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ATYPICAL BOVINE PESTIVIRUSES

(review)

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Abstract

Increasingly frequent outbreaks of atypical viral infections, detection of new viruses, modified isolates and quasispecies with a confirmed or potential emergence have become a worrying feature of the last decades characterized by extremely close international dealings. For the cattle industry, they pose real and serious threat because of a tendency to spread widely and quickly due to globalization and the use of standardized zootechnical and veterinary protocols. The *Flaviviridae* family comprises several genera of which the genus *Pestivirus*, including four viruses, i.e. the cattle viral diarrhea — mucosal disease (VD-MD) virus types 1 and 2, swine fever virus and sheep border disease virus, are important to farm animals (http://ictvonline.org/virusTaxonomy.asp). The characteristics of a new group of viruses genus *Pestivirus* of the *Flaviviridae* family, allocated in the period from 2000 to 2014 from the buffalo and cattle, as well as fetal calf serum used for cell cultures and vaccines production harvested in Australia, Canada, Mexico, Brazil and the United States and packaged in Europe (H. Schirrmeier et al., 2004; A. Cortez et al., 2006; E. Bianchi et al., 2011; B. Rodrigues et al., 2011; H. Xia et al., 2011; H. Xia et al., 2012; S. Peletto et al., 2012) are submitted in the review. The virus has been isolated in Thailand, Bangladesh and China (L. Liu et al., 2009; L. Mao et al., 2012; N. Haider et al., 2014). Messages on the isolation of the agent in other European countries, North America, Russia, India and Australia are absent (F.V. Bauermann et al., 2013). The widespread use of contaminated biological products can facilitate the penetration of the virus in different regions of continent causing their potential emergence for cattle. Strains of viruses presented cytopathogenic and non-cytopathogenic biotypes not officially classified and have a variety titles in literature: a third type of viral diarrhea-mucosal disease in cattle (BVDV), an atypical pestivirus (HoBi-like), the fifth type of *Pestivirus* genus (N. Decaro et al., 2012). Based on phylogenetic analysis were identified two genetic groups: Brazilian and Thai, which differ from the prototype member of the genus - the BVD virus but having a great similarity in the manifestation of clinical signs, the ability to infect the foetus of cattle and buffalo (F.V. Bauermann et al., 2013). In cattle a spontaneous or experimental infection caused by HoBi-like virus is very similar to the cattle VD-MD and manifests as diarrhea, abortion, respiratory syndrome, persistent infection (F.V. Bauermann et al., 2013). The situation is aggravated by the fact that they like the BVDV are able to induce persistent infection and forms permanent epizootic foci (M.N. Weber et al., 2014). The discovery of this group of viruses requires a critical assessment of the diagnostic tools and vaccines against the BVDV. To date, the there are no tests for the detection of ruminants' pestiviruses or their antibodies, particularly due to high variability of this virus group. That is why their laboratory diagnosis should not rely on the use of a single test. The best approach would be serological diagnosis of the herd followed by the identification of persistently infected animals, the virus isolation and molecular analysis (F.V. Bauermann et al., 2013). Given the lack of HoBi-like infection diagnostics, these viruses can remain unnoticed and, presumably, compromising the effectiveness of control or eradication programs of BVDV realized in certain European countries and the United States (K. Stehl et al., 2004; J.F. Ridpath, 2010).

Keywords: pestiviruses, viral diarrhea-mucosal disease, atypical viruses, sequencing, genetic subgroups, fetal serum, buffalo, molecular diagnostics, control programs.

The increasingly frequent outbreaks of atypical viral infections, detection of new viruses, modified isolates and quasispecies with a confirmed or potential emergence for animals, including bovine cattle, have become a worrying feature

of recent decades, which are characterized by almost non-limited expansion of international trade relations. For the herding, they pose a real and serious threat because of the tendency to a wide and rapid spread between regions and continents due to globalization and international shipments of large batches of animals.

Moreover, the use of contaminated biological materials in relevant standardized protocols in zootechnics and veterinary medicine may result in expansion of viruses, including atypical ones.

In this regard, viruses from the *Flaviviridae* family are of special interest as they have a wide range of obligatory hosts (due to a great mutagenic activity) and are capable to cross the species barriers and infect heterologous animals.

This family comprises several genera, with the *Pestivirus* genus being the most significantly harmful among them for farm animals; this genus includes four viruses, i.e. bovine viral diarrhea-mucosal disease (BVD-MD) virus type 1 and 2; classical swine fever virus and (sheep) border disease virus (http://ictvonline.org/virusTaxonomy.asp).

Several new types have been discovered recently. Thus, the giraffe virus H138 was isolated from a giraffe in Kenya, the Pronghorn virus from a blind prong-horned antelope in the USA, the porcine Bungowannah virus from swine in Australia during a natimortality outbreak; also, a «HoBi»-like virus has been described [1-5]. The last one is of greatest concern for epizootologists and virologists due to its potential emergence and similarity to BVDV. The established strains of this virus presented both with cytopathogenic and non-cytopathogenic biotypes have been allocated into a separate species, which is designated variously in the literature, namely an atypical pestivirus, a bovine viral diarrhea virus type 3 (BVDV3, «HoBi»-like), the fifth type of the genus *Pestivirus* [6-9]. The International Committee on Taxonomy of Viruses (ICTV) has not accepted their official classification yet.

Manifestation peculiarities of pestivirus infections as exemplified in BVD. In 90 % cases, the diseases caused by pestiviruses in ruminants occur as acute subclinical and persistent infection. The major characteristic of this group of viruses is the ability to induce immune suppression associated with leukopenia, decreased lymphocyte proliferation, depletion of the lymphoid tissue, decreased chemotaxis and phagocytic activity, increased production of prostaglandin E2 and altered inflammatory cytokine production, which may be transient (2 to 3 weeks) in acute forms or prolonged in persistently infected (PI) animals [10-13]. Rare clinical signs of acute forms of the disease include gastrointestinal, respiratory and reproductive effects, such as diarrhea, fever, leukopenia, nasal and ocular discharge, abortion at all stages of pregnancy, delivery of PI calves. The PI animals are diagnosed with mucosal disease [14].

The BVDV is considered to be a prototype member of the genus *Pestivirus*. The disease caused by this virus is common throughout the world. The incidence of the infection in cattle is 60-85 % and is found to be region-specific [1, 2, 12, 13, 15]. The presence of PI animals in the herd increases this figure up to > 90 %. The economical losses are estimated as US$ 88 per animal [16].

Pestiviruses have a single-stranded RNA(+) genome of 12.3 thousand nucleotides. It consists of a single open reading frame (ORF) of approximately 4,000 codons in length, which encode 12 structural and nonstructural polypeptides (Npro-C-Erns-E1-E2-p7-NS2/NS3-NS4A-NS4B-NS5A-NS5B) and flanked by 5′- and 3′- untranslated regions (UTR) (5′-UTR and 3′-UTR) [17].

The virus is prone to mutations caused by errors of RNA-dependent RNA polymerase and recombination [17-20]. Due to frequent mutations, which occur during RNA replication, the virus exists as a pool of different but closely related mutants (quasispecies) subjected to continuous selection. Therefore,

strain pathogenicity varies considerably [10, 20].

The nucleotide sequence of genomic RNA is considered to be the most reliable tool for species and generic identification of pestiviruses. Most often, a highly conserved region suitable for amplification 5'-untranslated region (5'-UTR) and the N-terminal auto-protease (N^{pro}) gene are investigated. For phylogenetic analysis, the sites of the E_2 (the most variable) and E^{rns} genes are additionally investigated. The need to investigate several gene sites is associated with genome recombination [20-23].

In cattle, the disease may be induced by two viruses, BVDV type 1 and 2. Today at least 15 subspecies of BVDV type 1 (1a to 2o) and no fewer than 5 subspecies of BVDV type 2 (2a to 2e) are distinguished [4, 17]. Both types of the pathogen cause the same pathology in animals; however, the strains of the virus type 2 are more virulent and less common [15, 24-27]. Both viruses are represented with cytopathogenic (CP) and non-cytopathogenic (NonCP) biotypes, and NonCP types are prevailing [26, 27].

«HoBi»-like viruses (atypical pestiviruses). The first strain of the HoBi virus (D32/00_HoBi) later accepted as the prototype has been isolated in Switzerland from fetal calf serum, imported from Brazil [7]. Thereafter several more isolates have been obtained, e.g. two isolates from fetal serum in South America, CH-Kaho/cont from cell culture, Brz buf from samples of buffalo biological material, two isolates from aborted fetuses in Brazil, Th/04_Khonkaen from calf blood serum in Thailand [28]. In Italy in 2010, the virus was isolated from calves during a respiratory disease outbreak and also in the persistent form of infection [29, 30]. There was a report from Brazil in 2014 on the first case of the disease resembling the mucosal disease in BVD MD, with associated recovery of the CP strain of the virus from calves [31].

These viruses have also been detected in fetal serum, which was packaged in Europe though harvested in Australia, Canada, Mexico and the United States. According to some estimates, more than 30 % fetal calf serum batches shipped to Europe from South America are contaminated with the HoBi virus [9, 32]. Increased demand for fetal bovine serum contributes to the penetration of the virus in different regions.

The disease has been recorded in Bangladesh [33]. There were no reports on the isolation of virus from this group in other countries of Europe, North America, in Russia, India or Australia.

The origin of «HoBi»-like viruses is unknown. Currently there are two proposed anticipations. The first says that they are newly evolved or historically existed in South America and were brought to other countries and continents with biological products (fetal serum and vaccines). Another explanation is that these viruses have passed to cattle from buffalo and adapted as a result of multiple transspecies transmission. This anticipation explains the occurrence of these viruses in the regions with significant populations of buffaloes, such as Brazil or Thailand [9].

The phylogenetic analysis results demonstrate that all isolates of «HoBi»-like viruses identified at the moment have a great similarity and are grouped together. Considering the high genetic variability of pestiviruses, it is suggested that the emergence of «HoBi»-like viruses in South America and their subsequent spread to other regions is quite a recent event from the point of view of evolution. In the meantime, the way the isolate Th/04_KhonKaen from Southeast Asia differ from other «HoBi»-like viruses may suggest the independent evolution of at least two genetic subgroups of «HoBi»-like viruses (Brazilian and Thai), which has been confirmed by phylogenetic analysis of the strains [9, 28, 34].

The discovery of this group of viruses requires a critical reassessment

of existing diagnostic tools and vaccines against the BVD-MD and other viral diseases. As long as there are no routine lab tests of HoBi virus infection in most countries and available diagnostic tests lack specificity, these viruses may remain unnoticed and supposedly exist in other countries. The situation is aggravated by the fact that they like the BVDV are able to induce persistent infection and form permanent epizootic foci [35].

In Brazil, Italy and Thailand, in the herds, where these viruses might have already spread, they may lead to economic losses associated with the clinical manifestation of infection, reduced productivity and decrease in immunity (independently from the BVD virus circulation or concurrently).

The HoBi-positive status of countries may present a challenge in the international trade of animals and biological products derived from them (sperm, fetal serum, embryos) with countries free from these viruses.

As long as «HoBi»-like viruses were isolated from many species of ruminants on several continents and tend to the global spread, they represent the greatest threat for the herding among all new pestiviruses detected in 2000-2014 [9, 36].

Clinical manifestation of «HoBi»-like virus infection. In cattle, a spontaneous or experimental infection caused by «HoBi»-like viruses is very similar to the BVD-MD and manifests as diarrhea, abortion, respiratory syndrome, and persistent infection [9].

Natural infection. The first report on natural infection with Brazilian buffalo virus was received in 1990. The «HoBi»-like viruses isolated from samples of biological material from two aborted fetuses in Southeast Brazil were characterized in 2006 [34]. Three isolates of the virus were identified and sequenced in the same country in 2011. The isolate SV713/09 was obtained from a sample of a bull-producer's sperm, the use of which in herds resulted in multiple cases of blind calves born. The isolates SV241/10 and SV311/10 were identified from white blood cells of cattle and buffaloes with reproductive disturbances in the southern part of the country. In the mid-west region of Brazil the «HoBi»-like virus was identified from a calf spleen during a gastrointestinal disease [6, 35].

During an outbreak of a respiratory disease in 6-7-month old calves in southern Italy in 2009-2010, fever (39.4-40.1 °C), cough, serous nasal discharge, leukopenia, high heart and respiratory rates were reported. The autopsy of two animals revealed tracheitis and bronchopneumonia affecting apical lobes of the lungs. The virus was detected in nasal swab samples from six calves and in the lungs of the dead animals by quantitative RT-PCR (reverse transcription—polymerase chain reaction) and isolated from lungs in the MDBK cow calf kidney cell culture (Italy-1/10-1 and Italy-1/10-2 strains) [29].

In mass abortions in cattle, it was found possible to identify virus RNA and antigen in the tissues of aborted fetuses. The molecular research to identify other possible etiologic agents of the pathology produced negative results. The detected virus demonstrated a close affinity to the Italian, Australian and South American strains, though differed from the Thai one [29, 37, 38].

The site of the gene encoding the glycoprotein E2 and the 5′-UTR region in the Thai isolate revealed 99 % affinity to the Italian strain Italy-1/10-1. Titers of the relevant neutralizing antibodies were considerably higher than titers to BVDV type 1 and 2. The non-cytopathogenic «HoBi»-like virus was isolated in biological material samples of two aborted fetuses. Moreover, the first CP virus isolate was also identified in this herd, and it was obtained from the lungs of a heifer that died after a respiratory disease [39].

M.N. Weber et al. in 2014 reported clinical signs caused by the «HoBi»-like virus in cattle in Brazil, similar to the mucosal disease [31]. Sequencing and phylogenetic analysis of 5′-UTR, N^{pro} and E_2 regions revealed circulation of

four different strains of the virus in the herd. The main clinical signs and conditions involved respiratory and gastrointestinal tracts. Moreover, skin effects and corneal opacity were reported. The mucosal disease symptoms were identified in one cow calf, with subsequent isolation of the CP virus isolate. This paper gives the first case report on a condition resembling mucosal disease, which was associated with natural infection with a «HoBi»-like pestivirus [31].

Experimental infection. After the administration of the strain HoBi_D32/00, calves and pigs developed clinically asymptomatic seroconversion. Furthermore, mild fever and slight leukopenia were recorded in calves. The virus was detected in leukocytes on day 5, with its recovery over days 3 to 5 after infection [34].

In 1-month old cow calves, the Italian strain (Italy-1/10-1) caused moderate hyperthermia, serous nasal discharge and lymphocytopenia, while in lambs nasal discharge and minor lymphocytopenia only. Pigs displayed no clinical symptoms in response to virus challenge. All animals are reported to develop virus seroconversion on day 21 after infection; however titers of specific antibodies were higher in calves [40].

A comparative pathogenicity study of the Thai isolate and a highly virulent strain of BVDV revealed that the disease caused by the HoBi virus occurred in a milder form and manifested as bilateral conjunctivitis, serous nasal and ocular discharge, coughing, and thrombocytopenia on day 7, and lymphocytopenia on days 2 to 5, which returned to physiological values on day 14 after infection [41].

Diagnostic approaches in the identification of «HoBi»-like viruses. *Fetal serum testing*. Fetal calf serum is widely used in cell cultures and often found to be contaminated with pestiviruses. To obtain it, serum from many fetuses is used. The increased number of fetuses in a commercial batch contributes to the risk of inclusion any persistently infected fetus. The use of serum even with low-level virus contamination may result in cell culture infection and growth of a non-cytopathogenic strain in it. To rule out contamination, it is necessary to develop and use antigen-binding types of ELISA (enzyme-linked immunosorbent assay), qualitative and quantitative PCR. To determine the virus extension, all fetal serum batches should be tested, and not just those shipped from regions with documented cases of infection with viruses of this group [42-44].

Robust, highly sensitive test systems for the virus detection in animals and products of animal origin are warranted. This is particularly important for international trade. Currently there are no diagnostic tests for the detection of all ruminant pestiviruses or antibodies to them. Their development is particularly difficult due to high variability of this virus group, and therefore the laboratory diagnosis should not rely on the use of a single test only [9].

The «HoBi»-like virus antigen or genome can be detected in leukocytes, serum and nasal secretions of persistently infected animals. For virus isolation according to standard protocols, it may be appropriate to use free of pestivirus contamination primary trypsinized and finite septum cell lines (M-17) and the MDBK cell lines. The phylogenetic analysis of virus strains is recommended at the final stage of laboratory test [9, 21, 45, 46].

Identification of infected animals. Commercial diagnostic test kits for the detection of antibodies to BVDV using ELISA method produce false-negative results on the calf blood serum samples infected with «HoBi»-like viruses. A comparative study of BVDV-1, BVDV-2 and the HoBi virus using a commercial ELISA test kit and a serum neutralisation test found that cross reactions between epitopes of E^{rns} and NS2/3 proteins were higher than glycoprotein E_2. These results suggest that diagnostic tests for the detection of all three viruses should be

based on E^{rns} and NS2/3 epitopes, with the variability of the E_2 gene used for their differentiation [18, 47].

An additional problem is the time needed to achieve a detectable antibody titers concentration in infected animals. Furthermore, these test kits do not allow differentiating between antibodies to both viruses and the immune response to them. The neutralization test using CP strains of two viruses may be promising. Although the neutralization test is quite expensive in terms of time and results calculation, requires trained personnel and cell line stock in the laboratory, currently it is considered to be the most appropriate method for the detection and/or differentiation of animals exposed to infection with BVDV and/or «HoBi»-like viruses. However, the method requires validation.

The best diagnostic approach would be a serological survey in the herd, identification of PI animals, with virus isolation and subsequent molecular diagnostics [9].

Atypical pestiviruses and effectiveness of BVD-MD control programs. The BVD control or eradication programs, which are brought into effect in a number of countries, rely on three principles, and these are the identification and removal of PI animals from the herd; the prevention of including infected animals in the herd along with monitoring; and vaccination, the use of which depends on the disease prevalence in a region [48-50]. The successful implementation of such programs and maintenance of the free of BVDV status in the herd demand reliable diagnostic tests capable of differentiating persistently and transiently infected animals and identifying all viral quasispecies.

The existence of «HoBi»-like viruses requires special attention. They were isolated from commercial pools of blood serum used for cell culture and production of biological products, and pose a risk in view of the potential spread into new regions, possible reduced effectiveness of vaccines, diagnosticums and consequently the BVD-MD eradication programs. Moreover, animals intended for sale may also pose a threat.

Thus, the ability to infect calves, the severity of the disease, and the development of respiratory distress, intermittent fever, leukopenia, and lymphopenia caused by strains of «HoBi»-like viruses give evidence of significant similarities with the symptoms attributable to a typical bovine viral diarrhea-mucosal disease (BVD-MD). Given that all currently known strains of «HoBi»-like viruses were isolated from cattle or buffalo (if not adapted to other animal species in case of experimental infection), it suggests that they are obligate hosts of the virus. These novel, not yet fully characterized pestiviruses may affect the BVD-MD control and eradication programs and pose a hazard as being emergent pathogens for the cattle worldwide. The occurrence of the «HoBi»-like and other pestiviruses in ruminants, animal products and biopharmaceuticals should be considered and controlled.

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DEFORMED WING VIRUS IN *Apis mellifera* L.: PREVALENCE, MORPHOLOGY, AND PATHOGENICITY

(review)

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Abstract

Viral infections are not considered the most dangerous honeybee (Apis mellifera L.) diseases though being rather harmful. Virus-caused honeybee pathologies are mainly symptomless (N.J. Dimmock et al., 1987; A.C.F. Hung et al., 1996), nevertheless, a rapid replication of the viruses can be triggered leading to clinical manifestation and even death of the insects (R. Singh et al., 2010). In honeybee families a simultaneous circulation of several viruses can occur. A*cute* bee *paralysis virus* (ABPV), deformed wing virus (DWV) in Europe, and Kashmir bee virus (KBV), Israeli acute bee paralysis virus (IABPV) and DWV in the United States seem to be related to honeybee family collapse. This review summarizes the data about one of the most prevalent honeybee viruses, DWV (D. Tentcheva et al., 2004; O. Berènyi et al., 2006; S.L. Nielsen et al., 2008; S. Ruba et al., 2012). Surveys showed DWV in European countries. In Austria, France and Denmark the DWV was found in 91, 97 and 57% of the apiaries surveyed; in the Czech Republic 31% of sampled bees were infected with DWV (note, other viruses in Austria and France were less frequent, i.e. 68 and 58%, respectively, for ABPV; 49 и 86% for sacbrood virus, SBV; 30 and 86% for black queen cell virus, BQCV; and 10 and 28% for chronic bee paralysis virus, CBPV) (D. Tentcheva et al., 2004; O. Berényi et al., 2006). DWV predominated in the apiaries of all studied regions of Russia (A. Kalashnikov et al., 2012), and in Moscow Province only DWV and SBV were revealed. DWV is detected in Apis florea and A. dorsata (X. Zhang et al., 2012). Free DWV dissemination was indicated among some insects other than Apis (Bombus terrestris, B. pascuorum, B. huntii Green) (E. Genersch et al., 2005; J. Li et al., 2011; A.L. Levitt et al., 2013). DWV, a RNA virus with monocistronic genome, is a member of the genus Iflavirus (Iflaviridae family, Picornavirales) (G. Lanzi et al., 2006). Its phylogenetic relationship with Kakugo virus (T. Fujiyuki et al., 2004; A. Rortais et al., 2006) has been confirmed. The identity of the RNA nucleotide sequences of virus isolates from different geographic locations is 98-99 % (O. Berènyi et al., 2007). Its structural proteins VP1-3 are similar to the corresponding picornavirus structural proteins, while a low molecular weight protein VP4 is not found (G. Lanzi et al., 2006). The main targets of deformed wing virus are reproductive organs and digestive tract of bees (Y.P. Chen et al., 2006; J. Fievet et al., 2006). The viral RNA is also found in the wings, head, thorax, hemolymph, fat body (J. Fievet et al., 2006; H.F. Boncristiani et al., 2009). It can be detected during all life stages of honeybee (Y.P. Chen et al., 2005). The brood and adults with clinical manifestations of the disease die (L. Bailey et al., 2010). The worker bees are most sensitive to DWV. The bee colonies are weakened; they are characterized by reduced size and prone to sudden collapse (G. Lanzi et al., 2006; R.M. Johnson et al., 2009). The peak incidence is in the autumn. In addition to vector transmission a horizontal per os and also a vertical transovarial transmission of the virus are possible (C. Yue et al., 2005; C. Yue et al., 2007). The virus can cause a latent infection without visible symptoms of the disease with prolonged persistence of the pathogen in the host and vertical virus transmission or subclinical shorter form with high rate of viral replication and more pathogenic horizontal transmission. For clinical outbreak of DWV infection followed by colony collapse a strong trigger is required, such as immunosuppression by mites Varroa destructor or V. destructor as biological vector. The apiaries with V. destructor infestation are often infected by DWV.

Keywords: deformed wing virus, honeybee *Apis mellifera* L., bee family, collapse, virus transmission by vectors, per os transmission, vertical transovarial transmission.

It is currently estimated that there are over 18 viruses capable to infect the *Apis mellifera* L. honeybee. Although viral infections are not listed among

the most dangerous honeybee diseases, they may cause significant damage. Acute bee paralysis virus (ABPV) and deformed wing virus (DWV) (the latter is considered to be one of the most common honeybee viruses) in Europe and Kashmir bee virus (KBV), Israeli acute bee paralysis virus (IABPV) and DWV in the United States seem to be related to honeybee family collapse [1].

The viruses are morphologically similar (they have spherical or slightly oval shape and consist of single-stranded RNA enclosed in a 17-30 nm diameter icosahedral protein capsid) and referred to different families of the order *Picornavirales* [2]. Picornaviruses have no lipid envelope and replicate in the cytoplasm of the contaminated cell. Exclusions are DNA-containing filament virus of ellipsoidal shape [3], icosahedral iridovirus synergic with c *Nosema ceranae* [4], and RNA-containing chronic paralysis virus of different morphology with polycistronic genome [5, 6]. Most viral infections are asymptomatic [7, 8], however, in certain conditions, rapid replication of the viruses is possible, which leads to the manifestation of visible disease signs and often to death of honeybees [6]. Simultaneous circulation of several viruses can occur in honeybee families.

Deformed wing virus (DWV) is referred to the family *Iflaviridae*, genus *Iflavirus* [9]. It has been noted that this virus is serologically similar to Egypt bee virus [10] and remotely similar to human picornaviruses (poliomyelitis virus, rhinovirus) [11, 12]. DWV prevalence in *Apis mellifera* L. in European countries is confirmed by the studies carried out by different groups of scientists [13-16]. In Austria and France, DWV was found in 91 and 97 % of the surveyed apiaries, respectively; less frequent are acute bee paralysis virus (ABPV; 68 and 58%, respectively), sacbrood virus (SBV; 49 and 86 %, respectively), black queen cell virus (BQCV; 30 and 86 %, respectively) and chronic bee paralysis virus (CBPV; 10 and 28 %, respectively) [13, 14]. The rate of pupa infection in French apiaries was 94 % for DWV, 80 % for SBV, and 23 % for ABPV and BQCV [13]. All apiaries infested by *Varroa destructor* are contaminated by deformed wing virus. The seasonal variations of DWV prevalence were noted: in spring, summer and autumn, the rate of infection by the virus was 56, 66, and 85 %, respectively, for adult bees and 16, 38, and 54 %, respectively, for pupae. In apiaries in Denmark, SBV and DWV were primarily identified among six detected viruses (SBV, DWV, ABPV, CBPV, BQCV, KBV) [15].

The predominance of DWV was noted in apiaries in Maikop District of the Republic of Adygeya (Russia); it is followed by sacbrood virus, while black queen cell virus is the rarest (17). In Moscow Region, only DWV and SBV were found. The homology of DWV nucleotide sequences is 98 % [17].

Deformed wing virus was found in *Apis florea* and *A. dorsata* [18]. Free virus dissemination was noted among some arthropods not belonging to the genus *Apis* (*Bombus terrestris*, *B. pascuorum*, *B. huntii* Greene) [19-21].

According to the Baltimore Classification of Viruses, DWV is referred to group IV or (+)ssRNA viruses containing one single-stranded (+)RNA molecule [22]. Such viruses are characterized by the synthesis of (–)RNA which serves as a template for (+)mRNA formation [23]. DWV genetic make-up is similar to that of picornaviruses. The coding sequence of genomic RNA is conventionally divided into three regions: P1 codes structural proteins VP1, VP2, VP3, VP4; P2 and P3 code the proteins needed for cell reprogramming and replication. The 5′-end of genomic RNA is covalently bonded to protein VPg (viral protein genome linked) responsible for 5′-end stabilization in replication and translation processes. VPg consists of 23 amino-acid residues. A polyadenylate «tail», poly(A), is attached to the 3′-end. It is followed by a coding sequence flanked by a 5′-untranslated leader sequence (UTR, 1144 nucleotides) and a 3′-untranslated

region (317 nucleotides). The genomic RNA has a single open reading frame coding polyprotein with the molecular weight of 328 kDa. The full viral genome, including the poly(A) «tail», contains 10,140 nucleotides: adenine — 29.5 %, uracil — 32.5 %, guanine — 22.4 %, cytosine — 15.8 %. Of single nucleotide polymorphism (SNP), transitions make 82 % [11].

RNA molecule in a DWV particle is surrounded by protein coat. Three basic structural proteins VP1 (44 kDa), VP2 (32 kDa) and VP3 (28 kDa) correspond to structural proteins VP1, VP2 and VP3 in *Picornaviridae*. Low molecular weight protein similar to protein VP4 located within the capsid of picornaviruses has not been found [11]. The N-terminal region of polyprotein begins with the leader peptide (L-protein) which is more variable than other proteins. It is followed by structural proteins [24]. VP1 and VP3 amino-acid residues are located at polyprotein positions 486-880 and 902-1064, respectively. VP2 amino-acid residues are located to the left of VP1 (positions 256-448). Such boundary line may be hypothetical [11], and a large size of protein VP1 is rather an exclusion. The molecular weight of its homologue does not exceed 35 kDa, and more often it is much smaller [25]. Because VP1 is the integral component of the viral particle, it is still an open question how the additional amino acids located at its C-end can fit into the protein coat while not disturbing the spherical shape of the capsid with icosahedral symmetry. The C-terminal region contains the typical picornavirus nonstructural proteins: RNA helicase, chymotrypsin-like 3C-protease, RNA-dependent RNA polymerase [11]. Maybe, DWV does not use such a translation model when the synthesized giant polypeptide is decomposed by host cell proteases into smaller proteins [10]. It is noted than 28 % of nucleotide substitutions in the coding region lead to changes in amino-acid residues [11]. Insignificant changes in the amino acid composition are sufficient for the manifestation of viral tissue tropism [26, 27].

The identity of nucleotide sequences in the genome of viruses from different geographical areas (Austria, Poland, Germany, Slovenia, Hungary, Nepal, Sri Lanka, UAE, Canada) is 98-99 % [24], or 98 % in case of isolates from Italy and Pennsylvania (USA) [11]. The phylogenetic study revealed the genetic segregation of DWV and Kakugo virus (KV), as well as *Varroa destructor* virus 1 (VDV-1) [24]. The identity of nucleotide sequences is 84 % for DWV and VDV-1 (28) and 97-98 % for DWV and KV [9, 29, 30]. The evolutionary relationship of the three viruses has been confirmed. In spite of high homology at a nucleotide level, DWV and KV have distinct structural features [9, 29]: the genome of both viruses is characterized by polymorphism in the assumed leader region associated with viral pathogenesis [11]. It is reported that the combination of the three viruses (VDV-1, DWV and KV) may cause a collapse of honeybee colonies [30].

The main targets of DWV are the reproductive and digestive organs of honeybees [26, 27]. Viral RNA replication was found in samples of wing, head, thorax, leg and intestine biomaterial, as well as in hemolymph [31]. Low virus titer in ovaries contributes to the extension of the latent period [32]. The viral RNA is frequently found in neurocytons, and its presence in glial cells is not ruled out [33]. A strong specific reaction to DWV was registered in the cytoplasm and plasma membrane of most fat body cells [27]. The infection of the fat body, where nutrients are stored, metabolic products are accumulated and antimicrobial peptides are synthesized, can lead to disturbance of physiological processes and weakening of the immune system [34], and have an impact on the production of the main precursor of egg yolk, vitellogenin (monomeric phosphoglycoprotein), the concentration of which in hemolymph correlates with a physiological status of the insect and egg-laying capacity [27, 35].

DWV is identified at all development stages of the honeybee [36]. It is noted that infection rate in honeybee families with clear signs of infection is equal to 100 % for adult worker bees, 95 % for pupae, 80 % for larvae and 47 % for adult male bees. Virus titer variability at various honeybee development stages may reflect different capability to resist virus infection.

Deformed wing virus often exists in a latent or subclinical form. The onset of clinical symptoms in infected pupae leads either to their death or appearance of adults with deformed wings [34], distended short abdomens and no pigmentation [10]. Such bees become unviable and die [37]. The honeybee families infected by DWV are weakened, characterized by lesser number due to large reduction in life expectancy [38] and are prone to a sudden collapse [11, 39]. The killed families have much more worker bees with deformed wings as compared to the survived ones [40]. The number of worker bees with visible signs of DWV can be a marker of the upcoming death of the family [40].

In laboratory conditions, DWV introduction directly into the hemolymph of field bees led to greater replication of the virus within the period from day 3 to day 5 after injection [41]. A high degree of infection of different segments (maximum in abdomen, then, in descending order, in thorax and head), worsening of sensory perception, an increase in sensibility to water and low sucrose concentration, and a disorder of associative learning and memory have been noted with the absence of DWV clinical manifestations. Other researchers indicate changes in the behavior of morphologically normal field bees [42]. Such bees exhibit lower learning ability, leave the heave for a longer time and come back less frequently. The question whether deformed wing virus causes the aggressive behavior of bees or not is still disputable. A number of scientists do not associate aggression with the viral infection [9] while others express an opposite opinion [41, 43].

The viral infection combined with mite parasitism in bees causes immunosuppression [44], increased sensitivity to opportunistic pathogenic microorganisms [34], progressive decrease in bee family number and a complex of the diseases which are also determined by other pathogens [13, 45-47]. Mites contribute to wider distribution of the virus in a bee population and increase the DWV titer [1]. The rate of DWV infection positively correlates with mite number and duration of invasion by ectoparasites [40].

The mite parasitism significantly inhibits the gene expression of such antibacterial peptides as abaecin, defensin and hymenoptaecin, and the activity of the enzymes involved in immune response, such as phenol oxidase, glucose dehydrogenase, glucose oxidase and lysozyme [34, 42]; it also leads to an increase in DWV quantity in bees with bacterial infections [34]. The combination of the mite parasitism and presence of DWV and pathogenic microorganisms reduces the survival capacity of bees [48]. Negative correlation between the virus titer and phenol oxidase expression is noted [34]. It is believed that the death of the deformed wing bees infested by mites, which occurred within 24 h, is associated with the insufficient activity of phenol oxidase [48].

There are two hypotheses explaining the negative influence of ectoparasitic mites in case of virus infection. The first one assumes that *Varroa destructor* mites are biological vectors of DWV [49, 50]. Receiving the virus from infected bees, they accumulate it and then contaminate healthy individuals, in which morphological changes develop or which die within a certain period of time. Positive correlation between the number of the mites parasitizing a particular bee and the manifestation of morphological changes in bees or their death is noted [51]. In infected bees with asymptomatic disease development, DWV titers are

much lower than in the bees which exhibited the visible signs of the disease or died at the pupa stage. The degree of infestation by mites, rate of virus replication in mites and other factors have an influence on bee infection rate. The detection of viruses in mites and high viral load count in favor of this hypothesis [49, 52, 53]. In the female mites parasitizing infected pupae, the registered DWV titer is several times higher than in the contaminated pupae [51]. In *V. destructor*, picornaviruses were most frequently found in the cytoplasm of mid-intestine diverticle cells and sometimes in membrane-associated vesicles or long tubular membrane structures of cytoplasm. Q. Zhang et al. [54] believe that such subcellular localization may be an indicator of virus replication. DWV replication in the mid-intestine of mites indicates virus transmission via the hemolymph of infected bees and pupae [55]. In the mites that were able to induce an active form of infection, the number of DWV genome equivalents per individual was 10^{10}-10^{12}, while in those not causing the visible signs of wing deformation it did not exceed 10^{8} [56]. I.e. the development of deformed wings depends not only on *V. destructor* virus transmission, but also on the replication rate and DWV titer in the parasitizing mites. At the same time, the virus has no negative impact neither on the infected female mite, nor its offsprings.

According to the other hypothesis, mites are mechanical vectors. Due to the puncture of the cuticle, they introduce viral particles into bee hemolymph (reinfection). This is confirmed by data on virus reactivation after inoculation [57]. In addition to the mite parasitism, the virus reactivation may be influenced by infection by bacteria and protozoal agents [58], environmental contamination by the chemical compounds harmful to bees [59, 60], and other factors.

Epidemiological studies and laboratory experiments have demonstrated a correlation between the virulence of some viruses and the *V. destructor* mites acting as virus distributors between and inside bee colonies, and also as activators of virus reproduction in larvae and adult individuals [61].

Investigating the differences of gene expression in *Varroa*-sensitive and *Varroa*-tolerant bee families, M. Navajas et al. [42] concluded that mite parasitism causes changes in the expression of the genes associated with embryonic development, cell metabolism and immunity. The bees resistant to *V. destructor* primarily demonstrated changes in the expression of the genes controlling neuronal sensitivity and olfaction. Differences in olfaction may be due to increased grooming (ectoparasite removal by worker bees) and hygienic behavior (ability of bees to get rid of infected offsprings). The offsprings which are infested by the virulent mites causing visible DWV signs are removed in larger quantity than those infested by less virulent mites or intact offsprings [62]. Selective hygienic behavior (HB) and *Varroa* sensitive hygiene (VSH) help bees to manage mite parasitism [62, 63]. Interrelations of bees and identification of ill individuals may be influenced by a change in the hydrocarbon profile of the cuticle due to immunostimulation [64]. According to other data [65], on the background of mite parasitism, there is no visible correlation between changes in the hydrocarbon profile of the cuticle and behavior of bees within a social group.

The clinical manifestations of DWV in infected bee families had been attributed to mite parasitism for a long time [66-68] until scientists paid their attention to bees with the typical signs of the disease in the absence of mites [69, 70] and to correlation between the development of symptoms and the value of DWV titer [52, 71, 72]. Deformed wing virus can be a major factor of bee family death in the winter period independently from the presence of *V. destructor* mites [73].

J. Iqbal et al. (41) believe that, in natural environment, hemolymph

infection by *V. destructor*-like mites is the most probable way of DWV transmission. The studies by other scientists indicate that other virus transmission ways exist, too.

The presence of DWV in eggs and larvae at the development stages when infestation by mites was not noted [36, 74, 75], as well as the presence of viral RNA in the queen bee and its reproductive organs [26, 27] are indicative of vertical transmission. Among the offsprings from infected queen bees, DWV^+ eggs, larvae and adult bees constituted 100, 65 and 13 %, respectively. The excrements preliminarily taken from such queen bees were 90 % infected, hemolymph and ovaries were 100 % infected, and seminal receptacles were 80 % infected [27]. Laying DWV^+ eggs by queen bees with infected reproductive organs after artificial insemination confirms a transovarian vertical transmission [76]. The presence of viruses in ovaries and eggs is considered as a firm evidence of such virus transmission [26]. It has been established that the queen bees fertilized by infected sperm laid infertile eggs without DWV (DWV^-) and DWV^+ fertile eggs [76, 77]. Male bees and worker bees of generation F_1 were also DWV positive.

The horizontal transmission of deformed wing virus to larvae via infected feed is indicated by the infection of hypopharyngeal glands in worker bees [50, 77]. Although the assumption of DWV transmission via infected feed has not been confirmed by experiment (sucrose with virus lysate per os within 7 days), its longer consumption, for example, in winter, may be a source of virus infection [41]. The presence of deformed wing virus in the seminal receptacle assumes veneral horizontal transmission among individuals of one generation during mating [26, 36]. DWV in the epithelial cells of accessory glands and in testicles explains the presence of viral RNA in sperm [27, 77]. Its intensive replication in seminal vesicles may have a negative impact on the fertility of male bees [27]. In more resistant male bees, the virus was also found in most of the epithelial cells of the proventriculus, proctodeum and in the mature columnar cells of mid-intestine epithelium [27]. The mite parasitism inhibits the spermatogenesis [78].

Thus, deformed wing virus (DWV) is one of the most common viruses of the honeybee, *Apis mellifera* L. In Austria, France, and Denmark, the virus was found in 91, 97 and 57 % of apiaries, respectively; in the Czech Republic, 31 % of selected honeybees were contaminated by deformed wing virus. DWV prevalence is noted in most of the studied regions of Russia. Deformed wing virus was found in *Apis florea* and *A. dorsata*. In addition, DWV is spread among the arthropods not belonging to the genus *Apis* (*Bombus terrestris*, *B. pascuorum*, *B. huntii* Greene). DWV is characterized by the monocistronic genome. The virus is identified at all development stages of honeybees, however, worker bees are the most sensitive. Insects with deformed wings are unviable and die. In spite of evolutionary relationship with Kakugo virus, DWV differs from it with regard to virulence, tropism, clinical manifestations and geographical distribution. The identity of genome nucleotide sequences for DWV from different geographical areas is 98-99 %. DWV itself can cause a latent form of infection (with persistence of the pathogen in the host organism without clinical manifestations and with vertical transmission) or a subclinical form (shorter, with high rate of virus replication and more pathogenic horizontal transmission). An infected family with predominance of individuals without visible signs of the disease and with vertical transmission will normally develop, allowing the virus to exist in the population for a long time. For a clinical outbreak, infection transition to a lethal active form and a subsequent collapse of the bee family, a trigger is required, such as immunosuppression due to the infestation by *Varroa destructor* or the presence of the mite as a biological vector.

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PERSPECTIVE OF ZONA-FREE METHOD USE IN FARM ANIMAL CLONING

(review)

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Abstract

The review is dedicated to one of the relevant and widely discussed topics of modern biotechnology, namely cloning of mammals. Particularly, the success and problems of the somatic nuclear transfer (SCNT) are discussed herein. The advantages and disadvantages of a commonly used SCNT and zona-free modification are compared based on special publications and the data obtained in our experiments. The most promising targets for the SCNT are reproductive cloning, therapeutic cloning and fundamental science. Conservation of rare and endangered species is also in focus. Nevertheless, to date the cloning application is still relatively limited. One of the reasons is a low yield of healthy offspring in mammals, for example, average yield in cattle is about 9 % of cloned embryo transfers result in birth of healthy offspring. It is assumed that deviations in the development of cloned fetuses are caused by disorders in genomic reprogramming of a somatic cell nucleus, which results in significant disturbance of gene expression particularly in placenta. Even though there are several practical techniques that allow to increase effectiveness of SCNT, reprogramming of the nucleus demands further study as one of the fundamental problems of developmental biology. Second problem that hinders practical application of SCNT method is complexity of the conventional technique, which was introduced about 30 years ago by S.M. Willadsen (1986). Since then the technique has been applied almost without any variations. At the same time significant progress has been achieved in the so called zona-free nuclear thansfer method (zona-free NT), where oocytes are freed from zona pellucida before enucleation. This method was successfully applied for the first time by T.T. Peura et al. (1998). They used blastomeres of bovine embryo for electrofusion. The method was also effective for creation of cloned embryos of pig (P.J. Booth, 2001), sheep (T.T. Peura, 2003), cow (P.J. Booth et al., 2001), horse (C. Galli et al., 2003) using somatic cells. We have improved zona-free NT method for cattle embryo cloning (G.P. Malenko et al., 2006). In the available publications there are no references about zona-free NT use by other researches in Russia while worldwide it is considered more simple, effective and reproducible method compared to a conventional one (I. Lagutina с соавт., 2007; B. Oback с соавт., 2007). Preparation of cytoplasts by enucleation of zona-free oocytes can be carried out without fluorescent dyes with effectivness of 95-100 % and preservation of 96-97 % of ooplasm volume (M.I. Procofiev et al., 2007). Electrofusion rate of zona-free cytoplasts and somatic cells is 95-100 % (I. Lagutina et al., 2007; G.P. Malenko et al., 2007) compared to 60-70 % achieved during conventional cloning (I. Lagutina et al., 2007). Blastocyst yield is equal or higher then yield produced by the conventional method. Embryo transfer results are comparable for both methods. Zona-free NT method may further increase the output of cloned embryos and offspring of the farm animals due to the simplicity and high effectiveness. Since the unique gene combinations of elite bulls' genotypes can not been copied by natural reproduction, their cloning is promising, particularly by means of zona-free NT—SCNT. SCNT application also seems to be the most prospective in animal transgenesis.

Keywords: somatic cell nuclear transfer, zona-free method, enucleation, electrofusion, farm animals.

The first publication on successful cell nuclear transfer in mammals was issued back in 1981 [1]. The authors reported the birth of three mice resulting from transplantation of embryoblast cell nuclei into enucleated zygotes. However, no one, including the authors, has been able to reproduce this experiment. A few years later S.M. Willadsen of Cambridge University, a specialist in farm animals' embryology, made a breakthrough obtaining the first cloned lamb as a result of 8- and 16-cell embryo blastomeres and enucleated sheep ovum fusion [2]. Using the S.M. Willadsen's technique, cloned offspring of cattle and pigs [3, 4]. A report on the birth of Dolly the sheep published in 1997 [5] became a historical moment in the development of cloning technology which demonstrated a possibility of a complete reprogramming of a differentiated somatic cell nucleus by the cytoplasm of enucleated oocyte recipient. Next, numerous publications appeared on embryo cloning based on somatic cell nuclear transfer (SCNT) followed by transplantation of these embryos and by the birth of offspring in cattle [6, 7], mice [8], goats [9], pigs [10, 11], rabbits [12], horses [13] and other animal species.

The most promising targets for the SCNT are reproductive cloning, therapeutic cloning and fundamental science. In the practice of animal husbandry, the «copies» of elite bulls possessing a unique combination of genetic material can be created with the help of cloning which is impossible with the natural reproduction. Perspective of cloning method use for the preservation of rare and endangered species is in focus. SCNT application seems to be most prospective in animal transgenesis. The efficiency of gene constructs microinjection in male zygote pronucleus was very low when applied to farm animal species [14]. At the same time, cloning makes it possible to produce transgenic animals using previously transfected somatic cells in vitro as nuclei donors. As a result, efficiency is increased tenfold, which is very important when it comes to large farm animals [6]. Such cattle transgenic fetal fibroblasts retain their competence as nuclei donors in the SCNT technique [15]. The birth of calves transgenic for the human blood clotting factor IX (hFIX) in an experiment using this approach has been reported [16].

Transgenesis based on the use of somatic cells is compatible with target genetic modifications applying «zinc-finger nucleases» (ZFNs) that make it possible to produce specific endogenous gene knockout individuals [17]. Currently, intensive studies on transgenesis of pigs useful as donor organs for xenotransplantation are conducted in the world [18]. Transgenic pigs, the models of a number of human diseases, have been obtained [19, 20].

However, the method of animal cloning is not widespread yet, mainly due to a low yield of healthy offspring. In cattle, it averages about 9 %. Abnormalities in the development of cloned fetuses are mainly due to impaired reprogramming of the donor cell genome which results in a significantly disrupted gene expression, in particular, in the placenta [21, 22]. Even though there are several practical techniques that allow increasing SCNT effectiveness [23-29], reprogramming of the nucleus demands further study as one of the fundamental problems of developmental biology.

Another problem that hinders the development of cloning is the complexity of a number of SCNT stages implementation. A significant progress has been achieved in the so-called zona-free nuclear transfer method (zona-free NT), where oocytes are freed from zona pellucida before enucleation. First, it was successfully applied back in 1998 in cattle cloning when blastomeres were the nuclei donors [30]. The approach appeared to be effective with the use of somatic cells for creation of cloned embryos of pig [31], sheep [32, 33], cattle

[33-38], and horse [13].

We have improved the zona-free NT method for cattle embryo cloning [39]. In the available publications there are no references about zona-free NT use by other researches in Russia while worldwide it is considered a more simple, effective and reproducible method compared to the conventional ones [40, 41]. In this paper, based on publications and the data obtained in our experiments, we tried to perform a comparative analysis of the basic stages of SCNT technology under the commonly used and zona-free methods of cattle cloning.

The key SCNT process of the reprogramming of a differentiated somatic cell nucleus is the result of the direct oocyte cytoplasm effect on the karyoplast nuclear material. In vitro matured enucleated oocytes are usually used as recipient cytoplasts. The cytoplasm of cattle and pig oocytes contains numerous lipid granules resulting in invisibility of the metaphase plate under the microscope. When 20-30 % of the volume of oocyte cytoplasm adjacent to the first polar body (PB1) is removed, only 42-60 % of oocytes are enucleated [42-44], so that this method is recognized not to be suitable for the preparation of cattle cytoplasts [45].

Intravital oocyte staining with fluorescent nuclear Hoechst 33342 stain makes it possible to accurately determine the location and ensure the efficient (to 100 %) removal of maternal chromosomes. However, even the short-term exposure to ultraviolet (UV) radiation may affect the quality of cytoplasts adversely [44]. Cattle oocyte enucleation controlled by the Oosight imaging system doubled SCNT efficiency compared to Hoechst 33342 in combination with UV [46]. Demecolcine provided a high degree of enucleation (95.7 %) without any apparent negative effects [44].

Regardless of how the maternal chromosomes are located, oocyte enucleation by the traditional method is performed using a micromanipulator with a holding pipette and a manipulating pipette with an end sharpened at an angle, preferably with an additional spike. This stage requires a great skill of the performer which is acquired as a result of long practice.

In accordance with the zona-free NT technique, oocytes are freed from zona pellucida before enucleation by treatment with a pronase solution. Further enucleation can be performed manually or using a micromanipulator. Using the hand-made cloning (HMC) methods, oocytes are cut in halves with a sharp razor under a stereomicroscope [30, 33, 35, 47, 48]. Oocyte halves are stained with Hoechst 33342, and cytoplasts without nuclear material are identified under a microscope.

According to another enucleation technique used in HMC, oocytes without zona pellucida are treated with demecolcine solution which enhances the formation of a cytoplasmic membrane protrusion on the oocyte surface at the metaphase plate location which is clearly visible under a stereomicroscope. This part of the oocyte is cut away with a razor, which allows selecting cytoplasts without staining with Hoechst 33342 and further UV irradiation [49]. However, in this case, approximately 25-30 % of the oocyte cytoplasm volume is lost under enucleation as well, so two cytoplasts are required to produce one reconstructed embryo using the HMC technique. Reconstructed embryos can contain up to three types of mitochondrial DNA (mitochondrial heteroplasmy).

Using a micromanipulator, enucleation of oocytes without zona pellucida is performed without a holding pipette. At this, the number of enucleated oocytes for a certain period of time is 2-3 times higher compared to common enucleation of oocytes with zona pellucids [41], and the amount of cytoplasm removed with maternal chromosomes is less than 4 % of the oocyte volume [38]. Therefore, an enucleated oocyte is used as a cytoplast which is especially important in animals in

which the limited number of oocytes is available, for example in horses [41]. At this, the location of oocyte chromosomes and reliability of enucleation were also controlled by Hoechst 33342 staining followed by using UV [32, 38, 41].

We proposed a modified method of blind enucleation of cattle oocytes without zona pellucida. Oocytes were freed from cumulus cells and zona pellucida after 16 hours from the start of in vitro maturation. Oocytes with polar bodies were immediately selected for enucleation. The remaining cells were returned back to the maturation medium and viewed every 30 minutes, PB1 oocytes being selected each time. According to the published findings, the removal of cumulus cells 15 hours after the beginning of cattle oocyte maturation in vitro does not reduce the degree of nuclear maturation and no has effect on the further development of parthenogenetically activated or reconstructed embryos [50, 51]. According to our results, 16.5 hours after the beginning of maturation, about 25 % of cattle oocytes of cattle have separated PB1; in 18 hours, about 50 % of the oocytes reached the stage of MII. At this, the removal of cumulus cells using Vortex in hyaluronidase solution and enzymatic removal of zona pellucida in these periods did not result in the separation of PB1 from the surface of mature oocytes [52]. Since with the absence of zona pellucida, the first polar body PB1 stays on the oocyte surface only under the linkage with the metaphase plate, PB1 is the pointer of the precise location of maternal chromosomes [53].

Oocyte enucleation was performed using a holding pipette and manipulating micropipette with an evenly cut end of a diameter of 20-25 microns. The pipette end was led up to PB1, and the latter was aspirated along with a small portion of the adjacent cytoplasm. That is, enucleation was performed by a blind technique without additional treatment with demecolcine or nuclear dye Hoechst 33342 and UV. Enucleation efficiency was 97-100 %, cytoplastic lysis was virtually not observed, and the oocyte cytoplasm loss did not exceed 3 % of its original volume [52].

To obtain reconstructed embryos, microinjection of an isolated nucleus or the whole somatic donor cell in cytoplast is used in some cases. However, the most common technique of combining the donor cell nucleus and cytoplast is electrofusion. The common cloning technique includes placing a somatic cell from a micropipette under zona pellucida using a holding pipette, if possible close to the plasma membrane of a cytoplast. The close contact of cell membranes is one of the most important conditions for successful electrofusion. However, this cytoplast-somatic cell contact in the perivitelline space under zona pellucida is not always obtained, as the cells are significantly different in size. Typically, the electrofusion rate is 50-70 % of the constructs prepared and exposed to an electropulse. According to I. Lagutina et al. [41], electrofusion efficiency in cloning using the conventional method does not exceed 60-70 % in cattle, and 65-83 % in horses

Following the zona-free NT technique, cytoplast-somatic cell constructs are prepared manually under a stereomicroscope using a phytohemagglutinin solution. We oriented each of these constructs in the electrofusion chamber relative to electrodes manually without exposure to alternating electric field, both prior to and after the pulse. In our experiments, the electrofusion rate was up to 95-100 % [52]. According to I. Lagutina et al. [41], following the zona-free NT technique, this value was 96-100 % in cattle and horses.

When using phytohemagglutinin, there is also a possibility to automatically orientate a group of several «cytoplast-somatic cell» constructs under the effect of an alternating electric field in a chamber with rectangular in section parallel electrodes 35 mm long spaced 3 mm from one another [37, 38, 54].

Karyoplasts contained in reconstructed embryos are subjected to the

combined effect of the oocyte cytoplasm, and as a result, the original core material of a differentiated somatic cell may undergo the reprogramming and acquire the properties of a totipotent cell. It is the cytoplasm of oocytes at MII stage that contains the factors that promote reprogramming of differentiated cell nuclei [2, 5].

In vivo embryo development is triggered by an activation process, when the amount of maturation promoting factor (MPF) decreases in the cytoplasm of a mature oocyte under the influence of a wave of increasing calcium ion concentration caused by the penetration of a sperm cell. Reconstructed embryos should be artificially activated for which (regardless of the cloning method) both chemicals and physical impact are used. At the same, starting from the stage of activation, reconstructed embryos without zona pellucida require individual placement to prevent their aggregation. At the stage of activation when the reconstructed embryos are incubation for 4 hours in the medium containing 2 mM DMAP (6-dimethylaminopurin), this problem is solved by placing them by one in medium microdroplets of 5 mcl coated with vaseline oil.

Later, reconstituted cattle embryos should be cultured for 6-7 days until the early stages of preimplantation development suitable both for cryopreservation and for non-surgical transplantation. Individual cultivation of cattle embryos is successfully carried out in small drops of medium [30, 38, 55, 56, 57]. However, there are data indicating that the effectiveness of embryo development in individual culturing may significantly decrease compared to culturing in groups. In addition, embryos without zona pellucida may lose some blastomeres in the drops on a flat surface of the dish bottom prior to the stage of compaction. Therefore, the Well of the Well (WOW) system is a promising one for the cultivation of embryos without zona pellucida [58].

The technique of preparation of small recesses in the bottom of plastic Petri dishes by a cold dissecting needle pressing against the bottom under a medium drop was proposed back in 1993 [59]. In this study and in the following ones [60], the system was used for aggregating embryonic stem cells with mouse embryos when creating chimeras. Small-sized recesses were comfortable cells for individual placement of dividing mouse embryos freed from zona pellucida. The conical shape of the recess bottom contributed to the contact of blastomeres and stem cells. Moreover, a small volume of medium of approximately 0.04 mcl was in close proximity to the embryo in such a system. With this, the dilution of autocrine factors is apparently limited during cultivation which affects the embryo development positively [49, 61].

In the WOW system, the total medium volume is 0.5 ml per a 4-cell well that makes the embryo culturing without medium change possible. A disadvantage of the WOW system is the necessity to prepare recesses in plate wells by hand, as the plates of such type are not currently manufactured in the world. In our experiments (zona-free NT technique), the output of cloned cattle blastocysts under culturing embryos without zona pellucida in the WOW system ranged from 33 to 48 % of the total number of reconstructed embryos [52].

Transgenic sheep with increased content of omega-3 fatty acids in milk have been obtained using the zona-free NT technique [62]. The authors also noted that the zona-free NT technique is not inferior to the conventional SCNT method in efficiency but is less expensive and easier to implement. The staff of the institute where a horse was cloned first in the world using the zona-free NT technique (Istituto Sperimentale Italiano Lazzaro Spallanzani, Italy) [13], compared this method with the conventional one for cloning of cattle, horse, sheep, and pig embryos and offspring based on their own research and on the published data. According to the authors, the lack of zona pellucida in oocytes facilitates

the enucleation stage greatly and increases the efficiency of electrofusion of cytoplasts and somatic cells significantly. Blastocyst yield is equal or higher than the yield produced by the conventional method; survival after cryopreservation and transfer results are comparable for both methods.

Thus, when the zona-free nuclear transfer (zona-free NT) technique is used, cytoplasts are prepared from mature oocytes previously freed from zona pellucida. In general, this technique is easier to implement and provides higher efficiency compared to conventional methods, and this is the reason why the zona-free NT technique is regarded as a promising one for production of cloned embryos and viable offspring of farm animals. In our technique modification, oocyte enucleation is performed using a micromanipulator with an easy to manufacture microtool without nuclear fluorescent stains. At this, enucleation rate is 95-100 % while retaining 96-97 % of the ooplasm volume and without cytoplast lysis. Electrofusion of cytoplast-somatic cell constructs prepared using phytohemagglutinin is up to 95-100 %.

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HEAT STRESS IN POULTRY. II. METHODS AND TECHNIQUES FOR PREVENTION AND ALLEVIATION*

(review)

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Abstract

An adverse effect of heat stress in poultry depends on both the external factors such as diet, water supply, rearing technology, birds' population density, air humidity and flow rate, etc., and the internal factors, particularly, poultry species and breed specificity, physiological conditions, etc. Herein, the approaches to prevention and alleviation of heat stress in poultry are summarized and discussed. Different strategies were proposed for lowering of body heat production and for better heat dissipation, thus maintaining productivity and product quality and minimizing losses for poultry farms. These strategies include the increase in energetic level of a diet in accordance with decrease in feed consumption due to stress (N.J. Daghir, 2009) and inclusion of higher (up to 4-5 %) levels of fat (B.L. Red, 1981; N. Usayran et al., 2001; A.A. Ghazalah et al., 2008); decrease by 2-4 % of dietary crude protein (Q.U. Zaman et al., 2008) and carbohydrate levels (metabolization of fat produces less heat than protein and carbohydrates) (N.A. Musharaf, J.D. Latshaw, 1999; N.J. Daghir, 2008); changes in amino acid profile of a diet (diets imbalanced in amino acids may increase heat production; moreover, requirements in lysine and sulfur-containing amino acids are much higher in heat stressed poultry) (R.M. Gous, T.R. Morris, 2005; S. Syafwan et al., 2011; O. Vjreck, M. Kirchgessner, 1980); supplementation with additional 250 ppm of vitamin C (M. Ciftci et al., 2005; A. Kavtarashvili, T. Kolokolnikova, 2010), 200 ppm of vitamin E (Z.Y. Niu et al., 2009; A.A. Rashidi et al., 2010), 8000 IU/kg of vitamin A (H. Lin et al., 2002), minerals or proper premix of vitamins and minerals (V.I. Fisinin et al., 2009), supplementation of feed or drinking water with electrolytes $NaHCO_3$, KCl, $CaCl_2$, NH_4Cl (R.G. Teeter et al., 1985; T. Ahmad et al., 2005); pelleting of diets (R.M. Gous, T.R. Morris, 2005; A. Kavtarashvili, T. Kolokolnikova, 2010); special regimes of feeding (K. Hiramoto et al., 1995; M.H. Uzum, H.D. Oral Toplu, 2013) and intermitted lighting (A. Kavtarashvili, T. Kolokolnikova, 2010; D. Balnave, S.K. Muheereza, 1998); periodic (in 7-day periods) substitution of soda ($NaHCO_3$) for 50-80 % of dietary salt (P.S. Silva et al., 1996; A. Kavtarashvili et al., 2010); feeding of mixture of ground mussel and lime (1:1) from separate feeders with simultaneous decrease in dietary Ca level; inclusion of dietary enzyme preparations (V.I. Fisinin et al., 1999) and probiotic strains of *Lactobacillus* (P.T. Lan et al., 2004); the use of special anti-stress additives and preparations (P. Surai et al., 2012; P. Surai et al., 2013); moistening of enzyme-supplemented diets (H. Lin et al., 2006; M.A. Khoa, 2007); increase in air velocity in poultry houses up to 2.0-2.5 m/s (J. Donald, 2000); tunnel ventilation systems (M. Czarick, B.L. Tyson, 1989); systems of evaporative air cooling (J. Donald, 2000; E.S. Mailyan, 2007); the use of heat-insulating and light-reflective roof materials, sprinkling of roof with cold water (S. Yahav et al., 2004); 15-20 % decrease in stock density (T. Ahmad et al., 2006); a decreased litter thickness (to 3-5 cm) (Salah H.M. Esmail, 2001); decrease in any disturbing activity (vaccination, repopulation etc.) during the hottest hours; providing poultry with constant access to water including days when poultry is vaccinated via water; elimination of spray vaccines during heat stress (O. Mikhailovskaya et al., 2010); regular cleaning and disinfection of drinking water and drinking systems; acidification of drinking water (A. Kavtarashvili, 2013); regular refilling of drinking system with fresh and cold water; isolation and shading of water tanks and pipes exposed to direct sunlight; cooling of drinking water (S. Yahav et al., 1996); thermal training of embryos during 2nd half of embryogenesis (Y. Piestun et al., 2008) and 3-day chicks (S. Yahav et al., 2001; S. Yahav et al. 2004); genetic improvements in thermal tolerance (A.V. Miftahutdinov, 2011) including activated expression of naked neck gene *Na* and frizzle feather gene *F* (N. Deeb et al., 2001; M.V. Raju et al., 2004).

Keywords: temperature, heat stress, poultry farming, productivity, methods of prevention and alleviation.

* «Heat stress in poultry. I. Danger, related physiological changes and symptoms (review)» see in Agricultural Biology, 2015, vol. 50, № 2, pp. 162-171 (doi: 10.15389/agrobiology.2015.2.162rus, doi: 10.15389/agrobiology.2015.2.162eng).

Heat stress results in behavioral, physiological, and immunological changes in poultry, which has a negative impact on its health, feed consumption, productivity, and quality of products [1, 2]. This inevitably results in significant financial losses. The severity of the adverse effect of heat stress in poultry depends on external (diet, water supply, rearing technology, birds' population density, air humidity, airflow rate, etc.) and internal (poultry species and breed specificity, physiological condition, etc.) factors [3].

Currently, various methods and techniques for the prevention and alleviation of heat stress in poultry are known.

Feeding methods for dealing with heat stress. Under the high temperature conditions, feed intake is reduced [2], the acid-base balance in the body changes [4, 5], the secretion and activity of endogenous enzymes decreases [6], the intestine absorbing capacity is broken [7], the passage of feed through the gastrointestinal tract is accelerated due to the 3-5 times increased water intake [8, 9]. As a result, a deficiency of nutrients, vitamins (especially C and E), and some macro- and trace elements arises in the bird's body [10, 11].

To reduce the negative impact of heat stress, researchers suggest various strategies for feeding in poultry.

It is believed that the daily rate of nutrients under conditions of heat stress should be maintained by increasing the density of fodder proportional to the reduction of its consumption [12, 13]. For example, if the expected reduction in feed consumption is 10 %, the content of all nutrients (including vitamins, mineral compounds, and trace elements) must be increased by 10 %. But the use of this strategy for certain substances is sometimes limited with the production capability. Thus, the addition of more than 6-8 % fat to the feed mixture is not always acceptable. In addition, the doses of certain feed additives cannot be changed without knowing how it can affect the health of poultry. In many cases, the reduction in food intake is so great that it cannot be compensated by increasing the amount of nutrients.

Some authors claim that under heat stress, the increase in the proportion of crude protein in the diet at the background of low feed intake plays a positive role [14, 15]. Other researchers report the dangers of feeding broilers with the feed high protein content at high ambient temperature [16-18]. According to Q.U. Zaman et al. [19], in the heat stress conditions (32-39 °C), the diet with low crude protein (190 g/kg) and high content of metabolizable energy (12.55 MJ/kg) with standard values of essential amino acids contributes to greater efficiency of broiler chicks than the feed with high amounts of protein (210 g/kg and 230 g/kg) and metabolizable energy (12.55 MJ/kg).

The inclusion of 5 % of fat [20] in the diet of broiler chicks, 5 % of fat [21] and 4 % of soybean [22] or palm oil [23] for young hens at high temperature makes it possible to improve poultry productivity. This is because, first, fat enhances the palatability of fodder and encourages its consumption, and second, less endogenous heat is produced in fat metabolism than in protein and carbohydrate metabolism [14, 24, 25].

To reduce the endogenous production of heat in poultry under heat stress, V. De Basilio et al. [26] recommend using two diets: during the hottest period of the day it is a diet with higher (by 4-5 %) fat content and lower (by 2-4 %) amounts of crude protein, in the cooler period it is a diet with a reduced fat content and a higher crude protein proportion.

Positive results are obtained by a change in the proportion of fat and carbohydrates feed energy that is the ratio of effective and metabolizable energy [27]. In practice, the possibility of applying this method is somewhat limited due to the use of feed ingredients that the farms are able to purchase [12].

The balance of amino acids is also important. Consuming fodder of an unbalanced amino acid composition, poultry produce more heat per 1 g of fodder [11, 12]. The inclusion of additional amino acids in the diet results in the increase of oxidative processes in the follicular tissue, growth and development of follicles, and thus to the increase in poultry productivity [28]. When the ambient temperature increases, the consumption of lysine to maintain 1 kg of live weight of chickens and sulfur amino acids for the maintenance of egg production grows significantly [29]. Many authors [30, 31] find that fodder unbalanced in its amino acid composition increases the content of nitrogen compounds in the litter, which leads to the accumulation of ammonia in the poultry house and negatively affects the productivity, health, and thermoregulation in poultry.

Positive results were obtained with supplementation of fodder with additional 250 mg/kg of vitamin C [32, 33]. At higher doses (500 mg/kg and 750 mg/kg of feed) the effect was negative [34]. When ascorbic acid is added to water, not to the fodder, water intake increases [35].

Z.Y. Niu et al. [36] found that under heat stress (38 °C) broilers treated with vitamin E (200 mg/kg of feed) had higher macrophage activity and increased levels of IgM and IgG compared to the poultry that were not given the vitamin. There is evidence [37] of a significant intensification of phagocytosis, an increase in the number of T-lymphocytes and SRBC (sheep red blood cells) antibodies, spleen weight, and bursa of Fabricius in broiler chickens in cases of supplementation of fodder with vitamin E (100 IU/kg feed) during heat stress (32 °C). Other researchers [38] noted that at 33 °C a supplement of vitamin E in a dose of 200 mg/kg of fodder increased SRBC antibody titers in egg-production chickens. Vitamin E is an essential component of antioxidant protection, but recent studies have shown that it is not always able to improve the situation [39, 40].

The harmful effects of heat stress on egg laying can also be avoided by supplementation of fodder with vitamin A (8,000 IU/kg of feed) [41]. The greatest effect is achieved when complexes of vitamins, for example C and E [42], A and E [43], or the relevant vitamin-mineral premixes are fed [40].

High efficiency was shown for fodder or water supplements of various salts electrolytes ($NaHCO_3$, KCl, $CaCl_2$, NH_4Cl) [4, 44]. Supplementing feed with sodium bicarbonate at the rate of 4-10 kg/t helps restore the acid-base balance lost in alkalosis resulting from hyperpnoea in birds in the heat. An additional amount of electrolytes such as potassium chloride (0.25-0.5 % in drinking water or 0.5-1.0 % in feed) restores the electrolyte balance [33]. Drinking electrolyte solutions should be given in the morning before the rapid rise of air temperature [45]. Excessive potassium is better endured by birds than excessive sodium [46]. Under heat stress, the body tends to retain more electrolytes (Na, K, Cl) to maintain the acid-base balance. The amount of electrolytes in the urine is dependent on their content in the feed and on the ambient temperature. Water consumption is associated with the poultry age, with the K, Na, and Cl uptake with feed and has a direct effect on the litter moisture content and changes in rectal temperature [47, 48].

Fodder structure may affect the amount of energy spent for its consumption. For example, the intake of pelleted feed requires one-third less time compared with the same amount of spilled feed and allows birds to save about 6 % of energy [12], which can be beneficial for reducing heat production. Furthermore, pelleting increases the physical density of feed providing greater intake of nutrients [12, 33, 49].

Special feeding regimes provide positive results. Some scientists [50-52] recommend limiting feeding poultry 4-6 hours prior to the heat stress. During

this time, the remains of food are evacuated from the gut, and the increase of heat associated with feed consumption in the hottest period of the day is reduced. It is advisable to move the time of the main feeding to the morning and evening or use the «night feeding principle» [53]. To do this, we recommend implementing modes of intermittent illumination providing night illumination for 2 hours, dark periods of 3-4 hours in the hottest time of the day and night feeding [49, 54]. Other authors propose to add gradually (15 minutes per week) one hour of illumination at night during the hot period of the year (for example, from midnight to 1:00 a.m. but not less than after 4 hours since the end of the main illumination period). The regular illumination regime mode is restored in the fall, gradually reducing the «night feeding» in the same way. However, the use of such technology in poultry may shift the circadian rhythm of egg laying [55, 56], which may lead to some reduction in productivity. D. Balnave and S.K. Muheereza [57] reported that under a regime of intermittent illumination of 3L:1D (L means light D means darkness, h) compared with 16L: 8D at high temperature (32 °C), feed intake, body weight, egg weight, thickness, and strength of egg shell increased significantly. The positive effect of intermittent illumination is due to the fact that birds move less in the dark and, thus producing less heat energy. It was found that at high temperature in poultry houses, feed intake can be stimulated by increasing the multiplicity of its distribution up to 5 times or more with periodic idle starts of feeding system lines [49].

The adverse effect of heat stress can be alleviated by replacing the salt in the fodder (50-80 %) with baking soda (periodically for 7 days), in the most severe cases, the amount of soda supplement is allowed to reach 2-4 kg per 1 ton of fodder mixtures [53, 58]. There is evidence that supplementing the fodder with chromium at a dose of 600 mg/kg [59] or chromium (400 mg/kg) in combination with ascorbic acid (250 mg/kg) [60] increases the bodyweight of broilers. Positive results are obtained with the feeding of a mixture of ground mussel and lime (1:1) from separate feeders with a simultaneous decrease in dietary Ca level [53, 61].

To improve nutrient digestibility under heat stress, the inclusion of dietary enzyme preparations in the diet is recommended [53, 61]. Enhance feed palatability and nutrient digestibility is also enhanced by feed moistening using exogenous enzymes [49, 62, 63].

Probiotic strains of *Lactobacillus* can enrich the diversity of flora in the jejunum and cecum of chickens and restore the microbial balance in broiler chicks after heat stress [64]. During heat stress, special feed supplements and drugs may be used — ProviGard, Catosal (Germany), Betfin S1, OptiPro, zinc bacitracin, feed antibiotics, osmo-protective supplements, etc. [3, 40, 65-67].

While the feeding methods recommended to reduce the effects of heat stress have shown their potential benefits, none of them can be considered as the only ideal method or as effective as reducing the heat load on poultry using a variety of technological methods [12].

Reducing air temperature in poultry houses. As it is known, body temperature is determined by heat generation (heat production) and heat dissipation (heat release). At high temperatures, poultry suffer difficulties in achieving a balance between heat production and heat loss. If heat production exceeds the maximum loss for a long time, birds may die. Thus, a temperature increase of 4 °C causes the death of broilers [13].

To reduce the temperature in poultry houses, an increase in air velocity to 2.0-2.5 m/s and in the amount of fresh air to 6-7 m^3 per 1 kg of body weight per hour is recommended, which creates a feeling of coolness in birds [53, 68]. At this, the use of tunnel ventilation maximizes the convective heat loss, espe-

cially in high humidity conditions [69]. Farms should have spare power supply systems for the event of disruptions in hot periods. It is necessary to equip premises with evaporative cooling systems in which air passes through paper napkins moistened with water and enters the poultry house in cooled condition. With it, even at the ambient temperature above 35-38 °C, a temperature of 24-28 °C and below can be maintained in poultry houses [68, 70]. But one should keep in mind that the higher the humidity, the less effective evaporative cooling [62].

Misting systems (fine mist generated under high pressure inside the premises) increases the effectiveness of ventilation to 50 %, but it is important to remember that poultry tolerates the impact of high temperatures at high relative humidity much worse, so in such conditions, it is not allowed to moisture floor, bathe birds, etc. Humidity in poultry houses at high temperature in the absence of adequate ventilation should not exceed 50 % [68, 71].

The effectiveness of heat-insulating and reflective roofing materials (e.g., aluminum-plastic foil), irrigation of roofs with cold water, etc. has been reported [72, 73]. According to some authors, at high ambient temperatures, it is advisable to reduce the stocking poultry density by 15-20 % [33, 47]. According to V. Holik (74), the stocking density in the floor and cage poultry at the temperature of 25 °C should be 5.5 birds per sq. m and 450 cm^2 per bird; at 30 °C, 4.5 birds per sq. m and 550 cm^2 per bird; at 35 °C, 3.5 birds per sq. m and 650 cm^2 per bird. To reduce the release of biological heat of decomposing components, the thickness of the bedding used should be not more than 3-5 cm [70, 75]. The frequency of litter removal from houses should also be increased [70].

Handling birds under heat stress. When handling birds during heat stress, the following rules should be followed [45]: birds should not be disturbed during the hottest period of the day; vaccination, transportation of livestock to poultry houses for adult birds should be performed during the cool time of the day (early morning or late evening); under vaccination via drinking water, water supply should not be interrupted; spray vaccination should be excluded; prevention of bacterial infection should be performed (breathing with open mouth, there is no air filtration through nasal passages, secondary bacterial infections gets in the body, which results in bird wasting).

Features of drinking water preparation and providing water to poultry during periods of high temperature. At high ambient temperatures, favorable conditions for the growth of microorganisms, pathogenic bacteria, fungi, and algae arise in drinking systems due to the presence of mineral and organic impurities in water. They accumulate and form a so-called biofilm. In addition, as a result of mineral substances deposition, a lime peel is formed in pipes that serves as a refuge for micro-organisms and disrupts the normal operation of drinking systems [76].

At high temperatures, it is necessary to clean and disinfect water and drinking systems using products that contain a mixture of various organic (formic, acetic, citric, fumaric, etc.) acids. Acidification of water contributes to the sanitation of oral cavity, nose, and the entire digestive system in poultry, favors beneficial bacteria, and inhibits pathogens (*Salmonellae*, *Escherichia coli*, mold and yeast fungi) in the gastrointestinal tract. The acidic environment also helps the production of pancreatic enzymes, enhances the conversion of pepsinogen to pepsin, and inhibits the passage of chyme through the gastrointestinal tract [76]. In addition, it is advisable to ensure systematic draining from drinking systems for filling them with fresh cold water; insulation of water tanks and water pipes located in the sun, their protection by shade; cooling of drinking water to 5-18 °C (increases feed consumption by 5-11.6 %) (8, 77); providing unlimited access of birds to water and increasing drinking system by 20-25 %; timely re-

placement of water filters [45]. It was found that cold-water intake (10 °C) helps poultry stand the ambient temperature of 42.2 °C for 11.5 h [8].

Heat training. The results of heat stress can be effectively alleviated by heat training. The eggs of the meat chicken Cobb were incubated at standard temperature and humidity regime (37.8 °C/56 %, control group) [78]. In the experimental group, the eggs were subjected to training from the 7^{th} to the 16^{th} days of incubation for 12 or 24 hours at the temperature of 39.5 °C and air humidity of 65 %. At the 35-th day of cultivation, chickens of all groups were subjected to a thermal load (35.5 °C for 5 hours), as a result, higher heat resistance of broiler chickens of experimental groups was shown.

It was also found that heat training of 3-day old chicks (36-37.5 °C for 24 hours) increased poultry resistance to high temperatures in the later period of growth [56, 79]. Usually, right after the exposure to heat, the growth is slowed down but followed by compensatory growth contributing to the formation of greater body weight in broilers at the end of cultivation compared with poultry not subjected to heat training [79].

This phenomenon can be explained with heat training imprintingin developing embryos and with formation of epigenetic thermal adaptation in them manifested in the increased resistance of poultry to high temperatures [80]. A similar opinion is shared by other authors [81, 82] who have found that if poultry eggs are subjected to temperature stress during incubation, hatched individuals demonstrate changes in thermo sensitivity of hypothalamic neurons throughout their life.

Selection and breeding. Increasing of thermostability in poultry by selection and breeding can be considered a promising area of prevention and alleviation of heat stress effects [83]. However, one should remember that the actual heritability of the sign of poultry resistance to high temperatures is very low [84, 85]. In addition, selection for the increase of thermos-tolerance can cause a reduction in growth potential in a comfortable temperature conditions. Therefore, it is necessary to clarify the relationship and interaction of selection signs used in different temperature environments [86].

Results obtained in the study of the effect of heat load (35 °C/50 %/14 days, temperature/humidity/duration) in 38-week-old chickens of the egg crosses Hy-Line Brown, W36, and W98 prove their different thermos-tolerances [87]. Within the period of the thermal stress factor exposure, the reduction in egg production, feed intake, and egg shell thickness was 31.0 %, 35.0 %, and 0.07 mm, respectively, in the brown cross; 19.7 %, 29.0 %, and 0.04 mm in the W36 cross; and 13.0 %, 27.0 %, and 0.05 mm in the W98 cross, and mortality, on the contrary, increased, respectively, by 16.0 %; 4.0 %, and 8.0 %. The consistency of changes in the shell thickness and calcium in the gut was detected: in brown cross individuals the absorption decreased by 52.5 %, while in other crosses it decreased by 30 % only. The findings suggest that thermos-tolerance in layers of investigated genotypes varies, and the cross W98 livestock has the highest thermal stability. This provision based on the analysis of productivity values is also confirmed by the data on the known markers of the heat stress condition. Thus, polypnoea intensity in W98 is lower as indicated by the value of blood pCO_2: in W36, it decreased by 24 %; in brown cross, by 17 %; in W98, by 13 % only.

In a recent study [88], thermo-tolerance was evaluated in five commercial chicken genotypes (Lohmann Brown, Lohmann White, New Hampshire, dwarf White Leghorn, and White Leghorn) who were divided into two subgroups. Chickens of the first subgroup of each genotype were kept at a comfortable temperature for a long time (18-20 °C), chickens of the second group were kept under the heat stress conditions (30-32 °C). Feed intake, body weight, egg production, egg

weight, shell thickness, and strength in all birds of the first subgroup were higher than in those subjected to hyperthermia. The most stable body weight, egg weight, and feed conversion were observed in New Hampshire chickens, and the intensity of egg production and egg mass rate per 1 bird/day proved to be the most stable in White Leghorn chickens. This study showed that the negative manifestation of heat stress depends on the genotype.

The use of genes that contribute to thermal stability, such as the naked neck gene (*Na*) and the frizzled feather gene (*F*), in poultry breeding is noteworthy. The naked neck gene (*Na*) increases the growth rate of broiler chickens, the production of pectoral muscles, the heat loss through the neck, with decreased fat deposits in the skin and pectoral muscle [89, 90]. In heterozygous (*Na*/*na*) and homozygous (*Na*/*Na*) individuals, featherweight relative to body weight is by 20 % and 40 % lower, respectively, compared to fully feathered birds [91]. The frizzled feather gene (*F*) makes it possible to reduce bird feather insulation. The favorable effect of *F* gene on the body weight of broilers at high temperature is lower than the effect of *Na* gene. However, there is a positive effect of the simultaneous use of heterozygous genes (*Na*/*na* и *F*/*f*) in broiler chickens [92].

Thus, feed intake is reduced and water intake is increased under heat stress resulting in the reduction of poultry productivity. To alleviate the effects of heat stress, appropriate adjustments in the diet should be made, which will help reduce the heat production of the bird body and maintain the required intake of feed nutrients. Physical activity of birds needs to be minimized during the hottest time of the day. Prevention and alleviation of heat stress in poultry require an integrated approach and can include the following: selection for heat tolerance; changes in feeding, watering and lighting regimes; changes in the energy-protein ratio and the balance of amino acids in the diet; use of special feed or water supplements (vitamins, trace elements, anti-stress supplements, enzymes, probiotics); poultry houses redesign; improvement of ventilation and cooling systems; special technological methods. The choice of appropriate measures depends on many factors, such as intensity, duration, and diurnal heat variation; genetic features of poultry; features of feeding and housing (floor or cellular housing) of poultry; poultry age and productivity; relative air humidity; design of poultry houses, etc. The timely forecast of heat stress onset and adequate measures for its prevention will help neutralize its negative effects on poultry and associated economic losses.

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REPRODUCTIVE FUNCTION IN HYBRID POULTRY. II. AN IMPACT OF BREEDING FOR TRAITS OTHER THAN PRODUCTIVITY*

(review)

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Abstract

Herein, the data are summarized on the impact of such selection criteria as poultry cannibalism, ascites resistance, stress responsiveness, and primary immune response to reproductive function in poultry. The selection for one of these signs changes the productivity and other body functions. High-productive meat crosses differ from slow-growing chicks in a susceptibility to ascites (R. Wideman, 2001). Broilers' mortality because of ascites makes 5-10 %, and at the lowered temperature of the environment can reach 50 % calculated from world production (H. Pavlidis et al., 2007). Prerequisites to development of the disorder are formed during embryogenesis (E. Decuypere et al., 2000). In the embryos who are the descendants of ascite resistant poultry (L^-), a relative heart weight is reliable higher, and pipping shells and a hatching occur earlier, than in L^+ genotype lines for which the hypothyroidism and tachycardia are characteristic (D. Luger et al., 2002). An observed increase in egg incubation period in L^+ lines can be caused by lower activity of thyroid gland and an increased pCO_2 in egg air camera, and as a result, the embryos suffered from hypoxia. A positive correlation is revealed between the development of lungs and the thyroid gland activity during embryogenesis, i.e. the lungs volume is the larger the higher thyroidal hormone rate, and vice versa (M. Hassanzadeh et al., 2008). In the chickens with better developed lungs, grown up under chronic hypoxia, the mortality from ascites was reliably lower. In L^+ and L^- broilers the mortality was 93.2 and 9.0 %, respectively (S. Druyan, 2009). The heart beating in L^+ and L^- 1-day chickens differed, being on average 435 and 404 beats per minute, respectively, but to the day 17 the difference practically leveled (419 and 417 beats per minute, respectively) due to a decreased rate in L^+ and an increased rate in L^-. Divergent selection for the feather pecking behavior causes differentiation in reactivity of the neuro-endocrine and immune systems (A. Buitenhuis, 2006). There are the evidences that the optimized incubation protocols for meat hens can prevent broiler chicks from ascites and improve safety of the poultry. Feather pecking reduced in the course of selected is associated with improvements in egg production (number and weight of eggs), but the deterioration in the quality of hatching eggs, the results of incubation, the state of derived chickens and changes of stress responsiveness and(or) immune response. So, the feather pecking rate was reliable lower in L^- than in L^+ poultry, and the number and weight of eggs laid during a month are higher (i.e. 0.38 and 2.01 feather pecking per hour, 1223 and 1132 g, 24.4 and 18.3 eggs, respectively). However, the egg quality in L^+ hens was better compared to L^-, with the Haugh units of 73.0 and 64.9, shell thickness of 38.1 and 37.0 mm, and yolk ration of 30.6 and 29.5 %, respectively (G. Su, 2006). Selection for humoral immune response causes the metabolic changes and influences on the synthesis of proteins which are key factors for both immune protection and ensuring reproductive function and egg production, so there is an imbalance between a potential of antibody response, growth, development and reproductive function. Under the influence of divergent selection for primary immune response, body weight, time of puberty and egg production were higher in the L^- layers compared to L^+ hens, while the L^+ hens surpassed them in egg quality, such as height and pH of the egg white, Haugh units, the number of two-yolk eggs. The changes in reproductive function due to poultry targeted selection should be compensated by genotype-specific optimization of feed rations and rearing technologies for adult hens, and by an adjustment of egg incubation conditions.

Keywords: poultry, genotype, selection, breeding, traits, ascytes, stress responsiveness, feather pecking, antibody response, egg's quality and incubation, embryo metabolism.

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Poultry breeding for the increase in productivity has led to the dysfunction of reproductive capacity, resulting in a decrease in the quality of hatching eggs, embryo metabolism disorders, deterioration of the results of incubation and the state of young poultry [1, 2]. Changes are observed in chemical composition and the ratios of egg ingredients, shell permeability, deviations in development and integration of life support body systems (neuroendocrine, cardiovascular, immune systems, etc.), as well as in the duration of individual stages of development, particularly in the terminal period of embryogenesis.

In contrast to the initial genotypes, aggressiveness is manifested [3, 4], stress resistance and to infection is reduced [5, 6] in highly productive hybrid poultry. In addition, it is susceptible to so-called technological diseases — new metabolic disorders, including the sudden cessation of egg production syndrome, hysteria, ascites syndrome, sudden death, etc. [7]. Predisposition to them in different genotypes varies. Selection for reduction of the severity of these disorders provides a positive result [8-10], but has an effect on the reproductive function.

This article provides a review of studies on the reproductive function in different genotypes of highly productive poultry when breeding was performed for traits other than productivity. Particular attention is paid to the early period of ontogenesis in the offspring with different susceptibility to metabolic disorders.

Ascites syndrome. Broilers' mortality because of ascites makes 5-10 %, and at the lowered temperature of the environment it can reach 50 %, calculated from world production [11, 12]. High-productive meat crosses differ from slow-growing chicks in their susceptibility to this syndrome [13-16].

According to G. Havenstein [17], the visceral system organs have not changed as much as the vegetative system organs in such hybrids as a result of many years of breeding in their parents. Broilers of modern crosses having a live weight of 2 kg or more by the 40-42-day-old age need more oxygen for their growth and activity, so their heart must work inadequately for its capabilities [18]. Hypoxia developing on the back action scheme causes compensatory increase in the intensity of heart function. Hypertrophy of cardiomyocytes and thickening of the muscular wall arise. Due to the lack of oxygen, some of them die, and the remaining ones become elongated and thin, which ultimately leads to heart failure and paralysis of the heart with ascites symptoms [19]. Chronic heart failure is the main cause of hypoxemia and hypertension of pulmonary circulation and the resulting ascites syndrome in broilers [20].

Prerequisites to development of the disorder are formed during embryogenesis [21, 22]. In the embryos who are the descendants of ascite resistant poultry, the relative heart weight is significantly higher, and pipping shells and a hatching occur earlier, than in the susceptible genotype of the embryos of which hypothyroidism and tachycardia are typical [23]. The interval between the perinatal period stages I and II is identical in them, and the interval between stages II and III is shorter in the emryos not susceptible to ascites. At the incubation day 18, reduced pO_2 and increased pCO_2 in egg air chamber in birds susceptible to ascites compared to these parameters in hens not susceptible to ascites was recorded. According to the authors, a longer incubation of eggs from hens susceptible to ascites can provoke hypothyroidism and increased value of pCO_2 in the air chamber which results in embryo hypoxia. These data are consistent with the features of thyroid gland functioning in chickens with ascites [24].

The degree of prenatal hypoxia affects the development of ascites in the postnatal period [14, 25]. Moreover, embryo hypoxia even in the genotypes of the same production type (egg or meat poultry) varies due to different shell permeability and, consequently, the value of pO_2 and pCO_2 in the egg air chamber [12, 26].

In the environment with high CO_2 in the last week [27, 28] and/or first 10 days of incubation [29, 30] embryos displayed earlier than under the standard CO_2 level.

Half of eggs from commercial Cobb cross flock and paternal SAS-Hybro line with a tendency to the development of ascites was incubated for 10 days in non-ventilated (NV) setters which resulted in an increase in air CO_2 concentration from 0.05 to 0.7 %, the rest eggs were incubated in the setters at standard ventilation (SV) regimen, when the content of this gas was less than 0.1 % [30]. In both genotypes, from incubation day 11 to day 14 pCO_2 in the air chamber was significantly higher with NV than at SV. For example, it was 17.9±1.1 and 15.1±0.9 mmHg, respectively, at day 13 in Cobb and 16.2±1.1 and 13.2±0.9 mmHg in SAS-Hybro. Hatchability in SAS-Hybro-NV was 88.00 %, while in SAS-Hybro-SV it was just 76.84 %. The period from the start of incubation to each of the three stages of perinatal period was shorter in Cobb than in SAS-Hybro in all versions of the experiment: Stage I with the NV mode lasted for 7.33±0.46 and 13.29±0.50 hours, respectively, while with the SV mode it was 10.49±0.41 and 11.54±0.45 hours. Hatching in Cobb-NV was completed 2.5 hours earlier than in Cobb-SV, and 0.5 hours earlier than in SAS-Hybro. From day 17 to hatching, plasma T_3, T_4 and corticosterone levels in Cobb were significantly higher compared to SAS-Hybro under both incubation modes. In Cobb and SAS-Hybro, dependence of thyroid hormones concentration from pCO2 was not found, but the dynamics of corticosterone level was significantly defined by both incubation regime and genotype. While a sharp increase in this hormone level was observed at SV from day 11 to day 17 in both genotypes followed by its further decrease, at NV maximum corticosteroid level was reached at day 15. The latter was lower in Cobb-NV compared to SAS-Hybro-CB, while the dependence on pCO_2 was not found in SAS-Hybro.

N. Buys et al. [31] incubated two groups of eggs from two parent forms of meat hens susceptible (AS) and resistant to ascites (AR). During days 1-13, the CO_2 content of 0.2 % was maintained in all setters, and during days 14-19 it was increased to 0.4 % in one of them as a result of less active ventilation. AS embryos reached the perinatal stage I significantly later compared to AR embryos in both experimental versions. At 0.2 % CO_2, it was reached after 470.67±0.78 and 464.17±0.70 hours of egg incubation. AR embryos that were developing at 0.2 %, hatched significantly earlier than AS (in 492.52±1.11 and 500.97±1.17 hours, respectively), but this difference leveled at 0.4 % (494.12±0.58 and 493.47±0.70 hours). A significantly lower decrease in T_4 and T_3 was observed in AS genotypes than in AR. In embryos of both genotypes incubated under 0.4 %, plasma T_3 concentration was higher and mortality from ascites lower than under 0.2 %. The ratio of the right ventricle weight to the total ventricle weight in AS and AR embryos was greater at 0.2 % CO_2 than at 0.4 %.

Heterochrony of internal organs was identified in the embryos of Arbor Acres meat hens compared to the freely mate Athens-Canadian population, which was expressed, inter alia, in a decreased relative heart weight [32].

In embryo offspring of the Ross × Cobb cross chickens heart heterochrony was shown to be not only due to the genotype and hypoxia in embryos, but also to an increase in incubation temperature [33]. Control group of eggs was incubated at the shell temperature of 37.8 °C, experimental group was incubated at 38.9 °C from incubation day 7 until the end of incubation. Egg hatching was almost similar (94.5±0.57 and 92.5±1.04 %). Heart, body, and residual yolk weights at hatching were significantly lower in the experimental group compared to control (by 26 %, 3.4 and 0.5 g, respectively). Total mortality and mortality from ascites were significantly greater in the experiment than in control

(12.5±1.16 against 8.4±1.28 %; and 6.6±1.02 against 2.8±0.65 %, respectively) for the 42-day growing period. Therefore, incubation of eggs at a temperature increased from incubation day 7 by 1.1 °C compared with the standard, provokes heterochrony of heart development and an increase in overall chicken mortality by 4.1 %, and in mortality from ascites by 3.8 %.

As a result of successful divergent selection of maternal livestock for ascites resistance, S. Druyan et al. [9, 34] obtained offspring lines susceptible (L^+) and resistant (L^-) to ascites. Under provocative conditions, mortality of broilers was 93.2 and 9.0 %, respectively. The heart rate in L^+ and L^- 1-day chickens differed, being on average 435 and 404 beats per minute, respectively, but to the day 17 the difference practically leveled (419 and 417 beats per minute, respectively) due to a decreased rate in L^+ and an increased rate in L^-. Moderate hypoxia is known to initiate the acceleration of the heart rate in birds [35], as evidenced by increased pCO_2 in L^+ egg air chamber in the terminal period of incubation.

The below data make it possible to conclude that the reasons and conditions for ascites development arise in the incubation period, and embryogenesis in egg from L^+ and L^- hens differs (Table 1).

1. Features of embryogenesis in meat hens genotypes with different susceptibility to ascites syndrome [23, 27, 30, 34]

Parameter	Genotype	
	susceptible	resistant
Relative heart weight	Reduction	Increase
Egg air chamber at incubation day 18:		
pO_2	Reduction	Increase
pCO_2	Increase	Reduction
Plasma T_3, T_4 level	Reduction	Increase
Duration of incubation	Increase	Reduction
Heart rate in 1-day old chicks	Increase	Reduction

N o t e. The absolute values of the embryogenesis parameters in compared genotypes published by independent researchers vary but a regularity in the changes of these parameter related to one another can be identified. For example, the pCO_2 value in susceptible genotype was lower compared to that in resistant.

Hypothyroidism typical of the meat hens genotype predisposed to ascites [23, 24, 36, 37] also develops due to the breeding for the increase in growth rate and feed [22]. Apparently, it is the decreased thyroid functional activity that determines the processes resulting in the deficit of oxygen in the tissues of broilers which causes myocardial hypertrophy due to the intensified heart function, the lack of cardiovascular efficiency, and development of ascites syndrome as a consequence.

A direct correlation was found in the parameters characterizing lung development and thyroid function in embryogenesis, i.e. the lungs volume is the larger the higher thyroidal hormone rate is, and vice versa [7, 15]. In the chickens with better developed lungs grown under chronic hypoxia, mortality from ascites was significantly lower.

The impact of prenatal hypoxia on the manifestation of ascites syndrome in postnatal period has been proven. Embryogenesis at rather high air CO_2 is completed earlier than at its standard and low levels which is due to the increased thyroid under hypoxic conditions [22, 23, 31, 36]. Paradoxically, in chickens hatched from eggs incubated under hypoxic conditions, a decrease in the incidence of ascites has been recorded [28, 27]. One reason for this phenomenon can be a reduction of the period of embryogenesis which results in an earlier change of allantoic respiration to pulmonary respiration, transition to active life and improvement of tissue supply with oxygen.

There are the evidences that the optimized incubation protocols for meat hens appropriate for their genotype can prevent broiler chicks from ascites and

improve safety of poultry.

Stress resistance and aggressiveness. An increase in corticosterone content in egg white and yolk of eggs produced by stressed laying hens has been proven [38, 39]. The egg white in the eggs produced by hens after immobilization was reported to contain a significantly increased concentration of corticosterone from 1.4-1.5 to 1.7-2.0 ng/g [40]. Same regularity was found under different regimes of hyperthermia, as well as under the transfer of chickens in new types of cells and changes in the stocking density.

Hormones accumulated in eggs affect embryogenesis adversely [41-43]. Thus, corticosterone injections (10 or 20 ng/ml) into an egg result in increased embryo mortality, reduction of embryogenesis duration, and development bilateral asymmetry in tarsus length [44].

In stress simulation in Japanese quail (*Coturnix coturnix japonica*) by introducing implants with or without corticosterone, a correlation of the amount of the hormone entering the body and accumulating in the yolk of laid eggs was found [45]. Growth parameters in the offspring from the mothers with such implants appeared to be worse compared to control, and the reactivity of hypothalamic-pituitary-adrenocortical system (HPA) in response to immobilization was more significant.

Age and sex dimorphism was identified in Japanese quails in the parameters of growth and stress reactivity in response to corticosterone injections in egg yolk prior to incubation [46]. A growth slowdown was seen in males but not females, and a reduction in stress reactivity was found in adult quail females, but not quail males.

A reduction of embryogenesis period arises as a result of selection for the trait of stress resistance in quails, particularly young L^+ hatch 3.7 hours earlier than L^- [47]. This was confirmed in experiments with implantation of empty (control) and containing corticosterone implants (experiment) with L^- (control) 397.8±0.5 hours > L^- (experiment) 395.9±0.7 hours > L^+ (control) 393.8±0.3 hours > L^+ (experiment) 391.2±0.4 hours [48].

The feather-pecking (FP) behavior is one of the most common defects of egg hen behavior recorded in 40-80 % of industrial poultry livestock [49, 50]. There are mild (ignored by a recipient) and severe forms of FP [4, 51]. FP intensity increases with the start of egg laying due to increased release of sexual hormones. Cases of FP are more common under poultry selection for productivity [52].

As a result of selection of 5 generations of White Leghorns for FP, plasma serotonin levels in L^+ became significantly higher than in L^- (0.059 and 0.037 mmol/l, respectively) [53]. These data are consistent with the results obtained by H. Cheng et al. [54] who have proved that high levels of this neurotransmitter in chickens showing a tendency to FP is associated with low safety due to cannibalism.

Selection for the FP decrease is effective [51, 55, 56], but its effects on reproductive and other functions are not clear enough. Comparison of stress reactivity in the 6^{th} generation of White Leghorn hens selected for FP traits and freely mate control line (CL) that originated from the same population revealed the average plasma corticosterone of 1.6 ng/ml at rest [52]. In males, it was significantly higher than in females (1.9 and 1.5 ng/ml, respectively). In response to the stress factor (immobilization), corticosterone level in L^+ hens with severe defects of FP behavior and in L^- individuals increased unevenly and was 11.0 and 7.9 ng/ml with the intermediate value of this parameter (10.2 ng/ml) in CL.

A negative phenotypic correlation of mild FP and stress reactivity (–0.11±0.03), weight of eggs from 44- (–0.18±0.07) and 50-week (–0.16±0.06)

hens, and egg shell deformation in eggs from 50-week individuals (–0.16±0.07) was found in White Leghorn hens [57]. For such behaviors as soil eating, a direct correlation with the deformation of the shell of eggs obtained from 50-week-old chickens (r = 0.63±0.26), and a reverse correlation with the strength of the shell (r values for the eggs from 35-, 44- and 50-week-old chickens was –0.86±0.29, –0.81±0.20, and –0.76±0.24, respectively) were found.

G. Su et al. studied the reproductive function in five generations of White Leghorn hens selected for FP trials [58]. The feather-pecking rate was significantly lower in L^- than in L^+ poultry, and the number and weight of eggs laid during a month were higher (0.38 and 2.01 feather pecking per hour, 1223 and 1132 g, 24.4 and 18.3 eggs, respectively). However, the egg quality in L^+ was better compared to L^- due to the Haugh units of 73.0 and 64.9, shell thickness of 38.1 and 37.0 mm, and yolk ration of 30.6 and 29.5 %. The parameters of CL egg quality were of intermediate values. Hence, the egg quality parameters dropped as a result of breeding for FP reduction which caused a statistically significant decrease in pecking in the 5th generation along with an increase in some quantitative parameters of egg production.

Selection for FP traits determines the differentiation of livestock related to the degree of sympathicoadrenal system (SA) and HPA reactivity. Selection for the increase in stress reactivity results in a reduced embryogenesis period [47, 48]. The positive effect of hen selection for FP reduction causes the deterioration of egg quality [58]. In addition, the state of immune system changes [53]. Thus, a significant increase in the response to the vaccination against the Gumboro disease virus compared to L^- and CL was observed. WBC was greater in L^- compared to L^+ and CL. Consequently, such divergent selection affects the poultry health.

This should be considered in industrial poultry when as a result of high livestock crowding infectious diseases and stress due to the so-called technological stress factors are likely, as well as the propensity of chickens for FP.

Selection for immune response. Selection for humoral immune response causes the metabolic changes and influences the synthesis of proteins which are the key factors for both immune protection and ensuring reproductive function and egg production [59]. For example, selection in 14 hen generations for the primary antibody response impacts the growth rate and egg production [60]. Young Ls^- have greater body weight and mature individuals have lower body weight and lay their first egg earlier, while the number of produced eggs (and the number of two-yolk eggs) is greater compared to that in L^+.

Selection in 22 hen generations for the change in antibody response has a significant impact on the quality of hatching eggs [61]. Egg weight in laying hens of the same age was 59.44 g in CL, 55.50 g in L^-, and 54.15 g in L^+. The relative weight of egg shell, its thickness, egg white height and pH were lower in L^-, intermediate in CL and the greatest in L^+. Egg white height decreased with age in all laying hens, and the regularities of these changes were dependent on the genotype.

As a result of successful divergent selection for primary immune response in 37 generations in L^+ and L^- White Leghorn chickens from the same population, growth and reproductive functions changed for which selection was not conducted [62]. Thus, in the 16th to 37th L^+ generations, the antibody titer (log 2) increased from 8.27±1.64 to 18.20±6.10, and decreased in L^- from 4.12±2.07 to 2.0±1.30, respectively. In 24th generation, the first egg was laid by the individuals of the L^+ and L^- in the age of 181.0±20.9 days and 161.1±6.5 days, respectively. L^+ chickens had lower body weight in the age of 1 month compared to L^- of the same age. The body weight and antibody titers correlation

depends on the genotype and was direct in L^- and inverse in L^+.

Egg laying in L^- was proved to start 11.67±3.53 days earlier and layers had a lower body weight (–169.46±40.20 g) than in L^+ as a result of selection in 36 generations of White Leghorn hens for antibody response [63, 64]. At this, egg parameters were significantly greater in L^+ (shape index by 4.12±0.55 %, egg white height by 0.27±0.12 mm and egg white quality in Haugh units by 1.89±0.91 %). Egg weight and yolk coloration were similar in both genotypes, but the shell quality was better in L^-, its weight and thickness were 0.66±0.09 g and 0.03±0.01 mm greater compared to that in L^+.

Data on the regularities of the weight and shell thickness changes in eggs from L^+ and L^- published by A. Martin et al. [60] and H. Albrecht et al. [64] are contradictory. Probably, the reasons are the unequal duration of selection (22 and 36 generations respectively) and the differences between the genotypes of initial populations.

Human serum albumin (HSA) and lipopolysaccharides were simultaneously administered in the 30th generation chickens aged 7 and 12 weeks in the selection for the humoral immune response to sheep erythrocytes [65]. A rise in plasma antibodies specific to HSA and natural antibodies binding keyhole limpet hemocyanin and greater susceptibility to immune modulation by lipopolysaccharides were reported in L^+ individuals compared to L^-. A delayed puberty and lower egg productivity was found in L^+ livestock.

The above data suggest that selection of highly productive poultry for humoral immune response causes an imbalance between the potentials of antibody genesis, growth and development, and reproductive functions. Table 2 shows that layers L^- are superior to L^+ in body weight, puberty age (first egg) and egg production. However, Ls^+ are superior to Ls^- in egg quality (height and pH of egg white, Haugh units, number of two-yolk eggs). The main parameters that characterize the quality of hatching eggs are regulated by the industry standard OST 10 321-2003 [66].

2. Changes in the parameters characterizing the reproductive function of hens under the influence of divergent selection for traits of the primary immune response

Parameter	Direction of change		Reference
	L^+ line	L^- line	
Onset of puberty (first egg)	Later	Earlier	[60, 62, 63-65]
Egg productivity	Decreased	Increased	[60, 65]
Egg-laying intensity	Decreased	Increased	[63, 64]
Egg weight	Decreased	Increased	[61]
Egg shape index	Increased	Decreased	[64]
Height and pH of egg white, Haugh units	Increased	Decreased	[61, 64]
Number of two-yolk eggs	Less	More	[60]

N o t e. The absolute values of the parameters characterizing the reproductive function in compared genotypes published by independent researchers vary, but a regularity in the changes of these parameter related to one another can be identified. For example, the number of two-yolk eggs in L^+ is less compared to this parameter in L^-.

Thus, redistribution and the elimination of certain gene pools as a result of poultry selection for the traits other than productivity (ascites syndrome, tendency to FP, stress reactivity and immune defense) cause a reproductive system dysfunction in livestock. It is worth noting that directional selection causes correlated modifications for different trait groups. Thus, in selection for FP, a correlation of stress reactivity, immune response, and egg quality was found [57]. Apparently, under the selection for traits of stress reactivity due to the factors of various nature (social, physical, biological), the changes in reproductive function may have their distinctive features. This assumption is based on the fact that the number of individuals that exhibit different behavior strategies (coping styles) when dealing with stress varies in populations as a result of selection for relevant

traits [5, 67]. This type of dichotomy was found in different classes of animals, including birds, fish and mammals [68, 69].

The findings of the studies on the quality of hatching eggs, embryos metabolism, results of incubation and the state of young generations in poultry that were selected for traits other than productivity, prove that such a selection can cause deterioration of not only reproductive, but other functions as well. For example, successful breeding for reduction of feather pecking is associated with improvements in egg production, but with the deterioration of hatching eggs quality and with the changes in stress reactivity and(or) immune response. In ascites-susceptible genotypes, embryo offspring is characterized with heart heterochrony and tachycardia, hypothyroidism, and longer duration of development before hatching. Let us note that these changes are taking place as a result of selection for productive traits [2].

It appears that the identified regularities should be used to develop ways to offset the decline in reproductive function due to selection. Promising techniques include optimizing diet composition [70] and the conditions of the parent stock housing [6, 50], including prevention of the development of stress [71-74] and selection for the improvement of egg quality [84]. Producing the offspring, it is necessary to individualize the regimes of incubation of eggs from each genotype [75-78], regulate embryogenesis through introduction of biologically active substances in the hatching eggs [79] and thermo contrast embryo training [80-83].

Thus, selection of poultry for traits other than productivity (resistance to ascites, pecking, stress factors, and primary immune response) affects the reproductive and other physiological functions. For example, at the tendency to the development of ascites syndrome, the individuals' response to vaccination against the virus that causes the Gumboro increases significantly, and heart heterochrony and tachycardia, hypothyroidism, prolonged time to hatching are observed in embryos. Selection of egg incubation regimes may be one way of prophylaxis of ascites and enhancing the safety of livestock. Corticosterone is accumulated in egg white and yolk of eggs from hens that are under stress that, in particular, adversely affects embryo metabolism and the results of incubation. With the positive effect of selection of chickens for the reduction of their propensity for pecking and plucking, deterioration of egg quality occurs, the state of the immune, sympathicoadrenal and hypothalamic-pituitary-adrenocortical systems changes. Selection for traits of humoral immune response causes changes in energy metabolism and protein synthesis that are critical to both immune defense, and reproductive and productive functions. Under the influence of divergent selection for primary immune response, ascites-susceptible and resistant layers differed in their body weight, time of puberty, and egg quality (height and pH of egg white, Haugh units, the number of two-yolk eggs). It has been noted that highly productive poultry often show aggression, behavior defects, reduced resistance to stress and diseases. This should be considered in poultry industry in the crowded livestock when the probability of infection and the effects of technological stressors increase. Continuing the study of factors affecting the reproductive function in modern genotypes of poultry, we should, in particular, pay attention to the age of the parents and the prevention of stress caused in them by various factors. Conditions of storage of hatching eggs are also of interest as such a factor. Based on our results, the particularities of expression of the genes that determine the pleiotropic effect of selection can be identified.

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THE STUDY OF FACTORS AFFECTED THE GENE TRANSFER EFFICIENCY IN CHICKEN EMBRYONIC CELLS BY APPLICATION OF LENTIVIRAL VECTORS

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Abstract

Lentivirus-mediated gene transfer is being the one of the attractive method for genetic modification of chicken (S.C. Chapman et al., 2005; C.A. Smith et al., 2009; N.A. Volkova et al., 2013). However, the efficiency of thansgenesis of the chicken embryonic cells has been shown to be relatively low. Therefore, a large number (60,000 to 100,000) of embryonic cells at the start of incubation and the virus preparations with high titers (about 10^9 particles per milliliter) remain one of a crucial problem the researchers are facing with when try to achieve a satisfactory transgene introduction. The aim of the present study was to determine the optimal conditions for production and application of the modified lentiviral vector system of second generation for the transgenesis of chicken embryos. The vector system consisted of three different plasmids: psPAX2, containing gag-pol genes; pLPG, coding envelop glycoprotein G of vesicular stomatitis virus (VVC-G) and pWPXL, the self-inactivated lentiviral vector, carrying *eGFP* (enhanced green fluorescence protein) gene under control of promoter region of the human elongation factor 1 alpha-encoding gene. To produce the recombinant virus particles and to determine the virus titers we used human cell line 293T. The injections of the virus preparations into the chicken embryos were performed at the different stages: from 20 to 24 hours (group 1) and from 50 to 55 hours (group 2) of incubation. To detect the transgenesis efficiency and the number of the integrated copies of the transgene the total DNA was extracted from embryos on the day 7 of incubation and analyzed for the presence of specific *eGFP* sequences by real-time PCR. The maximal titers of the virus preparations were produced by the ratio of the psPAX2, pLPG and pWCAG plasmids equal 1:1:3 and were 2.4×10^7 CFU/ml before ultracentrifugation and 6.2×10^8 CFU/ml after concentration by ultracentrifugation. The efficiency of genetic transformation which was evaluated as the part of the transformed cells from the overall number of analyzed cells was 78.0 and 31.0 % in the groups 1 and 2, respectively. It was shown, that the alteration in the ratios between components of the vector system comparing to the standard scheme allows significantly increase the titers of the produced virus preparations. The biological titers of the virus preparations of 10^8 CFU/ml are sufficient to infect up to eighty percent of cells at the earlier stages of embryo development. Presumably, at earlier embryogenesis the cells were infected with more viral particles resulting in different number of the transgene copies integrated into cell genome in the groups 1 and 2. From the obtained results, the efficiency of transgenesis by means of the lentiviral vectors ranged from 30.0 to 34.3 % and varied slightly depending on time after the embryos incubation began. These results indicate that only a part of embryonic cells is usually available for viral infection. Injection of embryos at different intervals of incubation by viral preparations with similar titers produced the populations of embryonic cells with different amounts of vector copies in the cell genome. So the efficiency of hen embryo thransgenesis does not depend on the stage of its development at least for 55 hours of the embryogenesis and can be predictable.

Keywords: lentiviral vectors, molecular cloning, transfection, transgenic animals.

By now, the prospects of the use of retroviral and lentiviral vector sys-

tems for genetic modification in poultry has been shown in several studies [1-6]. Therefore, a large number (60,000 to 100,000) of embryonic cells at the start of incubation and the need for virus preparations with high titers (about 10^9 particles per ml) remains one of the crucial problems the researchers are facing when trying to achieve satisfactory transgene introduction. The use of constitutive promoter enhancers such as promoter enhance of early human cytomegalovirus genes (CMV) and promoter enhancer of bird β-actin gene makes it possible to achieve significant expression (up to a few milligrams of protein per 1 ml of serum and egg protein) in mosaic form (G_0) obtained from embryos after transgene introduction. However, expression is significantly reduced in next generations (G_1 and G_2) [5]. The reasons for this are not understood. Probably, high expression of a foreign protein acts as a selective factor in various organs and tissues. Furthermore, it has been noted that the expression level is directly correlated with the gene dose (number of viral copies per cell genome), i.e. the possibility of various physiological defects in the most promising producers is greatly increased [2, 7-9]. Attempts were also made to achieve tissue-specific transgene expression using regulatory elements that control the synthesis of egg ovalbumin [10-14]. According to the authors, expression of transgenes in several generations of animals was relatively stable, yet the reached levels of protein synthesis were approximately 20-50 times lower than when using constitutive promoters.

Vector systems derived from integrative viruses such as lenti- and retroviruses are an effective tool for the introduction and expression of genes due to a number of unique features: the members of the family are able to stably integrate into the genome of the host cell; relatively small genome sizes allow them to be easily manipulated *in vitro*; the internal genome sequences can be removed in such a way that all the functions required for replication are provided in trans; using surface virus glycoproteins that are tropic to a broad host range, it is possible to infect almost any species and type of vertebrate cells with hybrid virions.

The first stage of our study of the features of gene introduction and expression was to optimize the standard protocol of vector production based on lentiviral system, which made it possible to significantly increase the titer of the viral preparation used. The findings of our study of the conditions of chick embryo infection with lentiviral preparations give grounds to suppose the predictability of the result.

The purpose of the research presented was to evaluate the effect of the number and proportion of the lentiviral vector system components on physical and biological titers of the virus produced, as well as of the time of introduction of the resulting lentiviral vector in chick embryos on the effectiveness of transgenesis in vivo.

Technique. The research was made with the chicks of the Ptichnoe cross. We used a modified lentiviral vector system of the second generation which included three different plasmids, psPAX2, containing *gag-pol* genes, pLPG encoding surface glycoprotein G of vesicular stomatitis virus (VVC-G), and pWPXL, the self-inactivated lentiviral vector, carrying the *eGFP* (enhanced green fluorescence protein) gene under the control of human PNA-polymerase II elongation factor 1 (hEF1α) [15]. The pWPXL plasmid was used to construct the pWCAG vector. Viral vector was constructed using standard molecular cloning techniques [16].

To produce recombinant virus particles and to determine virus titers, we used the 293T human cell line. Cells of this line have the ability to divide rapidly and can be transfected with plasmid DNA with high efficiency. Cells were cultured in the DMEM medium (Dulbecco's Modified Eagle's Medium) containing fetal calf serum (10 %), L-glutamine (2 mM), penicillin (100 U/ml), strep-

tomycin (100 μg/ml) in the atmosphere of 5 % CO_2 at 37 °C. Lentiviral vector was introduced into the 293T cell line by calcium phosphate precipitation [16]. At the same time, 1.7×10^6 cells were placed in a vial with substrate area of 25 cm^2 for 1 day before the start of the experiment. At day 2, the culture medium was replaced with 1 ml of fresh medium containing DNA of all three plasmids of the vector system in different ratios and incubated for 6 hours in the presence of calcium phosphate buffer. Then the cells were chemically shocked by treatment with 20 % DMSO (dimethyl sulfoxide) for 5 minutes, then washed and placed in fresh medium. Next day, the culture medium was replaced with fresh media and incubation was continued for 48 hours, after which the culture supernatant containing virus was collected, clarified by centrifugation and aliquots were frozen at –80 °C. For concentrating virus preparations, culture supernatants were ultracentrifuged (70,000 g, 120 min, +4 °C) and pellets were resuspended at +4 °C in a small volume of TNE buffer (50 mM Tris-HCl, pH 7.8, 130 mM NaCl, 1 mM Na_2-EDTA).

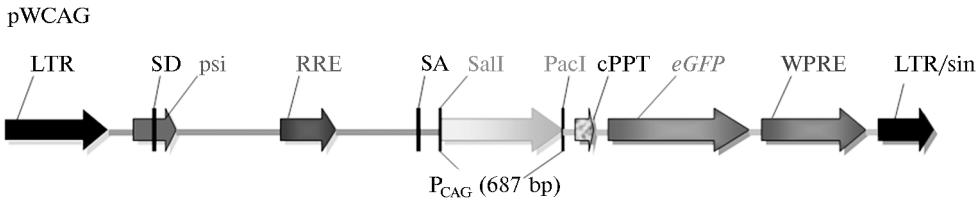
To determine the physical titer of aliquot viral vectors (5-50 μl) of culture supernatants containing the virus, viral RNA was isolated using a Qiagen (USA) kit. Viral RNA served as matrix for cDNA synthesis with reverse primer specific for the *eGFP* gene sequences. Then, the number of vectors in the real-time PCR (RT-PCR) was determined using cDNA synthesized and primers specific to the *eGFP* gene. A series of 2-fold dilutions of the viral vector plasmid DNA in the desired range was the standard for evaluating the number of gene copies. RT-PCR was conducted using a MiniOpticon™ apparatus (Bio-Rad, USA). Biological virus titer was determined in accordance with the description given by G. Tiscornia et al. [17]. The supernatant collected at culturing 293T cell clones transfected with retroviral vectors was centrifuged to remove cells and debris (10 min, 3,000 g). Infectioning was conducted immediately after virus collection. Recipient 293T cells were planted at a density of 3×10^5 in Petri dishes 60 mm in diameter 1 day prior to infecting. At day 2 the medium was replaced with 1 ml of fresh medium containing polybrene (8 mcg/ml) and aliquots of test virus. After 6 hour incubation, medium was changed by fresh one. After 48-72 hour incubation, cells were detached from the substrate with trypsin, resuspended in PBS and EGFP fluorescence intensity was measured using a flow cytometer FACSCanto (BD, USA) with a set of 480-490 nm (excitation) and 510 nm (emission) filters.

Chromosomal DNA was isolated from chick embryos by salt method [16]. DNA concentration was measured spectrophotometrically (Hitachi U-1100, Japan) at $\lambda = 260$ nm ($OE_{260} = 1$ corresponds to 50 μg of double-stranded DNA). The purity of DNA preparations was evaluated by calculating the ratio of optical density at wavelengths of 260 nm and 280 nm. Normalization of DNA samples was performed using RT-PCR with primers and hybridization probe specific to DNA encoding 18S rRNA in *Gallus gallus*. The number of DNA vector copies was determined with primers and hybridization probes specific of *eGFP*. To prepare standards, we used plasmid viral vector DNA transferred into the linear shape at a unique restriction site titred in a previously determined range. PCR-products were produced in the reaction mixture of 25 μl containing 1× buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.08 % Nonidet P400), 2 mM $MgCl_2$, 0.2 mM dNTPs, primers (0.25 μM for each), hybridization probe (0.125 μM), Taq-polymerase (1.25 U, Fermentas, Lithuania), and 5 ng of matrix. Amplification conditions were 95 °C, 5 min; 45 cycles of 94 °C, 30 s; 50 °C (varies), 30 s; and 72 °C, 1 min. Quantitative RT-PCR and experimental selection of optimal oligonucleotide annealing temperatures was also performed using a MiniOpticon™ unit (Bio-Rad, USA).

The analysis of eGFP expression in embryo tissues was performed using a flow cytometer (FACSCanto, BD, USA).

Results. We used a lentiviral vector system to determine the conditions for the effective introduction of transgenes in embryonic chicken cells. In particular, the modified system of the second generation was chosen which, as already mentioned, included three different plasmids: the first one encoded the vesicular stomatitis virus (VVS-G) surface glycoprotein G, the second one was a classic «packager» and contained the *gag* and *pol* genes, the third one was a self-inactivated vector (SN, self-inactivated) [15].

To study the efficiency of introduction of gene expression in poultry cells, the *eGFP* gene was used as a structural and hybrid CAG enhancer promoter of early human cytomegalovirus genes (hCMV, human cytomegalovirus) promoter and chicken β-actin promoter. A construct with the eGFP gene under the control of CAG promoter was produced using these sequences based on self-inactivated lentiviral vector genome.



Structure lentiviral expression vector pWCAG. Description of the viral vector construction is given in the text. LTR, LTR/sin (LTR, long terminal repeat; sin, self-inactivating), lentiviral long terminal repeats: 5′-LTR — wild type, 3′-LTR/sin — self-inactivating version. SD, SA (splice donor, splice acceptor) — donor and acceptor splicing sites; psi — region responsible for packaging of viral genomic RNA into virion; RRE (rev responsible element) — site of binding of Rev-protein responsible for the transportation of genomic RNA molecule from nucleus to cytoplasm; cPPT (central polypurine tract) — central polypurine tract involved in transportation of pre-integrative complex to cell nucleus; *eGFP* — enhanced green fluorescence protein gene; WPRE — woodchuck post-transcriptional regulatory element. P_{CAG} — hybrid regulatory element containing early human cytomegalovirus gene enhancer and chicken β-actin gene promoter. SalI, PacI — restriction sites used to clone regulatory elements.

The structure of lentiviral vector is shown in the figure. To obtain pWCAG vector, a fragment of 687 bp with the hybrid CAG enhancer-promoter contained in the psPAX2 plasmid [13] was amplified, gel purified and cloned by SalI-PacI restriction sites as part of the pWPXL plasmid (15).

The inter-component ratio in transfection was optimized experimentally.

As already mentioned, in production of viral preparation, plasmids psPAX2, pLPG, and pWCAG forming a ternary vector system, were introduced into human cells (line 293T) by $CaPO_4$ precipitation. Using plasmids in different proportions and changing their total number (while maintaining the proportion) and medium composition, conditions were selected to ensure the high titer output of viral particles.

Table 1 summarizes the data illustrating the effect of different ratios between the components of the vector system on physical and biological virus titer. As is evident, a 2-fold decrease or increase in the number of all three plasmids compared to the original version did not have a significant impact on the viral titer (change of no more than 2 times). At the same time, an increase in the packer (pLPG) and vector (pWCAG) amount compared to the standard version (1:2:3, psPAX2:pLPG:pWCAG) resulted in more than a 10-fold titer drop. Interestingly, a 2-fold decrease in the number of packer allowed increasing titer more than 4-fold relative to the standard. We used this option in subsequent experiments for obtaining viral preparations.

1. Titers of virus preparation used for chicken cells transformation in vivo depending on the quantitative ratio between the components of the vector system used to obtain it

Virus titer	1:2:3	(1:2:3)/2	1:4:3	1:4:6	2:2:3	1:1:3
Physical, number of RNA copies/ml	5.6×10^9	2.9×10^9	6.9×10^8	5.1×10^8	5.2×10^9	1.9×10^{10}
Biological, CFU/ml	6.7×10^6	3.4×10^6	8.3×10^5	5.8×10^5	6.3×10^6	2.4×10^7

N o t e. Quantitative plasmid ratio (psPAX2, pLPG, and pWCAG, respectively).

As a result, we obtained titers of 2.4×10^7 CFU/ml for the vector with CAG-promoter. Biological virus titer was determined in 293T cells by eGFP fluorescence. The amount of virus in the preparation was evaluated by specific RNA content in RT-PCR (Bio-Rad, USA). The vector titer was 6.2×10^8 CFU/ml after concentration by ultracentrifugation.

2. Evaluation of the efficiency of the pWCAG lentiviral vector in vivo introduction in embryonic cells of the Ptichnoe cross chickens depending on embryo age

Group (incubation time, hour	Sample number	Number of vector copies per diploid cell genome	Proportion of embryonic cells expressing eGFP,%
Group I (20-24)	1-1	0.96	
	1-2	1.86	
	1-3	0.33	
	1-4	0.10	
	1-5	0.36	
	1-6	0.14	
	1-7	5.52*	
	1-8	0.68	
	1-9	0.44	
	1-10	2.20	
mean efficacy		0.78 (78 %)	
	1-11		44.8
	1-12		33.1
	1-13		28.4
	1-14		32.8
	1-15		32.5
mean efficacy			34.3
Group II (50-55)	2-4	0.30	
	2-5	0.30	
	2-6	0.38	
	2-7	0.00	
	2-8	0.70	
	2-9	0.34	
	2-10	0.15	
mean efficacy		0.31 (31 %)	
	2-11		15.9
	2-12		42.7
	2-13		13.7
	2-14		37.8
	2-15		39.7
mean efficacy			30.0

N o t e. In determining average efficiency, samples marked with an asterisk (*), were not taken into account.

The resulting virus preparation was used for introduction into embryonic cells at different times:, i.e. chicken embryos of group I were infected after 20-24 hours of incubation, and in embryos of group II it was after 50-55 hours of incubation (Table 2). At incubation day 7, eggs were opened, embryos tissue fragments were collected as the samples for chromosome DNA extraction and analyze of eGFP expression. DNA samples with the value of $OE_{260/280}$ of not less than 2 were the benchmark for normalization of other samples. All calculations were made based on the fact that the molecular weight of diploid *Gallus gallus* genome was 2.5 pg.

According to RT-PCR results, the average efficiency of lentiviral vector introduction in chick embryo cells in group I was 0.78 copies per diploid cell genome, i.e. 78.0 % of embryonic cells contained an integrated copy of DNA

vector. In group II the value was 2.5 times lower (31.0 %). At the same time, there were 0.10 to 5.52 copies per cell genome in embryo group I, and in four of 10 samples this value was at least one proviral copy per diploid genome, while in group II the range was much lower making from 0.00 to 0.70 proviral DNA copies. The study of marker *eGFP* gene expression in embryonic tissues using flow cytometry revealed it in 34.3 % of embryonic cells on average in group I and in 30.0 % of embryonic cells in group II. Thus, in group II, indicators of the introduction efficiency for the vectors produced using RT-PCR and flow cytometry were almost identical (31.0 and 30.0 %, respectively), while in group I they differed 2.3 times (78.0 and 34.3 %, respectively; see Table 2).

Based on the results, the average efficiency of gene transfer using lentiviral vectors was 30.0-34.3 %, slightly varying with the changes in the time of introduction after the beginning of embryo incubation. Probably, only part of embryonic cells was available for virus infection. Since viral preparations with similar titers were used with both incubation periods, the observed difference in the number of vector copies in cell genome in embryo groups I and II was probably due to the fact that more viral particles penetrated embryo cells at earlier stages (see Table 2). This suggests that chicken embryos can be infected with lentiviral preparations with predicted efficiency via varying the viral preparation titres and the time of introduction after the beginning of embryo incubation, thereby producing transgenic chickens with the expected gene «dose» (number of copies) according to the experimental purpose. Thus, the efficiency of chicken embryo thransgenesis using lentiviral vectors does not depend on the stage of embryonic development at least for 55 first hours of incubation and can be predictable.

Thus, the study of the conditions of lentiviral vector introduction in chicken embryonic cells showed that biological titers of viral preparations of about 10^8 CFU/ml were sufficient to infect up to 78 % of the cells in the early stages of embryo development. Changing in the ratios of the vector system components compared to the standard scheme allows to significantly increase the titers of the virus preparations produced. The selected conditions may be used as a tool for the study of the factors influencing the level and stability of transgene expression in chicken cells in vivo in future experiments.

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NEW PROBABILISTIC STATISTICAL AND DYNAMIC MODELS TO CONTROL LIFE CYCLE IN LACTING COWS

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Abstract

Reproduction in dairy herds recently has become increasingly important. The tendency to its reduction occurs everywhere in all countries with a developed dairy husbandry. On average, the number of lactations per cow is diminishing and now close to 3 while a genetic potential of many cattle breeds is over 10 lactations. To resolve this issue, a theoretical base should be developed using latest progress in various related sciences. The aim of the present study is the first theoretical justification for a key stage of the general concept of lactating cows' health management, proposed in our previous paper (I.M. Mikhailenko, 2014). Herein we suggest an approach to programming cow's life cycle from the first to the last economically reasonable lactation. As a result, the risk of animal culling and unnecessary costs are minimized. The problem is solved for the first time in biological science. Our theory is based on developed dynamic and probabilistic statistical models. At its core, this approach provides a science-based standard of animal feeding for optimized lactation during life cycle. The dynamic models for lifetime annual yields reflect animal age and nutrient intake with diet, and the probabilistic statistical modeling, used to control cows' culling due to ill health and diseases, is the most important feature of the developed approach to life-cycle control. All physiological states, from a heifer to the last lactation, are considered, and all the flows within the dairy herd and possible causes for culling are identified. These mathematical models allow assessing the risk of possible livestock losses, which are minimized due to optimized annual diet. The developed algorithm allows to specify adjustments in annual feeding rations during the cow's life cycle (the feeding strategies for dairy cattle), which ensure optimal reproduction rate, optimal number of possible lactation per cow and optimal annual yields. Thus, the use of a lactating cow is normalized resulting in healthy livestock and maximized profitability of milk production. Since the individual approach to cows' feeding is a substantial reserve for increasing profitability of a dairy herd as a whole, the task of life cycle control is regarded at two levels, for an individual and for the herd on average. For a particular herd, the choice to one of the levels depends on whether there are the means to provide individual health control and dosing concentrated feed and food additives. Practically, the use of proposed mathematical models is mainly limited by lack of long-term (10-12 years) surveys of animal health as depended on the actual diet, since these data are necessary for identification and validation of the algorithms, but an experimental model such as 100-150 cows' dairy farm, equipped with systems for health monitoring and feed composition control, could improve the situation.

Keywords: dairy husbandry, reproduction, concept of lactating cows' health control, health, life cycle, lactation period, control algorithms, mathematical modeling.

An increase in milk production on the best dairy farms in a number of regions of the Russian Federation observed within the last decades which was due to the genetic progress, is not accompanied in general by an increase in cows' productive longevity and improvement in the quality of products obtained. Experts estimate the 65 % of profits in dairy cattle breeding to be determined by the duration of the economic use of cows. In Canada, it is 5 lactations for the whole country, in the US it is 4, in the best breeding centers of the Russian Federation it is 3.8, in many commercial farms it is 2,5 or less, while the estimated biological and economic optimum is 7-8 lactations [1-7].

In Scandinavia, attempts to breed cows producing 100 tons of milk in

10 lactations are made. In our country, under the existing conditions, the extension of the productive use of cows by 6 months is equivalent in the economic effect to increasing the number of dairy herd by 12 %. It means that there is a significant resource to enhance the efficiency of dairy cattle breeding due to increased lifetime productivity of cows [8, 9].

Losses from the shortfall in herd replacements in dairy husbandry occupy one of the first places among all economic losses. In addition, each barren cow causes farm loses of at least 25 % of its milk yield per lactation. To this, the cost of treatment, numerous unsuccessful insemination and losses associated with premature culling of valuable, often young cows, should be added [10].

In our previous study [11], a general description of the problem of reproduction in dairy husbandry was given which is the central problem in this important sector for all countries with developed agriculture. In this regard, the general concept of animal health control was proposed [8]. According to this concept, the purpose of control should not to achieve maximum efficiency as it is widely accepted now, but to maximize the profits earned over the lifetime of an animal. This goal can be achieved following the three inter-related requirements: i) providing the duration of the entire life cycle from first to last genetically and economically feasible lactation; ii) obtaining the conditional maximum profit on each lactation life cycle; iii) individual correction of the conditional maximum profit for the cows and the herd in real time.

This paper discusses the theoretical basis for solving the problem of the life cycle control. At the same time, two approaches are possible depending on the technical and technological basis of a particular farm. In the first case, when such a basis takes into account the health status of each cow, the life cycle of individual animals is managed. This is achieved even without the full-scale personal feeding of an animal without creating special conditions, and can be only due to individual dosage of concentrated fodder, vitamins and premixes. In the second case, when the health assessment is performed selectively, and this figure is determined as the average one for the herd, an additional need to manage the average life cycle of the herd arises.

The main condition for the implementation of the full life cycle of a cow is preservation of the reproductive function which can be lost due to inadequate care and feeding and as a result of diseases and disorders. The most common reasons for the culling of lactating cows are metabolic disorders, diseases of digestive system, udder, genitals, postpartum complications, mastitis, joint diseases, and other diseases associated with the peculiarities of feeding and housing of animals. In total there are more than 20 types of diseases resulting in culling of cows and in termination of their life cycle [10].

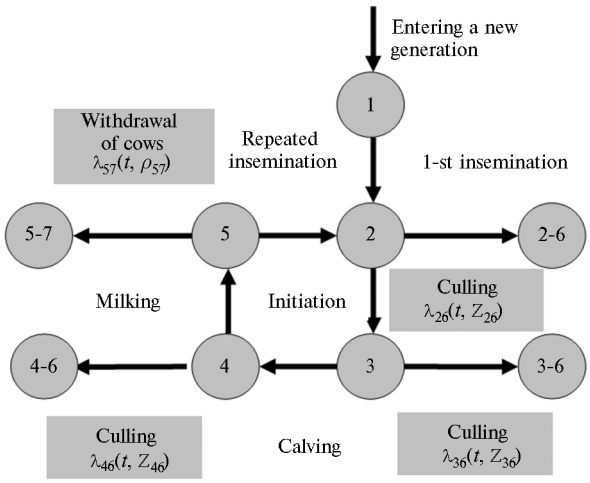
Since the appearance of any disease is probabilistic in its nature, the onset of any next lactation is regarded as a random event (continued individual life cycle). This results in generation of random flows in the general herd passing from one state to another (Fig.). Of all the possible states, we are interested only in those for which culling is likely, resulting in the termination of the life cycle of an animal and in the changes the structure of the herd.

We introduce the notation of culling flows λ:

from state s = 2 (springer heifers) — $\lambda_{26}(t, Z_{26})$;
from state s = 3 (dry cows) — $\lambda_{36}(t, Z_{36})$;
from state s = 4 (newly calved cows) — $\lambda_{46}(t, Z_{46})$;
from state s = 5 (lactating cows) — $\lambda_{57}(t, \rho_{57})$,

where $\lambda_{ij}(t, Z_{ij})$ is the number of cows culled per unit of time (day), which is generally regarded as the intensity of culling; Z_{ij} is nosological culling vectors which include all types of diseases characteristic of the respective condition lead-

ing to culling; ρ_{57} is a rule for the exclusion of lactating cows from circulation.



Graph diagram of livestock turnover in the herd: 1— heifers, 2 — springer heifers, 3 — dry cows, 4 — newly-calved cows, 5 — lactating cows, 6 — culled cows, 7 — cow excluded from turnover based on the efficiency criterion.

If the culling flows in the states of s = 2, 3, 4 is the result of imperfection and inadequacy of the herd state on average control, the formation of the culling flow of lactating cows (s = 7) is the local control component directly focused on obtaining additional profits.

General algorithms of lactating cows' life cycle control. At individual control of lactating cows life cycle, one should keep in mind that of all the states in which the animal can be, s = 5 (lactation) and s = 6 (culling) are most important for the criterial assessment. Each of these states is random and depends on the health of the animal. Therefore, the individual life-cycle control of an individual animal should minimize the risk of losses related to the non-occurrence of the next lactation.

The expression for the risk of the health control in an individual animal is as follows:

$$R(n^*, U_n) = \sum_{n=1}^{n^*-1} [M_n^* - (1 - p_n(Z)) \cdot (c_n \Pi(U_{1n}) - r(U_{1n}))] - p_n(Z) c_k,$$
$$\Pi_n(U) \le \Pi_n^*; p_n \lhd 0{,}5; p_{n=1} = 0, \quad (1)$$

where n = 1, 2, ..., n^* are the numbers of lactations (from the first to the last economically feasible one); M_n^* is a defined program of profiting from a cow throughout the life cycle; c_n is milk price projections, $\Pi(U_{1n})$ is the annual milk yield throughout the life cycle (function of control vector U_{1n}, the components of which are the expenses of all types of nutrients in the diet); $p_n(Z)$ is a likelihood of culling n-th lactation cows, depending on the disease vector Z; $r(U_{1n})$ is the annual cost of breeding per cow (function of control vector U_{1n}); c_k is the cost of a culled cow; Π_n^* is genetic program of productivity of the appropriate breed.

Expression (1) is the difference between the income at the time of culling ($n = n^*$) and the damage from the animal loss because of culling which is weighted to the state probabilities.

Achieving the goal of the life cycle control in cows corresponds to the minimization of the risk in the number of lactations n^* and the sequence of control vectors over all lactations:

$$R(n^*, U_{1n}) \xrightarrow[n^*, U_{1n=1} \ldots U_{1n=n^*}]{} \min,$$
$$\Pi_n(U) \le \Pi_n^*, \; p_n \lhd 0{,}5; \; p_{n=1} = 0. \quad (2)$$

In the life cycle control in herd on average, the expression for the risk will be as follows:

$$R(n^*, \tilde{U}_n) = \sum_{n=1}^{n^*-1} [\tilde{M}_n^* - (1 - \tilde{p}_n(Z_n)) \cdot (c_n \tilde{\Pi}_n(\tilde{U}_{1n}) - r(\tilde{U}_{1n}))] - c_k \tilde{p}_n(Z_n),$$
$$\tilde{\Pi}_n(U_n) \le \Pi_n^*;\ \tilde{p}_n \lhd 0{,}5;\ \tilde{p}_{n=1} = 0\ , \tag{3}$$

where $\tilde{p}_n(Z)$ is a likelihood of non-occurrence of the n-th lactation (culling) on average for the herd defined by the disease vector Z for «average for the herd» state of health; $\tilde{\Pi}_n$ is annual milk yield on average for the herd; $\tilde{U}_{1n}$ is the vector of control in the corresponding period of lactation (on average for the herd); $r(\tilde{U}_{1n})$ is the expenses for diet (on average for the herd).

Like for the individual life cycle control, the control on average for the herd corresponds to criterion minimization (1) according to the number of cycles and the sequence of control vectors in individual lactation periods:

$$R(n^*, \tilde{U}_{1n}) \xrightarrow[n^*, \tilde{U}_{1n}=1 \ldots \tilde{U}_{1n}=n^*]{} \min\ ,$$
$$\tilde{\Pi}_n(\tilde{U}_n) \le \Pi_n^*;\ p_n \lhd 0{,}5;\ \tilde{p}_{n=1} = 0\ . \tag{4}$$

The solution of (2) and (4) is a sequence of control vectors for all lactation periods U_{1n} and $\tilde{U}_{1n}$, as well as the optimal number of lactations n^*.

This will require mathematical models that take into account all the parameters of states for the estimation of the risk of life cycle control. According to the analysis of domestic and foreign publications, nothing like that has been presented yet [12-27]. The main focus of such development is creation of approximating functions maximally reflecting the shape of maximum lactation curves, but they are not suitable for control tasks. New models that reflect age dynamics of cows and culling processes are proposed below.

Models of individual and herd on average lifetime milk yield are:

$$\begin{aligned}
&\Pi_{n+1} = a\Pi_n + b^T U_{1n} + c^T f[n],\\
&n \in (1, n^* - 1),\ \Pi(1) = \Pi_0;\\
&f^T[n] = [n\ n^2\ n^3],\\
&U_{1n} = HR_n,
\end{aligned} \tag{5}$$

where Π is annual milk yield, kg; U_1 is the vector of nutrient elements spent per year, R is the vector of nutrient components (diet), H is a matrix of nutrient content in fodder ingredients; with $f^T[n] = [n\ n^2\ n^3]$ as the time functions vector taking into account the age of the animal.

Models of individual and herd on average cow culling are:

$$\begin{aligned}
&X_n = DU_n,\\
&Y_n = X_n - \Delta,\\
&C_n = WY_n,\\
&z_n = 0, \text{ if } \rho_z = h_z^T C_n \lhd \delta_z;\\
&z_n = z_n, \text{ if } \rho_z = (h_z^T C_n) \ge \delta_z\ ;\\
&n = n^*, \text{ if } z_n \ne 0\ ;\\
&p(z_n) = \frac{\rho_z}{\delta_z}\ .
\end{aligned} \tag{6}$$

$$p_n(Z) = \sum_{z=1}^{N_z} p(z_n)\ , \tag{7}$$

where X_n is the vector of health status parameters of lactating cows at the n lactation (average value of health status in the interval of lactation $\tilde{X}_n$); Y_n is the vector of diagnostic features equal to the dimension of the vector of health status parameters; Δ is the vector of allowable health values, deviations of which are

the diagnostic indicators; C_n is the vector of pathological syndromes which is a linear combination of vectors of diagnostic symptoms; W is a matrix of linear combinations of diagnostic symptoms; h is the vectors of discriminant functions of disease detection resulting in culling; $P\{(h_Z^T C_n \cdot t_\Sigma) \in \Omega_Z\}$ is a probability of discriminant function values hitting in the range of permissible values; t is total time of the health status parameter exceeding allowable values; Ω_Z is the areas of allowable values of disease detection discriminant values.

The algorithm of individual solutions for the animal. The meaning of the task is to minimize the risk

$$R(n^*, U_{1n}) = \sum_{n=1}^{n^*-1} [M_n^* - (1 - p_n(Z)) \cdot (c_n \Pi_n(U_{1n}) - r(U_{1n}))] - p_{n^*}(Z) c_k, \quad (8)$$
$$\Pi_{ni}(U) \le \Pi_n^*; p_n \lhd 0,5; \; p_{n=1} = 0$$

to the following dynamic system:

$$\begin{aligned} &\Pi_{n+1} = a\Pi_n + b^T U_{1n} + c^T f[n], \\ &n \in (1, n^* - 1), \; \Pi(1) = \Pi_0; \\ &f^T = [n \; n^2 \; n^3], \\ &U_{1n} = HR_n. \end{aligned} \quad (9)$$

The Hamiltonian of the system (8), (9) is:

$$H_n = (M_n^* - (1 - p_n(Z))(c_n \Pi(R_n) - C_R^T R_n) - \frac{1}{n^*} p_n(Z_n) c_k) + \\ + \lambda(a\Pi_n + b^T HR_n + c^T f[n]), \quad (10)$$

with the corresponding conjugate variable model:

$$\lambda_{n+1} = -\frac{\partial H_n}{\partial \Pi} = -a\lambda_n + (1 - p_n(Z) c_n), \quad (11)$$

and the optimization problem is to minimize the criterion (8) by using the Hamiltonian (10) and the conjugate variable (11). A detailed algorithm of this solution is shown below.

In the above problem, the program for profit lactation M_n^*, the price of milk for lactation periods c_n, cows cost c_k, the price of fodder and fodder additives are the initial information which determines the cost of feeding per lactation (U_{1n}) and the parameters of mathematical models of a, b^T, c^T, h^T, δ_Z. In addition, there are «phase» restrictions due to health, defining genetic limitations and the possibility of culling:

$$\Pi(U_{1n}) \le \Pi_n^*; p_n \lhd 0,5; \; p_{n=1} = 0 .$$

With recent notes, the optimization problem of the individual life cycle is as follows:

$$R(n^*, R_n) = \sum_{n=1}^{n^*-1} [M_n^* - (1 - p_n(Z)) \cdot \\ \cdot (c_n \Pi_n(R_n) - r(R_n))] - p_n(Z_n) c_k \xrightarrow[R_1, \ldots R_{n^*} \in \Omega_R]{} \min . \quad (12)$$

Incremental algorithm. Thus, there is the resulting algorithm.

Step 0. Cyclic algorithm variable $i = 0$, initial duration of life cycle, that is the maximum possible number of lactations n_i^*, initial program of «life feeding» as a sequence of the animal diet vectors R_{ni} with $n = 1, 2, \ldots n_i^*$, and the sequence of culling probabilities p_{ni} are set.

Step 1 for i = 1. For the interval of $n = 1, 2, \ldots n^*$ the system is solved:

$$
\begin{aligned}
&\Pi_{n+1,i} = a\,\Pi_{n,i} + \mathrm{b}^{\mathrm{T}}\mathrm{U}_{1n,i} + \mathrm{c}^{\mathrm{T}}\mathrm{f}[n],\\
&n \in (1, n_i^* - 1),\ \Pi_{n,i}(1) = \Pi_{0i};\\
&\mathrm{f}^{\mathrm{T}}[n] = [n\ n^2\ n^3],\\
&\mathrm{U}_{1n,i} = \mathrm{HR}_{n,i};\\
&\mathrm{X}_{n,i} = \mathrm{DU}_{n,i},
\end{aligned}
\tag{13}
$$

then the sequence of culling probabilities for all lactations is computed

$$
\begin{aligned}
&\mathrm{Y}_{n,i} = \mathrm{X}_{n,i} - \Delta,\\
&\mathrm{C}_{n.i} = \mathrm{WY}_{n.i},\\
&z_{n,i} = 0, \quad \text{if} \quad \rho_{zn,i} = (\mathrm{h}_z^{\mathrm{T}}\mathrm{C}_{n,i}) \lhd \delta_z,\\
&n_{i+1}^* = n_i^*, \quad \text{if} \quad z_{n,i} = 0,\\
&z_{n,i} = z_n, \quad \text{if} \quad \rho_{zn,i} = (\mathrm{h}_z^{\mathrm{T}}\mathrm{C}_{n,i}) \geq \delta_z,\\
&n_{i+1}^* = n_i, \quad \text{if} \quad z_{n,i} \neq 0,\\
&p(z_{n,i}) = \frac{\rho_{z,i}}{\delta_z},\\
&p_{n,i}(\mathrm{Z}) = \sum_{z=1}^{N_z} p_i(z_n).
\end{aligned}
\tag{14}
$$

Step 2. Equation for the conjugate variable for each lactation is solved in backward time:

$$
\begin{aligned}
&\lambda_{n+1,i} = -\frac{\partial H_{n,i}}{\partial \Pi} = -a\lambda_{n,i} + (1 - p_{n,i}(\mathrm{Z})c_n),\\
&n \in (n_i^* + 1, 1); \lambda(n_{i+1}^*) = 0,
\end{aligned}
\tag{15}
$$

and $\lambda_{n,i}$ array is formed.

Step 3 for i = 1. Partial derivatives of the Hamiltonian to vector of diet for each lactation are calculated:

$$
g_{n,i} = \frac{\partial H_{n,i}}{\partial \mathrm{R}} = (1 - p_{n,i})\mathrm{C}_{\mathrm{R}} + \lambda_{n,i}\,\mathrm{H}^{\mathrm{T}}\,\mathrm{b}\,. \tag{16}
$$

Step 4 for i = 1. Sequence of vectors of diet is specified by a lactation:

$$
\begin{aligned}
&\mathrm{R}_{n,i+1} = \mathrm{R}_{n,i} + \Delta_{n,i} g_{n,i}, \quad \text{if} \quad \Pi_{n,i+1} \leq \Pi_n^*,\\
&\mathrm{R}_{n,i+1} = \mathrm{R}_{n,i}, \quad \text{if} \quad \Pi_{n+1} \rhd \Pi_n^*,
\end{aligned}
\tag{17}
$$

followed by transition back to Step 1.

Step 5 for i > 1. Partial derivatives of the Hamiltonian to vector of diet for lactations are calculated:

$$
\begin{aligned}
&g_{n,i} = \frac{\partial H_{n,i}}{\partial \mathrm{R}} = (1 - p_{n,i}(\mathrm{Z}))\mathrm{C}_{\mathrm{R}} + \lambda_{n,i}\,\mathrm{H}^{\mathrm{T}}\,\mathrm{b} +\\
&+ (c_n \Pi_{n,i} - \mathrm{C}_{\mathrm{R}}\mathrm{R}_{n,i} - \frac{c}{n_{n,i}^*})\frac{\partial p_{n,i}(\mathrm{Z})}{\partial \mathrm{R}}\ ;
\end{aligned}
\tag{18}
$$

$$
\begin{aligned}
&\frac{\partial p_{n,i}(\mathrm{Z})}{\partial \mathrm{R}} = \sum_{z=1}^{N_z} [\frac{\partial p_{i,n}(z_n)}{\partial \mathrm{R}}] = \sum_{z=1}^{N_z} [\frac{\partial \rho_{z,i}}{\partial \mathrm{R}}],\\
&\frac{\partial \rho_{z,i}}{\partial \mathrm{R}} = \mathrm{H}^{\mathrm{T}}\,\mathrm{D}^{\mathrm{T}}\,\mathrm{W}^{\mathrm{T}}\,\mathrm{h}\,.
\end{aligned}
\tag{19}
$$

Step 6 for i > 1. Sequence of diet vectors is specified:

$$R_{n,i+1} = R_{n,i} + \Delta_{n,i} g_{n,i}, \quad \text{if} \quad \Pi_{n,i+1} \le \Pi_n^*, \text{ или } p_n(Z) \le 0,45;$$
$$R_{n,i+1} = R_{n,i}, \quad \text{if} \quad \Pi_{n+1} \triangleright \Pi_n^*, \text{ или } p_n(Z) \triangleright 0,45, \tag{20}$$

with transition back to Step 2 if $R^*_{n,i} \ge \delta_p$; if this condition is not satisfied, the process is terminated.

The result of the problem solution is a sequence of optimal annual diets for all lactation periods R^*_n, the limit of lactation number n^*, and optimum annual milk yield Π_n^*, which are used as integral constraints in the management of each lactation period.

The algorithm for the life cycle control averaged for the herd differs from individual algorithm in the state variables only. As a result, in addition to the average herd optimal annual feed rations for all lactation periods R^*_n and optimal average annual milk yields Π_n^* the expected number of retirement cows $N_n^* = p_n(\tilde{Z})N$, with N as total herd livestock at a farm, and the overall risk for dairy cattle herd $\tilde{R}(n^*)$ are obtained. Due to the fact that the article deals with only a general theoretical approach, we do not describe the content of all the vectors of state and control. This can be most clearly shown in practical examples which will be done in next papers.

Thus, the theory of the lactating cows' life cycle control is grounded. In essence, it means minimizing risk of losses from culling and costs of breeding. The theory is based on new dynamic models of annual milk yield and probabilistic statistical models of culling due to cows' diseases and economic feasibility. At its core, the solution of this problem provides science based standards for the animal feed for all lactations of the life cycle ensuring the given rate of reproduction. The problem of the practical implementation of the proposed theory is the lack of information (monitoring) concerning the health status of animals for a prolonged period (at least 10 years) depending on the actual feed rations. This is due to the fact that in the modern farm veterinary services the relevant functions are lost. The situation can be improved only by creating a model dairy farm of 100-150 animals equipped with all necessary systems for monitoring the lactating cows' health and the biochemical composition of feed. Identification of the proposed mathematical models by monitoring information will make it possible to put into practice the lactating cows' life cycle control and solve the reproduction problems in dairy husbandry.

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MAIN FACTORS AFFECTING MARE INSEMINATION WITH CRYOPRESERVED DOMESTIC AND FOREIGN SPERM

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Abstract

Artificial insemination (AI) has the following advantages over natural mating: fast selection effect, easy transportation, wide dissemination of valuable genetic material, economic use of semen and rational use of sires, preventing sexually transmitted infections, reserving sperm in cryobanks. Among the factors influencing the result of AI in horse breeding are time, frequency rate and insemination repetition, quality and quantity of semen, the depth of insertion of semen into the uterus of a mare, the observance of temperature mode and sanitary regulations during AI procedure, the reproductive performance of mares. There are two main approaches to freezing of stallion sperm and thus two AI technologies for semen packaging and equipment for its introduction into the uterus of mares. The first approach worked out and used in Russia is based on a sparing mode of semen cryopreservation in large volumes, 20-25 ml per dose. The second method, developed abroad, provides pre-treatment of sperm by centrifugation and maximum removal of semen plasma. In another procedure currently used in Russia the small volumes (5-6 ml) of semen are cryopreserved after centrifugation, removal of 50-60 % of semen plasma and thickening semen by dialysis. In recent years frozen semen of stallions from Europe and America is being actively imported to Russia. Despite the technological differences, domestic and foreign approaches to the cryopreservation of stallion's semen provide its similar qualitative characteristics after thawing. The aim of our research was to identify the most significant factors affecting pregnancy rates in artificially inseminated mares when domestic and foreign protocols were used to freeze sperm. Herein, an impact of five such factors (i.e. sperm packing/cryopreservation technology, time of insemination, reproductive state and gynecological soundness of the mares, the activity of sperm) on the effectiveness of AI with frozen semen was estimated in mares of various breeds (Arabian, Akhal-Teke, Trakehner, Hanoverian, Russian Riding horse, American Standardbred, Russian Trotter and Orlov Trotter). Experiments were carried out in 2012 to 2014 at Tersk stud, at a private farm (Mr. A.A. Kazakov the owner) and at experimental stable of the All-Russian Research Institute for Horse Breeding. We compared the data on 106 estrus cycles of 53 mares, artificially inseminated with domestic (granules and tubes of 5 ml and 15-25 ml) and foreign frozen semen (0.5 ml in straws). All used sperm doses in various packages were divided into two groups with the activity above and below 1.5 points (15 %). The time recommended for AI with frozen semen was divided into three intervals (12 h before ovulation, during ovulation, 6 h after ovulation). The animals were conventionally grouped as barren and maiden mares, lactating mares, mares after late (6 or more months of pregnancy) abortion, and also with regard to absence or presence of gynecological pathologies such as vaginal discharge, fluid and air in the uterus, mating-induced endometritis. The effectiveness of artificial insemination was evaluated with regard to pregnancy rate, occurred abortions number and successful foaling. So, two of five factors analyzed were found out to determine reliably a successful AI with frozen semen at high significance level ($p < 0.001$). They were the sperm activity of ≥ 15 % and healthy mares with no pathology observed. Cryopreservation technology, the type of sperm doses' packaging and also the mare's reproductive status (i.e. barren, maiden, lactating or after late abortion) are of secondary importance. It is shown that mares' artificial insemination with one dose of semen during the 6-hour period after ovulation provides the same pregnancy rate (67.7 %) as insemination during the 12-hour period before ovulation (65.0 %) or insemination at the time when the ovum is released from a follicle.

Keywords: mare, sperm, cryopreservation, artificial insemination.

Attempts to fertilize females by transferring male sperm into their reproductive tracts have been made by ancient Assyrians (1000 BC). However, this technique reached the scope of zootechnical production method in animal hus-

bandry only at the beginning of the last century thanks to I.I. Ivanov [1]. The contribution of Russian and Soviet scientists in the world practice of artificial insemination (AI) of farm animals is enormous. The names of V.K. Milovanov, A.V. Kvasnitsky, I.I. Sokolovskaya, and I.V. Smirnov are well known abroad. Scientific publications and achievements of the All-Union (now All-Russian) Institute of Horse Breeding (Breeding Institute) on AI and cryopreservation of stallion semen make up the golden fund of domestic science, are marked by state awards and are among the world's priorities. In 1954, P.N. Skatkin and his student T.P. Il'inskaya obtained the world's first foals from frozen semen; V.G. Parshutina and E.S. Kruzhkova (1956), V.A. Rumyantseva (1958), E.M. Platova and S.Ya. Rombe (1967), A.I. Naumenkova and N.K. Roman'kova (1969) conducted studies on the selection of composition of diluents, cryoprotectants, refrigerants, methods of packing and processing regimes for stallion semen to ensure its fertilizing capacity and maximum storage time [2, 3]. In the 1970s, the Cryobank was established at the Research Institute of Horse Breeding which currently holds about 3,000 doses of semen of outstanding stallions of 15 horse breeds. It should be emphasized that such a record time of stallions' semen storage has not been reported wherever in the world yet. Periodic inspections of fertilizing capacity and obtaining progeny from the semen stored in liquid nitrogen for 16, 25, 30, 33, 35, and 38 years prove that technologies developed by Russian scientists withstand the test of time successfully [4-6].

Currently, research in the area of artificial insemination of horses in Russia and abroad is carried out at a completely new level using the modern methods of computer analysis of semen, flow cytometry, transmission electron microscopy, etc. New technical opportunities have allowed scientists to delve deeper into the study of intracellular interactions and ultrastructural characteristics of germ cells in connection with cryostability and fertilizing capacity of sperm after freezing and thawing [7-10].

The unconditional advantages of AI compared to natural mating are fast selection effect, easy transportation, wide dissemination of valuable genetic material, economic use of semen and rational use of sires, prevention of sexually transmitted infections, reserving sperm prominent individuals and representatives of endangered breeds using cryobanks.

Dealing with frozen semen requires attention and care, as the qualitative characteristics of sperm are largely deteriorated as a result of cryopreservation stress. Insemination with such sperm reduces the pregnancy rate compared to fresh and cooled semen. According to foreign sources, the pregnancy rate in mares after insemination with frozen semen averages 35-50 % with the fluctuations of 31 to 73 % in the first cycle [11]; in Russia, these figures are approximately the same [12, 13].

Among the factors influencing the result of AI in horse breeding are time, frequency rate and insemination repetition, quality and quantity of semen, the depth of insertion of semen into the uterus of a mare, the observance of temperature mode and sanitary regulations during AI procedures [14-18]. In addition, experts pay serious attention to the state of reproductive system of mares, especially after foaling, abortion, and in the presence of signs of abnormalities [14, 15, 19, 20] associated directly with pregnancy rates. Many foreign publications deal with the problem of mating-induced endometritis [11, 21, 22].

The main requirements on the quality of sperm after thawing are currently the following: semen activity at least 2.5 points, or 25 % according to Russian standards [23, 24] and 30 % according to foreign standards [19, 25, 26]; the number of spermatozoa with rectilinear translational motion (RTM) in the dose should not be less than 200-300 million (12, 19, 25); in domestic technolo-

gies of cryopreservation, quality indicators also include the survival of sperm of not less than 96 hours [23, 24].

Currently, there are two main approaches to freezing of stallion sperm and thus two AI technologies for semen packaging and equipment for its introduction into the uterus of mares. The first approach worked out and used in Russia is based on a sparing mode of semen cryopreservation in large volumes, 20-25 ml per dose [27]. The second method, developed abroad, provides pretreatment of sperm by centrifugation and maximum removal of semen plasma, i.e. the increase in spermatozoa content and reduction of the dose volume down to 1-6 ml [28, 29]. Under this approach, the so-called low volume foreign technologies also differ in the composition of diluents, cryoprotectants and methods of preparation of sperm for freezing. In other procedures used in Russia, small volumes (5-6 ml) of semen are cryopreserved after centrifugation, removal of 50-60 % of semen plasma [30] and thickening semen by dialysis [31]. In the 1970s, a method of semen cryopreservation (without centrifugation) in granules [32] packaged in tubes with the final volume of 20-25 ml per dose was developed in the USSR.

Depending on the method of cryopreservation, different types of semen packaging are provided: aluminum tubes of 5 and 15-25 ml (domestic technology) [27, 30, 31]; plastic straws of 0.25; 0.5 and 5 ml (foreign technology) [26, 29]. In the first option, one tube makes one dose for insemination, and in the second option, a dose may be from one straw of 5 ml to 2-12 and more straws of 0.25 and 0.5 ml.

It should be emphasized that stallions are very strictly selected for semen cryopreservation abroad, despite the breeding value and the outstanding workability of procreaters. Therefore, all semen in straws in the international market usually has high quality indicators. There is no such choice of stallions in Russia, so our experts commissioned by horse owners sometimes have to work with the semen of lower quality to save valuable breed lines. Domestic technology [27] makes it possible as it lacks the centrifugation stage that reduces the damaging effect and keeps the fertilizing capacity of sperm to an acceptable level.

The issue of the optimal interval between insemination and ovulation is the cornerstone of any AI technology. Domestic and foreign recommendations for the use of cryopreserved semen are slightly different. For example, in Russia insemination of mares with frozen and thawed semen starts in the 3rd-4th degree of follicle maturity with an interval of 12-16 hours prior to the ovulation. Russian experts recommend not to inseminate mares after ovulation [14-17, 20, 24]. Abroad, mares are inseminated with frozen and thawed semen (based on rectal and ultrasound signs of approaching ovulation) no earlier than 12 hours prior to ovulation and not later than 6 hours after ovulation [11, 19, 25, 26, 33].

In comparing domestic and foreign approaches to cryopreservation of stallion semen and insemination of mares with frozen and thawed semen, their benefits and drawbacks should be highlighted. Semen thickening by centrifugation and semen packaging in straws or tubes of small volume makes it possible to store and transport a larger number of doses in Dewar flasks and saves liquid nitrogen. However, this approach requires a high quality of the original semen material and is associated with its loss during processing (removing the supernatant after centrifugation) and as a result with the increased amount of semen per dose. The domestic so-called large-volume technology (15-25 ml) is more gentle, therefore allowing to work with the sperm of less quality and use the entire original amount of semen without loss. In terms of automation, computerization and full provision of the production process with specially developed tools, foreign technology can be probably preferred. However, the cost of production

lines, dependence on imported media and consumables, as well as the need to create certain conditions for work limit their use to the scope of specialized reproductive centers or large studs. Simple and inexpensive domestic technology, on the contrary, makes it possible to successfully freeze stallion semen even at small private farms. Despite the above differences, both approaches are not inferior to each other in terms of semen quality after thawing and provide about the same performance in the artificial insemination of mares.

Stallion sperm, cryopreserved according to foreign technology, appeared in Russia fairly recently. The high cost of the semen (from some hundred to some thousand euros per one dose of 1.5-6 ml), expensive imported tools, lack of national experts with the necessary skills slow down the process of active introduction of foreign technology in the practice of Russian horse breeding. Therefore, it was not possible to collect credible data on the results of its use in our country until recently.

The purpose of this study was to analyze the indicators of mare reproduction after artificial insemination with semen frozen on different technologies, and figuring out the decisive factors.

Technique.. Experiments were carried out at the Tersk stud (Stavropol region), Mr. A.A. Kazakov's private farm (Ryazan region) and experimental stable of the All-Russian Research Institute for Horse Breeding in 2012 to 2014. Conditions and feeding conformed zootechnical standards at all farms. Stallion cryopreserved semen frozen on different technologies and with different activity, including the below the recommended ones, was used for insemination.

Mares of riding (Arabian, Akhal-Teke, Trakehner, Hanoverian, Russian Riding horse) and trotter (American Standardbred, Russian Trotter and Orlov Trotter) breeds were divided into groups based on the following factors: insemination with sperm in different packaging (granules, tubes, straws), insemination with sperm with different activity, time of insemination relative to ovulation, reproductive group of mares (barren and maiden, lactating, after a late abortion), initial gynecological state of mares (with no visible signs of abnormalities, with visible signs of abnormalities).

Semen packaging involves the technology of its cryopreservation. Semen doses frozen using three domestic technologies and semen of foreign production (Germany, France, Italy, and United States) were included in the experiments. For this purpose, semen packed in granules stored in the Cryobank of the Breeding Institute (according to E.M. Platonov's) (Group 1), in tubes of 15-25 ml (according to A.I. Naumenkov and N.K. Romanjkova) (Group 2), in tubes of 5 ml (according to E.L. Fomina) (Group 3) was used. Imported semen (Group 4) in straws of 0.5 ml (3 to 8 straws in a dose) was obtained from farms and private customers for the insemination of their mares.

Semen activity after thawing was determined using a standard method in accordance with the recommendations [24]. All used sperm doses (domestic and foreign) in various packages were divided into two groups with the activity of $\geq$ 1.5 points and $<$ 1.5 points. Sperm samples with low activity cryopreserved according to the domestic technology, when compared with imported semen, were additionally combined into two subgroups with the activity above and below 2 points (20 %).

Follicle maturity was monitored by rectal and ultrasound diagnostics with an interval of 6 hours. The period recommended for artificial insemination with frozen semen was divided into three intervals (12 hours prior to ovulation, during ovulation, 6 hours after ovulation) and results of insemination were evaluated for each of them. Mares were inseminated using the appropriate tools. The result of insemination was determined using ultrasound diagnostics (from

days 14-15 after ovulation) and rectal (from days 18-20 after ovulation) method. Pregnancy rates (%) and successful foaling (%) were registered based on the data for the year not only relative to the number of pregnant mares but also to the number of inseminated mares as the total measure of the breeding stock efficiency. Additionally, abortions (or cases of fetal death) were taken into account.

Three groups of mares were identified based on the functional state of their reproductive system: Group I — barren and maiden mares (mating for the first time), Group II — lactating mares (nursing), Group III — mares after late (6 or more months of pregnancy) abortion.

Mares were divided into two groups based on their gynecological state— with abnormalities and without abnormalities. Visible signs (diagnosed with rectal, ultrasound or vaginoscopic examinations) of abnormalities included vaginal discharge, fluid and air in the uterus, and mating-induced endometritis.

The data were processed by the conventional method of calculation of statistical characteristics evaluating the significance of intergroup differences using the Student-Fischer *t*-test (34).

Results. We processed the data of three breeding seasons of 106 estrus cycles (53 mares) when artificial insemination was carried out with cryopreserved semen, prepared by Russian and foreign technologies.

Comparison of the effectiveness of insemination with sperm in a different packaging showed that the pregnancy rate in the mares of Group 4 (straws of 0.5 ml) was similar to that in Group 1 (granules), but was significantly ($p < 0.05$) greater versus that in Group 2 (5-25 ml tubes) (Table 1).

Since the activity of imported semen was never below 2-2.5 points, while in Group 2 it did not exceed 0.5 points in some cases, we divided Group 2 into two subgroups (with sperm activity above and below 2 points). As a result, the pregnancy rate in the subgroup with high semen quality increased to 69.6 % and was no longer significantly different from that in Group 4, it dropped to 43.5 % in the subgroup with semen activity of less than 2 points, but the differences between subgroups were not significant.

1. **Results of artificial insemination (AI) of mares of riding and trotting breeds with cryopreserved sperm in various packaging and of unequal activity** (Tersk stud, Mr. A.A. Kazakov's private farm, All-Russian Research Institute for Horse Breeding, 2012-2014)

Variant	AI, cycles	Pregnant		Abortions, specimens	Successful foaling ($M \pm m$), %	
		specimens	$M \pm m$, %		of the number of pregnant	of the number of inseminated
Granules (Group 1, activity of 1-1.5 points)	7	5	71.4±17.10	1	80.0±17.90	57.1±18.70
Tubes of 15-25 ml (Group 2): total	46	26	56.5±7.31[a]	5	80.8±7.70	45.7±8.00
in Subgroup with the activity of ≥ 2 points	23	16	69.6±9.59	4	75.0±10.80	52.2±10.40
in Subgroup with the activity of < 2 points	23	10	43.5±10.34	1	90.0±9.50	39.1±10.20[d]
Tubes of 5 ml (Group 3, activity of ≥ 2 points)	3	2	66.7±27.15	1	50.0±35.40	33.3±27.20
Straws of 0.5 ml (Group 4, activity of ≥ 2 points)	50	37	74.0±6.20[b]	4	89.2±5.10	66.0±6.70[c]

[a, b] $p < 0.05$; [c, d] $p < 0.05$ (respectively, significance of differences between groups with indices a and b and between groups with indices c and d).

The percentage of successful foaling versus the number of inseminated mares had some tendency to increase in Group 4. It differed significantly ($p < 0.05$) from that in the subgroup with semen activity of < 2 points. However, if the percentage of successful foaling relative to the number of pregnant mares is considered, the difference is leveled. Intergroup differences were insignificant

in all other cases.

In general, the effectiveness of insemination of mares with semen frozen according to different technologies and packed in different containers (subject to the skilled procedure of insemination) ranged within 69-72 % and had no significant differences.

2. Results of artificial insemination (AI) of mares of riding and trotting breeds depending on the timing of insemination with cryopreserved sperm and its activity (Tersk stud, Mr. A.A. Kazakov's private farm, All-Russian Research Institute for Horse Breeding, 2012-2014)

AI, cycles	Pregnant		Abortions, specimens	Successful foaling ($M\pm m$), %	
	specimens	$M\pm m$, %		of the number of pregnant	of the number of inseminated
Time of insemination					
12 hours prior to ovulation					
20	13	65.0±10.67	1	92.3±7.40	60.0±10.95
During ovulation					
21	11	52.4±10.90	2	81.8±11.56	42.9±10.80
6 hours after ovulation					
65	44	67.7±5.80	7	84.1±5.51	56.9±6.14
Sperm activity					
≥ 1.5 points					
59	46	78.0±5.40[a]	7	84.8±5.30	66.1±6.20[c]
< 1.5 points					
28	5	17.9±7.20[b]	1	80.0±17.90	14.3±6.60[d]

[a, b] $p < 0.001$; [c, d] $p < 0.001$ (respectively, significance of differences between groups with indices a and b and between groups with indices c and d).

In comparing effectiveness depending on the time of insemination (12 hours prior to ovulation, during ovulation, 6 hours after ovulation) no significant differences in pregnancy rates were observed between variants (Table 2), and the average values were not beyond 52-68 %. There were no significant differences in the percentage of successful foaling as well (see Table. 2). Meanwhile, it is well known that the timing of follicle maturation and ovulation in mares is often (especially in the spring transition period) unpredictable even for experts, so the attempts of insemination prior to ovulation are always followed by a risk to waste a semen dose at the price from 500-5,000 euros and above. The studies confirm high pregnancy rate in mares inseminated with single doses of sperm frozen in tubes and straws, within 6 hours after ovulation.

Analysis of pregnancy rates after insemination of mares with semen with the activity below and above 1.5 points (15 %) regardless of the type of packing (see Table 2) revealed highly significant intergroup differences ($p < 0.001$). This means that semen quality affects the outcome of the procedure dramatically. Differences in the proportion of successful foalings versus the number of inseminated mares appeared to be significant as well ($p < 0.001$). However, the abortion rate in the groups of pregnant mares was similar.

The functional state of the reproductive system of mares at the time of insemination is directly related to the conditions for the survival of semen and the embryos in the uterus and hormonal regulation of sexual function, and, consequently, may affect the pregnancy rate. However, significant differences between three reproductive groups in the pregnancy rate have not been identified (Table 3).

Although the pregnancy rate in Group III was 31.3 % higher than that in group II, the number of mares in Group III, was probably not sufficient to consider such differences significant.

Insemination with and without signs of abnormalities of the reproductive system in mares revealed highly significant differences in subsequent pregnancy rate ($p < 0.001$) and successful foaling ($p < 0.01$) in favor of ani-

mals with good reproductive health. However, the increased proportion of abortions in pregnant mares with abnormalities was not significantly confirmed despite the considerable difference of this value in healthy and problem mares.

3. Results of artificial insemination (AI) of mares of riding and trotting breeds depending on the reproduction group and their gynecological state (Tersk stud, Mr. A.A. Kazakov's private farm, All-Russian Research Institute for Horse Breeding, 2012–2014)

AI, cycles	Pregnant		Abortions, specimens	Successful foaling ($M \pm m$), %	
	specimens	$M \pm m$, %		of the number of pregnant	of the number of inseminated
Reproductive group					
Group I (barren and maiden)					
46	26	56.5±7.31	5	80.8±7.72	45.7±7.34
Group II (lactating)					
25	13	52.0±10.00	1	92.3±7.40	48.0±10.00
Group III (after late abortion)					
6	5	83.3±15.23	2	60.0±21.90	50.0±20.40
Gynecological state					
No visible abnormalities					
18	16	88.9±7.40[a]	3	87.2±9.20	72.2±10.60[c]
Abnormalities (vaginal discharge, fluid and air in the uterus, mating-induced endometritis)					
18	7	38.9±11.50[b]	3	57.1±11.70	22.2±9.80[d]

[a, b] $p < 0.001$; [c, d] $p < 0.01$ (respectively, significance of differences between groups with indices a and b and between groups with indices c and d).

Thus, two of the five factors analyzed, most important and determining the outcome of insemination with frozen semen can be identified. These are semen quality (activity of not less than 1.5 points) and the initial gynecological state of mares. Cryopreservation technology, the type of sperm doses' packaging and also the mare's reproductive status are of secondary importance subject to professional insemination. It is shown that mares' artificial insemination with one dose of semen within 6 hours after ovulation provides the same pregnancy rate as insemination within 12 hours prior to ovulation or at the time when the ovum is released from a follicle. This means rectal and ultrasound diagnosis of mare ovarian during maturation of the follicle can be limited to a 6-hour interval, and insemination should be immediately conducted in fact of ovulation. Such a regime saves the dose of expensive semen and ensures high pregnancy rates in mares.

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INDIVIDUAL REACTIVITY OF GRANULOCYTIC SYSTEM OF NEWBORN CALVES AND ITS ROLE IN PATHOGENESIS OF INFLAMMATORY DISEASES OF RESPIRATORY AND GASTROINTESTINAL TRACTS

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Abstract

Inflammatory diseases of the respiratory and gastrointestinal tracts are the actual problem of veterinary medicine. Innate or natural immunity plays the leading role in the initial reactions to microbial agents contaminating sterile mucous membranes of newborn animals. It is implemented through the activation of cellular and humoral factors of nonspecific resistance. Leukocyte concentration, leukogram, content of cationic proteins in neutrophils, blood serum lysozyme activity (BSLA) were studied in 20 red-motley calves in 1 hour after their birth on the days 2, 5-7 and 14-15 of life, together with an impact of these indices on the terms of appearance and clinical course of inflammatory diseases of the respiratory and gastrointestinal tracts, under conditions of a large dairy complex environment (Voronezhpishcheprodukt Co Ltd, Novousmansky district, Voronezh region) in 2014. Blood sampling was implemented in the morning before feeding from the jugular vein. Body temperature, pulse and respiratory rate, state of the visible mucous membranes, time of appearance and intensity of sucking reflex, the presence and nature of cough, dyspnea, nasal expiration, reaction to palpation of the larynx, trachea, intercostal spaces, the abdominal wall, percussion and auscultation of the chest were determined in calves. The markers of intestinal inflammation are soluble protein, erythrocytes (hemoglobin), leukocytes (leukocyte elastase), pH. They were identified in feces by dry-chemistry method during the same period and also selectively at the age of 1-1.5 months. According to clinical and hematological indices the animals were retrospectively divided into 2 groups: group A with leukocyte concentration of (11.0-18.3)×10^9/l and segmentonuclear neutrophils (SN) more than 4×10^9/L at birth; group B with leukocyte concentration of 6.5-11.3×10^9/l and SN lesser than 3.9×10^9/l at birth. Granulocyte blood concentration dynamics in calves of these groups significantly differed both among themselves and from physiological dynamics (adaptive norm) during the first 15 days of their life. This was determined by various phases of adaptation syndrome (mobilization, resistance, exhaustion) and various possibilities of calves' granulocytic system reaction to irritants impact according to J. Wilder's law of initial values (1957). The increased blood level of SN and rod neutrophils (RN) in animals of group A at birth indicates existing activation of bone marrow and may not significantly increase in response to a rather intensive stimulus. Initial level of SN and RN in calves of group B was close to the physiological one, it significantly increased on the day 2 and decreased by the days 5-7 of life. Cationic protein concentration and BSLA in all the calves during the research period were lower than the indices typical of the animals of this age. Diarrhea developed in all the calves on the day 2 of life. Its duration in animals of groups A and B was 8.6±1.1 and 4.2±0.6 days, respectively. When diarrhea symptoms disappeared, intestinal inflammation markers were identified in calves' feces during 1-1.5 months. During the first 14 days of life the first bronchitis symptoms (induced cough) were registered in all the animals. The course of the disease was light in most of the calves. The mechanisms of changes of granulocytic system reactivity, leading to its decompensation and respiratory tract inflammation development, are discussed.

Keywords: calves, leukocytes, segmentonuclear neutrophils, cationic proteins, feces analysis, phases of biological systems reactivity, inflammatory diseases.

Inflammatory diseases of the respiratory and gastrointestinal tracts are still an urgent problem of veterinary medicine: they affect from 50 to 100 % of calves, starting from the first days of life [1, 2]. Numerous studies of newborn's

immune status mainly cover the issues related to formation of the colostral and lymphocytic components of the adaptive immune system and its disorders in case of inflammatory diseases [3-5]. At the same time, innate or natural immunity plays a leading role in the initial reaction of the organism to the microbial agents contaminating the sterile mucous membranes of newborn animals [6-8]. It is implemented through the activation of the cellular and humoral factors of nonspecific resistance: segmentonuclear neutrophils (SN's), macrophages, as well as the antimicrobial and regulatory substances which are secreted by them and, in turn, initiate a cascade of reactions involving lymphocytes (immune response as such) [9, 10].

SN's represent the first barrier of the antimicrobial protection which is provided by high content of such cells in blood in the newborn in the first hours of life and in first-yield colostrum [11-14]. SN activation is accompanied by the intensification of metabolic processes (respiratory explosion) and the secretion of large amount of antibacterial substances differing in chemical nature [15-17]. Such stereotyped reaction occurs to metabolites of microbial origin, for example, lipopolysaccharides (LPS's) of gram-negative bacteria [18], as well as to hormones, blood and tissue cell mediators, and various kinds of radiation [19-22].

On mucosal surface and in mucosal secretions, SN's perform their function not only by phagocytosis, but also due to the release of DNA and bactericide granules forming neutrophil extracellular traps [23, 24]. SN malfunction on the mucosal surface (often due to the loss of bactericide potential during migration from bone marrow, in the circulatory channel) is considered as one of the factors associated with the formation of pathological microbial communities leading to the development of dysbioses and inflammation of various localization [25, 26].

We were the first who identified the patterns of quantitative changes for SN's and band neutrophils (BN's) in peripheral blood in single-breed cattle within 2 weeks after birth and substantiated the relationship between the natural immunity cellular component reactivity phase at birth and the pattern of pathology development.

The work was aimed at investigating the individual dynamics of the content of granulocytic-series blood cells and their bactericide activity in calves from day 1 to day 15 of life in connection with the onset and subsequent course of respiratory and gastrointestinal tract inflammatory diseases.

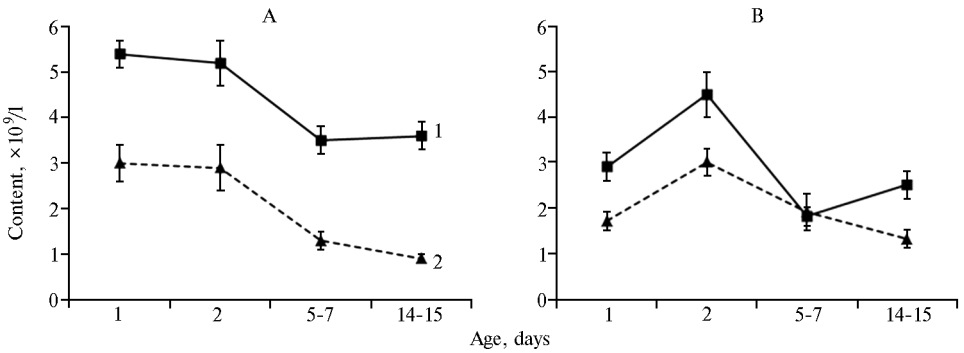
Technique. The studies were carried out in Voronezhpishcheprodukt LLC (Novousmansky District, Voronezh Region) in March—May 2014. A total of 20 red-and-white calves were randomly selected. The animals were kept in a preventorium by 5-6 heads per cage within 10-20 days. Then they were transferred to the group cages of a calf shed (by 5-8 heads) where they stayed until the age of 2-4 months. Newborn calves received colostrum from their mothers from suckling bottles 3 times per day. Within 10 days, colostrum (then milk) was given in the amount of $^1/_{10}$ of animal weight; from day 10 to day 20, this amount was equal to $^1/_5$, and from day 21, whole milk substitute or skim milk was used. The calves were trained to eat hay from the age of 10-12 days and concentrated feedstuffs from the age of 18-20 days.

The animals were examined 1 hour after birth (before the first colostrum feeding), on days 2, 5-7 and 14-15 of life. Body temperature, pulse and respiratory rate were measured; condition of visible mucous membranes, time of appearance and intensity of sucking reflex, and the presence and nature of cough, dyspnea and nasal discharges were assessed; palpation of the larynx, trachea, intercostal spaces and abdominal wall was carried out along with auscultation and

percussion of the chest. Blood was sampled in the morning, before feeding, from the jugular vein using a vacuum system with ethylene diamine tetraacetic acid (EDTA); feces samples were taken directly from the rectum. The total number of leukocytes in blood was measured using a Micros-60 analyzer (Horiba ABX, France); leukogram was calculated using a standard method after Romanowsky blood smear staining. Cationic protein concentration in neutrophils was assessed by the microscopic study of the blood smears stained using Fast Green FCF and by counting of a total cytochemical factor (TCF) [27]. BSLA was determined as described [28]. The rapid analysis of feces was conducted by a «dry-chemistry» method [29] with the use of 10EA test strips for urine analysis (Arkray, Japan) to test the following: presence of soluble protein, erythrocytes, leukocytes (leukocyte elastase) bilirubin (semiquantitative estimation), as well as pH. The clinical study and analysis of feces of 1-1.5 month old animals were also carried out on a selective basis.

Statistical processing of the results was carried out using a correlation analysis and *t*-test for independent variables in the Statistica v. 8.0 software (StatSoft Inc., USA).

Results. The amount of leukocytes and neutrophils in blood in calves on day 1 of life varied from 6.5×10^9/l to 18.3×10^9/l (total leukocytes), from 1.6×10^9/l to 7.2×10^9/l (segmentonuclear neutrophils) and from 1.0×10^9/l to 4.4×10^9/l (band neutrophils).



The dynamics of segmentonuclear (1) **and band** (2) **neutrophil content in peripheral blood in red-and-white calves from day 1 to day 15 of life:** A — group A, B — group B (Voronezhpishcheprodukt LLC, Novousmansky District, Voronezh Region, 2014).

With regard to the amount of leukocytes in blood at birth, the animals were divided into two groups: group A with values from 11.0×10^9/l to 18.3×10^9/l and SN more than 4.0×10^9/l (n = 11) and group B with values from 6.5×10^9/l to 11.3×10^9/l and SN less than 3.9×10^9/l (n = 9). The dynamics of segmentonuclear and band neutrophil content in blood in calves from both groups demonstrated a close correlation (correlation coefficient $r = +0.76$ at $p < 0.05$, see Fig.).

SN amounts in calves from groups A and B statistically significantly differed not only on day 1 of life, but also at later observation stages (Table).

Cationic protein concentration in neutrophils (according to TCF) in calves on day 1 was also characterized by significant variability (from 0.22 to 0.72) and was substantially lower than in adult animals (1.02-1.37) [30]. At later dates, both increase and decrease of TCF were noted in different animals as compared to the value on day 1, however this parameter did not correlate with the amount of segmentonuclear or band neutrophils and did not reach the age norm.

BSLA concentration throughout the whole period of study was within

0.1-0.3 rg/ml, while the norm for calves of such an age is 0.3-0.5 rg/ml [32, 33]. This indicates a low bactericide potential of SN's in the examined animals [34].

The content of segmentonuclear (SN) **and band** (BN) **neutrophils** ($\times 10^9$/l) **in blood in red-and-white calves from groups A and B, differing in the amount of leukocytes at birth, within the period from day 1 to day 15 of life** ($X \pm x$, Voronezhpishcheprodukt LLC, Novousmansky District, Voronezh Region, 2014)

Age, days	SN		BN	
	Group A (n = 11)	Group B (n = 9)	Group A (n = 11)	Group B (n = 9)
1	5.4±0.33*	2.9±0.32	3.0±0.39*	1.7±0.17
2	5.2±0.48	4.5±0.45[d]	2.9±0.54	3.0±0.34[d]
5-7	3.5±0.27*	1.8±0.24[d]	1.3±0.20[d]	1.9±0.39[d]
14-15	3.6±0.34*	2.5±0.33[d]	0.9±0.12	1.3±0.22

* $p < 0.05$ as compared to a similar indicator in Group B.
[d] $p < 0.05$ as compared to the previous period of study within the group.

The clinical condition of calves from groups A and B was significantly different. Prticularly, diarrhea duration was 8.6±1.1 and 4.2±0.6 days, respectively; time of appearance of the first signs of bronchitis (induced cough) was 3.7±0.7 and 7.6±1.6 days of life, respectively. In all calves from group B, the course of bronchitis was mild, while 2 of 11 calves in group A demonstrated a moderate course. Upon the onset of diarrhea symptoms, erythrocytes (++++), leukocytes (++), soluble protein (++, +++) and, in several cases, bilirubin at pH 5-7 were found in feces. These parameters were not normalized with the disappearance of clinical signs of diarrhea. All intestinal inflammation markers (soluble protein, erythrocytes, leukocytes) persisted, however, manifestations were less pronounced (+, ++). This was observed in calves not only at the age up to 15 days, but also 1-1.5 months, which, in our opinion, indicates the transformation of acute intestinal inflammation into chronic one.

In the first days of calf's life, all organs and systems actively adapt to extrauterine existence. Within days 5-7, physiological changes are observed in various parameters of vital activity and metabolism [33, 35]. The granulocytic system of calves in the first 24 hours is characterized by the high number of leukocytes (up to 11×10^9/l) and segmentonuclear neutrophils (up to 64 %), which rapidly decreases starting from day 2 of life [11]. The percentage of band neutrophils from day 1 to day 15 varies from 4 to 5 % without significant peaks and troughs. The absolute values of these parameters depend on the breed of animals and season of study [35, 36].

The analysis of our results in groups A and B has revealed two different patterns of granulocyte dynamics other than physiological one. Group A demonstrated initially high SN and BN content lasting within 2 days with subsequent decrease by days 5-7 and stabilization by day 15. The initial number of these cells in group B was 1.8 and 1.7 times lower, but it sharply increased on day 2, which reflected the release of the cells from bone marrow in response to its irritation. Because a diarrhea syndrome developed in all calves on day 2, we think that it is legitimately to associate the response of the granulocytic system with an inflammatory process in the intestine. However, it is still questionable why it is so different in the calves of the same breed, sex and age, which are kept in the same conditions.

The individual features of the organism response are determined by the initial values of parameters under study [37]. The dependence of a response to a stimulus on a value of the studied parameter in initial state was first described in 1957 by J. Wilder as a "Law of Initial Value" (LIV) [37]. According to the LIV, a change in any parameter (difference between final and initial values) is the less, the higher the initial value is. The higher the initial level is, the more often paradoxical responses to stimuli, which usually increase the studied parameter, are

observed, i.e. an increase in the initial parameter leads to a reduction in capabilities for the further stimulation of the function.

With regard to the granulocytic system, this is expressed in the fact that initially high number of SN's in blood (irrespective of the causes) cannot significantly increase even in response to rather intensive stimuli. Because peripheral blood leukocytes represent a transient pool reflecting the intensity of cell migration from bone marrow into tissues, a change in their number characterizes the reserve of mature cells and proliferative activity of the bone marrow. The high content of BN's in blood and appearance of earlier precursors indicate the significant activation of bone marrow, however, its proliferative activity, with retention of humoral stimulation, will expectedly pass, in accordance with a general adaptation syndrome phase sequence, from the mobilization stage to the resistance stage and then to the exhaustion (areactivity) stage, which will manifest as a decrease in leukocyte content in peripheral blood with the remaining or new focus of inflammation. It is the variant which was observed in the group A calves demonstrating a decrease in granulocyte number by days 5-7 against the background of diarrhea and appearance of the first symptoms of bronchitis.

In spite of the polyethiological nature of diarrhea in calves [1, 3], it is always combined with an intestinal inflammation and wall permeability disorder [38], and with an increase in translocation of intestinal microflora endotoxins, in particular, LPS, into the system blood flow [39]. At the same time, load on the functions of the liver and SN's binding LPS increases. According to the results of feces analysis, the cessation of diarrhea in calves was not accompanied by the elimination of enteritis. The inflammatory process in the intestine remained in the subclinical form which was verified only by laboratory testing. We believe that chronic endotoxinemia is the main cause maintaining the continuous additional load both on the SN function and the whole granulocytic system [40-44]. Against such background, any, even nonspecific, stimulus, for example, stress in case of regrouping of calves or abnormal parameters of microclimate, may cause the immune system to pass from the resistance stage to the decompensation stage [37, 42]. This will manifest as inflammatory diseases affecting, first of all, the respiratory passages and lungs which are always in contact with pathogenic and opportunistic pathogenic environmental microflora [45]. In spite of the fact that the mucous membranes of the respiratory passages contain the biggest part of the SN marginal pool [7], their bactericide activity may not be sufficient for efficient protection due to a decrease in such activity as far back as in peripheral blood, which is indicated by the results of cationic protein and BSLA studies.

In calves from group B, granulocyte content on day 1 was close to a physiological norm, therefore, in response to humoral stimulation, a pronounced increase in SN's and BN's in accordance with the LIV was noted on day 2. It is likely that the reactive potential of the system was one of the reasons behind the fact that the duration of diarrhea in these calves was half as long as in animals from group A, and the first signs of bronchitis appeared much later.

Thus, at birth, the innate immunity cellular component reactivity in clinically healthy calves is in different phases. The causes of it are undoubtedly associated with a functional and metabolic status of mother cows during the pregnancy period, however, the molecular mechanisms of humoral influence on the granulocytic system of the fetus are almost unknown. As in case of animals with depressed reactivity, the calves having a highly reactive granulocytic system at birth are susceptible to respiratory and gastrointestinal tract inflammatory diseases, the only differences are in the onset time and severity of the pathological process. Therefore, the strength of the combined specific (bacterial) and nonspe-

cific (stress) factors activating the granulocytic system of newborn calves turns out to be excessive and is rapidly transferred at cellular and tissular levels from the stage of mobilization and resistance to the stage of exhaustion, i.e. immunodeficiency. Because the granulocytic component is functionally linked to the lymphocytic, macrophage and humoral components, immunodeficiency may manifest in different forms and degrees. The use of immunostimulators for correction of secondary immunodeficiencies, as well as vaccines with adjuvanticity, often leads to exacerbations of inflammatory diseases or even death of animals because the stimulation of the system at the exhaustion stage causes a failure of adaptation. In our opinion, pathogenetically substantiated methods for prevention and therapy in such cases can be those aimed at releasing the immune system from excessive (by frequency and strength) irritation by specific and nonspecific agents.

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EFFECTS OF EXTERNAL γ-RADIATION ON ADENYLATE CYCLASE ACTIVITY IN SHEEP BLOOD CELLS

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Abstract

In the mechanisms of action of various adverse factors on mammals a special part is assigned to the regulatory systems. The main regulatory system of cellular metabolism is the cAMP system. Exposure of animals to external γ-radiation results in the modification of different biochemical processes in cells. In studying diversified intercellular disorders after irradiation it is, therefore, necessary to assess functioning of the cAMP system and its key enzyme, the adenylate cyclase. Note that the data published on the effect of γ-irradiation are mainly obtained with laboratory animals which are significantly different from farmed animals in the body features, whereas the effect in highly productive animals is of special interest. We studied an influence of γ-irradiation on cAMP in Tsygai sheep for the first time and showed a cAMP modification both in the lymphocytes susceptible to radiation and in the thrombocytes which are relatively resistant. In this paper the data are shown on the basal and prostaglandin E_1 stimulated activity of adenylate cyclase in radiosensitive blood cells of sheep exposed to total external γ-radiation at a dose of 4 Gy ($LD_{50/30}$) for 15 days. In the intact sheep lymphocytes a basal and E_1 stimulated adenylate cyclase activity was 2.82±0.64 pmol/(min × 10^6 cells) and 2.49±0.43 pmol/(min × 10^6 cells), respectively, and in the thrombocytes it amounted 10.90±1.90 pmol/(min × 10^8 cells) and 15.70±5.70 pmol/(min × 10^8 cells), respectively. From the first day after exposure, changes have been revealed in all activity components of this enzyme in the lymphocytes and thrombocytes of sheep. The lymphocytes showed a 1.7-4.3-fold increase in the basal adenylate cyclase activity on days 1-15 and 1.3-3.8-fold increase in the stimulated activity on days 1-10. In thrombocytes the basal activity of adenylate cyclase increased 2.7 and 3.5 times on days 1 and 7, respectively, and the prostaglandin E_1 stimulated activity of adenylate cyclase grew 6.9 and 5.7 times on days 1 and 7 after exposure, respectively. In all other days the adenylate cyclase activity components of interest didn't practically differ from the initial level. This suggests that i) modification of adenylate cyclase activity is caused by postradiation alteration of the structural-functional condition of plasma membranes in these blood cells, and ii) in the peripheral blood there is a prevalence of more resistant to radiation damage subpopulation of lymphocytes and thrombocytes with increased adenylate cyclase activity.

Keywords: sheep, external γ-radiation, lymphocytes, thrombocytes, cyclic adenosine monophosphate system (cAMP), basal and prostaglandin E_1 stimulated activity of adenylate cyclase.

Messenger (regulatory or signal transduction) systems play a special role in mechanism of action of various adverse factors on mammalians [1-3]. The systems of cyclical adenosine and guanosine monophosphate (cAMP, cGMP), Ca^{2+}-calmodulin and inositol phosphate are considered the most important intracellular modulators while signal molecules (hormones, mediators, transmitters) are thought as the most important extracellular ones [4-8]. The signal of many hormones, mediators and other primary messengers is transduced by so-called signaling pathways. For example, cAMP-dependent signaling is mediated by functional interaction between membrane β-adrenoreceptor, G-proteins and adenylate cyclase, the key enzyme of cAMP system [5, 9]. The signal from a primary messenger (signal molecule) activates the respective cytoplasmic membrane receptor, which modifies the conformation of G-protein molecules. Depending on whether G_s- or G_i-protein is activated, adenylate cyclase is either activated or inhibited, thus resulting in either stimulation or inhibition of cAMP

(secondary messenger) synthesis [10-12]. Then, cAMP acts as a secondary intracellular messenger activating or inactivating cellular protein kinases, which phosphorylate effector proteins and modulate (increase or decrease) their activity. The resulting metabolic and functional changes specific for a particular hormonal signal modulate the respective cell functions [3, 13, 14]. The cAMP-dependent system of transmembrane signal transmission regulates cell metabolism, proliferation and differentiation. It is also important in hematopoiesis, cell immunity, apoptosis regulation, viral infections and other processes [15-23].

Intracellular messenger systems were shown to be involved in hemostasis and in platelet activation during aggregation [24]. Experiments showed that adenylate cyclase signaling participates in stimulation of biochemical processes resulting in changes of RBC pliability. Thus, the deformed RBCs are more effective in providing of oxygen to organs and tissues. The need to activate calcium signal cascade to increase the membrane and overall cellular stability was also found [25-29]. Furthermore, potential cross-interaction between cAMP and Ca^{2+}-dependent signaling was demonstrated [30].

Experiments showed that animal exposure to γ-rays alters the organ and tissue levels of catecholamines, corticosteroids, serotonin and other biologically active substances, which serve as signal molecules activating membrane receptors of the respective signal paths [5, 31]. Laboratory [3-35] and livestock animals [36-38] showed post-radiation injury of enzymatic activity of some messenger systems in several cell populations.

External γ-radiation of mammals in the dose of 2-10 Gy first injures the hematopoietic system causing the loss of bone marrow and peripheral blood cells [31]. Thus, a question arises whether this exposure activates the cAMP-dependent signaling in radiosensitive peripheral blood cells. It should be noted that most publications on this topic are the studies in laboratory animals, which significantly differ from livestock in their physiology and resistance to radiation.

We examined basal and prostaglandin E_1 stimulated activity of adenylate cyclase in sheep lymphocytes and platelets after whole-body external exposure to γ-rays.

Technique. The study was held in tsygay breed sheep (n = 16) of the live weight 32.35±0.08 kg/animal, kept in vivarium of All-Russian Research Institute of Radiology and Agrarian Ecology. The animal feed was balanced according to the norms set by All-Russian Research Institute of Animal Husbandry (Moscow Region). The control group comprised 8 animals kept in conditions similar to those of experimental animals. The experimental animals (8 sheep) were exposed to whole-body external γ-radiation in a half-lethal dose ($LD_{50/30}$) of 4 Gy, dose intensity being 1 Gy/h delivered by instrument GUZh-24 (Russia) (^{137}Cs as radiation source; γ-quant energy of 0.67 meV). The radiation dose and its homogeneity were controlled by dosimeter VAJ-18 (Germany) with spherical ionization chamber VAK-253 (Germany). Homogeneity of γ-field was within ±15 %.

Blood was taken from jugular vein before irradiation and at 1, 3, 5, 7, 10 and 15 days after it. Sodium citrate (0.38 %) was used as anticoagulant.

Sheep platelet and lymphocyte populations were isolated by a previously developed method of ours [39]. The isolated cells were twice washed in solution containing NaCl, KCl, K_2HPO_4, $MgCl_2$, dextrose and N-(2-hydroxyethyl)piperazine-N′-(2-ethane sulfonic acid) (145, 5, 0.5, 1, 3 and 10 mM, respectively, pH 7.4). Cells in these suspensions were counted in Goryaev's chamber. The platelet aliquot was frozen before the enzymatic reaction; the lymphocyte aliquot was lyzed in hypotonic medium at 4 °C for 30 min.

Adenylate cyclase activity in cell lysates was determined according to conditions described previously [40]. [^{14}C]-ATP and [^{3}H]-cAMP (GE Health-

care, UK) were used as labeled enzymatic reaction substrates. Enzymatic reaction products were separated by thin-layer chromatography using Silufol UV-254 plates (Chemapol, Czech Republic). The incubation medium for enzyme assay included ATP, GTP, $MgSO_4$, ethylene-glycol-di/β-aminoethyl ester/-N,N-acetic acid (EGTA), creatine phosphate, Tris-HCl at 0.5, 0.1, 10, 2, 5 and 50 mM, respectively, as well as creatine kinase (40 units/ml) and 37 kBq of ^{14}C-ATP (pH 7.4). Reaction was carried out in cell homogenates in the presence of EGTA, phosphodiesterase inhibitor, at 30 °C. Hormone-stimulated adenylate cyclase activity was assayed in the presence of prostaglandin E_1 (10^{-5} M) by the difference between the results with and without the activator. Sample radioactivity was assayed by liquid scintillation counter SL-4220 (Intertechnique, France).

The statistical processing was carried out using Student's *t*-test and Microsoft Excel 2003 package. Differences between control and experimental group results were deemed significant at $p < 0.05$.

Results. The viability of isolated cell population was 90-95 % as determined by trypan blue testing.

The basal adenylate cyclase activity is the component of the activity of cAMP-forming enzyme associated with its catalytic subunit in the absence of cell stimulation and inhibition by physiologically active compounds [41]. This activity in lymphocytes from non-irradiated sheep lymphocytes was 2.82±0.64 pmol/(min × 10^6 cells). These data are similar to this parameter in bovine livestock: 2.0±0.4 pmol/(min × 10^6 cells) [38]. Control animals showed virtually no changes in the enzyme activity throughout the study.

External γ-radiation caused an increase in basal adenylate cyclase activity in sheep lymphocytes since the day 1 after exposure (Table). At this term, it was 1.73 times higher than in intact animals. The basal activity of this enzyme in lymphocytes increased 2.15-fold and 4.32-fold on day 3 and day 5, respectively. The elevated values of this parameter were also recorded on day 7, day 10 and day 15 when they were 2.22 times, 2.94 times, and 3.26 times higher, respectively. Thus, the basal adenylate activity in sheep lymphocytes after radiation exposure stayed 1.7-4.3 times above the baseline during the whole study.

The basal and stimulated activity of adenylate cyclase in lymphocytes and platelets of intact and irradiated tsygay breed sheep ($X \pm x$; vivarium of All-Russian Research Institute of Radiology and Agrarian Ecology, Obninsk)

Day after exposure	Lymphocytes, pmol/(min × 10^6 cells)		Platelets, pmol/(min × 10^8 cells)	
	basal activity	stimulated activity	basal activity	stimulated activity
Baseline (n = 16)	2.82±0.64	2.49±0.43	10.90±1.90	15.70±5.70
Control (non-irradiated), n = 8				
1	2.66±0.64	2.53±0.42	10.40±1.20	17.20±3.30
3	2.97±0.55	2.34±0.36	10.10±0.08	16.50±2.30
5	2.64±0.52	2.33±0.31	11.20±1.30	15.40±0.90
7	3.03±0.44	2.47±0.28	10.20±0.07	17.20±0.80
10	2.92±0.36	2.32±0.44	9.80±1.20	13.30±0.54
15	3.11±0.45	2.45±0.33	9.60±1.10	13.50±0.70
Test animals, n = 8				
1	4.88±0.56*	7.35±0.26*	26.80±7.10	107.50±27.50*
3	6.05±1.62	7.33±0.11*	9.70±3.00	16.60±6.30
5	12.17±1.44*	21.80±5.16*	5.10±0.60*	13.40±3.40*
7	6.25±1.10*	5.31±1.01*	38.60±8.60*	89.50±16.50
10	8.30±1.90*	3.22±0.58	9.50±2.10	9.80±4.50
15	8.20±0.60*	6.91±0.51*	9.30±0.70	8.30±0.30

* $p < 0.05$ compared with control animals.

The stimulated adenylate cyclase activity is measured in the presence of effector, prostaglandin E_1 and it is related to the function of hormonal subunit of the enzyme [41]. The mean value of this parameter in lymphocytes of all 16 animals before irradiation was 2.49±0.43 pmol/(min × 10^6 cells). The stimulated adenylate cyclase activity was 2.95 times higher than the value in intact animals on

day 1 after exposure. It increased 2.94 and 3.76 times on day 3 and day 5, respectively, and 1.83 and 1.29 times on day 7 and day 10, respectively. This parameter increased 2.78 times on day 15. Thus, the adenylate cyclase activity stimulated by prostaglandin E_1 in lymphocytes after γ-radiation exposure remained elevated 1.3-3.8 times during the entire study.

As the basal adenylate cyclase activity is associated with catalytic subunit and the stimulated activity is associated with hormonal subunit, the progression of radiation injury in sheep resulted in increased function of both subunits in adenylate cyclase enzymatic complex of lymphocytes.

Basal activity of adenylate cyclase in platelets of all 16 intact animals was 15.70±5.70 pmol/(min × 10^8 cells) similar to 13.3±3.0 pmol/(min × 10^8 cells) reported for bovine livestock [38]. The basal activity of the enzyme in sheep erythrocytes increased 2.73-fold on day 1 after irradiation and returned to baseline on day 3. It decreased 2.14-fold on day 5 but again elevated 3.54 times on day 7. The further values were similar to those in control animals. Prostaglandin E_1-stimulated adenylate cyclase activity in platelets increased 6.85 and 5.70 times on day 1 and day 7 after exposure, respectively. It was similar to the control values in other days.

Thus, whole-body external γ-irradiation in sheep modified adenylate cyclase activity in all examined blood cell types. Namely, radiation induces the activation of adenylate cyclase signaling both in radiosensitive lymphocytes and in platelets, which are moderately radioresistant. In lymphocytes of irradiated animals, the activity of both adenylate cyclase components increases since day 1; in platelets, the increase is observed in days 1 and 7. The processes observed in the enzymatic complex of both cell types involve both catalytic and regulatory subunits of adenylate cyclase. The functional activity of adenylate cyclase first depends on macromolecule conformation and its interactions with cytoplasmic cell membrane component because adenylate cyclase is an integral part of this membrane [41]. Biological membranes are one of ionizing radiation cell targets [42-44]. Therefore, the altered activity of adenylate cyclase in lymphocytes and platelets is surely caused by changes in structure and function of their plasmatic membranes induced by radiation exposure.

The observed increase in adenylate cyclase activity after external γ-irradiation may be also explained by other causes. The progression of radiation injury is known to be accompanied with lymphocyte death in interphase [41]. It is manifested by changes in lymphocyte subpopulations in peripheral blood of animals: the massive loss of the most radiosensitive B-lymphocytes (D_0 is 1.2-1.8 Gy) and the increase in relative proportion of more radioresistant T-lymphocytes (D_0 is 2.0-2.5 Gy for the majority of subpopulation and it exceeds 10 Gy in 3-8 % of cases) [45]. Therefore, lymphocyte population circulating in blood after radiation-induced cell death at initial and main stages of radiation injury mostly comprises the most radioresistant T-lymphocytes persisting in peripheral blood for a long time (lifespan of 200-300 days) [45, 46]. It may be reasoned that increased adenylate cyclase function in lymphocytes is related not only to the cytoplasmic membrane post-radiation modification but it is mostly is a feature of blood cells remaining in circulation after loss of the most radiosensitive lymphocyte subpopulation. It is evident that only the most radioresistant lymphocytes persist after γ-radiation in sheep peripheral blood. These cells may have different cytoplasmic membrane properties, higher content of protein [47] and intracellular calcium [48], and elevated adenylate cyclase activity.

Platelets are even more radioresistant than T-lymphocytes. The normal lifespan of platelets in circulation is 5-10 days; therefore, the peripheral blood platelet population is largely replaced within 7 days [49]. While γ-radiation effects on adenylate cyclase activity in platelets at initial period of radiation injury

are mostly mediated by cytoplasmic membrane alterations, the enzyme activity changes on day 7 seem to be related to release of new cells with new qualitative properties from the bone marrow pool into the circulation. The new platelet population shows significantly higher adenylate cyclase activity, both basal and stimulated with prostaglandin E_1.

Thus, the onset of acute radiation injury after whole-body external γ-radiation of sheep in a half-lethal dose demonstrated the modified function of adenylate cyclase both in radiosensitive lymphocytes and in relatively radioresistant platelets. Lymphocytes showed increased basal and stimulated (by prostaglandin E_1) activity during the entire study duration, i.e. for 15 days (the maximum was observed on day 5). In platelets, basal and stimulated adenylate cyclase activity increased 2.7- and 3.5-fold on day 1 and 6.9- and 5.7-fold on day 7 while no significant changes were observed at other time points. Therefore, adenylate cyclase signaling was activated in both cell types. The detected modification of enzyme activity seems to be caused by post-radiation alterations in structure and function of cell cytoplasmic membranes that affect enzymes of adenylate cyclase signaling cascade, and by changes in peripheral blood morphology where the cell sub-population more resistant to radiation injury and having an increased adenylate cyclase activity predominates.

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MYCOTOXIN CONTAMINATION OF MEADOW GRASSES IN EUROPEAN RUSSIA

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Abstract

A toxicity of plants eaten by animals at grazing land is shown to be complicated with various causes and clinical manifestations. In addition to phytotoxins and «infection» factors carried onto the plants by the insects (e.g., bacterial corynetoxins), the toxic metabolites of endophytic and epiphytic fungi are considered to play the significant role. Based on early understanding, the local risks for cattle, sheep and horses during grazing and stable periods were caused mainly by ergotism, myrothecio- and fusariotoxicoses. Then, for a long time a mycotoxicological evaluation of local grass feeds was not carried out. To date, there is the only one study indicating differences between contaminations of the wild-growing gramineous plants and cultivated cereal crops (A.A. Burkin et al., 2010). The aim of the paper was to summarize our data of assaing 517 meadow grass samples from natural pastures and haying places in European Russia undertaken for the first time to determine contamination with mycotoxins. The spikes of fescue *Festuca* sp., couch grass *Elytrigia repens* (L.), timothy *Phleum* sp., and other locally occurring gramineous plants were selected in North Karelia, Tverskaya, Leningradskaya and Astrakhanskaya regions for July-October 2011. For summer and autumn 2014 collections the aboveground parts of gramineous plants and legumes were taken from Moskovskaya, Tverskaya, Astrakhanskaya regions and North Karelia. The average samples of the field sets of hay were obtained from the animal farms of Moskovskaya region from December 2013 up to April 2014. A multiple combined contamination of grassland gramineous plants and legumes by the mycotoxins was detected, particularly we have found the *Fusarium* fungi metabolites T-2 toxin (T-2), diacetoxyscirpenol (DAS), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FUM), the *Alternaria* metabolite alternariol (AOL), the *Myrothecium* metabolite roridin A (RoA), the «storage fungi» metabolites aflatoxin B_1 (AB_1), sterigmatocystin (STE), cyclopiazonic acid (CPA), emodin (EMO), ochratoxin A (OA), citrinin (CIT), mycophenolic acid (MPA), PR-toxin (PR), and also the ergot alkaloids (EA). The common trend to changing the component composition and content of mycotoxins was revealed for collected samples of gramineous plants from Moskovskaya and Tverskaya regions (June-September) such as reed grass *Calamagrostis* sp., crested dog's tail *Cynosurus cristatus* L., sweet vernal grass *Anthoxanthum odoratum* L., cock's foot *Dactylis glomerata* L., bromegrass *Bromus* sp., bluegrass *Poa* sp., oats *Avena sativa* L., fescue *Festuca* sp., bentgrass *Agrostis* sp., couch grass *Elytrigia repens* (L.), timothy *Phleum* sp., bristly foxtail grass *Setaria* sp. The complex of all examined mycotoxins was found to be formed in plants during early growing period (June). Moreover, AOL, STE, CPA and EMO were detected almost in all locations (80-98 % of samples), whereas DAS, EA, AB_1, OA, STE and MPA occurred rarely (50-70 %). This period, in contrast to subsequent ones, was characterized by low level of T-2 (≤ 40 μg/kg), ZEN (≤ 56 μg/kg), EA (≤ 64 μg/kg), AOL (≤ 200 μg/kg) and EMO (≤ 315 μg/kg) and its negligible (no more than 10-fold) variations in all mycotoxins with the exception of RoA. In the second collection of samples (July) AOL and EMO remained the significant contaminants (89 and 100 %) with an increased incidence of T-2, DON, ZEN and a wider range of the fusariotoxins, CPA and EA amounts. During continued vegetation (August-September) there were stable high indices of prevalence and accumulation of T-2 (up to 795 μg/kg), AOL (up to 10000 μg/kg), EMO (up to 5620 μg/kg), a decreasing incidence of AB_1, CPA, OA, CIT, MPA, PR, DAS, DON, FUM, ZEN fusariotoxins, and super high level of ZEN (up to 5750 μg/kg) occurred occasionally. The peculiarities of contamination of the legumes, such as meadow clover *Trifolium pratense* L., white clover *Trifolium repens* L., narrow-leaved vetch *Vicia* sp., wood vetch *Vicia sylvatica* L., meadow peavine *Lathyrus pratensis* L., the meadow grasses and the hay of various botanic compositions are discussed. For the first time a contamination of

herbage with STE has been shown. The data obtained on RoA are especially important due to limited information of its prevalence.

Keywords: meadow grasses, gramineous plants, pod-bearing plants, hay, mycotoxins.

The toxicity of plants eaten by animals in pastures has been shown to be a complicated problem with various causes and clinical manifestations. In addition to phytotoxins and «infectious» factors carried onto the plants by the insects (e.g., bacterial corynetoxins), the toxic metabolites of endophytic and epiphytic fungi are considered to play a significant role [1]. Endophytes of meadow grasses that produce ergot alkaloids caused considerable damage to livestock in the United States and other countries [2, 3]. This problem was managed in recent years only due to the successful introduction of variety substitution technology [4-7]. Toxins of epiphytic fungi that infect vegetative forage plants are quite diverse and include phomopsines (*Phomopsis leptostromiformis*), diplodiatoxines (*Diplodia maydis*), slaframine (*Rhizoctonia liguminicola*), paspalines (*Claviceps paspali)*, ergot alkaloids (*Claviceps purpurea*), trichothecenes, and zearalenone [8-10].

The studies of etiology of mycetogenetic animal intoxication started in our country in the 1930s revealing the general risks for cattle, sheep and horses during grazing and stable periods caused mainly by ergotism, myrothecio- and fusariotoxicoses. At the first signs of poisoning, inspection of pastures for ergot and *Myrothecium* infection was recommended, as well as avoiding grazing on stubble in the fall and early spring in the meadows with the remains of overwintered crops and the young grass damaged with ground frosts. At the same time, experts noted drastic changes in the accumulation of mycotoxins due to unknown causes. Often, extensive fungal infections did not signify hazard, and, on the contrary, in the absence of visible signs plant infection, acute toxicity could arise [11, 12]. These observations are still the most convincing argument for the need for toxicological monitoring of grass fodder.

We performed the first study of meadow grass in August 2009 in North Karelia at the Arctic zone border along the White Sea coast within an area of about 150 km in length located between the biological stations of Saint-Petersburg State University and M.V. Lomonosov Moscow State University. Tops of sand lyme-grass, couch grass and timothy grass collected from several closely spaced plants were free from ochratoxin A, citrinin and T-2 fuzariotoxin, deoxynivalenol, zearalenone, but contained alternariol that is rare for grain (one of the toxic metabolites of *Alternaria* fungi) and diacetoxyscirpenol (trichothecene fuzariotoxin) [13]. In this way, the first confirmation of differences in contamination of wild grasses and cultivated crops has been obtained earlier.

Continuing our research, we were the first in Russia to perform the random mycotoxicological estimation of meadow grass stands the results of which are summarized in this article. At the same time, the data obtained on red clover (*Trifolium pratense* L.) fundamentally changed the conception of local risks associated with it as a common pasture culture. Different contamination of herbage and hay with sterigmatocystin was first described. The data on roridin A are of particular interest as the information on its prevalence is very limited.

The purpose of this study was to investigate vegetating meadow grass samples from natural pastures and hayfields in European Russia to determine contamination with mycotoxins.

Technique. In July and October 2011 spikes were selected of fescue *Festuca* sp., couch grass *Elytrigia repens* (L.), timothy grass *Phleum* sp. and other locally occurring grasses, particularly, reed canary grass *Phalaroides arundinacea* (L.) Rauschert, sand lyme-grass *Leymus arenarius* (L.) Hochst. in North Karelia (Loukhskii District); reed grass *Calamagrostis* sp., cock's foot *Dactylis*

glomerata L., bromegrass *Bromus* sp., bluegrass *Poa* sp., common reed *Phragmites australis* (Cav.) Trin. ex Steud. in Tver' Province (Vyshnevolotskii Region); reed grass, canary reed grass, cock'sfoot, bromegrass, bluegrass, rye *Secale* sp. in Leningrad Province (Luzhskii, Priozerskii, Pushkinskii regions), and bromegrass, bluegrass, pampas grass *Cortaderia* sp., and common reed in Astrakhan' Province (Enotaevskii Region). In summer and fall of 2014, aerial parts of gramineous (reed grass, crested dog's tail *Cynosurus cristatus* L., sweet vernal grass *Anthoxanthum odoratum* L., canary reed grass, cock's foot, bromegrass, lyme-grass, foxtail *Alopecurus* sp., bluegrass, oats *Avena sativa* L., fescue, bentgrass *Agrostis* sp., couch grass, ryegrass *Lolium* sp., rye, timothy grass, bristly foxtail grass *Setaria* sp.) and leguminous plants (narrow-leaved vetch *Vicia* sp., wood vetch *Vicia sylvatica* L., red clover *Trifolium pratense* L., white clover *Trifolium repens* L., meadow peavine *Lathyrus pratensis* L.) were conducted in Moscow Province (Kashirskii, Noginski, Ruzskii regions in June to September), Tver' Ptovince (Vyshnevolotskii Region, in July and September), Astrakhan' Province (Enotaevski Region, bank of the Volga river in October) and in North Karelia (Loukhski District, the White Sea coast in August). The plants were cut at a height of 3-5 cm from the soil surface and dried at 50 °C. Average samples from production batches of hay were provided by agricultural enterprises of Moscow region in the period from December 2013 to April 2014.

Ground air-dried plant material was extracted with a mixture of acetonitrile and water in the volume ratio 86:14; extraction agent consumption of 10 ml per 1 g of sample weight. Extracts 10-fold diluted with a buffer solution were used for indirect competitive enzyme-linked immunosorbent assay (ELISA). Content of T-2 toxin (T-2), diacetoxyscirpenol (DAS), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FUM), ergot alkaloids (EA), alternariol (AOL), roridin A (RoA), aflatoxin B_1 (AB_1), sterigmatocystin (STE), cyclopiazonic acid (CPA), emodin (EMO), ochratoxin A (OA), citrinin (CIT), mycophenolic acid (MPA), and PR-toxin (PR) was evaluated using certified enzyme immunoassay [14]. The lower limit of quantification was determined by the 85 % level of antibody binding.

Results. AOL was found in all the spikes collected in July-October 2011. DAS was found in one sample from Tver' region only (Table 1).

1. Contamination of spikes with fusariotoxins, alternariol, and ergot alkaloids in wild grass in different regions of the European part of Russia (collected in 2011)

Mycotoxin	n^+/minimal-maximal content, rg/kg			
	Tver' Province, July (n = 29)	North Karelia, August (n = 19)	Leningrad Province, September (n = 65)	Astrakhan Province, October (n = 48)
T-2	23/14-450	1/10	19/8-125	5/8-60
DAS	1/100	–	–	–
DON	2/126, 225	–	1/150	–
ZEN	–	–	–	–
EA	19/10-69,000	9/19-16,980	27/17-5,250	2/10, 80
AOL	11/40-560	5/343-8,310	52/40-1,320	39/45-1,260

N o t e. T-2 — T-2 toxin, DAS — diacetoxyscirpenol, DON — deoxynivalenol, ZEN — zearalenone, EA — ergot alkaloids, AOL — alternariol; n — number of samples studied, n^+ — number of positive samples. Dash means that no positive samples were found.

In two areas (North Karelia, Astrakhan' Province), grass rarely contained small amounts of T-2, in other areas (Tver' and Leningrad provinces) T-2 in the amount of more than 100 μg/kg was common, DON was rare. In Tver' and Leningrad Provine and in North Karelia, within the range of ergot, extensive contamination of spikes with ergot alkaloids (EA) was observed, and ultrahigh accumulation of EA could be the consequence of sclerotia maturation. Further extended analysis of 29 samples from Tver' Province (16 mycotoxins) showed extensive contamination of spikes with EMO (24 samples, 80-4,680 μg/kg) and less frequent contamination with OA, STE (7 samples, 8-25 mcg/kg), and CPA

(3, 125-160 μg/kg). This meant that not only toxigenic fungal species of *Fusarium* and *Alternaria* and producers of ergot alkaloids, but also the representatives of other micromycetes allegedly belonging to the *Aspergillus* and *Penicillium* genera or perhaps a number of other genera played their role in the infection of growing plants.

Further examination of meadow grass was performed using a panel of 16 analytical test systems, and, in most cases, legume samples were collected along with grass. At the beginning of the growing season (June), multiple concomitant contaminations of grass with the all analyzed mycotoxins were observed in Moscow Province (Table 2). The amount of EA was not great (beyond 64 μg/kg). AOL, STE, CPA, and EMO were widely distributed, the rate of DAS, OA, CIT, MPA, AB_1 and EA was around 50 %, and this value was 35 and 23 % for RoA and PR, respectively. The frequency of T-2, ZEN, and DON detection was inferior to DAS, and FUM was found in 2 % of samples only.

2. Mycotoxin incidence and content in meadow grasses in different regions of the European part of Russia, depending on the timing of the growing season (collected in 2014)

Mycotoxin	Incidence,%/minimal-maximal content, rg/kg							
	Moscow Region				Tver' Province		North Karelia	Astrakhan Province
	June (n = 92)	July (n = 55)	August (n = 46)	September (n = 17)	July (n = 21)	September (n = 16)	August (n = 22)	October (n = 18)
T-2	32/3-40	65/4-760	70/4-795	65/4-630	86/4-2,510	69/5-540	23/3-630	33/3-220
DAS	52/104-500	22/89-500	2/125	–	29/117-400	6/220	–	6/160
DON	15/126-315	31/117-930	2/125	12/78.400	24/79-400	6/120	5/570	6/500
ZEN	17/27-56	27/52-145	11/25-5,750	12/79, 1,520	29/49-100	–	–	–
FUM	2/66, 85	4/126, 215	–	–	–	–	–	–
EA	70/2-64	53/2-52,200	35/3-2,000	24/2-870	67/4-33,000	31/2-125	100/2-10,000	-
AOL	98/19-200	89/28-830	100/28-2,240	100/31-10,000	86/25-135	94/24-9,770	100/40-4,680	83/40-2,510
RoA	35/8-265	20/2-80	–	–	24/21-50	–	5/7	–
AB_1	51/2-16	15/4-10	2/5	–	10/6, 8	–	23/4-10	6/3
STE	80/10-53	33/9-95	59/8-190	71/13-80	29/32-70	75/13-135	68/12-45	44/12-40
CPA	95/151-660	49/115-1,380	11/166-330	–	76/200-515	–	18/132-280	11/155, 158
EMO	87/33-315	100/73-3,890	100/56-5,620	100/72-5,130	86/59-330	100/59-5,370	95/63-870	94/51-2,500
OA	55/8-28	36/8-21	13/7-9	–	57/6-20	–	14/9-13	17/10-370
CIT	50/33-160	27/66-295	9/42-70	–	19/91-175	–	–	–
MPA	60/14-84	40/14-80	15/10-150	24/12-25	67/18-45	–	41/20-170	28/23-35
PR	23/105-400	24/120-430	2/165	–	19/200-250	–	–	–

Note. T-2 — T-2 toxin, DAS — diacetoxyscirpenol, DON — deoxynivalenol, ZEN — zearalenone, FUM — fumonisins, EA — ergot alkaloids, AOL — alternariol, PoA — roridin A; AB_1 — aflatoxin B_1, STE — sterigmatocystin, CPA — cyclopiazonic acid, EMO — emodin, OA — ochratoxin A, CIT — citrinin, MPA — mycophenolic acid, PR — PR-toxin; n — number of samples studied. Dash means that no positive samples were found.

The amount of most mycotoxins varied insignificantly: it was within the same range in DAS, DON, ZEN, FUM, STE, CPA, MPA, and PR, or it was in the same range in all the others, except RoA. Presumably, different grasses respond to infection-producing fungi similarly in the initial stage of plant development. The same feature was observed in Leningrad Region in the seeded grass herbage prior to the first cut [15].

In a month, in July, the incidence index decreased in DAS, RoA, AB_1, STE, CPA, OA, CIT, and MPA. At that, there was an increase in both fusariotoxins (most dramatic in T-2) and EA, AOL, STE, CPA, and EMO content range. It was during this period that the ultrahigh amount of EA was observed in plants, up to 52,200 μg/kg.

In August and September, the T-2 contamination in plants remained the same, while DAS was detected even rarer. It is essential to note the cases of ZEN quantities accumulation greater than 1,000 μg/kg, as this conceivably could be due to *Fusarium* species that tend to change their metabolic response under the influence of environmental factors [16]. Prevalence of AOL and EMI re-

mained widespread, the intensity of AOL accumulation progressed smoothly, and the amount of this toxin could reach 10,000 μg/kg in September. EMO content was stable with the limit value of about 5,000 μg/kg. The incidence of many other toxins decreased. Thus, RoA was not found in grasses in August, and AB_1, OA, CIT, CPA, MPA, and PR were rarer compared to in July. AB_1, OA, CIT, CPA, and PR were never found.

The dynamics of DON and STE incidence and content were unstable. The maximum DON amount was 930 μg/kg. Content lower than 10 μg/kg was typical of STE, and this value was rarely exceeded only in the later stages the growing season (August and September).

Some of the trends described for grasses in Moscow Region, were the same in Tver' Province (see Table 2). Thus, the incidence of most mycotoxins decreased, the range of AOL and EMO increased, and ultra-high amounts of EA and ZEN were not observed in September compared to in July. In North Karelia (August) and Astrakhan Province (October), grasses were poor in mycotoxins with a dominant spread of AOL and EMO, but in smaller quantities compared to that in September in Moscow and Tver' provinces. A low infectious load in the place of growth could be the reason for this.

Seasonal changes in contamination of grasses, various in different mycotoxin groups, were likely the result of processes in the composition of microbiota. It could be of a regular character, but the effect of climatic or environmental factors could not be excluded. The most important future purpose for researchers is obtaining statistically significant information on the nature of meadow and pasture grass contamination with micromycetes and mycotoxins within the entire period of their economic use from the extended database. The results presented in this study is the first attempt to perform a mycotoxicological estimate of natural herbage grasses which will later make it possible to move on to exploration of some of the most common plant species.

3. Contamination of samples of meadow leguminous plants with mycotoxins in Moscow and Tver' provinces, and North Karelia (2014)

Mycotoxin	n^+/minimal-maximal content per sample, μg/kg				
	red clover (n = 35)	white clover (n = 5)	narrow-leaved vetch (n = 11)	wood vetch (n = 12)	meadow peavine (n = 6)
T-2	29/3-108	4/3-4	8/3-50	3/5-6	1/6
DAS	19/122-550	–	2/132, 133	–	–
DON	11/89-405	–	–	3/126-140	1/67
ZEN	15/31-190	1/90	–	–	–
FUM	18/42-300	3/56-245	–	2/56, 56	1/100
EA	35/2-490	5/4-9	5/3-62	12/2-15	3/3-8
AOL	35/30-830	5/129-400	11/19-310	12/23-100	6/20-540
RoA	17/7-185	1/100	3/35-125	–	1/4
AB_1	29/3-22	4/5-15	3/5	4/2-6	5/2-6
STE	28/18-200	4/42-115	10/25-2,320	11/15-45	6/10-70
CPA	35/190-2,455	5/176-540	6/158-525	9/107-560	2/155, 165
EMO	35/260-27,540	5/155-5,500	11/63-1,585	11/51-1,000	6/50-150
OA	35/7-105	4/10, 11	5/11-15	4/8-16	1/10
CIT	30/42-340	3/72-90	3/91-125	–	–
MPA	33/14-130	5/22-40	3/28-40	2/16, 16	–
PR	35/148-910	3/158-315	3/176-400	2/133, 150	–

Note. T-2 — T-2 toxin, DAS — diacetoxyscirpenol, DON — deoxynivalenol, ZEN — zearalenone, FUM — fumonisins, EA — ergot alkaloids, AOL — alternariol, PoA — roridin A; AB_1 — aflatoxin B_1, STE — sterigmatocystin, CPA — cyclopiazonic acid, EMO — emodin, OA — ochratoxin A, CIT — citrinin, MPA — mycophenolic acid, PR — PR-toxin; n — number of samples studied, n^+ — number of positive samples. Dash means that no positive samples were found.

In red clover samples selected in Moscow and Tver' provinces and in Northern Karelia, the complex of mycotoxins usually consisted of 12-15 ingredients (Table 3). AOL, EMO, OA, PR, CIT, and MPA were widespread or were of close prevalence, PoA was quite substantially sizeable (in half of samples). An ul-

tra-high content of EMO (up to 27,540 μg/kg) was observed in this plant in different habitats in July, August and September.

Our findings fundamentally change the existing ideas about local risks associated with this major pastoral culture. Earlier, clover contamination with *Rhyzoctonia leguminicola* fungus observed in some regions of the world (particularly in the US) and «slobber» syndrome in cattle and horses caused by slaframine and swainsonine were considered of economic importance [17]. Extensive multiple mycotoxin contamination characteristic of a variety of fungi species is not in complete agreement with a developed system of biochemical protection of these plants against fungal infection [18].

Despite the smaller sample size of white clover, as a whole it has the same features of extensive contamination as red clover. Widespread or close incidence of the same group of mycotoxins was observed, but the mycotoxin limit accumulation did not reach the values typical of red clover.

The number of narrow-leaved vetch, meadow pea-vine, and wood vetch samples was also small (6 to 12). In these plants, AOL, STE, and EMO were found almost everywhere, and they were inferior to both clover species in the content of other mycotoxins. Similarities in T-2 common incidence and rare DAS and CIT were observed in narrow-leaved vetch and clovers. A possibility of intensive CTE contamination (up to 2,320 μg/kg) is one of the *Vicia* sp. features.

In general, the accumulation of the amounts of T-2 exceeding 100 μg/kg was not observed in the late stages of vegetation in leguminous plants, unlike grasses. Unfortunately, the small sample sizes of these plants provide just a general idea of mycotoxin contamination. To verify the data and evaluate the seasonal variation in legume contamination, it is necessary to have more extensive biomaterial. It is important to expand the species composition of the crops studied including economically valuable plants, such as alfalfa, sainfoin, bird's foot deer vetch, goatэs rue, sweet clover, and others.

4. Mycotoxin incidence and content in meadow grass and hay samples in Moscow Province (collected in 2014)

Mycotoxin	Incidence, %/minimal-maximal content, rg/kg	
	meadow grasses (n = 227)	hay (n = 120)
T-2	54/3-795	94/3-1,410
DAS	32/89-550	19/100-1,445
DON	19/78-930	28/87-1,620
ZEN	21/25-5,750	45/20-10,000
FUM	4/66-300	8/97-250
EA	57/2-52,200	83/2-3,160
AOL	96/19-0000	98/21-10,000
RoA	23/2-265	13/5-65
AB_1	30/2-6	25/2-25
STE	64/8-200	88/6-7,940
CPA	60/115-2,455	67/63-5,130
EMO	95/33-27,540	100/33-17,780
OA	41/7-105	14/5-30
CIT	35/33-340	33/28-515
MPA	46/10-150	88/15-10,000
PR	23/105-790	26/50-1,070

N o t e. T-2 — T-2 toxin, DAS — diacetoxyscirpenol, DON — deoxynivalenol, ZEN — zearalenone, FUM — fumonisins, EA — ergot alkaloids, AOL — alternariol, PoA — roridin A; AB_1 — aflatoxin B_1, STE — sterigmatocystin, CPA — cyclopiazonic acid, EMO — emodin, OA — ochratoxin A, CIT — citrinin, MPA — mycophenolic acid, PR — PR-toxin; n — number of samples studied.

Identifying distinct seasonal dynamics in cereal contamination and interspecific differences in mycotoxins content in leguminous plants, we felt it appropriate to generalize the results of Moscow Provicne obtained within the entire period of observation, and to compare them with the data on the dry grass fodder contamination harvested in the same area a year before. All 16 of mycotoxins participated in grass contamination in summer and fall providing multi-

component contamination with varying amounts of T-2, ZEN, EA, AOL, and EMO within two to three orders of magnitude (Table 4).

Frequent detection of fusariotoxins in herbs (20-96 %), as well as EA, AOL, and RoA can be attributed to the spread of «field» fungi. In contrast, high values of this parameter for all other mycotoxins (23-90 %) were quite unexpected, and a special study on the identification of micromycetes providing biosynthesis of these substances is required. So far, it is assumed that the probability of accumulation of mycotoxins typical of «storage fungi», OA in particular, is extremely low in vegetative plants [19].

The frequency of detection of the majority of mycotoxins in herbs and hay was quite comparable, but there was a significant (approximately 2-fold) increase in this parameter in T-2, ZEN, and MPA in hay, and the range of content clearly shifted towards higher values. We have already discussed possible sources and causes of accumulation of significant amounts of ZEN in vegetative grasses and hay [16]. In selective toxicological evaluation of hay from different regions of the European part of the country, the upper T-2 and MPA limits exceed 2,000 μg/kg [20]. An abnormally high level of STE (2,510, 3,550, and 7,940 μg/kg) was found in three samples of perennial hay grasses and motley grass. Differences in vegetative herbage and hay on the intensity of STE contamination were found for the time. Their reasonable interpretation can be started only after the completion of the search of micromycetes responsible for STE biosynthesis. It is obvious that these fungi not only continue to develop in the drying phase, but also intensify toxin formation under these conditions. In addition, the causes of significant STE accumulation may be associated with the peculiarities of the botanical composition of the forage.

Hay AOL contamination remained high; there were no significant changes in DON, FUM, AB_1, OA, and CIT contamination, the limit amounts of EA and RoA were lower compared to herbs. The situation of EA can be explained by ergot sclerotia shedding during tedding, stacking hay in bales or stacks, as well as during transportation. Reduced incidence of PoA (from 23 % in plants to 13 % in hay) was accompanied by a decrease in the average value of accumulation from 48 to 20 μg/kg. These results are of particular value, since the information on RoA prevalence is very limited. RoA incidence and content in cultivated herbs in multi-mowing fields was low [8]. Cases of accumulation of 100 μg/kg of RoA were rare in meadow herbs. Of all the samples containing this toxin ($n^+ = 51$), its content was 100 to 265 μg/kg in seven samples only. In this context, the possibility of myrotheciotoxicosis in ruminants in the territories studied raise doubts.

In contrast, a high-limit content of T-2, DAS, ZEN, and EA in meadow plants in a number of areas of European Russia, as well as in dry green fodder, undoubtedly represents a threat to animal health and, consequently, fusariotoxicosis and ergotism can be considered as a real problem. Indeed, in the late 1990s, when calves were pastured in Kursk Province with frostbitten corn herbage heavily contaminated with *F. sporotrichioides,* massive acute toxicosis with sores in mouth was diagnosed in some areas. Cases of ergotism, claviceps-toxicosis and zearalenone-toxicosis in cattle, sheep and horses have not been recorded in our country, but such examples have been described in other countries [21-24]. Unfortunately, the negative effects of the intake of AOL, EMO, STE, CPA, MPA, and PR at thousands or tens thousands micrograms per 1 kg have not been evaluated experimentally, but the threat may be very serious due to the combination of various forms of their direct and long-term toxic effects [25].

Thus, many grass and leguminous forage species are a source of complex mycotoxin combinations which is subject to changes in vegetation, and has its

own features of composition and ratio of individual components in different crops. Herbage T-2, zearalenone, mycophenolic acid, and sterigmatocystin contamination prior to drying may result in extremely intensive contamination of hay. The selective evaluation of meadow herbage mycotoxin contamination performed in our country for the first time indicates the need for increased monitoring studies, studies of other plants, as well as for toxicological experiments to assess the risk of combined effects of mycotoxin intake with grass and leguminous fodder on animals. In the future, it is also very important to pay attention to mycological and mycotoxicological assessment of *Asteraceae* meadows of economic importance, sedge herbage that make up the bulk of hay in the northern regions of the forest zone (slender sedge, water sedge, etc.), as well as of the plants belonging to other botanical families widely represented at natural forage lands.

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DIHYDROQUERCETIN, THE BIOACTIVE SUBSTANCE, TO BE USED AGAINST PATHOGENIC MICROORGANISMS AS AN ALTERNATIVE TO ANTIBIOTICS

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Abstract

According to WHO reports, since 2000 a microorganism resistance to antimicrobials has become a serious threat to global public health. Thereby, more strict control for used antibiotics and novel antibacterial substances are considered helpful. Of that, the development of new antimicrobial compounds seems to be most perspective, seeing the high yielding animals are much more susceptible to diseases, and animal products are poorly stored because of microbial contamination. Among compounds possessing antimicrobial properties the dihydroquercetin, a bioflavonoid is of special interest due to wide range of biological activity, including antioxidant activity. Dihydroquercetin is used widely in food industry and medicine, but in animal farming its use is a novel project aimed to provide for animal welfare and quality of livestock products. We compared in vitro antimicrobial effect of different antibiotics (tetracycline, chloramphenicol, streptomycin, bacitracin, grisin, benzyl penicillin at 3.0, 5.0, 10.0, 16.0, 19.0, 24.0 and 48.0 μg/ml each) and 0.5, 1.0, 2,0 and 5.0 % dihydroquercetin to pathogenic, opportunistic, and probiotic microorganism *Staphylococcus epidermidis* ATCC 14990, *Micrococcus luteus* (*lysodeicticus*) ATCC 4698, *M. luteus* ATCC 10240, *Escherichia coli* VL-613, *Pseudomonas aeruginosa* 98. In gel diffusion test with a series of dilutions the diameters of growth inhibition zone (D) were measured. *St. epidermidis* was found to be high sensitive to 5.0 % dihydroquercetin (D of 21.33±0.82 mm) but low sensitive to all the tested concentrations of bacitracin at the Ds ranged from 14.40±0.27 to 18.80±0.42 mm, and to grisin at 3 to 10 μg per milliliter concentration with zone diameters of 18.30±0.22 to 19.80±0.22 mm. Probiotic *E. coli* and nonpathogenic *M. luteus* (*lysodeicticus*) ATCC 4698 и *M. luteus* ATCC 10240 of the gastrointestinal microflora seem to be insensitive to 0.5 до 2.0 % dihydroquercetin (D of 12.20±0.84 to 19.75±0.73 mm) but high sensitive to all tested antimicrobial drugs (D of 20.20±0.22 to 54.80±0.22 mm).

Keywords: antibiotic resistance, antibiotic sensitivity, dihydroquercetin, *Staphylococcus epidermidis*, *Micrococcus luteus* (*lysodeicticus*), *Micrococcus luteus*, *Escherichia coli*, *Pseudomonas aeruginosa*.

World Health Organization (WHO) notes that microbial resistance poses a growing threat in the last twenty years. This resistance develops due to injudicious antibiotic use in healthcare and animal husbandry [1, 2]. WHO report on April 30, 2014, presented data on microbial resistance to antimicrobial drugs in more than 114 European and African countries for the first time (http://www.who.int/drug-resistance/documents/surveillancereport/en/).

Antibiotics in feed are widely used to stimulate the growth of healthy farm animals and to prevent their diseases. However, the use of antimicrobial drugs in large animal populations may result in spread of bacteria resistant to antibacterial drugs and may cause drug-resistant infections [3-6]. The problem is even more acute for pedigree cattle.

The higher number of resistant bacterial strains, which are common infectious pathogens for humans and animals, has the greatest epidemiological importance. Humans may be infected through contaminated food, after direct contact with animals, or though environment. In Europe, use of glycopeptides (e.g.,

avoparcin) to stimulate animal growth resulted in the spread of vancomycin-resistant enterococci in symbiotic flora and meat of animals as well as in symbiotic flora of healthy people [1]. Fluoroquinolones (e.g., enrofloxacin) induced ciprofloxacin-resistant *Salmonella*, as well as *Campylobacter* and *E. coli* causing human and animal diseases, which are difficult to treat [7, 8]. In EU countries, resistance to erythromycin is unevenly distributed among *Campylobacter* strains isolated from poultry and pigs; it is probably explained by differences in use of antimicrobial products. *E. coli* strains resistant to β-lactam antibiotics due to β-lactamase activity are isolated both from human patients and from farm animals [8, 9]. European Center for Disease Prevention and Control (ECDC) warns that growth of bacterial resistance to antibiotics poses a threat to humankind survival [10]. Moreover, microbial contamination contributes to losses of animal produce during storage [11].

When developing medicines, prebiotics, and probiotics, special attention is paid to biologically active substances. These substances include dihydroquercetin, an active antioxidant, immunomodulator, natural acceptor of free radicals, liver-protective drug, and drug protective against ionizing radiation. It has anti-inflammatory and analgesic properties, promotes vasodilation, impedes the progression of atherosclerotic plaques, and decreases cholesterol synthesis [12-16]; it also eliminates heavy metals, including radionuclides from the body because of its high complex properties. Dihydroquercetin is used in medicine and food industry; however, the available literature does not mention its use in animal husbandry as an alternative to antibiotics.

In this publication, we present for the first time the information on dihydroquercetin ability to suppress the growth of facultative pathogens without negative effects on principal species of normal flora of animals. These effects may form the basis for innovative program of replacing the feed antibiotics with this biologically active substance of natural origin.

The aim of this research was to study susceptibility of obligate and facultative pathogenic microorganisms to some antibiotics and dihydroquercetin in vitro.

Technique. Experiments were held with museum collection strains of *Staphylococcus epidermidis* ATCC 14990, *Pseudomonas aeruginosa* 98 (Tarasevich State Research Institute of Standardization and Control of Medical and Biological Products), *Escherichia coli* VL-613 (All-Russian Collection of Industrial Microorganisms, State Research Institute of Genetics, Moscow), *Micrococcus luteus* (*lysodeicticus*) ATCC 4698 (Research Center for Expert Review of Medical Goods of the Ministry of Health of Russia, Moscow), *M. coccus luteus* ATCC 10240 (All-Russian State Center of Quality and Standardization of Feeds and Veterinary Medicines, Moscow). The purity of cultures was confirmed before use by morphological, cultivation, physiological, and biochemical parameters. To prepare the test microbial inoculum, we inoculated several tubes with slanted meat-peptone agar with microbial culture and kept them in thermostat for 24 hours at 37±1 °C. Test culture was washed with 5 ml of sterile 0.9 % NaCl. The suspension density was adjusted to 3.3 by McFarland standard (equivalent to 1×10^9 CFU/ml). Inoculum was used within 15 min after its preparation.

Antibiotic standards produced according to specifications, in-house standards, state Reference Standards (RS; manufactured by VGNKI, Moscow) and added to the industry list were used in this study. The ampoules of tetracycline, chloramphenicol, streptomycin, bacitracin, grisin, or benzylpenicillin were weighed and reconstituted with sterile 0.9 % NaCl. Working solutions of the following concentrations were made: 3.0, 5.0, 10.0, 16.0, 19.0, 24.0, and 48.0 μg/ml. To prepare dihydroquercetin working standard, 0.5 g of the product (Ametis, Russia)

was dissolved in 10 ml of sterile distilled water. Working solutions of the following concentrations were made: 0.5 %, 1.0 %, 2.0 %, and 5.0 %. The obtained cultures were steam sterilized (1 atm, 112 °C, 15 min) and cooled to room temperature.

The microbial susceptibility to antibiotics and dihydroquercetin was determined by diameter of growth inhibition due to diffusion of antimicrobial compounds into the agar [17]. Test culture was added (0.5 ml per 50 ml of the medium) into the melted growth medium for determining the microbial susceptibility to antibacterial drugs, prepared according to instructions (State Research Center of Applied Microbiology and Biotechnology, Moscow Province), and cooled to 40-45 °C; 20 ml aliquots were poured into Petri dishes. Agar was dried in a thermostat for 1-2 hours; then 5 holes were made using drill of 8 mm (±0.1 mm) outer diameter, 6 mm (±0.1 mm) inner diameter, and height of 10 mm (±0.1 mm).

Each sample of antibacterial drug and dihydroquercetin was tested in 4 dishes. To the first hole, 0.06 ml of 0.9 % NaCl was introduced; into other holes, 0.06 ml of antibiotic or dihydroquercetin solution in the concentration tested was added. Dishes were put into a thermostat for 24 hours at 37±1 °C. After incubation, they were placed bottom up on a dark matt surface, and diameters were measured in reflected light at incidence angle of 45°. Growth inhibition diameters were measured with accuracy up to 1 mm, using vernier caliper. Cultures were deemed resistant if diameter of growth inhibition was less than 15 mm, of intermediate susceptibility if diameter was 16-19 mm, and susceptible if diameter exceeded 20 mm [17, 18].

MS Excel software and parametric methods were used for statistical data processing. This paper presents thee arithmetic means and the mean errors.

Results: The following table shows the obtained data.

Growth inhibition diameters ($X \pm x$, mm) of microbial test cultures exposed to antibiotics and dihydroquercetin

Antimicrobial agent, concentration	*Escherichia coli* VL-613	*Micrococcus luteus* (*lysodeicticus*) ATCC 4698	*Micrococcus luteus* ATCC 10240	*Staphylococcus epidermidis* ATCC 14990	*Pseudomonas aeruginosa* 98
Dihydroquercetin (%)					
0.5	12.20±0.84	15.00±0.71	13.22±0.16	12.00±0.35	11.60±0.17
1.0	12.40±0.27	16.46±0.28	16.50±0.32	16.67±0.41	14.40±0.23
2.0	14.80±0.55	19.75±0.73	17.90±0.46	19.67±0.82	15.70±0.16
5.0	15.60±0.42	23.25±0.71	20.80±0.42	21.33±0.82	17.50±0.18
Benzylpenicillin (μg/ml)					
3.0	11.80±0.22	45.40±0.27	45.60±0.27	17.20±0.22	27.20±0.22
5.0	12.80±0.22	—	46.60±0.27	18.60±0.27	30.80±0.22
10.0	15.60±0.27	47.00±0.35	49.60±0.27	20.20±0.22	32.00±0.50
16.0	15.60±0.22	49.60±0.27	50.80±0.42	20.80±0.22	32.80±0.22
19.0	15.80±0.27	50.80±0.42	—	—	—
24.0	18.00±0.18	54.80±0.22	54.60±0.27	22.40±0.27	37.40±0.27
48.0	11.80±0.22	45.40±0.27	45.60±0.27	17.20±0.22	27.20±0.22
Grisin (μg/ml)					
3.0	15.00±0.18	21.40±0.27	24.20±0.22	18.30±0.22	14.60±0.27
5.0	15.20±0.22	23.20±0.22	24.80±0.22	18.60±0.27	15.80±0.42
10.0	15.80±0.22	—	26.00±0.35	19.80±0.22	18.00±0.61
16.0	16.00±0.00	23.60±0.27	26.40±0.27	20.00±0.00	18.60±0.27
19.0	16.60±0.27	26.20±0.22	—	20.60±0.27	—
24.0	—	27.67±0.41	28.00±0.18	—	19.80±0.22
48.0	18.20±0.22	29.67±0.41	30.00±0.35	23.60±0.27	21.80±0.22
Tetracycline (μg/ml)					
3.0	—	29.88±0.36	—	11.80±0.29	—
5.0	—	31.60±0.27	—	11.80±0.22	—
10.0	—	35.80±0.22	—	14.00±0.35	—
16.0	—	36.60±0.27	—	14.70±0.14	—
19.0	—	39.40±0.27	—	14.70±0.22	—
24.0	—	42.67±0.33	—	–	—
48.0	—	50.67±0.33	—	16.50±0.29	—
Streptomycin (μg/ml)					
3.0	18.80±0.22	23.33±0.22	26.00±0.32	24.40±0.27	15.80±0.42
5.0	19.40±0.27	—	34.60±0.27	26.00±0.50	17.00±0.50

Table continued

10.0	19.80±0.22	24.67±0.22	35.73±0.13	27.00±0.35	23.80±0.22
16.0	20.20±0.22	24.67±0.22	39.67±0.29	27.80±0.22	23.80±0.22
19.0	—	29.00±0.58	—	29.80±0.22	—
24.0	20.60±0.27	35.33±0.33	39.90±0.09	—	25.00±0.00
48.0	26.76±0.17	37.33±0.33	44.67±0.29	30.90±0.55	27.80±0.55
Bacitracin (μg/ml)					
3.0	26.67±0.37	38.75±0.29	39.67±0.37	14.40±0.27	12.90±0.23
5.0	27.60±0.27	40.67±0.41	42.67±0.41	15.80±0.22	15.40±0.21
10.0	28.60±0.27	—	44.00±0.35	17.40±0.27	17.00±0.18
16.0	—	41.67±0.41	—	—	—
19.0	30.60±0.27	—	45.67±0.41	18.80±0.42	18.40±0.45
24.0	—	42.67±0.41	—	—	—
48.0	—	—	—	—	—
Chloramphenicol (μg/ml)				—	
3.0	25.40±0.27	32.20±0.22	—	30.60±0.27	22.80±0.42
5.0	25.60±0.27	–	—	34.20±0.42	—
10.0	26.60±0.45	33.60±0.27	—	34.80±0.22	26.40±0.42
16.0	28.40±0.27	34.80±0.22	—	39.80±0.22	28.20±0.27
19.0	—	36.60±0.27	—	40.60±0.27	—
24.0	30.40±0.27	37.40±0.27	—	—	30.40±0.27
48.0	31.60±0.27	37.60±0.27	—	41.00±0.35	30.20±0.22

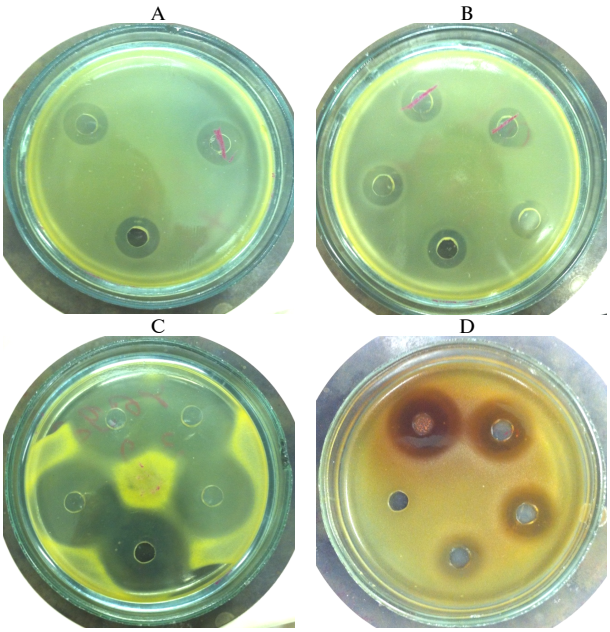
N o t e. Agar diffusion method was implemented. Cultures were deemed resistant if diameter of growth inhibition was less than 15 mm, of intermediate susceptibility if diameter was 16-19 mm, and susceptible if diameter exceeded 20 mm. Dashes mean that the respective parameter was not measured.

St. epidermidis are gram-positive cocci. It showed maximum susceptibility to all concentrations of chloramphenicol (growth inhibition diameter 30.60±0.27 mm to 41.00±0.35 mm) and streptomycin (24.40±0.27 mm to 30.90±0.55 mm) and to high concentrations (16-48 μg/ml) of grisin (20.00±0.00 mm to 23.60±0.27 mm) and benzylpenicillin (20.20±0.22 mm to 22.40±0.27 mm). The test culture demonstrated resistance to tetracycline (Fig. A) and bacitracin (3.0-48.0 μg/ml), to low levels (3.0-10.0 μg/ml) of grisin and benzylpenicillin. At the same time, *St. epidermidis* was poorly susceptible to 0.5 % and 1.0 % dihydroquercetin (growth inhibition diameter was 12.00±0.35 mm and 16.67±0.41 mm, respectively) (see Fig. B) but highly susceptible to 2.0 % (19.67±0.82 mm) and 5.0 % dihydroquercetin solutions (21.33±0.82 mm).

Ps. aeruginosa are gram-negative facultative pathogenic bacteria. The tested strain showed high susceptibility to chloramphenicol (growth inhibition diameter 22.80±0.42 mm to 30.20±0.22 mm), benzylpenicillin (27.20±0.22 mm to 37.40±0.27 mm), streptomycin (23.80±0.22 mm to 27.80±0.55 mm), grisin in concentration of 48 μg/ml (21.80±0.22 mm), and 2.0 % and 5.0 % dihydroquercetin (15.70±0.16 mm and 17.50±0.18 mm, respectively). However, the test culture was poorly susceptible to low concentrations of grisin and streptomycin and to all bacitracin concentrations.

E. coli is used in livestock farming as a probiotic culture [19, 20]. In our tests, growth inhibition diameter for *E. coli* VL-613 exposed to 0.5 % dihydroquercetin was 12.20±0.84 mm, the antimicrobial effect being lower than with the tested antibiotics in the applied concentrations; as for the use of 1.0 % dihydroquercetin, the parameter (12.40±0.27 mm) was similar to that after benzylpenicillin concentration of 5.0 μg/ml. The effect of 2.0 % dihydroquercetin solution (14.80±0.55 mm) was approximately similar to that observed with grisin in concentration of 3 μg/ml (15.00±0.18 mm) and benzylpenicillin in concentration of 10 μg/ml (15.60±0.27 mm). Growth inhibition diameters of 5.0 % dihydroquercetin solutions (15.60±0.42 mm) were approximately equal to those of streptomycin at concentration of 3 μg/ml (18.80±0.22 mm), grisin at 5 μg/ml (15.20±0.22 mm), and benzylpenicillin at 16 μg/ml (15.60±0.22 mm). *E. coli* was highly susceptible to all chloramphenicol and bacitracin concentrations; the minimum tested level (3 μg/ml) produced growth inhibition diameters of 25.40±0.27 mm and 26.67±0.37 mm, respectively, being significantly higher than

the respective parameter with the maximum concentration of dihydroquercetin.



The susceptibility of *Staphylococcus epidermidis* ATCC 14990 (upper row) **and *Micrococcus luteus* ATCC 4698 strains** (lower row) **to tetracycline** (A — 48 μg/ml; C — 3 μg/ml) **and dihydroquercetin** (B — 1.0 % solution, D — 0.5-5.0 % solution).

M. luteus are gram-positive immotile cocci. They play a minor role in human or animal diseases. Our experiments showed that the test strain of *M. luteus* (*lysodeicticus*) ATCC 4698 was highly susceptible even to minimum antibiotic concentrations. Thus, growth inhibition diameter for antibiotics (3.0 μg/ml) ranged from 21.40±0.27 mm to 45.40±0.27 mm (see Fig. C), while even the maximum concentrations of dihydroquercetin (2.0-5.0 %) were less inhibitory against this culture (growth inhibition diameter 19.75±0.73 mm to 23.25±0.71 mm) (see Fig. D). The activity of antibiotics was similar against *M. luteus* ATCC 10240. At 3.0 μg/ml, growth inhibition diameter ranged from 24.20±0.22 mm to 39.67±0.37 mm and 30.00±0.35 mm to 54.60±0.27 mm at the maximum level (48.0 μg/ml). At the same time, the culture was poorly susceptible to all tested concentrations of dihydroquercetin (growth inhibition diameter was 13.22±0.16 mm and 20.80±0.42 mm for 0.5 % and 5.0 % solutions, respectively).

Thus, facultative pathogens such as *Staphylococcus epidermidis* ATCC 14990 and *Pseudomonas aeruginosa* 98 demonstrated poor susceptibility to all bacitracin concentrations and to some grisin concentrations (3-16 μg/ml), while probiotic *Escherichia coli* VL-630 and nonpathogenic *Micrococcus luteus* (*lysodeicticus*) ATCC 4698 and *M. luteus* ATCC 10240 were highly susceptible to these drugs. These results cast doubt on feasibility of use of the above substances as antibiotics added to animal feed. However, dihydroquercetin solutions effectively suppressed facultative pathogens, being without negative effects on probiotic cultures. Thus, *St. epidermidis* and *Ps. aeruginosa* showed high susceptibility to 2.0 % and 5.0 % dihydroquercetin, while *E. coli* VL-630, *M. luteus* (*lysodeicticus*) ATCC 4698, and *M. luteus* ATCC 10240 showed low susceptibility. We think that dihydroquercetin may be suggested as alternative to feed antibiotics because this compound of biological origin is able to inhibit the growth and development of facultative pathogens without negative effects on key species present in normal flora of animals.

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