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Soybean Lecithin-Based Extender Improves the Quality of Chilled Canine Sperm

Mingkun Sun[a], Wangcheng Song[a], Changrong Lv[a], Yu Li[b] and Song Hua[a*]

[a]College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi Province 712100, China
[b]College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi Province 712100, China

Mingkun Suna and Wangcheng Song contributed equally to this work

[*]**Corresponding author:** Dr. Song Hua, Ph.D., College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi Province 712100, China, Tel: +86 29 87091117, E-mail: hs863@nwsuaf.edu.cn

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Abstract

Semen preservation technique includes chilling at 4-5 °C and cryopreservation at -196 °C. The role of extender enriched with protectant is essential for storing sperm. In order to evaluate the effects of different concentrations of soybean lecithin (SL) in Tris-based extender on quality parameters of chilled canine semen and subsequent potential. The Tris-based extenders with 0.5%, 1.0% or 2.0% SL (w/v) were used to store the sperm of French Bulldog; meanwhile, Tris-based extenders supplemented with 20.0% (v/v) egg yolk (EY) were used as the control group. Sperm samples in each group were stored at 4°C for 168 hours period. The results showed that the percentages of sperm viability and progressive motility, and acrosome and plasma membrane integrity in 1.0% SL group were higher than those in the 20.0% EY group. These may be attributed to the phospholipids contained in SL, which has the function of anti-oxidation and protecting sperm plasma membrane. Meanwhile, the whelping rates(total puppies full-term born alive/number of bitches with full-term pregnancies) of bitches inseminated with sperm stored in 1.0% SL extender were significantly higher than those inseminated with sperm stored in 20.0% EY extender. These results suggest that Tris-based extender supplemented with 1.0% SL can be used to chill canine semen. These results have important reference significance to promote the commercialization of semen extender in canines.

Keywords: Sperm Chilled Storage; Canine; Soybean Lecithin; Pregnancy Rate; Whelping Rate

Introduction

Artificial assisted breeding of pet dogs is widely studied [1-3]. Particularly, the study and application of cold-stored canine semen for artificial insemination (AI) in pet dog breeding has become more and more concerned [4,5]. Semen extender has a vital role in the preservation of sperm quality. Although there are various commercial and noncommercial extenders available for chilling canine semen, one of the most commonly used extenders in veterinary practice is Tris-glucose/fructose buffer supplemented with 20% egg yolk (EY) [6], in which Tris-glucose/fructose buffer is used as basal buffer, and EY is considered as protective agent. But detailed ingredients in EY are not defined. Moreover, EY is an animal-origined production, it has potential risk of carrying disease microorganism and contamination [7,8]. Therefore, it is required to explore an alternative extender that can replace EY as protective agent to chill semen.

Several studies reported that soybean lecithin (SL) is a promising option as a substitute for EY to chill semen because SL has a similar composition (i.e. low-density lipoprotein) to that contained in EY, and may provide protection to the sperm plasma membrane during cold-shock [1,9-12]. A study reported that the sperm motility and viability were better after chilling canine semen up until day 8 in the extender with SL than with 20% EY [13]. Another study reported that sperm motility, plasma membrane integrity and mitochondrial membrane potential were superior after canine sperm was chilled in SL extender than chilled in 20% EY extender over 10 days of storage [14].

Though the replacement of EY with SL to chill canine semen was studied previously, all of these researches evaluated the quality of chilled sperm just from sperm motility, plasma membrane integrity and so on; they did not evaluate fertility of canine chilled sperm basing on whelping rate and litter size. While the birth rate and litter sizes are the golden standard to measure sperm quality in canines [15].

In the present study, French Bulldogs came to our animal breeding center for AI during estrus period in the past two years were inseminated with semen chilled in Tris-glucose/fructose buffer with either EY or SL, and semen quality, whelping rate and litter size were compared.

Material and Methods

Unless otherwise specified, all chemicals were purchased from Sigma Chemical Company (USA).

Animals

A total of five healthy male 2-4-year-old French Bulldogs (10.2±0.46 kg) with normal fertility were used to collect sperm through manual massage. Forty eight female French Bulldogs (one to three years old, 9.6±0.69 kg), gave birth to only one litter in natural oestrus were used for artificial insemination. All canines were fed nutritionally balanced adult commercial dog food (D&C Full Dog Food, Rizhao Bafang Pastoral Agriculture Science and Technology Co. Ltd). The temperature inside kennels was maintained (27.0 ± 2.0) °C with a relative humidity level of (60.0 ± 5.0) %. They had free access to water, and outdoor free activities in the morning and evening for one hour each. The owners of bitches signed an informed consent forms (permission of their bitches to be used for the determination of blood hormones during estrus, and to be used to inseminate with chilled semen, and permission of the data obtained in this process to be published), and all experimental operations related with animals were approved by the Animal Care and Use Committee of Shaanxi Province (Approval No.201902D6).

Semen recovery

Semen was collected every other day from each dogs with two days off on weekends. The sperm-rich (second) fraction of the ejaculate was collected into a sterile plastic vial with scale by digital manipulation in the presence of an estrous bitch. Immediately after

collection, each ejaculate was analyzed to determine semen motility, concentration, and abnormal morphology using a computer-assisted semen analysis (CASA) system (MAILANG CHINA SJ-TMDI100JZ) according to the description reported previously [16]. The sperm with concentration more than 200×10^6 sperm/ml, abnormal morphology less than 5%, and viability more than 90% were employed to further experiment.

Semen chilling

The composition of the Tris-based extenders was 30.25 g/L Tris, 17.0 g/L sodium citrate, 12.5 g/L fructose, 0.6g/L penicillin, 1.0 g/L streptomycin. The commercial SL (Type II-S,14-23% pure; Beijing Solarbio Science &Technology Co., Ltd., CAS:L8050) was added into Tris-based extenders to make up 0.5%, 1.0% and 2.0% SL extenders(w/v). After stirred with a blender for 10 minutes and centrifuged at 5 000 r/min for 15 min, the supernatant solution passed sequentially through 1.0 μm and 0.45 μm filters to remove remaining solids [17,18]. At the same time, 20.0% EY extender (v/v) prepared according to the method reported by [14] was used as control group.

Pooled semen was centrifuged at 720 g for 5 min to discard the supernatants [18]. The pellets were resuspended in three different concentrations of SL extenders (0.5%SL, 1.0%SL and 2.0%SL), and one concentration of EY extender (20% EY), and the final sperm concentration was 10.0×10^7 sperm/ml [19,20]. After that, the diluted semen was divided into 0.5 ml using centrifuge tubes (1.5 mL), and was placed in a plastic box and cooled down gradually to 4 °C [21] and then stored at 4 °C up until 168 hours.

Assessment of sperm motility and viability

Sperm quality parameters were analyzed at 24 h intervals. The percentages of sperm that exhibit rapid, linear movement were assessed using the CASA system as reported by [22]. The percentages of sperm viability were measured using eosin–nigrosin stain. Briefly, a smear was consisted of one drop (10 μl) of semen and three drops (30 μl) of the stain on a preheated slide at 37 °C. The stained slide was placed under a phase-contrast microscope, and a total of 1000 sperm was calculated at 400×magnification. Sperm that absorbed the stain was considered dead, whereas those that did not absorb the stain were alive.

Measurement of sperm acrosome and plasma membrane integrity

The acrosome integrity rate was assessed using the fluorescent dye FITC-conjugated lectin (L7381-1MG; Sigma-Aldrich, Co. St. Louis MO, USA) from peanut (FITC-PNA) and DAPI staining solution according to the previous article [23] with some modifications. Briefly, FITC-PNA powder (1 mg) was diluted to 20 μg/ml by Dulbecco's phosphate--buffered saline (DPBS). A semen sample of 30 μL was evenly smeared on a clean glass slide and left to air dry. After it was fixed in methanol for 30 s to permeabilize the sperm membranes, 30 μL FITC-PNA dye solution was added to the slide, and it was placed in a wet box at 37 °C and incubated in the dark for 30 min. After rinsing three times with PBS, 30 μL DAPI dye solution (10 μg/ml) was added to the slide, and incubated at room temperature for 10 min. After unbound dyes were removed, antifade mounting medium was added and a cover slip was then used and the edges were sealed with colorless nail varnish. Sperm cells displaying intense green, uniform and well demarcated fluorescence over the acrosomal cap were considered as acrosome intact, yet those sperm cells without green fluorescence, or with green fluorescence only along its outline or acrosomal fringe were considered as damaged acrosome [24] (Figure 1). A total of 1000 were counted under 400 × magnification in three replicates, and the calculation formula as follows: percent acrosome integrity = the number of acrosome integrity sperm ÷ the total sperm count.

The plasma membrane integrity of sperm was detected using SYBR-14/PI Dual Fluorescent Staining Kit (HL14057, Shanghai Haring Biotechnology co., LTD), and detailed procedures were done according to the manufacturer's instructions. The bright green fluorescence of the whole sperm head was regarded as intact plasma membrane, and red or orange fluorescence was considered as damaged plasma membrane (Figure 2). A total of 1000 were counted under 400 × magnification in three replicates, and the calculation formula as follows: percent plasma membrane integrity = the number of intact plasma membrane sperm ÷ the total sperm count.

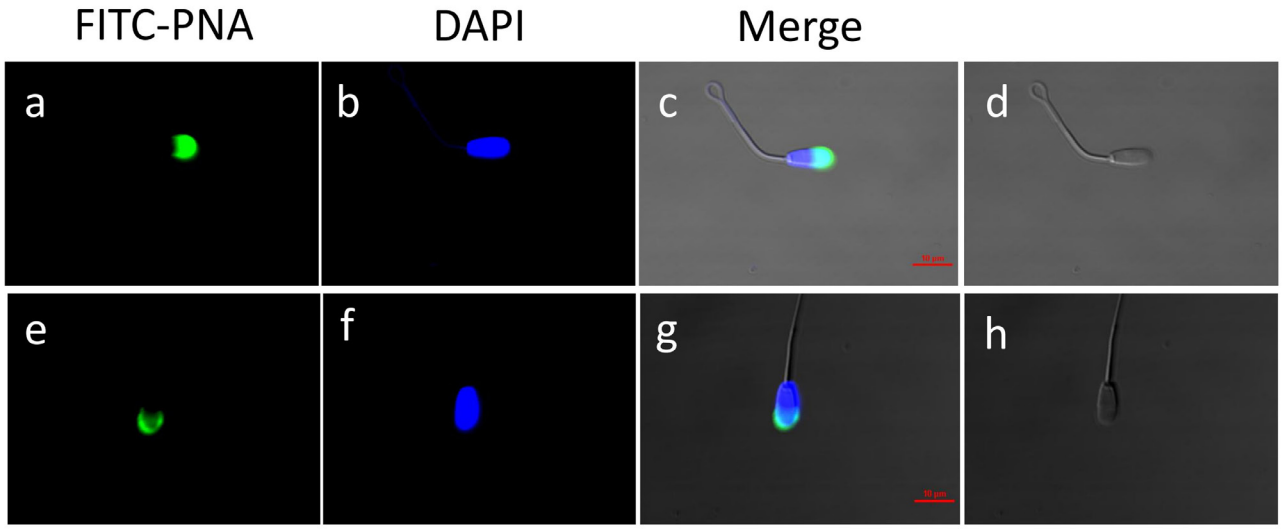

Figure 1: Acrosomal integrity analysis of sperm with the FITC-PNA and DAPI staining solution under a confocal laser scanning microscope **(a)** Sperm with intact acrosome; **(b)** Sperm nucleus stained with DAPI; **(c)** Two images merged one; **(d)** Image taken under ordinary visible light; **(e)** Sperm with damaged acrosome; **(f)** Sperm nucleus stained with DAPI; **(g)** Merged image; **(h)** Image taken under ordinary visible light

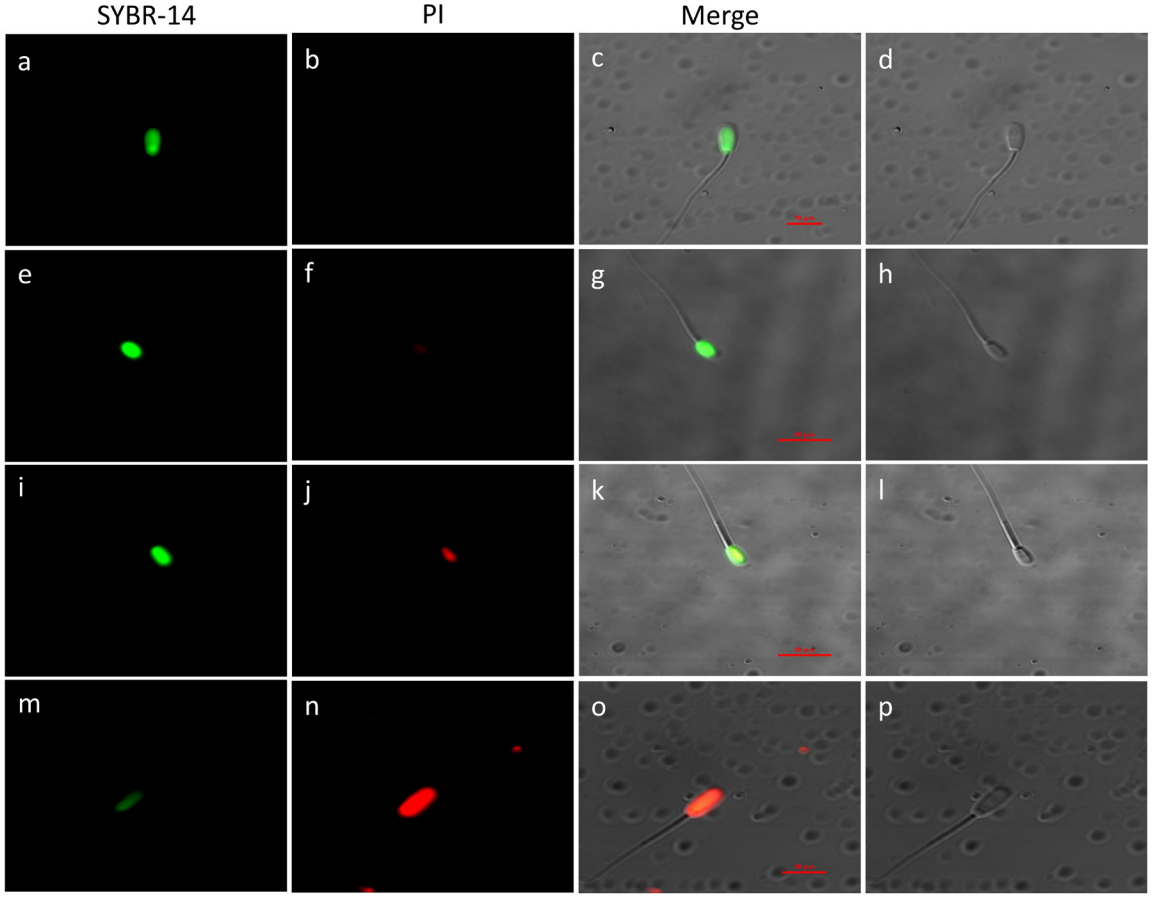

Figure 2: Sperm plasma membrane integrity analysis with SYBR-14/PI Dual Fluorescent Staining Kit **(a)** Sperm with intact plasma membrane; **(b)** Sperm with intact plasma membrane were not stained by PI; **(c)** Two images merged one; **(d)** Image taken under ordinary visible light; **(e)** Sperm with partially damaged plasma membrane; **(f)** The partial nucleus of sperm with partially damaged plasma membrane was stained with PI; **(g)** Merged image; **(h)** Image taken under ordinary visible light; **(i)** Sperm with extensively damaged plasma membrane; **(j)** The more nucleus in sperm with extensively damaged plasma membrane was stained with PI; **(k)** Merged image; **(l)** Image taken under ordinary visible light; **(m)** Sperm without plasma membrane; **(n)** Sperm nucleus without plasma membrane were stained by PI; **(o)** Two images merged one; **(p)** Image taken under ordinary visible light

Artificial insemination

A total of 113 naturally estrous French Bulldogs came to our animal breeding center (Shaanxi Agricultural University Pet Home Management Co. Ltd) for AI in the past two years were inseminated with semen chilled in Tris-glucose/fructose buffer with either EY or SL, 48 dogs (only one childbirth history) inseminated with sperm cooled in 1.0% SL extender (n=24) and 20.0% EY extender (n=24) were selected randomly to statistically analyze. Moreover, the fertility results of 27 female French Bulldogs inseminated with fresh semen were used as control. Detailed insemination operations were performed with the help of endoscopic-assisted transcervical catheterization (EIU) according to previous report [25]. The day of estrus bleeding was considered as the 0 day, and bitches were inseminated on the 12th day of estrus with semen chilled for 48 h; then inseminated again at 13th day using semen chilled for 72 h. Each insemination dose was 2.0 ml containing 10×10^7sperm. All operations were performed by an experienced veterinarian. Digital Radiography (DG3650) was used to diagnose pregnancy 45 days after artificial insemination.

Statistical analysis

The experiment of sperm quality analysis was repeated at least three times. All values of sperm parameters and the litter size were represented by Mean ± SD. As all data are normally distributed, one-way ANOVA using SPSS 18.0 was statistically compared. When difference was significant after one-way ANOVA, individual means were further tested by Tukey's multiple comparison tests. At the same time, values of conception rate and whelping rate were statistically compared using the chi-square test. In addition, effects of the canine age and the reproduction seasons on conception rates and litter sizes were compared by Fisher's exact test, and $P<0.05$ was considered significant.

Results

Sperm viability and sperm motility

Raw semen concentration of five male dogs was between 3.1×10^8 to 1.8×10^9, and abnormal morphology sperm was 3.5%-5.0%, and sperm viability was between 94.3% to 97.8%.

After the cold storage, the percentage of sperm viability in the 1.0% SL group was similar to that in the 20.0% EY group after chilling storage for 120 h. The results were shown in Figure 3a. But from the 144 h after chilling storage, the percent sperm viability was significantly higher in 1.0%SL group than that in any other group. In addition, the percentage of sperm with progressive motility was significantly higher in 1.0% SL group as compared to any of the other three groups from 24 h after chilling. The results were shown in Figure 3b.

Sperm acrosome and plasma membrane integrity

No significant difference in the acrosome integrity rate was observed between the 1.0% SL and the 20.0% EY groups 48 h after chilling. And the percentages of acrosome integrity in the 1.0% SL group were always higher than those in any other group from the start of 72 h. The results were shown in Figure 3c.

Seventy two hours following chilling storage, the percentages of sperm with intact plasma membrane in 1.0% SL group was similar to that in 20.0% EY group. The results were shown in Figure 3d. From the start of 96 h, the percentage of sperm with intact plasma membrane in 1.0% SL group was significantly higher than that in the 20.0% EY groups.

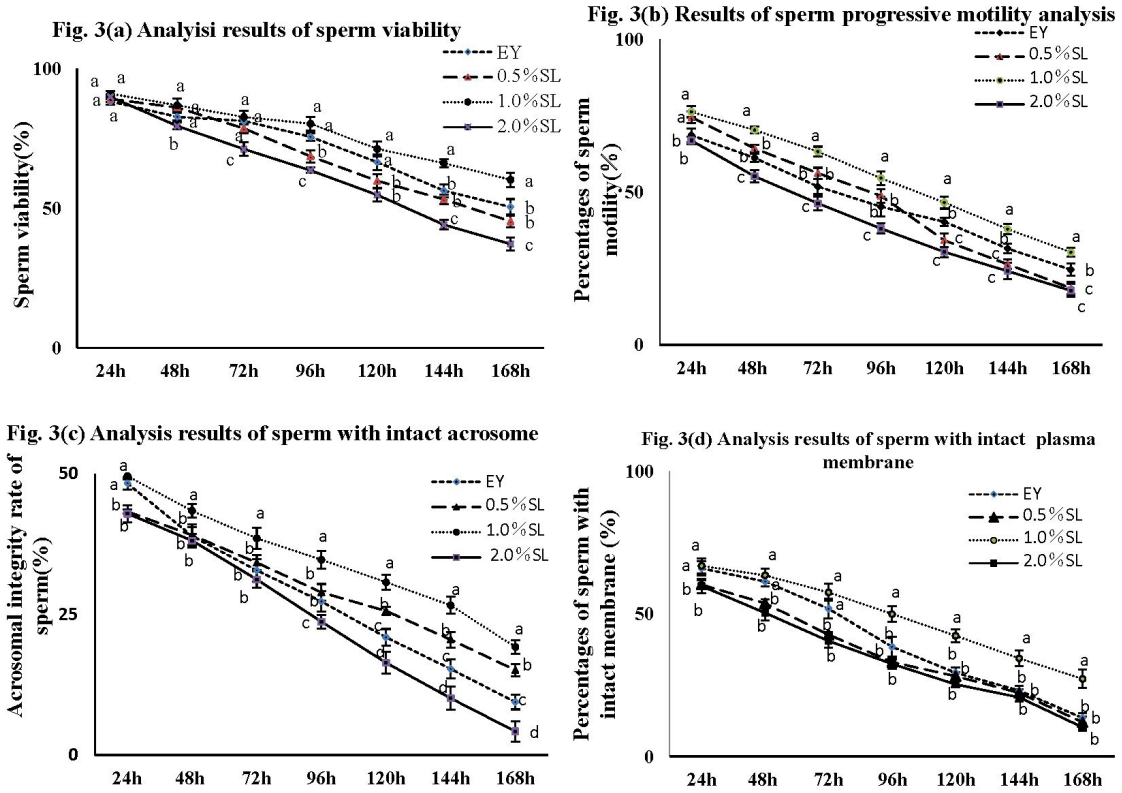

Figure 3: Analysis results of sperm quality parameters **(a)** Percentages of sperm viability at various time points after chilled storage using different concentrations of soybean lecithin and 20 % egg yolk. All values came from three repetitions, and different lowercase superscripts at the same time point indicate significant differences, P<0.05. EY stands for semen diluent containing 20.0% of egg yolk. 0.5% SL, 1.0% SL and 2.0% SL stand for semen extender containing 0.5%, 1.0% and 2.0% of soybean lecithin, respectively; **(b)** Progressive motility of sperm at various time points after storage using different concentrations of soybean lecithin and 20% egg yolk at 4 °C. All values came from three repetitions, and different lowercase superscripts at the same time point indicate significant differences, P<0.05; **(c)** Percentage sperm with an intact acrosome at various time points after storage using different concentrations of soybean lecithin and 20% egg yolk at 4 °C. All mean values came from four repetitions, and different lowercase superscripts at the same time point indicate significant differences, P<0.05; **(d)** Percentages of sperm with an intact plasma membrane at various time points after storage using different concentrations of soybean lecithin and 20% egg yolk at 4 °C. All mean values came from four repetitions, and different lowercase superscripts at the same time point indicate significant differences, P<0.05

Pregnancy diagnosis and whelping

Out of 24 bitches inseminated with sperm chilled storage in 1.0% SL, 17 bitches gave birth. Yet another 24 bitches inseminated with sperm chilled storage in 20.0% EY, only 15 bitches gave birth (Table 1). The conception rates diagnosed at day 45 after insemination were similar between the 1.0% SL group and 20.0% EY group(70.8% vs. 62.5%, *P*>0.05), though both of them were significantly lower than that in the fresh semen group. Whereas no significant difference in litter size was found among the three groups. Moreover, the whelping rates were similar in bitches inseminated with semen cooled in 1.0% SL extender and in bitches inseminated with fresh semen, yet both of them were significantly higher than that bitches inseminated with semen cooled in 20.0% EY extender.

Also, no significantly difference was observed in conception rates and litter sizes for French bulldog with different ages or different reproduction seasons (Table 2).

	Inseminated canines (n)	Conception rate (%)	Average litter size	Whelping rate (%)
EY	24	62.5% (15/24)[b]	5.5±2.1[a]	533.33% (80/15)[b]
1.0%SL	24	70.8% (17/24)[b]	4.8±2.4[a]	576.47.3% (98/17)[a]
Fresh semen	27	85.2% (23/27)[a]	5.3±1.9[a]	590.90% (130/22)[a]

*EY stands for semen diluent containing 20.0% of egg yolk. 1.0%SL stands for semen diluent containing 1.0% of soybean lecithin. Out of 24 bitches inseminated with sperm diluted in 1.0% SL, 17 bitches gave birth. Yet another 24 bitches inseminated with sperm diluted in 20.0% EY, only 15 bitches gave birth. The conception rate is equal to the number of bitches determined pregnant 45 days after artificial insemination by using Digital Radiography (DG3650) divided by the number of inseminated bitches. Whelping rate is equal to total puppies full-term born alive/number of bitches with full-term pregnancies. Litter size is equal to the number of offspring delivered at one birth by a bitch. Different lowercase superscripts within the same column indicate significant differences, P<0.05.

Table 1: Pregnancy diagnosis and delivery results after insemination using sperm stored in extenders containing 1.0% soybean lecithin (SL) and 20.0% egg yolk (EY) at 4 °C*

	Canine age			Reproduction seasons	
Main factor	One year old	Two years old	Three years old	Spring	Autumn
Conception rate	64.7%(11/17)[a]	66.7%(10/15)[a]	67.7%(11/16)[a]	62.9%(17/27)[a]	71.4%(15/21)[a]
Litter size	4.2±1.3[a]	5.2±1.8[a]	5.6±2.4[a]	6.2±2.4[a]	4.8±2.5[a]

*Conception rates assessed by Digital Radiography 45 days after the last artificial insemination. No significant differences (P > 0.05) were found for canine age and reproduction season factors which has been indicated with same letter (a). Moreover, no significant differences were found for litter size(litter size is equal to the number of offspring delivered at one birth by a bitch), P<0.05

Table 2: Variation factors of pregnancy rate and letter size*

Discussion

In this study, the replacement of egg yolk with SL to store canine semen at 4 °C was systematically studied. The addition of 1.0% SL in Tris-based extender showed positive effects on the quality of chilled sperm parameters and whelping rates after insemination. Although there are previous reports about replacing egg yolk with soybean lecithin for preservation of canine semen [26,27], yet the insemination trials had not been conducted. The commercial SL contains only 14%-23% lecithin, whether other ingredients in SL affect the effect of the extender is unknown. Moreover, the exact amount of phosphatidylcholine in SL is not reported in most studies, as it might be a mainly contributing factor [18].

Others studies reported that the phospholipids in SL are able to replace the phospholipids of the sperm membrane, which contributes to stabilize the structure and function of sperm [27] and reduce sensitivity to the cold shock during chilling process [28]. Meanwhile, the phospholipids from SL along with phospholipids from the sperm membrane could form a protective membrane that protects the sperm from harmful elements [12,29]. Moreover, egg yolk contains certain ingredients that are easily oxidized. Conversely, lecithin is an antioxidant, and better results are obtained when lecithin samples contain a high concentration of phosphatidylcholine [13,30,31]. However, in the present study 0.5% or 2.0% SL in the Tris-based extender was not conducive to the sperm quality. This suggests that the protective function of SL on sperm is closely related to its concentration. Previous study found that the viscosity is one factor affecting sperm quality, and high concentrations of SL increased the viscosity of the extender and reduced fertility [32]. This may be the reason why the results of the present study were inconsistent with previous results reported by Nguyen et al. (2019) [7]. On the contrary, extenders containing less than 1.0% SL might be insufficient to provide protection for sperm, which is consistent with the results reported by Dalmazzo et al.(2019) [33], who found that 0.1% SL in extender can not replace yolk to protect canine sperm.

As the current gold standard of sperm quality evaluation is fertilization capacity and subsequent conception rate and parturition rate, the results concerning whelping rate after bitches inseminated with sperm chilled in 1.0% SL extender were superior to those chilled in 20.0% EY extender, indicating that 1.0% SL extender is completely feasible to chill canine sperm at low temperature 4 °C.

Conclusion

In summary, replacing egg yolk with 1.0% lecithin is completely feasible to store French Bulldogs sperm at 4 °C. With the extension of storage time, the quality of the sperm stored in 1.0% lecithin extender is superior to that in egg yolk extender. These results have important reference significance to promote the commercialization of semen extender in canines.

Conflict of Interest

All authors declare no competing interests.

Acknowledgements

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Author Contributions Statement

Sun Mingkun and Song WC performed the experiments, Lv CR performed the tests of sperm quality parameters. Li Y was responsible for feeding management of canines. Hua S conceived the experiments and analyzed data, and prepared the manuscript. All authors reviewed the manuscript.

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