DOI:10.22059/IJVM.2022.351558.1005335 Iranian Journal of Veterinary Medicine Original Article

Online ISSN: 2252-0554

Supplementation of Cooling Extender with L-carnitine Preserves Ram's Sperm During Chilling Storage

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Abstract

BACKGROUND: 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 h. Due to the special physiological characteristics of
25 small ruminant spermatozoa, the cooling procedure decreases reproductive ability in this species.

OBJECTIVES: This study aimed to determine the effect of supplementing l-carnitine (LC) to the cooling extender on the quality of ram's sperm following cooling preservation at 4ºC.

METHODS:The collected sperm samples were diluted and divided into four groups with varying doses of LC supplementation (0, 1, 5 and 10mM LC).The samples were kept at 4ºC for up to 48
30 hours. At 0, 24, and 48 hours of cooling, sperm total motility, progressive motility, viability, lipid peroxidation, membrane integrity, and mitochondrial activity were all measured.

RESULTS: The results showed that different treatments had no effect ($P>0.05$) on the quality of semen samples at time 0 of cooling storage.Cooling medium supplemented with 5 mM LC demonstrated improved ($P\leq0.05$) total motility, progressive motility, viability, membrane
35 integrity, and mitochondrial activity compared to the other groups after 24 and 48 hours of cooling. Furthermore, after 24 and 48 hours of storage, 5 mM LC produced less lipid peroxidation ($P\leq0.05$) compared to the other treatments.

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CONCLUSIONS: In conclusion, reinforcing ram's cooling storage medium with 5 mM LC is a useful method for protecting ram semen samples against cold-induced structural and functional
40 impairment throughout 24 and 48 h storage.

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

Introduction

Sperm preservation at a cool temperature reduces sperm metabolism while preserving sperm viability and reproductive potential. Researchers have sought to extend semen
45 preservation effectiveness for more than 24 h(Bucak *et al.,* 2007). Due to the special physiological characteristics of small ruminant spermatozoa, the cooling procedure decreases reproductive ability in this species(Bucak andTekin, 2007).

It is required to improve the effectiveness of the protection strategy for ram's sperm storage periods because ram's sperm may be exposed to cooling injuries that result in membrane
50 damage and a corresponding 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 an auxiliary additive to this extender. Reactive oxygen species (ROS), which interfere with spermatozoa structure, can be produced during the chilling process
55 (Bucak *et al.,* 2007).

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L-carnitine (LC) possesses metabolic and antioxidant functions, prompting us to investigate. It 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). As an antioxidant, LC stabilizes the membrane of mitochondria and protects the DNA structure against reactive oxygen species (Fattah *et al.,* 2017a). This 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 dietary LC increased sperm antioxidant enzyme activity, such as superoxide dismutase and glutathione peroxidase (Neuman *et al.,* 2002), which are effective in scavenging ROS in cooled spermatozoa. The suitable effects of LC were recorded in sperm motility in a rooster (Fattah *et al.,* 2017a) 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. In order to determine the effect of LC on total motility, progressive motility, viability, mitochondrial activity, lipid peroxidation, and membrane functioning of chilled ram's sperm, the current study was conducted.

Materials and methods

Semen processing

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75 Using an artificial vagina, semen samples were taken from five adult Zandi rams during the breeding season. Samples were evaluated and chosen if the following criteria were fulfilled. Semen volume is between 1 and 2 ml, sperm overall motility is 75%, and sperm aberrant morphology is 15%. Sperm concentration is 3×10^9 spermatozoa/ml. Appropriate semen samples were combined with the absence of certain male effects. 20% (v/v) egg yolk, 1.0 g fructose, 1.4 g 80 citric acid, 2.71 g Tris, 100 IU penicillin, and 1 mg streptomycin made up the chilling medium. 7.2 and 320 mosm/kg of water were chosen as the PH and osmolarity settings. The samples were then split into four equal portions: extender without LC (LC0), extender with 1 mM (LC1), extender with 5 mM (LC5), and extender with 10 mM (LC10) LC. The samples were put in a rack and refrigerated from 37 to 4ºC for 30 minutes in a cold cabinet before being maintained at 85 4ºC. The final concentration was 400×10^6 sperm/ml. At 0 (start time), 24, and 48 hours after storage, MDA levels, viability, mitochondrial activity, and membrane functioning were all evaluated (Dadashpour Davachi *et al*., 2022).

Semen quality evaluation

For the examination of the sperm motility parameters, the sperm class analysis 90 programme (Version 5.1; Microptic, Barcelona, Spain) was employed. A pre-warmed chamber slide was filled with 5 µl of diluted semen (38 ºC, Leja 4; 20 mm height; Leja Products, Luzernestra at B.V., Holland). Each sample was read in an average of 5 seconds over six fields

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containing at least 400 sperm. Both the PM (%) and the TM (%) were reported (Masoudi *et al.,* 2020a).

95 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/partially stained(Fattah *et al.,* 2017a).

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

100 a phase-contrast microscope (×400). Approximately 300 sperm cells were counted. Sperm with swollen tails was recorded as a functional membrane.

During a flow cytometry investigation employing the FACS Calibur 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.,

105 2020b). Approximately 10,000 events were analyzed for each assay at a flow rate of 100 cells/s. The data was processed with the Flow Jo programme (Treestar, Inc., San Carlos, CA). The technique was carried out in accordance with Masoudi *et al.* (2020b).

Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) concentrations via the reaction of thiobarbituric acid at 532 nm by a spectrophotometer set (UV-1200, Japan)

110 (Esterbauer and Cheeseman, 1990), and the concentrations were recorded as nmol/ml. The

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procedure was performed according to the method described by Esterbauer and Cheeseman (2020).

Statistical analysis

The data in the current study (six replicates) were analyzed by the GLM procedure of SAS
115 9.1 software. Tukey's test was used to determine statistical differences between groups. The differences were statistically significant if P values were ≤0.05 ($P \leq 0.05$).

Results

Total motility and progressive motility

The effect of LC on the TM and PM of ram's sperm is displayed in Table 1. There were no
120 significant differences across treatments at 0 hours, 24 hours, and 48 hours after chilling. However, LC5 had considerably ($P \leq 0.05$) 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
125 at low temperatures. At time 0, there was no difference between treatments regarding sperm viability and lipid peroxidation. LC5 had a higher ($P \leq 0.05$) viability rate after 24 and 48 hours of chilling than the other treatments. There were no differences ($P > 0.05$) between LC0, LC1, and

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LC10. The MDA concentration at 24 h and 48 h storage was lower ($P \leq 0.05$) in LC5 compared to the other treatments, but there was no difference between the other treatments ($P > 0.05$).

130 **Mitochondrial activity and membrane functionality**

The observations revealed (Table 3) the influence of LC on the mitochondrial activity and membrane function of chilled sperm. At time 0, there were no significant differences in mitochondrial activity or membrane integrity between the treatments. At 24 h and 48 h of chilling storage, LC5 demonstrated increased ($P \leq 0.05$) mitochondrial activity and membrane 135 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); therefore, sperm samples must be diluted with a 140 suitable medium containing sufficient protective chemicals(Sharafi *et al.,* 2015).At 4ºC 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

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145 overall motility, progressive motility, viability, mitochondrial activity, and membrane integrity, as well as a reduced MDA content throughout 24 and 48 h of sperm cooling storage.

Previous research has indicated that dietary LC improves the quality of rooster sperm(Neuman *et al.,* 2002);LC supplementation of extenders was also effective(Fattah *et al.,* 2017b); however, no research has been conducted on rams. In the present study, sperm viability,
150 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,
155 higher concentrations of LC were detected in sperm plasma than in blood plasma(Jeulin and 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
160 revealed a decrease in lipid peroxidation(Derin *et al.,* 2004).The lipid peroxidation results from this study confirmed the data obtained for sperm viability, motility, and membrane integrity because lipid peroxidation was reduced in LC5, which is consistent with earlier findings of the

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

165 A correlation was found between mitochondrial activity and motility. Sperm motility is relatively reliant on mitochondrial function(Shahverdi *et al.,* 2015). It may be a result of 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 and Erecińska, 1997). The Na+ enhances ATP depletion by activating Na-ATPase pumps (Silver and Erecińska, 1997); hence the positive
170 effects of LC may be attributable to the elimination of Na+(Gibb *et al.,* 2015). The Na+ reduction in the medium reduces sperm energy needs, resulting in a slower rate of ATP depletion, so successfully preserving and enhancing sperm mitochondrial function and viability for extended storage durations(Gibb *et al.,* 2015).

Conclusion

175 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 realistic technique for transporting the ram's sperm to distant farms for reproductive purposes without a substantial drop in quality and productive capacity.

Acknowledgement

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180 The authors would like to thank the Animal Science Research Institute of Iran for supporting the current study with number 34-13-1318-048-980661.

Conflict of Interest

The authors declare that they have no conflict of interest.

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

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چکیده

زمینه مطالعه: ذخیره سرمایی اسپرم متابولیسم اسپرم را کاهش می‌دهد درحالیکه قابلیت باروری و زنده‌مانی اسپرم حفظ می‌شود. محققان تلاش بسیاری کرده اند تا بتوانند اسپرم را بیش از 24 ساعت زنده نگه دارند. به

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علت ویژگی‌های خاص اسپرم نشخوارکنندگان کوچک، فرایندسردسازی توانایی باروری را در این گونه‌ها کاهش می‌دهد.
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هدف: در این مطالعه اثر افزودن ال-کارنیتین به محیط سردسازی بر کیفیت اسپرم قوچ طی فرایند ذخیره سرمایی در دمای 4 درجه سانتیگراد ارزیابی شده است.

روش کار: نمونه های اسپرم پس جمع آوری و رقیق سازی به چهار قسمت تقسیم شده و مقادیر 0، 1، 5 و 10 میلی مولار ال-کارنیتین را دریافت نمودند. سپس نمونه ها در دمای 4 درجه سانتیگراد سرد شده و طی 48 ساعت ذخیره شدند. جنبایی کل و پیشرونده، زنده مانی، پراکسیداسیون لیپیدها، سلامت غشا و فعالیت
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میتوکندری در زمان های 0، 24 و 48 ساعت ذخیره سرمایی مورد ارزیابی قرار گرفتند.

نتایج: در زمان 0، تیمارها تاثیری بر کیفیت نمونه های اسپرم نداشتند ($P>0.05$). در زمان های 24 و 48 ساعت از ذخیره سرمایی، تیمار 5 میلی مولار ال-کارنیتین مقادیر بالاتر ($P\leq0.05$) جنبایی کل و پیشرونده، زنده مانی، سلامت غشا و فعالیت میتوکندری را نسبت به سایر گروه ها نشان داد. همچنین تیمار 5 میلی مولار ال-
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کارنیتین موجب پراکسیداسیون لیپیدی کمتر ($P\leq0.05$)در زمان های 24 و 48 ساعت از ذخیره سرمایی نسبت به سایر گروه ها شد.

نتیجه‌گیری نهایی: در نتیجه، استفاده از 5 میلی مولار ال-کارنیتین در محیط ذخیره سرمایی اسپرم بز می تواند راهی مناسب برای محافظت از اسپرم بز در هنگام 24 و 48 ساعت سردسازی در مقابل آسیب های ساختاری و عملکردی طی ذخیره سرمایی باشد.

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

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285 **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]

Different letters within the same column show significant differences among the groups (P≤0.05).

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

Treatments	Viability (%)			MDA 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]

300 Different letters within the same column show significant differences among the groups (P≤0.05).

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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]

Different letters within the same column show significant differences among the groups (P≤0.05).

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