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Importance of L-carnitine as Biochemical Marker for Semen Quality Preservation after Cryopreservation in Stallions

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ABSTRACT

Background: The aim of this work is to study the significance of carnitine fractions and NO metabolites for the assessment of spermatozoa resistance to cryopreservation, for which purpose these parameters were investigated in seminal plasma of animals divided according to the indices of spermatozoa of stallions subjected to cryopreservation.

Methods: There were 18 breeding stallions with an average age of 11.28±5.46 years, all 18 were of Arabian breed. Using cluster analysis of indicators of semen quality, animals were identified into two groups: with high and low sperm resistance to cryopreservation-thawing. We assessed standard quality parameters in fresh and cryopreserved sperm, as well as carnitine and its fractions and NO metabolites in stallions' seminal plasma, steroid hormones level and carnitine fractions in blood serum. Data were analyzed using JASP. Differences at $p<0.05$ were considered statistically significant.

Result: Seminal plasma obtained from animals from the cluster with low motility after cryopreservation-thawing was characterized by a lower concentration of nitric oxide metabolites in 1,79 time (p=0.0415) and free carnitine in 1,64 time (p=0.0349). Positive moderate strength correlation were established between the level of seminal plasma free carnitine and the percentage of live spermatozoa after sperm cryopreservation-thawing.

Key words: Cryopreservation, L-Carnitine, NO, Seminal plasma, Stallions.

INTRODUCTION

Cryopreservation is a practical method that is currently widely used in the field of reproductive medicine (Liu and Li, 2020) and horse breeding (Kumar *et al*., 2011). However, significant adverse effects of sperm freezing and subsequent thawing on the structural and functional parameters of spermatozoa have been established (Contreras *et al*., 2023). In our previous work, it was established that cryopreservation of stallion spermatozoa is accompanied by decrease in the number of sperms with intact heads by 19.7%, the number of spermatozoa with acrosome hypoplasia and the absence of internal content after cryopreservation increases by 20.9%, the share of spermatozoa with normal mitochondrial structure decreases by 6.5%, the share of spermatozoa with normal axoneme after cryopreservation decreases by 4.4% (Atroshchenko *et al*., 2019).

Studies have shown that many adverse effects are associated with increased reactive oxygen species (ROS) content during the freezing-thawing process of spermatozoa (Hezavehei *et al*., 2018; Martin Muñoz *et al*., 2015). Normally, there is a balance between antioxidant activity and ROS production in the reproductive tract. Disturbance of this balance leads to oxidative stress (Fujii *et al*., 2003; O'Flaherty and Scarlata, 2022; Peña *et al*., 2019; Shitikova *et al*., 2023). Cryopreservation induces excessive generation of ROS and causes damage to spermatozoa with extreme sensitivity to ROS due to limited cytoplasm, low antioxidant capacity and higher levels of polyunsaturated fatty acids (PUFAs) in the sperm plasma membrane (Hezavehei *et al*., 2018; Peris-Frau *et al*., 2020).

Some studies have shown the high concentration of L-carnitine in epididymis and its potential in stabilizing sperm plasma membrane, increasing sperm survival, as well as decreasing intracellular sperm L-carnitine levels after cryopreservation (Cooper, 1986; Grizard *et al*., 1992; Manee-In *et al*., 2014). Also addition of L-carnitine to the cryopreservation medium can compensate for its loss in sperm and improve sperm parameters during the freezing-thawing process (Ghorbani *et al*., 2021; Longobardi *et al*., 2017; Rezaei *et al*., 2020).

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L-carnitine is best known as a vitamin-like compound that plays an important role as a fatty acid transporter from the cytoplasm to the mitochondria to support β-oxidation processes (Mongioi *et al*., 2016). L-carnitine also plays a major part in protecting cellular membranes, preventing fatty acid accumulation, modulating ketogenesis and glucogenesis and in the elimination of toxic metabolites (Virmani and Cirulli, 2022). Thus, the existing background suggests the role of carnitine as part of a universal adaptation mechanism (Sharma *et al*., 2008). This aspect of the study of the biological properties of L-carnitine remains poorly studied to date and seems interesting in the framework of the study of the possibilities of L-carnitine and its derivatives as biomarkers and potential therapeutic agents in various types of pathology and, possibly, in fertility disorders (Gibb *et al*., 2015; Li and Zhao, 2021).

The effects identified in studies are most often attributed to the well-established antioxidant properties of L-carnitine and its role in sperm metabolism as a mediator of energy production, while anti-apoptotic and anti-inflammatory properties are also mentioned (Kooshesh *et al*., 2023). L-carnitine significantly increases sperm motility and viability during cryopreservation by reducing plasma membrane lipid peroxidation by inhibiting the ROS formation, chelating iron ions necessary for the formation of hydroxyl radicals and mediating free radical scavenging (Kooshesh *et al*., 2023).

It is known that the content of L-carnitine correlates with the level of NO production in endothelium in a lamb model of pulmonary hypertension (Sharma and Black, 2009; Sharma *et al*., 2013). No similar studies have been found on stallions. Nevertheless, a number of experimental studies suggest that the correlation may be universal and is also relevant for the reproductive system of stallions (Atroschenko *et al*., 2022; Zvyagina *et al*., 2018). Along with this, it was found that the level of NO production in stallions correlates with the preservation of sperm motility after cryopreservation-thawing (Ortega Ferrusola *et al*., 2009). In this regard, the role of L-carnitine as a possible biomarker of spermatozoa resistance to cryopreservation is increasing (Ortega Ferrusola *et al*., 2009).

Therefore, the aim of this study was to investigate the relationship between the content of L-carnitine fractions and NO levels in stallions‘ seminal plasma and serum and quality indicators of cryopreserved-thawed semen.

MATERIALS AND METHODS

Animals and semen collection

The study was carried out at All-Russian Research Institute of Horse Breeding (ARRIH, Ryazan Region, Russia) and the Tersk Stud Farm N169 (Stavropol Region, Russia), Perevozsky and Pochinkovsky studs (Nizhny Novgorod region, Russia). The principles of laboratory animal care were followed and all procedures were conducted according to the ethical guidelines of the All-Russian Research Institute of Horse Breeding. The protocol was approved by the Commission for the Control of the Keeping and Use of Experimental Animals (Commission on Bioethics) of the All-Russian Research Institute of Horse Breeding (Protocol Number: 2021/11/2) and the Law of the Russia Federation on Veterinary Medicine No. 4979-1 (14 May 1993).

There were 18 breeding stallions with a mean age of 11.28±5.46 years, all 18 were of Arabian breed.

Clinically healthy stallions were kept in individual stalls. The stallions received hay, oats and granulated compound feed with added minerals in accordance with the established standards and were exercised for at least 1 h daily.

Ejaculates were obtained during the breeding season of the year 2022 (February- April). Ejaculates were collected with an interval of 48 hours using an artificial vagina (ARRIH model, Ryazan, Russia) in the presence of a mare in heat. After a long period of sexual rest (10 days or more), five ejaculates were collected from each stallion at 48-hour intervals.

Sperm evaluation

Immediately after semen collection, gel was removed from the ejaculates using a sterile guaze filter. Then the sperm was filtered using a sterile gauze filter. Thereafter the volume of the ejaculate, concentration of spermatozoa, total and progressive motility were recorded. The ejaculate volume after filtration was determined using measuring cylinder. Concentration of spermatozoa was measured using the SDM1 photometer (Minitube GmbH, Tiefenbach, Germany). The assessment of progressive motility (PM) and (total) TM was implemented using an Argus CASA system (ArgusSoft LTD., St. Petersburg, Russia) and a Motic BA 410 microscope (Motic, Hong Kong, China) in a Mackler chamber at 37°C. From this, total number of spermatozoa (TNS) and total number of spermatozoa with progressive motility (TNS PM) were assessed.

Morphological abnormalities of spermatozoa in the fresh ejaculates were determined using eosin staining (2% aquous solution). The smears were examined at magnification of 1000× using an Olympus BX41 phase-contrast microscope (Olympus Corporation, Japan). Percentage of viable spermatozoa were calculated after counting at least 200-300 cells in the smear.

From this, total number of spermatozoa (TNS) and total number of spermatozoa with progressive motility (TNS PM) were assessed.

The viability of spermatozoa in frozen-thawed semen samples was assessed. Sperm smears were eosin stained and live:dead-ratio was examined using an Olympus BX41 phase contrast microscope (Olympus Corporation, Japan). At least 300 spermatozoa were evaluated in each smear.

To assess the survival of spermatozoa (in hours) during hypothermic storage of sperm, their progressive motility was determined at 24-hour intervals until the PP

decreased to 5%. The principle of the method is to determine the number of sperm with progressive motility (PM) during hypothermic storage of diluted or frozen sperm. After dilution or thawing sperm was stored at a temperature of +40°C. Every day, at 24-hour intervals, the progressive motility of spermatozoa was determined using light microscopy, up to a decrease in their PM to 5%. The period of time from dilution or thawing of sperm to a decrease in sperm PP is called sperm survival and is measured in hours.

Cryopreservation of semen and thawing

Lactose-chelated citrate-yolk medium containing 3.5% glycerin in a volume ratio of 1:3 was used to dilute sperm. After a period of equilibration (for 2 hours at a temperature of +4°C), the diluted sperm was packaged in labeled aluminum tubes with a volume of 20 ml. Sperm was frozen in liquid nitrogen vapor, the freezing time is 7 minutes. The sperm tubes were placed on a polyurethane stand, cooled from +4°C to -127°C for 420 seconds at a freezing rate of 3.2°C per second. The distance from the bottom surface of the sperm tubes to the surface of liquid nitrogen is 20 mm (Naumenkov and Roman'kova, 1971). Sperm was transferred to liquid nitrogen after freezing in liquid nitrogen vapor. Frozen sperm was stored in liquid nitrogen in a bioproduct storage facility (Russia) at a temperature of -196°C.

The frozen sperm was quickly thawed in a water bath at +40°C for 90 sec. After thawing the semen, total and progressive sperm motility, sperm viability and survival were determined at +4°C.

Seminal plasma

Another part of the ejaculate was centrifuged at 2000 g using an ELMI CM-6M centrifuge (SIA ELMI, Riga, Latvia) for 20 minutes. After examination of the supernatant by microscopy, aliquots of seminal plasma that did not contain spermatozoa were frozen at a temperature of -18°C prior to analysis.

Blood serum samples

A blood sample from each stallion from the jugular vein was also taken once during the sperm collection period during the breeding season. Vacuum tubes for taking venous blood "Vacuette" (5 ml, 13×100 mm) series "Premium" with a clot activator and gel (Greiner Bio-One GmbH, Austria) were used for this aim. Blood samples were taken before morning feeding. Blood samples were centrifuged at 400 g for 20 min and serum was stored at 20°C until analysis was performed.

Determination of steroid hormones in serum

Hormonal blood serum analysis-determination of testosterone, estradiol and cortisol was performed on a chemiluminescent analyzer Immulite 2000 (Siemens Healthcare Diagnostics Inc., USA). Dihydrotestosterone was measured using a commercial ELISA kit (DRG Instruments, Marburg, Germany) on a Multiskan ELISA analyzer ("Thermo Labsystems OU", Vantaa, Finland). The ratio of testosterone to dihydrotestosterone [(T/D)*100] was also calculated.

Biochemical investigations in seminal plasma and blood serum

The concentration of carnitine in seminal plasma and blood serum was determined on a Stat Fax 3200 analyzer (Awareness Technology, USA) by Wan and Hubbard method (1998) based on the formation of free CoASH that non-enzymatically reacts with 5,5-dithiobis-2-nitrobenzoate (DTNB) to form stained 5-thio-2-nitro-benzoate, the staining intensity of which was measured spectrophotometrically at λ = 410 nm (Wan and Hubbard, 1998).

The total level of NO metabolites (NOx) in seminal plasma was photocolorimetrically evaluated on Stat Fax 3200 analyzer (Awareness Technology, USA) using Griss reagent (Neva Reactiv, Russia) and vanadium chloride (III) (AcrosOrganics, USA) to determine the sum of nitrites and nitrates (Metelskaya and Gumanova, 2005).

Statistical analysis

The study was of a pilot nature. Data were analyzed using JASP (Version 0.18.1). Parameters characterizing the overall semen quality (ejaculate volume, sperm concentration, TNS PM, proportion of normal spermatozoa), as well as motility (PM, TM) and survival rate of spermatozoa in diluted and thawed semen at +4°C, percentage of live spermatozoa in thawed semen were used for hierarchical cluster analysis. As a result of clustering, all the samples studied were divided into two groups: stable and unstable to cryopreservation-thawing.

The distribution in the groups was evaluated using the Shapiro-Wilk test. In the case of normal distribution, Welch's t-test was used for pairwise comparison, because the sizes of clusters differed. In the case of distribution different from normal, the Mann-Whitney test was used. Correlation analysis of the studied parameters was determined using Spearman's criterion. Differences at $p<0.05$ were considered statistically significant. The results were expressed as Me [Q1;Q3] and Mean±SD, respectively, under nonparametric and parametric distribution.

RESULTS AND DISCUSSION

The groups of animals identified in the cluster analysis differed statistically significantly in terms of progressive and total motility both in the initial biomaterial and after cryopreservation according to the results presented in Table 1.

The performed analysis showed that the levels of sex hormones and cortisol were not statistically significantly different in the samples obtained from different clusters (Table 2). The analysis showed that the levels of sex hormones and cortisol were not statistically

significantly different in samples obtained from different clusters (Table 2).

Due to the increasing number of publications in recent years investigating the role of antioxidants in maintaining sperm motility (Halo *et al*., 2023; Catalán *et al*., 2024), it was decided to analyze the content of carnitine fractions of seminal plasma and blood serum. We also decided to evaluate the content of NO metabolites in seminal plasma, since it was previously found that carnitine may be associated with the process of NO formation in the epididymis, which plays an important role in sperm maturation (Zvyagina and Belskikh, 2022; Zvyagina and Belskikh, 2021; Zvyagina *et al*., 2022).

We found that the seminal plasma of the cluster characterized by higher sperm motility after cryopreservation had statistically significantly higher content of free carnitine and higher level of NO metabolites (Table 3). At the same time, serum carnitine fractions were not statistically significantly different.

To investigate the relationship between the indices of sperm motility, the content of NO metabolites and free carnitine in seminal plasma, a correlation analysis was performed between the indices of biosamples of all studied animals in fresh diluted semen and after its freeze-thawing (Fig 1).

According to the results obtained, the level of free carnitine in seminal plasma significantly positive correlated with sperm concentration and inversely correlated with ejaculate volume, while the level of NO metabolites was statistically significantly correlated with sperm concentration (Fig 1).

It also was observed that the % of Living sperm after thawing statistically significantly positive correlated with free carnitine level (Fig 2).

As it follows from the results of Table 1, statistically significant differences in progressive and total sperm motility of cryopreserved spermatozoa reflected one of the key parameters that are important for effective cryopreservation. Therefore, the next step in the search for factors determining these differences was the evaluation of serum levels of sex hormones and cortisol, which are important for spermatogenesis and sperm function. Our earlier study demonstrated the influence of hormonal background as a factor that can have a significant impact on semen quality parameters (Shitikova *et al*., 2022). Therefore, it was hypothesized that the selected groups of

Table 1: Sperm counts in the studied groups.

Semen parameter	Cluster 1, n=11	Cluster 2, n=7	p	p Shapiro-wilk	
				Cluster 1, n=11	Cluster 2, n=7
Age of stallions,years	10.00 [7.50;12.50]	17.00 [6.00;19.50]	0.3634	0.4901	0.0425*
Sperm volume, mL	21.36±7.50	28.29±8.99	0.1177	0.4234	0.3270
Sperm concentration, million / ml	364.00 [280.0;414.0]	174.0[168.00;181.0]	0.0066*	0.7987	0.0016*
TNS, billion	7.26±3.70	5.14±1.79	0.1250	0.6773	0.4476
TNS PM, billion	5.06±2.81	2.86±1.26	0.0386*	0.6941	0.1354
Progressive motility (FS), %	68.6±10.24	53.30±7.58	0.0023*	0.5759	0.3274
Total motility (FS), %	76.58±9.98	58.31±7.85	0.0006*	0.4961	0.2337
Sperm survival rate at + 4°C (FS), hours	156.0 [132.0;160.0]	120.0 [90.0; 132.0]	0.0395*	0.0175*	0.4449
Spermatozoa of normal morphology (FS), %	76.91±7.05	81.43±7.35	0.2195	0.0996	0.7237
Progressive motility (TS), %	44.06±8.55	17.46±12.21	0.0006*	0.0956	0.6116
Total motility (FS), %	53.65±12.85	25.34±15.97	0.0023*	0.2247	0.2083
Sperm survival rate at +4°C (TS), hour	92.73±31.79	56.57±24.70	0.0163*	0.3480	0.0906
Living sperm (TS), %	31.64±4.7597	20.43±6.99	0.0043*	0.5133	0.6480

Note: Cluster 1- Resistant to cryopreservation-thawing; Cluster 2- Unstable to cryopreservation-thawing, FS- Fresh sperm, TNS- Total number of spermatozoa, TNS PM- The total number of sperm with progressive motility, TS- Thawed sperm. *Significant at p<0.05.

Table 2: Serum steroid hormone values in the studied clusters.

Parameter	Cluster 1, n=11	Cluster 2, n=7	p	p Shapiro-wilk	
				Cluster 1, n=11	Cluster 2, n=7
Testosterone, nmol/L	1.5064±0.7518	2.6143±1.5523	0.1171	0.0924	0.4528
Dihydrotestosterone, pg/mL	109.3182±56.9378	148.8571±92.7100	0.3376	0.0613	0.1746
(T/D)*100	1.13 [0.75; 2.64]	1.63 [1.54; 1.99]	0.3649	0.0249*	0.0216*
Estradiol, pmol/l	142.00 [143.50; 158.00]	146.00 [143.50; 158.00]	0.1337	<0.0001*	0.2860
Cortisol, nmol/L	106.8091±18.1224	110.2714±23.6921	0.7479	0.2754	0.4210

Note: Cluster 1- Resistant to cryopreservation-thawing; Cluster 2- Unstable to cryopreservation-thawing, *Significant at p<0.05.

animals may differ in the profile of sex hormones. Testosterone and its derivatives are known to have a positive effect on sperm motility parameters (Hoffmann and Landeck, 1999). The relationship of blood cortisol and estrogen levels with morphofunctional indices of spermatozoa is still insufficiently studied - there are reports about both positive influence of steroid hormones and the absence of influence (Deichsel *et al*., 2015; Spitzer *et al*., 2022).

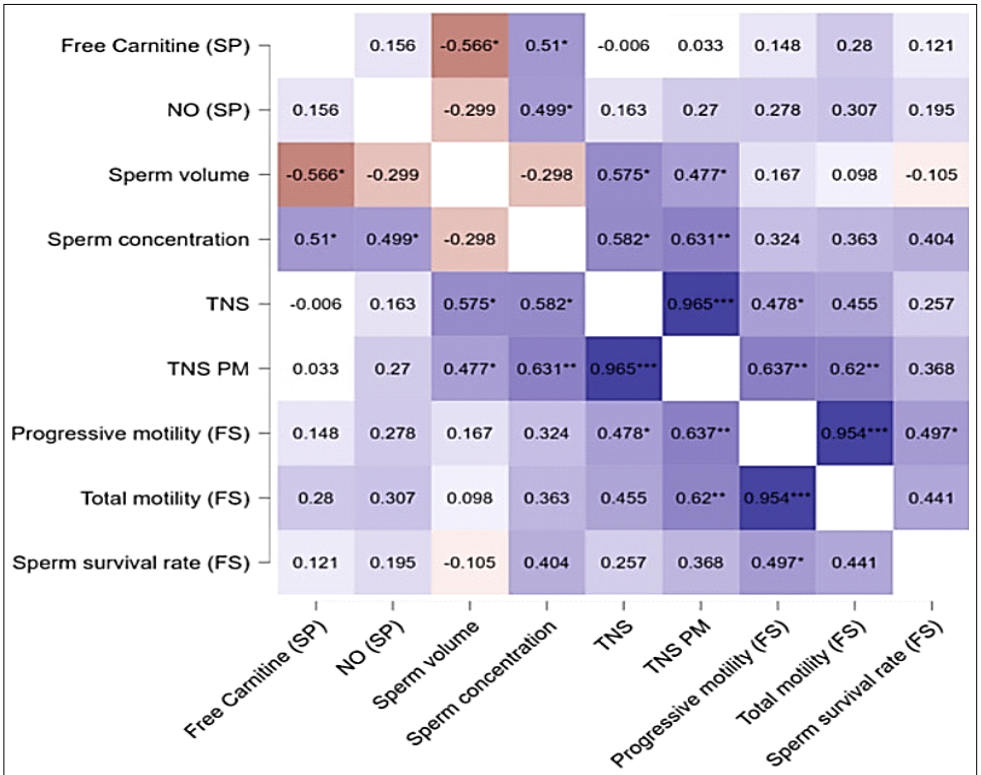
The analysis showed that the levels of sex hormones and cortisol were not statistically significantly different in samples obtained from different clusters (Table 2). This suggested that in the absence of a factor related to changes in sex hormone levels, sperm resistance to freezing stress may be due to factors related to the microenvironment of epididymal contents containing high concentrations of carnitine (Elbashir *et al.,* 2021).

Notably, the level of total sperm plasma carnitine tended to decrease in cluster 2 (Table 2). The comparable level of carnitine in serum indicates that the studied stallions did not have carnitine deficiency (Table 2).

Table 3: Carnitine and nitric oxide metabolites levels in the studied clusters.

Indicator	Cluster 1, n=11	Cluster 2, n=7	p	p Shapiro-wilk	
				Cluster 1, n=11	Cluster 2, n=7
Total carnitine (SP), µmol/L	760.23 [710.84;790.16]	490.96[410.7;720.63]	0.0937	0,0008*	0,7574
Free carnitine (SP), µmol/L	670.98±130.42	410.53±250.54	0.0349*	0,7143	0,9981
Bound carnitine (SP), µmol/L	70.93 [20.97; 70.93]	70.0 [60.33; 150.03]	0.7242	0,007*	0,3654
NO (SP), µmol/L	32.30 [25.57; 43.00]	18.05 [14.00; 26.30]	0.0415*	0,0207*	0,0981
Total carnitine (BS), µmol/L	160.30 [150.30; 250.20]	250.96 [210.30; 280.91]	0.1349	0,0005*	0,5713
Free carnitine (BS), µmol/L	90.63 [70.40; 140.93]	120.70 [120.33; 140.47]	0.3749	0,0252*	0,5752
Bound carnitine (BS), µmol/L	70.93 [60.16; 190.46]	90.40 [50.40; 160.43]	1.0000	0,0321*	0,4621

Note: Cluster 1- Resistant to cryopreservation-thawing; Cluster 2- Unstable to cryopreservation-thawing; SP- Seminal plasma, BS- Blood serum. *Significant at p<0.05.



Note: SP- Seminal plasma, FS- Fresh sperm, TNS- Total number of spermatozoa, TNS PM- The total number of sperm with progressive motility. *Significant at p<0.05, ** - at p<0.01, *** - at p<0.001.

Fig 1: Correlation matrix between the studied sperm parameters in fresh diluted semen and the level of free carnitine and NO metabolites in seminal plasma.

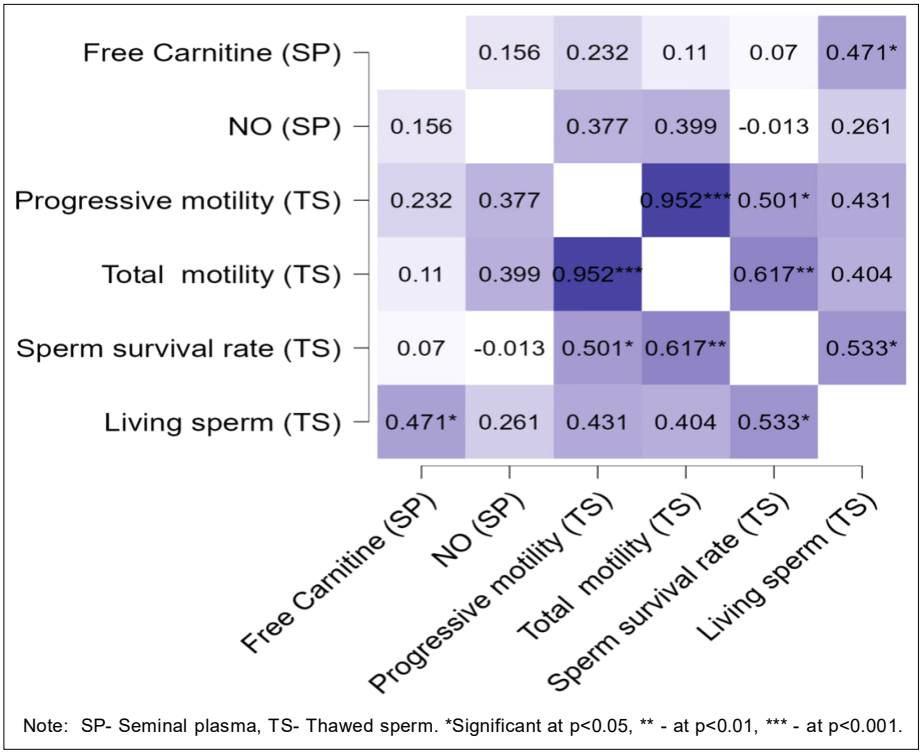


Fig 2: Correlation matrix between the studied sperm parameters after freezing-thawing and the level of free carnitine and NO metabolites in seminal plasma.

It is known that carnitine in the reproductive system is concentrated in the epididymis and the predominance of the free fraction has been established (Cooper, 1986). In this regard, a lower level of free carnitine in biomaterial from stallions of cluster 2 can probably be considered as a possible marker of epididymal dysfunction, which, in turn, is associated with both impaired sperm motility and viability (Elbashir *et al*., 2021). On the other hand, the observed differences in seminal plasma free carnitine levels may indicate the potential role of this compound as a protective factor for spermatozoa under cryopreservation conditions.

Multitude of research indicated number of factors related to motility, acrosomal reaction and apoptosis have been discussed for NO in relation to spermatozoa, these factors are mainly concentration-dependent (Dutta *et al*., 2022; Wang *et al*., 2014). The results obtained suggested that the level of NO production, indirectly determined by its metabolites, may contribute to sperm cryostability (Table 3).

It should be noted that the study has a limitation due to the pilot nature and the small sample size. The study on a larger sample will allow a more detailed analysis of the importance of L-carnitine fractions for preserving sperm motility after cryopreservation.

CONCLUSION

In this work, we confirmed the positive role of L-carnitine for sperm quality, which opens up further prospects for its determination for assessing the quality of frozen sperm.

The results obtained set the stage for further studies of free seminal plasma L-carnitine as a marker of epididymis function and a potential marker of sperm cryoresistance.

Detection of the positive relationship between the level of NO metabolites and L-carnitine content in seminal plasma of stallions suggests an important role of mitochondria in maintaining the viability of spermatozoa under cryopreservation conditions.

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Conflict of interest

The authors declare that there is no conflict of interest.

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