Effect of different concentrations of pentoxifylline on Cryopreservation of German Shepherd dog

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Abstract

To investigate the Effect of adding different concentrations of extract pentoxifylline 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 years In 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 rawSemen (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 ofSemen were followed after cooling and after freezing (post-thawing). In the first experiment, ten ejaculates were collected. Dilution ofSemen was performed with Tris supplemented with two different concentrations of pentoxifylline, T1(Tris + (1) mmol/ml. of pentoxifylline / 5 ml diluent). T2(Tris + 5 mmol/ml. of pentoxyfylline / 5 ml diluent).Diluted semen samples (C, 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 48 and 72hr 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). Semen samples were cooled and frozen, and physical evaluation was performed after cooling and freezing. The results of the first experiment proved for cooling and freezing, the individual motility percentage of sperm was significantly (P<0.05) higher in T3, which was quite different (P<0.05)from T2 and all the values were very (P<0.05) different from control T1. Meanwhile, matters of dead sperms Percentage show the lowest in T1, which is significantly (P<0.05) different from T2 after cooling, while after freezing, the lowest value in T1 and T3, and the highest in T2 with significantly (P<0.05) other. Abnormal sperm percentage was most deficient in T1 after cooling with significant (P<0.05) differences from T2 and T3. After freezing lowest value in T1 and T3 with a significant (P<0.05) difference from T2. Hypo osmotic swelling test (HOST) Percentage shows a considerable difference (P<0.05) Among the groups after cooling at different hours (48, 72, 96, 120). Still, after freezing, the highest value was in T3, with a significant difference from T2 and control T1. Regarding the Effect of cooling and freezing on semen quality of German Shepherd dogsSemen, the results show the addition of green tea extract act as a protective factor for sperm to withstand the detrimental Effect of cooling and freezing in liquid Nitrogen by preventing the significant (P<0.05) decrease of sperm progressive percentage and also prevent the substantial increase in dead, abnormal and defect Hypo osmotic swelling test (HOST) percentage in after freezing compared with after cooling.

Key words: sperm, German Shepherd dog, cryopreservation and pentoxifylline

Introduction

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 canineSemen (Hori *et al.*, 2011). Next to insemination with fresh, chilled, and frozen-thawed ejaculated sperm(Hori *et al.*, 2003; Hori *et al.*, 2004; Hori *et al.*, 2011).

The request from dog-owners to store theSemen 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 cryopreservedSemen 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

doi.org/10.31838/hiv23.02.68 Received: 22.10.22, Revised: 23.11.22, Accepted: 09.12.22.

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 colour 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 bulbusglandis (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, 2010).

Pentoxifylline (PTX) is a methylxanthine derivative and phosphodiesterase (PDE) non-specific inhibitor. (PTX) leads to increased intracellular concentrations of cyclic adenosine monophosphate (cAMP) and increases sperm motility and progressive motility. PTX has been used as an additive toSemen, as reported by Maxwell *et al.* (2002).

Materials

Solution Preparation

Preparation of Pentoxyfylline(PX).

This solution was prepared by dissolving 10 mg of PX powder (Sanofi Aventis, Egypt) in 10 ml of PBS (0.1%) and stirring until dissolved. These concentrations were prepared daily under sterile conditions using U/V light and Millipore filter (0.45µM) P (AL-Dujaily *et al.*,2012).

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)

Animal 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 Risopatron *et al.*, (2000). The 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 pieces 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 and abnormal sperm percentage and Hypo osmotic swelling test (HOST)in the following procedures.

-physical properties

1- Ejaculate volume

From the graduated measuring plastic tube, ejaculate volume was determined after collection(Freshman, 2001).

2-Semen color: i

t 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), 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) 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

a- The active ingredient of the stain

b- Eosin –y- 1.67gm
c- 2- 10 gm of nigrosine
d- 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 the 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 desig

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 Pentoxyfylline(PX) and 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 freezing in 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)

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

Addition of antioxidants: the diluted semen sample was allocated into three equal parts using a plastic test tube.
T 1 control (Tris)
T1 Tris + (1) mmol/ml. of pentoxyfylline / 5 ml diluent.
T2 Tris + 5) mmol/ml. of pentoxyfylline / 5 ml diluent.
OR
T1 control contains only (Tris-citric egg yolk-glycerol (TCEG). The three test tubes were placed in a beaker containing water at about (30-32 C°), and the cup was kept at room temperature for 30 minutes.

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).

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).

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

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). as fig.(3-3, A).

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). as fig. (3-3, B), Then, on each shelf, pick the straw, dip it into special cups containing liquid Nitrogen, and pass them to a tub of liquid Nitrogen as fig.(3-3, C).

Thawing of diluted Semen. After 48,72,96, and 120 hours of storage, thawing is carried out by placing the straw into a water bath at 37°C for 30 seconds, as fig.(3-3, D). 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 pentoxifylline on

Motility and plasma membrane intact cells (hypo-osmotic swelling test) parameters measured by CASA in ejaculated German Shepherd dog spermatozoa during various steps of processing (after dilution, after cooling, and post thawing) at 48 hr

The results of Concentration x10^6/ml of ejaculated German Shepherd dogs treated with different concentrations of pentoxifylline (table:4-1) after dilution was in T1(211.77±0.29), T2(195.03±0.33), and T3(199.97±0.33) respectively. The results proved the highest value significant ($p<0.05$) was in T1(211.77±0.29) and T3(199.97±0.33), and the lowest critical value ($p<0.05$) was in T2(195.03±0.33). Values of Concentration x10^6/ml with different concentrations of pentoxifylline after cooling and post-thawing were in T2 (184.02±0.19), T2(177.63±0.184), andT3 (188.95±0.30), T3(183.69±0.33), respectively (table: 1). The result showed that the highest value significant ($p<0.05$) was in T3 (188.95±0.30)and (183.69±0.33), and the lowest effective ($p<0.05$) were in T2 (184.02±0.19) and (177.63±0.184).

The results of the Progressive motility percentage of ejaculated German Shepherd dogs treated with different concentrations of pentoxifylline (table: 1) after dilution were T1 (60.65±0.25%), T2(53.44±0.21%), and T3(56.57±0.20%)respectively. The results showed no significant changes in T2(53.44±0.21%)and T3(56.57±0.20%). The result indicates a decrease of Progressive motility percentage after cooling and post-thawing with a significant ($P<0.05$) decrease in the value in T2 only compared with values of T1 and T3.

Values of sperms' Total motility percentage during different steps of freezing (after dilution, cooling, and post-thawing) are depicted in (table1).The results after dilution are T1 (86.57±1.23 %) ,T2 (82.11±0.01%) , and T3(83.82±1.03 %) respectively .The values proved a non-significant ($P<0.05$) in the Total motility percentage of german Shepherd dogsSemen after dilution compared with the control T2and T3 groups but with no significant rise in T2 groups after cooling and post-thawing. Values of Total motility percentage with different concentrations of pentoxifylline after cooling and post-thawing were in T3 (79.05±0.75%) (75.85±1.09 %), respectively (table1). The results showed highest significant ($p<0.05$) value was after cooling (79.05±0.75%), and the lowest significant ($p<0.05$) was in T3post thawing (75.85±1.09 %).

Values of VAP with two concentrations of pentoxifylline after dilution were in T2 (148.04±0.0052) and T3 (153.31±0.0065), respectively (Table 1). The results showed the highest significant ($p<0.05$) value was in T3 (153.31±0.0065), and the lowest powerful ($p<0.05$) was in T2 (148.04±0.0052). Values of the current study after cooling are T2(145.60±0.0075)andT3 (148.13±0.0047),(129.24±0.0031), respectively. In contrast, the cooling process has no significant difference in T2 and T3 compared with post-thawing. Results of the Effect of cooling and freezing steps within each treatment are depicted in the (table1) The values proved a non –significant ($P<0.05$) of VSL of german Shepherd dogsSemen after dilution and after cooling in T2 compared with control. Meanwhile, the T3 group showed no significant rise in T3 groups after cooling and post-thawing. The values referred to a non-significant ($P<0.05$) in VCL after dilution, after cooling, and post-thawing in the T2 group. Meanwhile, in VCL, after dilution and after cooling in T3 groups with no significant increase in the T3 group.

After dilution, The results of the study regarding ALH of ejaculated German Shepherd dogs treated with different concentrations of pentoxifylline (table: 1) after dilution was in T1(5.50±0.02 T2(4.63±0.02) and T3(4.79±0.58)respectively. The results proved the value non-significant ($p<0.05$) in T2 and T3. After cooling and post-thawing, no significant increase values ($p<0.05$) of ALH in T3(4.69±0.58) compared with T2 (4.33±0.01), with the highest value significant ($p<0.05$) was in T3 (4.34±0.55) compared with T2 (4.06±0.08). The importance of the current study after dilution of LIN in ejaculated German Shepherd dogs treated with two concentrations of pentoxifylline indicated that the lowest significance ($p<0.05$) was in T2 (0.86±0.0032)and the highest value significant ($p<0.05$) were in T3 (0.90±0.0048). While after cooling and post-thawing no considerable rise in the T2 group.

Values of STR after dilution, cooling, and post-thawing are depicted at 48 hr in table (1). Results after cooling and post-thawing are (85.47±0 .40), (83.53±0 .395), and (87.25±0.006),(85.28±0.0057) in T2 and T3 . the results proved no significant difference at ($P<0.05$) in the values of STR between T2and T3 groups. In contrast, values of STR post thawing, the development of the current study proved significantly at ($P<0.05$) lowest values attained in T2 and the highest importance in T3 followed by T1. while WOB(µm/s), after dilution and After cooling the result of the current study proved non-significantly at ($P<0.05$) in T2 and T3 and Highest value of STR attained in T3 (0.71±0.022) and lowest values in T2 (0.67±0.021) at post thawing.

The results of sperm Total abnormality percentage after cooling and post-thawing during treatment of diluted semen with two concentrations of pentoxifylline are shown in table (2). Values after cooling were (11.40±0.14 %)and (9.87±0 .29%) in control, T2, and T3, respectively. The lowest Percentage of Total abnormal sperms after cooling was observed in T3, which differed significantly at ($P<0.05$) from T2 and control (T1). Meanwhile, post thawing, the Total abnormality of sperm is (13.06±0.19%) and (11.23±0 .26%) in power, T2, and T3, respectively. The result of the current study proved significantly at ($P<0.05$), the lowest values attained in T3and T1 the highest values in T2.

Percentages of plasma membrane intact cells (hypo-osmotic swelling test) for german Shepherd dogsSemen treated with different concentrations of pentoxifylline table (2) after dilution was in T1(79.46±0.97%), T2(70.40±0.79 %) and T3(74.01±1.47%) respectively. The results proved the

highest value significant ($p<0.05$) was in T1(79.46±0.97%) and T3(74.01±1.47%), and the lowest significant value ($p<0.05$) was in T2(70.40±0.79 %). Values of hypo-osmotic swelling Percentages with different concentrations of pentoxifylline after cooling and post-thawing were in T2 (61.79±0.82, 58.60±0.65%), T3 (66.64±0.92, 64.31±0.97%), respectively (table1). The result showed that the highest value significant ($p<0.05$) was in T3 (66.64±0.92, 64.31±0.97%), and the lowest effective ($p<0.05$) were in T2 (61.79±0.82, 58.60±0.65%).

Results of the study related to dead sperms Percentage through different phases of frozen semen processing are presented in table (2). Values of the current study after cooling and post-thawing are T2 (11.07±0.0054),(14.82±0.0053)and T3 (10.37±0.0044),(12.90±0.0069), respectively. Dead sperms were observed 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 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), Total abnormality(%) and dead sperm after cooling and post thawing in T2 and lowest value (significant) were in cooling and post thawing in T3 table (1 and 2).

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

Analysis	T1- control (Tris)	Step of freezing T2(1mM/ml)			Step of freezing T3(5mM/ml)		
		After dilution	After cooling	Post thawing	After dilution	After cooling	Post thawing
Concentration x106/ml	211.77±0.29 Aa	195.03±0.33 Bb	184.02±0.19 Cc	177.63±0.184 Dd	199.97±0.33 Ba	188.95±0.30 Cb	183.69±0.33 Cc
Progressive motility (%)	60.65±0.25 Aa	53.44±0.21 Bb	49.09±0.21 Cc	45.06±0.24 Dc	56.57±0.20 Bb	52.18±0.30 Bb	48.97±0.293 Cc
Total motility (%)	86.57±1.23 Aa	82.11±0.01 Aa	78.22±0.01 Bb	72.11±0.01 Bb	83.82±1.03 Aa	79.05±0.75 Bb	75.85±1.09 Cc
VAP (µm/s)	157.61±0.007 8 Aa	148.04±0.005 2 Bb	145.60±0.007 5 Ba	139.26±0.007 0 Cb	153.31±0.006 5 Aa	148.13±0.004 7 Ba	142.33±0.008 8 Ba
VSL(µm/s)	149.41±0.001 4 Aa	127.48±0.001 5 Ba	124.44±0.002 8 Ba	116.32±0.003 9 Cb	138.56±0.002 Bc	129.24±0.003 1 Ca	121.38±0.002 9 Ca
VCL(µm/s)	185.42±0.003 6 Ba	200.05±0.005 6 Aa	205.07±0.005 4 Aa	207.84±0.004 8 Aa	194.06±0.004 1 Bb	194.91±0.002 7 Bb	200.46±0.012 Ab
ALH(µm/s)	5.50±0.02 Aa	4.63±0.02 Ba	4.33±0.01 Bb	4.06±0.08 Cb	4.79±0.58 Ba	4.69±0.58 Ba	4.34±0.55 Ca
LIN(µm/s)	0.95±0.0045 Aa	0.86±0.0032 Bb	0.80±0.0034 Cb	0.78±0.0034 Cb	0.90±0.0048 Aa	0.86±0.0047 Ba	0.81±0.0039 Ca
STR(µm/s)	94.80±0.016 Aa	86.11±0 .343 Bb	85.47±0 .40 Ba	83.53±0 .395 Ba	90.29±0.007 Aa	87.25±0.006 Ba	85.28±0.0057 Ba
WOB(µm/s)	0.85±0.03 Aa	0.74±0.019 Bc	0.71±0.0201 Bc	0.67±0.021 Cc	0.79±0.022 Bb	0.76±0.026 Bb	0.71±0.022 Cb

- 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 pentoxifylline 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 T2(1mM/ml)			Step of freezing T3(5mM/ml)		
		After dilution	After cooling	Post thawing	After dilution	After cooling	Post thawing
Dead sperms (%)	7.66±0.00 58 Dc	8.66±0.007 1 Ca	11.07±0.005 4 Ba	14.82±0.005 3 Aa	8.31±0.041 Ca	10.37±0.004 4 Bb	12.90±0.006 9 Ab
Total abnormality(%)	5.78±0 .244 Dc	9.59±0.24 Ca	11.40±0.14 Ba	13.06±0.19 Aa	7.54±0 .170 Cb	9.87±0 .29 Bb	11.23±0 .26 Ab
HOS (%)	79.46±0.9 7 Aa	70.40±0.79 Bc	61.79±0.82 Cc	58.60±0.65 Dc	74.01±1.47A b	66.64±0.92 Bb	64.31±0.97 Cb

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

Effect of different concentrations of pentoxifylline on Motility and plasma membrane intact cells (hypo-osmotic swelling test) parameters measured by CASA in ejaculated German Shepherd dog spermatozoa during further steps of processing (after dilution, after cooling, and post thawing) at 72 hr

The results of Concentration x10^6/ml of ejaculated German Shepherd dogs treated with different concentrations of pentoxifylline (table:-3) after dilution was in T1(198.93±0.31), T2(189.92±0.29), and T3(197.24±0. 19) respectively. The results proved the highest value significant ($p<0.05$) was in T1(198.93±0.31) and T3(197.24±0. 19), and the lowest critical value ($p<0.05$) was in T2(189.92±0.29). Values of Concentration x10^6/ml with different concentrations of pentoxifylline after cooling and post-thawing were in T2 (182.65±0.31), T3 (187.11±0.23), and T2(180.08±0.25),

T3(184.56±0.35), respectively (table:4-2). The result showed that the highest value significant (p<0.05) was in T3 (187.11±0.23)and (184.56±0.35), and the lowest effective (p<0.05) were in T2 (182.65±0.31) and (180.08±0.25).

The results of the Progressive motility percentage of ejaculated German Shepherd dogs treated with different concentrations of pentoxifylline at 72 hr (table:3) after dilution were T1 (58.53±0.20%), T2(51.85±0.89%)and T3(55.52±0.24 %)respectively. The results proved highest significant value (p<0.05) was in T1 (58.53±0.20%), and the lowest considerable value (p< 0.05) was in T2(51.85±0.89%)in comparison with others. Values of Progressive motility percentage with different concentrations of pentoxifylline after cooling were in T2 (47.66±0.27%) and T3 (51.83±0.21%), respectively (table:3). The results showed the highest significance (p<0.05) value was in T3 (51.83±0.21%), and the lowest powerful (p<0.05) was in T2 (47.66±0.27%) in comparison with others. After freezing, values of Progressive motility percentage are T2(42.81±0.23%) and T3 (46.58±0.21%), respectively, Highest sperms Progressive motility 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 sperm's Total motility percentage during different steps of freezing (after dilution, cooling, and post-thawing) are depicted in (table:3). The results after dilution are T2 (76.89±0.18%) and T3(79.49±0.15%), respectively. Highly sperms Total motility percentage was attained in T3, which differed significantly (P<0.05) from T2. Values of Total motility percentage with different concentrations of pentoxifylline after cooling were in T2 (70.21±0.185%) and T3 (74.50±0.18%), respectively (table:4). The results showed the highest significance (p<0.05) value was in T3 (74.50±0.18%), and the lowest powerful (p<0.05) was in T2 (70.21±0.185%). At post-thawing, values of Total motility percentage are (66.30±0.204%) (and 69.29±0.21 %) in T2 and T3, respectively. The highest sperms Total motility percentage was observed in T3, which differed significantly (P<0.05) from T2(66.30±0.204%), and the lowest value was in T2 after cooling and freezing with significant differences (P<0.05) from others.

Can be observed for VCL values after cooling and post-thawing highest significant (p<0.05) value was in T2(208.51±0.0043) and (231.69±0.0047), which differed significantly (P<0.05) from T3(204.85±0.0039) and(216.18±0.0031), which proved an increase in VCL(μm/s) after freezing compared to after cooling in T2, while there was no significant rise in the T3 compared to T1.

Values of VAP and VSL with different concentrations of pentoxifylline after dilution were in T2 (144.64±0.0089),(120.92±0.0030), and T3 (148.83±0.013),(127.58±0.0016), respectively (table:4-2). The results showed highest significant (p<0.05) value was in T3 (148.83±0.013),(127.58±0.0016), and the lowest important (p<0.05) was in T2 (144.64±0.0089),(120.92±0.0030). Values of the current study after cooling are T2(137.85±0.0115), (111.64±0.0047) and (141.11±0.0067),(116.85±0.0015), respectively. Lowest VAP and VSL sperms were observed in T2, which differed significantly at (P<0.05), in control with significant differences (P<0.05) between T2 and T3. Meanwhile, post thawing, the VAP and VSL sperms are (122.53±0.0090), (102.57±0.0026)and (127.85±0.011), (109.36±0.76) in control, T2, and T3, respectively. The highest value of VAP and VSL sperms was attained in T1, 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.

WOB values in a table after dilution, cooling, and post-thawing at 72 hr (3).Results after dilution are (0.79±0.0045), (0.72±0.0025) and (0.74±0.0035) inT1 , T2 and T3 respectively . after dilution, the results proved a significant difference at (P<0.05) in the values of STR between T2and T3 groups, lowest values attained in T2 and the highest values in T3 followed by T1.while After cooling and post thawing, the result of the current study proved significantly at (P<0.05). The highest value of WOB was attained in T3 (0.69±0.0081),(0.59±0.0037), and the highest values in T1 followed by T3, which differed significantly (P<0.05) from T2. Regarding STR(μm/s), a result of the Effect of different steps of frozen semen processing (after cooling and freezing) is shown in table (2). The values referred to no significant (P<0.05) increase in STR(μm/s) after dilution and after cooling between T2 and T3.

The results of the study regarding ALH of ejaculated German Shepherd dogs treated with different concentrations of pentoxifylline (table3), after dilution and after cooling were in T2(5.23±0.37),(4.39±0.55) and T3(5.44±0.01),(4.64±0.01) .as can no significant (p<0.05) between T3and T2. Regarding LIN, the results indicate no significant differences at (P<0.05) increase in the T1 and T2 groups.

The results of sperm Total abnormality percentage after dilution, after cooling, and post-thawing during treatment of diluted semen with two concentrations of pentoxifylline are shown in(table:3). Values after cooling were (16.12±0.23%)and (14.33±0.30%) in T2 and T3, respectively. The lowest Percentage of Total abnormality sperms after cooling was observed, which differed significantly at (P<0.05) from T2 and control (T1). Meanwhile, post thawing, the Total abnormal sperm are (19.30±0.21%) and (16.62±0.18 %) in T2 and T3, respectively. The result of the current study proved significantly at (P<0.05), the lowest values attained in T3 and T1 the highest values in T2.

Hypo-osmotic swelling Percentage for german Shepherd dogsSemen treated with different concentrations of pentoxifylline (table:4-2) after dilution was in T1(75.47±0.27%), T2(63.42±0.57%),

and T3(66.21±0.19%) respectively. The results proved the value non-significant (p<0.05) between T2 and T3. Values of hypo-osmotic swelling Percentages with different concentrations of pentoxifylline after cooling and post-thawing were in T2 (53.78±0.29),(48.59±0.22%), T3 (59.65±0.28, 53.96±0.28%), respectively (table:3). The result showed that the highest value significant (p<0.05) was in T3 (59.65±0.28),(53.96±0.28%), and the lowest significant (p<0.05) were in T2 (53.78±0.29),(48.59±0.22%).

The result of the study of dead sperm percentage during different steps of frozen semen processing are shown in table (4); after cooling, the values areT2 (13.66±0.0049)and T3 (11.154±0.0097) in control, respectively. The lowest Percentage of dead sperm was recorded in T1 and T3, and all these values differed significantly (P<0.05) from T2 after cooling and freezing (table 4).

Table (3)Effect of different concentrations of pentoxifylline on Motility parameters measured by CASA in ejaculated German Shepherd dogs spermatozoa during further processing (after dilution, after cooling and post thawing) at 72 hr (Mean± SE).

Analysis	T1- control (Tris)	Step of freezing T2(1mM/ml)			Step of freezing T3(5mM/ml)		
		After dilution	After cooling	Post thawing	After dilution	After cooling	Post thawing
Concentration x10⁶/ml	198.93±0.31 Aa	189.92±0.29 Bb	182.65±0.31 Cc	180.08±0.25 Cc	197.24±0. 19 Aa	187.11±0.23 Bb	184.56±0.35 Cb
Progressive motility (%)	58.53±0.20 Aa	51.85±0.89 Bc	47.66±0.27 Cc	42.81±0.23 Dc	55.52±0.24 Bb	51.83±0.21 Cb	46.58±0.21 Db
Total motility (%)	83.02±0.22 Aa	76.89±0.18 Bc	70.21±0.185 Cc	66.30±0.204Dc	79.49±0.15 Bb	74.50±0.18 Cb	69.29±0.21 Db
VAP (µm/s)	154.62±0.013 Aa	144.64±0.0089 Bc	137.85±0.0115 Cc	122.53±0.0090 Dc	148.83±0.013 Bb	141.11±0.0067 Cb	127.85±0.011 Dd
VSL (µm/s)	137.86±0.00178 Aa	120.92±0.0030 Bc	111.64±0.0047 Cc	102.57±0.0026 Dc	127.58±0.0016 Bb	116.85±0.0015 Cb	109.36±0.76 Db
VCL (µm/s)	±0.32194.70 Cc	203.13±0.0061 Ba	208.51±0.0043 Bc	231.69±0.0047 Aa	200.86 ±0.0062 Bb	±0.0039204.8515 Ba	216.18±0.0031 Ab
ALH (µm/s)	5.75±0.02 Aa	5.23±0.37 Aa	4.39±0.55 Bb	3.98±0.51 Cc	5.44±0.01 Aa	4.64±0.01 Bb	4.04±0.02 Cb
LIN (µm/s)	0.89±0.0029 Aa	0.84±0.0031 Aa	0.81±0.0028 Aa	0.70±0.0031 Bc	0.86±0.0056 Aa	0.83±0.0031 Aa	0.78±0.0035 Bb
STR (µm/s)	89.16±0.0054 Aa	83.60±0.42 Aa	80.99±0.45 Aa	66.34±0.005 Bc	85.72±0.003 Aa	82.81±0.0016 Aa	75.19±0.0016 Bb
WOB(µm/s)	0.79±0.0045 Aa	0.72±0.0025 Bb	0.66±0.0015 Cb	0.53±0.0016 Db	0.74±0.0035 Ab	0.69±0.0081 Bb	0.59±0.0037 Cb

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

Table4/ Effect of different concentrations of pentoxifylline 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	T1- control (Tris)	Step of freezing T2(1mM/ml)			Step of freezing T3(5mM/ml)		
		After dilution	After cooling	Post thawing	After dilution	After cooling	Post thawing
Dead sperms (%)	9.71±.00051 Db	10.09±0.0038 Ca	13.66±0.0049 Ba	14.43±0.049 Aa	9.05±0.0050 Cb	11.154±0.0097 Bb	13.064±0.0030 Aa
Sperm abnormality(%)	7.92±0.20 Dc	13.51±0.25 Ca	16.12±0.23 Ba	19.30±0.21 Aa	10.80±0.21 Cb	14.33±0.30 Bb	16.62±0.18 Ab
HOS (%)	75.47±0.27 Aa	63.42±0.57 Bb	53.78±0.29 Cc	48.59±0.22 Dc	66.21±0.19 Bb	59.65±0.28 Cb	53.96±0.28 Db

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

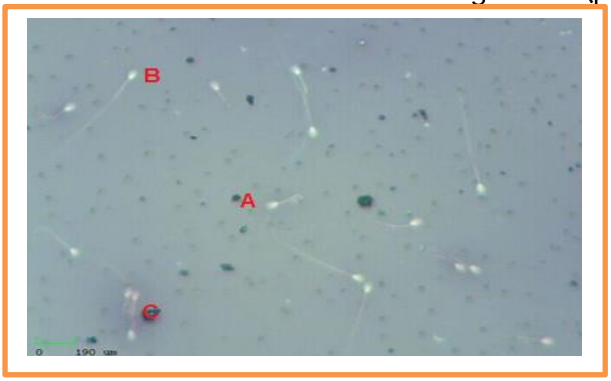

Figure (1): Sperms of german Shepherd dogs (control group), stained with eosin-nigrosin (10x), after cooling. (A=Alive Sperm with coiled tail, B= Alive sperm with normal and C= Dead abnormal sperm with Bent tail

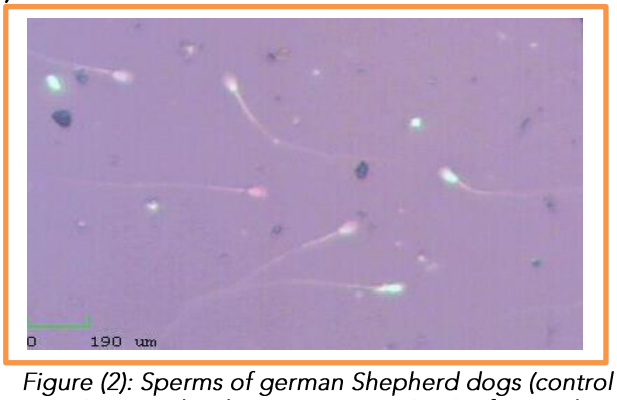

Figure (2): Sperms of german Shepherd dogs (control group), stained with eosin-nigrosin (10x), after cooling. (A=Alive Sperm with coiled tail, B= Alive sperm with normal and C= Dead abnormal sperm with Bent tail .

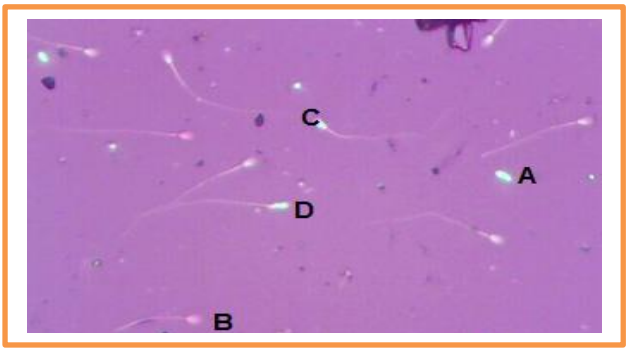

Figure (3): Sperms of German Shepherd dogs stained with eosin-nigrosin (10x), after thawing. (A= Alive Sperm with Detached head, B= Dead abnormal sperm with Bent tail and C,D= Alive sperm with normal).

Discussion

The Effect of different steps of processing (after dilution, after cooling and post thawing) of ejaculated german Shepherd dogs within each treatment, the study in indicated gradually significant decreased ($p<0.05$) 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),Total abnormality(%) and dead sperm after cooling and post thawing in T2 and lowest value (significant) was in cooling and post thawing in T3 table (1),according to Setyawan *et al.*, 2015may include frozen-thawedSemen is characterized by lower motility and viability than freshly ejaculated Frozen-thawed semen is characterized by a reduction of post-thaw motility and longevity with respect to newly ejaculated or chilledSemen; such a reduced post-thaw longevity is considered one of the causes of the poorer conception rates obtained with frozenSemen than with fresh, chilledSemen, or with natural mating. SpermSetyawan *et al.*, 2015.

Additionally, Cryopreservation induces crystallization and osmotic changes, which can cause cellular damage. An intact plasma membrane is essential for maintaining sperm cell integrity and the events associated with oocyte fertilization. (Stănescu and Bîrțoiu, 2011). However, most freezing-induced injuries in spermatozoa affect the plasma membrane (Bailey et al., 2000; Lucio *et al.*, 2017). Despite the advantages above of Cryopreservation, its process may directly or indirectly affect severe and irreversible damage to the spermatozoa for the thermal shock (cold shock), formation n of intracellular ice crystals, osmotic shock, the stress of cryoprotectants and generator of reactive oxygen species (ROS) (Holt, 2000; Rhemrev *et al.*, 2001; Watson, 2000). Because the spermatozoa are highly differentiated, are very structural, and are functionally motile, these kinds of damage results in the loss of viability, motility, and fertility of spermatozoa (Stănescu and Bîrțoiu, 2012).

The concentration of spermatozoa in the semen is defined as the number of spermatozoa/mL and is measured in sperm-rich fraction using the hemocytometer counting chamber(Brito *et al.*, 2017; Hori *et al.*, 2015)

In the sperm morphology (MOR) and viability (VIA) analysis, the spermatozoa should be stained with various staining methods such as Eosin-Nigrosin, Bengal Rose, Victoria Blue B, and Williams. After that, the total number of 100-500 stained spermatozoa is smeared and is observed under a light microscope at × 1000 magnification with emersion oil to record the Percentage (%) of alive spermatozoa, and defective spermatozoa in the head, neck, midpiece, and tail; abnormal spermatozoa can be recognized by a pear-shaped head, narrow head, detached head, bent neck, cytoplasmic droplet at midpiece, bent tail, coiled tail and broken tail (Hori *et al.*, 2004; Mota Filho *et al.*, 2014; Prapaiwan *et al.*, 2016; Rhemrev *et al.*, 2001; Varesi *et al.*, 2014). In particular, further study needs to secure more than 60% morphologically normal spermatozoa population to prevent the reduction of the fertility of canine spermatozoa (Varesi *et al.*, 2013)

The sperm motility (MOT) is conducted under a light microscope, and warm plate at 37-38 ℃ with a scale of 0-4 (0, absent; 1, weak; 2, definite; 3, sound; 4, vigorous; a score of 3 or 4 indicates progressive motility) or with the percentage (%) of progressively motile (PMOT) sperm (+, active movements; ±, slow motion; -, motionless) (Hori *et al.*, 2015) It was suggested that 30-50% was acceptable in the cryopreserved canine semen (Umamageswari *et al.*, 2012).

The development of computer-assisted sperm analysis (CASA) has been very useful for evaluating the parameter of spermatozoa movement including total motility (%), progressive motility (%), mean velocity (VAP, µm/s; the mean trajectory of the spermatozoa per unit of time), linear velocity (VSL, µm/s; the straight course of the spermatozoa per unit of time), curvilinear velocity (VCL, µm/s; the instantaneously recorded sequential progression along the whole trajectory of the spermatozoon per unit of time), amplitude of lateral head displacement (ALH, µm; mean width of sperm head oscillation), beat cross frequency or frequency of head displacement (BCF, Hz; the number of lateral oscillatory movements of the sperm head around the mean trajectory), mean coefficient (straightness, STR, %; (VSL/VAP) × 100) and linear coefficient (linearity, LIN, %; (VSL/VCL) × 100), wobble coefficient (WOB, %; the oscillation of the curvilinear trajectory upon the mean course; (VAP/VCL) × 100), distance curved line (DCL, mm; the actual distance that the sperm cell moved during the analysis period), distance straight line (DSL, mm; the distance from the point in which the cell was first found to the last location in a straight line), distance average path (DAP, mm; the measured distance using a smooth line as a reference), and average orientation change (AOC, degrees; the average number of degrees that the head of the

sperm moved from left to right during the analysis) (Martins *et al.*, 2009; Martins *et al.*, 2012; Rhemrev *et al.*, 2001). Of particular, some of the parameters above can be applied to determine the fertilization ability of spermatozoa; high values of VAP, VSL, and VCL of spermatozoa were correlated with their capability for oocyte fertilization; in addition, VCL and ALH, inversely proportional to LIN, indicated the hyperactivation of spermatozoa, implying successful penetration to the zona pellucida of oocytes (Schäfer-Some and Aurich, 2007; Silva *et al.*, 2006; Verstegen *et al.*, 2002).

Sperm motility can decrease due to changes in plasma membrane permeability in the tail region of spermatozoa and the formation of ice crystals in mitochondria and axonemes during Cryopreservation (Rasul *et al.*, 2001; Yu *et al.*, 2002; Prapaiwan *et al.*, 2016). spermatozoa are accurately and rapidly assessed using computer-assisted sperm analysis systems (CASA). These systems can detect even minor changes in sperm motility and are handy tools for improving the quality of cryopreserved canine semen (Rijsselaere *et al.*, 2012). In addition, it is possible to visualize the trajectory of movement of individual spermatozoa, enabling a detailed description of parameters related to motility and type of movement (Antończyk *et al.*, 2010). Furthermore, sperm fertilization potential maintenance also depends on spermatozoa's plasma membrane integrity and mitochondrial membrane potential (Fraser *et al.*, 2002; Dziekońska *et al.*, 2009). Various substances have been used to improve the quality of cryopreserved semen.

The most popular motility stimulants include methylxanthines such as caffeine (CAF) and pentoxifylline (PTX) which are competitive and non-selective inhibitors of cyclic adenosine monophosphate (cAMP) phosphodiesterase that increase intracellular cAMP concentration and enhance tyrosine phosphorylation in the sperm tail region (Yunes et al., 2005; Esteves *et al.*, 2007; Brie *et al.*, 2016).

PTX inhibits tumor necrosis factor-alpha, which is responsible for DNA fragmentation and programmed cell death. It also reduces superoxide anions and lipid peroxidation associated with sperm membrane damage (Peeker *et al.*, 1997; Mundle *et al.*, 1999; Zhang *et al.*, 2005).2′-deoxyadenosine (DX), an adenosine analog with a modified ribose ring, delivers similar effects. 2′-deoxyadenosine stimulates motility via a different molecular mechanism which probably involves the activation of A2-receptor-mediated adenylate cyclase, which increases the intracellular concentration of cAMP (Milani *et al.*, 2010)

Ibrahim *et al.* (2015) reported that 5.0 mM PTX protects normal sperm during Cryopreservation. PTX inhibits cAMP phosphodiesterases, thus increasing the intracellular concentration. A rise in cAMP concentration causes an increase in the cAMP-dependent processes of spermatozoa, such as motility, capacitation, and acrosome reaction.

Leite *et al.* 2010showed that PTX can also increase sperm motility by inhibiting phosphodiesterase enzyme activity by increasing the intracellular concentration of cAmp, glycolysis, and energy production. It is possible to summarize three main mechanisms of action for PTX: (i) inhibition of phosphodiesterase, (ii) calcium efflux, and (iii) antagonism of the adenosine receptor (Leite *et al.*, 2010).

Motility stimulants such as pentoxifylline are already known to enhance the motility of ejaculated sperm by inhibiting phosphodiesterase activity, thus enhancing cAMP at the level of the sperm tail. 2′-Deoxiadenosine, an analog of adenosine, has a similar effect. In the dog species, pentoxifylline has been proven to impact the progressive motility of freshmen significantly. In frozen-thawedSemen, its addition at the moment of thawing is helpful (Milani *et al.*, 2010). Various extenders and cryopreservation protocols have been developed over the years, but the whelping rates of frozen dog semen are still unsatisfactory in comparison with freshmen (Uchoa *et al.*, 2012; Rodenas *et al.*, 2014; Johnson *et al.*, 2014). In dogs, the success of AI with cryopreserved semen is considerably limited due to problems associated with insemination timing (Alhaider and Watson, 2009). The selection of the appropriate extender, the freezing process, and the thawing protocol are critical for the success of AI and the quality of cryopreserved canine sperm (Silva *et al.*, 2006; Karger *et al.*, 2017). Furthermore, the success of AI with fresh or cryopreserved canine spermatozoa depends on the AI technique, accuracy of ovulation detection, the time for AI, and the number and concentration of spermatozoa(Eilts, 2005).

The hyperosmotic swelling test (HOST) is applied to evaluate the membrane functionality of spermatozoa. The sets of serial concentrations of distilled water are exposed to spermatozoa. After that, spermatozoa (%) with a functional membrane are counted when the sperm has a curled tail(Mota Filho *et al.*, 2014).

❖ Reference

❖ **Aitken, R.J.; De Iuliis, G.N. 2010**:On the possible origins of DNA damage in human spermatozoa. Mol. Hum. Reprod. 16, 3–13.

❖ **Al-Daraji, H.J., 2011.** Effect of diluent supplementation with different levels of green tea on roosters' semen quality during in vitro storage. Int. J. Pl. Anim. environ. Sci., 3: 51-56.

❖ **Alhaider AK, Watson PF.2009:** Cryopreservation of dog semen: the effects of Equex STM paste on plasma membrane fluidity and the control of intracellular free calcium. *Anim Reprod Sci.* 110(1-2):147–161.

❖ **Antończyk A., Niżański W., Twardoń J., Kozdrowski R., Ochota M., Błasiak K., Mikołajewska N., Stańczyk E. 2010**. Current views on computer assisted sperm analy-sis. Med. Weter., 66: 663–667.

❖ **Bani-Hani S.A. 2011.**Semen quality

and chemical oxidative stress; quantification and remedia-tion. ETD Archive, 24,.

❖ **Banihani s.a., abu-alhayjaa r.f. 2016:** The activity of seminal creatine kinase is increased in the presence of pentoxifylline. Andrologia, 48: 603–604.

❖ **Bailey J.L., Bilodeau J.F., Cormier N. 2000:** Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J. Androl., 21: 1-7.

❖ **Bansal AK., Bilaspuri GS. 2011.** Impacts of oxidative stress and antioxidants on semen functions. **Veterinary Medicine International** .

❖ **Brie D., Sahebkar A., Penson P.E., Dinca M., Ursoniu S., Serban M.C., Zanchet- ti A., Howard G., Ahmed A., Aronow W.S., Muntner P., Lip G.Y., Wong N.D., Rysz J., Banach M. 2016:** Effects of pentoxifylline on inflammatory markers and blood pressure: a systematic review and meta-analysis of randomized controlled trials. J. Hypertens., 34: 2318–2329.

❖ **Brito M.M., LA cio C.F., Angrimani D.S., Losano J.D., Dalmazzo A., Nichi M., and Vannucchi C.I. 2017**: Comparison of Cryopreservation Protocols (Single and Two-steps) and Thawing (Fast and Slow) for Canine Sperm. Anim. Biotechnol. 28: 67-73.

❖ **Centola G.M., Cartie R.J., Cox C. 1995:** Differential responses of human sperm to varying concentrations of pentoxifylline with demonstration of toxicity.

❖ **Cheema, R.S., Bhakri, G., Gandotra, V.K. and Dhanju, C.K. (2011):** Characterization of mongrel dog seminal plasma proteins and their correlation with semen characteristics, J. Reprod. Stem Cell Biotechnol. 2: 55-63.

❖ **Chen L, Yang X, Jiao H, Zhao B.2003:** Tea catechins protect against lead-induced ROS formation, mitochondrial dysfunction and calcium dysregulation in PC12 cells. Chem Res Toxicol . 16: 1155-1161.

❖ **Chyu, K.Y., Babbidge, S.M., Zhao, X., Dandillaya, R., Rietveld, A.G., Yano, J., Dimayuga, P., Cercek, B. and Shah, P.K., 2004.** Differential effects of green-tea derived catechin on developing versus established atherosclerosis in apolipoprotein E-null mice. Circulation, 109: 2448 2453.

❖ **Dziekońska A, Fraser L, Strzeżek J 2009:**Effect of differ-ent storage temperatures on the metabolic activity ofspermatozoa following liquid storage of boar semen.J Anim Feed Sci 18: 638-649.

❖ **Esteves S.C., Spaine D.M., Cedeno A.P. 2007:** Effects of pentoxifylline treatment before freezing on motility, viability and acrosome status of poor quality human spermatozoa cryopre-served by the liquid nitrogen vapor method. Braz. J. Med. Biol. Res., 40: 985–992.

❖ **Eilts B.E. 2005:**Theoretical aspects of canine semen cryopreservation. Theriogenology 64: 692-697.

❖ **Esterhuizen, A.D.; Franken, D.R.; Lourens, J.G.; Prinsloo, E.; van Rooyen, L.H.2000:** Sperm chromatin packaging as an indicator of in-vitro fertilization rates. Hum. Reprod. 15, 657–661.

❖ **Fraser L., Lecewicz M., Strzeżek J. 2002:** Fluorometric assessments of viability and mito-chondrial status of boar spermatozoa following liquid storage. Pol. J. Vet. Sci., 5: 85–92.

❖ **Freshman, J.L., 2002.** Semen collection and evaluation. Clinical Techniques in Small Animal Practice 17 (3), 104-107.

❖ **Gradil C.M., Ball B.A..2000:** The use of pentoxifylline to improve motility of cryopreserved equine spermatozoa. Theriogenology, 54: 1041–1047.

❖ **Goodrowe KL., Walker SL., Ryckman DP., Mastromonaco GF., Hay MA., Bateman HL., Waddell WT. 2000.** Piecing together the puzzle of carnivore reproduction. Animal Reproduction Science 60-61:389-403.

❖ **Hewitt, D.A., Leahy, R., Sheldon, I.M. and England, G.C.M., 2001.** Cryopreservation of epididymal dog sperm. Animal Reproduction Science 67, 101-111.

❖ **Holt W.V. 2000:** Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenology 53: 47-58.

❖ **Hong, H.M.; Sim, G.Y.; Park, S.M.; Lee, E.J.; Kim, D.Y.2018:** Ameliorative effect of chitosan complex on miniature pig sperm cryopreservation. J. Anim. Reprod. Biotech. 33, 337–342.

❖ **Hori T., Atago T., Kobayashi M., and Kawakami E. 2015:** Influence of different methods of collection from the canine epididymides on post-thaw caudal epididymal sperm quality. J. Vet. Med. Sci. 77: 625-630.

❖ **Hori T., Ichikawa M., Kawakami E., and Tsutsui T. 2004:** Artificial insemination of frozen epididymal sperm in beagle dogs. J. Vet. Med. Sci. 66: 37-41.

❖ **Hori, T., Haguida, K., Kawakami, E. and Tsutsui, T., 2004.** Unilateral intrauterine insemination with prostatic fluid-sensitized frozen caudal epididymal sperm in Beagle dogs. Theriogenology 63, 1573-1583.

❖ **Hori, T., Matsuda, Y., Kobayashi, M., Kawakami, E. and Tsutsui, T., 2011.** Comparison of fertility on intrauterine insemination between cryopreserved ejaculated and cauda epididymal sperm in dogs. The journal of Veterinary Medical Science 73, 1685-1688.).

❖ **Hyon, S.H., 2004:** A non-frozen living tissue bank for allotransplantation using green tea polyphenols. Yon med. J., 45: 1025-1034.

❖ **Ibrahim A., Barakat, H., Mohamed, A.; Danfour, F. , Galewan, A. M. and Mohamed ,A. 2015:.**Effect of Various Concentrations of Caffeine, Pentoxifylline, andKallikrein on Hyperactivation of Frozen Bovine Semen, BioMed Research Internationa, Article ID 948575, pages7.

❖ **Johnson A.E., Freeman E.W., Wildt D.E., Songsasen N. 2014.** Spermatozoa from the maned wolf (Chrysocyon brachyurus) display typical canid hyper-sensitivity to osmotic and

freez-ing-induced injury, but respond favorably to dimethyl sulfoxide. Cryobiology, 68: 361–370.

❖ **Jiřina, K. and Anton, K., 2013.** Effect of green tea extract on motility parameters of rabbit sperm. *J. Microbiol. Biotech. Fd. Sci.*, 2: 1-13.

❖ **K a y V.J., C ou t ts J.R.T., R o b e r t s o n L. 1993:** Pentoxifylline stimulates hyperactivation in hu-man spermatozoa. Hum. Reprod., 8: 727–731.

❖ **L e w i s S.E., M oo h an J.M., T h om p so n W. 1993:** Effects of pentoxifylline on human sperm mo-tility in normozoospermic individuals using computer assisted analysis. Fertil. Steril., 59: 418–423.

❖ **L ó p e z F.J., A l v a ri ñ o J.M. 2000:** Effects of added caffeine on results following artificial insemination with fresh and refrigerated rabbit semen. Anim. Reprod. Sci., 58: 147–154.

❖ **Lanzafame F., Chapman M.G. Guglielmino A., Gearon C.M., Forman R.G. 1994:** Pharmacological stimulation of sperm motility Hum. Reprod., 9: 192–199.

❖ **Leite, T.G., do Vale Filhoa, V.R., Arruda ,R.P., Andrade, A.F.C., Emericka, L.L., Zaffalon, F.G., Zaffalon, J.A.M., Martinsa, J.A.M., and de Andrade, V.J. 2010:** Effects of extender and equilibration time on post-thaw motility and membrane integrity of cryopreserved Gyr bull semen evaluated by CASA and flow cytometry. Anim Reprod Sci. 120:31–38.

❖ **Lomeo AM, Giambérsio AM. 1991.** Water-test: a simple method to assess sperm-membrane integrity. Int J Androl, 14:278-282.

❖ **Lucio C.F., Angrimani D.S.R., Brito M.M., Vannucchi C.I. 2017:** Oxidative stress challenges during the sperm cryopreservation in dogs. J. Vet. Androl., 2: 1-7.

❖ **M b i z v o M.T., J oh n st o n R.C., B a k e r G.H.W. 1993:** The effect of the motility stimulants, caf-Bereitgestellt von Uniwersytet Warminsko Mazurski | Heruntergeladen 23.09.19 14:08 UTC Effect of motility stimulants on frozen-thawed canine semen 745feine, pentoxifylline, and 2-deoxyadenosine on hyperactivation of cryopreserved human sperm. Fertil. Steril., 59: 1112–1117.

❖ **Manitawittayarat,Ayaito,Taichikimura,Zha onamula,Vien Vietluu,Lanh Thi Kimdo,Yokosato,Masayasu,Taniguchi Andtakeshigeotoi 2013**:Effects of green tea polyphenol on the quality of canine semen after long-term storage at 5 °C. Volume 13, Issue 3, September , Pages 251-254.

❖ **Martins M., Justino R.C., Sant'anna M.C., Trautwein L.G., and Souza F.F. 2012**: Comparison of two different extenders for cryopreservation of epididymal dog sperm. Reprod. Domest. Anim.: 293-294.

❖ **Martins M.I., Padilha L.C., Souza F.F., and Lopes M.D. 2009:** Fertilizing capacity of frozen epididymal sperm collected from dogs. Reprod. Domest. Anim.: 342-234.

❖ **Maxwell, D. T., Jacobson, J. D. , King, A. and Chan, P. J.2002** :Effect of pentoxifylline on tumor suppressor and proto-oncogene apoptosis in sperm. J. Assist. Reprod. Genet. 19(6): 279-283.

❖ **Michael, A.; Alexopoulos, C.; Pontiki, E.; Hadjipavlou-Litina, D.; Saratsis, P.; Boscos, C.2007:** Effect of antioxidant supplementation on semen quality and reactive oxygen species of frozen-thawed canine spermatozoa. Theriogenology ., 68, 204–212.

❖ **Milani C., Fontbonne A., Sellem E., Stelletta C., Gérard O., Romagnoli S. 2010:** Effect of post-thaw dilution with caffeine, pentoxifylline, 2'-deoxyadenosine and prostatic fluid on motility of frozen-thawed dog semen. Theriogenology, 74: 153–164.

❖ **Mota Filho A.C., Silva H.V., Nunes T.G., de Souza M.B., de Freitas L.A., de Araújo A.A., and da Silva L.D. 2014:** Cryopreservation of canine epididymal sperm using ACP-106c and TRIS. Cryobiology 69: 17-21.

Mundle S.D., Reza S., Ali A., Mativi Y., Shetty V., Venugopal P., Gregory S.A., R a z a A. 1999: Correlation of tumor necrosis factor alpha (TNF alpha) with high Caspase 3-like activity in myelodysplastic syndromes. Cancer Lett., 140: 201–207.

Naresh, S.; Atreja, S.K. 2015: The protein tyrosine phosphorylation during in vitro capacitation and cryopreservation of mammalian spermatozoa. Cryobiology , 70, 211–216.

P e e k e r R., A b r am s s o n L., M a r kl u nd S.L. 1997: Superoxide dismutase isoenzymes in hu-man seminal plasma and spermatozoa. Mol. Hum. Reprod., 3: 1061–1066.

❖ **Park, B.J.; Lee, H.J.; Lee, S.L.; Rho, G.J.; Kim, S.J.; Lee, W.J.2018**: Establishment of normal reference data of analysis in the fresh and cryopreserved canine spermatozoa. J. Anim. Reprod. Biotechnol. 33, 75–84.

❖ **Park, S.H.; Jeon, Y.; Yu, I.J.** 2017:Effects of antioxidants supplementation in porcine sperm freezing on in vitro fertilization and the glutathione and reactive oxygen species level of presumptive zygotes. J. Anim. Reprod. Biotechnol32, 337–342.

❖ **Pena, A.I.; Barrio, M.; Becerra, J.J.; Quintela, L.A.; Herradon, P.G.2012** Motile sperm subpopulations in frozen-thawed dog semen: Changes after incubation in capacitating conditions and relationship with sperm survival after osmotic stress. Anim. Reprod. Sci. 133, 214–223.

❖ **Peña, F.J., Núñez-Martínez, I., Morán, J.M., 2006.** Semen technologies in dog breeding: an update. Reproduction of Domestic Animals 41, 21-29.

❖ **Ponce A.A., Fiol de Cuneo M., Ruiz R.D., Vincenti L.M., Santillá M.E., Stutz G., L a c u a r a J.L. 1999:** Influence of pentoxifylline on sperm membrane functional integrity. Arch. Androl., 43: 77–84.

❖ **Prapaiwan N., Tharasanit T., Punjachaipornpol S., Yamtang D., Roongsitthichai A., Moonarmart W., Kaeoket K., and Manee-In S.**

2016:Low-density Lipoprotein Improves Motility and Plasma Membrane Integrity of Cryopreserved Canine Epididymal Spermatozoa. Asian-Australas. J. Anim. Sci. 29: 646-651.

❖ **Quintero-Moreno, A., Rigau, T. and Rodriguez-Gil, J.E., 2004:** Regression analyses and motile sperm subpopulation structure study as improving tools in boar semen quality analysis. Theriogenology, 61: 673–690.

❖ **Rasul Z., Ahmad N., Anzar M. 2001**: Changes in motion characteristics, plasma membrane integrity, and acrosome morphology during cryopreservation of buffalo spermatozoa. J. Androl., 22: 278–283.

❖ **Rijsselaere T., Van Soom A., Maes D., Niżanski W. 2012:** Computer-assisted sperm analysis in dogs and cats: an update after 20 years. Reprod. Domest. Anim., 47: 204–207.

❖ **Rodenas C., Parrilla I., Roca J., Martinez E.A., Lucas X. 2014:** Effects of rapid cooling prior to freezing on the quality of canine cryopreserved spermatozoa. J. Reprod. Dev., 60: 355–361.

❖ **Rhemrev J.P., Lens J.W., McDonnell J., Schoemaker J., and Vermeiden J.P. 2001:** The postwash total progressively motile sperm cell count is a reliable predictor of total fertilization failure during in vitro fertilization treatment. Fertil. Steril. 76: 884-891.

❖ **Rota, A., Milani, C., Capianca, G. and Martiri, N. 2006.** Comparison between glycerol and ethylene glycol for dog semen cryopreservation. Theriogenology, **65**: 1848-1858.

❖ **Setyawan E.M., Kim M.J., Oh H.J., Kim G.A., Jo Y.K., Lee S.H., Choi Y.B., Lee B.C. 2015**:Maintaining canine sperm function and osmolyte content with multistep freezing protocol and different cryoprotective agents. Cryobiology, 71: 344–349.

❖ **Schäfer-Somi S., and Aurich C. 2007.** Use of a new computer-assisted sperm analyzer for the assessment of motility and viability of dog spermatozoa and evaluation of four different semen extenders for predilution. Anim. Reprod. Sci. 102: 1-13.

❖ **Sharma R.K., Tolentino M.V. Jr, Thomas A.J. Jr, Agarwal A. 1996:** Optimal dose and duration of exposure to artificial stimulants in cryopreserved human spermatozoa. J. Urol., 155: 568-573.

❖ **Silva A.R., Cardoso R.C.S., Silva L.D.M., Chirinea N.H., Lopes M.D., and Souza F.F. 2006**:Prognostic value of canine frozen thawed semen parameters on in vitro sperm oocyte interactions. Theriogenology 66: 456-462

❖ **Stănescu M., and Bîrțoiu A.I. 2012:**Freezing of dog's sperm: a review. Rom. Biotechnol. Lett. 17: 1109-7716.

❖ **Stephens T.D., Brooks R.M., Carrington J.L., Cheng L., Carrington A.C., Porr C.A., Splan R.K. 2013:** Effects of pentoxifylline, caffeine, and taurine on post-thaw motility and longevity of equine frozen semen. J. Equine Vet. Sci., 33: 615-621.

❖ **Tesarik J., Thèbault A., Testart J. 1992**: Effect of pentoxifylline on sperm movement char-acteristics in normozoospermic and asthenozoospermic specimens. Hum. Reprod., 7: 1257–1263.

❖ **Uchoa D.C., Silva T.F., Mota Filho A.C., Silva L.D. 2012:** Intravaginal artificial insemination in bitches using frozen/thawed semen after dilution in powdered coconut water (ACP-106c). Reprod. Domest. Anim., 47: 289–292.

❖ **Umamageswari J., Joseph C., Kulasekar K., Kalatharan J., and Sridevi P. 2012**:Effects of different cooling rates using programmable freezer on post thaw survival of dog spermatozoa. Indian J. Anim. Reprod. 33: 19-22.

❖ **Varesi S., Vernocchi V., Faustini M., and Luvoni G.C. 2013**:Quality of canine spermatozoa retrieved by percutaneous epididymal sperm aspiration. J. Small Anim. Pract. 54: 87-91.

❖ **Varesi S., Vernocchi V., Morselli M.G., and Luvoni G.C. 2014:** DNA integrity of fresh and frozen canine epididymal spermatozoa. Reprod. Biol. 14: 257-261.

❖ **Verstegen J., Iguer-Ouada M., and Onclin K. 2002:** Computer assisted analyzers in andrology research and veterinary practice. Theriogenology 57: 149-179.

❖ **Watson P.F. 2000**:The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci. 60-61: 481-49

❖ **Watson, P.F., and W.V. Holt. 2001.** Cryobanking the Genetic Resource: Wildlife Conservation for the Future? Taylor and Francis, New York.

❖ **Wittayarat M, Ito A, Kimura T, Namula Z, Luu VV, Do LTK, Sato Y, Taniguchi M, Otoi T.2013:** Effects of green tea polyphenol on the quality of canine semen after long-term storage at 5oC. Reprod biol . 13: 251-254.

❖ **Wydooghe, E.; Snoeck, F.; Van Soom, A.2016:** Establishment of live birth following intravaginal artificial insemination with chilled epididymal dog semen collected postmortem: Case report. In Proceedings of the 8th International Symposium on Canine and Feline Reproduction ISCFR, Paris, France, 25 June.

❖ **Yildiz, C.; Ottaviani, P.; Law, N.; Ayearst, R.; Liu, L.; McKerlie, C.2007:** Effects of cryopreservation on sperm quality, nuclear DNA integrity, in vitro fertilization, and in vitro embryo development in the mouse. Reproduction

❖ **Yovich J.M., Edirisinghe W.R., Cummins J.M., Yovich J.L. 1990:** Influence of pentoxi-fylline in severe male factor infertility. Fertil. Steril., 53: 715–722.

❖ **Yu I., Songsasen N., Godke R.A., Leibo S.P. 2002**:Differences among dogs in response of their spermatozoa to cryopreservation using various cooling and warming rates. Cryobiology, 44: 62–78.

❖ **Yunes R., Fernández P., Doncel**

G.F., A c o s t a A.A. 2005: Cyclic nucleotide phosphodies-terase inhibition increases tyrosine phosphorylation and hyper motility in normal and pathological human spermatozoa. Biocell., 29: 287–293.

❖ Z h a n g X., S h a rm a R.K., A ga r wa l A ., F a lc o ne T. 2005:Effect of pentoxifylline in redu- cing oxidative stress-induced embryotoxicity. J. Assist. Repro

❖ d. Genet., 22: 415–417.