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Adding Different Concentrations of Pomegranate Peels Alcoholic Extract and its Effect on Characteristics of Awassi Ram Semen Preserved at Cooling

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ABSTRACT
The current study was conducted with an aim to know the effect of adding different concentrations of pomegranate peels alcoholic extract (PPAE) on the traits of Awassi ram semen stored at 5°C. Eight ejaculations from three Awassi rams were collected, mixed and diluted with TRIS extender. The semen samples were divided into four equal parts, and then the alcoholic extract of pomegranate peels was added at concentrations of 0, 100, 200 and 300 mg/ 1 ml of extender, which represented each of the treatments C, T1, T2 and T3, respectively. The samples were stored at 5 ° C and semen examinations were performed during periods 0, 24, 48, 72 and 96 hours after collection. Semen traits included calculation percentage of individual motility, viability, abnormalities, plasma membrane integrity, and sperm acrosome integrity. The results indicated that treatment T3 better than control group C in the percentage of individual sperm motility for all preservation periods. The results also showed that treatment T3 were significantly higher than control group C in the percentage of sperm viability for the 72-hour period, reaching 75.25 ± 3.90 and 54.00 ± 7.42%, respectively. The results showed that there were no significant differences in the percentage of sperm abnormalities for all treatments and for all periods. The results showed an improvement in the percentage of plasma membrane integrity and acrosome integrity for T3 treatment compared with control group C during for periods 24, 72 and 96 hours of preservation. We conclude from the present study that the addition of PPAE at a concentration of 300 mg/ 1 ml of TRIS extender improved most parameters of preserved semen of Awassi rams during different preservation periods.

Keywords: Pomegranate peel extract, semen, ram, preservation, 5°C.

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INTRODUCTION

The artificial insemination process in sheep and goats is one of the important processes to increase the economic return and maintaining the quality and viability of the semen used for this purpose for the longest possible period, whether by cooling or freezing, is the most important step for the success of the artificial insemination process and obtaining the desired results (Mahfouz *et al.*, 2009; Rodello, 2006; Maia, 2006; Azevedo, 2006). Low temperatures, whether cooling or freezing, affect sperm viability (Jones and Mann 1976; White, 1993; Buhr *et al.*, 1994). The oxidation processes lead to damage to the sperm, which occurs as a result of the oxidation of fats in the plasma membrane of the sperm, and this step is considered natural or as natural byproducts of the metabolic processes that occur inside the sperm throughout the preservation period (Griveau and LeLannou, 1997; Baumber *et al.*, 2000). As a result, the processes of oxidative metabolism must be reduced to maintain sperm biomarkers for as long as possible during different conservation periods (Maxwell and Salamon, 1993; Gibb and Aitken, 2016; Yoshida, 2000). Therefore, a number of antioxidants are usually added to semen extenders that reduce and regulate oxidative stress during different preservation periods (Maneesh and Jayalekshmi, 2006; Sikka, 2004). The sperms themselves have a defense system against oxidative agents and are both enzymatic antioxidants such as catalase, glutathione reductase and non-enzymatic agents such as methionine, ascorbic acid and α-tocopherol (Bucak *et al*., 2012; Mara *et al.*, 2005). Adding fruit and vegetable extracts to Semen Extenders is one of the recent additions that have contributed greatly to maintaining the quality of semen because they contain natural antioxidants, and pomegranate is one of the most important fruits used medically in many treatments (Aviram *et al.*, 2000). Pomegranate is very rich in antioxidants that are very important in reducing oxidative processes, and the most important active substances in them are polyphenols (Seeram *et al.*, 2005; Tezcan *et al.*, 2012). Phenolic compounds are among the most important antioxidants in pomegranate fruit to counteract free radicals (Seeram *et al.*, 2008). Where researcher El-Ghazzawy *et al.* (2011) indicated that the use of pomegranate juice greatly contributed to confronting structural changes in the rat epididymis that interfere with testicular function in the production of sperm and thus reduce sperm abnormalities. Al-Daraji (2015) also indicated a remarkable improvement in the fertilization capacity of the preserved sperm and a reduction in the oxidative processes that occur during the period of preservation of the rooster's sperm for a period of up to 36 hours. Therefore, this study aims to investigate the effect of adding different levels of PPAE on the parameters of semen stored at cooling of Awassi rams.

MATERIALS AND EMTHODS

This study was conducted in the animal field belonging to the Department of Animal Production - College of Agricultural Engineering Sciences - University of Baghdad, for the period from March to May 2017.

Semen collection and evaluation

The semen sample was collected by the artificial vagina and kept in a clean, graduated tube, then, semen was mixed and pooled in order to remove the individual differences between the rams. The semen samples were taken directly to the laboratory for the necessary tests. Where the ejaculate volume was calculated immediately after the collection and the mass motility was calculated on the basis of the velocity and density of the moving

waves according to Evans and Maxwell (1990) method. Semen was diluted 1:10 with TRIS extender prepared according to the method of Moce *et al.* (2010), and then the percentage of individual sperm motility was calculated at 40X magnification power according to Walton (1933). The percentage of sperm dead was calculated based on the method of Swanson and Beardon (1951), with an estimate of the percentage of sperm abnormality according to what was reported by Hancock (1951). The Hypo-osmotic swelling test (HOST) was conducted according to the method of Zubair *et al.* (2013). Acrosome integrity test was also performed using Giemsa dye according to Hancock (1946) method. Fresh pomegranate peels were obtained from the local market, then, alcoholic extraction of pomegranate peels and the determination of the active components where done, they were dried, ground, and then alcohol extraction was performed according to Al-Gazali and Al-Shukree (2018). After that, a chemical analysis of pomegranate peel extract was performed to estimate the active compounds in the Food Contamination Laboratory / Environmental and Water Research and Technology Department / Science and Technology Department / Ministry of Higher Education and Scientific Research - Iraq, using high-performance liquid chromatography (HPLC) technology.

Preparing Semen Extender

Tris extender was prepared to preserve ram's semen samples, where it was divided into four groups that included control group C and treatments T1, T2 and T3, which included the addition of 0, 100, 200 and 300 mg of PPAE/ 1 ml of semen extender.

Statistical analysis

The statistical analysis system - SAS (2012) program was used in statistical analysis, based on Completely Randomized Design (CRD), to study the effect of the mentioned treatments on different semen traits, and the significant differences between the averages were compared with the Duncan (1955) polynomial test.

RESULTS

1- Chemical analysis of active compounds in pomegranate peel alcoholic extract

Table (1) indicates the results of the chemical analysis of the alcoholic extract of pomegranate peels using the High-Performance Liquid Chromatography (HPLC) technique, where the components were analyzed according to the retention time for Each of them depends on the molecular weight of the components of the substance that will pass through the column, where the less weighted components pass more quickly than the heavier components or depending on the polarity of the components, where the polar components are faster than the non-polar components. The components, and in light of the holding time for each part of the material that will be compared with the retention time of the standard substance, and by this the name of this or that component is identified, and the device also gives the area of each component of the material as well as the area of the standard material, and that this test is done according to fixed working conditions.

Table 1. Chemical analysis of active compound types and their concentrations in the alcoholic extract of pomegranate peel

Active compounds	Concentration (µg / g)
Ellagic acid	1.48
Tannic acid	39.9
Punicalin	3.4
Catechin	31.1
Gallic acid	362

2- Sperm Individual motility percentage

The results of the current study showed that there were no significant differences in the percentage of individual sperm motility at the time (0) for all treatments. Where, the results indicated that there were significant differences (P <0.05) between treatment T3 and control C treatment during the periods 48, 72 and 96 hours of stored at 5 ° C. It is worth noting that the decrease in the individual movement of the sperm with the progression of days was higher in the control group than in the groups to which the pomegranate peel extract was added.

Table 2. Effect of adding different levels of pomegranate peel alcoholic extract to semen extender of Awassi rams on percentage of sperm individual motility stored at 5°C (mean ± standard error)

Treatments	Time (hours)					Level of significance
	0	24	48	72	96	
C	93.75±1.25 A a	75.00±3.53 B b	61.25±4.26 BC b	47.50±6.29 CD b	32.50±7.50 D b	**
T1	92.50±1.44 A a	82.5±2.50 B ab	73.75±3.75 B a	61.25±3.14 C ab	50.00±3.53 D ab	**
T2	91.25±2.39 A a	81.25±4.26 AB ab	72.50±3.22 BC ab	61.25±6.25 CD ab	48.75±8.51 D ab	**
T3	93.75±1.25 A a	86.25±2.39 AB a	80.00±3.53 BC a	72.50±4.78 CD a	63.75±5.54 D a	**
Level of significance	NS	*	*	*	*	---

Averages that carry different small letters within a single column (between treatments) and capital letters within a single row (between times) differ significantly between them.
* (P <0.05), ** (P <0.01), NS: not significant.

3- Sperm viability percentage

The results of the sperm viability percentage showed no significant differences between the four treatments at storage periods 0, 24, 48 and 96 hours for all treatments. As for the 72-hour period of stored, the results indicated that there were significant differences (P <0.05) between the treatments, where treatment T3 excelled the percentage of live sperms, reaching 75.25 ± 3.90% compared to control group C, which amounted to 54.00 ± 7.42% (Table 3).

Table 3. Effect of adding different levels of pomegranate peel alcoholic extract to semen extender of Awassi rams on percentage of sperm viability stored at 5°C (mean ± standard error)

Treatments	Time (hours)					Level of significance
	0	24	48	72	96	
C	84.75±2.25 A a	73.75±3.40 A a	73.75±4.26 A a	54.00±7.42 B b	40.00±6.16 B a	**
T1	85.00±1.35 A a	77.75±2.49 A a	74.00±3.40 AB a	66.00±1.63 B ab	53.25±6.79 C a	**
T2	84.75±4.32 A a	78.75±4.25 AB a	73.75±3.75 AB a	65.50±7.92 AB ab	56.50±11.70 B a	*
T3	87.00±3.76 A a	82.75±4.06 A a	79.25±4.15 AB a	75.25±3.90 AB a	64.75±7.43 B a	*
Level of significance	NS	NS	NS	*	NS	---

Averages that carry different small letters within a single column (between treatments) and capital letters within a single row (between times) differ significantly between them. * (P <0.05), ** (P <0.01), NS: not significant.

4- Sperm abnormality percentage

The results did not show any significant differences in the percentage of sperm abnormalities between the different treatments as a result of adding different levels of pomegranate peel extract to all periods of preservation, Table (4).

Table 4. Effect of adding different levels of pomegranate peel alcoholic extract to semen extender of Awassi rams on percentage of sperm abnormality stored at 5°C (mean ± standard error)

Treatments	Time (hours)					Level of significance
	0	24	48	72	96	
C	21.25±2.39 B a	27.00±3.24 AB a	30.25±3.63 AB a	33.75±4.09 A a	37.25±4.42 A a	*
T1	19.75±2.01 C a	24.50±3.61 BC a	24.50±3.61 BC a	33.25±3.01 AB a	38.25±3.83 A a	**
T2	18.75±1.49 C a	22.75±2.59 BC a	27.75±2.95 AB a	31.25±2.65 A a	35.00±2.67 A a	**
T3	18.25±3.14 B a	23.25±4.49 AB a	26.50±4.11 AB a	30.75±3.49 A a	34.50±3.66 A a	*
Level of significance	NS	NS	NS	NS	NS	---

Averages that carry different small letters within a single column (between treatments) and capital letters within a single row (between times) differ significantly between them. * (P <0.05), ** (P <0.01), NS: not significant.

5- Plasma membrane integrity percentage

The results did not show any significant differences in the integrity of plasma membrane between the different treatments at time 0 and 48 of preservation at 5 ° C, Table (5). Where, the results indicated a significant superiority (P <0.05) for treatment T3 compared with control group C for periods of 24 and 72 hours of preservation. While treatment T2 significantly (P <0.05) outperformed the two treatments C and T1 during the preservation period of 96 hours Table (5).

Table 5. Effect of adding different levels of pomegranate peel alcoholic extract to semen extender of Awassi rams on percentage of sperm plasma membrane integrity HOST stored at 5°C (mean ± standard error)

Treatments	Time (hours)					Level of significance
	0	24	48	72	96	
C	76.25±2.39 A a	65.00±2.88 A b	62.50±4.83 A a	46.50±7.10 B b	34.75±5.93 B b	**
T1	77.00±0.91 A a	69.25±1.49 A ab	69.25±1.49 A a	57.75±1.60 B ab	46.75±7.56 C b	**
T2	77.50±3.57 A a	72.50±4.85 A ab	68.00±5.35 A a	58.75±8.25 A ab	58.75±10.75 A a	NS
T3	80.50±2.78 A a	77.25±3.42 A a	72.75±4.95 AB a	68.00±5.27 AB a	60.25±8.01 B ab	*
Level of significance	NS	*	NS	*	*	---

Averages that carry different small letters within a single column (between treatments) and capital letters within a single row (between times) differ significantly between them. * (P <0.05), ** (P <0.01), NS: not significant.

6- Sperm acrosome integrity percentage

It has been shown that adding pomegranate peel extract to semen extenders has a significant effect on preserving the acrosome of the sperm and this effect continues during all periods of preserving semen by cooling at a temperature of 5 ° C. The treatment T3 significantly (P <0.05) excelled the treatments T1 and T2 in the percentage of healthy sperm acrosome at 0 time (Table 6). After 24 hours of preservation, the treatment T3 showed a significantly excelled (P <0.05) for the sperm acrosome integrity, where it recorded the highest value which were (93.75 ± 0.25%) compared to the control treatment that amounted to (91.00 ± 0.57%). At the 48-hour preservation period, (P <0.01) treatment T1, which amounted to (92.25 ± 1.18%), recorded the highest level of sperm acrosome integrity for each of the two treatments C and T2, which was recorded (88.25 ± 0.25 and 90.25 ± 0.25%). Whereas the results of treatment T3 showed a significantly excelled (P <0.01) in the percentage of the sperm acrosome integrity on the rest of the treatments for 72 and 96 hours of preservation (Table 6).

Table 6. Effect of adding different levels of pomegranate peel alcoholic extract to semen extender of Awassi rams on percentage of sperm acrosome integrity stored at 5°C (mean ± standard error)

Treatments	Time (hours)					Level of significance
	0	24	48	72	96	
C	94.50±0.86 A ab	91.00±0.57 B b	88.25±0.25 C c	85.50±0.28 D c	83.00±0.40 E c	**
T1	92.25±0.75 A c	92.25±0.75 A ab	92.25±1.18 A a	88.50±0.50 B b	86.50±0.50 B b	**
T2	93.25±0.62 A bc	92.75±0.47 A ab	90.25±0.25 B bc	88.00±0.40 C b	86.25±0.47 D b	**
T3	95.75±0.47 A a	93.75±0.25 B a	92.00±0.70 C ab	90.50±0.50 C a	88.75±0.47 D a	**
Level of significance	*	*	**	**	**	---

Averages that carry different small letters within a single column (between treatments) and capital letters within a single row (between times) differ significantly between them. * (P <0.05), ** (P <0.01), NS: not significant.

DISCUSSION

The process of semen preservation at cooling is one of the most operations that are increasing the production of ROS in semen extenders (Rosato and Iaffaldano, 2011). In addition, natural sources of food often improve the movement and fertility of the sperm by reducing the harmful effects of ROS, because they contain phenolic compounds, vitamins, minerals and other antioxidants that play an important role in removing free radicals (Burdock, 1998).

The alcoholic extract of pomegranate peels was used in the present study as a natural source rich in antioxidants and which contains high concentrations of bioactive compounds such as phenols and tannins (Ismail *et al*, 2010; Reddy *et al*, 2007). The improvement in the percentage of individual sperm motility may be due to the effect of gallic acid, which was found in the alcoholic extract of pomegranate peel in the case study at a concentration of 362 g / g, as it improves the mitochondrial function of the sperm and thus improves its motility. This is in agreement with Gungor *et al* (2019) who indicated that adding different concentrations of Gallic acid and Carnosic acid to the Ram's semen extenders improved the individual motility and mitochondrial functions of the sperm. The improvement in the percentage of sperm viability in the treatment T4 compared with the control group as a result of treatment with pomegranate peel extract is in agreement with Türk *et al* (2008), who concluded that drinking pomegranate juice improves motility and reduces sperm abnormalities in male rats. In addition, the improvement in the percentage of plasma membrane integrity and acrosome integrity in the sperm of Awassi rams may be due to the presence of gallic acid in pomegranate peel extract, and this is in agreement with Gungor *et al* (2019) who indicated that adding Gallic acid to the liquid extender of the rams may be reduce the percentage of acrosome damage and damage to the plasma membrane of the sperm after freezing, and it is worth noting that the integrity of the acrosome is an important factor for completing the fertilization process because it contains the enzymes necessary to penetrate the egg.

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