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Effect of Addition of Pomegranate Juice (*Punica Granatum*) in Tris-based Extender on Physical, Kinetic Parameters of Spermatozoa in Cryopreserved Ossimi Ram Semen

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ABSTRACT

Pomegranate juice (PJ) contains potent antioxidant activity against lipid peroxidation. The current study's objective was to ascertain the effects of various pomegranate juice concentrations (2.5, 5, 7.5 and 10%) in a tris-based extender on physical and kinetic spermatozoa post-dilution, equilibration time, and freezing of Ossimi ram semen. Semen samples were taken once each week for seven weeks from five rams. Semen was diluted at a rate of 1:20, equilibrated for 4 hours at 5 °C, and then was loaded into 0.25 mL straws before being frozen in liquid nitrogen. By using spectroscopic assays, it was possible to measure the physical, kinetic, enzyme, and antioxidant capacities of spermatozoa. After thawing, the 10% pomegranate juice addition to the extender significantly (p 0.05) improved the morphology of the semen (CASA, total motility, progressive motility, VAP, VSL, VCL, STR and LIN). It is concluded that the addition of 10% pomegranate juice to tris-based extender enhanced post-thawing semen characteristics and CASA motion dynamics of cryopreserved Ossimi ram semen while also increasing overall antioxidant capacity and reducing MDA concentration.

1. INTRODUCTION

Artificial insemination (AI) is a reproductive biotechnology used in farm animals to improve genetic potential, lower the danger of sexually transmitted diseases, and control calving intervals to minimize the milk supply-demand gap **(Eaglesome and Garcia, 1997; Ax *et al.*, 2000)**. However, the sperm cryopreservation method is what ultimately determines if AI using frozen semen will be successful **(Wang *et al.*, 2015)**.

Reactive oxygen species (ROS) production, intracellular ice crystal formation, and cold shock are all mainly caused by semen cryopreservation **(Holt *et al.*, 1992; Holt and North 1994)**, Additionally, the balance between antioxidant and pro-oxidant activities is lost, leading to an increase in ROS, oxidation of polyunsaturated fatty acids in sperm membranes to form **(Del Olmo *et al.*, 2015)**. All these harmful alterations promote specific functional and physical harms, which ultimately cause the spermatozoa to lose their viability, motility, and capacity to fertilize **(Wang *et al.*, 1997; Bailey *et al.*, 2000; Salamon and Maxwell, 2000; Watson 2000; Medeiros *et al.*, 2002; Tekin 2006; Bernardini *et al.*, 2011)**. Antioxidant supplementation is a sensible course of action to prevent the negative effects of cryopreservation and ultimately increase the quality of semen **(Ansari, *et al.*, 2012)**.

Pomegranate (*Punica granatum* L.) belongs to Punicaceae family, is a nutrient-dense food source rich in phytochemical compounds, including tannins and other phenolic compounds, flavonoids-anthocyanins, and other complex flavanoids and hydrolyzable tannins (punicalagin, gallic, and ellagic acid) **(Gil *et al.*, 2000; Seeram *et al.*, 2006; Miguel *et al.*, 2010; Elfalleh *et al.*, 2011)**, as well as vitamins A, C and E, which have high antioxidant activity and may provide Hydrolysable tannins account for around 92% of the antioxidant activity in pomegranates **(Passamonti *et al.*, 2003)**.

Pomegranate juice (PJ) was found to have strong antioxidant action against lipid per oxidation **(Malik *et al.*, 2005)**. Various levels from PJ were used to maintain semen quality when used in semen extenders as (2%, 4%) into rooster semen extender **(Al-Daraji, 2015)**, (2.5%, 5%, 7.5% and 10%) into Nili Ravi buffalo semen extender **(Javed *et al.*, 2019)** and (10%, 20%, 30%, 40% and 50%) into cattle semen extender **(El-Sheshtawy *et al.*, 2016)**.

Objective of this study was to study the effect of adding different levels of the pomegranate juice (PJ) 2.5, 5, 7.5 and 10% to semen extender on semen quality of Ossimi ram.

2. MATERIALS AND METHODS

From September 2021 to March 2022, the current study was conducted in collaboration between the Animal production department, Faculty of agriculture, Tanta university, Egypt, and the Animal production research institute (APRI), Agricultural research center (ARC), Ministry of agriculture, Egypt at the animal production research station, Sakha, Kafrelsheikh Governorate, located in the northern part of the Nile Delta (latitude 31°15'N and longitude 31°45'E).

2.1 Pomegranate juice preparation

Pomegranates from market, cleaned, peeled and the red grains were gathered in a spick and span plate. Gauze was used to press the grains, producing a clear, watery liquid. The juice was purified and kept at -18 °C until used **(Aviram, *et al.*, 2000)**.

2.2 Semen extender preparation

The control extender contained 3.025g Tris, 1.66g citric acid monohydrate, 1.25g glucose, 1% soybean lecithin, 5% glycerol, 100 IU/mL penicillin, and 100µg/mL streptomycin.

Semen was further divided into 5 aliquots including free-extender (C), and extenders supplemented with PJ at levels of 2.5, 5, 7.5 and 10% (T1, T2, T3 and T4, respectively). After the supplementation of extracts, the extender was gently shaken and warmed in a water bath to 37 °C. The osmolarity and pH

were measured and adjusted to 280-300 mOsmol/L and 6.8-7.2.

2.3 Semen collection

A Five mature sexually mature Ossimi rams (75-85 kg, 2-4 y), kept in the same environment, fed 1.250 kg (14% CP) of concentrate feed mixture (CFM) and 1 kg of Berseem hay/head, and had had access to trace mineralized salt lick blocks and free water, were used in this study.

Using an artificial vagina, semen was collected once weekly for 7 weeks from all 5 rams before morning feeding. Samples with ≥70% motility were admitted, pooled in order to have sufficient semen for a replicate, diluted at rate of 1:20 (semen/extender), equilibrated for 4 hours at 5 °C and then being loaded into 0.25 mL straws and placed on 4 cm over liquid nitrogen vapor for 10 min before being immersed in liquid nitrogen until thawing at 37 °C in an aqueous bath for 30s.

2.4 Semen quality assessment

The assessment of semen quality was undertaken on after dilution, equilibration period and freeze-thawing of Ossimi ram spermatozoa.Semen was visually evaluated for physical sperm parameters including sperm progressive motility according to **(Graham et al., 1970)**, sperm livability according to **Moskovtsev and Librach (2013)**, the morphological abnormalities of the spermatozoa (abnormal heads, tails, and cytoplasmic droplets) were identified on the same slide **(Menon et al., 2011)** and plasma membrane integrity using hypo-osmotic solution (osmolarity level of 75 mOsml) for 30 min. **(Neild et al., 1999)**.

2.5 Sperm motility parameters by CASA

Computer assisted semen analysis (CASA, SPERMOLAB®, Cairo, Egypt) was applied to evaluate semen. A drop of semen (5 µL) extended with different levels of extracts was loaded into a pre-warmed slide (disposable Leja). Before the analysis, sample was allowed to settle on the mini-thermal heating stage (38°C). For each specimen, about 200 spermatozoa from 2-3 drops of each sample were evaluated. The final analysis was done for each sample, including the followingParameters:

Percentages of total sperm motility (TSM), progressive sperm motility (PSM), rapid progressive sperm motility (RSM), slow progressive sperm motility (SSM), non-progressive sperm motility (NSM), and immotile spermatozoa (IMS). Where: TSM = PSM + NSM; PSM = RSM + SSM; IMS= 100 – TSM.

2.6 Sperm kinetic parameters by CASA

- Curve linear velocity (VCL): Average velocity of the sperm through its real path, (reference value > 45 µm/s).
- Straight linear velocity (VSL): Average velocity of the sperm through the straight line connecting the first position of the last track (reference value > 25 µm/s). - Average path velocity (VAP): Average velocity of the sperm through its average trajectory (reference value > 35 µm/s).
- Linearity (LIN%): The straightness of the sperm path.LIN=VSL/VCL x100
- Straightness (STR%): The righteousness of motion. STR=VSL/VAP x100
- Wobble (WOB%): Is the degree of oscillation of the actual path of the sperm head in his relationship with the VAP. WOB = VAP/VCL x 100

2.7 Antioxidant assay and enzyme activity in the extenders of thawed semen

Antioxidant capacity parameters including levels of total antioxidant capacity (TAC) and malondialdehyde (MDA) was determined in post-thawed semen according to **(Ohkawa et al., 1979; Aebi, 1984; Koracevic et al., 2001)**. Aspartate transaminase (AST) and alanine transaminase (ALT) activities were measured as described by **Reitman (1957)**.

All assays were achieved by using a spectrophotometer (Spectro UV-VIS Auto, UV-2602, Labomed, Los Angeles, CA, USA) and commercial kits (Biodiagnostic, Giza, Egypt) according to the manufacturer's instructions.

2.9 Statistical analysis

Using a software application, the acquired data were statistically analyzed using a one-way ANOVA design (SAS, 2007). Duncan's multiple range test (Duncan, 1955) was used to test for significant differences among groups at $p<0.05$.

3. RESULTS AND DISCUSSION

3.1 Visual sperm characteristics

Data analysis the effect of supplementation pomegranate juice (PJ) in Tris-extender on sperm characteristics (%) in post dilution, equilibration period and thawing of Ossimi ram semen is presented in Table 1. Results revealed that extender in T4 was significantly higher ($p<0.05$) in the percentages of visual progressive motility, livability, abnormality, and membrane integrity of ram spermatozoa in post dilution, equilibration period and thawed semen as compared to control-extender and other treatments (C, T1, T2 and T3). Type of sperm progressive motility (CASA analysis): Data in Table 2 shows that the effect of supplementing Tris -extender with Pomegranate juice on type of sperm motility (CASA analysis) in post-dilution, post-equilibration period and post-thawing of Ossimi ram semen. Results revealed that T4 (10 % PJ) increasing significantly ($P<0.05$) in the percentages of rapid, slow, and total progressive sperm motility, livability and total motility while decreasing significantly ($P<0.05$) in the percentages of non-progressive motility percentage and immotile sperm in post-dilution, post-equilibration period and post-thawing of semen as compared to other types of extenders (C, T1, T2 and T3).

Table 1: Effect of levels of Pomegranate juice in Tris-extender on sperm characteristics in post- dilution, equilibration period and thawing of Ossimi ram semen

Item	Sperm progressive motility (%)	Sperm livability (%)	Sperm abnormality (%)	Membrane integrity (%)
Post-dilution				
C (Control)	83.57±1.42^{ab}	82.42±1.08^{ab}	10.57±0.71^{b}	82.57±0.64^{b}
T1 (2.5 % PJ)	82.14±1.48^{b}	80.71±0.56^{b}	12.60±0.57^{a}	83.28±0.74^{b}
T2 (5 % PJ)	80.71±0.71^{b}	79.71±0.91^{c}	12.54±0.64^{a}	82.85±0.50^{b}
T3 (7.5 % PJ)	84.28±1.70^{ab}	82.75±0.77^{ab}	10.14±0.40^{b}	85.85±1.18^{a}
T4 (10 % PJ)	87.14±1.01^{a}	84.85±0.73^{a}	10.28±0.68^{b}	86±0.81^{a}
Post-equilibration				
C (Control)	72.85±2.14^{d}	70.71±0.86^{c}	23.85±0.67^{a}	71.14±1.48^{c}
T1 (2.5 % PJ)	74.28±1.30^{d}	71.85±0.50^{c}	21±0.59^{b}	73.14±0.40^{c}
T2 (5 % PJ)	77.14±1.01^{c}	74.57±0.57^{c}	19±0.37^{c}	72.57±0.57^{c}
T3 (7.5 % PJ)	80.71±1.70^{b}	78.14±0.82^{b}	17.42±0.48^{d}	77.85±1.12^{b}
T4 (10 % PJ)	84.28±1.30^{a}	81.42±0.48^{a}	15.85±0.70^{e}	81.85±0.67^{a}
Post-thawing				
C (Control)	43.14±0.63^{b}	41.14±0.63^{c}	37.85±0.67^{a}	38.28±1.56^{c}
T1 (2.5 % PJ)	42.57±0.78^{b}	42±0.37^{c}	31±1.63b^{c}	40.57±0.52^{c}
T2 (5 % PJ)	44.71±0.28^{b}	45±0.95^{b}	32.80±0.34^{b}	43.52±0.50^{b}
T3 (7.5 % PJ)	52.14±0.67^{a}	52.57±0.52^{a}	31.71±0.42^{bc}	50.42±0.64^{a}
T4 (10 % PJ)	51.42±1.42^{a}	52.32±1.37^{a}	29.57±0.48^{c}	51.85±0.67^{a}

a-e Means denoted within the same column with different superscripts are significantly different at P<0.05.

Table 2: Effect of supplementation different levels of Pomegranate juice in Tris-extender on type of sperm motility (CASA analysis) in post dilution, equilibration period and thawing of Ossimi ram semen

Item	Type of sperm motility (%)					
	Rapid progressive	Slow progressive	Total progressive	Non progressive	Total motility	Immotility
Post-dilution						
C (Contro	59.13±0.73^{b}	14.53±1.51^{b}	73.66±0.88^{b}	9.90±0.49^{c}	83.56±1.10^{b} ^{c}	16.43±1.10^{a} ^{b}
T1 (2.5 % PJ)	56.43±0.66^{d}	11.50±0.43^{c}	67.93±0.99^{c}	15.76±1.17^{a}	83.70±1.25^{b} ^{c}	16.30±1.25^{a} ^{b}
T2 (5 % P	57.60±0.87^{c}	10.93±0.40^{c}	68.53±0.49^{c}	12.56±0.57^{b}	81.10±0.66^{c}	18.90±0.66^{a}
T3 (7.5 % PJ)	60.23±0.95^{a} ^{b}	18.63±0.53^{a}	78.86±0.52^{a}	7.73±0.56^{d}	86.60±1.08^{b}	13.40±1.08^{b}
T4 (10 % PJ)	61.96±0.33^{a}	17.66±0.21^{a}	79.63±0.50^{a}	11.43±0.47^{b}	91.06±0.17^{a}	8.93±0.17^{c}
Post-equilibration						
C (Contro	50.13±0.44^{c}	13.13±0.18^{c}	63.26±0.31 ^{b}	20.06±0.71 ^{a}	83.33±0.63 ^{a}	16.66±0.63 ^{c}
T1 (2.5 % PJ)	48.83±0.76^{d}	11.63±0.12^{c} ^{d}	60.46±0.68 ^{c}	14.26±0.75 ^{c}	74.73±1.24 ^{d}	25.26±1.24 ^{a}
T2 (5 % P	48.98±0.73^{d}	20.06±0.78^{a}	69±0.28^{a}	13.26±0.65 ^{d}	82.26±0.46 ^{b}	17.73±0.46 ^{b}

T3 (7.5 % PJ)	52.23±0.64[b]	10.56±0.93[d]	62.80±0.30[b]	19.06±0.84[b]	81.86±0.68[b]	18.13±0.68[b]
T4 (10 % PJ)	53.76±0.70[a]	15.83±1.05[b]	69.60±0.41[a]	10.53±0.92[e]	80.13±1.18[c]	19.86±1.18[b]
Post-thawing						
C (Contro	31.10±0.44[d]	9.80±0.30	40.93±0.64[c]	7.36±0.60[b]	48.30±0.05[c]	51.70±0.05[a]
T1 (2.5 % PJ)	28.75±0.32[c]	11.03±0.08	39.80±0.40[d]	6.32±0.41[b]	46.14±0.08[c]	53.86±0.08[a]
T2 (5 % P	29.96±0.76[c]	10.43±0.83	40.40±1.60[c]	8.04±1.51[b]	48.42±0.12[c]	51.54±0.14[a]
T3 (7.5 % PJ)	33.17±0.52[b]	10.35±0.42	43.53±0.93[b]	12.05±1.61[a]	55.56±0.68[a]	44.42±0.68[c]
T4 (10 % PJ)	34.63±0.71[a]	9.90±0.62	44.60±0.11[a]	7.81±0.18[b]	52.43±0.09[b]	47.55±0.10[b]

a-e Means denoted within the same column for each stage with different superscripts are significantly different at P<0.05.

3.2 Sperm kinetic parameters (CASA analysis)

From results of sperm kinetic parameter spostdilution, equilibration period and thawed semen was illustrated in Table 3. It was investigated that VCL, VSL, VAP, LIN, STR and WOB were affected significantly (P<0.05) by types of extender, the highest in group T3 and the lowest in group T1 as compared to other groups in post-diluted ram semen, while VCL, VSL, VAP, LIN, STR and WOB were differ significantly (P<0.05) the highest by control group (c) and the lowest by T4 as compared to other groups in post equilibration period and thawing semen.

Table 3: Effect of supplementation different levels of Pomegranate juice in Tris-extender (on kinetic sperm parameters in post dilution, equilibration and thawing of Ossimi ram semen

Item	Sperm kinetic parameters					
	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)
Post-dilution semen						
C (Control)	77.60±0.97[b]	38.26±0.71[ab]	59.56±0.97[a]	48.96±0.95	76.13±0.58[b]	62.13±0.50[c]
T1 (2.5 % PJ)	70.60±0.55[c]	33.76±0.66[c]	53.10±0.92[c]	47.86±0.95	76.33±0.60[b]	63.40±0.75[b]
T2 (5 % PJ)	75.83±0.73[b]	36.80±0.65[b]	56.66±0.88[b]	49.90±1.00	78.03±0.73[ab]	65.30±0.77[ab]
T3 (7.5 % PJ)	80.23±0.76[a]	39.86±0.71[a]	61.86±0.82[a]	50.16±0.31	79.26±0.61[a]	66.93±0.73[a]
T4 (10 % PJ)	70.66±0.63[c]	33.76±0.31[c]	53.43±0.58[c]	48.10±0.36	76.23±0.55[b]	63.90±0.72[b]
Post-equilibration						
C (Control)	75±0.10[a]	37.06±0.71[a]	57.85±0.77[a]	49.26±0.71[a]	76.96±0.61[a]	63.25±0.93
T1 (2.5 % PJ)	65.50±0.15[b]	31.33±0.58[b]	49.76±0.71[b]	47.53±0.92[b]	76.53±1.38[a]	63.13±1.51
T2 (5 % PJ)	56.46±0.71[d]	25.93±0.60[d]	43.23±1.19[c]	46.16±0.77[c]	74.24±0.72[b]	61.80±1.09
T3 (7.5 % PJ)	60.06±0.03[c]	28.26±0.81[c]	45.34±0.62[c]	46.80±1.05[c]	75.06±0.69[ab]	61.93±0.90
T4 (10 % PJ)	55.80±0.05[d]	26.30±0.65[d]	41.75±0.69[d]	45.03±0.65[d]	72.93±0.75[c]	63.10±0.95
Post-thawing						
C (Control)	63.72±0.38[a]	20±0.35[c]	48.22±0.73[a]	46.90±0.05[a]	75.53±0.62	63.30±0.78[a]
T1 (2.5 % PJ)	62.27±0.61[a]	19.03±0.33[d]	46.90±0.75[a]	46.13±0.62[a]	73.80±0.80	62.96±0.21[b]
T2 (5 % PJ)	62.90±0.07[a]	20.16±0.55[c]	47.94±0.82[a]	47.44±0.65[b]	75.10±0.40	62.05±0.68[b]

T3 (7.5 % PJ)	63.88±0.05[a]	21±0.75[b]	47.13±0.62[a]	47.81±0.60[b]	75.60±0.65	63.60±0.75[a]
T4 (10 % PJ)	51.84±0.71[b]	22±0.45[a]	39.36±0.88[b]	44.25±0.59[c]	73.96±0.62	58.92±0.71[c]

a-e Means denoted within the same column for each stage with different superscripts are significantly different at P<0.05. VCL: Curve linear velocity (VCL). VSL: Straight linear velocity. VAP: Average path velocity. LIN (%): Linearity =VSL/VCL x100. STR (%): Straightness = VSL/VAP x100. WOB (%): Wobble = VAP/VCL x100

3.3 Sperm abnormality (CASA analysis)

Data of morphological sperm abnormality (Table 4) showed significantly (P<0.05) effect of different levels of PJ supplementations on normal forms and head, neck, and tail abnormalities of sperm cells in post-dilution, equilibration period and thawing semen. Whereas, in post-dilution semen, the highest normal forms of spermatozoa were achieved significantly higher (P<0.05) in (control and T1 groups) as compared to other groups, while the percentages of normal form of spermatozoa post-equilibration period was significantly higher (P<0.05) in T1 and T4 than in other treatments, also the percentages of normal form of spermatozoa in all treatment groups was significantly higher (P<0.05) as compared to control group in post thawing semen.

The percentages of the neck, head and tail abnormality in post-dilution and the head and tail abnormality in post thawing semen we reinvestigated significantly higher (P<0.05) in T4 than in other groups. Also, in post-equilibration period, the percentage of abnormal neck forms of spermatozoa in group T3, abnormal head forms of spermatozoa in group T2 and abnormal tail forms of spermatozoa in group T4 were achieved significantly higher (P<0.05) as compared to other groups.

Table 4: Effect of supplementing Tris-extender with Pomegranate juice (PJ) on sperm abnormalities in post-diluted, post-equilibrated and post-thawed Ossimi ram semen

Item	Normal forms (%)	Sperm abnormalities (%)		
		Neck	Head	Tail
Post-dilution				
C (Control)	76.33±0.38[a]	23.20±0.35[b]	7.66±2.90[c]	12.63±3.17[b]
T1 (2.5 % PJ)	77.10±1.56[a]	20.23±0.88[c]	14.70±5.86[a]	10.83±1.56[b]
T2 (5 % PJ)	72.20±0.76[b]	21.73±1.78[c]	12.13±5.83[b]	9.63±1.96[c]
T3 (7.5 % PJ)	70.36±0.77[b]	25.60±0.49[b]	11.60±4.93[b]	18.96±2.65[a]
T4 (10 % PJ)	69.10±0.90[b]	28.60±1.50[a]	13.56±4.89[a]	16.83±4.93[a]
Post-equilibration				
C (Control)	59.23±0.38[b]	35.60±0.55[a]	22.73±1.08[c]	18.53±0.60[e]
T1 (2.5 % PJ)	64.06±0.72[a]	34.13±0.61[a]	36±0.54[a]	22.13±0.68[d]
T2 (5 % PJ)	57.37±0.60[c]	31.52±0.63[b]	22.80±0.65[c]	25.26±0.37[b]
T3 (7.5 % PJ)	54.55±0.86[d]	34.84±0.71[a]	26.70±0.50[b]	23.94±0.70[c]
T4 (10 % PJ)	63.60±1.05[a]	18.12±0.60[c]	22.03±0.62[c]	26.82±0.51[a]
Post-thawing				
C (Control)	42.32±1.20[b]	34±0.57[a]	32.63±0.08[b]	25.82±0.44[b]
T1 (2.5 % PJ)	49±3.05[a]	20.70±0.05[d]	33.60±0.05[b]	26.33±0.12[b]
T2 (5 % PJ)	47±0.57[a]	29.62±0.03[b]	31.40±0.10[c]	27.14±0.45[b]
T3 (7.5 % PJ)	48±0.60[a]	25.45±0.24[c]	30.16±0.12[c]	25.60±0.11[b]
T4 (10 % PJ)	50.68±0.88[a]	24.44±0.23[c]	36.30±0.15[a]	35.47±0.14[a]

a-e Means denoted within the same column for each stage with different superscripts are significantly different at P<0.05.

3.4 Plasma and sperm lipid per oxidation level and antioxidant enzyme activities

Means and standard errors of Total antioxidants capacity, MDA, AST and ALT

concentrations in post-thawing of Ossimi ram semen in different types of extender are presented in Table 5. Analysis of variance revealed that total antioxidant capacity was not affected significantly (P<0.05) by different levels of Pomegranate juice. While MDA concentration was lower which was affected significantly (P<0.05), being the highest in control group (C) and the lowest in T4 group. Enzymatic activity, AST and ALT concentrations in extender were affected significantly (P<0.05) by different types of extender, being the highest concentration of AST was in control group (C) and the lowest in T2 group and the highest concentration of ALT was in T3 group and the lowest in T2 group.

Table 5: Total antioxidants capacity and MDA concentrations, enzymatic activity in different types of extender post-thawing of Ossimi ram semen

Treatment	TAC (mM/L)	MDA (nmol/ml)	AST (U/L)	ALT (U/L)
C (Control)	2.82±0.57	55.64±5.77^{a}	62.33±0.66^{a}	11.33±0.33^{e}
T1 (2.5 % PJ)	3.44±0.55	29.70±0.57^{c}	43.66±7.05^{b}	35.33±5.04^{c}
T2 (5 % PJ)	3.12±0.48	45.07±1.50^{b}	41.66±6.35^{b}	24.66±6.96^{d}
T3 (7.5 % PJ)	3.35±0.44	30.50±1.52^{c}	42.61±6.11^{b}	61.33±5.48^{a}
T4 (10 % PJ)	3.87±0.50	12.78±0.05^{d}	53±1.15^{ab}	44±6.08^{b}

a-e Means denoted within the same column for each stage with different superscripts are significantly different at P<0.05

DISCUSSION

Artificial insemination with good semen quality is in need for a physiological limit of ROS in order to fulfill its function. High levels of ROS, on the other hand, are associated with a reduction in sperm's capacity to fertilize in order to produce enough semen for a replication ***(Capucho et al.,* 2012)**. Changes in function and structure of sperm membrane were caused by ROS in concomitant with antioxidant defense mechanisms are also altered ***(Bilodeau et al.,* 2001)**. Seminal plasma has an antioxidant system that appears to be particularly important for sperm protection in order to combat the harmful effects of ROS **(Alvarez and Storey, 1982)**. Spermatozoa unfortunately have relatively little antioxidant capacity to defend themselves against ROS. To increase the viability and spermatozoa's potential to fertilize later, antioxidants might be added to semen extenders **(Gadea *et al.,* 2008)**. Supplementing antioxidants to extenders to prevent ROS effects has been studied in a numerous research **(Uysal and Bucak, 2007; Bucak *et al.,* 2008)**. Lipid per oxidation damages the lipid components of sperm membranes, resulting in decreased sperm viability due to axonemal damage, greater mid-piece morphological abnormalities, decreased intracellular energy generation, and lipid per oxidation of sperm membranes **(Henkel, 2005)**. The acrosome and plasma membrane are essential components that regulate extracellular exchanges and the fertilization process **(Flesch and Gadella, 2000)**. The lipids in the sperm membrane are thought to be the main factor in viability, motility, and cryosurvivability **(Hammerstedt *et al.,* 1990)**.

This study aimed to evaluate the effect of the supplementing semen extender with different levels of pomegranate juice (PJ) at levels of 2.5, 5, 7.5 and 10% on the motility, livability, normality, plasma membrane integrity, and kinetic parameters of ram spermatozoa in diluted, equilibrated and thawed Ossimi ram semen. In cryopreserved cattle semen, PJ supplementation reduced total sperm abnormalities, increased post-thaw sperm motility, membrane integrity, and viability, and enhanced chilled semen sperm motility. The antioxidant potential of PJ depends on other antioxidant-rich components such tannins and flavonoids in addition to its vitamin C concentration. However, the

combined activity of a multitude of components determines PJ's antioxidant capacity. Additionally, fresh PJ has 1.5% pectin, ascorbic acid, polyphenolic, flavonoids, 10% total sugars and the essential amino acids (glutamic and aspartic acid). These phytochemicals could control bacterial populations in the body or in the environment and function as antioxidants. Additionally, PJ is abundant in vitamins A, C, and E, all of which increase both men's and women's sexual libido **(Blesbois *et al.*, 1999; Lampe, 1999; Aviram and Dornfeld, 2001; Longtin, 2003; Virgili and Marino, 2008; El-Sheshtawy *et al.*, 2016)**.

The obtained results indicated beneficial effects of supplementing Tris-extender of ram semen with 10% PJ in post-diluted, post-equilibrated and post-thawed semen to improve the visual sperm characteristics (progressive motility, livability, normality, and membrane integrity) and different types of motility (rapid, slow, total progressive, total motility) and decreasing sperm abnormalities in head, neck, or tail. In agreement with our results, sperm plasma and the acrosome membrane of semen dosages cryopreserved with 10% PJ showed a substantial improvement. Due to the ant-oxidative impact of chemicals found in the PJ, which has decreased lipid peroxidation and improved plasma membrane integrity, post-thaw plasma and acrosome membrane integrity have improved **(Iqbal *et al.*, 2016b; Javed *et al.*, 2019)**. Addition of 10% PJ to chilled cattle semen helped to sustain sperm motility percentage over the course of 10 days and dramatically boosted the post-thaw motility and live percentages **(El-Sheshtawy *et al.*, 2016)**, significantly improved semen quality, CASA motion dynamics and Nili Ravi buffaloes field fertility **(Iqbal *et al.*, 2016a; Iqbal *et al.*, 2016b; Naz *et al.*, 2018; Javed *et al.*, 2019; Naz *et al.*, 2019; Zarepourfard *et al.*, 2019)**.

Semen quality evaluation using motion analysis is crucial because of its positive stress and per oxidative damage, which this antioxidant defense mechanism guards against **(Koksal *et al.*, 2003; Turner and Lysiak, 2008)**.

correlation with male fertility and because it is one of the factors that are most impacted by cryopreservation. However, cell collision, occlusion, and missed detection make sperm tracking exceedingly difficult. Because it enables spermatozoa to move from their introduction source to the site of fertilization, motility is a crucial factor to take into account when assessing sperm for artificial intelligence **(Pereira *et al.*, 2017)**. It is a requirement for showing how sperm work. This result is in line with other studies that showed the usage of antioxidants to preserve the body's mobility during cryopreservation **(Zanganeh *et al.*, 2013; Najafi *et al.*, 2014; Sharafi *et al.*, 2015)**, as well as an inverse association between sperm motility and the rate of lipid per oxidation **(Aitken and Fisher, 1994).**

Computer-assisted sperm analysis (CASA) is a distinct, in-depth, and all-encompassing method for assessing the various sperm motility features, which directly relate to bovine fertility **(Kathiravan *et al.*, 2008)**. In similarity of our results, **Javed et al. (2019)** showed that the inclusion of 10% PJ in tris-based extender exhibited significantly improved post-thaw sperm CASA motility properties (total motility, progressive motility, and kinematics), which comparable to those of earlier research on cattle **(El-Sheshtawy *et al.*, 2016)**, goat **(Zarepourfard *et al.*, 2019)**, rats **(Türk *et al.*, 2008; Mansour *et al.*, 2013)** and rooster **(Al-Daraji, 2015)** semen. The antioxidants found in the PJ (polyphenols, vitamin C, E, anthocyanins, punicalagin, ellagic, and gallic acid) may be responsible for the improvement in post-thaw sperm motility measures ***(Seeram et al., 2006; Seeram et al.*, 2008)**. It is shown that the increased antioxidant enzyme profile in spermatozoa and ROS scavenging activity following cryopreservation may be the causes of the PJ antioxidant benefits. The primary causes of spermatogenic dysfunctions are oxidative

Antioxidants in the seminal plasma play a role in antioxidant defence mechanisms. Antioxidantsscavenge oxygen radicals to protect spermatozoa. Total antioxidant capacity (TAC) and ROS production are balanced in fertile men **(Agarwal *et al.*, 2014)**. Spermatozoa are therefore vulnerable to ROS from LPO due to relatively low levels of scavenging enzymes or non-enzymatic antioxidants in the cytoplasm and high levels of PUSFA in membranes **(Sanocka and Kurpisz, 2004)**.

As known, there is a negative relationship between TAC and MDA, when total antioxidant capacity was higher, MDA concentration was lower. In this study, however, total antioxidant capacity was not affected significantly by different concentrations of Pomegranate juice, 10% PJ resulted the lowest MDA concentration when TAC was highest compared with other groups. These results followed the same trend as the findings of **(Yüce and Aksakal, 2007; Guo *et al.*, 2008; Türk *et al.*, 2008; Rad *et al.*, 2010)** who reported that oral consumption of pomegranate juice provides significant reduction in testicular tissue MDA level and also significantly improves sperm count, motility, and abnormal sperm rate in non-stressed healthy laboratory animals. Malondialdehyde (MDA) is a biomarker of an advanced oxidative status that is used to measure the degree of per oxidation damage in spermatozoa **(Tavilani *et al.*, 2005)**. It is which consider as an important indicator for the per oxidation of PUSFA in sperm cells **(Motlagh *et al.*, 2014)**. It is a parameter for measurement of oxidative stress **(Zanganeh *et al.*, 2013)** and an objective parameter of sperm quality **(Ball *et al.*, 2001)**. The ROS and ultimately cytotoxic secondary products, especially MDA increased during sperm cryopreservation as a result of removing the balance between antioxidant and pro-oxidant activities **(Del Olmo *et al.*, 2015)**. The MDA male reproduction. The World Journal of Men's Health, 32, 1-17.

Aitken, J. and Fishe, H. (1994). Reactive oxygen species generation and human produces plasmatic and acrosome membranes, mitochondria and the axonemal sheath of spermatic cell damages, which causes by a large number of ROS, that effects on viable cells **(Salamon and Maxwell, 2000)**.

Good quality ejaculates had much lower AST and ALT levels than poor quality ejaculates, according to analysis of these enzyme profiles **(Perumal *et al.*, 2016)**. In our study, the Control group (C) had the highest levels of AST and ALT in T3 (7.5% PJ), whereas T2 (5% PJ) had the lowest levels. Due to the fact that they exhibit a positive association with sperm concentration and a negative correlation with semen volume, the testes or epididymides are assumed to be the potential source of these enzymes **(Kareskoski and Katila, 2008)**. The extracellular fluid after ejaculation contained large amounts of the enzyme due to structural damage, increased cell membrane permeability, and destabilization of the membrane integrity of the acrosome, plasma, mitochondria, and flagella of the sperm, as shown by the higher activity of AST and ALT in poor quality ejaculates seminal plasma **(Mostari *et al.*, 2019)**.

It was concluded that in post diluted, equilibrated and thawed Ossimi ram semen, 10% PJ significantly increased sperm motility, membrane integrity and viability, function, velocity, and decreased total sperm abnormalities. PJ antioxidants had a positive impact on oxidative stress damage, which preserved the integrity and viability of spermatozoa after freezing and thawing and increased fertility following artificial insemination.

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تأثير إضافة عصير الرمان لمخفف السائل المنوي على الخصائص الفيزيائية والحركية للحيوانات المنوية في السائل المنوي المجمد للكباش الأوسيمي

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مجلة العلوم الزراعية والبيئية المستدامة

الكلمات المفتاحية:

الملخص العربى

يعتبر عصير الرمان ذو نشاط قوي مضاد للأكسدة ضد اكسدة الليبيدات حيث كان الهدف من هذه الدراسة هو تحديد التأثير المضادة للأكسدة لمستويات مختلفة من عصير الرمان (2.5-5-7.5-10%) في مخفف السائل المنوي على الخصائص الفيزيائية والحركية للحيوانات المنوية في السائل المنوي للكباش الاوسيمى بعد التخفيف والموازنة و الإسالة. تم جمع السائل المنوي من 5 كباش مرة في الأسبوع لمدة 7 أسابيع باستخدام المهبل الصناعي. تم تخفيف السائل المنوي بمعدل 1:20) المخفف/السائل المنوي) وتمت موازنة السائل المنوي المخفف لمدة 4 ساعات عند 5 درجات مئوية قبل وضعه في قشات 0.25 مل ثم تم التجميد في النيتروجين السائل. تم فحص الخصائص الفيزيائية والحركية للحيوانات المنوية وأنشطة الإنزيمات والقدرة المضادة للأكسدة. أدت إضافة 10٪ من عصير الرمان في المخفف إلى تحسين جودة السائل المنوي بشكل ملحوظ وتشكل الحيوانات المنوية بعد الإسالة والحركة الكلية والحركة التقدمية ومقاييس الحركة باستخدام CASA كما أن هناك تحسن في القدرة المضادة للأكسدة وانخفاض تركيز المالوندا يألديهيد.

استنتج أن إضافة 10٪ من عصير الرمان في المخفف يحسن خصائص السائل المنوي بعد الإسالة وديناميكيات الحركة باستخدام CASA في السائل المنوي المجمد للكباش الاوسيمى مما يحسن الخصوبة بعد التلقيح الصناعي لها.