Analysis of seminal plasma biochemical parameters and sperm cryostability in different age groups of stallions

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Abstract. We studied sperm of stallions of different age groups of the Arab breed at the age of 4 to 21 (n=36). The concentrations of total protein (TP), albumin, globulins, glucose, urea, phospholipids, ionized calcium (Ca²⁺), and enzyme activity of creatine kinase (CK), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), amylase, lactate dehydrogenase (LDH) were determined in seminal plasma. After sperm freezing and thawing the progressive sperm motility and viability at +4 °C were studied. We found that progressive motility and viability of spermatozoa after freezing and thawing in stallions at the age of 6-10 years was statistically significantly higher than that of stallions at the age of 4-5 (p=0.004 and p=0.02), 11-15 (p=0.04 and p=0.04) and 16-21 (p=0.01 and p=0.01, respectively). The concentration of sperm in the ejaculates of older stallions (16-21 years old) is significantly lower than in the ejaculates of stallions aged 4-5 (p=0.04), 6-10 (p=0.003), 11-15 (p=0.04). The level of urea in seminal plasma of older stallions (16-21 years old) was statistically significantly higher than in the group of stallions aged 6-10 (p=0.01). There is a negative correlation between progressive sperm motility after cryopreservation and urea concentration in stallion seminal plasma (r=-0.48; p<0.05).

1. Introduction
The method of artificial insemination has a number of advantages over natural mating. The sperm preservation of the most outstanding sires and representatives of endangered species has become possible through the establishment of cryobanks. The cryobank of The All-Russian Research Institute for Horse Breeding currently contains the genetic material of 45 outstanding stallions of 15 horse breeds [1]. The success of cryopreservation is influenced by such factors as cryopreservation technology, a diluent for sperm freezing, breed, and individual characteristics of the animal [2], among which age is of particular interest, because there is no clear information at the moment about the influence of this factor on the result of cryopreservation.

Seminal plasma is a microenvironment of spermatozoa and creates the necessary complex of conditions for their normal functioning, and a set of enzymes and metabolites found in seminal plasma largely affects the quality and cryostability of sperm [3].
The aim of the research was to study the quality of cryopreserved semen and some biochemical parameters in seminal plasma of stallions of different age groups.

2. Material and methods
36 stallions of purebred Arabian breed aged 4 to 21 were used in the experiments. The conditions of stallion keeping and feeding complied with the established norms.

Semen from stallions was obtained during the breeding season (February-May) with an interval of 48 hours. Parameters of the first two stallion sperm ejaculates after a period of sexual rest were not used in the treatment. At least 5 ejaculates from each stallion were received. Determination of seminal plasma biochemical parameters were conducted in one of the most typical for each stallion ejaculate.

The volume and concentration of sperm were determined in each ejaculate after receiving sperm. Further, the ejaculate was divided into two parts, one part was diluted with lactose-chelate-citrate-yolk (LCHCY) medium in a ratio of 1:3, determined the progressive motility of sperm, as well as the viability of sperm in hypothermic sperm storage at a temperature of +4 °C. Then we froze sperm in vapors of liquid nitrogen according to the standard technology of the All-Russian Research Institute for Horse Breeding [4]. Frozen sperm was stored in liquid nitrogen at a temperature of −196 °C. Thawing of cryopreserved sperm was carried out in a water bath at a temperature of +40 °C for 90 seconds. Progressive motility and viability of sperm were also determined after thawing of cryopreserved semen at a temperature of +4 °C.

Another part of the ejaculate was centrifuged at 3000 rpm for 15 min. After microcopy of the supernatant the aliquots of the seminal plasma free of sperm were frozen in Eppendorf tubes (2.0 ml) at a temperature of -18 °C before studies. In the resulting seminal plasma we determined the concentration of total protein (TP), albumin, globulins, glucose, urea, and the enzyme activity of creatine kinase (CK), alanine aminotransferase (ALT), aspartate aminotransferase (AST), amylase, lactate dehydrogenase (LDH), alkaline phosphatase (ALP) in biochemical analyzer AU 680 (Beckman Coulter, USA) at standardized photometric methods of clinical laboratory studies, the concentration of phospholipids - in biochemical analyzer AU 480 (Beckman Coulter, USA), ionized calcium (Ca²⁺) – in electrolyte analyzer EASYLYTE CALCIM (MEDIA CORP., USA).

Statistical processing was carried out using the program Statistica 10.0 and "Microsoft Office Excel 2016". The nonparametric Mann – Whitney U-test was used for estimation of statistical significance in the study groups and Spearman coefficient was used for estimating the rank correlation. The median and upper and lower quartiles were determined for each sample. The results were presented in Me [Q1/Q3] format. Differences were considered statistically significant at p<0.05.

3. Results and discussion
All samples were divided into 4 groups depending on the age of the stallions and analyzed (table 1). The first group included ejaculates of stallions at the age of 4-5 years, the second group – at the age of 6-10 years, the third – 11-15 years, and the fourth group – at the age of 16 to 21 years.

It was found that the viability of diluted sperm at a temperature of +4 °C in stallions aged 6-10 (group 2) was statistically significantly higher than these indicators compared with the 1st (p=0.01), 3rd (p=0.02) and 4th (p=0.01) groups. It was also found that the progressive motility of native sperm of stallions aged 6-10 (group 2) and 11-15 (group 3) was statistically significantly higher than these indicators compared with the 1st (p=0.01 and p=0.01) and 4th (p=0.01 and p=0.004, respectively) groups.

High rates of sperm activity and viability after freezing and thawing are one of the most important characteristics of successful cryopreservation. It was found in our study that the progressive motility of sperm after cryopreservation in stallions aged 6-10 (group 2) significantly exceeded these indicators compared to the 1st (p=0.004), 3rd (p=0.04) and 4th (p=0.01) groups. It was also found that the viability of sperm at a temperature of +4 °C after cryopreservation in stallions aged 6-10 (group 2) is also significantly higher than these indicators compared with the 1st (p=0.02), 3rd (p=0.04) and 4th (p=0.01) groups.
Table 1. Semen quality and biochemical parameters in seminal plasma of stallions of different age groups, Me [Q1/Q3], (n=36)

| Indicator                                      | Group 1                  | Group 2                  | Group 3                  | Group 4                  |
|------------------------------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| n                                              | 6                        | 11                       | 11                       | 8                        |
| Age (years)                                    | 4-5                      | 6-10                     | 11-15                    | 16-21                    |
| Volume (ml)                                    | [31.0 [24.8/33.5]         | [30.0 [20.5/34.0]         | [20.0 [17.5/26.5]         | [28.0 [26.5/33.5]         |
| Protein (g/L)                                  |                          |                          |                          |                          |
| Sperm concentration \( \times 10^6 \text{ ml}^{-1} \) | [288.5b]                 | [265.0b]                 | [295.0b]                 | [167.0]                 |
| Progressive motility (%)                       | [47.5a]                  | 60.0                     | 60.0\(^{c}\)            | 50.0\(^{a}\)            |
| The viability at a T +4\(^{0}\)C (hour)        | [120.0\(^{a}\)           | 164.0                    | 156.0\(^{b}\)           | 120.0\(^{b}\)           |
| Progressive mobility in cryopreserved sperm (%) | [18.5\(^a\)]            | 30.0                     | 25.0\(^{a}\)            | 20.0\(^{a}\)            |
| The viability at a T +4\(^{0}\)C in cryopreserved sperm (hour) | [54.0\(^a\)] | [108.0]                  | 60.0\(^{a}\)            | 48.0\(^{a}\)            |
| Total protein (g/L)                            | 15.1 [11.7/17.3]         | 14.3 [10.8/19.0]         | 17.5 [12.8/18.7]         | 12.7 [8.9/24.9]         |
| Albumin (g/L)                                  | 1.0 [0.6/1.1]            | 1.2 [0.7/1.5]            | 1.2 [0.7/1.6]            | 1.0 [0.5/1.9]            |
| Globulins (g/L)                                | 14.0 [11.1/16.4]         | 13.4 [10.6/17.6]         | 15.8 [12.0/17.1]         | 11.8 [8.4/22.9]         |
| Glucose (mmol/L)                               | [0.3 [0.3/0.5]           | 0.5 [0.1/0.6]            | 0.3 [0.2/1.0]            | 0.7 [0.5/0.8]           |
| Phospholipids (mmol/L)                         | 0.7 [0.6/1.0]            | 0.6 [0.5/0.7]            | 0.5 [0.4/1.0]            | 0.5 [0.4/1.0]           |
| Urea (mmol/L)                                  | 5.5 [4.6/5.9]            | 4.4\(^{b}\) [4.3/4.6]   | 4.6 [4.3/5.0]            | 5.1 [4.9/5.3]           |
| ALT (IU/L)                                     | 3.5 [1.9/4.6]            | 3.0 [1.6/4.3]            | 4.7 [2.4/5.6]            | 3.3 [1.8/4.8]           |
| AST (IU/L)                                     | 154.1 [97.6/229.7]       | 111.0 [74.0/322.4]       | 185.9                    | 165.5                   |
| Amylase (U/L)                                  | 3.8 [3.2/5.3]            | 5.3 [2.8/6.1]            | 4.9 [3.0/5.8]            | 4.2 [2.3/6.7]           |
| Creatine kinase                               | 279.4                    | 252.7                    | 352.4 [93.9/522.9]       | 554.0                   |
| (U/L)                                          | [117.7/390.3]            | [152.5/649.4]            | [135.8/1000.2]           |                         |
| LDH (IU/L)                                     | 640.8                    | 445.8                    | 491.2                    | 218.0                   |
| (U/L)                                          | [235.9/712.2]            | [166.8/836.0]            | [150.9/970.7]            | [164.5/1037.1]          |
| ALP (IU/L)                                     | 24644.0                  | 24610.0                  | 36949.0                  | 14765.0                 |
| (U/L)                                          | [21128.3/34175.5]        | [20950.0/26021.0]        | [16471.0/45542.0]        | [9765.0/19450.0]        |
| Ca\(^{2+}\) (mmol/L)                          | 1.7 [1.2/2.7]            | 1.9 [1.5/2.0]            | 3.0 [1.3/5.0]            | 2.7 [1.1/4.2]           |

\(^{a}\) - statistically significant differences from group 2 (6-10 years old), p < 0.05;  
\(^{b}\) - statistically significant differences from group 4 (16-21 years old), p < 0.05;  
\(^{c}\) - statistically significant differences from group 1 (4-5 years old), p < 0.05.

There was a statistically significant increase in the urea level in group 4 (16-21 years old) compared to group 2 (6-10 years old) \((p=0.01)\). In addition, there was a decrease in the concentration of total protein in group 4 (16-21 years old) compared to other groups, but this indicator did not give statistical
significance. Possible described changes are related to restructuring of the catabolic metabolism in older horses. In addition, Koskinen E. et al. (2002) found that the highest concentration of TP is observed in ejaculates the most "rich" in sperm [5], and in our earlier studies, the ejaculates with sperm concentration of less than 150 million/ml had low content of TP [6]. In this study the described assumptions are confirmed, since the 4th group (16-21 years old) has not only the lowest concentration of TP, but also the sperm concentration in the ejaculates of stallions of this group is statistically significantly lower compared to the concentration in the other study groups. The concentration of albumin and globulins did not differ significantly in the examined groups, and it might make sense for the study of advanced protein spectrum in seminal plasma.

There were no statistically significant differences between the groups with respect to such indicators of carbohydrate metabolism as LDH and amylase activity, as well as glucose concentration. However, it is worth noting that the 4th group (16-21 years old) showed the lowest activity of LDH and the highest concentration of glucose compared to other groups. Despite the fact that the main method of ATP production in stallion sperm is oxidative phosphorylation [7], glycolysis is used to generate energy in the process of acrosomal reactions [8]. Inhibition of LDH activity leads to inhibition of glycolysis [9], which may be accompanied by an increase in glucose concentration. Apparently, in stallions of the older age group (16-21 years old) ATP level is maintained by creatine phosphate, since the activity of creatine kinase in group 4 (16-21 years old) is higher than in the other groups, but the difference is not statistically reliable.

The activity of alkaline phosphatase in seminal plasma is considered as a marker for ejaculation in stallions [10]. A significant difference in the activity of ALP in fertile and subfertile stallions was found in the study Kareskoski A. M. et al. [11]. We found no statistically significant differences in the activity of alkaline phosphatase in the study groups, however, in the 4th group (16-21 years) the lowest value of this indicator was observed.

Some authors believe that the high activity of intracellular enzymes such as ALT and AST in seminal plasma may indicate damage of sperm membranes [12, 13]. In this study we didn’t find statistically significant differences in the activity of these enzymes.

Of particular interest to scientists is the study of the content of phospholipids in seminal plasma, as it is believed that they are a target for the action of free radicals [14], and their content in sperm and seminal plasma decreases with fertility disorders in men [15]. The age of horses did not affect the value of this indicator in our study.

Calcium is a universal regulator of cellular processes, including its participation in the process of reproduction. Calcium ions are involved in capacitation, acrosomal reaction [16], progressive motility, hyperactivation, as well as directly or indirectly involved in the regulation of apoptosis in ejaculated and epididymal gametes [17]. According to Pesch S. et al. (2006) 60-75% of total calcium in seminal plasma have on ionized calcium [18]. No statistically significant differences in this indicator were found in the studied groups.

We also found a negative correlation between the progressive sperm motility after cryopreservation and the concentration of urea in the seminal plasma of the studied stallions (r=-0.48; p<0.05).

4. Conclusions

1. Viability of diluted sperm in stallions aged 6-10 (group 2) is statistically significantly higher than these indicators compared with the 1st (p=0.01), 3rd (p=0.02) and 4th (p=0.01) groups. Progressive motility of native sperm in stallions aged 6-10 (group 2) and 11-15 (group 3) is statistically significantly higher than these indicators compared with the 1st (p=0.01 and p=0.01) and 4th (p=0.01 and p=0.004, respectively) groups.
2. Progressive motility and viability of spermatozoa after freezing and thawing in stallions at the age of 6-10 years is statistically significantly higher than that of stallions at the age of 4-5 (p=0.004 and p=0.02), 11-15 (p=0.04 and p=0.04) and 16-21 (p=0.01 and p=0.01, respectively).
3. The concentration of sperm in the ejaculates of older stallions (16-21 years) is significantly lower than in the ejaculates of stallions aged 4-5 (p=0.04), 6-10 (p=0.003), 11-15 (p=0.04).
4. The level of urea in seminal plasma of stallions of older age (16-21 years) was statistically significantly higher than in the group of stallions 6-10 years (p=0.01).

5. There is a negative correlation between progressive sperm motility after cryopreservation and urea concentration in stallion seminal plasma (r=-0.48; p<0.05).

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References
[1] Lebedeva L F, Atroschenko M M, Burmistrova S A 2015 Main factors affecting mare insemination with cryopreserved domestic and foreign sperm Agricultural Biology 50(2) 476–485 (doi: 10.15389/agrobiology.2015.4.476eng)

[2] Singh M, Ghosh S K, Prasad J K, Kumar A, Ramteke S S, Bhure S K 2013 Heparin binding proteins of buffalo bulls seminal plasma and their relationship with semen freezeability Indian Journal of Animal Sciences 83(7) 700–704

[3] Mráčková M, Zavadilová M, Sedlinská M 2015 Assesment of the effect of selected components of equine seminal plasma on semen freezeability Macedonian Veterinary Review 38(1) 91–96 (doi: 10.14432/j.macvetrev.2015.01.037)

[4] Naumenkov A I, Roman'kova N K 1971 Laktozo-helato-citratno-zheltochnyj razbavitel' spermy zherebca Teoriya i praktika sovershenstvovaniya porod loshadei XXV Divovo 128–132 [In Russ.]

[5] Koskinen E, Karlsson M, Reilas T, Sankari S, Andersson M, Güvenc K, Katila T 2010 Alkaline and acid phosphatase, β‐glucuronidase and electrolyte levels in fractionated stallion ejaculates Reproduction in Domestic Animals 45(6) 369–374 (doi: 10.1111/j.1439-0531.2009.01579.x)

[6] Attia Y A, Kamel K I 2012 Semen quality, testosterone, seminal plasma biochemical and antioxidant profiles of rabbit bucks fed diets supplemented with different concentrations of soybean lecithin Animal 6(5) 824–833 (doi: 10.1017/S175173111002229)

[7] Turner R M O, McDonnell S M 2003 Alkaline phosphatase in stallion semen: characterization and clinical applications Theriogenology 60(1) 1–10

[8] Kareskoski A M, Reilas T, Sankari S, Andersson M, Güvenc K, Katila T 2010 Alkaline and acid phosphatase, β-glucuronidase and electrolyte levels in fractionated stallion ejaculates Reproduction in Domestic Animals 45(6) 369–374 (doi: 10.1111/j.1439-0531.2009.01579.x)

[9] Atroschenko M M, Zaitcev A M, Kalashnikov V V, Chetverikov L A, Rustakova E A, Kulakov V V, Saitanov E O 2017 Study of biochemical indices of plasma of stallion’ sperm Konevodstvo i konnyi sport 4 24–27 [In Russ.]

[10] Gibb Z, Aitken R J 2016 The Impact of Sperm Metabolism during In Vitro Storage: The Stallion as a Model BioMed Research International 2016(2) 1–8 (doi: 1–8 10.1155/2016/9380609)

[11] Attia Y A, Kamel K I 2012 Semen quality, testosterone, seminal plasma biochemical and antioxidant profiles of rabbit bucks fed diets supplemented with different concentrations of soybean lecithin Animal 6(5) 824–833 (doi: 10.1017/S175173111002229)

[12] Katila T 2001 In vitro evaluation of frozen-thawed stallion semen: a review Acta Veterinaria Scandinavica 42(2) 199–217 (doi: 10.1186/1751-0147-42-199)

[13] Kirilenko E A, Onopko V F 2017 Oxidative stress and male fertility: modern view on the problem Acta Biomedica Scientifica 2(2) 102–108 [In Russ.] (doi: 10.12737/article_59a614fd84d146.40261567)
[15] Antonov M P, Zhigulina V V 2012 Effect of biochemical changes of spermatozoon and spermoplasma lipids on ejaculate fertility *Upper Volga Medical Journal* **10**(3) 47–50 [In Russ.]

[16] Breitbart H 2002 Intracellular calcium regulation in sperm capacitation and acrosomal reaction *Molecular and Cellular Endocrinology* **187**(1–2) 139–144 (doi: 10.1016/S0303-7207(01)00704-3)

[17] Mendoza F J, Perez-Marín C C, García-Marín L, Madueño J A, Henley C, Aguilera-Tejero E, Rodríguez M 2012 Localization, distribution, and function of the calcium-sensing receptor in sperm *Journal of Andrology* **33**(1) 96–104 (doi: 10.2164/jandrol.110.011254)

[18] Pesch S, Bergmann M, Bostedt H 2006 Determination of some enzymes and macro- and microelements in stallion seminal plasma and their correlations to semen quality *Theriogenology* **66** 307–313 (doi: 10.1016/j.theriogenology.2005.11.015)