Research Article

International Journal of Indigenous Herbs and Drugs
Content Available at www.saap.org.in ISSN: 2456-7345

Effect of different concentrations of green tea on Cryopreservation of German Shepherd dog

Nagham y.Abood[1], Asmaa Sh.Aliawy[2]
[1] Department of Surgery, College of Veterinary Medicine,University of Basrah,Iraq
[2] Department of Surgery, College of Veterinary Medicine,University of Basrah,Iraq

Article Info :
Article History
Received on: 02-03-2023
Revised on: 16-03-2023
Accepted on: 28-04-2023

Abstract

To investigate the Effect of adding different concentrations of green tea extract as an antioxidant on specific physical semen characteristics of German Shepherd dogs during various steps of frozen semen processing this study was carried out. A total (of 20) good Ejaculate semen samples were collected in a plastic bag from German Shepherd dogs aged 3-4 yearsIn different locations (dog breeding).To achieve the current study's main objectives, two experiments were designed through periods extended from the beginning of December 2021 until the end of May 2022. The investigations included studying the physical properties of raw Semen (ejaculate volume, semen color, consistency, motility percentage, progressive motility percentage, dead sperm percentage, sperm abnormalities percentage, and hypo-osmotic swelling test (HOST). After that, semen samples were diluted with Tris supplemented with antioxidants. The physical properties of Semen were followed after cooling and after freezing (post-thawing). In the experiment, ten ejaculates were collected. Dilution of Semen was performed with Tris supplemented with two T1, T2, and T3) were cooled slowly up to 5C and equilibrated, packed into 0.5 ml straws, and put in vapor nitrogen (-196C) for 5-10 minutes after that, stored for 48and 72 hr in liquid Nitrogen (-196C). Progressive motility percentage, dead sperm percentage, sperm abnormalities percentage, and hypo-osmotic swelling test (HOST) were evaluated after cooling and post-thawing (Submerging the straws in the water bath at 37C for 30 seconds). It can be concluded from the present study that adding green tea extract as an antioxidant to the semen extender improves the ability to freeze, especially in the treatment T3 (0.01 mg/5 ml) and the T2 (0.05 mg/5 ml). This additive can decrease the negative Effect of freezing in liquid Nitrogen compared to the control group.

Keywords: *Sperm, German Shepherd Dogs Semen, Cryopreservation, Green Tea.*

***Corresponding Author**
Nagham y.Abood
DOI: https://doi.org/10.46956/ijihd.v8i2.447

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Introduction

The German Shepherd (GS) is one of the dog breeds most frequently utilized for working purposes (Ozcan et al. 2009; Parr and Otto 2013). Working dogs are indispensable part of military and police organs in many countries and are still more needed e.g. due to increasing terroristic attacks (detection, guard, and tracking dogs) and natural disasters (rescue dogs). Preservation of spermatozoa is a critical tool to preserve genetic diversity and assist in species reproduction. The family Canidae has many representatives that may benefit from using semen preservation as a tool for helping conservation (Goodrowe *et al.,* 2000; Watson and Holt, 2001). Preservation of spermatozoa is a critical tool to preserve genetic diversity and assist in species reproduction. The family Canidae has many representatives that may benefit from using semen preservation as a tool for helping conservation (Goodrowe*et al.,* 2000; Watson and Holt, 2001).

The interest in dog breeding has increased considerably during the last decades. Dogs have a

higher demand for assisted reproduction (Peña *et al.,* 2006). The growing number of artificial inseminations performed on dogs calls for more research into different techniques for storing canine Semen (Hori *et al.,* 2011). Next, insemination with fresh, chilled, and frozen-thawed ejaculated sperm(Hori *et al.,* 2003; Hori *et al.,* 2004; Hori *et al.,* 2011).

The request from dogowners to store the Semen of their valuable pet becomes problematic when a collection of ejaculated sperm is no longer possible, for example, because of the unexpected death of the male dog (Wydooghe *et al.,* 2016). In dogs, using cryopreserved Semen reduces the problems concomitant with natural breeding, animal transportation (Michael *et al.,*2007; Park *et al.,* 2018), and international trade (Esterhuizen*et al.,*2000).

However, the freezing process can exert specific detrimental changes in the morphology of sperm, resulting from thermal, mechanical, chemical, osmotic, and oxidative damage (Park *et al.,* 2017). These changes cause lower post-thaw sperm motility, decrease the integrity of the plasma and acrosomal membrane (Hewitt *et al.,* 2001;), and damage

The factors responsible for reduced fertility of post-thaw sperm include ice formation, high osmotic pressure (Pena*et al.,* 2012), reactive oxygen species (ROS) generation (Park *et al.,* 2017; Naresh*et al.,* 2015 and; Hong *et al.,*2018), and apoptotic pathway activation (Aitken and De Iuliis, 2010).

Dogs ejaculate in three fractions. The first fraction is the pre-sperm fraction, which originates from the prostate gland. Usually, it is clear or slightly cloudy, and the volume ranges from 0.5 to 20 ml or more (Freshman, 2001). The second fraction is called sperm-rich, which is usually opaque, milky-white in color, and ranges from 0.5 to 2.0 ml (Johnston *et al.,* 2001). The third or prostatic fraction usually is precise and may consist of more volume, depending on how long pressure is maintained proximal to the bulbusg landis (Johnston *et al*., 2001).

Antioxidants prevent free radicals, or their reactive Antioxidants can also reduce the impact of oxidative stress during the sperm storage process and generally improve the quality of liquid-stored boar semen (Bansal, 2011). Green tea polyphenols may exert antioxidative effects on canine spermatozoa during long-term storage Manita et al., 2013. The Hypo-Osmotic swelling test using distilled water has been effectively used to assess the functional integrity of sperm plasma membrane (Lomeo and Giambersio, 1991). During the HOS test, spermatozoa with a biochemically active plasma membrane, when exposed to the hypo-osmotic solution, will increase in volume due to intracellular influx of water, which is the sign of membrane integrity and regular activity of spermatozoa (Rota *et al.,* 2006; Cheema, 2012).

Materials

Extraction of green tea

Green tea leaves were dried and ground to a particle size of 0.75 micrometers in a grinding machine. Green tea powder was soaked by maceration using 96% ethanol solvent, allowed to stand for three days, and covered with aluminum foil. The soaked substance was squeezed through filter paper, evaporated at 50°C in a rotary evaporator at 45 rpm to obtain a thick extract, and then freeze-dried and stored at –20°C until required **Susilowati *et al.* 2018**.

Extender preparation

The stock solution consisted(The Tris extender contained 3.028 g of Tris-hydroxymethyl-aminomethane, 1. 78 g of monohydrated citric acid, and 1.25 g of fructose dissolved in 100 ml of distilled water (Silva *et al.,* 2000). pH 6.6. Then, 20% of the solution was substituted by egg yolk, and soon after, experimental treatments were established with the three final concentrations of glycerol to be tested: 4, 6, and 8%. Lincomycin (300/600 µg/mL), Tylosin (100 µg/mL), Gentamicin (500 µg/mL)

Methods

Animals of study

Twenty German Shepherd dogs were selected with a semen-freezing program for use in this study. Their average age was 4.5 years. Determined by the breeding record, Semen was collected from the dog by stimulating the bulbous gland, the dog was stimulated by penis massage, and the sperm-rich portion was contained in a plastic bag intended for the semen collection procedure Kutzler, 2005The study extended from the beginning of December 2021 to the end of March 2022.

Ejaculates

Ejaculate estimated (50% to 65% of Progressive motility (%) were taken daily. A total of 20 ejaculates were evaluated during the period of study. Semen samples were collected in a plastic bag. After collection, transferred the samples immediately sent to the laboratory located a few meters from the place of assembly and kept in a water bath at (37-38 C°) to determine the physical properties of semen samples,

ejaculate volume, semen color, and sperm concentration, Progressive motility (%), Dead sperm andabnormal sperm percentage and Hypo osmotic swelling test (HOST)in the following procedures.

Evaluation of semen quality

Physical properties:

1- **Ejaculate volume**:
From the graduated measuring plastic tube, ejaculate volume was determined after collection (Freshman, 2002).

2- **Semen color:** it was described as pearly, white, bloody, and yellow or green Johnston *et al*., 2001.

3- **Sperm concentration:** sperm concentration of ejaculates was evaluated by computer-assisted semen analysis (CASA) ((Baracaldo *et al*.,2007)

4- **Motility assays:** Motion parameters were determined using a computer-assisted sperm analysis (CASA) (Zaid ,2015andNoor ,2021)previously validated in our laboratory Gadea*et al.,* 2005 The studied CASA-derived motility characteristics were the Percentage of motile spermatozoa (%motil), Percentage of motile progressive spermatozoa (%motil prog), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity of the curvilinear trajectory (LIN, ratio of VSL/ VCL, %), straightness (STR, Percentage of VSL/VAP, %), the amplitude of lateral head displacement (ALH, μm) and wobble of the curvilinear trajectory (WOB, ratio of VAP/VCL, %). A 5μl drop of the sample, before (just ejaculated) and after sperm treatment, was placed on a warmed (37 °C) Slide and covered with a cover slip. Which spermatozoa had to be present to be counted.

5- **Dead sperm percentage:** it was determined according to (Bearden *et al*.,2004,Falah ,2017 and Sumayah ,2017) by placing 1-2 drops of the fresh semen sample and 1-2 drops of eosin-nigrosine (pre-warmed) on a clean Slide, then an edge of the second Slide applied to mix the semen sample with stain and also used to drag the mixture along the surface of clean Slide after she smears was dried examination had been done under a light microscope at (400X). Eosin is used to stain the dead sperms, whereas nigrosine is used to stain the background, so the dead sperms take the red color while the live sperms don't.

The stain was prepared as the following,The active ingredient of the stain

1. Eosin –y- 1.67gm
2. 2- 10 gm of nigrosine
3. 3- 2.9gm sodium citrate.

Dissolving in 100ml double distilled water followed by mixing, boiling, and filtration.The eosin-nigrosine stain was pre-warmed at 37 C° (water bath), and the Microscope of the study had a heated stage

6- **Sperm abnormalities percentage:** The Slide used for counting at least 200 sperm to determine dead sperm percentage is also used to estimate abnormal sperm percentage. The study of sperm abnormalities focused on identifying head, mid-piece, and tail abnormalities under a light Microscope (400X) (Bearden *et al.,*2004).

7- **Plasma Membrane Integrity**
An essential property of the sperm cell membrane is its ability to permit selective transport of molecules. Hypo-osmotic swelling test (HOST) is critical to analyzing the functional integrity of the sperm membrane because these characteristics are crucial for spermatozoa's viability and fertilizing ability (Jeyendran et al., 1984). Hypo-osmotic swelling test for sperm membrane integrity was assessed using the hypo-osmotic swelling test according to the methods described by Correa and Zavos (1994). Hypo-osmotic solution (Sodium citrate- 0.735 g; Fructose- 1.351 g; Millipore water- 100 ml and Osmolality- 150 mOsm kg-1) was mixed with 0.1 ml of Semen and incubated at 37°C for one hour. Following incubation, a well-mixed solution was placed on a clean, dry glass slide and covered with a cover slip. Sperm tail curling was recorded as an effect of swelling due to the influx of water. About 200 spermatozoa were counted in different fields with 40× objectives under a Microscope. The total proportion of swollen spermatozoa was calculated.

Experimental design Experiment Semen samples were collected weekly from german Shepherd dogs with the aid of a plastic bag; the samples were evaluated physically and then diluted with Tris-citric egg yolk-glycerol) diluter. The same diluent was supplemented

with green tea at two different concentrations and stored in liquid Nitrogen at (-196 C°) during December and January. Twenty ejaculates were collected from study animals and prepared for the freezing process according to the following steps.

1- **Evaluation:** Ejaculate were evaluated physically

a. Ejaculated volume (ml)
b. Semen color and consistency
c. Sperms concentration X106/ml
d. Progressive motility percentage
e. Dead sperm percentage
f. Abnormal sperm percentage
g. Hypo-osmotic swelling test (HOST percentage)

2-Dilution: Ejaculates were diluted by Tris diluent according to sperm concentration, he ejaculates, and the diluter in a water bath at 37 C°.

3-Addition of antioxidants: the diluted semen sample was allocated into three equal parts using a plastic test tube.

T1 control contains only (Tris-citric egg yolk-glycerol (TCEG).

T2 add green tea with 0.05 mg

T3 add green tea with 0.1 mg

The three test tubes were placed in a beaker containing water about (30-32 C°), and the cup was kept at room temperature for 30 minutes.

4-Cooling 5 ° C:- All diluted Semen containing different concentrations of antioxidants was transferred into the cold beaker, allowed to reach the stable degree 5 ˚C in about 1-1.5 hours to control the time of cooling diluted Semen in the cold cup, ice cubes were added to the beaker when the temperature of the water in the beaker reached 20° C, so should be below 5° C in a controlled manner, this can be done by the aid of sensitive thermometer to determine the degree of the temperature.

5-Equilibration: Diluted Semen containing different concentrations of antioxidants at the temperature of 5° C was performed for 4 hours at the same temperature (5 ° C).

6-Evaluation: Semen was evaluated physically for the Percentage of motility, dead, abnormalities, and Hypoosmotic swelling test (HOST percentage).

7-Packing: samples of the study were filled in straws (0.5 ml) and sealed at (5 C°) using a filling and sealing manual machine placed in the cold cabinet (Tris step was carried out during the equilibration time).

8-Freezing of straws: were kept horizontally, then placed in a container containing liquid Nitrogen to be exposed to liquid nitrogen vapor (2-3 cm) in the form for 5-10 minutes (Yu *et al.,* 2002). , Then, on each shelf, pick the straw, dip it into special cups containing liquid Nitrogen, and pass these cups to a tub of liquid Nitrogen().

9- Thawing of diluted Semen. After 48and 72 hours of storage, thawing is carried out by placing the straw into a water bath at 37°C for 30 seconds, Straws were cut to remove the straw vacuum, and the first drop and the second one on the Slide to start the diluted Semen were evaluated in the same measured way diluted Semen and after the cooling sample**.** (rota *et al.*2001 and cardoso *et al.* 2003).

Statistical Analysis

Using the post hoc method from the Statistical Application Program, ANOVA was subjected to data, version 22 (SPSS 22). Differences between After dilution, cooling, and post-thawing in Semen VCL (track velocity), VAP (path velocity), VSL (straight line velocity), LIN (linearity), STR (straightness), WOB (wobble), and ALH (amplitude of lateral head displacement). Live Spermatozoa (%)and dead spermatozoa, Total Sperm Abnormality (%), and Hypo osmotic swelling test (HOST) examination within and between groups were considered to be significant at $P<0.05$, expressed as the mean ± standard error (SE).

Results

Effect of different concentrations of green tea on Motility assays and hypo-osmotic swelling test of ejaculated German Shepherd dogs spermatozoa during further processing steps (after dilution, after cooling, and post thawing) at 48 hr.

The results of Concentration $x1^{0}6$/ml of ejaculated German Shepherd dogs treated with different concentrations of green tea (table:1), the result indicate a decrease of Concentration x106/ml post thawing with a significant ($P<0.05$) increase in the value in the control group (T1) only in comparison with values of T1, T2, and T3 which show no significant changes during the Effect of different steps of semen processing (cooling and post thawing)

The results of the Progressive motility percentage of ejaculated German Shepherd dogs treated with different concentrations of green tea (table:1) after dilution were T1 (68.99±0.37%), T2(60.60±0 .33%), and T3(63.81±0.31%)respectively. The results proved highest significant value ($p<0.05$) was in T1 (68.99±0.37%), and the lowest considerable value ($p< 0.05$) was in

T2(60.60±0 .33%)in comparison with other Values of Progressive motility percentage with different concentrations of green tea after cooling were in T2 (51.98± 0.38%) and T3 (58.05±0.28%), respectively (table:4.2.1).The results showed highest significant (p<0.05) value was in T3 (58.05±0.28%) and lowest critical (p<0.05) was in T2 (51.98± 0.38%) in comparison with others. After freezing, values of Progressive motility percentage are T2(47.79±0 .41%) and T3 (53.78±0.44%), respectively; the highest sperms Progressive motility percentage was observed in T1, which differed significantly (P<0.05) from T2 and T3 and the lowest value was in T2 after cooling and freezing with substantial differences (P<0.05) from others.

Values of sperms Total motility percentage during different steps of freezing (after dilution, cooling, and post-thawing) are pictorial in (table:1) . The results after dilution are T1 (95.79±0.39%), T2 (88.63± 0.30%), and T3(91.58±0.33%) respectively. Highly sperms Total motilitypercentage was attained in T1 (95.79±0.39%), which differed significantly (P<0.05) from T2 and T3. Values of Total motility percentage with two concentrations of green tea after cooling and post-thawing were in T2; The results showed a nonsignificant (p<0.05) value in T2 between after and after cooling and post-thawing. Meanwhile, matters of Total motility percentage. The highest sperms Total motility percentage was observed in T3, which differed significantly (P<0.05) from others, and the lowest value was in T2 after cooling and freezing with significant differences (P<0.05) from others.

The results of sperm Total abnormality percentage after cooling and post-thawing during treatment of diluted Semen with two green tea concentrations are shown in table (2). Values after dilution were T2 (8.38±0.004%)and T3 (7.83±0.002%), respectively. The lowest Percentage of Total abnormality sperms after dilution was observed in T3 (7.83±0.002%), which differed significantly at (P<0.05) from T2(8.38±0.004%). Meanwhile, after cooling and post-thawing, the Total abnormality sperm areT2 (10.77±0.0035, 12.32 ±0.004%) and T3(9.89±0.002, 9.01±0.043%), respectively. The result of the current study proved significantly at (P<0.05) lowest values attained in T3 and T1 different from T2, withT3 with no differences (P<0.05) between after cooling and post-thawing.

Hypo-osmotic swelling Percentages for german Shepherd dogs Semen treated with two concentrations of green tea (table2) after dilution was in T1(86.32±0.0044%), T2(80.43±0.0043%), and T3(84.66±0.0051%) respectively. The results proved the highest value significant (p<0.05) was in T1(86.32±0.0044%) and T3(84.66±0.0051%), and the lowest important values (p<0.05) was in T2(80.43±0 .0043%). Values of HOS Percentages with two concentrations of green tea after cooling and post-thawing were in T2 (72.53±0.0044, 65.86±0.0058%), T3 (76.14±0.0056, 67.86±0.0073%), respectively (table:4.2.1). The result showed that the highest value significant (p<0.05) were in T3 and the lowest important (p<0.05) were in T2.

Values of VAP and VSL with different concentrations of green tea after dilution were in T2 (162.14±0.43),(146.39±0.25) and T3 (169.13±0.28),(154.30±0.15), respectively (table:1). The results showed highest significant (p<0.05) value was in T3 and lowest effective (p<0.05) was in T2.Values of the current study after cooling are T2(154.09±0.36), (134.77±0.49) and T3 (158.24±0.37),(138.54±0.16), respectively. Lowest VAP and VSL sperms were observed in T2(154.09±0.36), (134.77±0.49), and. The highest value of VAP and VSL sperms was attained in T3 (158.24±0.37),(138.54±0.16). Meanwhile, post thawing, the VAP and VSLsperms are T2(145.66±0.38), (122.61±0.31), and T3 (147.86±0.29), (128.56±0.20) in control, T2 and T3 respectively. The highest value of VAP and VSL sperms was attained in the control group, which differed significantly at (P<0.05) in comparison with T2 and T3 groups, and the lowest value in T2, which significantly differed at (P<0.05) from T3.

Values of STR values at 48 hr in table (1). Results Control and after dilution areT1(96.04±0.32) and T2 (90.07±0.28) in control and T2, respectively. After dilution, the results proved the nonsignificant difference at (P<0.05) in the values of STR between the T1and T2 groups. While After cooling and post-thawing, the development of the current study proved nonsignificant at (P<0.05) in T3. Meanwhile, after dilution, The results of the survey regarding WOB of ejaculated German Shepherd dogs treated with different concentrations of green tea (table:2), after dilution were in T2(.970. ± 0.002) and T3(0.93±.002), respectively. The results proved the highest significant value (p<0.05) was in T2 (.970. ± 0.002), and the lowest critical value (p<0.05) was

in T3(0.93±.002). After cooling, the lowest significant values ($p<0.05$) of WOB were in T2(0.88±0.011) highest significant value ($p<0.05$) was in T3 (0.87±0.01). At post-thawing WOB in ejaculated German Shepherd dogs treated with two concentrations of green, there were no significant differences ($P<0.05$) between T2 and T3.

After dilution, The results of the study regarding ALH and LIN of ejaculated German Shepherd dogs treated with two concentrations of green tea (table:4.2.1)values proved a significant ($P<0.05$) increase in ALH and LIN(µm/s)ofGerman Shepherd dogs Semen after freezing compared with after cooling in T1 and T3 group but with no significant rise in T2 and T3 groups.

Regarding the Effect of different steps of processing (after dilution, after cooling, and post thawing) of ejaculated German Shepherd dogs within each treatment, the study indicated a gradually significant decrease ($p<0.05$) in Progressive motility (%), Total motility (%), VAP (µm/s), VSL(µm/s) and HOS (%). Meanwhile, the result indicates a decrease in Concentration x106/ml, ALH(µm/s), LIN(µm/s), STR(µm/s), WOB(µm/s)) post-thawing with a significant ($P<0.05$) increase in the value in the control group (T1) only in comparison with values of T1, T2 and T3 which show no significant changes during the Effect of different steps of semen processing (cooling and post thawing).concerning the impact of cooling and freezing on the physical characters (abnormal sperm percentage) and dead sperm and VCL(µm/s), the result depicted in which proved an increase in after freezing compared to after cooling in all treatments but the significant rise was recorded in the control group only while no considerable surge in the remaining group (T1, T2, and T3).

Table(1)/ Effect of different concentrations of green tea on Motility parameters measured by CASA in ejaculated German Shepherd dogs spermatozoa during other processing steps (after dilution, after cooling, and post thawing) at 48 hr (Mean± SE).

Analysis	T1- control (Tris	Step of freezing T1(0.05 mg)			Step of freezing T2(0.1 mg)		
		After dilution	After cooling	Post thawing	After dilution	After cooling	Post thawing
Concentration x10⁶/ml	291.44±0.39337 Aa	271.60±0.37 Ba	258.50±0.22 Bb	232.37±0.38 Bc	283.83±0.50 Ab	265.25±0.33 Ac	242.64±0.53 Ad
Progressive motility (%)	68.99±0.37 Aa	60.60±0.33 Bc	51.98±0.38 Cc	47.79±0.41 Dc	63.81±0.31 Bb	58.05±0.28 Cb	53.78±0.44 Db
Total motility (%)	95.79±0.39 Aa	88.63±0.30 Ab	80.56±0.25 Bb	75.05±0.26 Bb	91.58±0.33 Aa	84.72±0.34 Ba	78.92±0.25 Ca
VAP (µm/s)	171.12±0.28 Aa	162.14±0.43 Ab	154.09±0.36 Ba	145.66±0.38 Ca	169.13±0.28Aa	158.24±0.37 Ba	147.86±0.29 Ca
VSL(µm/s)	164.34±0.16 Aa	146.39±0.25 Bb	134.77±0.49 Ca	122.61±0.31 Db	154.30±0.15 Ba	138.54±0.16 Ca	128.56±0.20 Da
VCL(µm/s)	172.83±0.43 B	174.88±0.42 C	180.02±0.30 B	199.78±0.62 A	173.91±0.57B	177.36±0.49 B	195.10±0.27 A
ALH(µm/s)	4.55±0.005 Aa	4.02±0.006 Aa	3.64±0.005 Ba	3.15±0.004 Ba	3.89±0.002 Bb	3.77±0.003 Ba	3.35±0.004 Ba
LIN(µm/s)	0.96±0.002 Aa	0.90±0.005 Aa	0.86±0.003 Ba	0.84±0.003 Ba	0.91±0.002 Bb	0.88±0.003 Ba	0.86±0.003 Ba
STR(µm/s)	96.04±0.32 Aa	90.07±0.28 Aa	86.78±0.31 Ba	84.62±0.39 Cb	91.06±0.29 Ba	87.55±0.52 Ca	87.08±0.29 Ca
WOB(µm/s)	**0.96±0.003 Aa**	**0.97±0.002** Aa	0.011 Ba±0.88	0.76±0.01 Ca	0.93±.002 Aa	0.87±0.01 Ba	0.73±0.011 Ca

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- **Different capital letters mean significant (p<0.05) other within a column**.
- **Different small letters mean significant (p<0.05) other between columns.**

Table2/Effect of different concentrations of green tea on Dead sperms,Total abnormality(%), and HOS (%) parameters measured by CASA in ejaculated German Shepherd dog spermatozoa during other steps of processing (after dilution, after cooling, and post-thawing) at 48 hr (Mean± SE).

Analysis	T1- control (Tris	Step of freezing T1(0.05 mg)			Step of freezing T2(0.1 mg)		
		After dilution	After cooling	Post thawing	After dilution	After cooling	Post thawing
Dead sperms (%)	2.96±0.0072	3.15±0.0048	8.64±0.0072	13.05 ±.0054	2.12±0.0055	5.39 ±0.0041	12.60 ±0.0081
Sperm abnormality (%)	5.37±0.003 Db	8.38±0.004 Ca	10.77 ±0.0035 Ba	12.32 ±0.004 Aa	7.83±0.002 Bb	9.89±0.002 Ab	9.01±0.043 Ab
HOS (%)	86.32±0.004 4 Aa	80.43±0.004 3 Ab	72.53±0.004 4 Bb	65.86±0.005 8 Cb	84.66±0.005 1 Aa	76.14±0.005 6 Ba	67.86±0.00 73 Ca

- **Different capital letters mean significant (p<0.05) other within a column**.
- **Different small letters mean significant (p<0.05) other between columns.**

The results of Concentration x1^{0}6/ml of ejaculated German Shepherd dogs treated with different concentrations of green tea (table1) after dilution was in T1(275.89±0.281), T2(259.67±0.50), and T3(269.52±0.29) respectively.The results proved the highest significant value (p<0.05) was in T1(275.89±0.281)and T3(269.52±0.29), and the lowest critical value (p<0.05) was in T2(259.67±0.50). Values of Concentration x1^{0}6/ml with different concentrations of green tea after cooling and post-thawing were inT2 (243.29±0.33,229.19±0.23) and T3(251.08±0.42,230.90±0.31), respectively (table:4-2.2). The result showed that the highest value significant (p<0.05) was in T3(251.08±0.42, 230.90±0.31), and the lowest important (p<0.05) was inT2 (243.29±0.33, 229.19±0.23).

The results of the Progressive motility percentage of ejaculated German Shepherd dogs treated with two concentrations of green tea (table1) after dilution were T1 (64.71±0.35%), T2(58.99±0.30%), and T3(63.42±0.28%)respectively. The results proved highest significant value (p<0.05) was in T1 (64.71±0.35%), and the lowest considerable value (p< 0.05) was in T2(58.99±0.30%)in comparison with other Values of Progressive motility percentage with two concentrations of green tea after cooling were in T2 (50.73±0.23%) and T3 (54.78±0.38%), respectively (table:4.2.2).The results showed highest significant (p<0.05) value was in T3 (54.78±0.38%)and lowest critical (p<0.05) was in T2 (50.73±0.23%) in comparison with others. After freezing, values of Progressive motility percentage are T2(40.79±0.34%) and T3 (46.79±0.36%), respectively; the highest sperms Progressive motility percentage was observed in T1, which differed significantly (P<0.05) from T2 and T3 and the lowest value was in T2 after cooling and freezing with substantial differences (P<0.05) from others.

Values of sperms'Total motility percentageduring different steps of freezing (after dilution, cooling, and post-thawing) are pictorial in (table1) . The results after dilution are T1 (92.69±0.24%), T2 (84.87±0.32%), and T3(88.10±0.29%) respectively. The highest Total motilitypercentage was attained in T1 (92.69±0.24%), which differed significantly (P<0.05) from T2 (84.87±0.32%) and T3(88.10±0.29%). Values of Total motility percentage with two concentrations of green tea after cooling were in T2 (75.85±0.33%) and T3 (79.40±0.22%), respectively (table1). The results showed the highest significance (p<0.05) value was in T3 and lowest critical (p<0.05) was in T2. At post-thawing, values of Total motility percentage are (69.31±0.24%) (and 71.53±0.27%) in T2 and T3, respectively. The highest sperms Total motility percentage was observed in T3, which differed significantly (P<0.05) from others, and the lowest value was in T2 after cooling and

freezing with significant differences (P<0.05) from others.

The results of sperm Total abnormality percentage after cooling and post-thawing during treatment of diluted Semen with two green tea concentrations are shown in table (1). Values after dilution were T2 (13.17±0.003, 15.33 ±0.003%)and T3 (11.01±0.002, 13.44±0.003%) respectively . the result of the current study proved nonsignificant values attained in T1 and T3.

Results of the study related to dead sperm Percentage through different phases of frozen semen processing are presented in table (2). Values of the current study after cooling are T2 (10.94 ±0.0106%) and T3 (9.59±0.0048%), respectively. The lowest Percentage of dead sperms was observed in T3 (9.59±0.0048%), which differed significantly at (P<0.05) from T2. Meanwhile, after freezing, the Percentage of dead sperms is T2 (15.32 ±0.0098%) and T3 (14.03±0.0053%), respectively. The highest value of dead sperms was attained in T2 (15.32 ±0.0098%) which differed significantly at (P<0.05) compared to T3. After dilution, the results proved the highest significant value (p<0.05) was in T2 (4.31 ±0.0089%) and T3 (2.13±0.0056%). The results confirmed the highest significant value (p<0.05) was in T2 (4.31 ±0.0089%), and the lowest considerable value (p<0.05) was in T3. The values proved a significant (P<0.05) increase in dead sperm Percentage of German Shepherd dogs Semen after freezing compared with after cooling

Percentages of plasma membrane intact cells (hypo-osmotic swelling test) for german Shepherd dogs Semen treated with different concentrations of green tea (table2) after dilution was in T1(84.75±0.0050%) T2 (73.64±0.0078%) and T3 (76.42±0.0060%) respectively. The results proved the highest significant value (p<0.05) was in T1 (84.75±0.0050%), and the lowest critical value (p<0.05) was in T2 (73.64±0.0078%). Values of hypo-osmotic swelling Percentages with different concentrations of green tea after cooling and post-thawing were in T2 (62.21±0.0046, 51.42±0.0083%), T3 (66.23±0.0084, 57.31±0.0072%), respectively (table: 2). the result showed that the highest value significant (p<0.05) were in T3 and the lowest effective (p<0.05) were in T2. Values of VAP and VSL with different concentrations of green tea after dilution were in T2 (154.49±0 .21),(127.74±0.26) and T3 (157.98±0.26), (131.67±0.34), respectively (table2).After dilution, The results proved the highest value significance (p<0.05) was in T1(162.87±0.30) and T3(157.98±0.26), and the lowest significance (p<0.05) was in T2(154.49±0 .21). Values of current study after cooling and post thawing are T2 (141.14±0 .41, 139.19±0 .37), (100.14±0.40, 87.57±0.44) and T3 (145.77±0.53, 142.65±0.32), (110.76±0.24,93.92±0.37), respectively. The value T3 shows no significant changes between after cooling (145.77±0.53, 142.65±0.32) and post-thawing (110.76±0.24, 93.92±0.37).

Values of STR and WOB values after dilution at 72 hr in table(1).After dilution, results proved no significant difference at (P<0.05) in the importance of STR(μm/s) between T1 (89.59±0.22) and T2 (82.52±0.45). In contrast, After cooling and post-thawing, the result of the current study proved significantly at (P<0.05) lowest values attained in T2(71.13±0.42, 62.16±0.41), and the highest valuesT3(75.98±0.30,66.19±0.32). The results of WOB (μm/s) after cooling and freezing during the treatment of diluted Semen with two green tea concentrations are shown in table (1). The values indicate a decrease in WOB(μm/s) after freezing in comparison with after cooling, with a significant reduction in the control group only at (P<0.05) compared with (T1, T2, and T3) which proved non significant (P<0.05) decrease.

After dilution, The results of the study regarding ALH and LIN of ejaculated German Shepherd dogs treated with two concentrations of green tea (table:1), after dilution was in T1(4.32±0.004, 0.91±0.004)T2(3.92±0.003, 0.81±0.003) and T3(4.12±0.006, 0.83±0.005) respectively. ALH (μm/s), the results proved the highest significant value (p<0.05) was in T1 (4.32±0.004). While after dilution, freezing compared with after cooling in T2 and T3 groups with no significant rise in T3, which with no differences significantly (P<0.05) between T2 and T3.While LIN The results proved no significant differences (p<0.05) in T1(0.91±0.004) and after dilution T2 (0.81±0.003). After cooling, the lowest important values (p<0.05) of LIN were in T2 (0.70±0.003).highest significant value (p<0.05) was in T3 (0.77±0.003). At post-thawing LIN in ejaculated German Shepherd dogs treated with different concentrations of green tea indicated that the lowest significance (p<0.05) was in T2 (0.62±0.004), and the highest value significance (p<0.05) was in T3 LIN (0.66±0.003).

Semen spermatozoa had significant differences (P<0.05) after dilution observed for VCL values. The results showed a substantial (p<0.05) value in T2 (185.79±0.16) and T3 (175.56±0.16), respectively (table: 4.2.2).The results showed the highest significant (p<0.05) value was in T2 (185.79±0.16) and lowest critical (p<0.05) was in T3(175.56±0.16). Values of the current study after cooling and post-thawing are T2 (202.61±0.22, 229.83±0.24) and T3(188.14±0.15,210.89±0.24). The results

showed highest significant (p<0.05) value was in T2(202.61±0.22, 229.83±0.24), and the lowest adequate (p<0.05) was in T3(188.14±0.15, 210.89±0.24).

Regarding the Effect of different steps of processing (after dilution, after cooling, and post thawing) of ejaculated German Shepherd dogs within each treatment, the study indicated a gradually significant decrease (p<0.05) in Concentration x106/ml, Progressive motility (%), Total motility (%), VAP (μm/s), VSL(μm/s), ALH(μm/s), LIN(μm/s), STR(μm/s), WOB(μm/s) and HOS (%) in T2(1mM/ml)after dilution, after cooling and post thawing in all treatments, and the highest value significant (p<0.05) was in VCL(μm/s) and Total abnormality(%) after cooling and post thawing in T2. The lowest value (significant) was in cooling and published thawing in T3 and, While Dead sperms (%), the study proved non significantly values attained in T1, T2and T3. Table(1 and 2)

effect of different concentrations of green tea on Motility assays and hypo-osmotic swelling test of ejaculated German Shepherd dogs spermatozoa during further processing steps (after dilution, after cooling, and post-thawing) at 72 hr.

The results of Concentration x1^{0}6/ml of ejaculated German Shepherd dogs treated with different concentrations of green tea (table 3) after dilution was in T1(275.89±0.281), T2(259.67±0.50), and T3(269.52±0.29) respectively.The results proved the highest significant value (p<0.05) was in T1(275.89±0.281)and T3(269.52±0.29), and the lowest critical value (p<0.05) was in T2(259.67±0.50). Values of Concentration x1^{0}6/ml with different concentrations of green tea after cooling and post-thawing were inT2 (243.29±0.33,229.19±0.23) and T3(251.08±0.42,230.90±0.31), respectively (table:4-2.2). The result showed that the highest value significant (p<0.05) was in T3(251.08±0.42, 230.90±0.31), and the lowest important (p<0.05) was inT2 (243.29±0.33, 229.19±0.23).

The results of the Progressive motility percentage of ejaculated German Shepherd dogs treated with two concentrations of green tea (table: 3) after dilution were T1 (64.71±0.35%), T2 (58.99±0.30%), and T3 (63.42±0.28%) respectively. The results proved highest significant value (p<0.05) was in T1 (64.71±0.35%), and the lowest considerable value (p< 0.05) was in T2(58.99±0.30%)in comparison with other Values of Progressive motility percentage with two concentrations of green tea after cooling were in T2 (50.73±0.23%) and T3 (54.78±0.38%), respectively (table:3).The results showed highest significant (p<0.05) value was in T3 (54.78±0.38%)and lowest critical (p<0.05) was in T2 (50.73±0.23%) in comparison with others. After freezing, values of Progressive motility percentage are T2(40.79±0.34%) and T3 (46.79±0.36%), respectively; the highest sperms Progressive motility percentage was observed in T1, which differed significantly (P<0.05) from T2 and T3 and the lowest value was in T2 after cooling and freezing with substantial differences (P<0.05) from others.

Values of sperms Total motility percentageduring different steps of freezing (after dilution, cooling, and post-thawing) are pictorial in (table 3) . The results after dilution are T1 (92.69±0.24%), T2 (84.87±0.32%), and T3(88.10±0.29%) respectively. The highest Total motilitypercentage was attained in T1 (92.69±0.24%), which differed significantly (P<0.05) from T2 (84.87±0.32%) and T3(88.10±0.29%). Values of Total motility percentage with two concentrations of green tea after cooling were in T2 (75.85±0.33%) and T3 (79.40±0.22%), respectively (table 3). The results showed the highest significant (p<0.05) value was in T3 and lowest critical (p<0.05) was in T2. At post-thawing, values of Total motility percentage are (69.31±0.24%) (and 71.53±0.27%) in T2 and T3, respectively. The highest sperms Total motility percentage was observed in T3, which differed significantly (P<0.05) from others, and the lowest value was in T2 after cooling and freezing with significant differences (P<0.05) from others.

The results of sperm Total abnormality percentage after cooling and post-thawing during treatment of diluted Semen with two green tea concentrations are shown in table (4). Values after dilution were T2 (13.17±0.003, 15.33 ±0.003%) and T3 (11.01±0.002, 13.44±0.003%) respectively . the result of the current study proved nonsignificant values attained in T1 and T3.

Results of the study related to dead sperm Percentage through different phases of frozen semen processing are presented in table (4). Values of the current study after cooling are T2 (10.94±0.0106%) and T3 (9.59±0.0048%), respectively. The lowest Percentage of dead sperms was observed in T3 (9.59±0.0048%), which differed significantly at (P<0.05) from T2. Meanwhile, after freezing, the Percentage of dead sperms is T2 (15.32±0.0098%) and T3 (14.03±0.0053%), respectively. The highest value of dead sperms was attained in T2 (15.32±0.0098%), which differed significantly at (P<0.05) compared to T3. After dilution, The results proved the highest significant value (p<0.05) was in T2(4.31 ±0.0089%) and T3 (2.13±0.0056%). The results confirmed

the highest significant value (p<0.05) was in T2(4.31 ±0.0089%), and the lowest considerable value (p<0.05) was in T3. The values proved a significant (P<0.05) increase in dead sperm Percentage of German Shepherd dogs Semen after freezing compared with after cooling.

Percentages of plasma membrane intact cells (hypo-osmotic swelling test) for german Shepherd dogs Semen treated with different concentrations of green tea (table4) after dilution was in T1(84.75±0.0050%)T2 (73.64±0.0078%) and T3 (76.42±0.0060%) respectively. The results proved the highest significant value (p<0.05) was in T1 (84.75±0.0050%), and the lowest critical value (p<0.05) was in T2 (73.64±0.0078%). Values of hypo-osmotic swelling Percentages with different concentrations of green tea after cooling and post-thawing were in T2 (62.21±0.0046, 51.42±0.0083%), T3 (66.23±0.0084,57.31±0.0072%), respectively (table:4). The result showed that the highest value significant (p<0.05) were in T3 and the lowest effective (p<0.05) were in T2.

Values of VAP and VSL with different concentrations of green tea after dilution were in T2 (154.49±0 .21),(127.74±0.26) and T3 (157.98±0.26),(131.67±0.34),respectively (table 3).After dilution, The results proved the highest value significance (p<0.05) was in T1(162.87±0.30) and T3(157.98±0.26), and the lowest importance (p<0.05) was in T2(154.49±0 .21). Values of current study after cooling and post thawing are T2 (141.14±0 .41, 139.19±0 .37), (100.14±0.40, 87.57±0.44) and T3 (145.77±0.53, 142.65±0.32), (110.76±0.24, 93.92±0.37), respectively. The value T3 shows no significant changes between after cooling (145.77±0.53, 142.65±0.32) and post-thawing (110.76±0.24, 93.92±0.37).

Values of STR and WOB values after dilution at 72 hr in a table (3).After dilution, results proved no significant difference at (P<0.05) in the importance of STR(µm/s) between T1 (89.59±0.22) and T2 (82.52±0.45). In contrast, After cooling and post-thawing, the result of the current study proved significantly at (P<0.05) lowest values attained in T2(71.13±0.42, 62.16±0.41), and the highest valuesT3(75.98±0.30,66.19±0.32). The results of WOB (µm/s) after cooling and freezing during the treatment of diluted Semen with two green tea concentrations are shown in table (3). The values indicate a decrease in WOB(µm/s) after freezing in comparison with after cooling, with a significant reduction in the control group only at (P<0.05) compared with (T1, T2, and T3) which proved non significant (P<0.05) decrease.

After dilution, The results of the study regarding ALH and LIN of ejaculated German Shepherd dogs treated with two concentrations of green tea (table 3), after dilution was in T1(4.32±0.004, 0.91±0.004)T2(3.92±0.003, 0.81±0.003) and T3(4.12±0.006, 0.83±0.005) respectively. ALH (µm/s), The results proved the highest significant value (p<0.05) was in T1(4.32±0.004). While after dilution, freezing compared with after cooling in T2 and T3 groups with no significant rise in T3, which with no differences significantly (P<0.05) between T2 and T3.While LIN The results proved no significant differences (p<0.05) in T1(0.91±0.004) and after dilution T2 (0.81±0.003). After cooling, the lowest significant values (p<0.05) of LIN were in T2 (0.70±0.003).highest significant discounts (p<0.05) were in T3 (0.77±0.003). At post-thawing LIN in ejaculated German Shepherd dogs treated with different concentrations of green tea indicated that the lowest significant (p<0.05) was in T2 (0.62±0.004), and the highest value significance (p<0.05) was in T3 LIN (0.66±0.003).

Semen spermatozoa had significant differences (P<0.05) after dilution observed for VCL values. The results showed a substantial (p<0.05) value in T2 (185.79±0.16) and T3(175.56±0.16), respectively (table:4.2.2).The results showed the highest significant (p<0.05) value was in T2(185.79±0.16) and lowest critical (p<0.05) was in T3(175.56±0.16). Values of the current study after cooling and post-thawing are T2(202.61±0.22, 229.83±0.24) and T3(188.14±0.15,210.89±0.24). The results showed highest significant (p<0.05) value was in T2 (202.61±0.22, 229.83±0.24), and the lowest adequate (p<0.05) was in T3 (188.14±0.15, 210.89±0.24).

Regarding the Effect of different steps of processing (after dilution, after cooling, and post thawing) of ejaculated German Shepherd dogs within each treatment, the study indicated a gradually significant decrease (p<0.05) in Concentration x106/ml, Progressive motility (%), Total motility (%), VAP (µm/s), VSL(µm/s), ALH(µm/s), LIN(µm/s), STR(µm/s), WOB(µm/s) and HOS (%) in T2(1mM/ml)after dilution, after cooling and post thawing in all treatments, and the highest value significant (p<0.05) was in VCL(µm/s) and Total abnormality(%) after cooling and post thawing in T2. The lowest value (significant) was in cooling and published thawing in T3 and, While Dead sperms (%), the study proved non important values attained in T1 T2and T3. Table (3 and 4).

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Table(3)/ Effect of different concentrations of green tea on Motility parameters measured by CASA in ejaculated German Shepherd dog spermatozoa during other processing steps (after dilution, after cooling, and post thawing) at 72 hr (Mean± SE).

Analysis	Step of freezing (0.05 mg)				Step of freezing (0.1 mg)		
	Control	After dilution	After cooling	Post thawing	After dilution	After cooling	Post thawing
Concentration x10^6/ml	275.89±0.28 Aa	259.67±0.50 Bb	243.29±0.33 Cb	229.19±0.23 Da	269.52±0.29 Aa	251.08±0.42 Ba	230.90±0.31 Ca
Progressive motility (%)	64.71±0.35 Aa	58.99±0.30 Bb	50.73±0.23 Cb	40.79±0.34 Db	63.42±0.28 Aa	54.78±0.38 Ba	46.79±0.36 Ca
Total motility (%)	92.69±0.24 Aa	84.87±0.32 Bb	75.85±0.33 Cb	69.31±0.24 Db	88.10±0.29 Aa	79.40±0.22 Ba	71.53±0.27 Ca
VAP (µm/s)	162.87±0.30 Aa	154.49±0.21 Bb	141.14±0.41 Cb	139.19±0.37 Db	157.98±0.26 Ba	145.77±0.53 Ca	142.65±0.32 Ca
VSL(µm/s)	145.77±0.33 Aa	127.74±0.26 Bb	100.14±0.40 Cb	87.57±0.44 Db	131.67±0.34 Aa	110.76±0.24 Ba	93.92±0.37 Ba
VCL(µm/s)	170.36±0.24 Db	185.79±0.16 Ca	202.61±0.22 Ba	229.83±0.24 Aa	175.56±0.16 Cb	188.14±0.15 Bb	210.89±0.24 Ab
ALH(µm/s)	4.32±0.004 Aa	3.92±0.003 Ab	3.62±0.003 Ab	3.16±0.003 Ab	4.12±0.006 Aa	3.90±0.004 Aa	3.43±0.003 Aa
LIN(µm/s)	0.91±0.004 Aa	0.81±0.003 Aa	0.70±0.003 Bb	0.62±0.004 Cb	0.83±0.005 Ba	0.77±0.003 Ca	0.66±0.003 Da
STR(µm/s)	89.59±0.22 Aa	82.52±0.45 Aa	71.13±0.42 Bb	62.16±0.41 Cb	83.66±0.55 Aa	75.98±0.30 Ba	66.19±0.32 Ca
WOB(µm/s)	0.91±0.004 Ab	0.88±0.004 Aa	0.75±0.004 Ba	0.66±0.005 Ca	0.85±0.003 Ab	0.72±0.004 Bb	0.62±0.004 Cb

- **Different capital letters mean significant (p<0.05) other within a column**.
- **Different small letters mean significant (p<0.05) other between columns.**

Table(4)\Effect of different concentrations of green tea on Dead sperms, Total abnormality(%), and HOS (%) parameters measured by CASA in ejaculated German Shepherd dog spermatozoa during other steps of processing (after dilution, after cooling, and post thawing) at 72 hr (Mean± SE).

Analysis	Step of freezing (0.05 mg)				Step of freezing (0.1 mg)		
	Control	After dilution	After cooling	Post thawing	After dilution	After cooling	Post thawing
Dead sperms (%)	3.27±0.0080	4.31 ±0.0089	10.94 ±0.0106	15.32 ±0.0098	2.13±0.0056	9.59±0.0048	14.03±0.0053
Sperm abnormality(%)	7.65±0.004 Cb	11.85±0.004 Aa	13.17±0.003 Aa	15.33±0.003 Aa	9.77±0.003 Bb	11.01±0.002 Ab	13.44±0.003 Ab
HOS (%)	84.75±0.0050 Aa	73.64±0.0078 Bb	62.21±0.0046 Cb	51.42±0.0083 Db	76.42±0.0060 Ba	66.23±0.0084 Ca	57.31±0.0072 Da

- **Different capital letters mean significant (p<0.05) other within a column**.
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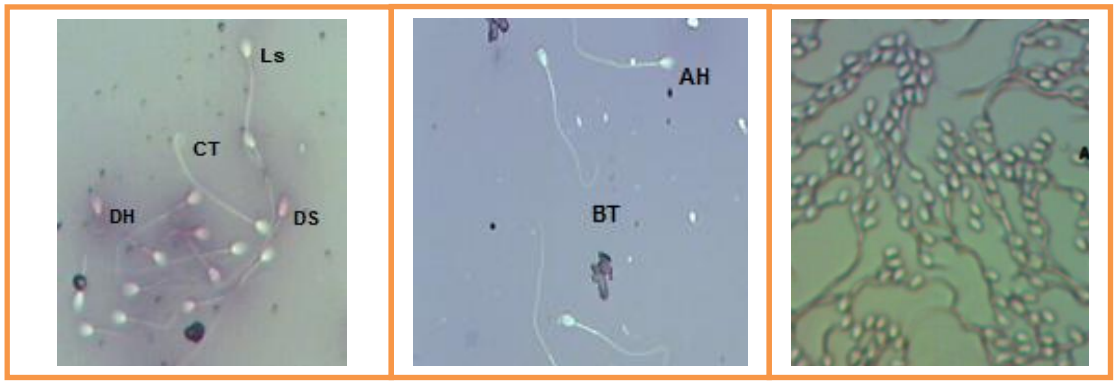

Discussion

Effect of green tea concentrations on Motility and plasma membrane intact cells (hypo-osmotic swelling test) parameters measured by CASA in ejaculated German Shepherd dog spermatozoa during other processing steps (after dilution, after cooling, and post-thawing). Generally, German Shepherd dog's spermatozoa undergo two different dilution steps for Cryopreservation. A dilution extender with green tea is used to produce a rapid phase transition during the freezing process of spermatozoa, and hence there is a radical increase in ROS. The increased ROS level causes poor motility, viability, morphology, and plasma membrane intact cells of frozen-thawed spermatozoa. Still, these results could be connected to its antioxidant qualities; Bailey *et al.* 2000 reported that cryoinjury might be induced by ROS activity generated during all freezing processing steps. Therefore, the present study evaluated the antioxidative Effect of green tea added dilution. This is the first report on green tea's antioxidative and related impacton boar sperm freezing and IVF parameters. Wittayarat et al.,2012 green tea has been tested for long-term semen storage (four weeks at 5 °C). The supplementation of Semen with extract concentrations ranging from 0.5 to 1 mg/mL increased sperm motility and viability during the storage period.

Consequently,Adding GTE improved the sperm quality parameters, including motility, viability, and acrosome integrity. Chen *et al*. (2003) reported that catechins presented in GTE significantly conserved cell viability and enhanced the mitochondrial membrane potential of cells. GTE contains several phenolic components, including catechins, that might positively affect sperm parameters (Chen *et al*., 2003).Green tea has also been employed for the Cryopreservation of ram semen Mehdipour *et al.*,2016 by adding to the semen extender three extract concentrations (5, 10, and 15 mg/L). These plant extracts positively affected sperm viability, motility, plasma membrane integrity, and mitochondrial activity. Sperm motility in T1- control (Tris)and T3(0.1 mg)groups supplemented with GTE was significantly higher than that of the T2(0.05 mg). The reduction of sperm motility due to Cryopreservation may be because of oxidative damage from excessive or ridiculous formation of ROS. In the canine species, it was demonstrated that the addition of green tea extract to the tris-egg yolk extender enhanced the motility and viability of chilled canine sperm; it was clarified that green tea had a dominant antioxidant effect against lipid peroxidation that was naturally occurring during Cryopreservation of avian Semen, thus improved the avian semen quality. Based on these studies and current studies, it was evident that a low concentration of green tea exerted an excellent effect on semen extender to enhance post-thaw quality parameter (Wittayarat *et al*., 2013; Jirina and Anton, 2013; Al-Daraji, 2011*)*

Green tea extract contains an ample amount of catechin polyphenols the antioxidant characteristics and is strongly associated with the fight against oxidative stress (Chyu *et al*., 2004).Considering the current findings and earlier studies, it seems reasonable that the positive effects of green tea extract on semen quality may be ascribed to catechin polyphenols content on reduction of the oxidative stress that naturally occurred during Cryopreservation of Semen, despite the difference in an experimental animal model. The functional mechanism through which green tea polyphenol is involved in inhibiting oxidative stress during semen preservation is unclear and remains to be elucidated. However, it was demonstrated that polyphenols might bind to components of the sperm membrane and would have prevented the lipid membrane oxidation induced by free radicals (Hyon, 2004). Additionally, the other potential factors might be attributed to the association of green tea polyphenols with the inhibition of egg yolk oxidation in semen

extender (Ponglohapan *etal*., 2004). In most animal studies, including ours, egg yolk extenders were widely used to preserve Semen (Wittayarat *et al*., 2013; Quintero-Moreno *et al*., 2004).

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