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Sperm Kinematics and Morphology of Bali Bull (*Bos javanicus*) after Freezing and Thawing Treated with Green Tea Extract in Extender

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ABSTRACT

Green Tea Extract, or GTE, is a powerful antioxidant that can be added to the extender to maintain the quality of Bali bull semen before and after freezing. This study aimed to evaluate the impact of the GTE addition in the extender on the sperm kinematic and sperm morphology from frozen semen of Bali bull. Five Bali bulls from the NAIC, were used as semen sources. In this study, there were four different treatments: a control (Tris-egg yolk without GTE), a GTE0.05 (100 mL tris egg yolk with 0.05 mg of GTE), a GTE0.10 (100 mL tris egg yolk with 0.10 mg of GTE), and a GTE0.15 (100 mL tris egg yolk with 0.15 mg of GTE). A sperm analyzer (CASA) was used to observe the sperm kinematics and morphology. Compared to the control group, adding different concentrations of GTE to the semen extender did not affect the sperm's morphology. Before freezing, adding GTE0.05 increases total and progressive motility as well as VAP, VCL, and VSL after thawing. GTE0.15 increases STR and ALH. The conclusion is that GTE additions at doses of 0.05 mg/100 mL and 0.15 mg/100 mL can increase sperm kinematics parameters, but sperm morphology is not affected by the GTE.

Keywords: Bali bull; green tea extract; kinematics; livestock and gene bank; sperm cryopreservation

INTRODUCTION

In Indonesia, artificial insemination (AI) has become an important method for breeding and propagating genetically superior Bali bulls (Sonjaya *et al.*, 2021). Long-term storage of superior bull genetic material has been made possible by cryopreservation of semen and artificial insemination with this material (Avdatek *et al.*, 2018). Freshly collected Bali bull semen undergoes additional treatment by adding extenders, freezing, and thawing prior to insemination into the receptive cows (Tethool *et al.*, 2021). However, freezing and thawing procedures can reduce sperm motility, viability, and fertilization capacity (Len *et al.*, 2019). The process of semen cryopreservation can cause thermal shock and trigger the production of reactive oxygen species (ROS) (Chowdhury *et al.*, 2014). Several studies have shown that freezing and thawing processes increase sperm DNA fragmentation, apoptosis, and ROS formation from dead sperm (Bollwein & Bittner, 2018). It has been shown that elevated ROS levels after cryopreservation can lead to acrosome damage, DNA fragmentation, decreased sperm motility (Pilane *et al.*, 2019), reduced normal sperm morphology, and increased infertility in males (Topraggaleh *et al.*, 2014). This process is mediated by lipid peroxidation of the sperm plasma membrane, cold shock, osmotic pressure, and intracellular ice formation (Evangelista-Vargas & Santiani, 2017).

Supplementation of semen extender with enzymatic and non-enzymatic antioxidant compounds during freezing and thawing is a strategy for preventing oxidative damage (Gadani *et al.*, 2017). Several studies have shown that antioxidants added to the extender can improve sperm functions such as motility and sperm membrane integrity, induce endogenous antioxidant activity, and protect sperm against oxidative damage (Bucak *et al.*, 2009; Sapanidou *et al.*, 2016). The inclusion of antioxidants in the semen provides far-reaching

benefits in protecting sperm from the harmful effects of ROS (De Oliveira *et al.,* 2013; Kandiel *et al.,* 2017; Nasiri *et al.,* 2012; Samir *et al.,* 2020). Green tea extract (GTE) contains a high amount of polyphenols, especially epigallocatechin gallate (EGCG), which has been shown to possess antioxidant properties (Inanç *et al.,* 2019). Catechin, epicatechin, gallocatechin, epigallocatechin, gallocatechin gallate, and epigallocatechin gallate (EGCG) are all found in GTE (Tsai & Chen, 2016). Due to its potent antioxidant activity, EGCG represents the most potent bioactive compounds in green tea extract. EGCG compounds prevent lipid oxidation, reduce ROS levels, and activate the body's defense systems, increasing sperm motility, viability, and fertility (Park & Yu, 2017). The beneficial effect of GTE on the maintenance of semen quality has been demonstrated in dogs, pigs, bulls, rats, and, recently in buffaloes (Ahmed *et al.,* 2020).

Sperm motility testing at the National Artificial Insemination Center (NAIC) has recently been performed using the Computer-Assisted Sperm Analysis (CASA) system, which provides a more accurate and objective assessment of sperm motility and morphological parameters (Setiyono *et al.,* 2020). Conventional statistical analysis of sperm kinematics using CASA devices is based on the average value of thousands of sperm recordings (Amann & Waberski, 2014; Yániz *et al.,* 2018). The heterogeneous subpopulations of motile sperm in the ejaculate, indicating the specific kinematic patterns, are widely recognized. However, the biological significance of these different sperm subpopulations has not been extensively studied (Barbas *et al.,* 2018). The patterns of sperm motion can be examined using CASA to obtain curvilinear velocity (VCL), linearity (LIN), amplitude of lateral displacement (ALH), total motility, and progressive motility scores (Hinrichs & Loux, 2012). Compared to subjective assessment of sperm quality, CASA provides more detailed assessment results and a high degree of accuracy (Shojaei *et al.,* 2012). High VCL, ALH values, and low LIN rates indicate that the sperms exhibit hyperactive movement. The ability of sperm to penetrate the cervical mucus and the zona pellucida is influenced by the values of VCL, ALH, and total motility (Bernecic *et al.,* 2019). This study aimed to determine the effect of the GTE addition in the extender on the sperm kinematic and sperm morphology from frozen semen of Bali bull.

MATERIALS AND METHODS

Ethical Approval

At the Singosari National Artificial Insemination Center (SNAIC) in Malang, East Java, Indonesia, this study was carried out in accordance with the standard operating procedures of SNI ISO 9001:2015 and SNI ISO 37001:2016, and it was supervised by veterinarians employed by the organization. Guidelines and ethical approval were provided for the responsible use of bulls for collecting fresh sperm by AI Singosari Malang's Ethics Committee.

Animals, Experiment Location, and Semen Collection

Five Bali bulls that participated in the study were aged between 4 and 10 years. They had an average weight of 654 ± 23 kg, high libido, good physical health, and a 27.12 ± 2.45 cm testicular circumference. The Bali bulls were housed in individual cages and were routinely cared for. This included cleaning the cage, bathing the bulls, and feeding them grasses, silage, and concentrate twice daily according to their nutritional requirements. Nutritional supplements in the form of mineral blocks and drinking water were also provided ad libitum. This study was conducted at the Singosari National Artificial Insemination Center (SNAIC) in Singosari, Malang, East Java, Indonesia. Semen was collected twice weekly in the morning using an artificial vagina (AV) from Minitüb, Tiefenbach, Germany (Schenk, 2018). The ejaculate was processed into frozen semen according to the Indonesian National Standard SNI of 4869-1:2021 (analysis of bull frozen semen) of the Indonesian National Standardization Agency.

In the biomolecular laboratory of the Faculty of Veterinary Medicine at Universitas Airlangga, the freeze-dry procedure and the preparation of green tea extract were performed. The collected semen was then taken to the laboratory and stored in a water bath at 33-34 °C. Semen samples were macroscopically examined for their volume, color, consistency, and pH. Microscopic examination was also performed to assess mass motility, individual motility, and abnormalities. The criteria for fresh semen to be used in this study were fresh semen with motility over 80%, having a volume of above 2 mL (2-15 mL), a concentration of 500 x 10^6 sperm/mL until 1500 x 10^6 sperm/mL, and abnormalities under 10% (Khattak *et al.,* 2022).

Green Tea Extract Preparation

Green tea leaves were dried and ground into a powder to prepare the green tea extract. After maceration for three days, the powder of green tea leaves was soaked in 96% ethanol and covered with aluminium foil. The soaked green tea leaf powder was crushed and collected using filter paper. Then, the green tea filtrate was evaporated in a rotary evaporator at 50 °C and a speed of 45 rpm to obtain the soluble components from the green tea extract concentrate to obtain a thickened concentrate (Alqawasmeh *et al.,* 2021). To obtain a pure GTE extract in the form of freeze-dried (powder), the resulting solid GTE extract should be subjected to a freeze-drying procedure to remove any remaining residues. When the green tea extract was added to the extender, it was homogenized by the freeze-drying process. The powder of green tea extract was kept sterile before use (Swari *et al.,* 2020).

Mixture Extender and Green Tea Extract

Fresh semen that met the criteria was added with an extender, namely tris-based egg yolk. The Tris-based egg yolk extender was composed of several ingredients including 20% egg yolk, 1.6% Tris aminomethane,

0.9% citric acid, 1.4% lactose, 2.5% raffinose pentahydrate, 100,000 IU/100 mL Penicillin, 0.1 g/100 mL Streptomycin, 80% distilled water, and 13% Glycerin (Yekti *et al.,* 2020). Tris aminomethane, lactose, citric acid, and raffinose pentahydrate were mixed with a stirrer and homogenized with 80 mL of distilled water for ten minutes at 100 °C. The temperature was then lowered to 37 °C. After lowering the temperature, penicillin and streptomycin were added to the solution and homogenized for 10-15 minutes. Then, up to 20 mL of the solution was taken and mixed with egg yolk solution and homogenized for ± 30 minutes. All prepared extenders were stored under sterile conditions in the laboratory according to the protocols of Susilowati *et al.* (2021). The three stages of the extender, namely extender solutions A1 and A2, which contained extender and green tea extract, and extender solution B, which contained extender, green tea extract, and 13% glycerol, were used to supplement the extender with green tea extract. Extender A1 was added to the semen in a 1:1 ratio between the volumes of semen and the extender. The semen mixed with extender A1 was placed in a cooling cabinet (4-5 °C). At the same temperature, extender A1 was supplemented with extender A2. The sperm solution and extender A were kept at a constant temperature. Extender B is added to extender A at a similar temperature. Half of the total volume was extender B, which contained 13% glycerol. The arrangement is mixed slowly through the cylinder walls in different volumes according to the given extender equation. The semen solution was placed in the refrigerator at 4-5 °C with a maximum waiting time of hours for equilibration. The freezing system was completed immediately after the equilibration time was finished.

After the *in vitro* examination, each semen ejaculate that fulfilled the criteria was added to the respective extenders to achieve a concentration of 25 × 10^6 sperm per insemination dose of 0.25 mL (MiniStraw). Straws were filled and sealed with diluted semen depending on ejaculate volume and sperm concentration per mL per ejaculate according to the routine procedures. The straws filled with diluted semen were gradually cooled to 4 °C and frozen using the following protocol −5 °C per minute from +4 to −10 °C, −40 °C per minute from −10 to −100 °C, and thereafter −20 °C per minute from −100 to −140 °C in a programmable freezer (IMV Technologies), followed by immersion and storage in liquid nitrogen at −196 °C until the use.

Experimental Design

In this study, the treatments were divided into four different groups. The first was the control group (CTR), in which the semen was tested with a Tris-based egg yolk extender without adding GTE. The second group is sample of sperm contained a Tris-based egg yolk extender with a GTE of 0.05 mg/100 mL (GTE0.05), the third group is sample contained a GTE of 0.10 mg/100 mL (GTE0.10), and the fourth group is sample contained a GTE of 0.15 mg/100 mL (GTE0.15). The extended semen was then placed into a MiniStraw 0.25 mL (IMV® Technologies L'Aigle Cedex, France), equilibrated, frozen, and stored in liquid nitrogen. Assessment of sperm kinematics and spermatozoa morphology was done before freezing and after thawing. Only the sperm samples with more than 40% viability determined immediately after thawing were used for the experiments.

Sperm Kinematics and Morphology Variables

Sperm Analyzer IVOS IITM (Hamilton Thorne Inc., Beverly, USA) Computer-Assisted Semen Analysis (CASA) software was used to study sperm morphology and kinematic parameters. Frame rate (60 Hz), Frames obtained (30), Minimum contrast (35), Minimum cell size (5 Pixels), Cell size (9 Pixels), and Cell intensity (110 Pixels) were the CASA settings used to evaluate sperm motility of Bali bull. The frozen Bali bull sperm samples were thawed at 37 °C for 30 seconds (Zhong *et al.,* 2019). For each semen sample, 5 µL was placed onto a prewarmed (38 °C) counting chamber. Sperm motility was observed 10× negative phase, 100× brightfield for morphology with a value between 0% to 100% (Chen *et al.,* 2019). At least 1000 sperms were analyzed. Kinematics and motility parameters such as total motility (TM), progressive motility (PM), average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), straightness (STR), linearity (LIN), the amplitude of lateral head displacement (ALH), beat/cross-frequency (BCF), and wobble (WOB). Sperm morphology was observed and measured for normal morphology (NM), abnormal morphology (AM), bent tail (BT), coiled tail (CT), proximal droplets (PD), distal droplets (DD), and distal midpiece reflex (DMR) (Sinha *et al.,* 2021). The description and measurement unit of each parameter can be found in Table 1.

Data Analysis

The Kolmogorov-Smirnov test showed that the parameters were normally distributed. Levene's test was used to assess the homogeneity of variance of the data. Mean ± SEM, n= 5 was used to represent the parameters of the sperm quality. Parameters such as total motility (TM %), Progressive motility (PM %), Average path velocity (VAP; µm/sec), Straight-line velocity (VSL; µm/sec), Curvilinear velocity (VCL; µm/sec), STR (%), LIN (%), ALH (µm), BCF (Hz), WOB (%), normal morphology (%), Abnormal morphology (%), ben tail (BT %), coiled tail (CT %), proximal droplets (PD %), distal droplets (DD %), and distal midpiece reflex (DMR %) were analyzed using one-way analysis of variance (ANOVA) using SPSS Version 23 (IBM, USA), where $p < 0.05$ indicates statistical significance.

RESULTS

GTE had a significant effect on total motility (TM), especially with GTE0.05 ($p<0.05$), and on progressive motility (PM) with GTE0.05 and GTE0.15 ($p<0.01$) before freezing. However, the other kinematic parameters (VAP, VSL, VCL, STR, LIN, ALH, BCF, and WOB) and sperm morphology were not affected by the GTE. In addition, GTE0.05 had a significant effect on the increased

Table 1. Bali bull sperm kinematic and morphology definitions and their corresponding definitions

Variables	Unit	Definitions
Sperm kinematic descriptor		
Total motility (TM)	(%)	Total percentage of motile sperms
Progressive motility (PM)	(%)	Total percentage of progressive motile sperms
Average path velocity (VAP)	(μm/s)	Per unit of time, the sperm head's average velocity along its average trajectory
Straight-line velocity (VSL)	(μm/s)	Per unit of time, the sperm head's average path velocity in a straight line from its first to its last position.
Curvilinear velocity (VCL)	(μm/s)	The typical way speed of the sperm head along its actual direction per unit time
Straightness (STR)	(%)	The ratio between VSL and VAP (x 100)
Linearity (LIN)	(%)	The ratio between VSL and VCL (x 100)
Amplitude of lateral head displacement (ALH)	(μm)	The standard deviation of the sperm head's extreme side-to-side movement during each beat cycle.
Beat cross frequency (BCF)	(Hz)	Beat cross frequency based on VCL crossing VAP per second
Wobble (WOB)	(%)	The ratio between VAP and VCL (x 100)
Sperm morphology descriptor		
Normal morphology (NM)	(%)	The percentage of normal sperm
Abnormal morphology (AM)	(%)	The percentage of abnormal sperm
Ben tail (BT)	(%)	Based on the number of ben tails, the percentage of abnormal sperm was determined.
Coiled tail (CT)	(%)	Amount of abnormal sperm determined by counting the number of coiled tails
Proximal droplets (PD)	(%)	Percentage of proximal droplets manifest as a swelling at the sperm head-tail junction
Distal droplets (DD)	(%)	The proportion of sperm with distal cytoplasmic droplets
Distal midpiece reflex (DMR)	(%)	The proportion of sperm with distal midpiece reflex

Table 2. Means ± SEM of sperm kinematic and sperm morphologic of Bali bull in extender before freezing with different doses of green tea extract

Variables	Treatments				p value
	CTR	GTE0.05	GTE0.10	GTE0.15	
Sperm kinematics					
TM (%)	76.54 ± 3.30^{b}	86.20 ± 1.95^{a}	82.04 ± 2.79^{ab}	83.36 ± 2.23^{ab}	< 0.05
PM (%)	54.88 ± 0.84^{b}	66.80 ± 1.48^{a}	57.78 ± 0.85^{b}	62.50 ± 2.50^{a}	< 0.01
VAP (μm/sec)	156.47 ± 10.59	162.35 ± 7.05	152.15 ± 7.77	164.56 ± 6.19	NS
VSL (μm/sec)	121.19 ± 10.35	128.25 ± 8.96	109.98 ± 5.35	125.46 ± 7.68	NS
VCL (μm/sec)	303.93 ± 17.28	318.21 ± 13.50	307.80 ± 10.87	315.62 ± 7.28	NS
STR (%)	79.32 ± 3.06	84.08 ± 2.01	81.43 ± 1.26	80.00 ± 1.34	NS
LIN (%)	39.06 ± 1.85	43.18 ± 3.52	39.70 ± 1.92	41.37 ± 3.02	NS
ALH (μm)	12.90 ± 0.23	12.83 ± 0.63	12.23 ± 0.22	12.95 ± 0.40	NS
BCF (Hz)	24.09 ± 0.76	24.62 ± 1.77	23.90 ± 0.56	25.63 ± 1.89	NS
WOB (%)	51.32 ± 0.67	53.11 ± 3.09	50.43 ± 1.56	52.74 ± 2.54	NS
Sperm morphology					
NM (%)	95.76 ± 0.59	96.70 ± 0.69	96.04 ± 1.07	94.84 ± 0.68	NS
AM (%)	4.24 ± 0.59	3.3 ± 0.69	3.96 ± 1.07	5.08 ± 0.63	NS
BT (%)	1.86 ± 0.47	0.9 ± 0.20	1.06 ± 0.17	1.6 ± 0.39	NS
CT (%)	0.66 ± 0.23	0.72 ± 0.15	0.46 ± 0.30	0.32 ± 0.09	NS
DMR (%)	1.42 ± 0.41	1.22 ± 0.50	2.04 ± 0.78	2.82 ± 0.45	NS
PD (%)	0.2 ± 0.13	0.18 ± 0.18	0	0	NS
DD (%)	0.6 ± 0.11	0.48 ± 0.05	0.74 ± 0.38	0.6 ± 0.11	NS

Note: Data show all mean ± SEM (n=5). Means in the same column with different superscripts a, b, and c differ significantly (p<0.05). SEM=Standard error of means, NS=Not significant
CTR= Control, without addition of green tea extract; GTE0.05= With addition of 0.05 mg green tea extract into 100 mL extender; GTE0.10= With addition of 0.10 mg green tea extract into 100 mL extender; GTE0.15= With addition of 0.15 mg green tea extract into 100 mL extender.
GTE= green tea extract; TM= total motility (TM); PM= progressive motility; VAP= average path velocity; VSL= straight-line velocity; VCL= curvilinear velocity; STR= straightness; LIN= linearity; ALH= amplitude of lateral head displacement; BCF= beat/cross-frequency, WOB= wobble NM= normal morphology, AM= abnormal morphology, BT= bent tail, CT= coiled tail, PD= proximal droplets, DD= distal droplets, DMR= distal midpiece reflex.

number of all kinematic parameters, especially the PTM parameters ($p<0.05$) and PM ($p<0.01$), in the CASA examination before freezing, but the ALH parameter decreased compared to the control. GTE0.10 significantly affected the increasing TM, PM, and LIN, but the other kinematic parameters decreased compared to the control. GTE0.15 had a positive effect on all kinematic parameters, especially the PM parameter, which was significant ($p<0.01$) compared to the control.

The results of sperm morphology observations in all doses of the GTE treatment showed fluctuating values compared to control. There was no significant difference between all these values. The normal and abnormal morphology values were consistent with the standard sperm assessment at NAIC (Table 2 and Figure 1).

After thawing, sperm kinematics and morphology were re-tested and gave different results from the test before freezing. GTE did not cause a significant difference in TM and PM compared to the control ($p>0.05$). The VAP and VCL parameters showed that GTE had a significant effect compared to the control ($p<0.05$), with GTE0.05 showing the highest value. The VSL parameter showed a significant difference in the effect of GTE ($p<0.01$) compared to the control, with GTE0.05 showing the highest value. For the STR and ALH parameters, there was a significant difference between the GTE and control ($p<0.05$), with the GTE0.15 giving the highest value. The sperm morphology observations showed no significant difference between the GTE and control. When comparing sperm morphology before freezing and after thawing, there was a decrease in normal morphology and an increase in abnormal morphology, which still met the standard value of SNAIC for sperm quality (Table 3 and Figure 2).

DISCUSSION

In assisted reproductive technology (ART), sperm cryopreservation is used for male fertility preservation and long-term semen storage (Hezavehei *et al.*, 2018). However, after freezing and thawing, sperm motility, morphology, viability, and DNA integrity will decrease (Said *et al.*, 2010). In addition, cryopreservation has the potential to alter the structure of cells, increase membrane damage, and DNA fragmentation (Estrada *et al.*, 2017; Tatone *et al.*, 2010), and increase the production of free radicals, particularly hydrogen peroxide (H_2O_2), leading to lipid peroxidation and changes such as apoptosis and DNA oxidation (Trzcińska & Bryła, 2015). Sperm are susceptible to oxidative pressure when exposed to long-term incubation and cryopreservation, suggesting a link between ROS concentrations and lipid peroxidation (Bansal & Bilaspuri, 2011). To protect bovine sperm from the effects of freezing and thawing cycles, cryo-diluent media must contain exogenous antioxidants (Mahmoudi *et al.*, 2018). Several studies showed that the addition of GTE to the extender improves sperm quality in some animals, including buffalo, cattle, cats, rats, and boar (Martínez-Pastor *et al.*, 2011; Park & Yu, 2015; Wittayarat *et al.*, 2022). GTE,

Table 3. Means ± SEM of kinematics and morphology of Bali bull sperm in extender with different doses of green tea extract after thawing

Variables	Treatments				p value
	CTR	GTE0.05	GTE0.10	GTE0.15	
Sperm kinematics					
TM (%)	66.38 ± 2.11	60.76 ± 2.20	63.38 ± 3.34	67.80 ± 3.01	NS
PM (%)	46.16 ± 1.99	44.90 ± 1.85	46.08 ± 2.53	43.42 ± 1.21	NS
VAP (μm/sec)	79.84 ± 14.81	140.51 ± 9.94	103.71 ± 11.20	127.09 ± 9.62	< 0.05
VSL (μm/sec)	67.69 ± 12.48	121.86 ± 9.49	88.09 ± 9.76	109.10 ± 7.37	< 0.01
VCL (μm/sec)	148.67 ± 27.95	256.42 ± 22.21	177.26 ± 23.05	226.32 ± 17.75	< 0.05
STR (%)	63.13 ± 7.59	85.91 ± 1.90	83.79 ± 6.26	88.22 ± 2.62	< 0.05
LIN (%)	37.81 ± 5.95	48.59 ± 4.06	54.82 ± 5.42	53.20 ± 4.79	NS
ALH (μm)	6.71 ± 1.09	10.65 ± 0.83	8.20 ± 1.61	11.27 ± 1.05	< 0.05
BCF (Hz)	18.28 ± 2.42	24.43 ± 2.65	22.68 ± 1.91	19.34 ± 0.98	NS
WOB (%)	43.62 ± 6.40	56.07 ± 3.74	61.59 ± 4.32	60.00 ± 3.94	NS
Sperm morphology					
NM (%)	92.56 ± 1.86	93.90 ± 1.10	94.62 ± 1.40	93.84 ± 1.21	NS
AM (%)	7.44 ± 1.86	6.1 ± 1.10	5.38 ± 1.40	6.16 ± 1.21	NS
BT (%)	3.98 ± 1.34	2.88 ± 0.77	3.58 ± 1.91	2.64 ± 0.62	NS
CT (%)	1.34 ± 0.50	0.88 ± 0.38	0.46 ± 0.28	0.36 ± 0.31	NS
DMR (%)	1.56 ± 0.47	2.08 ± 0.16	1.24 ± 0.57	2.74 ± 0.47	NS
PD (%)	0	0	0.12 ± 0.12	0.08 ± 0.08	NS
DD (%)	1.12 ± 0.32	0.48 ± 0.21	0.28 ± 0.12	0.48 ± 0.23	NS

Note: Data show all mean ± SEM (n=5). Means in the same column with different superscripts a, b, and c differ significantly ($p<0.05$). SEM=Standard error of means, NS=Not significant
CTR= Control, without addition of green tea extract; GTE0.05= With addition of 0.05 mg green tea extract into 100 mL extender; GTE0.10= With addition of 0.10 mg green tea extract into 100 mL extender; GTE0.15= With addition of 0.15 mg green tea extract into 100 mL extender.
GTE= green tea extract; TM= total motility (TM); PM= progressive motility; VAP= average path velocity; VSL= straight-line velocity; VCL= curvilinear velocity; STR= straightness; LIN= linearity; ALH= amplitude of lateral head displacement; BCF= beat/cross-frequency, WOB= wobble NM= normal morphology, AM= abnormal morphology, BT= bent tail, CT= coiled tail, PD= proximal droplets, DD= distal droplets, DMR= distal midpiece reflex.

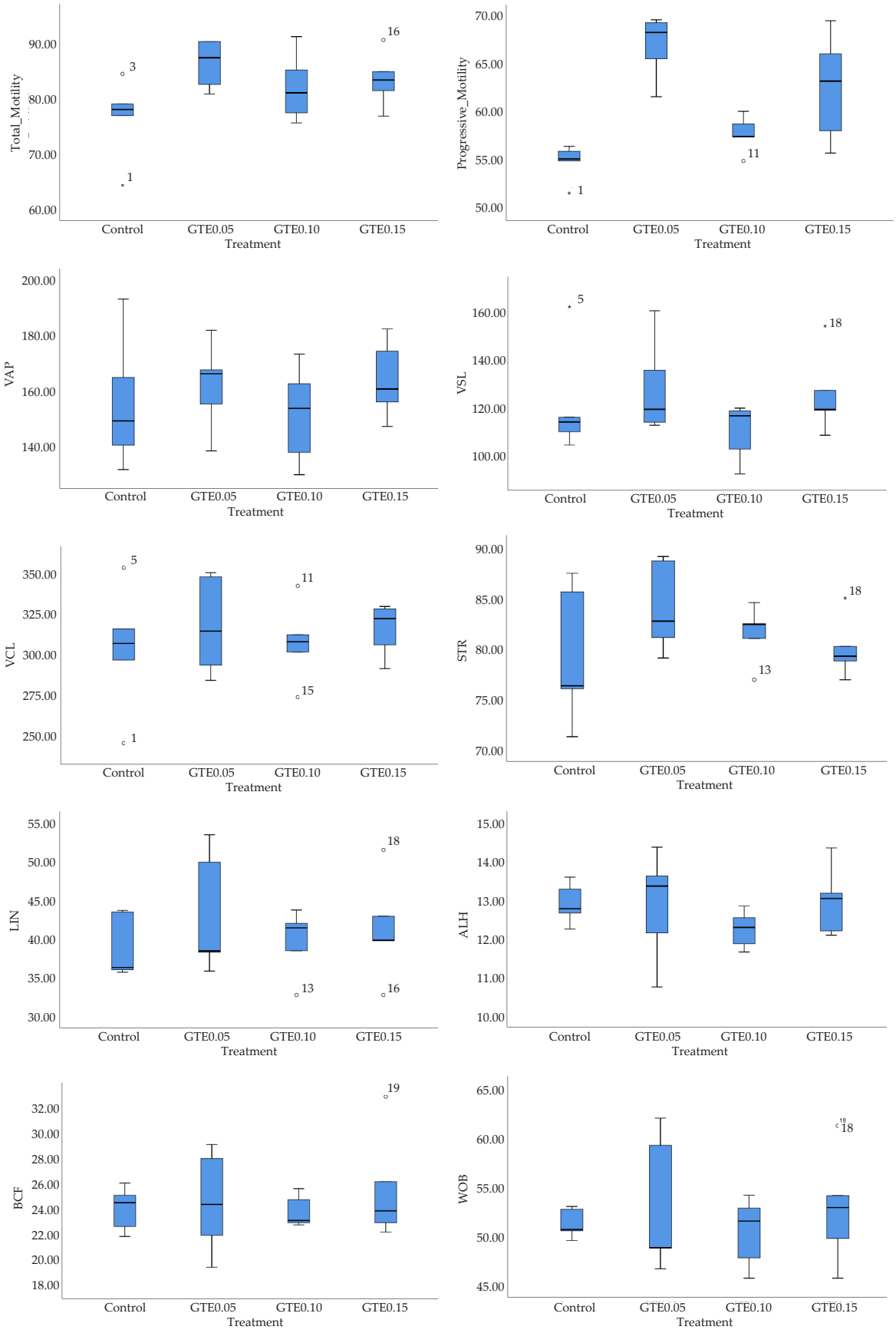

Figure 1. Boxplots showing the kinematics motility of Bali bull sperm before freezing and after dilution without (control) and with green tea extract (GTE) addition (0.05 g/100 mL, 0.10 g/100 mL, 0.15 g/100 mL extender) in extender measured by CASA. Data are presented as the mean ± SEM. Each experiment was repeated five times (five bulls). VAP= average path velocity; VSL= straight-line velocity; VCL= curvilinear velocity; STR= straightness; LIN= linearity; ALH= amplitude of lateral head displacement; BCF= beat/cross-frequency, WOB= wobble.

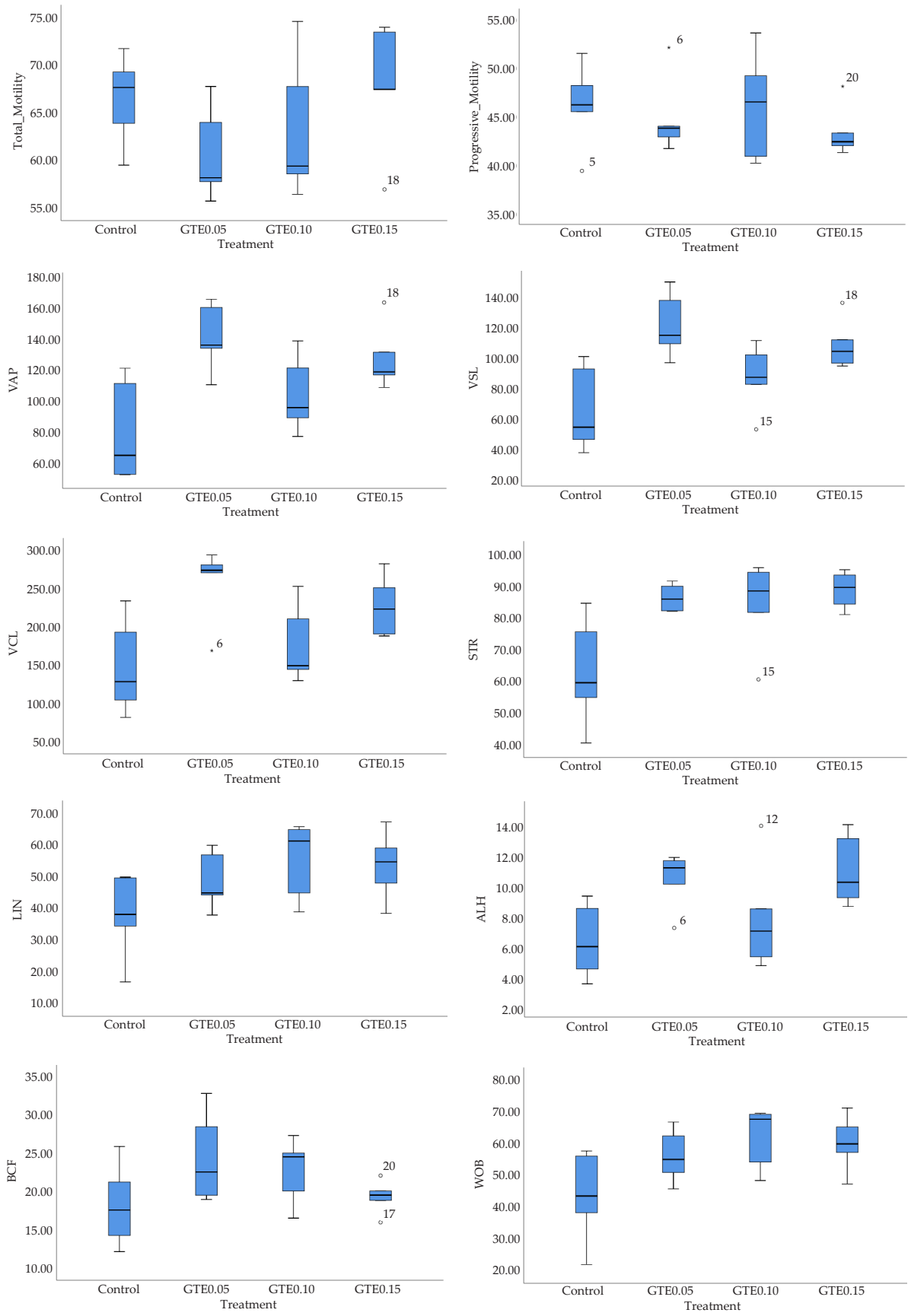

Figure 2. Boxplots showing kinematics motility of Bali bull sperm before freezing and after thawing without (control) and with green tree extract (GTE) addition (0.05 g/100 mL, 0.10 g/100 mL, 0.15 g/100 mL extender). Data are presented as mean ± SEM. Each experiment was repeated five times (five bulls). VAP= average path velocity; VSL= straight-line velocity; VCL= curvilinear velocity; STR= straightness; LIN= linearity; ALH= amplitude of lateral head displacement; BCF= beat/cross-frequency, WOB= wobble.

as an antioxidant in addition to extender components, can protect against oxidative stress during the freezing process (Dorado *et al.,* 2011). Our study was conducted to examine the effect of GTE antioxidants on Bali bulls and to find the most appropriate dosage to obtain the best kinematic parameters and sperm morphology.

CASA enables advanced multivariate statistics for recorded kinematic and morphometric parameters as a technology for animal reproduction. In the past, kinematic (wild pig, canine, fox, hare, solea, and steed) and morphometric (wild hog, bull, goat, llama, slam, and red deer) parameters have been studied to determine the sperm movement as a fertility test of males animals (Anel-López *et al.,* 2015; Del Olmo *et al.,* 2016; Martí *et al.,* 2012; Soler *et al.,* 2014).

Higher fertility in bulls is indicated by a high proportion of sperm with fast and direct movements and elongated shapes. However, in another study (Yániz *et al.,* 2015), sperm morphometric and kinematic parameters were not observed. A strong positive correlation exists between sperm motility and sperm flagellum length, head length, tail length, and total sperm length. Both sperm length and velocity are heritable traits (Lavara *et al.,* 2013).

CONCLUSION

the addition of 0.05 mg/100 mL of green tea extract to the extender improved total and progressive motility before freezing. In post-thawing, this addition enhanced specific sperm kinematics parameters—average path velocity, straight-line velocity, and curvilinear velocity. At a concentration of 0.15 mg/100 mL, green tea extract increased the straightness and amplitude of lateral head displacement. Importantly, no significant impact on sperm morphology was observed.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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