Indian Journal of Biotechnology
Vol. 19, April 2020, pp 131-139

Assessment and comparison of four lab made tris-base extenders for preservation of Labrador retriever dog semen at 4ºC

Sukhjeet Kaur[1], Ranjna S Cheema[1*], Gurjot K Mavi[2], M Honparkhe[1], Ashwani K Singh[1] and V K Gandotra[1]

[1]Department of Veterinary Gynecology and Obstetrics, [2]Department of Veterinary Animal Husbandry Extension Education, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141004, Punjab, India

Received 3 October 2019; revised & accepted 6 January 2020

The present study was aimed to develop a most effective extender for long term storage of Labrador dog semen at 4°C. Tris-citric acid-fructose was supplemented with egg yolk (TEY), egg yolk plasma (TEYP), low density lipoproteins (TLDL) and coconut water (TCW) @10, 15 and 20; 10, 15 and 20; 11-15; and 25, 50 and 75 percent, respectively. Extended semen was stored at 4°C and analyzed for sperm attributes and lipid peroxidation. Values for motility, viability and plasma membrane integrity remained significantly ($p < 0.05$) higher in 15% TEY, 15% TEYP, 13% TLDL and 25%/50% TCW from 0 hr to 72 hrs of storage. It indicated superiority of 15% TEY, 15% TEYP, 13% TLDL and 25% /50% TCW over other concentrations for storage of Labrador dog semen at 4°C. A significant ($p < 0.05$) decline in motility, viability and membrane integrity was observed from 0-72 hrs of preservation in all extenders, but sperm attributes were still >50% at 72 hrs of preservation. Decline was comparatively less in 15% TEY, 15% TEYP, 25%/50% TCW and 13% TLDL compared to other concentrations. Values for acrosome integrity also differ significantly ($p > 0.05$) among different concentrations of extenders except TEY. Lipid peroxidation did not vary among different extenders except TEY. In conclusion, both 15% TEY and 15% TEYP were better than 13% TLDL and 25% TCW. TEY extender may be substituted with TEYP for preservation of dog semen at 4°C and further interventions may improve TLDL and TCW extenders.

Keywords: Chilled semen, tris-base extenders, sperm attributes, lipid peroxidation, Labrador dog semen,

Introduction

The use of artificial insemination (AI) with chilled semen has become very popular among the canine breeders[1]. AI with preserved semen collected from study dogs of documented high quality provides the breeder with a circle for dispersal of the desired germ pool[2]. It is easier to transport chilled than frozen semen, which requires liquid nitrogen, and moreover, intra-vaginal insemination is possible rather than intra-uterine[3]. Gestation rate is also higher after artificial insemination using chilled semen than with frozen semen[4]. Commercial extenders commonly used for storage of chilled dog semen at 5°C promises the maintenance of good sperm quality[5-6]. But, still poor post thaw conception rates have been reported in dog[7]. Semen quality is indicated by sperm motility and vigor, membrane integrity, acrosome integrity, morphology, sperm cell DNA quality and ability to bind and fertilize oocytes.

For a long time, the standard semen extenders used in dogs were made with chicken egg yolk. However, egg yolk presents numerous disadvantages. It is associated with a health risk as egg yolk is an excellent microbial culture medium and can promote the onset of uterine infection following insemination of the bitch. Egg yolk also poses problems for analyses using a CERROS type image analyser or during biochemical assays[8]. Egg yolk (EY) contains granules, minerals and high density lipoproteins (HDL) that make sperm respiration difficult and may lead to decrease motility. Removal of these substances may improve the quality of preserved semen. The cryoprotective effect of EY on chilled and cryopreserved semen has been attributed to low density lipoproteins (LDL). Attempts have been made to use natural LDL in lieu of EY in semen extenders with promising results[9-10]. The inclusion of LDL and egg yolk plasma (EYP) in the extenders for dog semen is evaluated[11-12]. The use of LDL instead of EY in extenders improved canine sperm survival following freezing-thawing process[13-14]. Coconut water is characterized by its high contents of antioxidants[15]. Coconut water (CW) has been used successfully in cryopreservation of caprine[16], cattle[17] and chilling of ram[18], pig[19] and dog semen[20]. Most

*Author for correspondence
Tel: +91-161-2414003; Fax: +91-161-2400822
E-mail: ranjna.cheema@gmail.com

diluents for canine semen contain 10-20% egg yolk[21]. In spite of the progress that was made regarding the cryobiology of dog semen, no standardized freezing-thawing method appears to be ideal for all dogs and all ejaculates[22]. Moreover, there are only a few studies testing different concentrations of EY, EYP, LDL and CW to be added to extenders for preservation of canine semen. Therefore, the main objective of this study was to compare four different lab made tris base extenders: tris-base egg yolk (TEY), tris-base egg yolk plasma (TEYP), tris-base low density lipoproteins (TLDL) and tris-base coconut water (TCW) for storage of Labrador dog semen at 4°C.

Materials and Methods

Experimental Design

Effect of different concentrations of egg yolk (EY), egg yolk plasma (EYP), low density lipoproteins (LDL) and coconut water (CW) supplemented to tris-citric acid-fructose buffer (T) was observed on Labrador dog semen stored at 4°C from 0-72 hrs. EYP and LDL were purified from EY in the laboratory. Three ejaculates from each of the six dogs were analyzed for sperm attributes before initiating the actual trials. Four trials per extender were performed at July, 2018 to January, 2019. Best concentrations of EY, EYP, LDL and CW obtained from above trials were again compared in separate trials.

Maintenance of Dogs and Semen Collection

All the procedures were approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi vide F. No 25-19-2018-CPCSEA dated 22/11/2018. Six Labrador retriever dogs were maintained in individual pens. They were daily fed 500 g cooked feed twice daily and water provided ad libitum. Dogs were given regular exercise in the form of walking / running for one hour daily in the morning and evening. Dogs were dewormed and vaccinated for rabies, CDV, CAV2, CPV, CPI and CAV1 and after 15 days of vaccination, semen collection was initiated. Semen was collected by digital manipulation method twice a week.

Purification of Egg Yolk Plasma and Low Density Lipo Proteins

Egg yolk was separated from albumen and diluted with 0.17 M NaCl (1:2), stirred for 1 h at 4°C, and pH was noted. The mixture was then centrifuged at 10,000 g, 4°C for 45 min. The pellet containing granular portion of the egg yolk was discarded and the supernatant was again centrifuged twice as above. Supernatant as egg yolk plasma was stored in aliquots at -20°C for further use. The pH of the EYP was adjusted to 8.7 with 1 M sodium hydroxide solution. Ammonium sulphate solution (80%) was added drop wise (20 to 30 min) to plasma at 4°C in the ratio of 1:1 to achieve 40% final concentration. The mixture was maintained at 4°C, with continuous stirring, for another 1 hr. The mixture was then centrifuged as above and the supernatant dialyzed against Milli-Q water for overnight, following by centrifugation of the dialysate. The upper floating part containing the LDL was carefully withdrawn from the centrifuge tube, avoiding contamination by the fluid portion located at the bottom of the tube. The extracted LDL was stored in aliquots at -20°C for further use. Protein composition of egg yolk, EYL and LDL extracted was analysed by SDS-PAGE to check the purity[22].

Preparation of Coconut Water

Coconut shell was removed**;** water was extracted and collected in a sterilized beaker. Coconut water (CW) was filtered through 0.2 µ membrane filter and stored in aliquots at -20°C till use.

Preparation of Extenders for Extension of Semen

Tris - citric acid-fructose buffer (Tris, 3.08 g; citric acid, 1.78 g; fructose, 1.25 g 100 ml^{-1}, pH 7.2, gentamycin, 5 mg) was prepared and autoclaved at 15 lb for 15 min. Tris-citric acid-fructose buffer was filtered through 0.2 µ membrane filter and supplemented with different concentrations of egg yolk (TEY: 10%, 15% and 20%), egg yolk plasma (TEYP: 10%, 15% and 20%), low density lipoproteins (TLDL; 6- 9% and 11-15%) and coconut water (TCW: 25%, 50% and 75%. Extenders were always prepared fresh and again filtered through 0.2 µ membrane filter.

Standardization of Concentration of Extracellular Cryoprotectants for Chilling and Storage of Semen at 4°C

Semen of dogs exhibiting >70% motility was pooled for each trial and divided into required aliquots. Each aliquot was diluted with respective concentration of extender, equilibrated at 37°C for 10 min. Semen-extender mixture was centrifuged at 960 g for 3 min. Loose pellet was re-suspended in respective extender to get a final sperm concentration between 150-200 spermatozoa ml^{-1}. Sperm suspension was again equilibrated at 37°C for 10 min and evaluated for motility, viability, membrane and acrosome integrity. Samples in tubes were shifted to a

container containing warm water (37°C) and placed in a cooling cabinet at 4°C. Semen was analyzed for motility, viability and membrane integrity after every 24 hrs till 72 hrs of storage. Semen was also analyzed for acrosome integrity and lipid peroxidation at 72 hrs of storage.

Comparison Between Concentrations of Extracellular Cryoprotectant's for Chilling and Storage of Semen at 4°C

It took almost four months to standardize the concentrations of EY, EYP, LDL and CW. Semen quality varies from ejaculate to ejaculate and season to season. Good quality fresh semen will result in best parameters after chilling and preservation at 4ºC and vice versa. To overcome this effect, best concentrations were compared at one time in separate four trials. Pooled semen was divided into four fractions and each fraction was mixed with standardized concentration of TEY, TEYP, TLDL and TCW extenders. Samples were further processed and analyzed as mentioned above.

Analysis of Semen

Ejaculated, extended and preserved semen was analyzed for volume, individual motility (MOT), viability (VIA), plasma membrane integrity (PMI), acrosome integrity (AI) and lipid peroxidation (LPO)[23]. Volume of semen was noted in a graduated tube and sperm concentration was calculated with the help of haemocytometer. Motility was evaluated by wet mount and track method (Fig. 1A). Morphology and viability were assessed by staining the spermatozoa with syber green-propidium iodide stain kit (Sigma, Fig. 1B). Hypo-osmotic swelling test (HOST) was performed to analyze the integrity of sperm membrane. One drop of semen incubated in 60 mOsm hypo-osmotic swelling (HOS) solution for 30 min was placed on a slide, covered with cover slip and examined under bright field microscope (Olympus) at 400X for coiled tailed spermatozoa. A control was also run in phosphate buffer saline (PBS, pH 7.4). The number of coiled tailed spermatozoa in

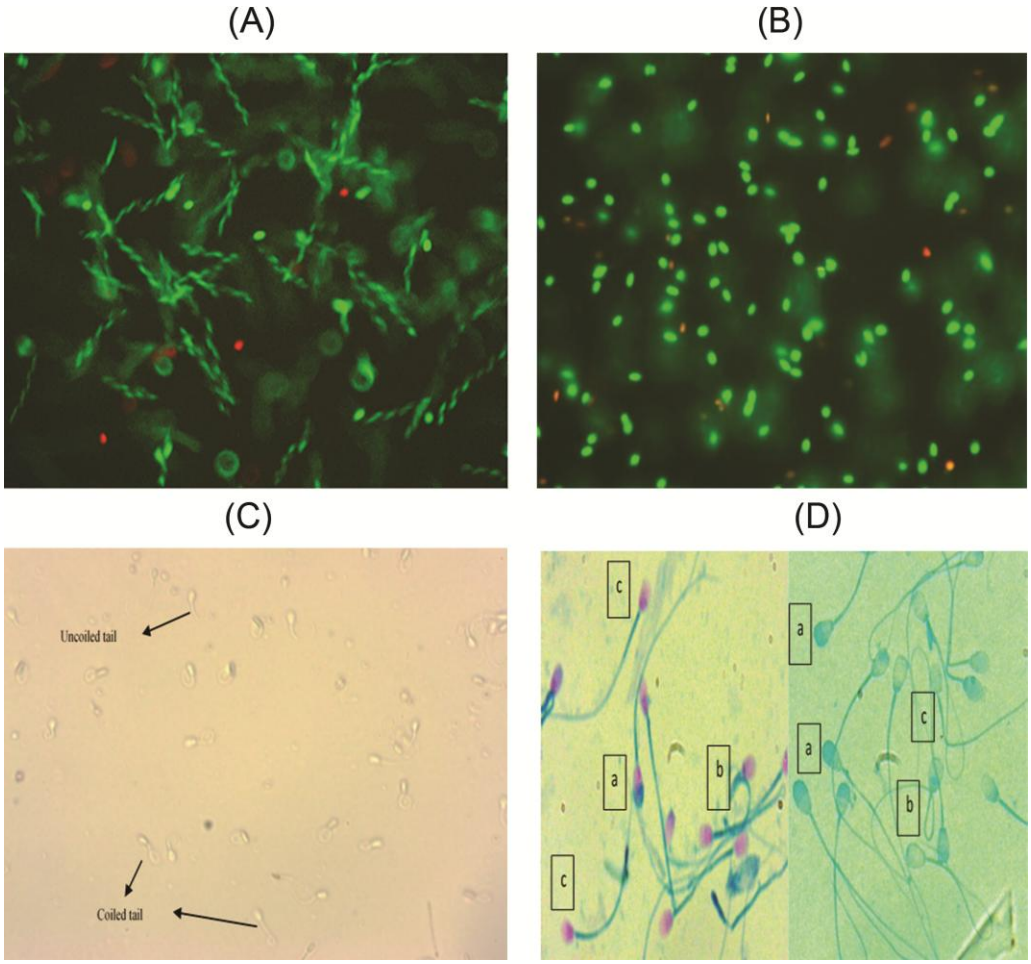

Fig. 1 — Showing A) Motility (track method), B) Viability (syber green propidium iodide stained red (dead), green (live) spermatozoa, C) Hypo osmotic swelling test and D) Acrosome integrity (spermatozoa with, intact (a), partially (b) and fully (c) damaged acrosomes).

PBS was deducted from the number in HOS and the resultant figure was taken as the HOS-reactive spermatozoa (Fig. 1C). For acrosome integrity, sperm smears prepared on clean glass slides were stained with spermac stain kit (Minitube) as per manufacturer's instructions (Fig. 1D). Spermatozoa with normal and abnormal morphology (MOR) were evaluated from the eosin-nigrosin stained sperm smears only in fresh semen. About 200 spermatozoa were counted in different fields for all sperm attributes and percentage of motility, viability, plasma membrane and acrosome integrity was calculated.

Malondialdehyde concentration (end product of LPO) was estimated by the method of Buege and Steven[24-25]. Briefly, semen was washed twice with PBS (pH 7.4) by centrifugation at 865 g for 5 min. Sperm pellet was suspended in 0.2 ml PBS, mixed with 0.1 ml of 150 mM Tris HCl (pH 7.1) and incubated at 37°C for 20 min. After incubation, 0.5 ml 10% trichloroacetic acid (TCA) and 1.0 ml 0.375% thiobarbituric acid (TBA) were added and kept for 20 min in the boiling water bath. Thereafter, mixture was centrifuged for 15 min at 2500 g and absorbance of supernatant was taken at 532 nm. The molar extinction coefficient for MDA was calculated as below:

$$\text{MDA content (units)} = \frac{\text{OD x volume of assay mixture}}{\text{Volume of sample taken x coefficient extinction}}$$

Statistical Analysis

Significant differences (5% level) in sperm attributes among the extenders were tested by t-test using SPSS 16 program (student version for windows, SPSS Inc. 233 South Wacker Drive, Chicago, IL 60606-6412). Normality of the data was assessed using the Shapiro-Wilk test and homogeneity of variances was evaluated using the Levene test.

Results and Discussion

Characterization of Proteins in EY and LDL

The floating residue collected after centrifugation of EYP dialysate treated with ammonium sulphate, presented the LDL. The bands of 240, 190, 130, 120, 110, 80, 65, 60, 50, 45, 40, 28, 22 and 20 kDa were fractionated from LDL on 10% acrylamide gel (Fig. 2). An additional band of 15 kDa was detected in EYP. Bands of 130, 28 and 22 kDa were not detected in EYP. Proteins of LDL are composed of six apo proteins[26]. Major apo protein that constitutes 70% of the apo proteins is of 130 kDa. Second apo

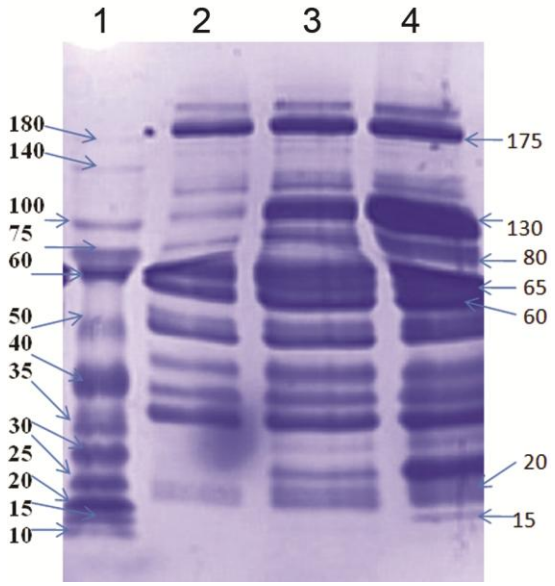

Fig. 2 — SDS-PAGE of egg yolk (EY), egg yolk plasma (EYP) and low density lipoproteins (LDL). Samples were run on 10% acrylamide gels and stained with comassie brilliant blue.
Lane # 1) standard, 2) EYP, 3) LDL and 4) EY.

protein between 15-20 kDa represents about 20% of the apoproteins. Four other minor apoproteins are identified between 60-95 kDa. Therefore, six major apoproteins of 175, 130, 80, 65, 60 and 20 kDa were present in the purified LDL. Mousa *et al* (2002) fractionated 175, 140, 80, 65, 60 and 15 kDa bands from purified LDL, which correspond to six major apoproteins of LDL.

Standardization of Extracellular Cryoprotectant's Concentration

The sperm parameters viz. MOT (70.0±5.8% - 85.0±2.8%), VIA (81.7±2.9% - 94.7±1.7%), abnormalities (12.9±4.4% - 37.3±0.9%), PMI (64.0 ± 4.5% - 85.0 ± 1.5%) and AI 80.0±4.7%-93.7±3.8%) in the fresh semen of freshly ejaculated semen of six dogs were within the physiologic range for dogs[27]. Our study employed subjective and track method for evaluation of motility rather than computer assisted semen analysis (CASA). Observations of Kustritz[28] revealed that semen quality results obtained by subjective and CASA methods are significantly correlated.

To test the suitability of extenders for chilled canine semen, it is essential to study sperm attributes such as MOT, VIA, PMI and AI after long term storage. Effect of storage period on semen extended in different concentrations of TEY, TEYP, TLDL and TCW extenders is shown in Tables 1-4. Although, 6-15% LDL concentrations were evaluated during the present study, but sperm attributes were not different

Table 1 — Effect of different concentrations of egg yolk (%) in tris-citric acid-frutose buffer (TEY) and storage time on sperm attributes at 4°C

Sperm attributes	0 Hr^{A}			24 Hrs^{B}			48 Hrs^{B}			72 Hrs^{C}		
	10	15	20	10	15	20	10	15	20	10	15	20
MOT (%)	71.2^{a} ± 5.9	71.2^{a} ± 5.9	71.2^{a} ± 5.9	59 .0^{b} ± 2.1	65.7^{a} ± 2.8	56.8^{b} ± 5.9	58.3^{b} ± 3.3	59.7^{a} ± 0.7	44.1^{c} ± 2.3	45^{a} ± 2.9	47.0^{a} ± 1.0	40.1^{b} ± 3.8
VIA (%)	74.2^{a} ± 6.2	74.2^{a} ± 6.2	74.2^{a} ± 6.2	69.7^{b} ± 1.5	75.3^{a} ± 0.3^{f}	65.9^{b} ± 4.4	65.0^{b} ± 0.0	71.3^{a} ± 0.7^{e}	67.2^{b} ± 3.0	53.3^{b} ± 4.4	58.3^{a} ±3.2^{c}	54.8^{b} ± 1.9
PMI (%)	71.2^{a} ± 9.5	71.2^{a} ± 9.5	71.2^{a} ± 9.5	48.3^{b} ± 4.4	53.3^{a} ±4.7	33.8^{c} ± 9.8	43.3^{a} ± 7.3	44.7^{a} ± 5.3	34.3^{b} ± 6.3	25.2^{b} ±2.2	33.8^{a} ± 8.9	13.9^{c} ±3.8
AI (%)	77.6^{a} ± 3.1	78.8^{a} ± 2.1	75.5^{a} ± 2.6	--	--	--	--	--	--	58.5^{b} ± 2.2	58.2^{b} ± 1.01	56.1^{b} ± 2.2
MDA (µM/10^{9} sperms)	--	--	--	--	--	--	--	--	--	31.6^{a} ± 3.3	16.1^{b} ±1 .8	35.4^{a} ± 4.0

Capital superscripts (A, B, C) indicate significant ($p<0.05$) effect of storage time on sperm attributes
Small superscripts (a, b, c) indicate significant ($p<0.0.5$) effect of egg yolk concentration on sperm attributes
MOT- motility, VIA- viability, PMI- plasma membrane integrity, AI- acrosome integrity, MDA-malondialdehyde

Table 2 — Effect of different concentrations of egg yolk plasma (%) in tris-citric acid-fructose buffer (TEYP) and storage time on sperm attributes at 4°C

Sperm attributes	0 Hr^{A}			24 Hrs^{B}			48 Hrs^{B}			72 Hrs^{C}		
	10	15	20	10	15	20	10	15	20	10	15	20
MOT (%)	73.3^{b} ± 3.3	80.0^{a} ± 2.9	75.0^{b} ± 2.9	68.3^{b} ± 3.3	76.7^{a} ± 4.1	68.3^{b} ± 4.4	65.0^{b} ± 5.0	70.0^{a} ± 2.9	61.7^{b} ± 4.4	53.3^{c} ± 6.0	68.3^{a} ± 1.7	61.7^{b} ± 3.3
VIA (%)	78.5^{b} ± 3.32	86.4^{a} ± 4.0	81.0^{b} ± 2.8	74.5^{b} ± 4.1	83.1^{a} ± 3.3	74.6^{b} ± 4.5	71.67^{b} ± 4.65	76.3^{a} ± 3.52	68.1^{b} ± 4.0	58.9^{c} ± 5.8	78.1^{a} ± 4.1	69.9^{b} ± 3.5
PMI (%)	78.8^{a} ± 3.1	76.4^{a} ± 2.6	72.2^{b} ± 3.9	73.0^{a} ± 2.2	74.2^{a} ± 4.4	68.6^{c} ± 4.8	58.90^{b} ± 5.85	63.7^{a} ± 3.1	56.7^{c} ± 3.9	60.2^{a} ± 4.5	63.2^{a} ± 4.6	57.8^{c} ± 2.7
AI (%)	66.0^{b} ± 5.4	81.2^{a} ± 2.9	70.1^{b} ± 3.5	--	--	--	--	--	--	56.1^{c} ± 3.2	70.8^{a} ± 3.5	63.4^{b} ± 2.3
MDA (µM/10^{9}sperms)	--	--	--	--	--	--	--	--	--	30.7^{a} ±11.2	25.5^{a} ± 3.6	30.4^{a} ± 4.2

Capital superscripts (A, B, C) indicate significant ($p<0.05$) effect of storage time on sperm attributes
Small superscripts (a, b, c) indicate significant ($p<0.05$) effect of egg yolk plasma concentration on sperm attributes
MOT- motility, VIA- viability, PMI- plasma membrane integrity, AI- acrosome integrity, MDA-malondialdehyde

Table 3 — Effect of different concentration of low density lipoproteins (LDL, %) in Tris-citric acid –fructose (TLDL) buffer and storage time on sperm attributes at 4°C

Sperm attributes	0 Hr[A]				24 Hrs[B]				48 Hrs[B]				72 Hrs[C]			
	11	12	13	14	11	12	13	14	11	12	13	14	11	12	13	14
MOT (%)	75.0^{a} ± 2.9	71.7^{b} ± 1.7	71.67^{b} ± 1.7	71.7^{b} ± 1.7	68.3^{a} ± 3.3	61.7^{b} ± 1.7	70.0^{a} ± 2.9	61.7^{b} ± 1.7	63.3^{a} ± 1.7	58.3^{b} ± 3.3	65.0^{a} ± 2.9	58.3^{b} ± 3.3	48.3^{b} ± 3.3	48.3^{b} ± 1.6	55.0^{a} ± 0.0	48.3^{b} ± 1.7
VIA (%)	76.3^{b} ± 0.3	76.4^{b} ± 3.71	80.3^{a} ±1.8	77.4^{b} ± 1.2	68.1^{b} ± 0.4	73.0^{a} ± 3.2	7 4.7^{a} ± 2.8	67.0^{b} ± 1.9	60.8^{b} ± 1.3	66.9^{b} ± 2.1	70.1^{a} ± 2.9	64.0^{b} ± 3.4	53.2^{b} ± 2.8	52.9^{b} ± 2.5	59.5^{a} ± 0.4	54.8^{b} ± 1.8
PMI (%)	75.0^{b} ± 1.2	73.07^{b} ± 0.8	77.2^{a} ± 1.3	75.2^{b} ± 1.3	70.2^{b} ± 2.8	74.8^{a} ± 2.3	74.8^{a} ± 1.74	69.2^{b} ± 0.5	61.9^{b} ± 0.5	65.5^{a} ± 2.4	67.7^{a} ± 0.9	63.6^{b} ± 2.1	57.2^{b} ± 2.0	55.5^{b} ± 3.3	60.5^{a} ± 2.4	57.9^{b} ± 2.7
AI (%)	72.9^{b} ± 2.5	73.4^{b} ± 1.1	76.2^{a} ± 2.2	72.8^{b} ± 2.0	--	--	--	--	--	--	--	--	50.1^{b} ± 0.4	51.8^{b} ± 6.7	55.0^{a} ± 0.3	51.6^{b} ± 1.2
MDA (µM/0^{9} sperms)	--	-- -- --- --				--	--	--	--	--	--	--	28.2^{a} ± 2.2	26.8^{a} ± 4.4	23.3 ±1.5	25.7^{a} ± 2.7

Capital superscripts (A, B, C) indicate significant ($p<0.05$) effect of storage time on sperm attributes.
Small superscripts (a, b, c) indicate significant ($p<0.05$) effect of LDL concentration on sperm attributes
MOT- motility, VIA- viability, PMI- plasma membrane integrity, AI- acrosome integrity, MDA-malondialdehyde

Table 4 — Effect of different concentration of coconut water (%) in tris-citric acid fructose buffer (TCW) and storage time on sperm attributes at 4°C

Sperm attributes	0 Hr[A]			24 Hrs[B]			48 Hrs[B]			72 Hrs[C]		
	25	50	75	25	50	75	25	50	75	25	50	75
MOT (%)	73.3^{b}± 3.3	81.7^{a}± 1.7	73.3^{b} ± 1.7	68.3^{a}± 3.3	63.3^{a}± 8.3	56.7^{b}± 4.4	65.0^{a} ± 5.0	65.0^{a} ±10.0	55.0^{b}± 5.0	53.3^{a}± 6.0	55.0^{a} ± 5.8	45.0^{b} ± 5.8
VIA (%)	78.5^{b}± 3.3	87.1^{a}± 1.7	80.3^{b} ± 1.4	74.5^{a}± 4.1	67.4^{b}± 8.6	61.1^{b} ± 4.0	71.7^{a}± 4.6	71.6^{a}± 10.71	59.8^{b}± 5.4	58.9^{a}± 5.8	60.8^{a} ± 5.2	48.8^{b}± 5.6
PMI (%)	78.8^{b} ± 3.1	84.6^{a} ± 2.1	77.3^{b} ± 3.2	73.0^{a} ± 2.2	66.4^{b}± 6.1	59.9^{c} ± 4.2	69.9^{a} ± 2.2	69.5^{a} ± 7.5	56.5^{b} ± 3.8	60.2^{a} ± 4.5	60.5^{a} ± 6.1	48.3^{b} ± 4.3
AI (%)	76.9^{a} ± 1.6	79.3^{a} ± 1.1	72.0^{a} ± 2.1	--	--	--	--	--	--	67.5^{b} ± 1.3	68.8^{b} ± 2.9	58.2^{b} ± 2.3
MDA (µM/10^{9}sperms)	--	--	--	--	--	--	--	--	--	23.9^{a} ± 3.9	21.4^{a} ± 2.6	25.7^{a} ± 4.5

Capital superscripts (A, B, C) indicate significant ($p<0.05$) effect of storage time on sperm attributes.
Small superscripts (a, b, c) indicate significant ($p<0.05$) effect of coconut water concentration on sperm attributes
MOT- motility, VIA- viability, PMI- plasma membrane integrity, AI- acrosome integrity, MDA-malondialdehyde

among 6-9% concentration. Therefore, only 11-15% concentrations were compared in this study. Values for MOT, VIA and PMI were also remained significantly ($p<0.05$) higher in 15% TEY, 15% TEYP, 13% TLDL and 25/50% TCW from 0 to 72 hrs of storage. It indicated superiority of 15% TEY, 15% TEYP, 13% TLDL and 25/50% TCW over other concentrations for storage of Labrador dog semen at 4°C. A significant ($p<0.05$) decline in MOT, VIA and PMI was observed from 0-72 hrs of preservation in all extenders, but sperm attributes were still >50% at 72 hrs of preservation. There was comparatively less decline in MOT, VIA and AI in 15% TEY, 15% TEYP, 13% TLDL and 25/50% TCW compared to other tested concentrations of respective extenders. A significant ($p<0.05$) decline in percent AI was also evident after 72 hrs of storage in all concentrations of TEY, TEYP, TLDL and TCW. During the present study, MOT, VIA, PMI and AI were similar to the observations in semen pooled from seven breeds and stored in tris-egg yolk extender at 4°C after 72 hrs of preservation[29]. Contrarily, 20% EY, 20% EYP, 20% CW and 6/8% LDL have been reported most suitable for long term storage of canine semen at 4°C[30-32]. Difference in most effective concentrations may be due to the fact that we performed experiments on pooled semen from a single breed. It indicates that different dog breeds respond inconsistently to the same extender. Stănescu and Bîrțoiu (2012) concluded in their review that in spite of the progress that was made regarding the cryobiology of dog semen, no standardized freezing-thawing method appears to be ideal for all dogs and all ejaculates. Cooling lowers sperm metabolism, but unlike freezing, it does not cause inactivity. Thus, reduced sperm attributes as a result of storage time is likely due to factors such as sperm metabolite accumulation, which may alter osmolarity and pH of the diluent, compromising sperm functionality and motility. Moreover, energy depletion may contribute to the negative consequences of prolonged storage time. MDA production ($\mu M/10^9$ spermatozoa) was comparatively low in 15% TEY, 13% LDL and 25/50% TCW in comparison to other tested concentrations of extracellular cryoprotectants. However, differences were significant ($p<0.05$) only among different concentrations of egg yolk in TEY extender (Table 1).

Comparative evaluation of TEY, TEYP, LDL and TCW Extenders for Long Term Storage of Semen at 4°C

MOT, VIA and PMI were significantly ($p<0.05$) higher in 15% TEY and 15% TEYP than 13% TLDL and 25% TCW from 24 hrs to 72 hrs of storage (Table 5). Percentage of AI was also significantly higher ($p<0.05$) in 15% TEY and 15% TEYP than 13% TLDL and 25/50% TCW at 0 hr and 72 hrs of storage. MDA ($\mu M/10^9$ spermatozoa) remained significantly ($p<0.05$) low in 15% TEY, 15% TEYP and 13% TLDL than 25/50% TCW at 72 hrs of storage (Table 5). Although, 15% TEY and 15% TEYP did not differ from each other with regard to sperm attributes during the present observations on Labrador dog semen, but visibility of spermatozoa was considerably better in TEYP than TEY extender. It may be contributed to the non-appearance of granules in EYP. Post-thaw sperm motility and integrity of membrane and acrosome were superior ($p < 0.05$) for mongrel dog semen extended with 20% EYP than with 20% EY. Egg yolk contains phospholipids and LDL which ensures efficient protection of the sperm membrane and nutritional support for these cells. Although, sperm attributes were comparatively low in 13% TLDL than 15% TEY and 15% TEYP after 72 hrs of storage, but still comparable to the standard parameters of dog semen required for artificial insemination. The replacement of egg yolk by purified low density lipoproteins (LDL, animal or vegetal) was beneficial for sperm motility and membrane integrity[33-34].

However, 25% TCW extender too maintained the sperm parameters close to 50% in Labrador dog semen stored at 4°C for 4 days, although less than TEY, TEYP and TLDL. Essential constituents such as sugar, vitamins, minerals, potassium, magnesium, fibre, and proteins[35] and antioxidant properties[36] of coconut water may be responsible for the maintenance of sperm attributes up to a desirable level.

Canine spermatozoa contain antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx)[37] as protection against the cytotoxic effects of internally generated reactive oxygen species (ROS). Excessive ROS impair motility and fertilization capacity. The production of ROS by sperm is a normal physiological process, but balance between ROS generation and scavenging activity is detrimental to the sperm and leads to decreased MOT, VIA, AI and increase in mid piece morphological defects and deleterious effects on capacitation and acrosome reaction. Therefore, decrease in sperm characteristics during long term storage of semen at 4°C may be attributed to generation of ROS. However, low level of LPO and high values of sperm

Table 5 — Comparative evaluation of best concentrations of TEY, TEYP, TLDL and TCW extenders for storage of semen at 4°C for 72 hrs.

Sperm attribute	0 Hr[A]				24 Hrs[B]				48 Hrs[B]				72 Hrs[C]			
	Extender				Extender				Extender				Extender			
	TEY	TEYP	TLDL	TCW	TEY	TEYP	TLDL	TCW	TEY	TEYP	TLDL	TCW	TEY	TEYP	TLDL	TCW
MOT (%)	$78.3^{a}\pm 1.7$	$78.3^{a}\pm 1.7$	$76.7^{a}\pm 3.3$	$73.3^{b}\pm 1.7$	$71.7^{a}\pm 1.7$	$70.0^{a}\pm 2.9$	$65.0^{b}\pm 2.9$	$63.3^{b}\pm 1.7$	$63.3^{a}\pm 1.7$	$61.7^{a}\pm 1.7$	$58.3^{b}\pm 1.7$	$56.7^{b}\pm 1.7$	$53.3^{a}\pm 1.7$	$53.3^{a}\pm 1.7$	$48.3^{b}\pm 1.7$	$46.7^{b}\pm 3.3$
VIA (%)	$81.5^{a}\pm 2.5$	$82.2^{a}\pm 2.1$	$80.1^{a}\pm 2.1$	$77.9^{b}\pm 1.6$	$74.6^{a}\pm 1.2$	$74.5^{a}\pm 1.9$	$70.9^{b}\pm 1.1$	$68.7^{b}\pm 1.2$	67.8 ± 1.0	$67.7^{a}\pm 0.4$	$64.5^{b}\pm 1.30$	$61.4^{b}\pm 1.1$	$58.10^{a}\pm 0.8$	$58.4^{a}\pm 2.1$	$53.7^{b}\pm 2.2$	$48.4^{c}\pm 2.9$
PMI (%)	$77.9^{a}\pm 3.1$	$78.4^{a}\pm 2.43$	$74.9^{b}\pm 2.5$	$74.1^{b}\pm 1.9$	$70.6^{a}\pm 1.0$	$70.7^{a}\pm 0.8$	$67.7^{b}\pm 1.5$	$65.4^{b}\pm 2.1$	$66.6^{a}\pm 0.5$	$67.0^{a}\pm 1.5$	$61.9^{b}\pm 1.1$	$59.1^{b}\pm 0.5$	$60.2^{a}\pm 0.5$	$60.3^{a}\pm 2.8$	$53.4^{b}\pm 1.5$	$48.8^{b}\pm 1.9$
AI (%)	$77.8^{a}\pm 1.5$	$78.8^{a}\pm 0.8$	$75.2^{a}\pm 1.1$	$73.4^{a}\pm 1.2$	--	--	--	--	--	--	--	--	$57.7^{b}\pm 2.5$	$58.2^{b}\pm 1.0$	$52.2^{b}\pm 1.0$	$49.0^{b}\pm 0.4$
MDA (μM/10^{9} sperms)	--	--	--	--	--	--	--	--	--	--	--	--	$16.8^{b}\pm 1.9$	$16.1^{b}\pm 1.8$	$18.8^{b}\pm 0.4$	$22.4^{a}\pm 1.5$

Capital superscripts (A, B, C) indicate significant ($p<0.05$) effect of storage time on sperm attributes.

Small superscripts (a, b, c) indicate significant ($p<0.05$) effect of extender on sperm attributes

MOT- motility, VIA- viability, PMI- plasma membrane integrity, AI- acrosome integrity, MDA-malondialdehyde

parameters in semen preserved in 15% TEY and 15% TEYP than 13% TLDL and 25% TCW revealed that EY and EYP gave more protection to Labrador dog spermatozoa than LDL and CW during preservation at 4°C for a longer period.

Sperm attributes were ≥50% even after 72 hrs of preservation during present study. Semen characteristics can be used to predict the conception potential of semen in the dog. Keeping this statement in consideration, higher conception rate may be expected upon AI with Labrador dog semen preserved in 15% TEY, 15% TEYP, 13% LDL and 25/50% TCW at 4°C for 72 hrs. In conclusion, both 15% TEY and 15% TEYP were better than 13% TLDL and 25% TCW. TEY extender may be substituted with TEYP for preservation of dog semen at 4°C and further study may improve TLDL and TCW extenders.

Acknowledgements

This research was supported by Department of Biotechnology, Ministry of Science and Technology, New Delhi: No. SAN No.102/IFD/SAN/5331/2017-2018.

References

1 Smith-Carr S, Canine artificial insemination, *Vet Tech*, 27 (2006) 474-486.

2 Rota A, Linde-Forsberg C, Vanozzi I, Romagnoli S & Rodriguez-Martinez H, Cryosurvival of dog spermatozoa at different glycerol concentrations and freezing-thawing rates, *Reprod Domest Anim*, 33 (1998) 355-361.

3 Rota A, Srom B & Linde-Forsberg C, Effects of seminal plasma and three extenders on canine semen stored at 4°C, *Theriogenology*, 44 (1995) 885-900.

4 Rota A B, Strom C, Linde-Forsberg C & Rodriguez-Martinez H, Effects of equex STM paste on viability of frozen-thawed dog spermatozoa during *in vitro* incubation at 38°C, *Theriogenology*, 47 (1997)1093-1101.

5 Ponglowhapan S, Esse´n-Gustavsson, B & Linde Forsberg C, Influence of glucose and fructose in the extender during long-term storage of chilled canine semen, *Theriogenology*, 62 (2004) 1498-1517.

6 Prinosilova P, Rybar R, Zajicova A & Hlavicova J, DNA integrity in fresh, chilled and frozen-thawed canine spermatozoa, *Vet Med*, 57 (2012)133-142.

7 Stănescu M & Bîrţoiu A, Freezing of dog's sperm: A review, *Romanian Biotechnological Letters*, 17 (2012) 7709-7716.

8 Wall R J & Foote R H, Fertility of bull semen frozen and store in clarified egg yolk-Tris-glycerol extender, *J Dairy Sci*, 82 (1999) 817-821.

9 Moussa M, Martinet V, Trimèche A, Tainturier D & Anton M, Low density lipoproteins extracted from hen egg yolk by an easy method: Cryoprotective effect on frozen–thawed bull semen, *Theriogenology*, 57 (2002) 1695-1706.

10 Hu JH, Tian WQ, Zhao XL, Zan LS, Wang H *et al*, The cryoprotective effects of ascorbic acid supplementation on bovine semen quality, *Anim Reprod Sci*,121 (2010) 72-77.

11 Varela Junior AS, Corcini CD, Ulguim RR, Alvarenga MVF, Bianchi I *et al*, Effect of low density lipoprotein on the quality of cryopreserved dog semen, *Anim Reprod Sci*, 115 (2009) 323-327.

12 Corcini CD, Goularte KL, Bongalhardo DC, Lucia Jr TJardim RD *et al*, Effect of egg yolk plasma on dog sperm cryopreservation, *Andrologia*, 47 (2015) 1-2.

13 Bencharif D, Amirat L, Anton M, Schmitt E, Desherces S *et al*, The advantages of LDL (low density lipoproteins) in the cryopreservation of canine semen, *Theriogenology*,70 (2008) 1478-1488.

14 Neves M M, Heneine L G D & Henry M, Cryoprotection effectiveness of low concentrations of natural and lyophilized LDL (low density lipoproteins) on canine spermatozoa, *Arqu Brasil de Medi Vet Zootec***,** 66 (2014) 769-777.

15 Yong J W H, Ge L, Fei Ng Y & Tan S N, The chemical composition and biological properties of coconut (*Cocos nucifera* L.) water, *Molecules*,14 *(*2009) 5144-5164.

16 Campos A C N, Monteiro A W U, Diogenes B O P, Pinheiro J H T & Nunes J F, Uso da aÂgua de cococriopreservadacomodiluidor de congelac Ëa Áodose Ãmencaprino. In: XXVI Congresso Brasileiro de Medicina Veterinaaria, Campo Grande,(1999).

17 Ibrahim R, El-Sheshtawy, El-Nattat W S & Ali G A D, Cryopreservation of cattle semen using coconut water extender with different glycerol concentration, *Asian Pac J Reprod*, 6 (2017) 279-282.

18 Oliveira L F, Evangelista J J F, Bezerra M B & Nunes J F, Âgua de coÃco in naturaadicionadaounaao de gemadeovo e sob a forma estabilizada de gel comodiluidor do se Ãmenovino. In: XXIII Congresso Brasileirode Medicina Veterina Âria, *Olinda-Pernambu*, (1994) 561.

19 Silva C G, Cunha E R, Blume G R, Malaquias' J V, BáoS N *et al*, Cryopreservation of boar sperm comparing different cryoprotectants associated in media based on powdered coconut water, lactose and trehalose, *Cryobiology*,70 (2015) 90-94.

20 Cardoso R C S, Silva A R, Uchoa D S & da Silva L D M, Cryopreservation of canine semen using a coconut water extender and three different glycerol concentrations, *Theriogenology*, **59** (2003) 743-751.

21 Silva A R, Cardoso R, De C S, Uchoa D C, Machado D A *et al*, Effect of tris-buffer, egg yolk and glycerol on canine semen freezing, *Vet J*, 164 (2002) 244-246.

22 Laemmli U K, Cleavage of structural proteins during the assembly of the head of bacteriophage $T_{4,}$ *Nature*, 220 (1970) 680-685.

23 Jeyendran R S, Van der Ven H H, Perez-Pelaez M, Crabo B G & Zaneveld L J D, Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics, *J Reprod Fertil*, 70 (1984) 219-228**.**

24 Buege J A & Steven A D, Microsomal lipid peroxidation, *Methods Enzymology*, 51 (1978) 302-310.

25 Le Denmat M, Anton M & Beaumal V, Characterisation of emulsion properties and of interface composition in oil, in water, emulsions prepared with hen egg yolk, plasma and granules, *Food Hdrocol*, 14 (2000) 539-549.

26 Johnston S, Kustritz M R & Olson P, Semen collection, evaluation and preservation, in the canine and feline, Theriogenology, edited by Johnston S, Kustritz M R & Olson P (WB Saunders, New York) 2001), 287-307.

27 Kustritz R, The value of canine semen evaluation for practitioners, *Theriogenology*,68 (2007) 329-337.

28 Bencharif D, Amirat-Briand L, Le Guillou J, Vialtelle C, Anton M *et al*, Refrigeration of canine sperm at +4°C: Comparative study of four different extenders for the refrigeration of canine sperm at +4°C: LDL, tris egg yolk, Equex®, and INRA96®, *Revue de Méd V ét*, 164 (2013) 252-62.

29 Da Cunha, I C N, de Ascençao Rocha A1, Quirino C R & Gimenes A L L, The efficacy of *in vitro* sperm tests in predicting pregnancy success after artificial insemination in the bitch, *Andrology (Los Angel*, 6 (2017) 190-194.

30 Pan C, Wu Y, Yang Q & Ye J, Effects of seminal plasma concentration on sperm motility and plasma and acrosome membrane integrity in chilled canine spermatozoa, *Polish J Vet Sci*, 21 (2018) 133-138.

31 Puja K, Made Sawttri N I, Maharani N, Gunawan W N F, Surya L G S *et al*, A comparative study on the effects of coconut water based extenders on the Quality of Kintamani dog semen preserved at 4^{o}C, *Adv Anim Vet Sci*, 6 (2018) 192-196.

32 Bencharif D, Amirat L, Pascal O, Anton M, Schmitt E *et al*, The advantages of combining LDL (Low Density Lipoproteins) with glutamine for the cryopreservation of canine semen, *Reprod Domest Anim*, 45 (2010) 189-200.

33 Bencharif D, Amirat-Briand L, Garrand A, Anton M *et al*, The advantages of using a combination of LDL and glutamine in comparison with tris egg yolk and Equex® STAMP extenders in the cryopreservation of canine semen, *Res Vet Sci*, 93 (2011) 440-447.

34 Silva P S & Bamunuarachchi A, Manufacture of carbonated tender coconut water and development of a process for the utilization of coconut flesh, *Asian J Food Agro-indust*, 2 (2009) 210-213.

35 Mantena K, Jagadish, Badduri S R, Siripurapu K B & Unnikrishnan M K, *In vitro* evaluation of antioxidant properties of *Cocos nucifera* Linn. Water Sudheer, *Nahrung / Food*, 47 (2003) 126-131.

36 Strzeżek R, Koziorowska-Gilun M, Kowalówka M & Strzeżek J, Characteristics of antioxidant system in dog semen. *Polish J Vet Sci*, 12 (2009) 55-60.