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CYTOGENETIC STATUS OF MARES (*Equus caballus*) OF UKRAINIAN RIDING BREED INFLUENCES THEIR FERTILITY

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Abstract

Cytogenetic studies of mares are widely used in practice (in case of embryonic death this is a mandatory test) in countries with developed horse breeding. Genetic evaluation of *Equus caballus* is also widely performed. Nevertheless, in the available literature, we could not find publications on the relationship between cytogenetic disorders in mares and the effectiveness of artificial insemination with frozen and thawed sperm. This paper is the first report on the impact of the cytogenetic status of mares *E. caballus* of Ukrainian horse breed on their sexual cycle and the efficacy of the artificial insemination by Kharkov technology. It has been shown that in case of ovary hypofunction caused by an increased chromosomal variability, as estimated by the per cent of aberrant metaphases with no genome mutations and the transmitted cytogenetic disturbances found, it is necessary to divide the mares into three groups: up to 5 %, from 5 to 10 % and over 10 % overall chromosomal instability. This allows better characterization of mares' physiological condition to optimized treatment and the artificial insemination procedure by Kharkov technology. When cooled semen used, the fertility of the mares having more than 10 % aberrant metaphases was the lowest, by 29.81 and 31.86 % less ($p < 0.01$) compared to mares from the groups with lower chromosomal instability. When thawed semen was used, the fertility was the highest in the mares with the chromosomal instability up to 5 %, that is, on average 14.93 % higher ($p < 0.05$) compared to the mares with more than 5 % of metaphases with aberrations. The influence of cytogenetic status on the fertility in the insemination was clearly seen when the cryopreserved semen was used as compared to cooled semen. The fertility of the mares inseminated with cryopreserved sperm averaged was 71.60 % in group 1 (up to 5 % aberrant metaphases), 56.67 % in group 2 (5-10 % aberrant metaphases), and only 37.04 % in group 3 (> 10 % aberrant metaphases). So, estimation of cytogenetic status ensures optimization of artificial insemination and an increase in mares' fertility when used cooled and frozen-thawed semen.

Keywords: *Equus caballus*, cytogenetic status of mares, fertility, aberrations

Genetic evaluation of *Equus caballus* is performed in many countries [1-3]. Nevertheless, we could not find in the available literature any publications on the relationship between cytogenetic disorders in mares and the effectiveness of artificial insemination with frozen and thawed sperm. Cytogenetic studies are more practically used in countries with developed horse breeding than in Ukraine, and in mandatory manner in case of embryonic death [4-6]. It should be noted that intensive selection in the Ukrainian horse breeds without sufficient fundamental studies could result in spread of cytogenetic anomalies which are able to reduce reproductive function [7-9]. Probably, for this particular reason foal crop across the Ukrainian horse breeding industry does not exceed 50 % lacking the required minimum of breeding reproductive stock for majority of the raised breeds. Only 3 out of the 12 *E. caballus* breeds officially registered in the Ukraine have sufficient quantity of breed animals; herewith the total number of horse stock at beginning of 2015 had decreased to 320-330 thousands [10]. Thus,

the fundamental objective of the horse breeding development program in Ukraine is to preserve and to increase the number of stock by 2020. Although use of modern reproduction biotechnology methods has its role to play [11, 12], they are ineffective upon diagnosis of anovulatory sex cycle in mares caused by hypovarianism or cytogenetic disorders.

It should be noted that over the last years we have witnessed decrease in fertility in all animal species (just like in humans) and growth of a number of obstetric-gynecologic diseases that, possibly, is partially due to deterioration of the ecologic situation [13-16]. Cytogenetic studies are wider used in the context of zoological gardens to preserve the appropriate gene pool and to ensure the effective animal reproduction [17-18].

These findings point out to the need for use of cytogenetic studies to find the reasons of anovulatory sex cycles and to increase fertilization rates in mares both in the natural horsing and after artificial insemination. First of all, it is necessary to perform cytogenetic tests on mares with rectally confirmed hypovarianism as the most spread pathology. However, only few cytogenetic tests were performed on *E. caballus* in Ukraine as regards to the reproductive function, without studying the link with effectiveness of the artificial insemination [19, 20].

In this paper we have for the first time shown that fertilization of horses of the Ukrainian roadster breed depends on the instability degree of their chromosomes apparatus (provided lack of genome mutations and balanced genetically transmitted cytogenetic disorders), provided that this effect is manifested stronger if mares are inseminated by frozen-thawed, rather than by frozen sperm.

Purpose of this study was establishment of the impact of the cytogenetic status in mares *E. caballus* of the Ukrainian horse breed on their sexual cycle and the efficacy of the artificial insemination by Kharkov technology.

Techniques. Tests on 143 mares of the Ukrainian roadster breed were conducted during 3 years (2012-2015) at one and the same farming units (private stud farms and breed reproducing farms in Kharkov, Poltava, Dnipropetrovsk, Zaporozhian, and Kiev regions of the Ukraine).

Blood for cytogenetic tests was collected weekly in sterile vials with heparin from the jugular vein before insemination by commonly accepted methods with adherence to the aseptic and antiseptic regulations. Lymphocytes were cultivated during 48-56 hours in sterile conditions in Eagle medium or 199 medium (Sigma, USA) with addition of the inactivated calf serum, Phytohaemagglutinin (Sigma, USA) and antibiotics (penicillin and streptomycin, 100 mg/cm^3) at temperature of 37 °C, following which preparations based on metaphase lymphocyte plates were produced ([21, 22]. Medicines were examined by optical microscope Jenaval (Carl Zeiss, Germany) under oil immersion at magnification of ×1000.

Based on cytogenetic data, mares were divided into groups prior to the horsing and insemination period depending on the percentage of metaphases with aberrations (up to 5 %, 5-10 % and over 10 %). Afterwards, a set of zootechnical and veterinary actions were taken for improvement of fertility (better feeding and raising, longer motion, use of developed complex for individual care) in mares with established hypovarianism. Animals with genome mutations and balanced genetically transmitted cytogenetic anomalies were excluded from the studies.

Optimal insemination time was comprehensively diagnosed by ultrasound scanner Aquila Pro (Esaote, Spain) for veterinary purpose with rectal linear probe (6-8 mHz).

Artificial insemination was performed in the first full-value ovulatory sex cycle by Kharkov technology [10] with the use of our instrument [23]. Cooled and frozen-thawed sperm was used. Prior to insemination, cooled stud sperm

was stored for no more than 48 hours in the household refrigerator at temperature of 2-4 °C. Sperm was frozen by Kharkov technology [10], spermatozoids were packed up in tube syringes of 5 cm^3 with sperm concentration of 150-200 million/cm^3. One sperm dose was used for one insemination. Spermatozoids were unfrozen (tube syringes) in water bath at temperature of 38-40 °C during 2-3 minutes. After defrosting, only such spermatozoids were used for insemination purposes, in which sperm mobility rate scored over 3 (at least 30 % of spermatozoon with rectilinear motion). Donor studs had acceptable chromosomal variability (at least 5 % of metaphases with aberrations) and no genome mutations and balanced cytogenetic disorders.

Study results were statistically processed by commonly accepted methods [24], as well as with the use of specialized program SPSS (IBM, USA). Mean (*M*) values and standard errors of the mean (±SEM) are provided in table below. Deviations were assessed by Student's *t*-test and were considered statistically significant at $p < 0.05$.

Results. The paramount problem occurring at preservation of the Ukrainian gene pool of *E. caballus* breed is in decrease of the number of mares with anovulatory sex cycles as fertilization becomes impossible in such animals [25]. At complex diagnostics of the state of reproductive range, deviations from the physiological norm were noted in animals with chromosomal variability exceeding 5 %. Physiological pre-ovulation mare follicle at acceptable number of metaphases with aberrations and hypo-ovaria in species with high percentage is presented in Fig. 1 below.

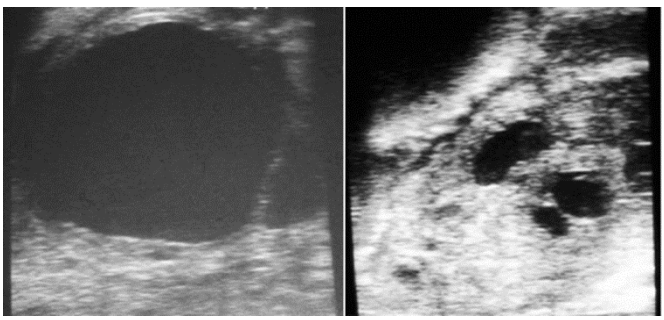

Fig. 1. Physiological follicle of mare (*Equus caballus*) of Ukrainian roadster breed with acceptable cytogenetic status (on the left) **and hypo-ovaria at high percentage of metaphases with aberrations** (on the right)**.**

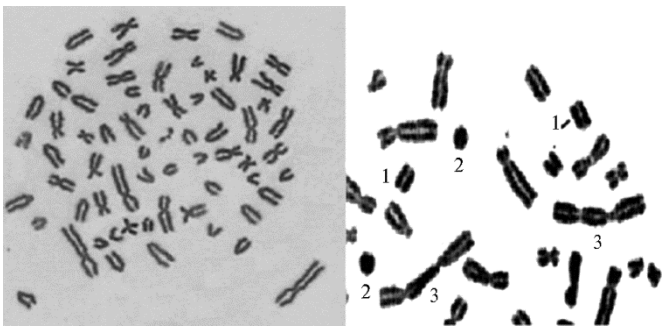

Fig. 2. Metaphase plates in mares (*Equus caballus*) of Ukrainian roadster breed without aberrations (on the left) **and high chromosomal instability** (on the right)**:** 1 — paired fragments, 2 — circular bodies, 3 — dicentrics.

Normal metaphases and observed cytogenetic disorders are illustrated in Fig. 2.

Data of the table 1 show that the mares with total chromosomal instability of up to 5 % and physiological sex cycle had metaphases with aberrations by 5.31 % less ($p < 0.001$) than animals from the group with 5-10 % aberrations, and by 17.09 % less ($p < 0.001$) metaphases with aberrations as compared to mares among which over 10 % of aberration metaphases are registered.

Upon use of defrost sperm, effect of cytogenetic status in mares on their fertilization by way of insemination was more displayed than in case of use of the cooled sperm, which correlates to other study results of use of frozen-thawed

stud sperm [26, 27]. Insemination was performed in the first full-value ovulation sex cycle. Fertilization rate upon use of the cooled sperm (by percentage of newborn studs) in mares with cytogenetic status of over 10 % metaphases with aberrations was the least as follows: in average by 29.81 and 31.86 % lower ($p < 0.01$) than in animals of the groups with 5-10 % and < 5 % metaphases with aberrations, respectively. Upon insemination by cooled sperm, fertilization rates in mares from the first (up to 5 % of metaphases with aberrations) and the second (5-10 % of metaphases with aberrations) groups lacked valid deviations.

1. Cytogenetic status of mares (*Equus caballus*) of the Ukrainian roadster breed and their fertilization at artificial insemination by Kharkov technology (*M*±SEM, private horse breeding plants and breeding reproducing centers of the Ukraine, years 2012-2015)

Indicator	Metaphases with aberrations		
	< 5 %	5-10 %	> 10 %
Number of studied metaphases	2418	2486	2930
Number of mares in a group	44	45	54
Metaphases with aberrations	1.48±0.08	4.40±0.11***	10.67±0.22***
Relative number of metaphases with aberrations, %	2.70±0.14	8.01±0.22***	19.79±0.46***
Number of studs born from the cooled sperm, %	92.00±1.15	90.00±1.11	60.19±0.93**
Number of studs born from the thawed sperm, %	71.60±1.14	56.67±1.11*	37.04±1.85**

*, **, *** Deviations are statistically significant as compared to the first group of mares (up to 5 % of metaphases with aberrations) at $p < 0.05$; $p < 0.01$ and $p < 0.001$, respectively.

Fertilization rates by insemination by thawed sperm was the highest in the group with total chromosomal instability of < 5 %, having in average exceeded by 14.93 % ($p < 0.05$) the rates in mares from the second group and by 34.56 % ($p < 0.01$) in mares with the highest percentage of metaphases with aberrations (> 10 %). Other researchers also note that cytogenetic status in mares is especially important upon use of the unfrozen sperm [28, 29].

2. Structure of chromosomal aberrations in mares (*Equus caballus*) of the Ukrainian roadster breed with different degree of chromosomal instability (*M*±SEM, private horse breeding plants and breeding reproducing centers of the Ukraine, years 2012-2015)

Indicator	Metaphases with aberrations		
	< 5 %	5-10 %	> 10 %
Total aberrations	1.95±0.10	6.69±0.27***	14.41±0.33***
Structure of aberrations, %:			
single fragments of chromosomes	70.08±3.78	47.57±2.10***	43.02±1.85***
paired fragments of chromosomes	23.11±5.10	27.75±1.65	23.11±0.77
circular chromosomes	0.00±0.00	10.36±1.70	16.84±1.20**
gaps and breaks in chromosomes	6.81±5.29	13.74±1.95	16.27±1.03
dicentrics	0.00±0.00	0.58±0.66	0.76±0.87

*, **, *** Deviations are statistically significant as compared to the first group of mares (up to 5 % of metaphases with aberrations) at $p < 0.05$; $p < 0.01$ and $p < 0.001$, respectively.

Studies of the structure of chromosomal aberrations (Table 2) had shown that their total number in mares from the first group (with acceptable total chromosomal instability) was in average by 4.74 less ($p < 0.001$) than in the second group, and by 12.46 less ($p < 0.001$) than in the third group. Single fragments of chromosomes had prevailed amongst the aberrations, whereas in the first group such indicator was by 22.51 % higher ($p < 0.001$) than in the second group and by 27.06 % higher ($p < 0.001$) than in the third group, where number of anovulation cycles was the highest. Percentage of paired chromosome fragments was in average practically similar in mares with total chromosomal instability of up to 5 % and over 10 %, and in the second group such indicator had invalidly exceeded thereof in the first and third groups by 4.64 %. Circular chromosomes were not found in animals from the first group and in the third group it was by 6.48 % more ($p < 0.01$) than in the second one. Number of gaps

and breaks in the studied metaphase plates was the least in mares from the first group that in average was by 6.93 % less than in the second group and by 9.46 % less than in the third group. No dicentrics were found in the studied mares with the acceptable degree of chromosomal instability (up to 5 %), their highest percentage was established in animals from the third group with over 10 % metaphases with aberrations that was by 0.18 % more than in the second group with total chromosomal instability 5-10 %.

Therefore, we have studied the impact of cytogenetic status of mares *Equus caballus* of the Ukrainian roadster breed on full-value of their sex cycle and fertilization by Kharkov technology. It is established that in order to increase fertilization rates in mares it is reasonable to separate them depending on their cytogenetic status (with number of metaphases with aberrations of up to 5 %, 5-10 % and over 10 %). Such division allows the breeders to increase the fertilization rates both naturally and at artificial insemination. Herewith, fertilization of mares by unfrozen sperm with number of aberration metaphases of up to 5 % in average comprises 71.60 %, with 5-10 % of such metaphases — 56.67 %, over 10 % — only 37.04 %. Accordingly, it is very important to account for the cytogenetic status of mares upon use of the unfrozen sperm.

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