Plant Archives Vol. 19 No. 2, 2019 pp. 3971-3976 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

EFFECT OF ADDING LYCOPENE TO THE TRIS EXTENDER ON AWASSI RAMS SEMEN QUALITY PRESEVEDED AT COOLING AND FREEZING STORAGE

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Abstract

Oxidative stress induced by reactive oxygen species (ROS) is associated with an impaired fertilization ability of spermatozoa. This experiment was designed to investigated the effect of adding lycopene (LYC.) to Tris extender and its effect on some Turkish Awassi ram semen characteristics for different preservation intervals (cooling at 5°C for 0, 24, 48, 72 hrs. and cryopreservation at liquid nitrogen (-196°C) for one week). The study was executed at Ruminants Researches Station in Agargof, which belong to the State Board of Agricultural Researches/Ministry of Agriculture, in a cooperation with college of agriculture engineering sciences at University of Baghdad during the period from 13th of January to 19th of April, 2018 using five Awassi rams ages 3-3.5 years, Semen sample were collected by artificial vagina as one ejaculate/ram/week. The study was including preliminary experiments to select three concentrations are studying in experiment. The experiment including four treatments were as follow: L1 (Tris + 0.1% LYC), L2 (Tris + 0.2% LYC), L3 (Tris + 0.3% LYC) and C as control group. The results were showed that L3 treatment was showed a significant improvement ($P \leq 0.01$) in sperm's individual motility and a significant decrease ($P \leq 0.01$) in dead, abnormal sperm percentage, percentage of sperm acrosomal damage compared with control groups and L1 during 72 hours at 5°C and post cryopreservation for one week, on the other hand the result did not showed any significant differences in the pH value of stored Turkish Awassi rams semen at all storage periods. In conclusion, adding 0.3% lycopene to Tris extender have a good role in improving most of ram semen characteristics. These improvements achieved when preserving the semen at 5°C for 72 hrs. and 1 week post cryopreservation in liquid nitrogen which can be developed the fact of artificial insemination in sheep especially in middle east and Iraq.

Introduction

The small ruminants are playing a large important economic role at many world countries (Rajashri, 2015) especially in the Middle East, such as Iraq, Syria, Jordan, Palestine and some surrounded countries such as Turkey because of the peoples in those countries have a big shortage in the consume of red meat and those animals does not have any form of competition with Humans food (Bashawat, 2015) Recently, new biotechnologies have been developed that are on the verge of revolutionizing reproductive processes in humans and animals. In agriculture, modern techniques in assisted reproductive technology (ART) are being used for improvement animals' reproductive efficiency (Salamon and Maxwell, 2000). ART like semen cryopreservation and artificial insemination plays a major role in genetic widespread dissemination of semen from superior manipulation sires. It has a reasonable cost with a big control in preventing the spreader diseases (Salamon and Maxwell, 2000, Munyai, 2012). However, the success of artificial insemination depends on the quality of the semen that used (Rekha *et al*., 2015, Stefanov *et al*., 2015, Barra, 2017). It has been shown that the storage of semen at low temperatures resulted damaging in spermatozoa in away reducing the motility, vitality, chemicals and biological changes in plasma membrane activity, all these changes lead to decline sperm's ability to fertile mature oocytes, although it was found that the best degree of

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the storage is ranged between 4-5°C for 10 days of preservation compared to the frozen of semen (Gundogan *et al*., 2011). It was shown that, the oxidative stress and reactive oxygen species (ROS) have the largest part of the biological damage that occurs during the storage (Khumran *et al*., 2015, Tvrda *et al*., 2017). Recently, there are many materials have ability to scavenge free radicals that formed during semen storage at cooling and frozen stages these materials aim to restore the balance between ROS and sperm antioxidants (Barbas and Mascarenhas, 2009). Some of the antioxidants are natural, such as vitamins (Safa *et al*., 2016) or carotenoids such as lycopene. Lycopene is a plant nutrient with antioxidant properties. It's a pigment gives red and pink color to the fruits, such as tomatoes, watermelons and pink grapefruit (Naviglio *et al*., 2008). Although it belongs to the carotenoid group, but it does not have a vitamin A activity (Choi and Seo, 2013, Seifi *et al*., 2013, Durairajanayagam *et al*., 2014).

In this study, the effect of adding lycopene to Tris based extender on the characteristics of turkish awassi rams semen was studied during chilling and freezing states at different periods.

Material and methods

Animals and semen collection

Semen samples were collected from five Turkish Awassi rams ages 3-3.5 years and 84-96 kg of weight by artificial vagina (41-42°C) during 6 weeks as one ejaculate/ram/week. The rams were housed and fed as a semi extensive system at the Ruminants Researches Station in Agargof, which belong to the State Board of Agricultural Researches/Ministry of Agriculture/Iraq. The study was carried out from the 13th of January to 19th of April 2018. All the rams were in a good health. The semen, after collection, was transported to the laboratory at 37°C immediately or after 5 min from collection in a thermos flask. Ejaculates were pooled to eliminate individual differences and kept at 37°C in water bath and then evaluated performed on fresh pooled semen and equally divided into four groups within the experiment.

Semen analysis

The volume of each ejaculates were recorded. Sperm cells were counted in five squares of a Neubauer Haemocytometer Chamber after 1:200 dilution of semen by Smith and Mayer (1955) procedure and pH was determine by pH meter (Inolab, South African) the percentage of the mass activity and individual motility was determined as the method described by Chemineau *et al*., (1991). Percentages of dead and abnormal sperm was performed by Eosin–Nigrosin staining method described by Swanon and Bearden (1951) modified by AL-Sarray (2012) and at less 200 sperm from different field were examined under a microscope (400x). The percentage of acrosomal Damage was estimated by Gemsa stain (Hancock, 1946).

Semen dilution and Processing

Semen samples were diluted with Tris basted extender contained hydroxyl methyl amino methane (3.63g), glucose (0.50g), citric acid (1.99g), egg yolk (14%), glycerol (6%), penicillin (100000 IU) and streptomycin (100 mg) dissolved in 100 mL of double distilled water (Evans and Maxwell, 1987). Semen was split into four parts and diluted with tris extender contain different amounts of lycopene 0 (control groups), 0.1, 0.2, 0.3% and samples were investigated after dilution and dividing into two groups. The first group was storage at cooling (5°C) for 0, 24, 48 and 72 hours, the remaining group was packaged into 0.25 ml straws (IMV, France) then the Diluted semen was cooled near to +5°C within 4 hours prior exposing to exposed to the liquid nitrogen vapor the semen straws were placed at 4-5 centimeter above liquid nitrogen surface level for 10 min. They were then immerged in liquid nitrogen and stored for one week. The straws were thawed in water bath at 37°C for two min. All procedures were used in dilution, cooling, freezing, thawing of semen were described by Evans and Maxwell (1987).

Statistical analysis

Data were analyzed by SAS (2012) computer program using completely randomized design (C.R.D) to study the effect of treatment for any time of storage according to model :

$Yij = \mu + Ti + eij$

Where, Yij is the value of observation j to treatment i, μ the general mean of the semen characteristic, Ti effect of treatment i, eij: random error. Duncan (1955) test was used to compare the significant differences between means.

Results

Evaluation of fresh semen samples of Turkish Awassi rams

The overall mean of ejaculates as a volume was 1.06±0.04 mL while the pH value was 6.78±0.04. The percentage of sperm mass activity and individual motility were averaged 86.41±0.96, 87.16±1.07% respectively. The overall mean of dead and abnormal sperm was 7.39±0.34, 9.04±0.49%, respectively and the overall mean of sperm concentration/ml was $3.096\pm0.27\times10^9$ sperm/mL of fresh semen.

Table 1. Effect of addition of lycopene to the tris extender on Turkish awassi pH semen post cooling and freezing storage (mean±SEM)

Treatment	Post dilution	Cooling intervals at 5°C (Hrs.)				Post cryopreservation
		0	24	48	72	
C (control)	6.78±0.05 [a]	6.79±0.06 [a]	6.76±0.09 [a]	6.64±0.08 [a]	6.57±0.10 [a]	6.73±0.04 [a]
L1 (0.1%)	6.77±0.08 [a]	6.76±0.09 [a]	6.71±0.13 [a]	6.63±0.09 [a]	6.51±0.09 [a]	6.75±0.01 [a]
L2 (0.2%)	6.71±0.04 [a]	6.76±0.07 [a]	6.73±0.12 [a]	6.63±0.09 [a]	6.52±0.12 [a]	6.73±0.05 [a]
L3 (0.3%)	6.69±0.07 [a]	6.70±0.10 [a]	6.72±0.11 [a]	6.52±0.09 [a]	6.47±0.13 [a]	6.66±0.03 [a]
p-value	N.S.	N.S.	N.S.	N.S.	P≤0.01	P≤0.01

Means in the same period with different superscript letter are significantly different.

Table 2. Effect of addition of lycopene to the tris extender on Turkish awassi rams sperm's cell individual motility post cooling and freezing storage (mean±SEM)

Treatment	Post dilution	Cooling intervals at 5°C (Hrs.)				Post cryopreservation
		0	24	48	72	
C (control)	81.25±3.14 [a]	72.50±2.78 [a]	61.50±3.88 [a]	55.00±3.35 [a]	45.00±3.40 [b]	22.50±2.44 [b]
L1 (0.1%)	80.00±3.53 [a]	75.00±3.40 [a]	67.50±3.22 [a]	58.25±4.80 [a]	50.00±4.56 [ab]	29.25±2.49 [b]
L2 (0.2%)	81.25±3.14 [a]	78.75±3.75 [a]	73.00±3.62 [a]	64.75±4.38 [a]	53.75±4.15 [ab]	29.75±3.25 [b]
L3 (0.3%)	82.50±3.22 [a]	80.50±3.20 [a]	74.00±4.02 [a]	70.50±3.22 [a]	65.50±2.54 [a]	35.50±3.33 [a]
p-value	N.S.	N.S.	N.S.	N.S.	P≤0.01	P≤0.01

Means in the same column with different superscript letter are significantly different.

Table 3. Effect of addition of lycopene to the tris extender on Turkish awassi dead sperm percentage post cooling and freezing storage (mean±SEM)

Treatment	Post dilution	Cooling intervals at 5°C (Hrs.)				Post cryopreservation
		0	24	48	72	
C (control)	10.02±0.97 [a]	14.95±0.81 [a]	20.01±0.65 [a]	24.76±0.81 [a]	31.89±1.00 [a]	41.58±0.37 [a]
L1 (0.1%)	10.87±0.49 [a]	13.80±0.46 [a]	18.37±1.01 [ab]	21.46±1.61 [a]	26.41±1.43 [b]	41.71±0.50 [a]
L2 (0.2%)	10.80±0.41 [a]	14.29±0.88 [a]	17.80±0.43 [ab]	22.28±1.72 [a]	27.72±1.00 [b]	41.32±0.88 [a]
L3 (0.3%)	10.58±0.32 [a]	13.16±0.47 [a]	17.21±0.68 [b]	20.79±1.32 [a]	26.25±0.99 [b]	39.34±0.55 [b]
p-value	N.S.	N.S.	P≤0.05	N.S.	P≤0.01	P≤0.01

Means in the same column with different superscript letter are significantly different.

Effect of addition different concentrations of Lyc. of some Turkish Awassi rams semen characteristics during storage at cooling and frozen state for different periods

The results showed that there were no significant differences in the pH of semen at all storage periods at chilling and freezing states and after dilution (table 1). Also, the results showed no significant difference (P≤0.01) in sperm's cell individual motility after dilute it and storage at 5°C for 0, 24 and 48 hr. However, a significant difference (P≤0.01) was observed at 72 hour between L3 and control group and no significant differences were found between the L1 and L2. The control L1, L2 and L3 as a sperm's individual motility for the four treatments at 72 hour recorded 45.00±3.40, 50.00±4.56, 53.75±4.15 and 65.50±2.54%, respectively. In addition, the results showed a significant improvement (P≤0.01) in sperm's individual motility was recorded by L3 after one week at liquid nitrogen compared with the other treatments and control groups (table 2). On the other hand, the results showed that the significant differences (P≤0.01) appeared at 72 hour interval between the treatments and control groups in dead sperm percentage. The control group recorded 31.89±1.00%, while L3 recorded 26.25±0.99%. L3 showed a significant difference (P≤0.05) compared with the other treatments groups at post cryopreservation for one week (39.34±0.55%). Followed by L2 (41.32±0.88%) while L1 and control groups recorded 41.71±0.50 and 41.58±0.37% respectively (table 3). The results indicated no significant differences between the control group and the other treatment groups during the first evaluated interval (after dilution) in the abnormal sperm percentage. However, the group L3 recorded a significant decrease (P≤0.01) during the periods of 0, 24 and 72 hour at preservation (5°C) compared with other treatment and control groups. In addition, the results showed that a significant superior (P≤0.01) was recorded by L3

Table 4. Effect of addition of lycopene to the tris extender on Turkish awassi abnormal sperm percentage post cooling and freezing storage (mean±SEM)

Treatment	Post dilution	Cooling intervals at 5°C (Hrs.)				Post cryopreservation
		0	24	48	72	
C (control)	10.65±0.46[a]	12.89±0.64[a]	17.79±0.17[a]	21.30±0.34[a]	28.42±1.30[a]	32.90±0.38[a]
L1 (0.1%)	9.97±0.51[a]	12.58±0.81[a]	16.96±0.32[ab]	18.89±0.22[b]	24.51±0.59[b]	30.42±0.25[b]
L2 (0.2%)	10.71±0.49[a]	12.68±0.33[a]	16.58±0.15[b]	18.45±0.46[b]	24.39±0.96[b]	29.76±0.23[b]
L3 (0.3%)	9.12±0.67[a]	10.11±0.77[b]	15.33±0.47[c]	18.20±0.71[b]	21.61±0.76[c]	28.82±0.28[c]
p-value	N.S.	P≤0.01	P≤0.01	P≤0.01	P≤0.01	P≤0.01

Means in the same column with different superscript letter are significantly different.

Table 5. Effect of addition of lycopene to the tris extender on Turkish awassi sperm acrosomal damage percentage post cooling and freezing storage (mean±SEM)

Treatment	Post dilution	Cooling intervals at 5°C (Hrs.)				Post cryopreservation
		0	24	48	72	
C (control)	3.99±0.21[a]	17.19±0.41[a]	22.04±0.62[ab]	27.81±0.33[a]	36.43±0.34[a]	35.80±0.31[a]
L1 (0.1%)	4.22±0.39[a]	15.41±0.46[b]	21.49±0.93[a]	26.29±0.24[b]	30.69±0.17[b]	35.20±0.26[ab]
L2 (0.2%)	4.14±0.25[a]	15.55±0.33[b]	19.08±0.22[ab]	24.44±0.59[c]	30.32±0.15[bc]	34.59±0.41[b]
L3 (0.3%)	3.67±0.08[a]	12.70±0.98[c]	17.08±0.61[bc]	25.23±0.68[c]	29.95±0.08[c]	33.52±0.33[c]
p-value	N.S	P≤0.01	P≤0.01	P≤0.01	P≤0.01	P≤0.01

Means in the same column with different superscript letter are significantly different.

(28.82±0.28%) compared with the other treatments and control groups after 1 week at cryopreservation. While the control group, L1 and L2 recorded 32.90±0.38, 30.42±0.25 and 29.76±0.23%, respectively. There is no any significant difference between L1 and L2 at this interval (table 4). The results of table 5 showed that there is no significant difference between the experimental groups in percentage of sperm acrosomal damage at the first interval (after dilution|), while the high significant differences (P≤0.01) were showed between the control and the other studied groups at 48 and 72 hour of preservation. However, there were no significant differences between L2 and L3 at 48 hour period. In addition, the results showed that there was a significant decrease (P≤0.01) in L3 compared with the other groups at freezing stage for one week. While L1 and the control have not showed any significant differences, L1 and L2 have not recorded any significant differences at the same period as well.

Discussion

It is known that excessive amounts of ROS cause a state of oxidative stress, which resulted in sperm membrane lipid peroxidation, DNA damage and apoptosis, which is leading to decreased sperm viability and motility. (Durairajanayagam *et al*., 2014) Today, there are many materials have ability to scavenge free radicals and ROS that formed during semen storage at cooling and frozen stage. These materials aim to restore the balance between the types of reactive oxygen species and sperm antioxidants (Barbas and Mascarenhas, 2009). In this work, the results showed a significant improvement (P≤0.01) in sperm's individual motility and a significant decrease (P≤0.01) in dead, abnormal sperm percentage, percentage of sperm acrosomal damage that recorded in L3 (0.3% LYC) at 5°C for 72 hour compared with the control group. While, L1 and L2 show no any significant differences in sperm's cell individual motility, dead sperm, abnormal sperm and percentage of sperm acrosomal damage at post cryopreservation for one week. This may be because the usage of 0.1% and 0.2% of lycopene is very ineffective to improve sperm vitality (Hekimoglu *et al*., 2009, Zini *et al*., 2010). In this study, our work shows no any significant differences in pH of semen during preservation and cryopreservation periods. This could be as evidence that the addition of lycopene did not effect on the extenders chemical nature. L3 recorded sperm's cell individual motility less than Uysal and Bucak (2007). The percentage of abnormal sperm was higher than Uysal and Bucak (2007). The difference may be due to the differences in the type of extender, concentration of lycopene and animal breeds. Mangiagalli *et al*., (2012) described the possibility of adding lycopene to the diluted semen of rabbits where Rosato *et al*., (2012) and Kijpooncharoen *et al*., (2017). Were explained the need to add lycopene to the semen extenders of pigs. On the other hand, Tvrda *et al*., (2017) pointed to the improvement of the frozen bulls' semen characteristics and this may be due to the several things including the lycopene as a stronger anti-oxidant. Lycopene will

scavenge the free radicals and prevent the oxidation of cholesterol and lipoproteins (Montesano *et al.*, 2008, Alshatwi *et al*., 2010 and Sarkar *et al*., 2012). Lycopene also has the ability to be dissolved in fat and distribute inside it, leads to protect cells and counteract oxidative factors. On the other hand, Pratik *et al*., (2007) and Malviya (2014) were indicated that lycopene had a functional activity to work as oxidation factors that are twice as beta-carotene. Lycopene has about ten times the effectiveness of tocopherol (vitamin E). Sperm membranes in most animals' species contain high rate of unsaturated fatty acids and cholesterol (Al-Subaihawi *et al*., 2017) that Lycopene playing a protectant factor. It is the first working mechanism of lycopene known as oxidative mechanisms. Lycopene has conjugated double bonds, for that it's rich of electrons which can be donated to free radicals (Barbas and Mascarenhas, 2009) resulting in their neutralization (Pratik *et al*., 2007 and Malviya, 2014). In this way, lycopene acts as an antioxidant to trap free radicals and halt the propagative chain reactions (Mangiagalli *et al*., 2012) Lycopene reduces ROS burden and alleviating oxidative stress, thus preventing oxidative damage to lipids, proteins and DNA (Rao *et al*., 2006). Lycopene suppresses free radicals like H_2O_2, nitrogen dioxide and hydroxyl root (Krishnamoorthy *et al*., 2011). On the other hand, lycopene have another mechanism known as non-oxidative mechanisms by which lycopene could exert its effects include the following: modulating gene expression, regulating the cell cycle and enhancing the sperm immune system (Rao *et al*., 2006, Palozza *et al*., 2012). In conclusion, adding of 0.3% lycopene to tris based extender showed to have a good role in improving most of ram semen characteristics. These improvements can be achieved when the semen preserves at 5°C for 72 hour or 1 week at Cryopreservation in liquid nitrogen can be developed the fact of artificial insemination in sheep especially in Iraq.

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