Journal of Animal and Poultry Production

Journal homepage & Available online at: www.jappmu.journals.ekb.eg

Effect of Aqueous and Methanolic Green Tea Extracts on Motility, Normal Forms, And Kinetic Parameters of Spermatozoa in Rahmani Ram Semen Equilibrated at 5 °C for 4 Hours

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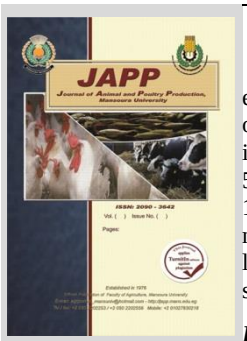

ABSTRACT

Green tea extract is plant–derived natural antioxidant. The purpose of this study was to investigate the effect of the supplementing semen extender (Tris-citric-soybean lecithin) with different levels of aqueous (AGTE) or methanolic (MGTE) green tea extract (0.5, 1.0 and 1.5%) on the motility, livability, normality, plasma membrane integrity, and kinetic parameters of ram spermatozoa in diluted and equilibrated semen. Semen was collected from 5 rams once a week for 7 weeks using an artificial vagina. Results showed positive effect of extender containing 1% MGTE on visual motility, livability, abnormality, membrane integrity, rapid motility, sperm velocity, and normal form in post-equilibrated semen. It is possible to infer that the inclusion of methanolic green tea extract at a level of 1% in semen extender had a substantial effect on sperm quality, normality, function, and velocity of ram spermatozoa in Rahmani semen after dilution and equilibration for 4 h at 5ºC.

Keywords: Aqueous and Methanolic green tea extract, ram spermatozoa, equilibration, motility CASA analysis.

INTRODUCTION

The artificial insemination (AI) industry has long been concerned with enhancing the quality of the frozen sperm that is sold (Muiño *et al*., 2007). Slow cooling to 4-5 ºC is frequently followed by a varied equilibration period (from 30 minutes to 24 hours) at this low temperature before freezing semen (Leite *et al*., 2010). The standard definition of equilibration is the amount of time that spermatozoa are in contact with glycerol before they freeze. Glycerol enters the sperm cells at this point to create a balanced intracellular and extracellular concentration. The equilibration process affects not just glycerol, but also the other osmotically active extender components. As a result, the equilibration process can interact with the kind of extender utilized (buffer and cryoprotectant) as well as other cryogenic operations (Vishwanath and Shannon, 2000).

During equilibration period, the presence of lipid peroxidation (LPO) in spermatozoa is caused by a greater generation of reactive oxygen species (ROS) (Kumaresan *et al*., 2006). Spermatozoa are unable to re-synthesize their membrane component, hence there is always the potential of LPO by ROS (Alvarez and Storey, 1989). Lower quantities of ROS stimulated sperm capacitation, acrosome reaction, and sperm–oocyte fusion (Rivlin *et al*., 2004; Cocuzza *et al*., 2007). However, at greater concentrations, ROS acted as a genotoxic agent, reducing semen quality (motility and plasma/acrosome integrities) in humans (Baumber *et al*., 2000; Bilodeau *et al*., 2001), ram (Peris *et al*., 2007), and bovine (Hu *et al*., 2010).

Natural antioxidants in bovine sperm are inadequate to maintain sperm integrity against oxidative degradation during semen preservation (Nair *et al*., 2006; Nichi *et al*., 2006). By using antioxidants in the semen extender, oxidative stress caused by excessive ROS generation can be lessen to maintain the normal sperm activities. As a result, it is critical to select an appropriate antioxidant level to maintain the natural balance that occurs between ROS formation and scavenging activities throughout the preservation process (Memon *et al*., 2011).

In this concern, green tea extract (GTE) was reported to improve the quality of sperm cells in boar (Mehdipour *et al*., 2016), canine (Wittayarat *et al*., 2013), bovine (Khan *et al*., 2017), mouse (Abshenas *et al*., 2011) and rooster (Al-Daraji, 2011) semen.

Motion analysis on semen sample quality evaluation is critical for the favorable correlation with male fertility and because it is one of the most changed parameters after preservation. However, due to cell contact, occlusion, and missed detection, sperm tracking is highly difficult. Computer-assisted semen analysis (CASA) systems have been employed in a growing number of papers in last years, demonstrating the usefulness of objective approaches for evaluating semen quality and predicting fertility.

CASA systems are widely utilized for determining sperm quality from diverse species (Billard and Cosson, 1992; Dietrich *et al*., 2005), cryopreservation efficacy (Cueto *et al*., 2016), toxicity bioassays, predicting reproductive potential, or fundamental sperm biology (Muiño Otero, 2008; Buzón, 2013).

Objective of this study was to assess the effect of the supplementing semen extender with different levels of methanolic or aqueous green tea extract at levels of 0.5, 1.0 and 1.5 % on the motility, livability, normality, plasma membrane integrity, and kinetic parameters of ram spermatozoa in diluted and equilibrated semen.

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DOI: 10.21608/jappmu.2022.145028.1041

MATERIALS AND METHODS

The current study was conducted at the Animal Production Research Station, Sakha, Kafrelsheikh governorate, located in the northern part of the Nile Delta (latitude 31o 15'N and longitude 31o 45'E), belonging to Animal Production Research (APRI), Agricultural Research Center (ARC), Ministry of Agriculture, Egypt, in collaboration with the Department of Animal Production, Faculty of Agriculture, Tanta University, Egypt, from August 2021 to February 2022.

Extract preparation:

Preparation of methanolic green tea extract:

The extraction of methanolic green tea extract (MGTE) followed a standard methodology published by (Chan *et al*., 2007; Gale *et al*., 2015). In a nutshell, we extracted green tea by soaking dried commercial leaves in a laboratory mixer and then powdering the leaves. About 8 g of the powdered green tea leaves were kept at room temperature in 400 ml of methanol for 24 hours (25^{o} C). The solution was then transferred to falcon tubes (15 ml) and centrifuged (4000 rpm) at room temperature for 30 minutes. Before filtering, the supernatant was carefully separated from the pellets, then the extract (MGTE) was kept at -20^{o} C until use.

Preparation of aqueous green tea extract:

Aqueous green tea extract (AGTE) is accessible according to Malo *et al*. (2011). About 10 g of green tea powder was stirred with 100 ml deionized water for 24 hours before baking for 15 minutes at 100 °C. The mixture was filtered to guarantee clear extraction, and the extracts were condensed under vacuum on a rotary evaporator once the water had cooled to 25 °C.

Semen extender preparation:

The control extender contained 3.025 g Tris, 1.66 g citric acid monohydrate, 1.25 g glucose, 1% soybean lecithin, 5% glycerol, 100 IU/mL penicillin, and 100 g/mL streptomycin. Semen was further divided into 7 aliquots including free-extender (C), and extenders supplemented with AGTE at levels of 0.5, 1.0, and 1.5% (T1, T2, and T3) or MGTE at levels of 0.5, 1.0, and 1.5% (T4, T5, and T6), 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 7.2-7.3.

Semen collection:

A total of 5 sexually mature Rahmani rams, a native sheep breed in Egypt, with 70-80 kg live body weight and 2-4 years of age, were chosen as semen donors.

Semen was collected using an artificial vagina. All rams were kept in the same environment, given a concentrate feed mixture (CFM) at a level of 1.250 kg (14% CP) and 1.00 kg of Berseem hay, and had constant access to trace mineralized salt lick blocks and drinking water.An artificial vagina approach was used to collect semen from the donors before feeding at 7-8 am once weekly for 7 weeks. Semen ejaculates were immediately transported to the laboratory and deposited in a 37 °C water bath. Only 70% motility sperm was used for dilution.

A rate of 1:20 (semen/extender) was used for different extender types. then the diluted semen was equilibrated for 4 hours at 5 °C.

Semen evaluation:

Semen was visually evaluated for physical sperm parameters including progressive motility, vitality, abnormality, and membrane integrity. However, different sperm motility parameters and sperm kinetic parameters was determined by CASA analysis. Semen was evaluated after dilution and equilibration.

Physical parameters:

A 10 µL aliquot of diluted semen was placed on a heated slide and covered with a coverslip; the number of spermatozoa demonstrating forward movement (progressive motility) in a long semi-arc pattern was counted in five fields, each containing 200 sperm cells.

A phase-contrast microscope with a 37 °C heated stage (DM 500; Leica, Switzerland) was used for three times.On a glass slide, a smear of diluted sperm was stained with a dual staining procedure (5% eosin and 10% nigrosin) for determining 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).

The hypo-osmotic swelling test (HOS-t) was used to assess spermatozoa's functioning plasma membrane A solution of osmolarity of 75 mOsml in term hypo-osmotic swelling test (HOS-t) for 30 min was also assessed by adding 0.1 ml of semen sample in a tube contains 0.9 ml of HOS media (Neild *et al*., 1999).

Sperm motility parameters by CASA:

Computer assisted semen analysis (CASA, SPERMOLAB®, Cairo, Egypt) was applied to evaluate the diluted and equilibrated 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 following parameters:

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.

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 x100

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.

RESULTS AND DISCUSSION

Results

Visual sperm characteristics:

Results of visual sperm characteristics presented in Table 1 revealed that both extenders in T2 and T5, significantly (P<0.05) improved visual progressive motility, livability, abnormality, and membrane integrity percentages of ram spermatozoa in post diluted semen as compared to free-extender (C).

However, each of T1, T3 and T4 did not affect the visual sperm characteristics. On the other hand, T6 showed negative effects on all sperm characteristics studied, being poorer than in free-extender (C). In post-equilibrated semen, T5 showed significantly (P<0.05) the best sperm characteristics in terms of the highest sperm progressive motility, livability, and membrane integrity, and the lowest sperm abnormalities.

Table 1. Effect of supplementing Tris-extender with aqueous or methanolic extract of green tea on sperm characteristics (%) in post-diluted and post-equilibrated ram semen.

Item	Sperm progressive motility (%)	Sperm livability (%)	Sperm abnormality(%)	Membrane integrity(%)
		In post-diluted semen		
C (Control)	84.28±1.30^{b}	84.42±0.92^{b}	10.57±0.68^{bc}	82.42±0.20^{b}
T1 (0.5 % AGTE)	83.85±1.48^{b}	82.28±0.18^{b}	11.71±0.18^{b}	83±0.72^{b}
T2 (1.0 % AGTE)	86.42±1.42^{a}	87.71±0.18^{a}	8.14±0.34^{d}	85.28±0.77^{a}
T3 (1.5 % AGTE)	84.28±1.30^{b}	83.24±0.34^{b}	10.71±0.47^{bc}	82.57±0.64^{b}
T4 (0.5 % MGTE)	84.28±1.30^{b}	83.16±0.50^{b}	9.71±0.47^{c}	82±0.72^{b}
T5 (1.0 % MGTE)	87.14±1.48^{a}	87.10±0.70^{a}	8.28±0.18^{d}	85.42±0.64^{a}
T6 (1.5 % MGTE)	77.85±1.48^{d}	77.28±1.39^{c}	13.42±0.52^{a}	78±0.72^{c}
		In post-equilibrated semen		
C (Control)	71.42±2.10^{d}	71±1.04^{c}	23.28±0.74^{ab}	71.14±1.48^{d}
T1 (0.5 % AGTE)	72.85±1.48^{d}	72.85±0.40^{c}	20.57±0.20^{b}	73±1.00^{dc}
T2 (1.0 % AGTE)	82.85±1.48^{ab}	82.71±0.35^{a}	25.14±0.55^{a}	82±0.81^{ab}
T3 (1.5 % AGTE)	76.42±2.10^{c}	77±1.41^{b}	25.57±0.99^{a}	75.14±1.7^{c}
T4 (0.5 % MGTE)	79.28±1.30^{bc}	79.28±1.12^{b}	21.28±2.08^{b}	79.28±0.68^{b}
T5 (1.0 % MGTE)	84.28±1.30^{a}	84.14±0.63^{a}	15.42±0.52^{c}	83.42±0.48^{a}
T6 (1.5 % MGTE)	72.85±1.48^{d}	73.28±1.06^{c}	21±0.57^{b}	72.42±0.52^{dc}

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

Type of sperm progressive motility (CASA analysis):

Results of different types of sperm motility shown in Table 2 revealed that T2 significantly (P<0.05) increased percentages of rapid, slow, and total progressive sperm motility, livability, while significantly (P<0.05) decreased non-progressive motility percentage without any effect on total motility and immotile sperm percentages in post diluted semen as compared to free-extender (C). However, T5 significantly (P<0.05) increased only the percentage of rapid progressive motility with insignificant differences in other types of motility with the free-extender. Results showed that T6 (as found for visual sperm characteristics) and T1 revealed significantly (P<0.05) negative effect on most types of sperm motility in post-diluted semen. In post-equilibrated semen, T5 showed significantly (P<0.05) the highest rapid and total progressive sperm motility and the lowest non-progressive motility with significant effects on total motility and immotility in comparing with the free-extender, while T6 showed opposite trends.

Table 2. Effect of supplementing Tris-extender with aqueous or methanolic extract of green tea on type of sperm motility (CASA analysis).

Item	Type of sperm motility (%)					
	Rapid progressive	Slow progressive	Total progressive	Non progressive	Total motility	Immotility
			In post-diluted semen			
C (Control)	59.46±0.77^{b}	16.20±0.15^{c}	75.66±0.66^{c}	10.56±0.86^{b}	86.23±0.39^{a}	13.76±0.39^{c}
T1 (0.5 % AGTE)	54.73±0.83^{cd}	12.60±1.87^{d}	67.33±1.16^{d}	10.10±0.66^{b}	77.43±1.83^{c}	22.56±1.83^{a}
T2 (1.0 % AGTE)	63.56±0.85^{a}	19.90±1.18^{ab}	83.46±0.84^{a}	6.33±0.37^{c}	89.80±0.64^{a}	10.20±0.64^{c}
T3 (1.5 % AGTE)	57.06±1.29^{bc}	21.96±2.10^{a}	79.03±1.12^{b}	10.90±0.70^{b}	89.93±0.47^{a}	10.06±0.47^{c}
T4 (0.5 % MGTE)	56.63±1.29^{bc}	19.20±0.87^{bc}	75.83±0.88^{c}	12.36±0.53^{ab}	88.20±0.92^{a}	11.80±0.92^{c}
T5 (1.0 % MGTE)	62.83±1.42^{a}	14.73±2.14^{cd}	77.56±0.78^{bc}	11.96±1.12^{ab}	89.53±1.82^{a}	10.46±1.82^{c}
T6 (1.5 % MGTE)	51.70±0.98^{d}	16.16±0.89^{c}	67.86±0.94^{d}	14.33±1.10^{a}	82.20±1.68^{b}	17.80±1.68^{b}
			In post-equilibrated semen			
C (Control)	50.13±0.73^{c}	13.80±0.56^{c}	63.93±0.60^{d}	20.06±0.71^{b}	84.00±1.27	16.00±1.29
T1 (0.5 % AGTE)	45.06±0.87^{e}	21.80±0.26^{a}	66.86±1.10^{c}	19.36±1.26^{bc}	86.23±1.80	13.76±1.80
T2 (1.0 % AGTE)	53.56±0.91^{ab}	13.03±0.29^{d}	66.60±0.62^{c}	19.76±0.61^{b}	86.36±1.23	13.63±1.23
T3 (1.5 % AGTE)	49.06±0.67^{d}	16.66±0.17^{b}	65.73±0.69^{cd}	17.56±0.89^{c}	83.30±1.37	16.70±1.37
T4 (0.5 % MGTE)	52.30±0.55^{b}	21.16±0.53^{a}	73.46±1.09^{a}	13.26±1.20^{d}	86.73±1.69	13.26±1.69
T5 (1.0 % MGTE)	55.16±0.98^{a}	15.60±1.65^{bc}	70.76±0.67^{b}	12.40±0.65^{d}	83.16±1.32	16.83±1.32
T6 (1.5 % MGTE)	45.36±1.11^{e}	15.96±0.08^{bc}	61.33±1.03^{e}	24.76±0.76^{a}	86.10±1.65	13.90±1.65

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

Sperm kinetic parameters:

Results of sperm kinetic parameters in post-diluted semen illustrated in Table 3 cleared that VCL, VSL, VAP, and LIN were significantly (P<0.05) the highest by T6 and the lowest by T1 and T4 as compared to free-extenders (C). Treatment extenders including T2, T3, and T5 significantly (P<0.05) increased VCL and VAP, while did not affect significantly on VSL and LIN in comparing with free-extender.

However, STR and WOB were not affected significantly by type of supplementation.

In post-equilibrated semen, surprise to found that VCL, VSL, and VAP were maintained by T5 as in free-extender (C), being significantly (P<0.05) higher in free-extender (C) and T5 than in other treatment extenders (T1, T2, T3, T4, and T6), being the lowest in T4. However, LIN, STR, and WOB were not affected significantly by type of supplementation (Table 3).

Table 3. Effect of supplementing Tris-extender with aqueous or methanolic extract of green tea on kinetic sperm parameters in post-diluted and post-equilibrated ram semen.

Item	Sperm kinetic parameters					
	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)
			In post-diluted semen			
C (Control)	77.93±0.69^{c}	38.26±0.66^{b}	59.86±0.61^{c}	49.26±0.66^{ab}	64.26±0.88	77.16±0.72
T1 (0.5 % AGTE)	66.86±0.66^{d}	31.96±0.77^{c}	50.40±0.60^{d}	47.86±0.63^{c}	63.23±0.63	75.96±0.64
T2 (1.0 % AGTE)	81.40±0.65^{b}	40.46±0.86^{ab}	62.80±0.75^{b}	49.96±0.56^{ab}	64.80±0.57	77.46±0.82
T3 (1.5 % AGTE)	81.36±0.71^{b}	40.26±0.95^{ab}	62.86±0.63^{b}	49.76±0.72^{ab}	64.46±0.74	77.53±0.91
T4 (0.5 % MGTE)	62.26±0.66^{e}	29.36±0.63^{d}	46.76±0.92^{e}	47.26±0.91^{c}	62.96±0.97	75.23±0.66
T5 (1.0 % MGTE)	81.96±0.60^{b}	40.56±0.74^{ab}	63.16±0.64^{b}	49.86±0.85^{ab}	64.46±0.65	77.46±0.82
T6 (1.5 % MGTE)	84.36±0.77^{a}	42.16±0.92^{a}	65.20±0.87^{a}	50.26±0.67^{a}	64.96±0.89	77.86±0.71
			In post-equilibrated semen			
C (Control)	75.66±0.66^{a}	37.06±0.84^{a}	57.85±0.88^{a}	49.25±0.68	64.26±0.95	76.95±0.92
T1 (0.5 % AGTE)	70.73±0.68^{b}	34.16±0.92^{b}	53.73±0.56^{b}	48.47±0.73	63.76±0.84	76.47±0.62
T2 (1.0 % AGTE)	62.56±3.03^{cd}	31.73±0.68^{c}	49.14±0.54^{c}	47.94±0.82	63.90±0.76	75.88±0.71
T3 (1.5 % AGTE)	64.46±0.61^{c}	30.30±0.55^{c}	48.23±0.87^{c}	47.27±0.91	62.86±0.68	75.70±0.69
T4 (0.5 % MGTE)	59.93±0.68^{d}	27.96±0.91^{d}	44.73±0.67^{d}	46.73±0.76	62.73±0.92	74.94±0.82
T5 (1.0 % MGTE)	74.86±0.66^{a}	36.46±0.72^{a}	57.27±0.82^{a}	48.95±0.67	64.06±0.82	76.85±0.58
T6 (1.5 % MGTE)	66.16±0.75^{c}	31.36±0.89^{c}	49.96±0.59^{c}	47.46±0.98	62.96±0.78	75.87±0.79

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

Sperm abnormality (CASA analysis):

Data of morphological sperm abnormality (Table 4) showed insignificant effect of different green tea extract supplementations on normal forms, and head, neck, and tail abnormalities of sperm cells in post-diluted semen.

Normal form of spermatozoa was the highest and neck abnormality was the lowest in T5, while normality and head, neck, and tail abnormalities were lower in treatment extenders (T1, T2, T3, T4, and T6) than in free-extender (C), but these differences were not significant.

In post-equilibrated semen, the highest normal forms of spermatozoa were achieved significantly (P<0.05) by T5 with significantly (P<0.05) lower head, neck, and tail abnormalities than in free-extender (C).

However, T6 showed the significantly (P<0.05) the lowest normal forms and neck abnormality, and the highest tail abnormality as compared to free-extender and other treatment extenders (Table 4).

Table 4. Effect of supplementing Tris-extender with aqueous or methanolic extract of green tea on sperm abnormalities in post-diluted and post-equilibrated ram semen.

Item	Normal forms (%)	Sperm abnormalities (%)		
		Neck	Head	Tail
		In post-diluted semen		
C (Control)	73.86±8.16	24.20±0.65	8.33±3.52	13.30±3.78
T1 (0.5 % AGTE)	59.76±5.20	25.33±0.68	14.80±6.47	14.83±3.34
T2 (1.0 % AGTE)	70.43±8.19	21.73±3.71	16.86±6.44	14.03±2.96
T3 (1.5 % AGTE)	65.03±8.09	23.00±0.65	15.66±6.41	13.06±3.48
T4 (0.5 % MGTE)	78.50±8.50	29.16±6.35	10.10±3.74	16.96±4.73
T5 (1.0 % MGTE)	81.23±8.19	15.00±5.29	26.80±6.33	13.80±3.41
T6 (1.5 % MGTE)	56.13±8.32	21.96±6.40	16.96±6.11	13.46±3.48
		In post-equilibrated		
C (Control)	59.23±0.38^{d}	36.86±0.75^{a}	27.83±0.58^{a}	20.16±1.01^{c}
T1 (0.5 % AGTE)	67.36±0.71^{b}	34.00±0.86^{b}	20.06±0.71^{c}	23.16±0.41^{b}
T2 (1.0 % AGTE)	59.73±0.68^{d}	19.46±0.92^{e}	20.20±0.95^{c}	17.23±1.18^{d}
T3 (1.5 % AGTE)	58.10±0.95^{d}	23.23±0.57^{d}	28.23±0.88^{a}	27.06±0.58^{a}
T4 (0.5 % MGTE)	63.10±0.68^{c}	22.50±0.67^{d}	9.30±0.35^{e}	18.66±0.71^{cd}
T5 (1.0 % MGTE)	73.06±0.77^{a}	27.46±0.58^{c}	14.06±0.66^{d}	19.13±0.69^{cd}
T6 (1.5 % MGTE)	54.13±0.90^{e}	21.50±0.94^{d}	25.10±0.76^{b}	27.16±0.55^{a}

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

Discussion

The large concentration of polyunsaturated fatty acids in plasma membranes of ram spermatozoa may be inadequate to avoid lipid peroxidation during dilution and cooling for semen preservation (Bucak *et al*., 2013). Exposing the semen to cold shock and atmospheric oxygen during cooling raise up the level of lipid peroxidation (Bucak *et al*., 2012). Therefore, oxidative stress produced during dilution and equilibration due to high reactive oxygen species (ROS) is associated with low quality of seminal materials and death of sperm cells with abnormal morphology, thus resulting in a predominant impairment for profitable semen preservation (Bailey *et al*., 2000). Excess ROS has been shown to decrease sperm function (De Lamirande *et al*., 1997), and preservation exacerbates the imbalance between the semen's endogenous antioxidant system and the presence of ROS. This study aimed to evaluate the effect of the supplementing semen extender with different levels of aqueous or methanolic green tea extract at levels of 0.5, 1.0 and 1.5 % on the motility, livability, normality, plasma membrane integrity, and kinetic parameters of ram spermatozoa in diluted and equilibrated semen. The obtained results indicated beneficial effects of supplementing Tris-extender of ram semen with 1% AGTE or 1% MGTE in post-diluted semen and only for T5 in post-equilibrated semen (after cooling the diluted semen at 5ºC for 4 hours) to improve the visual sperm characteristics (progressive motility, livability, abnormality, and membrane integrity) and different types of motility (rapid, slow, total progressive, total motility). In addition, T5 increased sperm normality after dilution and equilibration by decreasing sperm abnormalities in head, neck, or tail. Motion analysis on quality assessment of semen samples is of great importance for the positive association with male fertility and because it is in one of the most affected parameters after cryopreservation. However, sperm tracking is quite complex due to cell collision, occlusion and missed detection. Because motility allows spermatozoa to migrate from their source of introduction to the site of fertilization, it is an important criterion to consider when evaluating sperm for AI (Pereira *et al*., 2017). It is a necessary parameter for demonstrating sperm function. This conclusion is consistent with earlier research that demonstrated the use of antioxidants to maintain motility 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 peroxidation (Aitken and Fisher, 1994). In accordance with the obtained results, Wittayarat *et al*. (2013) found that the inclusion of green tea polyphenols into the extender has demonstrated a strong protective impact on motility and viability of dog spermatozoa in chilled semen for up to four weeks. Also, several authors reported that green tea extract can increase motility in swine sperm (Park and Yu, 2015) and human sperm (De Amicis *et al*., 2012) at low concentrations. Furthermore, green tea supplementation increased motility and membrane integrity in bull sperm cryopreservation (Ali *et al*., 2014; Khan *et al*., 2017).

The assessment of plasma membrane integrity is an essential metric for structural and functional aspects, and it is connected to *in vivo* fertility in buffalo bull spermatozoa (Ahmed *et al*., 2016). sperm plasma membrane. Spermatozoa's plasma membrane shields organelles from mechanical damage and serves as a filter for the interchange of intracellular and extracellular substances. The plasma membrane's integrity is critical for spermatozoa because it impacts the metabolism associated with motility and viability. In agreement with our results, MGTE supplementation at 1% increased sperm plasma membrane integrity substantially more than control (Ahmed *et al*., 2019). A beneficial impact of plant extract during stages of semen preservation was demonstrated in term of an improvement in membrane integrity when rosemary extract was added to pig sperm. However, when natural chemicals are employed, it is necessary to standardize the extraction procedure before suggesting their usage as a freeze extender (Malo *et al*., 2011). Despite the positive impacts observed on motility, livability, and acrosome and membrane integrities. According to Gale *et al.* (2015), adding green tea extract to the cryo-medium of boar semen extender had no favorable impacts on motility, viability, acrosome integrity, or membrane integrity. Another boar sperm research looked at the toxicity of green tea extract on cooled spermatozoa. Although no harmful impact was identified, there was no difference in sperm quality metrics between the control and varied amounts of green tea extract supplementation (Parkand Yu, 2015). Furthermore, in multiple investigations on other species, the incorporation of natural antioxidants had no favorable impact (González *et al*., 2010; Gadea *et al*., 2011). However, in chilled dog sperm, the inclusion of green tea polyphenols into the extender has demonstrated a strong protective impact on motility and viability measures for up to four weeks (Wittayarat *et al*., 2013).

In the recent decades, CASA is considered as one of the newest techniques for semen evaluation (Hoogewijs *et al*., 2012; Amann and Waberski, 2014). Regarding CASA parameters, the present results in our study revealed positive impact of 1% MGTE on different types of sperm motility, especially rapid, slow, and total progressive motility. In this respect, Younan *et al.* (2021) found positive correlation between progressive motility and sperm fertility in rabbits ($r=0.811$, $P<0.01$ with pregnancy and $r=0.898$, $P<0.001$ with prolificacy. Results of CASA analysis indicated positive effect of 1.0 and 1.5% AGTE as well as 1.0 and 1.5% MGTE on most of kinetic sperm parameters (VCL, VSL, VAP, and LIN) in post-diluted semen, being the maximum for 1.5% MGTE. This finding may indicate impact of increasing level of AGTE or MGTE on sperm velocity as compared to the lower levels of each extender. However, no pronounced effects were found for different types of extracts on increasing sperm kinetic parameters after cooling the diluted semen at 5ºC for 4 hours. Contrary, all levels of AGTE and the lowest or the highest levels than 1% MGTE than 1% showed negative effect on sperm velocity in equilibrated semen. In accordance with the obtained results of parameters of sperm dynamics, some authors reported similar results on rabbit semen (Safaa *et al*., 2008; Błaszczyk *et al*., 2013).

Sperm functional impairment occurs by ROS attack, as the direct result of damage in sperm oxidative defense system resulting in the reduction of motility, mitochondria activity, deterioration of plasma membrane integrity and induction of sperm apoptosis, causing subsequently negative impact on *in vivo* fertilizing ability (Bucak *et al*.,

2013; Mata-Campuzano *et al*., 2014; Bucak *et al*., 2015; Sarıözkan *et al*., 2015). The observed impact of green tea extracts at a level below1% indicated insufficient level of 0.5% from both extracts either aqueous or methanolic. However, the impaired effect of the level higher than 1% may be associated with higher scavenging ability of this dose to decrease ROS (malondialdhyde) to minimal levels des despite a limited production of ROS in spermatozoa is needed to some normal physiological functions like sperm capacitation, hyperactivation and acrosome reaction (Aitken, 1995). Spermatozoa are incapable of re-synthesizing its membrane component due to which there is always a risk of ROS (Alvarez and Storey, 1989). Lower concentrations of ROS showed a stimulatory role in sperm capacitation (Rivlin *et al*., 2004), acrosome reaction and sperm–oocyte fusion (Cocuzza *et al*., 2007). However, at higher concentration, ROS behaved as a genotoxic agent that is believed to deteriorate semen quality (motility and plasma/ acrosome integrities) in human (Baumber *et al*., 2000; Bilodeau *et al*., 2001), ram (Peris *et al*., 2007) and bovine (Hu *et al*., 2010). Green tea (Camellia sinensis) is regarded as a dietary source of antioxidant compounds, chiefly comprising polyphenolic components like (Epicatechin Epicatechin gallate, Epicatechin-3-gallate, Epigallocatechin gallate, Epigallocatechin) (Gündüz and Özdemir, 2014; Rahman *et al*., 2018) which have shown high free radical scavenging activity (Nakagawa and Yokozawa, 2002; Michalak, 2006), anthocyanins, gallic acid derivatives, tannins, vitamin C, vitamin E and carotenoids, minerals (Selenium and Zinc) having physiological significance (Hashim *et al*., 2016), tea polyphenols (catechines, flavanols, flavanones, phenolic acids, glycosides and the aglycons of plant pigments) tea caffeine, amino acids and saponins (Pawlowska *et al*., 2006). Green tea extract (GTE) has beneficial effects on semen quality in boar (Mehdipour *et al*., 2016), canine (Wittayarat *et al*., 2013), bovine (Khan *et al*., 2017), mouse (Abshenas *et al*., 2011), Rooster (Al-Daraji, 2011) and dog (Wittayarat *et al*., 2013) semen. It has been reported that green tea has advantageous effects on canine sperm quality during long-term liquid storage at 5 °C (Wittayarat *et al*., 2013), and prevent oxidative damage to boar sperm at freezing (Gale *et al*., 2015). The superiority of MGTE than AGTE may be due to that methanol had been reported to be the most suitable solvent for extracting phenolic compounds from fresh young shoots of tea, compared with chloroform, ethyl acetate and water (Yao *et al*., 2006). Hot-water extraction of microwaved green tea resulted in a significantly lower TPC and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging than did methanol extraction. However, ferric-reducing antioxidant power (FRAP) and ferrous-ion chelating (FIC) abilities were similar for both methods of extraction. Methanol appears to be a more efficient solvent than is hot water (Chan *et al*., 2007). Yao *et al*. (2004) also reported that hot water extracted less catechins from tea than methanol. However, after repeated extraction, both solvents yielded similar amounts of polyphenols.

It is possible to infer that the inclusion of methanolic green tea extract at a level of 1% in semen extender had a substantial effect on sperm quality, normality, function, and velocity of ram spermatozoa in Rahmani semen after dilution and equilibration for 4 h at 5ºC.

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تأثير مستخلص الشاي الأخضر المائى و الميثانولى على حيوية و الشكل الطبيعى ومقاييس الحركة فى السائل المنوى للكباش الرحمانى بعد الموازنة على درجة حرارة ٥ درجة مئوية لمدة اربعة ساعات

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مستخلص الشاي الأخضر هو أحد مضادات الأكسدة الطبيعية المشتقة من النباتات. كان الغرض من هذه الدراسة هو معرفة تأثير اضافة مستويات مختلفة من مستخلص الشاي الأخضر المائي أو الميثانولي الى (٠,٥ – ١,٠ – ١,٥) مخفف السائل المنوي (ترس - سيتريك - ليثيسين فول الصويا) على الحركة و الحيوية و الشواذ و سلامة الغشاء البلازمى ومقاييس الحركة للحيوانات المنوية للسائل المنوي للكباش الرحمانى بعد التخفيف وبعد الموازنة. تم جمع السائل المنوي من ٥ كباش مرة في الأسبوع لمدة ٧ أسابيع باستخدام مهبل صناعي. أظهرت النتائج تأثيراً إيجابياً للمخفف الذى يحتوى على ١٪ مستخلص الشاي الأخضر الميثانولي على الحركة و الحيوية و الشواذ وسلامة الغشاء البلازمى والحركة السريعة وسرعة الحيوانات المنوية والشكل الطبيعي في السائل المنوي بعد الموازنة. يمكن الاستدلال على أن إضافة مستخلص الشاي الأخضر الميثانولي بمستوى ١٪ في مخفف السائل المنوي كان له تأثير كبير على جودة الحيوانات المنوية وطبيعتها ووظيفتها وسرعتها في السائل المنوي للكباش الرحماني بعد التخفيف والموازنة لمدة ٤ ساعات عند ٥ درجة مئوية.