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Effect of tris-citric acid-egg yolk-glycerol and vitrified medium with and without ascorbic acid on the quality of post- thaw canine semen

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Abstract

The study aimed to compare vitrified medium (Soya lecithin 1% and 0.25 M sucrose) with tris-citric acid fructose-egg yolk 20%-glycerol 3% (TCF-EY-GL) for freezing of canine semen and assess the impact of adding ascorbic acid to both extenders on the post-thawing semen quality. Semen was collected from 3 healthy and fertile German shepherd dogs by digital manipulation and stimulation of the bulbus glandis. Collected semen was initially evaluated by a computerized assisted semen analyzer (CASA). Ejaculates from the dogs were pooled and divided into 4 equal parts and diluted 1:1 with the following 4 extenders: TCF-EY-GL with ascorbic acid (Ex1), TCF-EY-GL without ascorbic acid (Ex2), vitrified medium with ascorbic acid (Ex3) and vitrified medium without ascorbic acid (Ex4). The extended semen allowed for equilibrating for 1 h in a refrigerator set at 4 ºC then packed into 0.5ml straws. Straws containing semen diluted in Ex1 and2 were frozen by the conventional method however, those containing semen diluted in Ex3 and Ex4 (vitrified media) were plunged directly in liquid nitrogen. Semen samples were evaluated by CASA immediately after thawing. Results revealed that progressive motility, normal morphology, and acrosome integrity were significantly higher in vitrified medium (Soya lecithin 1% and 0.25 M sucrose) than those obtained in TCF-EY-glycerol however, plasma membrane integrity was significantly improved in TCF-EY-GL compared with that observed in the vitrified medium. Addition of ascorbic acids to vitrified medium and TCF-EY-GL resulted in a significant increase in all semen quality parameters. Total sperm motility, progressive motility, and normal sperm morphology were significantly higher in vitrified medium with ascorbic acid than those observed in TCF-EY-GL with ascorbic acid. It was concluded that the addition of ascorbic acid to vitrified medium or TCF-EY-GL extender resulted in significant improvement in post-thawing canine semen quality and the vitrified medium can be used as a good alternative for TCF-EY-GL extender to facilitate freezing and avoid undesirable use of EY.

Keywords: Canine; Semen; Cryopreservation; Vitrification; Glycerol; Ascorbic acid

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1. Introduction

About 35000 dogs in Egypt are registered (Haron, 2012). Dogs provide humans with a variety of services, including companionship, hunting, load-pulling, protection, assistance with police and military work, and more recently, assistance to people with disabilities.

Artificial insemination (AI) is commonly employed in domestic dog breeding to protect threatened canid species, meet the needs of individual dog owners, and improve breed genetics. Worldwide adoption of AI has helped ex situ dog species conservation programs, according to (Jewgenow & Songsasen, 2014)

The viability and functional competence of the spermatozoa must be present in sufficient numbers for all the ovulated oocytes to be fertilized normally and for the spermatozoa to contribute to embryo development in order for semen samples to be considered fertile (Rodríguez-Martínez, 2003).

Sperm DNA damage, loss of membrane integrity, and changes to the acrosome's structure can all be brought on by cryopreservation (Karger et al., 2017; Kim et al., 2010) The aforementioned dangers call for novel approaches to improve canine spermatozoa post-thaw parameters, maximize oocyte fertilization, and boost contraceptive rates.

Sperm motility is one of the main elements defining the quality of frozen-thawed samples for AI (Van den Berghe et al., 2018). Positive results of fertility from using frozen canine semen is depended on the total number of progressively motile spermatozoa after thawing (Mason, 2017). Changes in spermatozoa's tail region's plasma membrane permeability and the formation of ice crystals in their mitochondria and axonemes during cryopreservation can both cause a decrease in sperm motility (Prapaiwan et al., 2016; RASUL et al., 2001; Yu et al., 2002).

Spermatozoa die during preservation due to a combination of cold shock and reactive oxygen species (ROS). ROS can cause lipid peroxidation, which leads to membrane loss, deoxyribonucleic acid (DNA) damage, decreased spermatozoa motility, and decreased spermatozoa fertilizing capacity (Lucio et al., 2016).

Sperm extenders are made up of extracellular and intracellular cryoprotectants like milk and egg yolk, as well as buffering agents like tris, sugars like sucrose, lactose, and glucose-fructose, salts like sodium citrate and citric acid, and antibiotics like streptomycin and amikacin penicillin. Several extenders, including soy lecithin, low-density lipoproteins (LDL), reduced glutathione, milk, liposomes, and coconut water powder, are advised for semen cryopreservation in canine species (Belala et al., 2016; Das et al., 2018; Kmenta et al., 2011; Lucio et al., 2017; Ogata et al., 2015)

ROS are produced by the spermatozoa as a result of semen cryopreservation, which stresses the sperm cells and prevents them from capacitation, which is important for fertilization (Lenzi et al., 2002) . However, oxidative stress can occur prematurely during the cryopreservation process, which affects sperm DNA, sperm motility, plasma membrane integrity, and proteins (Birben et al., 2012; Halliwell, 1991; Sharma et al., 2021).

The metabolic product is called a ROS, and it is generated by spermatozoa and leukocytes. ROS are normally well controlled by self-antioxidants within cells (Ashok Agarwal et al., 2003); however, imbalances between excessive production/accumulation of ROS and impaired antioxidant mechanisms (Oxidative stress) can harm spermatozoa, diminishing the quality of preserved semen by lowering sperm motility and the ability to fuse with oocytes and fertilize them (Griveau & Lannou, 1997; Michael et al., 2008), spermatozoa are extremely vulnerable to oxidative stress-related harm (Michael et al., 2008; Shiva et al., 2011). In order to counteract the harmful effects of oxidative stress on the canine semen, antioxidant such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and non-enzymatic antioxidants such vitamin C (ascorbic acid), uric acid, vitamin A, and vitamin E are used (Griveau et al., 1995; Michael et al., 2008).This study was designed to assess the effects of TCF-EY-Glycerol extender compared with Vitrified medium (Soya lecithin 1% and 0.25 M sucrose) on the quality of post-thaw canine semen and to measure the impact of the addition of ascorbic acid to both extenders on post-thaw canine sperm quality.

2- Materials and Methods

2.1. Animals.

Three healthy mature male dogs of German shepherd breed, aged from 2 to 4 years with a good fertility report (history of ability to induce pregnancy in fertile bitches and normal healthy off springs) were used in this study, each dog received a commercial dry canine diet (Mera Dog Active Dry Food) twice daily and unlimited amounts of water. All dogs had palpable normal genitalia and normal libido.

All treatments and pet animals care procedures were approved (Approval number: **DMU/vetmed-2023/009)** by the Institutional Animal Care and Use Committee in AU-IACUC, Damanhour University, Egypt. Authors declare that the procedures imposed on the dogs were carried out to meet the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals and birds used for scientific purposes.

2.2. Semen collection, pooling and initial evaluation.

Semen from dogs was collected by digital manipulation and stimulation of the Bulbus glandis (Linde-Forsberg, 1991) into a pre-warmed plastic bags and kept at water bath 37 ˚C until evaluation and processing (Christiansen et al., 1984; Sahashi et al., 2011) semen were initially evaluated by CASA (Computer Assisted Semen Analyzer), (CASA Sperm Vision, Germany) Dog ejaculates that met the criterion of having at least 70% motile spermatozoa, 70% morphologically normal spermatozoa, and a total sperm count of at least 250 million were used in this investigation.

2.3. Semen dilution and conventional Freezing process.

Collected semen were pooled and then divided into 4 parts each part diluted 1:1 (Linde-Forsberg, 2014) with one of the following 4 extenders: to represent 4 groups 3 samples used for each group.

Extender 1 = Tris citric fructose -egg yolk 20%- Glycerol 3% (TCF-EY- Glycerol 3 %) with ascorbic acid.

Extender 2 = Tris citric fructose -egg yolk 20%- Glycerol 3% (TCF-EY- Glycerol 3 %) without ascorbic acid.

Extender 3 = Vitrified medium (Soya lecithin 1% and 0.25 M sucrose) with ascorbic acid.

Extender4 = Vitrified medium (Soya lecithin 1% and 0.25 M sucrose) without ascorbic acid.

(Addition of 50 M ascorbic acid to TCF-EY-GL or vitrified medium)

The extended semen in extender 1 and 2 (Table 1) was allowed to equilibrate for 1 hour in a refrigerator stetted at 4 ˚C (Pena and Linde-Forsberg., 2000), then, diluted semen was packed in 0.5 mL straws. Semen-containing straws that had been diluted in extenders 1 and 2 were put 5 cm over liquid nitrogen vapor at -70 C for 10 min before being submerged in liquid nitrogen at -196 C. The straws were kept in liquid nitrogen for at least 48 hours before being thawed. (Hermansson & Forsberg, 2006; Verstegen et al., 2005). Semen straws were thawed in 37 ˚C water bath for 60 sec after one week from freezing process (Hermansson & Forsberg, 2006; Silva et al., 2003) and after thawing, semen evaluated by CASA reveals sperm motility, progressive motility, morphology and acrosome integrity were evaluated in all samples (Figure 1).

2.4. The hypoosmotic swelling test (HOST)

The hypoosmotic swelling test (HOST), developed by(Khan & Ijaz, 2008), was used to evaluate the integrity and functionality of post-thawing sperm membranes. The proportion of spermatozoa with curled tails was identified as HOST positive spermatozoa (Figure 2).

2.5. Vitrification media and vitrification process

The vitrification process was done by adding one portion of the vitrification medium (Tris 2.4 gm + Citric Acid 1.4 g + Fructose 1.0 g + Streptomycin 5 µg + Penicillin 10 kIU is diluted in 100ml distilled Water with Soy lecithin at 1%(Andressa Dalmazzo et al., 2018) and 0.25 M sucrose) to one portion of fresh semen at 37 ˚C immediately after initial analysis (Pipan et al., 2020). After addition of vitrification media, vitrified semen was packed into straw straws (0.5 ml) are maintained at 5 °C for 30 min (equilibration time), then plunged directly into liquid N_2 (LN_2) for at least one week. Devitrification of Semen straws were done in 37 ˚C water bath for 60 sec after one week from Vitrification process (Figure 3).

2.6. Statistical analysis

The obtained results from the experiments were expressed as means ± SD and were analyzed by analysis of variance (ANOVA) using SPSS Statistics for Windows, version 23.0 (IBM Corp: Armonk, NY, U.S.A.). For vitrification results, data were analyzed by independent T-test. Differences were declared significant when ($P < 0.05$). ANOVA test "One-Way Analysis of Variance" was used to detect the statistical differences between the means of more than two groups simultaneously. However, ANOVA results do not identify which differences between pairs of means are significant, so TUKEY POST HOC test was used for the pairwise comparisons of the data, “t-test” was used to determine the significant differences between the means of the two groups regarding quantity.

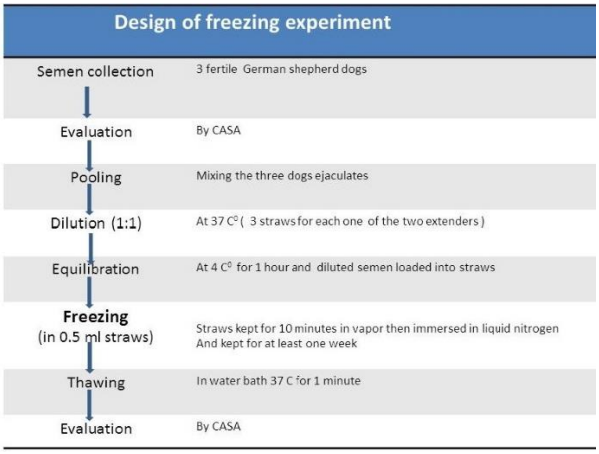

Figure 1. Conventional freezing of dog semen by extender 1,2

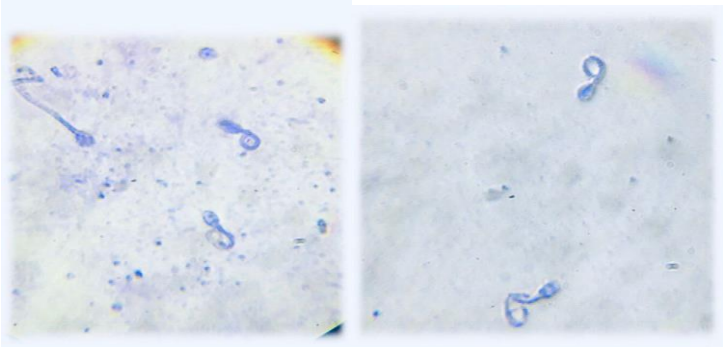

Figure 2. Spermatozoa showing coiled tail were determined as HOST positive spermatozoa.

Table 1. Composition of extender 1 and 2

Composition	Extender 1	Extender 2
Tris (g)	3.025 g	3.025 g
Citric acid (g)	1.7 g g	1.7g
Fructose (g)	1.25 g g	1.25 g
Glycerol 3% (ml)	3 ml	3 ml
Egg yolk 20% (ml)	20 ml	20 ml
Ascorbic acid	50 M	---
Penicillin (g)	0.1 g g	0.1 g
Streptomycin (g)	0.1 g g	0.1 g
Distilled Water (ml)	to 100 mlml	to 100 ml

All chemicals were purchased from El Gomhoria chemicals Company, Alex, Egypt.
Extender 1 = Tris citric fructose -egg yolk20%- Glycerol 3% (TCF-EY- Glycerol 3 %) with ascorbic acid. Extender 2 = Tris citric fructose egg yolk Glycerol 3% (TCF-EY- Glycerol 3 %) without ascorbic acid (Eulenberger et al., 2009; Ponglowhapan et al., 2004).

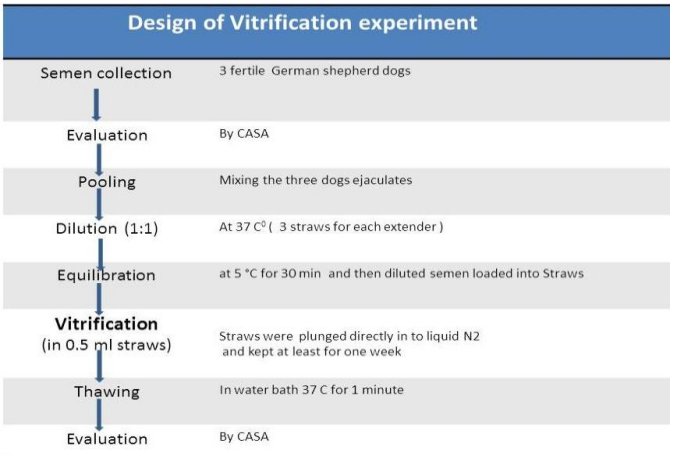

Figure 3. Vitrification process of dog semen by extender 3 and 4.

4. Results

Results of the initial examination of raw semen immediately after collection and pooling were presented in Table1. The average ejaculate volume was 8 ml with 78 × 10^6 sperm concentration/ml. Progressive motility recorded 89% while total motility exceeded this value by about 5%. 91% of sperms were morphologically normal. Acrosome and cell membrane integrity percentage of sperm cells were 93 and 88% respectively.

Results illustrated in Table 2 revealed that progressive motility, normal morphology, and acrosome integrity were significantly higher in vitrified medium (Soya lecithin 1% and 0.25 M sucrose) than those obtained in TCF-EY-glycerol however, plasma membrane integrity and total motility were significantly improved in TCF-EY-GL compared with those observed in vitrified medium.

Data presented in Table 3 showed that addition of ascorbic acid to TCF-EY-glycerol extender resulted in a significant improve in quality of frozen-thawed canine semen. Total motility, progressive motility, normal morphology, acrosome integrity and plasma membrane integrity were significantly increased as a result of addition of ascorbic acid to TCF-EY-glycerol extender.

Data presented in Table 4. Indicated that addition of ascorbic to vitrified medium (Soya Lecithin 1% and sucrose) resulted in a significant increase in total and progressive motility. A positive impact for ascorbic acid was also recorded in other evaluation parameters of frozen-thawed canine semen. It resulted in a significant increase in normal morphology and acrosome integrity which was assessed objectively by CASA. Sperm cell membrane integrity % which was estimated by positive HOST was significantly higher in vitrified medium with ascorbic acid than in ascorbic acid-free vitrified medium.

Results illustrated in Table 5 indicated that post-thawing total motility, progressive motility, and normal morphology percentages were significantly higher in vitrified medium with ascorbic acid than those observed in TCF-EY-GL extender with ascorbic acid.

4-Discussion

The purpose of present study was to compare the effects of TCF-EY-Glycerol extender to Vitrified medium (Soya lecithin 1% and 0.25 M sucrose) on the quality of post-thaw canine sperm, as well as the effect of adding ascorbic acid to both extenders on post-thaw canine sperm quality. Before conducting our experiment, initial quality parameters of fresh semen were measured immediately after collection and pooling. The average ejaculate volume in our study was 8ml, which was like the result obtained by Shalini and Antoine (2018).

In our study, the initial progressive motility rate was 89 %. This finding was similar to that of (Gunay et al., 2003), who reported 84.42±5.3 % initial motility of the first ejaculate, and Shalini & Antoine (2018), who reported 83.3±0.79 % initial motility. (Ray et al., 2019) reported a lower (76.333±2.728 % progressive motility) than the above values. The estimated acrosome integrity (93%) in this study is higher than that obtained by Shalini & Antoine, (2018) and Umamageswari et al., (2015). In fresh dog sperm, they found 76.83± 2.63 % and 79.0± 0.83 % acrosome integrity, respectively.

Table 1. Initial examination of fresh semen after collection before freezing

Volume	Conc. Sperm/ml	Total motility %	Progressive Motility %	Morphology%	Acrosome Integrity %	HOST% positive
8 ml	278 × 10^6	94 %	89 %	91%	93%	88%

Table 2. The effect of TCF-EY-glycerol and vitrified medium (Soya lecithin 1% and 0.25 M sucrose) on the evaluation parameters of frozen-thawed canine sperm.

Parameters	Total motility %	Progressive motility %	Normal Morphology%	Acrosome integrity %	Host %
TCF-EY-Glycerol	50.33±1.52 [a]	44.00±2.00 [b]	61.33±1.52 [b]	62.33±2.51 [b]	68.67±1.52 [a]
Vitrified medium	50.00±1.00 [b]	44.67±1.53 [a]	67.00±1.00 [a]	63.67±1.53 [a]	65.67±1.53 [b]

Means within the same row not sharing the same letter were significantly different at $P < 0.05$.

Table 3. The effect of ascorbic acid addition to TCF-EY-glycerol on the evaluation parameters of frozen-thawed canine sperm

Parameters	Total motility %	Progressive motility %	Normal Morphology%	Acrosome integrity %	Host %
TCF-EY-Glycerol Without ascorbic acid	50.33±1.52 [b]	44.00±2.00 [b]	61.33±1.52 [b]	62.33±2.51 [b]	68.67±1.52 [b]
TCF-EY-Glycerol With ascorbic acid	55.00±2.00 [a]	48.00±2.00 [a]	63.67±3.05 [a]	66.67±2.08 [a]	69.67±2.52 [a]

Means within the same row not sharing the same letter were significantly different at $P < 0.05$.

Table 4. The effect of ascorbic acid addition to vitrified medium (Soya lecithin 1% and 0.25 M sucrose) on the quality of frozen-thawed canine sperm

Parameters Vitrified medium	Total motility %	Progressive motility %	Normal Morphology%	Acrosome integrity %	Host %
Without Ascorbic acid	50.00±1.00 [b]	44.67±1.53 [b]	67.00±1.00 [b]	63.67±1.53 [b]	65.67±1.53 [b]
With Ascorbic acid	57.33±1.53 [a]	52.67±1.53 [a]	72.33±1.53 [a]	68.00±1.00 [a]	72.33±2.08 [a]

Means within the same row not sharing the same letter were significantly different at $P < 0.05$.

Table 5. The effect of TCF-EY-glycerol and vitrified medium (Soya lecithin 1% and 0.25 M sucrose) with ascorbic acids on the evaluation parameters of frozen-thawed canine sperm

Parameters	Total motility %	Progressive motility %	Normal Morphology %	Acrosome integrity %	Host %
TCF-EY-Glycerol	55.00±2.00 [b]	48.00±2.00 [b]	63.67±3.05 [b]	66.67±2.08 [a]	69.67±2.52 [a]
Vitrified medium	57.33±1.53 [a]	52.67±1.53 [a]	72.33±1.53 [a]	68.00±1.00 [a]	72.33±2.08 [a]

Means within the same row not sharing the same letter were significantly different at $P < 0.05$.

The incidence of HOST positive spermatozoa (88%) in the current study is similar (88-97%) to that observed by Borges, (2011) while, it is higher than that (74.8 ± 0.72%) observed by Shalini & Antoine, (2018), and this (80.166 ± 2.522) obtained by Ray et al., (2019). Percentage of positive HOST recorded in our work is somewhat lower than that recorded in the study of (A Rota et al., 1995) which was 93.6%. The total sperm abnormality was 9% however; it was 7-10%, 14.830 ± 2.386 and 7.25 ± 0.86% in data recorded by Kurien et al., (2012), Ray et al., (2019), and Shalini & Antoine, (2018), respectively.

In general, the evaluation parameters of freshly collected semen are consistent with Reference values cited in the article of Shalini & Antoine, (2018), which made the semen acceptable for processing experiments and indicated that these dogs were reproductively sound and did not suffer from any reproductive disorders. The cited reference values for ejaculate volume, initial motility, Sperm concentration, intact acrosome, and HOST positive spermatozoa were 1.91-8.68 ml, 78.30-85.00 %, 273-598 millions/ml, 75 - 85.10 %, and 70 - 82.10 %, respectively.

Our data showed progressive motility, normal morphology, and acrosome integrity were significantly higher in vitrified medium (Soya lecithin 1% and 0.25 M sucrose) than those obtained in TCF-EY-glycerol however, plasma membrane integrity was significantly improved in TCF-EY-GL compared with that observed in vitrified medium. Progressive motility, normal morphology, acrosome integrity, and plasma membrane integrity in TCF-EY-glycerol were 44.00±2.00, 61.33±1.52. 62.33±2.51 and 68.67±1.52 respectively, while the values were 44.67±1.53, 67.00±1.00, 63.67±1.53, and 65.67±1.53 in vitrified medium respectively. It is clear from our data superiority of vitrified medium over TCF-EY-GL in 3 of the evaluation parameters (Progressive motility, normal morphology, acrosome integrity) however the superior of TCF-EY-GL was in plasma membrane integrity. The most used cryoprotectant for freezing canine sperm is glycerol (GL). There is a wide range of GL concentrations used in dogs (1-16%), as well as different extenders and methods. All of these protocol variations result in a wide range of post-thawing motility rates (22-75 %) for frozen dog semen Okano, (2004); Olar et al., 1989; Ada Rota et al., 2010; Silva et al., 2006; Ström, (1997). In agreement with our findings, Futino et al., (2010) evaluated glycerol (GL) as cryoprotectant for canine semen cryopreservation at concentration 3% in egg yolk-TRIS extender. They observed that means for total motility, progressive motility, and morphologically normal spermatozoa were, respectively, 69.0 ± 5.4%, 61.0 ± 7.4%, and 57.1 ± 5.0%. In addition to this agreement there was convergence of values regarding normal morphology and results of host between our data and their data however, our values of total and progressive motility were lower than those assessed by Futino et al., (2010).

Post thawing total motility recorded in our study was 50.33±1.52 at GL 3% compared with 32.75 ± 1.88 and 28.50 ± 0.81% recorded in study of Kusum, (2012) as a result of using 4, 8% GL respectively. While 4 % glycerol and 4 % ethylene glycol showed 29.25 % similar findings were reported by Kurien, (2000). Morton & Bruce, (1989) obtained 41 percent post-thaw motility in frozen thawed dog semen.

In the current study, the post-thaw acrosome integrity in TCF-EY-GL was 62.33±2.51. Furthermore, the post-thaw plasma membrane integrity in TCF-EY-GL was 68.67±1.52. Although they used a different extender, in Mongrel dog sperm, Kurien, (2000) found values an average post-thaw intact acrosome percentage of 64.78 ±0.33, 64.43± 0.26, and 62.86 ±0.41 in Tris, Triladyl, and Laiciphos-488 extenders, respectively. These obtained values were close to our values. Lower than our values, Yildiz et al., (2000) found that using fructose and glucose, the mean percentage of damaged acrosomes was 44.6±3.2 and 47.9±3.1, respectively. Likewise, Umamageswari et al., (2015) discovered 46% post-thaw acrosomal integrity. Close to our value, Ponglowhapan et al., (2004) discovered 34.7±15.0 percent acrosomal loss in frozen sperm.

In the current study, we examined the impact of vitrified medium (Soya lecithin 1 percent and 0.25 M sucrose), which was free of egg yolk and any permeable cryoprotectants, on the post-thaw quality of canine semen. Progressive motility, normal morphology, acrosome integrity, and plasma membrane integrity were 44.67±1.53, 67.00±1.00, 63.67±1.53, and 65.67±1.53 in vitrified medium respectively. Our data show that vitrified medium is superior to TCF-EY-GL in three of the evaluation parameters (progressive motility, normal morphology, and acrosome integrity), but TCF-EY-GL is superior in plasma membrane integrity.

In sperm extenders, soybean lecithin can be utilized in place of egg yolk. A phospholipid component can take the place of the phospholipids and high molecular weight lipoprotein present in egg yolks (Layek et al., 2016). In a number of species, including dogs, soybean lecithin has been utilized to cryopreserve sperm (Axnér & Lagerson, 2016; Beccaglia et al., 2009; Caturla-Sánchez et al., 2018; Andressa Dalmazzo et al., 2018; Hidalgo, 2014; Nöthling et al., 2007).
dog semen has been successfully cryopreserved with 0.04% or 1.5% soybean lecithin (Beccaglia et al., 2009; Hidalgo, 2014), respectively. According to these two experiments, the quality of the sperm after thawing was comparable to that after freezing in a Tris egg yolk (TEY) extender. Axnér & Lagerson, (2016) and Pipan et al., (2020) concluded that for freezing dog semen, an extender with 1% soybean lecithin extender produced superior post-thaw sperm motility than 2% soybean lecithin.

We agree with Pipan et al (2020) and other studies in which some protein supply aids in survival during the initial cryopreservation cold shock. Sperm vitrification has been carried out in a variety of species both with and without the presence of animal proteins using sucrose as a non-permeable cryoprotectant (Isachenko et al., 2008; Pradieé et al., 2015).

Proteins like lecithin, BSA and egg yolk are renowned for preventing the initial cold shock that occurs during freezing. In addition to acting as an antioxidant, soy lecithin can shield semen from oxidative stress, increasing viability and motility after cryopreservation (A. Dalmazzo et al., 2018). By substituting soy lecithin for other proteins of animal origin in the extender as a cryoprotective agent, vitrified dog semen can be transported to all nations of the world more easily (Pipan et al., 2020).

Increasing the concentration of soy lecithin to 1 percent in the study of Pipan et al., (2020)produced encouraging results on post-thaw semen viability and acrosome integrity, which is consistent with earlier studies (Axnér & Lagerson, 2016; Andressa Dalmazzo et al., 2018; Hidalgo, 2014). Our finding backs up earlier claims that canine sperm characteristics such as motility kinetics require high concentrations of soy lecithin to be maintained during storage (Andressa Dalmazzo et al., 2018).

In line with our findings, Pipan et al., (2020) showed that the viability, motility, progressive motility, morphology, acrosome, sperm tail membrane integrity and DNA integrity of canine spermatozoa were all adequately preserved during vitrification in an extender containing soy lecithin and sucrose. These findings suggest that vitrifying sperm can be an efficient and effective way to freeze canine semen. This process is easy, quick and doesn't call for expensive machinery. Our findings are consistent with those obtained by Caturla-Sánchez et al., (2018) They found when compared to conventional freezing, vitrification of canine spermatozoa produced significantly more spermatozoa with intact acrosomes, indicating that the physio-chemistry of vitrification in the presence of disaccharides is advantageous for acrosome integrity.

Our data revealed that addition of ascorbic acid to vitrified medium or TCF-EY-GL resulted in a significant improvement in post-thaw canine semen quality. Post-thaw total, progressive motility and normal morphology was significantly higher in vitrified medium than those observed in TCF-EY-GL extender. The present results are consistent with study of Wittayarat et al., (2012). They demonstrated that supplementation with vitamin C increased the motility of spermatozoa in canine semen. In agreement to our finding, Asadpour et al., (2011) reported that the inclusion of vitamin C in sperm extenders may improve sperm performance by reducing cell damage caused by radical scavenging. In many earlier studies, the causes of the decreased semen quality following freezing and thawing were interpreted. When spermatozoa are frozen and then thawed, cryopreservation causes lipid

peroxidation and an increase in ROS, which upsets the delicate balance between free radicals and the antioxidant system and causes oxidative stress(Guthrie & Welch, 2012).

ROS reaction with spermatozoa enzymes, membrane phospholipid, and chromatin may result in spermatozoa structure and function damage (Aitken, 2017). According to Bui et al., (2018), Morielli & O'Flaherty, (2015), and Treulen et al., (2018), this results in lipid peroxidation, protein oxidation, and DNA fragmentation. Additionally, the use of the cryopreservation technique revealed that post-thawed semen samples had a high susceptibility to excessive ROS production and the disruption of antioxidant levels and function (Ball, 2008). As a result, Agarwal & Allamaneni, (2004) Oxidative stress is when there is an imbalance between the creation of ROS and the antioxidant system's protective function, which is in charge of neutralizing and removing them. All oxygen-derived radicals and non-radicals are categorized as ROS due to their high electron reactivity and instability. ROS can behave as electron givers or receivers in a variety of chemical reactions with various substances (Agarwal et al., 2005) and they are widely regarded as the primary cause of damage to living organisms (Bedard & Krause, 2007). ROS play a significant role in cellular signaling since they are naturally produced as byproducts of oxygen metabolism. They consist of peroxide, free radicals, and oxygen ions(Pinto, 2020).

5. Conclusion

The addition of ascorbic acid to vitrified medium or TCF-EY-GL extender resulted in significant improvement in post-thawing canine semen quality. Post-thawing total sperm motility, progressive sperm motility, and normal sperm morphology in vitrified medium were significantly higher than those observed in TCF-EY-GL extender.

Author contributions

All authors contributed to the conception and realization of the work. All the authors have contributed to the paper redaction and approved the final version of the manuscript.

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