Plant Archives Vol. 20, Supplement 1, 2020 pp. 604-608 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

EFFECT OF ADDING ASTAXANTHIN AND TAURINE AS ANTIOXIDANTS TO IMPROVE CHARACTERISTICS OF AWASSI RAM SEMEN AFTER FREEZING STORAGE

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Abstract

The aim of this study was to investigate the effect of different concentration of astaxanthin and taurine to improve the characteristics of Awassi ram semen after two months of liquid nitrogen freezing. This study was carried out at the animal farm, Department of animal production, Agricultural Engineering Sciences, College, University of Baghdad-Iraq, during the period from February to September 2019. In this experiment used three mature Awassi rams. The pooled semen was divided into nine treatments. Control group (without addition), T1 (1µM astaxanthin), T2 (0.5 µM astaxanthin), T3 (35 mM taurine), T4 (45 mM taurine), T5 (1µM (astaxanthin+35 mM taurine), T6 (1µM astaxanthin+45 mM taurine), T7 (0.5 µM astaxanthin+35 mM taurine) and T8 (0.5 µM astaxanthin+45 mM taurine). The results were showing a significant ($p \leq 0.01$) increasing of sperm individual motility after freezing-thawing processes by added 0.5 and 1 µM astaxanthin, to the diluted semen and by adding a combination of astaxanthin and taurine (T5). Adding of astaxanthin and taurine as a combination to the diluted semen decreased the total sperm abnormalities. However, a significant effect of hypo-osmotic swelling test (HOST) was shown in in T6. The group T1 showed a significant decreasing of plasma Malondialdehyde (MDA). In conclusion, adding astaxanthin and taurine to Tris extender improved semen characteristics of Awassi rams during freezing storage.

Keywords **:** Astaxanthin, taurine, antioxidants, freezing storage

Introduction

Semen cryopreservation technique is an important procedure in the livestock industry, especially the spread of genetic material, limiting sexually diseases, and breed improvement programs (Bailey *et al.*, 2000). The sperm concentration, motility, and morphology of sperm are the most important parameters in semen evaluation (Banaszewska *et al.*, 2015). Morphology is considered as an indicator of fertility (Phetudomsinsuk *et al.*, 2008). Sperm characteristics decreased during storage, thus leading to decrease of sperm survival chance in the female reproductive tract and reduced fertility (Kasimanickam *et al.*, 2007). Commercial extenders consist of nutrient substances to increase the sperm storage time. However, semen processing, such as dilution, semen manipulating under inappropriate conditions, may affect the sperm quality because of oxidative stress (Brouwers *et al.*, 2005). Added antioxidants in diluted semen can improve sperm quality during storage (Zhang *et al.*, 2015). Antioxidants remove oxidative substances to inhibit lipid oxidation of the sperm membrane, thereby, save sperm shape and function (Mortazavi *et al.*, 2014). Carotenoids and amino acids can be used as antioxidants for improving semen quality (Tirpák *et al.*, 2015). Carotenoids such as astaxanthin, canthaxanthin, and lycopene (Gong and Bassi, 2016). Astaxanthin penetrates cell membranes and protects the membrane fatty acids from lipid peroxidation (Dona *et al.*, 2013). The taurine is amino acid can be used as an antioxidant, taurine regulates mitochondrial protein synthesis defending the mitochondria against superoxide generation (Jong *et al.*, 2007). So, this study aimed to show the ability to use astaxanthin and taurine to improving the Awassi ram semen quality at freezing storage.

Materials and Methods

Animals of experiment

This study was carried out at the Animal production Department \ Agricultural Engineering Sciences, College \ Baghdad University, Iraq during the period from February to September 2019. In this experiment we used three mature Awassi rams (2.5-3 years old) and their live weight 55-60 kg.

Semen collection and experiments

Semen was collected from rams using an artificial vagina (Walton, 1945). Before the semen pooled, all ejaculates were evaluated by measuring the colour, ejaculate volume, sperm concentration and mass motility, after dilution the semen was evaluated by measuring the sperm individual motility and total sperm. The semen was diluted to at a rate 1:10 (100 µl fresh pooled semen and 900 µl Tris buffer solution). The extender was prepared according to the method that described by Salamon and Maxwell (2000).The diluted semen was divided into nine equal parts, each part was treated with two concentrations of astaxanthin (0.5 and 1 µM) and (35 and 45 mm) Turin: control group (without addition). T1 (1µM astaxanthin) T2 (0.5 µM astaxanthin) T3 (35 mM taurine) T4 (45 mM taurine) T5 (1µM astaxanthin+35 mM taurine). T6 (1µM astaxanthin+45 mM taurine). T7 (0.5 µM astaxanthin+35 mM taurine). T8 (0.5 µM astaxanthin+45 mM taurine).

Cryopreservation technique

After the semen dilution, 7% glycerol was added. The diluted semen was incubated at 32 ° C in the water bath, French straws 0.25 ml was used to package the semen. The semen equilibrated with glycerol at 5 ° C. For two hours after plunging it in the liquid nitrogen. The straws were placed on the rack at 10 cm above the liquid nitrogen surface for evaporation for 15 min. before plunging it in liquid nitrogen at -196 ° C for two months. (Evans and Maxwell, 1987).

Sperms mass activity

Mass activity was evaluated before the semen pooled by taking 20 µl of fresh semen and placed it on a warmed glass slide, mass motility was shown under the microscope with a magnification force of 100x. (Salisbury *et al.*, 1987).

Sperm concentration

Sperm concentration estimated based on method that described by (Salisbury *et al.*, 1943). The counting solution was prepared by dissolving 2 gram Eosin, 0.01% HgCl, 0.9 % NaCl and 1000 ml distilled water, for estimated the sperm concentration, fresh semen (0.05 ml) was added to 19.95 ml of the counting solution, final dilution was 1:200, one drop of final solution was placed on Neubauer Hemocytometer Counting Chamber, the sperm were counted in 5 squares based on the following equation:

$$\text{Sperms} \setminus \text{ml}^3 = \text{number of sperm} \setminus 80 \times 400 \times 10$$

X = number of sperm counted in 80 small squares; 80 = the number of squares in which the sperm were counted; 400 = the total number of squares of the glass slide; 400 = dilution rate; 10 = stage height of the slide.

Sperm individual motility

Sperm individual motility was estimated based on (Walton, 1933), by placing 30 µl diluted semen on warmed a glass slide and cover slip, the slide examined under the microscope 400x magnification.

Sperm abnormality Percentage

The sperm abnormality percentage had estimated by a modifying method that descried by (Hancock, 1951). Eosin Y (0.67 gram), nigrosine (10 gram) and sodium citrate (2.9 gram) with 100 ml distilled water was used to prepare the staining solution. The sperm abnormality percentage was calculated by counting 200 sperms and using the following mathematical formula:

$$\text{Sperm abnormality \%} = \text{Number of abnormal sperm} \setminus \text{total sperm} \times 100.$$

Hypo-osmotic swelling test (HOST)

The percentage (HOST) was estimated according to (Jeyendran *et al.* 1984) with the minor modifications, the Host solution was prepared by adding 1 ml of distilled water (0 mOsm / L) with 1 ml of normal saline (0.9 % NaCl) (308 mOsm / L), the solution was mixed and incubated at 37 °C. A 20 µl of diluted semen mixed with 180 µl of the hypo-osmotic solution and incubated at 37 °C for 15-20 minute. After that, two drops of eosin solution (2%) were added for the best visualization of sperm. One drop of the final solution was put on a slide and covered by coverslip and then observed under the microscope at a ×400 magnification. The coiling tail and swollen sperm were considered as an intact plasma membrane. A 200 sperms were calculated in different slide fields and the HOST percentage were calculated by using the following equation:

$$\text{HOST} = \text{Number of swollen and coiled tail sperm} / \text{Number of total sperm} \times 100$$

Plasma Malondialdehyde (MDA)

Plasma Malondialdehyde levels were estimated depending on the thiobarbituric acid methods that described by (Rao *et al.,* 1989). The seminal plasma was isolated from the sperm cells by centrifuge 2000 rpm for 10 minutes, the supernatant was used for the determination of plasma MDA.

Statistical analyses

The statistical analyses were performed using a statistical analysis system (SAS). Means with significant differences were compared using Duncan multiple range tests (Duncan, 1955). The statistical model for comparison among groups within each preservation time.

$$Y_{ij} = \mu + T_i + e_{ij}$$

Y_{ij}=dependent variable pertaining to the j observation of the i treatment., μ = overall mean, T_i= effect of the treatment, $e_{ij=}$ error term.

Results and Discussion

Semen analysis

Microscopic examinations used to estimate Awassi rams semen characteristics (table 1). Sperm concentration was 3.48×10^9/ cm^3, Benia *et al.* (2018) suggested the sperm concentration of Ouled-Djellal ram was 4.25×10^9 cm^3. The differences in the semen volume and sperm concentration between Awassi rams in this study and another studies may be due to several effected factors such as breed, season and food (Benia *et al.*, 2018). Mass activity and sperm individual motility were 86.5 and 83.75% respectively, and this in the range which referred by Al-Sarray (2019) 86.41%, and was higher than confirmed by Saieed (2016) which was 78.71%. Sperm abnormality percentage was 4.625 % and this result was closely to that recorded by Saieed (2016) 5.71%.

Table 1 : Semen analysis for experimental Awassi rams.

Parameters	Mean ± SE
Semen color	Milky
ejaculate volume ml \ ram	0.743+0.041
sperm concentration $\times10^9$ cm^3	3.48+0.237
Mass activity %	86.5+0.8
Sperm individual motility %	83.75+0.818
Sperm abnormalities%	4.625+0.46
P value	p≤0.05

The semen evaluation

The results of the sperm motility percentage after freezing – thawing process was shown in (table 2). Groups T8 (37.333%), T1 (37%), T5 (35.833%), and T2 (35.5%) were recorded a significant (P< 0.01) increasing of the sperm motility percentage compared with control (28.833 %). While, no significant difference was recorded between T7, T4, T6, and T3 groups. Freezing technique used to extend the sperm lifespan by reducing sperm metabolic activity (Manafi, 2011). These results were showed a significant increasing of sperm individual motility after thawing by using two concentrations of astaxanthin and Turin. This results were agreed with Farzan *et al.* (2014) that suggest adding 0.5 µM astaxanthin led to increase bull's sperm individual motility after thawing. Astaxanthin has antioxidant properties and it protects cell membrane (Soren *et al.*, 2017). Astaxanthin has the ability to penetrate cell membranes and protect fatty acids from lipid peroxidation (Dona *et al.*, 2013). Reddy *et al.* (2010) suggest that added 50 mm of taurine can lead to improve the sperm motility in buffalo semen during the freezing-thawing process. On the other hand, the ram sperm motility did not improve by 20, 50, and 80 mM taurine addition to the semen after the freezing-thawing process (Uysal *et al.*, 2006). The results of table (2) showed a significant variation (p≤0. 001) of sperm abnormality percentage between all treatments after thawing. The result was showed a significant (p≤0.001) decreasing of the sperm abnormality by using different concentration of astaxanthin

and taurine compared with control. While, group T6 (20.37%) was shown no significant variation compared with control that recorded 22.525%. The sperm abnormality was decreased maybe by adding astaxanthin and taurine to the Tris extender, Reddy *et al.* (2010) referred to the taurine has positive effect to protect buffalo sperm cell membrane. Banday *et al.*, (2017) suggest that added 40 mM taurine reduced oxidation in crossbred ram. Astaxanthin has the ability to penetrate cell membranes and protect fatty acids from oxidation (Goto *et al.*, 2001; Dona *et al.*, 2013) and this lead to protect sperm cell during the storage. Basioura *et al.* (2017) suggest that added 0.5 µM astaxanthin, to the boar diluted semen was shown a positive effect on sperm morphology.

Hypo-osmotic swelling test percentage results were shown in table (2), after thawing, the T6 (43.5%) recorded a significant increasing of HOST percentage ($p \leq 0.05$), on the other hand, no significant effect found between T1 (42.375%), T4 (42 %), T5 (42 %), T8 (41%), T7 (40.38%), T2 (39.625%), and control (38.875%), while, group T3 was recorded lowest percentage of HOST percentage by 38.25%. the variation of HOST percentage between rams caused by variation of season might be related to seasonal changes in melatonin (Ahmad, 2015). The additive of the taurine, glutathione, and trehalose to the ram diluted semen improved sperm motility, live sperm, and host percentage (Bucak and Tekin, 2007). Sarıözkan *et al.* (2009) showed the taurine did not effect of HOST percentage in the bulls diluted semen. Farzan *et al.* (2014) showed that used high concentration of astaxanthin (2 µM) can a damage sperm. These may be responsible for the decline in the HOST percentage.

Table 2 : Effect of adding astaxanthin and taurine to Tris extender on the Awassi ram semen charactestics after two months of freezing.

Treatments	% motility Mean ± SE	% Abnormalities Mean ± SE	% HOST Mean ± SE
Control	28.833± 0.47 B	22.525±0.795 A	38.875± 1.25 AB
T1	37 ± 2.12 A	16.5±0.755 CDE	42.375± 1.44 AB
T2	35.5 ± 1.23 A	15.375±1.252 E	39.625 ± 1.8 AB
T3	32.666 ± 2.04 AB	15±0.801 E	38.25± 0.49 B
T4	33.666 ± 2.84 AB	15.625±0.998 ED	42 ± 1.721 AB
T5	35.833 ± 2.38 A	18.5±1.101 BCD	42±1.388 AB
T6	32.833 ± 0.60 AB	20.375±1.426 AB	43.5 ± 1.679 A
T7	33.833 ± 1.53 AB	17.25±0.818 CDE	40.38 ± 2.282 AB
T8	37.333 ± 1.49 A	18.875±0.666 BC	41 ± 0.707 AB
P value	**	***	*

A, B, C, D=Different letters in the same column indicate significant differences. SE: Stander error; T1=1µM Astaxanthin, T2= 0.5µM Astaxanthin, T3 = 35mM Taurine, T4 = 45 mM taurine, T5 = 1µM Astaxanthin +35 mM Taurine, T6= 1µM Astaxanthin +45 mM Taurine, T7 = 0.5 µM Astaxanthin +35 mM Taurine, T8 = 0.5 µM Astaxanthin +45 mM Taurine. N.S. = non-significant. *= $p \leq 0.05$; **= $p \leq 0.01$; ***= $p \leq 0.001$.

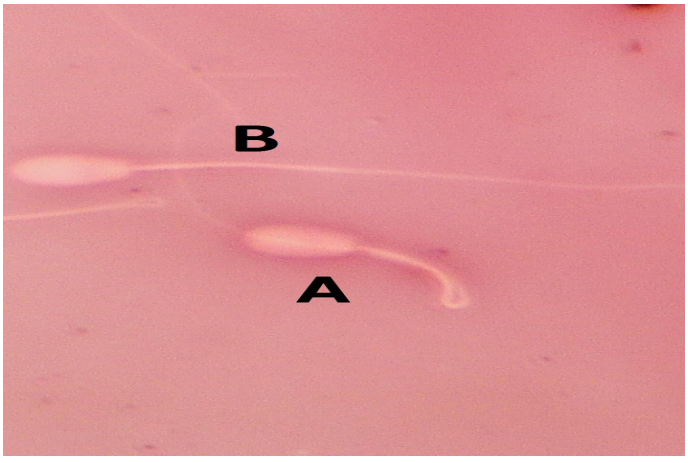

Fig. 1 : Abnormal tail of Awassi ram sperm. A: abnormal tail sperm, B: normal tail sperm.

The results were showing a significant effect to add 1 µM astaxanthin (T1) on the seminal plasma Malondialdehyde (MDA) values after thawing figure (2). The highest values of MDA were recorded in the control group (135.74 µM\ml) and T5 (135.74 µM\ml). The differences between treatments may be due to the role of astaxanthin and taurine as an antioxidant and synergistic between them. These results were agreed with Farzan *et al.* (2014) that showed 1 µM of astaxanthin can lead to preventing reactive oxygen species (ROS) production in bull diluted semen during the freezing-thawing process. Astaxanthin is a natural xanthophyll carotenoid, it is a powerful antioxidant and providing important biological properties (Ambati *et al.*, 2014).

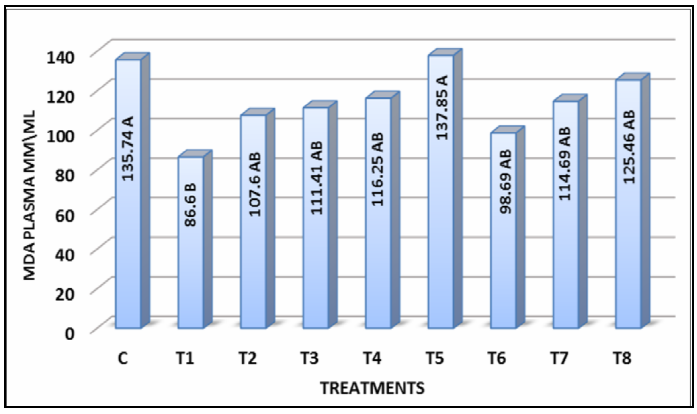

Fig. 2 : Effect of adding astaxanthin and taurine to Tris extender on the plasma malondialdehyde.

In conclusion, the results of the present study indicate the positive influence of astaxanthin and taurine on ram sperm parameters after thawing, the results were referred to the positive effect of astaxanthin and taurine separately and as a combination to improve individual sperm motility, the significant decrease the abnormal sperm morphology and MDA in the diluted ram semen. While, The Host percentage was increased by adding astaxanthin and taurine.

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