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Deep Nitinkumar Dudhatra

Department of Veterinary
Gynaecology and Obstetrics, College
of Veterinary Science and A.H.,
Kamdhenu University,
Sardarkrushinagar, Gujrat, India

Chandrakant Fuljibhai Chaudhari

Department of Veterinary
Gynaecology and Obstetrics, College
of Veterinary Science and A.H.,
Kamdhenu University,
Sardarkrushinagar, Gujrat, India

Ravjibhai Karshanbhai Chaudhari

Department of Veterinary
Gynaecology and Obstetrics, College
of Veterinary Science and A.H.,
Kamdhenu University,
Sardarkrushinagar, Gujrat, India

Tarunkumar Virabhai Sutaria

Department of Veterinary
Gynaecology and Obstetrics, College
of Veterinary Science and A.H.,
Kamdhenu University,
Sardarkrushinagar, Gujrat, India

Hareshbhai Chhotlal Nakhshi

Department of Veterinary
Gynaecology and Obstetrics, College
of Veterinary Science and A.H.,
Kamdhenu University,
Sardarkrushinagar, Gujrat, India

Babulal Natvarlal Suthar

Department of Veterinary
Gynaecology and Obstetrics, College
of Veterinary Science and A.H.,
Kamdhenu University,
Sardarkrushinagar, Gujrat, India

Bharatkumar Govindbhai Chaudhary

Dama Semen Production Unit, Banas
Dairy, Palanpur, Gujrat, India

Ketankumar Ganeshbhai Patel

Dama Semen Production Unit, Banas
Dairy, Palanpur, Gujrat, India

Corresponding Author:

Deep Nitinkumar Dudhatra

Department of Veterinary
Gynaecology and Obstetrics, College
of Veterinary Science and A.H.,
Kamdhenu University,
Sardarkrushinagar, Gujrat, India

Influence of L-Carnitine on Post-Thaw Semen Quality in Mehsana Buffalo Bulls with Different Freezability Levels

**Deep Nitinkumar Dudhatra, Chandrakant Fuljibhai Chaudhari,
Ravjibhai Karshanbhai Chaudhari, Tarunkumar Virabhai Sutaria,
Hareshbhai Chhotlal Nakhshi, Babulal Natvarlal Suthar, Bharatkumar
Govindbhai Chaudhary and Ketankumar Ganeshbhai Patel**

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Abstract

This study evaluated the effect of L-carnitine supplementation on post thaw semen quality and oxidative stress parameters in Mehsana buffalo bulls. Bulls were categorized into three groups based on freezability: poor, moderate and good. Results indicated no significant differences in individual motility among groups at post-dilution and post-thaw stages. Sperm viability showed no significant variation at post-dilution but differed significantly ($P<0.05$) at the post-thaw stage. Plasma membrane integrity and sperm morphology differed significantly ($P<0.05$) among groups at both stages. Oxidative stress markers (MDA and SOD) showed no significant differences at post-thaw stage. The study suggests that supplementation of L-carnitine (0.05 mg/ml) in semen extenders improves post-thaw semen quality and can be recommended for routine use in semen production units.

Keywords: Buffalo bulls, L-carnitine, MDA, Semen freezability, SOD

1. Introduction

Male fertility is a key determinant of reproductive efficiency in bovines, particularly under artificial insemination (AI) systems where a single bull contributes to the fertilization of numerous females. Therefore, accurate evaluation of semen quality is essential for predicting fertility potential. Semen analysis remains the most reliable and widely adopted method for assessing male reproductive performance (Bisla *et al.*, 2022; Patel *et al.*, 2024; Patel *et al.*, 2026) [3, 17, 16]. Parameters such as motility, concentration, morphology and viability are critical indicators of sperm function, and their impairment can lead to reduced conception rates and significant economic losses in the livestock sector. Hence, improving semen quality, especially during cryopreservation, is of considerable importance for enhancing breeding efficiency.

L-carnitine (γ -trimethylamino- β -hydroxybutyrate), a naturally occurring compound abundant in the epididymis, plays a vital role in sperm maturation and energy metabolism by facilitating the transport of fatty acids into mitochondria for β -oxidation and ATP production. Additionally, it exhibits antioxidant properties that help mitigate reactive oxygen species (ROS)-induced damage to sperm cells (Lenzi *et al.*, 1992; Kerner and Hoppel, 2000; Vaz and Wanders, 2002) [13, 12, 22]. Supplementation of L-carnitine has been reported to improve post-thaw semen quality in various species, including enhanced motility in stallions, improved viability in buffaloes, and overall semen quality in cattle (Bucak *et al.*, 2009; Gibb *et al.*, 2015; Longobardi *et al.*, 2017; Vaghasiya *et al.*, 2024) [5, 8, 14, 21]. However, its effect on semen freezability in Mehsana buffalo bulls with varying freezability levels has not been investigated. Therefore, the present study was undertaken to evaluate the effect of L-carnitine supplementation on semen quality and oxidative stress parameters in Mehsana buffalo bulls, with the objective of optimizing semen extenders and improving the efficiency of artificial insemination programs.

Materials and Methods

Grouping of Bulls

Nine healthy, sexually mature Mehsana buffalo bulls maintained at the Dama Semen Production Unit, Banas Dairy, Palanpur, were selected for the study. Based on their semen freezability, determined from one year of production records, bulls were categorized into three groups: poor (Group I), moderate (Group II), and good freezability (Group III). The classification was based on post-thaw semen straw discard rate (%), calculated as the proportion of ejaculates discarded due to less than 50% post-thaw motility. Bulls with discard rates above mean +1 SD were classified as poor, those below mean -1 SD as good, and the remaining as moderate freezability group.

Semen Collection

Prior to semen collection, preputial washing was performed using 0.9% normal saline. Semen was collected twice weekly in the morning using an artificial vagina (AV) with a male dummy, following three false mounts to ensure adequate sexual stimulation. A total of 108 ejaculates (12 per bull) were collected over a period of six weeks.

Semen Processing

Only ejaculates exhibiting initial motility $\geq 70\%$ were processed further. Semen was diluted to a final concentration of 20 million spermatozoa per 0.25 ml French mini straw (TBS, IMV, France) using standard diluents. The filled and

sealed straws were equilibrated at 4-5°C for 4 hours in a cold handling cabinet. Cryopreservation was carried out using a controlled freezing protocol: from 4°C to -10°C at 5°C/min, from -10°C to -100°C at 40°C/min, and from -100°C to -140°C at 20°C/min. After 24 hours of storage, straws were thawed at 37°C for 30 seconds for evaluation.

Semen Evaluation

Semen samples were assessed for individual motility, sperm viability, morphology and plasma membrane integrity at both post-dilution and post-thaw stages. Oxidative stress parameters, namely malondialdehyde (MDA) and superoxide dismutase (SOD), were evaluated at the post-thaw stage.

Statistical Analysis

Data were tested for normality using the Shapiro-Wilk test. Percentage data were arcsine transformed prior to analysis. Statistical comparisons among groups were performed using one-way ANOVA followed by Tukey's post hoc test. Results were expressed as mean $\pm$ Standard error (SE), with significance set at $P < 0.05$. Statistical analysis was conducted using GraphPad Prism version 6.

Results and Discussion

The semen samples were examined for individual subjective motility, viability, plasma membrane integrity and sperm abnormality at post dilution and post thawed stages (Table 1).

Table 1: Effect of L- carnitine on semen qualitative parameters (Mean $\pm$ S.E.) in Mehsana buffalo bulls at post dilution and post thawed stage

	Stage	Group I	Group II	Group III
Individual subjective motility (%)	PD	77.12 $\pm$ 0.03	76.44 $\pm$ 0.03	78.61 $\pm$ 0.04
	PT	58.94 $\pm$ 0.01	59.27 $\pm$ 0.01	61.51 $\pm$ 0.01
Viability (%)	PD	85.77 $\pm$ 0.01	85.91 $\pm$ 0.03	87.27 $\pm$ 0.02
	PT	63.93 $\pm$ 0.01 ^{ab}	64.32 $\pm$ 0.02 ^b	69.11 $\pm$ 0.01 ^c
Plasma membrane integrity (%)	PD	78.03 $\pm$ 0.02 ^c	81.64 $\pm$ 0.01 ^a	82.33 $\pm$ 0.02 ^{ab}
	PT	60.99 $\pm$ 0.01 ^{ab}	62.93 $\pm$ 0.01 ^b	66.44 $\pm$ 0.01 ^c
Morphology (%)	PD	93.25 $\pm$ 0.01 ^c	95.37 $\pm$ 0.01 ^a	95.07 $\pm$ 0.01 ^{ab}
	PT	89.39 $\pm$ 0.01 ^a	91.61 $\pm$ 0.01 ^b	89.94 $\pm$ 0.01 ^{ac}

Means bearing different superscripts (a,b,c) differ significantly within column ($p < 0.05$)

Individual subjective motility and sperm viability did not differ significantly among the three groups at the post-dilution stage. These findings are consistent with earlier reports indicating no significant effect of L-carnitine supplementation on semen quality immediately after dilution (El-Raey *et al.*, 2016; Hussein, 2018; Vaghasiya *et al.*, 2024). This suggests that the beneficial effects of L-carnitine may not be evident during the initial processing stage. However, at the post-thaw stage, sperm viability differed significantly ($P < 0.05$) among the groups, with improved values observed in bulls with better freezability. These results are in agreement with previous studies reporting enhanced post-thaw sperm viability following L-carnitine supplementation (El-Raey *et al.*, 2016; MA *et al.*, 2017; Wafa *et al.*, 2021). The improvement in viability indicates a protective role of L-carnitine against cryo-induced damage.

Plasma membrane integrity and sperm morphology showed significant differences ($P < 0.05$) among the groups at both post-dilution and post-thaw stages. The higher membrane integrity and reduced sperm abnormalities observed in the present study are supported by earlier findings, which demonstrated improved HOST response and reduced morphological defects following L-carnitine supplementation (Bucak *et al.*, 2010; Longobardi *et al.*, 2017; MA *et al.*, 2017)

[4, 14, 15]. These observations highlight the role of L-carnitine in maintaining membrane stability and structural integrity of spermatozoa during cryopreservation.

The beneficial effects of L-carnitine can be attributed to its involvement in sperm energy metabolism and antioxidant defense. Sperm motility is largely dependent on ATP generated through mitochondrial oxidative phosphorylation. L-carnitine facilitates the transport of fatty acids into mitochondria, thereby enhancing ATP production and supporting sperm motility and survival. Additionally, it reduces oxidative stress and minimizes capacitation-like changes during cryopreservation, leading to improved post-thaw semen quality (Longobardi *et al.*, 2017) [14]. Spermatozoa are highly susceptible to oxidative damage due to the high content of polyunsaturated fatty acids in their plasma membrane. Excessive lipid peroxidation during cryopreservation can compromise membrane integrity, reduce motility and impair fertility (Griveau *et al.*, 1995) [9]. The improved membrane integrity observed in this study suggests that L-carnitine may help protect sperm cells from such oxidative damage, thereby enhancing their functional competence after thawing.

Reactive oxygen species (ROS) are naturally generated by spermatozoa and play an essential role at physiological levels

in key fertilization processes such as capacitation, acrosome reaction, hyperactivation, and sperm-oocyte fusion (Ahmed *et al.*, 2020) [1]. However, excessive ROS production during cryopreservation induces lipid peroxidation, leading to structural damage of the sperm plasma membrane and compromised sperm function (Sharma and Agarwal, 1996) [18]. The beneficial effects observed in the present study may be attributed to the multifunctional role of L-carnitine. Its membrane-stabilizing properties, along with its role in maintaining osmotic balance, contribute to preserving sperm structural integrity during freezing and thawing (Deana *et al.*, 1984) [6]. Consequently, L-carnitine supplementation likely enhances sperm motility and increases the proportion of spermatozoa with intact plasma membranes at the post-thaw stage.

Table 2: Effect of L-carnitine on oxidative stress markers (Mean $\pm$ S.E.) in Mehsana buffalo bull semen at post thawed stage

Groups	MDA (nmol/ml)	SOD (ng/ml)
I	9.13 $\pm$ 0.40	21.87 $\pm$ 2.57
II	9.01 $\pm$ 0.33	22.45 $\pm$ 2.73
III	8.95 $\pm$ 0.43	23.11 $\pm$ 3.11

The antioxidant parameter malondialdehyde (MDA) did not differ significantly among the three groups at the post-thaw stage (Table 2). These findings are consistent with earlier reports, where no significant variation in MDA concentration was observed following L-carnitine supplementation in buffalo bull semen (Bucak *et al.*, 2010; Longobardi *et al.*, 2017; Vaghasiya *et al.*, 2024) [4, 14, 21]. This suggests that L-carnitine supplementation at the tested levels may not markedly influence lipid peroxidation as measured by MDA. Spermatozoa are highly susceptible to lipid peroxidation due to the abundance of polyunsaturated fatty acids in their plasma membrane. During cryopreservation, excessive generation of reactive oxygen species (ROS) leads to oxidative damage, disrupting membrane integrity and impairing sperm function (Bucak *et al.*, 2010) [4]. ROS-mediated lipid peroxidation is further exacerbated by iron-catalyzed reactions such as the Haber-Weiss and Fenton reactions, which accelerate the formation of highly reactive free radicals. L-carnitine, owing to its metal-chelating and antioxidant properties, may reduce iron-induced oxidative stress by limiting free radical generation (Gulcin, 2006) [10]. Although MDA levels were not significantly altered in the present study, the observed improvements in semen quality parameters suggest a protective role of L-carnitine against oxidative damage.

A similar non-significant trend was observed for superoxide dismutase (SOD) activity at the post-thaw stage. However, previous studies have reported significantly increased SOD activity following L-carnitine supplementation (MA *et al.*, 2017; Vaghasiya *et al.*, 2024) [15, 21]. SOD is a primary antioxidant enzyme that catalyzes the dismutation of superoxide radicals into hydrogen peroxide, thereby protecting sperm cells from oxidative stress (Barranco *et al.*, 2019) [2]. The resulting hydrogen peroxide is subsequently detoxified by other antioxidant enzymes such as catalase and glutathione peroxidase (Silvestre *et al.*, 2021) [19]. The observed trend toward higher SOD levels in L-carnitine-treated groups may be attributed to its multifaceted antioxidant action, including ROS scavenging, metal chelation, and reduction of oxidative enzyme activity (Surai, 2015) [20]. Additionally, L-carnitine has been reported to directly neutralize reactive species such as superoxide and

hydrogen peroxide (Gulcin, 2006) [10]. These combined effects may contribute to improved antioxidant defense and enhanced post-thaw sperm quality, even in the absence of statistically significant changes in oxidative stress markers.

Conclusion

L-carnitine supplementation significantly improved post-thaw sperm viability, plasma membrane integrity and morphological normality in Mehsana buffalo bull semen, with more pronounced effects observed in bulls with good freezability. These findings indicate that L-carnitine plays a beneficial role in reducing cryo-induced damage and enhancing membrane stability during semen cryopreservation in Mehsana buffalo bulls.

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Conflict of Interest

The authors declare no conflict of interest.

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