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Study of the Relationship between the Levels of L-carnitine and NO Metabolites in Blood Plasma and Seminal Plasma in Stallions

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ABSTRACT

Background: Artificial insemination with chilled sperm is a reproductive biotechnology often used in horse breeding. However, maintaining the acceptable sperm quality after cooling, which affects the frequency of pregnancy, remains an urgent problem at the present time. Therefore, clarifying the role of important physiological metabolites, such as L-carnitine and nitric oxide, is a significant aspect in terms of possible improvement of the quality of cryopreserved sperm.

Methods: The study included 35 breeding stallions, 27 of which were stallions of the Arabian breed and 8 were of the Soviet heavy draft breed. The content of total, free and bound carnitine and NO metabolites was determined by photometric method in the samples of seminal plasma and blood plasma. The correlation coefficient between the content of L-carnitine and the level of NO metabolites was also determined.

Result: We have found a direct moderate correlation between the level of total and free L-carnitine in the seminal plasma and blood plasma. The study has also revealed a positive correlation between the level of all L-carnitine fractions and NO metabolites in seminal plasma. In addition, there was a positive correlation between the total L-carnitine of blood plasma and NO metabolites in seminal plasma.

Key words: Horses, L-carnitine, Nitric oxide (NO), Seminal plasma.

INTRODUCTION

Artificial insemination with chilled sperm is a reproductive biotechnology often used in horse breeding (Olaciregui *et al.,* 2014). However, maintaining the acceptable sperm quality after cooling, which affects the frequency of pregnancy, remains an urgent problem at the present time (Baishya *et al.,* 2015; Pathak *et al*., 2020; Ugur *et al.,* 2019). The exact reasons for the decreased sperm motility after cryopreservation remain unclear. It is assumed that they may be associated with oxidative stress and the resulting imbalance between the reactive oxygen species (ROS) formed and the activity of antioxidant defense systems (Agarwal *et al.,* 2006; Pande *et al.,* 2019). In addition, disorders may occur due to differences in the content of substances in the seminal plasma that ensure the normal functioning of spermatozoa such as L-carnitine (Nery *et al.,* 2020). It plays an important role in the oxidation of long-chain fatty acids in the mitochondria participating in the production of energy necessary for sperm motility. In addition, it regulates the acyl-CoA/CoA ratio and also has an antitoxic effect due to binding and eliminating of poorly metabolized acyl groups in the form of carnitine esters (Mongioi *et al.,* 2016). L-carnitine may exhibit antioxidant, anti-inflammatory and anti-apoptotic effects in various pathophysiological conditions (Chiu *et al.,* 2004; Surai, 2015).

Free L-carnitine is absorbed from the blood plasma and transported to the epididymal plasma. Then it diffuses passively into the spermatozoa, where it accumulates as free and acetylated carnitine. The initiation of sperm motility is proportional to the concentration of L-carnitine in the lumen of the epididymis (Chiu *et al.,* 2004). The determination of free L-carnitine in seminal plasma serves as a marker for the diagnosis of obstructive azoospermia (Agarwal and Said, 2004). On the other hand, the reactive oxygen and nitrogen species (ROS/RNS) naturally generated by spermatozoa, including superoxide anion, hydrogen peroxide and nitric oxide (NO), are very important in regulating the sperm energy as well as the acrosome reaction that spermatozoa need to implement the possibility of fertilization (Buzadzic *et al.,* 2015).

But if the levels of RNS become excessively high, there are damaging effects that can affect the structure and function of proteins as well as the catalytic activity of enzymes. They change the organization of the cytoskeleton and disrupt the transduction of cellular signals. When NO interacts with the superoxide anion, peroxynitrite (ONOO-)

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is formed, which can induce lipid peroxidation and nitrosation like other RNS, but its effects are concentrated on tyrosine molecules, which usually act as mediators of enzyme functions and signal transduction (Bartesaghi and Radi, 2018).

Therefore, the aim of this research was to study the relationship between the levels of L-carnitine in the seminal plasma and blood plasma as well as to determine the correlation coefficient between the content of L-carnitine and NO metabolites in stallions.

MATERIALS AND METHODS

Animals and sperm collection

The study was conducted on the livestock of the All-Russian Research Institute of Horse Breeding (ARRIH, Ryazan Region, Russia) and Tersk Stud Farm No. 169 (Stavropol Territory, Russia), Perevozsky and Pochinkovsky stud farms (Nizhny Novgorod region, Russia). All procedures were carried out in accordance with the "European Convention for the Protection of Vertebrates Used for Experimental and Other Scientific Purposes" ETS No. 123 (March 18, 1986) and the Law of the Russian Federation "On Veterinary Medicine" No. 4979-1 (May 14, 1993). The protocol of the present investigation was approved by the Local Ethics Committee of the All-Russian Research Institute of Horse Breeding (ARRIH), Ryazan Region, Russia.

The research was carried out on 35 stud horses, 27 of which were stallions of the Arabian breed and 8 were of the Soviet Heavy Draft breed. The average age of the animals was 11.1±0.9 years, with a minimum of 4 years and a maximum of 20 years. The stallions were kept in separate stalls. The stallions received hay, oats and granulated mixed food with mineral supplements in accordance with the established norms and trained for at least 1 hour daily.

Sperm from stallions was obtained during the breeding season of 2021 (February- April) with the help of an artificial vagina (ARRIH model, Ryazan, Russia) of a mare over the rutting period. Five ejaculates were collected from each stallion at 48-hour intervals.

Laboratory studies and analysis of the data obtained were carried out in 2021 (May-October) at the All-Russian Research Institute of Horse Breeding (ARRIH), Ryazan Region, Russia and Ryazan State Medical University Named after Academician I. P. Pavlov, Ryazan, Russia.

Collection of seminal plasma and blood plasma samples

Immediately after receiving the sperm, the gel was removed. Then the sperm was filtered with the help of a sterile gauze filter.

To obtain seminal plasma, the aliquot of native sperm was immediately centrifuged at 1200 g for 20 minutes using an ELMI CM-6M centrifuge (SIA ELMI, Riga, Latvia).

The supernatant was taken and after light microscopy for the absence of spermatozoa with the use of the Olympus BX41 phase contrast microscope (Olympus Corporation, Japan), the samples were frozen and stored at a temperature of -20°C until the analysis was carried out.

A sample of each stallion's blood from the jugular vein was taken once during the sperm collection period. Blood samples were taken before morning feeding. Then they were centrifuged at 400 g for 15 minutes and the plasma was stored at a temperature of -20°C until the analysis was carried out.

Determination of total, free and bound (latent) carnitine and NO metabolites in seminal plasma and blood plasma

The content of total, free and bound carnitine and NO metabolites was determined in the samples of seminal plasma and blood plasma. The determination of NO metabolites was performed using the method modified by Metelskaya and Gumanova (2005) on a StatFax 3200 EI analyzer (Awareness Technology Inc., USA).

The test samples were preliminarily deproteinized by adding 96% ethyl alcohol in a ratio of 1:2 and then centrifuged at 300 g for 20 minutes. Equal volumes of the test sample were measured out into a dish for enzyme immunoassay. They were mixed with 10% solution of the Griess reagent (NevaReaktiv LLC, Russia) in 12% acetic acid ("Baza No. 1 Himreaktivov" CJSC, Russia) and 8% solution of VCl_3 (Acros Organics, USA) in 1 M HCl (prepared ex tempore). The mixture was incubated in a shaker at 37°C for 30 minutes. After that, the optical density was determined at a wavelength of 545 nm with a differential light filter of 630 nm against the control of reagents. The concentration of NO metabolites was calculated using a calibration curve constructed from a series of dilutions of an aqueous 1 M solution of $NaNO_2$.

The concentration of L-carnitine in the seminal plasma and blood plasma was determined by Wan L. and Hubbard R. W.'s method based on the formation of free CoASH, which undergoes nonenzymic reaction with 5.5-dithiobis-2-nitrobenzoate (DTNB) to form a colored 5-thio-2-nitrobenzoate, the intensity of which was measured spectrophotometrically at $\lambda = 410$ nm (Wan and Hubbard, 1998).

Preparation of samples to determine the concentration of total carnitine

200 µl of blood plasma or seminal plasma sample was mixed with 10 µl of 2 M KOH and incubated for 45 min at the indoor temperature for complete hydrolysis of the ester bonds. Then 40 µl of $HClO_4$ solution was added to the mixture, stirred for 10 seconds and 30 µl of 2 M KOH was added for neutralization. The resulting solution was centrifuged at 300 g for 5 minutes using the ELMI CM-70M-09 centrifuge (SIA ELMI, Riga, Latvia). The supernatant was used for determination.

Preparation of samples for determination of free carnitine concentration

40 µl of $HClO_4$ solution was mixed with 200 µl of blood plasma or seminal plasma sample and added to 40 µl of 2 M KOH for neutralization and centrifugation at 300 g for 5 min.

The incubation medium containing 180 µl of 50 mM HEPES/KH_2PO_4/K_2HPO_4 buffer with 0.02% DNTB (pH=7.5),

25 µl of the test sample and 20 µl of acetyl-CoA solution was placed into a microtube of the enzyme immunoassay analyzer. The reaction was initiated by the addition of 35 µl of carnitine acetyltransferase solution. The optical density was measured. The concentration of carnitine was calculated according to the calibration curve constructed from a series of dilutions of an aqueous 5 mM solution of L-carnitine. The concentration of latent carnitine was calculated as the difference between total and free carnitine.

The results were expressed in µmol/l of plasma (seminal plasma).

Statistical analysis

Statistical analysis was carried out using the program Statistica 10 and "Microsoft Office Excel 2016" (StatSoft Inc., USA). The nonparametric Mann - Whitney U-test and Spearman's coefficient (Rs) were used to estimate the rank correlation in the studied groups. The results were presented in M±SE format, where M is the mean value and SE is the standard error of the mean. The differences were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

The research has studied the relationship between the levels of total, free and bound L-carnitine in seminal fluid and blood plasma. As can be seen from the presented graphs (Fig 1), we have found a direct moderate correlation between the level of total L-carnitine in the seminal plasma and blood plasma of stallions as well as a similar positive correlation between the content of free L-carnitine in the seminal plasma and blood plasma of the studied animals and the absence of a statistically significant connection between the concentration of latent L-carnitine under the same conditions.

It is known from the literature that physiological concentrations of NO play an important role in the control of sperm physiology. Spermatozoa are the source of NO and the constitutive forms of NOS appear to be involved in sperm motility, condensation and acrosomal response (Herrero and Gagnon, 2001; Wang *et al.,* 2014). The type and degree of changes caused by NO depend, among the other things, on the redox potential of the cell and since L-carnitine has well-known antioxidant properties (Calo *et al.,* 2006; Surai, 2015), we decided to check whether there is interdependence between L-carnitine levels and NO metabolites.

In our study, we have found a strong positive correlation between the level of total L-carnitine and the content of nitric oxide metabolites in the seminal plasma (Fig 2A) as well as the absence of a statistically significant correlation between the concentration of total L-carnitine in the seminal plasma and the level of NO metabolites in the blood plasma.

Since NO is a short-lived, highly reactive molecule and its half-life in the blood is 0.05-1.8 ms (Rassaf *et al.,* 2002), NO cannot be transported by the blood over significant distances and therefore acts as an autocrine and paracrine regulator.

In addition, a direct interconnection has been found between the level of free L-carnitine and NO metabolites ($r=0.65$, $p=0.0000$) and the level of latent L-carnitine and nitric oxide (II) metabolites in seminal plasma of stallions ($r=0.59$, $p=0.0002$). It is also interesting that there is a direct relation between the level of total L-carnitine in the blood plasma and NO metabolites in the seminal plasma (Fig 2B). We have also found a direct correlation between the level of free L-carnitine in the blood plasma and NO metabolites in seminal plasma ($r=0.50$, $p=0.0023$). Our findings can be explained by two mechanisms. Firstly, one of the reasons is the antioxidant effect of L-carnitine and the possible prevention of the conversion of the nitrogen oxide molecule to peroxynitrite ($ONOO^-$). Secondly, it was previously demonstrated that the expression of the eNOS gene increases in cultured human endothelial cells after L-carnitine incubation (Abd-Elrazek and Ahmed-Farid, 2017). Thirdly, we can't exclude that carnitine participates in the excretion of degradation products of ADMA, which is a natural NO-synthase inhibitor. But all these assumptions require further research and confirmation.

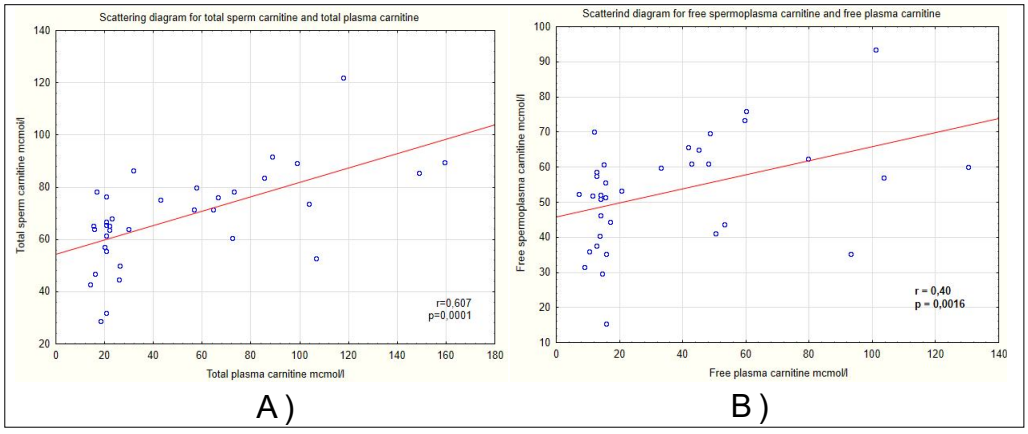

Fig 1: A) Scattering diagram for total blood plasma L-carnitine and total L-carnitine in seminal plasma. B) Scattering diagram for free blood plasma L-carnitine and free L-carnitine in seminal plasma.

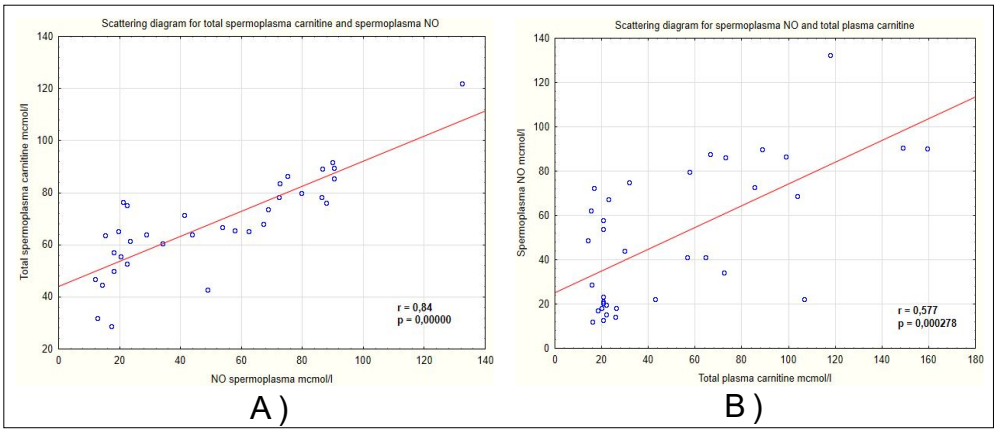

Fig 2: A) Scattering diagram for the total L-carnitine and the NO metabolites in seminal plasma. B) Scattering diagram for NO metabolites in seminal plasma and the total blood plasma L-carnitine.

CONCLUSION

We have found the statistically significant direct correlations between the main fractions of carnitine and NO metabolites, which indicate the presence of interdependence between the metabolism of nitric oxide and the level of carnitine in the seminal plasma of stud horses. The study of this relationship directly in the seminal plasma is of great significance for the identification of the functional role of L-carnitine and its interaction with nitric oxide (II). It is also important for understanding what the primary disorder in this tandem is. The study of the interaction between L-carnitine and nitric oxide (II) will open the prospect of using L-carnitine in the comprehensive therapy of fertility diseases aimed at potentiating the formation of NO, or preventing its degradation.

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Conflict of interest: None.

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