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ASSOCIATED CONNECTION OF ERYTHROCITARY ANTIGENS WITH CHARACTERISTICS OF STALLION SEMEN AFTER CRYOCONSERVATION

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Abstract

Cryopreservation of animal sperm is the most important way to preserve endangered breeds and species. This is of particular relevance in the horse breeding of Russia and Ukraine given the critical decrease in the livestock of pedigree animals to 1.3 million and up to 300 thousand heads, respectively. For example, in Ukraine only 3 out of 12 officially registered breeds have the minimum required number of breeding stallions and mares. Therefore, the increase in the characteristics of stallion sperm and the improvement of the ability to predict the effectiveness of cryopreservation are of great scientific and practical importance. This article presents the results of the studies on the associated dependence of erythrocyte antigens A, C, D and K of the blood groups of horses of Ukrainian selection. The associated relationship of the efficiency of cryopreservation of stallion sperm has been revealed depending on the antigenic characteristics of erythrocytes according to A, C, D, and K blood group systems. It has been shown that the obtaining of the average parameters of stallion spermatozoa motility and survival after cryopreservation of 2.5 points and 4 hours, respectively, was accompanied by the presence of alleles of erythrocyte antigens *bcm/cgm*, *bcm/de*, *bcm/dg*, *bcm/dk*, *cegm/cgm*, *cegm/d*, *cegm/dg*, *cegm/dk*, *cgm/*cgm, *cgm/d*, *cgm/dg*, *de/d*, *de/dk*, *dk/d*, *dk/de*, *dk/dk* of the blood group D system. High semen characteristics after cryopreservation, i.e. the motility more than 4 points and survival spermatozoa more than 4 hours were obtained from the stallions with alleles of erythrocyte antigens *bcm/d*, *cgm/de*, *dg/dk* of the blood group D. The degree of the influence of erythrocyte antigens of the blood group D on the cryoresistance of the stallion semen is 32.5 % ($p < 0.001$), on the motility of the thawed semen — 18.2 % ($p < 0.01$), and on the survival rate of the thawed spermatozoa — 25.2 % ($p < 0.001$). The absence of the blood group A antigens in the stallions was accompanied by a significant increase in the biological characteristics of the semen after cryopreservation. In the absence of erythrocyte antigens of the blood group A, the activity of the sperm in stallions was higher by 0.51 points ($p < 0.05$) as compared to the control, by 1.36 points ($p < 0.01$) as compared to the carriers of *a/–*, by 0.17 points as compared to the stallions with *ad/–*. It has been shown that the presence of *a/–* of the blood group C contributed to a significant ($p < 0.05$) impairment of the activity (by 0.33 points), of the survival (by 0.78 hours) and of the semen durability (by 6.26 %) as compared to the stallions in which there were no alleles of this blood group system. If the antigens of the blood group K were not inherited by stallions, the activity of the thawed sperm was 0.35 points higher ($p < 0.05$) than in the carriers of *a/–* of the K system, and by 0.40 points ($p < 0.05$) higher than in the control stallions in which the antigenic profile of erythrocytes was not determined. It has been found out that the degree of the influence of the alleles of the blood group K on the activity of spermatozoa after thawing was 1.4 % ($p < 0.05$), on the semen durability — 2.0 % ($p < 0.05$), on the absolute value of durability — 1.5 % ($p < 0.05$), on the spermatozoa preservation — 1.2 % ($p < 0.05$). The generalized dispersion analysis of the obtained data has shown that the degree of the influence of the antigenic characteristics of erythrocytes on sperm cryoresistance in the stallions of Ukrainian selection was 38.7 % ($p < 0.001$) for blood group D, 1.7 % ($p < 0.05$) for blood group A, 16.6 % ($p < 0.01$) for blood group C, and 12.9 % ($p < 0.01$) for blood group K.

Keywords: erythrocyte antigens, alleles of systems of blood groups, cryopreservation of se-

Cryopreservation of animal sperm is the most important way to preserve endangered breeds and species. This is of particular relevance in the horse breeding of Russia and Ukraine given the critical decrease in the livestock of pedigree animals to 1.3 million and up to 300 thousand heads, respectively [1-3]. For example, in Ukraine only 3 out of 12 officially registered breeds have the minimum required number of breeding stallions and mares [4]. Therefore, the increase in the characteristics of stallion sperm and the improvement of the ability to predict the effectiveness of cryopreservation are of great scientific and practical importance.

The modern physiology focuses more on the impact of microorganisms on the primary characteristics of semen after cryopreservation, which is explained by the subsequent application of the preserved semen doses in the system of artificial insemination of horses [5-7]. Many other factors are analyzed, which can affect the main spermogram parameters of stallions: endocrine profile, breed, age, time of year, overall physiological condition, mycotoxins, overall chromosomal instability, etc. [8-12]. Work is underway to evaluate the efficiency of cryopreservation of epididymal semen of stallions [1].

At the same time, the associated link between the erythrocyte antigens with ejaculate parameters after cryopreservation is almost not studied at all [13-15] in spite of the fact that their association with fertile potential of horses was proven as far back as in 1940-s [16-18]; only a few researches point out the necessity of such work [19-22]. Whereas 20 blood group systems of humans have been officially recognized today out of 35 existing groups [23], horses have only 9 such systems, where four are of particular practical value, A, C, D, and K, the associated link of which with spermogram parameters after cryopreservation has not been analyzed. The ability to improve mare fertilization rate during mating is displayed based on impact of immunogenetic markers; however, the association of these markers with stallion spermogram parameters have not been studied [24]. The only research of biological properties of stallion native semen properties in combination with the antigenic erythrocyte profile showed that these properties are associated, but their association with the cryopreservation performance was not taken into consideration [4].

We have compared the antigenic properties of A, C, D and K erythrocytes of horse blood group systems with the primary semen properties after cryopreservation for the first time and have proven that high mobility and survival rate of sperm cells after cryopreservation is observed in specimen with a certain set of D system erythrocyte antigens, and absence of A blood group system antigens is accompanied with a certain increase of biological properties of semen after cryopreservation.

The goal of the presented research was to identify the association links between erythrocyte antigens of A, C, D and K stallion blood group systems and properties of their semen after cryopreservation.

Techniques. The research was performed on 1676 fresh ejaculates, of which 1413 displayed cryogenic resistance. The semen was received from 69 stallions of 9 stud farm breeds (Ukrainian Saddle Horse, the Thoroughbred, the Trakehner, the Hanoverian, the Belgian, the Westphalian, the Arabian horse, the Orlov Trotter and Russian Trotter) of stud farms, pedigree breeding units, horse sports club of Kharkiv, Poltava, Zaporizhia, Luhansk, Kyiv, Zhitomir and Dnipropetrovsk region (Ukraine) during 10 years, starting with 2005. The ejaculates were selected onto sterile artificial vagina with a sterile semen receiving unit after sanitary treatment of stallions, and Kharkiv technology was used for selection and cryopreservation of semen [4, 9]. The activity of sperm cells in the de-

frosted semen was determined in points (1 point = 10% of sperm cells with rectilinear translation) visually in Jenaval light microscope (Carl Zeiss, Germany) with lens magnification of ×10-20, sperm cell survival rate (in hours) in the thermostat at 37 °C, the absolute survival rate indicator (in conventional units), preservation of sperm cells as percentage of the initial values in the native seamen using generally accepted methods [25]. The defrosted semen parameters were analyzed individually for alleles from blood group systems (D, A, C and K). The immunogenetic genotyping by erythrocyte antigens of stallion blood group systems was conducted in the direct agglutination immunoassay by application of standard monospecific serum reagents verified by international standard reagents and manufactured in the genetics laboratory of the Animal Breeding Institute of the National Academy of Agrarian Sciences of Ukraine (Aa, Ad, Ca, Da, Db, Dc, Dd, De, Dg, Dk, Ka) using generally accepted methodologies [13]. When determining stallion genotypes by blood groups in each system both alleles were identified, which were inherited from parents. The genotypes were marked delineated by a line: the allele before the line is inherited from a father, the allele after the line is inherited from the mother.

The statistical processing was performed using generally accepted methods of variation statistics, the statistical significance of differences was evaluated according to Student *t*-criterion [26]. The tables show mean (*M*) and mean deviations (±SEM). The dispersion analysis was performed using a specialized application program package SPSS for Windows (nonparametric statistics) (IBM, USA).

Results. The test data regarding identification of associative links between erythrocyte antigens of D blood group system with semen properties after cryopreservation are shown in Table 1.

1. The association of alleles of erythrocyte antigens of D (EA D) **blood group system with stallion semen properties after cryopreservation** (*M*±SEM, *n* = 1413)

EA D	Number of samples	Activity of sperm cells, points	Survival rate of sperm cells		Preservation of sperm cells, %
			at 37 °C, h	absolute, con.unit	
Low semen quality					
ad/bcm	17	2.12±0.23	2.12±0.23	4.62±0.66	42.94±4.57
ad/cgm	12	2.37±0.26	2.79±0.32	5.79±0.67	47.22±5.42
ad/d	16	1.93±0.29	1.93±0.29	4.50±0.71	38.54±5.91
ad/de	36	2.06±0.23	2.01±0.21	5.26±0.68	37.68±4.11
ad/dk	14	1.39±0.27	1.50±0.31	3.32±0.71	27.65±5.42
cgm/ceg	15	1.53±0.33	1.63±0.35	3.53±0.81	28.32±5.72
cgm/dk	45	2.51±0.09	2.84±0.12	7.57±0.47	47.52±1.90
de/cgm	8	2.10±0.29	2.25±0.34	5.1±0.79	40.90±6.10
dg/cgm	14	2.11±0.29	2.46±0.35	5.11±0.75	42.90±5.45
dg/di	16	1.47±0.31	1.50±0.32	3.63±0.85	26.14±5.10
Medium semen quality					
bcm/cgm	103	3.60±0.19**	3.00±0.15**	9.91±0.61**	50.13±2.47*
bcm/de	91	3.53±0.17**	3.11±0.14**	9.59±0.48**	52.57±2.17*
bcm/dg	65	3.38±0.13**	3.43±0.19**	10.40±0.68***	52.84±1.43*
bcm/dk	115	3.59±0.11**	3.70±0.11**	11.80±0.46***	54.30±1.36*
cegm/cgm	27	3.24±0.11**	3.68±0.16**	9.87±0.43**	54.63±1.27**
cegm/d	20	3.25±0.11**	3.55±0.13**	10.70±0.47***	52.60±0.84*
cegm/dg	27	3.18±0.12**	3.67±0.15**	9.67±0.45**	55.50±1.54**
cegm/dk	37	3.33±0.17**	3.54±0.25**	10.90±0.91***	57.31±2.46**
cgm/cgm	72	2.92±0.11*	3.37±0.15**	8.10±0.38**	51.87±1.79*
cgm/d	29	3.93±0.47**	2.17±0.19*	8.27±0.99**	49.80±5.70*
cgm/dg	58	2.98±0.17*	3.24±0.20*	9.49±0.73**	48.18±2.57*
de/d	27	3.80±0.48***	2.02±0.21*	7.59±0.95*	47.20±6.05
de/dk	88	3.18±0.13**	3.47±0.15**	10.24±0.55***	51.90±1.70*
dk/d	67	3.37±0.11**	3.60±0.14**	10.61±0.49***	53.30±1.54**
dk/de	25	3.34±0.12**	3.44±0.16**	11.36±0.73***	58.27±1.77**
dk/dk	30	3.74±0.17***	3.15±0.15**	12.35±0.91***	59.23±2.08**
High semen quality					
bcm/d	27	4.33±0.11***	5.03±0.12***	14.94±2.67***	67.12±0.87***
cgm/de	48	4.67±0.18***	5.19±0.21***	16.45±0.75***	61.90±2.29***
dg/dk	19	4.34±0.33***	4.71±0.36***	15.90±1.32***	56.34±4.30**

*, **, *** Differences with low quality semen are statistically significant respectively at $p < 0.05$; $p < 0.01$ and $p < 0.001$.

Based on the obtained data (see Table 1) it is apparent that erythrocyte antigen alleles *ad/bcm*, *ad/cgm*, *ad/d*, *ad/de*, *ad/dk*, *cgm/ceg*, *cgm/dk*, *de/cgm*, *dg/cgm*, *dg/di* of D blood group system in surveyed horses was accompanied by manifestation of low semen quality after cryopreservation (sperm cell mobility after defrosting below 2.5 points and low survival rate below 2.5 h). The average physiological characteristics of semen (mobility of sperm cells from 2.5 points and survival rate below 4 h) were noted when stallions of Ukrainian selection had alleles of erythrocyte antigens *bcm/cgm*, *bcm/de*, *bcm/dg*, *bcm/dk*, *cegm/cgm*, *cegm/d*, *cegm/dg*, *cegm/dk*, *cgm/cgm*, *cgm/d*, *cgm/dg*, *de/d*, *de/dk*, *dk/d*, *dk/de*, *dk/dk* of D blood group system. High performance of cryopreservation semen (mobility above 4 points and survival rate of 4 h) was observed in stallions with erythrocyte antigen alleles *bcm/d*, *cgm/de*, *dg/dk* of D blood group system.

It has been determined that the degree of impact of erythrocyte antigens of D blood group system on cryogenic resistance of semen was 32.5 % ($p < 0.001$), 18.2 % for mobility of defrosted sperm cells ($p < 0.01$), 25.2% ($p < 0.001$) for their survival rate, 24.5 % ($p < 0.01$) for absolute survival rate of defrosted semen and 12.2 % ($p < 0.05$) for preservation of defrosted semen.

2. The association of alleles of erythrocyte antigens of A (EA D) **blood group system with stallion semen properties after cryopreservation** (M±SEM, n = 1413)

EA A	Number of samples	Activity of sperm cells, points	Survival rate of sperm cells		Preservation of sperm cells, %
			at 37 °C, h	absolute, con.unit	
Test group					
–/–	101	3.42±0.11*	3.45±0.11**	10.88±0.49*	54.97±1.38*
a/–	8	2.06±0.29*	2.25±0.34	5.06±0.78**	40.88±6.09
ad/–	1059	3.25±0.05	3.26±0.05*	9.72±0.17*	50.92±0.60
Control group					
Not determined	245	2.91±0.09	2.71±0.08	8.09±0.31	46.21±1.27

*, **, *** Differences with the control group are statistically significant respectively at $p < 0.05$; $p < 0.01$ and $p < 0.001$.

The analysis of primary characteristics of cryopreserved semen of Ukrainian selection horses in comparison with alleles of A blood group system (Table 2) allows us to arrive at a conclusion that biological quality of this semen reliably increased when stallions did not have erythrocyte antigens of this system. For instance, the activity of sperm cells was 0.51 points higher ($p < 0.05$) than in the control group, 1.36 points ($p < 0.01$) higher as compared with the activity of carriers of *a/*-allel and 0.17 points higher compared with producers with *ad/*-allels. The survival rate of defrosted semen turned out highest for stallions with no A system antigens, i.e. 0.74 h higher than in the control group ($p < 0.05$), 1.2 h higher ($p < 0.001$) as compared to the carriers with *a/*-allel and 0.19 h higher for carriers of *ad*-allel. The inheritance by stallions of *a/–* erythrocyte antigen of A blood group system was accompanied with the worst preservation rate of sperm cells (below 41 %).

The degree of impact of erythrocyte antigens of A blood group system on cryogenic resistance constituted 3.4 % ($p < 0.05$), 1.2% ($p < 0.05$) on the mobility of sperm cells after defrosting, 2.4 % ($P < 0.05$) on survival rate of defrosted sperm cells, 2.1% ($p < 0.05$) on absolute parameter of survival rate of defrosted semen and 1.4 % ($p < 0.05$) on preservation rate of defrosted semen.

The results of analysis of key physiological characteristics of stallion semen depending on presence of erythrocyte antigens of C blood group system (Table 3) are indicative of the fact that presence of *a/–* accompanied with certain ($p < 0.05$) decline of activity (by 0.33 points), survival rate (by 0.78 h), absolute survival rate parameter (by 1.84 conventional units), preservation rate of sperm cells (by 6.26 %) as compared with similar parameters of stallions with no erythrocyte antigens of this blood group system.

It has been determined that the degree of impact of erythrocyte antigens

of C blood group system on activity of sperm cells of stallions of Ukrainian selection was 1.4 % ($p < 0.05$), 5.6 % ($p < 0.05$) on semen survival rate, 3.1 % ($p < 0.05$) on absolute survival rate parameter and 2.4 % ($p < 0.05$) on preservation of sperm cells.

3. The association of alleles of erythrocyte antigens of C (EA D) **blood group system with stallion semen properties after cryopreservation** ($M \pm$SEM, $n = 1413$)

EA C	Number of samples	Activity of sperm cells, points	Survival rate of sperm cells		Preservation of sperm cells, %
			at 37 °C, h	absolute, con.unit	
		Test group			
–/–	940	3.32±0.05*	3.42±0.05*	10.15±0.17*	52.42±0.57*
a/–	227	2.99±0.12	2.64±0.11	8.31±0.40	46.16±1.61
		Control group			
Not determined	245	2.91±0.09	2.71±0.08	8.09±0.31	46.21±1.27

*, **, *** Differences with the control group are statistically significant respectively at $p < 0.05$; $p < 0.01$ and $p < 0.001$.

For stallions without K blood group system antigens, the activity of defrosted semen was 0.35 points higher ($p < 0.05$) than of K system antigen *a*/–allel carriers, and 0.40 points ($p < 0.05$) higher than of the control horses whose antigen erythrocyte profile was not determined. The degree of impact of K blood group system erythrocyte antigens on the activity of sperm cells after defrosting was 1.4 % ($p < 0,05$), 2.0 % ($p < 0.05$) on semen survival rate, 1.5 % ($p < 0.05$) on absolute survival rate parameter and 1.2 % ($p < 0.05$) on preservation of sperm cells.

The summarizing dispersion analysis of the obtained data showed that the degree of impact of antigen characteristics of erythrocytes on cryogenic resistance of semen of stallions of Ukrainian selection constitutes 38.7 % ($p < 0.001$) for D blood group system, 1.7 % ($p < 0.05$) for A blood group system, 16.6% ($p < 0.01$) for C blood group system and 12.9 % ($p < 0.01$) for K blood group system.

Therefore, the horses of Ukrainian selection were for the first time surveyed for association link between erythrocyte antigens of A, C, D and K systems with key properties of semen after cryopreservation. It has been observed that presence of erythrocyte antigen alleles *ad*/*bcm*, *ad*/*cgm*, *ad*/*d*, *ad*/*de*, *ad*/*dk*, *cgm*/*ceg*, *cgm*/*dk*, *de*/*cgm*, *dg*/*cgm*, *dg*/*di* of D blood group system in the analyzed animals was accompanied with low mobility of sperm cells (below 2.5 points) and low survival rate (below 2.5 h). The cryopreserved semen of mean quality (mobility from 2.5 points and survival rate up to 4 h) was received from stallions with erythrocyte antigen alleles *bcm*/*cgm*, *bcm*/*de*, *bcm*/*dg*, *bcm*/*dk*, *cegm*/*cgm*, *cegm*/*d*, *cegm*/*dg*, *cegm*/*dk*, *cgm*/*cgm*, *cgm*/*d*, *cgm*/*dg*, *de*/*d*, *de*/*dk*, *dk*/*d*, *dk*/*de*, *dk*/*dk* of D blood group system. The high semen characteristics after cryopreservation (mobility of sperm cells above 4 points and survival rate above 4 h) were observed in stallions with erythrocyte antigen genotype for of D blood group system *bcm*/*d*, *cgm*/*de*, *dg*/*dk*. The percentage of impact of antigen characteristics of D blood group system on cryogenic resistance of sperm cells of stallions was 32.5% ($p < 0.001$), 18.2 % ($p < 0.01$) on mobility, 25.2 % ($p < 0.001$) on survival rate of defrosted semen, 24.5% ($P < 0.01$) on absolute survival rate parameter and 12.2% ($p < 0.05$) on preservation rate of defrosted semen. In absence of K blood group system antigens, the activity of defrosted semen was 0.35 points higher ($p < 0.05$) than activity of *a*/–allel carriers of K system, and 0.40 points higher ($p < 0.05$) than activity of control group producers, whose antigen erythrocyte profile was not determined. The presence of *a*/–allel (C blood group system) was accompanied with certain ($p < 0.05$) reduction of activity (by 0.33 points), survival rate (by 0.78 h), absolute survival rate parameter (by 1.84 conventional units), sperm cell preservation (by 6.26 %) as compared to stallion parameters

without erythrocyte antigen of this blood group system. The absence of A blood group system antigens resulted in certain increase of biological properties of semen after cryopreservation.

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