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Original Article

The effect of varying concentrations of chicken plasma egg yolk and glycerol on the viability of canine sperm following short and long-term preservation

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Abstract

Background: Plasma egg yolk (PEY), due to simple preparation and easier access, could be a suitable alternative to raw egg yolk for preserving canine semen. **Aims:** The present study investigated suitable concentrations of PEY and glycerol for preservation of canine semen. **Methods**: Semen was collected by digital manipulation (seven replicates from four dogs). Following initial raw semen evaluation, the semen was diluted in a tris-based extender supplemented with varying concentrations of chicken PEY (0, 20, and 40% v/v) and glycerol (3%; v/v). After cooling the specimen to 4°C within 1 h, the specimens were diluted with an equal volume of freezing extender consisting of similar concentrations of chicken PEY and 0 and 7% glycerol to reach the final glycerol concentration of 1.5 and 5% for short-term storage of canine semen. Samples with different concentrations of PEY and 5% glycerol were frozen. The sperm viability parameters including total motility, progressive forward motility, plasma membrane integrity, and live percentage of sperm were assessed following short and long-term storage. **Results:** Sperm viability parameters of semen extended in an extender supplemented with 20 or 40% chicken PEY with either 1.5 or 5% glycerol remained superior until 72 h after semen collection compared to the specimen that did not receive any PEY ($P<0.05$). Post-thaw sperm viability was also greater in samples extended in extender supplemented with either 20 or 40% PEY compared to 0% PEY. **Conclusion:** Tris-based extender supplemented with either 20% chicken PEY could be suitable for short and long-term preservation of canine semen.

Key words: Canine semen, Glycerol, Plasma egg yolk, Preservation

Introduction

Artificial insemination (AI) with preserved high-quality semen collected from pure breed, stud, and documented dogs provides the breeder with a tool for widening the desired gene pool (Silva *et al*., 2003). Many factors influence the quality of preserved semen including semen collection techniques (Linde-Forsberg, 1991; Kutzler, 2005), the type of the extender (Olar *et al*., 1989; Rota *et al*., 1995), the final concentration of sperm (Peña and Linde-Forsberg, 2000; Okano *et al*., 2004), semen processing (Platz and Seager, 1977; Rijsselaere *et al*., 2002), the cooling rate (Peña and Linde-Forsberg, 2000), and the thawing technique (Rota *et al*., 1997; Peña and Linde-Forsberg, 2000; Brito *et al*., 2017).

Throughout the cooling and freezing process, the sperm suffers cold shock resulting in the reduction of the number of live and motile sperm (Holt, 1997; Bencharif *et al*., 2008). Traditionally, whole egg yolk is used as a cooling protectant during semen preservation in different species (Holt, 1997; Wall and Foote, 1999; Bergeron and Manjunath, 2006; Bucak *et al*., 2008; Panahi *et al*., 2017; Bencharif *et al*., 2020). Nevertheless, whole egg yolk could be a potential risk of contamination, prevent respiration and decrease motility of sperm, affect biochemical assays, metabolic investigations and computer assisted sperm analysis (Wall and Foote, 1999; Moussa *et al*., 2002; Pillet *et al*., 2011). As a result, there have been increasing demands to substitute whole egg yolk with its cooling protective components in semen extender. Low density lipoproteins (LDL) is the main effective component in egg yolk (Watson and Marin, 1975) that could act via stabilization of the sperm membrane (Watson, 1975), formation of a defensive layer over the surface of the sperm (Quinn *et al*., 1980), replacement of membrane phospholipids (Foulkes *et al*., 1980), and thus reduction in the membrane phase transition temperature (Graham and Foote, 1987). Besides, LDL could inhibit lipid-binding proteins of seminal plasma (Vishwanath *et al*., 1992), and it could be responsible for removing cholesterol and

phospholipid from the sperm membrane (Bergeron and Manjunath, 2006), resulting in the improvement of sperm viability during preservation in liquid or frozen states (Bergeron and Manjunath, 2006). LDL was effectively used as a cooling protectant in dogs (Bencharif *et al*., 2008, 2010). Plasma egg yolk was used as a replacement to LDL due to its simple extraction and similar effects to LDL (Pillet *et al*., 2011; Panahi *et al*., 2017; Shah *et al*., 2017). Egg yolk from different avian species was used as a cooling protectant to complement semen extender during sperm preservation in bulls (Su *et al*., 2008), stallions (Clulow *et al*., 2007) and dromedary camels (Panahi *et al*., 2017). Silva *et al*. (2002), had shown that the combination of tris-chicken egg yolk-glycerol is useful for canine semen preservation. The variation in the reaction of sperm to the egg yolk of different avian species could be due to the species-specific composition of egg yolk like different concentrations of phospholipids, fatty acids (Graham and Foote, 1987; Trimeche *et al*., 1997; Bathgate *et al*., 2006), and cholesterol (Bair and Marion, 1978; Kaźmierska *et al*., 2005). More recently, we compared different types of avian plasma egg yolks including chicken, duck, and pigeon plasma egg yolks for cryopreservation of canine semen (Nazeri *et al*., 2022). Accordingly, due to the easy access and cost-effectiveness of chicken PEY, it could be considered as the best PEY for successful cryopreservation of canine semen (Nazeri *et al*., 2022). There is no study to determine a suitable concentration of chicken plasma egg yolk for the preservation of canine semen.

Glycerol is most commonly used as a cryoprotectant for freezing canine semen (Lopes *et al*., 2009; Futino *et al*., 2010). A balance between glycerol toxicity and its protecting effects could provide its ideal concentration in the extender (Fontbonne and Badinand, 1993). High concentration of glycerol could also disturb the fertilizing capacity of the sperm (Fontbonne and Badinand, 1993). Glycerol concentration could be affected by other components of the extender, cooling, freezing, and thawing methods (Watson, 1979). This study compared the effect of different concentrations of chicken plasma egg yolk and glycerol on the viability of canine sperm after short and long-term storage.

Materials and Methods

This study has received an approval from the Animal Ethics Committee of the Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran (SAT005/25.04. 2019).

Experimental location

The experiment was conducted at the Veterinary Research and Teaching Hospital, Faculty of Veterinary Medicine, University of Tehran (latitude: 35°39'8'N; longitude: 51°26'38'E; altitude: 1029 m).

Experimental animals

Healthy mature dogs (n=4), four years of age, including Golden Retriever, Husky, and Samoyed breeds with a sound history of fertility were selected. Commercial dry canine diet was provided in association with free access to water. Semen was collected twice a week. Only semen specimen with progressive forward motility of >90% and morphologically normal sperm of >75% were used throughout the study.

Preparation of extenders

Extender

The basis of the extender for this study was based on previous studies (Silvia *et al*., 2003; Hermansson and Forsberg, 2006; Bencharif *et al*., 2008; Belala *et al*., 2016). It contains tris (3.028 g; hydroxymethyl-aminomethan, Merck, Germany), fructose (1.25 g; Merck, Germany), citric acid (1.7 g; Sigma-Aldrich Inc., St Louis, USA), streptomycin sulfate (1000 mg/ml; Streptocin®, Jaber-Ebne-Hayyan Pharmaceutical Co., Iran), and penicillin G sodium (1000 IU/ml; Pen Sodium®, Jaber-Ebne-Hayyan Pharmaceutical Co., Iran) in 100 ml of sterile deionized water. The osmolality and pH of the extender were 352 mOsm/kg H_2O and 7, respectively. The extender was filter-sterilized by 0.22 µm syringe filter and kept in a refrigerator for a week.

Plasma egg yolk

Plasma egg yolk (PEY) was extracted from domestic chicken fresh egg (Sebright fowl: *Gallus gallus domesticus*, n=15) according to previous recommendations (Panahi *et al*., 2017). In brief, the fresh egg was manually broken and the albumen was discarded. The egg yolk membrane was then perforated and the yolk was placed in a beker surrounded by iced water. In the cooled room, the egg yolk was extended with tris-based extender (v/v; 1:1) and stirred for 1 h before centrifugation at 10,000 g for 45 min at 4°C. Centrifugation was carried out twice. The supernatant (plasma egg yolk) was stored in 2 ml glass vials and sterilized by gamma radiation (Iranian Atomic Energy Organization) at 3 kGy within the cooling chamber in order not to exceed the temperature above 35°C. The gamma radiated vials were stored at 4°C.

Semen collection

Semen was collected by digital manipulation, while the dog was on the standing position (Kutzler, 2005). Sperm rich fraction was used for the present study. Semen volume was recorded and sperm concentration was estimated using hemocytometer.

Assessment of semen viability

Motility

CASA system (Video Test, Russia) was used to evaluate total motility (TM) and progressive forward motility (PFM). CASA system was calibrated for dog semen using a diluted specimen (25×10^6 sperm/ml). Analysis was carried out with a phase contrast microscope (Model: BX51; Olympus, Japan) equipped with a stage warmer (Thermo Plate, Tokai HIT, Olympus, Japan) set at 38°C, 10X negative phase

objective and digital camera (50 frame/s). A total of 1000 sperms were assessed for each sample from random fields. The range of particle size between 6 and 25 μm^2 was considered. Sperms with a VCL between 60-400 and 0-15 μ/s were considered as progressive forward motile and immotile, respectively. Sperms with straightness ≥70% and ALH 0.5-6 μm were considered as progressive forward motile sperm.

Plasma membrane integrity

Hypo-osmotic swelling test was used to assess plasma membrane integrity (PMI) using sucrose solution (3.423% prepared in di-ionized water (osmolarity: 121 mOsm/kg H_2O; PH 7.86). Diluted semen (100 μL) was added to the pre-warmed (37°C) sucrose solution (1 ml) in a test tube and incubated for 45 min at 37°C. The assessment was carried out under a phase contrast microscope at a magnification of ×400 to determine the percentage of sperm with coiled tails.

Live percentage

Eosin B-fast green was applied to stain and count the live sperm using a dissecting microscope (×1000) (Mosaferi *et al*., 2005).

Experimental design

Following the analysis of the raw semen to meet the criteria for entering the experiment, the semen was centrifuged at 700 g for 10 min and the supernatant was removed. A small volume of seminal plasma (about 1 ml) was used to resuspend the pellet. An equal volume of semen was placed in water jacketed collection glass vessels (IMV, France) at room temperature. Next, the specimen was extended to reach 50-100 × 10^6 per ml, by the extender supplemented with varying concentrations of chicken PEY (0, 20, and 40% v/v) and 3% glycerol. Each vessel was transferred to a vaccine transport device (Model: LRVC-404; Capacity: 1.4 L; medea®, Tajhiz Teb Fanavaran, Iran) equipped with three ice packs. In these circumstances, semen was cooled to 4°C after 1 h. After reaching 4°C, samples were assigned equally into two vessels. At this point, the second cooled extender consisting of 7% and 0% glycerol, with the same concentration of PEY as in the initial part, was added to achieve the final glycerol concentrations of 5% and 1.5% glycerol, respectively. At this time, the semen was evaluated prior to freezing. Afterward, the samples with 1.5 and 5% glycerol and different concentrations of PEY were kept in 4°C and evaluated every day for 72 h (Experiment 1). Samples with different concentrations of PEY and 5% glycerol were loaded into 0.5 straws and frozen using a sperm freezing device (Cryogenic 5000, Australia). The freezing rate was 6°C/min from 4°C to -10°C and maintained for 5 min at -10°C then 5°C/min from -10°C to -30°C and maintained for 10 min at -30°C and 4°C/min from -30°C to -43°C. The straws were then plunged into liquid nitrogen. Semen straws were thawed at 37°C for 30 s. Semen viability was evaluated after thawing (Experiment 2). Overall, seven replicates from four dogs were included for this experiment.

Statistical analysis

The response variables with discrete nature (binomial distribution) were subjected to arcsin transformation. Changes in motility, plasma membrane integrity, and the live percentage of sperm were analysed for the effects of treatment, time, and treatment by time interaction using GLM procedure in SAS [SAS, 2016] with repeated measures included in the model. Between group differences at any given time were compared using analysis of variance followed by Least squares means within the GLM procedure in SAS (SAS, 2016). Data were presented as means±SEM.

Results

Experiment 1

Total motility

There was no treatment by time interaction (P>0.05). PEY concentrations of 20% and 40% with either 1.5 or 5% glycerol had greater TM compared to 0% PEY (P<0.005; Table 1). TM decreased throughout cooling to 4°C (88.7 ± 0.9; P<0.001), remained constant for 24 h (85.0 ± 1.2; P>0.05), and then declined continuously for 72 h after semen extension (50.5 ± 4.2; P<0.0001; Table 1).

Progressive forward motility

There was treatment by time interaction (P<0.0001). At 48 and 72 h after semen extension, PFM was superior in the semen sample supplemented with 20% and 40% chicken PEY, irrespective of glycerol concentrations, compared to the specimen without PEY (P<0.01; Table 2). PFM decreased steadily throughout the time from the first semen extension (76.9 ± 0.42) to 72 h after semen extension (30.8 ± 3.4; P<0.0001; Table 2).

Live percentage

There was no treatment by time interaction (P>0.05). The specimen without PEY supplemented with either 1.5 or 5% glycerol displayed inferior livability compared to other extenders (P<0.0001; Table 3). There was a reduction in livability throughout the time (P<0.0001) except for Time 2 (at 4°C: 91.4 ± 0.73) and time 3 (at 24 h: 87.7 ± 1.0; P>0.05; Table 3).

Plasma membrane integrity

There was treatment by time interaction (P<0.0001). At 72 h, the semen extended in 1.5% glycerol with either 20% (58.5 ± 9.1%) or 40% (57.5 ± 6.5%) chicken PEY had superior PMI compared with the semen extended in 5% glycerol and those specimens without PEY (P<0.0001; Table 4). There was a reduction in PMI throughout the time (Table 4; P<0.05).

Experiment 2

Total motility

There was treatment by time interaction (P<0.0001). After thawing, the specimen supplemented with 20% (52.7 ± 6.2%) and 40% (42.9 ± 5.6%) chicken PEY

Table 1: Effect of different concentrations of chicken plasma egg yolk (PEY) and glycerol (G) on total motility of canine semen after semen collection. Data were presented as mean±SEM

Ingredients		Assessments after					Extender effect
PEY (%)	Final G (%)	1st extender (G: 3%)	2nd extender (G: 0 or 7%)	24 h	48 h	72 h	
0	1.5	93.1±1.46 (88.3-98.2)	86.9±3.43 (68.5-46.4)	81.5±4.15 (58.5-90.7)	64.3±10.47 (28.2-88.1)	36.5±14.04 (0-82.3)	73.5±4.72[a] (0-98.2)
20	1.5	95.1±1.13 (91-98.7)	90.4±1.19 (85-94.7)	89.1±1.87 (83.8-92.9)	79.3±4.29 (55.7-90)	62.4±8.49 (36.1-89)	83.9±2.57[b] (36.1-98.7)
40	1.5	93.6±1.11 (90-97.4)	90.0±1.16 (84.2-93.7)	88.2±0.74 (84-90)	80.0±3.62 (59.5-87)	65.5±6.32 (44.6-84)	84.0±2.12[b] (44.6-97.4)
0	5	93.1±1.46 (88.3-98.2)	86.7±3.38 (67.7-94.7)	86.0±0.95 (83.3-89)	60.9±6.79 (38.2-84)	32.4±12.75 (0-78.3)	71.9±4.62[a] (0-98.2)
20	5	95.1±1.13 (91-98.7)	90.2±1.22 (84-93.8)	84.7±1.45 (77.6-89)	72.5±3.78 (57.4-87)	52.8±5.94 (33.5-70)	80.1±2.87[b] (33.5-98.7)
40	5	93.5±1.11 (90-97.4)	87.8±1.28 (82.7-91.9)	80.6±5.19 (50.1-90.1)	73.6±4.72 (56.1-86.3)	53.6±5.88 (37.8-78.5)	79.4±2.75[b] (37.8-97.4)
Time effect		93.9±0.49[a] (88.3-98.7)	88.7±0.88[b] (68.7-96.4)	85.0±1.20[b] (50.1-92.9)	71.8±2.59[c] (28.2-90)	50.5±4.15[d] (0-89)	

[a, b, c] Time effect and extender effect with different superscripts differ (P<0.01)

Table 2: Effect of different concentrations of chicken plasma egg yolk (PEY) and glycerol (G) on progressive forward motility of canine semen after semen collection. Data were presented as mean±SEM

Ingredients		Assessments after				
PEY (%)	Final G (%)	1st extender (G: 3%)	2nd extender (G: 0 or 7%)	24 h	48 h	72 h
0	1.5	76.9±0.71[Aa] (73-78.8)	71.0±4.15[Aab] (46.3-76.5)	60.0±5.15[Ab] (30.7-70.9)	36.9±8.19[Ac] (9.3-65.7)	15.6±7.73[Ad] (0-47.8)
20	1.5	77.4±1.54[Aa] (72-84.1)	74.1±1.95[Aa] (64-80)	70.3±2.58[Aab] (58.3-77.1)	57.2±5.16[Bbc] (35-76.1)	45.1±8.18[Bc] (20.8-74.3)
40	1.5	76.3±0.78[Aa] (73-79.2)	73.9±2.47[Aa] (60.6-82)	69.5±2.14[Aab] (59-76)	59.8±4.25[Bbc] (35.5-68)	44.7±5.65[Bc] (26.8-63.7)
0	5	76.9±0.71[Aa] (73-78.8)	69.3±3.90[Aab] (46-75.2)	60.5±5.23[Ab] (30.5-70.9)	32.5±3.67[Ac] (20.4-48)	9.8±4.48[Cd] (0-26.6)
20	5	77.4±1.54[Aa] (72-84.1)	74±2.25[Aa] (63.7-80.3)	69.2±2.27[Aa] (61.5-76.4)	51.5±4.66[Bb] (34.6-72.2)	35.1±6.04[ABb] (19.8-53.7)
40	5	76.3±0.78[Aa] (73-79.2)	72.6±1.87[Aa] (63.1-79)	66.8±2.56[Aab] (58-77.8)	53.9±4.85[Bb] (35.4-68.4)	34.3±6.03[ABc] (20.5-56.9)

[A, B, C] Values within columns with different superscripts differ (P<0.05), and [a, b, c, d] Values within rows with different superscripts differ (P<0.05)

Table 3: Effect of different concentrations of chicken plasma egg yolk (PEY) and glycerol (G) on live percentage of canine semen after semen collection. Data were presented as mean±SEM

Ingredients		Assessments after					Extender effect
PEY (%)	Final G (%)	1st extender (G: 3%)	2nd extender (G: 0 or 7%)	24 h	48 h	72 h	
0	1.5	96.4±0.57 (95-99)	89.7±2.92 (74-97)	85.0±3.38 (66-93)	70.4±8.01 (43-88)	45.5±12.5 (10-84)	78.8±4.08[a] (10-99)
20	1.5	97.1±0.63 (95-99)	93±1.13 (87-96)	91.1±1.35 (84-95)	84.5±2.68 (70-90)	70.8±6.89 (49-90)	87.8±2.01[b] (49-99)
40	1.5	96.7±0.47 (95-98)	92.3±1.06 (87-95)	90.1±1.07 (85-93)	84.8±2.17 (74-89)	72.7±4.66 (57-88)	87.8±1.66[b] (57-98)
0	5	96.4±0.57 (95-99)	89.3±2.58 (75-95)	88.4±3.92 (61-91)	68.1±5.24 (51-85)	41.5±12.43 (6-79)	76.7±4.09[a] (6-99)
20	5	97.1±0.63 (95-99)	93.1±0.93 (88-95)	88.7±1.22 (83-93)	79.8±2.34 (71-89)	65.2±4.69 (49-80)	85.4±2.14[b] (49-99)
40	5	96.7±0.47 (95-98)	91.3±1.22 (85-94)	87.7±1.22 (83-93)	79.8±3.69 (63-88)	63.8±5.82 (45-86)	84.5±2.28[b] (45-98)
Time effect		96.7±0.22[a] (95-99)	91.4±0.73[b] (74-97)	87.7±0.99[b] (61-95)	77.9±2.00[c] (43-90)	59.9±3.81[d] (6-90)	

[a, b, c] Time effect and extender effect with different superscripts differ (P<0.01)

Table 4: Effect of different concentrations of chicken plasma egg yolk (PEY) and glycerol (G) on plasma membrane integrity of canine semen after semen collection. Data were presented as mean±SEM

Ingredients		Assessments after				
PEY (%)	Final G (%)	1st extender (G: 3%)	2nd extender (G: 0 or 7%)	24 h	48 h	72 h
0	1.5	86.3±0.86^{Aa} (83-89)	79.1±3.46^{Aa} (59-86)	70.3±5.79^{Aa} (38-86)	49.1±7.08^{ACb} (18-70)	24.3±9.18^{ACb} (3-64)
20	1.5	86.7±0.94^{Aa} (84-90)	84±1.25^{Aab} (80-89)	80.6±1.97^{Aab} (73-88)	67.8±4.61^{Abc} (48-86)	58.5±9.09^{BCc} (32-88)
40	1.5	86.6±0.89^{Aa} (84-90)	83.1±1.07^{Aa} (78-87)	79.4±1.75^{Aa} (73-87)	71.0±4.09^{Ba} (51-84)	57.5±6.46^{Bb} (35-73)
0	5	86.3±0.86^{Aa} (83-89)	77.1±3.11^{Aa} (60-85)	69.7±5.97^{Aa} (37-85)	45.6±3.47^{Cb} (33-55)	19.1±8.35^{Ac} (1-54)
20	5	86.7±0.94^{Aa} (84-90)	82.8±1.40^{Aa} (79-88)	79.4±2.20^{Aab} (73-88)	62.8±4.10^{ABCbc} (47-77)	46.5±7.86^{Cc} (29-76)
40	5	86.6±0.89^{Aa} (84-90)	82.4±1.50^{Aab} (77-87)	77±1.98^{Aab} (71-86)	65.0±4.92^{ABCbc} (48-84)	47.0±8.42^{Cc} (29-81)

$^{A, B, C}$ Values within column with different superscripts differ (P<0.05), and $^{a, b, c, d}$ Values within rows with different superscripts differ (P<0.05)

Table 5: The effect of different concentrations of PEY on total motility at different times after semen collection. Data were presented as mean±SEM

Plasma egg yolk	After 1st extender	After 2nd extender	After thawing
0	93.1±1.46^{Aa} (88.3-98.2)	86.7±3.38^{Aa} (67.7-94.7)	11.2±3.59^{Ab} (0-25)
20	95.1±1.13^{Aa} (91-98.7)	90.3±1.22^{Aa} (84-93.8)	52.7±6.16^{Bb} (32-76)
40	93.5±1.11^{Aa} (90-97.4)	88.7±1.29^{Aa} (82.7-91.9)	42.9±5.57^{Bb} (23-60)

$^{A, B, C}$ Values within columns with different superscripts differ (P<0.05), and $^{a, b, c}$ Values within rows with different superscripts differ (P<0.05)

Table 6: The effect of different concentrations of PEY on progressive forward motility at different time after semen collection. Data were presented as mean±SEM

Plasma egg yolk	After 1st extender	After 2nd extender	After thawing
0	76.9±0.71^{Aa} (73-78.8)	66.3±3.91^{Ab} (46-75.2)	1.9±0.84^{Ac} (0-5.2)
20	77.4±1.55^{Aa} (72-84)	74±2.26^{Aa} (63.7-80.3)	36.8±4.84^{Bb} (19.5-56.5)
40	76.3±0.79^{Aa} (73-79.2)	72.6±1.88^{Aa} (63.1-79)	30.9±4.45^{Bb} (15.7-47.7)

$^{A, B, C}$ Values within columns with different superscripts differ (P<0.05), and $^{a, b, c}$ Values within rows with different superscripts differ (P<0.05)

Table 7: The effect of different concentrations of PEY on live percentage at different time after semen collection. Data were presented as mean±SEM

Plasma egg yolk	After 1st extender	After 2nd extender	After thawing
0	96.4±0.57^{Aa} (95-99)	89.3±2.59^{Ab} (75-95)	20.8±4.94^{Ac} (0-38)
20	97.1±0.63^{Aa} (95-99)	93.1±0.94^{Aa} (88-95)	63±5.13^{Bb} (43-80)
40	97.7±0.47^{Aa} (95-98)	91.3±1.23^{Ab} (85-94)	57.1±4.36^{Bc} (44-75)

$^{A, B, C}$ Values within columns with different superscripts differ (P<0.05), and $^{a, b, c}$ Values within rows with different superscripts differ (P<0.05)

showed superior TM compared with that extended without PEY (11.2 ± 3.6; P<0.05; Table 5).

Progressive forward motility

There was treatment by time interaction (P<0.0001). After thawing, 20% (36.8 ± 4.8%) and 40% (30.9 ± 4.5%) chicken PEY showed superior PFM compared with the semen diluted with 0% chicken PEY (1.9 ± 0.8; P<0.05; Table 6).

Live percentage

There was treatment by time interaction (P<0.0001). Following thawing, 20% (63 ± 5.13%) and 40% (57.1 ± 4.4%) chicken PEY showed superior live percentage compared with the semen diluted with 0% chicken PEY (20.8 ± 4.9; P<0.05; Table 7).

Plasma membrane integrity

There was treatment by time interaction (P<0.0001). Just prior to freezing and after thawing, the semen extended in 20% and 40% chicken PEY displayed better PMI compared with the specimen that did not have PEY (P<0.05; Table 8).

Table 8: The effect of different concentrations of PEY on plasma membrane integrity at different time after semen collection. Data were presented as mean±SEM

Plasma egg yolk	After 1st extender	After 2nd extender	After thawing
0	86.3±0.86^{Aa} (83-89)	77.1±3.11^{Ab} (60-85)	5.8±3.18^{Ac} (0-9)
20	86.7±0.94^{Aa} (84-90)	82.8±1.40^{Aa} (79-88)	46.3±4.03^{Bb} (33-63)
40	86.5±0.89^{Aa} (84-90)	82.4±1.5^{Aa} (77-87)	40.7±4.41^{Bb} (23-58)

$^{A, B, C}$ Values within columns with different superscripts differ (P<0.05), and $^{a, b, c}$ Values within rows with different superscripts differ (P<0.05)

Discussion

In the present study, the effectiveness of the tris-based extender supplemented with varying concentrations of chicken PEY (0, 20, and 40%) and glycerol (1.5 and 5%) for short and long-term preservation of canine semen was investigated. Tris-based extender supplemented with 20 or 40% chicken PEY with either 1.5 or 5% glycerol sustained the sperm viability parameters for 72 h after semen collection. Moreover, post-thaw sperm viability was also greater in canine semen samples extended in tris-based extender supplemented with both 20 and 40% PEY. Tris based extender supplemented with chicken egg yolk and glycerol was suggested for semen preservation of canine semen (Silva *et al*., 2002). Apparently, different species revealed different response to plasma egg yolks extracted from avian species. Duck egg yolk enhanced TM and PFM of frozen-thawed stallion semen compared with chicken egg yolk (Clulow *et al*., 2007). Superior cryoprotective effect of pigeon egg yolk was detected in bull (Su *et al*., 2008), ram (Gholami *et al*., 2012) and dromedary camel (Panahi *et al*., 2017). Among different types of avian plasma egg yolks, chicken PEY was found to be the best PEY for successful cryopreservation of canine semen (Nazeri *et al*., 2022). In the present study, 20% chicken PEY was found to be effective for short and long-term preservation of canine sperm.

Glycerol is a permeable polyhydric alcohol that is being used most frequently for semen freezing in different species (Colas, 1975; Watson *et al*., 1992; Silva *et al*., 2003). However, it could exhibit toxic effects on sperm, resulting in the reduction of fertility (Curry, 2000; Holt, 2000). The ideal concentration of glycerol in the extender represents a balance between its toxic and protective effects (Silva *et al*., 2003). Concentrations of 4% or greater had an adverse effect on fresh diluted canine sperm during incubation at 39°C (Olar *et al*., 1989; Cardoso *et al*., 2003). The recommended concentrations of glycerol for freezing canine sperm are between 2 and 8% (Olar *et al*., 1989). In the present study, 1.5 and 5% glycerol was able to maintain sperm quality for 72 h at 4°C and 5% glycerol was efficient for long-term preservation of canine semen at frozen state.

In some protocols for cryopreservation of canine sperm, semen is extended in one step with glycerol because canine semen seems to be less sensitive to osmotic damage caused by single addition of glycerol (Fontbonne and Badinand, 1993; Silva *et al*., 2003). However, in other protocols, a two-step dilution is performed in which the freezing extender added in the second step of dilution contains a high glycerol concentration (Rota *et al*., 1997; Ström *et al*., 1997). The second extender is added just before freezing so that its higher glycerol concentration does not exert a detrimental effect on the sperm. Peña and Linde-Forsberg (2000) have shown that addition of glycerol (3 and 5%) in 1 or 2 steps had no significant effect on the post-thaw viability of canine sperm, and this is in agreement with other reports (Andersen, 1975; Fontbonne and Badinand, 1993). Moreover, glycerol concentration of 3.2% has been reported to be sufficient for a good cryoprotection effect without presenting risk of toxicity in freezing canine semen (Fontbonne and Badinand, 1993; Belala *et al*., 2016). In another study, using a coconut water extender, it was reported that 4, 6, or 8% glycerol could be used successfully for cryopreservation of canine sperm (Cardoso *et al*., 2003).

Sperm motility is the main criterion to assess the quality of extenders, particularly after cryopreservation (Ivanova-Kicheva *et al*., 1997). The motility of 30-50% is acceptable for canine frozen semen (Silva *et al*., 2003). Post-thaw motility of 20-30% resulted in successful pregnancies (Cardoso *et al*., 2003). Therefore, post-thaw progressive motility of 36.8% (with 20% PEY) and 30.9 (with 40% PEY) in the present study could be sufficient to obtain pregnancy following artificial insemination. Great individual variation in sperm survival following thawing (Silva *et al*., 2002) could be due to different methodologies used to freeze canine semen (Peña *et al*., 1998; Silva *et al*., 2003).

In conclusion, tris-based extender supplemented with 20% chicken PEY and 1.5% glycerol for cold preservation and 5% glycerol for freezing could provide a suitable extender for preservation of canine semen.

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Conflict of interest

The authors declare no conflict of interest.

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