Original Article

Supplementation of Cooling Extender With L-carnitine and Preserving Ram's Sperm During Chilling Storage

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ABSTRACT

Background: Sperm preservation at a cool temperature reduces sperm metabolism while preserving its viability and reproductive ability. Researchers have sought to extend semen preservation effectiveness for more than 24 hours. Due to the particular physiological characteristics of small ruminant spermatozoa, the cooling procedure decreases its reproductive ability.

Objectives: This study aimed to determine the effect of adding L-carnitine (LC) to the cooling extender on the quality of the ram's sperm following cooling preservation at 4°C.

Methods: The collected sperm samples were diluted and divided into 4 groups with varying doses of LC supplementation (0, 1, 5, and 10 mM). The samples were kept at 4°C for up to 48 hours. At 0, 24, and 48 hours of cooling, the sperms' total motility, progressive motility, viability, lipid peroxidation, membrane integrity, and mitochondrial activity were assessed.

Results: The results showed that different treatments did not affect the quality of semen samples at time 0 of cooling storage (P>0.05). Cooling medium supplemented with 5 mM LC demonstrated improved total motility, progressive motility, viability, membrane integrity, and mitochondrial activity compared to the other groups after 24 and 48 hours of cooling (P≤0.05). Furthermore, after 24 and 48 hours of storage, 5 mM LC produced less lipid peroxidation (P≤0.05) than the other treatments.

Conclusion: In conclusion, reinforcing ram's cooling storage medium with 5 mM LC protects ram semen samples against cold-induced structural and functional impairment throughout 24- and 48-h storage.

Keywords: Chilling, L-carnitine, Ram, Quality evaluation, Sperm

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Introduction

Sperm preservation at a cool temperature reduces sperm metabolism while preserving sperm viability and reproductive potential. Researchers have sought to extend semen preservation effectiveness for more than 24 hours (Bucak & Tekin, 2007). Due to the particular physiological characteristics of small ruminant spermatozoa, the cooling procedure decreases their reproductive ability (Bucak & Tekin, 2007).

It is necessary to improve the protection strategy efficacy for the ram's sperm storage period because cooling may damage the ram's sperm, leading to membrane damage and a reduction in reproductive potential. The tris-based extender, which contains Tris, fructose, and citric acid, is a typical medium for ram's sperm conservation (Zarei et al., 2021). The protection rate of the ram's sperm against cooling damages may also be effectively increased by adding another additive to this extender. Reactive oxygen species (ROS), which interfere with spermatozoa structure, can be produced during chilling (Bucak & Tekin, 2007).

L-carnitine (LC) possesses metabolic and antioxidant functions, prompting us to investigate its properties. LC serves an essential function in sperm cell energy production by promoting the transfer of fatty acids into mitochondria (Heidari et al., 2021). As an antioxidant, LC stabilizes the membrane of mitochondria and protects the DNA structure against reactive oxygen species (Fattah et al., 2017a) which restricts procedures to store sperms for extended periods of time for artificial insemination of commercial flocks. This study was conducted to evaluate the suitability of adding L-carnitine (LC. As an antioxidant, LC stabilizes the membrane of mitochondria and protects the DNA structure against reactive oxygen species (Fattah et al., 2017a). This outcome is due to LC's ability to decrease the availability of lipids for peroxidation by transporting fatty acids into mitochondria for oxidation (Fattah et al., 2017b). Previous studies have shown that dietary LC increased sperm antioxidant enzyme activity, such as superoxide dismutase and glutathione peroxidase (Neuman et al., 2002), effectively scavenging ROS in cooled spermatozoa. The suitable effects of LC were recorded in sperm motility in a rooster (Fattah et al., 2017a) which restricts procedures to store sperms for extended periods of time for artificial insemination of commercial flocks. This study was conducted to evaluate the suitability of adding L-carnitine (LC and boar (Yeste et al., 2010).

No study has yet been undertaken to determine the effect of supplementing the cooling extender with LC on the quality of sperm during storage under refrigeration. The current study was conducted To assess the impact of LC on total motility, progressive motility, viability, mitochondrial activity, lipid peroxidation, and membrane functioning of chilled ram's sperm.

Materials and Methods

Semen processing

Using an artificial vagina, semen samples were taken from 5 adult Zandi rams during the breeding season. Samples were evaluated and chosen if the following criteria were fulfilled. Semen volume was between 1 and 2 mL, sperm overall motility was 75%, and sperm aberrant morphology was 15%. Sperm concentration was 3×10^9 spermatozoa/mL. Appropriate semen samples were combined with the absence of specific male effects. The chilling medium consists of 20% (v/v) egg yolk, 1.0 g fructose, 1.4 g citric acid, 2.71 g Tris, 100 IU penicillin, and 1 mg streptomycin. The PH and osmolarity settings are 7.2 and 320 mOsm/kg of water. The samples were then split into four equal portions: Extender without LC (LC0), extender with 1 mM (LC1), 5 mM (LC5), and 10 mM (LC10) LC. The samples were put in a rack and refrigerated from 37°C to 4°C for 30 minutes in a cold cabinet before being maintained at 4°C. The final concentration was 400×10^6 sperm/mL. At 0 (start time), 24, and 48 hours after storage, malondialdehyde (MDA) levels, viability, mitochondrial activity, and membrane functioning were evaluated (Dadashpour Davachi et al., 2022).

Semen quality evaluation

For the examination of the sperm motility parameters, the sperm class analysis program (Version 5.1; Microptic, Barcelona, Spain) was employed. A pre-warmed chamber slide was filled with 5 µL of diluted semen (38°C, Leja 4; 20 mm height; Leja Products, Luzernestra at B.V., Holland). Each sample was read in an average of 5 s over 5 fields containing at least 400 sperm. Both the progressive motility (PM) (%) and the total motility (TM) (%) were reported (Masoudi et al., 2020a).

The eosin-nigrosine staining was used to assess sperm viability by counting 200 sperm cells using a phase-contrast microscope (×400). The heads of active cells were unstained, while the heads of dead cells were completely or partially stained (Fattah et al., 2017a) which restricts procedures to store sperms for extended periods of time for artificial insemination of commercial flocks. This

study was conducted to evaluate the suitability of adding L-carnitine (LC.

The hypo-osmotic swelling test (HOST) investigated the functioning of the sperm membrane (Masoudi et al., 2021). Following 30 min incubation, the samples were examined using a phase-contrast microscope (×400). Approximately 300 sperm cells were counted. Sperm with swollen tails was recorded as a functional membrane.

Using the FACSCalibur flow cytometer, mitochondrial activity was assessed using Rhodamine 123 (R123; Invitrogen TM, Eugene, OR, USA) and propidium iodide (PI) (Becton Dickinson, San Khosoz, CA, USA) (Masoudi et al., 2020b). Approximately 10000 events were analyzed for each assay at a flow rate of 100 cells/s. The data were processed with the FlowJo program (Treestar, Inc., San Carlos, CA). The technique was carried out following Masoudi et al. (2020b).

Lipid peroxidation was evaluated by measuring MDA concentrations via the reaction of thiobarbituric acid at 532 nm by a spectrophotometer set (UV-1200, Japan) (Esterbauer & Cheeseman, 1990), and the concentrations were recorded as nmol/mL. The procedure was performed according to the method described by Esterbauer and Cheeseman (2020).

Statistical analysis

The data in the current study (6 replicates) were analyzed by the GLM procedure of SAS software, version 9.1. Tukey test was used to determine statistical differences between groups. The differences were statistically significant if P were ≤0.05.

Results

Total motility and progressive motility

Table 1 presents the effect of LC on the TM and PM of ram's sperm. There were no significant differences across treatments at 0 hours, 24 hours, and 48 hours after chilling. However, LC5 had considerably (P≤0.05) higher TM and PM than the other groups. There was no statistically significant difference (P>0.05) between the other treatments.

Viability and lipid peroxidation

Table 2 displays the effects of LC on sperm viability and lipid peroxidation during storage at low temperatures. At

Table 1. Effects of LC on ram's cooled sperm TM and PM

Treatments	TM (%)			PM (%)		
	0 h	24 h	48 h	0 h	24 h	48 h
LC0	87.4±0.9	54.2±1.0^{b}	22.8±1.1^{b}	68.5±0.8	26.4±1.1^{b}	11.6±1.4^{b}
LC1	88.0±0.9	55.5±1.0^{b}	24.6±1.1^{b}	70.1±0.8	28.5±1.1^{b}	13.1±1.4^{b}
LC5	86.9±0.9	60.3±1.0^{a}	29.5±1.1^{a}	69.4±0.8	35.0±1.1^{a}	17.3±1.4^{a}
LC10	88.4±0.9	54.0±1.0^{b}	23.3±1.1^{b}	69.8±0.8	26.1±1.1^{b}	10.4±1.4^{b}

$^{a, b}$Significant differences among the groups (P≤0.05).

Table 2. Effects of LC on ram's cooled sperm viability and lipid peroxidation

Treatments	Viability (%)			Malondialdehyde Concentration (nmol/mL)		
	0 h	24 h	48 h	0 h	24 h	48 h
LC0	89.8±1.0	58.2±0.9^{b}	25.3±1.2^{b}	2.48±0.12	4.38±0.20^{b}	9.25±.0.22^{b}
LC1	90.7±1.0	59.0±0.9^{b}	26.0±1.2^{b}	2.62±0.12	4.21±0.20^{b}	9.10±0.22^{b}
LC5	90.4±1.0	64.7±0.9^{a}	30.2±1.2^{a}	2.55±0.12	3.66±0.20^{a}	8.52±0.22^{a}
LC10	91.6±1.0	57.4±0.9^{b}	24.0±1.2^{b}	2.55±0.12	4.45±0.20^{b}	9.35±0.22^{b}

$^{a, b}$Significant differences among the groups (P≤0.05).

Table 3. Effects of LC on ram's cooled sperm mitochondrial activity and membrane functionality

Treatments	Mitochondrial activity (%)			Membrane functionality (%)		
	0 h	24 h	48 h	0 h	24 h	48 h
LC0	95.2±1.2	64.5±1.9^{b}	28.8±1.0^{b}	91.0±0.7	62.5±1.3^{b}	28.0±1.0^{b}
LC1	94.0±1.2	66.4±1.9^{b}	29.9±1.0^{b}	91.7±0.7	63.4±1.3^{b}	29.1±1.0^{b}
LC5	93.4±1.2	70.7±1.9^{a}	33.0±1.0^{a}	91.2±0.7	66.9±1.3^{a}	33.1±1.0^{a}
LC10	94.7±1.2	63.6±1.9^{b}	28.5±1.0^{a}	92.0±0.7	61.2±1.3^{b}	27.6±1.0^{b}

$^{a, b}$Significant differences among the groups (P≤0.05).

time 0, there was no difference between treatments regarding sperm viability and lipid peroxidation. LC5 had a higher (P≤0.05) viability rate after 24 and 48 hours of chilling than the other treatments. There were no differences (P>0.05) between LC0, LC1, and LC10. The MDA concentration at 24 h and 48 h storage was lower (P≤0.05) in LC5 compared to the other treatments, but there was no difference between the other treatments (P>0.05).

Mitochondrial activity and membrane functionality

The observations revealed the influence of LC on the mitochondrial activity and membrane function of chilled sperm (Table 3). At time 0, there were no significant differences between the treatments in mitochondrial activity or membrane integrity. At 24 h and 48 h of chilling storage, LC5 demonstrated increased (P≤0.05) mitochondrial activity and membrane functioning than the other treatments. There was no statistically significant difference between the groups (P>0.05).

Discussion

Cooling preservation of spermatozoa is a technique used to preserve sperm for reproductive purposes in small ruminants (Gibb et al., 2015) restricting the commercial viability of these animals and necessitating the development of a chemically defined room temperature (RT. Therefore, sperm samples must be diluted with a suitable medium containing sufficient protective chemicals (Sharafi et al., 2015). At 4°C for 48 hours, the beneficial effects of LC in the ram's sperm chilling medium were investigated in the current study. The results revealed a time-dependent decrease in the motility, mitochondrial activity, viability, and membrane functioning of cooled sperm; however, this decrease was less pronounced in the LC5 group than in the other groups. Using 5 mM LC resulted in greater overall motility, progressive motility, viability, mitochondrial activity, membrane integrity, and reduced MDA content throughout 24 and 48 hours of sperm cooling storage.

Previous research has indicated that dietary LC improves the quality of rooster sperm (Neuman et al., 2002). Despite the effectiveness of LC supplementation of extenders (Fattah et al., 2017b), no research has been conducted on rams. In the present study, sperm viability, motility, and membrane function enhancements may be attributable to LC's metabolic functions (Banihani et al., 2012). Facilitating the transport of fatty acids across the inner mitochondrial membrane via LC improves ATP production viaβ-oxidation (Steiber et al., 2004), thereby enhancing the energy supply for spermatozoa motility. Moreover, LC may effectively scavenge accumulated ROS and reduce sperm storage damage (Fattah et al., 2017b). Moreover, higher concentrations of LC were detected in sperm plasma than in blood plasma (Jeulin & Lewin, 1996), demonstrating the crucial role of LC in energy production via the pyruvate cycle (Gibb et al., 2015). As an antioxidant, LC neutralizes free radicals, inhibits xanthine oxidase activity, and eliminates metal chelation and hydrogen peroxide (Gülçin, 2006). On the other hand, research reveals a decrease in lipid peroxidation (Derin et al., 2004). The lipid peroxidation in this study confirmed our findings with regard to sperm viability, motility, and membrane integrity because lipid peroxidation was reduced in LC5, consistent with earlier findings of the positive effects of LC on many cell types (Di Giacomo et al., 1993) and spermatozoa (Banihani et al., 2012).

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ings of the positive effects of LC on many cell types (Di Giacomo et al., 1993) and spermatozoa (Banihani et al., 2012).

A correlation was found between mitochondrial activity and motility. Sperm motility is relatively reliant on mitochondrial function (Shahverdi et al., 2015). It may result from the osmolyte function of LC in the extension. To preserve isotonicity, adding LC to a diluent causes a portion of the Na^+ to be removed (Silver & Erecińska, 1997) sodium, and calcium gradients out of equilibrium. [Na^+](i. The Na^+ enhances ATP depletion by activating Na-ATPase pumps (Silver & Erecińska, 1997). Hence, the positive effects of LC may be attributable to the elimination of Na^+ (Gibb et al., 2015) restricting the commercial viability of these animals and necessitating the development of a chemically defined room temperature (RT. The Na^+ reduction in the medium reduces sperm energy needs, resulting in a slower rate of ATP depletion, successfully preserving and enhancing sperm mitochondrial function and viability for extended storage durations (Gibb et al., 2015) restricting the commercial viability of these animals and necessitating the development of a chemically defined room temperature (RT).

Conclusion

Adding LC to ram sperm cooling media protects sperm quality by reducing lipid peroxidation and preserving mitochondrial function. Therefore, supplementing the ram's chilling medium with 5 mM LC is a practical technique for transporting the ram's sperm to distant farms for reproductive purposes without a substantial drop in quality and productive capacity.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of the Animal Science Research Institute of Iran.

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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مقاله پژوهشی

افزودن ال-کارنیتین به رقیق‌کننده باعث محافظت از اسپرم قوچ در فرایند سردسازی می‌شود

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چکیده

زمینه: ذخیره سرمایی اسپرم، متابولیسم اسپرم را کاهش می‌دهد، درحالی‌که قابلیت باروری و زنده‌مانی اسپرم حفظ می‌شود. محققان تلاش بسیاری کرده‌اند تا بتوانند اسپرم را بیش از ۲۴ ساعت زنده نگه دارند. به‌علت ویژگی‌های خاص اسپرم نشخوارکنندگان کوچک، فرایندسردسازی توانایی باروری را در این گونه‌ها کاهش می‌دهد.

هدف: در این مطالعه اثر افزودن ال-کارنیتین به محیط سردسازی بر کیفیت اسپرم قوچ طی فرایند ذخیره سرمایی در دمای ۴ درجه سانتی‌گراد ارزیابی شده است.

روش کار: نمونه‌های اسپرم پس از جمع‌آوری و رقیق‌سازی به ۴ قسمت تقسیم شدند و مقادیر ۰، ۱، ۵ و ۱۰ میلی‌مولار ال-کارنیتین دریافت کردند. سپس نمونه‌ها در دمای ۴ درجه سانتی‌گراد سرد و طی ۴۸ ساعت ذخیره شدند. تحرک کلی و پیشرونده، زنده‌مانی، پراکسیداسیون لیپیدها، سلامت غشا و فعالیت میتوکندری در زمان‌های ۰، ۲۴ و ۴۸ ساعت ذخیره سرمایی مورد ارزیابی قرار گرفتند.

نتایج: نتایج نشان داد تیمارهای مختلف بر کیفیت نمونه‌های اسپرم در زمان صفر نگهداری خنک‌کننده، تأثیری نداشتند ($P>0/05$). در زمان‌های ۲۴ و ۴۸ ساعت از ذخیره سرمایی، تیمار ۵ میلی‌مولار ال-کارنیتین مقادیر بالاتر ($P\leq0/05$) تحرک کلی و پیشرونده، زنده‌مانی، سلامت غشا و فعالیت میتوکندری را نسبت به سایر گروه‌ها نشان داد. همچنین تیمار ۵ میلی مولار ال-کارنیتین موجب پراکسیداسیون لیپیدی کمتر ($P\leq0/05$) در زمان‌های ۲۴ و ۴۸ ساعت از ذخیره سرمایی نسبت به سایر گروه‌ها شد.

نتیجه‌گیری نهایی: درنتیجه استفاده از ۵ میلی‌مولار ال-کارنیتین در محیط ذخیره سرمایی اسپرم بز می‌تواند راهی مناسب برای محافظت از اسپرم بز در هنگام ۲۴ و ۴۸ ساعت سردسازی در مقابل آسیب‌های ساختاری و عملکردی طی ذخیره سرمایی باشد.

کلیدواژه‌ها: ذخیره سرمایی، ال-کارنیتین، قوچ، ارزیابی کیفیت، اسپرم.

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