with polyvinyl chloride (PVC) powder and dried. After straws passed equilibration period, straws were frozen horizontally on metal rack presented in foam box, then liquid nitrogen (LN2) vapor just 6 cm above the surface of LN2 for 10 minutes before they were plunged in to LN2. The frozen straws were then transferred to liquid nitrogen container. The straws were thawed at 37ºC for 60 seconds after of LN2 storage and then characteristics of progressive motility, live sperm, normal sperm, integrity acrosome and LPO concentration were examined after 24 hours.

Freezability of spermatozoa and recovery rate:

The freezability of spermatozoa characteristics (recovery rate) determinated by comparing the sperm characteristics pre-freezing (SCPF) and frozen- thawing sperm characteristics (FTSC) use the formula which described by **(Khalifa *et al.*, 2016).**

Recovery rate (%) = FTSC / SCPF ×100.

Fertility assessments of frozen-thawing semen extenders:

During a period of about six weeks, from the first of May to the middle of June 2017, a total of forty (10 ewes /treat antioxidant) Rahmani ewes were inseminated artificially (AI) to compare the conception rate between P1, P2, P3 and P4 extenders. The clinically and healthy ewes as well as in average bodyweight, with apparent signs of heating, selected by a teaser ram were used and inseminated by a speculum method (at insemination time, the speculum lubricated with glycerol then, inserted into ewe vagina to open ewe vagina after that, the opening thawing straw was placed semen dilution on the front of Os-cervix using insemination gun). In general, the oestrus cycle was checked twice a day with a time interval of about 12 hours using a teaser ram. If ewe appears heating, it will inseminate twice then, AI was recommended at 12 and 24 hours after detection of standing oestrus. To detect ewes returning to oestrus, a teaser ram was checked daily all inseminated ewes. The ewes not returning to oestrus (from 12 to 25 days after insemination) were considered pregnant and recorded as the percentage ewe. Consequently, conception rate (%) was determinate by comparing the number of ewes pregnant / number of ewes' inseminated ×100.

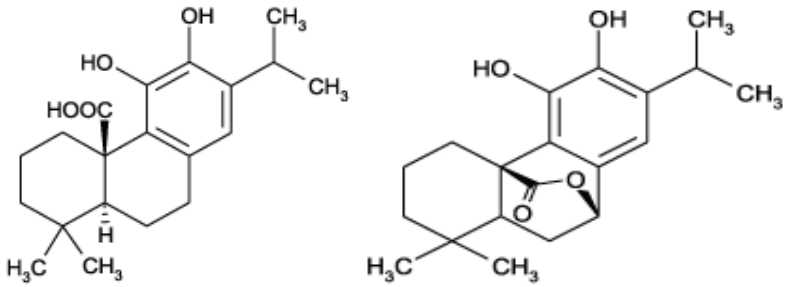
Statistical Analysis:

All values were expressed as mean ± SEM. Statistical evaluation of significant difference between means was performed by one-way analysis of variance (ANOVA) followed by the Duncan post hoc test to determine significant differences in all the parameters among all extender types using the SPSS/PC computer program **(Version 22.0 SPSS, 2013).** The significance level considered was $P<0.05$. Furthermore, results of conception rate were calculated according to the Chi-square test.

RESULTS AND DISCUSSION

Analysis of dry rosemary leaves:

Rosemary extracts are contained several compounds which have been shown to exert antioxidative functions. These compounds belong mainly to the classes of phenolic acids, flavonoids, diterpenoids and triterpenes. The principal antioxidative components of the extracts are the phenolic diterpenes carnosol and carnosic acid Fig. (1). **According to EFSA (2008)** provided that, the analytical profile of dry rosemary leaves as shown in (Table 2).



Carnosol **Carnosic acid**

Fig. (1): Chemical structure of the two major antioxidative compounds in rosemary extracts.

Table (2): Analytical profile of dry rosemary leaves.

Parameter	Unit	level	Parameter	Unit	level
Phenolic diterpenes:			**Anions:**		
Carnosic acid	**%w/w**	**9.7**	**Fluoride**	**mg/kg**	**<47**
Carnosol	**%w/w**	**0.3**	**Chloride**	**mg/kg**	**3000**
Triterpenes:			**Bromide**	**mg/kg**	**<50**
Betulin	**mg/g**	**< 4.76**	**Nitrate**	**mg/kg**	**<50**
Amyrin	**mg/g**	**< 0.5**	**Phosphate**	**mg/kg**	**1809**
Triterpenic acids:			**Sulfate**	**mg/kg**	**3571**
Betulinic acid	**mg/g**	**65.2**	**Cations:**		
Sum oleanic + ursolic acid	**mg/g**	**148.1**	**Cadmium**	**mg/kg**	**<0.23**
Organic acids:			**Chromium**	**mg/kg**	**4.76**
Citric acid	**mg/g**	**<0.5**	**Copper**	**mg/kg**	**22.4**
Malic acid	**mg/g**	**<0.5**	**Nickel**	**mg/kg**	**5.2**
Flavonoids:			**Zinc**	**mg/kg**	**90**
Genkwanin	**mg/kg**	**2.9**	**Volatiles:**		
Tannins:			**1.8-Cineole**	**mg/g**	**56.1**
Expressed as gallotannin	**mg/g**	**177.6**	**Camphor**	**mg/g**	**25.2**
Polyphenols:			**Borneol**	**mg/g**	**10.0**
Expressed as gallic acid	**mg/g**	**262.9**	**Verbenone**	**mg/g**	**2.24**
Protein:			**Bonyl acetate**	**mg/g**	**1.00**
Total nitrogen x 6.25	**%**	**23.3**	**Antioxidant /Volatiles ratio**	**mg/g**	**0.1**
Lipophilic substances:			**Polysaccharides:**		
Hexane-extractable matter	**%**	**43.3**	**Expressed as starch**	**mg/g**	**104.8**

Characteristics of Rahmani ram spermatozoa under fresh condition

Semen physical characteristics of fresh Rahmani ram spermatozoa are represented in (Table 3). Similarly, **Shamia *et al.* (2015)** indicated that, the mean of semen physical quality of Rahmani rams was ranged from 80 to 85 % in motility, 87.5 to 92.0% in live sperm, 1.75 to 2.25% in abnormal sperm, 6.0 to 7.0% in acrosome reaction as dead-reacted and 1.71 to

2.25×10^9 / ml in sperm cells concentration using four. In addition our results were found to similar the study of **(Khalifa *et al.,* 2016)** who reported that, the individual values of the differences in all physical semen characteristics were not significant in Rahmani ram breeds. Additionally, the provided authors defined that values of motility, live sperm, normal sperm, damage acrosomes and concentration of sperm were ranged from 85.00 to 86.25%, 87.5 to 88.00%, 90.00 to 90.25%, 8.50 to 9.25% and 3.95 to 3.98×10^9/ml, respectively.

Table (3): Mean (± SE) characteristics of pooled ejaculates in Rahmani ram.

Items	Semen physical characteristics,%				
	Progressive motility	Live sperm	Normal sperm	integrity acrosome	Concentration $n\times10^9$/ml
Values	89.38±1.48	92.88±0.69	93.00±0.66	95.62±0.32	3.53±0.05

Characteristics of Rahmani ram spermatozoa post- equilibration condition

Table (4) showed that, the highest progressive motility, live sperm, normal sperm and integrity acrosome attained in P2, P3 and P4 diluents compared to P1 diluent after storage at 5°C for up to 3 hours as equilibration period. However, a characteristic as live sperm (91.38%) were higher ($P<0.05$) in P4 diluent than P2 (89.12%) and P3 (87.50%). Otherwise, spermatozoa diluted in P2 and P4 extenders observed higher significant intact acrosome than spermatozoa diluted in P3 extenders. In the present study, we found that exposure of sperm to the vitamin E (P2) resulted in improvement characteristics spermatozoa compared to control (P1). These results are in agreement with **Mahnaz *et al.* (2016)** who obtained that vitamin E levels at 0, 1, 2 and 3 m/M in extender during short-term storage at 5ºC with 2 hours had progressive motility at 68.6, 76.4, 82.2 and 75.6%, livability at 76.0, 84.0, 91.6 and 83.6%, normal sperm 81.6, 89.2, 88.8 and 77.8% and damage acrosome at 4.8, 4.4, 1.6 and 2.8%, respectively. Generally, **João *et al.* (2018)** recorded that vitamin E had reported positive effects of storage sperm parameters. In addition, glutathione (GSH) in diluents is sufficiently effective to maintain the percentage of spermatozoa motility stored at 5 ºC **Grymak and Vovk (2014)** revealed that 5.0 µM and 0.0 µM level of GSH indicated equilibration motility at 84.46 and 73.83%, respectively. The current study was carried out to evaluate the effectiveness of rosemary extract on protecting stored ram spermatozoa **(Motlagh *et al.,* 2014)** demonstrated that rosemary aqueous extract can improve chilling and post-thawed ram spermatozoa characteristics. On the other hand, **Haniyeh and Mohammad (2017)** obtained

that rosemary extract level at 50.0µg/ml improves (P<0.05) sperm quality such as motility (73.69%), livability (71.95%), integrity membrane (73.69%) and LPO (0.93 nM /10×10^6 sperm cells) of rooster spermatozoa during storage at 4 ºC up to 48 hours compared to 58.35, 69.08, 70.60% and 1.15nM at 0.0µg/ml, respectively. Additionally, rosemary extract is poor in copper **Tabassomi and Alavi-Shoushtari (2013)** indicated that copper is the metal co-factor for a variety of enzymes (such as amine oxidase, dependent superoxide dismutase, cytochrome oxidase and tyrosinase) and involved in dismutation of hydroxylation and oxygenation reactions however, excess copper can oxidize proteins, lipids (which bind to nucleic acids), enhance the production of free radicals, reduces the oxidative processes and glucose consumption, which reduces or abolishes sperm motility. The same authors defined that sperm parameters after equilibration time such as motility, viability, integrity membrane and damaged DNA were77.30, 80.60, 81.70 and 3.70% using copper sulphate at 0.016 mg/L compared to 71.40, 78.00, 79.00 and 3.00% using 0.000 mg/L in buffalo bulls , respectively.

Table (4): Characteristics of Rahmani ram spermatozoa post- equilibration condition.

Extender Types	Characteristics of spermatozoa, %			
	Progressive motility	Live Sperm	Normal sperm	Integrity acrosome
P1	81.88±0.92^{b}	84.75±0.49^{c}	83.62±0.65^{b}	91.50±0.19^{c}
P2	84.38±0.63^{ab}	89.12±0.88^{b}	89.38±0.84^{a}	93.88±0.29^{a}
P3	83.12±0.91^{b}	87.50±0.63^{b}	88.50±0.73^{a}	92.50±0.18^{b}
P4	85.62±1.13^{a}	91.38±0.91^{a}	90.12±0.81^{a}	94.25±0.16^{a}

P1: diluent without antioxidant; P2: diluent within vitamin E; P3: diluent within glutathione; P4: diluent within rosemary extract.

Different superscript letters (a: c) within the same column showed significant differences among the diluents (P<0.05).

Characteristics of Rahmani ram spermatozoa post frozen-thawing condition:

Rosemary extract adjusted in P4 extender was provided higher action of the spermatozoa characteristics post-thawing than other extenders contained vitamin E (P2), glutathione (P3) and control (P1). Reduction (P<0.05) percentages of normal sperm and intact acrosome can be observed in P1extender. However, among P2and P4 extenders are achieved non-significant affected on normal sperm and intact acrosome except P3 shows (Table 5). Generally, the

current results reported that vitamin E might be ameliorated sperm characteristics post-thawing. This observation is confirmed by the report of recently study by **Daramola *et al.* (2017)** which showed that supplementation of vitamin E at 6.0 mM in extender under frozen-thawingconditionhas motility,integrityacrosome,integrity membrane and abnormalities up to 61.33, 82.5, 91.5 and 0.83%, respectively. Glutathione addition to the extender could protect sperm against the freezing-thawing damage and has exhibited a high cryoprotective effect on frozen-thawed spermatozoa. These results are in line with **Wang and Dong (2017)** who stated that spermatozoa parameters including motility, acrosomal integrity, sperm-membrane integrity and sperm morphology of frozen-thawed was 43.01, 36.16, 36.94 and 6.29% using glutathione at 8.0 mmol / L compared to 36.47, 32.16, 32.06 and 9.46 in control extender, respectively. Concerning to addition rosemary extract in frozen-thawing sperm quality, **Motlagh *et al.* (2014)** who indicated that supplementation of different levels at 0, 2, 4, 6 and 8% of rosemary extract to ram extender could be achieved total post-thawing up to 33.85, 54.28, 51.86, 42.44 and 43.22%, respectively. Inclusion rosemary extract contains zinc as essential constituents of semen extenders; it has been proved to improve the quality of cryopreserved spermatozoa and could give a better sperm preservation upon freezing processes. Hence, **Binsila *et al.* (2018)** revealed that zinc had higher motility, viability, sperm membrane integrity, acrosome reaction as well as lower abnormal morphology and also involved in the antioxidant capacity. In addition, copper supplementation (located in rosemary extract) of semen extenders might help preserve the sperm quality. Similarly, **Tabassomi and Alavi-Shoushtari (2013)** reported that 0.036 mg / L of copper sulphate showed frozen-thawing motility, viability, membrane integrity and damaged DNA at 51.90, 65.70, 62.90 and 10.10 % compared to 40.50, 60.10, 56.60 and 11.80% in control extender, respectively.

Table (5): Characteristics of Rahmani ram spermatozoa post- thawing condition.

Extender Types	Characteristics of spermatozoa, %			
	Progressive motility	Live sperm	Normal sperm	Integrity acrosome
P1	45.00±1.64^{b}	40.75±1.35^{b}	63.88±0.67^{c}	74.25±0.66^{c}
P2	50.62±1.75^{a}	58.12±0.77^{a}	71.12±1.45^{ab}	79.88±0.83^{ab}
P3	50.00±1.63^{a}	57.50±0.63^{a}	69.75±1.25^{b}	78.75±0.53^{b}
P4	54.38±1.75^{a}	63.88±1.59^{a}	73.62±1.07^{a}	80.88±0.48^{a}

P1: diluent without antioxidant; P2: diluent within vitamin E; P3: diluent within glutathione; P4: diluent within rosemary extract.

Different superscript letters (a : c) within the same column showed significant differences among the diluents (P < 0.05).

Furthermore, the sustained integrities (as frozen-thawing sperm characteristics) in this study could also be attributed to important components (as phenolic) of rosemary that are necessary for reduction free radical to keep sperm cell survival under freezing conditions. Confirmation, **Kashyap *et al.* (2017)** recommended that rosemary extracts have a radical scavenging activity up to 95.1% with approximately 90% of the antioxidant activity attributed to carnosol and carnosic acid. Also, the same author defined that carnosol at level 100 - 400 mg/kg has been shown to enhance activity of glutathione transferase (GT) and also it has significant antioxidant activity with antimutagenic activity similar to ascorbic acid.

Freezability of sperm characteristics (recovery rate)

Recovery rate of sperm characteristics are shown in (Table 6). The highest (P<0.05) sperm characteristics as motility, viability, normal spermatozoa and integrity acrosome recovery rate were observed in P2, P3 and P4 extenders compared with P1 extender.

Table (6): Freezability of sperm characteristics (recovery rate) of Rahmani rams spermatozoa.

Extender Types	Recovery rate %			
	Progressive motility	Live Sperm	Normal sperm	Integrity acrosome
P1	50.52±2.31^{b}	43.87±1.38^{b}	68.68±1.52^{c}	77.90±1.22^{b}
P2	56.74±2.13^{a}	62.62±1.02^{a}	76.48±1.49^{ab}	82.63±1.45^{a}
P3	56.09±2.22^{a}	61.94±1. 22^{a}	75.02±1.39^{b}	82.49±1.51^{a}
P4	60.83±1.73^{a}	68.68±4.63^{a}	79.18±1.11^{a}	84.05±1.46^{a}

P1: diluent without antioxidant; P2: diluent within vitamin E; P3: diluent within glutathione; P4: diluent within rosemary extract.

Different superscript letters (a: c) within the same column showed significant differences among the diluents (P < 0.05).

Biochemical assay as lipid peroxidation (LPO) concentration

The effects of P1, P2, P3 and P4 on oxidative status of LPO as malondialdehyde acid (MDA) post-equilibration and post- frozen thawed Rahmani semen are shown in (Table 7). In general, P2, P3 and P4 extenders included different antioxidant types could be decreased (P<0.05) MDA concentration compared with the P1 as control extender post either equilibration or thawing processes. These results were supported the role of antioxidant in maintaining sperm parameters during cryopreservation **(Chaudhary *et al.*, 2018)**. Thus, the previous observation was clearly with **Daramola *et al.* (2016)** who evidenced that antioxidants are compatible in improving sperm viability parameters by enhanced percentage of motility, integrity acrosome, integrity sperm membrane, acrosome reaction and reduced MDA concentrations of cryopreserved spermatozoa. Moreover, the obtained results showed that MDA level was lower in P4 extender (contains rosemary extract) under equilibration and thawing conditions. This observation is further confirmed by **Motlagh *et al.* (2014)** who stated that rosemary extract at 6% could protect sperm due to its strong antioxidant activities by inhibiting the lipid peroxidation up to 2.74 nmol/ mL compared to 3.61 nmol/ mL in control extender of ram semen during freezing-thawing processes. Indeed, vitamin E (in P2 extender) addition improved efficiency of LPO during cryopreservation condition **(Daramola *et al.*, 2017)** revealed that vitamin E adjusted at 4mM has lower MDA concentration up to 0.22 nmol/ml than 0.38 nmol/ml in control extender. At the same time, glutathione (in P3 extender) showed

a higher resistance of LPO concentration under cryopreservation conditions **(Wang and Dong, 2017)** indicating that glutathione addition at 8 mmol/ L could cause higher ($P<0.05$) inhibiting degree of MDA up to 41.16 than 33.47 nmol/mL in control diluent.

Table (7): Concentration of lipid peroxidation as malondialdehyde acid post-equilibration and post- frozen thawed Rahmani semen.

Extender Types	Concentration of lipid peroxidation as malondialdehyde acid, µM	
	Post-equilibration	Post- frozen thawed
P1	2.88±0.24^{a}	3.75±0.14^{a}
P2	1.25±0.14^{bc}	2.75±.032^{b}
P3	1.88±0.23^{b}	2.00±0.20^{c}
P4	1.00±0.20^{c}	1.88±0.24^{c}

P1: diluent without antioxidant; P2: diluent within vitamin E; P3: diluent within glutathione; P4: diluent within rosemary extract.

Different superscript letters (a : c) within the same column showed significant differences among the diluents ($P < 0.05$).

Fertility assessments of frozen semen extenders

Values for pregnancy rates are presented in (Table 8). There were differences values in total conception rate of frozen-thawing ram spermatozoa between P1, P2, P3 and P4 extenders; it was reached to 37.50, 53.33, 53.33 and 57.14%, respectively. Actually, cryopreserved cells are subjected to stress that leads to membrane changes; these changes during freeze-thawing process obstruct the transbilayer phospholipids asymmetry of mammalian sperm and damage to plasma membrane increases susceptibility to lipid peroxidation due to high production of ROS. These harmful effects eventually lead to impairment of sperm motility and functional membrane integrity. Then, it was reported that low motile sperm (in P1 extender) injection may have a negative effect on fertilization and pregnancy rates **(Câmara *et al.,* 2016)**. Thus, artificial insemination (AI) was performed to determine whether the beneficial effects of test antioxidants (as vitamin E, glutathione and rosemary extract) and also manifested as improved fertility. Our results agreed with those previously published studies **(Daramola *et al.*, 2017 and Wang and Dong, 2017)**, where addition levels of antioxidants in extender were found to be associated with increased sperm quality and decreased formation of MDA which leads to improve pregnancy rate.

Table (8): Conception rate of frozen semen extenders contained different type of antioxidants.

Items	Antioxidant types in extender			
	P1	P2	P3	P4
No. of inseminated ewes (n)	10	10	10	10
No. of pregnant ewes (n) at 1st service at first estrous	4	5	5	6
Conception rate, %	40.00	50.00	50.00	60.00
No. of inseminated ewes (n) at 2nd service at return estrous	6	5	5	4
No. of pregnant ewes (n) at 2nd service	2	3	3	2
Conception rate, %	33.33	60.00	60.00	50.00
Total inseminated ewes (n) at 1st and 2nd services	16	15	15	14
Total pregnant ewes (n) at 1st and 2nd services	6	8	8	8
Total conception rate at 1st and 2nd services, %	37.50	53.33	53.33	57.14

P1: diluent without antioxidant; P2: diluent within vitamin E; P3: diluent within glutathione; P4: diluent within rosemary extract.

CONCLUSION

Our results demonstrated that supplementing the Tris dilution / refrigeration / frozen media with vitamin E, glutathione and rosemary extract observed a positive impact on activity; surviving and fertilizing capacity of ram frozen-thawed sperm than extender media free of antioxidant. In particularly, rosemary-treated within extender lead to an improvement in plasma membrane functionality, decrease of LPO levels and decelerated loss of sperm viability in comparison to the control extender group pre or post- freezing.

REFERENCES

Akalin, P. P., Bülbül, B., Coyan, K., Baspinar, N., Bucak, M. N., Güngör, S. and Öztürk, C. (2015): Relationship of blood and seminal plasma ceruloplasmin, copper, iron and cadmium concentrations with sperm quality in Merino rams. Small Ruminant Research, 133: 135-139.

Binsila, B. K., Selvaraju, S. Somashekar, L., Archana, S. S., Arangasamy, A., Ravindra, J. P. and Bhatta, R. (2018): Molecular advances in semen quality assessment and improving fertility in bulls. Indian Journal of Animal Reproduction, 39 (1):1-10.

Câmara, D. R., Pinto, L. C., Pinto, M. M. C. M., Kastelic, J. P., Nunes, J. F., Barbosa, J. M. P. and Guerra, M. M. P. (2016): Influence of catalase and pre-freezing equilibration on post-thaw semen quality and conception rate in ewes laparoscopically inseminated. Animal Reproductive, 13 (1): 21-27.

Chaudhary, P. J.,Dhami, A.J.,Chaudhari, D. V.and Pathan, M. M.(2018): Leakage of transaminases during cryopreservation of cattle and buffalo semen in egg yolk tris and soya bean milk based extenders. Indian Journal of Animal Reproduction, 39 (2): 32-35.

Daghigh-Kia, H., Olfati-Karaji, R., Hoseinkhani, A. and Ashrafi, I. (2014): Effect of rosemary (*Rosmarinus officinalis*) extracts and glutathione antioxidants on bull semen quality after cryopreservation. Spanish Journal of Agricultural Research, 12 (1): 98-105.

Daramola, J. O., Adekunle, E. O., Oke, O. E., Onagbesan, O. M., Williams, T. J., Iyasere, O. S., James, I. J., Oyewusi, I. K. and Oyewusi, J. A. (2017): Effects of pyridoxine in combination with different antioxidants on viability and oxidative stress parameters of cryopreserved goat buck semen. Archivos de Zootecnia, 66 (253): 15-21.

EFSA, (2008): Scientific opinion of the panel on food additives, flavourings, processing aids and materials in contact with food on a request from the commission on the use of rosemary extracts as a food additive. The European Food Safety Authority Journal, 721:1-29.

Gad, A. S. and Sayd, A. F. (2015): Antioxidant properties of rosemary and its potential uses as natural antioxidant in dairy products. Food and Nutrition Sciences, 6: 179-193.

Grymak, C. and Vovk, S. (2014): The impact of addition of reduced glutathione and bovine serum albumin to the extender on the qualitative indexes of the frozen–thawed ram sperm. Acta Science Polonorum Zootechnica, 13 (3): 47-54.

Haniyeh, R. and Mohammad, R. A. M. (2017): Storage of rooster semen in liquid form using alcoholic extract of rosemary. Iranian Journal of Animal Science, 48 (1): 51-59.

João, D. A. Losano, Daniel, S. R. Angrimani, Andressa, D., Carolina, C. Rocha, Maíra, M. Brito, Eduardo, G. A. Perez, Roberta, H. Tsunoda, Paola, A. A. Góes, Camilla, M. Mendes, Mayra, E. O. A. Assumpção, Valquiria, H. Barnabe and Marcilio, N. (2018): Effect of vitamin E and Polyunsaturated Fatty Acids on Cryopreserved sperm quality in Bos Taurus Bulls under testicular heat stress. Animal Biotechnology, 29 (2):100 -109.

Kashyap, D., Kumar, G., Sharma, A., Sak, K., Tuli, H. S. and Mukherjee, T. K. (2017): Mechanistic insight into carnosol-mediated pharmacological effects: Recent trends and advancements. Life Science, 15 (169): 27-36.

Khalifa, E. I.,Abedel-Hafez, M, A. M. and Ghobashy, H.(2016): Impact of lactate dehydrogenase addition to ram semen stored at 5°C on sperm quality and fertility rate. Egyptian Journal of Sheep and Goat Sciences, 11(3): 163-176.

Mahnaz, A. H., Abdol-Mansour, T., Abbas, A. N. and Yousef, J. A. (2016): Evaluation of vitamin E on microscopic parameters of chilled and frozen stored ram semen. Der Pharma Chemica, 8 (6):16-22.

Motemani, M., Chamani, M., Sharafi, M. and Masoudi, R. (2017): Alpha-tocopherol improves frozen-thawed sperm quality by reducing hydrogen peroxide during cryopreservation of bull semen. Spanish Journal of Agricultural Research, 15 (1): 1-7.

Motlagh, M. K., Mohsen S., Mahdi, Z., Abdollah, M. S., Malak, S., Masoud, S. and Saeed, Z. (2014): Antioxidant effect of rosemary (Rosmarinus officinalis L.) extract in soybean lecithin-based semen extender following freeze-thawing process of ram sperm. Cryobiology, 69 (2014): 217-222.

Nikolovski, M., Ljupco, M., Monika, D., Vladimir, P., Branko, A. and Toni, D. (2014): Influence of glutathione on kinetic parameters of frozen-thawed spermatozoa from Ovchepolian Pramenka rams. Macedonian Veterinary Review, 37 (2): 121-128.

NRC (2007): Nutrient requirements of small ruminants: Sheep, goats, cervids, and new world camelids, National Academies Press, Washington, D.C., U.S.A.

Oliveira, J. R., Daiane, J. And Luciane, D. O. (2017): *Rosmarinus officinalis* l. (Rosemary) extract decreases the biofilms viability of oral health interest. Brazilian Dental Science, 20 (1): 64 - 69.

Sarlós, P.,Molnár,A.,Kókai, M., Gábor, G. Y. and Rátky,J.(2002): Comparative evaluation of the effect of antioxidants in the conservation of ram semen. Acta Veterinaria Hungarica, 50 (2): 235-245.

Shamia, S. M., Saifelnasr, E. O. H., Eetidal, H. El-Sayed, Abdel-Khalek,A.E., Ashmawy, T. M. and El-Gendy, M. E. (2015): In vitro induction of the acrosome reaction in local Egyptian rams spermatozoa by calcium ionophore (A23187). Egyptian Journal Animal Production, 52(2):129-134.

Solouma, G. M. A. (2013): The influence of adding glutathione on semen characteristics of Sohagi rams. Egyptian Journal of Sheep and Goat Sciences, 8 (2): 61- 68.

Soltanpour, F.,Moghaddam, G., Asadpour,R. and Rafat,S.A.(2014): Effect of Antioxidant combinations on sperm quality of cross breed rams during liquid storage. International journal of Advanced Biological and Biomedical Research, 2 (3):732-740.

Souza, H. M., Arruda, L. C. P., Monteiro, M. M., Nery, I. H. A.V., Araújo, S. R. A. J., Batista, A. M. and Guerra, M. M. P. (2017): The effect of Canthaxanthin on the quality of frozen ram spermatozoa. Biopreservation and Biobanking, 15 (3): 220-227.

SPSS, (2013): Statistical package for social sciences, IBM®SPSS Statistics Data Editor 22.0 License Authorization Wizard, Chicago, USA.

Tabassomi,M.andAlavi-Shoushtari, S.M.(2013): Effects of in vitro copper sulphate supplementation on the ejaculated sperm characteristics in water buffaloes (*Bubalus bubalis*). Veterinary Research Forum, 4 (1): 31-36.

Wang, Y. and Dong, S.(2017): Glutathione in combination with trehalose has supplementary beneficial effects on cryopreserved red deer (*cervus elaphus*) sperm. American Journal Reproductive Immunology. 77 (1): 1 - 4.

Zeitoun, M. M. and Al-Damegh, M. A.(2015): Effect of nonenzymatic antioxidants on sperm motility and survival relative to free radicals and antioxidant enzymes of chilled-stored ram semen. Open Journal of Animal Sciences, 5:50-58.