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Cryopreservation of canine semen using plasma egg yolk of three avian species

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Abstract

Present study investigated a suitable source of plasma egg yolk (PEY) to supplement tris-based extender for cryopreservation of canine semen. Collected semen by artificial vagina was diluted to reach 50-100×10^6 per ML by Tris-based extender, supplemented with 20% PEY of three avian species (domestic chicken, domestic duck and pigeon) and 3% glycerol. After cooling specimen to 4°C for 1 hr, the specimens were diluted with equal volume of freezing extender consisting of 20% PEY, similar to initial PEY, and 7% glycerol to achieve the final glycerol concentration of 5%. The sperm viability parameters including total motility, progressive forward motility, plasma membrane integrity and live percentage of sperm were assessed following semen collection, after adding the first and the second part of semen extender and post thawing. Chicken PEY had better plasma membrane integrity (80.9±2.0 %) compared to pigeon PEY (76.6±3.08 %; P<0.01). There was not any other significant difference in semen viability parameters between chicken PEY (total motility: 85.4±2.72; progressive forward motility: 71.9±3.24; live percentage: 89.7±1.66) and other plasma egg yolks. In conclusion, due to the ease of availability and superiority in some sperm viability parameters, chicken PEY at the concentration of 20% could provide beneficial effect for cryopreservation of canine semen.

Key words: Plasma egg yolk, Semen, Canine, Cryopreservation

Introduction

During the cooling and freezing process, the sperm undergoes cold shock resulting in the decrease in the number of live and motile sperm (Bencharif et al, 2008; Holt, 1997). Whole egg yolk is a conventional cooling protectant during semen preservation in different specious (Holt, 1997; Wall & Foote, 1999; Bergeron & Manjunath, 2006; Bucak et al, 2008; Panahi, 2017). However, whole egg yolk could represent a potential risk of contamination, inhibit respiration, reduce motility of sperm, and interfere with biochemical assays, metabolic investigations and computer assisted sperm analysis (Wall & Foote, 1999; Moussa et al, 2002; Pillet et al, 2011). Therefore, in the last few years, there have been increasing demands to replace whole egg yolk with its cooling protective components in semen

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extender. The main effective component in egg yolk is low density lipoproteins (LDL; Watson & Marin, 1975). LDL could act through stabilization of the sperm membrane (Watson, 1975), formation of a protective layer over the surface of the sperm (Quinn et al, 1980), replacement of membrane phospholipids (Foulkes et al., 1980) and thereby decrease in the membrane phase transition temperatures (Graham & Foote, 1987). Moreover, LDL could interact with lipid-binding proteins of seminal plasma (Vishwanath et al, 1992) that could induce cholesterol and phospholipid removal from the sperm membrane (Bergeron & Manjunath, 2006), resulting in the enhancement of sperm viability during storage in liquid and frozen states (Bergeron & Manjunath, 2006). Recently, plasma egg yolk was introduced to replace LDL due to its simple extraction and similar effect to LDL (Pillet et al, 2011; Shah et al, 2017, Panahi et al, 2017). Egg yolk from different avian species was used as a cooling protectant to supplement semen extender during sperm storage in bull (Su et al, 2008), stallion (Clulow et al, 2007) and dromedary camel (Panahi et al, 2017). There is variation in the reaction of sperm to the egg yolk of different avian species. It might be related to the species' specific composition of egg yolk such as varying concentrations of phospholipids, fatty acids (Graham & Foote, 1987; Trimeche et al, 1997; Bathgate et al, 2006) and cholesterol (Bair & Marion, 1978; Kaźmierska et al, 2005). To the best of our knowledge, there is no research on the effect of plasma egg yolk of different avian specious on cryopreservation of canine semen. The objective of the present study was to compare the effect of plasma egg yolk of chicken, duck and pigeon on the viability of canine semen following cryopreservation.

Materials and Methods

Present study was approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran (SAT005/25.04.2019).

Experimental location

This study was carried out at 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=3; 4 years of age) of golden Retriever, Husky, Samoyed breeds with a sound history of fertility, were selected. On a daily basis, each dog received commercial dry canine diet and had free access to water. Semen collection was conducted twice a week. Semen samples with progressive forward motility of >90% and morphologically normal sperm of >75% were included in the present study.

Preparation of extenders

Tris-based extender

Tris-based extender was prepared according to previous studies (Silvia et al, 2003; Hermansson & Forsberg, 2006, Bencharif et al, 2008 and Belala et al, 2016). It includes Tris (3.028 gr; hydroxymethyl-aminomethan, Merck, Germany), Citric Acid (1.7 gr; Sigma-Aldrich Inc., St Louis, USA), Fructose (1.25 gr; Merck, Germany), Penicillin G sodium (1000 IU/ml; Pen Sodium®, Jaber-Ebne-Hayyan pharmaceutical company, Iran), Streptomycin sulfate (1000 mg/ml; streptocin®, Jaber-Ebne-Hayyan pharmaceutical company, Iran) in 100 ml of sterile deionized water. The respective osmolality and pH of Tris extender was 352 mOsm/kg H_2O and 7. The extender was filter-sterilized by 0.22 µm syringe filter and kept in a refrigerator for a week.

Plasma egg yolk

Fresh laid eggs from domestic chicken (Sebright fowl: *Gallus gallus domesticus*, n=10), domestic duck (Pekin duck: *Anas platyrhynchos domesticus*, n = 10), pigeon (Fantile pigeon: *Columba livia*, n = 16) were collected from Barajin National Park, Qazvin, Iran. Plasma egg yolk (PEY) was

extracted according to the previous recommendation (Panahi et al, 2017). In brief, fresh avian eggs were manually broken and the albumen was discarded. Egg yolk was carefully rolled on filter paper to remove albumen and chalazes adhering to the vitelline membrane. This membrane was then perforated to collect yolk in a beaker cooled in iced water. Egg yolk was diluted with Tris-based extender (v/v) at the ratio of 1:1 and stirred for 1hr before centrifugation at 10,000 x g for 45 min at 4 °C. Centrifugation was carried out twice. The supernatant (plasma egg yolk) was collected, stored in 2 mL glass containers, Gamma radiated (3 kGy at cooling chamber not exceeding 35 °C) and preserved at 4 °C for further use.

Semen collection

Semen was collected by digital manipulation in standing position (Feldman & Nelson, 1996). In some occasions, a bitch on estrus was available; although its presence was not essential. The second semen fraction (sperm rich fraction) was collected for this study. Following semen collection, the volume was assessed and sperm concentration was estimated following semen dilution using haemocytometer.

Assessment of semen viability

Motility

Total motility (TM) and progressive forward motility (PFM) were assessed using CASA system (Video Test, Russia) calibrated for dog semen after further diluting the specimen to achieve 25×10^6 sperm/ml. Accordingly, 4 µL sample was deposited on the counting chamber (Spermtrac®, Proiser, Spain) and covered with special grid of 100 squares (each square had a dimension of 100 ×100 µm). The depth between slide and coverslip was 10 µm. Analysis was carried out with the phase contrast microscope (Model: BX51; Olympus, Japan) equipped with 10 negative phase objective and stage warmer (Thermo Plate, Tokai HIT, Olympus, Japan) set at 38°C. The image capture speed was 50 frame/sec. The motility status of at least 1000 cells was recorded for each sample from random fields. Range size particles to capture sperm head were defined between 6 and 25 μm^2 in the CASA settings. With respect to the setting parameters for calibrating the software, sperm with a VCL between 60-400, 15-60 and 0-15 µ/sec were considered as progressive, low motile and immotile, respectively. Sperm with straightness of ≥ 70% and ALH of 0.5-6 µm were considered as progressive.

Plasma membrane integrity

Plasma membrane integrity (PMI) was assessed using hypo-osmotic swelling test. Accordingly, sucrose solution (3.423% prepared in di-ionised water; 121 mOsm/kg; PH:7.86) was prepared on a daily basis. Diluted semen (100 µL) was added to the pre warmed (37 °C) sucrose solution in a test tube (1 mL), which was then incubated for 45 min at 37 °C. Assessment was performed under a phase contrast microscope at a magnification of 400 X to determine the percentage of sperm with coiled/swollen tails (normal sperm) out of 200 counted sperm.

Live percentage

The total number of live and dead sperm was assessed using the Eosin B-fast green stain (Mosaferi et al, 2005). Diluted semen (10 µL) was added to 10 µL of Eosin B-fast green stain. The smear was dried immediately on the stage warmer. A total number of 100 sperms were counted with dissecting microscope (1000 X) and the percentage of live sperm was recorded.

Experimental design

Five replicates from three dogs were included in this experiment (Total: 15 samples). Following semen collection, the viability of raw semen was analyzed. Then the specimen was centrifuged at 700 g for 10 min and the supernatant was removed.

Equal volume of specimen was placed in water jacketed collection glass vessels (IMV, France) at room temperature. Then the specimen was immediately diluted to reach 50-100×10^6 per ML by Tris-based extender, supplemented with 20% PEY of three avian species (domestic chicken, domestic duck and pigeon) and 3% glycerol. The diluted samples were pipetted three times to homogenize the diluted semen and the semen was analyzed. Every single vessel was then placed in vaccine transport device (Model: LRVC-404; Capacity: 1.4 lit; medea®, Tajhiz Teb Fanavaran, Iran) equipped with three ice packs. Subsequently, semen was cooled to 4 °C for 1 hr. After reaching to 4°C, the specimens were diluted with equal volume of freezing extender consisting of 20% PEY, similar to initial PEY, and 7% glycerol to achieve the final glycerol concentration of 5%. At this time, the semen was evaluated prior to freezing. Afterward, the samples were frozen using 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 seconds. Semen viability was evaluated after thawing.

Statistical analysis

The response variables had a discrete nature with the binomial distribution; therefore, all percentage data were subjected to arcsin transformation. Changes in the progressive forward 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, 2014) with repeated measures included in the model. Between groups differences at any given time were compared using Analysis of Variance followed by Least squares means within the GLM procedure in SAS. Data were presented as means± standard error of the mean (SEM) and percentages.

Results

Raw se men total motility, progressive forward motility, live percentage and plasma membrane integrity were 93.7±1.01%, 82.7±2.05%, 94.8±1.16% and 89.6±1.03%, respectively.

Total motility

There was no treatment by time interaction (P>0.05) and treatment effect (P>0.05). All experimental groups showed the same pattern over time where there was reduction in TM from Time 2 (after 1st extender: 91.3 ± 0.88 %) to Time 3 (after 2nd extender: 86.7 ± 1.93 %), and Time 4 (after thawing: 63.9±2.99 %; P < 0.05; Table 1).

Table 1. The effect of varying kind of avian plasma egg yolk on total motility of canine semen at different time after semen collection. Data were presented as mean ± SEM

Plasma egg yolk	After 1st extender	After 2nd extender	After thawing	Extender effect
Chicken	91.3±1.71	87.4±2.64	69.2±5.91	85.4±2.72
Duck	91.5±1.44	87.9±1.02	64.2±4.91	84.3±2.99
Pigeon	91.2±1.74	84.9±2.36	58.4±4.51	82.0±3.47
Time effect	91.3±0.88^{ab}	86.7±1.93^{b}	63.9±2.99^{c}	

^{abc} Values within rows with different superscript differ (P<0.05).

Progressive forward motility

There was no treatment by time interaction (P>0.05) and treatment effect (P>0.05). There was reduction in PFM from Time 2 (1st extender: 81.0 ± 1.02) to Time 3 (after 2nd extender: 75.0 ± 1.01) and Time 4 (after thawing: 44.2±2.20; P<0.001; Table2).

Live percentage

There was no treatment by time interaction (P>0.05) and treatment effect (P>0.05). Live percentage of sperm decreased after adding the 2nd extender (89.9 ± 0.59) and after thawing (73.6±1.92; P<0.01; Table 3).

Plasma membrane integrity

There was no treatment by time interaction (P>0.05). There was significant difference in PMI among experimental groups (P < 0.01; Fig. 1D); where, chicken PEY (80.9±2 %) had better PMI compared to pigeon PEY (76.6±3.08 %; P<0.01); but there was not any significant difference among other PEYs (P > 0.05). PMI decreased throughout the time (after 1st extender: 83.5 ± 0.93, after 2nd extender: 79.8 ± 0.82, after thawing: 60.9±2.02; P < 0.01; Table 4).

Table 2. The effect of varying kind of avian plasma egg yolk on progressive forward motility of canine semen at different time after semen collection. Data were presented as mean ± SEM.

Extender	after 1th extender	after 2nd extender	after thawing	Extender effect
Chicken	80.2±1.73 (75.9-84)	74.8±2.20 (68.1-82)	58.2±87.2 (40.1-63.7)	71.9±3.24 (40.1-90)
Duck	82.5±2.44 (76-90)	76.3±1.50 (70.9-80)	43.3±2.43 (37.3-49.4)	71.2±3.86 (37.3-90)
Pigeon	80.5±1.11 (77.3-84)	73.8±1.71 (68.5-79)	39.4±3.34 (31.2-48)	69.1±4.13 (31.2-90)
Time effect	81.0±1.02[a] (75.9-90)	75.0±1.01[b] (68.1-82)	44.2±2.20[c] (31.2-63.7)	

[abc] Values within rows with different superscript differ (P<0.001).

Table 3. The effect of varying kind of avian plasma egg yolk on Live percentage of canine semen at different time after semen collection. Data were presented as mean ± SEM.

Extender	after 1th extender	after 2nd extender	after thawing	Extender effect
Chicken	93.4±1.57 (90-97)	90.6±1.75 (87-97)	80.0±3.40 (72-88)	89.7±1.66 (72-97)
Duck	93.4±1.17 (90-96)	89.6±0.40 (89-91)	72.8±1.93 (68-79)	87.6±2.10 (68-97)
Pigeon	93.2±1.36 (89-96)	89.6±0.51 (88-91)	68.2±2.42 (60-74)	86.4±2.55 (60-97)
Time effect	93.3±0.73[a] (89-97)	89.9±0.59[b] (87-97)	73.6±1.92[c] (60-88)	

[abc] Values within rows with different superscript differ (P<0.01).

Table 4. The effect of varying kind of avian plasma egg yolk on plasma membrane integrity of canine semen at different time after semen collection. Data were presented as mean ± SEM.

Extender	after 1th extender	after 2nd extender	after thawing	Extender effect
Chicken	84.4±1.50 (74-88)	81.4±1.86 (75-86)	68.2±2.65 (62-78)	80.9±2.0[a] (62-92)
Duck	84.0-2.17 (78-91)	78.4±1.21 (75-81)	59.2±1.83 (53-64)	77.8±2.73[ab] (53-92)
Pigeon	82.2±1.24 (78-85)	79.6±1.03 (76-82)	55.2±3.21 (46-65)	76.6±3.08[b] (46-92)
Time effect	83.5±0.93[b] (78-91)	79.8±0.82[c] (75-86)	60.9±2.02[d] (46-78)	

[abc] Values within row (Time effect) and column (Extender effect) with different superscripts differ (P<0.01).

Discussion

This study was conducted to investigate the viability of canine sperm during long-term storage in tris-based semen extender supplemented with varying type of plasma egg yolk extracted from three avian species (chicken, duck and pigeon). Most viability parameters of canine sperm following freeze-thaw process were similar among

extenders supplemented with 20% PEY extracted from three type of avian species. Only plasma membrane integrity was superior in chicken PEY compared to pigeon. Silva et al. (2002) found that tris-chicken egg yolk-glycerol combination was beneficial for canine semen preservation. 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 (44.06% and 31.63%) compared with chicken egg yolk (38.54% and 27.38%; 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).

There is a relationship between the fatty acid ratio of the phospholipids of sperm and their susceptibility to cold shock (Poulos et al, 1973). Composition of domestic duck and chicken egg yolk showed a similarity in basic components of moisture, total protein and total fat (Bathgate et al, 2006), but there is variation in phospholipids, fatty acids (Graham & Foote, 1987; Trimeche et al, 1997; Bathgate et al, 2006) and cholesterol (Bair & Marion, 1978; Kaźmierska et al, 2005) among egg yolks of different avian species. Duck egg yolk had more monounsaturated fatty acids (MUFA) and contained more phosphotidylinositol than chicken egg yolk.

Sperm motility is the main criteria to evaluate the quality of extenders, particularly after cryopreservation (Ivanova-Kicheva et al, 1997). The sperm motility of 30-50% in frozen semen is biologically acceptable (Silva et al, 2003); however, the least sperm motility of 40-50% is necessary for successful artificial insemination with canine frozen semen (Silva et al, 2003). Pregnancies have been obtained by frozen semen with 20-30% post-thaw motility (Cardoso et al, 2003). Therefore, post-thaw motility of 40.1-63.7% with chicken plasma egg yolk achieved in the present study could be considered ideal to obtain pregnancy following artificial insemination. The wide range of post thaw motility with the same extender (33 to 75%) indicated that there is a great individual variation in the capacity of sperm survival after thawing (Silva et al, 2002). This could also be due to the wide diversity of methodologies used to freeze canine semen (Silva et al, 2003; Peña et al, 1998).

In conclusion, due to the ease of availability and low cost of chicken PEY, it could be considered as the best PEY for successful cryopreservation of canine semen. Accordingly, tris-based extender, supplemented with 20% PEY of domestic chicken and 5% glycerol could provide a suitable extender for cryopreservation of canine semen. Further studies are underway in our lab to examine the effect of varying concentrations of chicken plasma egg yolk and glycerol for the short and long term preservation of canine semen.

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Conflict of interest

The authors declare that they have no conflict of interest.

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مقایسه‌ی تأثیر استفاده از پلاسمای زرده‌ی تخم سه گونه مختلف از ماکیان در انجماد اسپرم سگ

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چکیده

در مطالعه‌ی حاضر به بررسی تأثیر پلاسمای زرده‌ی سه گونه‌ی مختلف از ماکیان در رقیق کننده‌ی پایه‌ی تریس جهت انجماد اسپرم سگ پرداخته شد. نمونه‌ی اسپرم اخذ شده بلافاصله توسط رقیق کننده‌ی تریس حاوی ۲۰ درصد پلاسمای زرده گونه‌های مختلف ماکیان شامل: مرغ خانگی، اردک خانگی و کبوتر به همراه ۳ درصد گلیسرول به ترتیبی رقیق گردید که غلظت اسپرم به ۵۰ تا ۱۰۰ میلیون در هر میلی‌لیتر برسد. پس از رسیدن به دمای ۴ درجه‌ی سانتی‌گراد در مدت یک ساعت، هم حجم نمونه، رقیق کننده انجماد حاوی ۲۰ درصد پلاسمای زرده مشابه مرحله‌ی اول و ۷ درصد گلیسرول اضافه گردید تا غلظت نهایی گلیسرول پنج درصد باشد. پارامترهای زنده‌مانی اسپرم شامل: حرکت کل، حرکت پیشرونده مستقیم، یکپارچگی غشاء پلاسمایی و درصد اسپرم‌های زنده بلافاصله پس از جمع‌آوری نمونه، بعد از افزودن رقیق کننده اول و دوم و پس از یخ گشایی مورد ارزیابی قرار گرفتند. یکپارچگی غشای پلاسمایی در گروه آزمایشی حاوی پلاسمای زرده‌ی تخم مرغ خانگی (۲٫۰±۸۰/۹ درصد) به طور معنی‌دارای بالاتر از گروه حاوی پلاسمای زرده‌ی تخم کبوتر بود (۳/۰۸±۷۶/۶ درصد؛ P<۰/۰۱). اختلاف معنی‌داری در سایر پارامترهای مختلف اسپرم در بین گروه حاوی پلاسمای زرده‌ی تخم کبوتر (حرکت کل: ۲/۷۲±۸۵/۴؛ حرکت پیشرونده مستقیم: ۳/۲۴±۷۱/۹؛ درصد اسپرم‌های زنده: ۱/۶۶±۸۹/۷) و سایر گروه‌های آزمایشی وجود نداشت. به طور کلی به دلیل در دسترس بودن و برتری در برخی از فراسنجه‌های زنده‌مانی اسپرم، پلاسمای زرده‌ی تخم مرغ خانگی در غلظت ۲۰ درصد می‌تواند تأثیر مناسبی در انجماد اسپرم سگ داشته باشد.

کلمات کلیدی: پلاسمای زرده تخم ماکیان ،اسپرم، سگ، انجماد

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