



The modern methods of reproduction physiology of horses

A.V. Tkachev¹, O.L. Tkacheva², B.V. Gutyj³

¹Belgorod state agricultural university named after V. Gorin, Vavilova Str., 1, Office 306, pos. Mayskiy 308503, Belgorod region, Russia

²Institute of Animal Science of National academy of agrarian sciences of Ukraine, Tvarynykiv Str. 1-A, Kharkiv, 61026, Ukraine

³Stepan Gzhytskyi National University of Veterinary Medicine and Biotechnologies Lviv, Pekarska Str., 50, Lviv, 79010, Ukraine

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Correspondence author

Aleksandr Tkachov

Tel.: +7(4722) 39-28-09

E-mail: sasha.sashaola2017@gmail.com

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Abstract

The concept of development of horse breeding in Ukraine until 2020 provides for an increase in the number of horses through the intensive use of modern methods of reproduction biotechnology. However, the imperfection of these methods hinders their widespread use in practice. The aim of the work was to draw attention to the most important problems of the physiology of horse reproduction in Ukraine and to show ways to solve them that have already been proposed by domestic and foreign researchers. The development strategy of the physiology of horse reproduction in order to increase its effectiveness should take into account the least studied aspects that were discussed above. The article shows that taking into account the influence of micromycetes, the absolute number of colony forming units of *E. coli* in semen of stallions; immuno- and cytogenetic features; new methods of sanitary preparation of horses for sperm and insemination; the effect of permissible levels of feed mycotoxins on physiological functions improves the efficiency of equine reproduction physiology methods. However, we first discovered new physiological features of the effect of erythrocyte antigens of blood groups of horses of Ukrainian selection on the indicators of their native sperm. In the presence of ad/bcm and dg/cgm alleles of the blood group D system in stallions, sperm motility is on average less than 5 points; alleles ad/cgm, ad/d, ad/de, ad/dk, bcm/d, bcm/de, bcm/dg, bcm/dk, cegm/cgm, cegm/d, cegm/dg, cegm/dk, cgm/ceg, cgm/cgm, cgm/dg, cgm/dk, de/cgm, de/dk, dg/di, dk/d, dk/de, dk/dk is accompanied by sperm motility from 5 to 7 points; alleles bcm/cgm, dg/dk, de/d, cgm/d, cgm/de sperm motility is observed more than 7 points. The results obtained allowed us to develop for practice ways to increase the efficiency of sperm cryopreservation by immunogenetic parameters. In addition, open physiological correlations can increase the fertility of mares during mating.

Key words: physiology of reproduction, horses, sperm cryopreservation, artificial insemination, methods, perspectives.

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1. Introduction

Modern horse breeding in Ukraine is in a difficult condition. The number of horses in 2018 decreased to 300.000 heads, compared to 720000 in 1992. The vast majority of factory breeds of horses, out of 12 officially registered, do not have the minimum required number of breeding stock. Only 3 breeds have the minimum allowable number of breeding stallions and mares. The yield of foals on average in the horse breeding industry of Ukraine does not exceed 50% (Tkachev et al., 2017). Of course, there are separate farms with foals over 80%, but their number is insignificant. The concept of development of horse breeding in Ukraine until 2020 provides for an increase in the number of horses through the intensive use of modern methods of reproduc-

tion biotechnology. However, the imperfection of these methods hinders their widespread use in practice.

The aim of the work was to draw attention to the most important problems of the physiology of horse reproduction in Ukraine and to show ways to solve them that have already been proposed by domestic and foreign researchers.

2. Results and discussion

One of the directions in the development of methods of physiology of horse reproduction may be the transition from payet and aluminum bags to the use of the syringe tube we offer (Sushko et al., 2010; Tkachov, 2013).

In the world there are 3 forms of sperm doses in which stallion sperm is frozen: 1) sequins with a capacity of 0.25–0.5 ml, are used in Western European biotechnology firms

IMV (France) and Minitub (Germany); 2) aluminum bags with a volume of 10–20 ml, which are used in the technology of the All-Russian Research Institute of Horse Breeding (Naumenkova & Vasilyeva, 2006; Naumenkova & Vasilyeva, 2007; Atroshchenko & Bragin, 2011; Naumenkova et al., 2012; Atroshchenko & Kanashchenkov, 2014; Atroshchenko et al., 2016; Naumenkova et al., 2016; Atroshchenko et al., 2017); 3) open sperm granules of 0.25 ml, which are no longer used in practice, but sometimes the semen of stallions is frozen in the form of open granules during scientific research (Sushko et al., 2010; Pojprasath et al., 2011; Sushko & Tkachov, 2015).

The most common form of sperm in the world are 0.5 ml sequins. Such a volume makes it possible to obtain high cooling and freezing rates of sperm, which increases their physiological characteristics after deconservation. A major practical drawback of payet is the inconvenience of their use during the artificial insemination of mares. It is necessary before artificial insemination of each mare: 1) to defrost 8–10 payet and either to seed 8–10 times in a row, which is very inconvenient; 2) either drain 8–10 payet into one container, put into a syringe, connect it to the instrument and introduce sperm into the mare's uterus, which contributes to the contamination of sperm with microorganisms. Another important practical drawback of payet is the need to purchase special, expensive equipment for freezing them, which is intended only for stationary work.

The second most common form of stallion sperm doses is aluminum bags. A large volume of this form of sperm dose has both advantages and disadvantages. The advantage of a large sperm dose is the expectation of an increased likelihood of successful fertilization. An important practical advantage is that freezing of aluminum bags does not require special expensive equipment and they are convenient for expeditionary work. The disadvantage is the inability to ensure a uniform decrease in temperature throughout the sperm dose. As a result, the physiological characteristics of sperm in different places of the sperm dose may vary.

Our studies (Sushko et al., 2010; Tkachov, 2013; Sushko & Tkachov, 2015) proved that the use of sperm doses in the form of syringes with a volume of 4–5 ml allows us to correct the shortcomings of sequins and aluminum bags, to obtain physiological characteristics of semen stallions after deconservation on a par with payets. Syringe tubes do not require special expensive equipment for freezing, do not require sterilization costs, because already sterile syringes can be purchased at any pharmacy.

The next important direction in the development of physiology of horse reproduction in Ukraine may be the correct timing of artificial insemination and an unambiguous interpretation of fertilization. Let's start with the need for an unambiguous interpretation of the effectiveness of mating or artificial insemination (Naumenkova & Vasilyeva, 2006; Naumenkova & Vasilyeva, 2007; Atroshchenko & Bragin, 2011; Naumenkova et al., 2012; Atroshchenko & Kanashchenkov, 2014; Atroshchenko et al., 2016; Naumenkova et al., 2016; Tkachov et al., 2016; Atroshchenko et al., 2017). In practice, the following phrases are used: 1) “yield foals” in percent; 2) “prosperous foal” in percent; 3) “foaming” or “mares that have gotten horse”. An analysis of the patterns of the mating-foal showed that domestic stud farms have 80–100% of “safe foraging”, because it is calculated as the percentage of foals born from mares that got horse-drawn. This is a deliberate distortion of the effectiveness of breed-

ing and breeding work, because the diagnosis of pregnancy in practice is practically not conducted. The stud farms knowingly do not calculate the “foals yield” – foals that were born from the number of all the mares that were seeded naturally or artificially. If you calculate exactly the “yield of foals”, then it will be at best 50–60%.

For an unambiguous interpretation of the effectiveness of artificial insemination, we propose the use of such an expression as “true fertility” and “general fertilization” (Sushko & Tkachov, 2015; Tkachov et al., 2016). By “true fertility” we should understand the percentage of foals born from those mares in which a full reproductive cycle with ovulation was observed and inseminated. Only in this way can one objectively characterize the fertilizing ability of semen stallions. If the horse was planned to be inseminated but not inseminated due to the fact that she did not have ovulation, then they should not be taken into account when establishing “true fertility”. For example, it was planned to inseminate 10 mares, only 8 out of which had ovulation (they were inseminated) from which 5 foals were born. “True fertility” is $5 \times 100/8 = 62.5\%$ – this is an objective characteristic of the fertilizing ability of semen stallions. “General fertility” in this case is $5 \times 100/10 = 50\%$ – this characteristic rather characterizes the effectiveness of breeding and breeding work in stud farms and breeding breeders. We propose the simultaneous use of the expressions “true fertility” and “general fertility” in scientific papers and official statements of the mating-foal of the subjects of breeding. Some foreign researchers are already taking a similar path (Cleys et al., 2003; Morris, 2004).

The next strategic direction in the development of physiology of horse reproduction is the development of methodological approaches to reduce bacterial (Tkachov et al., 2011; Atroshchenko & Kanashchenkov, 2014; Tkachev, 2014; Tkachov & Sheremeta, 2016) and micro-mycetic (Tkachov et al., 2011; Tkachov & Sheremeta, 2016) semen contamination. Most researchers believe that it is not even worth investigating the effect of bacterial contamination on the quality of semen stallions, because this issue has been studied for a long time and no longer has unexplored aspects. This is a misconception. Let me explain why. In conditions of prolonged use of antibacterial drugs, the microflora gets used to them and sanitary and hygienic measures become ineffective. For more than 40 years, the veterinary and sanitary instructions for sperm sanitation and the preparation of stallions have not been reviewed. This confirms that today it is time for such studies. We noticed that veterinary-sanitary measures were ineffective when we observed an increase in the total bacterial contamination of sperm at various biotechnological stages: obtaining sperm – cooling – after thawing (Tkachov et al., 2011; Tkachov & Sheremeta, 2016). We have developed methods to reduce the contamination of prepuce (Ukrainian patent for the invention No. 109846) and sperm (Ukrainian patent for the invention No. 112473), which can reduce the number of bacteria by 56 times, and micromycetes by 40 times due to the use of Miramistin, Dekasan, Chlorhexidine bigluconate and Candida.

An unexplored issue remains the effect of the absolute number of colony forming units of *Escherichia coli* on the physiological characteristics of sperm of stallions and other animals (Tkachov et al., 2011; Tkachov & Sheremeta, 2016). In the semen of stallions and other animals, only the maximum allowable amount of coli titer is determined (up

to 1:10). We noticed that if the titer is **the volume of fluid (sperm) in which there is Escherichia coli**. That is, this is a relative value that cannot be processed statistically. The coli titer does not reflect the absolute number of colony forming units of *E. coli*. We were the first to establish the absolute number of colony forming units of *Escherichia coli*, which does not reduce the quality of sperm and its fertilizing ability in horses. Based on the results obtained, a method for increasing the fertility of mares was first proposed by the absolute number of colony forming units of bacteria of the *Escherichia coli* group (Ukrainian patent for invention No. 110180). During artificial insemination of mares using stallion sperm in cm³ of which contains up to 250 colony forming units of *E. coli* bacteria, the fertilizing ability of chilled sperm increased by 11.77% ($P < 0.01$), thawed sperm – by 15.68% ($P < 0.01$). When using stallion sperm with colony forming units of *E. coli* group bacteria (BECG) from 250 to 3500 per cm³, mare feverability from chilled and frozen-thawed sperm practically did not change compared to sperm with an acceptable coli titer level (Tkachov et al., 2011; Tkachov & Sheremeta, 2016).

We propose to determine the absolute number of colony forming units of *E. coli* at the same time as determining the total bacterial contamination of sperm and to calculate the sum of these indicators, which should not exceed 5000 CFU/cm³.

An insufficiently unexplored issue is the effect of micromycetic contamination on the physiological parameters of stallion sperm. Not a single state standard for sperm of stallions in the world normalizes the maximum allowable amount of saprophytic fungal microflora and generally does not contain such words as “micromycetes” or “mushrooms”. Our studies have proved that the fungal microflora of stallion sperm has a stronger negative effect on sperm quality and its biotechnological suitability for cryopreservation than general bacterial contamination. Most stallion sperm dilution media do not contain antifungal agents. We have proposed a number of measures to reduce the negative impact of the fungal microflora of stallion sperm on its physiological characteristics (Tkachov, 2011; Tkachov et al., 2011).

Foreign researchers have noted an insufficient level of research on the influence of micromycetes on the quality of sperm of stallions (Meachem et al., 2005; Plante et al., 2012; Aitken et al., 2015; Alghamdi et al., 2015), which negatively affects the fertility of mares under conditions of artificial insemination (Tkachov, 2015). Our studies have established the maximum allowable levels of total micromycetic contamination of semen of stallions of different breeds. To increase the fertility of mares, thawed semen of stallions of Ukrainian riding breed in which up to 250 CFU/cm³ of micromycetes should be used; thoroughbred horse and Trakenens breed – up to 40 CFU/cm³; Westphalian breed – up to 80 CFU/cm³; Belgian breed – up to 150 CFU/cm³; Arab breed – up to 120 CFU/cm³; trotter breeds – up to 250 CFU/cm³; Hanover breed – up to 100 CFU/cm³ of the total number of micromycetes (Ukrainian patent No. 110125). To more effectively determine the total fungal contamination, we developed a method for the accelerated determination of micromycetic contamination of semen of stallions and males of other animal species (Ukrainian patent No. 110173), which allows 2–2.5 times faster determination of the total fungal contamination of semen of stallions and males of other animal species.

The next important direction in the development of physiology of horse reproduction may be an increase in the number of studies on the effect of their cytogenetic profile on reproductive function. The relationship of the cytogenetic profile with the reproductive function of horses is not well studied, both in Ukraine (Tkachova et al., 2014; Rossokha & Tkachova, 2018) and abroad (Baarends et al., 2005; Baumann et al., 2011). It has been established for humans that the physiological level of general chromosomal instability should not exceed 5%. The single physiological level of general chromosomal instability for horses is almost unknown, as different authors give different data, which vary from 3 to 10%. Our studies have shown that for physiology and physiology of horse reproduction, a level of up to 10% of total chromosomal instability can be considered acceptable. Indeed, cryoresistance of semen of stallions with a general chromosomal instability of 5–10% is greater than 3 points (Tkachov & Tkachova, 2017).

The use of cytogenetic studies in reproduction allows to increase the fertility of mares in the conditions of mating and artificial insemination (Ukrainian patent No. 112459). It was found that when using stallions of the Ukrainian horse breed in mating or their semen in artificial insemination, fertility increased only if the level of total chromosomal instability did not exceed 3%, the number of metaphases with aberrations and the total number of aberrations did not exceed 2, the number of aberrations per 100 cells did not exceed 3, the number of single aberrations was not less than 55%, the number of paired aberrations was not more than 45%, there were no ring aberrations and chromatid gaps, the relative length of the fourth auto pair som is not less than 4.4%, the relative length of the eighth pair of autosomes is not more than 3.65%, the relative length of the tenth pair of autosomes is not less than 3.0%, the relative length of the fourteenth pair of autosomes is not less than 4.5%. When using stallions of the Russian trotter, trotter, Belgian, Hanoverian, thoroughbred horse and New Aleksandrovsky heavy-carriage breeds or their sperm, fertility increased only if their level of total chromosomal instability did not exceed 4%. When using stallions of Westphalian, Arabian and Trakenen breeds or their semen, fertility increased only if their level of total chromosomal instability did not exceed 6%. The proposed method for the first time allows to increase the fertility of mares during mating by 21.76% ($P < 0.001$), artificial insemination with chilled sperm by 25.05% ($P < 0.05$), thawed sperm – by 25.13% ($P < 0.05$) taking into account the total number of aberrations, the number of aberrations per 100 cells, the number of single, paired, annular aberrations and the number of gaps in aberrant metaphases for stallions and mares, as well as the relative length of autosomes (Tkachov & Tkachova, 2017).

The level of general chromosomal instability affects the effectiveness of treatment of hypofunction of ovaries of mares (Ukrainian patent No. 109754). The use of the developed new method for treating hypofunction of ovaries of mares makes it possible to effectively treat the mild form of severity of ovarian hypofunction from 2.45 days, the moderate form of severity for 6.42 days, the severe form of severity for 17.4 days due to the use of specialized hormonal drugs. It allows to increase true fertility from natural mating to 84.25%, from artificial insemination with chilled sperm to 92.05%, thawed sperm to 74.45%. Such effectiveness in treating hypofunction of ovaries of mares is achieved by dividing mares into three groups depending on the level of

general chromosomal instability. It is proposed to consider the mild form of the course of ovarian hypofunction with a level of general chromosomal instability of up to 5%; moderate severity of hypofunction – 5–10%; severe mild ovarian hypofunction with more than 10% of total chromosomal instability. This confirms the need for wider use of cytogenetic studies in the reproduction of horses (Tkachev, 2015; Tkachov et al., 2018). However, it should be borne in mind that the cytogenetic profile of horses may depend on the presence of mycotoxins in the feed (Tkachov, 2015). Which, in its turn, can reduce the fertility of mares by 32.8% ($P < 0.001$), the insemination rate of chilled sperm by 29.5% ($P < 0.001$), thawed sperm by 25.1% ($P < 0.001$) when eaten feed with the maximum permissible concentrations of zearalenone, T-2 toxin, deoxynivalenol, aflatoxin for four or more weeks (Tkachov & Zhukova, 2015). One of the revealed mechanisms of the negative influence of permissible levels of mycotoxins in feed for horses is to reduce the resistance (Tkachov, 2014) and the hormonal profile of horses (Tkachov, 2014). If mycotoxins enter the semen, the minimum toxic dose of zearalenone and T-2 toxin is 0.01 mM. At the same time, the biological difference between the semen of stallions and sperm of bulls is shown. The joint presence of zearalenone and T-2 toxin is more toxic to stallion sperm, for bulls only the T-2 toxin is more toxic, and not their combined penetration into semen (Tkachov & Tkachova, 2017).

Taking into account the abovementioned, the problem arises of increasing the safety of sperm membranes during freezing of semen of stallions. The highest percentage of live intact sperm (about 40%) is observed when IMV, Minitub and the Thinner developed by us are used for freezing, which ensures a foal yield of 80–85%. The use of HCFC provides no more than 19% of sperm with intact membranes, which contributes to a decrease in foals up to 55–60% (Tkachov, 2013).

The insufficiently studied problem of increasing the methods of physiology of horse reproduction is the influence of immunogenetic factors (Khrabrova & Kisilev, 2016; Khrabrova, 2017). A decrease in the yield of foals can be caused by immunological incompatibility in the system of a stallion-mare-fetus similar to a Rhesus conflict in humans. When the foal inherits erythrocyte antigens that are not found in the mare, the body of the latter begins to produce antibodies. The clinical manifestations of such an immunogenetic conflict may be abortion, a decrease in the foal yield and neonatal isoerythrolysis as a result of which the foal may die if it is not separated from the mother in time (Khrabrova et al., 2015; Khrabrova & Alekseeva, 2015; Khrabrova & Trufanov, 2015; Khrabrova & Kisilev, 2016; Khrabrova, 2017).

In the practical horse breeding of Ukraine, immunogenetic factors are not taken into account. In the countries of Western Europe, when compiling parental pairs, testing of erythrocyte antigens of 5 blood group systems (A, C, D, Q, P) is mandatory to eliminate possible immunogenetic conflict. Researchers from the All-Russian Research Institute of Horse Breeding (Divovo, Ryazan Oblast) drew attention to this problem; the results obtained by them and us prove that taking the immunogenetic profile into account allows increasing the foal yield by 12–20% (Tkachov, 2013; Khrabrova et al., 2015; Khrabrova & Alekseeva, 2015; Khrabrova & Trufanov, 2015; Khrabrova & Kisilev, 2016; Khrabrova, 2017).

However, we first discovered new physiological features of the effect of erythrocyte antigens of blood groups of horses of Ukrainian selection on the indicators of their native sperm. In the presence of ad/bcm and dg/cgm alleles of the blood group D system in stallions, sperm motility is on average less than 5 points; alleles ad/cgm, ad/d, ad/de, ad/dk, bcm/d, bcm/de, bcm/dg, bcm/dk, cegm/cgm, cegm/d, cegm/dg, cegm/dk, cgm/ceg, cgm/cgm, cgm/dg, cgm/dk, de/cgm, de/dk, dg/di, dk/d, dk/de, dk/dk is accompanied by sperm motility from 5 to 7 points; alleles bcm/cgm, dg/dk, de/d, cgm/d, cgm/de sperm motility is observed more than 7 points (Tkachev et al., 2017). The results obtained allowed us to develop for practice ways to increase the efficiency of sperm cryopreservation by immunogenetic parameters. In addition, open physiological correlations can increase the fertility of mares during mating.

The insufficiently studied problem of reproduction of horses is the negative effect of helminth invasion. It has been proven that helminthiasis are widespread in Europe, Russia, Ukraine and other countries, but studies of their negative effects on reproductive function are not enough. In veterinary medicine, 3 levels of helminth infestation are recognized. Low level (up to 200 eggs/g feces). The average preclinical level (200–500 eggs/g feces). High clinical level (more than 500 eggs/g feces). In this case, helminth invasion is determined for each type of parasite separately. We drew attention to this. For example, finding in the feces of a horse 4 types of parasite of 150 eggs/g, the laboratory concludes that the parasitological situation is preclinical. If $4 \times 150 = 600$ eggs/g, which is the clinical level of invasion. Having studied the complex effect of the association of three types of intestinal nematodes strongylidae, parascaris equorum and oxyuris equi, we showed that up to 50 eggs/g of feces should be considered a low level of invasion; medium – 50–300 eggs/g feces; high – more than 300 eggs/g of feces. We proposed such levels of total helminth invasion on the basis that, at a low level (up to 50 eggs/g feces), the foal yield from chilled sperm was $90.3 \pm 1.40\%$; at an average level (50–300 eggs/g feces) – $75.64 \pm 1.28\%$; at a high level (more than 300 eggs/g of feces) – $50.72 \pm 1.45\%$. The yield of foals from frozen sperm was 77, 58, and 42%, respectively (Tkachov, 2014).

3. Conclusions

Thus, the development strategy of the physiology of horse reproduction in order to increase its effectiveness should take into account the least studied aspects that were discussed above. The article shows that taking into account the influence of micromycetes, the absolute number of colony forming units of *E. coli* in semen of stallions; immunogenetic and cytogenetic features; new methods of sanitary preparation of horses for sperm and insemination; the effect of permissible levels of feed mycotoxins on physiological functions improves the efficiency of equine reproduction physiology methods.

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