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Research Article

Visual Examination and Computer Assessed Sperm Analysis (Casa) of Goat Semen Diluted by Tris-Extender Supplemented with Aqueous Extract of *Moringa Oleifera* Leaves

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Abstract:

The aim of the current study was to assess the impacts of aqueous extract of Moringa oleifera leaves (MOLE) on semen quality and sperm freezing ability of bucks. 5 sexually mature Damascus bucks (65-75 kg live body weight, 2-4 years of age) were used for collecting semen once per week for seven weeks. Only semen with a mass motility of ≥ 70% was pooled. Pooled semen had been divided into 4 aliquots, the free extender (C=control, no MOLE) and 3 extenders supplemented with MOLE at levels of (100, 200, and 300 µl/mL) as an antioxidant. Tris-citric acid-extender was used to dilute semen at a rate of 1:20 using different MOLE levels, then chilled to 5 °C for two hours (equilibration period), placed into 0.25 mL straws and finally kept in liquid nitrogen at -196 oC for two weeks. After dilution, equilibration, and thawing, semen was assessed visually and via CASA. The results showed that MOLE, at a level of 300 µl/ml, had a beneficial effect on all sperm characteristics, all types of sperm motility, and normal forms assisted by CASA in post-diluted semen, after equilibration and thawing. The highest MOLE level (300 µl/ml) considerably improved the sperm kinetic indexes and all sperm velocity types (VCL, VSL, and VAP). In conclusion, Moringa oleifera leaves aqueous extract (300 µl) in semen extender of bucks is good tool for improving semen quality and sperm freezing ability of bucks.

1. Introduction

Motility, livability and plasma membrane integrity are crucial for semen cryopreservation which determines the success of artificial insemination (AI) with frozen semen (Wang et al., 2015). Measurement of sperm functional indicators i.e. sperm with potential fertilizing capacity of the cryopreserved processed semen (Huynh et al., 2000; Rodriguez-Martinez, 2003; Rodríguez-Martínez, 2006).

Dilution, equilibration, and freezing are the main processes for semen cryopreservation used for AI. Cooling and freezing processes cause cold shock and reactive oxygen species (ROS) production during semen cryopreservation (Holt et al., 1992; Holt and North, 1994). Increasing pro-oxidant activities increase ROS, and oxidation of polyunsaturated fatty acids in sperm membranes then induce lipid peroxides (Del Olmo et al., 2015). Lipid peroxidation during cryopreservation resulted in a reduction in motility, respiratory function, enzyme activity, DNA damage of spermatozoa (White, 1993). The strength of antioxidants was reduced during cryopreservation (Bilodeau et al., 2000) to be not sufficient to defend sperm cells against oxidative stress (Nichi et al., 2006). Antioxidants supplementation to the semen extender helps in improving sperm cryopreservation techniques and prevent ROS and lipid peroxidation (LPO) by protecting sperm cells from membrane damage, which can assist in retain the characteristics and function of cryopreserved sperm cells (Budai, 2014). Numerous natural antioxidant compounds have been demonstrated in recent years to shield sperm from the damaging effects of ROS and enhance post-thaw sperm motility, viability, and fertility (Atessahin et al., 2008; Anel-López et al., 2015; Sarıözkan et al., 2015). Plant-derived antioxidants are currently receiving a lot of attention since they exhibit reduced cytotoxicity and are thought to be superior than synthetic antioxidants (Gupta and Sharma, 2006; Nagulendran et al., 2007; Sen et al., 2010; Ibrahim et al., 2014). Several plant-derived compounds, most notably carotenoids and flavonoids, have been investigated for their antioxidant potential to increase fertility as components of in vitro culture medium (Moretti et al., 2016). Some researchers have revealed the beneficial effects of herbal antioxidants derived from medicinal plant species (Kulisic et al., 2005; El-Nekeety et al., 2011; Roby et al., 2013). *Moringa oleifera* (MO) is a plant contains polyphenols, tannins, anthocyanins, glycosides, and thiocarbamates. It is considered as a fodder crop in dry season (Nouman et al., 2013). The compounds in MO leaves appear to have antioxidant activity by scavenging the free radicals and increasing the activity of antioxidant enzyme (Luqman et al., 2012). Significant protection against oxidative stress is provided by water extract of MO leaves, which has powerful antioxidant activity

against free radicals, prevents oxidative damage for important biomolecules, and prevents oxidative stress (Chumark et al., 2008; Sreelatha and Padma, 2009). Experimental proof of MO's as a natural antioxidant had ability to shield cells and organisms from oxidative DNA damage has been published. MO leaves total antioxidant potential may be due to the presence of poly-phenolic substances (Singh et al., 2009; Sreelatha and Padma, 2009).

Therefore, the aim of the present study was to evaluate the impacts of aqueous extract of *Moringa oleifera* leaves on semen quality and sperm freezing ability in bucks.

2. Materials and Methods

This study was carried out in frame of the scientific cooperation between Animal Production Research Institute (APRI), Agricultural Research Center and Animal Production Department, Tanta University, Egypt during the period from January to April 2022.

Moringa oleifera leaf extract preparation

Fresh MO leaves were gathered, dried for 48 hours in the shaded air then for 2 hours at 40°C in the oven, and finally pulverized using an electric household blender (Ramluckan et al., 2014). In a flask, 300 g of finely ground MOL was added to 1.5 L of distilled water. The resulted mixture was completely homogenized, sieved through cheese cloth, and then filtered through Whatman filter paper (24 cm). The resulting filtrate was kept at -20°C in the freezer until use (Ojo and Abdurahman, 2017).

Animals:

As semen donors, five sexually mature Damascus bucks were kept at Animal Production Research Station, Sakha, Kafrelsheikh Governorate, belonging to APRI. Bucks were (65-75 kg live body weight, 2-4 years of age), were raised in the same environment, and given a free access to drinking water, trace mineralized salt lick blocks, and concentrate feed mixture (CFM) at a level of 1 kg (14% CP) and 0.750 kg of Berseem hay.

Semen collection

Semen ejaculates were collected consistently prior to morning feeding collected once per week at 7-8 a.m. for 7 weeks using an artificial vagina, then taken to the lab immediately and put in a 37 °C water bath. Only semen with a mass motility of ≥ 70% was pooled.

Experimental extenders

Tris-citric-glucose-soybean lecithin extender containing Tris (3.025 g), citric acid monohydrate (1.66 g,), glucose (1.25 g), 1% soybean lecithin, 5% glycerol, penicillin 100 IU/mL, and streptomycin (100 µg/mL) was used as a based-extender (Sigma Chemical Co., St. Louis, MO, USA). The collected pooled semen was divided into 4 aliquots including free extender (C= control, no MOLE) and extenders supplementing with MOLE, as an antioxidant, at levels (100, 200, and 300 µl/mL).

Before usage, the extenders were given a gentle shake and warmed in a 37 °C water bath. Before the addition of MOLE, the osmolarity and pH were measured and set to a range of 6.8–7.2 (PH/mV Temperature Meter, Jenway 3510, Jenway, Staffordshire, UK) and 280–300 mOsmol (Micro-Osmometer, Loser Type 6, Löser Messtechnik, Berlin, Germany) respectively.

Semen freezing

Pooled semen was diluted at a ratio of 1:20 (semen/extender), chilled to 5 °C for 2 hours (equilibration period), then put into 0.25 mL straws. Straws were exposed to vapor of liquid nitrogen (LN) for 10 min, then inserted in LN (-196oC) for one week at least.

Semen evaluation

A coverslip was placed over a 10 µL portion of diluted semen that was placed on a heated slide in order to measure progressive motility percentage. The number of spermatozoa in five fields with 200 sperm cells each was counted in a long semi-arc pattern. The phase-contrast microscope (DM 500; Leica, Switzerland) was equipped with a heated stage set at 37 °C. The three repetitions of the blind analysis were carried out by the same expert investigator.

Livability was measured according to (Moskovtsev and Librach, 2013). Dual staining was used to color a smear of the diluted semen sample on a glass slide (5% eosin and 10% nigrosin). A light microscope (Leica DM 500; Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) was used to analyze 200 sperm cells from each sample at a magnification of 400, dead (red-stained) or alive (unstained). Furthermore, the morphological abnormalities of the spermatozoa (namely, abnormalities of the head, tail, and cytoplasmic droplets) were identified (Menon et al., 2011).

The functional plasma membrane of spermatozoa was assessed using the hypo-osmotic swelling test (HOS-t) (Neild et al., 1999). 10 µL of diluted semen were combined with about 100 µL of a hypo-osmotic solution (6.75 g/L fructose and 3.67 g/L sodium citrate, at an osmolality level of 75 mOsmol/L), which was then incubated for 30 min at 37 °C. A microscope slide was then mounted with a coverslip after 10 µL of the mixture was added to it. Swollen and coiled tails were examined using phase-contrast microscopyat 400x magnification on a field of 300 spermatozoa (from each sample).

CASA analysis

CASA analysis (SPERMOLAB®, Cairo, Egypt) was performed to evaluate different sperm motility, normality, and kinetic parameters of sperm cells in semen post dilution, equilibration, and thawing. Semen sample (5 µL) extended with different MOLE levels was placed into a pre-warmed dis-posable Leja slide. The material was let to settle on the mini-thermal heating stage (38 °C) prior to analysis. About 200 spermatozoa from two to three drops of each specimen were assessed and the results of CASA analysis were completed as the following:

Percentages of different types of sperm motility (total, progressive, non-progressive, and immotility). Also, sperm velocity parameters (Curve linear, VCL; straight linear, VSL; average path, VAP) and kinetic ratios (Linearity, LIN; Straightness, STR; Wobble, WOB) were analyzed.

Statistical analysis

A software program (SAS, 2007) was used to statistically evaluate the acquired data using a one-way ANOVA design. The Duncan's multiple range test (Duncan, 1955), with a P<0.05, was used to determine the significant differences between groups.

3. Results

3.1. Visual sperm parameters

3.1.1. In post-diluted semen

Supplementation of MOLE to semen extender significantly (P<0.05) affect the percentages of progressive motility (PM), sperm livability (SL), and membrane integrity (MI), while did not affect abnormal sperm (AS) percentage. The differences in PM and SL between semen supplemented with all levels of MOLE and free-semen were not significant. Meanwhile, sperm membrane integrity showed significantly (P<0.05) the highest percentage in semen supplemented MOLE (300 µl) as compared to free-semen (Table 1).

Table 1. Sperm parameters in buck semen after dilution as affected by MOLE.

Treatment	Sperm characteristics (%)			
	Progressive motility (PM)	Livability (SL)	Abnormality (AS)	Membrane integrity (MI)
Control	83.33±1.66^{ab}	84.00±1.52^{ab}	10.33±1.20	82.33±0.33^{b}
T1 (100 µl MOLE)	80.00±2.88^{b}	80.66±1.85^{b}	12.00±0.57	80.00±2.08^{b}
T2 (200 µl MOLE)	80.00±1.10^{b}	80.66±1.33^{b}	12.33±1.45	82.33±0.88^{b}
T3 (300 µl MOLE)	88.33±1.66^{a}	89.00±1.52^{a}	9.00±1.15	86.66±0.66^{a}
P-value	0.0402	0.0170	0.2186	0.0244

a and b: Significant treatment differences in each column at P<0.05.

3.1.2. In post-equilibrated semen

All sperm parameters after equilibrating were affected by MOLE (Table 2). PM, SL, AS, and MI percentages were significantly (P<0.05) improved only by 300 µl- MOLE supplementation as compared to control treatment.

3.1.3. In post-thawed semen

As recorded in Table 3, there is a significant effect for MOLE on all sperm characteristics studied in post-thawed semen. Sperm characteristics including progressive motility, livability, and membrane integrity were significantly increased, while sperm abnormality decreased only by MOLE supplementation (300 µl) in comparing with control and other MOLE treatments. Sperm livability, abnormality, and membrane integrity were significantly improved by other levels of MOLE as compared to control, but still to be significantly lower than the highest MOLE level (300 µl).

Table 2. Sperm parameters in buck semen after equilibration as affected by MOLE.

Treatment	Sperm characteristics (%)			
	Progressive motility (PM)	Livability (SL)	Abnormality (AS)	Membrane integrity (MI)
Control	70.00±2.88^{b}	70.66±1.85^{b}	23.66±1.20^{a}	70.33±2.33^{b}
T1 (100 µl MOLE)	75.00±1.48^{b}	75.33±2.18^{b}	22.33±2.60^{a}	75.33±2.18^{b}
T2 (200 µl MOLE)	73.33±1.48^{b}	73.66±1.20^{b}	19.66±0.66^{ab}	74.66±1.33^{b}
T3 (300 µl MOLE)	83.33±1.66^{a}	83.66±1.45^{a}	14.66±0.88^{b}	82.33±1.20^{a}
P-value	0.0210	0.0038	0.0142	0.0110

a and b: Significant treatment differences in each column at P<0.05.

Table 3. Sperm parameters in buck semen after thawing as affected by MOLE.

Treatment	Progressive motility (PM)	Livability (SL)	Abnormality (AS)	Membrane integrity (MI)
Control	40.00±2.88^{b}	40.33±1.76^{c}	37.66±1.20^{a}	38.66±2.84^{c}
T1 (100 µl MOLE)	46.66±1.66^{b}	47.00±1.00^{b}	32.66±1.60^{bc}	46.00±1.52^{b}
T2 (200 µl MOLE)	45.00±1.01^{b}	45.33±2.96^{b}	33.35±0.88^{b}	44.33±2.40^{b}
T3 (300 µl MOLE)	55.00±0.96^{a}	55.00±0.57^{a}	29.00±0.57^{c}	53.00±1.15^{a}
P-value	0.0086	0.0031	0.0052	0.0088

a, b and c: Significant treatment differences in each column at P<0.05.

3.2. Sperm motility parameters by CASA:

3.2.1. In post-diluted semen

Results in Table 4 showed that the effect of MOLE on percentages of all sperm motility types in post-diluted semen was significant. Supplementation of extender with MOLE at a level of 300 µl significantly increased progressive and total motility of sperm cells to the maximal values and significantly decreased non-progressive motility and immotility percentages to the minimal values as compared to control treatment.

Table 4. Sperm motility types in diluted buck semen extended with MOLE.

Treatment	Motility type (%)			
	Progressive motility (PM)	Non-progressive motility (NPM)	Total motility (TM)	Immotility (IM)
Control	67.86±0.08^{d}	11.33±0.66^{b}	79.20±0.57^{b}	20.80±0.52^{b}
T1 (100 µl MOLE)	61.70±0.23^{c}	13.56±0.52^{a}	75.26±0.43^{c}	24.74±0.43^{a}
T2 (200 µl MOLE)	73.16±0.58^{b}	11.13±0.69^{b}	84.30±0.92^{a}	15.70±0.92^{c}
T3 (300 µl MOLE)	74.66±0.33^{a}	9.90±0.49^{b}	84.56±0.80^{a}	15.44±0.80^{c}
P-value	<0.0001	0.0156	<0.0001	<0.0001

a, b, c and d: Significant treatment differences in each column at P<0.05.

3.2.2. In post-equilibrated semen

The effect of MOLE on percentages of all sperm motility types in post-equilibrated semen was significant (Table 5). As found in post-diluted semen, progressive and total motility of sperm cells were significantly the highest, while non-progressive motility and immotility percentages were significantly the lowest in post-equilibrated semen extended with MOLE at a level of 300 µl as compared to other MOLE levels or control treatment.

Table 5. Sperm motility in equilibrated buck semen extended with MOLE as an antioxidant using CASA.

Treatment	Progressive motility (PM)	Non-progressive motility (NPM)	Total motility (TM)	Immotility (IM)
Control	63.26±0.31^{bc}	19.40±0.71^{b}	82.66±1.27^{ab}	17.33±1.29^{bc}
T1 (100 µl MOLE)	64.43±0.61^{b}	13.80±1.26^{d}	78.23±1.80^{c}	21.76±1.80^{a}
T2 (200 µl MOLE)	62.20±0.57^{c}	20.80±0.61^{a}	81.43±1.37^{b}	18.56±1.37^{b}
T3 (300 µl MOLE)	66.13±0.38^{a}	15.30±0.89^{c}	83.00±1.23^{a}	17.00±1.23^{c}
P-value	0.0007	<0.0001	0.0002	0.0002

a, b,c and d: Significant treatment differences in each column at P<0.05.

3.2.3. In post-thawed semen

As indicated in post-diluted and post-equilibrated semen, all sperm characteristics studied in post-thawed semen were significantly improved by all MOLE levels, but supplementation of MOLE at a level of 300 µl showed significantly the highest positive impacts (Table 6).

3.3. Morphological sperm abnormality by CASA

The effect of MOLE on percentages of normal forms was significant in post-equilibrated and post-thawed semen (Table 7), whereas, percentage of normal forms was significantly improved by all MOLE levels, being the highest in semen extended with MOLE at a level of 300 µl as compared to other MOLE levels and control treatment.

Table 6. Sperm motility in post-thawed buck semen extended with MOLE as an antioxidant using CASA.

Treatment	Progressive motility (PM)	Non-progressive motility (NPM)	Total motility (TM)	Immotility (IM)
Control	40.26±0.12^{c}	8.03±0.16^{b}	48.30±0.57^{d}	51.70±0.57^{a}
T1 (100 µl MOLE)	45.53±0.57^{b}	10.06±0.55^{a}	55.60±0.71^{c}	44.40±0.71^{b}
T2 (200 µl MOLE)	46.36±0.06^{b}	9.80±0.57^{a}	56.16±0.12^{b}	43.83±0.12^{c}
T3 (300 µl MOLE)	48.50±0.23^{a}	6.30±0.30^{c}	56.80±0.15^{a}	43.20±0.15^{d}
P-value	<0.0001	0.0045	<0.0001	<0.0001

a, …..d: Significant treatment differences in each column at P<0.05.

Table 7. Sperm with normal forms in diluted, equilibrated and post-thawing buck semen extended with MOLE using CASA.

Treatment	Normal forms		
	Post-dilution	Post-equilibrated	Post-thawing
Control	71.16±8.81	50.56±0.38^{d}	42.33±1.20^{c}
T1 (100 µl MOLE)	68.16±5.20	56.70±0.71^{c}	47.33±1.66^{b}
T2 (200 µl MOLE)	66.56±8.19	58.06±0.68^{b}	46.66±0.88^{b}
T3 (300 µl MOLE)	72.66±8.09	63.10±0.77^{a}	51.00±0.57^{a}
P-value	0.4248	<0.0001	0.0052

3.4. Sperm kinetics parameters by CASA

3.4.1. In post-diluted semen

The effect of MOLE on sperm velocity (VCL, VSL, and VAP) and sperm kinetic index (LIN) in post-diluted semen was significant, however, kinetic indexes (WOB and STR) were not affected significantly by MOLE treatment (Table 8). Sperm velocity in terms of VCL, VSL, and VAP and LIN index were significantly increased by the highest MOLE level (300 µl), and decreased by other MOLE levels, as compared to control.

Table 8. Average of dynamic sperm characteristics in diluted buck semen extended with MOLE.

Treatment	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	WOB (%)	STR (%)
Control	77.3±0.14^{b}	37.6±0.57^{b}	59.2±0.61^{b}	48.6±0.66^{b}	76.5±0.72	63.6±0.88
T1 (100 µl MOLE)	64.3±0.05^{d}	40.4±0.54^{a}	48.3±0.60^{d}	47.3±0.63^{c}	75.2±0.64	63.1±0.63
T2 (200 µl MOLE)	68.3±0.05^{c}	32.4±0.86^{c}	51.7±0.75^{c}	47.4±0.56^{c}	75.6±0.82	62.6±0.57
T3 (300 µl MOLE)	79.4±0.71^{a}	39.1±0.95^{a}	61.8±0.63^{a}	51.2±0.72^{a}	76.7±0.91	64.2±0.74
P-value	<0.0001	<0.0001	<0.0001	<0.0001	0.104	0.094

a, b, c and d: Significant treatment differences in each column at P<0.05.

LIN=VSL/VCL, WOB=VAP/VCL, STR=VSL/VAP

3.4.2. In post-equilibrated semen

The effect of MOLE on sperm velocity (VCL, VSL, and VAP) in post-equilibrated semen was significant, however, kinetic indexes (LIN, WOB and STR) were not affected significantly by MOLE treatment (Table 9). Sperm velocity including VCL, VSL, and VAP were significantly increased by the highest MOLE level (300 µl), and decreased by other MOLE levels, as compared to control.

Table 9. Average of dynamic sperm characteristics in equilibrated buck semen extended with MOLE.

Treatment	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	WOB (%)	STR (%)
Control	74.1±0.61^{b}	35.8±0.55^{b}	56.3±0.87^{b}	48.4±0.41	76.2±0.69	63.5±0.68
T1 (100 µl MOLE)	68.1±0.68^{d}	32.5±0.92^{d}	51.3±0.56^{d}	47.8±0.53	75.5±0.62	63.3±0.84
T2 (200 µl MOLE)	73.2±3.03^{c}	35.5±0.68^{c}	55.7±0.54^{c}	48.5±0.62	76.1±0.71	63.7±0.76
T3 (300 µl MOLE)	75.0±0.66^{a}	36.4±0.84^{a}	57.2±0.88^{a}	48.6±0.58	76.3±0.92	63.6±0.95
P-value	<.0001	<.0001	<.0001	0.101	0.082	0.166

a, b and c: Significant treatment differences in each column at P<0.05.

LIN=VSL/VCL, WOB=VAP/VCL, STR=VSL/VAP

3.4.3. In post-thawed semen

The effect of MOLE on sperm velocity (VCL, VSL, and VAP) and sperm kinetic indexes (LIN, WOB and STR) in post-thawed semen was significant (Table 10). Sperm velocity and kinetic indexes were significantly improved by all MOLE levels. MOLE level at 300 µl showed significantly the highest values in comparing with other MOLE levels and control treatment.

Table 10. Average of dynamic sperm characteristics in post-thawed buck semen extended with MOLE.

Treatment	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	WOB (%)	STR (%)
Control	47.6±0.57^{d}	19.7±0.68^{a}	34.5±0.72^{c}	41.2±0.78^{c}	72.6±0.75^{c}	57.5±0.66^{c}
T1 (100 µl MOLE)	63.3±0.33^{b}	19.7±0.76^{a}	47.5±0.80^{b}	46.9±0.71^{b}	74.9±0.70^{b}	62.7±0.51^{b}
T2 (200 µl MOLE)	61.6±0.60^{c}	18.5±0.76^{b}	46.5±0.73^{b}	46.1±0.51^{b}	74.8±0.28^{b}	61.8±0.76^{b}
T3 (300 µl MOLE)	76.4±0.79^{a}	17.5±0.63^{c}	58.6±0.75^{a}	48.5±0.76^{a}	76.5±0.88^{a}	63.6±0.86^{a}
P-value	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

a, b, c and d: Significant treatment differences in each column at P<0.05.

LIN=VSL/VCL, WOB=VAP/VCL, STR=VSL/VAP

4. Discussion

In the present study, we assumed that MOLE, as a natural antioxidant, added to semen extenders may have positive impacts on buck sperm characteristics during different processes of cryopreservation. We added MOLE to Tris-base-extender at different levels (0, 100, 200, and 300 µl) and evaluated the visual sperm characteristics (manual analysis including PM, SL, AS, and MI) as well as CASA analysis parameters including various types of motilities, morphological normal forms, velocity, and kinetic indexes of buck spermatozoa after dilution, equilibration (at 5ºC for 2h), and thawing (at 37ºC for 15 s).

The obtained results indicated that MOLE at a level of 300 µl showed beneficial effects on visual sperm membrane integrity and all types of sperm motility assisted by CASA in post-diluted semen and also had positive impacts on all sperm characteristics, all types of sperm motility, and normal forms after equilibration and thawing. In addition, all sperm velocity types (VCL, VSL, and VAP) and sperm kinetic indexes were significantly increased by the highest MOLE level (300 µl). Similarly, Wahjuningsih et al. (2019) found significant ($P<0.05$) increase in PM, SL, and MI in semen extended with egg yolk-extender supplemented with MOLE. Addition of 5% MOLE provides the best outcomes when compared to other amounts. The sperm membrane may be kept in place and sperm damage might be prevented because to MOL's antioxidant component. Membrane integrity is crucial for certain changes in membrane components, particularly during fertilization, as well as for metabolism. Because of the loss of cellular components and the inactivation of a critical enzyme for proteins in the acrosome, damage in plasma membrane would result in a loss of sperm motility and the inability to conceive (Nalley and Arifiantini, 2013; Rajashri et al., 2017). The addition of MOLE improved TM and PM of sperm in the Bali bulls (Syarifuddin et al., 2017). There are positive correlations between different sperm motility parameters and fertility (Perumal et al., 2014), and it can be considered as a prediction for fertilizing ability of the sperm cells. Rabbit semen extended with 2 mg MOLE could maintain its quality in term of motility, livability and abnormality when stored for five days at cool temperature (Ghodiah, 2016).

There are three sperm motility patterns that are relevant to CASA parameters: transition groups, non-hyperactivate groups, and hyperactivate groups. Compared to the non-hyperactivated group, the hyperactivate group's fertility rates were more successful. VCL values and LIN percent are necessary for hyperactivate (Susilawati, 2011). In our study all sperm velocity values in buck semen, particularly in post-thawed semen, as progressive motility indicators (VCL, VSL, and VAP) significantly increased while LIN particularly in post-equilibrated, as sperm rigor was not affected by MOLE. Similarly, Syarifuddin et al. (2017) found that MOL supplementation enhanced sperm velocity in Bali bulls (VCL, VSL, and VAP), but reduced sperm LIN. In general, VAP and VCL levels are a good indicator of sperm's capacity for *in vitro* fertilization (Susilawati, 2011). Increasing the values of these parameters in our study have the possibility of producing more fertile sperm.

For each level of MOLE supplementation during cryopreservation, we observed variation in sperm quality before freezing and during thawing. Sperm's irreversibly damaged cell membrane phospholipids and a decline in cell membrane function cause the process to have a negative impact on the quality of the sperm (Ducha et al., 2012). Within sperm frozen, osmotic shock occurred, causing ice crystals to develop in cells and damage the plasma membrane's structure. This might explain why post-thawed semen has reduced PM, SL, and MI. Furthermore, sperm freezing and thawing increases ROS, resulting in impaired sperm function (Wahjuningsih et al., 2019).

The process of freezing and thawing reduces sperm viability, normal morphology, and motility while increasing DNA damage (Asturiano et al., 2007). Since the creation of adenosine triphosphate (ATP) is halted when the environment around sperm drops below zero degrees, sperm cells either hibernate or die as a result of ROS formation, which is a key factor in this event (Meryman, 2007). In addition, the oxidation of membrane lipids by free radicals renders mammalian spermatozoa extremely vulnerable to lipid peroxidation (Aitken et al., 1993). Antioxidants must thus be added to the semen before it is cryopreserved in order to maximize sperm quantity and quality following the thawing procedure (Topraggaleh et al., 2014). It's interesting to note that giving MOLE to bucks helped safeguard and enhance their cryopreserved spermatozoa. Increased post-thawing sperm motility, viability index, and membrane integrity, as well as reduced sperm abnormalities and acrosomal defects (Shokry et al., 2020), were indicated of these findings. Additionally, adding MOLE to the diet reduced the DNA fragmentation of post-thawed spermatozoa from bucks. The robust antioxidant potentials of MOLE, which decreased lipid peroxidation and enhanced antioxidant enzyme activities throughout the cryopreservation process, can be used to explain these favorable effects of MOLE on post-thawed sperms (Shokry et al., 2020). Previous studies reported an improvement in sperm DNA integrity by addition of antioxidants (trehalose and raffinose) in semen of Barki ram's semen (Elshamy et al., 2019), GPx and SOD in dog semen (Chatterjee and Gagnon, 2001), and GSH in Boer buck semen (Rawash et al., 2018).

In several in-vivo studies, Shokry et al. (2020) revealed that MOLE treatment increased the ram's sperm concentration and semen volume. These results corroborated those of earlier research in which MO enhanced the motility, concentration, morphology, and survivability of sperm from rabbits, rats, and goats (Akunna et al., 2012; Priyadarshani and Varma, 2014; Raji and Njidda, 2014; El-Harairy et al., 2016). In the

seminal plasma of rams treated with MOLE, lipid peroxidation was decreased and the antioxidant defense system was improved. Additionally, MOLE raised the ascorbic acid level in seminal plasma (Shokry et al., 2020) and ascorbic acid correlated positively with sperm motility or morphology in ram (Mahsud et al., 2013) and horse (Bucci et al., 2016; Talluri et al., 2017) and with sperm DNA integrity (Sierens et al., 2002). In rams, the powerful capacity of MOLE as antioxidant was credited with increasing the seminal plasma antioxidant defense system. The potent antioxidant components in MOLE included kaempferol, kaempferol malonyl glucoside, kaempferol hexoside, kaempferol-3-O-glucoside, kaempferol-3-O-acetyl-glucoside, cyanidin-3-O-hexoside, ellagic acid, quercetin, quercetin-3-O-glucoside, and apigenin glucoside (Mousa et al., 2019). Thus, these potent antioxidants could reduce the processes of lipid peroxidation and ROS formation during cryopreservation. These results were consistent with earlier research showing that antioxidants such trehalose and raffinose were added to Barki ram's semen (Elshamy et al., 2019), GPx and SOD in dog semen (Chatterjee and Gagnon, 2001), glutathione in Boer buck semen improves DNA integrity of spermatozoa (Rawash et al., 2018). Additionally, adding antioxidants lowers sperm DNA fragmentation while long-term incubation of human spermatozoa with exogenous ROS increases the proportion of sperm DNA fragmentation (Lopes et al., 1998). Flavonoids, saponins, alkaloids, tannins, carotenoids (particularly lutein and carotene), quercetin, and phenol are all antioxidants found in MOLE (Kasolo et al., 2010; Raji and Njidda, 2014).

5. Conclusions

Based on the foregoing results, supplementation of Tris-extender with *Moringa oleifera* leaves aqueous extract (300 µl/ml) is a good supplement for enhancing semen quality and sperm freezing ability of bucks by promoting positive effects on function, membrane integrity, normality, velocity, and kinetic index of cryopreserved buck spermatozoa.

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