Low-density Lipoprotein Improves Motility and Plasma Membrane Integrity of Cryopreserved Canine Epididymal Spermatozoa

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ABSTRACT: Cryopreservation of caudal epididymal spermatozoa is an effective technique to conserve genetic potentials of superior dogs when it is not possible to collect ejaculated spermatozoa. Although hen egg yolk is commonly supplemented into the semen extender, active substances within the egg yolk which protect sperm against cryoinjury remain to be discovered. Among its compositions, low-density lipoprotein (LDL) has been reported to have a cryoprotective property for sperm cryopreservation. However, the effects of LDL on dog epididymal spermatozoa during cryopreservation have not yet been investigated. This study aimed to investigate the effects of LDL on epididymal spermatozoa quality following cryopreservation and thawing. After routine castration of 12 dogs, caudal epididymides from individuals were separated from the testes and cut into a few pieces in a Tris-buffer. Spermatozoa recovered from each sample were examined at once for sperm quality and divided into six groups of extender: no LDL, 20% egg yolk, 4%, 8%, 16%, and 24% LDL, before cryopreservation. The sperm aliquots were then equilibrated and conventionally frozen. After thawing, sperm motility, morphology, plasma membrane integrity, and acrosome integrity were evaluated. The results revealed that 4% LDL and 20% egg yolk yielded significantly higher sperm motility (57.69% and 52.69%, respectively, $p<0.05$) than other LDLs. In addition, 4% LDL yielded the significantly highest plasma membrane integrity (70.54%, $p<0.05$). In conclusion, the supplementation of 4% LDL in Tris-glucose extender could be applied for cryopreservation of canine epididymal spermatozoa. (**Key Words:** Low-density Lipoprotein, Epididymal Spermatozoa, Cryopreservation, Dog)

INTRODUCTION

Spermatozoa cryopreservation is a method for preserving genetic material and maintaining genetic diversity in several species, including dogs. This extends the storage time and facilitates semen transportation over distance. Although ejaculated spermatozoa have been successfully cryopreserved, this technique encountered a limited success with epididymal spermatozoa (Hewitt et al., 2001). This probably results from the difference in morphology, function, and membrane stability between epididymal and ejaculated spermatozoa (England, 1993; Hewitt et al., 2001). Previous investigation in dogs demonstrated lower sperm motility, membrane integrity, and conception rate in post-thawed epididymal spermatozoa compared to ejaculated spermatozoa (Hori et al., 2004).

Since cryopreservation of epididymal spermatozoa is the technique of choice to preserve genetic potential of fertility-proven dogs when they unexpectedly die or fail to provide ejaculated sperm, it is, therefore, important to improve the freezing technique for sperm recovered from the caudal epididymis. In addition, it has been established that the

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composition of the semen extender plays a pivotal role in protecting sperm against cryoinjury which occurs during freezing and thawing processes (Hewitt et al., 2001).

Recent studies report that the sperm plasma membrane is the primary site of cryodamage during freezing and thawing due to changes in its lipid and protein structure (Gao and Critser, 2000). Egg yolk is frequently included in semen extenders because it provides long-term survival of spermatozoa during freeze-thaw process due to its protective effects against cold shock (Kampschmidt et al., 1953). A major components of egg yolk are low density lipoprotein (LDL) and high density lipoprotein (HDL) (Bencharif et al., 2008). LDL has been reported to have a cryoprotective effect, while HDL is disadvantageous for spermatozoa cryopreservation (Amirat et al., 2004). In bulls, spermatozoa cryopreserved with LDL-supplemented extender yielded 2 times higher motility than that with crude egg yolk semen extender (Moussa et al., 2002; Amirat et al., 2004). Similarly, LDL-based extender has been proven to enhance the cryopreservability of canine ejaculated spermatozoa (Varela Junior et al., 2009). Given that epididymal spermatozoa are more sensitive to cold stress than ejaculated spermatozoa, it is of interest to examine whether LDL-based extender protects epididymal spermatozoa against cryoinjury. This study aimed at investigating the effects of LDL-based semen extender on quality of epididymal spermatozoa following cryopreservation and thawing.

MATERIALS AND METHODS

The animal intervention was approved by the Faculty of Veterinary Science-Animal Care and Use Committee (FVS-ACUC), Mahidol University, protocol number MUVS-2010-16.

Experimental design

Canine spermatozoa were collected from caudal epididymides. Sperm motility, morphology, plasma membrane integrity, and acrosome integrity were evaluated immediately after sperm recovery. Sperm samples were centrifuged at 700×g for 6 min, the supernatant removed, and the sperm pellets diluted with 6 different Tris-glucose-based extenders: i) no LDL, ii) 20% egg yolk, iii) 4% LDL, iv) 8% LDL, v) 16% LDL, and vi) 24% LDL. The recovered spermatozoa were equilibrated in these primary semen extenders and then conventionally frozen. Sperm motility, plasma membrane integrity and acrosome integrity were evaluated at 0, 2, and 4 h after thawing.

Preparation of freezing medium

Freezing extender used in this study was prepared as followed: 2.4% (w/v) Tris (BDH, Leuven, Belgium), 1.4% citric acid (BDH, London, England), 0.8% glucose (Merck, Germany), 0.06% (w/v) sodium benzyl penicillin (M&H manufacturing co., Ltd., Samutprakarn, Thailand), 0.1% (w/v) streptomycin sulphate (M&H manufacturing co., Ltd., Thailand), and different concentrations of LDL (no LDL, 4%, 8%, 16%, 24% LDL) or 20% (v/v) egg yolk. These extenders were supplemented with 3% and 7% (v/v) glycerol (Sigma-Aldrich, St. Louis, MO, USA) for extender 1 and 2, respectively. Thawing medium used in this study was prepared according to Axner et al. (2004).

Low density lipoprotein preparation

The LDL was extracted from egg yolk according to the method essentially described by Moussa et al. (2002). Briefly, hen egg yolk was collected in a cooled beaker. Plasma fractionation was performed by diluting egg yolk twice with 0.17 M NaCl and stirred for 1 hour and were then twice centrifuged at 10,000×g for 45 min at 10°C. LDL were extracted by mixing the plasma with 40% ammonium sulfate (Sigma-Aldrich, USA) for 1 hour to precipitate livetins. The pH of the plasma was fixed at 8.7. Plasma was centrifuged at 10,000×g for 45 min at 4°C. Supernatant was dialyzed against distilled water for 6 hours to discard ammonium sulfate. After 6 hour, the solution was centrifuged at the same protocol. The floating LDL rich residue was obtained.

Animals and collection of epididymal spermatozoa

Epididymal spermatozoa were collected from 12 healthy dogs of various breeds that were routinely castrated, aged between 1 and 7 years old. Spermatozoa were collected from a pair of caudal epididymides from each dog. Caudal epididymides were cut into a few pieces and then placed in thawing medium for 10 min at 37°C (Tittarelli et al., 2006). After epididymal tissues were removed from that medium, spermatozoa suspensions from right and left caudal epididymides of individuals were pooled. The spermatozoa samples containing those having motility and normal morphology more than 70% were included in the experiment.

Spermatozoa cryopreservation and thawing

Spermatozoa cryopreservation was performed according to Manee-in et al. (2014). Briefly, the spermatozoa suspension was centrifuged at 700×g for 6 min; the spermatozoa pellet was then re-suspended in extender 1. After 1 h equilibration at 4°C, an equal volume of extender 2 was gently added (Axner et al., 2004). The spermatozoa suspension was loaded into a 0.5 mL straw and subsequently frozen by placing the straw horizontally 4 cm above liquid nitrogen vapors for 10 min. All straws were finally immersed in the liquid nitrogen (Andersen, 1975). Thawing procedure was performed by immersing the straw in warm water (37°C) for 15 seconds. The frozen-thawed spermatozoa suspension was then released into thawing medium for further investigations.

After the incubation in thawing medium at 37°C for 5 min, the sperm suspensions were evaluated for sperm motility, plasma membrane integrity, and acrosome integrity at 0 h, 2 h, and 4 h, respectively.

Spermatozoa evaluation

Spermatozoa samples were assessed immediately after collecting and thawing. Spermatozoa motility and progressive motility (scale 0 to 5) was blind evaluated under a phase-contrast microscope at 200× (Platz and Seager, 1978). Head morphology was estimated under a light microscope at 1,000× by counting 500 spermatozoa after Williams staining. Thereafter, a 2 μL of semen sample was fixed with formal saline for evaluating tail morphology from 200 spermatozoa under a phase-contrast microscope at 1,000×. Plasma membrane and acrosome integrities were evaluated by staining the spermatozoa with Ethidium homodimer-1 (EthD-1, Molecular probes Inc., Eugene OR, USA) and fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA, Sigma, USA), respectively. In brief, an aliquot of 5 μL sperm suspension was mixed with an equal volume of 2 μM EthD-1 for 10 min at 37°C. Subsequently, 5 μL of a 10 mg/mL salmon sperm DNA (Fluka, Munich, Germany) in phosphate buffered saline (PBS) was added to bind excessive EthD-1 for 3 min at 37°C. Supernatant was removed after centrifugation at 300×g for 5 min. The spermatozoa were then smeared on a glass microscopic slide and fixed with 95% (v/v) ethanol for 30 s. Fixed spermatozoa were stained with 100 μg/mL FITC-PNA in PBS in a humidified chamber for 30 min at 4°C, rinsed with 4°C distilled water, and allowed to air dry at 4°C. The fluorescently labeled slides were kept in the dark until evaluation. In total, 200 spermatozoa per slide were randomly evaluated and classified into 3 categories as described by Cheng et al. (1996) using an epifluorescent microscope at 1,000×.

Statistical analysis

All data were manipulated and statistically analyzed using the Statistical Analysis Systems software (SAS for windows, version 9, Cary, NC, USA). Normal distribution of residuals from the statistical models was tested using Shapilo-Wilk test. An analysis of variance was performed under the general linear model procedure. The statistical models included the effect of groups of LDL concentration, time, and interaction between LDL concentration and duration after thawing. Least-squares means were calculated and compared using Tukey-Kramer adjustment for multiple comparisons. All data were expressed as mean ±standard deviation. The level of significance was considered at p<0.05.

RESULTS

The characteristics of spermatozoa recovered from caudal epididymides (fresh spermatozoa) and after cryopreservation are shown in Tables 1 to 3. Motility, plasma membrane integrity, and acrosome integrity of post-thawed spermatozoa decreased over the time of examination. In fresh spermatozoa, an average percentage of normal head morphology, normal tail morphology, motility, progressive motility, plasma membrane integrity, and acrosome integrity were 82.77±6.25, 80.19±7.23, 80.77±4.94, 4.54±0.51, 74.88±5.86, and 55.50±16.67, respectively.

Moreover, LDL dose-specific was observed: 4% LDL-based-extender yielded the highest percentage of post-thawed sperm motility, plasma membrane integrity, and acrosome integrity (57.69±5.63, 70.54±12.84, and 46.58± 10.79, respectively) compared to other LDL concentrations. After thawing chronologically (0 h, 2 h, and 4 h), it was found that 4% LDL and 20% egg yolk groups yielded significantly higher sperm motility (57.69±5.63 and 52.69± 17.15, respectively), than other LDL groups (p<0.05). In addition, 4% LDL group provided the significantly highest motility at 2 h. For plasma membrane integrity, 4% LDL group resulted in the highest plasma membrane integrity (p<0.05) at 0 h after thawing. At 2 h and 4 h, membrane integrity of all groups (except no LDL group) was not significantly different (p>0.05). For acrosome integrity, 4% LDL group yielded the highest acrosome integrity. However, acrosome integrity in all LDL-supplemented groups was not significantly different (p>0.05) at 0 h, 2 h, and 4 h after

Table 1. Spermatozoa motility (%) and progressive motility of caudal epididymal spermatozoa before freezing and after thawing for 0 h (T0), 2 h (T2), and 4 h (T4) in each experimental group (mean±SD)

Group/time	Fresh		T0		T2		T4	
	Motility	Progressive motility	Motility	Progressive motility	Motility	Progressive motility	Motility	Progressive motility
No LDL	80.77±4.94[A]	4.54±0.51	3.03±4.80[B,c]	0.31±0.48	2.46±5.59[B,d]	0.08±0.28	1.00±2.77[B,d]	0.08±0.28
4% LDL	80.77±4.94[A]	4.54±0.51	57.69±5.63[B,a]	3.69±0.25	51.15±9.16[B,a]	3.04±0.52	28.46±11.07[C,a]	1.69±0.72
8% LDL	80.77±4.94[A]	4.54±0.51	38.46±15.86[B,b]	2.27±1.03	30.00±15.94[B,c]	1.81±0.97	12.69±9.71[C,b,c]	0.77±0.73
16% LDL	80.77±4.94[A]	4.54±0.51	34.46±15.40[B,b]	2.12±0.98	26.38±13.57[B,c]	1.77±0.81	9.23±8.06[C,c,d]	0.54±0.52
24% LDL	80.77±4.94[A]	4.54±0.51	32.92±18.91[B,b]	1.96±1.18	21.92±16.90[C,c]	1.27±1.07	9.08±8.48[D,c,d]	0.38±0.51
20% egg yolk	80.77±4.94[A]	4.54±0.51	52.69±17.15[B,a]	3.08±1.04	40.00±17.80[C,b]	2.38±1.08	21.54±11.62[D,a,b]	1.23±0.70

SD, standard deviation; LDL, low density lipoprotein.

Values within the same column followed by the different small letter superscripts, and within the same row followed by the different capital letter superscripts are significantly different (p≤0.05).

Table 2. Plasma membrane integrity (%) of caudal epididymal spermatozoa before freezing and after thawing for 0 h (T0), 2 h (T2), and 4 h (T4) in each experimental group (mean±SD)

Group/time	Fresh	T0	T2	T4
No LDL	74.88±5.86[A]	54.60±17.92[B,b]	45.62±19.24[B,C,b]	38.04±19.67[C,b]
4% LDL	74.88±5.86[A]	70.54±12.84[A,a]	62.19±12.42[A,a]	55.96±14.98[B,a]
8% LDL	74.88±5.86[A]	58.23±13.65[B,b]	53.85±11.42[B,a,b]	51.50±13.20[B,a]
16% LDL	74.88±5.86[A]	55.56±14.13[B,b]	52.27±11.30[B,a,b]	48.07±11.61[B,a,b]
24% LDL	74.88±5.86[A]	55.04±9.25[B,b]	52.19±11.55[B,a,b]	48.23±15.40[B,a,b]
20% egg yolk	74.88±5.86[A]	59.27±14.21[B,b]	54.77±12.42[B,C,a,b]	45.31±12.51[C,a,b]

SD, standard deviation; LDL, low density lipoprotein.
Values within the same column followed by the different small letter superscripts, and within the same row followed by the different capital letter superscripts are significantly different (p≤0.05).

thawing.

DISCUSSION

In the present study, the highest percentage of post-thawed spermatozoa motility was observed in 4% LDL group in all freezing times. However, that motility was not statistically different from 20% egg yolk-based extenders, which are routinely used for semen cryopreservation. Nevertheless, at 2 h post-thaw, spermatozoa motility from 4% LDL group was significantly higher than other groups. This was in agreement with the study on cryoprotective effect of LDL on bull semen motility, which demonstrated two folds higher motility from LDL supplementation than from egg yolk (Moussa et al., 2002; Amirat et al., 2004). Regarding the duration after freeze-thaw process, spermatozoa motility of all groups was highest at 0 h and declined chronologically. Cryoinjury of spermatozoa is caused by several aspects, including membrane disturbances and osmotic pressure changes, leading to cell volume change (Pena, 2007). The decrease in motility after freeze-thaw process might be caused by an active transport and a permeability alteration of plasma membrane in the tail region of spermatozoa (Yu et al., 2002). The previous study revealed that 8% LDL was the best concentration for ejaculated dog spermatozoa since it improved spermatozoa motility and plasma membrane integrity (Varela Junior et al., 2009). These results differed from our results where 4% LDL was the best concentration. This might be due to the differences

in composition of the plasma membrane between ejaculated and epididymal spermatozoa, especially in surface component changes during epididymal maturation and sperm capacitation (Oliphant and Singhas, 1979) which makes the difference in cold shock susceptibility of spermatozoa (England, 1993). Moreover, our result showed a (England, 1993; White, 1993) decrease in motility when higher concentration of LDL was added. This might have been caused by a decrease in osmotic pressure of the extender when LDL concentration increased (Amirat et al., 2004).

For the plasma membrane integrity, 4% LDL group provided the best result at 0 h of post-thawed spermatozoa, suggesting that LDL possessed a beneficial cryoprotective effect on spermatozoa plasma membrane (England, 1993). This effect was due to LDL adhering to the cell membrane of spermatozoa (Graham and Foote, 1987; Bergeron et al., 2004), forming an interfacial film between fatty acids and water (Anton et al., 2003), and building a complex with seminal plasma proteins. This made them unavailable to function in the membrane (Manjunath and Therien, 2002; Bergeron et al., 2004), and prevented the loss of membrane phospholipids (Amirat et al., 2004). This could help protect spermatozoa during freeze-thaw process, because the spermatozoa membrane is composed of phospholipid and cholesterol which maintains the correct membrane fluidity and membrane permeability (White, 1993). Cryoinjury induces phospholipid phosphatidylserine translocation from inner to outer leaflets of plasma membrane which relates to membrane degeneration (Pena, 2007). This corresponds with

Table 3. Sperm acrosome integrity (%) of caudal epididymal spermatozoa before freezing and after thawing for 0 h (T0), 2 h (T2), and 4 h (T4) in each treatment and control (mean±SD)

Group/time	Fresh	T0	T2	T4
No LDL	55.50±16.67[A]	18.96±14.64[B,b]	10.81±9.66[B,C,b]	3.85±4.28[C,b]
4% LDL	55.50±16.67[A]	46.58±10.79[A,a]	38.04±10.42[A,B,a]	28.77±15.53[B,a]
8% LDL	55.50±16.67[A]	37.38±15.23[B,a]	36.88±12.28[B,a]	24.50±14.32[C,a]
16% LDL	55.50±16.67[A]	36.62±14.59[B,a]	29.50±11.14[B,C,a]	21.96±11.67[C,a]
24% LDL	55.50±16.67[A]	38.19±18.38[A,a]	28.31±17.11[B,C,a]	22.88±14.91[C,a]
20% egg yolk	55.50±16.67[A]	39.46±19.25[B,a]	27.77±14.82[C,a]	19.77±10.79[C,a]

SD, standard deviation; LDL, low density lipoprotein.
Values within the same column followed by the different small letter superscripts, and within the same row followed by the different capital letter superscripts are significantly different (p≤0.05).

the previous studies conducted in boar, bull, and stallion semen cryopreservation (Moussa et al., 2002; Amirat et al., 2004; Jiang et al., 2007; Canisso et al., 2008).

The highest percentage of acrosome integrity was found in the 4% LDL group. At 0 h post-thawed, it provided the better protection for the acrosome; acrosome integrity decreased when the storage time extended. Likewise, mammalian spermatozoa must undergo the physiological changes of capacitation in the female reproductive tract in order to penetrate oocytes (Yanagimachi and Bhattacharyya, 1988). Sperm capacitation is followed by acrosome reaction, which involves the breakdown and the fusion of the sperm plasma membrane and outer acrosomal membrane covering the anterior portion of the sperm head (Bedford, 1968; Yanagimachi and Usui, 1974). Consequently, during freezing process, the spermatozoa might have undergone the acrosome reaction (Yanagimachi and Usui, 1974). Egg yolk contains progesterone (Bowden et al., 2001) which plays an important role in the capacitation of spermatozoa in cattle (Witte and Schafer-Somi, 2007), horses (Aitken and McLaughlin, 2007), and humans (Wistrom and Meizel, 1993; Meyers et al., 1995). Progesterone is responsible for the capacitation of spermatozoa and therefore prejudicial to the preservation of spermatozoa during freezing (Bencharif et al., 2010). It has been reported that LDL contained a lower progesterone level than egg yolk due to the filtering effect of the dialysis procedure (Bencharif et al., 2008). It is, therefore, hypothesized that the extender containing LDL provided good protection for acrosome due to it containing a low level of progesterone. The investigation of acrosomal integrity following freezing and thawing is therefore an important parameter providing a qualitative assessment of sperm fertility (Bencharif et al., 2010).

In conclusion, LDL-supplemented extender enhanced canine epididymal spermatozoa quality during cryopreservation. The results from this study showed that the 4% LDL supplementation was the most suitable extender and yielded the better results for all spermatozoa quality parameters, especially for plasma membrane integrity than other LDL concentrations and egg yolk. At 0 h post-thawed, it provided better results; spermatozoa quality, which then decreased chronologically.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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