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L-carnitine Supplemented Extender Improves Cryopreserved-thawed Cat Epididymal Sperm Motility

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ABSTRACT: Cryopreservation of epididymal sperm is an effective technique to preserve genetic materials of domestic cats and wild felids when they unexpectedly die. However, this technique inevitably causes detrimental changes of cryopreserved-thawed spermatozoa, for example, by physical damage and excessive oxidative stress. L-carnitine is an antioxidant that has been used to improve sperm motility in humans and domestic animals. This study aimed to investigate the effects of L-carnitine on cat epididymal sperm quality following cryopreservation and thawing. After routine castration, cauda epididymides were collected from 60 cat testes. The epididymal spermatozoa from 3 cauda epididymides were pooled as 1 replicate. Spermatozoa samples (16 replicates) were examined for spermatozoa quality and then randomly divided into 4 groups: 0 mM L-carnitine (control), 12.5 mM, 25 mM and 50 mM L-carnitine. The sperm aliquots were then equilibrated and conventionally frozen. After thawing, sperm motility, plasma membrane integrity, DNA integrity and acrosome integrity were evaluated. The 25 mM L-carnitine significantly improved sperm motility compared with a control group ($p<0.05$), although this was not significantly different among other concentrations. In conclusion, supplementation of 25 mM L-carnitine in freezing extender improves cauda epididymal spermatozoa motility. The effects of L-carnitine on the levels of oxidative stress during freezing and thawing remains to be examined. (**Key Words:** L-carnitine, Epididymal Spermatozoa, Cryopreservation, Cat)

INTRODUCTION

In recent years, cryopreservation is an attractive subject with the purpose of establishing an efficiently gene bank model to be used for threatened and endangered male breeders. This technique has a potential mean of enabling long-term storage and transport of viable genetic materials (Pena et al., 2003; Thuwanut et al., 2010). Cryopreservation has many benefits for long-term preservation, artificial insemination, breeder's death and over distance of breeder (Bailey et al., 2000). Although ejaculated spermatozoa have been successfully cryopreserved, this technique has met a limited success when applied to epididymal spermatozoa, probably because of the differences in morphology, function and also the membrane stability between the epididymal and ejaculated spermatozoa (Thuwanut and Chatdarong, 2009). Cryopreservation of epididymal spermatozoa is a technique of choice to preserve genetic potential of proven-fertility toms when they unexpectedly die or fail to obtain ejaculated spermatozoa. It is therefore important to improve freezing technique for feline spermatozoa recovered from caudal epididymis.

Previous studies demonstrated that freezing and thawing potentially induce cryoinjuries (Pena et al., 2005; Thomson et al., 2009; Zini et al., 2009; Kim et al., 2010b), all of which are critical for sperm morphology, acrosome integrity and survival rate (Cerolini et al., 2001). During cooling and freezing processes, cold shock frequently occurs if temperature is reduced too rapidly. This causes extensive

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intracellular ice formation and is detrimental to sperm plasma membrane (Hammerstedt et al., 1990). Suboptimal cooling and freezing rates would therefore increase the production of free radicals, oxidants and reactive oxygen species (ROS) resulting in decreasing of membrane fluidity, increase DNA fragmentation and decrease the fertilizability of spermatozoa (Bailey et al., 2000; Thomson et al., 2009; Kim et al., 2010a; Aitken and Koppers, 2011).

The antioxidant supplementation in freezing extender has been used to defend against free radicals that potentially reduce sperm fertilizing ability. L-carnitine is an antioxidant that plays a role in spermatozoa motility (Stradaioli et al., 2004; Abd-Allah et al., 2009), enhances cellular energy in mitochondria, protects sperm membrane and DNA from ROS induced apoptosis (Agarwal et al., 2004). L-carnitine and free L-carnitine are naturally present in epididymal seminal plasma to support spermatozoa motility and maturation. L-carnitine has been reported to uptake by boar epididymal spermatozoa by passive and active diffusion depending on regions and expose duration of substances (Jeulin et al., 1994; Kobayashi et al., 2007). L-carnitine supplementation could improve spermatozoa motility in stallions whose had spermatozoa motility less than 50% (Stradaioli et al., 2004). Oral administration of L-carnitine in human with idiopathic asthenozoospermia also demonstrated the improvement of spermatozoa quality (Costa et al., 1994). Until the present, the cryoprotective effect of L-carnitine during freezing and thawing remains unclear. Therefore, this study was performed to investigate the effects of different L-carnitine concentrations in the freezing extender on post-thaw quality of feline epididymal spermatozoa.

MATERIALS AND METHODS

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

Experimental design

Feline spermatozoa were collected from cauda epididymides. Spermatozoa morphology, motility, plasma membrane integrity, acrosome integrity and DNA integrity were evaluated immediately after spermatozoa extraction. After spermatozoa samples were centrifuged and the supernatant was removed, the sperm pellets were diluted with Tris-glucose based extenders supplemented with different concentrations of L-carnitine: i) 0 mM L-carnitine (control), ii) 12.5 mM L-carnitine, iii) 25 mM L-carnitine and iv) 50 mM L-carnitine. The spermatozoa were then conventionally cryopreserved. Spermatozoa motility, plasma membrane integrity, acrosome integrity and DNA integrity were evaluated at 0, 2 and 4 hours after thawing.

Preparation of freezing medium

Freezing extender used in this study was Tris-glucose based extender containing 2.4% (w/v) Tris (BDH, England), 1.4% (w/v) citric acid (BDH, England), 0.8% (w/v) 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., Samutprakarn, Thailand), and different concentrations of L-carnitine (Sigma-Aldrich, St. Louis, MO, USA) (0 mM L-carnitine, 12.5 mM, 25 mM, and 50 mM). This extender was further supplemented with 3% and 7% (v/v) glycerol for extender 1 and 2, respectively. Thawing medium used in this study was prepared similarly to freezing extender except that it did not contain egg yolk, L-carnitine or glycerol (Axner et al., 2004).

Animals and collection of epididymal spermatozoa

Testes were collected from 24 healthy cats of various breeds, aged between 1-7 years old by routine closed-technique castration. They were immediately kept in 0.9% (w/v) normal saline solution supplemented with penicillin-streptomycin at room temperature until epididymal spermatozoa extraction. The epididymal spermatozoa from 3 cauda epididymides were randomly pooled as 1 replicate in order to increase the sperm numbers and also to reduce individual variations. A total of 16 replicates were performed in this study. To obtain the spermatozoa, cauda epididymides were cut into few pieces and then placed in thawing medium for 10 min at 37°C (Tittarelli et al., 2006). The sperm samples that contained with more than 70% motility and normal morphology were only used in this experiment.

Spermatozoa cryopreservation and thawing

The sperm suspension was centrifuged at 700× g for 6 min, and the sperm pellet was resuspended in extender 1. After equilibration at 4°C for 1 h, an equal volume of extender 2 was slowly added (Axner et al., 2004). The spermatozoa were loaded into a 0.25 mL-straw and then frozen by placing the straw horizontally 4 cm above liquid nitrogen vapors for 10 min. The straw was finally immersed in the liquid nitrogen (Andersen, 1975).. Thawing procedure was performed by immersing the straw in warm water (37°C) for 15 s. The frozen-thawed semen was then released into a pre-warmed thawing medium (37°C). After incubation at 37°C for 5 min (0 h) and at 2 and 4 hours in thawing medium, the sperm suspensions were evaluated for sperm motility, plasma membrane integrity, acrosome integrity and DNA integrity.

Sperm morphology and motility evaluation

A five μL of sperm suspension was put on a pre-warmed slide and then covered with a coverslip at 37°C. Sperm

motility and progressive motility were blindly evaluated by the same person under a phase-contrast microscope at 200× for 8-10 fields following the criteria of Platz and Seager (1978). Regarding tail morphology, fresh sperm suspension (2 μL) was diluted with 100 μL of formal saline, and a total of 200 spermatozoa were evaluated under a phase-contrast microscope at 1,000×. For sperm head morphology, a total of 500 spermatozoa previously stained with William's dye were evaluated under a light microscope at 1,000× (Axner et al., 2002).

Plasma membrane and acrosome integrity evaluation

Plasma membrane integrity and acrosome integrity 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, St. Louis, MO, USA) (Kitiyanant et al., 2002). 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, a 5 μL of a 10 mg/mL salmon sperm DNA (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in phosphate buffered saline (PBS) was added to bind excessive EthD-1 for 3 min at 37°C. After centrifugation, the spermatozoa were then smeared on a glass microscopic slide and fixed in 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 then rinsed with 4°C distilled water and allowed to air dry at 4°C. The fluorescent labeled slides were kept in the dark until evaluation. A total of 200 spermatozoa per slide were randomly visualized using an epifluorescent microscope at 1,000× and then classified into 3 categories as previously described (Cheng et al., 1996).

DNA integrity evaluation

Sperm DNA integrity was evaluated by acridine orange dye as described by (Thuwanut and Chatdarong, 2009). In brief, sperm suspension was first smeared on the glass slide, allowed air dry and then fixed in freshly prepared Carnoy's solution (3:1 [v/v] methanol:glacial acetic acid) for overnight at room temperature. The slide containing spermatozoa were then stained with 1% (w/v) acridine orange in distilled water for 5 min. A total of 200 spermatozoa per slide were evaluated and classified as normal DNA integrity (green fluorescence) and denatured DNA (orange, yellow or red fluorescence).

Statistical analysis

Data was handled and statistically analyzed using the SAS statistical package (version 9, SAS Institute, Inc., 2002, Cary, NC, USA). Normal distribution of residuals from the statistical models was tested using UNIVARIATE procedure option NORMAL PLOT. Two-way analysis of variance was performed using PROC GLM procedure to compare the difference among mean numbers of percentage of spermatozoa motility, intact plasma membrane spermatozoa, intact acrosome spermatozoa and normal DNA integrity spermatozoa. Concentrations of L-carnitine, times and interaction between concentrations of L-carnitine and times were regarded as a fixed effect. Least-squares means were obtained from each class of the factors; and were compared by using least significant different test with Tukey-Kramer adjustment for multiple comparisons. All data was expressed as mean±SD. A p value of less than 0.05 was considered statistically significant.

RESULTS

The characteristics of spermatozoa recovered from cauda epididymis (fresh spermatozoa) are shown in Table 1. Post-thawed spermatozoa characteristics including motility, plasma membrane integrity, DNA integrity and acrosome integrity are shown in Table 2, 3, 4, and 5, respectively. The percentages of motility, plasma membrane integrity, DNA integrity and acrosome integrity fresh spermatozoa were 80.75±4.87, 78.40±5.24, 94.98±2.81, and 73.57±5.22, respectively. These values were significantly greater than those obtained after freezing and thawing ($p<0.05$), indicating that cryopreservation process caused lethal damage to the spermatozoa. However, significantly higher motility was observed in 25 mM L-carnitine group when compared with 0 mM L-carnitine (control) group at 0 h and 2 h after thawing ($p<0.05$). Moreover, sperm motility at 2 h after thawing of 12.5 mM and 25 mM L-carnitine groups were significantly higher than 0 mM L-carnitine group ($p<0.05$). Plasma membrane integrity, DNA integrity and acrosome integrity were not significantly different in all treatment groups and all examination times ($p>0.05$).

DISCUSSION

It has been accepted that oral administration of L-carnitine is an effective option to improve semen quality in humans and some domestic animals (Kozink et al., 2004; Balercia et al., 2005). This sperm technology is important especially when cryopreservation is performed. Indeed,

Table 1. Characteristics of fresh cauda epididymal spermatozoa (mean±SD)

Sperm parameters	Fresh spermatozoa
Motility (%)	81.67±3.83
Intact plasma membrane (%)	78.61±5.03
Normal DNA integrity (%)	95.17±2.74
Intact acrosome in live spermatozoa (%)	73.92±4.12

SD, standard deviation.

Table 2. Sperm motility (%) of post-thawed cauda epididymal spermatozoa after thawing for 0 h (T0), 2 h (T2) and 4 h (T4) in each experimental group (mean±SD)

Group/time	T_0	T_2	T_4
0 mM L-carnitine	51.25±10.25^{A,a}	43.13±13.02^{A,B,a}	37.19±14.49^{B,a}
12.5 mM L-carnitine	62.81±8.75 ^{A,a,b}	57.19±9.30^{A,B,b}	48.13±14.82^{B,a}
25 mM L-carnitine	64.69±7.41^{A,b}	58.44±9.78^{A,B,b}	48.75±15.00^{B,a}
50 mM L-carnitine	55.94±9.87^{A,a,b}	47.81±13.16^{A,B,a,b}	40.94±14.97^{B,a}

SD, standard deviation.
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 ($p \leq 0.05$).

Table 3. Plasma membrane integrity (%) of post-thawed cauda epididymal spermatozoa after thawing for 0 h (T0), 2 h (T2), and 4 h (T4) in each experimental group (mean±SD)

Group/time	T_0	T_2	T_4
0 mM L-carnitine	67.19±9.98^{A,a}	64.19±8.76^{A,a}	61.91±7.99^{A,a}
12.5 mM L-carnitine	69.56±6.70^{A,a}	67.69±10.73^{A,a}	65.09±6.22^{A,a}
25 mM L-carnitine	66.69±8.26^{A,a}	65.66±9.91^{A,a}	62.78±9.20^{A,a}
50 mM L-carnitine	68.56±8.44^{A,a}	65.31±10.11^{A,a}	61.69±11.10^{A,a}

SD, standard deviation.
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 ($p \leq 0.05$).

freezing and thawing frequently induce excessive generation of free radicals and ROS. Several kinds of antioxidant supplementation in semen extender have successfully used to improve epididymal spermatozoa quality during cryopreservation (Neuman et al., 2002; Pena et al., 2003). We therefore hypothesized that L-carnitine supplementation in a freezing extender would improve cat epididymal spermatozoa quality during cryopreservation. This is the first study to investigate the effect of L-carnitine supplementation in the freezing extender for cat epididymal spermatozoa.

Our results demonstrated that cryopreservation markedly reduced the spermatozoa quality, although the post-thawed spermatozoa quality was in an acceptable range (Tables 2 to 4). This result was also similar to previous reports (Axner et al., 2004; Thuwanut et al., 2010). In addition, sperm quality after cryopreservation, in terms of spermatozoa motility, intact plasma membrane and intact

Table 4. Sperm DNA integrity (%) of post-thawed cauda epididymal spermatozoa after thawing for 0 h (T0), 2 h (T2) and 4 h (T4) in each experimental group (mean±SD)

Group/time	T_0	T_2	T_4
0 mM L-carnitine	91.38±9.26^{A,a}	89.88±14.89^{A,a}	84.56±19.65^{A,a}
12.5 mM L-carnitine	89.34±14.82^{A,a}	92.56±9.81^{A,a}	91.56±11.80^{A,a}
25 mM L-carnitine	83.09±23.11^{A,a}	89.63±14.58^{A,a}	89.53±12.50^{A,a}
50 mM L-carnitine	86.88±18.97^{A,a}	92.34±10.93^{A,a}	92.34±8.69$^{A a}$

SD, standard deviation.
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 ($p \leq 0.05$).

Table 5. Sperm acrosome integrity (%) of post-thawed cauda epididymal spermatozoa after thawing for 0 h (T0), 2 h (T2) and 4 h (T4) in each treatment and control (mean±SD)

Group/time	T_0	T_2	T_4
0 mM L-carnitine	33.00±12.92^{A,a}	33.59±13.24^{A,a}	26.13±12.33^{A,a}
12.5 mM L-carnitine	34.84±13.99^{A,a}	31.66±15.03^{A,a}	26.09±13.06^{A,a}
25 mM L-carnitine	38.00±21.97^{A,a}	35.38±18.56^{A,a}	29.56±13.33^{A,a}
50 mM L-carnitine	33.81±15.03^{A,a}	29.16±14.79^{A,a}	30.81±13.74^{A,a}

SD, standard deviation.
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 ($p \leq 0.05$).

acrosome was decreased over the times of examination. It is worth noting that the post-thawed quality of epididymal sperm was generally lower than ejaculated spermatozoa possibly by the facts that they are different in structural membrane characteristics and the susceptibility to cold shock (White, 1993). The plasma membrane of mammalian spermatozoa is predominantly composed of polyunsaturated fatty acids (Alvarez and Storey, 1995), which can be reacted with free radical to damage the sperm cells. Therefore, the antioxidants are believed to prevent spermatozoa damage from aforementioned free radicals that may excessively generate during the freeze-thaw process. Although we did not find any protective effect of L-carnitine on plasma membrane integrity, the 25 mM L-carnitine group provided the significantly higher motility than control when examined at 0 h and 2 h post thawing. These results suggest that the damages induced by an oxidative stress may cause only subtle changes of the plasma membrane structure.

The improvement of sperm motility has also been reported in other species. For examples, L-carnitine could improve stallion semen quality in asthenozoospermic semen (Cavallini et al., 2004). The effect of L-carnitine on improving human sperm motility has also been reported in oligo-, astheno- and terato-zoospermia (Costa et al., 1994; Balercia et al., 2005). These findings reinforce the hypothesis that L-carnitine may promote the cellular energetics (Lenzi et al., 2003) by transports long-chain acyl groups of fatty acids into sperm mitochondria, thereby improving flagella movement (Jeulin and Lewin, 1996).

After thawing, the intact acrosome spermatozoa were decreased in all treatment groups. This result showed that L-carnitine supplementation in freezing extender could not protect cat cryopreserved epididymal spermatozoa against acrosomal damage. This result is similar to the results of Yeste et al. (2010) reporting that dietary L-carnitine supplementation could not improve the acrosome integrity in ejaculated boar spermatozoa. Also, L-carnitine had no effect on DNA integrity of frozen-thawed spermatozoa. It is possible that the free radical scavengers within the semen extender were sufficient to protect the spermatozoa from oxidative stress generated during cryopreservation. In fact, the normal sperm DNA is less susceptible to this process (Zini et al., 2009), and *in vitro* antioxidant supplementations to normal DNA integrity spermatozoa have a limited consequence in DNA protection from ROS (Hughes et al., 1998; Donnelly et al., 1999; 2000; Chi et al., 2008).

In conclusion, the 25 mM L-carnitine supplementation in cryopreserved sperm extender improves motility of cat epididymal spermatozoa during freezing thawing. The interactions between ROS and antioxidants supplemented into freezing extender in terms of subtle changes of plasma membrane and fertility should be further investigated.

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