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The Impact of Using Extenders Containing Low-density Lipoprotein Versus Egg Yolk with or without Ascorbic Acid on the Fertility Parameters of Post-thawed Chilled Canine Semen

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Abstract: Egg yolk is included in semen extenders because it prevents cold shock during chilling. This study aimed to assess the impact of substituting egg yolk with low-density lipoprotein (LDL) in Tris-Citric-Fructose (TCF) chilled extenders, with or without ascorbic acid, on the canine semen quality at 24, 48, and 72 hours of cooling. Semen from three German shepherd dogs was evaluated and extended at a 1:1 ratio using four extenders: Group A (TCF buffer, 20% egg yolk, with ascorbic acid), Group B (TCF buffer, 20% egg yolk without ascorbic acid), Group C (TCF buffer, 20% LDL, with ascorbic acid), and Group D (TCF buffer, 20% LDL without ascorbic acid). The extended semen was stored at 4°C and assessed after 24, 48, and 72 hours of cooling. The egg yolk extender resulted in less reduction in progressive sperm motility after 24, 48, and 72 hours of cooling; however, reduction in normal morphology, acrosome integrity, and cell membrane integrity were lower in TCF-LDL chilled extender than in TCF-EY chilled extender. Furthermore, after 24 hours of cooling, all evaluation parameters in both extenders were greater than 50%. In conclusion, the quality of chilled canine sperm was significantly improved by adding ascorbic acid to TCF-EY, but the improvement was only marginal when applied to TCF-LDL chilled extender.

Keywords: Extenders; Low density lipoprotein; Egg yolk; Ascorbic acid; Canine semen

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1. Introduction

In order to protect endangered canid species, satisfy the needs of individual dog owners, and enhance breed genetics, artificial insemination, or AI, is widely utilized in domestic dog breeding. Worldwide adoption of AI has helped *ex-situ* dog species conservation programs (Jewgenow & Songsasen, 2014). Frozen, chilled, or fresh semen can be used for canine artificial insemination. With its ability to be preserved for a longer duration of time, cooled semen is currently being used more often in dogs, as it solves issues related to AI timing and long-distance transportation. However, chilled sperm is more frequently used in AI than frozen semen because it recovers more viable and motile sperm (Rota, Ström, & Linde-Forsberg, 1995) and chilled semen is easier to prepare and requires inexpensive equipment and provides improved pregnancy rates (Ponglowhapan, Essén-Gustavsson, & Forsberg, 2004).

The sperm's metabolism peaks at body temperature and falls off at room temperature (24-29°C). The sperm plasma membrane undergoes a shift from the cooled crystalline to the gel phase when semen is stored at 4-8°C. Cellular metabolism decreases by 50% for every 10°C drop in temperature; at 5°C, sperm metabolic activity is only 10% of what it would be at body temperature (McKinnon, 2019). During cold storage, spermatozoa generally consume and metabolize the oxygen required to support their lifespan (Agarwal, & Said, 2003).

Reactive oxygen species (ROS) are created by spermatozoa and leukocytes when seminal plasma components and prostatic fluid are mixed in fresh semen samples. ROS are known to be detrimental to spermatozoa (Goericke-Pesch, Klaus, Failing, & Wehrend, 2012; Rota et al., 1995).

ROS along with cold shock cause spermatozoa to die during storage. Lipid peroxidation, which is brought on by ROS, can result in membrane loss, damage to DNA, spermatozoa motility, and diminished spermatozoa fertilizing ability (Lucio et al., 2016).
ROS are normally well controlled by natural antioxidants within cells (Agarwal, Prabakaran, & Allamaneni, 2006). However, imbalances between excessive production of ROS and impaired natural-antioxidant (oxidative stress) can result in damage to the spermatozoa, reducing preserved semen quality by reducing sperm motility and the sperm fertilizing capacity (Griveau & Lannou, 1997; Michael et al., 2008)

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. Several extenders, including low-density lipoproteins (LDL), soya lecithin, milk, reduced glutathione, liposomes and coconut water powder, are advised for semen cryopreservation in canine species (Belala et al., 2016; Das, Biswas, Deka, & Dutta, 2018; Kmenta, Strohmayer, Müller-Schlösser, & Schäfer-Somi, 2011; Lucio et al., 2017; Ogata et al., 2015).

Antioxidants such as vitamin C (ascorbic acid), uric acid, vitamin A, and vitamin E are defense systems to neutralize the toxic effects of oxidative stress in human and dog semen (Griveau & Lannou, 1997; Michael et al., 2008).

Therefore, the Objectives of this study were to evaluate the impact of Tris-Citric-Fructose-Egg Yolk (TCF-EY) and Tris-Citric-Fructose-low density lipoprotein (TCF-LDL) chilled extenders on the quality of canine semen at 24,48, and 72 hours of cooling. Assess the effect of the addition of ascorbic acid to TCF- EY extender and TCF-LDL extender on the quality of semen at 24, 48, and 72 hours of cooling.

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 offspring) were used in this study; each dog received a commercial dry canine diet (Mera Dog Active Dry Food) twice daily and provided unlimited amounts of water.

All treatments and pet animal care procedures were approved (Approval number: DMU/vetmed-2024/008) by the Institutional Animal Care and Use Committee in AU-IACUC, Damanhour University, Egypt. The authors affirm that the measures implemented for the dogs were done in accordance with 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, initial evaluation and pooling

Semen from the three Dogs was obtained through digital manipulation and activation of the Bulbus glandis (Linde-Forsberg, 1991) into a pre-warmed plastic bags and kept at water bath 26 ºC until evaluation and processing (Christiansen, Cleemann, & Schmidt, 1984; Sahashi et al., 2011). Semen was initially evaluated by CASA (Computer Assisted Semen Analyzer), (CASA Sperm Vision, Germany). Dog ejaculates that met the criterion of having at least 70% motile and morphologically normal spermatozoa, and a total sperm count of at least 250 million/ml were used in this investigation. After evaluation semen from the three dogs was pooled (Futino, Mendes, Matos, Mondadori, & Lucci, 2010; A. Peña, Barrio, Quintela, & Herradón, 1998).

2.3.Semen processing and chilling

Fractionating the ejaculate is the best course of action, but it's not always practical, and most of the time, getting varying volumes of prostatic fluid is unavoidable. Prostatic fluid needs to be removed by centrifugation since it is known to be harmful for long-term preservation of semen. According to a recent study, the best centrifugation protocol for cleaning canine sperm is 720 g for 5 minutes (Rijsselaere, Van Soom, Maes, & de Kruif, 2002). After removing the seminal plasma, the resulting pellet is suspended with the extenders at room temperature. The pooled semen was extended by 4 extenders by dilution rate (1:1) (Rota et al., 1995)., representing 4 groups 3 samples were assigned for each group. **Group A:** Extender A= tris citric fructose buffer, egg yolk 20%, with ascorbic acid =TCF-EY-with ascorbic acid., Group **B:** Extender B= tris citric fructose buffer, egg yolk 20%, without ascorbic acid= TCF-EY-without ascorbic acid., **Group C;** Extender C**=** tris citric fructose buffer, low density lipoprotein 20%, with ascorbic acid=TCF-LDL-with ascorbic acid **Group D:** Extender D = tris citric fructose buffer, low density lipoprotein 20%, without ascorbic acid =TCF-LDL-without ascorbic acid. Extended semen is stored at 4 ºC and is assessed after 24, 48, and 72 hours (Peña & Linde-Forsberg, 2000). Composition of the extenders used in chilling process showed in table 1.

The semen must be stored in a sterile tube in the dark. The tube is then chilled for 24 hours in a glass of room temperature water in the refrigerator in order to prevent excessive chilling. (Linde-Forsberg, 2010).

2.4.The hypo-osmotic 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

2.5.Statistical analysis

The experimental findings were presented as means ± standard deviation and subjected to analysis of variance (ANOVA) using IBM Corp.'s SPSS Statistics for Windows, version 23.0 (Armonk, NY, U.S.A.). When ($P < 0.05$) was reached, differences were considered significant. To concurrently find statistical differences between the means of more than two groups, the "One-Way Analysis of Variance" (ANOVA) test was employed. However, the TUKEY POST HOC test was employed for the pairwise comparisons of the data because the ANOVA results do not specify whether specific changes between pairs of means are significant. To identify the statistically significant variations between the two groups' means with respect to quantity, the "t-test" was employed.

Table 1. Composition of chilled extenders used in chilling process

Compositions	Extender A	Extender B	Extender C	Extender D
Tris (g)	3.025 g	3.025 g	3.025 g	3.025 g
Citric acid (g)	1.7 g	1.7 g	1.7 g	1.7g
Fructose (g)	1.25 g	1.25 g	1.25 g	1.25 g
Egg yolk 20% (ml)	20 ml	20 ml	-	-
LDL 20% (ml)	-	-	20 ml	20 ml
Ascorbic acid	50bM	-	50 M	-
Penicillin (g)	0.1 g	0.1 g	0.1 g	0.1 g
Streptomycin (g)	0.1 g	0.1 g	0.1 g	0.1 g
Distilled Water (ml)	to 100 ml	to 100 ml	to 100 ml	to 100 ml

All chemicals were purchased from El Gomhoria chemicals company, Alex, Egypt.

3. Results

Results of the initial examination of raw semen immediately after collection and pooling were presented in **Table 2**. The average ejaculate volume was 8ml with 278 × 10^6 sperm /ml. Progressive motility recorded 89% while total motility exceeded this value by about 5%. Morphologically normal sperms were 91%. Acrosome and cell membrane integrity percentage of sperm cells were 93 and 88%, respectively.

The quality parameters of pooled canine semen in 2 different chilled extenders at different storage cooling times were demonstrated in **Table 3** Data showed that progressive motility percentages were reduced after 24 hours of chilling by 21 and 26% in TCF-EY and TCF-LDL extenders respectively; however, the reduction after 48 hours of chilling was 36 and 49% respectively. After 72 hours, progressive motility of sperms was completely stopped.

After 24 hours of chilling, the percentages of acrosome integrity decreased by 33 and 24% in TCF-EY and TCF-LDL extenders respectively. The decrease after 48 hours of chilling was 53 and 29% respectively. After 72 hours of chilling acrosome integrity percentage was reduced by 61 and 43% in TCF-EY and TCF-LDL extenders respectively. Sperm cell membrane integrity was estimated at a positive HOST%. After 24 hours of chilling, percentage of cell membrane integrity was decreased by 33 and 23% in TCF-EY and TCF-LDL extenders respectively. The decrease after 48hours of chilling was 53and 30%. After 72 hours, positive HOST was reduced by 65 and 41 in TCF-EY and TCF-LDL extender.

After 24 hours-chilling, the percentage of normal morphology sperm cells decreased by 34 and 25% in TCF-EY and TCF-LDL extenders respectively however, after 48 hours-chilling; the decrease was 52 and 29% respectively. After 72 hours, the reduction was 60 and 38 in TCF-EY and TCF-LDL extenders respectively.

It was concluded that After 24, 48, and 72 hours of cooling, TCF-EY chilled extender resulted in less reduction in progressive sperm motility however, reduction in percentages of normal morphology, acrosome integrity, and cell membrane integrity was lower in TCF-LDL chilled extender than those estimated in TCF-EY chilled extender. Furthermore, all evaluation parameters were > 50% in both extenders after 24 hours of cooling.

Total motility, progressive motility, normal morphology, acrosome integrity, and sperm plasma membrane integrity % of canine pooled semen in 2 different chilled extenders with the addition of ascorbic acid at different storage cooled times as illustrated in **Table 4**. The results showed that progressive motility was 62 and 57 percent after 24 hours of cooling, respectively, and then decreased to 46 and 41 percent after 48 hours of cooling in the TCF-EY and TCF-LDL extenders respectively. After 24 hours of cooling, the percentages of canine pooled semen with normal morphology, acrosome integrity and sperm plasma membrane integrity ranged from 65 to 75 percent, with a slight decrease after 48 hours of cooling. They ranged between 58 and 63 percent. Although motility had completely stopped after 72 hours of cooling, the other previously mentioned parameters ranged from 41 to 57 percent.

The effect of addition of ascorbic acid to EY and LDL extenders on pooled canine semen quality after 24 hours of cooling was presented in **Table 5**. The addition of ascorbic acid to TCF-EY extender resulted in a 10% increase in progressive motility, but only a 3% increase in TCF-LDL + ascorbic acid. TCF-EY extender containing ascorbic acid was found to have a 14% higher percentage of acrosome integrity than the same extender without ascorbic acid. TCF-EY extender with ascorbic acid was found to have a 14 percent increase in acrosome integrity, compared to only a 4 percent increase in TCF-LDL extender without ascorbic acid. The percentage of sperm cell membrane integrity increased by 20% in TCF-EY extender with ascorbic acid compared to a 3% increase in TCF-LDL extender without ascorbic acid.

The effect of the addition of ascorbic acid to TCF-EY and TCF-LDL extenders on pooled canine semen quality after 48 hours of cooling was presented in **Table 6**. Progressive motility is increased by 9 and 10% as a result of the addition of ascorbic acid in TCF-EY and TCF-LDL extenders respectively**.** A marked increase in acrosome integrity and sperm cell membrane integrity percentages was attained due to the addition of ascorbic acid to TCF-EY extender compared with TCF-LDL extender (22 Vs. 3) and (27 Vs. 2%), respectively. The same effect of addition of ascorbic acid to TCF-EY extender and TCF-LDL extender, TCF-LDL extender which occurred in acrosome integrity and cell membrane integrity was achieved for sperm normal morphology. An increase in normal morphology by 24% was observed in the TCF-EY extender while the increase was only 3% in the TCF-LDL extender.

Table 2. Initial examination of fresh semen after collection before freezing

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

Table 3. Total motility, Progressive motility, Normal morphology, Acrosome integrity, and sperm plasma membrane integrity % of canine pooled semen in 2 different chilled extenders at different storage cooled times.

Extender		Total motility %	Progressive motility %	Normal Morphology %	Acrosome integrity %	Host %
Before cooling	**EY**	81±0.20 ^{b}	73±0.20 ^{b}	91±0.20 ^{a}	89±0.20 ^{a}	88±0.20 ^{a}
	LDL	84±0.20 ^{a}	80±0.20 ^{a}	87±0.20 ^{b}	85±0.20 ^{b}	88±0.20 ^{a}
After 24 hrs.	**EY**	59±0.20 ^{c}	52±0.20 ^{d}	57±0.20 ^{e}	56±0.20 ^{d}	55±0.20 ^{d}
	LDL	57±0.20 ^{d}	54±0.20 ^{c}	62±0.20 ^{c}	61±0.20 ^{c}	65±0.20 ^{b}
After 48 hrs.	**EY**	42±0.20 ^{e}	31±0.20 ^{g}	39±0.20 ^{g}	36±0.20 ^{f}	35±0.20 ^{f}
	LDL	36±0.20 ^{f}	39±0.20 ^{f}	58±0.20 ^{d}	56±0.20 ^{d}	58±0.20 ^{c}
After 72 hrs.	**EY**	0.00±0.00 ^{g}	0±0.20 ^{g}	31±0.20 ^{h}	28±0.20 ^{g}	23±0.20 ^{g}
	LDL	0.00±0.00 ^{g}	0±0.20 ^{e}	49±0.20 ^{f}	42±0.20 ^{e}	47±0.20 ^{e}

$^{a\text{-}h}$ Means with different superscripts letters are significantly different ($P < 0.05$).
EY: egg yolk; LDL: low-density lipoprotein.

Table 4. Total motility, Progressive motility, Normal morphology, Acrosome integrity, and sperm plasma membrane integrity % of canine pooled semen in 2 different chilled extenders at different storage cooled times with the addition of ascorbic acid

Extender		Total motility %	Progressive motility %	Normal Morphology %	Acrosome integrity %	Host %
Before cooling	**EY**	87±0.20 ^{a}	81±0.20 ^{a}	90±0.20 ^{a}	88±0.20 ^{a}	87±0.20 ^{a}
	LDL	85±0.20 ^{b}	81±0.20 ^{a}	88±0.20 ^{b}	87±0.20 ^{b}	90±0.20 ^{b}
After 24 hrs.	**EY**	68±0.20 ^{c}	62±0.20 ^{b}	72±0.20 ^{c}	70±0.20 ^{c}	75±0.20 ^{c}
	LDL	61±0.20 ^{d}	57±0.20 ^{c}	66±0.20 ^{d}	65±0.20 ^{d}	68±0.20 ^{d}
After 48 hrs.	**EY**	51±0.20 ^{e}	46±0.20 ^{d}	63±0.20 ^{e}	58±0.20 ^{e}	62±0.20 ^{e}
	LDL	46±0.20 ^{f}	41±0.20 ^{e}	61±0.20 ^{f}	59±0.20 ^{f}	60±0.20 ^{f}
After 72 hrs.	**EY**	0.00±0.00 ^{g}	0.00±0.00 ^{f}	57±0.20 ^{g}	49±0.20 ^{g}	44±0.20 ^{g}
	LDL	0.00±0.00 ^{g}	0.00±0.00 ^{f}	52±0.20 ^{h}	45±0.20 ^{h}	41±0.20 ^{h}

$^{a\text{-}h}$ Means with different superscripts letters are significantly different ($P < 0.05$).
EY: egg yolk; LDL: low-density lipoprotein.

Table 5. Effect of addition of ascorbic acid to EY and LDL extenders on pooled canine semen quality after 24 hours of cooling.

Extender	Total motility %	Progressive motility %	Normal Morphology %	Acrosome integrity %	Host %
TCF-EY with ascorbic acid	68±0.20 [a]	62±0.20 [a]	72±0.20 [a]	70±0.20 [a]	75±0.20 [a]
TCF-EY without ascorbic acid	59±0.20 [c]	52±0.20 [d]	57±0.20 [d]	56±0.20 [d]	55±0.20 [d]
TCF-LDL with ascorbic acid	61±0.20 [b]	57±0.20 [b]	66±0.20 [b]	65±0.20 [b]	68±0.20 [b]
TCF-LDL without ascorbic acid	57±0.20 [d]	54±0.20 [c]	62±0.20 [c]	61±0.20 [c]	65±0.20 [c]

[a-d] Means with different superscripts letters are significantly different ($P < 0.05$).
EY: egg yolk; LDL: low-density lipoprotein.

Table 6. Effect of addition of ascorbic acid to EY and LDL extenders on pooled canine semen quality after 48 hours of cooling

Extender	Total motility %	Progressive motility %	Normal Morphology %	Acrosome integrity %	Host %
TCF-EY with ascorbic acid	51±0.20 [a]	46±0.20 [a]	63±0.20 [a]	58±0.20 [b]	62±0.20 [a]
TCF-EY without ascorbic acid	42±0.20 [c]	37±0.20 [c]	39±0.20 [d]	36±0.20 [d]	35±0.20 [d]
TCF-LDL with ascorbic acid	46±0.20 [b]	41±0.20 [b]	61±0.20 [b]	59±0.20 [a]	60±0.20 [b]
TCF-LDL without ascorbic acid	36±0.20 [d]	31±0.20 [d]	58±0.20 [c]	56±0.20 [c]	58±0.20 [c]

[a-d] Means with different superscripts letters are significantly different ($P < 0.05$).
EY: egg yolk; LDL: low-density lipoprotein.

4. Discussion

Assessment of evaluation parameters of fresh canine semen is crucial before conducting any experiments regarding the effect of different extenders, additives such as antioxidants, or on the quality of chilled or frozen-thawed semen.

Before conducting our experiments to determine initial quality parameters immediately after pooling, it was critical to examine and evaluate freshly collected semen from three generally and reproductively healthy German shepherd dogs (GSD). The average ejaculate volume in our study was 8 ml, which was similar to the result (8.68±0.47 ml) obtained by (Shalini & Antoine, 2018). In our study, the initial progressive motility rate was 89±0.20 %, this finding was similar to that of (Gunay, Polat, Gunes, Soylu, & Kil, 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. However, the findings of (Ray, Jha, Biswas, & Basu, 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 (Umamageswari, Joseph, Kulasekar, Kalatharan, & Sridevi, 2012) and (Shalini & Antoine, 2018). In fresh dog sperm, they found (76.83± 2.63) % and (79.0± 0.83) % acrosome integrity, respectively.

The incidence of **HOST** positive spermatozoa (88±0.20%) in the current study is similar (88-97%) to that observed by (A. I. Peña, Barrio, M., Becerra, J. J., Quintela, L. A., & Herradón, P. G. , 2012). 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 Hos recorded in our work is somewhat lower than that recorded in the study of (Rota et al., 1995) which was 93.6%. The total sperm abnormality was 9% however; it was 7-10% and 7.25 ± 0.86% and 14.830 ± 2.386 in data recorded by (Kurien, Kathiresan, Selvaraju, & Pattabiraman, 2012) (Shalini & Antoine, 2018) and (Ray et al., 2019) respectively. In our work, sperm concentration was 278 million/ml. This value is nearly similar to values obtained by (Daiwadnya, Hukeri, & Sonawane, 1995; Ray et al., 2019) which were (264 million /mL and 274.333 ± 2.667 million /mL, respectively). However, more than this value (376 ± 13.6millions/ml) was observed by (Shalini & Antoine, 2018). The variation in sperm concentration may be due to the influence of many factors as the age of the animal, body weight, testicular weight and sexual activity (Amann, 1986). In general, the evaluation parameters of freshly collected semen are consistent with reference values cited in a recent article (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 HOS positive spermatozoa were (1.91-8.68 ml, 78.30-85.00 %, 273-598 millions/ml, 75 - 85.10 %, and 70 - 82.10 %, respectively).

Chilled sperm is commonly used in canine artificial insemination because it has higher spermatozoa motility and viability than frozen sperm (Rota et al., 1995). Additionally, bitches that are inseminated with chilled sperm have a higher whelping rate than bitches that are inseminated with frozen sperm (Linde-Forsberg & Forsberg, 1989). Chilling causes a variety of events, many of which are poorly understood. One such phenomenon is "cold shock," which is characterized by a reduction in spermatozoa motility and metabolism as well as a change in membrane permeability that impairs cellular interactions (Rota et al., 1995; Rota, Ström, Linde-Forsberg, & Rodriguez-Martinez, 1997). Changes resulting from cold shock are permanent and warming the sperm again won't repair it, however, it can be minimized with the use of a sperm extender. Other factors, such as storage temperature and refrigeration speed, can also aid in the preservation of chilled sperm. The storage period of time is considered when faced with the problem of sending semen over ever-greater distances. One of our research goals is to compare the effects of TCF-20% EY and TCF-20% LDL extenders on the quality of chilled canine sperm.

Our findings indicate that quality metrics gradually declined with cooling storage duration. After 24 hours-chilling, progressive motility percentages were reduced by (21 and 26%) in TCF-EY and TCF-LDL extenders, respectively). However, the reduction after 48hours-chilling was (36 and 49%, respectively). After 72 hours, progressive motility of sperms was completely stopped. The findings demonstrated that the decline in progressive motility was greater in TCF- LDL extender than that in TCF-EY and indicated the superiority of EY as a protector against semen chilling. In disagreement to our finding, (Hori, Ichikawa, Kawakami, & Tsutsui, 2004) shown that when canine semen samples are extended with egg yolk tris-citrate fructose (EYT-F) or glucose (EYT-G) and kept at a temperature between 4°C and 12°C, semen qualities can be maintained for up to 48 hours.

In agreement with our results, (Khye, Yusuf, Satrio, & Karja, 2021) showed significant decrease in spermatozoa motility after 24 h of chilling in Tris-egg yolk (TEY). In the above study, spermatozoa motility decreased from (83.0±2.7%) at 6 hours to (66.0±4.2%) and from (43.0±4.5%) at 48 hours to (8.0±2.7%). Results of the present study were consistent with those obtained by (Iguer-Ouada & Verstegen, 2001; Tsutsui et al., 2003) who noted that when chilled dog semen was stored at 4° C, the amount of motile spermatozoa gradually reduced. Spermatozoa motility has decreased as a result of an early increase in metabolism that consumes oxygen and energy and, in turn, caused an increase in lactic acid concentration and a drop in pH (Verstegen, Onclin, & Iguer-Ouada, 2005). In addition, Osmotic pressure rises during storage due to the accumulation of metabolic end

products (Salisbury & Lodge, 1962). Also, progressive motility of canine spermatozoa was sensitive to osmotic stress (Songsasen et al., 2002). For more explanation (Setiadi & Yulnawati, 2007) reported that despite reduction of chilled semen metabolism at 4° C, Spermatozoa can still perform metabolic processes, but at a reduced capacity. These processes result in the production of metabolites and free radicals, which have an impact on the osmolarity of the extender and the motility of spermatozoa. For interpretation of decreased sperm motility as a result of storing at refrigerated temperature, John Aitken, Clarkson, & Fishel (1989) reported that while spermatozoa require low levels of ROS to develop the ability to fertilize, excessive ROS production can harm sperm motility, sperm-oocyte fusion, and impair fertilization. Agarwal, & Said (2003) shown that the metabolism and consumption of oxygen are necessary for sperm survival during refrigeration which raises the quantity of ROS.

In contrast to our findings, Bencharif et al (2013) observed that after four days of storage in a refrigerator at +4°C, the percentages of motile canine spermatozoa were 53.1%, 44.2%, and 52.2% for the 6% LDL+ 20 mM glutamine, 20 % EY, and 6% LDL extenders, respectively, for all the dogs. The discrepancy is due to a difference in the percentage of LDL in the two studies. We used 20% LDL, while they used 6%. Although the percentage of EY was similar (20%) to that used in the Bencharif et al., (2013) study, the motility of all spermatozoa was completely stopped on day three of storage, but it was 44.2 percent on day four in the above study.

The results of the present work revealed that, after 24 hours, the acrosome integrity, plasma membrane integrity and normal morphology percentages were reduced by 33 and 24%, 33 and 23%, and 34 and 25% in TCF-EY and TCF-LDL extenders respectively. After 48 hours, the acrosome integrity, plasma membrane integrity, and normal morphology percentages were reduced by 53 and 29%, 53 and 30%, and 52 and 29% in TCF-EY and TCF-LDL extenders respectively. After 72hours, they were reduced by 61 and 43%, 53 and 30%, and 60 and 38. The results indicate the superiority of LDL in decreasing the reduction of evaluating parameters related to the integrity of canine sperm cells (the integrity of morphology, acrosome, and cell membrane). These findings agree with those of Bencharif et al (2008) and Demianowicz & Strzezek (1995) they demonstrated that low-density protein (LDL) has a protective effects against cold shock, improved sperm motility, acrosome and plasma membrane integrity, and more effective sperm DNA fragmentation protection. (Bencharif et al., 2008) interpreted LDL superiority as follows: Because LDL has less progesterone than the egg yolk due to the filtering effect of the dialysis procedure, the extender containing LDL provided good protection for acrosomes due to its low progesterone level.

The current study found that the overall quality of chilled sperm deteriorated gradually during storage at 4° C. This finding is consistent with the findings of Michael et al (2008 and 2009) who found that during storage at 4° C, canine spermatozoa motility, viability, plasma membrane integrity, and acrosome integrity all reduced. An increase in ROS concentration may be the reason for the decline in sperm quality (Michael et al., 2009).

Our findings show that acrosome integrity, plasma membrane integrity and normal morphology percentages were reduced by 33, 33, and 34 percent, respectively, in TCF-EY chilled semen at 24 and 48 hours. Our findings agree with those of Khye et al (2021) in terms of the deterioration of the overall quality of chilled semen with storage time, but the difference was in the rate of deterioration. It was faster in our investigation than in the study of Khye et al (2021). They discovered a slight decrease in acrosome integrity (97.60.7 at 6 h to 95.01.5 at 24h to 93.22.2 percent at 48 h) and plasma membrane integrity (90.44.6 at 6h to 85.75.2 at 48 h).

Chilled semen is more easily prepared and transported, less expensive, and results in less sperm damage than does freezing (Ponglowhapan et al., 2004). One limitation is that spermatozoa can only survive in chilled sperm for a limited amount of time. To keep spermatozoa for an extended period of time, reduce their metabolic activities by suspending them in a suitable medium and storing them at a low temperature. The traditional method for extending their survival period has been to add suitable extenders, which provide energy, maintain pH and osmolarity, and protect the acrosome and plasma membrane integrity from damage (Shahiduzzaman & Linde-Forsberg, 2007).

Vitamin C is thought to be the most abundant water-soluble antioxidant in plasma (Bast, Haenen, & Doelman, 1991). Vitamin C acts as an antioxidant in living organisms, protecting the body from oxidative stress, though it does require certain substances, such as α-tocopherol (Sharma & Agarwal, 1996). The results of the current work indicate that the addition of Ascorbic acid to the TCF-EY extender resulted in a 10% increase in progressive motility but only a 3% increase was achieved in TCF-LDL after passing 24 hours of chilling. Addition of the ascorbic acid to either TCF-EY extender or TCF-LDL extender resulted in an increase in acrosome integrity, cell membrane integrity and normal morphology however, the increases were greater in TCF-EY extender than those in TCF-LDL extender. The recorded increases were (14% vs. 4%), (20% vs. 3%), and (15 vs. 4) in acrosome integrity, cell membrane integrity and normal morphology, respectively.

After 48 hours of chilling, the addition of ascorbic acid to TCF-EY and TCF-LDL extenders increases progressive motility by 9 and 10%, respectively**.** A marked increase in acrosome integrity and sperm cell membrane integrity percentages was attained due to the addition of ascorbic acid to TCF-EY extender compared with TCF-LDL extender (22 Vs. 3%) and (27 Vs. 2%) respectively. An increase in normal morphology by 24% was observed in the TCF-EY extender while the increase was only 3% in the TCF-LDL extender. The remarkable improvement in all quality parameters of chilled semen in our study particularly by using TCF-EY indicated that ascorbic had positive impact as antioxidant for preserving of chilled canine semen for 48 hours and deterioration of semen was occurred after this particularly the motility which completely ceased, and this means complete loss of fertilizing capacity of semen.

5. Conclusion

The preliminary results of an evaluation of fresh semen collected from German shepherd dogs (GSD) born and raised in Egypt are consistent with those obtained by previous researchers in various parts of the world. TCF-EY chilled extender resulted in less reduction in progressive sperm motility after 24, 48 and 72 hours of cooling; however, reduction in percentages of normal morphology, acrosome integrity and cell membrane integrity were lower in TCF-LDL chilled extender than in TCF-EY chilled extender. Furthermore, after 24 hours of cooling, all evaluation parameters in both extenders were greater than 50%. The addition of ascorbic acid to TCF-EY resulted in a significant improvement in the quality of chilled canine sperm, but the improvement was only marginal when applied to TCF-LDL chilled extender.

Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

All the authors took part in the realization of this article.

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