



In vitro effects of L-carnitine and glutamine on motility, acrosomal abnormality, and plasma membrane integrity of rabbit sperm during liquid-storage ☆

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ABSTRACT

This study was designed to evaluate the in vitro effects of L-carnitine and glutamine (Gln) on the sperm quality parameters of liquid-stored rabbit semen maintained up to 24 h at 5 °C. Pooled and extended ejaculates were divided into two equal portions. L-Carnitine doses of 0.5, 1 and 2 mM were added to the first portion, and glutamine was added at the same doses to the second portion. All samples were cooled to 5 °C and examined at 0, 6, 12 and 24 h of liquid storage. Supplementation of the semen extender with three different doses of L-carnitine provided significant increases in the percentage of motile sperm at 12 h ($P < 0.01$), and 24 h ($P < 0.001$) and enabled significant protection of the sperm plasma membrane ($P < 0.01$) at 12 and 24 h of cool-storage, in comparison to the control samples. Only the 2 mM dose of L-carnitine significantly ($P < 0.01$) decreased the rate of acrosomal damage when compared to the control group. Furthermore, all doses of Gln caused a significant ($P < 0.01$) decrease in acrosomal damage at 6 h, and provided significant improvement ($P < 0.01$) in sperm motility, acrosomal and plasma membrane integrities at 12 and 24 h of liquid storage, when compared to the controls. In conclusion, the supplementation of liquid-stored rabbit semen with L-carnitine and Gln provided a protection for sperm against cool storage-induced functional and structural damages.

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Introduction

Artificial insemination (AI) programmes are essential for breeding and selection schedules aimed at increasing rabbit production. However, the ability of rabbit sperm to survive in vitro after chilled [23,33,36] or frozen storage [27] is limited. Thus, AI protocols involve the use of fresh or briefly stored refrigerated semen with outcomes comparable to those of natural mating [25,34]. However, liquid storage or the freeze–thawing process also produces physical and biochemical disturbances in the sperm membrane of different species of animals, which result in reduced sperm motility and viability, damaged membranes and loss of DNA integrity and premature acrosome reaction leading to reduced fertilizing ability [2,8,18,33]. In addition, the processing of

mammalian sperm causes an increase in reactive oxygen species (ROS) and leads to a detrimental effect on sperm motility, viability, acrosomal integrity and fertilizing ability, due to alterations induced in the sperm plasma membrane [1,6], as well as on DNA integrity [15]. In order to overcome these disturbances different additives have been added to semen diluted with different extenders during cool-storage.

L-Carnitine is a water-soluble vitamin-like amino acid that occurs naturally in microorganisms, plants, and animals [13,42]. Its concentration in animals varies widely according to the species, tissue type, and nutritional status of the animal [29]. L-Carnitine is an important amino acid and also an essential co-factor for fatty acid metabolism, and plays an important role in the generation of metabolic energy by facilitating the transport of fatty acids into the mitochondria. L-Carnitine is found at high concentrations in mammalian epididymides and sperm. The epididymal epithelium and sperm generate energy from L-carnitine present in the epididymal fluid [19,20,35]. L-Carnitine is one of the most powerful antioxidants. It functions by reducing the availability of lipids for

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peroxidation by means of transporting fatty acids into the mitochondria for β -oxidation to generate ATP energy [26,30]. This reduces the amount of lipids available for peroxidation [22].

Glutamine is the principal free amino acid found in the testes and semen of most mammalian species, and mainly occurs in the seminal plasma rather than in sperm [40]. It has been reported that Gln addition to semen improves the post-thaw motility and fertilizing potential of human sperm [31] and bull [4], dog [7], goat [9], ram [11] and stallion semen [23]. However, to the best of our knowledge, there are no data available on the effects of L-carnitine and Gln on sperm motility and membrane integrity in liquid-stored rabbit semen maintained at 5 °C up to 24 h. Therefore, this study was conducted to investigate the in vitro effects of L-carnitine and Gln on sperm motility, acrosomal damage, and plasma membrane integrity in rabbit semen diluted with a Tris-based extender during cool-storage at 5 °C up to 24 h.

Materials and methods

Chemicals

L-Carnitine (C0283) and glutamine (G8540) and all the chemicals used in this study were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

Animals, semen collection and processing

Ten sexually mature male New Zealand White rabbits were used as semen donors. They were obtained from the Experimental and Clinical Research Centre of Erciyes University, Kayseri, Turkey and maintained therein. Males were housed in individual cages under standard laboratory conditions (were exposed to a 12 h/12 h light/dark cycle at a room temperature of 22–24 °C and a relative humidity of 55–60%). A commercial pellet diet (Optima Food Co., Bolu, Turkey) and fresh drinking water were given *ad libitum*. The experimental protocols were approved by the local Experimental Animal Committee of Erciyes University (Kayseri, Turkey). The animal care and experimental protocols complied with the NIH Guide for the Care and Use of Laboratory Animals.

Semen was collected twice a week with an artificial vagina. After semen collection, any gel plug was removed. Wave motion was visually assessed under a light microscope with a stage heated to 37 °C at 400 $\times$ magnification. 10 μ L of the semen was placed in a glass slide without covering a coverslip and the wave motion of semen evaluated (0–5 scale) after judging five different microscopic fields. The sperm concentration was determined by means of a haemocytometer [8]. Only ejaculates with good wave motion (≥ 3 on a 0–5 scale), $\geq 300 \times 10^6$ sperm/mL and $\geq 75\%$ motile sperm were used in this study [34]. All ejaculates that met these criteria were pooled in order to eliminate individual differences and diluted with a Tris-based extender (313.8 mM Tris, 103.1 mM citric acid and 33.3 mM glucose) at 37 °C. Six pooled and diluted ejaculates were included in this study. Each of the pooled samples was divided into two equal portions. The first portion was further divided into four equal aliquots containing 0 (control), 0.5, 1 and 2 mM of L-carnitine, at a final concentration of approximately 40×10^6 sperm/mL (single step dilution), in 1.5 mL Eppendorf tubes. The second portion was divided into four equal aliquots containing 0 (control), 0.5, 1 and 2 mM of glutamine, at a final concentration of approximately 40×10^6 sperm/mL, in Eppendorf tubes. The semen samples were then placed in a rack and cooled from 37 to 5 °C, in a cold cabinet, and maintained at 5 °C. Sperm motility, acrosomal damage, and plasma membrane integrity were determined at 0 (at the beginning of the cooling process to 5 °C), 6, 12 and 24 h.

Evaluation of sperm motility and acrosomal damage

Sperm motility was subjectively assessed by visual estimation [34]. A drop of 10 μ L of semen was delivered onto a clean glass slide and covered with a coverslip. The preparation was then examined under a light microscope with a stage heated to 37 °C at 400 $\times$ magnification. Motility estimations were performed by examining five microscopic fields. The mean of the estimations was recorded as the final motility score and expressed as percentage.

For the assessment of acrosomal damage, 3 drops from each diluted semen sample were added to 1 mL of Hancock solution. One drop of this mixture was put onto a slide and covered with a cover slip. The percentage of acrosomal damage was determined by counting a total of 400 sperm under a phase-contrast microscope at 1000 $\times$ magnification [38].

Evaluation of sperm plasma membrane integrity

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm plasma membrane. HOST relies on the resistance of the membrane to the loss of permeability barriers under stress conditions of stretching in a hypo-osmotic medium. Sperm with resistant membranes exhibit a swelling around the tail, which results in the flagella becoming curled and the membrane maintaining a swollen ‘bubble’ around the curled flagellum [32]. The assay was performed by mixing 30 μ L of diluted semen with 300 μ L of 100 mOsm/kg hypo-osmotic solution (9 g fructose plus 4.9 g sodium citrate/L distilled water). This mixture was incubated at 37 °C for 1 h, where 0.2 mL of the mixture was placed onto a microscope slide and mounted with a cover slip and immediately evaluated under a phase-contrast microscope at 400 $\times$ magnification. From each sample, a total of 200 sperm cells were counted in at least five different microscopic fields, and the percentage of sperm with swollen and curled tails was recorded.

Statistical analysis

The study was replicated six times. Values are presented as mean $\pm$ S.E.M and the level of significance was set at $P < 0.05$. The Kruskal–Wallis test was used to compare the differences between the groups for sperm motility, plasma membrane integrity and acrosomal damage. Follow-up tests were conducted to evaluate pairwise differences between the groups, and Type I errors were controlled using the Bonferroni approach. All analyses were performed using the SPSS programme (Version 15, SPSS, Chicago, IL, USA).

Results

Effects of L-carnitine on sperm motility, acrosomal damage, and plasma membrane integrity

When compared to the control group, neither of the doses of L-carnitine had a significant effect on any of the sperm parameters assessed at 0 and 6 h or on acrosomal damage at 12 h. In comparison to the control group, the three different doses of L-carnitine provided a significant retention in sperm motility at 12 h ($P < 0.01$) and 24 h ($P < 0.001$) of cool-storage and a significant ($P < 0.01$) protection of plasma membrane integrity at 12 and 24 h of cool-storage. Only the 2 mM dose of L-carnitine significantly ($P < 0.01$) decreased the rate of acrosomal damage at 24 h when compared to the control group (Table 1).

Table 1
Mean $\pm$ SEM values of sperm motility, acrosomal abnormality, and plasma membrane integrity (HOST) in diluted rabbit semen supplemented with different concentrations of L-carnitine at different time points at 5 °C. ("n = 6" refers to six replications).

Groups	Time points (h)					
	0	6	12	24	HOST (%)	HOST (%)
n	6	6	6	6	6	6
Control	84.4 $\pm$ 1.1	76.3 $\pm$ 1.3	69.4 $\pm$ 2.0 ^a	58.1 $\pm$ 2.1 ^a	13.5 $\pm$ 0.4 ^a	50.2 $\pm$ 0.7 ^a
L-carnitine 0.5 mM	85.0 $\pm$ 1.3	81.3 $\pm$ 1.6	80.6 $\pm$ 1.5 ^b	73.1 $\pm$ 0.9 ^b	9.7 $\pm$ 0.4	61.3 $\pm$ 0.8 ^b
L-carnitine 1 mM	86.3 $\pm$ 1.3	81.9 $\pm$ 1.3	79.4 $\pm$ 1.1 ^b	76.3 $\pm$ 1.3 ^b	9.3 $\pm$ 0.9	61.0 $\pm$ 1.7 ^b
L-carnitine 2 mM	86.9 $\pm$ 1.6	82.5 $\pm$ 1.6	78.8 $\pm$ 1.3 ^b	77.5 $\pm$ 0.9 ^b	9.2 $\pm$ 0.5	60.2 $\pm$ 0.6 ^b
Statistical Significance	NS	NS	P < 0.01	P < 0.001	NS	P < 0.01

NS; Non-significant.

^{a,b}: Different superscripts within the same column demonstrate significant differences.

Effects of glutamine on sperm motility, acrosomal damage, and plasma membrane integrity

The extender supplemented with Gln at three different doses had no significant effect on sperm motility and plasma membrane integrity at 0 and 6 h when compared to the control group. Glutamine at three different doses caused a significant decrease in acrosomal damage at 6 h of storage in comparison to the control group ($P < 0.01$). In addition, the three different doses of Gln provided significant improvements in sperm motility, acrosomal and plasma membrane integrities at 12 and 24 h of the liquid storage period compared to the controls ($P < 0.01$) (Table 2).

Discussion

Semen cooling for a short period has been well-known to cause some structural and functional damages to sperm in a time-dependent manner in almost all mammalian species. To prevent or decrease these damages, the semen needs to be diluted with suitable extenders for liquid-storage. Tris-based extenders have been widely used in several studies that have demonstrated extenders to be beneficial in maintaining the sperm characteristics of liquid-stored rabbit semen [27,33,34]. Based on previous reports, in the present study, in all groups the semen samples were diluted with a Tris-based extender. However, it was observed that time-dependent losses still occurred in motile, viable and morphologically normal sperm in rabbit semen diluted with a Tris-based extender. In the present study, these losses were also observed during the storage period and in a time-dependent manner in all groups. Semen extenders used for the dilution of mammalian semen, including rabbit semen, have been supplemented with different additives either to decrease time-dependent damages during cool-storage [7,18,39], or to obtain better quality after the freeze-thawing process [4,9,11,23,24]. In the present study, we also investigated the possible protective effects of L-carnitine and Gln addition to rabbit sperm diluted with a Tris-based extender on motility, acrosomal damage, and plasma membrane integrity during liquid-storage at 5 °C up to 24 h.

L-Carnitine is found at higher concentrations in the epididymal plasma and sperm than in the blood plasma. Epididymal carnitine is used efficiently by epididymal spermatozoa [21]. This has been shown to stimulate oxygen consumption in the mitochondria of rat, rabbit and bull spermatozoa, therefore, it is suggested that carnitine protects the energy metabolism and quality of semen [16]. Furthermore, it has been indicated that the initiation of sperm motility occurs in parallel with the increase in the concentration of L-carnitine in the epididymal lumen [21]. It has been reported that the oral consumption of L-carnitine ameliorated the semen quality of oligoasthenospermic stallions [41], and it has also been ascertained that oral consumption of L-carnitine provided significant increases in sperm motility, viability and acrosomal integrity of extended rabbit semen during liquid-storage at 4 °C [18]. Deana et al. [16] reported that L-carnitine showed different action on the membranes of different cellular compartments of bovine sperm, and suggested that the addition of high (20 mM), but not low (2 mM), concentrations of L-carnitine resulted in an increase of cellular calcium transport and inhibition of sperm motility.

L-Carnitine supplementation of the semen extender and the use of extenders in semen cryopreservation have been tested in bull and goat semen, but not in rabbit semen. L-Carnitine doses ranging from 2.5 to 20 mM have been tested in previous research on semen extenders [10,12,16].

In the present study, we observed significant improvement in motility, acrosomal and plasma membrane integrity with the use of even the lowest dose of carnitine (0.5 mM) in rabbit semen at 12 h of liquid storage.

Table 2
Mean $\pm$ SEM values of sperm motility, acrosomal abnormality, and plasma membrane integrity (HOST) in diluted rabbit semen supplemented with different concentrations of glutamine at different time points at 5 °C. ("n = 6" refers to six replications).

Groups	0			6			12			24		
	Motility (%)	Acrosomal Abnormality (%)	HOST (%)	Motility (%)	Acrosomal Abnormality (%)	HOST (%)	Motility (%)	Acrosomal Abnormality (%)	HOST (%)	Motility (%)	Acrosomal Abnormality (%)	HOST (%)
n	6	6	6	6	6	6	6	6	6	6	6	6
Control	85.3 $\pm$ 1.5	3.5 $\pm$ 0.2	67.8 $\pm$ 1.3	78.1 $\pm$ 1.6	12.7 $\pm$ 0.4 ^a	64.2 $\pm$ 1.3	70.6 $\pm$ 1.8 ^a	14.3 $\pm$ 0.5 ^a	55.8 $\pm$ 1.0 ^a	51.3 $\pm$ 1.3 ^a	17.5 $\pm$ 0.9 ^a	49.7 $\pm$ 1.1 ^a
Glutamine 0.5 mM	87.5 $\pm$ 0.9	3.3 $\pm$ 0.3	68.3 $\pm$ 1.2	83.8 $\pm$ 0.8	9.0 $\pm$ 0.6 ^b	65.8 $\pm$ 1.7	80.0 $\pm$ 1.3 ^b	10.7 $\pm$ 0.6 ^b	62.2 $\pm$ 0.7 ^b	65.6 $\pm$ 2.2 ^b	11.8 $\pm$ 0.7 ^b	59.7 $\pm$ 1.3 ^b
Glutamine 1 mM	88.1 $\pm$ 1.3	3.2 $\pm$ 0.3	65.3 $\pm$ 1.3	83.1 $\pm$ 1.3	9.5 $\pm$ 0.7 ^b	64.5 $\pm$ 1.7	79.9 $\pm$ 1.4 ^b	12.5 $\pm$ 0.6 ^b	63.8 $\pm$ 0.6 ^b	74.4 $\pm$ 1.5 ^c	13.0 $\pm$ 0.7 ^b	60.5 $\pm$ 1.5 ^b
Glutamine 2 mM	86.5 $\pm$ 1.8	3.2 $\pm$ 0.4	66.5 $\pm$ 0.9	81.6 $\pm$ 1.8	7.7 $\pm$ 0.9 ^b	64.0 $\pm$ 1.4	77.5 $\pm$ 1.9 ^b	8.5 $\pm$ 0.7 ^c	62.0 $\pm$ 1.8 ^b	72.5 $\pm$ 1.3 ^c	12.3 $\pm$ 0.7 ^b	58.5 $\pm$ 1.8 ^b
Statistical Significance	NS	NS	NS	NS	P < 0.01	NS	P < 0.01	P < 0.01	P < 0.01	P < 0.01	P < 0.01	P < 0.01

NS: Non-significant.

a,b,c: Different superscripts within the same column demonstrate significant differences.

Bucak et al. [10] have reported that L-carnitine supplementation of the extender used in the dilution of goat semen provides significant improvement in sperm acrosomal integrity but not in sperm motility after freeze–thawing. Another study showed that the in vitro supplementation of the semen extender with L-carnitine provided significant improvement in sperm motility and acrosomal integrity in bull semen after the cryopreservation process [12].

L-Carnitine is a potent enzymatic antioxidant, which scavenges high concentrations of ROS that cause sperm pathology such as ATP depletion and lead to insufficient axonemal phosphorylation, lipid peroxidation and loss of motility and viability [17]. In a recent study, L-carnitine and its derivatives have been proposed for the treatment of male infertility [5]. In our previous study, we also observed that the liquid storage of rabbit semen at 5 °C up to 72 h causes significant elevation in ROS and lipid peroxidation [37]. In addition, it was demonstrated that L-carnitine supplementation of the semen extender significantly increased catalase enzyme activity in goat sperm [10], but not in bull sperm [12] after the freeze–thawing process. In the present study, the three different doses of L-carnitine provided a significant increase in sperm motility, and a significant protection of plasma membrane integrity at 12 and 24 h of cool-storage, in comparison to the control group. Furthermore, only the 2 mM dose of L-carnitine significantly decreased the rate of acrosomal damage when compared to the control group. Improvements observed in the sperm quality parameters following the supplementation of diluted rabbit semen with L-carnitine may be explained by the antioxidant property of L-carnitine together with its important role in sperm energy and fatty acid metabolism.

Gln has been reported to have a synergistic cryoprotective role with glycerol in the cryopreservation of sperm [23]. It has been suggested that testicular damage induced by several chemicals in mice can be prevented by the oral consumption of Gln, owing to its antioxidant and free radical scavenging properties [14]. It has been reported that Gln supplementation improves the post-thaw motility and fertilizing potential of sperm in many species [4,9,23,31], and also increases catalase activity in post-thaw ram semen [11].

Gln doses ranging from 2.5 to 120 mM have been tested in previous studies on semen extenders [4,10,23]. In one of these studies, it was shown that supplementing the extenders with 50 mM of glutamine had adverse effects on sperm motility and membrane integrity in cool-stored horse semen [28]. Another study demonstrated that the use of glutamine at a higher concentration (>100 mM) in the presence of glycerol was toxic [23].

In the present study, all doses of Gln caused a significant decrease in acrosomal damage at 6, 12 and 24 h of the liquid storage period compared to the controls. Although the exact mechanism by which Gln protects cells from cool-storage damage is not completely understood, the improvements observed in sperm quality in this study may be explained by Gln providing protection with an extra-cellular role [23] and inhibiting the cold shock-induced excessive generation of free radicals, which leads to damages in the sperm plasma membrane [3]. When applied within a dose range of 0.5–2 mM, Gln enabled significant improvements in sperm motility and plasma membrane integrity at 12 and 24 h of the liquid storage period compared to the control group ($P < 0.01$). On the other hand, Phetudomsinsuk et al. [28] reported that supplementation with Gln had adverse effects on sperm motility and membrane integrity in stallion semen during liquid storage at 5 °C up to 96 h.

In conclusion, the results of the present study show that the addition of L-carnitine and Gln to rabbit semen diluted in a Tris-based extender has positive effects on sperm motility, and acrosomal and plasma membrane integrities during liquid storage at 5 °C up to 24 h. Although the exact modes of action of L-carnitine

and Gln are not fully understood, their positive effects on the quality of liquid-stored rabbit sperm observed in this study may be related to the energy supplying role of L-carnitine, the extra-cellular role of Gln and also the antioxidant activities of both supplements.

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