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Effect of L-carnitine administration on the seminal characteristics of oligoasthenospermic stallions

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Abstract

The effect of orally administered L-carnitine on the quality of semen obtained from stallions with different semen qualities was investigated. Four stallions with proven fertility (high motility group, HM) and with normal seminal characteristics (>50% progressive motility and $>80 \times 10^6$ spermatozoa/ml), and four questionable breeders (low motility group, LM) with <50% of sperm progressive motility and $<80 \times 10^6$ spermatozoa/ml, received p.o. 20 g of L-carnitine for 60 days. Blood and semen samples were collected before treatment (T0) and after 30 (T1) and 60 days (T2). Semen evaluation were performed on five consecutive daily ejaculates ($n = 120$ ejaculates) and conventional semen analysis was carried out on each ejaculate, both at collection and after refrigeration for 24, 48, and 72 h. Furthermore L-carnitine, acetylcarnitine, pyruvate, and lactate concentrations, and carnitine acetyltransferase activity (CAT) were determined both in raw semen and seminal plasma. There were an increase in progressive motile spermatozoa only in the LM group (26.8 ± 12.9, 39.1 ± 15.5, and 48.8 ± 8.6 for T0, T1, and T2, respectively). Free seminal plasma carnitine concentration was higher in the LM group compared to the HM one. Both pyruvate and lactate were higher in the LM group. Raw semen and seminal plasma carnitine and acetylcarnitine levels correlate positively with both sperm concentration and progressive motility; moreover, acetylcarnitine content was positively correlated with total motile morphologically normal spermatozoa. In conclusion, oral administration of L-carnitine to stallions with questionable seminal characteristics

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may improve spermatozoa kinetics and morphological characteristics; whereas, it seem to be ineffective in normospermic animals.

Keywords: Stallion; Seminal plasma; Semen; L-Carnitine

1. Introduction

The most common assisted reproduction technique world-wide applied in the equine industry is artificial insemination, either with fresh or frozen semen. Good quality semen is the most important factor to implement breeding programs [1–3]. On stud farms, it is desirable to use stallions with high quality semen since a high proportion of viable cells are lost between semen collection and artificial insemination procedures, particularly in long-term storage of chilled semen or after freezing and thawing. Nevertheless, it is common to obtain poor-quality ejaculates, containing a large number of defective spermatozoa, from aged and/or sub-fertile stallions that are genetically excellent. Therapeutic treatments to improve semen quality could be applied that would allow these valuable animals to be used in breeding programs.

Carnitine has been found in stallion epididymal plasma [4] and represents nearly all the carnitine available in seminal plasma [5]. Carnitine has been claimed to be involved in the acquisition of sperm motility [6–8], and in human beings the uptake of carnitine into spermatozoa and its conversion into acetylcarnitine is considered evidence of good epididymal function [9,10]. Carnitine is taken from the blood stream and then released in epididymal lumen by active transporters [11,12], which are regulated by androgens [13,14] and depletion of epididymal carnitine caused a reduction in fertilizing capacity of hamsters spermatozoa [15]. Carnitine and acetylcarnitine modulate many sperm metabolic functions [16] such as the β-oxidation of fatty acids [17], the intramitochondrial AcetylCoA/free CoA ratio [18,19], which provide readily available acetyl groups [20,21] and increase the utilization of pyruvate and lactate as energetic substrates in mature spermatozoa [22,23]. In humans [24–26], rams [27], and stallions [28], seminal carnitines are correlated with spermatozoa count and progressive motility. Moreover, a reduction in the acetylcarnitine/L-carnitine ratio and seminal plasma carnitines levels has been observed in asthenospermic patients [29,30], severe testicular failures [31], and in infertile patients [26].

Clinical evidence indicates that administration of L-carnitine or acetylcarnitine to infertile patients affected by idiopathic oligoasthenospermia was followed by an increase in spermatozoa concentration and motility [32–34]. Recently, carnitine/acetylcarnitine complex administered to patients with abacterial prostato-vesciculo-epididymitis and elevated reactive oxygen species production improved forward motility and fertilizing ability of semen [35,36]. It has also been demonstrated in the rooster that L-carnitine was able to increase sperm concentration and to reduce lipid peroxidation [37], whilst in boars it improved the seminal characteristics [38]. To our knowledge, there are only two reports on the effect of carnitine administration on stallions. Herfen et al. [39] reported that carnitine had a positive effect on sperm motility, while Rosa Filho et al. [40] found that it had no effect on seminal characteristics.

The objective of the present study was to investigate whether oral administration of L-carnitine to stallion could improve quantitative and qualitative sperm parameters and to evaluate the treatment-response among fertile and oligoasthenospermic stallions.

2. Materials and methods

2.1. Animals and treatment

The experiment was conducted at two stud farms located in Umbria in central Italy (43°07′N, 12°47′W) over a 2-month period, November and January.

Eight healthy 4–12-years old stallions, weighing 450 ± 80 kg of various light-horse breeds, were assigned to the study. The stallions underwent breeding soundness evaluation and no congenital or acquired abnormalities of the genital tract were detected. Four stallions of proven fertility (conception rates of ≥70%; ≥10 mares) were classified as the high motility group (HM) presenting more than 50% of progressive motile spermatozoa, and more than 80×10^6 spermatozoa/ml. Four other stallions considered ''bad breeder'' due to their low semen parameters (<50% progressive motile spermatozoa, and $<80 \times 10^6$ spermatozoa/ml) were classified as the low motility group (LM).

All the experimental animals were maintained in individual stalls, had free access to tap water and received an identical diet based on hay and concentrate pellets. The pellets were administered twice a day mixed with a total of 20 g of L-carnitine (Sigma-tau s.p.a., Pomezia, Rome, Italy) for 60 days (about 40 mg/kg live weight); the hay ration was given only after all the pellets and carnitine had been consumed.

All procedures were carried out with respect to the Italian legislation on animal care (DL n.116, 27/01/1992).

2.2. Semen collection and evaluation

In order to minimize the possible effect of spontaneous variations in seminal characteristics within the animals, semen evaluation was performed on five consecutive daily ejaculates as outlined by Palmer and Fauquenot [41] collected before treatment (T0) and after 30 (T1) and 60 days (T2), for a total of 120 ejaculates.

The gel-free ejaculates were obtained using an oestrous jump-mare, and collected with the Colorado model artificial vagina (Animal Reproduction System, Chino, CA, USA). Immediately after collection, the gross motility of the spermatozoa was estimated using a prewarmed stage of phase contrast microscope (TMS, Nikon Corporation, Tokyo, Japan) at magnification 200×.

Sperm concentration was measured with an hemocytometer, then raw semen was diluted with E-Z Mixin-''CST'' extender (Animal Reproduction System) at 20×10^6 ml^{-1}. The diluted semen was thoroughly mixed, evaluated for progressive motility, and then subdivided into three aliquots. The aliquots, kept in sterile warmed 15 ml capped plastic vials (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA), were then transferred to a 250 ml plastic capped vessel containing 160 ml of 37 °C water which was then placed into a 4°C refrigerator. After 24, 48, and 72 h of storage, the samples were incubated for 30 min at 37 °C prior to progressive motility evaluation.

Immediately after semen collection and filtration, two smears of the native semen, stained with eosin–nigrosin (Sigma-Aldrich S.r.l., Milano, Italy), were prepared [42]. For each sample, 400 spermatozoa (200 per slides) were evaluated for viability under oil immersion bight-field illumination (1000×; Optiphot 2, Nikon Corporation, Tokyo, Japan), and clustered in cells with normal morphology, abnormal midpiece and tail, curved midpiece and tail, detached head and tail [43]. The percentage of morphologically normal (normal, cytoplasmic droplets, and eccentric tail implant) and the total number of motile morphologically normal spermatozoa (TMMNS) were calculated [44].

2.3. Preparation of samples for biochemical analysis

Two 1 ml aliquots of raw semen were stored at −20 °C until analysis for carnitines. Raw semen was also centrifuged at 600 × *g* for 15 min and, after filtration through a 45 μm disposable syringe filter (Millex-HV, Millipore s.p.a., Milano, Italy), two 1 ml aliquots seminal plasma were obtained and stored at −20 °C until carnitines determination.

Blood samples were collected by jugular venipuncture into evacuated heparinized tubes (Becton Dickinson) at T0, T1, and T2. Plasma was separated by centrifugation at 1500 × *g* for 10 min and aliquots were frozen at −20 °C until the analysis.

For lactate and pyruvate analysis, 200 μl of 35% $HClO_4$ were added to 1 ml duplicates of raw semen, seminal, and blood plasma.

2.4. Biochemical analysis

All materials utilized for biochemical analysis were purchased from Sigma-Aldrich S.r.l. unless stated otherwise.

2.4.1. Carnitine and acetylcarnitine

In order to determine free L-carnitine (LC) and acetylcarnitine (AC), 1 ml of 5% cold $HClO_4$ was added to 0.5 ml of blood plasma. The samples were centrifuged at 3000 × *g* for 10 min at +4 °C and analysis was carried out on the supernatant. Ten milliliters of absolute methanol were added to two aliquots of 1 ml of raw semen and seminal plasma and then centrifuged for LC and AC analysis; the methanol extracts were brought to dryness in a flow of N_2 and recovered with 1 ml bidistilled water. LC and AC concentrations were measured using spectrophotometric methods as indicated by Pearson et al. [45].

2.4.2. Pyruvate and lactate

The perchlorised raw semen and seminal plasma samples were centrifuged at 5000 × *g* for 1 h. The extracts were neutralized by adding 0.25–0.3 ml of $KHCO_3$ 3 M. The clear supernatant was analyzed by an automatic analyzer according to the spectrophotometric methods reported by Noll [46] and Lamprecht and Heinz [47].

2.4.3. Carnitine acetyltransferase activity

Carnitine acetyltransferase activity (CAT) in raw semen was measured radioenzymatically at 37 °C as previously described [48]. The medium (pH 7.4) was composed as follows: 0.25 mM of EDTA, 100 mM of Hepes, 0.08% (w/v) Triton X-100, 1 μg/ml of

antimycin A, 1 μg/ml of Rotenone, 0.5 mM (Acetyl-1-^{14}C-) coenzyme A (0.6 Ci/mol) (Amersham Pharmacia Biotech, Buckinghamshire, UK) and 12 mM L-carnitine (Sigma-tau s.p.a.). At 0.3 ml of the aforementioned medium, an aliquot of raw semen was added to assure a final concentration of 2 mg/ml of protein. Incubation was carried out for 2 min before and 2 min after the addition of LC. The reaction was stopped by the addition, under stirring, of 0.3 ml of 2 × 8 Dowex resin diluted 1:1 (w/v). Following the addition of the resin, the samples were placed in an ice bath for 5 min, shaken up three times and then centrifuged at 3000 × *g* for 10 min. The incorporation of (Acetyl-1-^{14}C-) into AC was evaluated on a 0.3 ml aliquot in a scintillation vial and radioactivity was determined by liquid scintillation counting.

2.5. Statistical analysis

Statistical analysis of seminal and biochemical data was performed using ANOVA. The treatment time (T0 versus T1 or T2), and the interaction between groups (HM versus LM) and groups by treatment were considered as main factors; differences between means were compared with the LSD procedure [49]. Coefficient of correlation was performed with a two tails Pearson model [49]. Results are presented as means ± S.D., and data relative to treatment time are presented either distinctly for the two groups ($n = 20$ for each group in each treatment time) or as pooled values ($n = 40$ for each of treatment time).

3. Results

Pellets concentrate mixed with carnitine were accepted readily and all daily rations were consumed. Oral carnitine administration caused a significant increase in blood carnitine levels at T1 and this effect was even more evident at T2 (Table 1). In the LM group, this increase was less pronounced, it was statistically significant only at T2. Acetylcarnitine levels were not significantly influenced by treatments; whereas the ratio with their non-acetylated counterpart showed the same pattern observed for carnitine.

3.1. Effects of treatment on seminal characteristics

In accordance with the experimental design, all the main seminal characteristics differed between the two groups (Table 2). In particular, considering the two groups irrespectively to treatment time, mean sperm concentrations were higher in the HM compared to the LM group ($P < 0.001$). There was no effect of experimental treatment on sperm concentration (Table 3), even if the concentration tended to increase in the LM group over the time ($P < 0.10$). The HM group also had a higher mean number of spermatozoa per ejaculate ($P < 0.001$). The percentage of progressive motile spermatozoa differed according to the groups (Table 2), treatment time (43.31 ± 19.52, 50.68 ± 17.04, and 55.75 ± 14.17 for T0, T1, and T2, respectively) and their interactions. In particular, LM was the only group that manifested a significant increase in the aforementioned parameter following carnitine administration (Table 3). The percentage of morphologically normal live spermatozoa was

Table 1
Carnitine, acetylcarnitine, and acetylcarnitine/carnitine levels (LS, means ± S.D.) in the blood of the experimental stallions considered together ($n = 8$) or subdivided into high (HM, $n = 4$) and low motility groups (LM, $n = 4$), determined before (T0) and after 30 (T1) and 60 days (T2) of oral carnitine administration

Items (nmol/ml)	T0	T1	T2
Overall			
Carnitine	18.63 ± 3.40	26.94 ± 3.05*	29.23 ± 9.05**
Acetylcarnitine	7.29 ± 1.99	6.56 ± 1.18	5.68 ± 1.26
Acetylcarnitine/carnitine	0.41 ± 0.14	0.24 ± 0.02***	0.20 ± 0.04***
HM group			
Carnitine	16.50 ± 3.55	25.91 ± 3.19***	23.92 ± 1.19**
Acetylcarnitine	8.06 ± 1.32	5.88 ± 0.63	5.40 ± 0.66
Acetylcarnitine/carnitine	0.50 ± 0.09	0.23 ± 0.02***	0.23 ± 0.03***
LM group			
Carnitine	20.76 ± 1.53	27.97 ± 2.96	34.55 ± 10.68*
Acetylcarnitine	6.53 ± 2.43	7.24 ± 1.28	5.97 ± 1.75
Acetylcarnitine/carnitine	0.32 ± 0.14	0.26 ± 0.02	0.18 ± 0.03*

* Means within the same row significantly differ between T0, and T1 and T2 for $P < 0.05$.
** Means within the same row significantly differ between T0, and T1 and T2 for $P < 0.01$.
*** Means within the same row significantly differ between T0, and T1 and T2 for $P < 0.001$.

higher in the HM group ($P < 0.001$) and increased with treatment time (44.45 ± 17.71, 50.74 ± 17.26, and 55.39 ± 14.55 for T0, T1, and T2, respectively). The total mean number of TMMNS was higher in the HM compared to the LM group ($P < 0.001$). There was an increasing trend in TMMNS at T2 ($P < 0.07$), irrespective of the group. Spermatozoa morphological features differed according to the animal group; furthermore, we also observed an effect of treatment duration on the percentage of detached heads and tails.

Table 2
Seminal characteristics (LS, means ± S.D.) of the stallions in the high (HM, $n = 4$) and low motility groups (LM, $n = 4$)

Items	Groups		P
	HM	LM	
Gel-free volume (ml)	68.65 ± 43.14	59.02 ± 43.54	
Sperm concentration (10^6 ml^{-1})	180.17 ± 100.49	56.46 ± 39.86	***
Total sperm concentration (10^9)	10.16 ± 6.71	3.12 ± 2.77	***
Progressive motility (%)	61.63 ± 10.67	38.20 ± 15.38	***
Morphologically normal unstained (%)	56.99 ± 12.43	40.39 ± 15.34	***
TMMNS	4.01 ± 3.21	0.53 ± 0.53	***
Abnormal head shape (%)	5.37 ± 1.68	6.38 ± 2.78	*
Abnormal midpiece and tail (%)	5.28 ± 3.76	8.86 ± 6.43	***
Curved midpiece and tail (%)	10.85 ± 6.64	14.60 ± 5.15	***
Detached head and tail (%)	2.02 ± 0.99	5.86 ± 3.85	***
Total live abnormal spermatozoa (%)	18.33 ± 7.21	22.31 ± 6.22	***

TMMNS: total number of motile morphologically normal spermatozoa (10^9).
* Probability of F for Group effect for $P < 0.05$.
*** Probability of F for Group effect for $P < 0.001$.

Table 3
Seminal characteristics (LS, means ± S.D.) of the stallions in the high (HM, $n = 4$) and low motility groups (LM, $n = 4$), evaluated before (T0) and after 30 (T1) and 60 days (T2) of oral carnitine administration

Items	HM			LM			Main effects and interaction		
	T0	T1	T2	T0	T1	T2	T	G	T × G
Gel-free volume (ml)	56.8 ± 35.9	80.5 ± 49.9	68.7 ± 41.3	81.5 ± 58.7	50.0 ± 36.5	45.6 ± 17.3			
Sperm concentration (10^6 ml^{-1})	191.8 ± 93.9	143.3 ± 88.3	205.5 ± 111.8	51.8 ± 43.7	53.9 ± 33.2	63.7 ± 42.8		***	
Total sperm concentration (10^9)	9.1 ± 4.5	9.5 ± 6.5	11.8 ± 8.5	3.5 ± 3.0	2.6 ± 2.5	3.2 ± 2.9		***	
Progressive motility (%)	59.9 ± 6.2	62.3 ± 8.7	62.8 ± 15.3	26.8 ± 12.9	39.1 ± 15.5[a]	48.8 ± 8.6[b]	***	***	**
Morphologically normal unstained (%)	58.0 ± 6.9	61.4 ± 11.1	60.5 ± 17.4	35.9 ± 14.4	40.0 ± 15.7[a]	50.3 ± 8.8[b]	***	***	*
TMMNS	3.1 ± 1.6	3.9 ± 3.2	5.0 ± 4.2	0.4 ± 0.4	0.5 ± 0.4	0.8 ± 0.7		***	
Abnormal head shape (%)	4.9 ± 1.8	5.5 ± 1.5	5.8 ± 1.7	7.0 ± 3.6	6.3 ± 2.1	5.9 ± 2.4		*	
Abnormal midpiece and tail (%)	5.6 ± 3.1	5.3 ± 4.6	5.0 ± 3.6	6.5 ± 4.3	8.7 ± 5.7	11.4 ± 8.0		***	
Curved midpiece and tail (%)	11.9 ± 8.4	9.9 ± 6.4	10.8 ± 4.8	16.0 ± 4.4	14.8 ± 5.9	13.0 ± 4.9		***	
Detached head and tail (%)	2.4 ± 1.3	2.1 ± 0.9	1.6 ± 0.5[a]	7.6 ± 4.7	6.2 ± 3.3	3.8 ± 2.3[b]	***	***	*
Total live abnormal spermatozoa (%)	18.8 ± 6.3	19.0 ± 8.2	17.2 ± 7.2	22.0 ± 6.9	21.8 ± 5.2	23.1 ± 6.6		***	

T: probability of F for Treatment effect; G: probability of F for Group effect; T × G: probability of F for Treatment × Group effect; TMMNS: total number of motile morphologically normal spermatozoa (10^9).

[a] Significantly differ between T0, and T1 and T2 within the same group ($P < 0.01$).

[b] Significantly differ between T0, and T1 and T2 within the same group ($P < 0.001$).

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

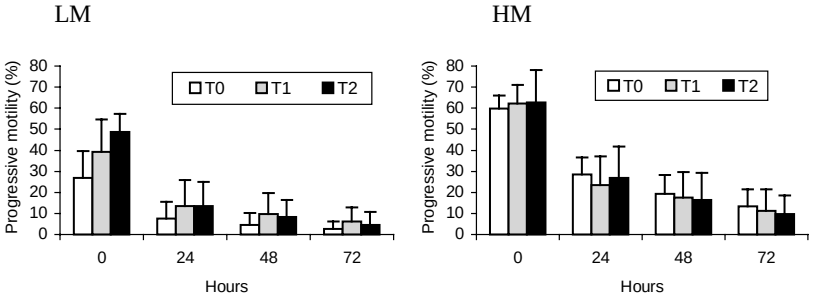

Fig. 1. Progressive motility of spermatozoa at collection and after storage for 24, 48, and 72 h at 4 °C, before (T0) and after 30 (T1) and 60 days (T2) of oral carnitine administration, in the low (LM, $n = 4$) and high motility groups (HM, $n = 4$).

Semen storage ability varied between animal group; but neither treatment time nor interaction between the groups induced any effect (Fig. 1).

3.2. Effects of treatments on biochemical parameters

Free seminal plasma carnitine mean concentrations were higher in the LM group compared to the HM one (Table 4; $P < 0.05$); no significant treatment effect was evidenced (Table 5). Inversely, acetylcarnitine content was higher in the HM group than in the LM one ($P < 0.001$), but treatment duration had no effect (Table 5). The acetylcarnitine/carnitine ratio was higher in the HM group ($P < 0.001$). Pyruvate and lactate behaved similarly; in particular, the pyruvate concentration was higher in the LM group ($P < 0.001$). Treatment decreased pyruvate level ($P < 0.05$) and this was more evident for the LM group ($P < 0.05$). Lactate was also more elevated in the LM group ($P < 0.05$). There was a decreasing trend as the treatment time increased ($P < 0.05$). Their ratio was higher in the LM group compared to the HM one ($P < 0.001$).

The values of carnitine, acetylcarnitine, pyruvate, and lactate concentrations, pyruvate/lactate and acetylcarnitine/carnitine ratios and CAT activity in raw semen are reported in Tables 4 and 6. No significant effect of treatment time, or animal group and their interaction was evidenced for carnitine content, apart from T0 which resulted higher in the HM group (Table 6; $P < 0.05$). Inversely, the mean levels of acetylcarnitine were higher in the HM group compared to the LM one (Table 4; $P < 0.001$). CAT activity was higher in the HM group than the LM one ($P < 0.001$). Similarly to the seminal plasma pattern, also the raw semen pyruvate concentration was influenced by treatment time, showing an overall decrease as treatment time increased (0.269 ± 0.443 nmol/ml, 0.161 ± 0.116 nmol/ml, and 0.123 ± 0.101 nmol/ml for T0, T1, and T2, respectively), for both animal groups and their interaction (Tables 4 and 6). Lactate concentration varied according to the animal group (Table 4), treatment time (5.68 ± 7.74 nmol/ml, 3.11 ± 2.84 nmol/ml, and 2.92 ± 2.76 nmol/ml for T0, T1, and T2, respectively) and their interaction (Table 6).

The correlation coefficients among seminal characteristics and biochemical data are reported in Table 7. Raw semen carnitine and acetylcarnitine levels correlate positively with sperm concentration and kinetic parameters both at collection and after storage.

Table 4
Carnitine, acetylcarnitine, pyruvate and lactate levels, pyruvate/lactate, acetylcarnitine/carnitine ratio, and CAT activity (LS, means ± S.D.) in both the seminal plasma and raw semen of the stallions in the high (HM, $n = 4$) and low motility groups (LM, $n = 4$)

Items (nmol/ml)	Groups		P
	HM	LM	
Seminal plasma			
Carnitine	553.40 ± 274.95	680.56 ± 346.59	*
Acetylcarnitine	70.21 ± 37.46	47.32 ± 19.38	***
Acetylcarnitine/carnitine	0.130 ± 0.040	0.078 ± 0.032	***
Pyruvate	0.113 ± 0.107	0.245 ± 0.220	***
Lactate	3.12 ± 3.44	5.41 ± 6.91	*
Pyruvate/lactate	0.043 ± 0.022	0.071 ± 0.037	***
Raw semen			
Carnitine	800.04 ± 465.46	723.51 ± 377.74	
Acetylcarnitine	152.32 ± 94.90	98.12 ± 55.19	***
CAT	3.35 ± 2.15	1.36 ± 2.41	***
Acetylcarnitine/carnitine	0.212 ± 0.098	0.152 ± 0.091	***
Pyruvate	0.107 ± 0.097	0.262 ± 0.363	***
Lactate	2.99 ± 2.84	4.81 ± 6.59	*
Pyruvate/lactate	0.042 ± 0.019	0.130 ± 0.155	**

CAT: carnitine acetyltransferase activity (nmol/min/10^6 spz).
* Probability of F for Group effect for $P < 0.05$.
** Probability of F for Group effect for $P < 0.01$.
*** Probability of F for Group effect for $P < 0.001$.

Similarly, seminal plasma acetylcarnitine was correlated with the spermatozoa count and motility; moreover, the acetylcarnitine of both samples were positively correlated with TMMNS. The pyruvate/lactate ratio in both raw semen and seminal plasma was negatively correlated with the motility both at collection and after different storage times. The acetylcarnitine/carnitine and pyruvate/lactate ratios were negatively correlated ($P < 0.01$; $n = 120$) both in raw semen ($r = -0.216$) and seminal plasma ($r = -0.445$).

4. Discussion

''Male factor'' infertility and sub-fertility, that have been partially overcome in human beings by the new assisted reproductive technologies made available in recent decades, are of great interest to the equine industry and, therefore, the development of new strategies for such therapy is needed. However, due to the difficulty in finding a sufficient number of sub-fertile stallions in the same time period with similar seminal defects and the reproductive seasonality of this species, controlled studies are scarce, so researchers are deprived of the opportunity of conducting long-term treatment and washout periods. In the study herein reported, we investigated the effect of oral administration of L-carnitine on four oligoasthenospermic stallions, but in order to minimize the effect of subjective variations on seminal characteristics we analyzed the ejaculate on five consecutive days for each animal

Table 5
Carnitine, acetylcarnitine, pyruvate and lactate levels, pyruvate/lactate and acetylcarnitine/carnitine ratio (LS means ± S.D.) in seminal plasma of the stallions in the high (HM, $n = 4$) and low motility groups (LM, $n = 4$), determined before (T0) and after 30 (T1) and 60 days (T2) of oral carnitine administration

Items (nmol/ml)	HM			LM			Main effects and interaction		
	T0	T1	T2	T0	T1	T2	T	G	T × G
Carnitine	658.5 ± 328.9	430.6 ± 210.9	571.1 ± 232.8	616.2 ± 479.3	687.8 ± 279.0	737.6 ± 239.9		*	
Acetylcarnitine	77.06 ± 40.79	50.84 ± 29.46	82.73 ± 34.88	46.17 ± 24.16	47.59 ± 13.06	48.21 ± 20.24		***	
Acetylcarnitine/carnitine	0.119 ± 0.034	0.121 ± 0.038	0.148 ± 0.043	0.089 ± 0.035	0.079 ± 0.035	0.066 ± 0.022		***	
Pyruvate	0.113 ± 0.112	0.157 ± 0.127	0.070 ± 0.052	0.351 ± 0.325	0.198 ± 0.122[a]	0.187 ± 0.108[a]	*	***	*
Lactate	3.38 ± 3.26	3.59 ± 4.37	2.39 ± 2.47	8.36 ± 10.20	4.22 ± 4.04	3.64 ± 3.74	*	*	
Pyruvate/lactate	0.037 ± 0.013	0.057 ± 0.025	0.036 ± 0.020	0.067 ± 0.030	0.069 ± 0.037	0.078 ± 0.043		***	

T: probability of F for Treatment effect; G: probability of F for Group effect; T × G: probability of F for Treatment × Group effect.
[a] Significantly differ between T0, and T1 and T2 within the same group ($P < 0.05$).
* $P < 0.05$.
*** $P < 0.001$.

Table 6
Carnitine, acetylcarnitine, pyruvate and lactate levels, pyruvate/lactate, acetylcarnitine/carnitine ratio, and CAT activity (LS, means ± S.D.) in the raw semen of the stallions in the high (HM, $n = 4$) and low motility groups (LM, $n = 4$), determined before (T0) and after 30 (T1) and 60 days (T2) of oral carnitine administration

Items (nmol/ml)	HM			LM			Main effects and interaction		
	T0	T1	T2	T0	T1	T2	T	G	T × G
Carnitine	925.1 ± 419.3	625.8 ± 430.1	849.3 ± 510.8	627.0 ± 487.6	764.6 ± 340.5	778.9 ± 273.1			
Acetylcarnitine	165.08 ± 105.01	120.41 ± 72.93	171.46 ± 99.95	90.54 ± 62.31	94.51 ± 42.29	109.33 ± 59.84		***	
CAT	2.75 ± 1.51	3.84 ± 2.28	3.39 ± 2.51	2.35 ± 3.89	1.16 ± 1.19	0.63 ± 0.63		***	
Acetylcarnitine/carnitine	0.192 ± 0.085	0.229 ± 0.121	0.219 ± 0.085	0.159 ± 0.061	0.152 ± 0.093	0.146 ± 0.089		***	
Pyruvate	0.086 ± 0.077	0.158 ± 0.130[a]	0.075 ± 0.044	0.451 ± 0.572	0.164 ± 0.104[b]	0.170 ± 0.120[b]	*	***	**
Lactate	3.12 ± 2.71	3.36 ± 3.45	2.50 ± 2.34	8.24 ± 10.09	2.86 ± 2.12[b]	3.33 ± 3.12[a]	*	*	*
Pyruvate/lactate	0.032 ± 0.009	0.056 ± 0.040	0.037 ± 0.015	0.080 ± 0.045	0.088 ± 0.090	0.141 ± 0.249		**	

T: probability of F for Treatment effect; G: probability of F for Group effect; T × G: probability of F for Treatment × Group effect; MSE: between subject mean square error; CAT: carnitine acetyltransferase activity (nmol/min/10^6 spz).

[a] Significantly differ between T0, and T1 and T2 within the same group ($P < 0.05$).

[b] Significantly differ between T0, and T1 and T2 within the same group ($P < 0.01$).

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

Table 7
Significant correlation coefficients among seminal and biochemical characteristics of the eight stallions ($n = 120$ ejaculates)

	Conc.	PM	PM 24	PM 48	PM 72	TMMNS
Raw semen						
Carnitine	0.569**	0.259**	0.321**	0.361**	0.374**	–
Acetylcarnitine	0.693**	0.433**	0.543**	0.559*	0.582**	0.479**
Acetylcarnitine/carnitine	–	0.259**	0.287**	0.244**	0.223*	0.388**
Pyruvate/lactate	–	–	−0.258**	−0.232**	−0.225*	–
Seminal plasma						
Carnitine	–	–	–	–	–	–
Acetylcarnitine	0.583**	0.268**	0.330**	0.297**	0.276**	0.227*
Acetylcarnitine/carnitine	0.303**	0.271**	–	–	–	0.364**
Pyruvate/lactate	–	−0.414**	−0.449**	−0.412**	−0.401**	−0.337**

Conc.: spermatozoa concentration ($\times 10^6$/ml); PM: progressive motility (%); PM 24, 48, and 72: progressive motility after storage for 24, 48, and 72 h; TMMNS: total motile morphologically normal spermatozoa.
* $P < 0.05$.
** $P < 0.01$.

throughout the experimental period. Moreover, to further characterize the efficacy of the treatment, we included four fertile normospermic stallions and, to reduce any seasonal effect, the treatment lasted 2 months in a period characterized by reduced variations in seminal characteristics [1,28].

Blood plasma carnitine levels before treatment were comparable to those previous reported for adult horses [28,50]; no significant differences were observed between the two groups, neither before nor during treatment, although the LM group tended to have a higher carnitine and a lower AC/LC ratio throughout the experiment. Thus, there was no detectable systemic deficit in carnitine availability for the LM group compared to the HM one, in agreement with previous observation in bovine, where those bulls exhibiting the highest blood testosterone and fertility levels also had the lowest concentrations of blood plasma carnitines [51]. Oral carnitine administration resulted in a 45 and 57% increase in plasma levels after 1 and 2 months of treatment, respectively. A doubling of the carnitine concentrations has been reported in horses by other authors [52,53]. These discrepancies could be due to differences in treatment doses, the length of the study, sample collection and analytical procedures, and stallions' age. The decrease in the ratio between acetylcarnitine and carnitine seem mainly due to the increase of the free carnitine concentrations.

The most evident effects of treatment on seminal characteristics, were an increase of sperm motility and morphologically normal live spermatozoa and a decrease in detached heads and tails in the LM group. These effects were time and group dependent, as evidenced by the significant interaction between the group and treatment. Among the previous reports, the experimental procedures differed from ours by treatment dose, semen collection frequency, and the seminal parameters evaluated, thus not allowing us to compare them with our results [39,40]. Most experiments with carnitine therapy intended to improve semen quality have been conducted on human beings [32–35,54–56]. An increase in spermatozoa motility has been observed in treated patients affected with idiopathic forms of oligospermia, asthenozoospermia, and oligoasthenoteratozoospermia

[32,33,53–56] or affected with bacterial inflammation of the accessory sexual gland [35,36]. In a recent double-blind crossover trial, carnitine administration was effective in increasing semen quality, mainly in patients with lower baseline levels; in particular, its greater effect on sperm motility was found in the most severe cases, such as those with the lowest initial forward motile sperm concentration [34]. These findings further fortify our observations; indeed, the stallions of our LM group were in some aspects similar to the oligoasthenozoospermic patients of the aforementioned papers. The HM group, on the other hand, presented seminal characteristics with normal values and this seemed to render them less responsive to treatment.

The increase in the percentage of motile and live morphologically normal spermatozoa caused an augmentation of TMMNS ($P < 0.07$), allowing an increase in the inseminating dose achievable by each stallion (from one to two doses per stallion in the LM group). In the LM group, sperm concentration tended to increase ($P < 0.10$), improving TMMNS. In roosters [37], the most evident effect of carnitine administration was an increase in sperm concentration just after 3–4 weeks of treatment; whereas in human, such an effect was obtained only after 3–4 months of therapy [32,33,54,56]. This increase could be related to a direct effect of carnitine on spermatogenesis, in accordance with the spermatogenic cycle length, which lasts 13–15 days in rooster and 75 days in human. Our findings, were unexpected, as the spermatogenic cycle length of stallions is 57 days and our treatment lasted only 60 days, although Lenzi et al. [34] obtained similar results in human after only 2 treatment months. This may be related to an unknown effect in Sertoli cell-spermatogenic line interaction, an action on postmeiotic phases of spermatogenesis, or an improvement in epididymal microenvironment leading to a reduced sperm phagocytosis. In effect, in the present study, we observed a decrease in the number of detached heads upon treatment in both groups (Table 2). It is well known that one of the major effects of epididymal transit is the stabilization of sperm head and tail structures, in particular nuclear protamine, mitochondrial ‘‘capsule’’ and the coarse outer fibers of flagella thought to be related to the formation of intra- and inter-molecular disulfides [57]. This process is essential for acquiring motility, ultrastructural stability, and fertilizing ability; thus, an improvement in epididymal microenvironment is likely to lead to an increase in sperm quality. Most of this stabilization process is due to oxidation of protein thiols (–SH) to form disulfides (S–S) [58] and an increase in the –SH and (–SH + S–S) ratio has been reported in asthenozoospermic patients, suggesting that this ‘‘over oxidation’’ reflects an abnormal maturation process of the epididymis [59]. In this context, it is intriguing to note, that carnitine also acts as a secondary antioxidant that repairs damage occurring after oxidative noxae [60] and its administration to aging rats improves the glutathione and overall thiols status, perhaps by exerting a sparing activity on thiol and methionine [61]. Therefore, a direct effect of carnitine on the functionality of Sertoli cells is also plausible, as observed by Palmero et al. [62] who reported an increase in both lipid oxidation and glucose utilization by in vitro cultured Sertoli cells in response to carnitine, and concluded that the improvement in semen quality reported after in vivo treatments could be related to its interactions with Sertoli cell functions. Carnitine’s protective role is further sustained by reducing toxicity and accelerating repair processes following physical [63,64] and chemical [65] damages on the testicular parenchyma. However, the positive effects of carnitine administration on the seminal characteristics of the LM group, were not reflected in a improved storage ability. Indeed, as the LM group

presented a minor semen dilution rate, stored sperm cells in the LM group contained more seminal plasma, which could negatively affected spermatozoa storage ability [66].

In the present study, no significant difference in seminal carnitines content were observed due to treatment. Previous studies reported contradictory data on semen carnitines concentrations following experimental administration [34,54,56]. However, even in other fields of medicine, carnitines activity is not related to their level in blood or other biological fluids [22,59,61]. Indeed, a slight increase in caritines levels in seminal fluid could be masked by the high concentration which naturally characterizes this compartment before treatments; moreover, the highly significant correlations we observed among carnitines and spermatozoa concentrations could only be due only to an increase in the intracellular pool of carnitine, although too slight to induce an increase of seminal levels. On the other hand, our findings on carnitines concentrations in both seminal plasma and raw semen demonstrated that the HM group had the higher values, mainly for the acetylated form and evenly considering the pretreatment values alone (data not shown). This also lead to an increase in the acetylcarnitine/L-carnitine ratio in the HM group, in agreement with a reduction of the acetylcarnitine/L-carnitine ratio and seminal plasma carnitines levels observed in several forms of human infertility or seminal deficit [26,29,30,31]. Furthermore, we observed positive correlations among semen motility at collection and after storage and carnitines concentrations, above all the acetylated one, thus confirming our previous observation comparing the first and second ejaculates [28].

To further characterize the effects of carnitine treatment on equine semen quality, we analyzed two indexes of carbohydrate metabolism: as lactate and pyruvate. The present study demonstrated that L-carnitine administration increased pyruvate utilization in both seminal plasma and raw semen in the LM group. Moreover, the seminal plasma and raw semen pyruvate/lactate ratio was negatively correlated to spermatozoa motility and storage ability. This observation suggests that L-carnitine administration improves pyruvate utilization, an elective energetic substratum for sperm motility [67]. In the LM group, the pyruvate/lactate ratio was inversely correlated to acetylcarnitine/carnitine ratio, in both raw semen and seminal plasma. The correct AcetylCoA/CoA ratio is fundamental in order to maintain the proper functionality of the Kreb's cycle for a sufficient production of ATP and the high levels of AcetylCoA inhibit pyruvic dehydrogenase enzyme activity; consequently, the metabolic flow of pyruvate into the Krebs cycle is slowed down. Through CAT activity, carnitine is transformed into acetylcarnitine (buffering effect), which reduces the AcetylCoA/CoA ratio [68] and improves the metabolic flux to Krebs Cycle, with an increased production of ATP, preserving a high motility of the spermatozoa. We observed a higher acetylcarnitine/carnitine ratio in raw semen than seminal plasma and a positive correlation among sperm motility after storage and carnitine and acetylcarnitine levels in the raw semen. The significantly lower seminal plasma acetylcarnitine levels in the LM group confirm the role of carnitine as a marker of semen quality in stallions [28].

In conclusion, oral L-carnitine administration to stallions with questionable seminal characteristics could be of benefit for horse breeders, by improving sperm kinetic and morphological characteristics, thus increasing the number of inseminating doses obtainable. However, L-carnitine administration seem to be ineffective in normospermic stallions. A direct effect of carnitine administration on quantitative spermatozoa traits also seems plausible, but a longer treatment period should be tested in order to reach definitive

conclusions. Our results should encourage more exhaustive research. Moreover, more conclusive findings could be obtained carrying out a study on a large number of sub-fertile stallions treated with carnitine over a longer period and that also includes field fertility trials.

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