Tikrit Journal for Agricultural Sciences (2024) 24 (3): 197-209
DOI: https://doi.org/10.25130/tjas.24.3.16

ISSN:1813-1646 (Print); 2664-0597 (Online)
Tikrit Journal for Agricultural Sciences
Journal Homepage: http://www.tjas.org
E-mail: tjas@tu.edu.iq

Effect of adding pomegranate juice to the extender on the cryopreserved semen quality of Awassi rams

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KEY WORDS:
pomegranate juice, semen , Tris extender

Received: 18/03/2024
Revision: 23/06/2024
Proofreading: 20/08/2024
Accepted: 25/08/2024
Available online: 30/09/2024

ABSTRACT

Sixty ejaculates of semen from 10 adult Awassi rams were collected biweekly at a rate of one ejaculate from each ram over a period of 3 months. Semen samples were collected from all rams, pooled and divided equally into four aliquots and diluted to 1: 2 Tris -citric acid-fructose egg yolk (TEYC) extender, the first aliquot is considered as control treatment consisting of extender without any addition , while the second, third and fourth were supplemented with 5 % , 10 % , 15% pomegranate juice PJ to the extender samples, respectively. Semen samples were stored and cryopreserved at 5 °C for (0 -24- 48- 72- 96) hours after collection. Assessment of semen qualities (individual sperm motility, live and dead sperms, abnormal sperms and sperm membrane integrity (HOST) were measured on after dilution and during cryopreservation periods. The results demonstrated that adding PJ into extender showed a significant ($p \leq 0.05$) increase in the percentage of sperm motility, viability, sperm membrane integrity and a significant ($p \leq 0.05$) decrease in sperm abnormalities percentage compared to control at different hours of storage periods, and the advantage of the 4th treatment supplemented with 15% pomegranate juice compared to other treatments. In conclusion, from this study revealed that adding pomegranate juice to the extender is suitable and good for preserving spermatozoa and improving semen characteristics by enhancing antioxidants and protecting the spermatozoa from reactive oxygen species (ROS) during cryopreservation storage.

تأثير اضافة عصير الرمان الى المخفف على نوعية السائل المنوي المبرد للكباش العواسية

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الخلاصة :

تم جمع ستين قذفة من السائل المنوي من 10 كباش عواسية بالغة كل اسبوعين , بمعدل قذفة واحدة من كل كبش وعلى مدى 3 أشهر. تم جمع عينات السائل المنوي المأخوذة من جميع الكباش وتقسيمها بالتساوي إلى أربعة معاملات , ثم إضافتها بنسبة 1 : 2 مخفف الترس ـ حامض الستريك –الفركتوز صفار البيض ، اعتبرت المعاملة الأولى معاملة سيطرة مكونة من المخفف دون أي إضافة ، بينما تم اضافة عصير الرمان بـنسبة 5 ٪ ، 10 ٪ ، 15 ٪ للمعاملات الثانية والثالثة والرابعة على التوالي . تم تخزين عينات السائل المنوي وحفظها بالتبريد عند درجة حرارة 5 C درجة مئوية للفترات صفر ، 24 ، 48 ، 72 ، 96 ساعة من التخزين. تم قياس صفات السائل المنوي (حركة الحيوانات المنوية الفردية ، والحيوانات المنوية الحية والميتة ، والحيوانات المنوية غير الطبيعية وسلامة غشاء الخلية (HOST) خلال فترات الحفظ بالتبريد. أظهرت النتائج ان اضافة عصير الرمان الى المخفف ادى الى حصول زيادة معنوية ($P\leq 0.05$) في النسبة المئوية للحركة الفردية , وحيوية الحيوانات المنوية وسلامة غشاء الحيوانات المنوية مقارنة بمعاملة السيطرة ، كما تميزت المعاملة الرابعة التي اضيف اليها عصير الرمان بنسبة 15٪ مقارنة بباقي المعاملات . نستنتج من هذه الدراسة أن إضافة عصير الرمان إلى المخفف مناسب وجيد للحفاظ على الحيوانات المنوية وتحسين معايير السائل المنوي من خلال تعزيز مضادات الأكسدة وحماية الحيوانات المنوية من نشاط انواع الاوكسجين التفاعلية أثناء الخزن بالتبريد.

مخفف الترس **الكلمات المفتاحية:** عصير الرمان , السائل المنوي ,

INTRODUCTION

The processes of dilution and cryopreservation of semen are linked to cold shock, which leads to an increase in reactive oxygen species (ROS) production and an imbalance between the antioxidant system and free radicals of the diluted semen. It is normal spermatozoa are not adapted to withstand low temperatures and reach near the freezing point of water , so the effect of cryopreservation these cells comes at the expense of normal function and cellular viability. The processes of storing semen, whether by cooling or freezing, reduce the metabolism in sperm cells and maintain the vitality of sperm for long periods of storage, however, there will be a deterioration in the quality of semen, a decrease in the vitality of sperm and damage to sperm during extended storage periods , and one of the reasons for this deterioration is the formation of reactive oxygen species resulting from metabolic processes that occur inside sperm cells and the oxidation of lipids in the sperm membrane throughout the preservation period (Perumal *et al*. 2011a; Perumal *et al*. 2011b) . Therefore, addition of vegetable and fruit extracts to semen extender is one of the modern additives that has made a major contribution to preserve semen quality, due to it contains natural antioxidants , in addition, it is considered one of the natural sources necessary to sustain the life of the organism through its contents of substances that have an impact on maintaining the health of the organism .

Pomegranate fruit one of the fruits thought to be beneficial for maintaining the health of the body (Aviram *et al*. 2000; Aviram *et al*. 2001). This is because it contains natural antioxidants such as polyphenols and flavonoids, which are considered powerful antioxidants to counteract

free radicals resulting from metabolic processes in the body of the organism (Jandal & Naji 2021; seeram *et al*. 2005). In addition to containing glucose sugar, fructose, citric acid and tannins(Gil *et al*. 2000; Longtin, 2003). Pomegranate also contains vitamins A , C , E (Seeram *et al*. 2006). Some recent studies has been conducted using of pomegranate juice in improving semen quality (Turk *et al*. 2008; Mansour *et al*. 2013), especially when it is added to the diluents of the stored semen, whether by refrigeration or freezing, for the purpose of preserving the quality and validity of semen for as long as possible and for the purpose of using it successfully in the artificial insemination technique later and getting intended results from it. Al-Daraji., (2015) pointed out that dietary antioxidants like pomegranate juice can contribute to reducing oxidative stress and the researcher obtained a significant improvement in the fertilization ability of Rooster sperm preserved for 36 hours of cryopreservation. Also Halvorsen *et al*. (2002) have pointed out that pomegranate juice contains high concentrations of antioxidants estimated at 11.33 mmol / 100 g, and the concentration of phenols and flavonoids as active antioxidants is several times greater than the concentration of vitamin E or C. (Cao *et al*.1998).

Thus, the objective of this study was aimed to determine the extent of the impact of adding pomegranate juice to the extender as a strong antioxidant to mitigate detrimental effects of lipid peroxidation that spontaneously happened during liquid storage on the quality of cryopreserved semen of Awassi rams at (5°c) and storage periods (0 – 96 hours).

MATERIAL AND METHODS

Ethical approval

Samples of this study were carried out with the agreement of the ethical and animal welfare committee under the number Um.Vet.2023.112 at 15/3/2023 College of Veterinary Medicine, University of Mosul.

Semen Collection

Semen ejaculates were collected biweekly from 10 adult local Iraqi Awassi rams aged 2.5-3 years by using electrical ejaculator for the duration of the experiment, which lasted 3 months. Semen ejaculates were transferred to the laboratory in glass test tubes, and placed immediately for 10 minutes in water bath at 37 ºC before dilution. Semen samples were admitted and pooled to eliminate individual ram effects and to have sufficient semen which split into 4 equal treatments and diluted with 1: 2 (semen / extender). Tris extender (TCYF) was used consisting of (Tris 3.36 g , Citric acid 1.99 g , Fructose 0.5 g , Penicillin 1000 IU , Streptomycin 1mg / mL , 15 ml egg yolk , distilled water 100 mL). The first treatment is considered as control consisting of semen diluted with TCYF extender without any addition, the second , third and fourth treatments, semen diluted with TCYF extender supplemented with 5%, 10%, 15% pomegranate juice PJ, respectively. To avoid a decrease in pH after adding pomegranate juice to the treatment samples, pH of the 4 treatment samples was adjusted to (6.8 – 7 pH) using buffer solution. S Semen samples were transferred from the water bath to the refrigerator and cryopreserved at 5

ºC temperature for zero (after two hours of storage), 24 , 48, 72, 96 hours of cryopreservation. Part of semen samples were taken during each of the previous storage periods for the measurements of individual motility , live and dead sperm , abnormal sperm and cell membrane integrity, where the percentage of individual motility was measured according to (Evans and Maxwell. 1987) using light microscopy, while viability (live and dead) sperms and defects in the spermatozoa's morphology (abnormal sperm heads, coiled or severed tails and cytoplasmic droplets) were assessed by using a drop of semen smear with warmed Eosin - Nigrosin stain placed in pre-warmed slide (Swanson and Beardon. 1951) and at less 250 spermatozoa in each slide were evaluated and unstained spermatozoa were considered as live by method of (Tabour and Taha. 2019). The functional integrity of the sperm membrane was assessed using the hypo-osmotic swelling test (HOST) after cryopreservation by the method of Zubiar *et al*. (2013).

Preparation of pomegranate juice :

Pomegranates (2 kg) were bought at local markets at ripened stage. It were washed, peeled and then separated the pomegranate grains from the husks manually , then the granules were inserted into an electric fruit blender in order to obtain pomegranate juice and pour the liquefied mixture through a strainer to remove any leftover pulp from the juice. After that, the juice was filtered using a gauze cloth in a glass jar, then the juice was taken and stored in deep freezer under a temperature of – 18 ºc until use (Aviram *et al*.2000)

Statistical analysis

The following model describes the statistical analysis using (SAS, 2003) technique in conjunction with the (CRD) design:

$Yijk = Fi + eijK + \mu$

The Duncan multiple rang test was used to calculate standard errors and differences between groups at $p \leq 0.05$ (Duncan. 1955).

RESULTS AND DISCUSSION

Results in (Tables 1 and 2) showed that adding pomegranate juice PJ to semen extender significantly ($p \leq 0.05$) increased individual motility and viability during different storage periods in all groups treated with PJ, where the highest individual motility and viability were in the T4 extender which supplemented with (15 %) pomegranate juice compare to control, while the difference between T2 and T3 were not significant, in addition, the results demonstrated that cryopreservation periods significantly ($p \leq 0.05$) affected on these two parameters and also the values of interaction between semen treatments and cryopreservation periods were differ significantly, where the T4 showed the highest individual motility and viability of spermatozoa during all the different storage periods.

Table 3 and 4 demonstrated that there are a significant ($p \leq 0.05$) decreases on percentage of dead and abnormal sperms in semen extenders supplemented with 5%, 10% , 15% pomegranate juice compared to control , and T4 gave significantly less percentage of dead sperm and sperm abnormalities compared to other treatments and the results also gave a significant ($p \leq 0.05$) increase in the ratio of dead and abnormal sperms by prolonging the cryopreservation

periods of semen, and also a significant ($p \leq 0.05$) impact of interaction between treatments and cryopreservation periods and T4 showed the lowest percentage of these parameters during all the different storage periods.

Table (1). Impact of adding pomegranate juice to semen extender on the percentage of Individual Motility of spermatozoa stored at 5ºC (means ± S.E.)

Storage / Treat.	0	24	48	72	96	Treat means
T1(0%PJ)	83.50 ±1.83b	72.25±6.08 d	62.00± 6.70e	51.50± 6.43g	43.58± 5.92h	62.57±15.38 C
T2(5%PJ)	84.58±1.88 b	77.58±4.60 c	69.67± 5.16d	61.67± 5.09e	53.08± 4.83g	69.32±12.07 B
T3(10%PJ)	85.83± 2.12b	79.17±4.26 c	72.08±4.89 d	65.00±4.57 e	56.83± 4.24f	71.78±11.05 B
T4(15%PJ)	89.58±1.78 a	83.92±3.34 b	77.42±4.08 c	70.50±4.17 d	62.75±3.39 e	76.83±10.15 A
Storage Means	85.88±2.97 A	78.23±6.18 B	70.29±7.59 C	62.17±8.58 D	54.06±8.37 E	

Means with capital letters indicate differences ($P \leq 0.05$) between treatments or storage periods.
Means with small letters indicate differences ($P \leq 0.05$) between (treatments×storage) interaction.

Table (2). Impact of adding pomegranate juice to semen extender on percentage of Sperm Viability stored at 5ºC (means ± S.E.)

Storage / Treat.	0	24	48	72	96	Treat means
T1(0%PJ)	81.17±3.13 d	69.58±4.46 g	57.17±4.47 j	47.42± 3.65 m	38.00±5.03 n	58.67±16.02 C
T2(5%PJ)	84.25±2.22 bc	74.08±2.61 f	63.33±2.46 i	54.08±1.98 k	44.92±4.60 m	64.13±14.36 B
T3(10%PJ)	86.25±2.05 ab	78.42±2.11 e	68.75±1.66 gh	59.92±3.94 j	51.08±3.92 l	68.88±12.98 B
T4(15%PJ)	88.33±3.31 a	82.17±2.21 cd	74.92±2.15 f	66.25±3.17 h	57.58±2.71 j	73.85±11.38 A
Storage Means	85.00±3.76 A	76.06±5.58 B	66.04±7.20 C	56.92±7.72 D	47.90±8.37 E	

Means with capital letters indicate differences ($P \leq 0.05$) between treatments or storage groups.
Means with small letters indicate differences ($P \leq 0.05$) between (treatments×storage) interaction groups

Table (3). Impact of adding pomegranate juice to semen extender on Dead Sperm Percentage stored at 5ºC (means ± S.E.)

Storage Treat.	0	24	48	72	96	Treat means
T1(0%PJ)	18.83±2.62 j	30.33±4.42 g	42.92±4.6 e	52.75±3.57 b	62.67±4.36 a	41.50±16.8 A
T2(5%PJ)	15.83±2.17 kl	25.92±2.61 h	38.17±4.00 f	45.83±2.08 d	55.17±4.45 b	36.18±14.3 B
T3(10%PJ)	13.83±1.99 lm	21.58±2.11 i	31.08±1.98 g	40.17±3.83 ef	48.83±4.06 c	31.10±12.6 C
T4(15%PJ)	11.67±3.31 m	17.50±1.98 jk	24.25±3.08 hi	32.75±4.71 g	41.58±2.71 e	25.55±11.21 D
Storage Means	15.04±3.65 E	23.83±5.62 D	34.1±7.92 C	42.88±8.23 B	52.06±8.75 A	

Means with capital letters indicate differences (P ≤ 0.05) between treatments or storage groups.
Means with small letters indicate differences (P ≤ 0.05) between (treatments×storage) interaction groups.

Table (4). Impact of adding pomegranate juice to semen extender on percentage of sperm abnormalities stored at 5ºC (means ± S.E.)

Storage Treat.	0	24	48	72	96	Treat means
T1(0%PJ)	5.25±0.97 j	8.08±1.38 h	11.67±1.15 de	14.50±1.45 b	17.83±1.99 a	11.47±4.71 A
T2(5%PJ)	4.83±0.72 jk	6.67±0.78 i	9.92±0.79 fg	12.17±1.27 cd	14.83±1.90 b	9.68±3.82 B
T3(10%PJ)	4.00±0.74 kl	5.83±0.72 ij	7.75±0.75 h	10.58±1.51 f	12.75±1.86 c	8.18±3.39 C
T4(15%PJ)	3.33±0.49 l	4.92±0.67 jk	6.75±0.97 i	9.25±0.97 g	10.83±0.94 ef	7.02±2.88 C
Storage Means	4.35±1.04 E	6.38±1.48 D	9.02±2.13 C	11.63±2.35 B	14.06±3.11 A	

Means with capital letters indicate differences (P ≤ 0.05) between treatments or storage groups.
Means with small letters indicate differences (P ≤ 0.05) between (treatments×storage) interaction groups.

Table 5. indicated that adding of PJ into extender resulted a significant ($p \leq 0.05$) increase in percentage of sperm membrane integrity (HOST) than control , and T4 showed highest percentage of sperm membrane integrity compared to T3, T2 and T1, and the second and third treatments also differed significantly from control treatment . Cryopreservation periods and the interaction between treatments and storage periods also affected significantly ($p \leq 0.05$) on integrity of the sperm cell membrane, where the T4 showed the highest percentage of sperm membrane integrity (HOST) during all the different storage periods.

Table (5). Impact of adding pomegranate juice to semen extender on the integrity percentage of sperm membrane (HOST) stored at 5ºC (means ± S.E.)

Storage Treat.	0	24	48	72	96	Treat means
T1(0%PJ)	82.42±0.9 c	73.67±1.78 f	65.42±1.62 h	54.92±2.97 k	45.67±2.31 l	64.42±13.31 C
T2(5%PJ)	84.17±1.19 bc	75.92±1.51 e	68.58±2.31 g	60.75±3.65 i	53.00±4.22 k	68.48±11.39 B
T3(10%PJ)	85.5±1.45 b	79.83±1.59 d	72.92±3.26 f	64.42±3.32 h	58.25±3.39 j	72.18±10.34 B
T4(15%PJ)	88.92±2.02 a	83.67±2.06 bc	77.08±2.50 e	69.25±3.25 g	64.17±2.66 h	76.62±9.46 A
Storage Means	85.25±2.79 A	78.27±4.21 B	71.00±5.07 C	62.33±6.18 D	55.27±7.56 E	

Means with capital letters indicate differences ($P \leq 0.05$) between treatments or storage groups.
Means with small letters indicate differences ($P \leq 0.05$) between (treatments×storage) interaction groups.

It is well known that one of the most common process that contributes to increase the reactive oxygen species generation (ROS) is the cryogenic or freezing storage of semen. The advance in the generation of (ROS) lead to decreases in sperms motility , viability and sperms capacity to fertilize. (Rosato and Iaffaldano, 2011) and lead to change in the composition and function of the sperm membrane in the concomitant change in the antioxidant defense systems (Ball *et al*., 2001), including a decrease in glutathione concentration as an antioxidant in intracellular sperm cell during storage (Gadea *et al*. 2004), and significant increase in lipid peroxidation (Al-Daraji., 2015). In addition to, prolonging the time of storage period of spermatozoa, whether by cryopreservation or freezing method, leads to less in nutrients substances in seminal plasma will resulted a decrease in the viability of spermatozoa. So, Addition of substances containing antioxidants to semen extender leads to a significant benefit and preservation of motility, viability and spermatozoa's potential to fertilize later (Gadea *et al*. 2007). Natural sources of nutrients supplementing semen extender increase spermatozoa motility and viability by reducing the detrimental effects of ROS, because they include antioxidant compounds like phenolic substance, vitamins that are essential for eliminating free radicals (Burdock., 1998). Pomegranate juice was utilized in this study as a naturally occurring source of antioxidant and high quantities of bioactive substances such tannins, polyphenol flavonoids, and polyphenols. The use of pomegranate juice in semen extenders is very suitable for preserving sperm, because pomegranates are superior to other fruits like pears and apples in terms of their high quantities of antioxidants and vitamin C (Rosalinda *et al*. 2021). The results of current study in (Tables 1, 2, 3, 4 5) demonstrated that the addition of pomegranate juice to the semen extender resulted a significant increase in the percentages of individual motility, viability and sperm membrane integrity, especially in the T4 with 15% PJ compare to control treatment (zero pomegranate juice) and reduced in the percentage of dead and abnormal sperms during different cryopreservation periods at (5°c) . These results agreed with Lukman *et al*. (2022) who obtained the highest progressive motility, sperm viability, and lowest percentage of abnormal sperms

when 10% pomegranate juice was added to the semen extender of male goats kept for a period of (0-2-4-6-8) hours at room temperature compared to control. Also our results agreed with Moazam *et al.* (2019) in obtaining the best individual motility in the extenders of male buffalo semen supplemented with (2.2 , 5 , 7.5 , 10%) pomegranate juice compared to control, and indicated that the addition of 10% PJ to extender resulted in the highest percentage of individual motility. Moreover the results also in agreement with Perumal and Rajkhowa, (2015), who recorded a positive significant ($p \leq 0.05$) effect of adding (6 , 8, 10 ml/ 100 ml extender) PJ to the extender on the percentage of sperm motility, viability and sperm membrane integrity and a lowest percentage of sperm abnormalities of bulls semen compared to control during cryogenic storage periods (0- 6 - 12- 24 -30) hours at 5°c, and the extender with addition of 8 ml pomegranate juice gave the best results compared to others. But, our finding did not agree with the results of Zina *et al*. (2021) who did not obtain a significant increase in individual motility of spermatozoa in semen extender supplemented with 0%, 5%, 10% and 15% PJ during preservation period (0 and 48)hours of cryogenic storage compared to control group. But the researcher found that inclusion 5 % PJ to Tris extender resulted a significant increase in the percentage of plasma membranes integrity of Awassi rams reached 64.5 % in the extender supplemented with 5% pomegranate juice compared to 60 % in the control group during 48 hours of cryopreservation. While El-Sheshtawy *et al*. (2016) stated that adding 10% and 20% PJ to the semen extender of bulls maintained the best individual motility and sperm viability in semen extender after freeze- thawing storage for a period of 1-10 days compared to the control, but the researcher pointed out that increasing the percentage of PJ to 40% –50% in extender failed to maintain the best percentage of sperm motility after freezing and thawing. However, no significant variations were observed between groups in the rate of abnormal sperms and plasma membrane integrity after freeze – thawing periods. Jabr *et al*.(2022) Also, obtained highest increases in the percentage of progressive motility, sperm viability and sperm membrane integrity in the extender supplemented with 10% pomegranate juice, after 4 hours of cryopreservation compared to extenders supplemented with (0, 2.5, 5, 7.5 %) PJ. In addition to , our finding agreed with Al-Daraji., (2015), who pointed out that the addition of (2 ml , 4 ml/100 ml) PJ to semen extender of Roosters led to a significant increase in percentage of sperm viability, sperm membrane integrity and a significant decrease in the percentage of dead and sperm abnormalities during (12 , 24 , 36 hours) periods of cryopreservation and the researcher attributed that these favorable results are due to the PJ contains high concentration of antioxidant substances. While our results did not agree with Gaurav *et al*.(2019) who obtained a deterioration in sperm viability and any improvement in sperm membrane integrity after frozen and thawed on semen extender of bulls supplemented with (1.5%) PJ compared to control.

Results about the impact of cryogenic storage periods on semen samples showed a significant decrease in the percentage of motility and viability of spermatozoa with different cryogenic periods, and the inclusion of PJ in semen extender, especially in T4 which supplemented with 15% PJ resulted a significant increase in sperm motility and viability, which reached 89.58±1.78% ,88.33±3.31% obtained at zero hour to 62.75±3.39% ,57.58±2.71% at 96 hour of cooling periods at 5°c respectively , and this value is considered acceptable and suitable for the

possibility of success of the artificial insemination (AI) process according to the result of Kusumawati.,(2016) how stated that at least 50% of sperm viability and motile spermatozoa can be used for AI . The results of our study agree with Abdulnasir., (2012) who obtained a significant effect of preservation periods (0 – 96) hours on Awassi Rams semen diluted with Tris extender. Al-daraji.,(2015) also found a significant effect of cryopreservation periods (0 – 36) hours on Rooster semen diluted with extender supplemented with PJ.

So, these improvements in extended semen parameters may be due to the fact that the combination of PJ is considered as a source of high concentration of antioxidants, especially containing a high amount of polyphenols (Haloho., 2015), includes tannins and flavonoids (Nge *et al*. 2015), and flavonoids content in pomegranate have antioxidant properties that protect sperm cell membranes from the damaging effects of free radicals, and able to sustain the quality of spermatozoa (Lukman et al. 2022). Moreover, consuming of PJ demonstrated strong antioxidant benefits and the active ingredients responsible for PJ's protection against oxidative stress and lipid oxidation were found to be polyphenols (Aviram *et al*. 2001), in addition to PJ contains high concentration of vitamins A, C and E, which plays an active antioxidant compounds in maintaining the sperm cell membrane and protecting it from harmful effects of free radicals by scavenging free radicals (Longtin., 2003 ; Seeram *et al*. 2006; El-Sheshtawy *et al*. 2016), and enhanced sperm resistant to lipid peroxidation (Al-Daraji., 2015), and preserving sperm cells from damage (Ganwar *et al*. 2015, Suleiman *et al*. 1996).

CONCLUSIONS

In conclusion, supplementing semen extender with 5% , 10% and 15% PJ, especially 15% PJ significantly increase in sperm motility, viability, sperm membrane integrity and a significant decrease in dead and abnormal sperms during cryopreservation periods. As pomegranate juice contains high concentrations of antioxidants, which had appositive effect on oxidative stress damage and considered necessary for spermatozoa by increased their function and ability to protect spermatozoa from free radicals, and lipid peroxidation which naturally occurred during storage. So, our finding confirm the protective effects of PJ against ROS and lipid peroxidation during liquid storage periods of ram semen and increased semen quality and subsequent fertilization following AI. Therefore, pomegranate juice is suitable as a good diluent medium and is excellent for preserving spermatozoa during cryopreservation.

CONFLICT OF INTEREST:

The authors reported no conflict of interest.

ACKNOWLEDGMENTS:

The authors are greatly indebted to the chancellorship of University of Mosul, dean of the College of Agriculture and forestry, also thanks to the head of animal production department and those working in animal production field for providing all the facilitations to conduct this work.

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