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Original Scientific Article

ASSESSMENT OF THE EFFECT OF SELECTED COMPONENTS OF EQUINE SEMINAL PLASMA ON SEMEN FREEZABILITY

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ABSTRACT

In this study, selected components of seminal plasma in equine semen were evaluated. Levels of enzymes, electrolytes, microelements and some other components were observed. The aim of this study was to find some important differences between the levels of these components and the total sperm motility after freezing and thawing (freezability of the semen). Total of 32 ejaculates from 7 stallions were collected, assessed and prepared in 0,5 ml straws for freezing. After thawing, the sperm motility was analyzed and ejaculates were divided into two groups: "good" freezable and "poor" freezable. The only statistically significant difference between groups of „good" and „poor" freezable ejaculates was in the concentration of vitamin E in the seminal plasma. In the group of „good" freezable ejaculates, the level of vitamin E was significantly lower ($p \leq 0,05$) than in the group of "poor" freezable ejaculates.

Key words: stallion, seminal plasma, semen freezing, vitamin E

INTRODUCTION

Seminal plasma is a very important part of the ejaculate and it is involved in many sperm functions, especially in the metabolism and in events preceding fertilization. But nevertheless, the physiological role of seminal plasma and its components is still not fully understood (1). Also, the effect of seminal plasma on sperm during storage, freezing or cooling is not completely clear.

So far, not many studies about the components of seminal plasma and the freezability of the ejaculate have been conducted. Until now, studies in horses have not shown that it is possible to predict the freezability of semen based on the concentration of some enzymes, microelements (2), total protein or composition of the protein spectrum (3).

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However, questionable or different information is found in certain studies or in studies on other animal species (4).

MATERIAL AND METHODS

Animals and semen collection

In this experiment, 7 licensed stallions were used. The stallions represented various breeds and ranged in age from 4 to 16 years old. During the semen collection, animals were stabled and fed at the Equine Clinic, University of Veterinary and Pharmaceutical Sciences Brno. All of them were in good clinical status and used in breeding with normal fertility. From each stallion 1 to 11 ejaculates were collected, but only 32 ejaculates were used in this study. Four ejaculates were excluded because they did not meet some of the basic quality requirements for semen freezing (2x low percentage of motile sperm, 2x urospermia).

The ejaculates were collected routinely using the Missouri model artificial vagina while stallions were mounted on a dummy with teasing mare standing in front of the phantom.

Evaluation of collected semen

Following collection, each ejaculate was filtered with non-woven semen filter (Minitüb,

Germany) to remove the gel fraction. Overall 36 ejaculates were collected and evaluated. After filtration a macroscopic examination was done: volume, color, odor and viscosity were evaluated. In the microscopic evaluation total sperm motility, concentration and morphology were included. Total sperm motility was assessed by light microscope with phase contrast (enlargement 400x) by one person to eliminate the influence of different observers. Concentration was counted in a Burker chamber and morphology was examined at high power under immersion oil (enlargement 1000x) using Giemsa stain.

Semen processing and freezing

After the macroscopic evaluation of each ejaculate, samples for microscopic evaluation were taken and the ejaculate was centrifuged (1000 G for 10 minutes). Seminal plasma was collected using a sterile syringe and the sediment of the sperm was diluted with French diluent in the concentration of 800 x 10^6 sperm per ml. The diluted semen was transferred to 0,5 ml straws and after 2 hours of equilibration in a fridge (+4°C), it was frozen in liquid nitrogen.

Samples with seminal plasma were kept frozen in a freezer (-18°C) until analysis. After the gathering of all samples, the samples were thawed and examined in the Large Animal Clinical Laboratory at UVPS Brno.

For biochemical analysis, the spectrophotometry analyzer LIASYS (Analyzer Medical System, Italy) was used.

The concentration of microelements was measured by using flame atomic absorption spectrometry 3500 Atomic Absorption Spectrometer (Thermo Electron Corporation).

Evaluation of thawed semen

Frozen-thawed ejaculate was evaluated at least 24 hours after freezing. The straws were thawed in a water bath at 38,5 °C for 30 seconds. Total sperm motility was assessed in a light microscope with phase contrast (enlargement 400x) immediately after thawing, in every case by one person. The total sperm motility after thawing was always the criterion for the evaluation of freezability. Ejaculates were divided into two groups (A, B):

Group A:

Ejaculates with post-thaw sperm motility ≥35% were classified as „good“ freezeble (n = 18).

Group B:

Ejaculates with post-thaw sperm motility <35 % were classified as“poor” freezable (n = 14).

Statistical evaluation

Results from group A and B were compared by a nonparametric Wilcoxon signed rank test for unpaired data. In cases where the result from the laboratory was disproportionately high or low, this result was not used in this study. If these extreme results were used, the overall result could be influenced and distorted by them.

RESULTS

Average values of basic parameters of ejaculates in both groups are shown in Table 1. The results of the measured selected parameters, which were monitored in the seminal plasma, are arranged in Table 2. Two parameters which were monitored, but not shown in the table, are cholesterol and vitamin A. In all samples the level of these parameters was zero.

Table 1. Average values of basic parameters of ejaculates in groups A and B (Motility II: motility after thawing)

	Group A (ejaculates with post-thaw motility ≥ 35 %)	**Group B** (ejaculates with post-thaw motility < 35 %)
	Mean ± SD	**Mean ± SD**
Volume (without gel fraction) (ml)	68,9±28,36	65,9±46,06
Motility after collection (%)	73,4±5,77	61,4±9,90
Sperm concentration (x10^6/mm^3)	123,6±80,54	129,1±108,74
Morphologically abnormal sperm (%)	28,0±5,88	33,1±13,52
Motility II (%)	41,7±7,07	19,1±12,92
Sperm concentration after thawing (x10^6/mm^3)	889,6±319,90	730,1±361,76
Morphologically abnormal sperm after thawing (%)	32,6±5,88	33,4±13,45

After the statistical analysis of both groups of ejaculates, the only significant difference ($p \leq 0,05$) was in the concentration of vitamin E in the seminal plasma. In the group of ejaculates where motility after thawing was ≥ 35%, concentration of vitamin E was significantly lower ($p \leq 0,05$) than in the group of ejaculates with motility < 35 % after thawing.

In this study we tried to find differences in the composition of seminal plasma between „good" and „poor" freezable ejaculates in stallions. The only monitored parameter: vitamin E showed statistically significant differences ($p \leq 0,05$) between the two groups listed above. It is interesting that higher levels of vitamin E were found in the group of „poor"

Table 2. Average values of selected biochemical parameters of ejaculates in groups A and B

	Group A (ejaculates with post-thaw motility ≥ 35 %)		**Group B** (ejaculates with post-thaw motility < 35 %)	
Monitored parameter	**n**	**Mean±SD**	**n**	**Mean±SD**
Total protein (g/l)	18	5,9±2,99	14	7,7±4,67
Creatinine (μmol/l)	18	54,3±19,42	13	53,2±21,23
Urea (mmol/l)	18	6,7±0,91	14	6,2±1,26
Alkaline phosphatase (ALP) (μkat/l)	13	65,9±28,96	8	56,4±38,29
Alanine amino-transferase (ALT) (μkat/l)	18	0,2±0,06	14	0,2±0,08
Aspartate-amino-transferase (AST) (μkat/l)	18	0,9±0,81	14	0,9±0,51
Creatinekinase (CK) (μkat/l)	13	2,1±1,56	13	2,2±1,13
Gama-glutamyl-transferase (GMT) (μkat/l)	11	54,6±14,61	6	47,7±18,74
Lactate-dehydrogenase (LDH) (μkat/l)	10	0,5±0,31	9	0,6±0,25
Na^+ (mmol/l)	18	109,6±17,97	14	109,1±20,49
K^+ (mmol/l)	18	21,9±5,77	14	24,5±10,55
Ca (mmol/l)	17	2,3±0,80	12	1,7±1,23
P (mmol/l)	18	0,7±0,44	13	0,8±0,45
Cl (mmol/l)	18	124,7±17,15	14	117,2±17,00
ZnS (μg/l)	18	4,8±2,02	12	4,9±2,29
Cu (μg/l)	17	4,1±2,17	12	5,2±3,11
Mg (μg/l)	18	1,8±0,94	14	1,3±0,63
Se (μg/l)	17	5,7±3,69	13	6,5±3,43
Vitamin E (mmol/l)	18	0,015±0,0269*	14	0,068±0,0720*

* $p \leq 0,05$

DISCUSSION

The results of some studies show that seminal plasma negatively affects sperm during short term and also long term storage (5-7). Some other studies denote that in humans (8), and also in stallions (9-11) the presence or adding specific amounts of seminal plasma before storage seems to improve sperm motility during cooled storage and also after thawing. However, the effect of seminal plasma differs significantly between individual stallions (12, 13).

freezable ejaculates (mean 68 μmol/l), while in the group of „good" freezable ejaculates the mean value of vitamin E was only 15 μmol/l. During semen cryopreservation in humans, the adding of vitamin E to extender in a dose 200 μmol has positive effects on sperm motility in samples from fertile and also subfertile men. When vitamin E in a dose 100 μmol was added, no positive effect on sperm motility was observed (14). Similar observations were made in boars (15), but adding vitamin E to the extender had no effect on sperm motility. When levels of vitamin E were measured in the fresh ejaculate of men (16),

no correlation between vitamin E (α-tocopherol) and sperm motility was found. It is a question if the difference between concentrations of vitamin E in our two groups of ejaculates is not just an accidental finding because of the very low concentration of this vitamin in stallion seminal plasma or if sperm motility after freezing and thawing is really influenced by vitamin E. This vitamin is well known as an antioxidant. It is possible that lower use of vitamin E in the antioxidant processes in „poor" freezable ejaculates is the reason for its higher level in this group of ejaculates. However, more samples and further studies are necessary for confirmation or refutation of the results of our study.

The antioxidant role of vitamin E and selenium is very closely related. In the study of Mahmoud et al. (17) it was demonstrated that supplementation of selenium and vitamin E in parenteral form in rams has a positive effect on the quality and quantity of semen. Similar observations were made in stallions (18). The authors proved that oral supplementation with selenium, vitamin E and zinc in stallions leads to the improvement of some quality parameters in ejaculate, especially the improvement of sperm motility. However, the results of another study did not show any improvement of sperm motility in fresh ejaculate and in ejaculate after 24 hours of storage at 5°C when stallions and pony stallions were supplemented with a combination of vitamin E, vitamin C, L-carnitine and folic acid (19). Over the last few years there has been a lot of work on antioxidants, fat acid or commercially available feed additive dietary supplementation, but the results are not very conclusive (20-22). Unfortunately, in none of the studies the levels of supplemented substances were not monitored in seminal plasma.

In this study not only vitamin E was determined, but also selenium. No statistically significant difference was found in the concentration of selenium. From the observations of Bertelsmann et al. (23) it is obvious that in stallions the level of selenium in the blood does not corresponded with the level of selenium in seminal plasma, but the concentration of selenium positively correlated with sperm motility in fresh ejaculate.

In this study, no correlation between the monitored elements was found. The monitored elements were Zn, Cu, Mg, Ca and P. This concurs with the work of Barrier-Battut et al. (2). In humans, a strong correlation between zinc concentration in seminal plasma and sperm motility exists (24, 25), but correlations with other elements has not been confirmed (26). From this view, a measurement of seminal plasma zinc concentration in fertile and subfertile stallions would be interesting.

Concentrations of Na, K and Cl did not significantly differ between both observed groups. The role of these ions during sperm cooling and freezing, as well as the influence on sperm motility before and after storage is still not very clear. There are some studies about these ions in seminal plasma, but the results differ. Also, connection or correlation with semen quality or freezability is still not clear enough (1, 27-30).

The total protein was higher in the group of „poor" freezable ejaculates, but the difference was not statistically significant. It is known that protein concentration is lower in pre-spermatic fraction of ejaculate and the highest protein concentration is in the first phase of spermatic fraction in stallions (31). The total protein seems not to be a very important parameter in stallion ejaculate, but further research of single proteins in seminal plasma could give us some valuable information (1).

Also enzyme concentrations which were monitored in this study are not useful for ejaculate freezability predictions. It has been seen that gama-glutamyl-transferase (GMT) has an important role in sperm protection against oxidative stress, so it was expected that this enzyme could be a good marker for semen freezability (32, 33). Pesch et al. (29) and Kareskoski and Katila (1) found a correlation between GMT concentrations in seminal plasma and sperm motility. In our study, the concentration of this enzyme was higher in the group of „good" freezable ejaculates, but without statistical significance.

A very strong positive correlation between the concentration of lactate-dehydrogenase (LDH) and sperm motility was found (29), but in our study LDH concentrations were almost the same in both groups of ejaculates. Another difference between the „good" and „poor" freezable group was in the concentration of alkaline phosphatase (ALP) but without statistical significance.

Concentrations of ALP were slightly higher in the group of „good" freezable ejaculates. In stallions, as well as in dogs, ALP is an indicator of ejaculation (34, 35). In the study by Kareskoski et al. (30) there was a clear difference between ALP concentrations in seminal plasma in fertile and subfertile stallions. The same result was found in dogs, where there was also a correlation between the concentration of ALP and ejaculate freezability (36). Average values of other monitored enzymes: aspartate-amino-transferase (AST), creatine kinase (CK) and alanine amino-transferase (ALT) was the same in both groups or the difference was minimal.

Creatinine and urea are well known markers for the detection of urine in ejaculate. In both groups, levels of creatinine and urea were almost the same. In all of the samples, the border for urospermia was not exceeded.

Evaluation of the effect of seminal plasma components on sperm and the freezability of ejaculate is complicated. Only little information is available about some of the components, while concerning other components information is not complete, clear or uniform. So, in this part of the research there is still a lot of work and verification to be done.

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